
Design and scalable manufacturing of mucus-penetrating liposomal systems for enhanced pulmonary delivery of ciclesonide and indacaterol maleate

Tuan Nghia Dinh
Pharmacist

Promotor : Professor **Géraldine Piel**

Doctoral thesis presented in partial fulfillment of the requirements for
a PhD in Biomedical and Pharmaceutical Sciences

Academic year 2025-2026

Jury

Professor Géraldine Piel	(Promotor, University of Liège, BE)
Professor Anne-Catherine Servais	(President of the Jury, University of Liège, BE)
Professor Brigitte Evrard	(Secretary of the Jury, University of Liège, BE)
Professor Didier Cataldo	(University of Liège, BE)
Doctor Bruno Grignard	(University of Liège, BE)
Professor Elisabeth Badens	(Aix-Marseille University, FR)
Professor Rita Vanbever	(Catholic University of Louvain, BE)

REMERCIEMENTS

Au terme de cette aventure aussi exigeante qu'enrichissante, je tiens à exprimer ma profonde gratitude à toutes les personnes qui, par leurs conseils, leur collaboration ou leur soutien, ont contribué d'une manière ou d'une autre à mener à bien ce travail.

Tout d'abord, je tiens à exprimer ma profonde gratitude à Madame la Professeure Géraldine Piel, promotrice de ma thèse, pour m'avoir offert l'opportunité de réaliser ce travail au sein de son équipe. Je lui suis extrêmement reconnaissant de la qualité de son encadrement, son suivi attentif et ses conseils avisés tout au long de ces quatre années. Son exigence scientifique alliée à une grande disponibilité ont grandement contribué à l'évolution de mes recherches.

Je tiens à exprimer ma sincère gratitude aux membres de mon comité de thèse, Madame la Professeure Anne-Catherine Servais, Madame la Professeure Brigitte Evrard, Monsieur le Professeur Didier Cataldo, Monsieur le Docteur Bruno Grignard, ainsi qu'à ma promotrice, pour leur accompagnement attentif, leurs conseils avisés et leurs précieux encouragements tout au long des séances. Je souhaite également remercier aux membres du jury, Madame la Professeure Elisabeth Badens et Madame la Professeure Rita Vanbever, pour leur dévouement et leur expertise dans l'évaluation de ce travail.

Cette thèse a été réalisée dans le cadre du projet « LNPulmo » et n'aurait pas été possible sans le généreux soutien financier de la Région wallonne, à laquelle je tiens à exprimer ma sincère gratitude. Je souhaite également remercier notre partenaire industriel, Aquilon Pharma, et l'ensemble de ses collaborateurs pour leur précieux soutien. J'adresse tout particulièrement mes sincères remerciements aux professeurs Didier Cataldo et Brigitte Evrard et Madame le Docteur Anna Lechanteur ainsi qu'à ma promotrice de thèse, pour la richesse de leurs échanges et la qualité de leur encadrement tout au long de la réalisation de cette thèse. Enfin, je tiens à exprimer ma profonde gratitude à mes deux collaborateurs de projet et de thèse, Benedetta Bottero et Laure-Anne Bya, pour leur dévouement et leurs précieuses contributions scientifiques à ce projet commun, ainsi que pour leur soutien durant la rédaction de la thèse.

Je souhaite également exprimer ma plus sincère gratitude à tous les membres, actuels et anciens, du Laboratoire de Technologie Pharmaceutique et Biopharmacie, qui ont contribué à créer une atmosphère conviviale et unique au sein du laboratoire. Je remercie particulièrement Laurence Collard et Françoise Léonard pour leur présence, leur soutien technique et scientifique, et le temps qu'elles ont consacré à la résolution des différents défis expérimentaux rencontrés dans le cadre de cette recherche. Je remercie également Manon Berger, Chloé Parulski, Eva Gresse, Laure-Anne Bya, Manon Degey, Giacomo Baudino et Alicia Curnel. Leur bonne humeur, leur soutien quotidien, ainsi que leurs pauses et les moments partagés sont inestimables.

Je tiens également à exprimer ma sincère gratitude à mes collègues du bureau qui m'ont soutenue au fil des ans. Le bureau a évolué, passant d'une petite équipe avec Noémie Penoy, Chloé Gauthy,

Nathan Koch et Olivier Jennotte à une équipe dynamique avec Quentin Bourcy, Lena Renauld et Berfin Dédé. Merci à tous d'avoir fait de ces quatre précieuses années une réussite, remplies de rires, d'anecdotes amusantes, de petites attentions, de soutien mutuel, d'encouragements et d'une écoute attentive dans les moments difficiles. Les moments passés en votre compagnie vont beaucoup me manquer.

Je tiens à exprimer ma gratitude à tous ceux qui ont collaboré aux recherches présentées dans cette thèse.

Je remercie au Docteur Noémie Penoy pour son aide précieuse dans les travaux concernant la production des liposomes par le CO₂ supercritique ainsi que pour ses conseils, ses orientations et nos nombreux échanges fructueux sur ce sujet. Je remercie également au Docteur Pierre-Yves Sacré pour son aide et sa volonté de me soutenir dans la réalisation et l'analyse des plans d'expérience concernant la production des liposomes par le CO₂ supercritique dans cette thèse.

Merci également à Monsieur le Professeur Eric Ziemons et à Madame le Docteur Amandine Dispas du Laboratoire de Chimie Analytique de l'Université de Liège pour leur aide dans le développement de la méthode analytique.

Merci à Madame Pauline Desdemoustier du plateforme In vitro-Santé pour la formation à des expériences cellulaires.

Merci à Madame le Docteur Sandra Ormenesse, Monsieur Stephan Raafat, Madame Céline Vanwinge et Monsieur Alexandre Hego du plateforme de cytométrie en flux et d'imagerie cellulaire de l'Université de Liège pour leur soutien dans l'analyse des cellules par la cytométrie en flux.

Merci à Madame le Docteur Erika Hendrickx et Madame le Docteur Louise Conrard, du Centre de microscopie et d'imagerie moléculaire de l'Université libre de Bruxelles, pour la réalisation des analyses de microscopie cryo-TEM.

Merci à Madame la Professeur Dominique Teye et Monsieur le Docteur Sebastien Calvo, du Laboratoire de génie chimique de l'Université de Liège, pour leur aide dans la compréhension de la production des liposomes par le CO₂ supercritique en salle blanche.

Enfin, je tiens à exprimer ma plus profonde gratitude à ma famille et à mes amis, qui m'ont toujours soutenu et cru en moi.

Du fond du cœur, merci à tous.

Tuan Nghia

ABSTRACT

According to the 2025 GINA Guidelines, the management of asthma remains fundamentally dependent on the combination of an inhaled corticosteroid (ICS) and a long-acting β 2-agonist (LABA). Clinical practice is increasingly shifting toward molecules with a balanced hydrophilic/lipophilic profile, such as budesonide and formoterol. This balance facilitates rapid dissolution in the airway lining fluid and efficient penetration across the mucus barrier to access their molecular receptors. While the high lipophilicity of newer ICS and LABA molecules enables convenient once-daily dosing regimens, this property renders them more susceptible to mucociliary clearance and phagocytosis by alveolar macrophages, thereby compromising their ability to reach the target cells. To address these pulmonary delivery limitations, this study focuses on the formulation development and production scale-up of an advanced liposomal carrier system for a new ICS-LABA combination: ciclesonide (CIC) and indacaterol maleate (IND).

Initially, we focused on the design of a liposome architecture that allows for mucus penetration, stable drug encapsulation, evasion of macrophage phagocytosis, and enhanced epithelial uptake. By meticulously optimizing the type and concentration of PEGylated lipids and phospholipids, an optimal formulation (DLPC/Cholesterol/DSPE-PEG) containing CIC and IND was identified. This formulation significantly improved the attenuation of airway hyperresponsiveness and inflammation in an *in vivo* acute asthma mouse model compared to non-liposomal drug formulation. Furthermore, the liposomal delivery of CIC and IND exhibited a substantial dose-sparing potential for both therapeutic agents.

Building upon the proof-of-concept for this optimized formulation, the second objective aimed to facilitate its production at a pilot scale without using organic solvents. This scale-up effort required a comprehensive application of Design of Experiments principles to characterize multiple critical process parameters, such as dispersion volume, pressure, agitation speed, and depressurization rate, within a pilot-scale, GMP-like environment. The rigorous optimization process resulted in the generation of liposomes exhibiting a mean particle size of 152 ± 8 nm and a Pdl of 0.36 ± 0.02 , while maintaining high drug encapsulation efficiencies (>90%). Furthermore, the detailed characterization of the liposomes manufactured at the pilot scale confirmed that the chosen method did not induce statistically significant increases in lipid oxidation or hydrolysis, confirming the chemical stability and industrial suitability of the process.

In conclusion, this study successfully met its primary goals. We first validated a highly effective liposomal platform that significantly enhances the anti-asthmatic efficacy of the CIC and IND combination *in vivo*. Second, we demonstrated the feasibility of transitioning this delivery system to a pilot manufacturing scale using the advanced, organic solvent-free PGSS process. These results provide a robust and highly viable platform for developing next-generation inhaled therapies for chronic respiratory diseases.

RÉSUMÉ

Selon les recommandations GINA 2025, la prise en charge de l'asthme repose fondamentalement sur l'association d'un corticostéroïde inhalé (ICS) et d'un bêta-2-agoniste de longue durée d'action (LABA). La pratique clinique s'oriente de plus en plus vers des molécules présentant un profil hydrophile/lipophile équilibré, telles que le budésonide et le formotérol. Cet équilibre facilite une dissolution rapide dans le liquide de surface des voies aériennes ainsi qu'une pénétration efficace à travers le mucus pour accéder aux récepteurs moléculaires. Bien que la lipophilie élevée des nouvelles molécules de ICS et de LABA permette une administration quotidienne unique, cette propriété les rend plus sensibles à la clairance mucociliaire et à la phagocytose par les macrophages alvéolaires, compromettant ainsi leur capacité à atteindre les cellules cibles. Pour remédier à ces limitations de l'administration pulmonaire, cette étude se concentre sur le développement de la formulation et la montée en échelle de la production d'un système de transport liposomal avancé pour une nouvelle association ICS-LABA: le ciclésone (CIC) et le maléate d'indacatérol (IND).

Dans un premier temps, nous nous sommes concentrés sur la conception d'une architecture de liposome permettant la pénétration du mucus, l'encapsulation stable des molécules actives, l'évasion de la phagocytose par les macrophages et une absorption épithéliale accrue. En optimisant le type et la concentration des lipides PEGylés et des phospholipides, une formulation optimale (DLPC/Cholestérol/DSPE-PEG) contenant du CIC et de l'IND a été identifiée. Cette formulation a considérablement amélioré l'atténuation de l'hyperréactivité bronchique et de l'inflammation dans un modèle murin d'asthme aigu *in vivo* par rapport à une formulation non liposomale. De plus, l'administration liposomale de CIC et d'IND a présenté un potentiel substantiel d'épargne de dose pour les deux molécules actives.

Fort de la preuve de concept de cette formulation optimisée, le second objectif visait à faciliter sa production à l'échelle pilote sans recours aux solvants organiques. Cet effort de montée en échelle a nécessité une application d'un plan d'expérience pour caractériser de multiples paramètres de processus critiques, tels que le volume de dispersion, la pression, la vitesse d'agitation et le taux de dépressurisation, au sein d'un environnement de type GMP à l'échelle pilote. Ce processus d'optimisation rigoureux a permis de générer des liposomes présentant une taille de particule moyenne de 152 ± 8 nm et un Pdl de $0,36 \pm 0,02$, tout en maintenant des efficacités d'encapsulation élevées (> 90 %). En outre, la caractérisation détaillée des liposomes fabriqués à l'échelle pilote a confirmé que la méthode choisie n'induisait pas d'augmentation statistiquement significative de l'oxydation ou de l'hydrolyse des lipides.

En conclusion, cette étude a atteint avec succès ses objectifs principaux. Nous avons d'abord validé une formulation liposomale qui améliore significativement l'efficacité anti-asthmatique de l'association CIC et IND *in vivo*. Deuxièmement, nous avons démontré la faisabilité de la transition de cette formulation de l'échelle du laboratoire vers une échelle de fabrication pilote en utilisant le procédé avancé PGSS, exempt de solvants organiques. Ces résultats offrent une plateforme robuste pour le développement de thérapies inhalées de nouvelle génération destinées aux maladies respiratoires chroniques.

TABLE OF CONTENTS

ABBREVIATIONS	1
INTRODUCTION	1
I. Inhaled corticosteroids and long-acting β_2-agonists for asthma management	1
I.1. Introduction to type 2-high asthmatic inflammation	1
I.2. Role of inhaled corticosteroids and long-acting β_2 agonists combinations for asthma management	5
I.2.1. β_2 adrenergic receptor agonists	5
I.2.1.1. Mechanism of action	5
I.2.1.2. Classification of β_2 adrenergic receptor agonists according to their duration of action	7
I.2.2. Inhaled corticosteroids	11
I.2.2.1. Mechanism of action	11
I.2.2.2. Inhaled corticosteroids used in the treatment of asthma	14
I.2.3. Combination of inhaled corticosteroids and long-acting β_2 adrenergic receptor agonists for asthma management– Advantages and challenges	17
II. Use of liposomes for lung administration	23
II.1. General introduction to liposomes	23
II.2. Liposomes for lung administration	29
II.2.1. Pulmonary administration: anatomical foundations and physiological barriers	29
II.2.1.1. Basic anatomical structure of the airway epithelium	30
II.2.1.2. Physiological barriers	32
II.2.2. Engineering of liposomes to overcome physiological barriers in the lungs	37
II.2.2.1. Engineering of liposomes to overcome mucociliary clearance	37
II.2.2.2. Engineering of liposomes to overcome the phagocytosis by alveolar macrophages	39
II.2.2.3. Engineering of liposomes to leverage the action of pulmonary surfactant	42
II.2.2.4. Critical quality attributes of liposomes containing ciclesonide and indacaterol maleate for improved therapeutic efficacy against asthma	43
II.2.3.5. Challenges linked to PEGylation	44
II.2.3. Challenges linked to liposomes stability and production methods	46
II.2.3.1. Structural stability and delivery-related constraints	46
II.2.3.2. Translational and manufacturing challenges	47
III. Large-scale production of liposomes: process engineering challenges and strategic choices	48
III.1. Limitations of conventional laboratory-scale methods	48
III.2. Current liposomes production methods at industrial scale: ethanol injection and microfluidics	49
III.2.1. Ethanol injection method	50

III.2.2. Microfluidic	52
III.1.3. Downstream processing: a critical yet limiting step	54
III.3. Transition toward solvent-free processes: rationale and limitations –	
Supercritical fluids-based approaches.....	57
III.3.1. Methods for producing liposomes assisted by supercritical carbon dioxide and organic solvent	58
III.3.2. Methods for producing liposomes assisted by supercritical carbon dioxide and without organic solvent	60
CONTEXT AND OBJECTIVES OF THE STUDY.....	67
I. Context of the study	69
II. Objectives of the study	71
EXPERIMENTAL PART.....	73
CHAPTER I Design of liposomes containing ciclesonide and indacaterol maleate for pulmonary delivery	75
I.1. Introduction to Chapter I	77
I.2. Optimizing the composition of liposomes containing ciclesonide and indacaterol maleate for pulmonary delivery	80
I.2.1. Introduction	84
I.2.2. Materials and methods	86
I.2.2.1. Materials	86
I.2.2.2. Liposomes preparation by the thin film hydration method	87
I.2.2.3. Characterization of liposomes physicochemical properties	87
I.2.2.4. Evaluation of liposomes mucopenetration properties	88
I.2.2.5. Cytotoxicity and cellular uptake	89
I.2.2.6. Ex vivo relaxant effect of indacaterol maleate on isolated tracheal rings from rats	89
I.2.2.7. In vivo evaluation in murine asthma model	90
I.2.3. Results	93
I.2.3.1. Impact of the PEGylated lipid type and concentration on key liposome properties	94
I.2.3.2. Impact of the type of phospholipid on key liposome properties	102
I.2.3.3. Impact of liposomes containing CIC and IND on the treatment of AHR and inflammation in a mouse model of mild eosinophilic asthma by pulmonary administration.....	107
I.2.4. Discussion	115
I.2.5. Conclusion	121
I.3. Complementary results	122
I.3.1. Morphology of liposomes containing ciclesonide and indacaterol maleate by cryogenic transmission electron microscopy	122
I.3.1.1. Method	122
I.3.1.2. Results and discussion.....	123
I.3.2. Physical stability of the optimal formulation of liposomes containing ciclesonide and indacaterol maleate	124
I.3.2.1. Method	124

I.3.2.1. Results and discussion.....	124
I.3.3. Potential of liposomes in improving ex vivo bronchodilatory effect of other drugs: case of formoterol fumarate dihydrate.....	125
I.3.3.1. Method	126
I.3.3.2. Results and discussion.....	126
I.4. Conclusion.....	128
CHAPTER II Scaling-up liposome production by the particles from gas-saturated solution process (PGSS)	131
II.1. Introduction to Chapter II.....	133
II.2. Adaptation and validation of the lab-scale PGSS process to the production of the optimized liposome formulation containing ciclesonide and indacaterol maleate.....	134
II.2.1. Method	134
II.2.2. Results and discussion	136
II.2.2.1. Adaptation of the PGSS parameters for liposomes containing ciclesonide and indacaterol maleate at the lab-scale by DoE approach	136
II.2.2.2. Validation of the PGSS process for the production of liposomes containing ciclesonide and indacaterol maleate at the lab-scale.....	137
II.3. Scaling-up the PGSS process to the pilot-scale production	139
II.3.1. Introduction	143
II.3.2. Materials and methods	145
II.3.2.1. Materials	145
II.3.2.2. Liposome production	145
II.3.2.3. Characterization of physicochemical properties of liposomes.....	148
II.3.2.4. Drugs quantification.....	149
II.3.2.5. Evaluation of lysophospholipids (LPC) content (hydrolysis products of phospholipids)	150
II.3.2.6. Determination of percentage of peroxidized lipids	150
II.3.2.7. Evaluation of liposomes morphology by cryo-TEM imaging	151
II.3.2.8. Statistical Analysis	151
II.3.3. Results and discussion	151
II.3.3.1. Risk analysis	151
II.3.3.2. Study of the impact of PGSS production parameters on liposome characteristics using the design of experiments approach	152
II.3.3.3. Validation of the optimal condition.....	158
II.3.3.4. Transfer of the optimal conditions to the production of drug-containing formulations	159
II.3.4. Conclusion.....	166
II.4. Conclusion.....	168
DISCUSSION	171
I. Ciclesonide and indacaterol maleate for asthma treatment: a new ICS-LABA combination and their clinical problems.....	174

II. Design of liposomes containing ciclesonide and indacaterol maleate for pulmonary delivery	176
II.1. Lipophilicity of inhaled active molecules: A paradox for free drugs, but an advantage for liposomes.....	176
II.2. Rational selection of PEGylated lipid is important to overcome the lungs barriers while preserving the safety of liposomes.....	178
II.3. Interplays between PEGylated lipids and phospholipid with challenges concerning PEGylation (PEG dilemma and immunogenicity)	180
II.4. Problems encountered with the use of an unsaturated phospholipid with a high concentration of PEGylated lipids	183
III. Particles from gas-saturated solution process as an organic solvent-free method for liposomes production.....	187
III.1. Advantages and disadvantages of the PGSS process over ethanol injection and microfluidic to produce liposomes at the industrial scale	187
III.2. Important parameters for the production of liposomes containing ciclesonide and indacaterol maleate by the PGSS method from the laboratory scale to the pilot scale	190
III.3. Further improvements to mitigate Joule-Thomson effect	192
CONCLUSION AND PERSPECTIVES	199
I. Conclusion	201
II. Perspectives	202
APPENDICES	207
I. Appendix I.....	209
I.1. Supplementary materials and methods	209
I.1.1. Materials	209
I.1.2. Calculation of test concentrations for in vivo study (section I.2.2.7.3).....	209
I.1.3. Airway responsiveness measurements (baseline measurement for section I.2.2.7.4)	210
I.1.4. Collection of BALF (for section I.2.2.7.5).....	210
I.1.5. Histological analysis (for section I.2.2.7.6)	211
I.1.6. Lung homogenates and protein extraction (for section I.2.2.7.7).....	211
I.1.7. Parameters of airway hyperresponsiveness (for section I.2.3.3.1).....	212
I.2. Supplementary results	213
II. Appendix II	224
REFERENCES	229

ABBREVIATIONS

AHR	Airway hyperresponsiveness	des-CIC	Desisobutyryl-ciclesonide
AIR	Anti-inflammatory reliever	Dexa	Dexamethasone
AMs	Alveolar macrophages	DLPC	1,2-dilinoleoyl-sn-glycero-3-phosphocholine
ApoH	Apolipoprotein H	DMG-PEG	1,2- dimyristoyl-rac-glycero-3-[methoxy(polyethylene glycol)-2000])
ASM	Airway smooth muscle	DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
AT1	Alveolar type I epithelial cells	DMPE-PEG	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000])
AT2	Alveolar type II epithelial cells		
β_2 -agonists	β_2 -adrenergic receptor agonists		
β_2 ARs	β_2 -adrenergic receptor		
BALF	Bronchoalveolar lavage fluid	DoE	Design of experiments
BDP	Beclomethasone dipropionate	DOPC	1,2-dioleoyl-snglycero-3-phosphocholine
BMP	Beclomethasone-monopropionate	DOPG	1,2-dioleoyl-sn-glycero-3-phosphoglycerol
BUD	Budesonide	DPoPC	1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine
C ₈ -PEG-Ceramide	N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]}	DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
cAMP	3'-5'-Cyclic adenosine monophosphate	DPPE-PEG	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000])
CBP	cAMP-response element-binding protein		
CF	Cystic fibrosis	DPPG	1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol
Chol	Cholesterol	DSG-PEG	1,2-distearoyl-rac-glycero-3-[methoxy(polyethylene glycol)-2000])
CIC	Ciclesonide	DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
COPD	Chronic obstructive pulmonary disease	DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000])
CPPs	Critical process parameters		
CQAs	Critical quality attributes		
Crs	Dynamic compliance		
Cryo-TEM	Cryo-transmission electron microscopy		
DCs	Dendritic cells		

DSPG	1,2-distearoyl-sn-glycero-3-phosphoglycerol	IP3	Inositol 1,4,5-trisphosphate
DNA	Deoxyribonucleic acid	IQ	Installation qualification
ECP	Eosinophil cationic protein	ISCRPE	Improved supercritical reverse phase evaporation
EDN	Eosinophil-derived neurotoxin	LABAs	Long-acting β_2 -agonists
EE	Encapsulation efficiency	LH Lip	Low hydrophilic liposomes
EMA	European medicines agency	LNPs	Lipid nanoparticles
EPX	Eosinophil peroxidase	LPC	Lysophospholipid
Ers	Elastance	MAPK	Mitogen-activated protein kinase
FDA	US food and drug administration	MART	Maintenance and reliever therapy
FOR	Formoterol fumarate	MBP	Major basic protein
FF	Fluticasone furoate	MCC	Mucociliary clearance
FP	Fluticasone propionate	MF	Mometasone furoate
GINA	Global Initiative for Asthma	MFI	Mean fluorescence intensity
GMP	Good manufacturing practice	MH Lip	Moderate hydrophilic liposomes
GRAS	Generally recognized as safe	MLCP	Myosin light chain phosphatase
GRs	Glucocorticoid receptors	mRNA	Messenger ribonucleic acid
H&E	Hematoxylin and eosin	NPs	Nanoparticles
HAT	Histone acetyltransferase	OLO	Olodaterol hydrochloride
HDAC2	Histone deacetylase 2	OQ	Operational qualification
HH Lip	High hydrophilic liposomes	OVA	Ovalbumine
HP β CD	Hydroxypropyl- β -cyclodextrin	PAS	Periodic acid schiff
HPH	High-pressure homogenization	PAT	Process Analytical Technology
IC	Inspiratory capacity	PBS	Phosphate-buffered saline
ICSs	Inhaled corticosteroids	PCAF	p30x0 and p300/CBP-associated factor
IFN	Interferon	PD	Pharmacodynamic
IgE	Immunoglobulin E	PdI	Polydispersity index
IL	Interleukin	PGSS	Particles from gas-saturated solutions
ILC2s	Group 2 innate lymphoid cells	PK	Pharmacokinetic
IND	Indacaterol maleate		

PKA	Protein kinase A	SP	Surfactant protein
PS	Pulmonary surfactant	SPC	Soy phosphatidylcholine
QbD	Quality by Design	T2	Type 2
RNA	Ribonucleic acid	TFF	Tangential flow filtration
Rrs	Airway resistance	TFH	Thin film hydration
SAB	Salbutamol/Albuterol sulfate	TGF- β	Transforming growth factor- β
SABAs	Short-acting β_2 -agonists	Th2 cells	T-helper type 2 cells
SAL	Salmeterol xinafoate	TNF- α	Tumor necrosis factor-alpha
SC-CO ₂	Supercritical carbon dioxide	T _m	Phase transition temperature
SCF	Supercritical fluid	TSLP	Thymic stromal lymphopoietin
SD-PGSS	Sequential-dispersion particles from gas-saturated solution	Ultra-LABAs	Ultra long-acting β_2 -agonists
		VIL	Vilanterol trifenate

INTRODUCTION

I. Inhaled corticosteroids and long-acting β 2-agonists for asthma management

I.1. Introduction to type 2-high asthmatic inflammation

According to the 2025 Global Initiative for Asthma (GINA) strategy report, asthma is fundamentally defined by a history of characteristic respiratory symptoms, specifically wheezing, breathlessness, chest tightness, and coughing, that vary in intensity and over time, accompanied by variable expiratory airflow [1]. Although these symptoms may resolve spontaneously or with medication, the disease is marked by the potential for episodic flare-ups, or exacerbations, which can be life-threatening and impose a heavy burden on patients and healthcare systems. According to 2025 GINA report, these exacerbations are defined as an acute or sub-acute progressive worsening of respiratory symptoms and a decrease in expiratory airflow from a patient's usual baseline status, signifying a deterioration severe enough to require a change in medical treatment [1]. Asthma is also usually associated with **chronic airway inflammation** and **airway hyperresponsiveness** (AHR) to direct or indirect stimuli, pathophysiological features that often persist even when clinical symptoms are absent or lung function appears normal. The heterogeneity of the disease means that it encompasses diverse clinical presentations (phenotypes), which often result from distinct underlying biological mechanisms (endotypes) [2,3].

Historically, asthma was primarily viewed through the lens of the adaptive immune system, with a focus on the response of T-helper type 2 cells (Th2 cells) and Immunoglobulin E (IgE) to specific allergens. However, recent evidence has shifted the paradigm towards an "epithelial era", positioning the airway epithelium as the central orchestrator of the immune response rather than merely a passive physical barrier (Figure 1) [4]. The epithelium functions as the primary interface between the host and the environment. In a healthy state, airway epithelial cells maintain immune tolerance and actively suppress dendritic cells (DCs), the immune system sentinels, to prevent unnecessary reactions to harmless environmental antigens. This suppression is maintained through physical contact, specifically via adherens junctions (such as E-cadherin) which send "tonic inhibitory signals" to the DCs located in the sub-epithelial layer [4,5]. As long as the epithelial barrier remains intact and these connections hold, DCs remain in a quiescent state, even if they physically encounter an allergen. However, when this epithelial barrier is breached or

INTRODUCTION

activated by environmental insults, the epithelial cells release a specific set of upstream cytokines known as "alarmins": Thymic Stromal Lymphopoietin (TSLP), Interleukin (IL)-25, and IL-33 [6]. These alarmins act as master regulators, filling the "inflammatory bucket" by activating downstream pathways. Current immunopathological understanding broadly categorizes these downstream pathways into two major endotypes: Type 2 (T2)-high inflammation and T2-low inflammation.

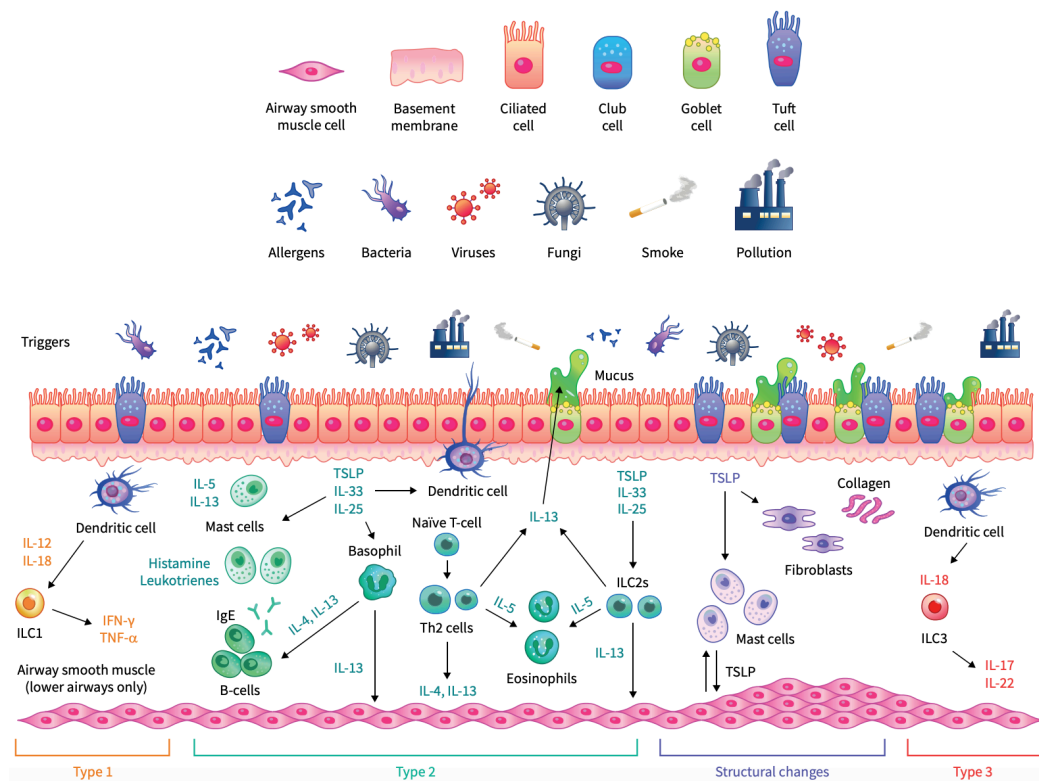


Figure 1. Schematic overview of asthmatic airway inflammatory endotypes and structural remodeling [7].

T2-high inflammation is the most common endotype, found in 50–70% of patients with severe asthma. It is driven by two distinct but complementary axes that often co-exist (Figure 1) [8]:

- **Adaptive immune response:** This pathway is the classic driver of allergic asthma. DCs capture the allergen, but they require specific instructions to determine the appropriate immune response. Epithelial alarmins derived from a damaged epithelial barrier (IL-33, IL-25, TSLP) provide this instruction [3]. DCs exposed to these alarmins then migrate to the

INTRODUCTION

lymph nodes. This process may be regulated by IL-13 secreted by Group 2 Innate Lymphoid Cells (ILC2s) and interferon (IFN) type I. They train naïve CD4⁺ T-cells to become Th2 cells. These Th2 cells then migrate back to the airways to secrete the hallmark T2 cytokines: IL4, IL-5, and IL-13.

- **Innate immune response:** In parallel with the adaptive pathway, the same epithelial alarmins directly stimulate ILC2s [3,6]. These cells respond immediately to the alarmins by producing large amounts of IL-5 and IL-13. This allows T2 inflammation to occur in case of non-allergic (intrinsic) asthma, even when no specific allergen is involved.

The pathophysiology of the T2 inflammation is orchestrated by a specific triad of cytokines: IL-4, IL-5, and IL-13. Each cytokine plays a distinct yet synergistic role in driving the hallmark features of asthma:

+ **IL-4:** IL-4 acts as a central regulator of the T2-high inflammatory endotype in asthma. Its primary function is to drive the differentiation of naïve CD4⁺ T-cells into Th2 cells. This process requires an initial pulse of IL-4 to polarize the immune response, which is often provided by basophils (sourced from the spleen, peripheral blood, or cord blood) [9,10] or by follicular Th cells, which are specialized T-cells that reside in lymphoid follicles [11]. Once Th2 cells are established, IL-4 continues to orchestrate the inflammatory cascade by acting on B-lymphocytes to induce immunoglobulin class switching to IgE [3]. IgE binds to high-affinity Ig E receptors on mast cells and basophils. Upon re-exposure to an allergen, receptor cross-linking triggers the release of histamine, neutral proteases (such as tryptase and chymase), lipid mediators, and additional Th2-associated cytokines [12]. This release reinforces the pro-inflammatory Th2 environment and causes immediate bronchoconstriction. IL-13 closely supports this cascade, as its signaling pathway shares a common receptor subunit with IL-4 (the IL-4 receptor alpha) [12]. In addition, IL-4 prepares the tissue for leukocyte infiltration by upregulating Vascular Cell Adhesion Molecule-1 on endothelial cells [13]. This allows eosinophils and T-cells to adhere to the blood vessel wall and migrate into the lung tissue.

+ **IL-13:** While IL-4 drives the immunological setup, IL-13 is the primary effector cytokine that acts directly on the structural cells of the airway to cause physiological dysfunction. It elicits goblet cell metaplasia and upregulates the expression of the mucin gene MUC5AC, altering the biophysical properties of mucus so that it becomes thick and viscous [12]. This leads to the formation of mucus plugs, a major cause of airflow

INTRODUCTION

obstruction and fatal asthma. IL-13 also acts directly on airway smooth muscle (ASM) cells promoting ASM hypertrophy, proliferation, and hypercontractility. This thickens the airway wall and drives the AHR [14]. It also drives lung fibroblasts to differentiate into myofibroblasts, stimulating them to proliferate and secrete excessive extracellular matrix components, such as collagen [15]. Together, these processes (subepithelial fibrosis, mucus plugging and ASM thickening) constitute the key structural features of fixed airway obstruction. On the other hand, IL-13 also contributes to eosinophil recruitment into the airways by increasing eotaxin synthesis at level of the bronchial epithelium [15] and upregulates inducible nitric oxide synthase, thereby increasing the airway levels of nitric oxide by airway epithelial and inflammatory cells [16]. Consequently, Fractional Exhaled Nitric Oxide serves as a specific biomarker for IL-13 activity in the airway compartment, distinguishing it from blood eosinophils which primarily reflect systemic IL-5 activity in asthma [17].

+ **IL-5:** Unlike IL-4 and IL-13, which have broad effects on structural and immune cells, IL-5 is highly specific to eosinophils. It plays a critical role in the terminal differentiation and maturation of eosinophils in the bone marrow and their subsequent release into the circulation [3]. Once eosinophils have migrated into the lung tissue (aided by IL-4/IL-13-induced adhesion molecules and eotaxins), IL-5 acts as a critical survival factor, inhibiting apoptosis and extending eosinophil longevity within the airways. Furthermore, IL-5 primes eosinophils for activation, triggering the release of cysteinyl leukotrienes, pro-fibrotic growth factors (such as Transforming Growth Factor- β (TGF- β)) and cytotoxic granule proteins, including Major Basic Protein (MBP), Eosinophil Peroxidase, Eosinophil Cationic Protein (ECP), Eosinophil Peroxidase (EPX), and Eosinophil-Derived Neurotoxin (EDN).

Under normal physiological conditions, these mediators are essential for host defense and tissue homeostasis. The highly charged cytotoxic granules such as MBP, ECP, and EPX evolve to kill invading helminth parasites and bacteria, EDN provides protective antiviral defense by degrading the RNA of single-stranded respiratory viruses, and growth factors like TGF- β facilitate normal tissue repair and regeneration after epithelial injury [18–20]. However, in the context of asthma, the chronic overaccumulation and excessive release of these mediators become pathological. The weapons designed to destroy pathogens instead cause profound host tissue damage: MBP and ECP disrupt airway epithelial cell membranes while EPX generates potent oxidizing species that drive severe oxidative stress in the tissue [3,19]. Furthermore, MBP acts as an endogenous antagonist of the M2

muscarinic receptor on airway nerves, thereby exacerbating vagally mediated bronchoconstriction [19,21]. Meanwhile, excessive cysteinyl leukotrienes act as extremely potent bronchoconstrictors (up to 10000-fold more potent than histamine), and the overproduction of TGF- β drives abnormal airway remodeling, leading to subepithelial fibrosis, excessive extracellular matrix deposition, and ASM hypertrophy [15,22].

I.2. Role of inhaled corticosteroids and long-acting β_2 agonists combinations for asthma management

The long-term goals of asthma treatment are to achieve good symptom control and minimize future risk, including preventing exacerbations, persistent airflow limitation, and medication side effects [1]. A variety of pharmacological groups have been used in asthma management to achieve these goals, including inhaled and oral corticosteroids, β_2 -adrenergic receptor agonists (β_2 -agonists), muscarinic 3 receptor antagonists, leukotriene receptor antagonists, phosphodiesterase inhibitors, macrolide antibiotics like azithromycin, and injected biologics targeting specific biomarkers. Inhaled corticosteroids (ICSs) and β_2 -agonists form the basis of asthma control in both domains (symptom control and future risk).

I.2.1. β_2 adrenergic receptor agonists

I.2.1.1. Mechanism of action

The contraction of ASM is driven by intracellular Ca^{2+} signaling that is initiated when bronchoconstrictive mediators bind to specific receptors on the surface of ASM cells (Figure 2). The process typically begins when contractile agonists, such as acetylcholine, histamine or cysteinyl leukotrienes, bind to their respective G-protein-coupled receptors of the G_q -subfamily (e.g., M3 muscarinic, H1 histamine or CysLT1 receptors) located on ASM cells membrane [23,24]. Once the agonist binds, the receptor undergoes a conformational change that enables coupling to and activates the associated $G_{\alpha q}$ protein. The activated G-protein then stimulates enzyme phospholipase C, which catalyzes the hydrolyze of phosphatidylinositol 4,5-bisphosphate to produce inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 diffuses through the cytosol and binds to its specific receptor (IP3R) which is located on the sarcoplasmic reticulum (the cell's internal calcium store). This prompts a rapid release of Ca^{2+} into the cytosol. This internal release is combined with Ca^{2+} release from ryanodine-sensitive stores and an influx of extracellular Ca^{2+} through channels on the plasma membrane. This leads to a sharp increase in the intracellular Ca^{2+} level. The

INTRODUCTION

elevated intracellular Ca^{2+} binds to calmodulin, forming a complex that stimulates an enzyme called Myosin Light Chain Kinase. Activated Myosin Light Chain Kinase then phosphorylates the 20-kDa regulatory myosin light chain. This crucial phosphorylation event activates myosin ATPase, which hydrolyzes adenosine triphosphate (ATP) to power the myosin cross-bridges to bind to actin filaments and cycle. This physically shortens the sarcomere and contracts ASM.

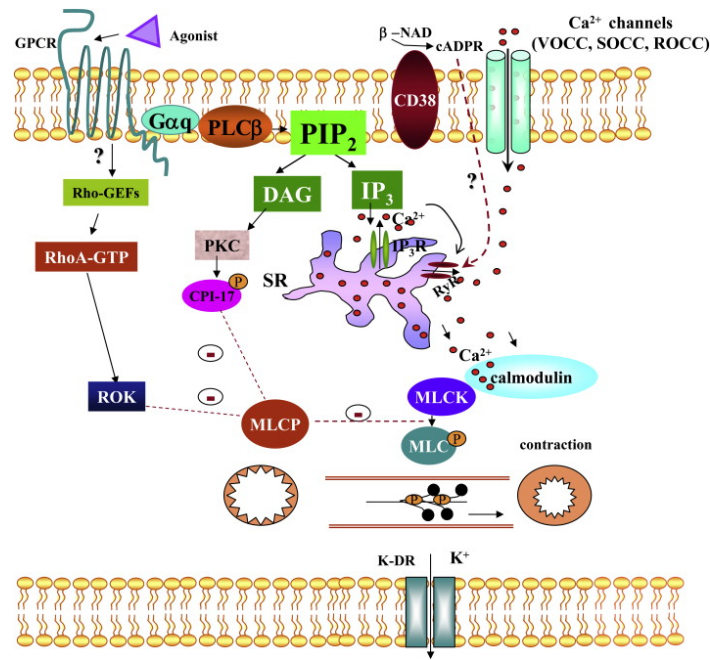


Figure 2. Signal transduction pathways involved in airway smooth muscle contraction [24].

Following the initial contraction, ASM cells can maintain a sustained, tonic contraction through ‘calcium sensitization’ mechanisms that prevent the dephosphorylation of myosin [24]. The main signaling mechanisms thought to be responsible for this process include the RhoA/Rho-kinase pathway and the PKC/CPI-17 effector system. These mechanisms converge to inhibit the enzyme Myosin Light Chain Phosphatase (MLCP), the enzyme that is normally responsible for removing the phosphate group from the regulatory myosin light chain to allow relaxation. Specifically, activated RhoA/Rho-kinase phosphorylates the myosin-binding subunit of MLCP, thereby inhibiting its enzymatic activity. Meanwhile, PKC phosphorylates the 17-kDa peptide CPI-17, converting it into a potent MLCP inhibitor. By inhibiting the phosphatase responsible for muscle relaxation, these parallel signaling pathways ensure that the myosin light chains remain phosphorylated. This sustains the

contracted state of the airways, enabling ASM to remain contracted even as intracellular Ca^{2+} levels drop to a steady-state plateau that is only slightly above baseline resting concentrations.

β_2 -agonists act as powerful bronchodilators, opening narrowed airways by targeting β_2 -adrenergic receptors ($\beta_2\text{ARs}$) which are a classic GPCR located on the surface of ASM cells [23,25,26]. Binding of β_2 -agonists to the $\beta_2\text{ARs}$ induces a conformational change in the receptor, causing the dissociation of the α -subunit of the associated stimulatory G-protein (G_s). The free G_s α -subunit then activates the transmembrane enzyme adenylyl cyclase, which catalyzes the conversion of ATP into 3'-5'-Cyclic adenosine monophosphate (cAMP). Historically, it is known that cAMP molecules bind to protein kinase A (PKA) which induces the dissociation of the catalytic and regulatory subunits from each other. The released PKA catalytic subunits then phosphorylate numerous targets in ASM cells, causing either reduced intracellular Ca^{2+} concentrations or decreased Ca^{2+} sensitivity:

- Inhibition of G_q -coupled receptor transmembrane signaling: PKA-mediated phosphorylation and inhibition of G_q -coupled receptors, $G_{\alpha q}$ or phospholipase C can reduce the production of IP3 and stop the initial mobilization of Ca^{2+} .
- Modulation of Ca^{2+} concentration: PKA may reduce the influx of extracellular Ca^{2+} by inhibiting store-operated Ca^{2+} channels on the cell membrane. It can also phosphorylate IP3R on the sarcoplasmic reticulum, which diminishes their sensitivity to IP3 and reduces further calcium release from internal stores. Furthermore, PKA facilitates the clearance of Ca^{2+} from the cytosol by promoting $\text{Ca}^{2+}/\text{Na}^+$ exchange and re-sequestration of Ca^{2+} into the sarcoplasmic reticulum via the sarco/endoplasmic reticulum Ca^{2+} -ATPase pump.

1.2.1.2. Classification of β_2 adrenergic receptor agonists according to their duration of action

β_2 -agonists are broadly classified into three main categories based on their duration of action: Short-Acting β_2 -agonists (SABAs), Long-Acting β_2 -agonists (LABAs) and Ultra-Long-Acting β_2 -agonists (ultra-LABAs) (Table 1). SABAs are characterized by a high hydrophilicity, allowing them to dissolve easily in the lung lining fluid after lung deposition and diffuse rapidly through different tissues to access the active site of $\beta_2\text{ARs}$ on the surface of ASM cells. For salbutamol sulfate (SAB), this results in an effective bronchodilation within 2 to 3 min and peak bronchodilation within 15 min of inhalation [27]. However, as it binds only weakly to the receptor and does not partition into the lipid cell

membrane to sustain local concentrations, it quickly diffuses away from the tissue [28]. Consequently, its duration of action is inherently brief, generally lasting only 3 to 6 h.

To address the limitations of SABAs and enable continuous asthma control, including overnight, LABAs were developed by modifying the molecules structure to increase their lipophilicity [26]. The introduction of two classic LABAs, salmeterol xinafoate (SAL) and formoterol fumarate (FOR), overcame this limitation by providing a prolonged duration of action over 12 h, enabling the clinical application of a twice-daily dosing regimen for the management of airway obstruction. However, while FOR still yields a rapid onset of action similar to SAB, SAL has a considerably slower onset of action [29]. The moderate lipophilicity of FOR is thought to enable its rapid transport through different tissues from the airways lumen to the surface of ASMs cells where it interacts with β_2 ARs [28,30]. SAL has a long aliphatic side chain and is therefore more lipophilic, leading to its almost complete partitioning in lipid membranes. This slows its diffusion through tissues before reaching ASMs and resulting in a slower onset of action [27]. The long duration of these drugs can be explained by two hypotheses. The "exosite" hypothesis proposes that the long aliphatic side-chain of SAL binds to a specific secondary binding site (an "exceptor" or exosite) on or near the β_2 ARs (Figure 3A) [28]. This anchoring is thought to tether the drug in place, allowing the active head to repeatedly angle on and off the active site of β_2 ARs. This could explain its prolonged 12-h duration. However, this hypothesis has been challenged by the fact that other molecules (including FOR) have this property despite lacking long side chains and by the finding that pre-treatment of ASMs with the pure aliphatic side chain of SAL failed to block its long duration [28,31]. A second hypothesis, known as the plasmalemma diffusion microkinetic model, suggests that the LABAs partition more highly into lipid membranes due to their greater lipophilicity than SABAs. This creates a local depot of these drugs in tissue from which they slowly leach out, continuously interacting with the β_2 ARs (Figure 3B) [28].

The latest advancement in the design of β_2 -agonists was the introduction of ultra-LABAs, such as indacaterol maleate (IND), vilanterol trifenate (VIL), and olodaterol hydrochloride (OLO), which can sustain the bronchodilation effect for up to 24 h [32]. This property laid the foundation for the clinical use of ultra-LABAs in a once-daily dosing regimen. The 24-h duration of ultra-LABAs is believed to be achieved through two distinct molecular mechanisms, namely the plasmalemma diffusion microkinetic model or the long residence time at β_2 ARs. The latter is more likely to be responsible for the observed long duration of

INTRODUCTION

action of OLO, as it has been proved to bind tightly to β_2 ARs, forming a specific ternary complex that stabilize this binding. This results in an exceptionally long dissociation half-life of 17.8 h [33]. However, the residence time of IND and VIL at β_2 ARs seems to be insufficient to explain their long duration. Instead, according to the diffusion microkinetic model, their extensive partitioning into the cell membrane to serve as a local depot has been considered a more rational explanation [34,35]. Interestingly, although IND and SAL have very similar lipophilicity, the longer duration of action of IND compared to SAL could be related to its higher partitioning into the lipid-raft micro-domains that are encompassing β_2 ARs (Figure 3B) [36]. Furthermore, the faster onset of action of IND compared to SAL could be driven faster membrane permeation of IND which is favored by its predominant presence as a neutral or zwitterionic form at physiological pH, rather than the predominantly cationic form of SAL.

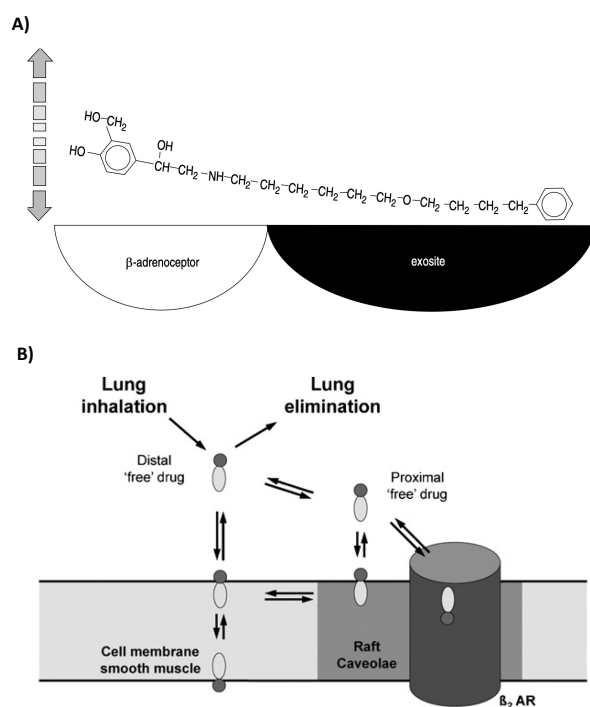


Figure 3. (A) Schematic illustration of the "exosite" hypothesis of salmeterol [28]. (B) Model to explain the pharmacological profile differences between salmeterol and indacaterol [36].

Table 1. Classification of β_2 -agonists according to their duration of action. *: still not approved for asthma treatment. **: used as a racemic mixture.

Category	β_2 -agonist	Chemical structure	LogP	Onset of action (min)	Duration of action (h) [29]	β_2 intrinsic efficacy (vs Isoprenaline)	Selectivity Ratio $\beta_2:\beta_1$ [25]
SABAs	Salbutamol** (Albuterol)		0.83 [37] (sulfate)	1.6 ± 0.3 [38]	3-6	0.47 ± 0.01 [39]	27
	Terbutaline**		1.02 [37] (sulfate)	<5 [40]	4-6	—	63
LABAs	Formoterol**		1.21 [41] (fumarate)	2.0 ± 0.3 [38]	12	0.95 ± 0.04 [35]	150
	Salmeterol**		3.08 [41] (xinafoate)	6.6 ± 0.3 [38] 8.3 ± 0.8 [35]		0.41 ± 0.01 [35]	3000
Ultra-LABAs	Indacaterol		3.65 [41] (maleate)	3.0 ± 0.2 [38]	24	0.86 ± 0.02 [35]	16
	Vilanterol		3.39 [42] (trifenatate)	3.1 ± 0.3 [35]		0.70 ± 0.03 [35]	2400
	Olodaterol*		1.84 [43] (hydrochloride)	~5 [44]		0.88 ± 0.02 [45]	65

1.2.2. Inhaled corticosteroids

1.2.2.1. Mechanism of action

ICSs exert their potent anti-inflammatory effects by crossing the cell membrane and binding to glucocorticoid receptors (GRs), specifically the GR α isoform, in the cytoplasm [43]. This binding induces a conformational change that causes GRs to detach from various chaperone proteins, such as heat shock protein 90, and to cross into the nucleus after binding to nuclear import proteins, such as importin α . Once inside the nucleus, the complex controls airway inflammation through several main mechanisms (Figure 4), including:

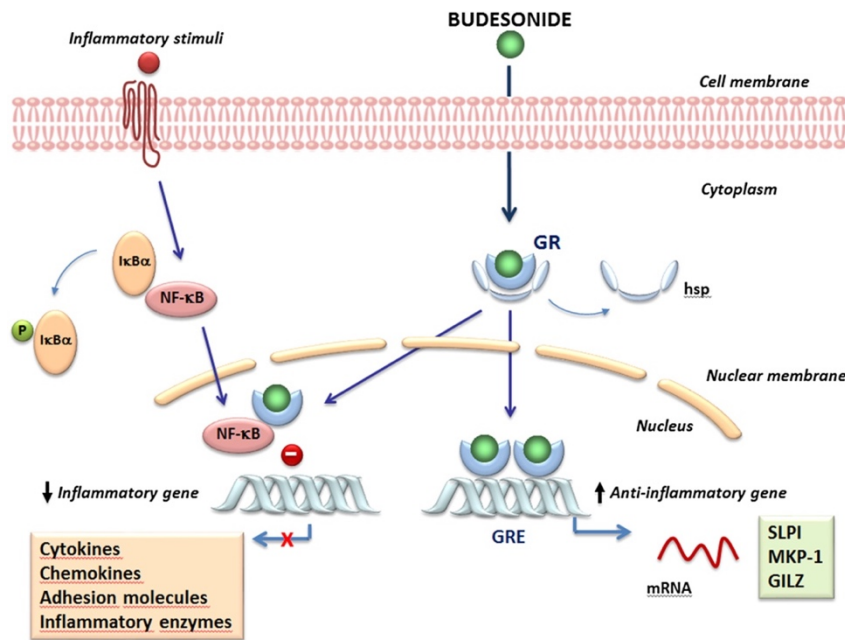


Figure 4. Mechanisms of action of corticosteroids in asthma [46].

- *Switching off inflammatory genes:* Inflammatory genes that encode for cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors are activated by pro-inflammatory transcription factors, such as nuclear factor κ B and activator protein 1 [47]. These pro-inflammatory transcription factors are activated in all inflammatory diseases and move to the nucleus to bind to specific DNA recognition sequences. This subsequently recruits massive coactivator molecules, such as cAMP-response element-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF) [48]. These coactivators function as molecular switches because they possess intrinsic histone acetyltransferase (HAT) activity.

HAT enzymes can acetylate lysine residues on the core histones, reducing their charge and allowing the chromatin structure of DNA to transform from a closed resting conformation to an activated open form. This enables RNA polymerase II to bind to DNA and initiate the transcription of multiple inflammatory genes.

In the monomer form, activated GRs in the nucleus can bind directly to the inflammatory coactivator molecules (like CBP and PCAF) to inhibit their intrinsic HAT activity, thereby reversing DNA unwinding around core histones and repressing inflammatory genes [48]. More importantly, activated GRs can recruit a crucial enzyme called Histone Deacetylase 2 (HDAC2) to the active inflammatory transcription complex. This enzyme can remove acetyl groups from the core histones, forcing DNA to wind tightly back around the histone core and physically blocking RNA polymerase II, thereby silencing the transcription of multiple inflammatory genes simultaneously. This mechanism explains why ICSs are so broadly effective in asthma without disrupting normal cellular genes that lack the aforementioned specific inflammatory coactivators.

- *Switching on anti-inflammatory genes*: This mechanism is initiated by the homodimerization of two activated GRs [48]. The resulting GR homodimer translocates to the nucleus, where it binds to specific DNA sequences in the promoter regions of steroid-responsive genes, known as Glucocorticoid Response Elements. Upon binding to the Glucocorticoid Response Elements, the homodimer recruits specialized coactivator molecules, including CBP, PCAF, and Steroid Receptor Coactivator-1, all of which possess HAT activity. These enzymes then acetylate core histones to open the chromatin at protective gene sites, initiating their transcription. Key genes induced by corticosteroids include those encoding β_2 AR, anti-inflammatory proteins, and mitogen-activated protein kinase phosphatase 1. The latter serves to inhibit mitogen-activated protein kinase (MAPK) signaling pathways.

- *Post-transcriptional effects (mRNA destabilization)*: Several inflammatory genes, such as those encoding TNF- α , IL-1 β , IL-6, and IL-8, produce mRNAs containing specific sequences in their 3'-untranslated regions known as Adenine-Uracil-rich elements [43]. Corticosteroids can stimulate the expression of zinc finger proteins, such as tristetraprolin, which bind specifically to the Adenine-Uracil-rich elements at the 3' ends of inflammatory mRNAs and mark them for rapid degradation. Simultaneously, the high level of mitogen-activated protein kinase phosphatase 1 production induced by corticosteroids can shut down the p38 MAPK pathway, which protects these inflammatory mRNAs from destruction.

These mechanisms enable ICSs to target various inflammatory cells in the respiratory tract, thereby mitigating the asthmatic inflammation. They trigger eosinophil apoptosis by suppressing the synthesis of their important survival factors like IL-3, IL-5 and granulocyte macrophage-colony stimulating factor [46,49]. ICSs also potently inhibit mast cells from releasing inflammatory mediators like IL-4, IL-6, and TNF- α [50]. ICSs not only clear out immune cells but also actively repair and alter the behavior of the structural cells that make up the respiratory tract, helping to reverse airway remodeling. At therapeutic concentrations, BUD can prevent the apoptosis of bronchial epithelial cells caused by the TGF- β -activated p38 MAPK pathway [51]. ICSs may also help restore the physical integrity of the airway barrier by promoting the redistribution of tight junction proteins [52]. Furthermore, ICSs can suppress the abnormal expansion of smooth muscle mass (hyperplasia), which translates directly into reduced AHR and limits the physical narrowing of the airway lumen [53].

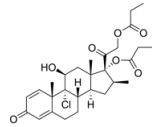
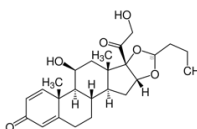
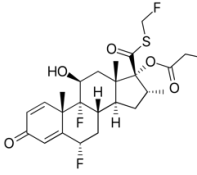
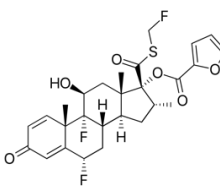
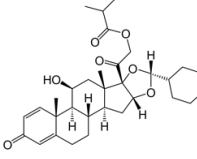
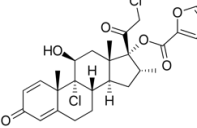
ICSs are clinically efficacious in improving lung function and preventing exacerbations in the majority of asthma patients. However, approximately 5–10% of patients respond poorly to even high doses of inhaled or systemic glucocorticoids [54]. In the context of T2-low asthma, which is frequently triggered by cigarette smoking, obesity, air pollution, and chronic respiratory infections, corticosteroid resistance is heavily driven by oxidative and nitrative stress. This environmental stress activates the phosphoinositide 3-kinase delta pathway, which subsequently phosphorylates and inactivates HDAC2 [43]. Without functional HDAC2, corticosteroid-activated GRs cannot deacetylate core histones successfully to rewind chromatin and silence inflammatory genes. Furthermore, T2-low asthma is dominated by Th1 and Th17 immune pathways, producing cytokines like IFN- γ , TNF- α , and IL-17. These specific cytokines may exacerbate resistance by phosphorylating GRs and over-activating competing pro-inflammatory transcription factors such as Activator protein 1 and nuclear factor κ B [43,54]. Conversely, corticosteroid resistance also emerges in severe T2-high asthma, where the airways are chronically filled with massive local concentrations of T2 cytokines (IL-2, IL-4, and IL-13) [43,48]. This intense cytokine milieu overactivates intracellular kinases, particularly p38 MAPK, which phosphorylate GRs, reducing their binding affinity for corticosteroids and blocking their nuclear translocation. Furthermore, alarmins such as TSLP and IL-33 can activate ILC2s together, converting them into a highly inflammatory state that is strongly resistant to corticosteroid-induced cell death [55].

1.2.2.2. Inhaled corticosteroids used in the treatment of asthma

The first ICS developed and introduced into clinical practice for asthma treatment was beclomethasone dipropionate (BDP) in the early 1970s [56]. This was a major breakthrough in asthma management, as it allowed patients with chronic asthma to successfully replace or reduce their systemic oral corticosteroids with inhaled therapy. Other ICSs currently recommended for clinical use in the 2025 GINA guideline include BUD, ciclesonide (CIC), fluticasone propionate (FP), fluticasone furoate (FF), and mometasone furoate (MF) (Table 2). While older-generation ICSs such as flunisolide (FLU) and triamcinolone acetonide were early alternatives in asthma care, they have largely been superseded and are no longer included in the current GINA guidelines [1]. This transition is likely driven by their inferior pharmacological profiles, specifically their lower GRs binding affinities and shorter durations of pulmonary retention [57]. Significantly higher oral bioavailability presents a higher risk of systemic adverse effects, making them less suitable for modern maintenance therapy.

Lipophilicity is the critical physicochemical property governing both the pharmacodynamic (PD) and pharmacokinetic (PK) profiles of ICSs used in asthma therapy. At the PD level, the lipophilicity of an ICS molecule is positively correlated with its binding affinity to GRs. Pharmaceutical evolution has intentionally introduced specific lipophilic substituents, such as acetals, esters, and halogens at various carbon positions on the basic corticosteroid ring, to strengthen this receptor binding strength [57,58]. Consequently, highly lipophilic ICSs, such as FF, MF, and FP, exhibit substantially greater intrinsic potency and anti-inflammatory capacity compared to their less lipophilic predecessors like FLU and BUD. Notably, fluticasone is available in two esterified forms: fluticasone propionate (FP) and fluticasone furoate (FF). FF is more lipophilic, has a greater affinity for GRs and a lower aqueous solubility than FP [58]. Once an ICS aerosol deposits on the airway surface, the particles must dissolve in the limited volume of the aqueous airway lining fluid before they can be taken up by epithelial cells and engage with intracellular GRs. Therefore, FF dissolves much more slowly in the airway lining fluid, remaining in the lung tissue for a significantly longer period and maintaining its anti-inflammatory effect for 24 h, which allows for once-daily dosing in clinical use.

Table 2. Inhaled corticosteroids used in asthma management and their properties.

Inhaled corticosteroid	Chemical structure	LogP [58]	GRs affinity (vs dexamethasone) [57]	Oral bioavailability (%) [58]	Inhaled half-life (h) [57]
Beclomethasone dipropionate (Beclomethasone monopropionate)		4.59 (3.27)	53 (1345)	15-20 (26-40)	(2.7)
Budesonide		2.32	935	11	2.0
Fluticasone propionate		3.89	1775	<1	14.4
Fluticasone furoate		4.17	2989	<1	23.7
Ciclesonide (desisobutyryl-ciclesonide)		3.20 (3.00)	12 (1200)	<1 (<1)	0.5 (4.8)
Mometasone furoate		4.73	2100	<1	—

INTRODUCTION

Beyond the lungs, when the drugs are swallowed into the gastrointestinal tract during the inhalation or enter the systemic circulation from the lungs, highly lipophilic drugs are more prone to systemic clearance thanks to their high protein binding rates and their high first-pass metabolism by the Cytochrome P450 3A4 in the liver [58]. This enhances their safety profile considerably compared to more hydrophilic options like BUD and FLU.

Two of the cited ICSs are prodrugs: BDP and CIC. Despite being highly lipophilic, they display very low binding affinity to GRs in their parent forms. Their metabolism by esterase enzymes produces the highly active metabolites beclomethasone-monopropionate (BMP) and desisobutyl-ciclesonide (des-CIC), which have a markedly higher affinity for GRs (Figure 5). The abundant presence of esterases in the lung epithelium, but not in the oropharynx, means that BDP and CIC are only activated when they reach the lower airways safely [57]. Once inside cells, des-CIC can undergo further reversible fatty acid conjugation to form des-CIC palmitate and des-CIC oleate, which potentially serve as a local depot steadily releasing des-CIC locally in the lungs over a prolonged period [59]. This process has also been found with BUD through the detection of BUD oleate *in vivo* in human lungs [60]. Although fatty acid esterification can be demonstrated both *in vitro* and *in vivo*, this mechanism has not been shown to provide an improved therapeutic index or a prolonged duration of action than agents that do not undergo esterification [61].

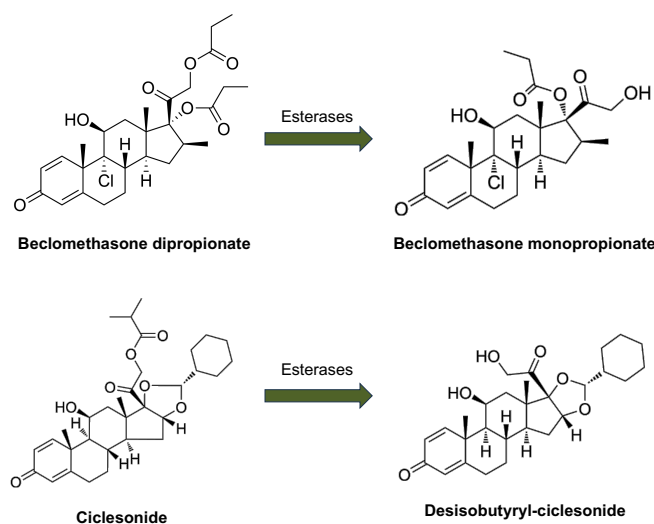


Figure 5. Metabolism of Beclomethasone dipropionate and Ciclesonide into active molecules by airways esterases.

I.2.3. Combination of inhaled corticosteroids and long-acting β_2 adrenergic receptor agonists for asthma management– Advantages and challenges

For the remainder of this thesis, three key terms must be clearly distinguished: a reliever is an inhaler that is taken as required to quickly relieve acute symptoms; a controller is any medication that actively targets current symptoms and the future risk of adverse outcomes; and maintenance treatment refers specifically to therapies that are prescribed to be taken regularly every day [1].

Until 2018, the clinical management of asthma, particularly mild asthma, relied on using low-dose ICSs as controllers and SABAs as relievers as-needed, or even using SABAs alone [62]. However, extensive research has demonstrated that the regular use of SABAs without concomitant ICSs therapy causes severe adverse effects, including increased AHR, worsened airway inflammation, and an increased risk of severe exacerbations [63]. A major reason for this phenomenon is that because SABAs provide almost immediate relief of acute symptoms, patients often mistakenly perceive these inhalers as sufficient to resolve their condition. Therefore, adherence to ICSs is poor in real life, leading to undertreatment of underlying inflammation and to an increased risk of exacerbations [63]. It should be noted that using a LABA as monotherapy is strictly contraindicated as it can exacerbate asthma and significantly increase the risk of severe exacerbations and asthma-related death [62]. Adding an ICS to a LABA therapy provides protective mechanisms that can prevent the β_2 ARs downregulation caused by long-term LABA use and mitigate cAMP-mediated inflammation [54,62]. Conversely, while ICSs are highly effective on their own, LABAs further enhances their efficacy by improving GRs nuclear translocation (steroid-sparing effect of LABA) [53]. In combination, ICSs and LABAs can synergistically inhibit ASMs cell proliferation and reduce collagen deposition, thereby actively preventing airway remodeling.

In order to prioritize patient safety, the 2019 GINA guidelines introduced a significant change: the treatment of asthma with SABAs alone is no longer recommended for adults and adolescents [63]. To bridge the gap between symptom relief and inflammation control while overcoming poor adherence to maintenance therapies, GINA fundamentally shifted the role of the ICS/LABA combination. They recommended the use of low-dose ICS-formoterol (ICS-FOR) as an as-needed therapy for mild asthma. The updated 2025 GINA guidelines continue to establish low-dose ICS-FOR as an anti-inflammatory reliever (AIR) in mild asthma and as both maintenance and reliever therapy (MART) in moderate-to-

severe asthma in Track 1 (Figure 6) [1]. This track is preferred because using ICS-FOR as a reliever significantly reduces the risk of severe exacerbations and the need for urgent health care compared with SABA-based regimens. It also favors patient compliance by simplifying the regimen to a single combined inhaler device across all steps. The alternative Track 2, which consists of maintenance controller therapy plus a SABA (or ICS-SABA) reliever, has been retained primarily because many low- and middle-income countries continue to lack affordable access to ICS-FOR inhalers [64].

Within Track 1, almost all evidence for the efficacy of AIR-only therapy (as needed in Steps 1-2) is derived from BUD-FOR [1]. For the MART applied in Steps 3 through 5, both BUD-FOR and BDP-FOR are fully approved and clinically supported. FOR is uniquely positioned for both the MART and AIR strategies because it has a rapid onset of action (1-3 min) that is comparable to that of SABAs for immediate rescue relief, as well as a prolonged 12-h duration of action [53]. Other ICS/LABA combinations cannot be used as relievers either due to a slower onset of action (e.g., ICS-SAL) or due to an absence of established safety and efficacy when taken more than once daily (e.g., ICS-VIL, ICS-IND) [1].

Among the ICSs, BUD is the primary ICS used in combinations with FOR. Because of its relatively moderate lipophilicity, BUD dissolves quickly in the airway lining fluid. This rapid dissolution allows BUD to prompt a rapid local anti-inflammatory effect within the lung tissue [57]. While a fraction of the drug is sustained locally in the airways through an intracellular esterification mechanism, the bulk of the deposited BUD is then rapidly absorbed into the systemic circulation, creating brief but relatively high plasma peaks. Researchers propose that these short pulses are highly beneficial because they temporarily suppress inflammatory precursors in the blood and bone marrow such as eosinophils and lymphocytes [65]. Furthermore, because BUD has a high systemic clearance rate and a short plasma half-life of 2 to 3 h, this transient systemic activity does not last long enough to trigger a corresponding level of severe metabolic adverse effects.

The emerging role of FOR and BUD in the preferred Track 1 of the 2025 GINA guidelines highlights a strategic clinical shift towards drugs with a balanced hydrophilicity/lipophilicity profile allowing them to rapidly dissolve and access their target sites in the lung tissue. Other ICS and LABA molecules, especially those designed for once-daily regimens due to their 24-h duration of action, continue to be used strictly for maintenance therapy in Track 2. While high lipophilicity successfully prolongs their duration of action, this property also

GINA 2025 Adults & adolescents 12+ years

Personalized asthma management
Assess, Adjust, Review
for individual patient needs

Symptoms
Exacerbations
Side-effects
Comorbidities
Lung function
Consider biomarkers
Patient (and parent/caregiver) satisfaction



Confirmation of diagnosis if necessary
Symptom control & modifiable risk factors
Comorbidities
Inhaler technique & adherence
Patient (and parent/caregiver) preferences and goals

Treatment of modifiable risk factors and comorbidities
Non-pharmacological strategies
Asthma medications including ICS
Education & skills training, action plan

TRACK 1: PREFERRED
CONTROLLER and **RELIEVER**
Using ICS-formoterol as the reliever*
reduces the risk of exacerbations
compared with using a SABA reliever,
and is a simpler regimen

STEPS 1 – 2
AIR-only*: low-dose ICS-formoterol as needed

STEP 3
MART* with
low-dose maintenance
ICS-formoterol

STEP 4
MART* with
medium-dose
maintenance
ICS-formoterol

STEP 5
Add-on LAMA
Refer for assessment of
phenotype. Consider trial
of high-dose maintenance
ICS-formoterol. Consider
anti-IgE, anti-IL5/5R,
anti-IL4Rα, anti-TSLP

RELIEVER: As-needed low-dose ICS-formoterol*

TRACK 2: Alternative
CONTROLLER and **RELIEVER**
Before considering a regimen
with SABA reliever, check if the
patient is likely to adhere to daily
controller treatment

STEP 1
Reliever only; if SABA,
take ICS with each dose

STEP 2
Low dose
maintenance ICS

STEP 3
Low dose
maintenance
ICS-LABA

STEP 4
Medium dose
maintenance
ICS-LABA

STEP 5
Add-on LAMA
Refer for assessment of
phenotype. Consider trial
of high-dose maintenance
ICS-LABA. Consider
anti-IgE, anti-IL5/5R,
anti-IL4Rα, anti-TSLP

RELIEVER: as-needed ICS-SABA*, or as-needed SABA

See GINA
severe
asthma guide

Figure 6. Asthma treatment steps in adults and adolescents in 2025 GINA guidelines [1]. Reproduced with permission from GINA.

makes them more vulnerable to elimination by physiological barriers like mucus and AMs. In an evaluation of aerosols containing different bronchodilators at 1 mM through a model of artificial mucus, the time taken for 50% of the deposited aerosols of IND ($\log P = 3.65$) to cross the mucus layer was 67 ± 11 min, far longer than that of FOR ($\log P = 1.21$) at 13 ± 3 min [66]. This physical entrapment and subsequent clearance have been demonstrated in a recent clinical evaluation of human lung PK [67]. FP dissolves very slowly and is therefore extensively retained in the airway lumen. This results in concentrations of FP in the epithelial lining fluid up to approximately 2100-fold higher than in systemic plasma. The airway to plasma ratio of FP is roughly 10 to 20 times higher than that of highly soluble drugs like SAB. Analysis of bronchoalveolar lavage cell pellets revealed that FP accumulates in these cells at much higher levels than SAB and SAL, which suggests that the phagocytosis of AMs plays a role in the uptake of poorly soluble compounds via inhalation. This kinetic vulnerability was also quantitatively demonstrated *in silico* using a mathematical model of pulmonary PK by Hartung and Borghardt [68]. Their model predicted that 28% of a deposited FP dose would be physically eliminated via the mucociliary clearance (MCC) in healthy volunteers, compared to only 6% of the BUD dose. In asthmatic patients, disease-induced airway obstruction shifts aerosol deposition towards the central airways where the MCC is most efficient. Consequently, the model predicted that FP elimination would increase up to 53%, compared to 29% for BUD. While this asthmatic clearance ratio was mathematically modeled, it aligns perfectly with findings in severe COPD patients. In a clinical trial analyzing spontaneously expectorated sputum, most of BUD in sputum was recovered within the first 2 h, whereas FP quantity continuously increased over 6 h [69]. This prolonged presence of FP in the airway lumen has been mechanistically linked to its significantly higher risk of severe pneumonia compared to BUD in COPD patients [70]. As FP persists for longer as undissolved particles in the mucus, higher local airway concentrations are achieved, where it can be taken up by AMs and impair their phagocytic function, which is essential for clearing bacterial pathogens.

Furthermore, in asthma, the slow diffusion of FP through the mucus may impair dose-response effect during severe airway obstruction [71]. PK studies indicate that during moderate-to-severe asthma exacerbations, approximately half of the inhaled FP dose is lost for clinical effect. This kinetic loss means that quadrupling the prescribed dose of FP during a severe flare-up may result in only a doubling (or less) of the dose that actually reaches the target airway receptors. In contrast, the rapid dissolution of BUD allows it to

evade this clearance, meaning a nominal increase in the inhaled dose reliably equals the amount available in the airways to counteract the exacerbation. It is important to note that escalating to medium or high doses of ICSs significantly increases the risk of major adverse cardiovascular events, pulmonary embolisms, and, hospitalizations for pneumonia [72].

Despite these significant kinetic hurdles, the continued use of highly lipophilic, ultra-long-acting ICS/LABA molecules in Track 2 remains clinically indispensable. The primary advantage of these molecules is their long duration of action which permits the once-daily dosing. Clinical evidence shows that reducing the dosing frequency from twice-daily to once-daily significantly improves patient adherence and maintains long-term treatment efficacy. For example, large-scale retrospective studies using health insurance claims data have shown that patients initiating a once-daily combination (such as FF/VIL) achieve a significantly higher proportion of days covered and have a substantially lower risk of treatment discontinuation compared to twice-daily alternatives like BUD/FOR or FP/SAL [73]. Similarly, real-world data collected via digital electronic medication monitors confirm that users of the once-daily regimen consistently maintain higher median daily adherence rates across almost all age groups in both asthma and COPD [74]. Furthermore, the once-daily dosing regimen can act as a crucial pharmacological safety net during periods of non-adherence. This protective benefit was quantitatively demonstrated using a mathematical PK/PD model that compared the bronchoprotective profiles of once-daily FF, twice-daily FP and twice-daily BUD over a 28-day period [75]. While all three drugs provide excellent airway protection under perfect 100% adherence, massive efficacy gaps emerge when patients miss doses. The model predicted that with a 50% adherence scenario, once-daily FF would maintain a clinically significant bronchoprotective effect for 75.7% of the time, while BUD and FP would reduce their protective efficacy to only 51.3% and 52.3% of the time, respectively. Under a simulated infrequent, as-needed regimen (dosing 3 to 4 times a week), FF still provided protection for 77.0% of the time, while BUD and FP decreased sharply to 26.2% and 25.5%. This superiority is likely to be driven by the prolonged GRs occupancy of highly lipophilic molecules like FF, which effectively bridges the gap between missed doses.

While combinations of ICS and ultra-LABA are a mainstay of asthma treatment, their clinical efficacy nevertheless depends on their ability to reach the target lung compartment. As their fate in the airways is strongly influenced by several physiological and immunological defense barriers specific to the lungs, it is crucial to develop advanced formulation

strategies that enable these agents to access the airway epithelium and their underlying target cells more efficiently. In this context, nanoparticles (NPs) have emerged as a versatile delivery platform and have already been used to enhance the therapeutic efficacy of ICSs via the pulmonary route [76–80]. The design strategies for NPs to achieve this improvement will be thoroughly described in the following section. If successfully implemented, the use of NPs can maximize the local therapeutic efficacy of long-acting ICS and LABA before a significant portion of the deposited dose is eliminated via the MCC. Furthermore, minimizing prolonged luminal entrapment reduces drug uptake by AMs, thereby mitigating the risk of local immunosuppression and severe pneumonia. Altogether, these strategies could potentially allow for a reduction in the administered dose. Lowering the nominal dose while maintaining high local airway efficacy would substantially decrease the amount of drug entering the systemic circulation, thereby alleviating adverse side effects in other organs. However, NPs have rarely been used to deliver an ICS/LABA combination simultaneously, which is a research gap that this study aims to address.

The ICS and LABA molecules chosen for this study are **CIC** and **IND**, respectively. The selection of CIC over other ICSs is primarily driven by its low risk of local side-effects (oral candidiasis, hoarseness, and pharyngitis), negligible oral bioavailability (< 1%), and its established approval as a once-daily monotherapy for asthma [58,81]. Complementing this, IND is an ultra-LABA characterized by a rapid onset of action and a sustained 24-hour bronchodilatory effect [38], making it an ideal candidate for this study. Crucially, the combination of CIC and IND has not yet been explored for asthma treatment. Although CIC and IND benefit from favorable pharmacological properties such as prolonged duration of action, their high lipophilicity can limit their effective delivery within the lungs by promoting retention within airway mucus and clearance by physiological defense mechanisms thereby compromising their therapeutic potential. To address these limitations, the development of formulation strategies capable of enhancing drug transport across pulmonary barriers is required. In this context, nanoparticle-based systems, and particularly liposomes, represent a promising approach to improve pulmonary drug delivery. By co-encapsulating CIC and IND within a nanocarrier-based formulation, this study seeks not only to evaluate a novel ICS/LABA therapeutic combination but also to implement an advanced delivery strategy designed to maximize the therapeutic potential of this synergy.

II. Use of liposomes for lung administration

The diversity of NPs used to develop treatments for lung diseases is reflected in the variety of materials used to construct these vesicles. These include lipid, polymer and inorganic components, which are used either individually or in combination to appropriately tailor the properties of the NPs. Of the various types of NPs, liposomes represent a highly promising nanocarrier for pulmonary delivery due to their structural and biological advantages over polymeric or inorganic materials. Their vesicular structure, featuring an aqueous core surrounded by one or more lipophilic bilayers, enables the encapsulation of both hydrophilic and lipophilic drugs. The encapsulation efficiency and release kinetics of these drugs can be tailored by rationally selecting the lipid components to tightly control the properties of the lipid bilayers. Because these components are primarily constituted of phospholipids and cholesterol (Chol), they normally exhibit remarkably low immunogenicity and toxicity [82]. The clinical viability of this approach is highlighted by the prominent example of Arikayce® (liposomal amikacin for inhalation) for the treatment of refractory nontuberculous mycobacterial lung disease. This medication was approved by the FDA in 2018 and subsequently by the EMA in 2020 as the first and currently only NPs-based formulation for inhalation. Its clinical success strongly validates the potential of lipid-based NPs to maximize local therapeutic efficacy while minimizing the systemic toxicity of inhaled molecules. This greatly encourages the development of novel inhaled liposomes containing therapeutic molecules other than antibiotics and targeting other severe respiratory conditions. The following sections serve to explain the strategies that can be applied to liposomes to help an ICS and LABA combination to overcome the lungs physiological barriers, as well as the challenges currently encountered when implementing these strategies.

II.1. General introduction to liposomes

Liposomes are spherical lipid vesicles that were first described in the mid-1960s. They have since become a highly successful and versatile class of drug delivery system [83]. Their core structure consists of one or more concentric lipid bilayers (lamellae) enclosing an internal aqueous compartment (Figure 7). Their compartmentalized structure allows them to carry a diverse range of therapeutic payloads, including hydrophilic and hydrophobic small molecules as well as proteins and genetic materials like DNA, RNA and proteins. Beyond delivering drugs to targeted tissue and cells and minimizing the drug exposure to

healthy, non-target tissues, liposomes can protect the encapsulated agents from enzymatic degradation, chemical inactivation, and rapid immune clearance.

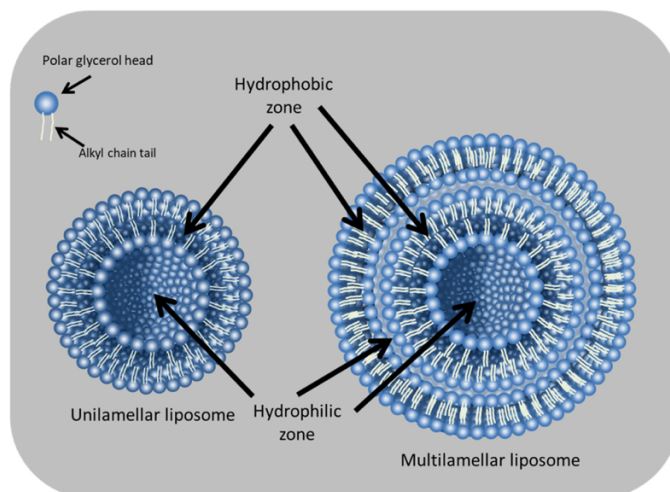


Figure 7. Structure of unilamellar and multilamellar liposomes [84].

The primary building blocks of liposomes are phospholipids which can be categorized into glycerophospholipids and sphingolipids (Figure 8) [85]. Glycerophospholipids are amphiphilic molecules containing a hydrophilic phosphate head group and a hydrophobic tail, which is typically made of two fatty acid chains. The phosphate group is often bonded to another organic molecule, such as choline or ethanolamine. This determines the lipid's specific class, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, or phosphatidylserine. Although natural phospholipids are typically derived from egg yolk or soybeans and contain mixture of phospholipids, researchers often use synthetic derivatives (such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-snglycero-3-phosphocholine (DOPC), 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine), to achieve greater chemical purity [86]. Sphingolipids, which are predominantly found in mammalian cell membranes, are also amphiphilic but comprise an 18-carbon amino-alcohol backbone called sphingosine [85]. Ceramide are formed when a fatty acid chain binds to sphingosine via an amide bond. These ceramides can then combine with a hydrophilic group, such as phosphorylcholine or a glucose molecule, to form sphingomyelin and glucosylceramide, respectively.

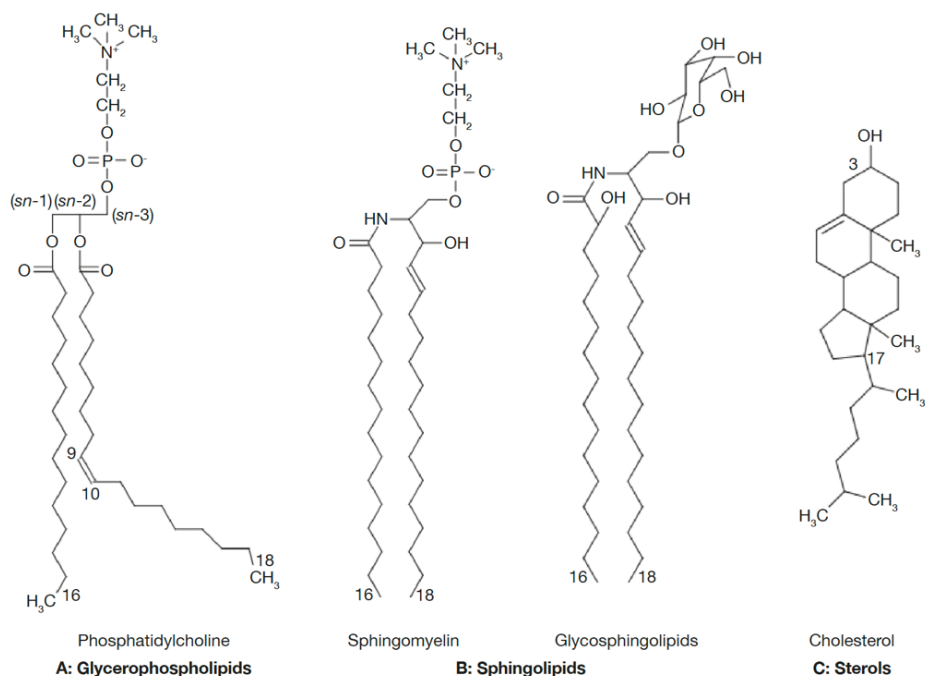


Figure 8. Chemical structure of (A) Phosphatidylcholine, (B) Sphingomyelin and Glycosphingolipids, and (C) Cholesterol [87].

The specific choice of phospholipids dictates the fundamental properties of liposomes, particularly their fluidity and permeability, which are governed by the lipid phase transition temperature (T_m). As the temperature rises, the lipid bilayer first reaches a pre-transition temperature, at which point it shifts from a rigid, tightly packed, quasi-crystalline gel (or solid-ordered) phase to an intermediate rippled gel phase (Figure 9) [82,88]. In this intermediate state, the alignment of the hydrophobic acyl chains is altered, causing the bilayer to adopt a periodically wavy or rippled physical structure. As heating continues and the temperature reaches the main T_m , the membrane ultimately melts from this rippled phase into a highly fluid and permeable "liquid-crystalline" (or liquid-disordered) phase. Because longer hydrocarbon chains can interact more strongly with one another, they increase the T_m . Conversely, introducing double bonds (unsaturation) creates "kinks" in the lipid tails that disrupt tight packing, thereby lowering the T_m . Researchers exploit this phenomenon to engineer temperature-sensitive liposomes that remain stable in the blood but release their therapeutic payload rapidly when heat is applied externally to a target tissue [89].

INTRODUCTION

Interestingly, the phospholipid components of liposomes can themselves exert intrinsic therapeutic effects. For example, empty DOPC liposomes attenuate *in vitro* TNF- α -induced IL-6 release in epithelial cells, reduce AHR, and decrease eosinophil infiltration in asthmatic models [90]. Similarly, DPPC can act as an immunomodulator that directly inhibits LPS-induced cytokine release from innate immune cells [91]. Furthermore, SPC selectively inhibits TLR4-mediated inflammatory signaling in vascular cells [92]. By leveraging these intrinsic biological activities, liposomes have the potential to act synergistically with encapsulated ICSs and LABAs to maximize the resolution of asthmatic airway inflammation.

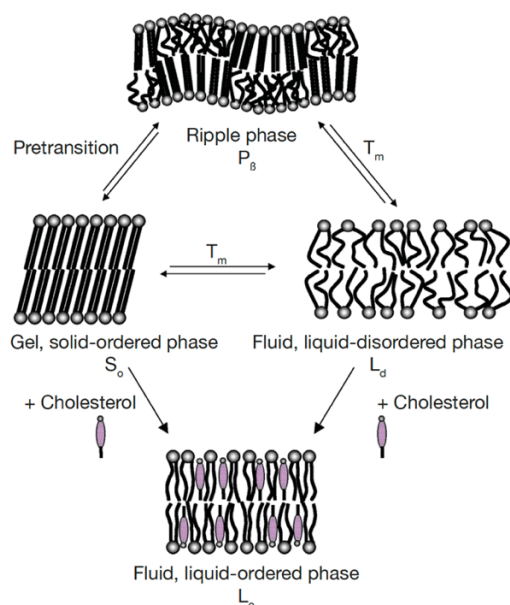


Figure 9. Different physical states adopted by a lipid bilayer in an aqueous medium [87].

Chol is another critical structural component that is extensively incorporated into liposomal formulations to modulate the properties of the lipid bilayer. It is an amphiphilic sterol that embeds itself within the phospholipid bilayer. Its rigid steroid ring and flexible hydrocarbon tail extend deeply into the hydrophobic core, maximizing van der Waals interactions with the fatty acyl chains [93,94]. Simultaneously, its polar hydroxyl group orients towards the aqueous interface, where it forms hydrogen bonds with the carbonyl and phosphate groups of the phospholipids. There are also weak charge pairs formed between its negatively charged oxygen atom and the positively charged choline methyl groups of the phospholipid

heads. Chol modulates membrane fluidity depending on the existing physical state of the host lipids (Figure 9). In gel-phase membranes (like pure DPPC below its T_m), Chol molecules disrupt the regular crystallization of the saturated chains, introducing disorder and increasing fluidity [95]. Conversely, in liquid-crystalline membranes (like POPC or DPPC above its T_m), Chol exerts a strong condensing effect by minimizing the area per lipid molecule and restricting the rotational mobility and wobbling diffusion of the acyl chains, thus making the bilayer much more rigid and ordered [94]. At high concentrations (e.g., 30–50 mol%), it induces a distinct “liquid-ordered” phase providing a unique balance of high lateral mobility and high conformational order. In this specific state, the lipid acyl chains are highly extended and tightly packed like a solid gel, however the molecules retain the mobility to move freely across the membrane [95,96].

Initially, conventional liposomes were simply formulated using natural or synthetic phospholipids and Chol. However, as soon as they enter a physiological environment such as bloodstream, plasma opsonin proteins quickly bind to their surface [83]. This process known as opsonization which tags the vesicles for destruction, causing them to be rapidly engulfed and cleared by the macrophages of the mononuclear phagocyte system. Consequently, the half-life of these first generation of liposomes in the bloodstream is very short, severely limiting their ability to deliver drugs effectively to target tissues like tumors [97]. To address the issue of rapid clearance, researchers developed a strategy to create a protective shield around the vesicle by coating liposomes surface with hydrophilic polymers [82]. This surface modification led to the development of a second generation of carriers, known as “stealth” or “long-circulating” liposomes.

Among various lipid-polymer conjugates explored for this purpose, PEGylated lipids, which are lipids covalently conjugated to poly(ethylene glycol) (PEG), are the most extensively used. PEG is overwhelmingly preferred because it is a non-toxic, highly water-soluble, and chemically inert synthetic polymer and is recognized as a GRAS (generally regarded as safe) polymer [98]. When PEGylated lipids are incorporated into the liposomes, the PEG chains extend outward from the lipid membrane into the aqueous environment, forming a “hydration cloud” around the vesicles. This hydration layer acts as a robust steric and entropic barrier that prevents self-aggregation and effectively repels the adhesion of opsonizing plasma proteins [99]. By preventing these proteins from binding, PEGylated lipids considerably reduce the immunogenicity and the uptake by macrophages, thereby

extending their circulation time in the bloodstream from mere minutes to several hours or even days [88].

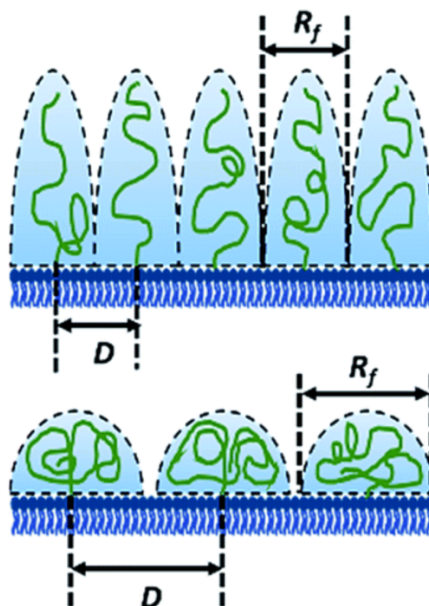


Figure 10. Brush-shaped (top) and mushroom-shaped (bottom) configurations of the PEG layer [100].

Notably, the structural conformation of the PEG layer significantly impacts both its shielding capabilities. This conformation is dictated by the surface grafting density of the PEGylated lipids and the PEG molecular weight. This can be expressed as the ratio between the polymer Flory radius (R_F , the size of the unperturbed polymer coil) to the average distance between adjacent PEG attachment points (D) (Figure 10) [98]. At low grafting densities (typically below 4–5 mol% for PEG₂₀₀₀), the distance between adjacent PEG attachment points is greater than the polymer Flory radius ($D > R_F$) [100]. In this state, neighbouring PEG chains do not interact laterally. Instead, they coil into independent, random half-spheres, forming the "mushroom" configuration. As these mushrooms do not overlap, there are exposed gaps on the liposome surface, meaning the underlying lipid bilayer is only partially shielded from interacting with blood proteins and immune cells. At these low concentrations, the PEG moieties do not significantly alter the cohesion of the lipids within the bilayer [96]. However, as the concentration of PEGylated lipids increases, the distance between attachment points becomes smaller than the Flory radius ($D < R_F$). In order to avoid overlap, the neighbouring PEG chains exert strong lateral steric repulsion against each other. This forces the polymers to uncoil and extend straight outwards from the

surface, forming a dense "brush" configuration [100]. This brush provides a continuous, highly effective steric shield that thoroughly masks the liposomes from opsonization and clearance.

II.2. Liposomes for lung administration

Pulmonary drug delivery is a highly attractive, non-invasive route for administering both local and systemic therapies. This approach offers a massive absorptive surface area, rapid systemic entry, avoidance of hepatic first-pass metabolism, and maximized local drug concentrations with minimal systemic side effects.

The value of liposomes lies not only in their vesicular structure, but above all in the ability to finely tune their composition in order to optimize their biological and biopharmaceutical behaviour. While liposomes represent highly promising nanocarriers for pulmonary drug delivery, their successful application relies on the ability to overcome a set of complex and interdependent challenges inherent to the respiratory environment. Indeed, once deposited in the lungs, liposomes must navigate through multiple biological barriers, including the airway mucus layer, the presence of pulmonary surfactant components, and the activity of immune cells such as AMs. In parallel, their physicochemical properties must be carefully engineered to ensure efficient transport, adequate interaction with target cells, and optimal drug retention.

Furthermore, the constraints associated with the route of administration, particularly aerosolization and lung deposition, impose additional requirements on formulation stability and performance.

Beyond these delivery-specific constraints, the transition toward a commercial product introduces substantial hurdles regarding large-scale manufacturing. Ultimately, achieving industrial scalability and batch-to-batch reproducibility remains a critical bottleneck for translating these liposomal systems into clinical applications.

II.2.1. Pulmonary administration: anatomical foundations and physiological barriers

Because the lung has a complex architecture, deposition in different regions, ranging from the mucus-lined main airways down to the surfactant-coated alveolar epithelium, determines specific and diverse interactions of drug with local biological components. The final fate of the inhaled drug, whether it achieves targeted delivery or is rapidly cleared by robust defense mechanisms like the MCC and AMs phagocytosis, is therefore entirely dependent on the unique microenvironment it first encounters.

II.2.1.1. Basic anatomical structure of the airway epithelium

The lungs are the main organ of the respiratory system. They have a highly intricate structure that enables them to conduct air in and out of the body and to exchange air between blood and the airways. Their structure is divided into 2 functional regions: the conducting airways and the respiratory area (Figure 11). Typically, drugs are deposited in the trachea in the conducting airways and then in the deeper parts until the respiratory regions. The trachea bifurcates into the left and right bronchi [101]. Each bronchus enters the lung at the hilum and continues to divide into lobar (secondary) bronchial branches which again ramify into segmental (tertiary) bronchi. Terminal bronchioles mark the ultimate division of the airway devoid of respiratory surfaces [102]. The respiratory region begins at the respiratory bronchioles, which have a structure very similar to that of the terminal bronchioles, except for the presence of occasional sac-like alveoli [102–104]. The respiratory bronchioles branch into alveolar ducts and alveolar sac, before finally becoming alveoli.

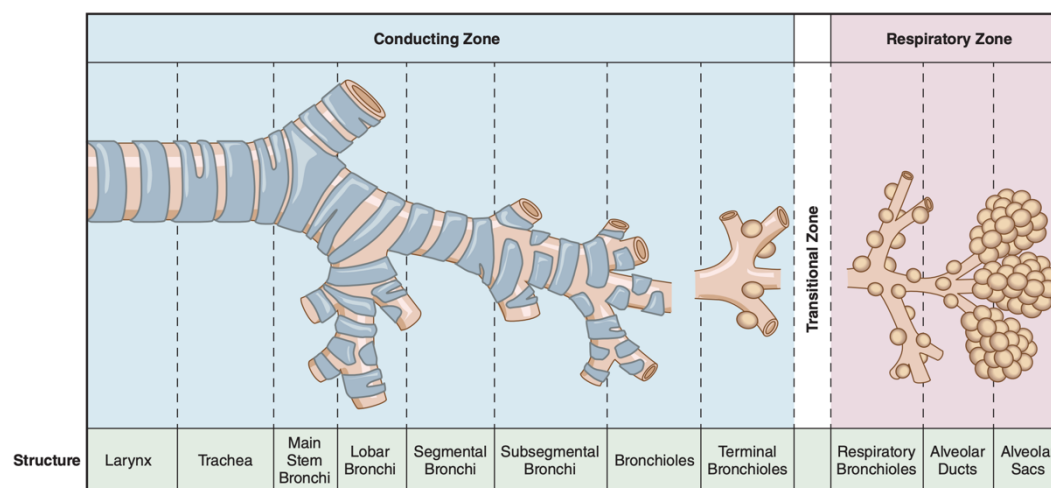


Figure 11. Main generations of the lower airway [105].

The lumen of the trachea and bronchi is lined with a pseudostratified columnar epithelium consisting in three major cell types: ciliated cells, non-ciliated secretory cells, and basal cells (Figure 12). Between the epithelium and the respiratory gases, there is a continuous film of liquid constituted of a watery periciliary solution (5-10 μm) situated between the densely packed cilia and an overlying layer of mucus [106,107]. The primary biological roles of the mucus include lubricating epithelial surfaces, humidifying inspired air,

preventing tissue dehydration, and acting as a highly selective barrier against environmental threats [108]. In the trachea and large bronchi, mucus is predominantly secreted by the seromucinous tracheal glands, which are in the connective tissue between the epithelial layer and the cartilage. It is also secreted by goblet cells, which are one of the secretory cell types [109,110]. The low viscosity of the periciliary layer enables the cilia to beat and propel the mucus towards the larynx, facilitating the removal of xenobiotics retained inside the mucus from the body. This process is termed MCC. As the bronchi branch into bronchioles, the epithelium gradually transitions from a pseudostratified morphology to a simple cuboidal layer. Additionally, there is a progressive reduction in the number of ciliated, goblet, basal cells as well as seromucous glands [111]. The number of goblet cells gradually declines to reach zero by the terminal bronchioles where fewer ciliated cells and more non ciliated Clara cells are present (now known as Club cells) [112]. It has been reported that Club cells provide an additional barrier against toxic vapors, as well as the defense against inhaled particles provided by the mucus in the larger airways [113].

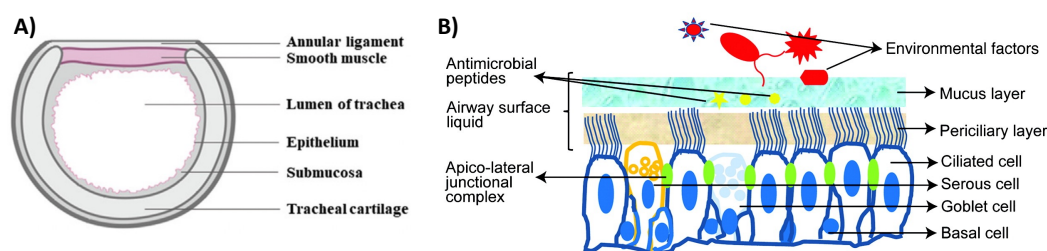


Figure 12. (A) Cross-sectional view of the trachea [114] and (B) structure of the tracheobronchial epithelium [115].

The alveolar epithelium is completely covered by an alveolar lining layer, characterized by a surfactant-rich film (0.1-0.2 μm) at the air-aqueous interface of a lower aqueous hypophase (Figure 13) [116,117]. The presence of pulmonary surfactant (PS) lowers surface tension at the air-liquid interface of alveolar tissue, thereby preventing alveolar collapse during expiration and minimizing the energy needed for inhalation [118]. Within the alveolar epithelium, two primary cell types predominate: alveolar type I and II epithelial cells (AT1 and AT2). Although AT1 cells account for only 10% of alveolar cells, they cover more than 90% of the alveolar surface due to their broad, flat shape [119]. Their broad surface coverage combined with a thickness of 0.1 μm results in a structural lining with a very short diffusion distance for gas exchange with the underlying endothelium [120]. On

INTRODUCTION

the other hand, the cuboidal AT2 cells cover only 5% of the alveolar surface and are progenitors of the AT1. Importantly, they contribute to alveolar homeostasis by secreting surfactant lipids and surfactant proteins [121,122]. Another important cell line in this region is the AMs within the alveolar space. They represent over 90% of the pulmonary macrophages alongside bronchial macrophages and interstitial macrophages [123,124]. Their main functions are to phagocytose and remove pathogens, pollutants, and dying cells and to help initiate and resolve of the immune response in the lung [125].

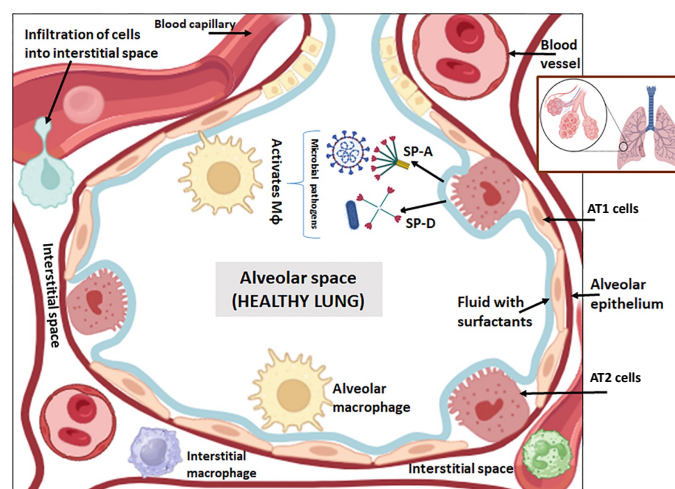


Figure 13. Structure of the alveolar epithelium [126].

II.2.1.2. Physiological barriers

As the lungs must continuously move ambient air into intimate contact with blood for gas exchange, human airways are constantly exposed to airborne particles of which many may be harmful to the respiratory system (dusts, bacteria, viruses, pollutants...). In order to protect them from pathogenic agents, the lungs have robust, natural defense mechanisms which operate in two highly coordinated phases: mechanical filtration during deposition, followed by biological clearance.

First, the complex, repeatedly branching architecture of the airways acts as an anatomical filter designed to actively trap inhaled particles [127]. Large particles travelling at high airflow velocities in the upper respiratory tract possess too much momentum to alter their trajectories when the air stream changes direction at airway bifurcations [128]. Consequently, they are efficiently eliminated early on by colliding with the upper airway

walls through a mechanism called inertial impaction. However, particles that are small enough to evade this initial impaction travel deeper into the lower airways and alveolar regions. As the total cross-sectional area of the lungs increases systematically in these deeper zones, the airflow velocity drops to nearly zero in the peripheral airways [127]. Because the air moves so slowly here, the remaining particles are ultimately forced to touch the airway surfaces through gravitational sedimentation (settling slowly onto the walls under the influence of gravity) and Brownian diffusion (the random movement of ultrafine particles) [128].

While optimizing the aerodynamic properties of drugs to successfully bypass this first anatomical filter is a major pharmaceutical challenge, this thesis focuses on the natural barriers that act after deposition.

Once a particle touches the pulmonary epithelium, it is recognized as a foreign entity. Its final fate and therapeutic efficacy are then entirely dictated by its interactions with secondary physical and immune defenses. These include entrapment by airway mucus and the MCC in the conducting airways, complex interactions with the PS and phagocytosis by the resident AMs in the deep lung.

II.2.1.2.1. Mucociliary clearance

The microscopic architecture of the airway mucus can be described by the "gel-on-brush" model. This consists of two distinct interacting layers: an overlying viscoelastic mucus gel layer that traps inhaled debris and a lubricating periciliary layer underneath (Figure 14A) [129]. The thickness of the mucus layer varies significantly depending on the anatomical location within the airways and the presence of pathological conditions. In healthy human airways, the mucus layer is generally estimated to range from 10 to 30 μm in the trachea, gradually decreasing to approximately 2 to 5 μm in the distal bronchi and bronchioles [108].

As mentioned above, foreign particles, allergens and pathogens can be moved from the airways by the MCC through a combination of entrapment within the mucus layer and the free movement of cilia. During the power stroke, the ciliary tips engage with and propel the mucus layer forward towards the pharynx to be swallowed or expectorated, while the recovery stroke occurs within the low-viscosity periciliary layer to prevent the mucus from being dragged backward [130]. In healthy subjects, ciliary motion occurs at an average flow rate of about 5 mm/min [131]. Because of this continuous and rapid transport, the luminal

INTRODUCTION

mucus layer has a short lifetime, giving to the "self-renewing" of the mucus layer approximately every 10 to 20 min [98].

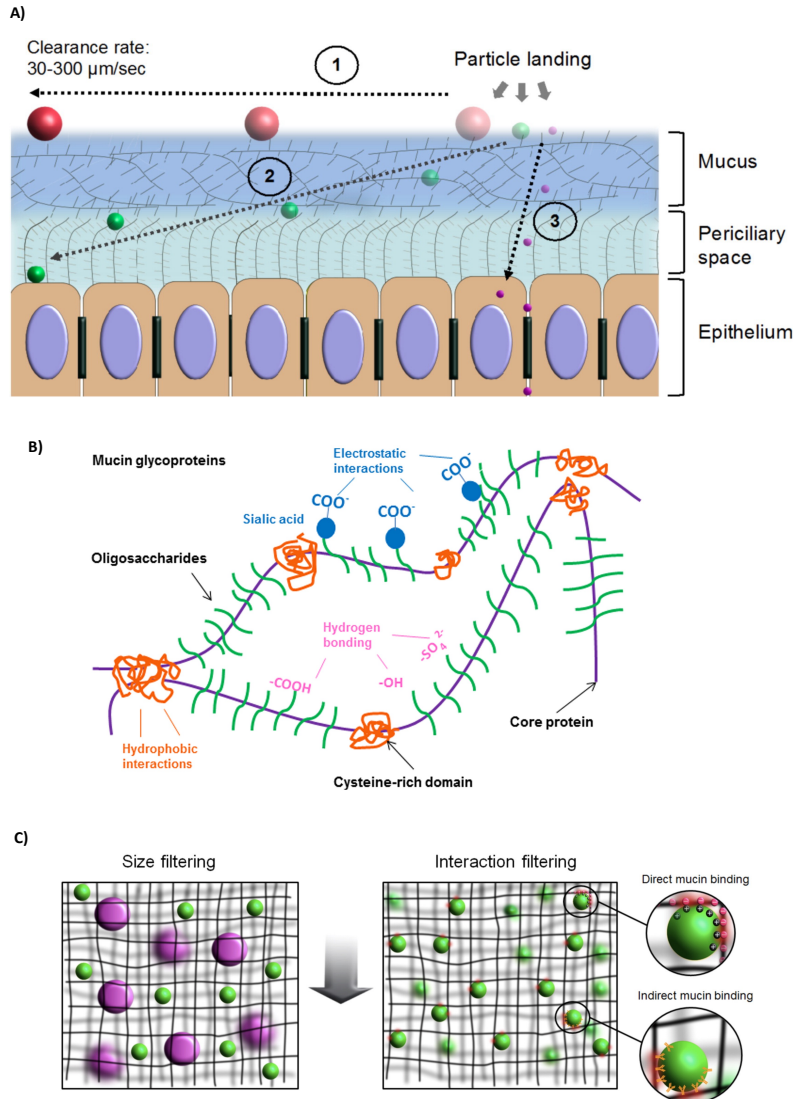


Figure 14. Schematic representation of (A) the mucociliary clearance, (B) the structure of mucin glycoproteins, and (C) the filtering properties of the mucus barrier [132,133].

Although water accounts for roughly 95% of the mucus total mass, its unique barrier properties and rheological behavior are strongly related to the structure and the arrangement of its solid components, particularly mucins [132]. Beside water and mucins ($\sim 2\text{-}5\%$, w/v), mucus also contains inorganic salts ($\sim 1\%$), lipids, extracellular DNA, non-mucin globular proteins (such as lysozyme, lactoferrin, and immunoglobulins), cellular

debris and trapped bacteria. Mucins are enormous, heavily O-glycosylated polymeric proteins with polypeptide backbones densely coated by oligosaccharide chains accounting for up to 80% of their dry weight (Figure 14B) [98]. These glycoproteins can establish a dense, three-dimensional mesh network. This mesh depends on the intermolecular cross-linking of mucin molecules via hydrophobic naked globular domains alongside the flanks of the polypeptide backbone. These domains are rich in cysteine, enabling the formation of multiple inter- and intramolecular disulfide bonds, which are responsible for the structural stabilization and polymerization of the mesh.

This mesh-like structure enables particles to penetrate the mucus at different rates, a process known as 'size-filtering' (Figure 14C). The mucin network contains heterogeneous pores, typically averaging between 100 and 500 nm in healthy airway mucus [98,132]. These pores physically impede the diffusion of large particles, bacteria, and macromolecules that exceed the mesh spacing, trapping them in the mucus for subsequent clearance. In addition to size exclusion, foreign particles can be retained inside the mucus through several kinds of adhesive interactions with different sites in the mucins structure, a process known as "interaction filtering" [98]. These include electrostatic interactions, which are heavily influenced by the negatively charged sialic acid and ester sulfate groups that terminate the glycan chains on the mucin brush. Due to this polyanionic nature, positively charged particles and molecules frequently bind tightly to the mucin network. Furthermore, the mesh utilizes hydrophobic interactions with periodic repeats of hydrophobic globular domains located along the mucin polypeptide backbone, which readily bind lipophilic particles and drugs. Hydrogen bonding between particle surfaces and the dense glycan coating of mucins further contributes to this chemical interaction filter.

The delicate balance of mucus hydration and composition is critically disrupted in pathological conditions such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), non-CF bronchiectasis, and severe asthma [134]. In these diseases, defects in epithelial ion transport, mucin hypersecretion or airway dehydration lead to hyperconcentrated mucus with a significantly higher percentage of organic solids. This dramatically increases the viscoelasticity and osmotic pressure of the mucus layer, compressing the underlying periciliary layer and reducing the mucin mesh pore size to less than 100 nm [134,135]. Furthermore, the accumulation of extracellular DNA and actin filaments from necrotic inflammatory cells (like neutrophils) heavily cross-links the mucus, stiffening the gel [132]. These rheological and structural changes can ultimately overwhelm

ciliary beating, abolishing the MCC and leading to the formation of static, adherent mucus plaques and plugs that obstruct airflow and provide a persistent nidus for chronic bacterial infection and inflammation [134].

II.2.1.2.2. Action of alveolar macrophages

AMs are the most abundant innate immune cells residing in the distal lung parenchyma. They are located on the luminal surface of the alveolar space [125]. Unlike many other macrophage populations that rely on constant replenishment from circulating bone marrow-derived blood monocytes, it is now well established that resident AMs are mainly formed prenatally from embryonic precursors, such as fetal monocytes derived from the yolk sac and fetal liver [136]. Once they colonize the alveolar niche during the first week of life, they become long-lived cells capable of maintaining their own population throughout adulthood via local, low-grade proliferation without the need for input from circulating adult monocytes.

In a healthy lung, AMs perform vital non-immune, tissue-specific homeostatic functions. A primary protective role involves the continuous internalization and catabolism of PS. If AMs fail to clear the PS, lipids and proteins accumulate in the airspaces, leading to a life-threatening condition known as pulmonary alveolar proteinosis [125]. Furthermore, AMs act as diligent scavengers of the alveoli, efficiently phagocytosing microbes, dead cells and other airborne particles to prevent unnecessary inflammation. Remarkably, AMs execute this phagocytic clearance in an immunologically "silent" manner, concealing and neutralizing foreign material without triggering an influx of neutrophils or provoking excessive inflammation that could irreversibly damage the extremely thin and delicate alveolar capillary membranes required for gas exchange [137].

To preserve this delicate microenvironment, AMs are maintained in a highly regulated, immunosuppressive state by continuous cross-talk with the surrounding respiratory epithelium. Alveolar epithelial cells constitutively express inhibitory ligands, such as Cluster of differentiation 200, which binds to Cluster of differentiation 200 receptors highly expressed on AMs, delivering negative signals that restrict pro-inflammatory pathways [138]. Similarly, PS proteins A and D (SP-A and SP-D) can bind to AMs to tonically suppress unnecessary phagocytic and inflammatory responses. Under these steady-state conditions, AMs actively prevent other immune cells from overreacting to harmless inhaled antigens and releasing a variety of anti-inflammatory mediators, which helps to resolve any minor inflammation and protect the fragile lung tissue from excessive, self-inflicted damage.

II.2.1.2.3. Action of pulmonary surfactant

PS is a complex mixture of surface-active lipoprotein that covers the entire alveolar epithelium. It forms a critical interface between inhaled air and the aqueous alveolar lining fluid. It is primarily secreted via exocytosis by AT2, although specific protein components are also produced by club cells in terminal bronchioles [122]. PS is structurally composed of approximately 90% lipids and 10% specific surfactant-associated proteins [139]. The lipid fraction is heavily dominated by phospholipids, accounting for 80-90% of the total lipid mass, alongside neutral lipids such as Chol [122]. The most abundant and functionally critical phospholipid is DPPC, a fully saturated lipid that constitutes roughly 40-50% of the total surfactant mass. DPPC possesses straight, saturated acyl chains that allow it to pack tightly at the air-liquid interface during exhalation, enabling the surfactant film to reduce surface tension to near-zero values. Other vital lipids include unsaturated phosphatidylcholines and anionic phospholipids, like phosphatidylglycerol and phosphatidylinositol, which maintain the fluidity of the surface film at body temperature and interact directly with surfactant proteins.

The remaining 8-10% protein fraction consists of four specific surfactant proteins (SP) that are divided into two distinct functional categories: the small, hydrophobic proteins (SP-B and SP-C) and the large, hydrophilic proteins (SP-A and SP-D). SP-B and SP-C facilitate the rapid transfer, adsorption, and spreading of phospholipids from secreted lamellar bodies into the interfacial monolayer, while also stabilizing the lipid layers during consecutive breathing cycles [140,141]. Conversely, SP-A and SP-D can help to recognize, bind, and agglutinate a wide variety of inhaled bacteria, viruses and fungi, specifically marking these pathogens for phagocytosis and clearance by AMs [118].

II.2.2. Engineering of liposomes to overcome physiological barriers in the lungs

II.2.2.1. Engineering of liposomes to overcome mucociliary clearance

Airway mucus restricts the transport of foreign NPs through the 'size filtering' and 'interaction filtering' mechanisms. To overcome the "size filtering" mechanism, NPs must be formulated to be smaller than the mucus mesh pores. For instance, nanospheres measuring 560 nm were found to be almost completely blocked from passing through CF sputum [139]. Even when coated with a dense, muco-inert PEG layer, particles around 500 nm in diameter remain sterically immobilized or experience strongly hindered transport in the respiratory mucus [141]. In contrast, PEGylated NPs sized between 100 nm and 200

nm exhibit rapid diffusion through native airway mucus and CF sputum [135]. Therefore, maintaining a particle size well below 200 - 300 nm is generally required to ensure that NPs are small enough to slip through the pores of the mucin meshwork [142].

However, size alone is not enough. Mucins possess glycosylated regions with high sialic acid and sulfate content that impart a strong negative charge, as well as hydrophobic globular domains that can readily trap particles via hydrophobic interactions. Formulating NPs with neutral or slightly negative surface charges has been shown to limit these electrostatic interactions with mucins, thereby improving their mucopenetration [143,144]. The encapsulation of the highly positively charged aminoglycoside, amikacin, within zwitterionic liposomes (such as the clinically used formulation Arikayce®) successfully demonstrated this concept. By shielding the drug positive charge within a lipid bilayer, these liposomes enhance mobility inside the mucus and achieve rapid penetration into non-tuberculous mycobacterial biofilms and infected macrophages [145,146].

Another highly efficient strategy to create mucus-penetrating particles is the PEGylation [98]. The hydrophilic shield created by the PEG layer effectively masks the underlying hydrophobic and charged domains of the carrier, neutralizing electrostatic and hydrophobic adhesive interactions with mucin fibers. The success of the PEGylation strategy in mediating mucus penetration depends on two critical biophysical parameters:

- **High grafting PEG density** (the "dense brush") (Figure 10): to completely shield the particle core, PEG must be grafted at a sufficiently high density [147]. At low densities, PEG chains collapse into a sparse "mushroom" conformation, leaving fragments of the hydrophobic NPs surface exposed to mucins. This results in rapid immobilization. Conversely, a high grafting density forces the flexible PEG chains to extend outward into a dense "brush" shape, which is absolutely required to confer muco-inert properties and slip past the mucin fibers.

- **Optimal PEG MW**: generally, low MW PEG (~ 2 - 5 kDa) is preferred for pulmonary mucus-penetrating particles [147]. Studies have demonstrated that while dense 2 kDa and 5 kDa PEG coating allows polymeric NPs to diffuse rapidly through mucus, higher MW PEG chains (e.g., 10 kDa and above) can physically entangle with the mucin fibers, rendering the particles mucoadhesive via interpenetrating network effects [98]. Interestingly, recent advancements in lipid nanoparticles (LNPs) for nucleic acid delivery have shown that using a mixture of different PEG species (rather than a single PEG

species) can improve LNPs diffusion in highly viscous CF mucus by up to 4-fold while maintaining robust transfection into CF human bronchial epithelial cells [148].

An alternative approach is to physically modify the mucus barrier itself using mucolytic agents. Administering NPs alongside chemical mucolytics like N-acetylcysteine, which cleaves the disulfide bonds linking mucin oligomers, or enzymatic mucolytics such as recombinant human DNase, which hydrolyzes accumulated DNA in diseased sputum, can reduce mucus viscoelasticity and enlarge the mesh pore size [142]. However, while mucolytics can improve the penetration of NPs, widespread alteration of the mucosal barrier may also compromise its natural protective function against viruses and bacteria [132].

II.2.2.2. Engineering of liposomes to overcome the phagocytosis by alveolar macrophages

Firstly, it is important to clarify that AMs are not always considered a barrier to be overcome in the lung delivery of NPs. Given their critical role in many pathological situations, such as providing a refuge for intracellular infections (like tuberculosis and Human immunodeficiency virus) and driving disease progression in inflammation and lung fibrosis, various nanoparticulate platforms have been developed to specifically target therapeutic agents to these cells [91,143,146,149]. However, in the context of this study in which we aim to target an ICS to the airway epithelium and underlying immune cells, and a LABA to ASMs cells, AMs-mediated clearance becomes a significant delivery barrier. This clearance mechanism significantly reduces the therapeutic dose that reaches the intended targets and drastically shortens the drugs residence time in the lungs [150].

The engulfment of NPs by AMs depends on a complex interplay of biophysical and biochemical properties:

- **Opsonization and the surfactant corona:** When NPs deposit in the deep lung, they immediately encounter the alveolar lining fluid and its PS. Surfactant lipids and proteins rapidly adsorb onto the particles surface, forming a biomolecular corona. The hydrophilic surfactant proteins SP-A and SP-D act as potent opsonins when they bind to NPs, interacting with specific immune receptors on AMs membrane, effectively marking the particles for rapid phagocytosis and clearance [151].
- **Particles size:** AMs exhibit strong size-dependency when recognizing targets. They are highly efficient at phagocytosing particles in the 1 to 6 μm range, with peak activity directed

at particles around 2 to 3 μm in size which closely mimics most naturally occurring bacteria [150,152]. Conversely, NPs smaller than 200 nm or larger than 6 μm are much less efficiently internalized by AMs.

- **Particle geometry and shape:** The physical shape of particles significantly affects their interaction with the AMs membrane. Spherical particles provide a highly favorable spatial orientation for membrane wrapping and internalization [150]. In contrast, particles with high aspect ratios or high surface curvatures are more difficult for AMs to engulf [142].

- **Surface charge and hydrophobicity:** AMs preferentially phagocytose particles bearing a highly negative or positive surface charge compared to electrically neutral ones [150]. Furthermore, highly hydrophobic surfaces promote the rapid adsorption of opsonizing proteins, which subsequently trigger macrophage recognition and uptake [142].

To maximize therapeutic efficacy and circumvent this AMs-mediated clearance, researchers have developed a variety of sophisticated bioengineering strategies. Focusing on spherical NPs and formulating them to maintain a size below 200 nm enable them to largely evade the 1 to 6 μm size-detection threshold of AMs [142]. More importantly, the PEGylation of NPs is widely used to bypass the rapid opsonization by PS proteins which is a key process of AMs phagocytosis. The presence of a sufficiently high amount of PEG provides the steric hindrance, which effectively limits the adsorption of opsonized proteins and hides them from AMs immune receptors [98]. Kaneko *et al.* showed that the addition of 9.1 mol% DSPE-PEG₂₀₀₀ to liposomes composed of hydrogenated Egg PC, Chol and dicetylphosphate significantly reduced the uptake of aerosolized liposomes by AMs. This was probably due to the reduced adsorption of proteins in the alveolar epithelial lining fluid to the surface of the liposomes. The presence of these liposomes in this compartment of rats was also more sustained than those without DSPE-PEG₂₀₀₀ [153]. Using similar liposomes containing 10% DSPE-PEG₂₀₀₀ in a mouse model of bleomycin-induced pulmonary fibrosis, Togami *et al.* confirmed that PEGylated liposomes exhibited a prolonged distribution on the alveolar surface by avoiding AMs uptake. However, their accumulation in lung fibroblasts and alveolar epithelial cells was equivalent to that of non-PEGylated liposomes [154]. Recently, a study investigated the effect of different polymer chains of DSPE-PEG and their ratio with other phospholipids like DOPC, DOTAP, and DPPG to optimize a stealth liposomal formulation [155]. This carrier was designed to deliver an antifibrotic peptide to subepithelial myofibroblasts by overcoming the mucus-like matrix of the alveolar edema fluid and AMs phagocytosis. *In vivo* experiments confirmed that the

primary benefit of the optimal formulation composed of DOPC:DSPE-PEG₂₀₀₀ (91.5:8.5, molar ratio) is evading AMs phagocytosis. When pulmonary macrophages were experimentally depleted in mice, the massive delivery advantage of the PEGylated liposomes over non-PEGylated ones diminished, proving the stealth function of the PEG layer. However, a lower concentration of DSPE-PEG₂₀₀₀ of around 3.7 mol% in a formulation with hydrogenated SPC and Chol was insufficient to protect liposomes from AMs uptake in healthy lungs of rats [156]. AMs uptake of liposomes becomes even more aggressive in a chronic bleomycin model of lung inflammation due to elevated AMs numbers.

The PEGylation was already applied to precisely tune the surface hydrophilicity of liposomes, dictating their PK destiny and maximizing the retention of encapsulated BUD in the lungs [76]. Researchers adjusted the surface hydrophilicity of liposomes by setting the weight ratios of SPC to DSPE-PEG₂₀₀₀ at 10:2, 10:4, and 10:8 to formulate the low, intermediate, and high hydrophilic liposomes (LH Lip, MH Lip, and HH Lip), respectively. LH Lip rapidly penetrated the alveolar epithelium for systemic absorption due to enhanced membrane avidity, multiple endocytosis pathways, and lysosome-bypassing transport (Figure 15). Conversely, HH Lip mostly accumulated in the alveolar lining fluid and bypass AMs-mediated clearance. They were also able to penetrate through the mucus in the bronchial region and accumulated in the periciliary layer, which shielded them from the mucus layer to further confirm their prolonged retention. When BUD was loaded into these liposomes, the enhanced pulmonary accumulation of HH Lip together with a sustained release of BUD allowed a single inhaled dose of this formulation to nearly restore asthmatic lung histology to a normal state and suppress inflammatory cytokines (IL-4, IL-5, and IL-13) more efficiently than their LH and MH counterparts and BUD free solution.

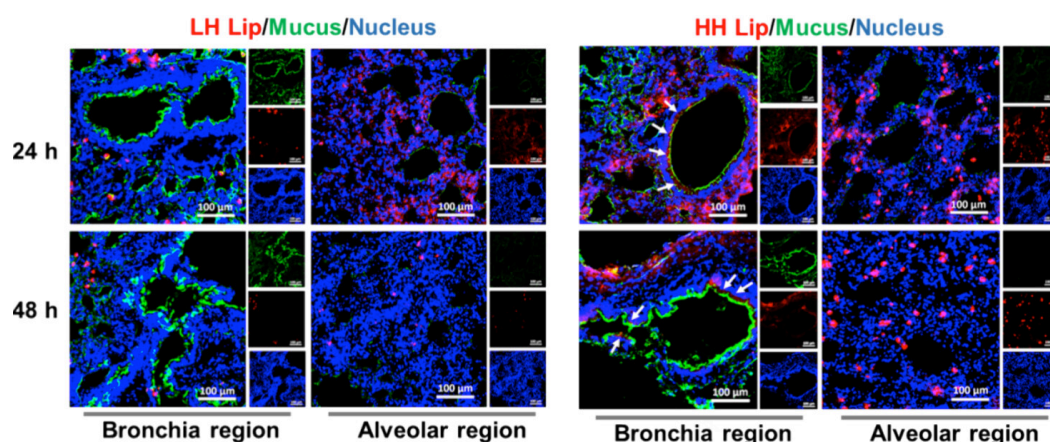


Figure 15. Confocal images showing colocalization of mucus and liposomes with different hydrophilicity on lung tissues for 24 or 48 h incubation, Legend: red, Dil-labeled liposomes; green, AF488-labeled WGA, mucus; blue, nucleus [76].

II.2.2.3. Engineering of liposomes to leverage the action of pulmonary surfactant

The formation of the biomolecular corona on NPs happens through the adsorption of proteins in the mucus or PS in the alveoli. One of the most well-documented consequences of the protein corona on NPs is the modulation of the innate immune response involving SP-A and SP-D. When they bind to the NPs surface, they alert the innate immune system and flag the carriers for phagocytosis by resident AMs [157–160]. Interestingly, SP-A exhibits a strong affinity for hydrophobic surfaces, particularly DPPC-containing membrane [161], while SP-D was shown to interact more readily with hydrophilic surfaces [118].

Recent extensive proteomic profiling has revealed that specific proteins recruited into the corona can actively dictate and significantly enhance the epithelial uptake. Subramaniam *et al.* studied the impact of proteins from bronchoalveolar lavage fluid (BALF) on the uptake of liposomes (composed of PC and dimethyldioctadecylammonium bromide (cationic lipid)) and cubosomes (Glyceryl monooleate, propylene glycol, and poloxamer 407) by different pulmonary cells [162]. Cubosomes, which possess an internal bicontinuous cubic phase and a hydrophilic poloxamer coating, recruited a high abundance of apolipoprotein H (ApoH) and various other hydrophilic molecular regulatory proteins into their protein corona. This ApoH-enriched corona significantly increased the uptake of cubosomes by A549 alveolar epithelial cells. In contrast, liposomes developed an albumin-rich protein corona which failed to enhance epithelial cell uptake, instead triggering rapid endocytosis followed by immediate exocytosis in macrophages.

Notably, the protein corona affects not only the cellular uptake of liposomes but also the bacterial killing activity of encapsulated antibiotics [163]. Cationic liposomes containing DOTAP and loaded with vancomycin exhibit a high binding affinity to methicillin-resistant *Staphylococcus Aureus* and its biofilms in PBS buffer solution thanks to their electrostatic interactions with the negatively charged bacterial surfaces. However, the incubation with mouse serum or BALF leads to the rapid adsorption of negatively charged proteins on their surface, shifting their surface charge to negative and resulting in a significant loss of binding ability. Conversely, anionic liposomes containing either DSPG or DPPG showed significantly increased binding to methicillin-resistant *Staphylococcus Aureus* when incubated in serum or BALF compared to PBS. This improvement was explained by the enrichment of the immune protein complement C3 and its degraded fragments in their protein corona. Because complement fragments naturally deposit on bacterial cell walls to flag pathogens for immune clearance, the anionic liposomes potentially hijack this native mechanism, using the acquired complement-enriched corona to directly bind to the bacteria and penetrate dense biofilms.

II.2.2.4. Critical quality attributes of liposomes containing ciclesonide and indacaterol maleate for improved therapeutic efficacy against asthma

Based on the physiological constraints of pulmonary drug delivery and the specific properties of CIC and IND, the design of liposomal systems must rely on carefully selected critical quality attributes (CQAs). Liposomes intended for inhalation should exhibit a nanometric size, typically **below 200 nm**, to enable efficient transport through the mucus mesh while limiting recognition and uptake by AMs. Their surface properties are equally critical, as a **hydrophilic and nearly neutral interface** is required to minimize adhesive interactions with mucins and reduce opsonization by PS proteins. This is commonly achieved through the incorporation of PEGylated lipids at controlled densities. In addition, the liposomal bilayer must favor **efficient incorporation of highly lipophilic compounds**, ensuring that drugs such as CIC and IND remain associated with the carrier during their diffusion across the airway lining. Altogether, these attributes must be finely balanced to promote mucus penetration, limit premature clearance, and favor access to target cells within the lung.

These considerations highlight the complexity of designing liposomal systems for pulmonary delivery and underline the existence of important trade-offs, which are discussed in the following section.

II.2.3.5. Challenges linked to PEGylation

Throughout the below sections of engineering liposomes to overcome the physiological barriers of the lungs, we can witness that the PEGylation is a common strategy help these vesicles overcome both the entrapment in the mucus layer and opsonin-mediated uptake in AMs. However, this strategy has several drawbacks that can critically impair therapeutic outcomes.

Firstly, the use of a high density of PEG component to adopt the brush-like configuration of the PEG layer can negatively **impact the liposomes structure and physicochemical properties**. Specifically, when PEGylated lipids are incorporated at high concentrations (typically above 8 mol%), the intense lateral steric repulsion between the tightly packed PEG chains severely perturbs the lipid bilayer [164]. This stress can induce a critical phase transition where the stable lamellar vesicles break down into mixed micelles. Even before reaching the point of complete micellization, high densities of grafted PEG can increase membrane permeability, potentially leading to premature drug leakage [99] and reducing the encapsulation efficiency (EE) of both hydrophobic and hydrophilic drugs [96,144,165].

Secondly, the dense PEG shield that helps liposomes to bypass the mucus and evade immune clearance could severely restrict their entry into epithelial cells. This paradox in nanomedicine is widely known as the "**PEG dilemma**" [166]. The first driving force behind this phenomenon is that the thick, hydrophilic, and neutral cloud of PEG sterically hinders liposomes from making direct contact with the target cell surface. Additionally, the reduced protein adsorption onto the liposome surface can deprive them of possible endogenous targeting mechanism [148].

A further critical aspect relates to the **immunological and inflammatory** consequences of PEGylation, particularly in the context of pulmonary diseases. Although PEG is generally considered biologically inert, it is widely recognized that 20% to over 70% of the general healthy population possesses circulating anti-PEG IgG and IgM antibodies due to routine environmental exposure (such as from cosmetics and pharmaceutical excipients), even without any prior treatment with PEGylated therapeutics [167]. When PEGylated liposomes are systemically administered, these pre-existing or treatment-induced antibodies actively recognize and bind to the polymer corona, leading to the dense opsonization of the liposomes with complement proteins and their subsequent rapid clearance via phagocytosis by the mononuclear phagocyte system. This process, which is called the accelerated blood clearance phenomenon, reduces the half-life of PEGylated liposomes in

the blood, preventing them from accumulating at their intended target sites and severely diminishing therapeutic efficacy with repeated use. Since systemically circulating antibodies are known to infiltrate mucosal secretions, researchers have used *ex vivo* animal models to demonstrate that when anti-PEG antibodies are present in the mucus, they can actively bind to the PEG coatings of inhaled NPs [168]. As Fc-domains of these antibodies form multiple transient, adhesive crosslinks with the surrounding mucin fibers, the NPs become physically immobilized inside the mucus mesh. In inflammatory environments such as asthmatic lungs, the altered composition of airway mucus and immune activation may further promote opsonization processes and accelerate the recognition and removal of PEGylated liposomes. Moreover, the interaction of PEGylated liposomes with activated immune cells and inflamed epithelium may exacerbate local immune responses or alter cytokine signaling, potentially impacting both safety and drug delivery performance.

One strategy to mitigate these limitations involves exploiting the dynamic nature of PEGylation, in particular through PEG shedding. As NPs enter the pulmonary environment, they are exposed to a complex mixture of biomolecules, including mucins, surfactant components, proteins, and extracellular DNA. The adsorption of these biomolecules onto the nanoparticle surface, leading to the formation of a bio-molecular corona, can facilitate the gradual desorption of PEG chains [142,169]. This transition from a highly shielded to a partially exposed surface may allow liposomes to retain their mucus-penetrating properties in the upper airways, while subsequently promoting cellular interactions in deeper regions of the lung.

Importantly, the kinetics of PEG shedding are governed by the strength of interactions between the hydrophobic anchors of PEGylated lipids and the lipid bilayer. Shorter or unsaturated acyl chains tend to weaken these interactions, resulting in faster PEG dissociation compared to longer and saturated chains [170]. Therefore, fine-tuning the structure of PEGylated lipids and the composition of the lipid bilayer is essential to balance PEG retention and release. Such optimization is particularly relevant for liposomes encapsulating highly lipophilic drugs such as CIC and IND, which preferentially localize within the membrane and whose delivery strongly depends on both particle integrity and surface properties.

Altogether, these considerations highlight the need to carefully balance PEG density, lipid composition, and NP–biological interactions in order to reconcile mucus penetration, immune evasion, and effective cellular delivery in the pulmonary environment.

II.2.3. Challenges linked to liposomes stability and production methods

II.2.3.1. Structural stability and delivery-related constraints

Beyond biological interactions, liposomes are inherently prone to physicochemical instability during storage and handling. Common issues include aggregation, fusion, coalescence, oxidation and hydrolysis of lipids, which can alter particle size, morphology, and distribution. A recurrent problem is the leakage of encapsulated drugs, either due to increased membrane permeability or degradation of the lipid bilayer, compromising therapeutic efficacy and dose reproducibility. Moreover, liposomes must also withstand the intense physical stresses associated with pulmonary administration. In particular, aerosolization processes such as nebulization or spray-drying subject the carriers to severe mechanical disruption. During nebulization, liposomes are exposed to high shear forces and air-liquid interfacial stress, which can physically tear the lipid membrane, induce vesicle aggregation, and cause the premature leakage of encapsulated drugs [171]. Spray-drying introduces additional challenges, including extreme heat, severe dehydration, and osmotic stress caused by a steep increase in solute concentration during droplet evaporation [172].

In addition, achieving efficient pulmonary delivery requires careful control of formulation properties that influence deposition behavior. The deposition of inhaled particles is strictly governed by their mass median aerodynamic diameter. To successfully bypass the upper respiratory tract and reach the deep alveolar regions, particles should have a mass median aerodynamic diameter between 1 and 5 μm [150]. Failure to control this parameter results in suboptimal delivery, as particles larger than 5 μm typically impact the oropharynx and trachea due to inertial impaction, whereas particles smaller than 0.5 to 1 μm lack sufficient inertia to settle and are frequently exhaled.

To mitigate these challenges, formulation strategies must carefully engineer the liposomes to ensure:

- Adequate membrane robustness through lipid composition optimization: to withstand the shear stress of aerosolization and prevent drug leakage, liposomes are normally formulated with high T_m phospholipids (such as DPPC or DSPC) combined with Chol [173].

Chol can increase the mechanical rigidity and structural stability of the bilayer, rendering it much less permeable and highly resistant to shear-induced disruption.

- Stability aided by protectant during high-stress processes: the addition of lyoprotectants, primarily disaccharides like sucrose, trehalose, or lactose, is paramount to protect liposomes from dehydration and osmotic stress during high-stress processes like spray-drying or freeze-drying. These sugars preserve liposomal integrity either by forming hydrogen bonds with the lipid headgroups, replacing lost water molecules, or by trapping the vesicles in a rigid, vitrified glassy matrix that prevents fusion and drug leakage [172]. For dry powder formulations, the co-spray drying of hydrophobic excipients like L-leucine or magnesium stearate can further protect the highly hygroscopic sugars from moisture-induced recrystallization, maintaining the long-term stability of powder [171].

- Compatibility with inhalation devices and delivery conditions: for liquid liposomal dispersions, vibrating-mesh nebulizers are highly preferred over traditional air-jet or ultrasonic nebulizers as they use lower energy and generate far less disruptive shear and heat [173]. For dry powder inhalers, engineering geometrically large and highly porous lipid microparticles can effectively weaken strong interparticle cohesive van der Waals forces, ensuring that the powder can be easily dispersed by inspiratory airflow of patients [174].

II.2.3.2. Translational and manufacturing challenges

Finally, the clinical translation of inhaled liposomal systems is critically dependent on the ability to develop scalable and reproducible manufacturing processes. Indeed, a formulation that shows *in vitro* or *in vivo* promises is not yet a mature therapeutic solution if its production remains difficult to reproduce or unsuitable for industrial-scale manufacturing. The challenge is therefore no longer limited to designing an effective formulation, but rather to demonstrating that it can be produced in a robust, reproducible manner that meets the requirements of scale-up. Conventional preparation methods often rely on organic solvents and batch processes that are difficult to scale up while maintaining tight control over critical quality attributes such as size, Pdl, and EE.

This limitation highlights the need for robust, scalable, and potentially solvent-free production technologies, capable of generating liposomes with consistent properties suitable for pulmonary delivery. Addressing these challenges is essential not only for ensuring product quality and regulatory compliance but also for enabling large-scale production required for clinical and industrial applications.

This scale-up is necessitated by the substantial material volumes required for the thorough investigation of pulmonary delivery performance. In particular, assessing the aerosolization stability and efficiency of these nanocarriers, whether through nebulization or their conversion into dry powders, is an inherently material-intensive process. Moreover, achieving a robust and reproducible manufacturing process is the cornerstone of clinical translation. Consequently, the investigation of a scalable production method capable of generating high-quality liposomes with consistent reproducibility is another objective of this study, as detailed in the following sections.

III. Large-scale production of liposomes: process engineering challenges and strategic choices

At the laboratory scale, numerous methods have been used for liposome preparation including thin film hydration (TFH), ethanol injection, reverse phase evaporation, detergent removal, microfluidic-based method and supercritical fluid-based methods [83]. However, few satisfy the rigorous demands of industrial-scale pharmaceutical manufacturing. At this level, high reproducibility, sterility, and precise control over important properties, such as mean particle size, Pdl, lamellarity, EE, and minimization of lipid degradants, are of paramount importance [175]. Regulatory agencies view lipid-based nanosystems as highly complex drug products, meaning the manufacturing process itself largely dictates the physicochemical attributes and the *in vivo* performance of the final formulation [176,177]. Consequently, establishing a path toward successful industrial application requires designing the research framework within a qualified environment from its very inception. Initiating development with a translation-ready mindset ensures that early-stage laboratory success can safely navigate strict regulatory boundaries later on. Furthermore, this approach must address the practical challenge of generating high volumes of liposomes, as massive quantities are required to feed downstream characterization and aerosolization pipelines. Investigation of a robust and scalable manufacturing platform is therefore essential to prevent batch-to-batch variability, ensure reliable clinical safety, and satisfy these high-throughput volume demands.

III.1. Limitations of conventional laboratory-scale methods

The most widely used method to produce liposomes at the laboratory scale is the TFH technique, which involves dissolving lipids in volatile organic solvents, removing the solvent via rotary evaporation to form a dry lipid film, and hydrating this film with an aqueous buffer

solution to generate heterogeneous vesicles [178]. To achieve a uniform size distribution suitable for drug delivery, these vesicles must subsequently undergo mechanical downsizing, typically through membrane extrusion or ultrasonic probe sonication. However, these conventional batch methods present severe inconveniences that fundamentally limit their translation to large-scale industrial manufacturing. First, the reliance on rotary evaporation to remove organic solvents becomes exceedingly complex, time-consuming, and difficult to scale up safely for large volumes [179]. Second, high-pressure extrusion introduces a major industrial challenge because forcing large volumes of viscous lipid suspensions through polycarbonate filters makes the system highly susceptible to membrane clogging and fouling, which severely reduces product yield, limits the maximum lipid concentration, and interrupts continuous aseptic processing lines [180]. Alternative downsizing methods like sonication are equally problematic at an industrial scale, as they suffer from heat-induced degradation of delicate lipids or drugs, and the risk of metal contamination from the sonicator probe [181].

Altogether, TFH and related batch processes illustrate a key limitation in liposome manufacturing: they lack process robustness and scalability, making them unsuitable as stand-alone strategies for industrial production. To overcome these severe scale-up bottlenecks and ensure consistent quality, the pharmaceutical industry has made significant efforts to gain more control over liposome properties in the two common continuous-flow methods, namely the ethanol injection method and microfluidics.

III.2. Current liposomes production methods at industrial scale: ethanol injection and microfluidics

The ethanol injection method is currently the most common technique for the industrial manufacture of parenteral liposomes containing small molecules, whereas microfluidics has become the dominant technology for manufacturing LNPs used in gene therapies and mRNA vaccines [182]. Both methods rely on the same principle of solvent exchange, also known as nanoprecipitation [175,182]. In this process, lipids and hydrophobic drugs are dissolved in a water-miscible organic solvent, typically ethanol due to its low toxicity as a Class 3 solvent. This organic lipid phase is then mixed with an aqueous phase containing hydrophilic drugs or nucleic acids. This mixing drastically increases the polarity of the solvent environment, thereby decreasing the solubility of the lipids and triggering rapid lipid precipitation. The physical transition from dissolved lipids to spherical vesicles is driven by

thermodynamic energy minimization. Theoretical models, such as the Bilayer Planar Fragment theory by Lasic and the kinetic non-equilibrium model by Zook and Vreeland, explain this self-assembly process by demonstrating that as the solvent environment becomes highly polar, precipitating lipid monomers self-associate to form intermediate planar bilayer discs to shield their hydrophobic acyl chains from the polar water-alcohol mixture [183,184]. While the flat surfaces of the discs are stable, the hydrophobic chains around the edges of these discs are increasingly exposed to the polar solvent as ethanol is further diluted, creating an unfavorable "edge energy" (or line tension). To eliminate this exposure and reach the most energetically favorable state, the planar discs eventually bend and close upon themselves, forming spherical liposomes and capturing a portion of the aqueous mixture within their core.

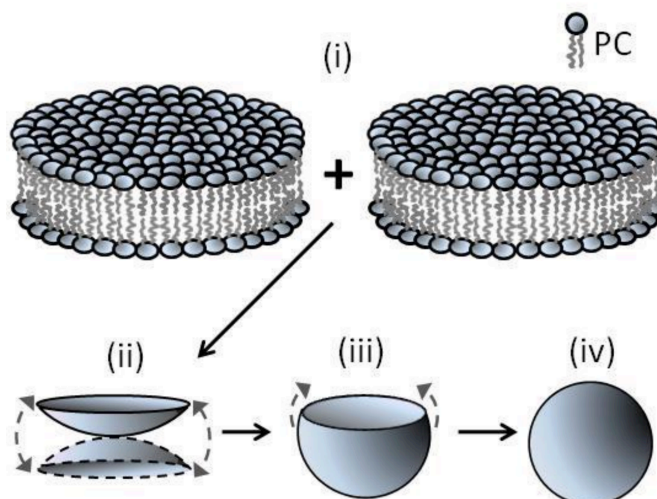


Figure 16. Hypothesized liposome formation mechanism, starting with (i) the aggregation of bilayer planar discs. These discs grow until they are large enough and the alcohol is sufficiently depleted so that they begin to (ii) bend and (iii) eventually rapidly close into (iv) spherical bilayer vesicles [184].

III.2.1. Ethanol injection method

Conventional ethanol injection relies on dispersing the ethanolic lipid solution into an aqueous phase under macroscopic stirring (Figure 17). The successful scaling up of this production method can be achieved by tightly controlling several key parameters such as stirring speed, lipid concentration, the volume ratio of organic solvent to aqueous phase, aqueous phase viscosity, and ethanol injection pressure [182]. This method is highly favored for commercial production due to its simplicity and the avoidance of highly toxic

solvents. However, the fundamental problem with this approach is that macroscopic agitation creates chaotic, turbulent fluid dynamics characterized by a distinct velocity gradient from the center of the mixing vortex out to the vessel side walls [182,185]. The resulting uneven mixing environment causes lipid molecules to experience different local ethanol concentrations and dilution rates depending on their physical location in the tank. According to kinetic models of vesicle assembly, intermediate lipid planar bilayer discs in slower-mixing zones near the tank walls remain stabilized by the alcohol for longer periods, allowing them to recruit more lipids and grow significantly larger before the thermodynamic edge energy forces them to bend and close into spheres [184]. Conversely, lipids in high-velocity zones experience instant dilution and close rapidly into much smaller vesicles.

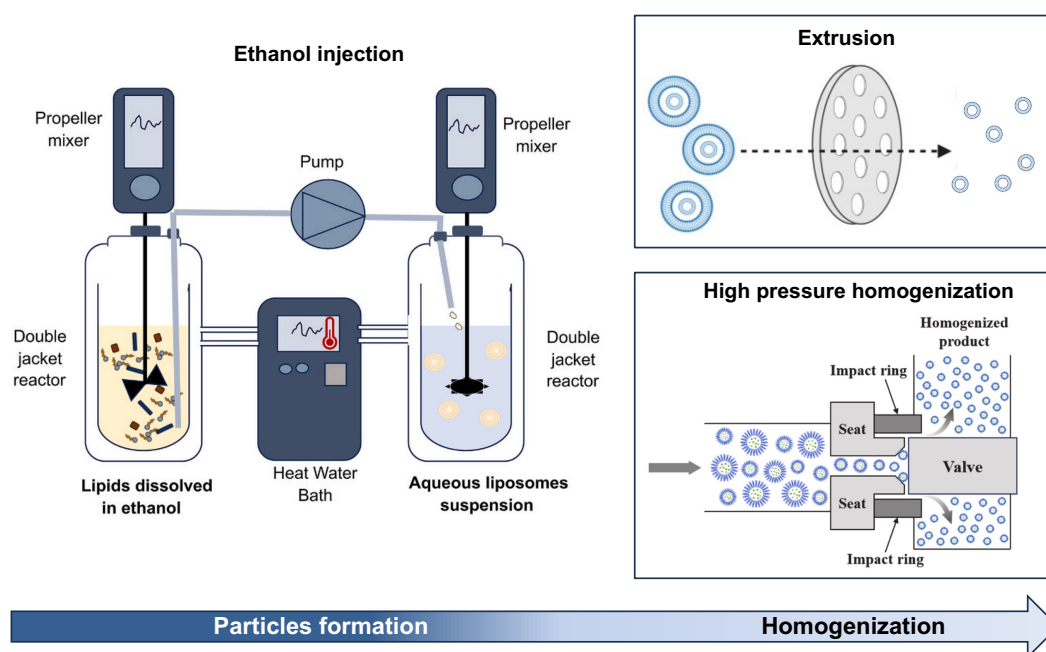


Figure 17. Schematic representation of liposomes formation by ethanol injection method, followed by homogenization by extrusion or high-pressure homogenization [182,186,187].

Consequently, this mixing inherently yields a highly heterogeneous, broadly distributed population of large, multilamellar vesicles [188]. To meet the strict particle size uniformity required for clinical applications, this conventional method must be followed by downstream size-reduction steps such as high-pressure extrusion or high-pressure homogenization (HPH). Extrusion is the most widely accepted technique which involves repetitively forcing the heterogeneous multilamellar vesicles through polycarbonate membrane filters with defined pore sizes under high pressure [175]. Although it yields final liposomes of a defined

mean size and acceptable Pdl (≤ 0.1), it remains a time-consuming, multi-stage operation associated with high equipment costs, high susceptibility to membrane clogging, and substantial product losses (often well over 50%) [182,189,190]. Alternatively, in HPH, the large vesicles are forced through a narrow gap under extreme pressure to break them into smaller vesicles [183]. However, HPH is generally less favored because it produces a broader size distribution than extrusion (Pdl ~ 0.2) [175]. Furthermore, the extreme shear forces, cavitation effects, and localized temporary heating generated in this process can chemically degrade sensitive lipids and therapeutic cargo [190].

III.2.2. Microfluidic

As an advanced adaptation of ethanol injection-based methods, microfluidic technologies have emerged as a dominant manufacturing platform. Microfluidics confines the mixing process to microchannels (typically 10-100 μm in diameter) where the physical forces governing the liquid fundamentally change [191]. At the macroscopic scale, fluid flow is dominated by volume forces, like inertia or gravity [192]. However, at the microscopic scale, surface forces and fluid friction (viscosity) become overwhelmingly dominant. This relationship is mathematically defined by the Reynolds number, which is the ratio of inertial forces of a fluid to its viscous forces (Figure 18A). In macroscopic tanks, the Reynolds number is very high ($\text{Re} > 2000$), meaning inertia takes over and creates chaotic turbulence [193]. In microfluidics, the Reynolds number drops drastically (typically $\text{Re} < 100$ and often $\text{Re} < 1$) [194]. In a low-Re environment, the system is forced into a laminar flow regime where the fluid layers slide perfectly in parallel (Figure 18B) [193]. Because there are no random perturbations or chaotic turbulence, the flow characteristics become mathematically definable, highly stable, and reproducible from batch to batch. Furthermore, because the lipid-ethanol stream and the aqueous buffer solution stream flow side-by-side in these parallel layers, they do not mechanically swirl together. Therefore, the only way for the two fluids to mix is across the liquid-liquid boundary which is driven entirely by molecular diffusion - the natural, thermally induced, random movement of molecules (Brownian motion) along a concentration gradient [195]. As the parallel streams travel down the channel, the ethanol molecules slowly diffuse outward into the water stream and the water molecules diffuse inward into the ethanol stream. This diffusional mixing creates a precise, steady and localized spatial gradient of solvent polarity which allows the lipid molecules to experience a uniform decrease in solubility. This triggers them to self-

assemble into intermediate disc-like fragments, which then grow and close into spherical vesicles.

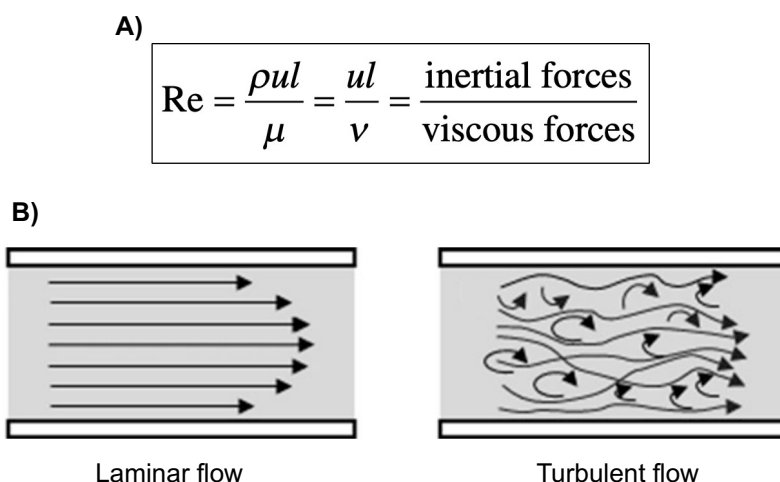


Figure 18. (A) Equation of Reynolds number. ρ is the density, u is the linear velocity, ν is the kinematic viscosity, l (a characteristic dimension) stands for the diameter (for flow inside a channel with cylindrical circular cross section) or for the hydraulic diameter in the case of non-circular cross section channels, and μ is the viscosity. (B) Flow patterns in a channel [193].

To achieve optimal mixing and produce finely tuned liposomes, various sophisticated microfluidic chip architectures have been engineered. Microfluidic hydrodynamic focusing is a foundational technique where a central lipid-solvent stream is hydrodynamically squeezed into a narrow sheet by two adjacent, co-flowing aqueous buffer streams (Figure 19A) [196,197]. This precise control of the interfacial area allows researchers to strictly tune liposome sizes between 50 and 150 nm simply by adjusting the flow rate ratio between the aqueous and organic phases [198]. Because pure microfluidic hydrodynamic focusing relies on molecular diffusion across laminar flow interfaces, specialized geometries like the Staggered herringbone mixer (SHM) were developed to incorporate microscopic grooves on the channel floor (Figure 19B). These structures induce chaotic advection, which repeatedly folds and stretches the fluid streams, significantly accelerating the mixing process [199]. For high-throughput industrial production, novel geometries such as toroidal mixers have been designed to handle massive continuous flow rates without clogging or losing the nanoscale precision of the liposomal product (Figure 19C) [191].

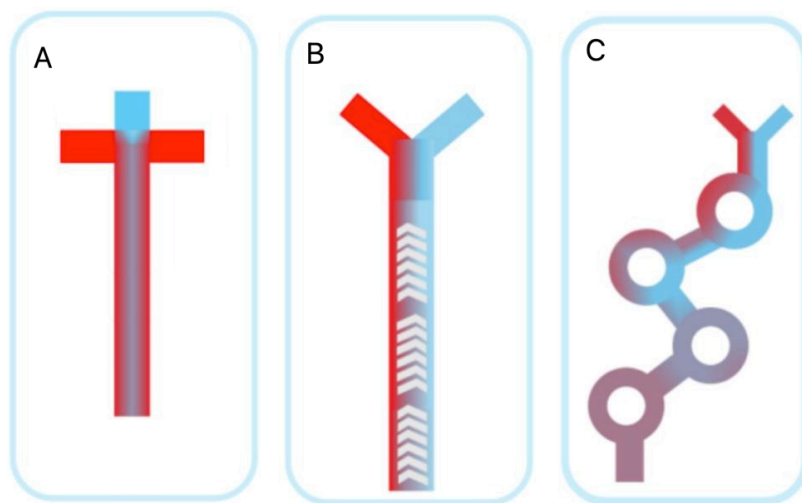


Figure 19. Commonly used micromixing geometries for the manufacturing of nanoparticles: **(A)** hydrodynamic flow focusing; **(B)** staggered herringbone geometry; **(C)** toroidal mixing device [200].

The most critical advantage of applying microfluidics to liposome production is the ability to bridge the gap between laboratory-scale discovery and Good manufacturing practice (GMP) commercial production. Webb et al. provided a case study illustrating this industrial scale-up using a novel toroidal mixer to formulate liposomes loaded with OVA protein [201]. They successfully scaled the production from a small benchtop flow rate of 12 mL/min up to a massive GMP-compliant 200 mL/min (12 L/h) using the exact same microfluidic parameter set points. Across all operating scales, the critical quality attributes remained perfectly consistent, maintaining an average particle size of 60–70 nm, a Pdl around 0.2, and stable protein loading without any mechanical size reduction.

Overall, while continuous-flow methods represent a major step forward, they involve trade-offs between control, throughput, and scalability, which must be carefully balanced depending on the intended application.

III.1.3. Downstream processing: a critical yet limiting step

After liposomes are formed via techniques like ethanol injection or microfluidic mixing, the resulting dispersion contains unencapsulated therapeutic agents and residual organic solvents, primarily ethanol. Removing of this residual ethanol is a critical downstream process. If ethanol remains in the formulation, it can perturb the lamellar spacing, negatively affect the internal order of the NPs and induce Ostwald ripening. This process involves the dissolution and merging of smaller vesicles with higher surface energy dissolve into larger

ones with lower surface energy. This shifts the size distribution over time and causing colloidal instability [202]. Furthermore, regulatory guidelines, such as those from the ICH, require that residual ethanol in parenteral products must be reduced to acceptable and safe levels, typically below 5000 ppm or 0.5% [203].

To achieve this at the industrial scale, manufacturers predominantly use tangential flow filtration (TFF), also known as cross-flow filtration or diafiltration (Figure 20). In TFF, the NPs suspension is circulated tangentially across a filter membrane, such as a polysulfone or polyethersulfone hollow fiber cartridge [188,204,205]. The NPs are retained within the retentate stream, while the smaller ethanol molecules, buffer components, and unencapsulated drugs pass through the microscopic pores as permeate and are discarded. TFF is highly advantageous because it allows for the simultaneous solvent removal, the buffer exchange to a physiological solution, and the concentration of the NPs formulation to the target therapeutic dosage.

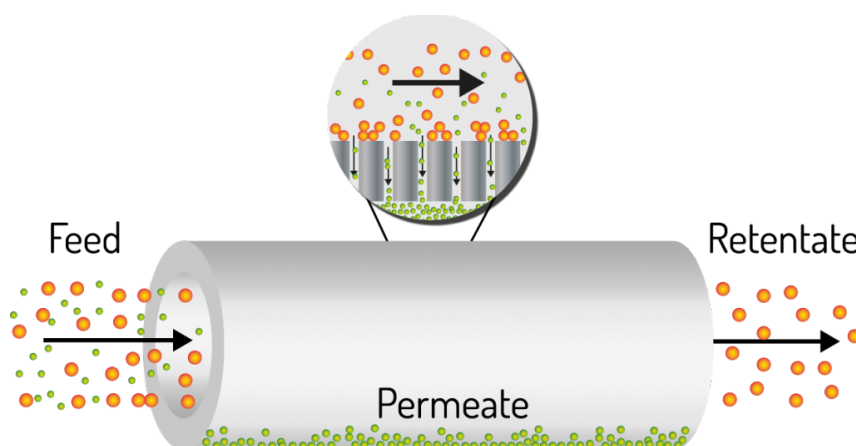


Figure 20. Schematic representation of Tangential Flow Filtration [206].

Despite its widespread adoption, TFF remains one of the slowest and most challenging stages in manufacturing scale-up [207], sometimes consuming up to 60% of the entire processing timeline [208]. Material accumulation leads to membrane fouling and a gel polarization layer, which reduces permeate flux and prolongs operations [208]. Because it dominates the timeline, TFF heavily drives overall operating costs. However, accelerating the process using larger pumps is counterproductive. Since liposomes are highly shear-sensitive, extreme shear forces damage the vesicles, causing structural degradation, drug leakage, and reduced encapsulation yields. Furthermore, Dimov et al. found that during the simultaneous removal of residual ethanol and an unencapsulated lipophilic drug (propofol)

using TFF, the drug was removed from the suspension much faster than the ethanol [209]. They concluded that the ethanol content is a crucial factor in determining the required number of diafiltration cycles. Recent evaluations have demonstrated that high concentrations of ethanol actively reduce the permeability of the hollow fibers used in TFF modules, thereby decreasing the overall efficiency of the filtration process [210]. This impairment is likely due to the ethanol causing the polymeric hollow fibers to swell, which introduces an unexpected partial rejection coefficient that resists the transport of the solvent itself across the membrane. To overcome this problem, manufacturers can implement a upfront dilution of the liposomal suspension to minimize initial exposure of the membrane to ethanol to preserve its structural permeability [210]. Another promising strategy is to develop a production method that uses only a small amount of ethanol or even eliminates this solvent entirely.

Following successful purification and concentration, the formulation mostly undergo sterilization, especially when the vast majority of liposomal drug products are intended for parenteral administration. As delicate, multiphase systems, liposomes are highly susceptible to structural damage from conventional terminal sterilization techniques, including autoclaving, dry heat, radiation, and gas exposure, which trigger lipid hydrolysis, vesicle aggregation, or premature drug leakage [182,211,212]. Consequently, aseptic sterile filtration through 0.22 μm membranes, configured with a 0.3–0.5 μm pre-filter to maximize throughput, is frequently used in the pharmaceutical industry [208,213]. To ensure process compliance, regulatory agencies like the FDA require that membrane pressure must be recorded during filtration and ensured not to exceed maximum membrane pressure while the filter member integrity test is required for each process filtration step [214]. In cases where sterile filtration is not feasible because of high viscosity of formulations or a mean particle size approaching or exceeding 200 nm, the entire manufacturing process must be conducted under strict aseptic conditions from start to finish.

In overall, while the vast majority of current industrial liposome manufacturing relies heavily on organic solvents such as ethanol, isopropanol to dissolve and mix lipid components [197], their use introduces severe safety, environmental, and manufacturing drawbacks. From a safety and environmental perspective, organic solvents pose significant flammability hazards within manufacturing facilities and contribute to photochemical air pollution (such as ozone and smog) upon emission [215]. This forces pharmaceutical

companies to implement rigorous and highly expensive tracking and ventilation systems for volatile organic compounds [216]. Furthermore, residual organic solvents can alter membrane properties, interfere with the efficient encapsulation of active drugs, and cause drug leakage from the vesicles [191]. Because these residual solvents compromise the stability of the liposomes and pose safety risks to human health, manufacturers are forced to implement the exhaustive, time-consuming, and costly downstream purification processes discussed previously. Crucially, as noted earlier, ethanol also impairs the performance and prolongs the duration of the TFF [209,210]. These extensive solvent-removal operations not only drastically increase the overall complexity and financial burden of the manufacturing line, but they can also cause physical damage to industrial equipment (like freeze-dryers) [215,216]. Consequently, overcoming these profound industrial and environmental hurdles requires a shift away from traditional organic solvent-based protocols. This has driven the exploration of alternative processing technologies that operate with minimal organic solvent volumes or eliminate them entirely from the fabrication pipeline.

III.3. Transition toward solvent-free processes: rationale and limitations – Supercritical fluids-based approaches

To overcome the limitations of conventional organic solvent-based methodologies, scientists have increasingly turned to Supercritical Fluid (SCF) technologies. Among these, supercritical carbon dioxide (SC-CO₂) has emerged as the most prominent green alternative because it is non-toxic, non-flammable, inexpensive, chemically inert, and operates at relatively mild critical parameters (31.1 °C and 73.8 bar) [217]. As a SCF, SC-CO₂ exhibits dual characteristics: it possesses the liquid-like density and solvating power of a fluid, combined with the gas-like diffusivity, high mass-transport capabilities, and penetrability of a gas. Most importantly, upon depressurization, the SC-CO₂ simply reverts to a gaseous state and evaporates completely, leaving no toxic solvent residue in the final product. This provides a cleaner, environmentally friendly and physiologically safe alternative for liposome production, aligning perfectly with green chemistry principles while preventing the solvent-induced degradation of sensitive drugs [211].

III.3.1. Methods for producing liposomes assisted by supercritical carbon dioxide and organic solvent

SC-CO₂ can serve multiple roles in liposome formulation. When acting as a primary solvent, SC-CO₂ directly dissolves the active molecules and lipids, which are then rapidly expanded into different media. If the supercritical solution is rapidly expanded into a low-pressure collection chamber filled with air or a gaseous stream, the process is known as the standard Rapid Expansion of Supercritical Solutions (Figure 21A) [218]. Upon sudden depressurization, the supercritical fluid instantly transitions to a gas, causing a loss of solvent power that triggers the rapid precipitation of solvent-free solid microparticles or NPs. However, conventional Rapid Expansion of Supercritical Solutions cannot form liposomes directly, as phospholipids strictly require an aqueous environment to self-assemble into vesicles [219]. This limitation can be overcome by expanding the supercritical mixture into an aqueous phase containing stabilizing agents or surfactants that prevent the NPs from aggregating within the expansion jet [220]. These modified approaches are called rapid expansion of a supercritical solution into a liquid solvent or rapid expansion of supercritical solutions into an aqueous solution. Because pure phospholipids exhibit limited solubility in non-polar SC-CO₂, a small amount of an organic co-solvent, such as ethanol, is frequently added to enhance its solvating power [217].

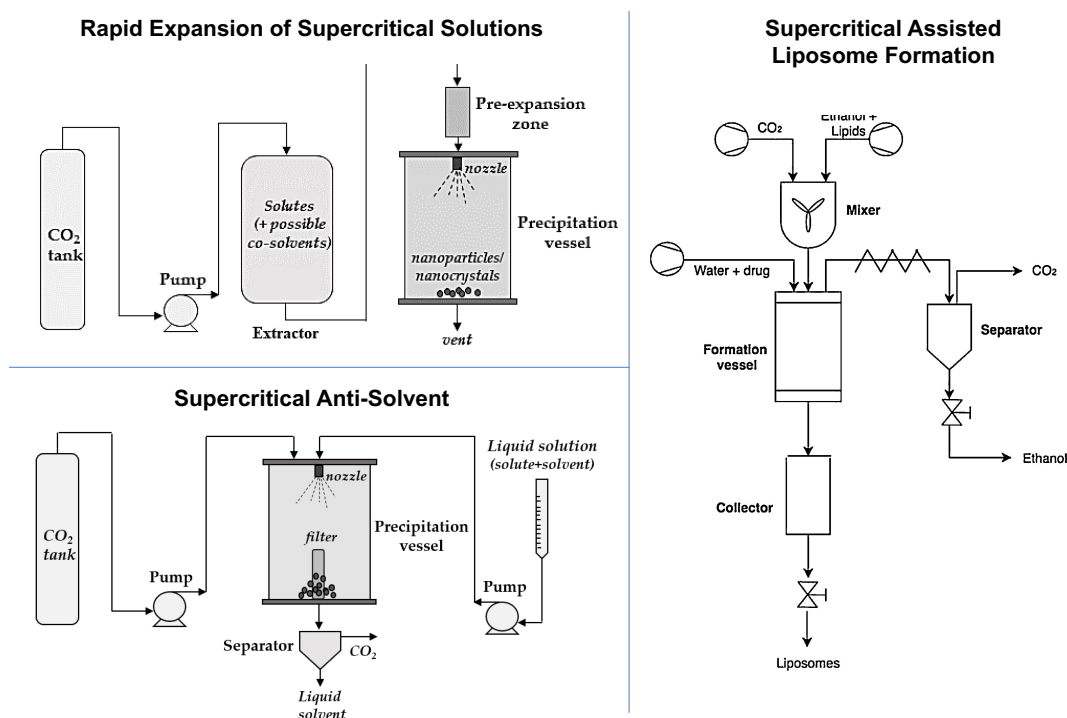


Figure 21. Schematic representation of (A) Rapid Expansion of Supercritical Solutions, (B) Supercritical Anti-Solvent, and (C) Supercritical Assisted Liposome Formation processes [221,222].

For compounds and lipids that are poorly soluble in SC-CO₂, techniques like the Supercritical Anti-Solvent processes utilize SC-CO₂ as an antisolvent to precipitate the lipid matrix (Figure 21B) [223]. In this process, the phospholipids and active molecules are first fully dissolved in an organic solvent. This organic solution is then continuously sprayed through a nozzle into a high-pressure vessel already filled with SC-CO₂. A rapid, two-way mass transfer immediately occurs: the highly diffusive SC-CO₂ dissolves rapidly into the liquid solvent droplets, while the organic solvent simultaneously evaporates into the supercritical phase. This mutual diffusion reduces the dissolving capacity of the organic solvent, triggering a supersaturation of phospholipids in the liquid droplets followed by the nucleation and aggregation of finely divided, dry phospholipid particles known as proliposomes. These proliposomes are typically collected on a filter located at the bottom of the high-pressure vessel, while the fluid mixture (supercritical fluid plus solvent) exits the vessel [218]. To ensure the product is nearly free of organic solvent residues, a washing step involving the continued flushing of pure SC-CO₂ through the vessel is performed to

extract the remaining solvent [224]. These dry proliposomes can then be easily hydrated with an aqueous solution to spontaneously form liposomes.

In the Supercritical assisted Liposome formation process, SC-CO₂ acts as a cosolvent alongside an organic solvent to bypass the poor solubility of lipids in pure SC-CO₂ (Figure 21C) [217]. Specifically, an ethanolic lipid solution is mixed with SC-CO₂ in a high-pressure saturator to generate a gas-expanded liquid. The core innovation of this process is the complete inversion of traditional liposome assembly: an aqueous solution containing the hydrophilic drug is atomized directly into this expanded liquid inside a formation vessel. The atomized water droplets are instantaneously surrounded by a layer of amphiphilic phospholipids, forming inverted micelles on the fly [225]. Because the hydrophilic drug remains strictly confined within the water droplets while the initial lipidic compartment forms around them, its loss is minimized to yield high encapsulation efficiencies [226]. As these precursor micelles fall into a continuous water pool located at the bottom of the vessel, they acquire a second lipid layer, completing the characteristic bilayer structure. Simultaneously, after depositing the lipids, the SC-CO₂ and ethanol are continuously vented and eliminated from the top of the formation vessel.

III.3.2. Methods for producing liposomes assisted by supercritical carbon dioxide and without organic solvent

Among various SCF-assisted techniques, Improved Supercritical Reverse Phase Evaporation (ISCRPE) and Particles from Gas-Saturated Solutions (PGSS) methods are two prominent methods that completely bypass the use of organic solvents by utilizing SC-CO₂ as a dispersing agent (Figure 22). In both techniques, an aqueous dispersion of lipid components and active molecules is initially loaded into a high-pressure autoclave and mixed with injected SC-CO₂ under agitation. Although SC-CO₂ and water exhibit limited mutual miscibility, a fraction of the CO₂ solubilizes within the aqueous phase during the mixing stage, establishing an equilibrium with carbonic acid. This carbonic acid partially dissociates into H⁺ and HCO₃⁻ ions. The HCO₃⁻ ions can interact with the hydrophilic head groups of the phospholipids, inducing a lateral expansion of the bilayer due to electrostatic repulsion between neighboring heads [227]. Simultaneously, the unhydrated SC-CO₂ can partition into the crude lipid aggregates, intercalating between the fatty acyl chains of the phospholipids. This intercalation disrupts the intermolecular van der Waals interactions, effectively dispersing the poorly soluble phospholipids and Chol. The intercalated SC-CO₂ also acts as a plasticizer that significantly depresses T_m of the lipids, rendering them highly

fluid and flexible even at mild processing temperatures [228]. Driven by these interactions, the dispersed lipids can migrate to the interface between the SC-CO₂ and the aqueous phase, stabilizing the formation of emulsion systems [227].

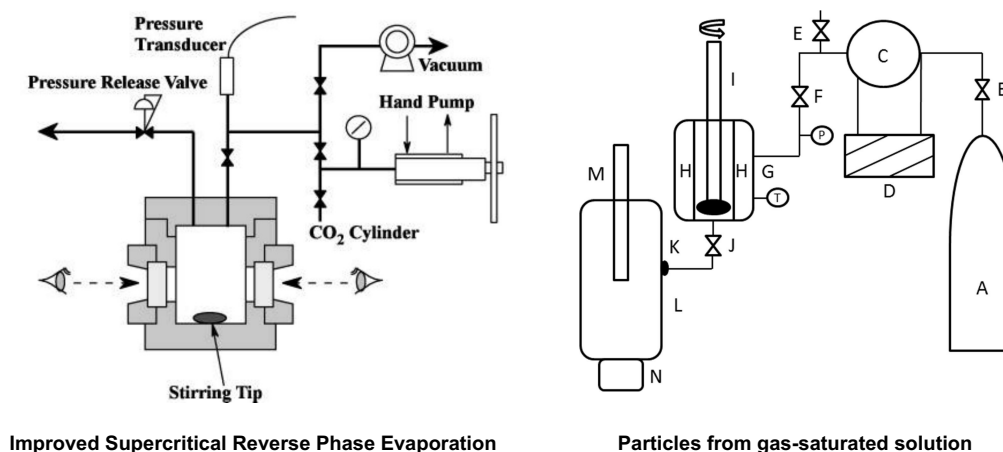


Figure 22. Schematic representation of Improved Supercritical Reverse Phase Evaporation and Particles from Gas-Saturated Solutions processes [229,230].

Depressurization of SC-CO₂ after the initial mixing step is what fundamentally differentiates the mechanism of liposome formation between ISCRPE and PGSS. In the ISCRPE method, depressurization is a static batch operation conducted within the same high-pressure vessel [229,231]. During the mixing stage, strong shear forces from vigorous magnetic stirring disrupt the phospholipid coagulates, efficiently forming a CO₂-in-water (CO₂/W) emulsion. Upon depressurization, the release of the SC-CO₂ gas drives a phase inversion from this CO₂/W emulsion into an aqueous liposomal dispersion. The published studies identify this specific phase inversion as the vital controlling step for liposomes formation. A slower, more controlled phase inversion allows for the assembly of larger vesicles which inherently possess a smaller surface-to-volume ratio and can therefore encapsulate more aqueous cargo using the same amount of phospholipids. Crucially, the complete absence of organic solvent in this process prevents the disruption of the assembling vesicles, thereby promoting a more stable liposome assembly and significantly enhancing drug trapping efficiency during the phase inversion.

Conversely, the PGSS method uses a dynamic spraying mechanism. The entire gas-saturated aqueous dispersion is forcefully expelled through a nozzle into a low-pressure expansion chamber. During this depressurization, the rapid expansion of SC-CO₂ into its

gaseous form fragments the plasticized lipid phase into small, disordered assemblies or even discrete molecules [232–234]. Subsequently, driven by hydrophobic interactions that minimize their exposure to the surrounding aqueous buffer solution, these dispersed lipids spontaneously self-assemble into bilayers and, ultimately, fully formed liposomes.

The PGSS method has gained the attention of many research groups, including ours, as a promising technique for producing liposomes without organic solvents at the laboratory scale (~6–14 mL). These studies have provided valuable insights into how manipulating processing parameters enables precise control over the physicochemical properties of liposomes. Pressure is a paramount parameter, as elevating the initial processing pressure (e.g. from 60 to 300 bar) significantly decreases the size and Pdl of liposomes [232,234]. Higher pressures increase the density and dispersibility of SC-CO₂, driving more unhydrated CO₂ to accumulate within the hydrophobic acyl chains of phospholipids. Upon depressurization, this higher concentration of intercalated SC-CO₂ produces a stronger mechanical dispersing effect to thoroughly shatters the lipid aggregates into discrete molecules which then reassemble into smaller, highly uniform vesicles. Regarding EE, pressure can exert complex effects depending on the active compound. While high pressure generally improves the uniformity of the dispersion, excessively high pressures can be detrimental for target drugs that are highly soluble in SC-CO₂ (such as Coenzyme Q10). In such cases, the elevated pressure can actively extract the drug into the supercritical fluid, preventing its incorporation into liposomes and thereby decreasing EE [234]. Conversely, for other compounds like lutein, elevated pressures favor a thorough dispersion of phospholipids and lutein aggregates into monomers, promoting a more uniformly distributed incorporation of lutein into liposomes [235].

The **depressurization rate** dictates the speed of CO_{2di} release and heavily influences the uniformity of vesicle size. A faster depressurization rate (e.g., 90 to 120 bar/min) triggers an explosive gas expansion that ensures a thorough fragmentation of lipid aggregates, thus lowering the Pdl and improving EE [232,235,236]. However, an excessively fast depressurization risks inducing a severe Joule-Thomson effect, which causes a drastic and sudden temperature drop within the mixture as the gas expands and leaves the liquid phase [237]. At a molecular level, this effect occurs because the rapid expansion significantly increases the distance between the CO₂ molecules [238]. Although attractive and repulsive intermolecular forces both decrease as the molecules separate, the repulsive forces diminish much more rapidly. In the context of the PGSS process, if CO₂ expands so rapidly

that the system cannot absorb enough external thermal energy to facilitate this separation, the CO₂ molecules must expend their own internal kinetic energy to overcome intermolecular attraction. Moderate cooling effect is beneficial, as it stabilizes the newly formed vesicles and protects heat-sensitive encapsulated compounds [233]. However, as cautioned in several articles, an excessively rapid depressurization (such as 200 bar/min) can exaggerate the Joule-Thomson effect and cause an extreme temperature drop [233,235]. This severe and sudden chilling can force the phospholipids to undergo a premature phase transition from a flexible liquid-crystalline state back into a rigid gel phase before the vesicles have properly assembled. Therefore, the depressurization rate should be carefully optimized at each production scale to balance lipid dispersion with appropriate cooling.

Temperature exerts two opposing physical effects on liposome formation [232,235,236]. On the one hand, elevated temperatures provide thermal energy that disrupts van der Waals interactions between the hydrophobic lipid tails. This enhances the flexibility and fluidity of phospholipids, creating a disordered state highly favorable for the spontaneous reassembly of smaller, more uniform vesicles. On the other hand, increasing temperatures reduce the SC-CO₂ density and its solubility within the aqueous phase. With less unhydrated SC-CO₂ intercalated into the lipid aggregates, the scattering effect during depressurization is considerably weakened. This tends to leave larger lipid aggregates intact, thereby increasing the overall particle size. Consequently, in some studies, temperature exhibits a biphasic effect on particle size, where an intermediate optimum (often around 50 °C) achieves the smallest diameter while still protecting thermolabile compounds.

The **composition and structure of phospholipids** also influence the liposomal properties. Phospholipids with shorter fatty acid chains (e.g., dimyristoyl-sn-glycero-3-phosphocholine (DMPC, C14:0)) possess lower phase transition temperatures, resulting in highly fluid bilayers and the formation of smaller particles [239]. Conversely, phospholipids featuring longer saturated hydrocarbon chains (such as DPPC (C16:0) and DSPC (C18:0)) tend to produce larger but more spherical and symmetrical liposomes. This enhanced morphological symmetry occurs because the longer acyl chains permit a greater number of SC-CO₂ molecules to intercalate between the hydrophobic tails, ensuring a superior dispersion of the lipid molecules during depressurization [240].

Furthermore, SC-CO₂ has emerged as a highly promising, non-thermal sterilization technology. It exhibits potent microbicidal efficacy against a broad spectrum of pathogens, including vegetative bacteria, fungi, viruses, and highly resistant bacterial endospores [241]. Its unique physical properties - combining gas-like diffusivity and low viscosity with liquid-like density - facilitate rapid penetration through complex microbial cell walls and membranes. Once in contact with the microbial cell, inactivation is achieved through several hypothesized mechanisms. First, the solubilization of pressurized CO₂ in the aqueous medium and intracellular water forms carbonic acid, which lowers the intracellular pH. This acidification inactivates key metabolic enzymes and disrupts protein synthesis and gene transcription, thereby inhibiting cellular metabolism [241]. Simultaneously, the highly diffusive SC-CO₂ extracts structural lipids from the cell wall and membrane, causing functional modifications that fatally increase membrane fluidity and permeability [211]. Finally, during the depressurization phase, the rapid volumetric expansion of the vaporizing CO₂ gas can trigger mechanical cell rupture and extraction of intracellular substances [224].

Using a QbD optimization strategy, our laboratory identified that the optimal PGSS conditions to achieve both sterility and acceptable vesicle characteristics were a temperature of 80 °C, a pressure of 240 bar, and a contact time of 30 min [242]. Under these specific conditions, the PGSS process successfully achieved the mandated sterility assurance level of 10⁻⁶ (greater than a 6-log reduction), effectively inactivating highly resistant *Bacillus atrophaeus* spores. Notably, these sterilizing conditions still yielded vesicles with a diameter of 200 nm or less and a Pdl between 0.30 and 0.35. Furthermore, the process demonstrated effective for both low (5 mM) and high (45 mM) lipid concentrations across various lipid compositions.

From a process engineering perspective, the PGSS method presents a highly advantageous, single-step manufacturing platform characterized by its completely solvent-free operation. By exploiting its ability to disperse both lipids and active molecules, the SC-CO₂ acts as a dispersing agent that spontaneously reverts to a gas and separates upon depressurization, thereby eliminating the need for costly and time-consuming downstream solvent elimination steps. Beyond simplifying the production layout, the high-pressure SC-CO₂ environment has intrinsic microbicidal properties capable of damaging microbial cell membranes, which offers the unique potential for simultaneous sterilization. Finally, the combination of an oxygen-removing inert atmosphere and the Joule-Thomson cooling

effect occurring during expansion makes PGSS compatible with sensitive compounds, effectively protecting those susceptible to degradation and oxidation from degradation.

However, significant challenges remain. The process is highly dependent on parameters such as pressure, temperature, and depressurization rate, which directly influence liposome size, Pdl, and drug EE. Furthermore, despite recent advancements in adapting the PGSS method for liposome formulation, its capability remains limited to relatively small volumes at the laboratory scale. Presently, there is a distinct lack of published data regarding the generation of larger batches, such as 100 mL or greater. Consequently, further experimental investigations and validations are necessary to clarify how system parameters shift during scale up. Addressing this gap is essential to promote the industrial adoption of PGSS as an organic solvent-free method for manufacturing liposomes.

Altogether, the development of effective pulmonary delivery strategies for ICS and LABA combinations is constrained by a series of interconnected challenges that operate across multiple scales. At the biological level, the primary limitation lies in the rapid clearance of inhaled drugs by airway mucus and AMs, which is further exacerbated by the high lipophilicity of long-acting compounds, leading to prolonged entrapment within the airway lumen and reduced access to target cells. At the formulation level, these constraints impose stringent requirements on particle design, as drug carriers must simultaneously achieve efficient mucus penetration, minimal immune recognition, and adequate interaction with epithelial and ASM cells, highlighting critical trade-offs in surface engineering (e.g., PEGylation) and physicochemical properties. At the technological level, ensuring the stability of such systems during aerosolization and achieving controlled deposition within the respiratory tract remain significant challenges. Finally, at the scale of industrial translation, the development of robust, reproducible, and scalable manufacturing processes represents a major bottleneck, as many laboratory-scale preparation methods fail to meet regulatory and production constraints.

CONTEXT AND OBJECTIVES OF THE STUDY

I. Context of the study

Asthma remains a chronic condition whose management relies primarily on the combination of an ICS and a LABA, in accordance with the GINA 2025 guidelines. Newer, highly lipophilic ICS and LABA molecules with long durations of action (~ 24h) allow once-daily dosing regimens, significantly improving patient compliance and providing sustained protection [73,74]. However, this high lipophilicity causes inhaled particles to dissolve more slowly in the airway lining fluid and increases their adhesion to mucus via hydrophobic interactions with mucins [66,67]. This prolonged luminal retention makes them more prone to elimination by MCC and AMs-mediated phagocytosis, before they can reach their target cells [68,71]. In this context, an inhaled delivery system that facilitates access to the targets of long-acting ICS and ultra-LABA combinations, via improved mucopenetration and the ability to evade AMs uptake, is of great interest. Furthermore, to maximize efficacy of an inhaled drug, this delivery system must bypass deposition in the oropharyngeal sites to reach both the central and peripheral airways. Given these limitations, it is essential to develop innovative delivery systems capable of overcoming physiological barriers in the lungs, improving penetration through mucus, and evading immune recognition. Liposomes, thanks to their ability to encapsulate both hydrophilic and lipophilic molecules, represent a promising solution for optimizing the local bioavailability of active ingredients and reducing systemic side effects. The challenge is also to ensure optimal distribution in the respiratory tract, avoiding oropharyngeal deposition and reaching the central and peripheral regions of the lungs.

These challenges led to the LNPulmo project funded by the Walloon Region (Win2Wal program, agreement No. 2210043), entitled 'Development of inhalable powder containing lipid nanovectors with improved bioavailability and therapeutic efficacy' (Figure 23).

This collaborative project, encompassing three distinct partners, is structured around several following objectives:

- The first phase focused on optimizing a liposomal formulation coencapsulating CIC and IND, evaluating key properties such as drug encapsulation, mucopenetration, evasiveness to AMs phagocytosis, and uptake in epithelial cells. The overall goal of this phase was to identify an optimal formulation that would enhance the therapeutic efficacy of both drugs while allowing for a reduction in dosage to minimize undesirable side effects. The optimization of the liposomal formulation in this study is part of an effort to transition to a production method compatible with industrial-scale manufacturing. Indeed, one of the main obstacles encountered in the development of new delivery systems is the "gap" between

basic research and industrial implementation (“bench-to-bedside”). The approach adopted here aims to bridge this gap by incorporating criteria for robustness, reproducibility, and compliance with industrial requirements (processes without organic solvents, GMP-like environment, Design of Experiments) from the optimization stage onward. This approach provides significant added value: it ensures that the developed formulation is not only therapeutically effective but also transferable and scalable by industrial partners, thereby accelerating patient access to innovation. All these formulations optimization and scale-up of the production method constitute the core focus of the present doctoral thesis, conducted by PhD candidate Tuan Nghia Dinh under the supervision of Professor Géraldine Piel at the Laboratory of Pharmaceutical Technology and Biopharmacy (LTPB), University of Liège (ULiège).

- The optimized liposomal formulation was subsequently used to optimize a spray-drying process, generating liposome-containing dry powders for inhalation. Various process parameters and types of protectants were systematically investigated to produce powders with favorable aerodynamic and aerosolization properties while preserving liposomal structural integrity. This specific research axis forms another doctoral thesis, conducted by PhD candidate Laure-Anne Bya under the supervision of Professor Brigitte Evrard (LTPB, ULiège).

- In parallel with the aforementioned studies, the *ex vivo* and *in vivo* therapeutic efficacies of the liposomal formulations as well as the pharmacokinetic profiles of the liposome-containing dry powders were evaluated to determine the biological activity and physiological fate of the drug-containing nanocarriers in animal models. These preclinical investigations comprise another doctoral thesis, conducted by PhD candidate Benedetta Bottero under the supervision of Professor Didier Cataldo at the Laboratory of Tumor and Development Biology, ULiège.

This project also involves the participation of Aquilon Pharma, a spin-off company of the ULiège founded in 2013 that specializes in enhancing the efficacy of inhaled medicines.

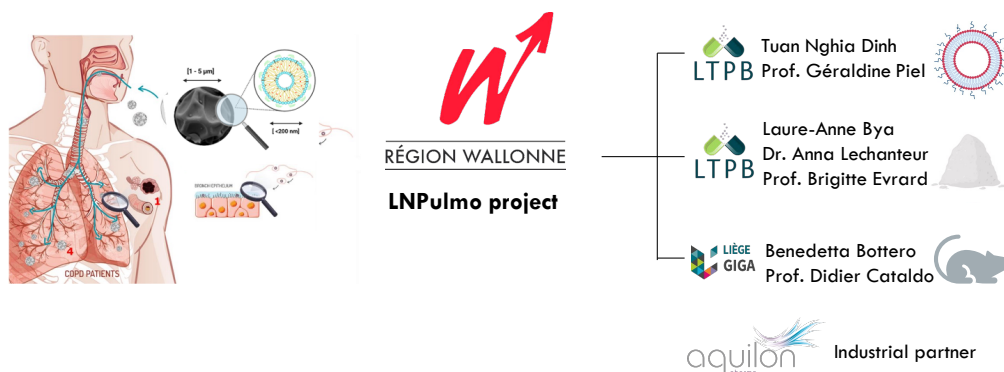


Figure 23. Partners of the LNPulmo projet.

II. Objectives of the study

The primary objective of this doctoral thesis is to develop and validate a liposomal formulation co-encapsulating CIC and IND for pulmonary delivery, with the aim of enhancing anti-asthmatic efficacy while enabling a reduction in administered doses. This work is designed from the outset to facilitate industrial translation, by optimizing both the formulation and the production process in accordance with the requirements of large-scale manufacturing.

- Objective I: Design and optimization of liposomes containing CIC and IND for pulmonary delivery

The first objective of this thesis is to rationally engineer a liposomal formulation for the co-delivery of CIC and IND that overcomes key pulmonary clearance mechanisms (MCC and AMs phagocytosis) and, ultimately, maximizes their *in vivo* anti-asthmatic efficacy. To achieve this, we aim to evaluate the effects of different phospholipid and PEGylated lipid compositions on the critical attributes required for efficient pulmonary delivery. Specifically, we will investigate how fatty acyl chain structures and molar concentrations of these lipid components govern key parameters, including drug encapsulation, mucopenetration, cellular cytotoxicity, AMs evasion, epithelial cell uptake, and relaxation effect on isolated tracheal rings. The capacity of the optimized formulation to enhance therapeutic efficacy will then be validated *in vivo* using an OVA-induced murine model of acute asthma by evaluating the alleviation of AHR and local inflammation. Once the *in vivo* effectiveness of this liposomal platform is established for CIC and IND, this delivery strategy will be translated to FOR. This extension aims to provide a broader perspective on the potential application of the developed liposomal system to a wider range of molecules currently employed in asthma therapy.

- Objective II: Scaling-up liposome production by the PGSS process

To bridge the gap between laboratory research and industrial application (“bench-to-bedside”), the second objective is to adapt and validate a solvent-free PGSS process for the scalable production of the optimized liposomal formulation. Particular attention will be paid to the influence of process parameters on CQAs, the reproducibility of the system, and compliance with GMP-like standards.

Within this section, the initial goal is to adapt the PGSS process parameters at the laboratory scale for the optimal formulation obtained in Objective I, building upon previously established conditions. This step serves to evaluate whether these operating parameters can be adjusted to mitigate the risk of lipid and drug degradation, particularly by reducing the high operational temperature of 80 °C. The resulting data will then serve as the foundation for the scale-up of the PGSS process from laboratory to pilot scale. By using a Design of experiments (DoE) model to simultaneously evaluate multiple parameters of the pilot-scale PGSS apparatus, we aimed to identify optimal operating conditions (lipid concentration, volume of dispersion, temperature, pressure, agitation speed, depressurization rate) to produce the optimal formulation. The target CQAs for this pilot-scale formulation include a mean particle size below 200 nm, a Pdl close to 0.30, drug EE comparable to those achieved at the laboratory scale, and minimized lipid hydrolysis and oxidation. Notably, this work was performed within the Installation qualification and Operational qualification (IQ/OQ-qualified), GMP-like zones of the IMPact Cleanroom platform at ULiège. This facility was specifically engineered to facilitate liposome production within a qualified environment, thereby ensuring the technology transfer to industrial partners.

By integrating formulation and process optimization within an industrial perspective, this thesis aims to deliver a therapeutic platform that is both scientifically innovative and ready for clinical and commercial development, thereby accelerating the translation of research into patient benefit.

EXPERIMENTAL PART

CHAPTER I

Design of liposomes
containing ciclesonide and
indacaterol maleate for
pulmonary delivery

I.1. Introduction to Chapter I

Within this part of the study, the objective was to determine the optimal lipid composition to enhance the therapeutic efficacy of co-encapsulated CIC and IND in an *in vivo* asthmatic model.

We hypothesize that a rationally engineered liposomal system can overcome the physiological barriers of the lung, namely mucus entrapment and AMs clearance, while ensuring efficient drug encapsulation, epithelial uptake, and improved bronchodilatory effect. The optimized formulation will be evaluated for its ability to enhance therapeutic efficacy in an acute asthma mouse model, with a focus on dose-sparing potential and physicochemical stability.

From a liposome design perspective, the lipid membrane serves as the localization site for these two hydrophobic drugs and the PEG shedding process is tightly governed by the interactions and anchoring effect of the lipid moieties within the bilayer. Therefore, it is critical to evaluate the impact of the structure of fatty acyl chains of both the phospholipid and PEGylated lipid, as well as their molar concentrations, on important physicochemical and biological properties of liposomes. These include drug EE, mucopenetration, cytotoxicity and cellular uptake by AMs and lung epithelial cells, the resulting *ex vivo* relaxation efficacy of IND, and *in vivo* anti-asthmatic efficacy of CIC and IND.

Before conducting experimental research, we aimed to establish a specific profile of physicochemical properties for liposomes to optimize the pulmonary delivery of CIC and IND for the following reasons:

- Size smaller than 200 nm: to effectively reach the pulmonary epithelium, liposomes must navigate two primary biological barriers: the airway mucus and AMs. As previously described in section II.2.2.1. *Engineering of liposomes to overcome mucociliary clearance*, maintaining a particle size below 200 - 300 nm is generally required to ensure that NPs are small enough to slip through the pores of the mucin meshwork [142]. Meanwhile, AMs preferentially recognize and phagocytose much larger particles, typically exhibiting peak clearance for particles in the 1 to 6 μm range, while the macrophage uptake of particles under 200 nm is significantly reduced [150]. Therefore, a size smaller than 200 nm was selected to help liposomes bypass both 'size filtering' mechanism of the mucus and the uptake of AMs.

- PdI < 0.30: a PdI value below 0.3 is the standard regulatory requirement to classify a liposomal formulation as monodisperse (homogeneous) in the pharmaceutical domain [178]. As vesicle size strongly influences the liposomes mucopenetration and their evasion of AMs, maintaining a low PdI is critical to guarantee consistent *in vitro* and *in vivo* performance, as well as successful clinical translation.

- Neutral or negative surface charge: the pulmonary mucus network is densely decorated with negatively charged mucin glycoproteins and DNA [98]. If the liposomes are positively charged, they will suffer from strong electrostatic attraction to these mucin fibers, causing them to immediately aggregate, adhere to the mucus, and be rapidly eliminated via the MCC. Therefore, using a neutral or negative surface charge is critical to shield liposomes from electrostatic entrapment, thereby facilitating their penetration through the mucus and minimizing AMs-mediated clearance [243]. The near-neutrality of liposomes is primarily due to the inherently zwitterionic nature of the PC derivatives and the dense PEG layer that can effectively shields and masks the underlying electrostatic charge [244].

- EE of the two drugs as high as possible: maximizing the EE is vital for both therapeutic and manufacturing reasons. From a clinical standpoint, a high EE ensures that the required therapeutic dose of the drug is successfully delivered while minimizing the total amount of lipid carrier administered, which helps avoid toxicity potentially induced by excessive amounts of lipid materials [245]. Furthermore, unencapsulated drugs in the suspension can trigger severe off-target side effects and toxicity [175]. From a manufacturing perspective, maximizing the EE minimizes the waste of expensive drug compounds and reduces the burden of performing time-consuming downstream purification steps to remove unencapsulated materials.

Based on these fundamental physicochemical properties, we continued to build up the optimal biological profile of a liposomal formulation to enhance the therapeutic efficacy of CIC and IND:

- Effective mucopenetration: to efficiently reach the underlying respiratory epithelium, liposomes must be engineered to avoid both the 'size filtering' and 'interaction filtering' mechanisms of the mucus. Maintaining a vesicle size smaller than 200 nm is expected to help them bypass steric obstruction within the mucin mesh. Furthermore, grafting a dense surface layer of PEG in a brush conformation to create a highly hydrophilic, muco-inert shield is a promising strategy to overcome adhesive entrapment.

In this study, the evaluation of these strategies to improve the mucopenetration of liposomes was performed using the Transwell® model, which is extensively used for this purpose in the literature. An initial screening test was conducted to select an appropriate artificial mucus model for this assay. While the artificial mucus formulation by Hamed and Fiegel [246] yielded a gel that was too viscous, causing the liposome suspension to remain on the mucus surface, the formulation by Ibrahim *et al.* [247] allowed the suspension to penetrate through it and was therefore selected for this assay. The precise composition of this artificial mucus is detailed in section 1.2.2.4. *Evaluation of liposomes mucopenetration properties.*

- Minimal toxicity with pulmonary cells, including AMs and lung epithelial cells: before tuning liposomal cellular uptake, it is imperative to ensure that the nanocarriers do not trigger significant cytotoxicity. Establishing this safety profile is particularly crucial for their application in treating chronic inflammatory respiratory diseases such as asthma. The MTT test was chosen as a widely used *in vitro* assay to evaluate the cellular viability and mitochondrial metabolic activity of the cells exposed to the formulations. To mimic the primary biological barriers of the respiratory tract, RAW 264.7, BEAS-2B, and A549 cell lines were selected as representative *in vitro* models for AMs, bronchial epithelial cells, and AT2 cells, respectively.

- Avoidance of AMs uptake and facilitation of lung epithelial cell internalization: while it has already been established that CIC needs to be internalized by lung epithelial cells to be enzymatically metabolized into its active therapeutic form (des-CIC), IND must traverse the epithelial barrier to interact with β_2 ARs located on the underlying ASM cells. Therefore, in the context of liposomal encapsulation, evading phagocytosis by AMs is critical to prevent off-target accumulation and immune clearance of both drugs. Meanwhile, promoting epithelial uptake serves as a targeted intracellular delivery mechanism for CIC. IND could also benefit from this epithelial entry, as its high lipophilicity together with its predominantly zwitterionic form at physiological pH has been proposed to facilitate its translocation across tissue to reach its target receptors on the underlying ASM [39].

While PEGylated lipids introduce a hydrophilic steric shield that improves mucus penetration and limits AMs phagocytosis, the potential interference of this polymer coat on epithelial internalization and the *ex vivo* bronchodilatory effect of IND must be systematically characterized.

Together with these *in vitro* experiments, the impact of the liposomal encapsulation on the *ex vivo* and *in vivo* therapeutic efficacy of CIC and IND was also integrated into the design process and conducted by PhD student Benedetta Bottero (LTBD, ULiège) as a collaboration within the framework of the LNPulmo project:

- *Ex vivo* relaxant effect of IND on isolated rat tracheal rings: this test assesses whether liposomal encapsulation alters maximal relaxant effect and half-maximal effective concentration of IND against methacholine-induced bronchoconstriction in isolated tracheal tissue. This serves as a critical assessment to finely tune the liposome composition to maximize the *ex vivo* relaxation effect of encapsulated IND.

- *In vivo* evaluation of CIC and IND in a murine model of acute asthma: this important assay aims to provide a proof-of-concept for the improved therapeutic efficacy of CIC and IND loaded within the liposomal formulation optimized from both *in vitro* and *ex vivo* experiments. The alleviation of AHR and the inflammatory state of OVA-induced asthmatic mice was thoroughly evaluated via the analysis of lung mechanics and inflammatory manifestations (inflammatory cells in BALF, inflammatory cytokines in whole-lung homogenates, and histological analysis).

Once the *in vivo* effectiveness of liposomal encapsulation in improving the therapeutic profile of IND was established, we sought to investigate whether this strategy could be translated to formoterol (FOR), a conventional LABA widely used in clinical asthma management. This extension aims to provide a supplementary overview regarding the potential application of liposomal delivery systems to a broader range of molecules currently employed in asthma therapy.

1.2. Optimizing the composition of liposomes containing ciclesonide and indacaterol maleate for pulmonary delivery

Introduction and overview of the results currently under peer review in International Journal of Pharmaceutics:

This study aims to evaluate the impact of the structure of fatty acyl chains of both the phospholipid and PEGylated lipid, as well as their molar concentrations, on the different key liposomes properties. Chemical structures of PEGylated lipids and phospholipids used in this optimization study are illustrated in Figure 24.

The investigated PEGylated lipids share an identical PEG chain length (PEG₂₀₀₀) but vary in their hydrophobic fatty acyl chain configurations and the presence of a phosphate group

within the polar linker region. Specifically, the phosphate-containing PEGylated lipids feature symmetric, saturated fatty acyl chains of 14 carbons (DMPE-PEG), 16 carbons (DPPE-PEG), and 18 carbons (DSPE-PEG). Conversely, the phosphate-free variants comprise symmetric, saturated chains of 14 carbons (DMG-PEG), 18 carbons (DSG-PEG), and an 8-carbon ceramidyl derivative (C₈-PEG-Ceramide).

Meanwhile, the phospholipids, all PC derivatives, varied in length and degree of unsaturation of the fatty acyl chain. This group includes two saturated phospholipids with 18-carbon and 16-carbon chains (DSPC and DPPC, respectively), along with three unsaturated phospholipids: two 18-carbon phospholipids containing either one double bond per chain (DOPC) or two double bonds per chain (DLPC), and a 16-carbon variant with one double bond per chain (DPoPC).

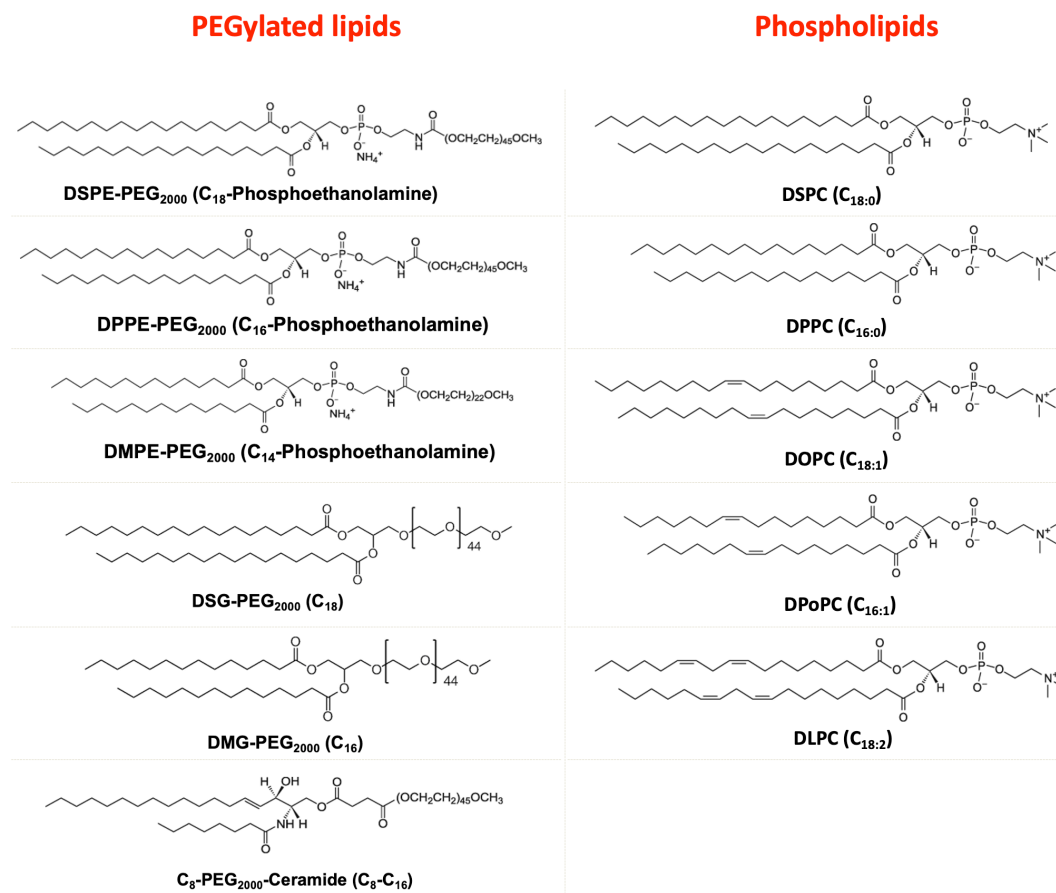


Figure 24. Chemical structures of PEGylated lipids and phospholipids used in the study.

Regarding the operational workflow, this part was divided into the sequential optimization of the PEGylated lipid and then the phospholipid components via *in vitro* and *ex vivo* experiments, before the optimal formulation was subjected to *in vivo* testing. The concentration of Chol was fixed at 30% for all experiments. For the initial optimization of the PEGylated lipid, SPC was used as the main phospholipid, while the PEGylated lipid was varied in terms of both type and concentration (0–30%). Varying the PEGylated lipid concentration across this broad range enabled a comprehensive evaluation of the polymer structural transitions, progressing from the low-density "mushroom" regime to the densely packed "brush" conformation needed for muco-inertness [248]. Following the identification of the optimal type and concentration of the PEGylated lipid, a similar optimization process was conducted to select the most suitable phospholipid.

It was demonstrated that within the investigated PEGylated lipids, DSPE-PEG was selected as the optimal one. Its phosphate group in the linker maximizes IND encapsulation via favorable amine-phosphate electrostatic interactions, while its hydrophobic 18-carbon anchor exhibits superior biocompatibility with macrophages compared to its shorter C14 (DMPE-PEG) and C16 (DPPE-PEG) analogs. Integrating DSPE-PEG at a concentration of 15% facilitated mucus penetration and AMs evasion, and significantly enhanced the *ex vivo* bronchodilatory efficacy of IND relative to the free drug solution.

Concurrently, DLPC was selected as the most appropriate main phospholipid. The high degree of acyl chain unsaturation within DLPC proved critical for achieving high encapsulation efficiencies for both hydrophobic payloads, promoting efficient intracellular internalization by airway epithelial cells, and maximizing the *ex vivo* relaxation potency of IND.

The optimal formulation (**DLPC/Chol/DSPE-PEG, 55/30/15**) simultaneously loaded with CIC and IND markedly attenuated AHR and suppressed the type 2 inflammatory cascade in the OVA-induced asthmatic model.

Published results in the International Journal of Pharmaceutics [249]:

Ciclesonide/indacaterol-encapsulated liposomes: optimizing corticosteroid-bronchodilator therapy for a more effective approach to asthma management

Tuan Nghia Dinh^{a,1}, Benedetta Bottero^{b,1}, Fabienne Perin^b, Laure-Anne Bya^a, Anna Lechanteur^a, Brigitte Evrard^a, Géraldine Piel^{a,*}, Didier Cataldo^{b,*}

^aLaboratory of Pharmaceutical Technology and Biopharmacy, Center for Interdisciplinary Research on Medicines (CIRM), University of Liege, Avenue Hippocrate 15, 4000 Liege, Belgium.

^bLaboratory of Tumor and Development Biology, GIGA-Cancer and CHU of Liège, University of Liège, Avenue Hippocrate 15, 4000 Liege, Belgium.

* Corresponding author: Géraldine Piel (geraldine.piel@uliege.be), Didier Cataldo (Didier.Cataldo@uliege.be)

¹ These authors contributed equally to this work

KEYWORDS:

Liposome, ciclesonide, indacaterol maleate, asthma, lipid, pulmonary delivery

ABSTRACT

Combination therapy with inhaled corticosteroids (ICSs) and long-acting β_2 -agonists (LABA) is the clinical cornerstone of asthma management. However, therapeutic efficacy is frequently compromised by high drug hydrophobicity, which enhances entrapment within the pulmonary mucus and promotes recognition and clearance from alveolar macrophages. This results in reduced drug availability at the target site, requiring higher doses or more frequent administrations. To overcome these biological barriers and improve the therapeutic outcomes, we report the systematic optimization of a liposomal formulation for the co-delivery of ciclesonide (CIC) and indacaterol maleate (IND). Among various PEGylated lipids, DSPE-PEG was chosen because its amine function maximizes IND encapsulation via amine-phosphate interactions and its 18-carbon chain exhibited superior biocompatibility with macrophages compared to C14 and C16 analogs. Incorporating

DSPE-PEG at 15% ensured robust mucopenetration and macrophage evasion, significantly improving the ex vivo relaxation effect of IND compared to the drug solution. DLPC was utilized as the main phospholipid since its high degree of unsaturation (two double bonds) was essential for achieving high EE of both hydrophobic payloads, efficient epithelial cell uptake and improved ex vivo efficacy and potency of IND. In an OVA-induced murine model, the optimized formulation markedly attenuated airway hyperresponsiveness and suppressed the type 2 inflammatory cascade, including upstream epithelial-derived alarmins, downstream inflammatory cytokines and inflammatory cell recruitment. Notably, liposomal encapsulation also provided a potential dose-sparing effect for both compounds, suggesting that efficient pulmonary delivery of ICS/LABA via lipid nanoparticles is a promising asthma management strategy and may be applicable to other respiratory diseases.

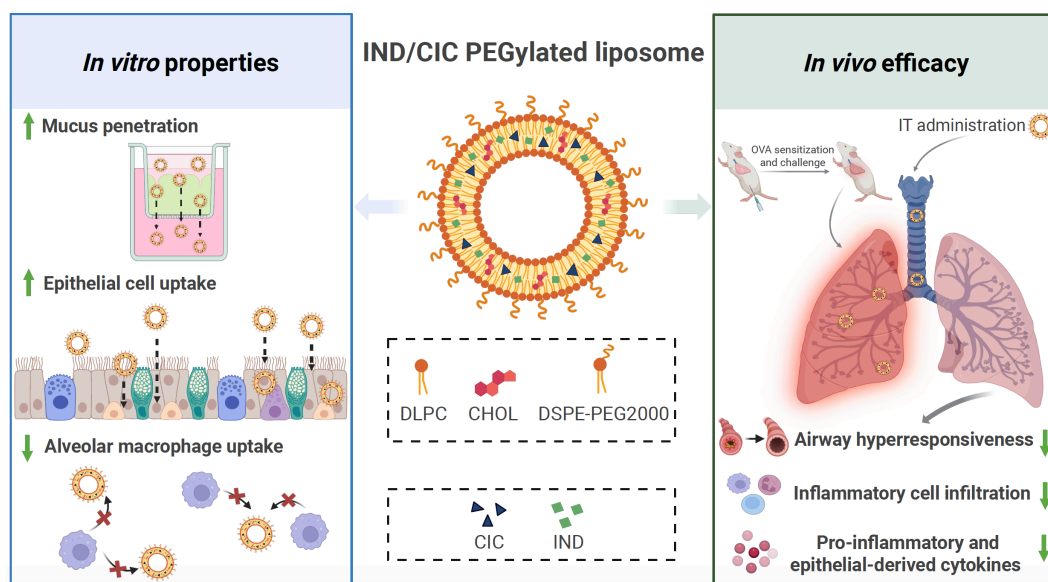


Figure 25. Schematic illustration of the use of liposomes to improve the therapeutic efficacy of CIC and IND, with good mucopenetration and the ability to evade phagocytosis by AMs.

I.2.1. Introduction

High type 2 immunity (eosinophilic) asthma is particularly prevalent, accounting for approximately 50% of adults with asthma and up to 80% of those with severe disease [8]. Inhaled corticosteroids (ICSs) remain the cornerstone of anti-inflammatory therapy for this endotype [250]. ICS therapy is often co-administered with long-acting bronchodilators via

the pulmonary route to provide symptomatic relief and control underlying inflammation. Pulmonary delivery allows drugs to reach their target cells rapidly (epithelial and immune cells for ICSs and airway smooth muscle (ASM) cells for bronchodilators) while reducing potential systemic effects [251]. However, inhaled drugs must first overcome the airway mucus layer, which protects the tracheobronchial epithelium from inhaled pathogens. If inhaled particles are trapped in mucus, they will be subsequently removed by mucociliary clearance [142]. Conversely, in alveolar regions where the mucus layer is absent, the primary clearance mechanism is mediated by alveolar macrophages, which can rapidly internalise particles by phagocytosis and can sequester and/or degrade inhaled particles [142]. In asthma, the increased secretion of mucus, its elevated mucin and DNA content and the infiltration of immune cells can make inhaling drug-loaded particles even more challenging [252,253].

Furthermore, the high hydrophobicity of small molecule drugs (e.g. indacaterol maleate IND) ($\log P = 4.05$), cinnarizine ($\log P = 5.5$)) has been shown to limit their ability to cross this barrier as compared to the more hydrophilic compounds [66,254]. In asthma patients, such impaired mucopenetration is proposed to result in a greater quantity of hydrophobic drugs, such as fluticasone propionate, being trapped within the airway mucus and subjected to mucociliary clearance compared to less hydrophobic molecules, such as budesonide [71]. This barrier, together with phagocytosis by alveolar macrophages and insufficient deep-lung deposition due to formulative constraints, can reduce the drug residence time and the fraction that reaches the site of action, contributing to the need for frequent administrations or higher dosages of currently available inhalable formulations [255–257]. Paradoxically, the high lipophilicity of modern long-acting ICSs and long-acting β_2 -agonists (LABAs) is the result of chemical engineering to try to enhance lung retention and prolong duration of action [26,57,58]. This underscores a critical need for strategies to enhance the delivery of these drugs to their intended cellular targets before they are eliminated by mucociliary clearance and macrophages-mediated degradation.

Liposomes are lipid-based vesicles which are mainly composed of cells-inspired components such as phospholipids and Chol have been widely studied to enhance the drug transmucosal delivery [156,243,258]. Generally, particles smaller than 200 nm are considered favorable for penetrating the mucin meshwork [142]. Moreover, the incorporation of polyethylene glycol-lipid conjugates (PEGylated lipids) provides a hydrophilic surface that limits hydrophobic interactions with cysteine-rich subdomains and electrostatic interactions with negatively charged sialic acid and sulfate moieties in the

structure of mucin, as well as with phosphoric acid residues of DNA [98,259]. The PEG layer can also protect liposomes from macrophage uptake thanks to its ability to reduce the adsorption of opsonized proteins on these vesicles [260].

Nevertheless, relatively few studies have explored liposomes for co-delivery of ICS and LABA. Penoy *et al.* successfully produced dry powders of liposomes containing budesonide and salbutamol at contents comparable to Pulmicort® and Ventolin® [261]. Other nanocarrier systems have also been investigated, such as polyamide-based nanocapsules loaded with fluticasone propionate and salmeterol, which exhibited extended drug release and improved aerodynamic behavior compared with Seretide Diskus® [262]. While these formulations demonstrated promising physicochemical and lung delivery characteristics, their efficacy in improving therapeutic outcomes of the combination ICS-LABA remains to be confirmed in relevant biological models. To address this gap, it remains to be elucidated how the choice of lipid composition in liposomes influences their overall performance, from drug encapsulation, mucus penetration and macrophage evasion to subsequent delivery of ICSs and LABAs to target cells *in vivo*.

In this study, we developed a liposomal formulation for the combined delivery of the long-acting ICS ciclesonide (CIC) and the LABA indacaterol maleate (IND), a previously unused ICS-LABA combination. We focused on how modulation of PEGylated lipids and phospholipids increases the efficacy of inhaled liposomal drugs. Specifically, we examined the role of lipid chain structure while keeping the hydrophilic headgroups constant (PEG₂₀₀₀ for PEGylated lipids and choline for phospholipids). After identifying the optimal final liposomal formulation via different *in vitro* and *ex vivo* tests, we thoroughly assessed its efficacy in reducing airway inflammation and bronchoconstriction in an *in vivo* model of eosinophilic asthma.

1.2.2. Materials and methods

1.2.2.1. Materials

Soy phosphatidylcholine with 95% L- α -phosphatidylcholine (SPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine (DPoPC), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (16:0 PE Rhod), cholesterol (Chol), 25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol (25-NBD CHOL), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (ammonium salt) (DSPE-PEG), distearoyl-rac-

glycero-3-methoxy [(polyethylene glycol)-2000]) (DSG-PEG), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DPPE-PEG), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DMPE-PEG), 1,2-dimyristoyl-rac-glycero-3-methoxy [(polyethylene glycol)-2000] (DMG-PEG) and N-octanoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (Cer-PEG) were purchased from Avanti Polar Lipids, Inc. (AL, USA). Micronized CIC (98%) was kindly provided from NewChem spa (Milan, Italy) and IND (99%) was purchased from LEAP Chem (Zhejiang, China).

1.2.2.2. Liposomes preparation by the thin film hydration method

Phospholipids, Chol (30%, molar ratio), PEGylated lipid, CIC and IND (5% of total lipids) were dissolved in ethanol in a round-bottom flask. The solvent was evaporated at 30 °C using a rotary evaporator (BÜCHI Labortechnik GmbH, The Netherlands), forming a thin lipid film. The film was rehydrated with HEPES buffer (10 mM, pH 7.4), and the resulting dispersion was sequentially extruded three times through polycarbonate membranes with pore sizes of 400 nm and 200 nm (Whatman, UK). The theoretical lipid concentration in the final liposome suspensions was 5 mM. To remove non-encapsulated CIC and IND, liposomes were dialyzed against HEPES buffer (1:20, v/v) for 4 h at 4 °C, with the external medium renewed every hour. Liposomes were stored at 4 °C until further analysis. For mucopenetration and uptake studies, empty liposomes were fluorescently labeled with 1% 25-NBD-Chol and 1% 16:0 PE-Rhod, respectively.

1.2.2.3. Characterization of liposomes physicochemical properties

1.2.2.3.1. Particles size, polydispersity index and zeta potential

Particle size and polydispersity index (Pdl) were determined by dynamic light scattering (DLS) using a Malvern Zetasizer® Nano ZS (Malvern Instruments, UK) at 25 °C and a fixed scattering angle of 90°. Samples were diluted in HEPES buffer (10 mM, pH 7.4) to a final lipid concentration of 0.45 mM before measurement. Zeta potential (mV) was measured with the same instrument. All measurements were performed in triplicate.

1.2.2.3.2. Encapsulation efficiency and drug loading

Encapsulation efficiency (EE) and drug loading (DL) of CIC and IND were determined by quantifying drug concentrations (µg/mL) before and after dialysis. For quantification, liposome samples were diluted ten-fold in methanol to disrupt the bilayer. EE and DL were then calculated according to the following equations:

Equation 1. EE determination

$$EE (\%) = \frac{\text{Drug concentration after dialysis}}{\text{Drug concentration before dialysis}} \times 100$$

Equation 2. DL determination

$$DL (\%) = \frac{\text{Drug mass after dialysis } (\mu\text{g})}{\text{Theoretical lipids mass } (\mu\text{g})} \times 100$$

1.2.2.3.3. Drugs quantification

CIC and IND concentrations were quantified using a High Performance Liquid Chromatography (HPLC) method. The HPLC system was an Agilent® 1100 (Santa Clara, USA) controlled by OpenLab CDS LC ChemStation software (version C.01.05). The stationary phase was a Xbridge BEH C18 column (3.5 μm , 3.0×50mm) maintained at 30 °C. The mobile phase consisted of an ammonium acetate buffer at pH 10 (A) and methanol (B) with the following gradient elution at a flow rate of 0.7mL/min: 0-1 min 80% A; 10-13 min 5% A; 13.5-20 min 80% A. The injection volume was 10 μL . The UV detector was set at 243 nm wavelength. The limit of quantification of CIC and IND are 3.205 and 2.031 $\mu\text{g/mL}$, respectively.

1.2.2.4. Evaluation of liposomes mucopenetration properties

24-well Transwell® plate (polycarbonate membrane, 3.0 μm pore size) was used to evaluate the diffusion of liposomes through an artificial mucus. The artificial mucus was prepared as previously described by stirring 25 μL of sterile egg yolk emulsion, 25 mg of mucin type II from porcine stomach, 30 μL of DTPA (1 mg/mL), 20 mg of calf thymus DNA, 25 mg of NaCl, 11 mg of KCl, and 100 μL of RPMI into 5 mL of water [263]. 75 μL of artificial mucus was stratified on the upper chamber of the semipermeable filter, while the bottom chamber was filled with 300 μL of simulated interstitial lung fluid (SILF). SILF (NaCl 0.1 M, KCl 4 mM, Na_2HPO_4 1 mM, Na_2SO_4 0.5 mM, CaCl_2 2.5 mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.5 mM, NaHCO_3 31 mM, CH_3COONa 7 mM, sodium citrate monohydrate 0.36 mM) was prepared as previously reported [264]. 75 μL of empty liposomes labeled by 25-NBD-Chol were then added to the upper chamber. The amount of permeated liposomes in the basolateral chamber was quantified by measuring the fluorescence intensity of the SILF at different time points (λ_{exc} / λ_{ems} : 466/519 nm).

1.2.2.5. Cytotoxicity and cellular uptake***1.2.2.5.1. Cells culture***

RAW 264.7, A549, and BEAS-2B cells were purchased from ATCC (VA, USA). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at 37 °C in 5% CO₂-humidified atmosphere.

1.2.2.5.2. Cell viability assay

RAW 264.7, BEAS-2B, and A549 cells were seeded in 96-well plates at a density of 2×10^4 , 1×10^4 , and 5×10^3 cells/well, respectively [265,266]. After 24 hours, cells were incubated with liposomes diluted in Opti-MEM™ (Gibco, USA) to 0.5, 0.25 and 0.05 mM for either 4 or 24 h. Following the incubation, the formulations were replaced with fresh culture medium containing MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent, and cells were incubated for an additional 3 h at 37 °C. A solution containing 10% sodium dodecyl sulfate and 0.01 M hydrochloric acid was then added, and incubation was continued for up to 24 h. Untreated cells served as the positive control, while cells treated with 20 mM hydrogen peroxide solution were used as the negative control. Absorbance was measured at 590 nm.

1.2.2.5.3. Cellular uptake

RAW 264.7, BEAS-2B, and A549 cells were seeded in 24-well plates at a density of 2×10^5 , 1×10^5 , and 1×10^5 cells/well, respectively. After 24 h (for RAW 264.7 and A549) or 48 h (for BEAS-2B), cells were incubated with 16:0 PE-Rhod-labeled liposomes diluted in Opti-MEM™ to 0.25 mM for 4 h. Following treatment, cells were washed twice with PBS, detached using Trypsin-EDTA®, and resuspended in PBS. Cells were then analyzed using a BD LSRFortessa™ flow cytometer (λ_{exc} / λ_{ems} : 561/585 nm).

1.2.2.6. Ex vivo relaxant effect of indacaterol maleate on isolated tracheal rings from rats***1.2.2.6.1. Animals***

Healthy male Wistar IGS rats (body weight 250-350 g), obtained from the University of Liege's animal facility (ULiège, Belgium), were used. The animals were housed under a 12-h light/dark cycle in a temperature- and humidity-controlled room with food and tap water *ad libitum*. All animal care and experimental procedures complied with the animal

protection and welfare guidelines and were approved by the Animal and Ethics Review Committee of the University of Liège (ethical approval number: 24-2712).

1.2.2.6.2. Preparation of Isolated Tracheal Rings from healthy Wistar rats

Healthy Animals were sacrificed with an intraperitoneal injection of sodium pentobarbital (200 mg/kg, Euthasol, Dechra Regulatory B.V, Netherlands). The trachea was excised and immediately placed in Krebs–Henseleit solution prepared from modified Krebs–Henseleit buffer powder (Sigma-Aldrich, USA), supplemented with CaCl_2 (2.5 mM) and NaHCO_3 (25 mM) [267]. After careful removal of surrounding tissues, tracheae were cut into 3–5 mm rings. Following calibration of force transducers (1 and 10 g), rings were mounted on triangular metal hooks in 20-mL organ baths (EMKA Technologies, France) containing Krebs–Henseleit solution maintained at 37 °C, pH 7.4, and continuously gassed with 95% O_2 /5% CO_2 [267,268]. Isometric tension was recorded using force displacement transducers connected to an IOX data acquisition system (EMKA Technologies). Tissue viability was confirmed by two successive additions of potassium chloride (KCl, 80 mM). Tracheal contraction was then induced with methacholine chloride (3×10^{-6} M; EC_{80}). After stabilization (10 min), cumulative concentrations of IND, dissolved in water or liposome-encapsulated, with or without CIC, were added. Empty liposomes and HEPES buffer were used as controls. At the end of each experiment, atropine sulfate (1 μM) was added to induce maximal relaxation, which was used for normalization. Relaxation responses were expressed as a percentage of atropine-induced maximal relaxation, and half maximal effective concentration (EC_{50}) values were calculated to compare relaxant potency among formulations.

1.2.2.7. In vivo evaluation in murine asthma model

1.2.2.7.1. Animals

In vivo experimental procedures using Balb/cJRj mice complied with the animal protection and welfare guidelines and were approved by the Animal and Ethics Review Committee of the University of Liège (ethical approval number: 24-2712). Balb/cJRj male mice aged 8 weeks-old (Janvier Labs, France) and weighing 20-25 g were randomly divided in groups of 6 per cage and housed under controlled conditions (12 h light/dark cycle, 25 °C ambient temperature, 50% humidity) with *ad libitum* access to food and water at the University of Liège's animal facility (Liège, Belgium). Mice were acclimated for 1 week before starting the model.

1.2.2.7.2. Preparation of ciclesonide and indacaterol maleate solution as non-liposomal formulation

Given the very low aqueous solubility of CIC, hydroxypropyl- β -cyclodextrin (HP β CD) was incorporated to promote its solubilization through inclusion complex formation. HP β CD was first dissolved in HEPES buffer (pH 7.40, without NaCl), after which CIC was added at approximately 60 °C and stirred for 3 h to allow complexation. Next, IND was added to the solution and the mixing was continued for 2 h. The resulting solution was then cooled to room temperature under agitation, and the final volume was adjusted to 100 mL with HEPES buffer. NaCl was subsequently incorporated to achieve isotonicity. The final concentration of HP β CD was 5% (w/v).

1.2.2.7.3. Murine model of asthma induced by allergen exposure

Male Balb/cJRj mice (9 weeks old) were sensitized with intraperitoneal injection of 10 μ g grade V ovalbumin (OVA) (>98% purity, Sigma, USA) mixed 1:1 (v/v) with Alum (Alhydrogel adjuvant 2%, InvivoGen) in 200 μ L of sterile PBS on days 1 and 8. Healthy mice received the same volume of sterile PBS mixed 1:1 with Alum. From day 24 to 26, mice were challenged with grade III OVA aerosol (1 mg/mL), administered for 1h in a total body exposure chamber coupled to an ultrasonic nebulizer (Fazzini, Italy), at a nebulization rate of 2 mL/min. The healthy control group (SHAM group) received saline (PBS) aerosol on the same schedule [269,270]. To assess the therapeutic effects of treatments, two doses were used: the low dose with 0.0326 mg/kg CIC and 0.0307 mg/kg IND and the high dose which was ten-fold higher. The calculation of these doses was described in the appendix I section 1.1.2. *Calculation of test concentrations for in vivo study*. These drugs were coencapsulated in the optimal liposomal formulation or prepared in solution as described in the section 1.2.2.7.2. *Preparation of ciclesonide and indacaterol maleate solution as non-liposomal formulation*. From day 21 to 26, and one hour before airway responsiveness measurements on day 27, each group was intratracheally administered 50 μ L of liposomes containing drugs at low (Lipos_{LOW} group) and high dose (Lipos_{HIGH} group), solution containing drugs at low (Sol_{LOW} group) and high dose (Sol_{HIGH} group), HP β CD solution at 50 μ g/mL (HP β CD group) and empty liposomes (Lipos_{EMPTY} group, administered at the same liposome concentration as Lipos_{HIGH} but without drugs). SHAM group receiving an equivalent volume of HEPES was used as negative control group.

1.2.2.7.4. Airway responsiveness measurements

Twenty-four hours after the final allergen challenge, and one hour after the last intratracheal administration, respiratory mechanics was assessed using the FlexiVent system (SCIREQ, Montréal, Québec, Canada) as previously described [271,272]. Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and tracheotomy was then performed. An 18-gauge polyethylene cannula was inserted into the trachea and connected to the FlexiVent via an FX adaptor Y-tubing. After baseline measurements (Appendix I, section 1.1.3. *Airway responsiveness measurements*), increasing concentrations of methacholine chloride (1.25, 2.5, 5, 10, 20, and 50 mg/mL) were aerosolized using an ultrasonic nebulizer (ICN Biomedicals), and delivered to the inspiratory line of the FlexiVent using a flow of medical air, following the manufacturer's instructions. Before each nebulization, two deep inflations to 30 cmH₂O for lung recruitment were performed. Fifteen seconds after nebulization, the SnapShot-150 and the Quick Prime-3 were performed eleven times in an alternating fashion, with 8 seconds of tidal breathing between each. The maximum value obtained after each nebulization was used to plot respiratory system parameters (airway resistance (R_{rs}), dynamic compliance (C_{rs}), elastance (E_{rs}), and inspiratory capacity (IC), tissue damping, elasticity, Newtonian resistance) as a function of methacholine concentration. Respiratory mechanics was evaluated using the latest version the FlexiVent operating software (flexiware version 8.1.3).

1.2.2.7.5. Cell differential analysis in BALF using Diff-Quik staining

The cellular pellets derived from BALF fractions (Appendix I, section 1.1.4. *Collection of BALF*) were resuspended in 1 mL of ice-cold PBS–EDTA (50 µM), and the total cell concentration was determined using a Coulter Beckman counter (Z2 Coulter Beckman particle count and size analyser, Analys). To analyze the immune cell types in BALF, cells were migrated at increasing concentrations onto a slide using a Cytospin centrifuge (Cytofuge 2, Beckman Coulter diagnostics) at 4400 rpm for 2 minutes. Cells were then stained with Diff-Quik staining solution according to the manufacturer's instructions. Eosinophils, neutrophils, lymphocytes, lung epithelial cells and macrophages were identified based on their morphology and counted under a microscope (Olympus CX31, Keyelce, Belgium). 300 cells per slide were counted in at least 3 different spots of the slide. Total cell count (cells/µL), percentage cell count (%) (Figure 58) and absolute cell count (cells/µL) for each cell type were reported.

I.2.2.7.6. Histological analysis

After BALF collection, the lungs were isolated. The right lung was snap-frozen immediately in liquid nitrogen (N₂) and stored at -80 °C for further analysis. The left lung was infused with 4% paraformaldehyde and embedded in paraffine for histological analysis. Sections of 5-µm thickness from all lobes were stained with hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS). H&E staining was used to assess airway inflammation and to grade the peribronchial infiltrates. PAS staining was used to identify mucus-containing epithelial cells (goblet cells) and to quantify goblet cell hyperplasia (Appendix I, section I.1.5. *Histological analysis*). All counts were performed by an observer blinded to the experimental groups and using a reproducible scoring system, as previously described [269,273]. Images were captured using the Hamamatsu scanner (NanoZoomer 2.0HT) at various magnifications.

I.2.2.7.7. Lung cytokines measurements

The right lungs from mice in the Lipos_{LOW}, Lipos_{HIGH}, Sol_{LOW}, Sol_{HIGH}, HpβCD, Lipos_{EMPTY} and SHAM groups were homogenized (Appendix I, section I.1.6. *Lung homogenates and protein extraction*) and IL-4, IL-5, IL-13, TSLP, and IL-33 (samples diluted 1:25 in Reagent Diluent) levels were measured using ELISA kits (BioTechne, Ireland), in accordance with the manufacturer's instructions. Cytokine concentrations were reported as absolute values in pg/mL. Lung total protein concentration (mg/mL) and cytokine concentrations normalized to total lung protein (pg/mg) are also provided in the Appendix I.

I.2.2.7.8. Statistics

All statistical analyses were conducted using GraphPad Prism 8.4.3 software (GraphPad Software Inc., San Diego, CA). Results are reported as mean ± standard deviation (SD) or standard error of the mean (SEM). Unpaired t-test was applied to compare two columns between them. For multiple comparisons, one-way or two-way ANOVA followed by Tukey or Holm-Sidak multiple comparisons post-tests were used. A *p* value <0.05 was considered statistically significant.

I.2.3. Results

In the first part of this study, we focused on the influence of liposome composition on their physicochemical characteristics and bioactivity. Specifically, we examined the role of two main lipid components, PEGylated lipids and phospholipids, whose chemical structures are shown in Figure 24. The concentration of Chol was maintained at 30% (molar ratio), a ratio

commonly used to ensure membrane stability [274]. Our approach consisted of first evaluating different types (DSPE-PEG, DPPE-PEG, DMPE-PEG, DSG-PEG, DMG-PEG and C₈-PEG-Ceramide) and concentrations (0, 5, 15 and 30%) of PEGylated lipids, using SPC as the primary phospholipid. The investigated PEGylated lipids share an identical PEG molecular weight (PEG₂₀₀₀) but differ in their hydrophobic fatty acyl chain configurations and the presence of a phosphate group within the polar linker region. Specifically, the phosphate-containing PEGylated lipids feature symmetric, saturated fatty acyl chains consisting of 14 carbons (DMPE-PEG), 16 carbons (DPPE-PEG), or 18 carbons (DSPE-PEG). Conversely, the phosphate-free variants include symmetric, saturated chains of either 14 carbons (DMG-PEG) or 18 carbons (DSG-PEG) and an 8-carbon ceramidyl derivative (C₈-PEG-Ceramide).

Once the most suitable PEGylated lipid had been identified, a second optimization step was performed by replacing SPC with synthetic phospholipids. All prepared liposomes exhibited the required physicochemical properties. All formulated liposomes exhibited the desired physicochemical properties, including a mean diameter below 200 nm (ranging from 80 to 190 nm), a Pdl below 0.30 (0.07 to 0.27), and a neutral or negative zeta potential (ranging from -2.2 to -1.7 mV).

1.2.3.1. Impact of the PEGylated lipid type and concentration on key liposome properties

1.2.3.1.1. Impact of PEGylated lipids on mucopenetrating properties

The first tests evaluated liposomes penetration through an artificial mucus layer in relation to the concentration of each PEGylated lipid. At 30 min and 1 h after deposition, most formulations containing 15% and 30% PEGylated lipids generally showed significantly higher percentages of permeated particles than those containing 0% or 5% PEG. The exceptions were DPPE-PEG and DMPE-PEG formulations, which only demonstrated a clear improvement at the 30% PEG concentration (Figure 26A). Furthermore, the 30% formulation was more effective than the 15% formulation in increasing the penetration at certain points, such as DSG-PEG after 30 min and C₈-PEG-Ceramide after 1 h.

When the performance of liposomes containing different concentrations (15% and 30%) of PEGylated lipids was compared over a period of 4 h (Figure 51), formulations containing DPPE-PEG and DMPE-PEG appeared to penetrate slower than the others. However, all PEGylated lipids were retained for further testing at 15% and 30%.

EXPERIMENTAL PART – CHAPTER I

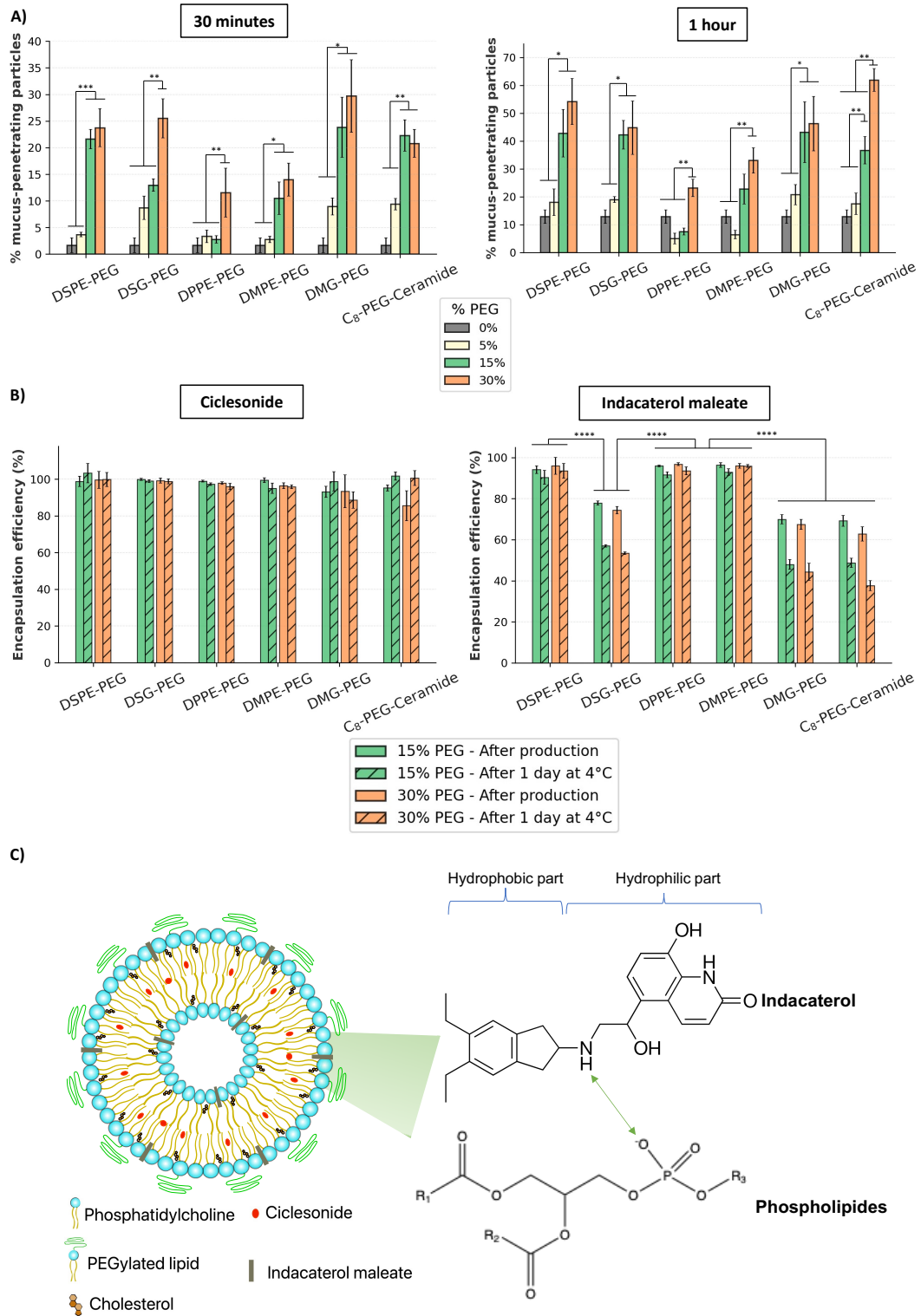


Figure 26. (A) Percentages of permeated liposomes after 30 min and 1 h. (B) Encapsulation efficiency of CIC and IND in function of type and concentrations of PEGylated lipids (n=3). Data are expressed as mean $\pm$ SD. One-way ANOVA followed by Holm-Šidák post test were performed: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (C) Proposed localisation of CIC and IND in liposomes and electrostatic interactions between amin functions of indacaterol with phosphate group of phospholipids.

1.2.3.1.2. Impact of the type and concentrations of PEGylated lipid on drugs encapsulation

CIC was well encapsulated in all formulations with EE above 90% (Figure 26B). IND displayed particularly high and stable encapsulation (~90%) in formulations containing PEGylated lipids containing phosphate groups, namely DSPE-PEG, DPPE-PEG, and DMPE-PEG, while the others had EE below 80% and displayed IND leakage of around 20% after only 1 day of storage at 4 °C (Figure 26B). The increased IND encapsulation in liposomes containing PEGylated phospholipid likely implies a specific interaction between IND and the phosphate groups of lipids.

For next stage of optimisation, formulations containing DSPE-PEG, DPPE-PEG, and DMPE-PEG were selected.

1.2.3.1.3. Impact of the type and concentrations of PEGylated lipid on liposomes uptake by macrophages and potential toxicity

Firstly, we assessed the potential toxicity of empty liposomes containing DSPE-PEG, DPPE-PEG, and DMPE-PEG on RAW264.7 macrophages. cells incubated with formulations without PEGylated lipids at 0.5 mM showed good viability after 24 h (Figure 27A). Liposomes containing 15% DSPE-PEG maintained 100% cell viability after 4 h, whereas those containing the same concentration of DPPE-PEG or DMPE-PEG decreased this value significantly to around 78% and 58%, respectively. This trend was also observed with formulations containing 30% PEGylated lipid (Two-way ANOVA test, $p < 0.05$). The toxic effect on macrophages was likely to be caused by the PEGylated lipids, as formulations without PEGylated lipid preserved cellular viability. Particularly, the results indicated that formulations containing DMPE-PEG exhibited the highest toxicity, followed by those containing DPPE-PEG. Notably, the toxicity increased when the concentration of DPPE-PEG and DMPE-PEG increased from 15% to 30%.

DSPE-PEG was the only PEGylated lipid retained for further optimisation steps. We then verified whether DSPE-PEG at the concentrations of 15% and 30% could reduce liposomes uptake by macrophages compared to a formulation without DSPE-PEG which was used as a negative control. Although all the groups had the same percentage of rhodamine positive cells at 100%, the results showed that macrophages incubated with both DSPE-PEG 15%

and 30% liposomes at 0.25 mM had significantly lower mean fluorescence intensities (MFI) of rhodamine compared to the control (Figure 27B). This proved the shielding effect of the PEG layer which effectively protected liposomes from being taken up by macrophages.

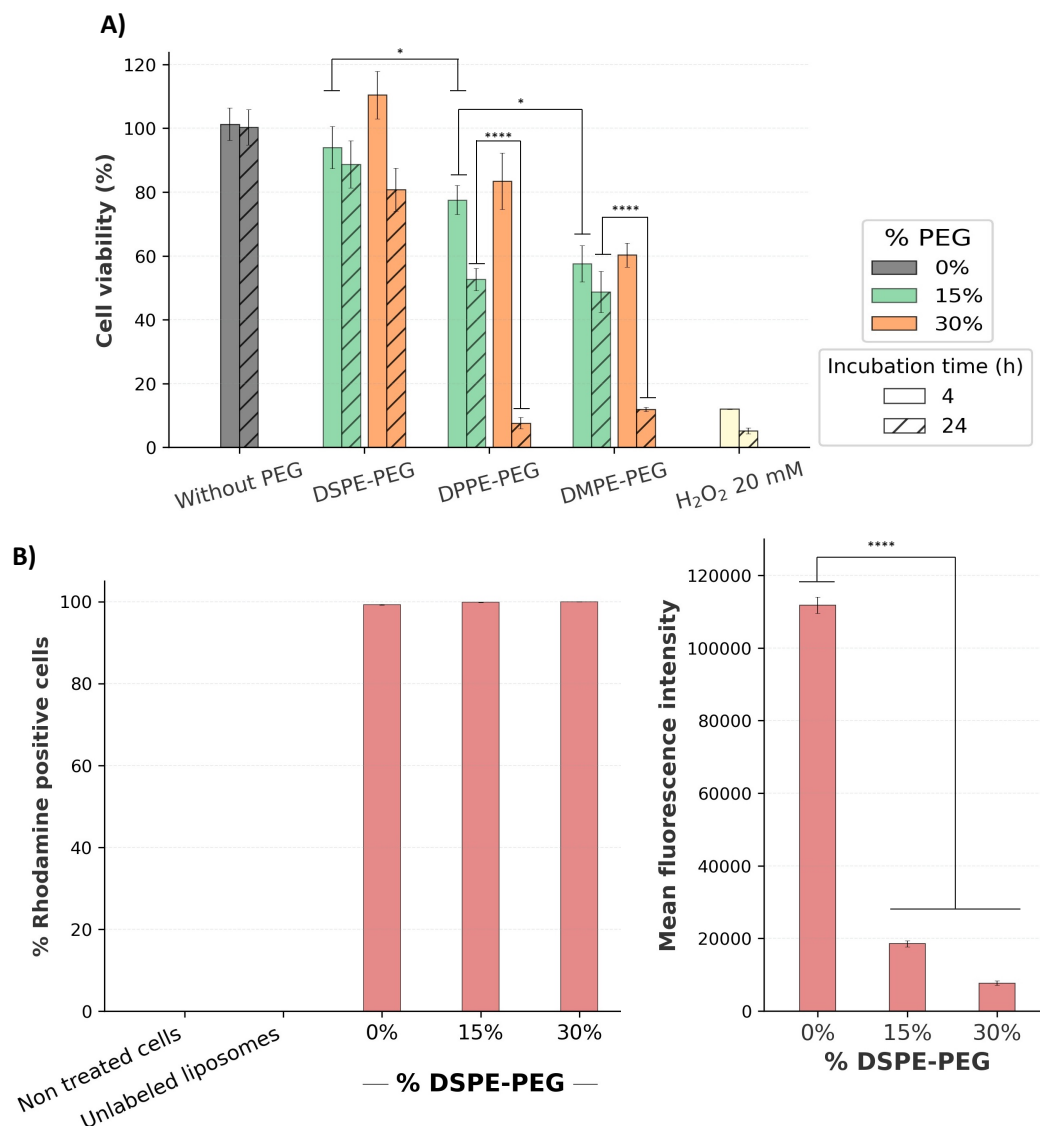


Figure 27. Effect of different PEGylated lipids on macrophages (RAW 264.7): (A) Viability of cells incubated with liposomes at 0.5 mM ($n=3$) and **(B)** Uptake of liposomes at 0.25 mM with 15% and 30% DSPE-PEG ($n=3$). Data are expressed as mean $\pm$ SD. One-way ANOVA (Fig. 27B) and Two-way ANOVA (Fig. 27A) followed by Holm-Šidák post test were performed: * $p < 0.05$, **** $p < 0.0001$.

1.2.3.1.4. Impact of the concentrations of DSPE-PEG on liposomes uptake by lung epithelial cells and potential toxicity

Lung epithelial cells are one of the targets of CIC which is metabolized by esterase into its therapeutic metabolite des-CIC [275]. Therefore, it is essential to study the toxicity and uptake of liposomes on these cells. BEAS-2B and A549 cells were selected as bronchial and alveolar lung epithelial cells, respectively. The main observation of the MTT test was that formulations without PEGylated lipid were not toxic to these cell types (Figure 28A). While formulations containing 15% DSPE-PEG did not exhibit significant toxicity towards these cells, those containing 30% DSPE-PEG significantly reduced the cellular viability after 24 h of incubation. Interestingly, this toxicity increased as the concentration of liposomes incubated with the cells decreased from 0.5 to 0.05 mM. Due to this toxicity, the formulation containing 30% DSPE-PEG was not subjected to the uptake test.

In the cellular uptake test, liposomes without DSPE-PEG were used as a positive control. Although the percentage of rhodamine-positive BEAS-2B and A549 cells after 4 h of exposure to 15% DSPE-PEG formulation were 3.0 and 1.2 times lower than the control group, respectively (Figure 28B, C), these results still revealed the internalisation of liposomes into these cells.

Overall, a concentration of DSPE-PEG at 30% was toxic to epithelial cells, whereas the 15% was not toxic and did not completely hinder liposome uptake by these cells.

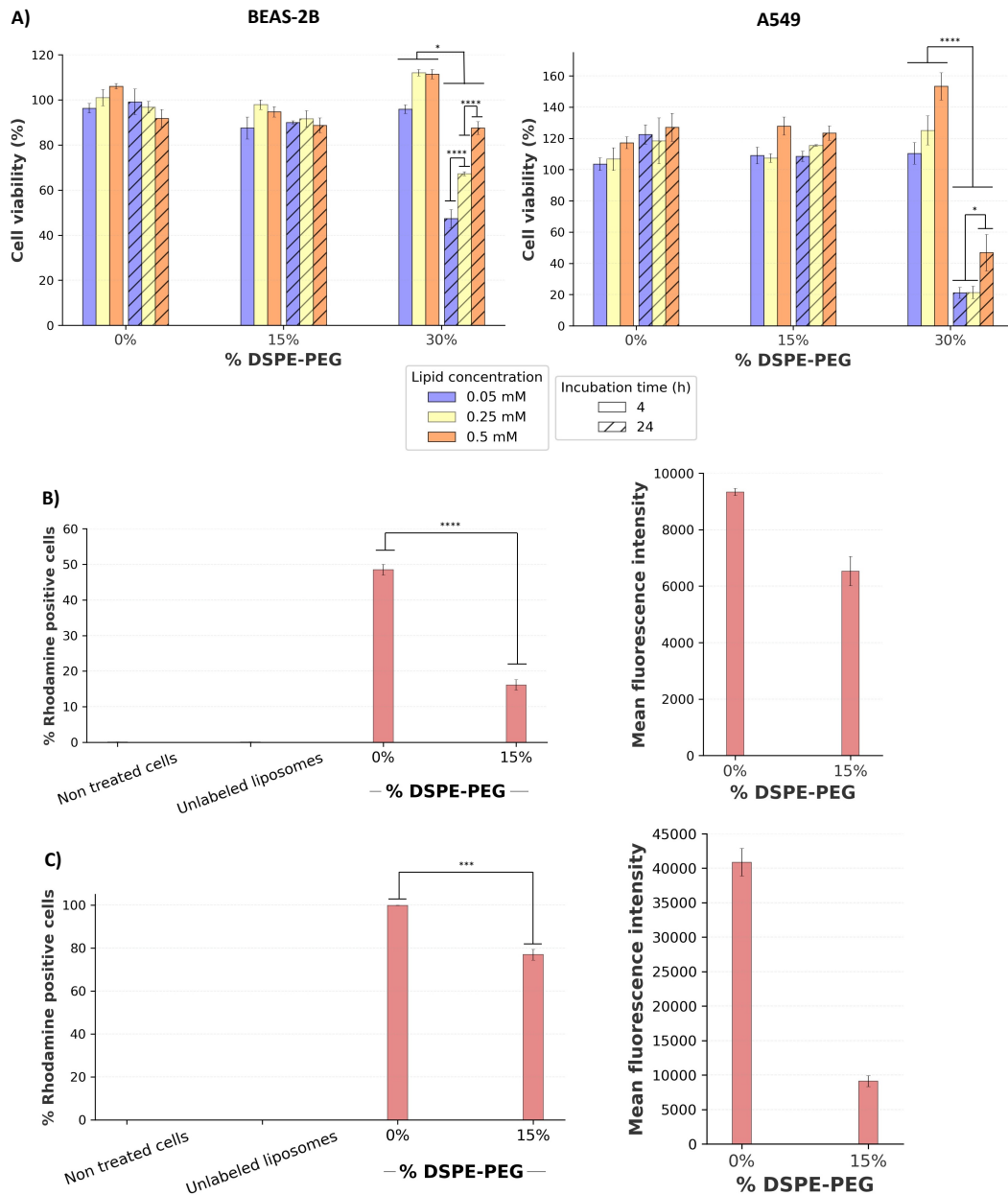


Figure 28. Effect of different concentrations of DSPE-PEG on lung epithelial cells (A549 and BEAS-2B): (A) Viability of cells incubated with liposomes at 0.5, 0.25 and 0.05 mM and uptake of liposomes at 0.25 mM on (B) BEAS-2B and (C) A549 (n = 3). Data are expressed as mean $\pm$ SD. T-test (Fig. 28B, C) and One-way ANOVA (Fig. 28A) followed by Holm-Šídák post test were performed: * p < 0.05, * p < 0.001, **** p < 0.0001.**

1.2.3.1.5. Impact of the concentrations of DSPE-PEG on relaxation effect of indacaterol maleate on isolated rat tracheal rings

Following the *in vitro* selection of PEGylated lipid compositions, we next assessed whether differences in DSPE-PEG content translated into altered bronchodilatory efficacy. Therefore, IND and CIC-loaded liposomes containing different DSPE-PEG proportions were evaluated *ex vivo* using isolated rat tracheal rings. The same *ex vivo* approach was subsequently applied to determine whether the nature of the main phospholipid further influenced the functional performance of IND and CIC-loaded liposomes.

Relaxations induced by the formulation containing 15% DSPE-PEG were significantly greater than those achieved by the IND solution in water, specifically in the range of high IND concentration from 10^{-6} to 10^{-5} M (Figure 29B). This improved efficacy translated into a 1.4-fold higher maximal effect ($E_{\max}$) and a 4.2-fold lower half-maximal effective concentration (EC_{50}) compared to the IND solution (Figure 29C). In contrast, liposomes containing 30% DSPE-PEG showed results similar to the control IND solution. The superior effect observed with the 15% DSPE-PEG formulation was confirmed to be independent of the presence or absence of the co-encapsulated CIC (Figure 53A). Importantly, empty liposomes themselves did not trigger a significant relaxation effect in this model, confirming that their action is purely a delivery role which enhance IND inherent property rather than adding their own therapeutic effect.

Overall, while the 30% DSPE-PEG formulation showed toxicity to epithelial cells and similar relaxation effects on isolated tracheal rings as the aqueous IND solution, the 15% formulation was not toxic with these cells and significantly improved the efficacy of IND. Therefore, the liposomes with 15% DSPE-PEG (SPC/Chol/DSPE-PEG, 55/30/15) were selected as the optimal component for PEGylated lipid.

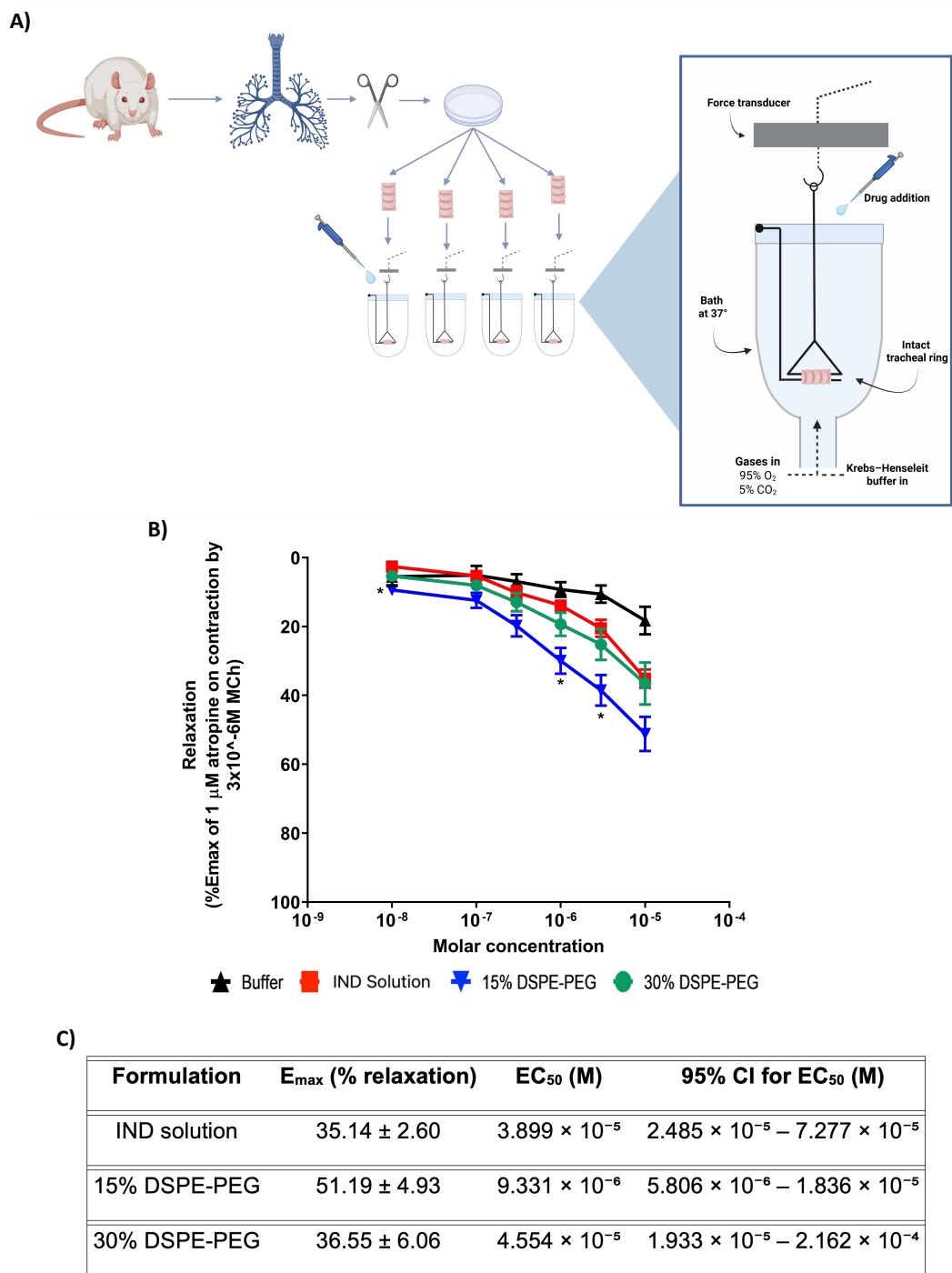


Figure 29. (A) Schematic illustration of the ex vivo organ bath test on isolated rat tracheal rings, image created with Biorender (adapted from Reprocell® and [267]). (B) Ex vivo concentration-response curve of 15% and 30% DSPE-PEG formulations and IND solution (n=5-8). Data are expressed as mean $\pm$ SEM. Two-way ANOVA followed by Holm-Šidák post test: * $p < 0.05$ versus

IND Solution. (C) Summary of the pharmacological parameters obtained from the concentration-response curves of 15% and 30% DSPE-PEG formulations and IND solution (n=5-8). E_{max} represents the maximal relaxation achieved by each formulation and is reported as mean $\pm$ SEM. EC_{50} represents the concentration required to produce 50% of the maximal effect and is reported as the fitted value obtained from nonlinear regression analysis, together with its 95% confidence interval (CI). Higher E_{max} values indicate greater efficacy, whereas lower EC_{50} values indicate greater potency.

1.2.3.2. Impact of the type of phospholipid on key liposome properties

Having identified DSPE-PEG (15%) as the optimal PEGylation strategy, further optimization focused on replacing SPC with a synthetic phospholipid to improve compositional control and scalability. Because SPC is a heterogeneous natural mixture prone to batch variability [276] and complicates future lipid quantification [177], synthetic phospholipids were preferred. Consequently, formulation screening was conducted using three unsaturated phospholipids with different acyl chain lengths, degrees of unsaturation, and T_m : DLPC (18:2, $T_m = -57$ °C), DPOPC (16:1, $T_m = -36$ °C), and DOPC (18:1, $T_m = -17$ °C) as unsaturated phospholipids and DSPC (18:0, $T_m = 55$ °C), DPPC (16:0, $T_m = 41$ °C) as saturated phospholipids [277].

1.2.3.2.1. Impacts of phospholipids structure on drugs encapsulation

Instead of EE, the amount of drugs encapsulated in formulations containing different phospholipids was compared since the total amount of drugs (both encapsulated and unencapsulated) was not similar among all the formulations. One day after the preparation, formulations containing saturated phospholipids (DSPC and DPPC) showed significantly lower encapsulation concentration of CIC than those with unsaturated phospholipids (DOPC, DPOPC, and DLPC) (Figure 30A). Not even a 1:3 molar ratio of DSPC and DOPC could prevent the leakage. This trend was also observed in IND encapsulation, with a greater difference in extent. Overall, only the unsaturated phospholipids were retained for further experiments, the saturated ones were eliminated due to the reduced drugs encapsulation.

EXPERIMENTAL PART – CHAPTER I

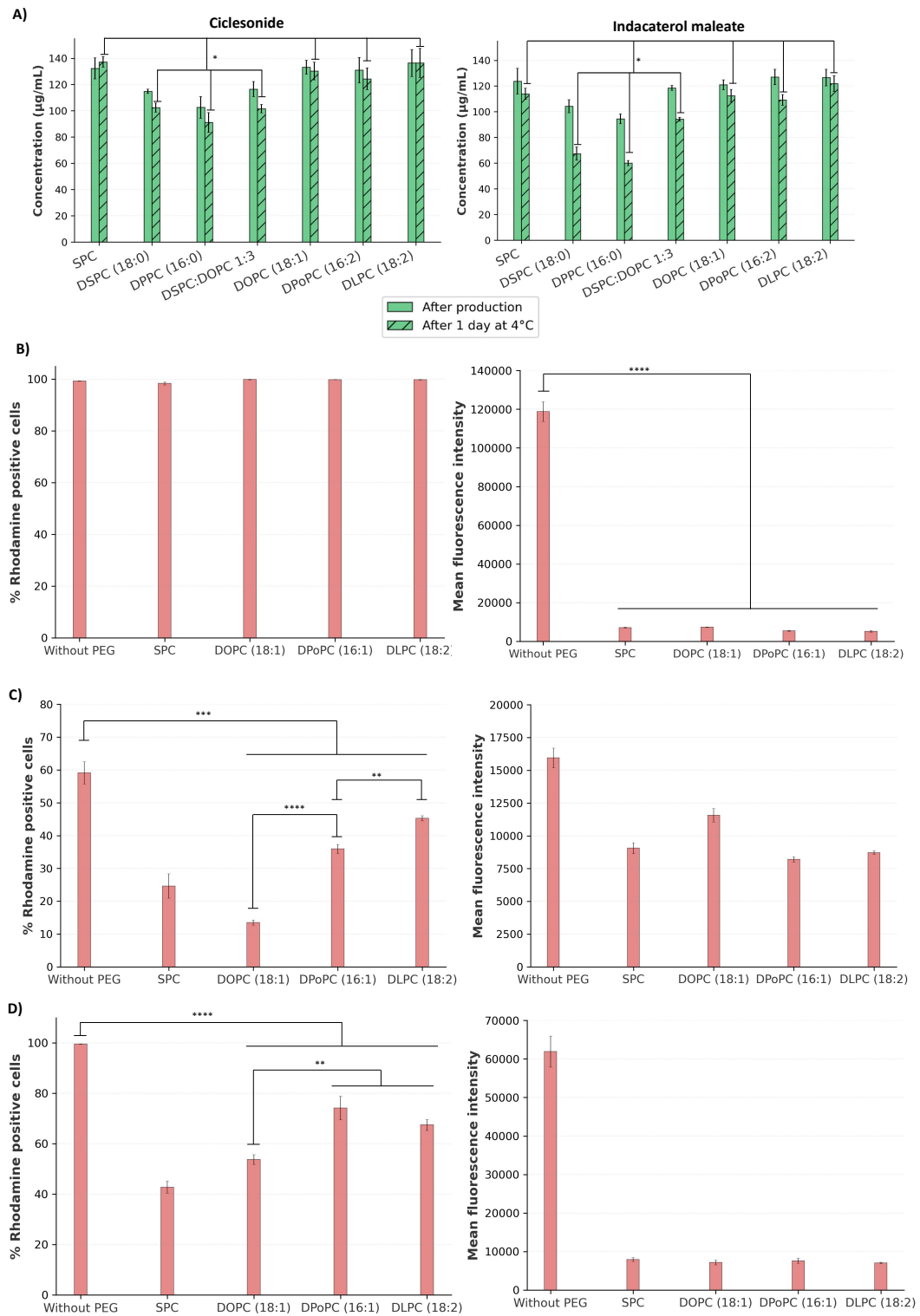


Figure 30. Effect of different phospholipids on (A) encapsulated concentration of CIC and IND and uptake of liposomes at 0.25 mM on (B) RAW264.7, (C) BEAS-2B, and (D) A549 cells (n = 3). Data are expressed as mean $\pm$ SD. One-way ANOVA followed by Holm-Šidák post test were performed: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

1.2.3.2.2. Impacts of phospholipids structure on liposomes toxicity and uptake on macrophages and lung epithelial cells

The structural differences in phospholipids between DOPC, DPOPC, and DLPC did not cause liposome toxicity to RAW 264.7, BEAS-2B, and A549 cells. All formulations maintained cell survival above 80% even after 24 hours of incubation with these cells (Figure 52).

Regarding the uptake on RAW 264.7 cells, nearly 100% of the cells across all groups tested were positive for the rhodamine signal. However, the MFI of cells incubated with the positive control liposomes (without DSPE-PEG) was approximately 10-fold higher than that of cells treated with 15% DSPE-PEG liposomes (Figure 30B). This result highlights the protective effect of DSPE-PEG against uptake by macrophages, regardless of the synthetic phospholipid used.

In BEAS-2B cells, the percentage of rhodamine-positive cells was 59% for the control liposome, followed by the DLPC formulation (45%), DPOPC (36%), and DOPC (14%) (Figure 30C). A similar trend was observed in A549 cells, without significant difference between the DLPC and DPOPC formulations (Figure 30D). These results suggest that the presence of 15% DSPE-PEG does not inhibit liposome uptake by epithelial cells and that DLPC results in the highest uptake among the three synthetic phospholipids.

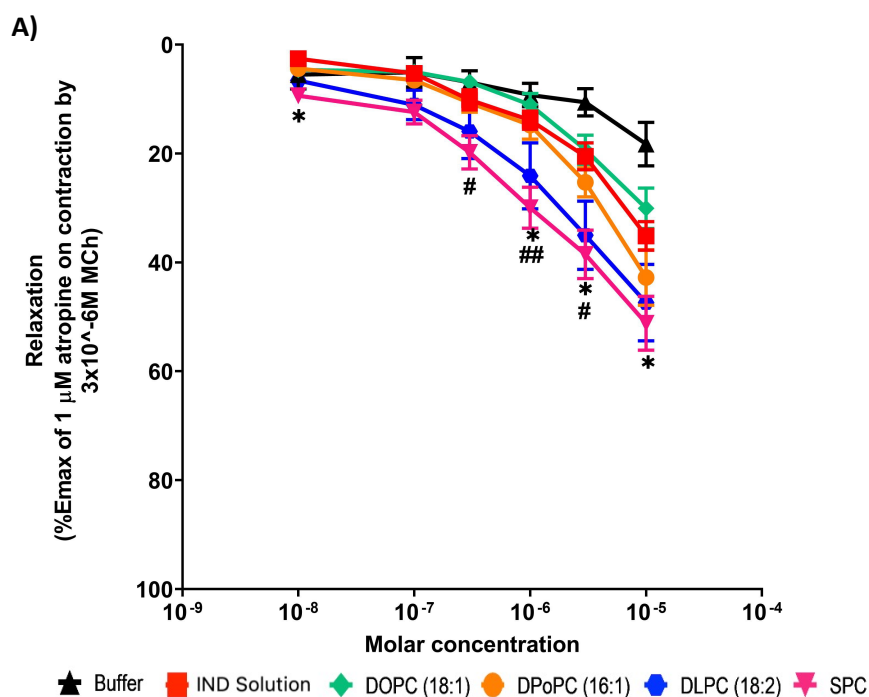
1.2.3.2.3. Impacts of phospholipids structure on relaxation effect of indacaterol maleate on isolated rat tracheal rings

The concentration–response curves demonstrated that liposomes formulated with DOPC exhibited reduced relaxation responses compared with the IND solution (Figure 31A), displaying the lowest $E_{\max}$ and the highest EC_{50} among all the samples in this section (Figure 31B). Conversely, liposomes formulated with DPOPC provided a slightly improved relaxant efficacy compared to free IND, especially at higher concentrations. The best improvement was observed with DLPC liposomes, which yielded a significantly greater relaxation of tracheal smooth muscle than the IND control. This enhanced response was reflected by a 1.3-fold higher $E_{\max}$ and 2.9-fold lower EC_{50} in comparison to the free IND solution. These results were not influenced by the presence or absence of CIC and did not

originate from an additional relaxation effect of the DLPC liposomes themselves (Figure 53B).

When compared to the results of SPC, the DLPC formulation gave a similar $E_{\max}$ and a 1.4-fold higher EC_{50} , meaning that SPC-based liposomes produced a slightly greater relaxation response. However, the overall relaxation profiles of SPC and DLPC liposomes were comparable, with closely overlapping concentration–response curves. Since the main objective of this section was to find a synthetic phospholipid to replace SPC for the reasons described initially, DLPC was chosen as the optimal phospholipid.

Overall, the optimal composition of liposomes was DLPC, Chol, and DSPE-PEG in a molar ratio of 55/30/15. Its physicochemical properties were resumed in the Table 11. This composition was applied to produce liposomes co-encapsulating CIC and IND for subsequent *in vivo* testing and will be called “liposomes” in next results.



B)

Formulation	$E_{\max}$ (% relaxation)	EC_{50} (M)	95% CI for EC_{50} (M)
IND solution	35.14 ± 2.60	3.899×10^{-5}	$2.485 \times 10^{-5} - 7.277 \times 10^{-5}$
DOPC (18:1)	30.06 ± 3.70	6.165×10^{-5}	$3.136 \times 10^{-5} - 1.809 \times 10^{-4}$
DPoPC (16:1)	42.78 ± 5.10	1.892×10^{-5}	$1.248 \times 10^{-5} - 3.525 \times 10^{-5}$
DLPC (18:2)	47.40 ± 6.99	1.325×10^{-5}	$6.708 \times 10^{-6} - 4.519 \times 10^{-5}$
SPC	51.19 ± 4.93	9.331×10^{-6}	$5.806 \times 10^{-6} - 1.836 \times 10^{-5}$

Figure 31. (A) *Ex vivo* concentration-response curve of DOPC, DPoPC, DLPC, SPC formulations and IND solution ($n=5-10$). Data are expressed as mean $\pm$ SEM. Two-way ANOVA followed by Holm-Šídák post test: * $p < 0.05$ versus IND solution, # $p < 0.05$ and ## $p < 0.01$ versus DOPC. (B) Summary of the pharmacological parameters obtained from the concentration-response curves of DOPC, DPoPC, DLPC, SPC formulations and IND solution ($n=5-10$). $E_{\max}$ represents the maximal relaxation achieved by each formulation and is reported as mean $\pm$ SEM. EC_{50} represents the concentration required to produce 50% of the maximal effect and is reported as the fitted value obtained from nonlinear regression analysis, together with its 95% confidence interval (CI). Higher $E_{\max}$ values indicate greater efficacy, whereas lower EC_{50} values indicate greater potency.

1.2.3.3. Impact of liposomes containing CIC and IND on the treatment of AHR and inflammation in a mouse model of mild eosinophilic asthma by pulmonary administration

We next evaluated the *in vivo* pharmacodynamic performance of liposomes containing CIC and IND using a mouse model of mild eosinophilic asthma induced by OVA. The aim was to study to what extent liposomal encapsulation might modify the bronchodilator and anti-inflammatory efficacy of IND and CIC compared with non-liposomal formulations. In this *in vivo* efficacy study, IND/CIC-loaded liposomes were evaluated at two dose levels: Lipos_{LOW}, containing 0.0326 mg/kg CIC and 0.0307 mg/kg IND, and Lipos_{HIGH}, comprising 0.326 mg/kg CIC and 0.307 mg/kg IND. Non-liposomal IND/CIC formulations were prepared using HP β CD to solubilize CIC and were administered at the same drug doses; these groups are referred to as Sol_{LOW} and Sol_{HIGH}, respectively. Control groups received HP β CD vehicle solution (50 μ g/mL), Lipos_{EMPTY}, corresponding to empty liposomes administered at the same liposome concentration as Lipos_{HIGH}, or HEPES buffer.

1.2.3.3.1. Measurement of AHR

One hour after the final IT administration (day 27), airway mechanics was assessed by invasive plethysmography using the SCIREQ FlexiVent system to evaluate treatments efficacy on airway bronchoconstriction and lung compliance. After baseline measurements, animals were challenged with increasing concentrations of methacholine, a muscarinic receptor agonist which induces airway smooth muscle contraction and is commonly used to assess airway hyperresponsiveness. This challenge reproduces a key functional feature of asthma, namely the exaggerated narrowing of the airways in response to a bronchoconstrictive stimulus, allowing the FlexiVent system to quantify the resulting mechanical changes in respiratory function. These changes were assessed by measuring airway resistance (Rrs), dynamic compliance (Crs), elastance (Ers), and inspiratory capacity (IC). The physiological relevance of these lung function parameters is briefly described in the Appendix I (section 1.1.7. *Parameters of airway hyperresponsiveness*).

Healthy mice (SHAM) did not exhibit a significant increase in the AHR, even at the highest methacholine concentration (50 mg/mL). In contrast, allergen-exposed mice treated only with HP β CD, the vehicle used for the non-liposomal formulations, showed marked bronchoconstriction, as indicated by increased Rrs and Ers together with decreased Crs and IC. These alterations were already evident at methacholine concentrations as low as 2.5–5 mg/mL, indicating the onset of bronchoconstriction (Figure 32B-E).

Treatment with all four drug-containing formulations (Lipos_{LOW}, Lipos_{HIGH}, Sol_{LOW}, and Sol_{HIGH}) attenuated methacholine-induced airway responses compared to Hp β CD-treated allergen-exposed controls. Notably, Lipos_{LOW} (0.0326 mg/kg CIC and 0.0307 mg/kg IND) and Lipos_{HIGH} (ten-fold higher) demonstrated superior efficacy over their concentration-matched non-liposomal solutions, almost restoring Rrs, Crs/Ers, and IC to healthy values following methacholine challenge (Figure 32B-E). Interestingly, Lipos_{LOW} not only outperformed Sol_{LOW} but also achieved efficacy comparable to, or even greater than Lipos_{HIGH} and Sol_{HIGH}. The same trend was also observed when analyzing the resistance and elasticity of the airways and lung tissue separately (Figure 56).

Although not statistically significant, Lipos_{EMPTY} also produced a mild improvement in AHR, partially alleviating airway responses across all measured parameters. This observation supports previous reports that liposomal carriers are not biologically inert but may exert bronchoprotective or anti-inflammatory effects depending on their lipid composition and surface properties [90,278]. Of note, this *in vivo* effect contrasts with our *ex vivo* findings, where empty liposomes did not induce direct relaxation of pre-contracted tracheal rings. This discrepancy suggests that the improvement in AHR observed *in vivo* is likely mediated by indirect anti-inflammatory mechanisms rather than a direct bronchodilator action.

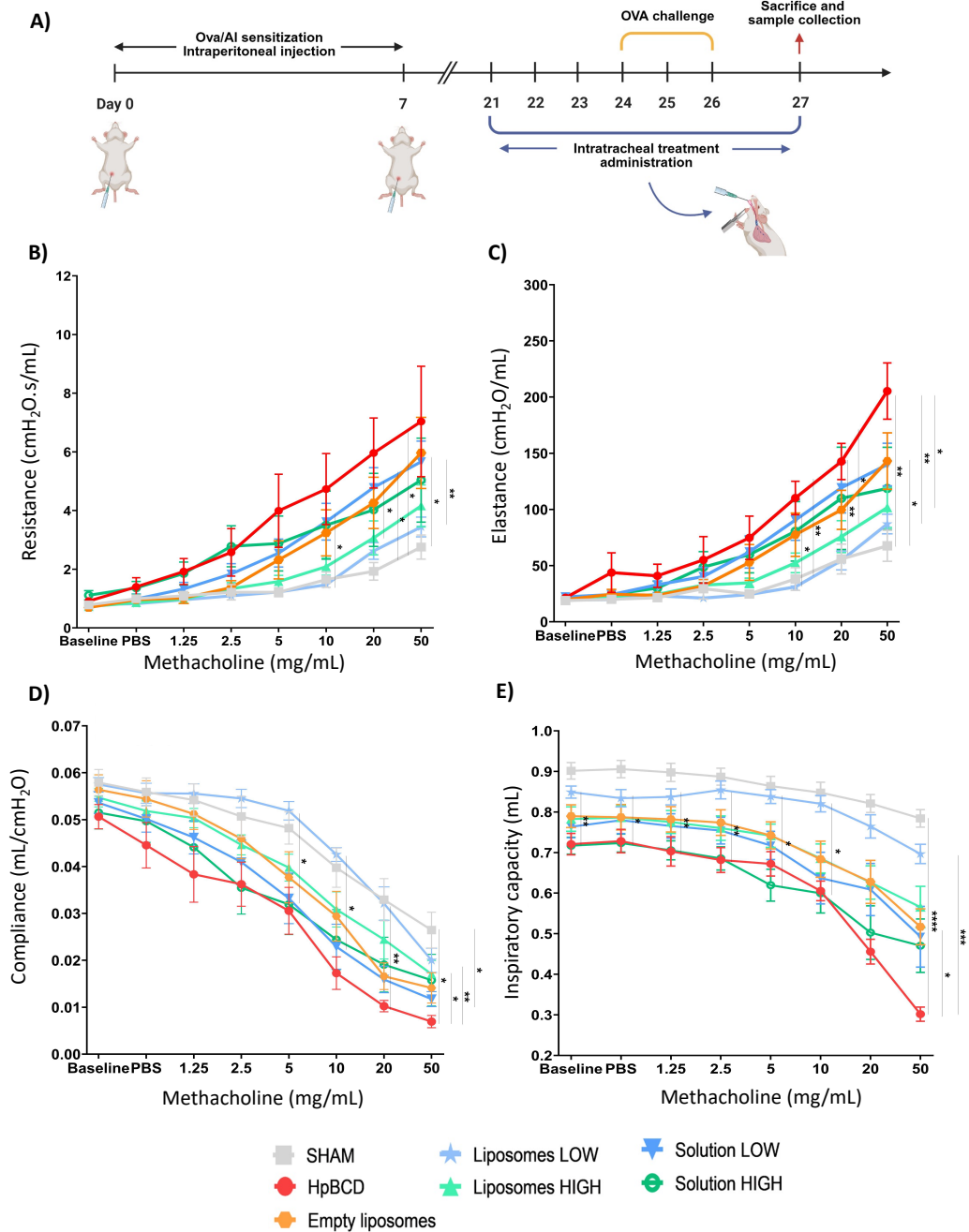


Figure 32. (A). Schematic illustration of the OVA-induced asthma mice model and treatment regimen in BALB/c mice (created with Biorender). (B) Airway resistance, (C) elastance, (D) compliance, and (E) inspiratory capacity of the mice subjected to the indicated treatments were assessed using the SCIREQ flexiVent system following stimulation with various concentrations of methacholine ($n=9-10$ biologically independent mice per group). Results are presented as mean $\pm$ SEM. Significant differences were assessed using Two-way ANOVA followed by Holm-Šidák post test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

1.2.3.3.2. Inflammatory cell counts in BALF

BALF and lung samples were collected at the end of the AHR assessment to evaluate immunological biomarkers associated with asthma responses, including the quantification of immune cells in BALF, inflammatory cytokines in lung homogenate and histological analysis.

Differential cell analysis with Diff-Quick staining revealed a marked increase in all inflammatory cell types in the BALF, particularly eosinophils, in the treated and non-treated allergen-exposed groups compared with healthy controls, confirming successful eosinophilic asthma induction (Figure 33A-D). Specifically, intratracheal treatment with both Lipos_{LOW} and Lipos_{HIGH} resulted in a significant reduction of eosinophil counts in the BALF compared to vehicle-treated and solution-treated controls. A marked reduction in neutrophils, macrophages, and lymphocytes was also observed in the Lipos_{LOW} group, despite these cells not being primary drivers of type 2-mediated inflammation. A similar, though less pronounced reduction was observed in the Lipos_{HIGH} group, which also exhibited more variability in cell counts. Overall, Lipos_{LOW} displayed higher anti-inflammatory efficacy than Lipos_{HIGH}, highlighting a dose-sparing effect already observed in AHR measurement results.

In contrast, only a modest decrease in the number of these cell types was observed in the Sol_{LOW} and Sol_{HIGH} groups, with Sol_{HIGH} displaying a slightly stronger response, especially in reducing eosinophil counts (Figure 33A-D). Interestingly, Lipo_{EMPTY} also showed a tendency toward decreased inflammatory cell infiltration, further supporting the notion that the liposomal carrier itself may exert a mild anti-inflammatory effect (Figure 33A, B, D). Similar trends were also observed in raw cell percentages (Figure 57).

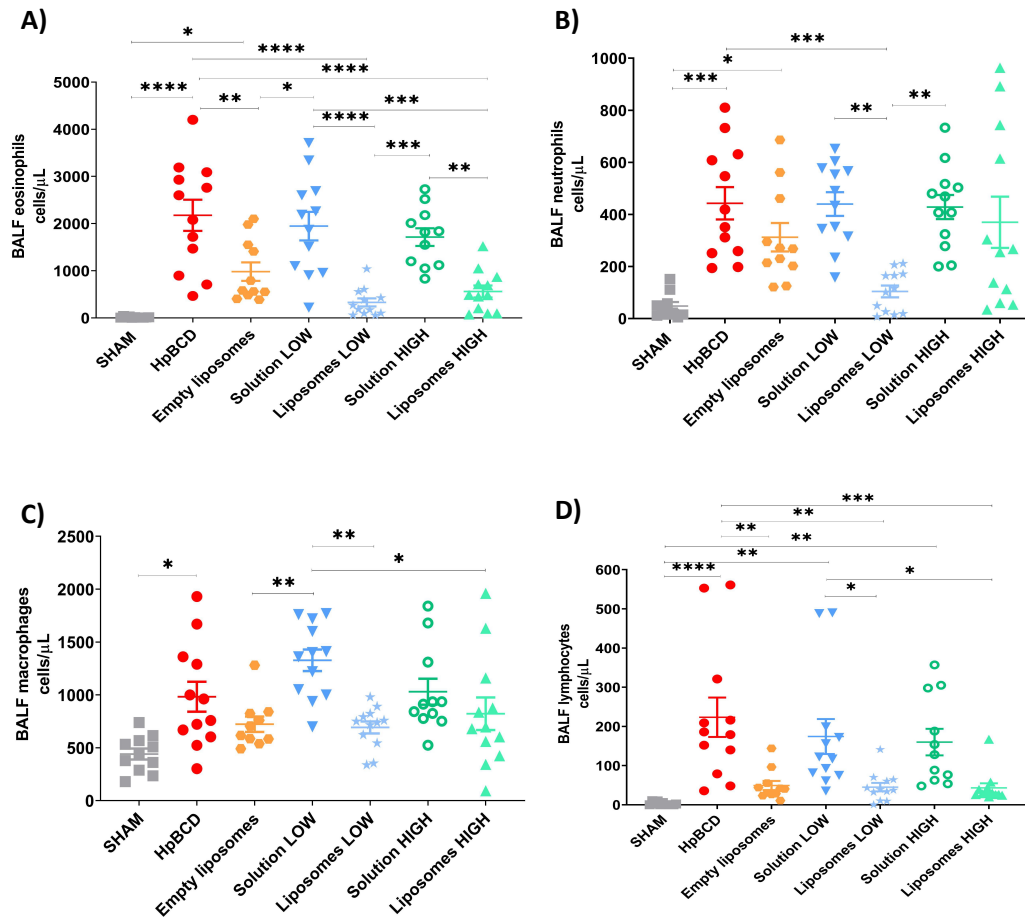


Figure 33. (A) Eosinophils, (B) neutrophils, (C) macrophages, and (D) lymphocytes counts in the BALF of mice following the indicated treatments were assessed using a Diff-Quick staining kit (n=11-12 biologically independent mice per group). Results are presented as mean $\pm$ SEM. Significant differences were assessed using One-way ANOVA followed by Holm-Šidák post test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

1.2.3.3.3. Cytokines quantification in whole lung homogenates

ELISA analysis of IL-4, IL-5, IL-13, IL-33, and TSLP showed that these cytokines were significantly increased in the vehicle-treated allergen-exposed mice compared with the healthy controls, confirming robust establishment of eosinophilic airway inflammation (Figure 34A-E).

Treatment with both liposomal formulations significantly reduced cytokine levels in comparison to their concentration-matched non-liposomal counterparts, with Lipos_{LOW} displaying once again the strongest pharmacodynamic efficacy. Notably, Lipos_{HIGH} effectively reduced all measured cytokines, suggesting that liposomal encapsulation

mitigated the reduced responsiveness observed at higher steroid concentrations [279–281]. Liposomal treatments were also particularly effective in suppressing epithelial-derived alarmins IL-33 and TSLP, indicating that nanoparticle-mediated delivery may enhance CIC access to epithelial compartments and improve regulation of upstream inflammatory signalling. Conversely, non-liposomal formulations induced only a moderate decrease in cytokine levels. Specifically, Sol_{HIGH} exhibited the lowest efficacy, showing minimal suppression of all tested cytokines. In contrast, Sol_{LOW} significantly reduced IL-13 and TSLP levels compared with vehicle-treated controls (Figure 34A-E), while only a trend toward reduction was observed for IL-4, IL-5, and IL-33. Similar trends were observed when normalizing cytokine levels to total lung protein concentration (Figure 58).

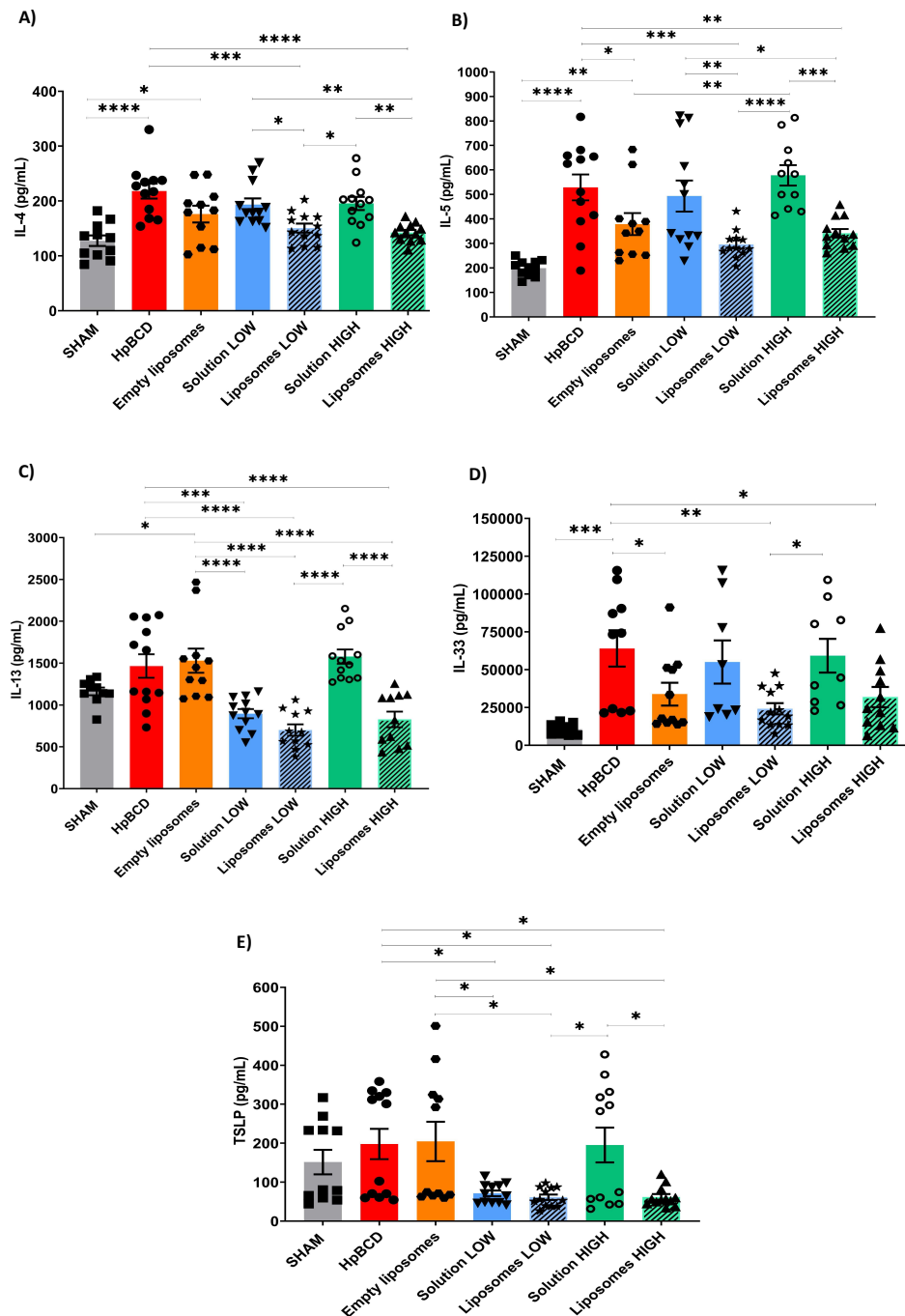


Figure 34. ELISA analysis of (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-33, and (E) TSLP levels in the whole lungs collected from the mice receiving the indicated treatments (n=10-12 biologically independent mice per group). Results are presented as mean $\pm$ SEM. Significant differences were assessed using One-way ANOVA with Holm-Šídák multiple comparisons test: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

1.2.3.3.4. Allergen-induced airway remodelling: inflammation score and goblet cell hyperplasia evaluation

Histological analysis of lung tissue revealed a reduction of airway inflammation, wall thickening, and goblet cells hyperplasia in mice treated with both liposomal and non-liposomal formulations compared to the allergen-exposed placebo-treated animals (Figure 35A).

To quantitatively assess airway inflammation, an inflammation score was calculated from the H&E-stained sections as previously described [272], reflecting the extent of peribronchial inflammatory cell infiltration (Figure 35B). The percentage of periodic acid Schiff-positive (PAS+) epithelial cells was also evaluated to quantify goblet cell hyperplasia (Figure 35C). Consistent with the differential cell count results, Hp β CD and Lipos_{EMPTY} groups exhibited markedly elevated inflammation scores and increased PAS+ cell percentages compared with SHAM and treated groups. Both Lipos_{LOW} and Lipos_{HIGH} treatments significantly reduced inflammation scores and PAS+ cell percentages compared to their corresponding non-liposomal formulations. Indeed, lung sections from liposome-treated allergen-exposed animals displayed a reduction of allergen-induced airway remodeling characterized by reduced inflammatory cell infiltration and decreased goblet cell hyperplasia (Figure 35A). Notably, Lipos_{LOW} achieved the greatest reduction in inflammation score and goblet cells hyperplasia, further supporting a potential dose-sparing effect of the liposomal formulation (Figure 35B, C). In contrast, treatment with Sol_{LOW} and Sol_{HIGH} resulted in a more modest reduction in peribronchial inflammatory infiltrates and goblet cell accumulation.

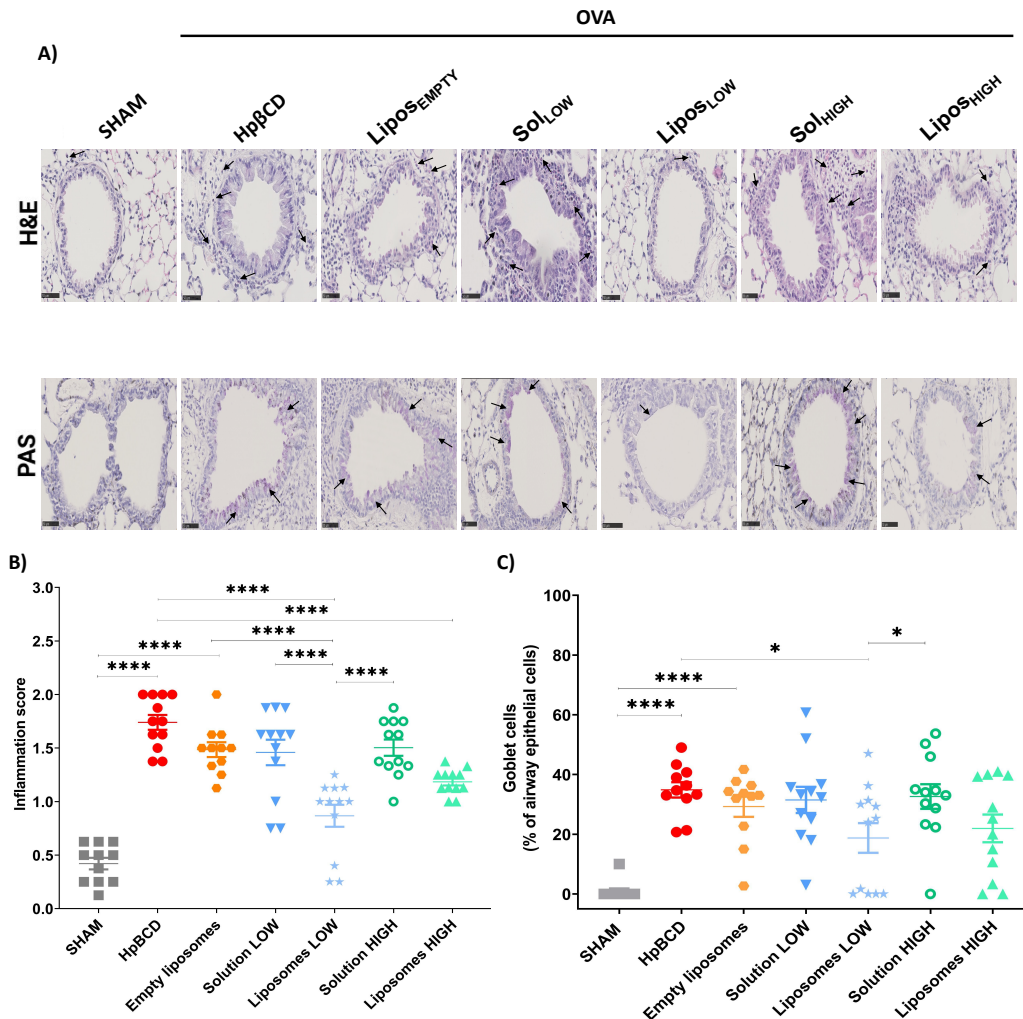


Figure 35. (A) Histological images of lung sections from mice subjected to the indicated treatments. Lung tissue was processed and stained using H&E and PAS to assess overall tissue morphology, calculate the inflammation score according to inflammatory cell infiltration, and to evaluate goblet cells hyperplasia. Black arrows indicate the presence of inflammatory and goblet cells, respectively. Scalebars, 50 μ m. Quantification analysis of histological staining in lung sections by calculating (B) inflammation score and (C) percentage of PAS positive epithelial cells (n = biologically 11-12 independent mice per group). Results are presented as mean $\pm$ SEM. Significant differences were assessed using One-way ANOVA with Holm-Šídák multiple comparisons test: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

I.2.4. Discussion

Asthma is a heterogeneous disease which is typically characterized by hyperresponsiveness of the airways that is the consequence of a chronic airway inflammation. As ICS-LABA therapy addresses both components simultaneously, this combination remains the gold standard across asthma phenotypes [1]. Although the high

lipophilicity of long-acting ICS and LABA is supposed to increase their residence time and duration of action in the lungs, it simultaneously hinders their ability to traverse the respiratory mucus layer. Consequently, to compensate for the significant drug loss caused by the MCC, the dosage and frequency of administration must be substantially increased to ensure that therapeutic concentrations of drugs reach target sites to elicit a significant therapeutic response [71,255–257]. This underlines the critical need to develop innovative treatment strategies to allow more lipophilic drugs to overcome these physiological barriers. While PEGylated liposomes have shown potential to penetrate mucus and evade macrophage clearance via the steric shielding of a PEG layer, their application in optimizing specific ICS-LABA combinations remained largely unexplored. To bridge this gap, we optimized a liposomal formulation for the co-delivery of CIC and IND in the airways through the strategic selection of PEGylated lipids and phospholipids. This optimization process revealed significant influences of lipid chemical structure and concentration on the resulting liposomal properties.

The chemical structure of the lipid components plays a crucial role for effective drug encapsulation. We observed that unsaturated lipid chains in the main phospholipid favored the encapsulation of both CIC and IND. The reduced encapsulation efficiency of hydrophobic drugs in formulations with saturated phospholipids is in line with previous reports indicating that these phospholipids tend to create a dense, tightly packed lipid bilayer which lacks the free volume necessary for drug accommodation and, therefore, makes the membrane physically resistant to the perturbation caused by the insertion of drug molecules [282,283]. When an unsaturated phospholipid was used, the encapsulation efficiency of CIC exceeded 90% regardless of the PEGylated lipid used (Figure 30A). In particular, the phosphate group in the PEGylated lipid linker maximized IND encapsulation. Given the amphiphilic nature of IND, it was previously assumed that its hydrophobic part would insert into the lipid membrane alongside the phospholipid acyl chains, while its positively charged amine group interacted electrostatically with the negative phosphate groups of the lipids [36,41,284]. This interaction likely accounts for the superior IND encapsulation observed in liposomes containing PEGylated phospholipids (Figure 26B). Based on these results, we propose a co-localization model: CIC is distributed deeply within the lipid bilayer, surrounded by fatty acyl chains, while IND is localized at the interface between the bilayer and the aqueous phase (Figure 26C). This spatial arrangement explains the high encapsulation efficiency maintained even when both drugs are loaded into the same compartment.

The choice of PEGylated lipid also significantly impacts cytotoxicity. In RAW 264.7 macrophages, the formulation without PEGylated lipid (SPC/cholesterol, 70/30 molar ratio) preserved cell viability at around 100% even after 24 hours (Figure 27A), implying the high biocompatibility of SPC and cholesterol with these cells. Among the formulations containing PEGylated lipids, only the one containing DSPE-PEG (18-carbon chain) generally preserved macrophage viability above 80% after 24 hours (Figure 27A). Shortening the fatty acyl chain length of the PEGylated lipid to 16 carbons (DPPE-PEG) and 14 carbons (DMPE-PEG) proportionally increased toxicity in these cells. This result differs from previous observations regarding the impact of PEGylated lipids on liposome toxicity in macrophages. For instance, the incorporation of 10 mol% DPPE-PEG was previously found to abolish the toxicity of cationic liposomes towards macrophages and monocyte-like U937 cells, which was explained by the reduced endocytosis of the PEGylated vesicles by these cells [285]. However, since we demonstrated that SPC and cholesterol do not inherently cause toxic effects on macrophages, the toxicity observed with liposomes containing DPPE-PEG and DMPE-PEG is more likely to stem from the specific PEGylated lipids themselves rather than the other membrane components. More recently, the immunogenicity of PEGylated nanoparticles in the body has been widely attributed to the recognition of PEG chains by anti-PEG antibodies [167]. In the context of pulmonary administration, the presence and binding of anti-PEG antibodies to PEGylated nanoparticles can effectively immobilize these carriers in the mucus through adhesive cross-linking with mucin fibers [168]. Nevertheless, since anti-PEG antibodies were not present in our *in vitro* MTT assay conditions, the toxicity of liposomes containing DPPE-PEG and DMPE-PEG cannot be attributed to this antibody-mediated immunogenic property of PEGylated lipids. In light of these differences, further experiments focusing on clarifying the specific impact of the PEGylated lipid's fatty acyl chain structure on macrophage toxicity are of great interest to fully explain our results. Overall, DSPE-PEG, with its phosphate group and long fatty acyl chains, emerged as the optimal candidate.

Furthermore, the concentration of DSPE-PEG is a critical parameter. Increasing its concentration enhances the shielding effect of the "brush-like" PEG layer, limiting hydrophobic interactions with mucin and preventing opsonization-mediated macrophage uptake [165]. This explains the improved mucopenetration (Figure 26A) and low macrophage uptake observed with 15% and 30% DSPE-PEG compared to 0% or 5% (Figure 27B). However, the 30% concentration triggered toxicity in bronchial epithelial (BEAS-2B) cells (Figure 28A) and failed to improve the *ex vivo* relaxation effect of IND,

unlike the 15% formulation (Figure 29B, C). The reasons why the formulation with 30% DSPE-PEG causes toxicity to BEAS-2B and A549 cells, and why this toxicity increases with decreasing liposome concentration incubated with the cells, still need to be clarified in the future. Consequently, 15% DSPE-PEG was selected as the optimal concentration to achieve superior pulmonary delivery characteristics.

Phospholipid unsaturation influences not only drugs encapsulation but also liposomal uptake by bronchial epithelial cells (BEAS-2B) and the *ex vivo* efficacy of IND. The increase in cellular uptake and relaxation effect of IND followed the order: DLPC > DPOPC > DOPC (Figure 30C, D). We hypothesize that these results are linked to a "PEG desorption" process, in which the structure of the lipid chain of the phospholipid may regulate the release of DSPE-PEG from the liposome surface. Several factors such as liposomes exposure to biomolecules and dilution in the *in vitro* and *ex vivo* tests could trigger the desorption of DSPE-PEG [286,287]. This process is influenced by the packing between the fatty acyl chains of DSPE-PEG (18-carbon, saturated) and the main phospholipid. Interactions become looser as the phospholipid chain length decreases and unsaturation increases [288]. We propose that DSPE-PEG would be released most readily from DLPC liposomes, followed by DPOPC and then DOPC liposomes. More the PEGylated lipid molecules are released, more the steric hindrance is reduced, promoting liposome uptake by epithelial cells. Once internalized, the liposomes could be degraded, releasing IND to reach the underlying ASM [289–291]. This uptake mechanism is quite consistent with the *ex vivo* results of the PEGylated lipid optimisation, where the 15% DSPE-PEG formulation improved IND effect while the 30% formulation did not (Figure 29B, C). This suggests that the PEG density at 30% might be too high to allow efficient cellular uptake even after the DSPE-PEG desorption. However, this formulation produced a similar response to the IND solution possibly because, when not internalized into cells, the liposomes were able to release IND which then migrated to the ASM in the same manner as the drug in solution.

A practical question arising from this hypothesis is whether a lower concentration of DSPE-PEG should be used to further promote liposome uptake. While the 10% DSPE-PEG formulation showed a muscle relaxant effect comparable to the 15% formulation (Figure 54), the latter exhibited a significantly better mucus permeation rate (Figure 55). Consequently, 15% DSPE-PEG was still considered the optimal choice for overall pulmonary delivery characteristics.

Our *in vivo* OVA-induced asthma model confirmed that the optimal formulation (DLPC/Chol/DSPE-PEG, 55/30/15) successfully increased therapeutic efficacy of both drugs compared to non-liposomal formulations. Treatment with liposomes significantly reduced the production of upstream epithelial-derived alarmins and downstream inflammatory cytokines, alongside decreasing inflammatory cell infiltration, goblet cells hyperplasia and airway hyperresponsiveness during methacholine stimulation (Figure 32- Figure 35). The dose-sparing effect, observed only with the liposomal formulations, suggests that improving delivery via liposomes is a more effective strategy. In our view, this improvement is likely due to optimized liposomes exhibiting all the expected characteristics mentioned above in the *in vivo* model, which may be closely related to the hypothesis of epithelial uptake via PEG desorption. When liposomes were located in the lungs, the DSPE-PEG desorption could be triggered by the interaction of the liposomal surface with biomolecules such as mucin, DNA, proteins... It could be hypothesised that the PEG layer initially exerted a shielding effect to assist the liposomes in overcoming the mucus barrier. However, as the particles penetrated the mucus, the PEG layer underwent desorption so that the resulting reduced PEG density did not prevent uptake by the underlying epithelial cells. Epithelial internalization is especially critical for CIC, as intracellular conversion to its active metabolite and engagement of the glucocorticoid receptor are required for anti-inflammatory efficacy [275]. IND also needs to pass through the epithelium to reach the underlying ASM [280]. The epithelial uptake of liposomes likely enhanced the absorption of encapsulated CIC and IND into the lung tissue although they could cross biomembranes thanks to their high hydrophobicity. Further evidence of effective epithelial targeting is provided by the pronounced reduction of IL-33 and TSLP, key epithelial cell-produced alarmins that activate dendritic cells and type-2 innate lymphoid cells, thereby amplifying eosinophilic inflammation [3]. Alarmins are generally associated to corticosteroids resistance, and indeed steroids ability to suppress these upstream mediators remains controversial [292,293]. The strong inhibition of alarmins expression observed in our model suggests that liposomal delivery might result in an effective CIC delivery to epithelial compartments. The simultaneous suppression of both alarmins and downstream type-2 cytokines may support a broader and more effective immunomodulatory action of our IND and CIC-loaded liposomes.

It is noteworthy that empty liposomes also exhibited an inherent anti-inflammatory effect, evidenced by a significant reduction in eosinophil and lymphocyte counts, as well as an attenuation of IL-5 and IL-33 levels compared to the HP β CD group. In existing literature,

the bioactivity of empty liposomes has primarily been documented in LPS-induced inflammation models, where specific phospholipids, such as DPPC, DOPC, and DLPC, played a functional role [90,91,294]. Specifically, DLPC has been suggested to exert anti-inflammatory effects through TLR4 antagonism [92,294]. However, since TLR4-mediated signaling is not the primary driver of inflammation in the OVA-induced model utilized here, it was expected that empty liposomes did not achieve the comprehensive anti-inflammatory and bronchodilatory effects observed with the drugs-loaded liposomes. Rather, these results suggest that the liposomal carrier acted synergistically with the encapsulated CIC and IND, enhancing their overall therapeutic efficacy.

In summary, our research demonstrated that the optimal liposomes (DLPC/Cholesterol/DSPE-PEG, 55/30/15) significantly improved the *in vivo* efficacy of CIC and IND against asthma. This resulted from a detailed optimization of the PEGylated lipid and phospholipid components via the evaluation of *in vitro* properties such as drug encapsulation, mucopenetration, evasion of macrophage uptake, and internalization by epithelial cells, as well as the *ex vivo* relaxation effect of encapsulated IND. To the best of our knowledge, this is the first time that a liposomal formulation has been shown to have the potential to improve ICS-LABA therapy for asthma. However, further investigations are needed to clarify the mechanisms of delivery of these liposomes in the lung environment, especially the role of PEG desorption hypothesis in a range of processes that liposomes undergo in the lungs (mucopenetration, interaction with macrophage, epithelial absorption, etc.). This can be accomplished by characterizing the distribution of liposomes and drugs in mucin-secreting epithelial cell models or even in the lungs after inhalation. For liposomes, this would help determine the extent of their epithelial uptake after the mucopenetration or the contact with macrophages and the potential presence of other mechanisms, such as prolonged retention between the mucus and the epithelial layer previously observed with liposomes containing high level of PEG [76]. These results may also provide clues about how the epithelial uptake of CIC and IND mediated by liposomes improved their efficacy although these hydrophobic drugs already have good membrane permeation. On the other hand, the clinical translation of this system will require the development of a finalized inhalation platform, necessitating a comparative evaluation of liquid nebulization versus dry powder formulation to optimize both liposomal stability and efficient lung deposition.

I.2.5. Conclusion

In conclusion, by systematically refining the liposomes composition (PEGylated lipid and phospholipid), we successfully designed a nanocarrier capable of penetrating the mucus barrier, evading macrophage clearance, being taken up by bronchial epithelial cells and improving the *ex vivo* bronchodilatory effect. The optimized formulation (DLPC/Chol/DSPE-PEG, 55/30/15) not only demonstrated superior attenuation of the airway hyperresponsiveness and pulmonary inflammation in an ovalbumine-induced acute asthma model compared to the non liposomal formulation but also exhibited a significant dose-sparing effect. These results offer strong proof-of-concept for the use of liposomes to enhance the efficacy of ICS/LABA combinations, a strategy that remains underexplored in the management of eosinophilic asthma, and also provide an established platform for the simultaneous, lung-targeted delivery of different categories of drugs for the treatment of other respiratory diseases, such as chronic obstructive pulmonary disease (COPD).

CRediT authorship contribution statement

Tuan Nghia Dinh: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Benedetta Bottero:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fabienne Perin:** Methodology, Data curation. Laure-Anne Bya: Methodology, Data curation. **Anna Lechanteur:** Writing – review & editing, Supervision, Funding acquisition. **Brigitte Evrard:** Writing – review & editing, Supervision, Funding acquisition. **Géraldine Piel:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Didier Cataldo:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgements

This research was funded by the Walloon Region, SPW-EER, DGO6 – Win2Wal – LNPulmo convention n°2210043 and supported by the 2024 classic credit from the Faculty of Medicine of the University of Liège, the FRS-FNRS (grants T.0036.25, J.0018.26 and Télévie 7.6504.24, 7.4528.25) (Belgium), the Foundation against cancer (grant 2022.192

and 2024.187) (Belgium), the Fondation Léon Fredericq (University of Liège, Belgium), the Fonds spéciaux of the University of Liège (Belgium) and the European Regional Development Fund (FEDER) - SYST-IMM project. Authors want to thank Aquilon Pharma (Liège, Belgium). Authors want to thank Stephan Raafat, Céline Vanwinge and Associate Professor Sandra Ormenesse from GIGA Flow Cytometry & Cell Imaging Platform (University of Liège, Belgium) for their help in the flow cytometry analysis. Authors want to thank Alicia Demanche and Chloé Vranken (Laboratory of Tumor and Development Biology, University of Liège, Belgium) for the technical support.

I.3. Complementary results

I.3.1. Morphology of liposomes containing ciclesonide and indacaterol maleate by cryogenic transmission electron microscopy

Cryo-transmission electron microscopy (Cryo-TEM) stands as a useful technique for the morphological characterization of liposomal systems. Unlike traditional TEM, cryo-TEM preserves the native, hydrated state of vesicles, avoiding structural artifacts caused by staining or dehydration. Evaluating the morphology of PEGylated liposomes is critical, as high levels of PEGylated lipids can exert massive lateral pressure that can strongly destabilize lipid bilayers.

I.3.1.1. Method

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 35s. Grids were then transferred to a Vitrobot Mark IV (ThermoFisher Scientific) for a plunge-freezing procedure. A double application of the sample was used: 3 μ L 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 μ L 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 $21 \text{ e}^-/\text{\AA}^2$. Liposomes were analyzed for a final lipid concentration of 5 mM.

1.3.1.2. Results and discussion

Almost all the vesicles observed by Cryo-TEM showed a nearly spherical to oval shape with unilamellar structure (Figure 36). Some bilamellar spherical vesicles were also observed. The nearly spherical to oval shape of the unilamellar vesicles is likely to be driven by the high fluidity and curvature induced by DLPC and DSPE-PEG at 15%. The unsaturated fatty acyl chains of DLPC introduce physical "kinks" in the *cis* conformation that interfere with tight hydrocarbon chain packing. This disruption reduces hydrophobic interactions and increases the fluidity and flexibility of the lipid bilayer compared to fully saturated lipids [295]. Conversely, DSPE-PEG at a high concentration of 15% strongly drives membrane curvature due to the high lateral repulsion of its bulky PEG chains at the surface [296]. To accommodate this bulkiness and minimize free energy, PEGylated lipid molecules preferentially pack into the outer monolayer facing the solvent rather than the highly constrained inner concave surface [297]. This asymmetric distribution of PEGylated lipids actively increases the spontaneous curvature of the membrane, driving the formation of spherical and oval-shaped unilamellar structures.

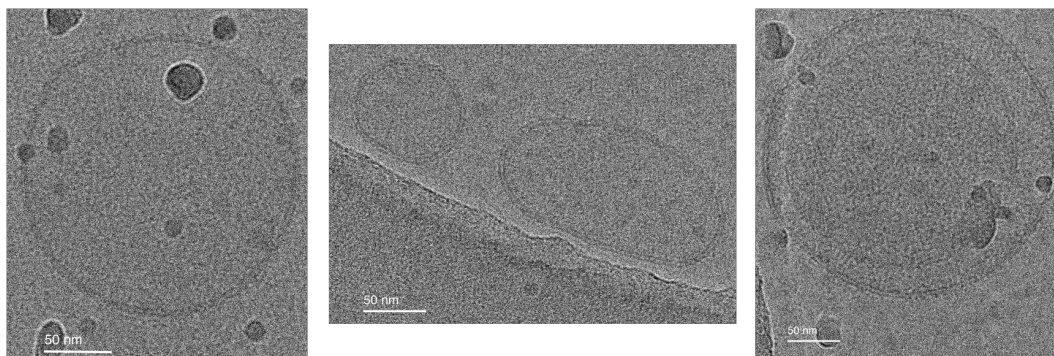


Figure 36. Morphology of liposomes (DLPC/Chol/DSPE-PEG, 55/30/15) containing CIC and IND.

Occasional bilamellar structures may arise from the high concentration of DSPE-PEG in the outer monolayer. This asymmetric distribution increases the area of the outer monolayer more than the inner monolayer. Liposomes adapt to this difference by generating local invaginations to minimize bending energy [298,299]. These invaginations may eventually pinch off, trapping a smaller vesicle inside the larger one and creating bilamellar structures.

I.3.2. Physical stability of the optimal formulation of liposomes containing ciclesonide and indacaterol maleate

I.3.2.1. Method

Liposomes preparation and characterization of physicochemical properties: the optimal formulation (DLPC/Chol/DSPE-PEG, 55/30/15) loaded with CIC and IND were prepared by the TFH and purified by the dialysis as described in sections *I.2.2.2. Liposomes preparation by the thin film hydration method* and *I.2.2.3. Characterization of liposomes physicochemical properties*, respectively. Purified liposomes were then characterized by DLS as described in section *I.2.2.3.1. Particles size, polydispersity index and zeta potential*. The liposomes were then stored at 4 °C. After a certain period, the liposomes were characterized by DLS and purified to survey encapsulated concentrations of CIC and IND.

I.3.2.1. Results and discussion

Over a 14-day stability study, the optimal formulation exhibited stable physicochemical properties in terms of size, Pdl, and ZP, as well as a consistent encapsulated concentration of CIC (Figure 37). In contrast, the encapsulated concentration of IND decreased significantly, falling from 126.5 ± 6.5 µg/mL immediately after production to 102.2 ± 5.7 µg/mL by day 14. The higher leakage of IND compared to that of CIC could be attributed to its lower lipophilicity, making it more susceptible to gradual partitioning and passive diffusion into the surrounding aqueous medium during liquid storage.

Crucially, this 14-day monitoring window was designed to verify the short-term stability of the optimal formulation for *in vivo* testing and subsequent downstream spray drying within the framework of LNPulmo project. Moreover, by transforming the liquid suspension into a solid-state dry powder through spray drying, this liquid-state leakage bottleneck can be effectively resolved. The dehydration process eliminates the surrounding aqueous phase that drives passive diffusion and locks the lipid vesicles and their payload within a stabilizing carbohydrate glass matrix, ultimately preserving liposomal integrity and extending the shelf life of the formulation [172].

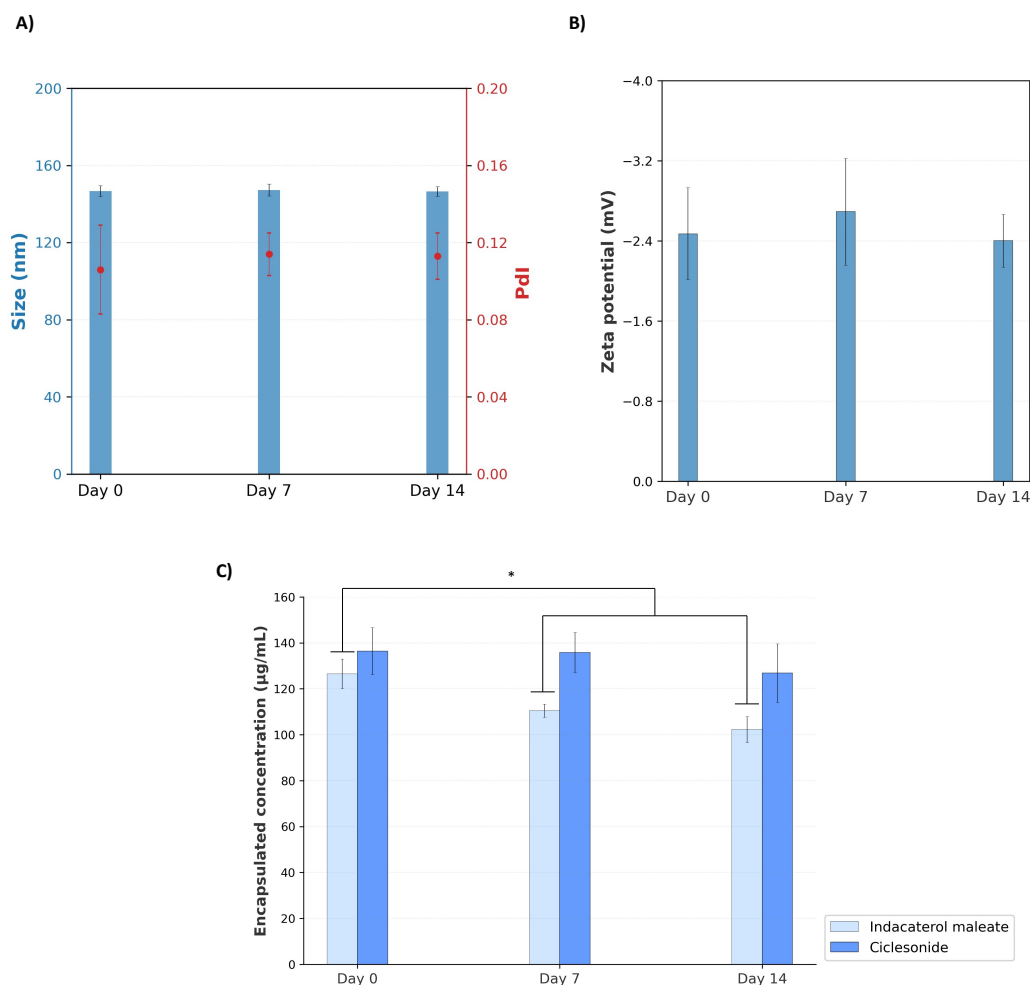


Figure 37. Stability of the optimal formulation (DLPC/Chol/DSPE-PEG, 55/30/15) via analysis of (A) size and PDI, (B) zeta potential, and (C) drugs encapsulation over 14 days (n = 3). One way ANOVA followed by Dunnett post test were performed: *p < 0.05.

I.3.3. Potential of liposomes in improving *ex vivo* bronchodilatory effect of other drugs: case of formoterol fumarate dihydrate

As the liposomal encapsulation of IND improved its bronchodilatory effect in both *ex vivo* and *in vivo* studies, we were also interested in whether these liposomes could also increase the activity of another LABA with different physicochemical properties. Therefore, we loaded FOR, which is less lipophilic than IND, into liposomes and compared its bronchodilatory effect on isolated tracheal rings to that of a FOR aqueous solution.

I.3.3.1. Method

- Liposomes preparation and characterization of physicochemical properties: FOR-loaded liposomes were prepared by the TFH method as described in the section 1.2.2.2. *Liposomes preparation by the thin film hydration method.* Removal of unencapsulated FOR was conducted via the dialysis of liposome against HEPES buffer solution at a ratio of 1:20 (v/v) during 4 h for the formulation at 5 mM and a ratio of 1:40 (v/v) during 5h for the formulation at 20 mM. Purified liposomes were then characterized by DLS as described in section 1.2.2.3.1. *Particles size, polydispersity index and zeta potential.*

- *Ex vivo* relaxation effect of FOR on isolated tracheal rings: similar to the method in section 1.2.2.6. *Ex vivo relaxant effect of indacaterol maleate on isolated tracheal rings from rats.*

- Quantification of FOR by HPLC: Quantification of FOR was performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a UV detector set at 243 nm [300]. Separation was made on a Waters XBridge BEH C18 column (3×50 mm, 3.5 μ m) at a constant temperature of 30 °C, with the autosampler maintained at 10 °C. The mobile phase, consisting of ammonium acetate buffer solution (pH 10) and methanol, was delivered at a flow rate of 0.7 mL/min with the following gradient mode: 0 min – 55/45 (v/v); 1 min - 55/45 (v/v); 2 min - 35/65 (v/v); 7 min - 35/65 (v/v); 8 min - 55/45 (v/v); 20 min - 55/45 (v/v). Acceptance limits were established at 10% within the range of 10 μ g/mL - 0.1 μ g/mL. the limit of detection was 0.1161 μ g/mL, with an limit of quantification of 0.9282 μ g/mL. The sample was diluted in Methanol/water (65/35, v/v) prior to analysis.

I.3.3.2. Results and discussion

FOR is a LABA with intermediate lipophilicity. This allows it to rapidly traverse tissue membranes to interact with β_2 ARs on ASM cells and exert a bronchodilatory effect for up to 12 h. Using liposomes composed of SPC/Chol/DSPE-PEG (55/30/15 molar ratio) at 5 mM, the mean size, Pdl, and zeta potential are 150 ± 3 nm, 0.08 ± 0.01 , and -1.8 ± 0.8 mV, respectively, which are satisfying the fixed CQAs. However, the EE of FOR was only $64.2 \pm 1.3\%$. This value decreased to $42.7\% \pm 3.0\%$ after just one day of storage at 4 °C. In the same formulation, IND exhibited a higher EE of approximately 90% after 1 day of storage at 4 °C (Figure 26B). This can be attributed to the higher lipophilicity of IND, which enables it to be retained more stably within the lipid bilayer than FOR. Despite this leakage, the *ex vivo* relaxation effect of liposomal FOR was significantly improved compared to its aqueous solution (Figure 38).

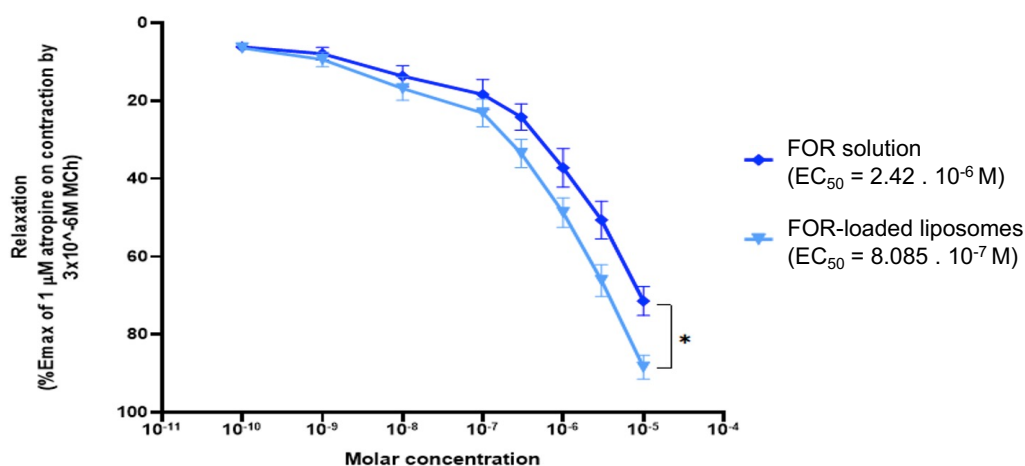


Figure 38. *Ex vivo* concentration-response curve of FOR-loaded liposomes and FOR aqueous solution (n = 5-8). Data are expressed as mean $\pm$ SEM. Statistical significance was determined by multiple t-tests using the Bonferroni-Dunn correction for multiple comparisons: * $p < 0.05$.

Despite the 20% drug leakage, the improved *ex vivo* performance underscores the need to finely tune the lipid composition to achieve more stable FOR encapsulation within liposomes. As FOR has an intermediate lipophilicity (Table 1), we hypothesize that it partitions between the lipid bilayer and the internal aqueous core. The aqueous fraction likely leaks readily into the external compartment, a phenomenon frequently observed with hydrophilic drugs. Interestingly, both FOR and SAB are proposed to be positively charged at physiological pH as they are nitrogenous bases [301–303]. A previous study showed that SAB entrapment in polymer NPs was improved using the negatively charged polymer poly(vinyl sulfonate-co-vinyl alcohol)-g-PLGA, which provided sustained release and extended the *in vivo* bronchoprotective effect against acetylcholine-induced bronchospasm up to 120 min while the effect of the free drug wore off at least 30 min earlier [304].

Applying this logic, we replaced SPC with dioleoyl-sn-glycero-3-phosphoglycerol (DOPG), an anionic phospholipid. This substitution successfully increased the initial EE of FOR, though it did not fully prevent leakage (Figure 39A). Increasing DOPG levels (compensated by DSPE-PEG at a fixed 30% Chol) showed no further improvement. However, increasing the total lipid concentration from 5 to 20 mM boosted the drug entrapment of all DOPG formulations to over 90%, which then stabilized at approximately 68% after 1 day of storage at 4°C. While all DOPG formulations maintained similar sizes (~150 nm) and a Pdl < 0.20, the zeta potential of the 20 mM formulations was nearly neutral and drastically less negative than the 5 mM counterparts (Figure 39B). We hypothesize that higher lipid concentrations

provide an increased surface area of negative charge to interact with the cationic FOR, thereby enhancing entrapment and neutralizing the anionic charge of the DOPG bilayer. Future evaluation of these optimized formulations on isolated tracheal rings is warranted to determine whether improved encapsulation enhances the *ex vivo* bronchodilatory response.

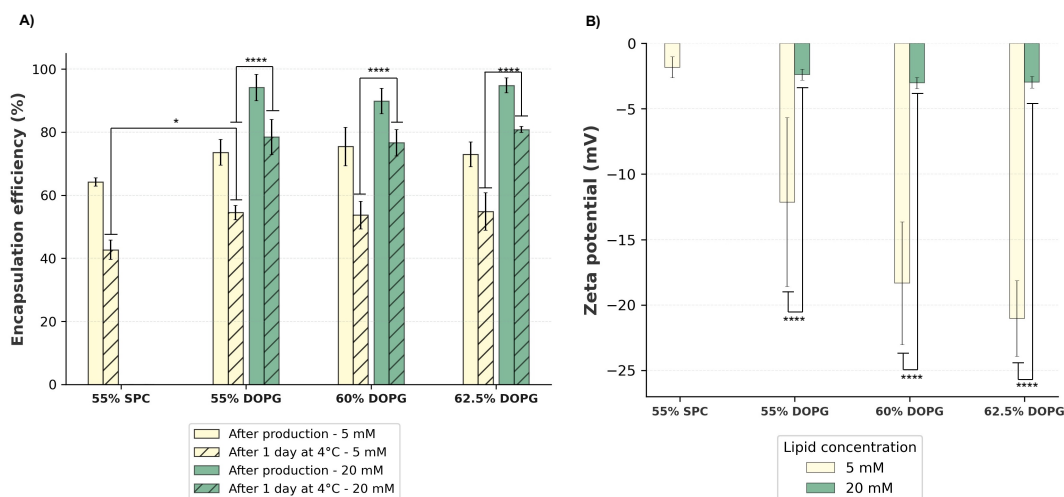


Figure 39. (A) EE and (B) of FOR-loaded liposomes with SPC (55%) or different concentrations of DOPG at either 5 or 45 mM ($n = 3$). Data are expressed as mean $\pm$ SD. One-way ANOVA followed by Šidák post-test were performed: * $p < 0.05$, **** $p < 0.0001$.

I.4. Conclusion

In summary, chapter I demonstrate that a rational selection of PEGylated lipid and phospholipid strongly influence the *ex vivo* and *in vivo* efficacy of liposomes containing CIC and IND. **DSPE-PEG** was identified as the optimal PEGylated lipid as its amine-phosphate interactions maximized IND encapsulation, while its C18 acyl chains provided superior macrophage biocompatibility compared to C14 and C16 analogs. The incorporation of 15% DSPE-PEG was important to ensure robust mucopenetration and macrophage evasion, significantly enhancing the *ex vivo* relaxation effects of IND over the free drug solution.

The high degree of unsaturation (two double bonds in each acyl chain) of **DLPC** was essential for achieving high EE, promoting efficient epithelial uptake, and improving the *ex vivo* potency of encapsulated IND.

Furthermore, 30% of **Chol** was likely to provide necessary bilayer resistance against the lateral pressure exerted by the 15% PEG chains and to stabilize the highly fluid nature of DLPC, resulting in nearly spherical and oval vesicles.

In the OVA-induced mouse model, this optimized formulation significantly reduced the AHR and inhibited the type 2 inflammatory response pathway, including upstream epithelial alarmins and downstream cytokines mobilization. Notably, the liposomal delivery of CIC and IND demonstrated significant dose-sparing efficacy for both.

Additionally, expanding this platform to FOR using a SPC/Chol/DSPE-PEG (55/30/15) formulation also yielded improved *ex vivo* efficacy compared to its aqueous solution. To mitigate FOR leakage, the neutral SPC was replaced with the anionic DOPG to foster electrostatic interaction with the drug, combined with an increase in total lipid concentration to 20 mM. These stabilized DOPG formulations provide a robust foundation for future *ex vivo* studies to further elucidate the relationship between encapsulation stability and therapeutic response.

DSPE-PEG and DLPC, combined with 30% Chol, enables the design of a robust liposomal formulation co-encapsulating CIC and IND, with superior mucopenetration, macrophage evasion, and epithelial uptake. This optimized formulation significantly improves both *ex vivo* and *in vivo* efficacy in asthma models, with a notable dose-sparing effect. The extension to formoterol and the adaptation of the lipid composition further illustrate the versatility of the platform. These findings provide a strong proof-of-concept for the use of liposomes to enhance ICS/LABA therapy in asthma and establish a solid foundation for industrial scale-up and future clinical translation. Further studies will be needed to assess long-term physicochemical stability and to validate the performance of this system in dry powder formulations and inhalation devices, paving the way for next-generation inhaled therapies.

CHAPTER II

Scaling-up liposome
production by the particles
from gas-saturated solution
process (PGSS)

II.1. Introduction to Chapter II

In Chapter I, we demonstrated that the rational optimization of the lipid composition yields an effective, dose-sparing liposomal formulation for pulmonary delivery of CIC and IND. It is now essential to consider scaling up this formulation to a production method compatible with industrial-scale manufacturing. The success of preclinical development does not guarantee the feasibility of large-scale manufacturing due to constraints related to process robustness, lipid stability, and preservation of critical formulation properties. Chapter II focuses on optimizing and validating a production method that uses SC-CO₂, the PGSS process. The objective is twofold: first, to ensure the reproducibility and stability of the liposomes during scaling up while maintaining essential quality attributes such as size, Pdl, EE, and chemical stability; and second, to pave the way for future industrial and clinical applications.

The PGSS process was implemented as a scalable and organic solvent-free alternative to the TFH method described in Chapter I. The primary objective of this phase was to transition the production of the previously optimized liposomal formulation from a lab-scale to a pilot scale. This scale-up was conducted within IQ/OQ-qualified, GMP-like zones at the IMPact Cleanroom platform (ULiège).

The PGSS process for production and sterilization of liposomes was previously validated in the laboratory [230,242]. This process resulted in two optimized production conditions at lipid concentration of 5 and 45 mM (Table 3). It was noted that a temperature of 80°C was necessary for successful liposome formation and sterilization [230,242,261]. Nevertheless, working at such a high temperature, in the presence of water and carbon dioxide, poses a substantial risk of chemical degradation for both phospholipids and thermolabile active molecules. As demonstrated in prior evaluations, the combination of a high temperature (e.g., 80 °C) and the acidic environment created by the formation of carbonic acid (from the interaction of CO₂ and water) significantly facilitates the hydrolysis of ester bonds of phospholipids, evidenced by an increased amount of lysophospholipids (LPC) after liposome production by the PGSS process [261]. At high concentrations, LPC forms mixed micelles with phospholipids, which can affect liposome stability by increased membrane fluidity and permeability, altering drug release and promoting liposome fusion or lysis [242]. Furthermore, high-temperature processing can strongly promote the oxidation of double bonds in the fatty acid chains of unsaturated lipids, thus creating structural defects in the bilayer.

Table 3. Previously optimized conditions for liposome production by PGSS at the laboratory scale [230].

Condition	Concentration (mM)	Volume of dispersion (mL)	Temperature (°C)	Pressure (bar)	Agitation speed (rpm)	Contact time (min)
1	45	14	80	240	500	30
2	5	10	80	156	500	30

To address these problems, saturated phospholipids can be used, as they are highly resistant to both hydrolysis and oxidation under harsh supercritical conditions [261]. However, the results from Chapter I showed that increasing the degree of unsaturation in the fatty acyl chains of phospholipids maximized the EE of CIC and IND and the *ex vivo* relaxant effect of IND. Consequently, to avoid the risks of oxidation and hydrolysis, Chapter II first focused on evaluating the possibility of lowering the temperature during liposome production.

These results served as the baseline for the subsequent process scaling from 50 mL to 500 mL reactor volume. Scale-up was achieved by fine-tuning critical process parameters, including pressure, dispersion volume, agitation speed, and depressurization rate via a DoE. Finally, the efficiency of the pilot-scale production was validated through a comprehensive characterization of the liposomes, focusing on particle size, Pdl, zeta potential, drug EE, and the presence of degradation products of phospholipids.

II.2. Adaptation and validation of the lab-scale PGSS process to the production of the optimized liposome formulation containing ciclesonide and indacaterol maleate

II.2.1. Method

Liposome production via the PGSS process at the laboratory scale was adapted from previously established protocols [230]. Briefly, the lipid and drug components were weighed and dispersed in HEPES buffer solution (10 mM, pH 7.4) at 60 °C for 15 min under constant stirring (200 rpm). This dispersion was subsequently transferred into the high-pressure reactor (Separex, France), where liquid CO₂ was introduced and brought to a supercritical state under the specific conditions. The SC-CO₂ was mixed with the lipid dispersion for 30

minutes at 500 rpm. Finally, the reactor was depressurized through a nozzle, and the resulting liposome suspension was collected in the expansion vessel.

Although the optimal formulation identified in Chapter I consisted of DLPC/Chol/DSPE-PEG (55/30/15, molar ratio), DLPC was replaced for this phase. SPC was selected as a cost-effective alternative as its fatty acid profile mainly consists of the same acyl chains that characterize DLPC [305]. The concentrations of CIC and IND remained fixed at 5 mol% relative to the total lipid content.

Table 4. Variables in the I-Optimal design for the adaptation of PGSS parameters at the laboratory scale.

Factor	Coded level		
<i>Independent variables</i>	Low level (-1)	Medium level (0)	High level (+1)
Lipid concentration (mM)	5	27.5	50
Dispersion volume (mL)	15	22.5	30
Contact time (h)	0.25	0.625	1
Agitation speed (rpm)	400	700	990
Temperature (°C)	35	57.5	80
Pressure (bar)	120	185	250
<i>Dependent variables</i>	Constraints		
Size (nm)	< 200		
Pdl	≤ 0.30		

While the primary goal of this stage was to determine whether the temperature could be reduced to less than 80 °C, other parameters such as lipid concentration, dispersion volume, contact time, agitation speed, and pressure were also included in this parametric optimization. Pressure was selected as a variable because this parameter, together with temperature, dictates the supercritical state and density of CO₂. Contact time is another important parameter to be studied, as prolonging the exposure of lipids to high temperature and pressure conditions can increase the risk of oxidation and hydrolysis. Dispersion volume and agitation speed are crucial factors that need to be carefully controlled to ensure efficient mixing of the SC-CO₂ and the lipid dispersion, especially since the properties of SC-CO₂ can change dramatically depending on temperature and pressure. Finally, lipid concentration is a crucial formulation variable because physicochemical properties of resulting liposomes were shown to vary depending on this parameter [239]. To evaluate influences of these parameters on liposome size and Pdl, an I-optimal DoE was generated

using JMP 19 software (SAS Institute, Cary, NC, USA). The independent and dependent variables are summarized in Table 4. The design evaluated main effects, interactions, and quadratic terms across three levels, totaling 36 experimental runs organized into blocks of three (Table 5).

Table 5. Experimental conditions and results of the 36 experiments from the I-optimal design.

Random Block	Lipid concentration (mM)	Dispersion volume (mL)	Contact time (h)	Agitation rate (rpm)	Temperature (°C)	Pressure (bar)	Size (nm)	Pdl
1	50	30	0.25	400	35	120	374.8	0.7975
	27.5	22.5	0.625	999	35	185	339.9	0.4306
	5	30	1	700	80	250	190.8	0.2536
2	27.5	22.5	0.25	999	57.5	185	236.5	0.6741
	50	30	1	400	80	120	178.6	0.3559
	27.5	15	0.625	700	80	250	168.9	0.2706
3	5	15	0.25	400	35	250	264.1	0.7425
	50	30	0.25	999	80	120	171.3	0.3131
	50	15	1	999	80	120	160.5	0.3026
4	27.5	22.5	1	700	80	185	154.2	0.3613
	50	15	1	400	57.5	250	215.4	0.6483
	50	15	0.625	400	35	185	319.4	0.5959
5	50	30	0.25	400	80	250	166.1	0.3536
	27.5	22.5	0.625	400	35	185	306.6	0.5034
	27.5	30	1	999	57.5	120	224.4	0.7324
6	5	30	1	400	35	120	340.9	0.7804
	5	15	1	400	80	185	132.8	0.338
	27.5	22.5	0.625	700	57.5	120	228.7	0.726
7	50	30	1	700	35	250	600.7	0.3546
	5	15	0.625	400	57.5	120	216.4	0.4123
	5	30	0.25	999	35	250	468.6	0.7372
8	5	15	0.25	999	80	250	129.2	0.3505
	27.5	30	0.625	700	57.5	185	232.2	0.6819
	50	22.5	0.625	999	35	120	414.3	0.9496
9	50	30	1	999	80	250	162.8	0.3616
	50	22.5	0.25	700	57.5	250	332.5	0.3866
	5	15	0.25	700	35	120	440	0.6133
10	5	15	1	999	35	250	222.9	0.4775
	5	30	0.25	400	80	120	161.8	0.3888
	50	15	0.25	400	80	120	150.3	0.3501
11	5	22.5	0.625	700	57.5	185	193.9	0.5981
	50	15	0.25	999	35	250	444	0.5062
	27.5	15	1	400	35	120	289.8	0.7122
12	27.5	30	0.625	400	57.5	250	221.4	0.462
	50	15	1	700	57.5	185	248.7	0.5206
	5	22.5	1	999	80	120	138.5	0.4426

II.2.2. Results and discussion

II.2.2.1. Adaptation of the PGSS parameters for liposomes containing ciclesonide and indacaterol maleate at the lab-scale by DoE approach

Of all investigated parameters, temperature exhibited the most significant impact on both the particle size and Pdl of the liposomes (Figure 40). Specifically, operating at 80 °C resulted in a sharp reduction of the Pdl to approximately 0.30 and the mean size to below

200 nm. This may be attributed to the lipids transitioning into a more fluid and disordered state at this temperature, which may have facilitated more efficient dispersion by the SC-CO₂ phase. Furthermore, temperatures below 80 °C may have been insufficient to adequately destabilize the crystalline state of the highly lipophilic drugs, CIC and IND, hindering their effective incorporation into the lipid matrix. The dominance of the temperature effect likely masked the influence of other parameters, potentially explaining why their effects were less discernible within this DoE. Taken together, these results demonstrate that maintaining a temperature of 80 °C is critical for the production of stable liposomal formulations co-encapsulating CIC and IND. The next step is to use the two conditions in Table 3 to produce the optimal formulation and compare the properties of the resulting liposomes with those produced by the TFH method.

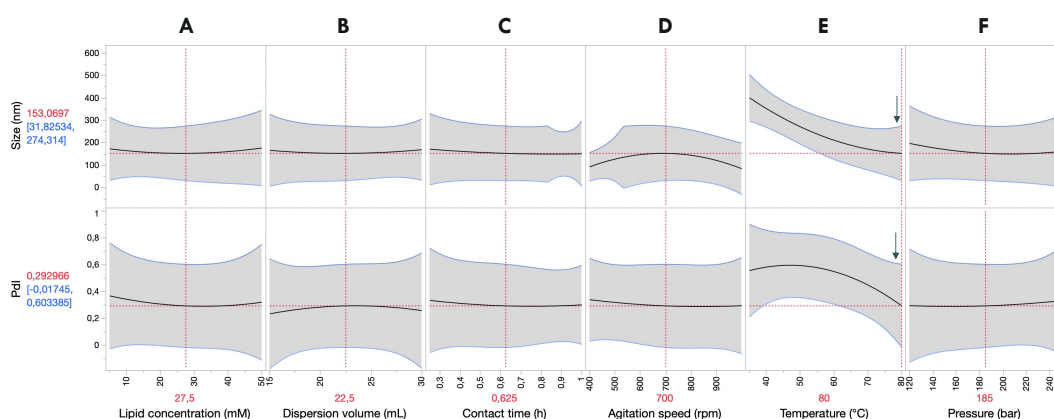


Figure 40. Prediction profiler of the I-optimal DoE: Illustration of the effects of (A) lipid concentration (mM), (B) dispersion volume (mL), (C) contact time (h), (D) agitation speed (rpm), (E) temperature (°C), and (F) pressure on the size and Pdl of liposomes.

II.2.2.2. Validation of the PGSS process for the production of liposomes containing ciclesonide and indacaterol maleate at the lab-scale

The two PGSS processing conditions defined in Table 3 were employed to produce the optimal formulation identified in Chapter I (DLPC/Chol/DSPE-PEG, 55/30/15) at two different lipid concentrations. The characteristics of the resulting liposomes were then compared to those of liposomes produced by the TFH method. While the condition 2 was applied to a lipid concentration of 5 mM as originally presented, the condition 1 was adjusted to 27.5 mM instead of the initial 45 mM. This modification was necessary because applying the condition 1 to produce the optimal formulation at 45 mM resulted in highly variable encapsulated drug concentrations after dialysis, which were sometimes even

higher than pre-dialysis levels (data not shown). This variability could be attributed to the excessively high lipid concentration (45 mM) and the correspondingly high concentration of lipophilic molecules (5% relative to lipids), which may have not been suitable for the condition 1. Therefore, the lipid concentration was reduced from 45 mM to 27.5 mM, a value that more closely aligns with the typical lipid concentrations found in industrially produced, ready-to-be-administered liposomal suspensions (5–25 mM) [197].

Liposomes produced by the PGSS process exhibited mean sizes below 150 nm, Pdl under 0.30, nearly neutral zeta potentials, and EE for both drugs exceeding 90% at both 5 and 27.5 mM of lipid concentrations (Table 6). These physicochemical characteristics closely mirrored those of TFH-produced liposomes, exception for Pdl. Liposomes produced by TFH showed a significantly lower Pdl (One-way ANOVA, $p < 0.05$), probably due to the extrusion-assisted homogenization step inherent to the TFH protocol.

These findings suggest that, despite the elevated temperatures employed, the optimized PGSS conditions established in previous studies remain effective for reproducibly producing the optimal formulation with the desired properties. Nevertheless, as these parameters may undergo further adjustment during the transition to pilot-scale equipment, monitoring lipid oxidation and hydrolysis products remains essential to confirm the chemical integrity of the liposomes post-production.

Table 6. Size, Pdl, Zeta potential, and EE of CIC- and IND-loaded liposomes produced by TFH and PGSS.

Method	Lipid concentration (mM)	Size (nm)	Pdl	Zeta potential (mV)	Encapsulation efficiency (%)	
					Ciclesonide	Indacaterol maleate
TFH	5	141 ± 3	0.09 ± 0.03	-2.7 ± 0.6	97.3 ± 0.2	94.6 ± 0.9
	27.5	141 ± 5	0.10 ± 0.02	-3.3 ± 0.4	99.3 ± 3.9	95.9 ± 3.5
PGSS	5	113 ± 3	0.26 ± 0.02	-4.3 ± 1.6	96.9 ± 0.7	96.1 ± 2.1
	27.5	126 ± 4	0.26 ± 0.05	-2.6 ± 0.5	96.2 ± 2.2	90.2 ± 2.7

II.3. Scaling-up the PGSS process to the pilot-scale production

Introduction and overview of the results currently under peer review in International Journal of Pharmaceutics:

The objective of this section was to scale up the PGSS production method previously validated at the lab-scale, to a pilot scale while continuing to meet the quality control criteria established for liposomes, namely an average particle size of less than 200 nm, a Pdl close to 0.30, and a high EE.

This study was conducted in parallel with another research project carried out in the laboratory, the NANOPDELEAR project, which focused on developing Dexa-loaded liposomes embedded within a hydrogel matrix for the localized treatment of sensorineural hearing loss. The study, conducted with my fellow PhD student, Chloé Gauthy, thus validated the new process using two distinct drug-loaded formulations. The first comprises the optimized formulation from Chapter I of this thesis: DLPC/Chol/DSPE-PEG (55:30:15) co-encapsulating CIC and IND. The second derived from the Nanopdelear project, consists of DSPC/Chol/DSPE-PEG (85:10:5) encapsulating Dexa [306].

Due to significant differences in lipid composition and active molecules between these two formulations, a simplified, empty liposomal alternative was used for the initial optimization of the pilot-scale PGSS parameters. This intermediate formulation consisted of SPC/Chol/DSPE-PEG (65:30:5). Our research group previously used this optimization strategy for the laboratory-scale optimization of PGSS. Specifically, a blank SPC/Chol (70:30, w/w) formulation was initially used to optimize laboratory-scale PGSS parameters [230] before successfully translating those optimal parameters to produce BUD- and SAB-loaded liposomes (SPC/Chol/DSPE-PEG, 55:30:15) [261].

The pilot-scale PGSS equipment shares the same basic workflow as the laboratory-scale PGSS system (Figure 42), where a dispersion of lipids and active molecules in a buffer solution is introduced into a high-pressure reactor, mixed with SC-CO₂ for a specific time, and then depressurized. However, the pilot-scale setup differs from the laboratory-scale equipment in several keyways. First, rather than being introduced as a cold liquid, the liquid CO₂ from the pressurized cylinder is heated before being pumped into the reactor. This preheating step enables better control over both temperature and pressure during large-scale operations. Additionally, the pilot-scale reactor is equipped with two sapphire viewing windows for visual observation. Furthermore, the reactor volume has been scaled up from 50 to 500 mL, and the agitator geometry was changed from a square blade to a pitched-

blade impeller. Crucially, the pilot-scale system allows for precise control over the depressurization rate, a feature that is absent in the laboratory machine.

To optimize the process parameters at the pilot scale, some parameters were fixed while others were investigated. The fixed parameters included lipid concentration, temperature, and contact time. Since this was the first large-scale investigation of the PGSS process which requires a large quantity of costly materials, from lipids to active molecules, we chose to perform this optimization at a low lipid concentration of 5 mM. This concentration minimized experimental costs while yielding valuable information on how PGSS operates at a large scale. The contact time was fixed at 30 min because previous optimization showed that this parameter did not obviously impact on the produced liposomes. Additionally, the temperature was fixed at 80 °C because previous laboratory-scale optimizations showed that this temperature was necessary to obtain liposomes with a size under 200 nm and a Pdl below 0.30, as well as to ensure efficient sterilization [230,242]. Moreover, results from the adaptation of the PGSS process at the laboratory scale showed that this temperature is paramount for the formation of liposomes in the presence of hydrophobic drugs like CIC and IND.

The following parameters were selected as variables for optimization: pressure (120–250 bar), agitation speed (10–70%), dispersion volume (100–200 mL), and depressurization rate (60–120 bar/min). An A-optimal design comprising 22 experiments was used to optimize these parameters, with particle size (target < 200 nm) and Pdl (target ~0.30) as the dependent variables. The results from this DoE approach showed that the combination of a high pressure (262 bar) and a low agitation speed (10%) likely provided a sufficiently high amount of SC-CO₂ and efficient mixing with the lipid dispersion. Moreover, a slow depressurization rate of 60 bar/min was critical to obtaining homogeneous liposomes. Since the dispersion volume did not display a remarkable effect on the properties of the liposomes, it was set to 100 mL to minimize material consumption under the optimal conditions. Using these optimized conditions, we successfully produced two drug-loaded formulations, with size under 200 nm, Pdl close to 0.30, and EE comparable to the formulations produced at the laboratory scale (for the formulation containing CIC and IND) or even slightly better (for the formulation containing Dexa). Importantly, these conditions did not significantly increase the hydrolysis or oxidation of the main phospholipids in either drug-loaded formulation. Future experiments will be needed to increase the lipid concentration closer to current industrial liposome manufacturing conditions, and to further reduce the Pdl below 0.30 to meet the strict requirements of regulatory authorities.

Manuscript under peer-review in the International Journal of Pharmaceutics [307]:

Pilot Scale Transfer and Optimization of an Innovative Supercritical CO₂ PGSS Method for the Production of Drug-Encapsulating Liposomes

Tuan Nghia Dinh^{a,1}, Chloé Gauthy^{a,1}, Hugo Magdalena Segura^a, Pierre-Yves Sacré^b, Erika Hendrickx^c, Louise Conrard^c, Brigitte Evrard^a, Brigitte Malgrange^d,
Géraldine Piel^{a*}, Noémie Penoy^a

^a *Laboratory of Pharmaceutical Technology and Biopharmacy, Center for Interdisciplinary Research on Medicines, University of Liège, 4000 Liège, Belgium*

^b *Research Support Unit in Chemometrics, Center for Interdisciplinary Research on Medicines, University of Liège, 4000 Liège, Belgium*

^c *Center for Microscopy and Molecular Imaging (CMMI), Université Libre de Bruxelles, 6041 Gosselies, Belgium*

^d *Developmental Neurobiology Laboratory, GIGA-Stem cells, University of Liège, 4000 Liège, Belgium*

* Corresponding author: Géraldine Piel (geraldine.piel@uliege.be)

¹ These authors contributed equally to this work.

Keywords: Liposomes, supercritical carbon dioxide, pilot scale, drug encapsulation, liposome production method.

Abstract

The industrial translation of liposome production processes remains challenging, particularly in achieving scalability while maintaining critical quality attributes and formulation stability. In this study, a supercritical CO₂-based PGSS process was successfully transferred from the laboratory to the pilot scale (50 to 500 mL reactor). Using a Quality by Design approach and a design of experiments on a simplified soy phosphatidylcholine formulation, we identified optimal production process parameters. These parameters were then validated and transferred to other drug-encapsulating liposome formulations, demonstrating process robustness. Under these conditions, liposomes measuring less than 200 nm and polydispersity indices close to 0.30 were reproducibly obtained at pilot scale. The process was successfully applied to drug-loaded formulations, achieving high encapsulation efficiencies while maintaining suitable physicochemical properties. Cryo-TEM confirmed the formation of consistent vesicular structures. Importantly, lipid chemical stability was preserved, with minimal hydrolysis and no significant increase in oxidation, even for unsaturated lipids. Overall, this study demonstrates the robustness, scalability, and relevance of the PGSS process as a solvent-free, single-step technology for liposome production.

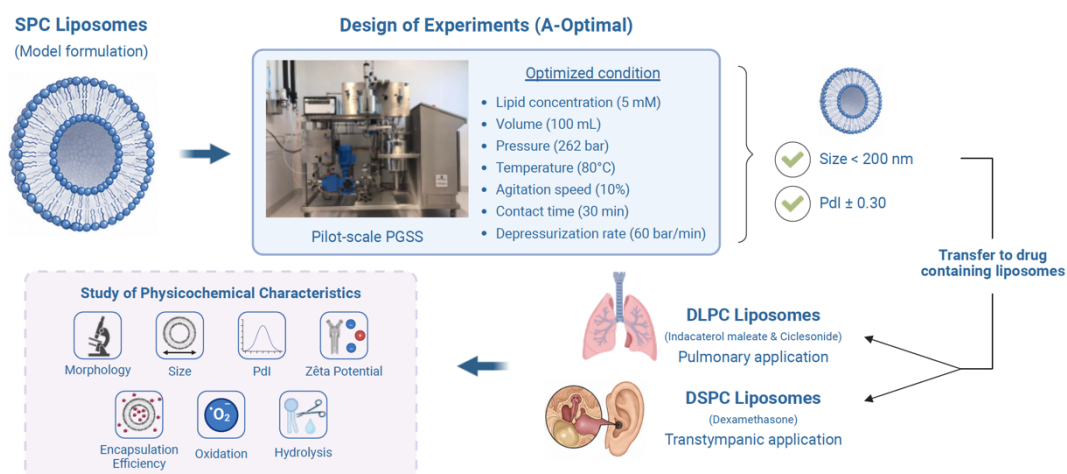


Figure 41. Schematic illustration of scaling up liposome production by the Particles from gas-saturated solution process at pilot scale.

II.3.1. Introduction

Liposomes are spherical vesicles composed of one or more phospholipid bilayers surrounding an aqueous core [308,309]. This unique architecture allows them to encapsulate, or co-encapsulate, both hydrophilic and hydrophobic active ingredients, making them a versatile delivery system for small molecules as well as biomolecules. While numerous methods have been developed for liposomes preparation at the laboratory scale (lab scale), few satisfy the rigorous demands of industrial-scale pharmaceutical manufacturing, where high reproducibility, sterility and precise control over critical quality attributes (CQAs) such as particle size, polydispersity index (Pdl), and encapsulation efficiency (EE) are of paramount importance [175]. Ethanol injection method is currently the most common technique for the industrial manufacture of parenteral liposomes containing small molecules, whereas microfluidics has become the dominant technology for manufacturing lipid nanoparticles (LNPs) used in gene therapies and mRNA vaccines [175,182]. Both methods rely on the same fundamental principle of solvent exchange: lipids and hydrophobic drugs are dissolved in a water-miscible organic solvent (typically ethanol) and mixed with an aqueous phase containing hydrophilic drugs. This mixing increases the polarity of the solvent environment, triggering lipid precipitation and self-assembly into vesicles. However, ethanol injection typically yields heterogeneous vesicles that require subsequent size reduction and homogenization steps, such as extrusion, to achieve a uniform particle size distribution. Furthermore, these production methods must be followed by organic solvent removal (e.g., via tangential flow filtration) and, if needed, a final sterilization step. One of the most challenging problems with these methods is the use of ethanol which necessitates complex removal steps and rigorous monitoring of residue levels in the final product [310]. These processes are both costly and time-consuming for industrial applications [311,312].

With the aim of developing a solvent free method for liposome production, our laboratory has focused for years on the Particles from Gas-Saturated Solution (PGSS) process using supercritical carbon dioxide (SC-CO₂). The primary role of SC-CO₂ in this process is to disperse the lipid molecules within the aqueous phase during pressurization, which then reassemble into lipid vesicles during the depressurization step [232]. The low critical parameters of SC-CO₂ (31.1 °C, 73.8 bar), combined with its non-flammable, non-toxic, chemically inert, and environmentally friendly nature, make it an ideal and cost-effective candidate for industrial scale applications [217,313].

In a previous study, Penoy *et al.* employed a Quality by Design (QbD) approach to optimize process parameters (temperature, pressure, agitation rate, dispersion volume, and contact time with SC-CO₂) as well as formulation parameters (lipid concentration) [230]. The goal was to produce liposomes at the lab scale exhibiting targeted CQAs, specifically a size below 200 nm and a Pdl close to 0.30. This strategy enabled one-step liposome production without organic solvents, and led to the identification of specific operating conditions that were reproducible and robust across multiple liposomal formulations, enabling efficient encapsulation of both hydrophilic and hydrophobic compounds without causing a considerable increase in hydrolysis and oxidation products of lipid components) [261]. The PGSS method has also gained attention from other research groups, as evidenced by studies investigating the impact of process parameters [232,233,235,236,314,315] and lipid composition [233,239] on liposomes formation and drugs loading.

In addition, SC-CO₂ exhibits notable microbicidal properties, offering a promising alternative for the sterilization of thermosensitive products such as liposomes [211]. More recently, the PGSS conditions optimized by Penoy *et al.* were shown to achieve a sterility assurance level compliant with pharmaceutical requirements, as a result of the combined effects of elevated temperature and pressure during processing [242].

Throughout these studies, PGSS has emerged as an innovative technology for the simultaneous production and sterilization of drug-encapsulated liposomes without the need for organic solvents. To move closer to industrial application, investigating its ability to produce liposomes at a pilot-scale is now of great interest. However, to the best of our knowledge, this subject has not yet been explored. To bridge this gap, our laboratory has worked on scaling up the PGSS process, transitioning from the lab scale to a pilot scale (50 to 500 mL reactor). This development was made possible by the use of a GMP-compliant PGSS system (IMPact Cleanroom, University of Liège).

However, the transition from the lab- to pilot-scale equipments involves significant mechanical and mechanistic differences, such as changes in reactor volume (from 50 to 500 mL), impeller design (from square to pitched blade impeller), and the ability to control the depressurization process of the pilot-scale device, making simple scaling up based solely on lab-scale conditions impossible. Therefore, the objective of this study is to conduct the first investigation on parameters for PGSS production at the pilot scale using a QbD approach. Based on our understanding from previous lab scale investigations, parameters such as lipid concentration, temperature, and contact time were fixed to facilitate the

investigation of others, including pressure, dispersion volume, agitation speed, and depressurization rate. Following a Design of Experiment (DoE) study, an optimal condition was applied to the production of two liposomal formulations containing drugs previously developed: a co-encapsulated indacaterol maleate (IND) and ciclesonide (CIC) formulation for pulmonary administration [249] and a dexamethasone (Dexa) formulation designed for intratympanic administration [306]. Then, the obtained liposomes were thoroughly analyzed in terms of size, Pdl, zeta potential, morphology, drugs encapsulation, as well as lipid hydrolysis and oxidation. These results confirmed the capability of the PGSS process to produce large quantities of liposomes with expected properties, further establishing it as a future industrial technology for solvent-free liposome manufacturing.

II.3.2. Materials and methods

II.3.2.1. Materials

Dexamethasone (Dexa) was purchased from Fagron (Belgium). Ciclesonide (CIC) was purchased from LEAPChem (China). Indacaterol maleate (IND) was purchased from NEWCHEM Spa (Italy). Cholesterol, chloroform, triethylamine and 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) $\geq 99.5\%$ were purchased from Sigma-Aldrich (Belgium). Soy phosphatidylcholine with 95% L- α -phosphatidylcholine (SPC) and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (18:0 LPC) were purchased from Avanti Polar Lipids, inc. (Alabaster, USA). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol-2000)] (DSPE-PEG2000) were purchased from SINOPEG (China). 1-linoleoyl-2-hydroxy-sn-glycero-3-phosphatidylcholine (18:2 LPC) was purchased from ABITEC (Sweden). HPTLC Silica gel 60 F254, orthophosphoric acid and copper sulfate (CuSO_4) were purchased from Supelco® (Germany), Merck (Germany) and Carl ROTH® (Germany), respectively. Liquid CO_2 with a purity of 99.7% was purchased from Air Liquide (Belgium). Ultrapure water was produced by a Milli-Q system (18.2 $\text{M}\Omega\cdot\text{cm}$ resistivity; Millipore, Bedford, MA, USA). Methanol and acetonitrile of (Ultra) Gradient HPLC Grade were purchased from J.T. Baker (Poland). Absolute ethanol from analytical reagent grade was purchased from VWR chemicals BDH® (France).

II.3.2.2. Liposome production

Table 7 shows the composition of the liposome formulations used in this study. To investigate the PGSS process parameters using a design of experiments (DoE) approach,

a formulation based on SPC, without any active pharmaceutical ingredient (API), was used. Two formulations loaded with an active ingredient were then used to evaluate the robustness and transferability of the production method during scaling up.

Table 7. Composition of liposomes.

Formulation	Lipid composition (mol%)	Drugs (5 mol% of total lipids)
SPC Formulation	SPC/Chol/DSPE-PEG (65/30/5)	/
DSPC Formulation	DSPC/Chol/DSPE-PEG (85/10/5)	Dexa
DLPC Formulation	DLPC/Chol/DSPE-PEG (55/30/15)	CIC and IND

II.3.2.2.1. PGSS method by the lab scale equipment

First, lipids and APIs were weighed and dispersed in HEPES buffer (10 mM, pH 7.4) at 60 °C for 15 min under stirring (200 rpm). Then, the dispersion was poured into the high-pressure reactor of the lab scale equipment (Separex, France) and liquid CO₂ was pumped into the reactor, where it reached the supercritical state under a previously optimized condition for the production of liposomes at 5 mM: 10 mL of lipid dispersion, 80 °C, 156 bar, 500 rpm of agitation, 30 min of contact [230]. SC-CO₂ was mixed with the lipid dispersion for 30 min at 500 rpm (Figure 42). Finally, the reactor was depressurized through a nozzle, and the resulting liposome suspension was collected in the expansion vessel.

II.3.2.2.2. PGSS method by the GMP pilot scale equipment

The liposome production was scaled up to fit the pilot scale GMP PGSS equipment, which features a high-pressure reactor with a tenfold larger volume. The procedure is more or less identical to that used at the lab scale. Briefly, lipids, APIs, and HEPES buffer (10 mM, pH 7.4) were first added to the reactor without preheating at 60 °C. This dispersion was mixed with SC-CO₂ under experimental conditions defined by a DoE as detailed below. Finally, the mixture was depressurized through a nozzle at a constant rate, also specified by the DoE, and the liposome suspension was collected from the expansion vessel.

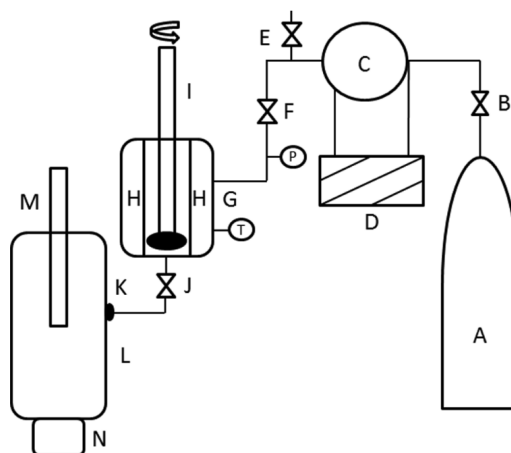


Figure 42. Schematic representation of PGSS equipments: (A) CO₂ bottle, (B, E, and F) on/off valves, (C) pump, (D) refrigerant, (G) high pressure reactor, (H) heating jacket, (I) Stirrer, (J) pneumatic valve, (K) nozzle, (L) expansion vessel, (M) CO₂ outlet, (N) sample recovery vial, (P) pressure gauge, (T) thermostat. Reprinted with permission from [230].

II.3.2.2.3. Design of Experiment (DoE): production parameters optimization

Building on previous optimization of the PGSS process at the lab scale, the temperature, contact time, and lipid concentration were fixed at 80 °C, 30 min, and 5 mM, respectively [230,242]. The investigated parameters included dispersion volume, pressure, agitation speed, and depressurization rate (Table 8). Their influence on the size and Pdl of the SPC formulation produced by the pilot scale equipment was evaluated using an A-optimal DoE generated by JMP 19 Software (SAS Institute, Cary, NC, USA). Main effects, interactions, and quadratic terms of these factors were evaluated at three levels, resulting in 22 experimental runs grouped into blocs of 2 (Table 12). The ranges for volume (100 to 200 mL) and pressure (120 to 250 bar) were based on previous studies [230,242], while the agitation speed was capped at 70% (10 to 70%) due to mechanical constraints of the pilot equipment. The depressurization rate (ΔP) was limited to 120 bar/min (60 to 120 bar/min) to mitigate the Joule-Thomson effect [233,235]. The objective of the DoE was to achieve a size below 200 nm and a Pdl close to 0.30.

Statistical analysis of the experimental data, including model fitting and evaluation of factor effects on the CQAs, was performed using JMP Pro 19. After analyzing the results using the Profiler in JMP, a Monte Carlo simulation was performed to account for factor variability and model uncertainty. This approach made it possible to assess the robustness of the previously identified optimal conditions by estimating the distribution of responses and the probability of meeting specifications (CQAs) under real-world conditions.

Table 8. Variables in the A-Optimal design for parameters investigation of PGSS at pilot scale.

Factors	Coded level		
	Low level (-1)	Medium level (0)	High level (+1)
Dispersion volume (mL)	100	150	200
Pressure (bar)	120	185	250
Agitation speed (%)	10	40	70
Depressurization rate (bar/min)	60	90	120
Responses	Constraints		
Size (nm)	< 200		
Pdl	≤ 0.30		

II.3.2.3. Characterization of physicochemical properties of liposomes

II.3.2.3.1. Size, Pdl, and zeta potential

The Z-average size (nm) and Pdl of the liposomes were determined by dynamic light scattering (DLS), while the zeta potential (ZP, mV) was measured by electrophoretic light scattering (ELS), using a Zetasizer Ultra Red (Malvern Panalytical, UK) at a scattering angle of 90° and at room temperature (25°C). Liposomes were diluted in HEPES buffer (10 mM, pH 7.4) to achieve a total lipid concentration of 0.5 mM, corresponding to a particle concentration suitable for measurement. Prior to data acquisition, an equilibration time of 120 seconds was applied to each sample. The dispersant (HEPES buffer) properties were defined using the standard parameters of water at 25°C: a refractive index of 1.332 and a viscosity of 0.8872 cP. The liposomal material was modeled with a refractive index of 1.450 and an absorption of 0.001. Data processing was performed using ZS Explorer software. For DLS analysis, the General Purpose model was utilized, with the number of runs per measurement automatically optimized by the software (typically ranging between 10 and 15 runs). For zeta potential measurements, the Auto mode analysis model was employed. All samples were measured in triplicate.

II.3.2.3.2. Encapsulation efficiency

Non-encapsulated drugs in DSPC and DLPC formulations were purified by dialysis at 4°C in HEPES buffer (10 mM, pH 7.4) (1:20, volume ratio) using a 20 kDa molecular weight cut-off cellulose membrane (Spectra/Por®, VWR Chemical) for 5h [306] and 4h [249], respectively, under constant agitation (200 rpm), with medium changes every hour. EE of Dexa in DSPC formulation and CIC and IND in DLPC formulation were determined by

quantifying drugs concentrations ($\mu\text{g/mL}$) before and after dialysis for the calculation by the following equation (3).

$$EE (\%) = \frac{\text{Drug concentration after dialysis}}{\text{Drug concentration before dialysis}} \times 100 \quad (3)$$

II.3.2.4. Drugs quantification

II.3.2.4.1. Quantification of Dexa

Dexa quantification was performed using a validated HPLC method [306]. The equipment consisted of an Agilent® 1100 system (Santa Clara, USA) equipped with OpenLab CDS LC ChemStation (version C.01.05). Separation was achieved on an RP-18 analytical column (250 x 4 mm, 5 μm) fitted with a matching RP-18 guard column (4 x 4 mm, 5 μm), using an isocratic mobile phase of water/acetonitrile (50:50, v/v) at a flow rate of 0.8 mL/min. The column temperature was kept constant at 35°C. Samples (10 μL) were injected at room temperature, with a total run time of 8 min, and Dexa was detected at 242 nm with a retention time of 4.9 min. Calibration curves were generated using standard solutions at 1, 10, 50, and 100 $\mu\text{g/mL}$. Samples were diluted 20-fold in absolute ethanol. This method was successfully validated using the accuracy profile approach and Enoval® software V3.0 (Arlenda, Liège, Belgium). The limit of quantification of Dexa is 1.868 $\mu\text{g/mL}$. The risk α was set at 5% with acceptance limits at 10%.

II.3.2.4.2. Quantification of CIC and IND

CIC and IND concentrations were quantified using a HPLC method [316]. The equipment consisted of an Agilent® 1100 system (Santa Clara, USA) equipped with OpenLab CDS LC ChemStation (version C.01.05). Separation was achieved on an Xbridge BEH C18 column (50 x 3 mm, 3.5 μm) maintained at 30°C. The mobile phase consisted of an ammonium acetate buffer at pH 10 (A) and methanol (B) with the following gradient elution at a flow rate of 0.7 mL/min: 0-1 min 80% A; 10-13 min 5% A; 13.5-20 min 80% A. The injection volume was 10 μL . The UV detector was set at 243 nm wavelength. The limits of quantification of CIC and IND are 3.205 and 2.031 $\mu\text{g/mL}$, respectively. Liposomes were diluted 10-fold in methanol prior to analysis.

II.3.2.5. Evaluation of lysophospholipids (LPC) content (hydrolysis products of phospholipids)

The evaluation of 18:0 LPC and 18:2 LPC, main hydrolysis products of DSPC and DLPC respectively, was performed by high-performance thin-layer chromatography (HPTLC) as previously reported [261,317]. Liposomes were diluted 2.5-fold in methanol. A lipid solution in methanol with the same composition and concentration was prepared as pre-PGSS control. Calibration curves of 18:0 LPC and 18:2 LPC were prepared in methanol at 25, 50, 100, and 150 µg/mL. Samples were applied onto silica gel HPTLC plates (Merck, 60 F254, 10 × 20 cm) using a semi-automatic LINOMAT 5 applicator (CAMAG, Switzerland) under the following conditions: 10 µL injection volume, 150 nL/s application rate, 5 mm band length, and a 15 mm distance from the lower edge of the plate. Dried plates were then developed in a development chamber (CAMAG ADC2, Switzerland) using a double-trough chamber (10 × 20 cm, CAMAG, Switzerland) containing 10 mL of mobile phase composed of chloroform, methanol, triethylamine, and water (30:35:34:8, v/v). Development was stopped when the solvent front had migrated 70 mm. Plates were immersed three times for 5 s in a 10% CuSO₄ solution acidified with 8% phosphoric acid for post-chromatographic derivatization, dried at room temperature for 20 min, and heated at 170°C for 30 min. Evaluation of 18:0 LPC and 18:2 LPC amounts was performed by measuring the intensity of the derivatized bands using CAMAG TLC 2 visualizer using visionCATS software, and LPC content was subsequently calculated using the equation , where LPC is LPC content (%), C_{LPC} is LPC concentration (µg/mL), and C_{phospholipid} is Initial phospholipid concentration (µg/mL).

$$LPC = \frac{C_{LPC}}{C_{phospholipid}} \times 100 \quad (4)$$

II.3.2.6. Determination of percentage of peroxidized lipids

The amount of oxidation was determined by measuring the absorbance of conjugated dienes, which appear in the oxidized form of phospholipids, by UV–visible spectrophotometry at 234 nm [318]. For the spectrophotometric measurements, liposomes were diluted in ethanol to a final phospholipid concentration of 0.5 mg/mL for DPLC formulation and 1 mg/mL for DSPC formulation. A lipid solution of identical composition and concentration was prepared to serve as a pre-PGSS control. The increase in the percentage of peroxidized lipids relative to the initial phospholipid content (%Δ Peroxidized

lipids) was calculated using the equation (5), where Abs_{234} and Abs'_{234} are the measured absorbances at 234 nm after and before the PGSS production, ϵ is the molar extinction coefficient of the conjugated dienes ($27000 \text{ M}^{-1} \cdot \text{cm}^{-1}$), D is the light path length of the quartz cell (1 cm), and C_{lipids} is the molar concentration of total lipids [318].

$$\% \Delta \text{ Peroxidized lipids} = \frac{Abs_{234} - Abs'_{234}}{\epsilon \cdot D \cdot C_{lipids}} \times 100 \quad (5)$$

II.3.2.7. Evaluation of liposomes morphology by cryo-TEM imaging

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 35s. Grids were then transferred to a Vitrobot Mark IV (ThermoFisher Scientific) for a plunge-freezing procedure. A double application of the sample was used: 3 μL of the particle suspension was applied to the grid, followed by blotting (blot force = 2, blot time = 4 s, wait time = 5 s). Immediately after the first blot, a second 3 μL aliquot of the same suspension was applied, followed by a final blot (blot force = 2, blot time = 4 s, wait time = 0 s). Vitrified grids were transferred into a Talos transmission electron microscope (ThermoFisher Scientific) and observed under cryogenic conditions at an accelerating voltage of 200 kV. Images were acquired using EPU software (ThermoFisher Scientific) and a Falcon III EC camera (ThermoFisher Scientific) at a magnification of 73,000x corresponding to a pixel size of 0.14 nm/pixel for an electron dose of $21 \text{ e}^{-}/\text{\AA}^2$. Liposomes were analyzed for a final lipid concentration of 5 mM.

II.3.2.8. Statistical Analysis

Data analysis was performed using GraphPad Prism software (version 8.4.3, La Jolla, CA, USA). Results are presented as mean values $\pm$ standard deviation (SD). Statistical differences between groups were assessed using unpaired t-tests. A p -value < 0.05 was considered statistically significant: $**p < 0.01$, $****p < 0.0001$. Non-significant differences are denoted as ns.

II.3.3. Results and discussion

II.3.3.1. Risk analysis

Before considering the scale-up of the liposome production process using PGSS, a comprehensive risk assessment was carried out to identify the parameters most likely to influence the key CQAs of the liposomes. Key process variables considered included pressure, temperature, agitation speed, dispersion volume introduced into the reactor, lipid

concentration, contact time with the SC-CO₂, and ΔP , as these parameters may significantly impact particle size, Pdl and EE.

In addition to the process parameters, the differences between the laboratory and pilot scale equipment were carefully evaluated. Scaling up from a 50 mL to a 500 mL reactor may affect heat and mass transfer, mixing efficiency, and internal fluid dynamics. Furthermore, potential sources of variability affecting process robustness and reproducibility were considered, including differences in impeller design and agitation geometry, the possibility of precisely controlling the ΔP , and the higher level of automation available at pilot scale.

Based on this risk analysis and previous lab scale findings, several parameters were fixed for the pilot scale study. The temperature was fixed at 80 °C, as it was identified as a decisive factor for both liposome formation and sterilization [230,242]. The contact time was maintained at 30 min, as it showed no significant impact on particles properties while ensuring acceptable industrial feasibility and process throughput. The total lipid concentration was set at 5 mM to limit raw material consumption and reduce production costs in view of large-scale manufacturing. The remaining parameters were selected as experimental variables for optimization, namely pressure (120–250 bar), agitation speed (10–70%), dispersion volume introduced into the autoclave (100–200 mL), and ΔP (60–120 bar/min). This systematic evaluation of variables via a DoE strategy was implemented in alignment with the QbD principles outlined in the ICH Q8 (R2) guideline. This guideline states that quality cannot be tested into products but must be built in by design [319]. Utilizing this DoE framework to screen variables, identify Critical Process Parameters, and discover their interactions with the liposomes CQAs directly addresses this expectation. In turn, this process understanding serves as the basis for the construction of design space in the future and facilitates technology transfer to commercial manufacturing [320].

II.3.3.2. Study of the impact of PGSS production parameters on liposome characteristics using the design of experiments approach

To facilitate the results analysis from the DoE, it is crucial to have a quick overview of previous hypotheses regarding the effect of SC-CO₂ on lipid components when using the PGSS process. Liposome production via this method involves two fundamental stages: first, SC-CO₂ is mixed with an aqueous lipid dispersion; then, the mixture is depressurized under controlled conditions (Figure 43) [232,233]. Although SC-CO₂ and water are only partially miscible, some CO₂ is solubilized in the aqueous phase during the mixing stage.

Simultaneously, the undissolved SC-CO₂ can partition into the crude lipid aggregates, intercalating between the fatty acyl chains of the phospholipids. This process disrupts the intermolecular van der Waals interactions, effectively dispersing the poorly soluble phospholipids and cholesterol. Furthermore, evidence suggests that dispersed lipids could migrate to the interface between the SC-CO₂ and the aqueous phase, leading to the formation of emulsion systems [227]. During the subsequent depressurization, the expansion of the CO₂ acts as a powerful mechanical dispersing agent. As the CO₂ transitions from a supercritical state to a gas, it fragments the plasticized lipid phase into small, disordered assemblies or even discrete molecules. Then, they spontaneously self-assemble into bilayers and finally liposomes, driven by hydrophobic interactions to minimize the hydrophobic surface area in contact with the aqueous buffer.

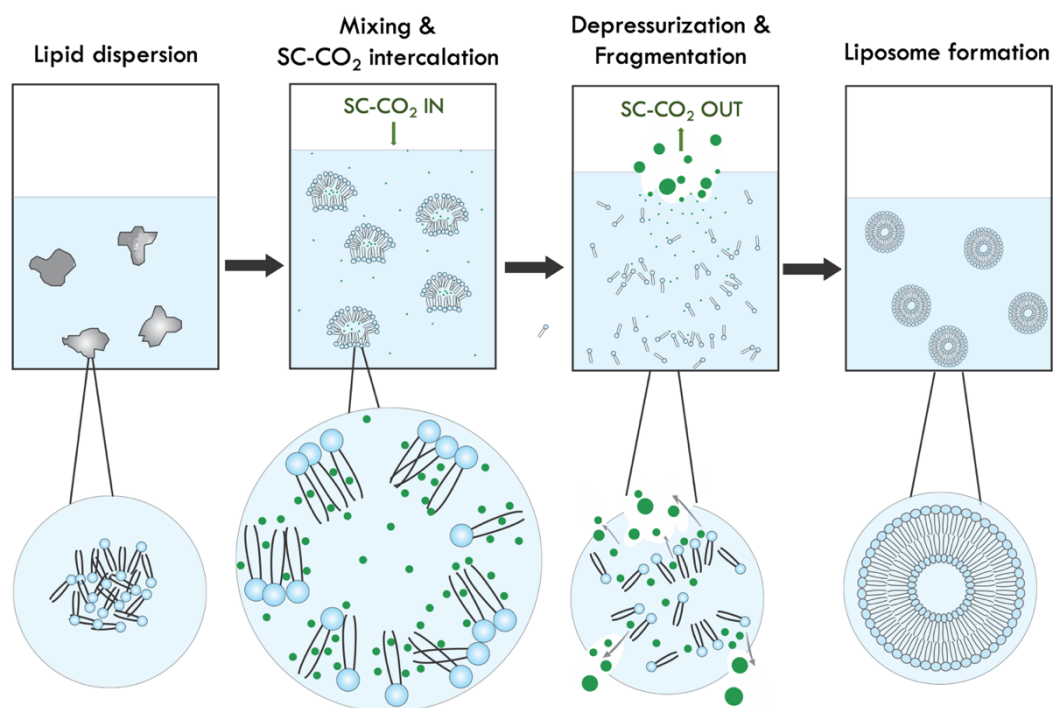


Figure 43. Schematic mechanism of liposomes formation by the PGSS method, inspired by the reference [232] with modifications: (A) Lipid aggregation in aqueous dispersion. (B) Intercalation of SC-CO₂ between hydrophobic regions of lipids during pressurization. (C) Lipid reassembly during depressurization. (D) Formation of liposome vesicles after depressurization.

As the final characteristics of the vesicles are dictated by the kinetics of this expansion, the ΔP is a decisive factor. An inadequate pressure drop can compromise liposome size and Pdl, even if the lipids were well dispersed during the mixing stage because phospholipids do not have sufficient time to reorganize into bilayers and subsequently into liposomes in

a reproducible manner. Consequently, the influence of the ΔP is analyzed first, followed by the remaining parameters governing the SC-CO₂-lipid mixing stage. This order of results interpretation was further supported by the significant influence of ΔP on the dependent variables, including its main effect, quadratic term, and interactions with other parameters (Figure 59). The profilers which showed the variation of size and Pdl of liposomes in function of the different investigated parameters at low (120 bar) and high (~ 250 bar) pressure conditions were used for the results interpretation (Figure 44).

II.3.3.2.1. Effect of depressurization rate

Under high-pressure conditions (262 bar), increasing the ΔP from 60 to 120 bar/min resulted in a significant increase in the Pdl, rising from approximately 0.30 to over 0.40 (Figure 44B). Similarly, at low pressure condition (120 bar), although the Pdl was already elevated (> 0.30) at 60 bar/min, increasing the ΔP to 90 bar/min exacerbated this further (Figure 44A). The same trend was observed for particle size, particularly when the ΔP was increased from 60 to 90 bar/min.

These findings contrast with those of previous optimization studies on the PGSS process for the production of small volumes of liposomes (6 mL) which suggested that rapid CO₂ release facilitated the disruption of phospholipid aggregates into discrete molecules, thereby promoting homogeneous reassembly into vesicles and lowering Pdl [232,233,235,236]. However, those studies also cautioned that applying high ΔP (e.g., 200 bar/min) to larger volumes would present significant challenges due to the Joule-Thomson effect [233,235]. During rapid depressurization, CO₂ molecules must overcome attractive intermolecular forces to expand [321]. When the expansion occurs too rapidly for heat to be absorbed from the surroundings, the CO₂ molecules are forced to consume their own internal heat energy, resulting in a severe temperature drop. In small-scale production, the high surface-to-volume ratio facilitates rapid heat transfer, which can potentially compensate for this cooling. However, at larger scales (100–200 mL), heat transfer would be more difficult and the uncompensated internal cooling could withdraw heat from the aqueous phase inducing lipid precipitation and increasing the Pdl. This would create bigger vesicles [315,322]. Furthermore, even when the depressurisation pipe is kept at 80°C, intense local cooling can pose a risk of blockage to the pipe or nozzle due to the formation of CO₂ ice [323,324]. Therefore, we set ΔP to 60 bar/min for subsequent analysis of the other results in the DoE.

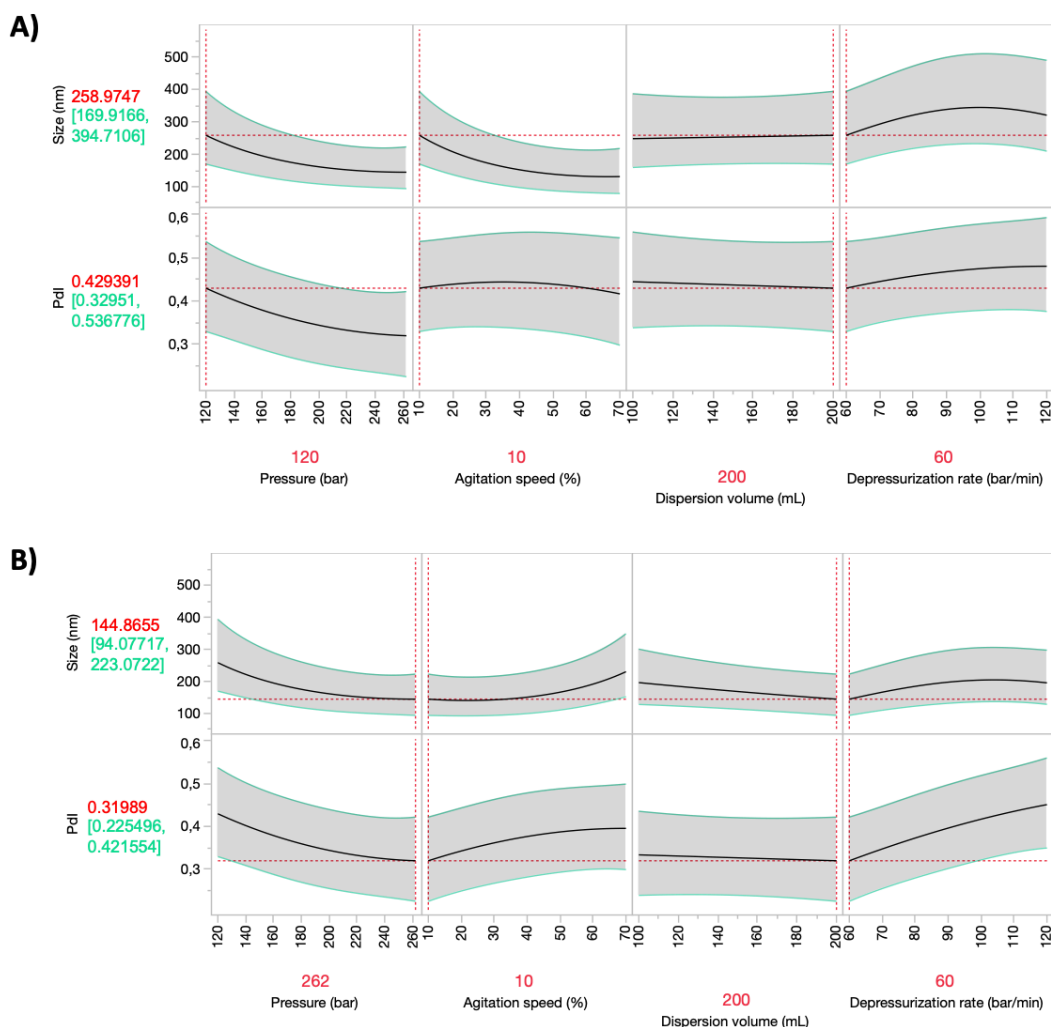


Figure 44. DoE prediction profilers at (A) low (120 bar) and (B) high (262 bar) pressure conditions: Illustration of the effects of process parameters on the size and Pdl of liposomes produced by the PGSS method at pilot scale. Fixed parameters include the lipid concentration at 5 mM, the temperature at 80 °C, and the contact time of 30 min. The grey shaded areas represent the prediction intervals of the model. Predicted values for each response and the corresponding prediction intervals are displayed on the left of each panel.

II.3.3.2.2. Effect of pressure and interaction with agitation

When the influence of pressure was analyzed independently, a clear trend emerged: an increase in pressure reduced the size and Pdl of the liposomes (Figure 44). This observation aligns with previous studies indicating that higher pressures favor narrower size distributions and smaller vesicles [233,236]. This can be attributed to the greater amount of CO₂ introduced into the reactor, which increases the concentration of unhydrated

CO₂ molecules intercalated between lipid aggregates, thereby enhancing the dispersing effect of SC-CO₂ on the lipids. Furthermore, at a constant depressurization rate, depressurizing from a higher initial pressure generated a stronger disruptive force on the lipid dispersion, effectively breaking aggregates into discrete molecules and promoting a more homogeneous reassembly into liposomes.

However, the interaction between agitation speed and pressure revealed a more complex behavior. Under the low-pressure condition, increasing the agitation speed reduced liposome size, but had a negligible impact on the Pdl, which remained consistently high (~0.40) (Figure 44A). In contrast, under the high-pressure condition, a size smaller than 200 nm and a Pdl around 0.30 were achieved only at the lowest agitation speed. Increasing agitation under this condition detrimentally increased both size and Pdl (Figure 44B).

For all volumes investigated, the 45° pitched impeller blades were fully submerged in the lipid dispersion. At low pressure (80°C, 120 bar), the lighter SC-CO₂ (296 kg/m³) formed a distinct layer above the denser aqueous phase (977 kg/m³) (Figure 45A) [325]. In this unbaffled reactor, agitation induces a central vortex that can facilitate the mass transfer of the lighter phase as small bubbles into the denser phase [326]. This process becomes significantly more efficient when the vortex is deep enough to reach the impeller (vortex ingestion), ensuring that the energy dissipated by the blades is mostly used to disperse the lighter phase [327]. Consequently, in this condition, increasing the agitation speed at low pressure deepened the vortex, which likely promoted the formation of SC-CO₂ bubbles in the aqueous phase and enhanced the turbulent dissipation rate responsible for bubble breakage, thereby improving CO₂ intercalation into the lipids [328].

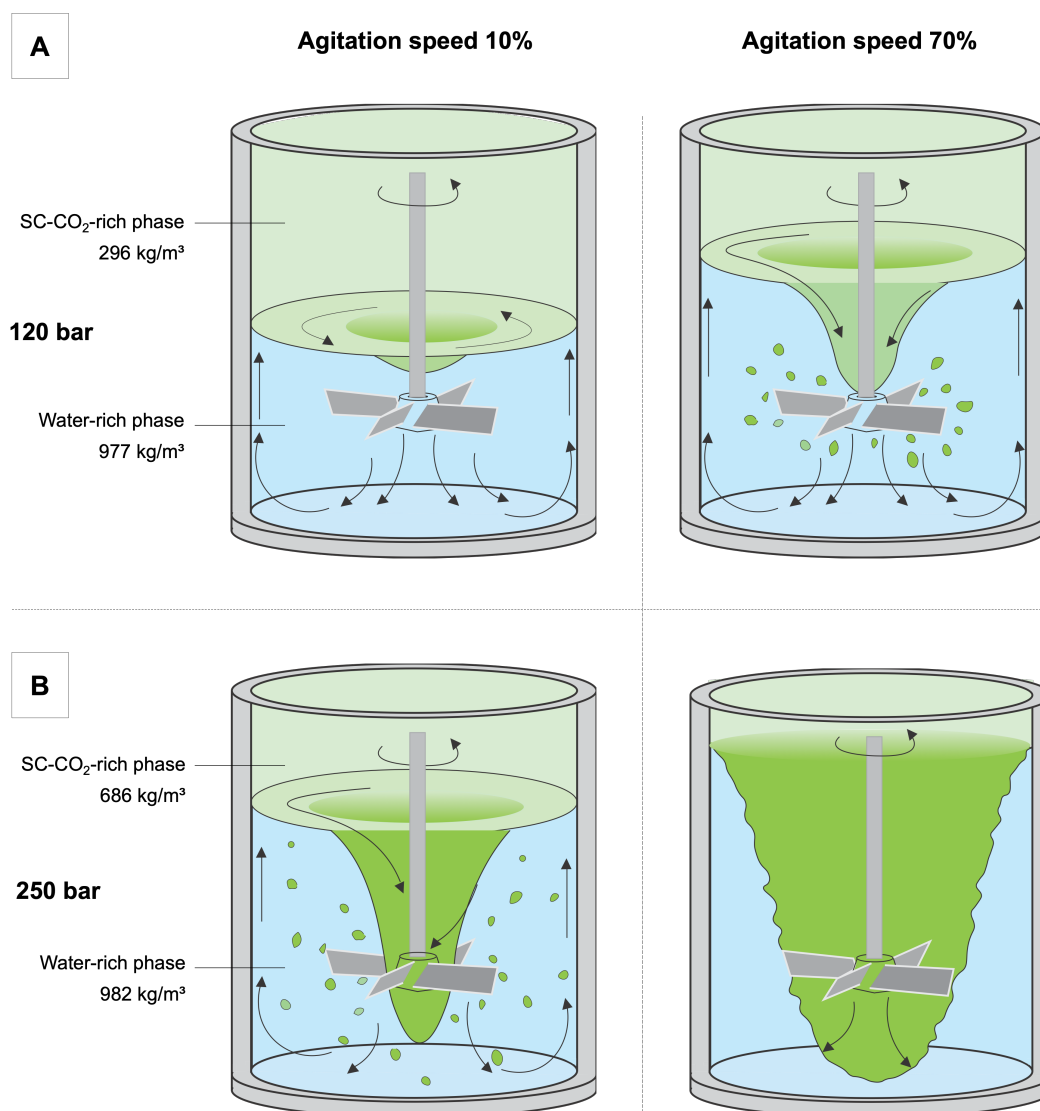


Figure 45. Schematic illustration of hypothetical effects of different agitation speeds, focused on the reactor bottom, under (A) low pressure and (B) high pressure conditions.

However, under high-pressure conditions, the density of the water remained stable at 982 kg/m³ while the density of the SC-CO₂ density increased considerably to 686 kg/m³ [325]. It has been established that increasing the density of the lighter fluid in a two immiscible fluids system enhanced the vortex effect due to a reduced density difference [326]. Therefore, we speculate that under high-pressure, vortex ingestion was more easily achieved even at a low agitation speed of 10%, resulting in a high concentration of SC-CO₂ bubbles in the aqueous phase (Figure 45B). However, when the agitation speed was increased further, the SC-CO₂ was likely swept down so aggressively by the axial flow that

the impeller became 'flooded', meaning the blades rotated primarily within a cavity of SC-CO₂ rather than interacting with the bulk aqueous phase. This phenomenon reduced the efficacy of transporting SC-CO₂ to the lipid dispersion, possibly explaining why increasing the agitation speed at high pressure markedly increased the size and Pdl of the liposomes. This hydrodynamic hypothesis should be clarified via further experiments.

II.3.3.2.3. Effect of the lipid dispersion volume

In the high-pressure condition, the volume of the lipid dispersion only had a minor influence on the size of the liposomes. Increasing the volume from 100 to 200 mL reduced the predicted size from ~200 nm to ~150 nm (Figure 44B). We propose 2 approaches to explain this result.

As mentioned above, the lipids dispersed by the SC-CO₂ can act as surfactants, stabilizing the interface to form emulsions between the water and the SC-CO₂ [227]. When the volume of the lipid dispersion is increased, the mechanical dissipation energy becomes more evenly distributed throughout the volume. This uniform distribution may facilitate the formation of smaller, more stable emulsion droplets than in the highly disturbed, chaotic environment of a lower volume. These stabilized droplets likely serve as templates for smaller vesicles during the depressurization phase.

In a fixed volume reactor at the same pressure, increasing the volume of the aqueous phase reduces the available headspace volume occupied by the SC-CO₂ phase not dispersed in the aqueous phase. The reduction in that SC-CO₂ fraction likely diminished the severity of the cooling effect exerted on the aqueous phase during depressurization. By attenuating the intensity of this thermal shock, the rapid, uncontrolled lipid precipitation that typically leads to the formation of large vesicles was minimized.

Altogether, this volume-dependent variation should be considered a potential tool for size tuning rather than a detrimental effect, particularly since the Pdl remained consistently close to 0.30 in the high pressure condition.

II.3.3.3. Validation of the optimal condition

The results obtained from the DoE enabled us to determine the optimal conditions for producing liposomes at a pilot scale using the PGSS method (Table 9). High pressure and low agitation speed were chosen to efficiently mix the lipid dispersion with SC-CO₂. The dispersion is then depressurized at a low rate to ensure the size homogeneity. As the dispersion volume did not greatly affect the liposome properties, we decided to continue in this initial investigation with the volume of 100 mL to minimize costs. Additionally, since the

results indicated that decreasing ΔP reduces the Pdl, we were interested in testing another condition with a ΔP of 30 bar/min while fixing the other parameters (additional condition).

Table 9. Optimal condition for the PGSS method at pilot scale, derived from the DoE, and additional condition for a more complete investigation of ΔP (* are fixed parameters).

	Optimal condition	Additional condition
Lipid concentration (mM)*	5	5
Dispersion volume (mL)	100	100
Pressure (bar)	262	262
Temperature (°C)*	80	80
Agitation speed (%)	10%	10%
Contact time (min)*	30	30
Depressurization rate (bar/min)	60	30

The SPC formulation produced under the optimal condition has an average size of 180 ± 4 nm (less than 200 nm), a Pdl of 0.34 ± 0.02 , which is close to 0.30, and ZP of -3.1 ± 1.0 mV (Figure 46). Reducing the ΔP to 30 bar/min under the additional condition yielded similar results for all three characteristics. Therefore, we selected the optimal conditions for evaluating the two drug-containing formulations.

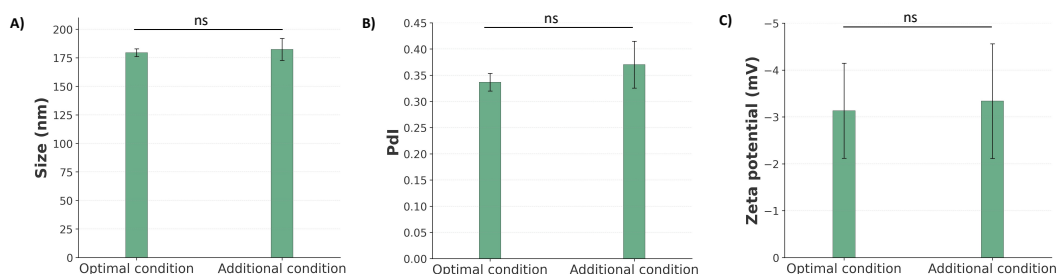


Figure 46. (A) Size, (B) Pdl, and (C) ZP of the SPC formulation produced under optimal and additional conditions (n = 3).

II.3.3.4. Transfer of the optimal conditions to the production of drug-containing formulations

The optimal conditions were then applied to produce liposomes containing phospholipids other than SPC and encapsulating an active ingredient. Two formulations developed in the laboratory were used: one co-encapsulating both IND and CIC, based on DLPC and suitable for pulmonary administration [249]; and the other encapsulating Dexa, based on DSPC and designed for transtympanic administration [306]. The physicochemical properties were compared with those of liposomes produced by the PGSS method at

laboratory scale under the condition described in *section II.3.2.2.1. PGSS method by the lab scale equipment*

II.3.3.4.1. Evaluation of physicochemical properties

When transitioning from the lab to the pilot scale, the mean particle sizes of the DLPC and DSPC formulations increased from 113 ± 3 nm to 152 ± 8 nm and from 152 ± 10 nm to 186 ± 8 nm, respectively (Figure 47A). The Pdl of the DLPC formulation rose from 0.26 ± 0.02 to 0.36 ± 0.02 , while that of the DSPC formulation increased from 0.31 ± 0.01 to 0.39 ± 0.01 (Figure 47B). Despite this upward trend, the sizes of both formulations remained below the 200 nm threshold with their Pdl remaining close to the target value of 0.30. A size less than 200 nm was important so that the liposomes could penetrate the pores of the mucus meshwork upon pulmonary administration (DLPC formulation) and cross the round window membrane after the transtympanic injection (DSPC formulation) [142,329]. A Pdl below 0.30 is generally considered as an indicator of a uniform size distribution [330].

Notably, the DLPC formulation exhibited high EE of CIC and IND across both scales (>90%) (Figure 47D). Meanwhile, the DSPC formulation produced at the pilot scale showed a significant improvement in DEXA EE, reaching $30.7 \pm 1.4\%$ compared to $23.9 \pm 1.0\%$ at the lab scale. These results demonstrate that the process is robust under these conditions, as the optimised pilot-scale conditions successfully produced liposomes of the expected size and Pdl with efficient drug encapsulation, despite the different lipid compositions.

However, a reduction in the Pdl of the pilot scale liposomes is still desirable. While both the FDA and the EMA classify particle size distribution as a CQA in their guidelines for liposomal medicines, they do not specify an acceptance criterion for the Pdl. This is because a complex assessment is required to evaluate the toxicity risks associated with the size distribution of particles in healthy tissues. This assessment depends on factors such as the route of administration, the drug properties, and the liposome composition [331]. Generally, a Pdl below 0.30 indicates a uniform size distribution, while values greater than 0.50 suggest a broader distribution [330,332]. In this study, the Pdl values for the pilot scale DLPC and DSPC formulations fell between 0.30 and 0.40, indicating a moderately uniform distribution that could be improved upon to reach the target value of 0.30. One potential approach to reduce Pdl is to increase the lipid concentration, as Zhao *et al.* previously reported that high phospholipid concentrations favor better particle uniformity at high temperatures [239]. This strategy is well aligned with the development of the PGSS method for the industrial scale, given that the total lipid concentration in commercially

available liposomal products typically ranges from 5 to 25 mM [197]. Furthermore, another approach to improve size uniformity is modifying the depressurization protocol itself. As previously demonstrated by Zhao and Temelli, maintaining a constant pressure inside the vessel by continuously pumping supercritical CO₂ while the liposome suspension is being discharged can significantly reduce the Pdl [232]. This constant-pressure protocol ensures that the concentration of unhydrated CO₂ inserted within the phospholipid bilayers remains stable throughout the entire depressurization stage. Consequently, every portion of the liquid phase experiences the same consistent dispersion force, preventing the formation of heterogeneous vesicles that commonly occurs when the pressure is allowed to drop gradually inside the vessel.

As such, another optimization process combines results obtained from the DoE of this study and the aforementioned strategies would be of great interest. In this fine-tuning step, an elevated pressure, a low agitation speed, and a low ΔP could be selected as the cornerstones to achieve a Pdl close to 0.30, while the addition of new parameters, such as lipid and the pumping rate of CO₂ during depressurization, could be introduced to reduce the Pdl to less than 0.30. Similar sequential DoE approaches were successfully applied to finely tune the parameters of microfluidic technology to produce liposomes with a Pdl of less than 0.30, therefore constituting a promising approach for the PGSS method [192,333]. If necessary, a downstream size homogenization step (e.g., extrusion or high-pressure homogenization) could be implemented after PGSS production to achieve the desired polydispersity.

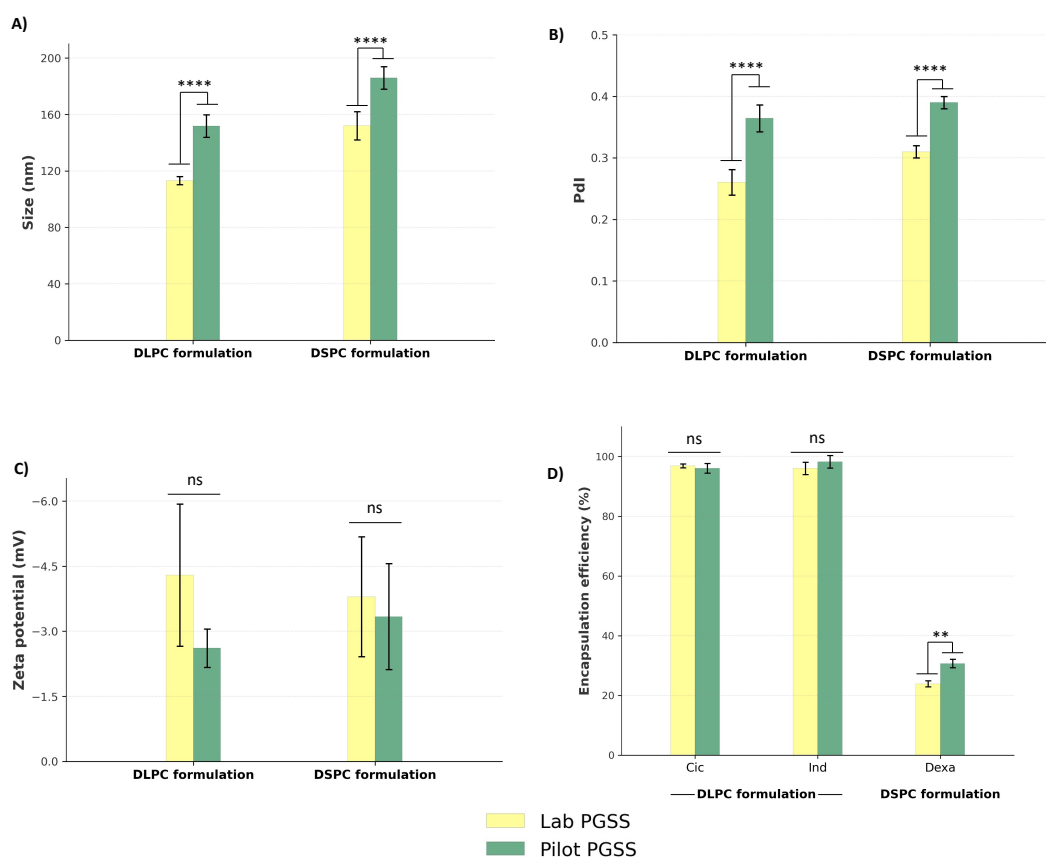


Figure 47. (A) Size, (B) Pdl, (C) ZP, and (D) EE of drugs for DLPC and DSPC formulations produced by the PGSS process at the lab and pilot scales (n=3).

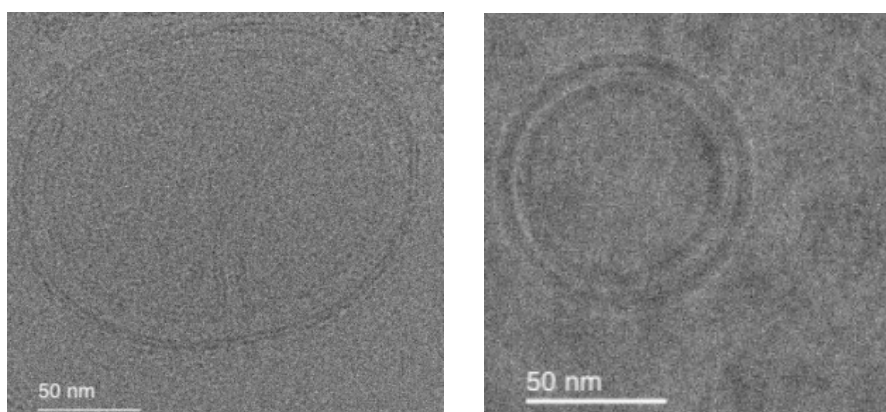
II.3.3.4.2. Morphology of drug-containing liposomes produced by the PGSS method at pilot scale

The DLPC formulation yielded spherical to oval-shaped vesicles with double bilayers (Figure 48A). This morphology increases the volume of the hydrophobic bilayer domain, providing greater capacity for the encapsulation of hydrophobic drugs such as CIC (logP = 4.08) and IND (logP = 2.33) [334,335]. Therefore, this structural advantage likely accounts for the high EE observed in this formulation.

In contrast, the DSPC liposomes displayed a quasi-spherical but distinctly angular morphology and occasionally exhibited oligolamellar structures (vesicle-in-vesicle) (Figure 48B). This angularity can be attributed to the saturated nature of the main lipid, DSPC (18:0), which leads to the formation of a rigid bilayer that resists smooth curvature. Drabik *et al.* explained that, in the solid phase of the lipid bilayer, shear elasticity drives the formation of rigid, flat patches that meet at sharp angles, resulting in 'oddly rectangular' or

faceted vesicles rather than smooth spheres [336]. Conversely, the smooth, spherical morphology of the DLPC liposomes is consistent with the high fluidity of lipid bilayers composed of unsaturated DLPC (18:2). Taken together, these results demonstrate that the pilot scale PGSS process successfully produces liposomes of varying compositions, yielding morphologies that are consistent with previous observations in the literature.

A)



B)

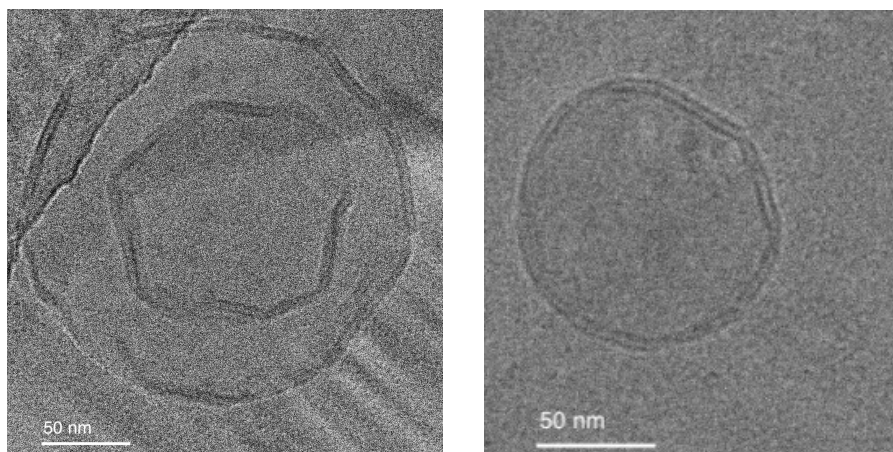


Figure 48. Cryo-TEM images of (A) DLPC and (B) DSPC formulations produced by the PGSS process at the pilot scale.

*II.3.3.4.3. Impact of the PGSS process on the phospholipid stability****Evaluation of phospholipid hydrolysis***

Phospholipids are inherently susceptible to hydrolysis when exposed to moisture, heat, or light, which cleaves the ester bonds to produce LPC, glycerophosphocholine, and free fatty acids. As the PGSS process involves high temperatures and pressures, combined with SC-CO₂ dissolution, which lowers the pH of the aqueous phase, it is crucial to evaluate the potential for phospholipids hydrolysis. Consequently, monitoring intermediate products like LPC serves as a critical control to quantify this degradation.

The main phospholipid, cholesterol, and DSPE-PEG were shown not to interfere with the peak of the LPC hydrolysis product during the HPTLC analysis (data not shown). The DSPC formulation showed no detectable peaks for 18:0 LPC before or after production, indicating that the degree of hydrolysis was less than 1.86% (corresponding to the minimum concentration of the calibration curve) (Figure 49B). In the DLPC formulation, although the peak density of 18:2 LPC increased significantly post-production compared to pre-production (t-test, $p < 0.05$), however, its level remained significantly below the lowest point of the calibration curve (t-test, $p < 0.05$), corresponding to only 2.9% (w/w) relative to DLPC (Figure 49A, C). Overall, the results are reassuring regarding the risk of hydrolysis but do not guarantee its complete absence. As such, it remains important to characterize other possible hydrolysis products such as fatty acids and glycerophosphocholine, as well as all lipid components in these liposomes, using an HPLC-based method to fully assess their chemical stability.

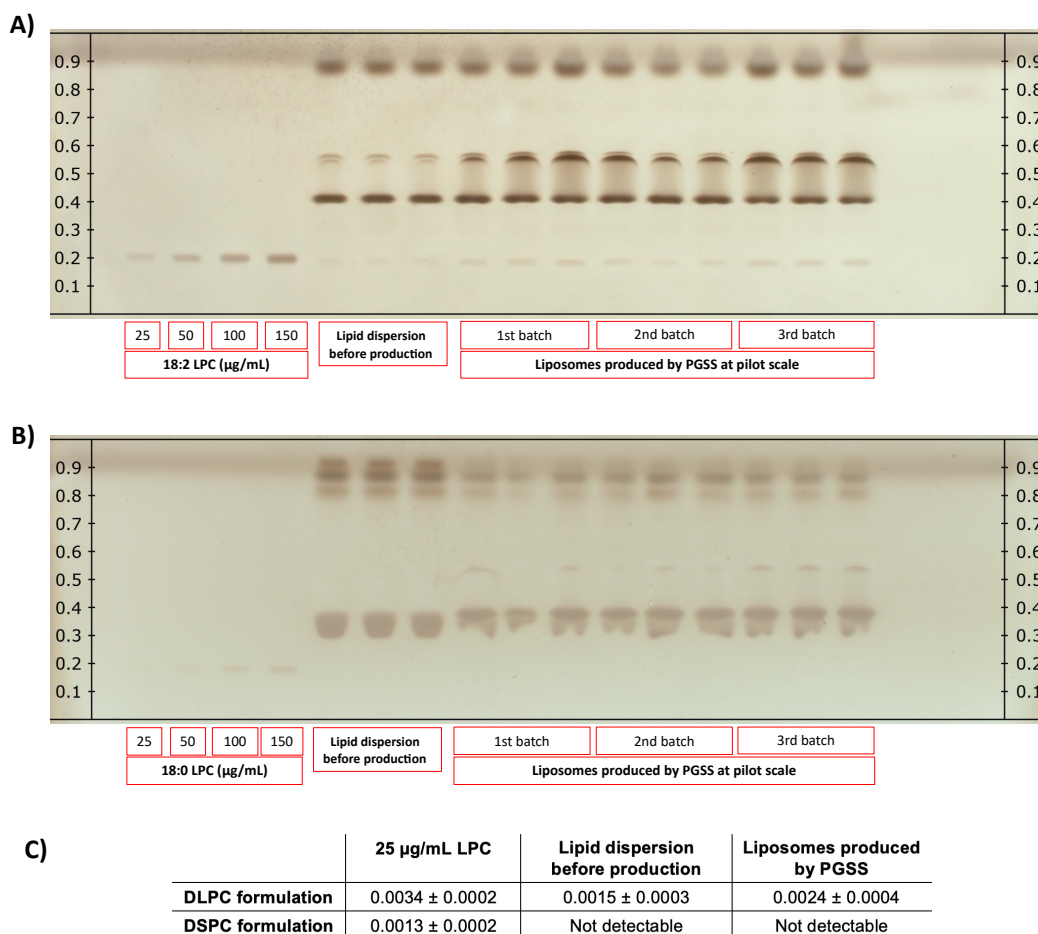


Figure 49. Images of the HPTLC plates for the hydrolysis evaluation of **(A)** DLPC and **(B)** DSPC formulations. **(C)** Optical intensity (AU) of LPC peak at 25 µg/mL and in DLPC and DSPC formulations before and after the production by the PGSS process at the pilot scale.

Evaluation of phospholipid oxidation

Because of its high degree of unsaturation, DLPC is particularly susceptible to oxidative degradation, which may be promoted under high-temperature processing conditions such as those used in PGSS. However, the percentage of peroxidized lipids in the DLPC formulation increased by only 0.004 ± 0.002 % after the production by the PGSS method at pilot scale. In addition, the DSPC formulation showed no oxidation results, which is consistent with the saturated state of DSPC. These results confirm that the PGSS method acts as a suitable platform for producing formulations containing oxidation-sensitive ingredients such as DLPC.

Overall, the DoE-derived conditions for the pilot-scale PGSS process allowed us to produce two drug-loaded liposomal formulations with sizes below 200 nm, Pdl approaching 0.30, and EE comparable to that of lab-scale liposomes. Notably, despite the elevated pressure and temperature conditions, the process had a negligible impact on lipid hydrolysis and oxidation. Beyond the physicochemical quality of the produced liposomes, the successful pilot-scale implementation of the PGSS method presents significant advantages for industrial translation. From an economic and practical perspective, although supercritical fluid technologies are traditionally thought to require specialized high-pressure equipment with higher initial capital expenditures compared to conventional low-pressure batch reactors, this initial investment is effectively offset by significantly lower operating expenses [220,337]. A major factor driving this cost-efficiency is that the production of liposomes by the PGSS process can be conducted without the use of any organic solvent. In widely adopted industrial-scale liposome production techniques, such as ethanol injection and microfluidics, the fundamental reliance on organic solvents introduces several severe drawbacks. These include high material costs for solvent consumption, flammability and explosion hazards, acute and chronic health risks to personnel, and prolonged downstream purification steps [210,338]. Furthermore, operating with solvents necessitates costly and rigorous analytical quality control to monitor residual solvent limits in the final product, and poses a significant negative environmental impact due to the carbon-intensive incineration of complex aqueous-organic waste streams [339,340]. In contrast, the PGSS method utilizes SC-CO₂ as a dispersing agent which is inexpensive, non-toxic, non-flammable, and simply reverts to a gaseous state upon depressurization. In light of this, demonstrating the feasibility of the PGSS method for large-scale liposome production paves the way to effectively resolve the economic, environmental, and safety hurdles related to organic solvent-based methods. To advance this technology closer toward full-scale industrial application, future priorities will include further fine-tuning the process parameters to reduce the Pdl, increasing the final lipid concentration of the suspensions, and integrating a closed-loop CO₂ recycling unit to maximize cost-efficiency and environmental sustainability.

II.3.4. Conclusion

In conclusion, the PGSS process was successfully transferred to a larger, pilot scale production, demonstrating its scalability and strong potential for industrial implementation. By applying a QbD approach supported by a structured DoE on a simplified liposome formulation, critical process parameters were systematically investigated and optimal operating conditions were defined. These conditions ensured good reproducibility and

enabled the consistent production of liposomes exhibiting CQAs within the targeted specifications.

The robustness of the process was further confirmed by successfully translating these operating conditions to more complex formulations containing APIs, while maintaining high EE. This highlights the versatility of the PGSS process and its suitability to produce functional liposomal systems. In addition, chemical stability studies demonstrated that the process does not significantly impact lipid integrity, with both hydrolysis and oxidation levels remaining minimal after processing, even under high-temperature conditions typically associated with this process.

Despite these promising results, further optimization is still required to improve size distribution, particularly to reduce the Pdl, which remains a key parameter for pharmaceutical applications. Moreover, the evaluation of higher lipid concentrations will be essential to better reflect industrial constraints and to assess the process performance under more concentrated and scalable conditions. These improvements are expected to enable this technology to produce liposome batches that comply with GMP standards, with a view to their application in Phase I or I/IIa clinical trials. Overall, these findings support the relevance of the PGSS process as a solvent-free, single-step, robust and scalable technology for liposomes and lipid-based system manufacturing.

CRedit authorship contribution statement

Tuan Nghia Dinh: Investigation, Methodology, Data curation, Conceptualization, Writing – original draft, Writing – review & editing. **Chloé Gauthy:** Investigation, Methodology, Data curation, Conceptualization, Writing – original draft, Writing – review & editing. **Hugo Magdalena Segura:** Investigation, Methodology, Data curation. **Pierre-Yves Sacré:** Methodology, Software, Writing – review & editing. **Erika Hendrickx:** Data curation. **Louise Conrard:** Data curation. **Brigitte Evrard:** Funding acquisition, Supervision. **Brigitte Malgrange:** Funding acquisition, Supervision. **Géraldine Piel:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing. **Noémie Penoy:** Investigation, Methodology, Data curation, Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

Acknowledgments

This research was funded by the Walloon Region, SPW-EER, DGO6 – Win2Wal – LNPulmo convention n°2210043 and NANOPDELEAR convention n°2210045, and supported by the 2025 Sectoral Research Credits in Health Sciences (University of Liège). Center for Microscopy and Molecular Imaging (CMMI) is supported by ERDF funding and the Walloon Region. Authors want to thank Aquilon Pharma and Dendrogenix (Liège, Belgium). Authors want to thank Professor Michel Frédérick and Delphine Etienne for their help in the high-performance thin-layer chromatography analysis. Authors also want to thank Professor Dominique Toye and Doctor Sebastien Calvo (Product Environment and Processes, Department of Chemical Engineering, University of Liège) for their help in interpreting the data.

II.4. Conclusion

In summary, Chapter II successfully optimized the PGSS process parameters to scale the production of the optimal formulation identified in Chapter I to a pilot-scale, GMP-like production environment. Preliminary laboratory-scale tests demonstrated that maintaining a temperature of 80 °C was essential to the formation of homogeneous liposomes co-encapsulating CIC and IND. Transitioning to the pilot scale, a DoE approach elucidated the synergistic effects of pressure, dispersion volume, agitation speed, and depressurization rate on liposome formation at 5 mM. To achieve a Pdl of around 0.30 and a particle size below 200 nm, a combination of high pressure (262 bar), low agitation speed (10%) - to facilitate efficient SC-CO₂ and lipid dispersion mixing - and a low depressurization rate (60 bar/min) was required. These parameters were applied to the optimal formulation, producing 100 mL of nearly spherical and oval liposomes with EE exceeding 90% for both CIC and IND. While the pilot-scale Pdl approached 0.30 (with the corresponding particle size distribution detailed in Figure 60), future work may incorporate a homogenization step to enhance size uniformity. Crucially, the pilot-scale PGSS process resulted in no significant DLPC oxidation and hydrolysis. Overall, this work highlights the robustness,

scalability, and viability of PGSS as a solvent-free, simple technology for liposome manufacturing.

DISCUSSION

DISCUSSIONS

Newer generations of ICSs, such as FP, FF, MF, and CIC, and ultra-LABAs, such as IND and VIL, have been structurally engineered with high lipophilicity to achieve sub-nanomolar receptor binding affinity, prolonged tissue retention, and extended duration of action that allow for convenient once-daily dosing. However, this extreme hydrophobicity simultaneously creates significant biopharmaceutical challenges, particularly concerning the MCC and phagocytosis by AMs. Liposomes are a promising delivery system to address these challenges, primarily because they can efficiently encapsulate these hydrophobic drugs within their lipid bilayers. Furthermore, the ability to modify both the type and concentration of the lipid components provides a powerful means to tune liposomal properties based on specific therapeutic targets. Given this versatility, it is essential design the lipid composition of the vesicles in a rational manner to overcome the physiological barriers of the lungs and enhance the anti-asthmatic efficacy of the ICS/LABA combination. Furthermore, a major gap in translating liposomal products from bench to bedside remains industrial-scale manufacturing. Large-scale production must be optimized to consistently maintain the precise physicochemical properties achieved during laboratory-scale optimization. Although the PGSS method represents an advanced, green alternative to produce liposomes without organic solvents, its application at a large production scale remains to be fully explored.

In light of these challenges, the primary objectives of this work are twofold: first, to establish a rational design framework for liposomal formulations that can bypass MCC and AMs uptake to enhance the anti-asthmatic efficacy of a long-acting ICS and ultra-LABA combination; and second, to investigate the scalability of the production method of this formulation using the PGSS method. To this end, the subsequent discussion details a systematic progression. First, it addresses the selection of the long-acting ICS and ultra-LABA combination alongside their corresponding clinical limitations. Next, it examines how the properties of these active molecules and lipid components dictate the engineering of liposomes designed to overcome respiratory barriers and enhance anti asthmatic efficacy. Finally, it evaluates the adaptation of the PGSS process from laboratory to pilot scale, demonstrating how the system can be scaled up to successfully produce liposomal formulations that retain their optimized laboratory-scale characteristics.

I. Ciclesonide and indacaterol maleate for asthma treatment: a new ICS-LABA combination and their clinical problems

In the current landscape of asthma management, the 2025 GINA guidelines strongly advocate for the use of moderately lipophilic ICSs and LABAs, specifically BUD-FOR and BDP-FOR combinations, for both maintenance and reliever therapy [1]. The actual use of these moderately lipophilic molecules is favored in this regimen because their physicochemical properties allow them to dissolve relatively quickly in the airway lining fluid, ensuring the rapid onset of action required for acute symptom relief while still providing robust anti-inflammatory control. However, a major clinical challenge with these therapies is that they typically necessitate twice-daily dosing. Real-world evidence demonstrates that poor patient adherence to frequent dosing regimens remains a primary driver of suboptimally controlled asthma, leaving patients at a much higher risk for asthma exacerbations and subsequent increased healthcare needs [73].

To address this critical compliance barrier, there is a strong pharmacological justification for using long-acting ICS and ultra-LABA therapies, normally characterized by high lipophilic active molecules. The inherently prolonged duration of action of these molecules accommodates a convenient once-daily dosing regimen, which has been shown to significantly improve patient adherence, increase treatment persistence, and ensure sustained 24-h bronchoprotection compared to twice-daily alternatives [73]. For this reason, the highly lipophilic prodrug CIC and IND represent a promising therapeutic pairing to be studied. The selection of CIC over other long acting ICSs is primarily driven by its unique prodrug nature, which minimizes the risk of local oropharyngeal side effects, such as oral candidiasis, hoarseness, and pharyngitis [341]. Furthermore, CIC offers a prolonged anti-inflammatory effect alongside a high systemic safety profile due to its negligible oral bioavailability (<1%) and rapid systemic clearance. Concurrently, IND delivers a rapid onset of action within minutes paired with a sustained 24-h bronchodilatory effect [32]. Crucially, despite their immense synergistic potential and their ability to maximize patient compliance through convenient once-daily administration, CIC and IND have never been used in combination in a commercialized product for asthma management. This highlights the significance of investigating this specific combination as a novel therapeutic modality to enhance the overall efficacy of ICS/LABA treatments for asthma.

However, harnessing the full clinical benefits of these advanced, once-daily molecules requires overcoming a profound biopharmaceutical hurdle known as the lipophilicity paradox. Both CIC (logP = 3.20) and IND (logP = 3.65) are characterized by high lipophilicity, a physicochemical property that acts simultaneously as a massive advantage for their duration of action and a severe barrier to their cellular targeting. The primary advantage of this high lipophilicity is its direct correlation with prolonged pulmonary retention and sustained receptor activation. The high lipophilicity of CIC favors its membrane permeation that is necessary for the subsequent metabolism to des-CIC. Once activated in the lungs, des-CIC undergoes reversible esterification to form fatty acid conjugates, creating an intracellular lipid depot that slowly releases the active drug over an extended period. Similarly, due to its lipophilic nature, IND partitions deeply into cell membranes in the lung tissue, forming a local drug reservoir from which drugs can be released the β_2 ARs in the ASM cells for extended periods.

Conversely, the drawback of this high lipophilicity is the pharmacokinetic bottleneck it creates in the airway lumen. Because highly lipophilic free molecules possess very limited aqueous solubility, they dissolve slowly in the limited aqueous volume of the pulmonary lining fluid. Rather than diffusing efficiently to their underlying cellular targets, these undissolved solid drug particles exhibit high hydrophobic adhesion and become heavily entrapped within the mucus layer. Consequently, they are actively swept out of the airways by the MCC or are rapidly phagocytosed and eliminated by AMs deep in the lungs. Ultimately, this paradox reveals that the exact hydrophobic properties required for sustained 24-h efficacy actively impede the diffusion of these drugs through the luminal mucus, predisposing them to pulmonary clearance and preventing optimal cellular targeting.

To address these problems, this study aims to develop a liposomal formulation coencapsulating CIC and IND that can help them to overcome the MCC and AMs phagocytosis, thereby enhancing their distribution to their targeted underlying cells and ultimately their therapeutic effect against asthma.

II. Design of liposomes containing ciclesonide and indacaterol maleate for pulmonary delivery

II.1. Lipophilicity of inhaled active molecules: A paradox for free drugs, but an advantage for liposomes

When loading CIC and IND into liposomes, we observed that their inherent lipophilicity was key to achieving high and stable encapsulation within the lipid bilayer. This was demonstrated by the robust encapsulation of the highly lipophilic CIC, contrasting with the less lipophilic IND, whose encapsulation requires additional electrostatic interactions with phospholipids. This trend implies that when the lipophilicity of a drug is sufficiently high, like in the case of CIC, the drug can be spontaneously embedded deeply within the lipid bilayer via hydrophobic interactions with the phospholipid acyl chains. In such cases, the primary requirement is the creation of sufficient 'kinks' within the bilayer to accommodate the drug, which was achieved in this study through the selection of specific phospholipids.

As lipophilicity decreases, the drug appears to distribute more toward the interface between the lipid bilayer and the aqueous phase [342]. Notably, Chol is also preferentially located at this interface, where its hydroxyl group can form hydrogen bonds with the carbonyl and phosphate groups of the phospholipids, alongside weak charge pairs with the choline headgroups [93,94]. Consequently, the localization of drugs at the bilayer interface often results in competition with Chol, as previously observed with intermediately lipophilic drugs like BUD [343] and Dexamethasone [344]. Indeed, the lipid membrane affinity of IND has been shown to decrease in the presence of Chol [36]. Since the amine and phenol groups of IND can form electrostatic interactions with phosphate groups and hydrogen bonds with carbonyl groups of phospholipids, respectively, we speculate that a competition for phospholipid interaction sites may occur between Chol and IND when they coexist in the bilayer. This likely explains why IND leaked from liposomes containing PEGylated lipids that lacked phosphate groups, despite the use of phospholipids as the main lipid component (Figure 26B). A full phospholipid membrane provided sufficient interaction sites for both IND and Chol, thereby maximizing IND encapsulation while maintaining the desired Chol level. Notably, although the concentration of Chol was not investigated in this study, maintaining it at 30% was considered essential to prevent the bilayer from rupturing into micelles - a risk typically associated with high DSPE-PEG concentrations and the use of highly unsaturated lipids in our optimal formulation [345–347].

When encapsulating FOR which is even less lipophilic than IND, the EE of FOR was lower and less stable compared to IND within the same formulation (SPC/Chol/DSPE-PEG, 55/30/15) (Figure 38A). Although the amin group of both FOR and IND is capable of creating electrostatic interaction with the negatively charged phosphate groups of phospholipids [41,348], the reduced EE of FOR is likely attributed to its lower lipophilicity which leads to a partial distribution of the drug outside the lipid bilayer, thereby increasing the risk of leakage. To circumvent this drawback, additional stabilization mechanisms have been often employed. Common strategies include the construction of rigid bilayers such as the DPPC:Chol (2:1 w/w) system used for the stable encapsulation even under the high shear stress of the nebulization and sustained release of amikacin sulfate in the lung environment of Arikayce[®] (liposomal amikacine for inhalation) [145]. Another strategy is the generation of solid drug nanocrystals or precipitates within the aqueous core to introduce a rate-limiting dissolution step. For instance, researchers have successfully employed freeze-thaw cycles followed by spray-drying to nucleate and form ciprofloxacin nanocrystals directly inside intact liposomes (Lipoquin[®]), remarkably prolonging the drug release profile in the lung [171]. In this study, we addressed this limitation by incorporating negatively charged DOPG and increasing total lipid concentration to provide an efficiently electrostatic interaction for FOR.

From the perspective of bilayer architecture, while the encapsulation of hydrophilic drugs typically requires saturated lipids to yield a rigid bilayer for cargo retention, our results show that the encapsulation of lipophilic drugs favors a highly fluid state. This holds true regardless of whether the drugs localize in the bilayer through purely hydrophobic forces (like CIC) or via supportive electrostatic interactions (like IND). As hypothesized by the "PEG desorption" hypothesis (section 1.2.4. *Discussion*), the high bilayer fluidity likely facilitates the desorption of DSPE-PEG from the liposomes, providing a critical balance between PEGylation for mucopenetration and de-PEGylation for sub-sequent epithelial uptake. Consequently, a series of interconnected consequences arise. The encapsulation of lipophilic drugs necessitates a disordered lipid bilayer, which in turn allows for the use of a high concentration of PEGylated lipid, such as 15% DSPE-PEG, for mucopenetration and to avoid AMs uptake. This process may also promote the de-PEGylation required for epithelial internalization. More broadly, this implies that by utilizing delivery systems like liposomes to bypass these physiological barriers, the future discovery of ICS and LABA molecules with even longer durations of action could no longer be restricted by the traditional limits of lipophilicity.

From a broader perspective, the high lipophilicity of active molecules is also a significant advantage when spray drying liposomes. Within the framework of the LNPulmo project, Bya et al. investigated the influence of the lipophilicity of active molecules, as well as the concentration of DSPE-PEG, on the properties of liposomes following spray drying [349]. When fixing the DSPE-PEG concentration at 2.5%, hydrophilic drugs like SAB, which localize in the aqueous core, exhibited massive leakage (~70%) as they were expelled along with the evaporating water during the drying process. Conversely, moderately lipophilic drugs like BUD and FOR were much better retained (~20–30% leakage), because their higher affinity for the lipid bilayer kept them anchored more strongly despite structural reorganizations. This leakage was further reduced to less than 20% when highly lipophilic molecules like CIC and IND were loaded. The DSPE-PEG concentration was fixed at 2.5% for this specific comparison because gradually increasing it up to 10% was shown to increase the leakage of BUD to approximately 35%. This phenomenon can be explained by the fact that the highly hydrophilic PEG chains, when combined with elevated drying temperatures, likely increase bilayer fluidity and compromise structural integrity, thereby forcing the lipophilic drug out of the membrane. Therefore, utilizing highly lipophilic molecules like CIC and IND represents a promising strategy to minimize drug leakage during spray drying. This approach may permit the adoption of the higher concentrations of DSPE-PEG that are crucial for facilitating liposomal penetration through the airway mucus and the evasion of AMs uptake.

II.2. Rational selection of PEGylated lipid is important to overcome the lungs barriers while preserving the safety of liposomes

The objectives of overcoming the mucus and avoiding AMs uptake impose several strict criteria regarding the physicochemical properties of the liposomes. First, a particle size of less than 200 nm is necessary to successfully bypass the dense mucus meshwork and to evade the optimal size range for AM phagocytosis (which preferentially targets larger microparticles in the 1 to 5 μm range) [150]. Furthermore, a neutral or slightly negative surface charge is highly beneficial to avoid electrostatic entrapment by negatively charged mucin fibers and to minimize recognition by AMs. Moreover, the Pdl must be kept below 0.30 to ensure a uniform size distribution.

Several key formulation and processing factors allowed us to meet these physicochemical criteria. The preparation of liposomes via TFH followed by extrusion through a 200 nm membrane serves as a reliable standard to obtain vesicles with a diameter below 200 nm and a Pdl below 0.30, as shown in Table 11. Moreover, the use of zwitterionic

phospholipids and Chol as the primary lipid components directly contributes to the near-neutral charge of the liposomes. This neutral surface charge is further ensured by the incorporation of PEGylated lipids as the resulting hydrophilic PEG polymer corona effectively can shields and masks residual charges on the underlying lipid surface [98].

Beyond basic physicochemical properties, PEGylated lipids also play a direct role in the mucopenetration of liposomes as well as their interaction with AMs [142]. Previous studies have demonstrated that using a sufficiently high PEGylated lipid concentration is crucial for the PEG layer to adopt a brush-like configuration, which efficiently limits the hydrophobic and electrostatic interactions of the liposome surface with the hydrophobic domains in the backbone of mucins. This same steric barrier also inhibits the adsorption of opsonins (serum and lung proteins) onto the liposome surface, effectively preventing them from being recognized, phagocytosed, and cleared by AMs. However, successfully attaining this protective brush configuration depends on several factors, namely the structure of lipid chains in the lipid bilayer, the MW of PEG, and the molar concentration of the PEGylated lipid. In this study, we focused on the interplay between the structure of the lipid bilayer and the concentration of the PEGylated lipid, while maintaining a constant PEG chain length (PEG₂₀₀₀). This specific MW was selected because this moderate-length chain has been shown to optimize the mucopenetration of liposomes without causing the physical entanglement with mucin fibers that severely immobilizes NPs coated with longer polymers (such as PEG₁₀₀₀₀) [98].

In this study, when using SPC as the main phospholipid and fixing Chol at 30% to investigate different PEGylated lipids (Figure 24), we observe that a PEGylated lipid concentration of 15% or 30% is necessary to significantly improve the mucopenetration of liposomes through an artificial mucus model, compared to liposomes containing only 5% or entirely lacking a PEGylated lipid (Figure 26A). These high concentrations of PEGylated lipids are also highly effective at limiting liposome uptake by AMs (Figure 27B). It should be noted that the use of unsaturated lipids, which creates a more loosely packed, liquid-disordered bilayer, requires a higher concentration of PEGylated lipid to induce the mushroom-to-brush transition. For example, when the Chol concentration is set at 40%, the conformational transition in rigid membranes containing hydrogenated SPC begins at 5% DSPE-PEG. In contrast, in fluid membranes containing egg PC, the transition is delayed and begins at approximately 6% DSPE-PEG [96]. Based on these observations, it is highly likely that at a 5% PEGylated lipid concentration, SPC-based formulation still retains the PEG layer in a mushroom configuration. This explains why liposomes containing

5% PEGylated lipid did not outperform those without PEGylated lipids during mucopenetration assays. Conversely, increasing the PEGylated lipid concentration to 15% and 30% successfully creates the protective brush conformation of the PEG layer, shielding the liposomes from mucin binding and opsonization-related AM uptake.

In addition to the above observations regarding the role of PEGylated lipids in mucopenetration and AMs uptake, which are consistent with previous results in the literature, our investigation of different PEGylated lipids revealed very interesting results about the variation of liposomes toxicity on AMs cells (RAW264.7) as a function of the fatty acyl chain length. We found that decreasing the length of the fatty acyl chain of PEGylated lipids increased the toxicity of the liposomes on RAW264.7 cells, as displayed in Figure 27A. Consequently, the formulations containing DSPE-PEG (C18) at 15% and 30% showed the best biocompatibility with RAW264.7 cells over those containing DPPE-PEG (C16) and DMPE-PEG (C14). More interestingly, we observed that this acyl chain length-dependent toxicity of PEGylated lipids strongly resembles the variation in the toxicity of LPS on cells via Toll-like receptor 4 (TLR4), which heavily depends on the structure of its lipid A moiety (Figure 52). Although this hypothesis still requires clarification via further experiments, it prompts us to fundamentally rethink the immunogenicity observed with PEGylated lipids, a concept that will be further discussed in the next section.

II.3. Interplays between PEGylated lipids and phospholipid with challenges concerning PEGylation (PEG dilemma and immunogenicity)

In the context of pulmonary delivery, the 'PEG dilemma' is the phenomenon wherein the dense, brush-like PEG layer that protects NPs from mucoadhesion and opsonization-mediated AMs uptake simultaneously acts as a steric barrier that prevents them from successfully interacting with target cells (epithelial cells in this study). Because PEGylated lipid molecules can be shed from liposomes after exposure to biomolecules in the lungs, the design of a sheddable PEG layer, which can initially protect liposomes from mucus binding and AM uptake but gradually detach once they reach the epithelium, is a promising strategy to overcome this 'PEG dilemma'.

Several of our results support this strategy. In Chapter I, when we optimized the concentration of DSPE-PEG in the *ex vivo* relaxation test, the formulation containing 15% DSPE-PEG performed better than the one containing 30% DSPE-PEG (Figure 29B, C). We also observed that, when maintaining DSPE-PEG at 15%, decreasing the T_m of the

phospholipids directly increased both the epithelial uptake of the liposomes and the ex vivo relaxant effect of the encapsulated IND (Figure 30C-F). These results strongly imply that a too-dense PEG layer combined with a highly rigid lipid bilayer may impede sufficient PEG desorption, thereby decreasing the access of the encapsulated IND to ASM cells located underneath the epithelium. This led to the 'PEG desorption' hypothesis that we proposed in section 1.2.4. *Discussion* to explain these results.

In vivo, in the tracheobronchial region where the epithelium is covered by the mucus layer, the PEG desorption likely allows liposomes that initially possess a high-density PEG layer to successfully penetrate the mucus. This PEG corona may then be shed during mucopenetration due to exposure to biomolecules such as mucins, DNA, and other proteins, ensuring that the steric barrier does not impede subsequent liposome uptake by epithelial cells. In the alveolar region, the PEG desorption process can also occur due to the interaction of liposomes with PS. However, the subsequent biological fate of the liposomes will likely depend heavily on the composition of the biomolecular corona formed on the liposome surface. For instance, the adsorption of large, hydrophilic surfactant proteins like SP-A and SP-D acts as an opsonin signal, promoting the rapid recognition and phagocytic clearance of the liposomes by AMs [157–160]. Conversely, the adsorption of ApoH has been shown to significantly enhance the intracellular uptake and cytosolic delivery of liposomes to both AMs and epithelial cells [162]. Because this study focused primarily on the selection of lipid components based on their influence on the biological properties of liposomes, the complex interplay between the biomolecular corona and cellular uptake in the lungs was not directly investigated. Therefore, this property, alongside the kinetics of PEG desorption in different lung environments, must be further clarified in future experiments to determine if PEG desorption effectively promotes epithelial uptake in both the bronchial and alveolar regions.

However, it should be noted that in the specific case of liposomes co-encapsulating CIC and IND, epithelial uptake in the alveoli is necessary for the anti-inflammatory action of CIC, but is not therapeutically useful for IND, as ASM is absent in this deep respiratory region. Moreover, alveolar epithelial uptake must be carefully controlled. While the primary goal is to maximize local therapeutic effects in the lungs, excessive alveolar absorption will facilitate the systemic entry of the drugs across the thin air-blood barrier, which can lead to increased systemic toxicity.

Regarding the PEG desorption, the literature demonstrates that this shedding process is heavily dependent on the acyl chain length of the PEGylated lipid anchor. For instance,

PEGylated lipids with shorter hydrophobic tails (such as C14) rapidly dissociate and shed from the lipid bilayer, whereas longer saturated tails (like C18) anchor the PEG much more rigidly into the membrane [169]. Therefore, using an easily sheddable PEGylated lipid theoretically allows for the use of a main phospholipid with less unsaturations, which would advantageously reduce the risk of lipid oxidation. However, our findings indicate that this approach is compromised as decreasing the length of the hydrophobic tail of the PEGylated lipid in the liposomal formulation directly increased its toxicity on AMs (Figure 27A).

Recent literature has raised cautions regarding the antigenicity (or immune reactivity) of PEGylated nanocarriers, characterized by the binding of pre-existing anti-PEG antibodies to their surface [167]. It should be noted that while anti-PEG antibodies are detectable in a large portion of the general population (approximately 72%), only a small fraction of individuals (roughly 8%) exhibit high titers (exceeding 500 ng/mL) sufficient to trigger rapid systemic clearance [350]. Furthermore, although direct clinical evidence of anti-PEG antibodies within the human lung environment is currently lacking, *ex vivo* studies highlight a potential cautionary mechanism at mucosal surfaces. These models demonstrate that, should these antibodies be present in respiratory secretions, they might immobilize nanoparticles within the mucus mesh via multiple low-affinity crosslinks between the Fc domains and mucin fibers [168]. Thus, while not a definitive drawback for the majority of patients, this potential antigenic interaction warrants careful consideration when developing inhaled PEGylated delivery systems.

Beyond these antibody-mediated concerns, our results regarding PEGylated lipid-related toxicity on AMs introduce a critical new factor that should be considered in the overall paradigm of PEGylation: its potential for innate self-immunogenicity. This implies that even in the complete absence of anti-PEG antibodies, PEGylated lipids may independently trigger an immunogenic response via direct interaction with the TLR4 receptor. Fortunately, our study highlights at least two practical strategies to control this innate toxicity. The first strategy is to maximize the length of the fatty acyl chains of the PEGylated lipids, which is proposed to reduce their binding affinity for the TLR4 receptor complex. Furthermore, we found that the toxicity of PEGylated lipids on RAW264.7 cells is directly proportional to their concentration. Increasing the concentration of DPPE-PEG or DMPE-PEG from 15% to 30% significantly exacerbated toxicity on RAW264.7 cells. This indicates that strictly modulating the administered dose of PEGylated liposomes serves as a second essential tool to minimize this immune toxicity. These strategies are extremely important in the context of treating chronic inflammatory respiratory diseases like asthma, as the introduction of any

additional inflammatory stimulants via the drug carrier itself would inadvertently exacerbate the underlying pathological state.

It is noteworthy that the empty liposomes (DLPC/Chol/DSPE-PEG, 55/30/15) demonstrated a minor anti-inflammatory effect and a mitigation of AHR in the acute asthmatic model, evidenced by a significant reduction in eosinophil and lymphocyte counts, as well as an attenuation of IL-5 and IL-33 levels compared to the HP β CD group. Within the existing literature, the intrinsic bioactivity of "empty" liposomes has predominantly been documented in LPS-induced models of inflammation, where specific phospholipid components play an active pharmacological role. For instance, phosphatidylcholines with unsaturated fatty acyl chains, particularly DLPC, have been shown to exert distinct anti-inflammatory effects by acting as specific antagonists to TLR4 signaling pathways [92,294]. However, the primary driver of airway inflammation in the OVA-induced asthma model utilized in this study is allergic (Th2-mediated) rather than endotoxin-driven. Because TLR4-mediated signaling is not the central mechanism in this specific pathological model, it is expected that the empty liposomes could not independently achieve the comprehensive anti-inflammatory and bronchodilatory effects observed with the drug-loaded formulation. Instead, these results strongly suggest that the mild intrinsic bioactivity of the DLPC liposomal carrier acted synergistically with the co-encapsulated active molecules (CIC and IND), effectively enhancing their overall therapeutic efficacy in the lungs. Moreover, these encouraging acute findings provide a strong rationale to further evaluate both the empty and drug-loaded liposomes over an extended treatment period. Confirming their sustained safety and efficacy in a chronic asthmatic model will be essential to establish the robustness and long-term tolerance of this formulation.

II.4. Problems encountered with the use of an unsaturated phospholipid with a high concentration of PEGylated lipids

As the use of unsaturated phospholipid like DLPC and a high concentration of DSPE-PEG at 15% is necessary to improve the therapeutic efficacy of CIC and IND, it is also crucial to consider possible drawbacks of this approach for the lung administration.

The first major drawback of this formulation stems from the chemical instability of the main phospholipid, DLPC. As a polyunsaturated lipid containing two cis double bonds in each of its linoleoyl chains, DLPC is more susceptible to lipid oxidation than saturated lipids. In the highly oxygenated environment of the lungs or during the storage in aqueous dispersions, these double bonds can undergo oxidative breakdown that generates reactive soluble

derivatives (such as aldehydes and ketones) and short-chain lipids [351]. This degradation can directly disrupt lipid packing, compromising the structural integrity and barrier function of the bilayer. Despite this inherent vulnerability, the results from the physical stability test over 14 days at 4 °C showed that almost all physicochemical properties of the optimal formulation, such as size, Pdl, ZP, and the encapsulated concentration of CIC, remained unchanged, with only a slight reduction in the encapsulated concentration of IND after 14 days (Figure 37). These results indicate that the liposomes did not suffer severe structural damage during this short period of refrigerated storage. Because these liposomes are designed to be administered via the pulmonary route, the strategy to ensure their long-term stability will ultimately depend on their intended physical state: either as an aqueous liquid dispersion (for nebulization) or as a solid dry powder (for dry powder inhalation).

Because the lipid membrane of the optimal formulation contains DLPC as the primary unsaturated phospholipid together with 15% DSPE-PEG, the physical state of this lipid bilayer is expected to be highly fluid and loosely packed. This state is attributed to the molecular "kinks" introduced by the multiple cis double bonds of the DLPC acyl chains, combined with the lateral steric repulsion forced by the dense, brush-like PEG layer on the lipid headgroups. This inherent fluidity is a critical vulnerability to consider during pulmonary administration, which typically requires aerosolization via nebulizers or the creation of dry powders via spray drying. While vibrating-mesh nebulizers are generally preferred for liposomal dispersions because they use lower energy and generate far less disruptive shear and heat than conventional air-jet and ultrasonic nebulizers, highly fluid lipid bilayers still lack the necessary mechanical rigidity to withstand this atomization stress [173]. This renders them highly prone to shearing, vesicle rupture, and massive drug leakage during aerosol generation. In light of this, administering the optimal formulation to the lungs via nebulization will likely be highly challenging. The spray drying of the liposomal dispersion into an inhalable dry powder could therefore serve as a promising alternative. On one hand, the solid-state powder significantly reduces the risk of lipid and drug hydrolysis often encountered during the long-term storage of aqueous dispersions [171]. On the other hand, the administration of liposome-embedded dry powders using a dry powder inhaler bypasses the intense shear forces of nebulization, thereby preventing the associated structural damage to the vesicles. However, the spray drying process itself typically subjects liposomes to high temperatures, osmotic stress, and shear forces during atomization, which also risk compromising their structural integrity. In this regard, optimizing the spray drying parameters along with the type and ratio of a protectant for the liposomes is of paramount importance. This specific optimization was conducted by PhD

student Laure-Anne Bya, a partner in the LNPulmo project, whose work in part showed that HP β CD protects liposomes during the spray drying process better than traditional disaccharide protectants like trehalose [349].

It is noteworthy that the intense lateral pressure and high curvature induced by high concentrations of PEGylated lipid, such as 15% DSPE-PEG in the optimal formulation, can act as destabilizing forces. In fluid liposomes composed solely of unsaturated phospholipids (like DOPC or Egg PC) without Chol, incorporating ~ 10% DSPE-PEG drives the vesicles to rupture into intermediate open bilayer discs, and concentrations of 15% and above induce a complete phase transition into mixed micelles [345]. However, when Chol is added to the lipid bilayer (normally between 30-50%) and packs tightly into the gaps between phospholipid acyl chains, it exerts a profound condensing effect, shifting lateral pressures toward the middle of the bilayer and increasing both the membrane stiffness and its elastic area expansion modulus (resistance to stretching) [345–347]. This cohesive strength provided by Chol was shown to counterbalance the lateral pressure of the PEG chains, making the bilayer highly resistant to forming curved surfaces or mixed micelles. Therefore, it is possible that the 30% Chol in the optimal formulation may help to prevent the vesicles from rupturing into micelles, allowing them to maintain their intact unilamellar structure despite the use of a highly unsaturated lipid like DLPC and the presence of 15 % DSPE-PEG. This stabilizing mechanism likely explains why we predominantly observe intact, nearly spherical to oval-shaped unilamellar structures for the optimal liposomes via cryo-TEM, without signal of small micelles (Figure 36).

In addition to the toxicity of PEGylated lipids on AMs discussed in previous sections, the influence of PEGylated liposomes on the physiological function of PS is another critical concern. A recent study simulating respiratory mechanics demonstrate that highly PEGylated liposomes can actively interfere with the physiological function of native PS although the PEGylation successfully reduced overall protein adsorption onto their surface [352]. Unlike PEG-free liposomes, those with 4% DSPE-PEG remain strongly associated with the surfactant film during the dynamic breathing cycle, causing an abnormal fluidization of the surfactant layer. This disruptive interaction could impair initial surfactant adsorption and prevent the native surfactant from effectively reducing alveolar surface tension to near-zero values during exhalation. Therefore, further experiments characterizing influences of optimized liposomes on the biophysical function of PS will be necessary to ensure that they do not inadvertently cause reduced lung compliance, impaired gas exchange, and the induction of respiratory distress.

Moreover, the biological fate and biodegradation of the massive PEG moiety might present a considerable long-term toxicological concern. Mammals have rarely been described to possess the specific etherase enzymes capable of fully degrading the polyether backbone of PEG [353]. Consequently, PEG chains can experience very slow clearance from the lungs depending on their molecular weight. Measurements of PEG concentrations in lung homogenates showed that very small PEGs (<1 kDa) were completely cleared from lung tissue within 24 hours, but 10–15% of the administered PEG dose $\geq$ 2 kDa remained in lung tissue for at least 7 days [354]. Moreover, PEGs chains can undergo pinocytosis and accumulate inside the lysosomes of AMs and other cells, leading to progressive cellular vacuolation [355–357]. High-molecular-weight PEGs (e.g., $\geq$ 30 kDa) cause more prolonged cellular vacuolation due to extensive lysosomal accumulation than low-molecular-weight PEG. Despite these structural changes to the cells, the general consensus in the literature is that PEG-induced cellular vacuolation is a non-adverse, adaptive physiological response rather than a toxic one [355]. Indeed, half of the approved PEGylated drugs have shown vacuolar formation in tissues, although this does not alter organ function [356]. However, the use of a high concentration of PEG remains a significant concern for chronic, long-term therapies. On one hand, *in vitro* studies have shown that high PEG concentrations can reduce the ability of macrophages to phagocytose bacteria and fungi [355]. On the other hand, there are theoretical regulatory concerns regarding the potential long-term impact of PEG accumulation in the brain on pediatric development, including possible adverse cognitive, functional, or metabolic effects [355]. These critical observations must be taken into careful consideration in the context of this study, where we aim to develop a daily-use pulmonary liposomal formulation containing a high concentration of DSPE-PEG.

Beyond the toxicological concerns associated with progressive PEG accumulation, the specific choice of lipid excipients presents a major regulatory hurdle for the clinical translation and commercialization of this formulation. In the inhalation field, only saturated lipids such as DPPC and DSPC, along with cholesterol, are considered GRAS because they are endogenous components of human pulmonary surfactant locally present in large quantities [358]. Indeed, the first and only FDA-approved inhaled liposomal product to date, Arikayce®, relies exclusively on a biocompatible matrix of DPPC and Chol. In contrast, in the optimal formulation, DLPC and DSPE-PEG currently lack regulatory precedent for the pulmonary route. Therefore, it will require toxicological evaluations to definitively prove their safety and local lung tolerance. On a positive note, *in vitro* studies have shown that DLPC possesses potent intrinsic anti-inflammatory effects on RAW264.7 macrophages, partly

supporting its tolerance in the lung environment [359]. Moreover, therapeutic formulations containing PEG or PEGylated lipids have recently successfully entered human clinical trials for pulmonary administration and demonstrated good tolerance. Notable examples include MRT5005, an inhaled mRNA LNPs containing DMG-PEG₂₀₀₀ evaluated in Phase 1/2 trials for cystic fibrosis [360], and inhaled PEGylated adrenomedullin investigated in Phase 2a/b trials for acute respiratory distress syndrome [361].

III. Particles from gas-saturated solution process as an organic solvent-free method for liposomes production

III.1. Advantages and disadvantages of the PGSS process over ethanol injection and microfluidic to produce liposomes at the industrial scale

Ethanol injection and microfluidics are two of the most widely used methods to produce liposomes at the industrial scale [175]. Both methods rely on mixing an organic phase (containing dissolved lipids and hydrophobic drugs) with an aqueous phase (containing hydrophilic agents). Conventional ethanol injection relies on dispersing the ethanolic lipid solution into an aqueous phase under macroscopic stirring. However, the fundamental problem with this approach is that macroscopic agitation creates chaotic, turbulent fluid dynamics that generate non-homogeneous liposomes [182,185]. Consequently, this mixing yields a highly heterogeneous, broadly distributed population of large, multilamellar vesicles. To meet the strict particle size uniformity required for clinical applications, this conventional method must be followed by downstream size-reduction steps, such as high-pressure extrusion or high-pressure homogenization. As an advanced adaptation of ethanol injection-based methods, microfluidic methods miniaturize the mixing of the two phases into microchannels, where chaotic turbulence is entirely replaced by a highly controlled fluid regime, allowing for the more homogeneous precipitation of NPs [197]. However, these two methods must typically be accompanied by the TFF to remove the organic solvent and unencapsulated drugs, as well as a sterilization step if needed.

The PGSS method is an advanced approach that is gaining increasing attention as an alternative to ethanol injection and microfluidics. In this process, the lipids and the drugs are dispersed in an aqueous phase, which is then mixed with SC-CO₂ under specific conditions of temperature, pressure, agitation, and time. SC-CO₂ plays the role of a dispersing agent that effectively disperses poorly soluble ingredients like lipids and

hydrophobic drugs [217]. The subsequent depressurization of SC-CO₂ releases it from the aqueous dispersion, scattering the lipids into small fragments and even individual molecules due to collision and shear forces, which allows them to homogeneously reassemble into liposomes.

One of the major advantages that the PGSS method provides over ethanol injection and microfluidics is bypassing the use of organic solvents to dissolve the lipids. Organic solvents used in liposome production are often fully miscible with water and can become physically entrapped deep within the NPs core. Removing these solvents requires diffusion-based techniques (like TFF) that provide enough time for water molecules to slowly penetrate the particles core and displace the organic solvent [340]. As separating water-miscible organic solvents from an aqueous stream remains a highly costly, energy-intensive industrial bottleneck [338], incineration is the most widely used method for solvent disposal, not to mention that non-flammable halogenated solvents cannot be removed by this manner [339]. Unfortunately, incineration has serious negative impacts on the environment because it generates an estimated amount of 6.7 kg of CO₂ for every 1 kg of organic carbon burned [338] and requires supplementary fuel to burn streams that have a high water content [362]. From the industrial view, operating with volatile organic solvents makes the production plants more complex due to the strict rules and regulations required to protect the production plant from severe flammability and explosion hazards, personnel from acute and chronic health risks, and the environment from photochemical pollution in the air such as ozone or smog [216,338].

In addition, the consumption of CO₂ in the PGSS process can be considered a highly sustainable reuse and recycling strategy, rather than a new source of greenhouse gas emissions. The liquid CO₂ used in these high-pressure operations is not manufactured specifically for liposome production. Instead, it is primarily sourced as an inexpensive byproduct or waste emission from other industrial operations or is gathered directly from the atmosphere [220]. Consequently, harnessing this byproduct for the PGSS process represents a zero-net carbon emission strategy, as it simply repurposes existing CO₂ without contributing additional greenhouse gases to the environment. Furthermore, the true environmental and economic importance of the PGSS method will lie in how the CO₂ is managed after the depressurization stage. While continuously venting large volumes of depressurized CO₂ directly into the atmosphere after a production cycle raises global warming concerns, modern supercritical fluid equipment can mitigate this issue by incorporating CO₂ recycling units [363]. Although the potential contamination of recycled

CO₂ requires further quality controls, implementing this in-process recycling can neutralize the environmental risk of atmospheric discharge and significantly reduce the overall treatment and operating costs of the PGSS process

Although a downstream purification step is always necessary to separate unencapsulated drug from the liposomes produced by the PGSS process, the absence of organic solvents makes this step significantly less time-consuming. A recent study has demonstrated that high concentrations of ethanol actively reduce the permeability of the hollow fibers used in TFF modules, thereby decreasing the overall efficiency of the filtration process [210]. Furthermore, the avoidance of treating complex aqueous-organic waste mixtures offers significant cost savings while minimizing the environmental impact of the production process. It also removes the need to routinely rely on complex, time-consuming analytical quantification methods such as Gas Chromatography to constantly monitor and validate residual solvent limits for batch release [340].

In addition, SC-CO₂ presents significant potential as an active sterilization method, as it can destroy pathogens, including viruses, fungi, and bacterial spores, by mechanically rupturing cells, lowering intracellular pH, and extracting vital cellular lipid [211]. However, SC-CO₂ cannot physically remove the destroyed microbial debris or any large, non-living particulate matter from the final product. Currently, sterile filtration through 0.22 µm membranes is the most widely used sterilization method. Nevertheless, from a strict biological safety standpoint, 0.22 µm filters cannot guarantee absolute sterility because they fail to retain viruses and ultra-small pathogenic bacteria, allowing these contaminants to pass directly into the final drug product [211]. In this regard, combining SC-CO₂ as a first step to completely eradicate a wide range of pathogens with downstream filtration to physically clear out large aggregates, dust, and inert particulates could serve as a complementary and much more thorough sterilization strategy.

However, one of the major areas where advanced continuous methods like microfluidics significantly outperform the PGSS method is the feasibility of industrial scale-up. Thanks to the ability to tightly control the fluid regime, microfluidic platforms can achieve massive throughput without sacrificing particle quality. This is primarily accomplished either by running dozens or even hundreds of identical microfluidic mixers in parallel arrays or by using continuous flow devices highly compatible with massive production volumes, such as confined impinging jet mixers and multi-inlet vortex mixers [245]. In contrast, the fundamental principle of the PGSS process dictates that increasing production capacity still relies on physically scaling up the size of the machinery. This volumetric scale-up can lead

to changes in heat transfer inside the reactor, altered mixing regimes, and variations in the depressurization process. Therefore, to fully realize the profound benefits of the absence of organic solvents in the PGSS process, there is a critical need to investigate liposome production using this method at a large scale.

III.2. Important parameters for the production of liposomes containing ciclesonide and indacaterol maleate by the PGSS method from the laboratory scale to the pilot scale

In the literature, process parameters that typically require thorough investigation when producing liposomes via the PGSS process include lipid concentration, dispersion volume, temperature, pressure, agitation speed, contact time, and depressurization rate (ΔP). Within our PGSS equipment at the laboratory scale, all of these parameters, except for ΔP , were previously optimized to produce liposomes at 5 and 45 mM with a size of less than 200 nm and a Pdl close to 0.30 (Table 3) [230]. Under both optimized conditions, the temperature was fixed at 80 °C, which is crucial for both liposome formation and simultaneous sterilization [242]. However, operating at such a high temperature can pose a risk of hydrolysis and oxidation of phospholipids, especially when the optimal formulation incorporates an unsaturated phospholipid like DLPC. Nevertheless, when performing a new DoE to find a possible new condition with a lower temperature for the production of the optimal formulation, we found out that maintaining the temperature at 80 °C is the most important factor to obtain a size of less than 200 nm and a Pdl close to 0.30 (Figure 40). This result may be due to lipids becoming more fluid and disordered at this temperature, which potentially facilitates more efficient dispersion by the SC-CO₂ molecules. Furthermore, it is possible that temperatures below 80 °C are insufficient to destabilize the crystalline state of highly lipophilic drugs, CIC and IND, impeding their efficient incorporation into the lipid bilayer. Given the importance of this high temperature for the production of the optimal formulation at the laboratory scale, the temperature for the pilot-scale optimization of other parameters was fixed at 80 °C. Oxidation and hydrolysis products of DLPC after the production were then characterized to control the chemical stability of this lipid.

Notably, the optimized pilot-scale condition of the PGSS process did not trigger a significant increase in the risk of hydrolysis and oxidation of DLPC in the optimal formulation. The minimized oxidation of DLPC is likely stems from the fact that the mixing of the lipid dispersion with SC-CO₂ in the reactor creates an inert environment that limits the contact of DLPC with oxygen, reducing the chance of oxidation of its unsaturated acyl chains [364].

Meanwhile, the minor hydrolysis of DLPC induced by the PGSS could potentially be linked to the state of the lipid in the reactor. Because the lipids and hydrophobic active molecules are insoluble in the aqueous buffer, they are hypothesized to initially exist as highly packed aggregates that sterically protect the internal ester bonds from water exposure. SC-CO₂ may require an initial time to diffuse through the water and partition into these lipid domains to disperse them. Therefore, it is plausible that the 30-min mixing step represents a tight kinetic window: it might be just long enough to achieve the well-dispersed state required for liposome formation, but brief enough to prevent the newly exposed ester bonds from undergoing extensive hydrolytic degradation before the depressurization. However, further mechanistic studies would be required to validate this assumption.

The optimization at the pilot scale of parameters including dispersion volume, pressure, agitation speed, and ΔP yielded several interesting observations on how these parameters influence the performance of the two stages in liposome production via the PGSS process. During the stage of mixing SC-CO₂ with the lipid dispersion, a high pressure is necessary to provide enough CO₂ molecules to act as a dispersing agent. Simultaneously, excessively high agitation speeds must be avoided to ensure efficient mixing with the lipid dispersion (Figure 44). The hypothesis proposed for the interaction between pressure and agitation speed (*II.3.3.2.2. Effect of pressure and interaction with agitation*) highlights the fact that the influence of agitation speed on the mixing efficiency of SC-CO₂ with the lipid dispersion should always be interpreted in relation to the density of SC-CO₂. Even within the supercritical region, the properties of SC-CO₂ can dramatically change from gas-like at lower pressures to liquid-like at higher pressures. This observation is particularly important because agitation speed has rarely been investigated in the literature, even during laboratory-scale optimization of the PGSS process.

On the other hand, although the depressurization happens at the end of the process, this stage seems to be the decisive factor in determining the final properties of the liposomes, regardless of the performance of the previous mixing step. In the optimization of the PGSS process at the laboratory scale, the ΔP is recognized as a critical parameter because it dictates the delicate balance between effective lipid dispersion and thermal stability. If the ΔP is too slow, the CO₂ diffuses out gradually, causing insufficient disruption of the lipid assemblies and resulting in large, non-uniform vesicles [232,235]. Conversely, while a very fast ΔP maximizes the scattering of lipids into discrete monomers, it simultaneously exacerbates the Joule-Thomson cooling effect. In the production of small volumes (e.g., around 6 mL) of liposomes by the PGSS method, this cooling effect can protect heat-labile

ingredients. However, in our DoE for the production of 100 to 200 mL of liposomes, increasing the ΔP (from 60 to 120 bar/min) resulted in an increase of both size and Pdl. This result likely implies that at a larger scale, the Joule-Thomson effect might pose a severe problem of heterogeneous lipid precipitation that leads to the formation of liposomes with heterogeneous sizes. This negative effect likely outweighs the thermal protection benefits previously proposed for the production of smaller volumes. Given that a rapid depressurization is generally a favorable factor to increase the size homogeneity of liposomes produced by the PGSS process, developing strategies to mitigate the Joule-Thomson effect should be the main subject for further improvements of the pilot-scale PGSS equipment.

III.3. Further improvements to mitigate Joule-Thomson effect

In section II.3.3.2.1. *Effect of depressurization rate*, we mentioned that a Joule-Thomson effect may occur during rapid depressurization, which can lead to heterogeneous lipid precipitation responsible for an elevated Pdl. Traditionally, the Joule-Thomson effect is well-known to happen when a pressurized fluid passes through a pipe under adiabatic conditions [238]. This could potentially happen within our system in the depressurization pipe, even though the temperature of this pipe was fixed at 80 °C. Furthermore, we can also speculate that this phenomenon occurs within the reactor during depressurization, especially if efficient heat transfer is not maintained throughout the entire mixing volume. The geometry of the impeller is a critical factor influencing heat transfer from the reactor wall to the bulk mixture. In this study, we employed a pitched blade impeller, which normally induces an axial flow. This type of impeller directs the fluid flow parallel to the impeller axis, typically creating a strong circulation that pushes the fluid down toward the bottom of the tank. Once the fluid reaches the bottom, it is forced to move radially outward before eventually ascending along the vertical thermal walls. As the solution moves along the vessel wall a hydrodynamic boundary layer is built and its thickness increases as the solution moves along the wall [365]. The boundary layer is the flow region adjacent to the wall in which the viscous effects are significant, creating velocity and temperature gradients [366]. The fluid temperature gradient at the wall will depend entirely on the rate at which the moving fluid carries the heat away. Because the fluid must traverse an extended circulation path before interacting with the heat transfer surfaces, the flow velocities rapidly decreases to low values as it moves away from the impeller [367]. Consequently, the lower speed of the moving liquid at the wall reduces the efficiency of the heat transfer to the bulk fluid.

In the case of a radial flow impeller, a considerable part of the fluid moves radially from the impeller directly toward the container wall, where it splits into two separate streams: an upward stream and a downward stream [365]. Consequently, the hydrodynamic boundary layers are built at the upper part of the wall and another separate one is built at the lower part. Because the distances the fluid in the two parts travel along the wall are shorter than in the axial flow configuration, the average thickness of these layers remains significantly smaller [365]. This reduced thickness of the hydrodynamic boundary layers, together with the fact that the radial impeller causes more turbulence and channels the fluid directly toward the walls of the tank, explains why the radial flow yields a more efficient heat transfer than the axial flow [365,368,369]. Therefore, future studies can consider impellers triggering radial flow to improve the heat transfer efficiency. However, the compartmentalization with two mixing zones can limit the overall homogeneity of the mixture and result in poor top-to-bottom mixing [370,371]. Furthermore, because flow velocities rapidly reduce to low values away from the radial impeller, the radial flow is prone to creating weak, quasi-static flow regions or localized dead zones, such as near the impeller shaft [372]. As such, when changing the impeller geometry, another DoE could be expected to thoroughly reinvestigate its impact on the physicochemical properties of the produced liposomes. Otherwise, an extrusion process can always be useful to attain a good homogeneity for the size distribution of liposomes.

Beyond optimizing the reactor's internal hydrodynamics by switching to a radial flow impeller, an alternative process engineering approach known as the Sequential-Dispersion PGSS (SD-PGSS) method can be employed. Unlike conventional PGSS operations that continuously spray the high-pressure mixture causing a constant, rapid drain of heat that exacerbates poor internal heat transfer, the SD-PGSS process alters the depressurization mechanism itself by using an automated, temperature-regulated double-valve dispersion line (Figure 50) [322]. During the depressurization, valve 1 opens, allowing a small, discrete volume (about 2.33 mL) of the pressurized suspension to flow into an enclosed space between two valves. Valve 1 remains open for 15 seconds so a high-pressure pump can restore and equilibrate the exact pressure and temperature of this trapped segment to match the main mixing vessel. Once equilibrated, valve 1 closes and valve 2 opens, sequentially discharging the trapped suspension through a nozzle into a collection vessel at atmospheric pressure. This design allows the fluid to be atomized in discrete, consecutive cycles rather than a continuous stream. Because the depressurization is broken into controlled cycles, the fluid is granted sufficient time to recover its thermal energy between individual sprays. However, this approach requires specific equipment

modifications to be implemented. Alternatively, based on this principle, depressurization within our existing setup could be performed while continuously pumping CO₂ into the reactor to compensate for the pressure loss. To implement this strategy effectively, the flow rate and temperature of the injected CO₂ could be important factors to be optimized.

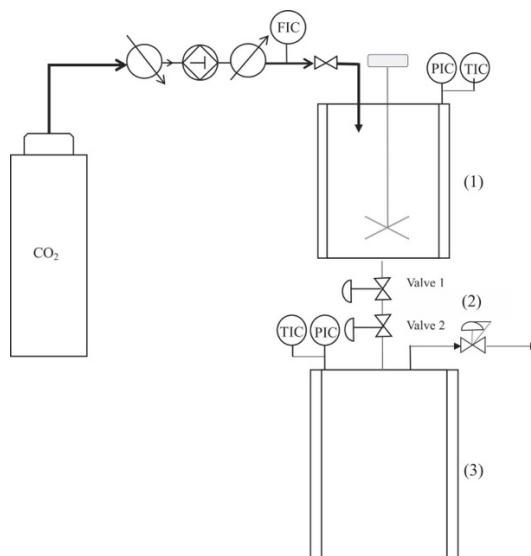


Figure 50. Schematic illustration of the SD-PGSS experimental setup. (1) mixing vessel, (2) dispersion line, (3) collection vessel [322].

III.4. Potential application of the PGSS method in continuous liposome production

The transition from traditional batch processing to continuous flow manufacturing (CFM) represents a paradigm shift in the industrial production of liposomal drug delivery systems [373]. Historically, the commercial-scale manufacturing of liposomes has heavily relied on conventional multi-step batch methods, such as ethanol injection followed by mechanical downsizing [182]. These conventional approaches involve multiple discrete unit operations with intermediate hold phases, which necessitate material testing at each stage and increase the risk of batch-to-batch variability. Furthermore, volumetric scale-up in batch manufacturing presents engineering challenges, as the use of larger reactor vessels often leads to unpredictable local variations in reagent concentrations, thermodynamics, and mixing efficiency [374]. Relying on extrusion to control vesicle size also poses a major bottleneck because this time-consuming "top-down" process is prone to severe membrane clogging and imparts intense mechanical stress on the formulation [180]. Recently, the evolution of continuous ethanol injection using crossflow modules and, especially, the

emerging prominence of microfluidics have facilitated the transition from batch manufacturing to CFM. By integrating all unit operations into a single, uninterrupted, and fully enclosed "end-to-end" production line, CFM minimizes manual interventions, eliminates hold phases, and reduces the risk of microbial contamination [182]. The well-controlled dynamics of continuous flow help to minimize batch-to-batch variability and yield highly uniform liposomal populations. From an economic perspective, CFM offers a drastically smaller facility footprint, lower capital expenditures, and maximized cost-efficiency [373].

Given the aforementioned advantages of CFM, adapting the PGSS method to a continuous flow paradigm represents a highly promising avenue for industrial translation. This adaptation would synergistically combine the scalability of continuous processing with the intrinsic safety and environmental benefits of PGSS, namely the complete elimination of organic solvents. To date, however, the PGSS production of liposomes has predominantly been operated in batch modes using large high-pressure vessels. Translating this method into a fully continuous process requires a fundamental reconfiguration of the operational layout. A recent study has demonstrated the feasibility of integrating SC-CO₂ technology with continuous-flow devices, such as high-pressure millifluidic systems [375]. Although this specific study utilized a co-solvent (ethanol) to dissolve phospholipids, its mechanical configuration provides a highly relevant engineering strategy for continuous SC-CO₂ expansion. In this specific approach, conventional large precipitation vessels are replaced by millimetric stainless-steel tubing equipped with a T-connection. A single feed solution (containing an aqueous buffer, siRNA, and phospholipids previously dissolved in ethanol) is continuously pumped to meet the SC-CO₂ stream at the T-junction, spontaneously forming an emulsion under high pressure. This mixture is then continuously depressurized through a micrometering valve. Crucially, to counteract the severe Joule-Thomson cooling effect caused by the rapid expansion of gas, the valve is continuously heated by a wrapping wire to immediately evacuate formed ice and prevent nozzle clogging. Furthermore, the authors also stated that production capacity can be seamlessly increased simply by "numbering-up" the fluidic devices, thus bypassing the unpredictable thermodynamic variations of large batch reactors. This innovative engineering approach can potentially serve as an inspiration for transforming the conventional batch-based PGSS method into a continuous manufacturing platform.

Furthermore, alongside the mechanical reconfiguration of the PGSS process, the successful implementation of continuous manufacturing fundamentally necessitates the

integration of QbD principles. Historically, pharmaceutical manufacturing relied heavily on end-product testing (Quality-by-Testing), a retrospective approach that is highly inefficient for dynamic continuous pipelines [182]. In contrast, the International Council for Harmonisation Q8(R2) guideline defines QbD as 'a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management' [319]. This regulatory paradigm asserts that quality cannot merely be tested into products, but must be built in by design from the product inception. In that context, to translate the continuous PGSS method into a viable industrial platform, it is imperative to establish a deep understanding of the interdependencies between Critical Material Attributes, (e.g., lipid composition, drug properties) and CPPs (e.g., pressure, temperature, lipid concentration, depressurization rate). The ultimate goal of this modeling is to establish a robust "design space", officially defined as the multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality [319]. Operating within this established design space is the cornerstone of continuous manufacturing, as movements within this space are not considered a process change. It is noteworthy that the DoE conducted in this study for the pilot scale PGSS process did not yield a preliminary design space because almost all the simulated conditions to construct it resulted in a Pdl of more than 0.40, thereby impairing the reliability of the interpretation of sizes from this simulation. Future optimization efforts aimed at reducing the Pdl to below 0.30, as previously mentioned, will be crucial to resolve this limitation and facilitate the construction of a design space for the PGSS process.

Building upon the successful establishment of a robust design space, the subsequent imperative for realizing a fully continuous liposome manufacturing platform is the deployment of Process Analytical Technology (PAT). Defined by regulatory agencies as a system for designing, analyzing, and controlling manufacturing through timely measurements of critical quality and performance attributes during processing, PAT shifts the industry paradigm away from retrospective end-product testing toward proactive, real-time quality assurance [376]. As the primary layer of this PAT framework, fundamental processing conditions must be strictly monitored using highly sensitive physical sensors. In-line pressure transducers, digital flowmeters, and thermocouples at different sites in the system are crucial to continually verify the hydrodynamic stability and immediately detect any early signs of nozzle clogging or fluidic blockages. Beyond these fundamental physical sensors, to meet the challenges of characterizing these dynamic dispersions, the integration of highly sensitive, non-destructive PAT tools to monitor CQAs (e.g., size, Pdl,

drug encapsulation efficiency, residual solvent levels) is essential to enable rapid process intervention and minimize the risk of batch failures. For morphological and physical assessment, spatially resolved dynamic light scattering technologies allow for the continuous, in-line measurement of particle size and Pdl directly within flowing and highly turbid nanosuspensions, completely eliminating the need for offline sampling and dilution [377]. For chemical characterization, near-infrared spectroscopy has proven to be an effective PAT tool for accurately quantifying residual organic solvents in real time during continuous downstream purification steps like tangential flow filtration [377]. This tool is particularly beneficial when a certain amount of organic solvent is used in the PGSS process to facilitate the dissolution of lipid components or highly hydrophobic drugs in the aqueous phase. Furthermore, probe-type Raman spectroscopy combined with partial least-squares analysis has emerged as an innovative method for the real-time, in-line quantitative monitoring of drug encapsulation, unencapsulated drug removal, and drug release kinetics [378]. Ultimately, integrating these continuous PAT sensors with digital control frameworks enables the real-time monitoring of operations, active feedback control, and the automatic diversion of out-of-specification materials [379,380]. By coupling a well-defined QbD design space with these advanced PAT monitoring strategies, manufacturers can ensure consistent liposomal product quality, reduce development timelines, and facilitate the successful transition to robust, GMP-aligned continuous nanomanufacturing.

CONCLUSION AND PERSPECTIVES

I. Conclusion

In the 2025 GINA guidelines, the preferred strategy across nearly all levels of asthma severity is an ICS-FOR combination. This dual therapy offers significant benefits: the ICS actively reduces chronic airway inflammation, while FOR as a LABA rapidly relaxes ASM, offering relief that can extend up to 12 h. More specifically, combining molecules with balanced lipophilicity/hydrophilicity, such as BUD-FOR, serves as both an AIR and a daily maintenance medication. Although highly lipophilic ICSs and LABAs benefit from their long duration of action (up to 24 h), they typically encounter several physiological clearance mechanisms related to the pulmonary route, such as the MCC and AMs phagocytosis. The primary objective of this study was to **develop a liposomal formulation that could help deliver CIC and IND - a combination of an ICS and a LABA never used in commercial products - to overcome these physiological barriers**. Through controlled refinement of the liposomal structure using PEGylated lipids and phospholipids, we developed a robust delivery platform that ensures the mucus penetration, avoid the recognition by AMs, and promote the uptake by bronchial epithelial cells, thereby enhancing the intrinsic bronchodilatory effect of IND. The optimized composition was determined to be DLPC/Chol/DSPE-PEG₂₀₀₀ at a 55/30/15 molar ratio. Notably, the initial inclusion of 30% Chol was likely to maintain vesicle structural stability, despite the use of the unsaturated DLPC and 15% DSPE-PEG. Critically, in an OVA-induced acute asthma model, the optimized liposomal formulation significantly outperformed non-encapsulated drug delivery, demonstrating superior attenuation of the AHR and airway inflammation as well as considerable dose-sparing effects for both drugs.

Building on the successful proof of liposomes role in enhancing the efficacy of CIC and IND in an *in vivo* model, we proceeded to the second objective: **scaling up the production of this formulation**. Starting from the laboratory scale, we replaced the initially used THF method with the PGSS method, which utilizes SC-CO₂ as a dispersing agent, thereby exempting the need for organic solvents. A DoE-assisted re-optimization of this process revealed that maintaining a temperature of 80 °C was crucial for forming a homogeneous population of liposomes. Utilizing these optimized conditions, the process successfully yielded the optimal formulation, achieving targeted CQAs with size < 200 nm, Pdl < 0.30, high EE for both drugs (> 90%) in a total lipid concentration of 5 and 27.5 mM. Scaling up to the pilot scale (a 10-fold increase in reactor volume to 500 mL), we investigated various CPPs including pressure, dispersion volume, agitation speed, and depressurization rate

CONCLUSION AND PERSPECTIVE

using another DoE. This study provided valuable insights regarding the individual and interactive effects of these CPPs on the formation of a model, drug-free formulation. The optimal condition derived from this comprehensive DoE (262 bar, 100 mL, 10% agitation, 60 bar/min depressurization, along with fixed parameters such as 80 °C, 30 min contact, and 5 mM total lipid) successfully produced the optimal formulation (size < 200 nm, PDI close to 0.30, and EE > 90% for both drugs). Furthermore, comprehensive characterization of the produced liposomes showed no statistically significant increase in oxidation or hydrolysis products of DLPC compared to before the production, confirming that the process has a negligible effect on the chemical integrity of this unsaturated lipid.

In summary, the study successfully achieved both critical objectives: demonstrating the superior *in vivo* anti-asthmatic efficacy of liposomes containing CIC and IND at reduced administered doses and establishing an organic solvent-free production method of this formulation at a pilot scale. This dual-delivery strategy is particularly innovative, as the specific combination of these two active molecules does not yet exist on the commercial market. By successfully unifying them within a single nanocarrier, this work opens up promising clinical perspectives for the treatment of asthma, even though certain limitations remain to be addressed, such as the reliance on lipid excipients that are not yet approved for pulmonary administration. Beyond the pharmacological novelty, the innovative nature of the PGSS method used to produce these liposomes represents a major engineering advantage for green, scalable manufacturing. Crucially, the versatility of these developed liposomes is further highlighted by their demonstrated capability to be formulated as a dry powder via spray drying, as investigated within the framework of the LNPulmo project. This successful downstream processing opens up highly interesting possibilities for the design of stable dry powder inhalation systems.

II. Perspectives

Building upon the results from the two primary axes of this thesis, our future focus should pivot from separated proof of concepts to the mechanistic clarification of liposome-bio interface interactions, moving toward validating these delivery systems within highly representative physiological models that mimic the native pulmonary environment. This transition requires a multi-pronged approach encompassing advanced biophysical characterization, sophisticated cellular modeling, and rigorous formulation engineering at a scale appropriate for industrial GMP.

CONCLUSION AND PERSPECTIVE

- Clarifying the PEG desorption hypothesis:

A critical priority is to investigate the 'PEG desorption' hypothesis. Surface PEGylation is widely known to facilitate the mucus penetration and avoid the phagocytosis by AMs. However, this same steric shield could heavily restrict subsequent uptake by target cells, such as pulmonary epithelial cells. The improved *in vivo* efficacy of CIC and IND in the optimal formulation likely implies that our liposomes effectively balance mucus penetration with epithelial uptake. This leads to an interest in evaluating the kinetics of PEG shedding from the perspective of different subsequent biological processes. This mechanistic clarity can be achieved by transitioning from traditional static cell cultures to advanced, three-dimensional, physiologically relevant models. Specifically, evaluating liposomal distribution using mucin-secreting bronchial epithelial cells cultured at the air-liquid interface (ALI) (e.g., Calu-3 ALI models) would provide a highly representative *in vitro* barrier. The monitoring of both liposomes and drugs can be obtained in this model by using high-resolution confocal laser scanning microscopy to achieve optical sectioning and three-dimensional tracking of fluorescently labeled liposomes, alongside quantifying the drug concentrations in different compartments of the model.

- Improvement of drug loading capacity:

Regarding the liposome formulation, it would be important to see if the drug loading in the actual formulation could be further improved. This optimization should be performed at a strategic concentration of liposomes towards industrial production. The concentration of 27.5 mM (total lipid concentration) already tested (section II.2.2.2. *Validation of the PGSS process for the production of liposomes containing ciclesonide and indacaterol maleate* is a concentration of interest for this purpose because it aligns closely with the upper limits of lipid concentrations typically found in ready-to-administer commercial liposomal medicines, which generally range between 5 and 25 mM [197]. While a traditional strategy to increase drug content in liposomes is simply to increase the initial amount of the drug added to the formulation, a more innovative approach involves altering the bilayer composition. Because we hypothesize that IND distributes preferentially at the interface between the lipid bilayer and the aqueous phase, the partial or entire replacement of Chol with IND could be very helpful to leverage IND in both a structural and therapeutic role. This replacement can effectively eliminate the possible competition for space within the bilayer between Chol and

CONCLUSION AND PERSPECTIVE

IND, freeing up the hydrophobic pockets and interfacial regions, allowing the liposomes to achieve a significantly higher encapsulation of IND.

However, because IND has never been used as a membrane stabilizer like Chol, the resulting liposomes must be thoroughly characterized in terms of bilayer structure and integrity. Fundamental parameters, such as size, Pdl, zeta potential, long-term encapsulated concentration of drugs, and vesicle morphology, will provide crucial initial observations regarding the impact of this replacement. To gain a more detailed look at the structure of the lipid bilayer, these lipid vesicles can be analyzed by advanced techniques such as Differential Scanning Calorimetry, Small-Angle X-ray Scattering, and Small-Angle Neutron Scattering. Differential Scanning Calorimetry analysis will verify if the incorporated IND can modulate the thermotropic behavior and thermodynamic parameters of the membrane, specifically the phase transition temperature (T_m) and enthalpy (ΔH) [95]. Meanwhile, Small-Angle X-ray Scattering and Small-Angle Neutron Scattering are powerful tools to precisely analyze lamellarity as long with the crystalline state of a loaded drug [309].

- Encapsulation of ICSs and LABAs with higher lipophilicity:

On the other hand, as elucidated in the discussion, our findings highlight a clear physicochemical trend: the more lipophilic the drug, the more likely it is to partition deeply into the hydrophobic acyl chains of the lipid bilayer, resulting in highly stable encapsulation. Consequently, it would be of significant interest to investigate whether this optimized liposomal platform could further improve the therapeutic efficacy of other ICS and LABA combinations that possess even higher lipophilicities than CIC and IND. In addition, to thoroughly validate the clinical translation of this approach, future *in vivo* studies should advance beyond the current acute setup to more complex, chronic asthmatic models. Specifically, evaluating these liposomes in models of severe neutrophilic asthma represents a critical next step. While T2-high asthma typically responds well to corticosteroids, neutrophilic airway inflammation, frequently observed in severe asthma phenotypes and COPD, is highly resistant to ICS therapy [47]. Therefore, determining whether this targeted liposomal delivery system can successfully restore corticosteroid sensitivity in this phenotype represents a vital objective for future research.

- Continued improvement of the PGSS method at pilot scale:

For the PGSS method, reducing the Pdl of liposomes produced at the pilot scale is one of the major goals for future experiments. As explained in the discussion, the change of the impeller geometry from an axial flow to a radial flow is a promising strategy to improve the

CONCLUSION AND PERSPECTIVE

heat transfer from the thermal wall to the mixing fluid, thereby decreasing the Joule-Thomson effect during the depressurization. In particular, it will be worth studying the impacts of these impeller geometries on the mixing efficiencies of the fluids inside the reactor. To thoroughly investigate these hydrodynamics without relying solely on difficult large-scale physical experiments, Computational Fluid Dynamics (CFD) should be extensively employed as a predictive design tool. CFD modeling allows researchers to virtually simulate the detailed 3D flow patterns, velocity fields, and temperature gradients generated by different impeller configurations [381,382]. By applying appropriate mathematical models to simulate the rotating action of the impellers and accurately resolve the resulting fluid turbulence, future studies can accurately map the turbulent energy dissipation rates and local heat transfer coefficients at the vessel wall for both axial and radial geometries. This will make it possible to visually and quantitatively assess if the radial flow successfully minimizes the Joule-Thomson cooling effect while simultaneously monitoring for any negative mixing trade-offs, such as the formation of stagnant "dead zones" [383,384].

Another crucial step in advancing this technology toward industrial commercialization is the development of the PGSS process into a fully continuous operation. Implementing a continuous manufacturing process is essential for industrial scale-up, as it ensures consistent product quality, improves batch-to-batch reproducibility, and facilitates compliance with GMP requirements [385]. To transition from a batch to a continuous process, the system can be engineered to continuously pump both the SC-CO₂ and the lipid dispersion into a high-pressure vessel or an in-line static mixer. Once a steady-state gas-saturated solution is achieved, the mixture can be continuously discharged and expanded through a depressurization nozzle into a collection chamber. Alongside this process intensification, the comprehensive characterization of the final product remains paramount. In this thesis study, we have already characterized the liposomes produced at the pilot scale in terms of size, Pdl, zeta potential, drug encapsulation, and the extent of lipid oxidation and hydrolysis. The exact quantification of lipid components and their specific oxidation or hydrolysis products can be determined much more accurately by using HPLC methods. Rigorous physicochemical characterization, including the exact quantification of these lipid components and their degradants, is a mandatory requirement outlined by regulatory agencies such as the FDA and EMA for liposomal drug products [176,177]. Because phospholipids naturally lack easily detectable chromophores, standard UV detection is often inadequate for accurate characterization. To accurately quantify the

CONCLUSION AND PERSPECTIVE

final lipid concentration, the pharmaceutical industry typically utilizes HPLC coupled to aerosol-based detectors, such as an evaporative light scattering detector or a charged aerosol detector [386]. These detectors allow for the rapid, direct, and simultaneous quantification of multiple lipid components in the final formulation without the need for complex lipid extraction or derivatization procedures.

Finally, to fully establish the clinical and commercial viability of the formulation, future research must include longer-term stability studies. Because liposomes in aqueous dispersions are highly prone to physical instability such as vesicle aggregation, fusion, and drug leakage, as well as chemical degradation over extended storage periods, evaluating the long-term stability of the product in a dry form will be essential.

APPENDICES

I. Appendix I

Supplementary materials of the article '**Ciclesonide/indacaterol-encapsulated liposomes: optimizing corticosteroid-bronchodilator therapy for a more effective approach to asthma management**'.

I.1. Supplementary materials and methods

I.1.1. Materials

4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) $\geq 99.5\%$, sodium chloride, ammonium acetate were purchased from Sigma Aldrich (Belgium). Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, MA, USA). Absolute ethanol from analytical reagent grade was purchased from VWR Chemicals (Rosny-sous-Bois-cedex, French), methanol of ultra-gradient HPLC grade was purchased from J.T. Baker (Fischer Chemical). Spectra/Por cellulose ester (CE) dialysis tubing with a molecular weight cut-off (MWCO) of 20 kDa was purchased from Spectrum Labs (Rancho Dominguez, USA).

I.1.2. Calculation of test concentrations for *in vivo* study (section I.2.2.7.3. Murine model of asthma induced by allergen exposure)

The doses of IND and CIC used in the *in vivo* mouse study were derived from the highest clinical doses reported for humans [1]. Indeed, we picked the dose of 300 $\mu\text{g/day}$ for IND and 320 $\mu\text{g/day}$ for CIC as starting doses for our calculations. The following formulation was used to convert these human doses to mouse-equivalent doses as described by Nair *et al.* [387]:

$$\text{Animal equivalent dose (AED) (mg/kg)} = \text{Human dose (mg/mg)} \times K_m \text{ ratio}$$

With a K_m ratio (human/mouse) = $37/3 = 12.3$ (Table 10) and an average human weight of 60 kg, the calculated AED doses in mice were 0.0652 and 0.0615 mg/kg for CIC and IND, respectively. From this reference dose, solutions (with HP β CD) with concentrations five times higher and five times lower were prepared and tested in a preliminary dose-response study (data not shown). Based on these results, the five-fold higher concentration was chosen as the high dose to assess the liposomes efficacy, while the ten-fold lower concentration was introduced as the low dose condition in the *in vivo* experiments. Therefore, at the high dose, we had 0.3260 mg/kg CIC and 0.3075 mg/kg IND, while at the low dose, we had 0.0326 mg/kg CIC and 0.0307 mg/kg IND.

1.1.3. Airway responsiveness measurements (baseline measurement for section 1.2.2.7.4. Airway responsiveness measurements)

The ventilator was set to standard parameters for murine ventilation: a positive end expiratory pressure (PEEP) at 3 cmH₂O, a tidal volume of 10 mL/kg, a respiratory rate of 150 breaths per minute. Baseline respiratory mechanical properties were measured 10 s after two deep inflations, required for lung recruitment, at a pressure of 30 cmH₂O. Respiratory mechanics was assessed using two distinct volume-perturbation maneuvers, referred to as the SnapShot-150 and the Quick Prime-3. Resistance (R_{rs} —cmH₂O.s/mL), elastance (E_{rs} —cmH₂O/mL) and compliance (C_{rs} —mL/cmH₂O) of the respiratory system were derived from the linear single-compartment model using the SnapShot-150, which is a volume perturbation providing a single 1-Hz sine wave oscillation. Quick Prime-3 applies a volume perturbation generated by a composite signal consisting of 13 sine waves with mutually prime frequencies (ranging from 1 to 20.5 Hz), each with distinct amplitudes and phases. This approach enables the calculation of Newtonian resistance (R_n , cmH₂O.s/mL), tissue damping (G , cmH₂O.s/mL), and tissue elastance (H , cmH₂O.s/mL) according to the constant phase model. Both Quick-Prime-3 and SnapShot-150 were repeated four times in an alternating fashion, each being intercalated by 8 s of tidal breathing to prevent desaturation. Inspiratory capacity (IC, mL) was also measured by performing a deep inflation maneuver, during which the lungs were inflated from end-expiration to a transrespiratory pressure of 30 cmH₂O, and the inspired volume was recorded. A coefficient of determination of 0.95 was the lower limit for accepting a measurement, and for each parameter, an average of four measurements was calculated at baseline. We also performed a partial P–V maneuver, which consisted of inflating the lungs by step increases in pressure from 3 to 30 cmH₂O and then deflating it by step decreases back to 3 cmH₂O. The changes in volume at the different holding (quasi-static) pressures were plotted for both the inflating and deflating limbs of the maneuver to form the P–V loop. Data of the expiratory branch of the curve were fitted using the Salazar–Knowles equation. Additional parameters were calculated from the quasi-static P–V loop were namely: quasi-static compliance (C_{st}), which is the derivative of a curve that fitted the data points from the deflation limb of the P–V loop to the Salazar–Knowles equation at 5 cmH₂O; hysteresis, which is the area within the P–V loop.

1.1.4. Collection of BALF (for section 1.2.2.7.5. Cell differential analysis in BALF using Diff-Quik staining)

At the end of the airway responsiveness measurements with the Flexivent, the mice in the acute asthma model were anesthetized, and bronchoalveolar lavage (BAL) was

immediately performed. The lungs were first flushed with 1 ml of ice-cold, sterile PBS containing 50 μ M EDTA, administered via a tracheal catheter with a 1 ml syringe. The lungs were gently massaged, and the fluid was recovered to obtain a first, more concentrated BAL fraction. Subsequently, the lavage was repeated again twice with 1 ml of PBS–EDTA solution, and the recovered fluid was collected in a separate tube as the second, less concentrated BAL fraction. Both resulting bronchoalveolar lavage fluid (BALF) fractions were centrifuged at 1250 rpm for 10 min at 4 °C to collect the cellular pellet. Supernatants from the concentrated and diluted fractions were carefully transferred into separate tubes and stored at -80 °C for further analysis.

1.1.5. Histological analysis (for section 1.2.2.7.6. Histological analysis)

The extent of peribronchial inflammation was evaluated using a semi-quantitative scoring system by quantifying the peribronchial mononuclear cells and eosinophils under the microscope. Briefly, for each section, a score ranging from 0 to 3 was assigned. A value of 0 was assigned when no inflammation was detectable; a value of 1 when occasional peribronchial cuffing with inflammatory cells was observed; a value of 2 when presence of a thin inflammatory cell layer (1–5 cells thick) surrounding most bronchi was detected and 3 when a thick inflammatory cell layer (>5 cells thick) surrounded most bronchi. For each animal, five to eight randomly selected lung sections were scored, and the mean score was calculated to obtain an inflammation index per mouse.

Goblet cell hyperplasia was evaluated by counting manually under a light-microscope the number of PAS positive cells (PAS+) and total epithelial cells. Goblet cell abundance was expressed as the percentage of epithelial cells exhibiting PAS positivity. Group comparisons were based on the mean goblet cell percentage and the mean inflammation score for each animal.

1.1.6. Lung homogenates and protein extraction (for section 1.2.2.7.7. Lung cytokines measurements)

The snap-frozen right lungs were thawed and placed in microtubes filled with ceramic beads (MagNa Lyser Green beads, Roche) containing 1 ml of Lysis buffer containing Complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail tablets (Roche, Camarillo, CA). Lungs were homogenated in the Precellys equipment (Precellys 24 Touch Homogenizer, LifeScienceProduct) through two cycles of 50 seconds at 7400 rpm. After that, the samples were centrifuged at 14000 rpm, 4°C for 15 minutes, and the supernatant was collected and stored at -80°C for protein concentration and cytokines measurement. Total protein concentration in the lung tissue homogenates was evaluated

as an additional inflammation marker, determined with a kit using the Lowry assay (DC protein assay kit, Biorad) and expressed in mg/mL [388–390].

1.1.7. Parameters of airway hyperresponsiveness (for section 1.2.3.3.1.

Measurement of AHR)

Airway resistance (Rrs), dynamic compliance (Crs), elastance (Ers), and inspiratory capacity (IC) were derived from the mechanical responses of the respiratory system to the application of a precisely controlled volume of air. Rrs will increase when the friction of the airflow through the lungs increases due to airway obstruction (e.g., bronchoconstriction, mucus plug) [391]. When the lungs stiffness increases (e.g. due to fibrosis or pulmonary edema), the tissue will resist expansion more strongly in response to the pressure of the air movement, and this will increase its intrinsic elastic recoil force that tends to make it retract and revert to its original shape. This impairment can be seen via a reduced Crs and its increased reciprocal Ers [392]. In addition, IC provides a measure of the available functional lung volume that can be inhaled from the end-expiratory position, and its reduction is indicative of significant volume restriction within the lungs.

Rrs, Crs and Ers reflect overall bronchoconstriction, lung flexibility, and stiffness of both upper and lower airways, respectively. In contrast, Newtonian resistance, tissue damping, and tissue elastance specifically describe upper airway bronchoconstriction, peripheral airway constriction, and lung tissue stiffness. Hysteresis, PV-loop area, and quasi-static compliance indicate, respectively, energy loss due to tissue resistance, total inspiratory effort, and intrinsic lung elasticity.

I.2. Supplementary results

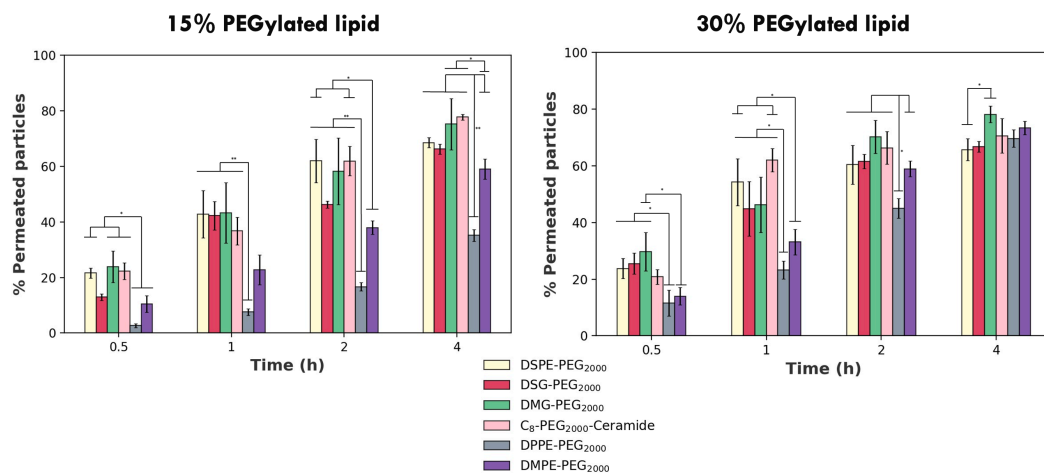


Figure 51. Percentages of permeated liposomes with different types of PEGylated lipids over 4h and at 15% or 30% (n = 3). Data are expressed as mean $\pm$ SD. One-way ANOVA followed by Holm-Šidák post test were performed: * $p < 0.05$, ** $p < 0.01$. Supplementary data for section I.2.3.1.1. *Impact of PEGylated lipids on mucopenetrating properties.*

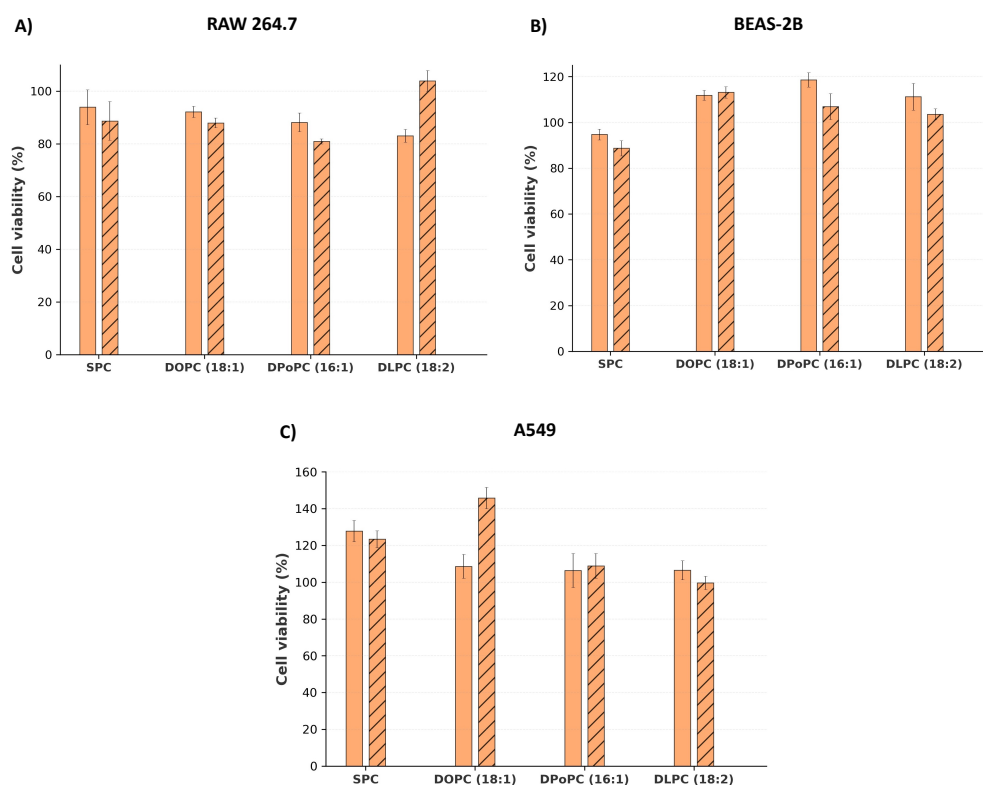


Figure 52. Effect of different phospholipids on toxicity of liposomes at 0.5 mM on (A) RAW 264.7, (B) BEAS-2B, and (C) A549 cells (n=3). Data are expressed as mean $\pm$ SD. Supplementary data for

section 1.2.3.2.2. *Impacts of phospholipids structure on liposomes toxicity and uptake on macrophages and lung epithelial cells.*

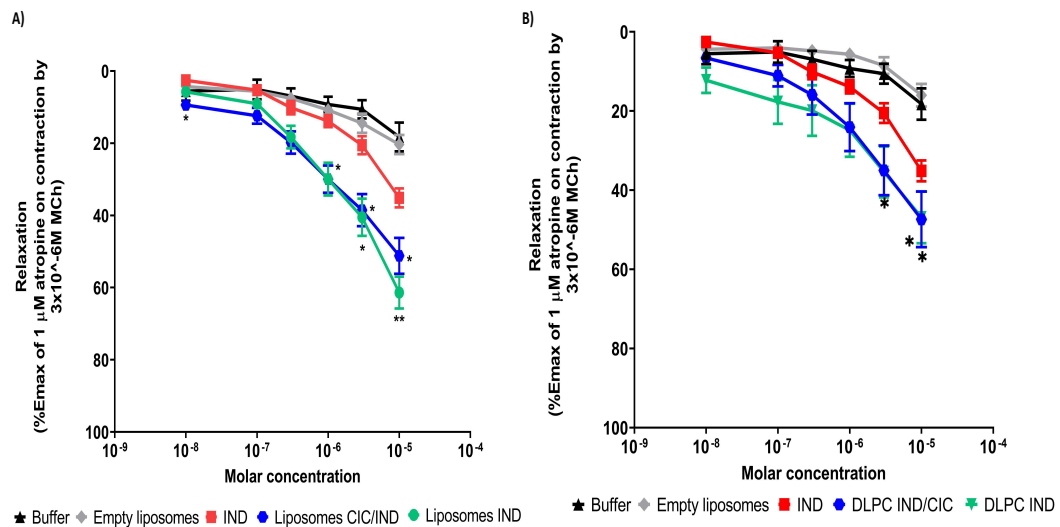


Figure 53. *Ex vivo* concentration-response curve of liposomes containing (A) SPC or (B) DLPC encapsulating only IND or with CIC (n=5-8). Data are expressed as mean $\pm$ SEM. Two-way ANOVA followed by Holm-Šídák post test were performed: * $p < 0.05$, ** $p < 0.01$ versus IND. Supplementary data for section 1.2.3.1.5. *Impact of the concentrations of DSPE-PEG on relaxation effect of indacaterol maleate on isolated rat tracheal rings* (A) and section 1.2.3.2.3. *Impacts of phospholipids structure on relaxation effect of indacaterol maleate on isolated rat tracheal rings* (B).

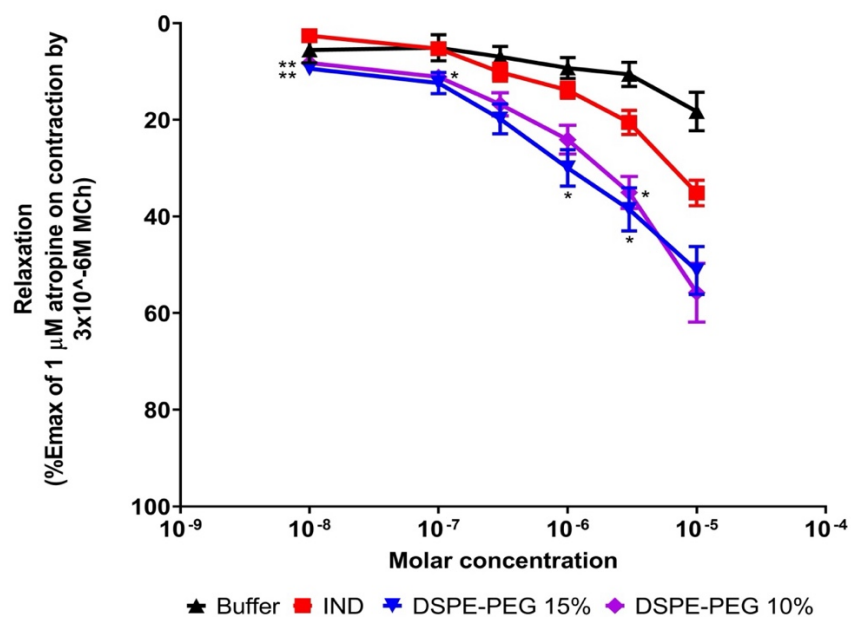


Figure 54. Effect of different DSPE-PEG concentrations on the *ex vivo* relaxation effect of IND (n= 3-10). Data are expressed as mean $\pm$ SEM. Two-way ANOVA followed by Tukey post test were performed: * $p < 0.05$, ** $p < 0.01$ versus IND. Supplementary data for section 1.2.4. Discussion.

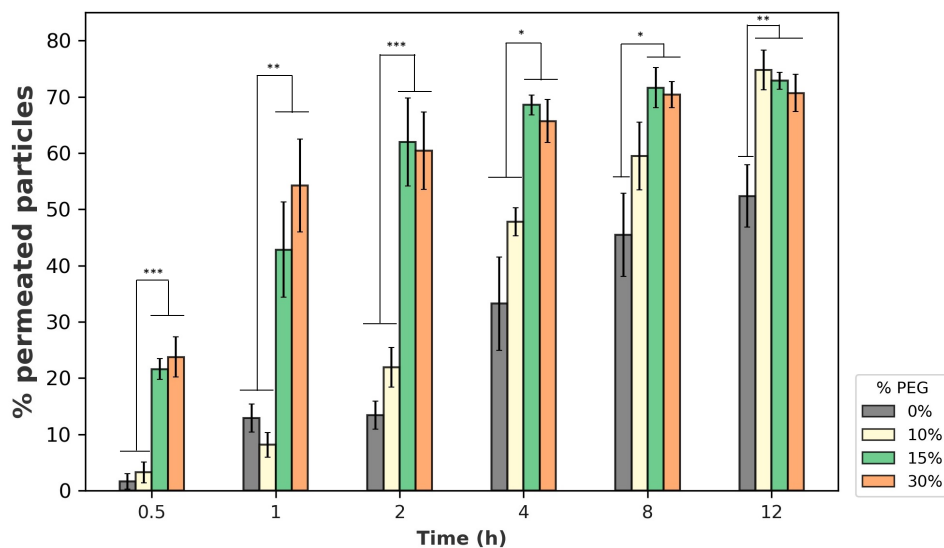


Figure 55. Effect of DSPE-PEG concentrations (0, 10, 15, and 30%) on the liposomes mucopenetration (n = 3). Data are expressed as mean $\pm$ SD. One way ANOVA followed by Tukey post test were performed: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Supplementary data for section 1.2.4. Discussion.

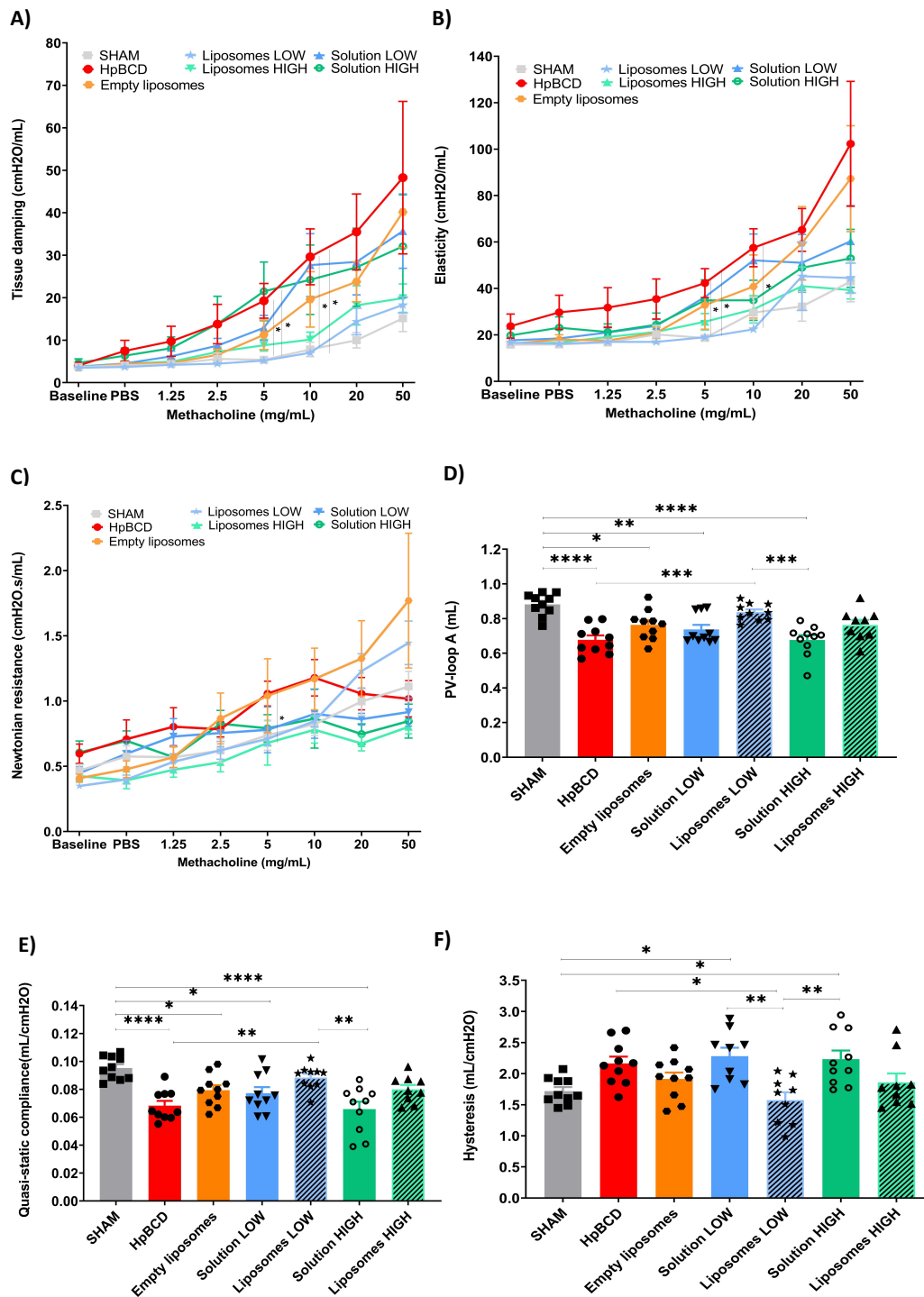


Figure 56. (A) Tissue damping, (B) elasticity, (C) Newtonian resistance, (D) PV-loop-A, (E) quasi-static compliance, and (F) hysteresis of the mice subjected to the indicated treatments were assessed using the SCIREQ flexiVent system following stimulation with various concentrations of methacholine (for tissue damping, elasticity, Newtonian resistance) and at baseline only for PV-loop A, quasi-static compliance, and hysteresis parameters (n=8-10 biologically independent mice per group). Results are presented as mean $\pm$ SEM. Significant differences were assessed using Two-way ANOVA (A-C) or One-way ANOVA (D-F) followed by Holm-Šídák post test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Supplementary data for section 1.2.3.3.1. *Measurement of AHR.*

In addition to resistance, compliance, elastance, and inspiratory capacity, tissue damping, tissue elastance, and Newtonian resistance were derived using the Quick-Prime 3 manoeuvre, while hysteresis, PV-loop area, and quasi-static compliance were assessed using the partial pressure–volume (P–V) manoeuvre. Resistance, compliance, and elastance reflect overall bronchoconstriction, lung flexibility, and stiffness of both upper and lower airways, respectively. In contrast, Newtonian resistance, tissue damping, and tissue elastance specifically describe upper airway bronchoconstriction, peripheral airway constriction, and lung tissue stiffness. Hysteresis, PV-loop area, and quasi-static compliance indicate, respectively, energy loss due to tissue resistance, total inspiratory effort, and intrinsic lung elasticity.

For Newtonian resistance, tissue damping, and tissue elastance we observed similar trends to resistance, compliance, elastance, and inspiratory capacity: healthy mice showed no increase in bronchoconstriction or lung tissue stiffness, whereas asthmatic mice (Hp β CD and Lipos_{EMPTY}) exhibited marked increases in these parameters. All four active formulations attenuated methacholine-induced airway responses compared to vehicle-treated asthmatic controls. Consistent with other respiratory parameters, Lipos_{LOW} and Lipos_{HIGH} were more effective than their corresponding free-drug solutions, with Lipos_{LOW} demonstrating equal or superior efficacy relative to Lipos_{HIGH} (Figure 56A-C).

By definition, a higher PV-loop A reflects greater inspiratory effort, whereas lower values indicate reduced effort. In this study, non-treated asthmatic groups exhibited markedly lower PV-loop A values compared to treated groups. Treatment increased PV-loop A toward levels observed in healthy controls, consistent with the bronchodilatory effect of indacaterol, which enhances inhaled air volume and total inspiratory work. All liposomal formulations produced slightly greater improvements than the corresponding drug solutions. Notably, Lipos_{LOW} showed significantly higher PV-loop A values compared with Hp β CD and Sol_{HIGH} (Figure 56D). Cst followed the same trend as PV-loop A: non-treated animals showed reduced compliance, while treated groups exhibited higher values, indicating improved lung flexibility. Lipos_{LOW} demonstrated significantly greater Cst than

Hp β CD and Sol_{HIGH} (Figure 56E). Non-treated and solution-treated groups showed higher hysteresis, reflecting greater energy loss and lower compliance. Treatment decreased hysteresis, with liposomal formulations producing the most pronounced effects. Among these, Lipos_{LOW} displayed the strongest improvement, surpassing even Lipos_{HIGH}. Significant differences were observed between Lipos_{LOW} and Hp β CD, as well as Sol_{LOW} and Sol_{HIGH} (Figure 56F).

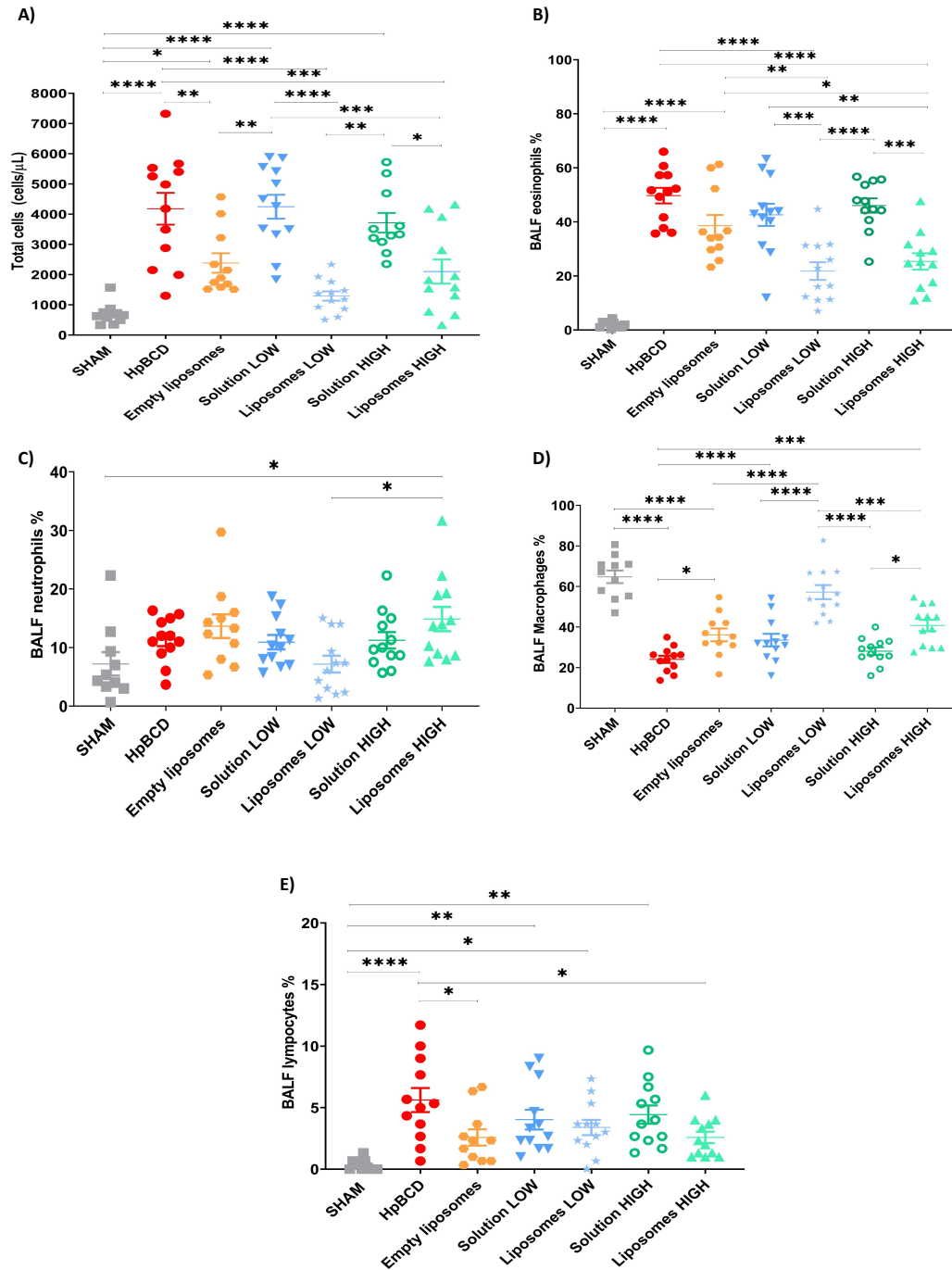


Figure 57. (A) Total cells count, (B) eosinophils, (C) neutrophils, (D) macrophages, and (E) lymphocytes percentages in the BALF of mice following the indicated treatments were assessed using a Diff-Quik staining kit (n=11-12 biologically independent mice per group). Results are presented as mean $\pm$ SEM. Significant differences were assessed using One-way ANOVA followed by Holm-Šídák post test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Supplementary data for section 1.2.3.3.2. *Inflammatory cell counts in BALF*.

The total number of inflammatory cells recovered from BALF was significantly increased in non-treated asthmatic groups compared with healthy controls. Solution-treated groups also exhibited elevated total cell counts relative to controls, although to a lesser extent than non-treated asthmatic animals. In contrast, all liposomal formulations markedly reduced the total inflammatory cell number, reaching values significantly lower than both non-treated and solution-treated groups (Figure 57A).

Considering the inflammatory cells percentages evaluated with differential cell count analysis, a pronounced reduction in eosinophil recruitment was observed in both liposomal groups compared with their non-liposomal counterparts. Lipos_{LOW} and Lipos_{HIGH} displayed comparable efficacy, with Lipos_{LOW} showing a slightly greater reduction. Similarly, Solution_{LOW} and Solution_{HIGH} induced equivalent decreases in eosinophil levels, with a marginal advantage for Solution_{LOW} (Figure 57B).

Comparable trends were observed for lymphocytes, which decreased following treatment (Figure 57E), and for neutrophils, although in this case Lipos_{HIGH} showed relatively higher percentages compared to Lipos_{LOW} and solution-treated groups (Figure 57C). In contrast, macrophages followed an opposite trend, showing higher relative proportions in the liposomal groups. Indeed, their absolute counts tended to be lower in the liposomes-treated mice compared with the solution-treated or untreated asthmatic ones, and similar to the count of the healthy mice group (Figure 33C). This reduction parallels the overall decrease in total BALF cellularity observed after treatment, reflecting the general resolution of airway inflammation. When expressed as percentages (Figure 57D), macrophages appeared proportionally higher in liposomes-treated and healthy mice than in solution-treated or untreated asthmatic groups. This apparent increase reflects a shift in the BALF cellular composition: in untreated asthmatic mice, the massive influx of eosinophils, neutrophils, and lymphocytes greatly expands the total inflammatory cell pool, thereby diluting the relative proportion of resident macrophages. Conversely, liposomal IND/CIC treatment limits the recruitment of these inflammatory cells, particularly eosinophils and neutrophils, thus reducing the overall cellular burden and restoring the macrophage-dominated profile characteristic of healthy airways. Together, these results indicate that IND/CIC liposomal treatment promotes the resolution of airway inflammation and the re-establishment of normal immune cell balance in the lungs more successfully than IND/CIC solution treatment. So simply, this apparent increase is likely due to the substantial reduction of recruited inflammatory cells (eosinophils, neutrophils, lymphocytes) in these groups, which shifts the cellular composition toward resident macrophages (Figure 57D).

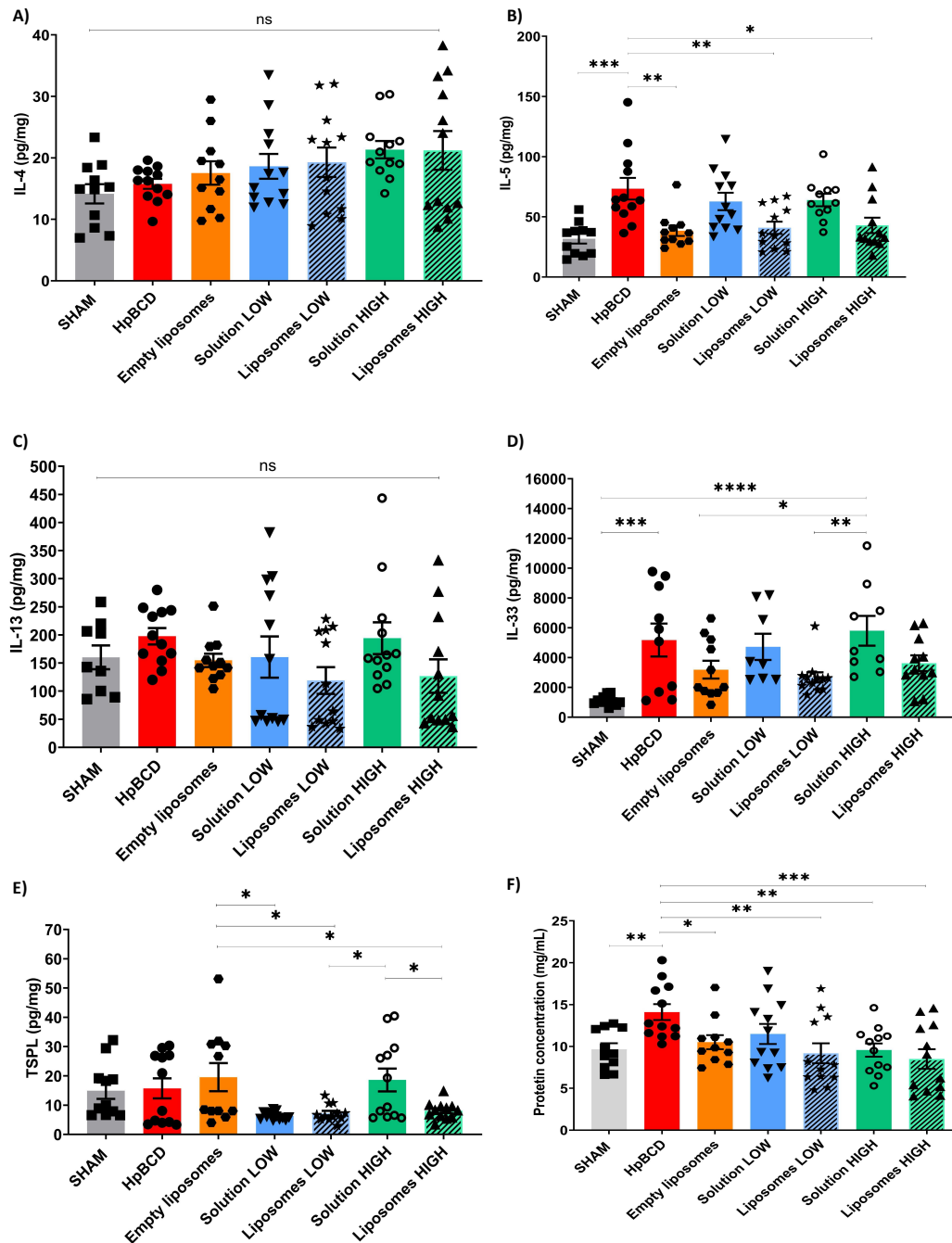


Figure 58. ELISA analysis of (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-33, and (E) TSP levels obtained from normalization of cytokines' concentrations to lungs' total protein concentrations, and (F) total lung protein concentration (n=10-12 biologically independent mice per group). Results are presented as mean $\pm$ SEM. Significant differences were assessed using One-way ANOVA followed by Holm-Šidák multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Supplementary data for section 1.2.3.3.3. Cytokines quantification in whole lung homogenates.

Both Th2-associated and epithelial-associated cytokines levels normalized to total lung protein concentration followed trends consistent with those observed for the absolute values reported in the main text. Treatment with IND/CIC formulations led to a general reduction in cytokines levels compared with non-treated asthmatic controls. However, the free-drug solutions induced only moderate decreases, with Sol^{HIGH} consistently showing the lowest efficacy, whereas Sol^{LOW} achieved greater reductions (Figure 58A-E).

In contrast, both liposomal formulations produced a markedly stronger reduction of the levels of all the cytokines analysed. Lipos^{LOW} demonstrated the most pronounced pharmacodynamic effect, mirroring the superior anti-inflammatory profile observed in other endpoints. Notably, Lipos^{HIGH}, in contrast to its non-liposomal counterpart, effectively lowered cytokine concentrations (Figure 58A-E).

As a general index of pulmonary inflammation and vascular leakage, total protein concentration was measured in lung homogenates (Figure 58F). Protein levels were significantly elevated in asthmatic non-treated groups compared with healthy control and markedly reduced by all treatments. Although both IND/CIC solution groups showed modest reductions relative to the non-treated asthmatic mice, the decrease was slightly more pronounced in the liposome-treated groups.

These results confirm that normalization to total protein mirrors the overall trend of treatment efficacy observed in the absolute cytokines' levels values and further supports the superior anti-inflammatory performance of the liposomal IND/CIC formulations.

Table 10. Table of K_m to calculate animal equivalent dose (AED, mg/kg) starting from human dose (mg/kg) of a drug [387].

Species	Average body weight (kg)	Body surface area (m ²)	To convert animal dose in mg/kg to HED in mg/m ² , multiply by K_m	To convert animal dose in mg/kg to HED in mg/kg, multiply animal dose by	To convert human dose in mg/kg to AED in mg/kg, multiply human dose by
Human	60	1.62	37	–	–
Mouse	0.02	0.007	3	0.081	12.3
Hamster	0.08	0.016	5	0.135	7.4
Rat	0.15	0.025	6	0.162	6.2
Ferret	0.30	0.043	7	0.189	5.3
Guinea pig	0.40	0.05	8	0.216	4.6
Rabbit	1.8	0.15	12	0.324	3.1
Dog	10	0.50	20	0.541	1.8
Cat	7	0.37	19	0.514	1.9
Monkeys (rhesus)	3	0.25	12	0.324	3.1
Marmoset	0.35	0.06	6	0.162	6.2
Squirrel monkey	0.60	0.09	7	0.189	5.3
Baboon	12	0.60	20	0.541	1.8
Micro pig	20	0.74	27	0.730	1.4
Mini pig	40	1.14	35	0.946	1.1

Table 11. Physicochemical properties of the optimal formulation (DLPC/Chol/DSPE-PEG, 55/30/15) (n = 3). EE: encapsulation efficiency, DL: drug loading, Conc: encapsulated concentration.

Particle size (nm)	Pdl	Zeta potential (mV)	Drugs encapsulation	
			Ciclesonide	Indacaterol maleate
147 ± 3	0.11 ± 0.02	-2.5 ± 0.5	EE = 97.3 ± 0.7 % DL = 2.7 ± 0.2 % Conc = 136.4 ± 10.2 µg/mL	EE = 94.6 ± 0.9 % DL = 2.5 ± 0.1 % Conc = 126.5 ± 6.5 µg/mL

II. Appendix II

II.1. Supplementary materials of the article ‘Pilot Scale Transfer and Optimization of an Innovative Supercritical CO₂ PGSS Method for the Production of Drug-Encapsulating Liposomes’

Supplementary Figure

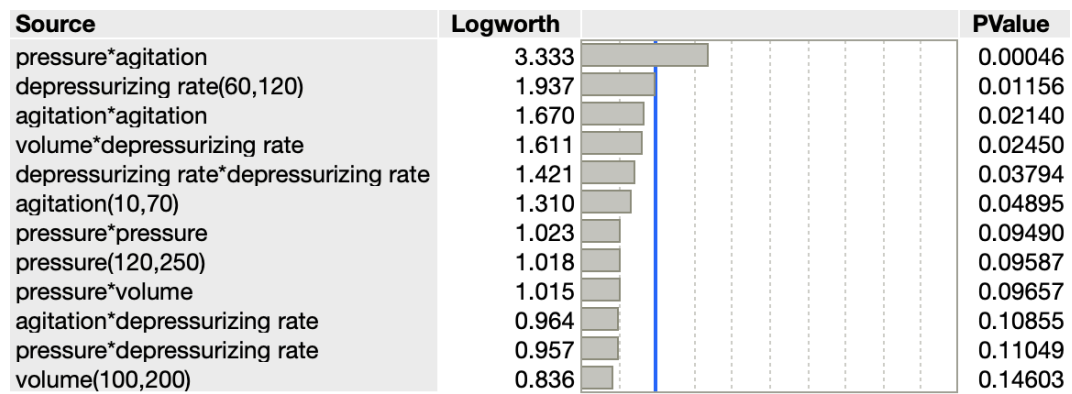


Figure 59. Evaluation of influences of different terms on the depend variables via *p*-value (for section II.3.3.2. *Study of the impact of PGSS production parameters on liposome characteristics using the design of experiments approach*).

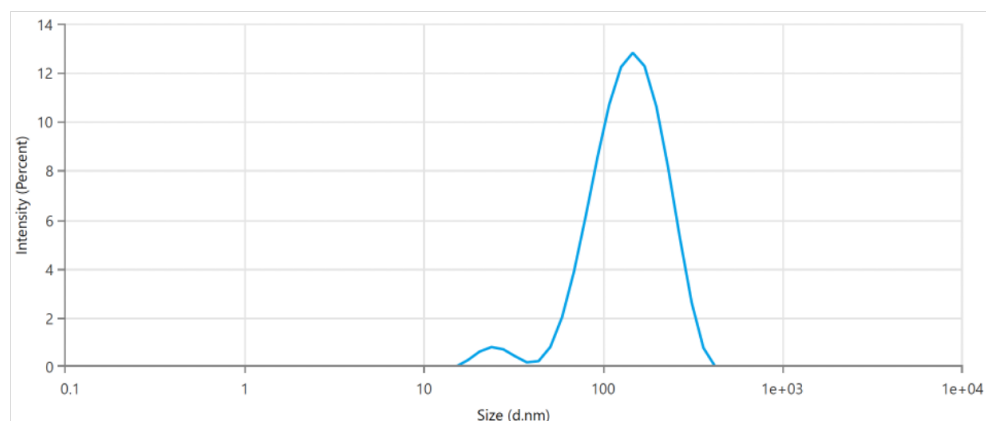
Supplementary Table

Table 12. Experimental conditions with results of 22 experiments from the A-optimal design (for section II.3.2.2.3. *Design of Experiment (DoE): production parameters optimization*).

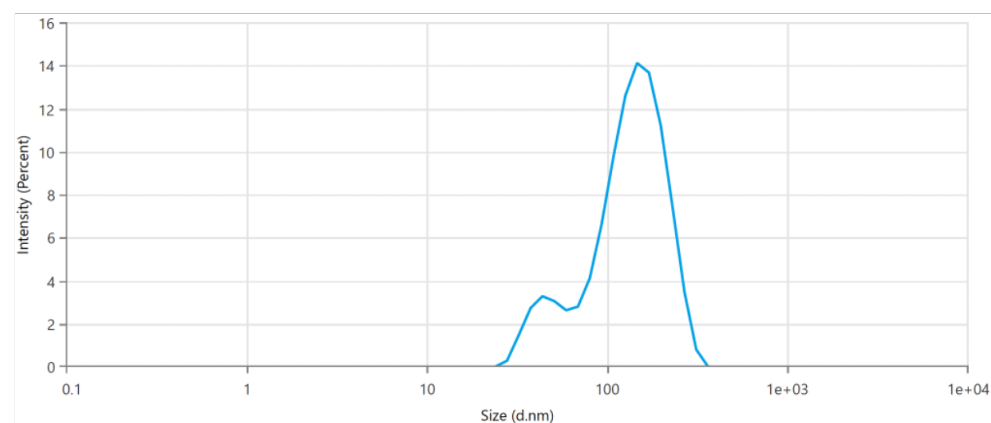
No.	Bloc	Pressure (bar)	Agitation (%)	Volume (mL)	Depressurisation rate (bar/min)	Size (nm)	Pdl
1	1	245	10	100	120	161.2	0.38
2		120	70	100	120	141.2	0.42
3	2	193	40	150	90	138.8	0.41
4		262	70	200	60	218.3	0.40
5	3	250	10	200	90	186.0	0.38
6		250	70	150	120	565.0	0.73
7	4	190	70	150	90	259.6	0.40
8		250	40	100	60	194.8	0.35
9	5	191	40	150	60	147.0	0.41
10		120	70	200	120	217.2	0.50
11	6	120	40	150	90	198.2	0.50
12		185	10	100	60	183.0	0.38
13	7	259	40	200	120	248.3	0.52
14		250	70	100	90	352.7	0.49
15	8	250	10	150	60	180.7	0.35
16		120	10	200	60	253.1	0.41
17	9	127	70	100	60	125.6	0.43
18		187	40	100	120	141.2	0.43
19	10	185	10	200	120	222.9	0.45
20		120	10	100	90	265.8	0.44
21	11	185	40	200	90	219.0	0.48
22		120	10	150	120	252.7	0.44

II.2. Size distribution plot of DLPC formulation produced by pilot-scale PGSS

Batch 1



Batch 2



Batch 3

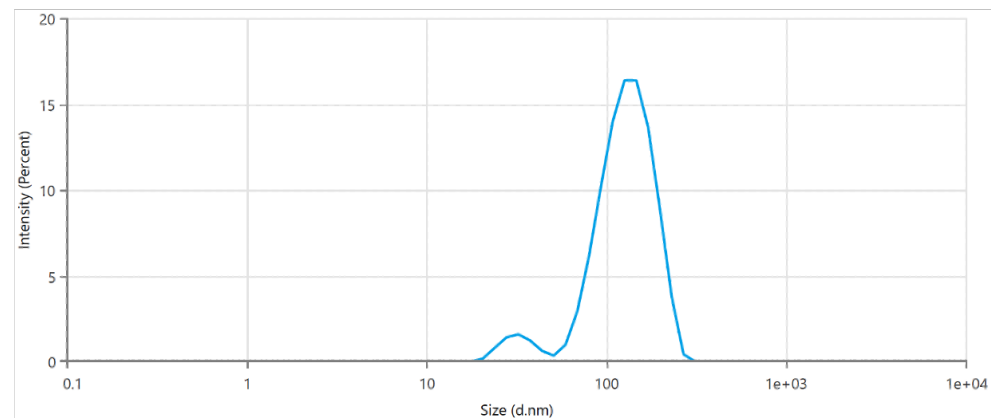


Figure 60. Intensity-weighted particle size distributions of DLPC formulation produced by pilot-scale PGSS, measured by DLS (for section II.4. Conclusion).

III. Appendix III

List of scientific publication:

1. L.A. Bya, B. Bottero, A. Coeurderoi, **T.N. Dinh**, P.Y. Sacré, E. Ziemons, D. Cataldo, G. Piel, B. Evrard, A. Lechanteur, Formulation of a dry powder for inhalation combining ciclesonide and indacaterol maleate using spray drying, International Journal of Pharmaceutics. 678 (2025), <https://doi.org/10.1016/j.ijpharm.2025.125696>.
2. L.A. Bya, **T.N. Dinh**, N. Penoy, P.Y. Sacré, B. Bottero, D. Cataldo, E. Hendrickx, L. Conrard, B. Evrard, G. Piel, A. Lechanteur. Optimizing spray-dried liposomes for pulmonary delivery: impact of lipids composition and of drying parameters using experimental design, European Journal of Pharmaceutics and Biopharmaceutics. 223 (2026) 115035, 10.1016/j.ejpb.2026.115035.
3. **T.N. Dinh**, B. Bottero, F. Perin, L.A. Bya, A. Lechanteur, B. Evrard, G. Piel, D. Cataldo. Ciclesonide/indacaterol-encapsulated liposomes: optimizing cortico-steroid-bronchodilator therapy for a more effective approach to asthma management, (2026). <https://doi.org/10.2139/ssrn.6765699>.
4. **T.N. Dinh**, C. Gauthy, H. Magdalena Segura, P.Y. Sacré, E. Hendrickx, L. Conrard, B. Evrard, B. Malgrange, G. Piel, N. Penoy. Pilot Scale Transfer and Optimization of an Innovative Supercritical CO₂ PGSS Method for the Production of Drug-Encapsulating Liposomes, (2026). <https://doi.org/10.2139/ssrn.6864554>.

List of scientific communication:

1. Liposomes for the treatment of COPD produced by supercritical carbon dioxide-based technologies (Oral presentation), 21st FORUM OF PHARMACEUTICAL SCIENCES (Belgian Society of Pharmaceutical Sciences), 2023.
2. Liposomes containing ciclesonide and indacaterol maleate for pulmonary delivery (Poster presentation), 17th edition of the EUROPEAN SYMPOSIUM ON CONTROLLED DRUG DELIVERY, Egmond aan Zee, the Netherlands, 2024.
3. Design of indacaterol maleate and ciclesonide-loaded liposomes for pulmonary delivery: a focus on roles of PEGylated lipids (Oral presentation), 10th annual meeting of SFNano, Toulouse, France, 2024.

4. Roles of PEGylated lipids in the design of indacaterol maleate and ciclesonide-loaded liposomes for pulmonary delivery (Oral presentation), 22nd FORUM OF PHARMACEUTICAL SCIENCES (Belgian Society of Pharmaceutical Sciences), 2025.
5. Impact of PEGylated lipids in the design of indacaterol maleate and ciclesonide-loaded liposomes for pulmonary delivery (Poster presentation), NanoMed Europe 2025, Barcelona, Spain, 2025.
6. Development of liposomes containing indacaterol maleate and ciclesonide for pulmonary delivery (Oral presentation), 2025 Annual Meeting and Exposition (Controlled Release Society), Philadelphia, United States of America, 2025.
7. Tailored design of lipid components of liposomes containing ciclesonide and indacaterol maleate for improved pulmonary delivery (Oral presentation), 2nd BNLF Meeting (CRS BeNeLux & France Local Chapter), Leiden, the Netherlands, 2025.

REFERENCES

REFERENCES

- [1] Global Initiative for Asthma, Global Strategy for Asthma Management and Prevention, 2025. https://ginasthma.org/wp-content/uploads/2025/11/GINA-2025-Update-25_11_08-WMS.pdf (accessed May 18, 2026).
- [2] F.L.M. Ricciardolo, G. Guida, F. Bertolini, A.D. Stefano, V. Carriero, Phenotype overlap in the natural history of asthma, *European Respiratory Review* 32 (2023). <https://doi.org/10.1183/16000617.0201-2022>.
- [3] H. Hammad, B.N. Lambrecht, The basic immunology of asthma, *Cell* 184 (2021) 1469–1485. <https://doi.org/10.1016/j.cell.2021.02.016>.
- [4] R.J. Russell, L.-P. Boulet, C.E. Brightling, I.D. Pavord, C. Porsbjerg, D. Dorscheid, A. Sverrild, The airway epithelium: an orchestrator of inflammation, a key structural barrier and a therapeutic target in severe asthma, *European Respiratory Journal* 63 (2024). <https://doi.org/10.1183/13993003.01397-2023>.
- [5] M.C. Nawijn, T.L. Hackett, D.S. Postma, A.J.M. van Oosterhout, I.H. Heijink, E-cadherin: gatekeeper of airway mucosa and allergic sensitization, *Trends in Immunology* 32 (2011) 248–255. <https://doi.org/10.1016/j.it.2011.03.004>.
- [6] C.M. Porsbjerg, A. Sverrild, C.M. Lloyd, A.N. Menzies-Gow, E.H. Bel, Anti-alarm-ins in asthma: targeting the airway epithelium with next-generation biologics, *European Respiratory Journal* 56 (2020). <https://doi.org/10.1183/13993003.00260-2020>.
- [7] C.E. Brightling, G. Marone, H. Aegerter, P. Chanez, E. Heffler, I.D. Pavord, K.F. Rabe, L. Uller, D. Dorscheid, the E.S.E. Group, H. Aegerter, A. Bourdin, C.E. Brightling, M. Caminati, P. Chanez, D. Dorscheid, J. Eckl-Dorna, I. Eguiluz-Gracia, E. Heffler, P. Hiemstra, G. Marone, O. Palomares, I.D. Pavord, R. Poto, K.F. Rabe, L. Uller, G. Varricchi, The epithelial era of asthma research: knowledge gaps and future direction for patient care, *European Respiratory Review* 33 (2024). <https://doi.org/10.1183/16000617.0221-2024>.
- [8] R.J. Kurukulaaratchy, H. Mistry, New Real-World Insights Into Severe Asthma: All About the Eosinophil?, *CHEST* 160 (2021) 789–790. <https://doi.org/10.1016/j.chest.2021.05.030>.
- [9] T. Yoshimoto, The hunt for the source of primary interleukin-4: how we discovered that natural killer T cells and basophils determine T helper type 2 cell differentiation in vivo, *Frontiers in Immunology* 9 (2018) 716. <https://doi.org/https://doi.org/10.3389/fimmu.2018.00716>.
- [10] T. Yoshimoto, K. Yasuda, H. Tanaka, M. Nakahira, Y. Imai, Y. Fujimori, K. Nakaniishi, Basophils contribute to TH2-IgE responses in vivo via IL-4 production and presentation of peptide–MHC class II complexes to CD4+ T cells, *Nat Immunol* 10 (2009) 706–712. <https://doi.org/10.1038/ni.1737>.
- [11] S.E.M. Olsthoorn, A. van Krimpen, R.W. Hendriks, R. Stadhouders, Chronic Inflammation in Asthma: Looking Beyond the Th2 Cell, *Immunological Reviews* 330 (2025) e70010. <https://doi.org/10.1111/imr.70010>.
- [12] C. Pelaia, E. Heffler, C. Crimi, A. Maglio, A. Vatrella, G. Pelaia, G.W. Canonica, Interleukins 4 and 13 in asthma: key pathophysiologic cytokines and druggable molecular targets, *Frontiers in Pharmacology* 13 (2022) 851940. <https://doi.org/https://doi.org/10.3389/fphar.2022.851940>.
- [13] K. Nakagome, M. Nagata, Involvement and Possible Role of Eosinophils in Asthma Exacerbation, *Front. Immunol.* 9 (2018). <https://doi.org/10.3389/fimmu.2018.02220>.
- [14] W.W. Busse, M. Kraft, K.F. Rabe, Y. Deniz, P.J. Rowe, M. Ruddy, M. Castro, Understanding the key issues in the treatment of uncontrolled persistent asthma with

REFERENCES

- type 2 inflammation, *European Respiratory Journal* 58 (2021).
<https://doi.org/10.1183/13993003.03393-2020>.
- [15] L. Sahnoun, K. Bajbouj, B. Mahboub, R. Hamoudi, Q. Hamid, Targeting IL-13 and IL-4 in Asthma: Therapeutic Implications on Airway Remodeling in Severe Asthma, *Clinic Rev Allerg Immunol* 68 (2025) 44. <https://doi.org/10.1007/s12016-025-09045-2>.
- [16] H. Rupani, B.D. Kent, Using Fractional Exhaled Nitric Oxide Measurement in Clinical Asthma Management, *Chest* 161 (2022) 906–917.
<https://doi.org/10.1016/j.chest.2021.10.015>.
- [17] S. Couillard, D.J. Jackson, I.D. Pavord, M.E. Wechsler, Choosing the Right Biologic for the Right Patient With Severe Asthma, *CHEST* 167 (2025) 330–342.
<https://doi.org/10.1016/j.chest.2024.08.045>.
- [18] C.N. McBrien, A. Menzies-Gow, The Biology of Eosinophils and Their Role in Asthma, *Front. Med.* 4 (2017). <https://doi.org/10.3389/fmed.2017.00093>.
- [19] K.R. Acharya, S.J. Ackerman, Eosinophil Granule Proteins: Form and Function, *Journal of Biological Chemistry* 289 (2014) 17406–17415.
<https://doi.org/10.1074/jbc.R113.546218>.
- [20] G. Varricchi, C.E. Brightling, C. Grainge, B.N. Lambrecht, P. Chanez, Airway remodelling in asthma and the epithelium: on the edge of a new era, *European Respiratory Journal* 63 (2024). <https://doi.org/10.1183/13993003.01619-2023>.
- [21] D.B. Jacoby, G.J. Gleich, A.D. Fryer, Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor., *J Clin Invest* 91 (1993) 1314–1318. <https://doi.org/10.1172/JCI116331>.
- [22] V. Capra, M.D. Thompson, A. Sala, D.E. Cole, G. Folco, G.E. Rovati, Cysteinyl-leukotrienes and their receptors in asthma and other inflammatory diseases: Critical update and emerging trends, *Medicinal Research Reviews* 27 (2007) 469–527.
<https://doi.org/10.1002/med.20071>.
- [23] C.K. Billington, O.O. Ojo, R.B. Penn, S. Ito, cAMP regulation of airway smooth muscle function, *Pulmonary Pharmacology & Therapeutics* 26 (2013) 112–120.
<https://doi.org/10.1016/j.pupt.2012.05.007>.
- [24] G. Pelaia, T. Renda, L. Gallelli, A. Vatrella, M.T. Busceti, S. Agati, M. Caputi, M. Cazzola, R. Maselli, S.A. Marsico, Molecular mechanisms underlying airway smooth muscle contraction and proliferation: Implications for asthma, *Respiratory Medicine* 102 (2008) 1173–1181. <https://doi.org/10.1016/j.rmed.2008.02.020>.
- [25] C.K. Billington, R.B. Penn, I.P. Hall, β_2 Agonists, in: C.P. Page, P.J. Barnes (Eds.), *Pharmacology and Therapeutics of Asthma and COPD*, Springer International Publishing, Cham, 2017: pp. 23–40. https://doi.org/10.1007/164_2016_64.
- [26] G. Xing, A.Y.-H. Woo, L. Pan, B. Lin, M.-S. Cheng, Recent Advances in β_2 -Agonists for Treatment of Chronic Respiratory Diseases and Heart Failure, *J. Med. Chem.* 63 (2020) 15218–15242. <https://doi.org/10.1021/acs.jmedchem.0c01195>.
- [27] M.R. Sears, J. Lötvall, Past, present and future— β_2 -adrenoceptor agonists in asthma management, *Respiratory Medicine* 99 (2005) 152–170.
<https://doi.org/https://doi.org/10.1016/j.rmed.2004.07.003>.
- [28] G.P. Anderson, A. Linden, K.F. Rabe, Why are long-acting beta-adrenoceptor agonists long-acting?, *European Respiratory Journal* 7 (1994) 569–578.
<https://doi.org/10.1183/09031936.94.07030569>.
- [29] W.T. Garzon-Siatoya, I. Carrillo-Martin, S.E. Chiarella, A. Gonzalez-Estrada, State-of-the-art beta-adrenoreceptor agonists for the treatment of asthma, *Expert Opinion on Pharmacotherapy* 23 (2022) 243–254.
<https://doi.org/10.1080/14656566.2021.1988074>.

REFERENCES

- [30] V. Rajesh, J. Augustine, R. Divya, M. Cleetus, Inhaled Formoterol-Fluticasone Single Inhaler Therapy in Asthma: Real-World Efficacy, Budget Impact, and Potential to Improve Adherence, *Canadian Respiratory Journal* 2020 (2020) 8631316. <https://doi.org/10.1155/2020/8631316>.
- [31] A. Bergendal, A. Lindén, B.-E. Skoogh, M. Gerspacher, G. p. Anderson, C.-G. Löfdahl, Extent of salmeterol-mediated reassertion of relaxation in guinea-pig trachea pretreated with aliphatic side chain structural analogues, *British Journal of Pharmacology* 117 (1996) 1009–1015. <https://doi.org/10.1111/j.1476-5381.1996.tb16690.x>.
- [32] M. Cazzola, P. Rogliani, M.G. Matera, Ultra-LABAs for the treatment of asthma, *Respiratory Medicine* 156 (2019) 47–52. <https://doi.org/10.1016/j.rmed.2019.08.005>.
- [33] P. Casarosa, I. Kollak, T. Kiechle, A. Ostermann, A. Schnapp, R. Kiesling, M. Pieper, P. Sieger, F. Gantner, Functional and Biochemical Rationales for the 24-Hour-Long Duration of Action of Olodaterol, *The Journal of Pharmacology and Experimental Therapeutics* 337 (2011) 600–609. <https://doi.org/10.1124/jpet.111.179259>.
- [34] D.A. Sykes, S.J. Charlton, Slow receptor dissociation is not a key factor in the duration of action of inhaled long-acting β 2-adrenoceptor agonists, *British Journal of Pharmacology* 165 (2012) 2672–2683. <https://doi.org/10.1111/j.1476-5381.2011.01639.x>.
- [35] R.J. Slack, V.J. Barrett, V.S. Morrison, R.G. Sturton, A.J. Emmons, A.J. Ford, R.G. Knowles, In Vitro Pharmacological Characterization of Vilanterol, a Novel Long-Acting β 2-Adrenoceptor Agonist with 24-Hour Duration of Action, *The Journal of Pharmacology and Experimental Therapeutics* 344 (2013) 218–230. <https://doi.org/10.1124/jpet.112.198481>.
- [36] D. Lombardi, B. Cuenoud, S.D. Krämer, Lipid membrane interactions of indacaterol and salmeterol: Do they influence their pharmacological properties?, *European Journal of Pharmaceutical Sciences* 38 (2009) 533–547. <https://doi.org/10.1016/j.ejps.2009.10.001>.
- [37] T. Zhou, Q. Cheng, C. Zou, T. Zhao, S. Liu, M. Pistolozzi, E. Tan, L. Xu, W. Tan, Simultaneous determination of bambuterol and its two major metabolites in human plasma by hydrophilic interaction ultra-performance liquid chromatography–tandem mass spectrometry, *Journal of Chromatography B* 967 (2014) 225–234. <https://doi.org/10.1016/j.jchromb.2014.07.022>.
- [38] R.G. Sturton, A. Trifilieff, A.G. Nicholson, P.J. Barnes, Pharmacological Characterization of Indacaterol, a Novel Once Daily Inhaled β 2 Adrenoceptor Agonist, on Small Airways in Human and Rat Precision-Cut Lung Slices, *The Journal of Pharmacology and Experimental Therapeutics* 324 (2008) 270–275. <https://doi.org/10.1124/jpet.107.129296>.
- [39] M. Cazzola, C.P. Page, P. Rogliani, M.G. Matera, β 2-Agonist Therapy in Lung Disease, *Am J Respir Crit Care Med* 187 (2013) 690–696. <https://doi.org/10.1164/rccm.201209-1739PP>.
- [40] B. Hu, L. Zhong, Y. Weng, L. Peng, Y. Huang, Y. Zhao, X.-J. Liang, Therapeutic siRNA: state of the art, *Sig Transduct Target Ther* 5 (2020) 1–25. <https://doi.org/10.1038/s41392-020-0207-x>.
- [41] S. Yan, D.E. Shaw, L. Yang, D.A. Sandham, M.P. Healy, J. Reilly, B. Wang, Interactions between β 2-Adrenoceptor Ligands and Membrane: Atomic-Level Insights from Magic-Angle Spinning NMR, *J. Med. Chem.* 60 (2017) 6867–6879. <https://doi.org/10.1021/acs.jmedchem.7b00205>.

REFERENCES

- [42] A. L., D.P. Beda, Implementation of quality by design and green chemistry principles to validate the stability indicating method on UHPLC for the simultaneous quantification of Glycopyrronium, Fluticasone, Vilanterol in inhaled formulation, *Green Analytical Chemistry* 11 (2024) 100160. <https://doi.org/10.1016/j.greeac.2024.100160>.
- [43] P.J. Barnes, Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease, *Journal of Allergy and Clinical Immunology* 131 (2013) 636–645. <https://doi.org/10.1016/j.jaci.2012.12.1564>.
- [44] G.T. Ferguson, G.J. Feldman, P. Hofbauer, A. Hamilton, L. Allen, L. Korducki, P. Sachs, Efficacy and safety of olodaterol once daily delivered via Respimat® in patients with GOLD 2–4 COPD: results from two replicate 48-week studies, *International Journal of Chronic Obstructive Pulmonary Disease* 9 (2014) 629–645. <https://doi.org/10.2147/COPD.S61717>.
- [45] T. Bouyssou, P. Casarosa, E. Naline, S. Pestel, I. Konetzki, P. Devillier, A. Schnapp, Pharmacological Characterization of Olodaterol, a Novel Inhaled β_2 -Adrenoceptor Agonist Exerting a 24-Hour-Long Duration of Action in Preclinical Models, *The Journal of Pharmacology and Experimental Therapeutics* 334 (2010) 53–62. <https://doi.org/10.1124/jpet.110.167007>.
- [46] G. Pelaia, A. Vatrella, M.T. Busceti, F. Fabiano, R. Terracciano, M.G. Matera, R. Maselli, Molecular and cellular mechanisms underlying the therapeutic effects of budesonide in asthma, *Pulmonary Pharmacology & Therapeutics* 40 (2016) 15–21. <https://doi.org/10.1016/j.pupt.2016.07.001>.
- [47] P.J. Barnes, I.M. Adcock, How Do Corticosteroids Work in Asthma?, *Ann Intern Med* 139 (2003) 359–370. https://doi.org/10.7326/0003-4819-139-5_Part_1-200309020-00012.
- [48] P.J. Barnes, Corticosteroid effects on cell signalling, *European Respiratory Journal* 27 (2006) 413–426. <https://doi.org/10.1183/09031936.06.00125404>.
- [49] X. Zhang, E. Moilanen, H. Kankaanranta, Enhancement of human eosinophil apoptosis by fluticasone propionate, budesonide, and beclomethasone, *European Journal of Pharmacology* 406 (2000) 325–332. [https://doi.org/10.1016/S0014-2999\(00\)00690-7](https://doi.org/10.1016/S0014-2999(00)00690-7).
- [50] Y. Zhao, P.C. Leung, K.S. Woo, G.G. Chen, Y.O. Wong, S.X. Liu, C.A. van Hasselt, Inhibitory effects of budesonide, desloratadine and dexamethasone on cytokine release from human mast cell line (HMC-1), *Inflamm. Res.* 53 (2004) 664–669. <https://doi.org/10.1007/s00011-004-1309-6>.
- [51] G. Pelaia, G. Cuda, A. Vatrella, D. Fratto, R.D. Grembale, P. Tagliaferri, R. Maselli, F.S. Costanzo, S.A. Marsico, Effects of Transforming Growth Factor- β and Budesonide on Mitogen-Activated Protein Kinase Activation and Apoptosis in Airway Epithelial Cells, *Am J Respir Cell Mol Biol* 29 (2003) 12–18. <https://doi.org/10.1165/rcmb.2002-0074OC>.
- [52] A. Sekiyama, Y. Gon, M. Terakado, I. Takeshita, Y. Kozu, S. Maruoka, K. Matsumoto, S. Hashimoto, Glucocorticoids enhance airway epithelial barrier integrity, *International Immunopharmacology* 12 (2012) 350–357. <https://doi.org/10.1016/j.intimp.2011.12.006>.
- [53] M. Tamm, D.H. Richards, B. Beghé, L. Fabbri, Inhaled corticosteroid and long-acting β_2 -agonist pharmacological profiles: effective asthma therapy in practice, *Respiratory Medicine* 106 (2012) S9–S19. [https://doi.org/10.1016/S0954-6111\(12\)70005-7](https://doi.org/10.1016/S0954-6111(12)70005-7).
- [54] I. Henderson, E. Caiazzo, C. McSharry, T.J. Guzik, P. Maffia, Why do some asthma patients respond poorly to glucocorticoid therapy?, *Pharmacological Research* 160 (2020) 105189. <https://doi.org/10.1016/j.phrs.2020.105189>.

REFERENCES

- [55] J. Kim, J. Ham, H.R. Kang, Y.-S. Bae, T. Kim, H.Y. Kim, JAK3 inhibitor suppresses multipotent ILC2s and attenuates steroid-resistant asthma, *Sci. Adv.* 9 (2023) eadi3770. <https://doi.org/10.1126/sciadv.adi3770>.
- [56] G. Crompton, A brief history of inhaled asthma therapy over the last fifty years, *Primary Care Respiratory Journal* 15 (2006) 326–331. <https://doi.org/10.1016/j.pcrj.2006.09.002>.
- [57] M.G. Matera, B. Rinaldi, L. Calzetta, P. Rogliani, M. Cazzola, Pharmacokinetics and pharmacodynamics of inhaled corticosteroids for asthma treatment, *Pulmonary Pharmacology & Therapeutics* 58 (2019) 101828. <https://doi.org/10.1016/j.pupt.2019.101828>.
- [58] J. Ora, L. Calzetta, M.G. Matera, M. Cazzola, P. Rogliani, Advances with glucocorticoids in the treatment of asthma: state of the art, *Expert Opinion on Pharmacotherapy* 21 (2020) 2305–2316. <https://doi.org/10.1080/14656566.2020.1807514>.
- [59] R. Nave, H. Watz, H. Hoffmann, H. Boss, H. Magnussen, Deposition and metabolism of inhaled ciclesonide in the human lung, *European Respiratory Journal* 36 (2010) 1113–1119. <https://doi.org/10.1183/09031936.00172309>.
- [60] K.I.M. Van Den Brink, M. Boorsma, A.J. Staal-van den Brekel, S. Edsbäcker, E.F. Wouters, L. Thorsson, Evidence of the in vivo esterification of budesonide in human airways, *British Journal of Clinical Pharmacology* 66 (2008) 27–35. <https://doi.org/10.1111/j.1365-2125.2008.03164.x>.
- [61] H.W. Kelly, Comparison of Inhaled Corticosteroids: An Update, *Ann Pharmacother* 43 (2009) 519–527. <https://doi.org/10.1345/aph.1L546>.
- [62] D.I. Suh, S.L. Johnston, The Wiser Strategy of Using Beta-Agonists in Asthma: Mechanisms and Rationales, *Allergy, Asthma & Immunology Research* 16 (2024) 217–234. <https://doi.org/10.4168/aaair.2024.16.3.217>.
- [63] H.K. Reddel, J.M. FitzGerald, E.D. Bateman, L.B. Bacharier, A. Becker, G. Brusselle, R. Buhl, A.A. Cruz, L. Fleming, H. Inoue, F.W. Ko, J.A. Krishnan, M.L. Levy, J. Lin, S.E. Pedersen, A. Sheikh, A. Yorgancioglu, L.-P. Boulet, GINA 2019: a fundamental change in asthma management: Treatment of asthma with short-acting bronchodilators alone is no longer recommended for adults and adolescents, *European Respiratory Journal* 53 (2019). <https://doi.org/10.1183/13993003.01046-2019>.
- [64] P. Venkatesan, 2025 GINA report for asthma, *The Lancet Respiratory Medicine* 13 (2025) e41–e42. [https://doi.org/10.1016/S2213-2600\(25\)00242-5](https://doi.org/10.1016/S2213-2600(25)00242-5).
- [65] R. Brattsand, O. Selroos, May a different kinetic mode explain the high efficacy/safety profile of inhaled budesonide?, *Pulmonary Pharmacology & Therapeutics* 77 (2022) 102167. <https://doi.org/10.1016/j.pupt.2022.102167>.
- [66] S. Alqahtani, C.J. Roberts, S. Stolnik, C. Bosquillon, Development of an In Vitro System to Study the Interactions of Aerosolized Drugs with Pulmonary Mucus, *Pharmaceutics* 12 (2020) 145. <https://doi.org/10.3390/pharmaceutics12020145>.
- [67] M.W. Sadiq, O. Holz, B.D. Ellinghusen, C. Faulenbach, M. Müller, P. Badorrek, U.G. Eriksson, M. Fridén, S. Stomilovic, A.J. Lundqvist, J.M. Hohlfeld, Lung pharmacokinetics of inhaled and systemic drugs: A clinical evaluation, *British Journal of Pharmacology* 178 (2021) 4440–4451. <https://doi.org/10.1111/bph.15621>.
- [68] N. Hartung, J.M. Borghardt, A mechanistic framework for a priori pharmacokinetic predictions of orally inhaled drugs, *PLoS Computational Biology* 16 (2020) e1008466. <https://doi.org/https://doi.org/10.1371/journal.pcbi.1008466>.
- [69] C. Dalby, T. Polanowski, T. Larsson, L. Borgström, S. Edsbäcker, T.W. Harrison, The bioavailability and airway clearance of the steroid component of budesonide/formoterol and salmeterol/fluticasone after inhaled administration in

REFERENCES

- patients with COPD and healthy subjects: a randomized controlled trial, *Respir Res* 10 (2009) 104. <https://doi.org/10.1186/1465-9921-10-104>.
- [70] T.P. Lodise, J. Li, H.N. Gandhi, G. O'Brien, S. Sethi, Intraclass Difference in Pneumonia Risk with Fluticasone and Budesonide in COPD: A Systematic Review of Evidence from Direct-Comparison Studies, *COPD Volume 15* (2020) 2889–2900. <https://doi.org/10.2147/COPD.S269637>.
- [71] S. Edsbäcker, P. Wollmer, O. Selroos, L. Borgström, B. Olsson, J. Ingelf, Do airway clearance mechanisms influence the local and systemic effects of inhaled corticosteroids?, *Pulmonary Pharmacology & Therapeutics* 21 (2008) 247–258. <https://doi.org/10.1016/j.pupt.2007.08.005>.
- [72] D.D. Sin, W.W. Busse, What Harm Are We Doing to Our Patients with Asthma by Using High-Dose Inhaled Corticosteroids?, *Am J Respir Crit Care Med* 211 (2025) 4–6. <https://doi.org/10.1164/rccm.202407-1428ED>.
- [73] C.M. Averell, R.H. Stanford, F. Laliberté, J.W. Wu, G. Germain, M.S. Duh, Medication adherence in patients with asthma using once-daily versus twice-daily ICS/LABAs, *Journal of Asthma* 58 (2021) 102–111. <https://doi.org/10.1080/02770903.2019.1663429>.
- [74] H. De Keyser, V. Vuong, L. Kaye, W.C. Anderson, S. Szeffler, D.A. Stempel, Is Once Versus Twice Daily Dosing Better for Adherence in Asthma and Chronic Obstructive Pulmonary Disease?, *The Journal of Allergy and Clinical Immunology: In Practice* 11 (2023) 2087–2093.e3. <https://doi.org/10.1016/j.jaip.2023.03.053>.
- [75] P. Daley-Yates, B. Aggarwal, Z. Lulic, S. Fulmali, A.A. Cruz, D. Singh, Pharmacology Versus Convenience: A Benefit/Risk Analysis of Regular Maintenance Versus Infrequent or As-Needed Inhaled Corticosteroid Use in Mild Asthma, *Adv Ther* 39 (2022) 706–726. <https://doi.org/10.1007/s12325-021-01976-4>.
- [76] X. Liu, L. Zhang, S. Li, L. Xing, M. Ni, M. Huang, Y. Huang, Harnessing Surface Hydrophilicity of Inhalable Nanoparticles for Precision Delivery of Glucagon-like Peptide-1 Receptor Agonists or Anti-Asthmatic Therapeutics, *ACS Nano* 19 (2025) 22357–22375. <https://doi.org/10.1021/acsnano.5c05745>.
- [77] A. Popov, L. Schopf, J. Bourassa, H. Chen, Enhanced pulmonary delivery of fluticasone propionate in rodents by mucus-penetrating nanoparticles, *International Journal of Pharmaceutics* 502 (2016) 188–197. <https://doi.org/10.1016/j.ijpharm.2016.02.031>.
- [78] Q. Xu, N.J. Boylan, S. Cai, B. Miao, H. Patel, J. Hanes, Scalable method to produce biodegradable nanoparticles that rapidly penetrate human mucus, *Journal of Controlled Release* 170 (2013) 279–286. <https://doi.org/10.1016/j.jconrel.2013.05.035>.
- [79] H.M. El-Laithy, A. Youssef, S.S. El-Husseney, N.S. El Sayed, A. Maher, Enhanced alveo pulmonary deposition of nebulized ciclesonide for attenuating airways inflammations: a strategy to overcome metered dose inhaler drawbacks, *Drug Delivery* 28 (2021) 826–843. <https://doi.org/10.1080/10717544.2021.1905747>.
- [80] Y. Yu, M. Ni, Y. Zheng, Y. Huang, Airway epithelial-targeted nanoparticle reverses asthma in inhalation therapy, *Journal of Controlled Release* 367 (2024) 223–234. <https://doi.org/https://doi.org/10.1016/j.jconrel.2024.01.044>.
- [81] H. Banat, R. Ambrus, I. Csóka, Drug combinations for inhalation: Current products and future development addressing disease control and patient compliance, *International Journal of Pharmaceutics* 643 (2023) 123070. <https://doi.org/10.1016/j.ijpharm.2023.123070>.
- [82] D. Guimarães, A. Cavaco-Paulo, E. Nogueira, Design of liposomes as drug delivery system for therapeutic applications, *International Journal of Pharmaceutics* 601 (2021) 120571. <https://doi.org/10.1016/j.ijpharm.2021.120571>.

REFERENCES

- [83] A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi, K. Nejati-Koshki, Liposome: classification, preparation, and applications, *Nanoscale Res Lett* 8 (2013) 102. <https://doi.org/10.1186/1556-276X-8-102>.
- [84] D. Gardea-Gutiérrez, E. Núñez-García, B.E. Oseguera-Guerra, M. Román-Aguirre, S.L. Montes-Fonseca, Asymmetric Lipid Vesicles: Techniques, Applications, and Future Perspectives as an Innovative Drug Delivery System, *Pharmaceuticals* 16 (2023) 777. <https://doi.org/10.3390/ph16060777>.
- [85] D.E. Large, R.G. Abdelmessih, E.A. Fink, D.T. Auguste, Liposome composition in drug delivery design, synthesis, characterization, and clinical application, *Advanced Drug Delivery Reviews* 176 (2021) 113851. <https://doi.org/10.1016/j.addr.2021.113851>.
- [86] N. Monteiro, A. Martins, R.L. Reis, N.M. Neves, Liposomes in tissue engineering and regenerative medicine, *J R Soc Interface* 11 (2014) 20140459. <https://doi.org/10.1098/rsif.2014.0459>.
- [87] M. Eeman, M. Deleu, From biological membranes to biomimetic model membranes, *Biotechnologie, Agronomie, Société et Environnement* 14 (2010). <https://orbi.uliege.be/handle/2268/81993> (accessed May 3, 2026).
- [88] E. Beltrán-Gracia, A. López-Camacho, I. Higuera-Ciapara, J.B. Velázquez-Fernández, A.A. Vallejo-Cardona, Nanomedicine review: clinical developments in liposomal applications, *Cancer Nano* 10 (2019) 11. <https://doi.org/10.1186/s12645-019-0055-y>.
- [89] T. Ta, T.M. Porter, Thermosensitive liposomes for localized delivery and triggered release of chemotherapy, *Journal of Controlled Release* 169 (2013) 112–125. <https://doi.org/10.1016/j.jconrel.2013.03.036>.
- [90] V. Komalla, V.S.R.R. Allam, P.C.L. Kwok, B. Sheikholeslami, L. Owen, A. Jaffe, S.A. Waters, S. Mohammad, B.G. Oliver, H. Chen, M. Haghi, A phospholipid-based formulation for the treatment of airway inflammation in chronic respiratory diseases, *European Journal of Pharmaceutics and Biopharmaceutics* 157 (2020) 47–58. <https://doi.org/10.1016/j.ejpb.2020.09.017>.
- [91] S. Arber Raviv, M. Alyan, E. Egorov, A. Zano, M.Y. Harush, C. Pieters, H. Korach-Rechtman, A. Saadya, G. Kaneti, I. Nudelman, S. Farkash, O.D. Flikshtain, L.N. Mekies, L. Koren, Y. Gal, E. Dor, J. Shainsky, J. Shklover, Y. Adir, A. Schroeder, Lung targeted liposomes for treating ARDS, *Journal of Controlled Release* 346 (2022) 421–433. <https://doi.org/10.1016/j.jconrel.2022.03.028>.
- [92] A. Ishikado, Y. Nishio, K. Yamane, A. Mukose, K. Morino, Y. Murakami, O. Sekine, T. Makino, H. Maegawa, A. Kashiwagi, Soy phosphatidylcholine inhibited TLR4-mediated MCP-1 expression in vascular cells, *Atherosclerosis* 205 (2009) 404–412. <https://doi.org/10.1016/j.atherosclerosis.2009.01.010>.
- [93] A.A. Jovanović, B.D. Balanč, A. Ota, P. Ahlin Grabnar, V.B. Djordjević, K.P. Šavikin, B.M. Bugarski, V.A. Nedović, N. Poklar Ulrih, Comparative Effects of Cholesterol and β -Sitosterol on the Liposome Membrane Characteristics, *Euro J Lipid Sci & Tech* 120 (2018) 1800039. <https://doi.org/10.1002/ejlt.201800039>.
- [94] W. Kulig, P. Jurkiewicz, A. Olżyńska, J. Tynkkynen, M. Javanainen, M. Manna, T. Rog, M. Hof, I. Vattulainen, P. Jungwirth, Experimental determination and computational interpretation of biophysical properties of lipid bilayers enriched by cholesterol hemisuccinate, *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1848 (2015) 422–432. <https://doi.org/10.1016/j.bbamem.2014.10.032>.
- [95] C. Demetzos, Differential Scanning Calorimetry (DSC): A Tool to Study the Thermal Behavior of Lipid Bilayers and Liposomal Stability, *Journal of Liposome Research* 18 (2008) 159–173. <https://doi.org/10.1080/08982100802310261>.

REFERENCES

- [96] O. Garbuzenko, Y. Barenholz, A. Priev, Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer, *Chemistry and Physics of Lipids* 135 (2005) 117–129. <https://doi.org/10.1016/j.chemphyslip.2005.02.003>.
- [97] S.-D. Li, L. Huang, Stealth nanoparticles: High density but sheddable PEG is a key for tumor targeting, *Journal of Controlled Release* 145 (2010) 178–181. <https://doi.org/10.1016/j.jconrel.2010.03.016>.
- [98] J.T. Huckaby, S.K. Lai, PEGylation for enhancing nanoparticle diffusion in mucus, *Advanced Drug Delivery Reviews* 124 (2018) 125–139. <https://doi.org/10.1016/j.addr.2017.08.010>.
- [99] M. Stepniewski, M. Pasenkiewicz-Gierula, T. Róg, R. Danne, A. Orlowski, M. Karttunen, A. Urtti, M. Yliperttula, E. Vuorimaa, A. Bunker, Study of PEGylated Lipid Layers as a Model for PEGylated Liposome Surfaces: Molecular Dynamics Simulation and Langmuir Monolayer Studies, *Langmuir* 27 (2011) 7788–7798. <https://doi.org/10.1021/la200003n>.
- [100] H. I. Labouta, M. Juliana Gomez-Garcia, C. D. Sarsons, T. Nguyen, J. Kennard, W. Ngo, K. Terefe, N. Iragorri, P. Lai, K. D. Rinker, D. T. Cramb, Surface-grafted polyethylene glycol conformation impacts the transport of PEG-functionalized liposomes through a tumour extracellular matrix model, *RSC Advances* 8 (2018) 7697–7708. <https://doi.org/10.1039/C7RA13438J>.
- [101] M. Beck-Broichsitter, O.M. Merkel, T. Kissel, Controlled pulmonary drug and gene delivery using polymeric nano-carriers, *Journal of Controlled Release* 161 (2012) 214–224. <https://doi.org/10.1016/j.jconrel.2011.12.004>.
- [102] S.C. Nata, M.V. Launico, Anatomy, Airway, in: StatPearls, StatPearls Publishing, Treasure Island (FL), 2026. <http://www.ncbi.nlm.nih.gov/books/NBK459258/> (accessed May 1, 2026).
- [103] Y.S. Khan, D.T. Lynch, Histology, Lung.[Updated 2023 May 1], StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing (2023).
- [104] F. Iñiguez Osmer, I. Sánchez, Development of the Respiratory System, in: P. Bertrand, I. Sánchez (Eds.), *Pediatric Respiratory Diseases: A Comprehensive Textbook*, Springer International Publishing, Cham, 2020: pp. 5–15. https://doi.org/10.1007/978-3-030-26961-6_2.
- [105] V.C. Margaret, The Lower Airways, in: *Respiratory Care: Cardiopulmonary Anatomy & Physiology*, 1st Edition, Jones & Bartlett Learning, Burlington, MA, 2022.
- [106] T. Mekonnen, X. Cai, C. Burchell, H. Gholizadeh, S. Cheng, A review of upper airway physiology relevant to the delivery and deposition of inhalation aerosols, *Advanced Drug Delivery Reviews* 191 (2022) 114530. <https://doi.org/10.1016/j.addr.2022.114530>.
- [107] J.H. Widdicombe, Regulation of the depth and composition of airway surface liquid, *Journal of Anatomy* 201 (2002) 313–318. <https://doi.org/10.1046/j.1469-7580.2002.00098.x>.
- [108] J. Leal, H.D.C. Smyth, D. Ghosh, Physicochemical properties of mucus and their impact on transmucosal drug delivery, *International Journal of Pharmaceutics* 532 (2017) 555–572. <https://doi.org/10.1016/j.ijpharm.2017.09.018>.
- [109] B.E.M. Brand-Saberi, T. Schäfer, Trachea: Anatomy and Physiology, *Thoracic Surgery Clinics* 24 (2014) 1–5. <https://doi.org/10.1016/j.thorsurg.2013.09.004>.
- [110] L. Reid, Measurement of the Bronchial Mucous Gland Layer: A Diagnostic Yardstick in Chronic Bronchitis *, *Thorax* 15 (1960) 132–141. <https://doi.org/10.1136/thx.15.2.132>.
- [111] C. Ehrhardt, B. Forbes, K.-J. Kim, In Vitro Models of the Tracheo-Bronchial Epithelium, in: C. Ehrhardt, K.-J. Kim (Eds.), *Drug Absorption Studies: In Situ, In Vitro*

REFERENCES

- and In Silico Models, Springer US, Boston, MA, 2008: pp. 235–257.
https://doi.org/10.1007/978-0-387-74901-3_10.
- [112] H. Fischer, J.H. Widdicombe, Mechanisms of Acid and Base Secretion by the Airway Epithelium, *J Membrane Biol* 211 (2006) 139–150.
<https://doi.org/10.1007/s00232-006-0861-0>.
 - [113] R.G. Crystal, J.B. West, The lung: scientific foundations, ICON Group International, 1991.
 - [114] Q. Zhou, Y. Saijo, Induced pluripotent stem cells for trachea engineering, *iPSCs in Tissue Engineering* (2021) 143–165. <https://doi.org/https://doi.org/10.1016/B978-0-12-823809-7.00007-4>.
 - [115] S. Ganesan, A.T. Comstock, U.S. Sajjan, Barrier function of airway tract epithelium, *Tissue Barriers* 1 (2013) e24997. <https://doi.org/10.4161/tisb.24997>.
 - [116] K.L. Leiby, M.S.B. Raredon, L.E. Niklason, Bioengineering the Blood-gas Barrier, *Comprehensive Physiology* 10 (2020) 415–452. <https://doi.org/10.1002/j.2040-4603.2020.tb00124.x>.
 - [117] B.C. Huck, X. Murgia, S. Frisch, M. Hittinger, A. Hidalgo, B. Loretz, C.-M. Lehr, Models using native tracheobronchial mucus in the context of pulmonary drug delivery research: Composition, structure and barrier properties, *Advanced Drug Delivery Reviews* 183 (2022) 114141. <https://doi.org/10.1016/j.addr.2022.114141>.
 - [118] R. Guagliardo, J. Pérez-Gil, S. De Smedt, K. Raemdonck, Pulmonary surfactant and drug delivery: Focusing on the role of surfactant proteins, *Journal of Controlled Release* 291 (2018) 116–126. <https://doi.org/10.1016/j.jconrel.2018.10.012>.
 - [119] M. Kubczak, S. Michlewska, M. Bryszewska, A. Aigner, M. Ionov, Nanoparticles for local delivery of siRNA in lung therapy, *Adv Drug Deliv Rev* 179 (2021) 114038. <https://doi.org/10.1016/j.addr.2021.114038>.
 - [120] L. Müller, M. Riediker, P. Wick, M. Mohr, P. Gehr, B. Rothen-Rutishauser, Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways, *J R Soc Interface* 7 (2009) S27–S40.
<https://doi.org/10.1098/rsif.2009.0161.focus>.
 - [121] D.F. Lee, F.J. Salguero, D. Grainger, R.J. Francis, K. MacLellan-Gibson, M.A. Chambers, Isolation and characterisation of alveolar type II pneumocytes from adult bovine lung, *Sci Rep* 8 (2018) 11927. <https://doi.org/10.1038/s41598-018-30234-x>.
 - [122] S. Han, R.K. Mallampalli, The Role of Surfactant in Lung Disease and Host Defense against Pulmonary Infections, *Ann Am Thorac Soc* 12 (2015) 765–774.
<https://doi.org/10.1513/AnnalsATS.201411-507FR>.
 - [123] R. Vlahos, S. Bozinovski, Role of Alveolar Macrophages in Chronic Obstructive Pulmonary Disease, *Front. Immunol.* 5 (2014).
<https://doi.org/10.3389/fimmu.2014.00435>.
 - [124] J. Balhara, A.S. Gounni, The alveolar macrophages in asthma: a double-edged sword, *Mucosal Immunology* 5 (2012) 605–609.
<https://doi.org/10.1038/mi.2012.74>.
 - [125] N. Joshi, J.M. Walter, A.V. Misharin, Alveolar Macrophages, *Cellular Immunology* 330 (2018) 86–90. <https://doi.org/10.1016/j.cellimm.2018.01.005>.
 - [126] S. Bindu, H. Yasmin, U. Barman, S. Pandit, K. Masmoudi, U. Kishore, Immunobiology of pulmonary fibrosis, *Front. Immunol.* 16 (2025).
<https://doi.org/10.3389/fimmu.2025.1708716>.
 - [127] J.E. Hastedt, P. Bäckman, A.R. Clark, W. Doub, A. Hickey, G. Hochhaus, P.J. Kuehl, C.-M. Lehr, P. Mauser, J. McConville, R. Niven, M. Sakagimi, J.G. Weers, Scope and relevance of a pulmonary biopharmaceutical classification system

REFERENCES

- AAPS/FDA/USP Workshop March 16-17th, 2015 in Baltimore, MD, AAPS Open 2 (2016) 1. <https://doi.org/10.1186/s41120-015-0002-x>.
- [128] T.C. Carvalho, J.I. Peters, R.O. Williams, Influence of particle size on regional lung deposition – What evidence is there?, *International Journal of Pharmaceutics* 406 (2011) 1–10. <https://doi.org/10.1016/j.ijpharm.2010.12.040>.
- [129] B. Button, L.-H. Cai, C. Ehre, M. Kesimer, D.B. Hill, J.K. Sheehan, R.C. Boucher, M. Rubinstein, A Periciliary Brush Promotes the Lung Health by Separating the Mucus Layer from Airway Epithelia, *Science* 337 (2012) 937–941. <https://doi.org/10.1126/science.1223012>.
- [130] J.C. Nawroth, A.M. van der Does, A. Ryan (Firth), E. Kanso, Multiscale mechanics of mucociliary clearance in the lung, *Philos Trans R Soc Lond B Biol Sci* 375 (2019) 20190160. <https://doi.org/10.1098/rstb.2019.0160>.
- [131] C. Karamaoun, B. Sobac, B. Mauroy, A.V. Muylem, B. Haut, New insights into the mechanisms controlling the bronchial mucus balance, *PLOS ONE* 13 (2018) e0199319. <https://doi.org/10.1371/journal.pone.0199319>.
- [132] X. Murgia, B. Loretz, O. Hartwig, M. Hittinger, C.-M. Lehr, The role of mucus on drug transport and its potential to affect therapeutic outcomes, *Advanced Drug Delivery Reviews* 124 (2018) 82–97. <https://doi.org/10.1016/j.addr.2017.10.009>.
- [133] X. Yang, K. Forier, L. Steukers, S.V. Vlierberghe, P. Dubruel, K. Braeckmans, S. Glorieux, H.J. Nauwynck, Immobilization of Pseudorabies Virus in Porcine Tracheal Respiratory Mucus Revealed by Single Particle Tracking, *PLOS ONE* 7 (2012) e51054. <https://doi.org/10.1371/journal.pone.0051054>.
- [134] D.B. Hill, B. Button, M. Rubinstein, R.C. Boucher, Physiology and pathophysiology of human airway mucus, *Physiological Reviews* 102 (2022) 1757–1836. <https://doi.org/10.1152/physrev.00004.2021>.
- [135] R. Pangen, T. Meng, S. Poudel, D. Sharma, H. Hutsell, J. Ma, B.K. Rubin, W. Longest, M. Hindle, Q. Xu, Airway mucus in pulmonary diseases: Muco-adhesive and muco-penetrating particles to overcome the airway mucus barriers, *International Journal of Pharmaceutics* 634 (2023) 122661. <https://doi.org/10.1016/j.ijpharm.2023.122661>.
- [136] E.Y. Bissonnette, J.-F. Lauzon-Joset, J.S. Debley, S.F. Ziegler, Cross-Talk Between Alveolar Macrophages and Lung Epithelial Cells is Essential to Maintain Lung Homeostasis, *Front. Immunol.* 11 (2020). <https://doi.org/10.3389/fimmu.2020.583042>.
- [137] H. Aegerter, B.N. Lambrecht, C.V. Jakubzik, Biology of lung macrophages in health and disease, *Immunity* 55 (2022) 1564–1580. <https://doi.org/10.1016/j.immuni.2022.08.010>.
- [138] T. Hussell, T.J. Bell, Alveolar macrophages: plasticity in a tissue-specific context, *Nat Rev Immunol* 14 (2014) 81–93. <https://doi.org/10.1038/nri3600>.
- [139] N. Sanders, C. Rudolph, K. Braeckmans, S.C. De Smedt, J. Demeester, Extracellular barriers in respiratory gene therapy, *Advanced Drug Delivery Reviews* 61 (2009) 115–127. <https://doi.org/10.1016/j.addr.2008.09.011>.
- [140] J.A. Whitsett, S.E. Wert, T.E. Weaver, Diseases of Pulmonary Surfactant Homeostasis, *Annual Review of Pathology: Mechanisms of Disease* 10 (2015) 371–393. <https://doi.org/10.1146/annurev-pathol-012513-104644>.
- [141] M. García-Díaz, D. Birch, F. Wan, H.M. Nielsen, The role of mucus as an invisible cloak to transepithelial drug delivery by nanoparticles, *Advanced Drug Delivery Reviews* 124 (2018) 107–124. <https://doi.org/10.1016/j.addr.2017.11.002>.
- [142] W. Wang, Z. Huang, Y. Huang, X. Zhang, J. Huang, Y. Cui, X. Yue, C. Ma, F. Fu, W. Wang, C. Wu, X. Pan, Pulmonary delivery nanomedicines towards circumventing physiological barriers: Strategies and characterization approaches, *Advanced*

REFERENCES

- Drug Delivery Reviews 185 (2022) 114309.
<https://doi.org/10.1016/j.addr.2022.114309>.
- [143] L. Ding, S. Tang, T.A. Wyatt, D.L. Knoell, D. Oupický, Pulmonary siRNA delivery for lung disease: Review of recent progress and challenges, *Journal of Controlled Release* 330 (2021) 977–991. <https://doi.org/10.1016/j.jconrel.2020.11.005>.
- [144] T. Yu, K.W.Y. Chan, A. Anonuevo, X. Song, B.S. Schuster, S. Chattopadhyay, Q. Xu, N. Oskolkov, H. Patel, L.M. Ensign, P.C.M. van Zijl, M.T. McMahon, J. Hanes, Liposome-based mucus-penetrating particles (MPP) for mucosal theranostics: Demonstration of diamagnetic chemical exchange saturation transfer (diaCEST) magnetic resonance imaging (MRI), *Nanomedicine: Nanotechnology, Biology and Medicine* 11 (2015) 401–405. <https://doi.org/10.1016/j.nano.2014.09.019>.
- [145] P. Meers, M. Neville, V. Malinin, A.W. Scotto, G. Sardaryan, R. Kurumunda, C. Mackinson, G. James, S. Fisher, W.R. Perkins, Biofilm penetration, triggered release and in vivo activity of inhaled liposomal amikacin in chronic *Pseudomonas aeruginosa* lung infections, *J Antimicrob Chemother* 61 (2008) 859–868. <https://doi.org/10.1093/jac/dkn059>.
- [146] J. Zhang, F. Leifer, S. Rose, D.Y. Chun, J. Thaisz, T. Herr, M. Nashed, J. Joseph, W.R. Perkins, K. DiPetrillo, Amikacin Liposome Inhalation Suspension (ALIS) Penetrates Non-tuberculous Mycobacterial Biofilms and Enhances Amikacin Uptake Into Macrophages, *Front. Microbiol.* 9 (2018). <https://doi.org/10.3389/fmicb.2018.00915>.
- [147] S.K. Lai, Y.-Y. Wang, J. Hanes, Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues, *Advanced Drug Delivery Reviews* 61 (2009) 158–171. <https://doi.org/10.1016/j.addr.2008.11.002>.
- [148] B. Tafech, M.-R. Rokhforouz, J. Leung, M.M. Sung, P.J. Lin, D.D. Sin, D. Lauster, S. Block, B.S. Quon, Y. Tam, P. Cullis, J.J. Feng, S. Hedtrich, Exploring Mechanisms of Lipid Nanoparticle-Mucus Interactions in Healthy and Cystic Fibrosis Conditions, *Advanced Healthcare Materials* 13 (2024) 2304525. <https://doi.org/10.1002/adhm.202304525>.
- [149] X. Wang, W. Wan, J. Zhang, J. Lu, P. Liu, Efficient pulmonary fibrosis therapy *via* regulating macrophage polarization using respirable cryptotanshinone-loaded liposomal microparticles, *Journal of Controlled Release* 366 (2024) 1–17. <https://doi.org/10.1016/j.jconrel.2023.12.042>.
- [150] X. He, J. Zou, W. Zhang, Y. Ma, W. He, Pulmonary inhaled drug delivery systems: From bench to bedside, *Journal of Controlled Release* 388 (2025) 114296. <https://doi.org/10.1016/j.jconrel.2025.114296>.
- [151] C.A. Ruge, U.F. Schaefer, J. Herrmann, J. Kirch, O. Cañadas, M. Echaide, J. Pérez-Gil, C. Casals, R. Müller, C.-M. Lehr, The Interplay of Lung Surfactant Proteins and Lipids Assimilates the Macrophage Clearance of Nanoparticles, *PLOS ONE* 7 (2012) e40775. <https://doi.org/10.1371/journal.pone.0040775>.
- [152] N. Doshi, S. Mitragotri, Macrophages Recognize Size and Shape of Their Targets, *PLOS ONE* 5 (2010) e10051. <https://doi.org/10.1371/journal.pone.0010051>.
- [153] K. Kaneko, K. Togami, E. Yamamoto, S. Wang, K. Morimoto, S. Itagaki, S. Chono, Sustained distribution of aerosolized PEGylated liposomes in epithelial lining fluids on alveolar surfaces, *Drug Deliv. and Transl. Res.* 6 (2016) 565–571. <https://doi.org/10.1007/s13346-016-0310-2>.
- [154] K. Togami, Y. Maruta, M. Nanbu, H. Tada, S. Chono, Prolonged distribution of aerosolized PEGylated liposomes in the lungs of mice with bleomycin-induced pulmonary fibrosis, *Drug Development and Industrial Pharmacy* 46 (2020) 1873–1880. <https://doi.org/10.1080/03639045.2020.1825473>.

REFERENCES

- [155] R. Lin, Z. Li, Y. Sun, J. Shen, X. Cao, Y. Lin, C. Fu, Y. Zhu, C. Wang, Z. Tan, Inhalable stealth liposomes improve peptide delivery for pulmonary fibrosis treatment, *Journal of Controlled Release* (2026) 114655. <https://doi.org/https://doi.org/10.1016/j.jconrel.2026.114655>.
- [156] S. Haque, O. Feeney, E. Meeusen, B.J. Boyd, M.P. McIntosh, C.W. Pouton, M. Whittaker, L.M. Kaminskas, Local inflammation alters the lung disposition of a drug loaded pegylated liposome after pulmonary dosing to rats, *Journal of Controlled Release* 307 (2019) 32–43. <https://doi.org/10.1016/j.jconrel.2019.05.043>.
- [157] C.A. Ruge, H. Hillaireau, N. Grabowski, M. Beck-Broichsitter, O. Cañadas, N. Tsapis, C. Casals, J. Nicolas, E. Fattal, Pulmonary Surfactant Protein A-Mediated Enrichment of Surface-Decorated Polymeric Nanoparticles in Alveolar Macrophages, *Mol. Pharmaceutics* 13 (2016) 4168–4178. <https://doi.org/10.1021/acs.molpharmaceut.6b00773>.
- [158] C.A. Ruge, J. Kirch, O. Cañadas, M. Schneider, J. Perez-Gil, U.F. Schaefer, C. Casals, C.-M. Lehr, Uptake of nanoparticles by alveolar macrophages is triggered by surfactant protein A, *Nanomedicine: Nanotechnology, Biology and Medicine* 7 (2011) 690–693. <https://doi.org/10.1016/j.nano.2011.07.009>.
- [159] M. Kendall, P. Ding, R.-M. Mackay, R. Deb, Z. McKenzie, K. Kendall, J. Madsen, H. Clark, Surfactant protein D (SP-D) alters cellular uptake of particles and nanoparticles, *Nanotoxicology* 7 (2013) 963–973. <https://doi.org/10.3109/17435390.2012.689880>.
- [160] J. Wang, P. Li, Y. Yu, Y. Fu, H. Jiang, M. Lu, Z. Sun, S. Jiang, L. Lu, M.X. Wu, Pulmonary surfactant–biomimetic nanoparticles potentiate heterosubtypic influenza immunity, *Science* 367 (2020) eaau0810. <https://doi.org/10.1126/science.aau0810>.
- [161] S. Carregal-Romero, H. Groult, O. Cañadas, N. A-Gonzalez, A.V. Lechuga-Vieco, B. García-Fojeda, F. Herranz, J. Pellico, A. Hidalgo, C. Casals, J. Ruiz-Cabello, Delayed alveolar clearance of nanoparticles through control of coating composition and interaction with lung surfactant protein A, *Biomaterials Advances* 134 (2022) 112551. <https://doi.org/10.1016/j.msec.2021.112551>.
- [162] S. Subramaniam, P. Joyce, L. Donnellan, C. Young, A. Wignall, P. Hoffmann, C.A. Prestidge, Protein adsorption determines pulmonary cell uptake of lipid-based nanoparticles, *Journal of Colloid and Interface Science* 641 (2023) 36–47. <https://doi.org/10.1016/j.jcis.2023.03.048>.
- [163] Q. Shao, T. Ding, F. Pan, G. Li, S. Shen, J. Qian, C. Zhan, X. Wei, Protein corona mediated liposomal drug delivery for bacterial infection management, *Asian Journal of Pharmaceutical Sciences* 17 (2022) 855–866. <https://doi.org/10.1016/j.ajps.2022.10.003>.
- [164] O. Tirosh, Y. Barenholz, J. Katzhendler, A. Priev, Hydration of Polyethylene Glycol-Grafted Liposomes, *Biophysical Journal* 74 (1998) 1371–1379. [https://doi.org/10.1016/S0006-3495\(98\)77849-X](https://doi.org/10.1016/S0006-3495(98)77849-X).
- [165] Y. Wu, W. Wang, Z. Yu, K. Yang, Z. Huang, Z. Chen, X. Yan, H. Hu, Z. Wang, Mushroom-brush transitional conformation of mucus-inert PEG coating improves co-delivery of oral liposome for intestinal metaplasia therapy, *Biomaterials Advances* 136 (2022) 212798. <https://doi.org/10.1016/j.bioadv.2022.212798>.
- [166] M. Berger, F. Toussaint, S.B. Djemaa, J. Laloy, H. Pendeville, B. Evrard, C. Jérôme, A. Lechanteur, D. Mottet, A. Debuigne, Poly (vinyl pyrrolidone) derivatives as PEG alternatives for stealth, non-toxic and less immunogenic siRNA-containing lipoplex delivery, *Journal of Controlled Release* 361 (2023) 87–101. <https://doi.org/https://doi.org/10.1016/j.jconrel.2023.07.031>.

REFERENCES

- [167] Y. Chen, Y.-C. Su, S.R. Roffler, Polyethylene glycol immunogenicity in nanomedicine, *Nat Rev Bioeng* 3 (2025) 742–760. <https://doi.org/10.1038/s44222-025-00321-6>.
- [168] C.E. Henry, Y.-Y. Wang, Q. Yang, T. Hoang, S. Chattopadhyay, T. Hoen, L.M. Ensign, K.L. Nunn, H. Schroeder, J. McCallen, Anti-PEG antibodies alter the mobility and biodistribution of densely PEGylated nanoparticles in mucus, *Acta Biomaterialia* 43 (2016) 61–70. <https://doi.org/10.1016/j.actbio.2016.07.019>.
- [169] V. Francia, R.M. Schiffelers, P.R. Cullis, D. Witzigmann, The Biomolecular Corona of Lipid Nanoparticles for Gene Therapy, *Bioconjugate Chem.* 31 (2020) 2046–2059. <https://doi.org/10.1021/acs.bioconjchem.0c00366>.
- [170] G.V. Azevedo, M.T.R. Alves, P.H.D.M. Prazeres, G.H.C. da Silva, L.H.F. Fujii, P.P.G. Guimarães, Impact of PEG-lipid structure and molar ratio on the physico-chemical and efficacy of pDNA-and mRNA-LNPs, *International Journal of Pharmaceutics* (2026) 126579. <https://doi.org/10.1016/j.ijpharm.2026.126579>.
- [171] I. Khatib, D. Khanal, J. Ruan, D. Cipolla, F. Dayton, J.D. Blanchard, H.-K. Chan, Ciprofloxacin nanocrystals liposomal powders for controlled drug release via inhalation, *International Journal of Pharmaceutics* 566 (2019) 641–651. <https://doi.org/10.1016/j.ijpharm.2019.05.068>.
- [172] P.T. Ingvarsson, M. Yang, H.M. Nielsen, J. Rantanen, C. Foged, Stabilization of liposomes during drying, *Expert Opinion on Drug Delivery* 8 (2011) 375–388. <https://doi.org/10.1517/17425247.2011.553219>.
- [173] F. Haddad, N. Mohammed, R.C. Gopalan, Y.A. Ayoub, M.T. Nasim, K.H. Assi, Development and Optimisation of Inhalable EGCG Nano-Liposomes as a Potential Treatment for Pulmonary Arterial Hypertension by Implementation of the Design of Experiments Approach, *Pharmaceutics* 15 (2023) 539. <https://doi.org/10.3390/pharmaceutics15020539>.
- [174] D. Cipolla, B. Shekunov, J. Blanchard, A. Hickey, Lipid-based carriers for pulmonary products: Preclinical development and case studies in humans, *Advanced Drug Delivery Reviews* 75 (2014) 53–80. <https://doi.org/10.1016/j.addr.2014.05.001>.
- [175] S. Shah, V. Dhawan, R. Holm, M.S. Nagarsenker, Y. Perrie, Liposomes: Advancements and innovation in the manufacturing process, *Advanced Drug Delivery Reviews* 154–155 (2020) 102–122. <https://doi.org/10.1016/j.addr.2020.07.002>.
- [176] European Medicines Agency, Reflection paper on data requirements for intravenous liposomal products developed with reference to an innovator liposomal product, European Medicines Agency, Amsterdam, Netherlands, 2013. https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-data-requirements-intravenous-liposomal-products-developed-reference-innovator-liposomal-product-revision-2_en.pdf (accessed May 18, 2026).
- [177] U.S. Food and Drug Administration, Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation, Center for Drug Evaluation and Research (CDER), Food and Drug Administration, Silver Spring, MD, 2018. <https://www.fda.gov/media/70837/download> (accessed May 18, 2026).
- [178] M. Mehta, T.A. Bui, X. Yang, Y. Aksoy, E.M. Goldys, W. Deng, Lipid-Based Nanoparticles for Drug/Gene Delivery: An Overview of the Production Techniques and Difficulties Encountered in Their Industrial Development, *ACS Mater. Au* 3 (2023) 600–619. <https://doi.org/10.1021/acsmaterialsau.3c00032>.

REFERENCES

- [179] C. Charcosset, A. Juban, J.-P. Valour, S. Urbaniak, H. Fessi, Preparation of liposomes at large scale using the ethanol injection method: Effect of scale-up and injection devices, *Chemical Engineering Research and Design* 94 (2015) 508–515. <https://doi.org/10.1016/j.cherd.2014.09.008>.
- [180] K. Turjeman, A.-N. Egler-Kemmerer, D. Shamrakov, C. Paulus, R. Thiermann, Y. Barenholz, R. Bleul, Comparing continuous micromixing and extrusion downsizing for PEGylated nanoliposomes remotely loaded with doxorubicin or the steroid pro-drug methylprednisolone hemisuccinate, *Journal of Controlled Release* 382 (2025) 113707. <https://doi.org/https://doi.org/10.1016/j.jconrel.2025.113707>.
- [181] H. Morad, R. Negarandeh, M. Rostamnezhad, N. Moein, Synthesis methods for liposomes and their scaling up for practical production, in: *Fundamentals and Therapeutic Applications of Liposomes*, Elsevier, 2026: pp. 31–83. <https://www.sciencedirect.com/science/chapter/edited-volume/pii/B9780443335488000148> (accessed May 18, 2026).
- [182] M. Biscaia-Caleiras, N.A. Fonseca, A.S. Lourenço, J.N. Moreira, S. Simões, Rational formulation and industrial manufacturing of lipid-based complex injectables: Landmarks and trends, *Journal of Controlled Release* 373 (2024) 617–639. <https://doi.org/10.1016/j.jconrel.2024.07.021>.
- [183] D.D. Lasic, Mechanisms of Liposome Formation, *Journal of Liposome Research* 5 (1995) 431–441. <https://doi.org/10.3109/08982109509010233>.
- [184] J.M. Zook, W.N. Vreeland, Effects of temperature, acyl chain length, and flow-rate ratio on liposome formation and size in a microfluidic hydrodynamic focusing device, *Soft Matter* 6 (2010) 1352–1360. <https://doi.org/10.1039/B923299K>.
- [185] A. Wagner, K. Vorauer-Uhl, G. Kreismayr, H. Katinger, The Crossflow Injection Technique: An Improvement of the Ethanol Injection Method, *Journal of Liposome Research* 12 (2002) 259–270. <https://doi.org/10.1081/LPR-120014761>.
- [186] L. Piwowarczyk, M. Kucinska, S. Tomczak, D.T. Mlynarczyk, J. Piskorz, T. Goslin-ski, M. Murias, A. Jelinska, Liposomal nanoformulation as a carrier for curcumin and pEGCG—study on stability and anticancer potential, *Nanomaterials* 12 (2022) 1274. <https://doi.org/https://doi.org/10.3390/nano12081274>.
- [187] Y. Tang, A. Zhou, S. Zhou, J. Ruan, C. Qian, C. Wu, L. Ye, Preparation of VC nanoliposomes by high pressure homogenization: Process optimization and evaluation of efficacy, transdermal absorption, and stability, *Heliyon* 10 (2024) e29516. <https://doi.org/10.1016/j.heliyon.2024.e29516>.
- [188] I. Maclachlan, L.B. Jeffs, E. Yaworski, K. Lam, Systems and methods for manufacturing liposomes, US9005654B2, 2015. <https://patents.google.com/patent/US9005654B2/zh> (accessed May 3, 2026).
- [189] Y. Richter, Y. Zelig, O. ELMALAK, D. Eyal, Liposome formulation and manufacture, US20140271813A1, 2014. <https://patents.google.com/patent/US20140271813A1/en> (accessed May 3, 2026).
- [190] H. Katinger, K. Vorauer-Uhl, A. Wagner, G. Kreismayr, Method and device for producing lipid vesicles, US6843942B2, 2005. <https://patents.google.com/patent/US6843942B2/fr> (accessed May 3, 2026).
- [191] Y. Cheng, C. D. Hay, S. M. Mahuttanatan, J. W. Hindley, O. Ces, Y. Elani, Microfluidic technologies for lipid vesicle generation, *Lab on a Chip* 24 (2024) 4679–4716. <https://doi.org/10.1039/D4LC00380B>.
- [192] R. Rebollo, F. Oyou, Y. Corvis, M.M. El-Hammadi, B. Saubamea, K. Andrieux, N. Mignet, K. Alhareth, Microfluidic Manufacturing of Liposomes: Development and Optimization by Design of Experiment and Machine Learning, *ACS Appl. Mater. Interfaces* 14 (2022) 39736–39745. <https://doi.org/10.1021/acsami.2c06627>.

REFERENCES

- [193] D.A. Lamprou, E. Weaver, eds., *Microfluidics in Pharmaceutical Sciences: Formulation, Drug Delivery, Screening, and Diagnostics*, Springer Nature Switzerland, Cham, 2024. <https://doi.org/10.1007/978-3-031-60717-2>.
- [194] A.P. Costa, X. Xu, M.A. Khan, D.J. Burgess, Liposome Formation Using a Coaxial Turbulent Jet in Co-Flow, *Pharm Res* 33 (2016) 404–416. <https://doi.org/10.1007/s11095-015-1798-8>.
- [195] M. Yu, A. Mathew, D. Liu, Y. Chen, J. Wu, Y. Zhang, N. Zhang, Microfluidics for Formulation and Scale-Up Production of Nanoparticles for Biopharma Industry, in: D.A. Lamprou, E. Weaver (Eds.), *Microfluidics in Pharmaceutical Sciences: Formulation, Drug Delivery, Screening, and Diagnostics*, Springer Nature Switzerland, Cham, 2024: pp. 395–420. https://doi.org/10.1007/978-3-031-60717-2_16.
- [196] M. Maeki, S. Uno, A. Niwa, Y. Okada, M. Tokeshi, Microfluidic technologies and devices for lipid nanoparticle-based RNA delivery, *Journal of Controlled Release* 344 (2022) 80–96. <https://doi.org/10.1016/j.jconrel.2022.02.017>.
- [197] D. Carugo, E. Bottaro, J. Owen, E. Stride, C. Nastruzzi, Liposome production by microfluidics: potential and limiting factors, *Sci Rep* 6 (2016) 25876. <https://doi.org/10.1038/srep25876>.
- [198] A. Jahn, W.N. Vreeland, D.L. DeVoe, L.E. Locascio, M. Gaitan, Microfluidic Directed Formation of Liposomes of Controlled Size, *Langmuir* 23 (2007) 6289–6293. <https://doi.org/10.1021/la070051a>.
- [199] C. Has, P. Sunthar, A comprehensive review on recent preparation techniques of liposomes, *Journal of Liposome Research* 30 (2020) 336–365. <https://doi.org/10.1080/08982104.2019.1668010>.
- [200] F. Mejzini, F. Erdmann, J. Kunert, H. Lucas, J. Kollan, S. Krüger, K. Mäder, Oscillation-generating micromixing (FDmiX) – a new method for manufacturing of mRNA-lipid nanoparticles with scalable high-throughput production, *Journal of Controlled Release* 393 (2026) 114783. <https://doi.org/10.1016/j.jconrel.2026.114783>.
- [201] C. Webb, N. Forbes, C.B. Roces, G. Anderluzzi, G. Lou, S. Abraham, L. Ingalls, K. Marshall, T.J. Leaver, J.A. Watts, J.W. Aylott, Y. Perrie, Using microfluidics for scalable manufacturing of nanomedicines from bench to GMP: A case study using protein-loaded liposomes, *International Journal of Pharmaceutics* 582 (2020) 119266. <https://doi.org/10.1016/j.ijpharm.2020.119266>.
- [202] K.D. Ristroph, N.M. Pinkerton, C.E. Markwalter, S.M. D’Addio, M.E. Gindy, R.F. Pagels, 20 years of Flash NanoPrecipitation – from controlled precipitation to global medicine, *Advanced Drug Delivery Reviews* 227 (2025) 115700. <https://doi.org/10.1016/j.addr.2025.115700>.
- [203] International Council for Harmonisation, ICH Q3C (R9) Guideline on impurities: guideline for residual solvents, European Medicines Agency / International Council for Harmonisation, Amsterdam, Netherlands, 2024. https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q3c-r9-guideline-impurities-guideline-residual-solvents-step-5_en.pdf (accessed May 18, 2026).
- [204] S. Bhamidipati, Z. Ahmad, I. Ahmad, Manufacturing process for liposomal preparations, US20060034908A1, 2006. <https://patents.google.com/patent/US20060034908A1/en?q=US+2006%2f0034908A1> (accessed April 11, 2025).
- [205] I. Maclachlan, L.B. Jeffs, L.R. Palmer, C. Giesbrecht, Liposomal apparatus and manufacturing methods, US11298320B2, 2022. <https://patents.google.com/patent/US11298320B2/en> (accessed May 3, 2026).

REFERENCES

- [206] Dynamics BioPharma, What is Tangential Flow Filtration and how can it benefit your process?, (2020). <https://biopharmadynamics.co.uk/blog/what-is-tangential-flow-filtration-and-how-can-it-benefit-your-process/> (accessed May 3, 2026).
- [207] M. Biscaia-Caleiras, D. Lopes, C. Henriques, A.S. Lourenço, A. Nunes, M. Bañobre, J.N. Moreira, S. Simões, Integrating microfluidics and streamlined remote drug loading: one step closer to continuous manufacturing of liposomal injectables containing small drugs, *International Journal of Pharmaceutics* 682 (2025) 125973. <https://doi.org/10.1016/j.ijpharm.2025.125973>.
- [208] H. Hartounian, D. Meissner, C.B. Pepper, Production of multivesicular liposomes, US9585838B2, 2017. <https://patents.google.com/patent/US9585838B2/en> (accessed May 3, 2026).
- [209] N. Dimov, E. Kastner, M. Hussain, Y. Perrie, N. Szita, Formation and purification of tailored liposomes for drug delivery using a module-based micro continuous-flow system, *Scientific Reports* 7 (2017) 12045. <https://doi.org/https://doi.org/10.1038/s41598-017-11533-1>.
- [210] R.D. Worsham, V. Thomas, S.S. Farid, Impact of ethanol on continuous inline diafiltration of liposomal drug products, *Biotechnology Journal* 18 (2023) 2300194. <https://doi.org/10.1002/biot.202300194>.
- [211] K.L. Delma, A. Lechanteur, B. Evrard, R. Semdé, G. Piel, Sterilization methods of liposomes: Drawbacks of conventional methods and perspectives, *International Journal of Pharmaceutics* 597 (2021) 120271. <https://doi.org/10.1016/j.ijpharm.2021.120271>.
- [212] V.G.S. Sainaga Jyothi, R. Bulusu, B. Venkata Krishna Rao, M. Pranothi, S. Banda, P. Kumar Bolla, N. Kommineni, Stability characterization for pharmaceutical liposome product development with focus on regulatory considerations: An update, *International Journal of Pharmaceutics* 624 (2022) 122022. <https://doi.org/10.1016/j.ijpharm.2022.122022>.
- [213] P. CARA, V.M.R. HENDERICKX, V.M.D. CUPERE, C.B.G.D. KESEL, Method of making a liposome composition, US10695424B2, 2020. <https://patents.google.com/patent/US10695424B2/en> (accessed May 3, 2026).
- [214] Y. Wang, D.W. Grainger, Regulatory Considerations Specific to Liposome Drug Development as Complex Drug Products, *Front. Drug Deliv.* 2 (2022). <https://doi.org/10.3389/fddev.2022.901281>.
- [215] M. Sameer Khan, G. Gupta, A. Alsayari, S. Wahab, A. Sahebkar, P. Kesharwani, Advancements in liposomal formulations: A comprehensive exploration of industrial production techniques, *International Journal of Pharmaceutics* 658 (2024) 124212. <https://doi.org/10.1016/j.ijpharm.2024.124212>.
- [216] L.V. ANDREASEN, G.K. WOOD, D. Christensen, Methods for producing liposomes, WO2013004234A2, 2013. <https://patents.google.com/patent/WO2013004234A2/en> (accessed May 3, 2026).
- [217] W. Bigazzi, N. Penoy, B. Evrard, G. Piel, Supercritical fluid methods: An alternative to conventional methods to prepare liposomes, *Chemical Engineering Journal* 383 (2020) 123106. <https://doi.org/10.1016/j.cej.2019.123106>.
- [218] J. Jung, M. Perrut, Particle design using supercritical fluids: Literature and patent survey, *The Journal of Supercritical Fluids* 20 (2001) 179–219. [https://doi.org/10.1016/S0896-8446\(01\)00064-X](https://doi.org/10.1016/S0896-8446(01)00064-X).
- [219] Z. Wen, X. You, L. Jiang, B. Liu, Z. Zheng, Y. Pu, B. Cheng, Liposomal incorporation of rose essential oil by a supercritical process, *Flavour and Fragrance Journal* 26 (2011) 27–33. <https://doi.org/10.1002/ffj.2012>.
- [220] L. Padrela, M.A. Rodrigues, A. Duarte, A.M.A. Dias, M.E.M. Braga, H.C. de Sousa, Supercritical carbon dioxide-based technologies for the production of drug

REFERENCES

- nanoparticles/nanocrystals – A comprehensive review, *Advanced Drug Delivery Reviews* 131 (2018) 22–78. <https://doi.org/10.1016/j.addr.2018.07.010>.
- [221] P. Franco, I. De Marco, Nanoparticles and Nanocrystals by Supercritical CO₂-Assisted Techniques for Pharmaceutical Applications: A Review, *Applied Sciences* 11 (2021) 1476. <https://doi.org/10.3390/app11041476>.
- [222] P. Trucillo, S. Cardea, L. Baldino, E. Reverchon, Production of liposomes loaded alginate aerogels using two supercritical CO₂ assisted techniques, *Journal of CO₂ Utilization* 39 (2020) 101161. <https://doi.org/10.1016/j.jcou.2020.101161>.
- [223] W.-C. Tsai, S.S.H. Rizvi, Liposomal microencapsulation using the conventional methods and novel supercritical fluid processes, *Trends in Food Science & Technology* 55 (2016) 61–71. <https://doi.org/10.1016/j.tifs.2016.06.012>.
- [224] I. Pasquali, R. Bettini, Are pharmaceuticals really going supercritical?, *International Journal of Pharmaceutics* 364 (2008) 176–187. <https://doi.org/10.1016/j.ijpharm.2008.05.014>.
- [225] P. Trucillo, R. Campardelli, M. Scognamiglio, E. Reverchon, Control of liposomes diameter at micrometric and nanometric level using a supercritical assisted technique, *Journal of CO₂ Utilization* 32 (2019) 119–127. <https://doi.org/10.1016/j.jcou.2019.04.014>.
- [226] P. Trucillo, R. Campardelli, E. Reverchon, A versatile supercritical assisted process for the one-shot production of liposomes, *The Journal of Supercritical Fluids* 146 (2019) 136–143. <https://doi.org/10.1016/j.supflu.2019.01.015>.
- [227] L. Lesoin, O. Boutin, C. Crampon, E. Badens, CO₂/water/surfactant ternary systems and liposome formation using supercritical CO₂: A review, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 377 (2011) 1–14. <https://doi.org/10.1016/j.colsurfa.2011.01.027>.
- [228] G.D. Bothun, B.L. Knutson, H.J. Strobel, S.E. Nokes, Liposome Fluidization and Melting Point Depression by Pressurized CO₂ Determined by Fluorescence Anisotropy, *Langmuir* 21 (2005) 530–536. <https://doi.org/10.1021/la0496542>.
- [229] K. Otake, T. Shimomura, T. Goto, T. Imura, T. Furuya, S. Yoda, Y. Takebayashi, H. Sakai, M. Abe, Preparation of Liposomes Using an Improved Supercritical Reverse Phase Evaporation Method, *Langmuir* 22 (2006) 2543–2550. <https://doi.org/10.1021/la051654u>.
- [230] N. Penoy, B. Grignard, B. Evrard, G. Piel, A supercritical fluid technology for liposome production and comparison with the film hydration method, *International Journal of Pharmaceutics* 592 (2021) 120093. <https://doi.org/10.1016/j.ijpharm.2020.120093>.
- [231] K. Otake, T. Shimomura, T. Goto, T. Imura, T. Furuya, S. Yoda, Y. Takebayashi, H. Sakai, M. Abe, One-Step Preparation of Chitosan-Coated Cationic Liposomes by an Improved Supercritical Reverse-Phase Evaporation Method, *Langmuir* 22 (2006) 4054–4059. <https://doi.org/10.1021/la051662a>.
- [232] L. Zhao, F. Temelli, Preparation of liposomes using a modified supercritical process via depressurization of liquid phase, *The Journal of Supercritical Fluids* 100 (2015) 110–120. <https://doi.org/10.1016/j.supflu.2015.02.022>.
- [233] L. Zhao, F. Temelli, Preparation of anthocyanin-loaded liposomes using an improved supercritical carbon dioxide method, *Innovative Food Science & Emerging Technologies* 39 (2017) 119–128. <https://doi.org/10.1016/j.ifset.2016.11.013>.
- [234] D. Villanueva-Bermejo, F. Temelli, Optimization of coenzyme Q10 encapsulation in liposomes using supercritical carbon dioxide, *Journal of CO₂ Utilization* 38 (2020) 68–76. <https://doi.org/10.1016/j.jcou.2020.01.011>.

REFERENCES

- [235] L. Zhao, F. Temelli, J.M. Curtis, L. Chen, Encapsulation of lutein in liposomes using supercritical carbon dioxide, *Food Research International* 100 (2017) 168–179. <https://doi.org/10.1016/j.foodres.2017.06.055>.
- [236] L. Zhao, F. Temelli, Preparation of liposomes using supercritical carbon dioxide via depressurization of the supercritical phase, *Journal of Food Engineering* 158 (2015) 104–112. <https://doi.org/10.1016/j.jfoodeng.2015.03.004>.
- [237] S.H. Soh, L.Y. Lee, Microencapsulation and nanoencapsulation using supercritical fluid (SCF) techniques, *Pharmaceutics* 11 (2019) 21. <https://doi.org/https://doi.org/10.3390/pharmaceutics11010021>.
- [238] J. Wang, Z. Wang, B. Sun, Improved equation of CO₂ Joule–Thomson coefficient, *Journal of CO₂ Utilization* 19 (2017) 296–307. <https://doi.org/10.1016/j.jcou.2017.04.007>.
- [239] L. Zhao, F. Temelli, J.M. Curtis, L. Chen, Preparation of liposomes using supercritical carbon dioxide technology: Effects of phospholipids and sterols, *Food Research International* 77 (2015) 63–72. <https://doi.org/10.1016/j.foodres.2015.07.006>.
- [240] F. Maqbool, P.M. Moyle, K.J. Thurecht, J.R. Falconer, Dispersibility of phospholipids and their optimization for the efficient production of liposomes using supercritical fluid technology, *International Journal of Pharmaceutics* 563 (2019) 174–183. <https://doi.org/10.1016/j.ijpharm.2019.03.053>.
- [241] A.N. Efaq, N.N.N.Ab. Rahman, H. Nagao, A.A. Al-Gheethi, M. Shahadat, M.O.Ab. Kadir, Supercritical Carbon Dioxide as Non-Thermal Alternative Technology for Safe Handling of Clinical Wastes, *Environ. Process.* 2 (2015) 797–822. <https://doi.org/10.1007/s40710-015-0116-0>.
- [242] N. Penoy, K.L. Delma, N. Homkar, A. Karim Sakira, S. Egrek, R. Sacheli, P.-Y. Sacré, B. Grignard, M.-P. Hayette, T.I. Somé, R. Semdé, B. Evrard, G. Piel, Development and optimization of a one step process for the production and sterilization of liposomes using supercritical CO₂, *International Journal of Pharmaceutics* 651 (2024) 123769. <https://doi.org/10.1016/j.ijpharm.2024.123769>.
- [243] J. Zhao, L. Qin, R. Song, J. Su, Y. Yuan, X. Zhang, S. Mao, Elucidating inhaled liposome surface charge on its interaction with biological barriers in the lung, *European Journal of Pharmaceutics and Biopharmaceutics* 172 (2022) 101–111. <https://doi.org/10.1016/j.ejpb.2022.01.009>.
- [244] M. Kowalska, M. Broniatowski, M. Mach, Ł. Płachta, P. Wydro, The effect of the polyethylene glycol chain length of a lipopolymer (DSPE-PEGn) on the properties of DPPC monolayers and bilayers, *Journal of Molecular Liquids* 335 (2021) 116529. <https://doi.org/10.1016/j.molliq.2021.116529>.
- [245] Y. Liu, G. Yang, Y. Hui, S. Ranaweera, C.-X. Zhao, Microfluidic Nanoparticles for Drug Delivery, *Small* 18 (2022) 2106580. <https://doi.org/10.1002/smll.202106580>.
- [246] R. Hamed, J. Fiegel, Synthetic tracheal mucus with native rheological and surface tension properties, *Journal of Biomedical Materials Research Part A* 102 (2014) 1788–1798. <https://doi.org/10.1002/jbm.a.34851>.
- [247] B.M. Ibrahim, S. Park, B. Han, Y. Yeo, A strategy to deliver genes to cystic fibrosis lungs: A battle with environment, *Journal of Controlled Release* 155 (2011) 289–295. <https://doi.org/10.1016/j.jconrel.2011.07.039>.
- [248] C. Allen, N. Dos Santos, R. Gallagher, G.N.C. Chiu, Y. Shu, W.M. Li, S.A. Johnstone, A.S. Janoff, L.D. Mayer, M.S. Webb, M.B. Bally, Controlling the Physical Behavior and Biological Performance of Liposome Formulations Through Use of Surface Grafted Poly(ethylene Glycol), *Biosci Rep* 22 (2002) 225–250. <https://doi.org/10.1023/A:1020186505848>.

REFERENCES

- [249] T.N. Dinh, B. Bottero, F. Perin, L.-A. Bya, A. Lechanteur, B. Evrard, G. Piel, D. Cataldo, Ciclesonide/indacaterol-encapsulated liposomes: optimizing corticosteroid-bronchodilator therapy for a more effective approach to asthma management, *International Journal of Pharmaceutics* (2026) 127217. <https://doi.org/10.1016/j.ijpharm.2026.127217>.
- [250] E. Marchi, T.S.C. Hinks, M. Richardson, L. Khalfaoui, F.A. Symon, P. Rajasekar, R. Clifford, B. Hargadon, C.D. Austin, J.L. MacIsaac, M.S. Kobor, S. Siddiqui, J.S. Mar, J.R. Arron, D.F. Choy, P. Bradding, The effects of inhaled corticosteroids on healthy airways, *Allergy* 79 (2024) 1831–1843. <https://doi.org/10.1111/all.16146>.
- [251] B. Wang, L. Wang, Q. Yang, Y. Zhang, T. Qinglai, X. Yang, Z. Xiao, L. Lei, S. Li, Pulmonary inhalation for disease treatment: Basic research and clinical translations, *Materials Today Bio* 25 (2024) 100966. <https://doi.org/10.1016/j.mtbio.2024.100966>.
- [252] Y. Wang, L. Liu, Immunological factors, important players in the development of asthma, *BMC Immunol* 25 (2024) 50. <https://doi.org/10.1186/s12865-024-00644-w>.
- [253] B.N. Lambrecht, H. Hammad, J.V. Fahy, The Cytokines of Asthma, *Immunity* 50 (2019) 975–991. <https://doi.org/10.1016/j.immuni.2019.03.018>.
- [254] L. Saaby, A. Müllertz, Transepithelial Transport in Calu-3 Cells Grown Under LCC and AIC Conditions: Impact of Mucus on Drug Permeability, (n.d.).
- [255] I.R. Woodward, C.A. Fromen, Recent Developments in Aerosol Pulmonary Drug Delivery: New Technologies, New Cargos, and New Targets, *Annual Review of Biomedical Engineering* 26 (2024) 307–330. <https://doi.org/10.1146/annurev-bioeng-110122-010848>.
- [256] B. Patel, N. Gupta, F. Ahsan, Barriers that Inhaled Particles Encounter, *Journal of Aerosol Medicine and Pulmonary Drug Delivery* 37 (2024) 299–306. <https://doi.org/10.1089/jamp.2024.27498.bp>.
- [257] E. Poplicean, A.F. Crişan, E. Tudorache, P. Hogeia, R. Mladin, C. Oancea, Unlocking Better Asthma Control: A Narrative Review of Adherence to Asthma Therapy and Innovative Monitoring Solutions, *Journal of Clinical Medicine* 13 (2024) 6699. <https://doi.org/10.3390/jcm13226699>.
- [258] C. Liu, Y. Liu, L. Xi, Y. He, Y. Liang, J.C.W. Mak, S. Mao, Z. Wang, Y. Zheng, Interactions of Inhaled Liposome with Macrophages and Neutrophils Determine Particle Biofate and Anti-Inflammatory Effect in Acute Lung Inflammation, *ACS Appl. Mater. Interfaces* 15 (2023) 479–493. <https://doi.org/10.1021/acsami.2c17660>.
- [259] A. Weston, S.-C. Vladescu, T. Reddyhoff, A. Griffiths, T. Crouzier, M. Fielden, J.A. Garnett, G.H. Carpenter, The influence of ions on the lubricative abilities of mucin and the role of sialic acids, *Colloids and Surfaces B: Biointerfaces* 227 (2023) 113327. <https://doi.org/10.1016/j.colsurfb.2023.113327>.
- [260] M.-S. Martina, V. Nicolas, C. Wilhelm, C. Ménager, G. Barratt, S. Lesieur, The *in vitro* kinetics of the interactions between PEG-ylated magnetic-fluid-loaded liposomes and macrophages, *Biomaterials* 28 (2007) 4143–4153. <https://doi.org/10.1016/j.biomaterials.2007.05.025>.
- [261] N. Penoy, K.L. Delma, H.A. Tonakpon, B. Grignard, B. Evrard, G. Piel, An innovative one step green supercritical CO₂ process for the production of liposomes co-encapsulating both a hydrophobic and a hydrophilic compound for pulmonary administration, *International Journal of Pharmaceutics* 627 (2022) 122212. <https://doi.org/10.1016/j.ijpharm.2022.122212>.

REFERENCES

- [262] M.H. Alyami, E.Z. Dahmash, D.K. Ali, H.S. Alyami, H. AbdulKarim, S.A. Alsudir, Novel Fluticasone Propionate and Salmeterol Fixed-Dose Combination Nano-Encapsulated Particles Using Polyamide Based on L-Lysine, *Pharmaceuticals* 15 (2022) 321. <https://doi.org/10.3390/ph15030321>.
- [263] R.M. Derbali, V. Aoun, G. Moussa, G. Frei, S.F. Tehrani, J.C. Del'Orto, P. Hildgen, V.G. Roullin, J.L. Chain, Tailored Nanocarriers for the Pulmonary Delivery of Levofloxacin against *Pseudomonas aeruginosa*: A Comparative Study, *Mol. Pharmaceutics* 16 (2019) 1906–1916. <https://doi.org/10.1021/acs.molpharmaceut.8b01256>.
- [264] M.R.C. Marques, R. Loebenberg, M. Almukainzi, Simulated Biological Fluids with Possible Application in Dissolution Testing, *Dissolution Technol.* 18 (2011) 15–28. <https://doi.org/10.14227/DT180311P15>.
- [265] Y. Hu, Y. Wang, J. Deng, X. Ding, D. Lin, H. Shi, L. Chen, D. Lin, Y. Wang, S. Vakkal, J. Wang, X. Li, Enzyme-instructed self-assembly of peptide-drug conjugates in tear fluids for ocular drug delivery, *Journal of Controlled Release* 344 (2022) 261–271. <https://doi.org/10.1016/j.jconrel.2022.03.011>.
- [266] K. Kolouchova, D. Jirak, O. Groborz, O. Sedlacek, N. Ziolkowska, M. Vit, E. Sticova, A. Galisova, P. Svec, J. Trousil, M. Hajek, M. Hruby, Implant-forming polymeric 19F MRI-tracer with tunable dissolution, *Journal of Controlled Release* 327 (2020) 50–60. <https://doi.org/10.1016/j.jconrel.2020.07.026>.
- [267] E. Menchi, C. El Khattabi, S. Pochet, O. Denis, K. Amighi, N. Wauthoz, Optimization of Long-Acting Bronchodilator Dose Ratios Using Isolated Guinea Pig Tracheal Rings for Synergistic Combination Therapy in Asthma and COPD, *Pharmaceuticals* 15 (2022) 963. <https://doi.org/10.3390/ph15080963>.
- [268] A.A.S. Albuquerque, M.T.M. Carvalho, P.M. Evora, T.R. de Nadai, A.C. Celotto, P.R.B. Evora, In vitro reactivity (“organ chamber”) of guinea pig tracheal rings—methodology considerations, *Annals of Translational Medicine* 4 (2016) 216–216. <https://doi.org/10.21037/atm.2016.05.18>.
- [269] D.D. Cataldo, K.G. Tournoy, K. Vermaelen, C. Munaut, J.-M. Foidart, R. Louis, A. Noël, R.A. Pauwels, Matrix Metalloproteinase-9 Deficiency Impairs Cellular Infiltration and Bronchial Hyperresponsiveness during Allergen-Induced Airway Inflammation, *The American Journal of Pathology* 161 (2002) 491–498. [https://doi.org/10.1016/S0002-9440\(10\)64205-8](https://doi.org/10.1016/S0002-9440(10)64205-8).
- [270] E. Di Valentin, C. Crahay, N. Garbacki, B. Hennuy, M. Guéders, A. Noël, J.-M. Foidart, J. Grooten, A. Colige, J. Piette, D. Cataldo, New asthma biomarkers: lessons from murine models of acute and chronic asthma, *American Journal of Physiology-Lung Cellular and Molecular Physiology* 296 (2009) L185–L197. <https://doi.org/10.1152/ajplung.90367.2008>.
- [271] G. Paulissen, M. El Hour, N. Rocks, M.M. Guéders, F. Bureau, J.-M. Foidart, C. Lopez-Otin, A. Noel, D.D. Cataldo, Control of Allergen-Induced Inflammation and Hyperresponsiveness by the Metalloproteinase ADAMTS-12, *J Immunol* 189 (2012) 4135–4143. <https://doi.org/10.4049/jimmunol.1103739>.
- [272] G. Bendavid, C. Hubeau, F. Perin, A. Gillard, M.-J. Nokin, O. Carnet, C. Gerard, A. Noel, P. Lefebvre, N. Rocks, D. Cataldo, Role for the metalloproteinase ADAM28 in the control of airway inflammation, remodelling and responsiveness in asthma, *Front Immunol* 13 (2022) 1067779. <https://doi.org/10.3389/fimmu.2022.1067779>.
- [273] M.M. Gueders, P. Bertholet, F. Perin, N. Rocks, R. Maree, V. Botta, R. Louis, J.-M. Foidart, A. Noel, B. Evrard, D.D. Cataldo, A novel formulation of inhaled doxycycline reduces allergen-induced inflammation, hyperresponsiveness and remodeling by matrix metalloproteinases and cytokines modulation in a mouse model of

REFERENCES

- asthma, *Biochemical Pharmacology* 75 (2008) 514–526.
<https://doi.org/10.1016/j.bcp.2007.09.012>.
- [274] M.-L. Briuglia, C. Rotella, A. McFarlane, D.A. Lamprou, Influence of cholesterol on liposome stability and on in vitro drug release, *Drug Deliv. and Transl. Res.* 5 (2015) 231–242. <https://doi.org/10.1007/s13346-015-0220-8>.
- [275] R. Nave, Metabolism of ciclesonide in the upper and lower airways: review of available data, *JAA* (2008) 11. <https://doi.org/10.2147/JAA.S4051>.
- [276] P. van Hoogevest, A. Wendel, The use of natural and synthetic phospholipids as pharmaceutical excipients, *European Journal of Lipid Science and Technology* 116 (2014) 1088–1107. <https://doi.org/10.1002/ejlt.201400219>.
- [277] Avanti Research, Phase Transition Temperatures for Glycerophospholipids, (n.d.). <https://www.avantiresearch.com/en-gb/support-hub/physical-properties/phase-transition-temps> (accessed January 18, 2026).
- [278] E. Maretti, F. Gioia, C. Rustichelli, S. Molinari, E. Leo, Inflammatory-Targeted Lipid Carrier as a New Nanomaterial to Formulate an Inhaled Drug Delivery System, *Molecules* 29 (2024) 1616. <https://doi.org/10.3390/molecules29071616>.
- [279] G. Caramori, F. Nucera, S. Mumby, F. Lo Bello, I.M. Adcock, Corticosteroid resistance in asthma: Cellular and molecular mechanisms, *Molecular Aspects of Medicine* 85 (2022) 100969. <https://doi.org/10.1016/j.mam.2021.100969>.
- [280] L.-M.L. Spies, N.J.D. Verhoog, A. Louw, Acquired Glucocorticoid Resistance Due to Homologous Glucocorticoid Receptor Downregulation: A Modern Look at an Age-Old Problem, *Cells* 10 (2021) 2529. <https://doi.org/10.3390/cells10102529>.
- [281] H.S. Nelson, D.Y.M. Leung, J.W. Bloom, Update on glucocorticoid action and resistance, *Journal of Allergy and Clinical Immunology* 111 (2003) 3–22. <https://doi.org/10.1067/mai.2003.97>.
- [282] H. Sakai, T. Gotoh, T. Imura, K. Sakai, K. Otake, M. Abe, Preparation and Properties of Liposomes Composed of Various Phospholipids with Different Hydrophobic Chains Using a Supercritical Reverse Phase Evaporation Method, *Journal of Oleo Science* 57 (2008) 613–621. <https://doi.org/10.5650/jos.57.613>.
- [283] S.-S. Hong, S.H. Kim, S.-J. Lim, Effects of triglycerides on the hydrophobic drug loading capacity of saturated phosphatidylcholine-based liposomes, *International Journal of Pharmaceutics* 483 (2015) 142–150. <https://doi.org/10.1016/j.ijpharm.2015.02.013>.
- [284] R.P. Austin, P. Barton, R.V. Bonnert, R.C. Brown, P.A. Cage, D.R. Cheshire, A.M. Davis, I.G. Dougall, F. Ince, G. Pairaudeau, A. Young, QSAR and the Rational Design of Long-Acting Dual D2-Receptor/ β 2-Adrenoceptor Agonists, *J. Med. Chem.* 46 (2003) 3210–3220. <https://doi.org/10.1021/jm020886c>.
- [285] M.C. Filion, N.C. Phillips, Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells, *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1329 (1997) 345–356. [https://doi.org/10.1016/S0005-2736\(97\)00126-0](https://doi.org/10.1016/S0005-2736(97)00126-0).
- [286] O.K. Nag, V.R. Yadav, A. Hedrick, V. Awasthi, Post-modification of preformed liposomes with novel non-phospholipid poly(ethylene glycol)-conjugated hexadecyl-carbamoylmethyl hexadecanoic acid for enhanced circulation persistence in vivo, *International Journal of Pharmaceutics* 446 (2013) 119–129. <https://doi.org/10.1016/j.ijpharm.2013.02.026>.
- [287] B.L. Mui, Y.K. Tam, M. Jayaraman, S.M. Ansell, X. Du, Y.Y.C. Tam, P.J. Lin, S. Chen, J.K. Narayanannair, K.G. Rajeev, M. Manoharan, A. Akinc, M.A. Maier, P. Cullis, T.D. Madden, M.J. Hope, Influence of Polyethylene Glycol Lipid Desorption Rates on Pharmacokinetics and Pharmacodynamics of siRNA Lipid Nanoparticles, *Molecular Therapy Nucleic Acids* 2 (2013). <https://doi.org/10.1038/mtna.2013.66>.

REFERENCES

- [288] A. Sarode, Y. Fan, A. E. Byrnes, M. Hammel, G. L. Hura, Y. Fu, P. Kou, C. Hu, F. I. Hinz, J. Roberts, S. G. Koenig, K. Nagapudi, C. C. Hoogenraad, T. Chen, D. Leung, C.-W. Yen, Predictive high-throughput screening of PEGylated lipids in oligonucleotide-loaded lipid nanoparticles for neuronal gene silencing, *Nanoscale Advances* 4 (2022) 2107–2123. <https://doi.org/10.1039/D1NA00712B>.
- [289] V. De Leo, S. Ruscigno, A. Trapani, S. Di Gioia, F. Milano, D. Mandracchia, R. Comparelli, S. Castellani, A. Agostiano, G. Trapani, L. Catucci, M. Conese, Preparation of drug-loaded small unilamellar liposomes and evaluation of their potential for the treatment of chronic respiratory diseases, *International Journal of Pharmaceutics* 545 (2018) 378–388. <https://doi.org/10.1016/j.ijpharm.2018.04.030>.
- [290] E. Mastrobattista, G. Storm, L. van Bloois, R. Reszka, P.G.M. Bloemen, D.J.A. Crommelin, P.A.J. Henricks, Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells, *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1419 (1999) 353–363. [https://doi.org/10.1016/S0005-2736\(99\)00074-7](https://doi.org/10.1016/S0005-2736(99)00074-7).
- [291] H. Hillaireau, P. Couvreur, Nanocarriers' entry into the cell: relevance to drug delivery, *Cell. Mol. Life Sci.* 66 (2009) 2873–2896. <https://doi.org/10.1007/s00018-009-0053-z>.
- [292] A.A. Calderon, C. Dimond, D.F. Choy, R. Pappu, M.A. Grimbaldston, D. Mohan, K.F. Chung, Targeting interleukin-33 and thymic stromal lymphopoietin pathways for novel pulmonary therapeutics in asthma and COPD, *European Respiratory Review* 32 (2023). <https://doi.org/10.1183/16000617.0144-2022>.
- [293] H. Kabata, K. Moro, K. Fukunaga, Y. Suzuki, J. Miyata, K. Masaki, T. Betsuyaku, S. Koyasu, K. Asano, Thymic stromal lymphopoietin induces corticosteroid resistance in natural helper cells during airway inflammation, *Nat Commun* 4 (2013) 2675. <https://doi.org/10.1038/ncomms3675>.
- [294] V.R. Thakur, J.V. Beladiya, K.K. Chaudagar, A.A. Mehta, An anti-asthmatic activity of natural Toll-like receptor-4 antagonist in OVA-LPS-induced asthmatic rats, *Clinical and Experimental Pharmacology and Physiology* 45 (2018) 1187–1197. <https://doi.org/10.1111/1440-1681.13002>.
- [295] O. Et-Thakafy, N. Delorme, C. Gaillard, C. Mériadec, F. Artzner, C. Lopez, F. Guyomarc'h, Mechanical Properties of Membranes Composed of Gel-Phase or Fluid-Phase Phospholipids Probed on Liposomes by Atomic Force Spectroscopy, *Langmuir* 33 (2017) 5117–5126. <https://doi.org/10.1021/acs.langmuir.7b00363>.
- [296] M. Ongun, A.G. Lokras, S. Baghel, Z. Shi, S.T. Schmidt, H. Franzyk, T. Rades, F. Sebastiani, A. Thakur, C. Foged, Lipid nanoparticles for local delivery of mRNA to the respiratory tract: Effect of PEG-lipid content and administration route, *European Journal of Pharmaceutics and Biopharmaceutics* 198 (2024) 114266. <https://doi.org/10.1016/j.ejpb.2024.114266>.
- [297] M. Rovira-Bru, D.H. Thompson, I. Szleifer, Size and Structure of Spontaneously Forming Liposomes in Lipid/PEG-Lipid Mixtures, *Biophysical Journal* 83 (2002) 2419–2439. [https://doi.org/10.1016/S0006-3495\(02\)75255-7](https://doi.org/10.1016/S0006-3495(02)75255-7).
- [298] E. Farge, P.F. Devaux, Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids, *Biophysical Journal* 61 (1992) 347–357. [https://doi.org/10.1016/S0006-3495\(92\)81841-6](https://doi.org/10.1016/S0006-3495(92)81841-6).
- [299] I.V. Zhigaltsev, P.R. Cullis, Morphological Behavior of Liposomes and Lipid Nanoparticles, *Langmuir* 39 (2023) 3185–3193. <https://doi.org/10.1021/acs.langmuir.2c02794>.

REFERENCES

- [300] E. Gresse, J. Rousseau, M. Akdim, A. du Bois, A. Lechanteur, B. Evrard, Enhancement of inhaled micronized powder flow properties for accurate capsules filling, *Powder Technology* 437 (2024) 119576. <https://doi.org/10.1016/j.powtec.2024.119576>.
- [301] J.J. Salomon, Y. Hagos, S. Petzke, A. Kühne, J.C. Gausterer, K. Hosoya, C. Ehrhardt, Beta-2 Adrenergic Agonists Are Substrates and Inhibitors of Human Organic Cation Transporter 1, *Mol. Pharmaceutics* 12 (2015) 2633–2641. <https://doi.org/10.1021/mp500854e>.
- [302] M. Mukherjee, D.I. Pritchard, C. Bosquillon, Evaluation of air-interfaced Calu-3 cell layers for investigation of inhaled drug interactions with organic cation transporters *in vitro*, *International Journal of Pharmaceutics* 426 (2012) 7–14. <https://doi.org/10.1016/j.ijpharm.2011.12.036>.
- [303] G. Horvath, E.S. Mendes, N. Schmid, A. Schmid, G.E. Conner, M. Salathe, A. Wanner, The effect of corticosteroids on the disposal of long-acting β_2 -agonists by airway smooth muscle cells, *Journal of Allergy and Clinical Immunology* 120 (2007) 1103–1109. <https://doi.org/10.1016/j.jaci.2007.08.034>.
- [304] E. Rytting, M. Bur, R. Cartier, T. Bouyssou, X. Wang, M. Krüger, C.-M. Lehr, T. Kissel, In vitro and in vivo performance of biocompatible negatively-charged salbutamol-loaded nanoparticles, *Journal of Controlled Release* 141 (2010) 101–107. <https://doi.org/10.1016/j.jconrel.2009.08.021>.
- [305] Avanti Research, Soy PC (95%) | 97281-47-5 | Avanti Research, (n.d.). <https://www.avantiresearch.com/en-gb/products/product/441601-soy-pc-95> (accessed April 2, 2026).
- [306] C. Gauthy, N. Cole, E. Spahic, R. Closset, E. Hendrickx, L. Conrard, Y. Bodadi, H. Magdalena Segura, V. Tallandier, P.-B. Van Lerberghe, L. Collard, N. Penoy, F. Boschini, B. Evrard, L. Delacroix, B. Malgrange, G. Piel, Development of a thermo-sensitive hydrogel containing liposomes for sustained dexamethasone delivery in sensorineural hearing loss, *International Journal of Pharmaceutics* 697 (2026) 126847. <https://doi.org/10.1016/j.ijpharm.2026.126847>.
- [307] T.N. Dinh, C. Gauthy, H. Magdalena Segura, P.-Y. Sacré, E. Hendrickx, L. Conrard, B. Evrard, B. Malgrange, G. Piel, N. Penoy, Pilot Scale Transfer and Optimization of an Innovative Supercritical CO₂ PGSS Method for the Production of Drug-Encapsulating Liposomes, (2026). <https://doi.org/10.2139/ssrn.6864554>.
- [308] H. Daraee, A. Etemadi, M. Kouhi, S. Alimirzalu, A. Akbarzadeh, Application of liposomes in medicine and drug delivery, *Artificial Cells, Nanomedicine, and Biotechnology* 44 (2016) 381–391. <https://doi.org/10.3109/21691401.2014.953633>.
- [309] S. Giordani, V. Marassi, A. Zattoni, B. Roda, P. Reschiglian, Liposomes characterization for market approval as pharmaceutical products: Analytical methods, guidelines and standardized protocols, *Journal of Pharmaceutical and Biomedical Analysis* 236 (2023) 115751. <https://doi.org/10.1016/j.jpba.2023.115751>.
- [310] S. Khadke, C.B. Roces, R. Donaghey, V. Giacobbo, Y. Su, Y. Perrie, Scalable solvent-free production of liposomes, *Journal of Pharmacy and Pharmacology* 72 (2020) 1328–1340. <https://doi.org/10.1111/jphp.13329>.
- [311] S. Deshpande, C. Dekker, On-chip microfluidic production of cell-sized liposomes, *Nat Protoc* 13 (2018) 856–874. <https://doi.org/10.1038/nprot.2017.160>.
- [312] S.M. Mortazavi, M.R. Mohammadabadi, K. Khosravi-Darani, M.R. Mozafari, Preparation of liposomal gene therapy vectors by a scalable method without using volatile solvents or detergents, *Journal of Biotechnology* 129 (2007) 604–613. <https://doi.org/10.1016/j.jbiotec.2007.02.005>.

REFERENCES

- [313] E. Badens, Y. Masmoudi, A. Mouahid, C. Crampon, Current situation and perspectives in drug formulation by using supercritical fluid technology, *The Journal of Supercritical Fluids* 134 (2018) 274–283. <https://doi.org/10.1016/j.supflu.2017.12.038>.
- [314] E. de Paz, Á. Martín, M.J. Cocero, Formulation of β -carotene with soybean lecithin by PGSS (Particles from Gas Saturated Solutions)-drying, *The Journal of Supercritical Fluids* 72 (2012) 125–133. <https://doi.org/10.1016/j.supflu.2012.08.007>.
- [315] S. Varona, S. Kareth, Á. Martín, M.J. Cocero, Formulation of lavandin essential oil with biopolymers by PGSS for application as biocide in ecological agriculture, *The Journal of Supercritical Fluids* 54 (2010) 369–377. <https://doi.org/10.1016/j.supflu.2010.05.019>.
- [316] L.-A. Bya, T.N. Dinh, N. Penoy, P.-Y. Sacré, B. Bottero, D. Cataldo, E. Hendrickx, L. Conrard, B. Evrard, G. Piel, Optimizing spray-dried liposomes for pulmonary delivery: impact of lipids composition and of drying parameters using experimental design, *European Journal of Pharmaceutics and Biopharmaceutics* (2026) 115035.
- [317] K.L. Delma, N. Penoy, A.K. Sakira, S. Egrek, R. Sacheli, B. Grignard, M.-P. Hayette, T. Issa Somé, B. Evrard, R. Semdé, G. Piel, Use of supercritical CO₂ for the sterilization of liposomes: Study of the influence of sterilization conditions on the chemical and physical stability of phospholipids and liposomes, *European Journal of Pharmaceutics and Biopharmaceutics* 183 (2023) 112–118. <https://doi.org/10.1016/j.ejpb.2023.01.002>.
- [318] R.S. Kim, F.S. LaBella, Comparison of analytical methods for monitoring autoxidation profiles of authentic lipids, *Journal of Lipid Research* 28 (1987) 1110–1117. [https://doi.org/10.1016/S0022-2275\(20\)38624-7](https://doi.org/10.1016/S0022-2275(20)38624-7).
- [319] International Council for Harmonisation (ICH), ICH guideline Q8 (R2) on pharmaceutical development - Step 5, ICH, Geneva, Switzerland, 2009. https://www.ema.europa.eu/en/documents/scientific-guideline/international-conference-harmonisation-technical-requirements-registration-pharmaceuticals-human-use-considerations-ich-guideline-q8-r2-pharmaceutical-development-step-5_en.pdf (accessed July 7, 2026).
- [320] N. Pippa, P.P. Dallas, D.M. Rekkas, Chapter 23 - Design of experiment approaches for liposome property optimization, in: S.G. Antimisariis (Ed.), *Liposomes in Drug Delivery*, Academic Press, 2024: pp. 583–611. <https://doi.org/10.1016/B978-0-443-15491-1.00013-4>.
- [321] B.-Z. Maytal, J.M. Pfothenhauer, The Joule-Thomson Effect, Its Inversion and Other Expansions, in: B.-Z. Maytal, J.M. Pfothenhauer (Eds.), *Miniature Joule-Thomson Cryocooling: Principles and Practice*, Springer, New York, NY, 2013: pp. 39–72. https://doi.org/10.1007/978-1-4419-8285-8_2.
- [322] M. Schneider, Y. Masmoudi, C. Girard, F. Matonti, C. Olmière, E. Badens, Sequential-Dispersion PGSS process applied to lutein encapsulation at moderate temperatures: A solvent-free method for thermosensitive compound encapsulation, *The Journal of Supercritical Fluids* 207 (2024) 106186. <https://doi.org/10.1016/j.supflu.2024.106186>.
- [323] J. Yang, O.N. Ciftci, Formation of hollow solid lipid micro- and nanoparticles using supercritical carbon dioxide, *Food and Bioproducts Processing* 98 (2016) 151–160. <https://doi.org/10.1016/j.fbp.2016.01.004>.
- [324] D.R. do Nascimento Junior, A. Tabernero, E.C. de M. Cabral Albuquerque, S.A.B. Vieira de Melo, Biopesticide Encapsulation Using Supercritical CO₂: A Comprehensive Review and Potential Applications, *Molecules* 26 (2021) 4003. <https://doi.org/10.3390/molecules26134003>.

REFERENCES

- [325] P.J. Linstrom, W.G. Mallard, eds., Thermophysical Properties of Fluid Systems, in: NIST Chemistry WebBook, NIST Standard Reference Database Number 69, National Institute of Standards and Technology, Gaithersburg MD, 2025: p. 20899. <https://doi.org/https://doi.org/10.18434/T4D303>.
- [326] M. Nolasco Araujo, T. Boucherès, N. Sheibat-Othman, T.F.L. McKenna, Gas–Liquid Mass Transfer in Unbaffled Multi-Impeller Stirred Tank Reactors for Vinylidene Fluoride Emulsion Polymerization under Supercritical Conditions: Experimental Pressure-Based Approach and CFD Simulations of Hydrodynamics, *Ind. Eng. Chem. Res.* 64 (2025) 24538–24550. <https://doi.org/10.1021/acs.iecr.5c04184>.
- [327] B. Prakash, T. Bhatelia, D. Wadnerkar, M.T. Shah, V.K. Pareek, R.P. Utikar, Vortex shape and gas-liquid hydrodynamics in unbaffled stirred tank, *The Canadian Journal of Chemical Engineering* 97 (2019) 1913–1920. <https://doi.org/10.1002/cjce.23433>.
- [328] C.A. Coulaloglou, L.L. Tavlarides, Description of interaction processes in agitated liquid-liquid dispersions, *Chemical Engineering Science* 32 (1977) 1289–1297. [https://doi.org/10.1016/0009-2509\(77\)85023-9](https://doi.org/10.1016/0009-2509(77)85023-9).
- [329] Z.H. Mok, The effect of particle size on drug bioavailability in various parts of the body, *Pharmaceutical Science Advances* 2 (2024) 100031. <https://doi.org/10.1016/j.pscia.2023.100031>.
- [330] S. Salave, D. Rana, P. Patel, R. Gupta, D. Benival, N. Kommineni, Development of generic liposome products for drug delivery, in: *Liposomes in Drug Delivery*, Elsevier, 2024: pp. 613–634. <https://www.sciencedirect.com/science/article/pii/B9780443154911000109> (accessed May 3, 2026).
- [331] Yu.A. Tereshkina, T.I. Torkhovskaya, E.G. Tikhonova, L.V. Kostryukova, M.A. Sanzhakov, E.I. Korotkevich, Yu.Yu. Khudoklinova, N.A. Orlova, E.F. Kolesanova, Nanoliposomes as drug delivery systems: safety concerns, *Journal of Drug Targeting* 30 (2022) 313–325. <https://doi.org/10.1080/1061186X.2021.1992630>.
- [332] L. Wu, J. Zhang, W. Watanabe, Physical and chemical stability of drug nanoparticles, *Advanced Drug Delivery Reviews* 63 (2011) 456–469. <https://doi.org/10.1016/j.addr.2011.02.001>.
- [333] G. Buttitta, S. Bonacorsi, C. Barbarito, M. Moliterno, S. Pompei, G. Saito, I. Oddone, G. Verdone, D. Secci, S. Raimondi, Scalable microfluidic method for tunable liposomal production by a design of experiment approach, *International Journal of Pharmaceutics* 662 (2024) 124460. <https://doi.org/10.1016/j.ijpharm.2024.124460>.
- [334] M. Rohrschneider, S. Bhagwat, R. Krampe, V. Michler, J. Breitzkreutz, G. Hochhaus, Evaluation of the Transwell System for Characterization of Dissolution Behavior of Inhalation Drugs: Effects of Membrane and Surfactant, *Mol. Pharmaceutics* 12 (2015) 2618–2624. <https://doi.org/10.1021/acs.molpharmaceut.5b00221>.
- [335] C. Tang, C. Ou-yang, W. Chen, C. Zou, J. Huang, C. Cui, S. Yang, C. Guo, X. Yang, Y. Lin, Q. Pei, G. Yang, Prediction of pharmacokinetic parameters of inhaled indacaterol formulation in healthy volunteers using physiologically-based pharmacokinetic (PBPK) model, *European Journal of Pharmaceutical Sciences* 168 (2022) 106055. <https://doi.org/10.1016/j.ejps.2021.106055>.
- [336] D. Drabik, G. Chodaczek, S. Kraszewski, M. Langner, Mechanical Properties Determination of DMPC, DPPC, DSPC, and HSPC Solid-Ordered Bilayers, *Langmuir* 36 (2020) 3826–3835. <https://doi.org/10.1021/acs.langmuir.0c00475>.
- [337] P. Trucillo, R. Campardelli, S. Iorio, P. De Stefanis, E. Reverchon, Economic Analysis of a New Business for Liposome Manufacturing Using a High-Pressure System, *Processes* 8 (2020) 1604. <https://doi.org/10.3390/pr8121604>.

REFERENCES

- [338] L. Cseri, M. Razali, P. Pogany, G. Szekely, Chapter 3.15-organic solvents in sustainable synthesis and engineering, *Green Chemistry* (2018) 513–553. <https://doi.org/10.1016/B978-0-12-809270-5.00020-0>.
- [339] J.D. Chea, A. Christon, V. Pierce, J.H. Reilly, M. Russ, M. Savelski, C.S. Slater, K.M. Yenkie, Framework for solvent recovery, reuse, and recycling in industries, in: *Computer Aided Chemical Engineering*, Elsevier, 2019: pp. 199–204. <https://www.sciencedirect.com/science/article/pii/B9780128185971500321> (accessed May 4, 2026).
- [340] L. Guerassimoff, J. Nicolas, Towards safer medicines: management of residual solvents and green alternatives, *Journal of Controlled Release* 391 (2026) 114643. <https://doi.org/10.1016/j.jconrel.2026.114643>.
- [341] H. Derendorf, Pharmacokinetic and Pharmacodynamic Properties of Inhaled Ciclesonide, *The Journal of Clinical Pharmacology* 47 (2007) 782–789. <https://doi.org/10.1177/0091270007299763>.
- [342] M. Kępczyński, K. Nawalany, M. Kumorek, A. Kobierska, B. Jachimska, M. Nowakowska, Which physical and structural factors of liposome carriers control their drug-loading efficiency?, *Chemistry and Physics of Lipids* 155 (2008) 7–15. <https://doi.org/10.1016/j.chemphyslip.2008.05.174>.
- [343] J.J. Parmar, D.J. Singh, D.D. Hegde, A.A. Lohade, P.S. Soni, A. Samad, M.D. Menon, Development and evaluation of inhalational liposomal system of budesonide for better management of asthma, *Indian Journal of Pharmaceutical Sciences* 72 (2010) 442. <https://doi.org/10.4103/0250-474X.73916>.
- [344] U. Bhardwaj, D.J. Burgess, Physicochemical properties of extruded and non-extruded liposomes containing the hydrophobic drug dexamethasone, *International Journal of Pharmaceutics* 388 (2010) 181–189. <https://doi.org/10.1016/j.ijpharm.2010.01.003>.
- [345] K. Edwards, M. Johnsson, G. Karlsson, M. Silvander, Effect of polyethyleneglycol-phospholipids on aggregate structure in preparations of small unilamellar liposomes, *Biophysical Journal* 73 (1997) 258–266. [https://doi.org/https://doi.org/10.1016/S0006-3495\(97\)78066-4](https://doi.org/https://doi.org/10.1016/S0006-3495(97)78066-4).
- [346] R. Lipowsky, The morphology of lipid membranes, *Current Opinion in Structural Biology* 5 (1995) 531–540. [https://doi.org/10.1016/0959-440X\(95\)80040-9](https://doi.org/10.1016/0959-440X(95)80040-9).
- [347] O.G. Mouritsen, Lipids, curvature, and nano-medicine, *European Journal of Lipid Science and Technology* 113 (2011) 1174–1187. <https://doi.org/10.1002/ejlt.201100050>.
- [348] C.T. Szlenk, J.B. Gc, S. Natesan, Membrane-Facilitated Receptor Access and Binding Mechanisms of Long-Acting β 2-Adrenergic Receptor Agonists, *Molecular Pharmacology* 100 (2021) 406–427. <https://doi.org/10.1124/mol-pharm.121.000285>.
- [349] L.-A. Bya, T.N. Dinh, N. Penoy, P.-Y. Sacré, B. Bottero, D. Cataldo, E. Hendrickx, L. Conrard, B. Evrard, G. Piel, A. Lechanteur, Optimizing spray-dried liposomes for pulmonary delivery: impact of lipids composition and of drying parameters using experimental design, *European Journal of Pharmaceutics and Biopharmaceutics* 223 (2026) 115035. <https://doi.org/10.1016/j.ejpb.2026.115035>.
- [350] Q. Yang, T.M. Jacobs, J.D. McCallen, D.T. Moore, J.T. Huckaby, J.N. Edelstein, S.K. Lai, Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the General Population, *Anal. Chem.* 88 (2016) 11804–11812. <https://doi.org/10.1021/acs.analchem.6b03437>.

REFERENCES

- [351] M. Xie, E.H.W. Koch, C.A. van Walree, A. Sobota, A.F.P. Sonnen, E. Breukink, J.A. Killian, J.H. Lorent, Two separate mechanisms are involved in membrane permeabilization during lipid oxidation, *Biophysical Journal* 122 (2023) 4503–4517. <https://doi.org/10.1016/j.bpj.2023.10.028>.
- [352] M. Piñol-Cancer, L. Fernández-Méndez, J. Carrillo-Romero, A. Urkola-Arsuaga, M. Azkargorta, F. Elortza, F. Goñi-de-Cerio, C. García-Mouton, C.M.-P. de Alejo, E. Ismalaj, O. Cañadas, J. Pérez-Gil, J. Ruíz-Cabello, S. Carregal-Romero, The role of PEGylation in the pulmonary delivery of antifibrotic liposomal therapies, *Journal of Controlled Release* 386 (2025) 114134. <https://doi.org/10.1016/j.jcon-rel.2025.114134>.
- [353] T. Peter L., Siekmann, PEG–protein conjugates: nonclinical and clinical toxicity considerations, in: *Polymer-Protein Conjugates*, Elsevier, 2020: pp. 61–101. <https://doi.org/10.1016/B978-0-444-64081-9.00004-8>.
- [354] H. Gursahani, J. Riggs-Sauthier, J. Pfeiffer, D. Lechuga-Ballesteros, C.S. Fishburn, Absorption of Polyethylene Glycol (PEG) Polymers: The Effect of PEG Size on Permeability, *Journal of Pharmaceutical Sciences* 98 (2009) 2847–2856. <https://doi.org/10.1002/jps.21635>.
- [355] A. Baumann, D. Tuerck, S. Prabhu, L. Dickmann, J. Sims, Pharmacokinetics, metabolism and distribution of PEGs and PEGylated proteins: quo vadis?, *Drug Discovery Today* 19 (2014) 1623–1631. <https://doi.org/10.1016/j.drudis.2014.06.002>.
- [356] X. Liu, B. Ucakar, K. Vanvarenberg, E. Marbaix, R. Vanbever, Impact of administration route and PEGylation on alpha-1 antitrypsin augmentation therapy, *Journal of Controlled Release* 382 (2025) 113643. <https://doi.org/10.1016/j.jcon-rel.2025.113643>.
- [357] I.A. Ivens, W. Achanzar, A. Baumann, A. Brändli-Baiocco, J. Cavagnaro, M. Dempster, B.O. Depelchin, A.R. Irizarry Rovira, L. Dill-Morton, J.H. Lane, B.M. Reipert, T. Salcedo, B. Schweighardt, L.S. Tsuruda, P.L. Turecek, J. Sims, PEGylated Biopharmaceuticals: Current Experience and Considerations for Non-clinical Development, *Toxicol Pathol* 43 (2015) 959–983. <https://doi.org/10.1177/0192623315591171>.
- [358] O. Ohgoda, I.N. Robinson, Toxicological evaluation of DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), *Fundamental Toxicological Sciences* 7 (2020) 55–76. <https://doi.org/10.2131/fts.7.55>.
- [359] Y. Son, J.H. Lee, N.-H. Kim, N.-Y. Surh, E.-C. Kim, H.-T. Chung, D.G. Kang, H.-O. Pae, Dilinoleoylphosphatidylcholine induces the expression of the anti-inflammatory heme oxygenase-1 in RAW264.7 macrophages, *BioFactors* 36 (2010) 210–215. <https://doi.org/10.1002/biof.87>.
- [360] S.M. Rowe, J.B. Zuckerman, D. Dorgan, J. Lascano, K. McCoy, M. Jain, M.S. Schechter, S. Lommatzsch, V. Indihar, N. Lechtzin, K. McBennett, C. Callison, C. Brown, T.G. Liou, K.D. MacDonald, S.Z. Nasr, S. Bodie, M. Vaughn, E.B. Meltzer, A.J. Barbier, Inhaled mRNA therapy for treatment of cystic fibrosis: Interim results of a randomized, double-blind, placebo-controlled phase 1/2 clinical study, *Journal of Cystic Fibrosis* 22 (2023) 656–664. <https://doi.org/10.1016/j.jcf.2023.04.008>.
- [361] C. Karagiannidis, D.F. McAuley, B.T. Thompson, T. Reimer, K. Shakery, S. Schmitz, M.N. Cortés, R. Ullrich, F. Meziani, A. Mercat, D. Chiumello, F. Duska, A. Combes, C. Karagiannidis, D.F. McAuley, B.T. Thompson, T. Reimer, K. Shakery, S. Schmitz, M.N. Cortés, R. Ullrich, F. Meziani, A. Mercat, D. Chiumello, F. Duska, A. Combes, K. Krenn, P. Eller, M. Joannidis, M. Joannidis, H. Koinig, R. Skulec, V. Cerny, J. Berousek, F. Pene, J.-D. Chiche, L. Papazian, S. Hraiech, N. Terzi, U. Günther, T. Welte, M. Witzernath, N. Weiler, M. Berger, G. Grasselli, A.B. Roig, C. de Haro Lopez, A.O. Calvo, R.F. Roca, R.M. Mendiluce, J. Bewley, A. Aswani, I.

REFERENCES

- Welters, M. Wise, R. Jackson, for the SEAL Trial Investigators, Safety and efficacy of inhaled PEG-ADM in ARDS patients: a randomised controlled trial, *Crit Care* 29 (2025) 448. <https://doi.org/10.1186/s13054-025-05617-y>.
- [362] D.J.C. Constable, C. Jimenez-Gonzalez, R.K. Henderson, Perspective on Solvent Use in the Pharmaceutical Industry, *Org. Process Res. Dev.* 11 (2007) 133–137. <https://doi.org/10.1021/op060170h>.
- [363] Md.S. Hossain, N.N. Nik Ab Rahman, V. Balakrishnan, A. F.M. Alkarkhi, Z. Ahmad Rajion, M.O. Ab Kadir, Optimizing supercritical carbon dioxide in the inactivation of bacteria in clinical solid waste by using response surface methodology, *Waste Management* 38 (2015) 462–473. <https://doi.org/10.1016/j.wasman.2015.01.003>.
- [364] W. Situ, X. Song, S. Luo, Y. Liang, A nano-delivery system for bioactive ingredients using supercritical carbon dioxide and its release behaviors, *Food Chemistry* 228 (2017) 219–225. <https://doi.org/10.1016/j.foodchem.2017.01.053>.
- [365] G.H. Sedahmed, Y.A. El-Taweel, M.H. Abdel-Aziz, H.M. El-Naqeara, Mass and heat transfer enhancement at the wall of cylindrical agitated vessel by turbulence promoters, *Chemical Engineering and Processing: Process Intensification* 80 (2014) 43–50. <https://doi.org/10.1016/j.cep.2014.04.004>.
- [366] A. Ferreira, F. Rocha, A. Mota, J.A. Teixeira, Characterization of industrial bioreactors (mixing, heat, and mass transfer), in: *Current Developments in Biotechnology and Bioengineering*, Elsevier, 2017: pp. 563–592. <https://www.sciencedirect.com/science/article/pii/B9780444636638000197> (accessed May 3, 2026).
- [367] A.A. Gulumyan, A.N. Verigin, N.V. Lisitsyn, Efficiency of mixed-flow heat exchangers, *Theor Found Chem Eng* 51 (2017) 94–106. <https://doi.org/10.1134/S0040579517010079>.
- [368] M. Cudak, J. Karcz, Distribution of local heat transfer coefficient values in the wall region of an agitated vessel, *Chem. Pap.* 62 (2008) 92–99. <https://doi.org/10.2478/s11696-007-0084-6>.
- [369] V. da Silva Rosa, M. Silva de Moraes, J. Tófanio de Campos Leite Toneli, D. de Moraes Júnior, External Heat Transfer Coefficient in Agitated Vessels Using a Radial Impeller and Vertical Tube Baffles, *Ind. Eng. Chem. Res.* 53 (2014) 13797–13803. <https://doi.org/10.1021/ie5008618>.
- [370] R. Sardeing, J. Aubin, C. Xuereb, Gas–Liquid Mass Transfer: A Comparison of Down- and Up-pumping Axial Flow Impellers with Radial Impellers, *Chemical Engineering Research and Design* 82 (2004) 1589–1596. <https://doi.org/10.1205/cerd.82.12.1589.58030>.
- [371] P. Vrabel, R.G.J.M. van der Lans, K.Ch.A.M. Luyben, L. Boon, A.W. Nienow, Mixing in large-scale vessels stirred with multiple radial or radial and axial up-pumping impellers: modelling and measurements, *Chemical Engineering Science* 55 (2000) 5881–5896. [https://doi.org/10.1016/S0009-2509\(00\)00175-5](https://doi.org/10.1016/S0009-2509(00)00175-5).
- [372] D. Posadas-Navarro, C. Palacios, A. Blancas-Cabrera, M.A. Trujillo-Roldán, M. Salinas-Vázquez, G. Ascanio, Flow Patterns of Multiple Axial-Radial Impellers for Potential Use in Aerated Stirred Tanks, *Chemical Engineering & Technology* 45 (2022) 860–867. <https://doi.org/10.1002/ceat.202100521>.
- [373] R.D. Worsham, V. Thomas, S.S. Farid, Potential of Continuous Manufacturing for Liposomal Drug Products, *Biotechnology Journal* 14 (2019) 1700740. <https://doi.org/10.1002/biot.201700740>.
- [374] A. Giorello, A. Nicastro, C.L.A. Berli, Microfluidic Platforms for the Production of Nanoparticles at Flow Rates Larger Than One Liter Per Hour, *Advanced Materials Technologies* 7 (2022) 2101588. <https://doi.org/10.1002/admt.202101588>.
- [375] M. Martino, A. Mouahid, M. Sergeant, C. Desgrouas, C. Badens, E. Badens, Supercritical millifluidic process for siRNA encapsulation in nanoliposomes for potential

REFERENCES

- Progeria treatment (*ex-vivo* assays), *Journal of Drug Delivery Science and Technology* 87 (2023) 104804. <https://doi.org/10.1016/j.jddst.2023.104804>.
- [376] S. Colombo, M. Beck-Broichsitter, J.P. Bøtker, M. Malmsten, J. Rantanen, A. Bohr, Transforming nanomedicine manufacturing toward Quality by Design and microfluidics, *Advanced Drug Delivery Reviews* 128 (2018) 115–131. <https://doi.org/10.1016/j.addr.2018.04.004>.
- [377] M. Sheybanifard, L.P.B. Guerzoni, A. Omidinia-Anarkoli, L.D. Laporte, J. Buyel, R. Besseling, M. Damen, A. Gerich, T. Lammers, J.M. Metselaar, Liposome manufacturing under continuous flow conditions: towards a fully integrated set-up with in-line control of critical quality attributes, *Lab Chip* 23 (2022) 182–194. <https://doi.org/10.1039/D2LC00463A>.
- [378] T. Sato, K. Haneishi, H. Hisada, M.Y. Fujii, T. Koide, T. Fukami, Real-Time Quantitative Evaluation of a Drug during Liposome Preparation Using a Probe-Type Raman Spectrometer, *Langmuir* 40 (2024) 7962–7973. <https://doi.org/10.1021/acs.langmuir.3c03872>.
- [379] C. Tetyczka, S. Celikovic, A. Azimi, S. Koncar, C. Glader, M. Zettl, Y. Wang, S. Stephan, P. Caisse, M. Spoerk, E. Roblegg, J. Khinast, Efficient process development and quality control for continuous nanomanufacturing: An impingement jet mixing case study, *Materials Today Nano* 33 (2026) 100786. <https://doi.org/10.1016/j.mtnano.2026.100786>.
- [380] L.X. Yu, G. Amidon, M.A. Khan, S.W. Hoag, J. Polli, G.K. Raju, J. Woodcock, Understanding Pharmaceutical Quality by Design, *AAPS J* 16 (2014) 771–783. <https://doi.org/10.1208/s12248-014-9598-3>.
- [381] C. Moholkar, V.S. Vitankar, C. Mathpati, J.B. Joshi, Heat Transfer Characteristics of Single and Multiphase Stirred Tanks, in: G.H. Yeoh, J.B. Joshi (Eds.), *Handbook of Multiphase Flow Science and Technology*, Springer Nature Singapore, Singapore, 2023: pp. 1369–1399. https://doi.org/10.1007/978-981-287-092-6_46.
- [382] L. Pakzad, F. Ein-Mozaffari, S.R. Upreti, A. Lohi, A novel and energy-efficient coaxial mixer for agitation of non-Newtonian fluids possessing yield stress, *Chemical Engineering Science* 101 (2013) 642–654. <https://doi.org/10.1016/j.ces.2013.07.027>.
- [383] P. Fiore, M. Coppola, P. Meninno, M. Sannino, G. De Marco, S. Sacco, A. Caravella, Transient multiphase CO₂ release from high-pressure tanks: Influence of phase changes, viscous heating, and Joule–Thomson effect, *The Canadian Journal of Chemical Engineering* 101 (2023) 3541–3558. <https://doi.org/10.1002/cjce.24712>.
- [384] J.E. Lugo Hinojosa, J.A. Yáñez Varela, A. Alonzo García, G. Rivadeneyra Romero, S.A. Martínez Delgadillo, Effect of Different Impeller Types on Mixing Efficiency in Mechanically Stirred Tanks with Tubular Baffles, *Processes* 14 (2026) 225. <https://doi.org/10.3390/pr14020225>.
- [385] L. Maja, K. Željko, P. Mateja, Sustainable technologies for liposome preparation, *The Journal of Supercritical Fluids* 165 (2020) 104984. <https://doi.org/10.1016/j.supflu.2020.104984>.
- [386] F. Weber, L. Rahnfeld, P. Luciani, Analytical profiling and stability evaluation of liposomal drug delivery systems: A rapid UHPLC-CAD-based approach for phospholipids in research and quality control, *Talanta* 220 (2020) 121320. <https://doi.org/10.1016/j.talanta.2020.121320>.
- [387] A. Nair, M.A. Morsy, S. Jacob, Dose translation between laboratory animals and human in preclinical and clinical phases of drug development, *Drug Development Research* 79 (2018) 373–382. <https://doi.org/10.1002/ddr.21461>.

REFERENCES

- [388] L.M. Boldrini-Leite, P.V. Michelotto Jr, S.A.B. de Moura, L.G.A. Capriglione, F.C.M. Barussi, F.Y.I. Fragoso, A.C. Senegaglia, P.R.S. Brofman, Lung Tissue Damage Associated with Allergic Asthma in BALB/c Mice Could Be Controlled with a Single Injection of Mesenchymal Stem Cells from Human Bone Marrow up to 14 d After Transplantation, *Cell Transplant* 29 (2020) 0963689720913254. <https://doi.org/10.1177/0963689720913254>.
- [389] T.H. Mahood, C.D. Pascoe, T.K. Karakach, A. Jha, S. Basu, P. Ezzati, V. Spicer, N. Mookherjee, A.J. Halayko, Integrating Proteomes for Lung Tissues and Lavage Reveals Pathways That Link Responses in Allergen-Challenged Mice, *ACS Omega* 6 (2021) 1171–1189. <https://doi.org/10.1021/acsomega.0c04269>.
- [390] X. Yue, J.J. Guidry, Differential Protein Expression Profiles of Bronchoalveolar Lavage Fluid Following Lipopolysaccharide-Induced Direct and Indirect Lung Injury in Mice, *International Journal of Molecular Sciences* 20 (2019) 3401. <https://doi.org/10.3390/ijms20143401>.
- [391] C. Prasad, S.K. Duraisamy, I.K. Sundar, Lung mechanics showing sex-based differences and circadian time-of-day response to bleomycin-induced lung injury in mice, *Physiological Reports* 11 (2023) e15828. <https://doi.org/10.14814/phy2.15828>.
- [392] A. Rojas-Ruiz, M. Boucher, R. Gill, L. Gélinas, F.-Q. Tom, L. Fereydoonzad, P. Graham, J. Soliz, Y. Bossé, Lung stiffness of C57BL/6 versus BALB/c mice, *Sci Rep* 13 (2023) 17481. <https://doi.org/10.1038/s41598-023-44797-x>.

According to the 2025 GINA Guidelines, the management of asthma remains fundamentally dependent on the combination of an inhaled corticosteroid (ICS) and a long-acting β 2-agonist (LABA). Clinical practice is increasingly shifting toward molecules with a balanced hydrophilic/lipophilic profile, such as budesonide and formoterol. This balance facilitates rapid dissolution in the airway lining fluid and efficient penetration across the mucus barrier to access their molecular receptors. While the high lipophilicity of newer ICS and LABA molecules enables convenient once-daily dosing regimens, this property renders them more susceptible to mucociliary clearance and phagocytosis by alveolar macrophages, thereby compromising their ability to reach the target cells. To address these pulmonary delivery limitations, this study focuses on the formulation development and production scale-up of an advanced liposomal carrier system for a new ICS-LABA combination: ciclesonide (CIC) and indacaterol maleate (IND).

Initially, we focused on the design of a liposome architecture that allows for mucus penetration, stable drug encapsulation, evasion of macrophage phagocytosis, and enhanced epithelial uptake. By meticulously optimizing the type and concentration of PEGylated lipids and phospholipids, an optimal formulation (DLPC/Cholesterol/DSPE-PEG) containing CIC and IND was identified. This formulation significantly improved the attenuation of airway hyperresponsiveness and inflammation in an *in vivo* acute asthma mouse model compared to non-liposomal drug formulation. Furthermore, the liposomal delivery of CIC and IND exhibited a substantial dose-sparing potential for both therapeutic agents.

Building upon the proof-of-concept for this optimized formulation, the second objective aimed to facilitate its production at a pilot scale without using organic solvents. This scale-up effort required a comprehensive application of Design of Experiments principles to characterize multiple critical process parameters, such as dispersion volume, pressure, agitation speed, and depressurization rate, within a pilot-scale, GMP-like environment. The rigorous optimization process resulted in the generation of liposomes exhibiting a mean particle size of 152 ± 8 nm and a PdI of 0.36 ± 0.02 , while maintaining high drug encapsulation efficiencies (>90%). Furthermore, the detailed characterization of the liposomes manufactured at the pilot scale confirmed that the chosen method did not induce statistically significant increases in lipid oxidation or hydrolysis, confirming the chemical stability and industrial suitability of the process.

In conclusion, this study successfully met its primary goals. We first validated a highly effective liposomal platform that significantly enhances the anti-asthmatic efficacy of the CIC and IND combination *in vivo*. Second, we demonstrated the feasibility of transitioning this delivery system to a pilot manufacturing scale using the advanced, organic solvent-free PGSS process. These results provide a robust and highly viable platform for developing next-generation inhaled therapies for chronic respiratory diseases.