The potential of inhaled albumin nanoparticles as a delivery strategy to improve the therapeutic profile of PDE4 inhibitors

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The potential of inhaled albumin nanoparticles as a delivery strategy to improve the therapeutic profile of PDE4 inhibitors

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Abstract

Background: Selective phosphodiesterase (PDE) 4 inhibitors have been developed as novel anti-inflammatory drugs. Despite the licensing of roflumilast (RFM) tablets for reducing exacerbations in patients with severe COPD associated with chronic bronchitis and a history of exacerbations, RFM use has been severely hindered by the dose-limiting side effects. It was hypothesised that delivery via the lungs may provide an improved therapy if the challenges of formulating a poorly soluble drug and retaining it in the lungs after inhalation can be overcome. Hence this work aimed to develop albumin nanoparticles encapsulating RFM as a delivery strategy for a prototype PDE4 inhibitor. This body of work focused on (i) fabrication and characterisation of RFM in albumin nanoparticles (HSA-NPs); (ii) formulation of the NPs within respirable microparticles; (iii) design and application of *in vitro* and *in vivo* models of acute lung inflammation.

Methods: To investigate the potential of HSA-NPs to deliver RFM effectively to the lungs, reducing exposure to the gastrointestinal tract, formulation approaches, *in vitro* assays and an *in vivo* guinea pig models were developed. NPs were formulated using a desolvation method and physiochemical characterisation performed using dynamic light scattering and nanoparticle tracking analysis. *In vitro* assays were used to determine the NP degradation and the drug release in the presence of lung fluid-levels of proteases. Spray-drying was adopted to convert NPs suspension into a respirable mannitol-based microparticles. *In vitro* and *in vivo* models were designed using lung epithelial cells and guinea pigs, respectively. Lipopolysaccharide (LPS) from *E. Coli* was used to trigger inflammation and RFM and dexamethasone (as a positive control) were used in an anti-inflammatory treatment protocol. For *in vivo* studies, a RFM suspension was developed for nebulisation to guinea pigs.

Results: HSA-NPs were manufactured successfully and shown to undergo degradation and drug release in a simulated lung environment. The use of LPS *in vitro* and *in vivo* resulted in robust acute lung inflammation models. *In vivo* the inflammation was shown to be suppressed by orally administered RFM, but not significantly via nebulisation, indicating that either an increase in the delivered dose or longer exposure to the drug may be required for anti-inflammatory activity. An alternative hypothesis is that PDE4 inhibitors need to be administered systemically to be effective as anti-inflammatory

agents. Promisingly, the nebulisation of NPs did not change particle size, polydispersity index (PDI) or zeta potential.

Conclusion: This study confirmed that HSA-NPs can be formulated into respirable microparticles and undergo degradation in a lung simulated environment, therefore representing a potential strategy to provide retained release in the lung. Despite lung delivery of RFM not resulting in a robust anti-inflammatory effect, hence preventing studies on the efficacy of nebulised drug-loaded NPs, this work highlights the potential value of pursuing HSA-NPs as a drug delivery strategy.

Declaration

The work in this thesis is based on research performed within the Institute of Pharmaceutical Science, Faculty of Life Sciences and Medicine, King's College London, UK and Vectura Group LTD, Chippenham, UK.

I hereby declare all the experimental work presented in this thesis (Chapter 2 – 6) was entirely carried out by myself. For some of the *in vivo* experiments described in this thesis, the surgical procedure only was carried out by Dr Sandra Rudman as stated in paragraph 5.3.3.2.

The Covid-19 pandemic partially affected my PhD project, as it reduced the amount of allocated time that I was supposed to spend at the industrial partner, Vectura Ltd. As a result, some experiments had to be carried out only once.

I hereby declare that no part of this thesis has previously been submitted in substance for any other degree or qualification, and it is the result of my own work unless otherwise stated in the text.

Conference Presentations

1. <u>Ivana Stolfa,</u> Ayasha Patel, Nick Childerhouse, Gemma Keegan, Clive Page & Ben Forbes, 2019. "Roflumilast-loaded albumin nanoparticles for pulmonary drug delivery". Poster presentation. *Drug Delivery to the Lungs (DDL), Edinburgh, UK.*

2. <u>Ivana Stolfa,</u> Ayasha Patel, Arcadia Woods, Nick Childerhouse, Gemma Keegan and Ben Forbes, 2019. "Albumin nanoparticles for pulmonary drug delivery". Poster presentation. *European Aerosol Conference (EAC), Gothenburg, SWE.*

3. <u>Ivana Stolfa</u>, Clive Page & Ben Forbes, 2020. "Development of an in vivo preclinical model of COPD in guinea pigs to study the anti-inflammatory effects of inhalaed roflumilast". Poster presentation. *Drug Delivery to the Lungs (DDL), Edinburgh, UK.*

4. <u>Ivana Stolfa</u>, Clive Page & Ben Forbes, 2021. "Development of an in vivo preclinical model of COPD in guinea pigs to study the anti-inflammatory effects of inhaled roflumilast". Oral presentation. *School of Cancer and Pharmaceutical Sciences PGR Symposium at King's College London, London, UK.*

5. <u>Ivana Stolfa</u>, Clive Page & Ben Forbes, 2021. "Development of a drug delivery system to the lungs: manufacture, *in vitro* and *in vivo* characterization of roflumilast albumin nanoparticles", 2021. LIDo iCASE Presentation Day, London, UK.

6. <u>Ivana Stolfa</u>, Clive Page & Ben Forbes, 2022. "Investigation of the potential of inhaled roflumilast albumin NPs for COPD treatment". Oral presentation. *School of Cancer and Pharmaceutical Sciences PGR Symposium at King's College London, London, UK.*

7. <u>Ivana Stolfa</u>, Clive Page & Ben Forbes, 2022. "Inhaled albumin nanoparticles as delivery strategy to improve the risk/benefit ratio of PDE-4 inhibitors". Poster presentation. *American Association Pharmaceutical Scientists (AAPS) 2022 PHARMSCI 360, Boston, MA*.

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List of Abbreviations

^{99m}Tc	Sodium Pertechnetate
ACN	Acetonitrile
API	Active Pharmaceutical Ingredient
BALF	Bronchoalveolar lavage fluid
BBB	Brain blood barrier
BCA	Bicinchoninic acid
BEAS-2B	Bronchial epithelial cells
BLM	Bleomycin
BSA	Bovine serum albumin
BW	Body weight
cAMP	Cyclic adenosine phosphate
cGMP	Cyclic guanosine monophosphate
CMC	Critical micellar concentration
COPD	Chronic obstructive pulmonary disease
CS	Cigarette-smoke
CSE	Cigarette smoke extract
CXCL8	Chemokine ligand 8
CYPs	Cytochromes P450
d_{ae}	Aerodynamic particle diameter
DD	Delivered dose
DLS	Dynamic Light Scattering
DMPE	Dimyristoylphosphatidylethanolamine
DMSO	Dimethyl sulfoxide
DPI	Dry powder inhaler
DPPC	Dipalmitoylphosphatidylcholine
DSC	Differential Scanning Calorimetry
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol)
DTX	Docetaxel
DVS	Dynamic Vapour Sorption
ECOPD	Exacerbation of chronic obstructive pulmonary disease
ED$_{50}$	Effective dose
EE	Encapsulation efficiency

ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FEV_1	Forced Expiratory Volume in one second
FITC	Fluorescin isothiocyanate
FPD	Fine particle dose
FPF	Fine particle fraction
FVC	Forced vital capacity
GLU	Glutaraldehyde
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GSD	Geometric standard deviation
GSK	GlaxoSmithKline
HARBS	High affinity rolipram-binding site
HCPT	10 – Hydroxycamptothecin
HPLC	High performance liquid chromatography
HSA	Human serum albumin
IC_{50}	Half maximal inhibitory concentration
ICS	Inhaled corticosteroid
IF	Inhalable fraction
IL-6	Interleukin-6
IL-8	Interleukin-8
Kcps	Kilo counts per second
LABA	Long-acting β_2 agonist
LAMA	Long-acting muscarinic antagonist
LARBS	Low affinity rolipram-binding site
LBP	Lipopolysaccharide binding protein
LHC-9	Laveck medium
LOD	Limit of detection
LOQ	Limit of quantification
LPNPs	Large porous nanoparticles
LPNs	Lipid nanoparticles
LPPs	Large porous particles
LPS	Lipopolysaccharide

mCD14	Membrane CD14
Methocel	Methyl cellulose
MMAD	Mass median aerodynamic diameter
MW	Molecular weight
N_A	Avogadro number
Nab™	Nanoparticle-albumin bound
NCLs	Nanostructured lipid carriers
NEMs	Nanoparticles in microparticles
NGI	Next Generation Impactor
NOAEL	No observed adverse effect level
NPs	Nanoparticles
NTA	Nanoparticle tracking analysis
OVA	Ovalbumin
p.o.	Oral administration
PAMAM	Poly (amidoamine)
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDI	Polydispersity index
PEG	Poly (ethylene glycol)- dimethyl ether
PEG_{400}	Polyethylene glycol 400
PGA	Polyglycolide
pI	Isolectric point
PLA	Polylactic acid
PLC	Polycaprolactone
PLGA	Poly lactic-*co*-glycolic acid
pMDI	Pressurised metered dose inhaler
PNPs	Polymeric nanoparticles
PO	Phosphodiester oligonucleotides
PSD	Particle Size Distribution
PVP	Poly N-vinyl pyrrolidone
QT	Quercetin
RFM	Roflumilast
RFM-HSA-NPs	Roflumilast albumin nanoparticles
RH	Relative Humidity

RMV	Respiratory minute volume
RSD	Relative standard deviation
SABA	Short acting β_2 agonist
SAMA	Short acting
SD	Standard deviation
SLNs	Solid lipid nanoparticles
SMIs	Soft-mist inhalers
SS	Stock solution
TFA	Trifluoroacetic acid
TiO$_2$	Titanium dioxide
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
UV	Ultraviolet
WR	Working reagent

1.1 General introduction

Pulmonary delivery represents an extremely useful but challenging way of administering therapeutics for both local and systemic activity. Successful aerosol particle delivery to the lungs is complex as there are many factors that will dictate the effective deposition of an aerosol into the lungs, including aerodynamic particle diameter, density, shape, pathological conditions that may have altered the physiology of the lungs as well as the clearance mechanisms (**Figure** 1.1). The use of pulmonary delivery represents an advantageous way to obtaining high dose exposure at the target site and reduction of side effects associated with systemic exposure. It also allows for drugs to avoid first pass metabolism which may lead to a reduction of the free fraction of the drug available to reach its target.
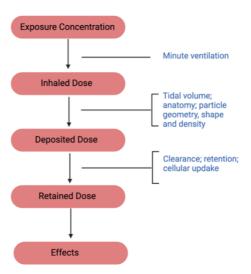
Figure 1.1 General overview of factors that may contribute to the active pharmaceutical ingredient (API) therapeutic activity after its delivery through the pulmonary route. Adapted from Oberdörster, 1993.

Roflumilast (RFM) is a phosphodiesterase (PDE) 4 inhibitor drug approved for the treatment of the exacerbations of chronic obstructive pulmonary disease (COPD) in patients with a chronic bronchitic phenotype. Prescribed as a once daily oral tablet, RFM

19   is characterised by anti-inflammatory activity, but has an unfavourable therapeutic index
20   due to gastrointestinal side effects and weight loss. The latter is greatest in patients with
21   most severe COPD that may be already experiencing reduction of weight due to the
22   respiratory conditions and the metabolic drive/energy to control the breathing (Jolley &
23   Moxham, 2009). Strategies to overcome the downsides of this drug class developed in
24   the past decade include screening and design of i) new molecules targeting only the
25   enzyme isoform responsible for the therapeutic activity rather than the side effects and ii)
26   new molecules exclusively for the inhalation route. However, those strategies have not
27   been completely successful so far, hence the need for pursuing new formulation
28   strategies.

29

30   Nanocarriers have shown great potential compared to 'conventional formulations' for the
31   delivery of hydrophobic drugs which can be otherwise very difficult to deliver.
32   Nanocarriers can protect their payload from unwanted degradation, they possess a high
33   surface area, and they allow modulation of the drug release rate. Human serum albumin
34   (HSA) based nanoparticles (NPs) represent one of these non-conventional formulation
35   approaches which has gained attention in the past years, especially after the approval of
36   Abraxane (paclitaxel loaded-HSA-NPs) as intravenous treatment for different types of
37   cancer.

38

39   The opportunity to combine nanotechnology with the advantages of pulmonary delivery
40   has been demonstrated by the success of the Arikayce®, Insmed Inc., an inhaled
41   (delivered via nebulisation) liposome amikacin formulation for the treatment of
42   Mycobacterium avium complex infections (Arikayce Liposomal; Khan & Chaudary,
43   2020). Therefore, the rationale behind this experimental work was to investigate the use
44   of HSA-NPs loaded with RFM via the pulmonary route as an alternative and new drug
45   delivery strategy to try and reduce the side effects associated with RFM, but also to
46   investigate the fate of HSA-NPs in the lungs and their potential as a drug carrier.

47

48   This thesis outlines the relevant background material in **Chapter 1**, focusing on the
49   pharmacology of PDEs inhibitors, the successes, and drawbacks of different type of
50   nanocarriers intended for pulmonary delivery, the challenges to overcome when
51   designing a carrier for pulmonary drug delivery, as well as their fate after inhalation. The

52 following chapters, **Chapters 2-6**, explore the potential of RFM-HSA-NPs for pulmonary

53 delivery using a variety of *in vitro* and *in vivo* experimental approaches.

54

1.2   Drug management and inhaled drug treatment of COPD

56

1.2.1   Diagnosis and initial assessment

58

59 COPD is a multifactorial clinical condition related mainly to the exposure to smoking and

60 characterised by persistent respiratory symptoms and airflow limitation due to airways

61 and alveolar abnormalities. The Global Initiative for Chronic Obstructive Lung Disease

62 (GOLD) reports are a useful tool used by clinicians for the diagnosis, management, and

63 prevention of COPD. The aims of the COPD assessment are to determine the degree of

64 airflow limitation, the disease severity, and the risk of future events as exacerbations,

65 hospital admission or death to guide therapy. The severity of airflow limitations is

66 evaluated via a spirometric assessment. Spirometry is performed after the administration

67 of a dose of at least one short-acting bronchodilator to minimise any variability of the

68 measurement.

69 Spirometry allows to measure the volume of air exhaled from the point of maximal

70 inspiration (forced vital capacity, FVC) and the volume of air exhaled during the first

71 second of this test (forced expiratory volume in one second, FEV_1). A ratio between FEV_1

72 and FVC < 0.70 corresponds to the presence of airflow limitation in the patient. Based on

73 this ratio, the severity of the airflow limitation is described by four categories (GOLD 1,

74 GOLD 2, GOLD 3 and GOLD 4): mild, moderate, severe and very severe (**Table** 1.1)

75 (Gold Initiative for Chronic Obstructive Lung Disease, Global Strategy for the Diagnosis,

76 Managment, and Prevention of Chronic Obstructive Pulmonary Disease, 2022).

77

Table 1.1 Classification of airflow limitation severity in COPD patientis with FEV_1/FVC < 0.70.

GOLD 1	**Mild**
GOLD 2	Moderate
GOLD 3	Severe
GOLD 4	Very Severe

78

79 The assessment of symptoms in COPD patients also requires the use of disease specific

80 health status questionnaires followed by the analysis of the patient's exacerbation history.

81 An exacerbation is defined as an acute worsening of respiratory symptoms, from the

82 stable state and beyond normal day-to-day variations that is acute in onset which results

83 in additional therapy (Rodriguez-Roisin, 2000; Vogelmeier et al., 2020).

84 A low risk of exacerbation is defined as one or less exacerbation in a year not resulting

85 in hospitalisation, whereas in presence of a minimum of two exacerbations in a year

86 resulting in hospital admission there is a high risk of exacerbation (Vogelmeier et al.,

87 2020).

88

89 The last step in the COPD assessment is the evaluation of the obtained values with the

90 "ABCD" assessment tool to achieve a classification of the patient. Four categories can be

91 defined based on the symptoms assessment score, the exacerbation history, and the

92 spirometry results (**Table** 1.2) (A. R. Patel et al., 2019).

Table 1.2 COPD patients classification according to symptoms and severity risk.

Group A	Less symptoms, low risk
Group B	More symptoms, low risk
Group C	Less symptoms, high risk
Group D	More symptoms, high risk

93

94 **1.2.2   COPD clinical phenotypes**

95

96 The concept of phenotype is referred to "those attributes of the disease alone or in

97 combination that describe the differences between individuals with COPD in relation to

98 parameters that have clinical significance" (MeiLan K. et al., 2010). Although there is

99 still no consensus on the exact number and definition of COPD phenotypes, those should

100 be used to classify patients in subgroups allowing a better diagnosis and determination of

101 the most appropriate therapy that results in meaningful outcomes. In 1989 Snider (Snider,

102 1989) represented the COPD phenotypes using a non-proportional Venn diagram in

103 which three subgroups of COPD were introduced: chronic bronchitis, asthma and

104 emphysema, with a common chronic airflow limitation (**Figure** 1.2) (Corlateanu et al.,

105 2020; Marsh et al., 2008).

106
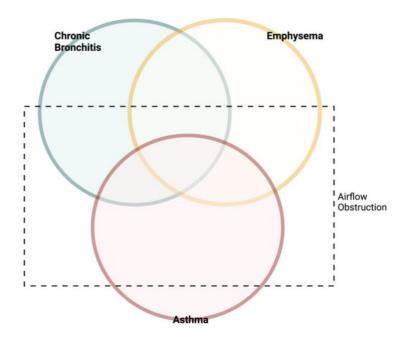
107 *Figure 1.2 Non-proportional Venn diagram of chronic obstructive pulmonary disease.*
108 *Adapted from Marsh et al., 2008*

109 Chronic bronchitis is associated with excess FEV_1, and it is defined as the presence of a
110 productive cough for three months in each of two successive years (V. Kim & Criner,
111 2015). Emphysema is the destruction of alveolar walls and the permanent loss of the
112 anatomical structure of the air spaces distal to terminal bronchioles. This condition led to
113 airflow limitation, progressive dyspnea and cough. Asthma is likely to be considered a
114 condition on its own and it is characterised by the narrowing of the airway lumen, with a
115 reduced expiratory airflow that results in symptoms such as wheeze and cough (Mannino
116 D., 2003). The use of a phenotypical approach in the nonpharmacological and
117 pharmacological management of COPD is a key aspect in a more personalised treatment
118 (Corlateanu et al., 2020) as very often those phenotypes overlap, representing a challenge
119 for clinicians.

120

121 **1.2.3 Pharmacological therapy for stable COPD patients**

122

123 The pharmacological therapy for COPD is aimed to treat symptoms, reduce the frequency
124 and severity of exacerbations, and improve health status of patients. Most of the drugs
125 are administered through the pulmonary route and therefore a proper technique in using

126 the inhalers is required by the patients. The classes of drugs commonly used in the

127 management of COPD patients are listed in **Table** 1.3.

128

129 The β_2 agonists bind the β_2 adrenergic receptors increasing the level of cyclic adenosine

130 monophosphate (cAMP) and leading to the relaxation of the airway smooth muscle. There

131 are short-acting (SABA) and long-acting (LABA) β_2 agonists, whereas those differ for

132 the duration of action (4-6 hours and 12 hours or more for SABAs and LABAs

133 respectively). Formoterol and salmeterol are LABAs and are administered twice daily.

134 Those significantly improve lung volumes, dyspnoea, exacerbation rate and number of

135 hospitalisations. In contrast, indacaterol is a LABA administered once daily that improves

136 breathlessness and exacerbations rate.

137

138 Antimuscarinic drugs block the M_3 muscarinic receptor expressed in airway smooth

139 muscle which activation induces a bronchoconstrictor effect. Short acting muscarinic

140 antagonists (SAMAs) (ipratropium bromide) can also block the M_2 muscarinic receptor

141 which can potentially cause vagally induced bronchoconstriction. Long-acting

142 muscarinic antagonists (LAMAs) as glycopyrronium bromide have prolonged binding to

143 M_3, but faster dissociation from the M_2 with a consequent prolonged bronchodilator

144 effect.

145

146 In the COPD management the degree of bronchodilation can be improved with

147 combinations of bronchodilators with different mechanism and duration of actions.

148 A pharmacological therapy with inhaled bronchodilators is key for the management of

149 symptoms (both prevention and reduction). Symptom reduction and FEV_1 improvement

150 are achieved with a regular and as-needed use of SABA and SAMA, with a combination

151 of those resulting in improved outcomes. Lung function, dyspnoea and exacerbation are

152 consistently reduced by a treatment with LABAs and LAMAs, with an improved

153 therapeutic effect provided by the combination therapy rather than the monotherapy.

154 Usually, SABAs are prescribed for immediate symptom relief and their use on a regular

155 basis is not recommended. In the case of patients with severe breathlessness a

156 combination therapy with LAMA/LABA can be offered as initial treatment or LABA/ICS

157 for patients with high risk of exacerbations and higher blood eosinophil counts. Although

158 regular treatment with only ICS does not modify the decline of FEV_1, a therapy based on

159 ICS in combination with LABA is more effective than the two compounds alone in the

33

160 improvement of lung function and exacerbations. ICS can also be included in a triple

161 inhaled therapy with LABA and LAMA with improved benefits compared to LABA/ICS,

162 LABA/LAMA or LAMA monotherapy.

163 The anti-inflammatory therapy in stable COPD patients is based on the use of inhaled

164 corticosteroids, oral glucocorticoids, PDE4 inhibitors, antibiotics and mucoregulators. A

165 regular treatment with inhaled corticosteroids has no effect on the long-term decline of

166 FEV_1, whereas a combination of this class of drugs with LABA is more effective than

167 either drug alone in the improvement of lung function and exacerbations in patients with

168 moderate to severe COPD.

169 Oral glucocorticoids are usually not adopted in the chronic daily treatment of COPD

170 because of their systemic effects but they play a role in the management of exacerbations.

171 Mucolytic are in some cases used in patients not receiving ICS to reduce the exacerbations

172 and slightly improve the health status.

173 Theophylline, the most used methylxanthine, is known to be a non-selective PDE

174 inhibitor. Its use in the COPD therapy management is not recommended unless other

175 long-term bronchodilators are not available. An additional PDE inhibitor with a selective

176 activity towards the isoform 4 of the enzyme is roflumilast. A more detailed description

177 of this drug is shown in paragraph 1.2.4.

178

Table 1.3 Maintenance medications in COPD patients.

Drug category		Generic Drug Name
β₂ agonists		
	Short-acting (SABA)	Fenoterol, Salbutamol
	Long-acting (LABA)	Formoterol, Indacaterol
Anticholinergics		
	Short-acting (SAMA)	Ipratropium bromide
	Long-acting (LAMA)	Glycopyrronium bromide
Methylxanthines		
	-	Theophylline
Phosphodiesterase 4 Inhibitor		
	-	Roflumilast
Mucolitic agents		
	-	Carbocysteine, N-acetylcysteine
Combination therapy		
SABA/SAMA	-	Fenoterol/ipratropium Salbutamol/ipratropium
LABA/LAMA	-	Formoterol/glycopyrronium Indacaterol/glycopyrronium
LABA/Inhaled corticosteroids (ICS)	-	Formoterol/beclomethasone Formoterol/budesonide

179

180 **1.2.4 Pharmacology of PDE inhibitors**

181

182 COPD is an airway disease characterised by progressive airflow obstruction associated

183 with an underling degree of chronic airways inflammation (Barnes et al., 2015). The use

184 of the PDE4 inhibitor RFM can be considered in patients with severe to very severe

185 airflow limitation, chronic bronchitis and exacerbations as addition to a treatment with

186 long-acting bronchodilators with or without ICS (Gold Initiative for Chronic Obstructive

187 Lung Disease, Global Strategy for the Diagnosis, Managment, and Prevention of Chronic

188 Obstructive Pulmonary Disease, 2022).

189   PDEs are a class of intracellular enzymes responsible for the degradation of cAMP and
190   cyclic guanosine monophosphate (cGMP) into the inactive 5' AMP and 5' GMP
191   respectively. cAMP and cGMP usually bind and activate protein kinase (A and G
192   respectively) which phosphorylate substrates that control different physiological
193   processes such as the release of inflammatory mediators and the airway smooth muscle
194   relaxation (**Figure** 1.3) (Beavo & Brunton, 2002). The inhibition of this degradation
195   pathway mediated by PDE inhibitors results in a wide range of cellular processes,
196   signalling and transcription of genes involved in both regulating the activation of
197   inflammatory cells and airway smooth muscle relaxation of relevance to COPD. The
198   resulting increased level of cAMP and cGMP and the following downstream activation
199   of phosphorylation cascades results in airway muscle relaxation and inhibition of
200   inflammatory pathways, depending on which PDEs are present in relevant cell types
201   (Conti et al., 2003).

202

203   The PDE family of enzymes has 11 isoforms (PDE1 to PDE11) which are differently
204   distributed in the body, and which possess different sensitivities for cAMP and cGMP.
205   The isoforms 4, 7 and 8 are highly selective towards cAMP only, whereas PDE 5, PDE 6
206   and PDE 9 for cGMP only. The remaining isoforms have activity for both cyclin
207   nucleotides (Houslay & Adams, 2003; Keravis & Lugnier, 2012) (Figure 1.3).

208
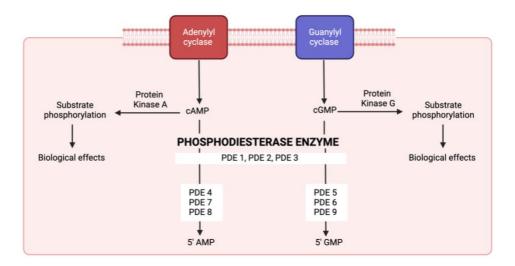
209   *Figure 1.3 Intracellular signalling of phosphodiesterase enzymes.*

210   The PDE4 family is comprised of four genes (A, B, C, D) whereas the enzymes
211   distribution is broad in the body with isoforms located in the brain, spleen, gastrointestinal
212   tract, hearth, testis, kidneys and lungs. Importantly, PDE4 is also expressed in many

213   inflammatory cells (T-cells, eosinophils, neutrophils, monocytes) involved in the
214   pathogenesis of COPD (Beavo et al., 2011).

215

216   Theophylline was the first PDE inhibitor discovered and has long been recognised for its
217   use in the treatment of airways diseases in part due to its non-selective inhibition of the
218   PDE enzymes (**Figure** 1.4). Theophylline is effective in the treatment of COPD but is
219   characterised by a weak and non-selective inhibitory activity on PDE enzymes. With the
220   recognition that airway smooth muscle relaxation mainly comes from PDE3 inhibition,
221   and that anti-inflammatory activity is a result of PDE4 inhibition, there was a major drive
222   to discover and develop more selective PDE inhibitors to improve the efficacy and safety
223   of theophylline (Boswell-Smith, Cazzola and Page, 2006). Theophylline has been
224   reported to reduce the proportion of neutrophils and interleukin-8 (IL-8 or chemokine (C-
225   X-C motif) ligand 8, CXCL8) levels in the sputum obtained from patients with COPD
226   (Culpitt et al., 2002). However, the absence of a PDE selectivity accounts in part for the
227   very narrow therapeutic window for theophylline as adverse effects usually occur when
228   the drug plasma concentration is > 20 mg/mL, although side effects may also develop at
229   lower plasma concentration levels (Barnes, 2013). The most frequent are nausea,
230   headache, and increased acid secretion. Higher concentrations can induce convulsions,
231   cardiac arrhythmias, and diuresis due to the additional adenosine receptor antagonist
232   activity of theophylline (Barnes, 2013). Hence, the use of theophylline is limited by the
233   unfavourable therapeutic window of this drug which necessitates careful titration and
234   plasma monitoring. In addition, due to the metabolism of theophylline mainly driven by
235   the cytochromes (CYP) (1A2 with a smaller contribution of 2E1 and 3A4) there are a
236   range of possible interactions with other drugs metabolised by the same CYP isoforms.

237

238   The wide range of side effects induced by theophylline highlighted the need for new drugs
239   with greater selectivity towards PDE3 and 4 (Figure 1.4). Rolipram is considered a first
240   generation PDE4 inhibitor and was characterised by a significant increase of the
241   selectivity towards the isoform 4, but this drug still had many gastrointestinal side effects
242   such as nausea and diarrhoea. An improved safety profile was subsequently obtained with
243   the second generation of PDE4 inhibitors. This new class was designed following the
244   discovery of two specific binding sites of PDE4. The high affinity rolipram-binding site
245   (HARBS) mainly located in the central nervous system and parietal glands and the low
246   affinity rolipram-binding site (LARBS) in immunocompetent cells. Indeed, rolipram

247   interacts with high potency with the HARBS ($K_D \sim 2$ nM) and lower with the LARBS
248   ($K_D \sim 200$ nm – 2000 nM) (Souness & Rao, 1997). Therefore the 2nd generation
249   molecules, RFM and cilomilast were designed to have an increased specificity for the
250   LARBS, to hopefully result in an improved therapeutic index compared to theophylline
251   (Boswell-Smith, Cazzola and Page, 2006).

252

253   However, despite the intention that the therapeutic index of the second generation of
254   PDE4 inhibitors would have an improved therapeutic window by reducing the affinity
255   towards the HARBS of PDE4, this has turned out to not be the case and it was
256   subsequently confirmed that the HARBS coincides with the holoenzyme responsible for
257   the enzyme catalysis (Laliberté et al., 2000). The catalytic machinery of PDE4 uses Mg^{2+}
258   as cation cofactor. The binding of this cofactor to the enzyme results in two coexisting
259   conformers that can bind inhibitors in a different way: the holoenzyme exists when the
260   enzyme binds the cation, whereas the apoenzyme is represented by the free enzyme
261   (Laliberté et al., 2000).

262
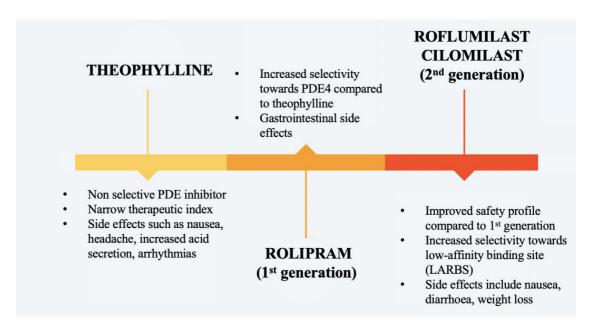
263   *Figure 1.4 PDE inhibitors: from theophylline to 2nd generation.*

264   Robichaud (Robichaud et al., 2002) suggested that the emesis caused by PDE4 inhibitors
265   is rather a result of the inhibition of the PDE4D subtypes of the enzyme. This finding
266   highlighted a new potential strategy to overcome the unfavourable therapeutic index by
267   designing a PDE4 subtype-selective activity inhibitor. However, considering that rodents
268   – usually used as species in preclinical models – are non-vomiting species, the potential

269 of those new selective PDE4 subtype inhibitors was investigated in a new model looking

270 at the effect of the drugs on the duration of anaesthesia induced by the xylazine/ketamine

271 combination as PDE4 plays a key role in the anaesthesia induced by α_2-adrenoreceptor

272 activation in mice. Interestingly, a positive correlation was found between the potency in

273 reducing the anaesthesia and the propensity of the molecule to induce emesis in ferrets.

274 This correlation was related to the proportion of the compound present in the brain

275 compared to plasma (Robichaud et al., 2002). RFM - as non-subtype selective PDE4

276 inhibitor - was demonstrated to have an improved therapeutic index compared to

277 cilomilast which in contrast has a PDE4D selectivity. Some suggested there was a need

278 to develop selective PDE4B inhibitors to improve the therapeutic window further, but to

279 date, no selective PDE4B inhibitors have been developed or approved (Ariga et al., 2004;

280 Phillips, 2020).

281

282 Considering this background discussed above, an improvement of the therapeutic index

283 of the PDE4 inhibitors could be perhaps achieved via following three different strategies:

284 i) identify new molecules that can bind the LARBS preferentially over the HARBS; ii)

285 target the isoform PDE4B rather than the isoform PDE4D which is considered to be

286 correlated with the onset of emesis; iii) identify new molecules that have a low penetration

287 of the blood brain barrier (BBB). However, considering that the postrema (which acts as

288 chemoreceptor trigger zone for emesis) is not protected by the BBB, this strategy, as well

289 as the others has been flawed and unsuccessful and has not resulted in any therapeutic

290 improvements.

291

292 RFM was developed as the most selective new PDE4 inhibitor with an improved

293 therapeutic window compared to the previous PDE inhibitors generations and as a once-

294 daily oral treatment therapy (500 µg) for a subset of COPD patients with severe and

295 recurrent exacerbations. RFM is a lipophilic and highly permeable molecule that is

296 rapidly absorbed after oral administration. It is metabolised in the liver to the metabolite

297 N-oxide by CYP3A4 and CYP1A2. Interestingly, the N-oxide metabolite also accounts

298 for the total therapeutic activity, and both metabolite and parent molecule are extensively

299 metabolised. The effective half-life for RFM is in the range of $8 - 31$ h, whether the

300 terminal half-life for the metabolite is in the range of $11 - 47$ h. Due to the long half-life,

301 the systemic exposure, and the potency of the metabolite, only one administration per day

302 is recommended for its clinical use. Although the metabolite is threefold less active than

303 RFM with respect to the PDE4 inhibition, it has a 10- to 12- fold higher plasma
304 concentration compared to RFM (Baye, 2012; European Medicines Agency, 2010).
305

306 Both RFM and its N-oxide possess selective inhibitory activity towards the PDE4
307 (Hatzelmann & Schudt, 2001). *In vivo*, orally delivered RFM has been demonstrated to
308 improve the inflammatory responses and the lung parenchymal destruction when
309 administered to mice exposed to cigarette smoke, as well as providing improvements of
310 the lung fibrotic damage induce by bleomycin (Hatzelmann et al., 2010).
311

312 In clinical studies, RFM – administered at the clinical dose of 500 µg once daily for 4
313 weeks - reduced by 39% the LPS-induced neutrophilia in patients compared to placebo
314 (Hohlfeld et al., 2008). Furthermore, RFM reduced the increased number of neutrophils
315 in the induced sputum of COPD patients by approximately 35% (Grootendorst et al.,
316 2007). Overall, the drug showed an improved safety profile compared to cilomilast, but
317 a variety of adverse effects were still reported. Compared to cilomilast, vomiting is rare
318 (Rabe et al., 2005) but other frequently reported side effects included diarrhoea, weight
319 decrease, nausea and headache (Calverley et al., 2009). Hence, despite the expectations
320 of an improved therapeutic index for this drug originally showed compared to previous
321 PDE4 inhibitors, there is still a low compliance and the risk/benefit ratio is highly
322 unfavourable which ultimately hinders the wider use of this drug (Mokry et al., 2021;
323 Singh et al., 2021).
324

325 Considering that these strategies to overcome the disadvantageous therapeutic index of
326 PDE4 inhibitors have failed, two new strategies, both based on the use of the inhalation
327 route have been developed: i) design of new molecules with a sub-type specificity that
328 are suitable for pulmonary delivery; ii) encapsulation of molecule with a less favourable
329 profile in 'non-conventional' formulation i.e. nanoparticles, microparticles, liposomes to
330 be administered via the pulmonary route.
331

332 So far, the investigation of new inhaled PDE4 inhibitors has not been successful (Phillips,
333 2020b; Singh et al., 2021)(**Table** 1.4). Throughout the last 20 years many molecules were
334 designed, and preclinical and clinical studies performed which, in most of the cases, was
335 followed by withdrawal from the companies' pipelines (Phillips, 2020a). This was the
336 case of the moderately potent AWD-12-281, (Elbion/GlaxoSmithKline (GSK) (half

337   maximal inhibitory concentration (IC$_{50}$) = 9.7 nM), tofimilast from Pfizer (IC$_{50}$ = 140

338   nM), UK-500,001 from Pfizer (IC$_{50}$ = 1 nM) and GSK256066 from GlaxoSmithKline

339   (IC$_{50}$ = 0.003 nM), which was designed as a very promising molecule having a HARBs

340   ratio (HARBs IC$_{50}$/LARBS IC$_{50}$) of at least 17, compared to 4.4 for RFM (Nials et al.,

341   2011; Tralau-Stewart et al., 2011).

342

343   In a wider study aimed to design new and improved PDE4 molecules, Astra Zeneca (de

344   Savi et al., 2014) attempted to rationalise the lack of success of UK-500,001 and

345   GSK256066 while conducting a bigger study screening new PDE inhibitor molecule (de

346   Savi et al., 2014). They suggested that in order to obtain a better drug, GSK designed one

347   with low aqueous solubility and oral bioavailability, whereas Pfizer's API was

348   characterised by high metabolic clearance and high plasma protein binding to achieve a

349   minimal systemic exposure, hence minimal side effects.

350

Table 1.4 Inhaled PDE4 and dual PDE3 – PDE4 inhibitors for the treatment of COPD and asthma and corresponding current development stage.

Molecule name	Company	Development stage
AWD-12-281	Elbion/GlaxoSmithKline	Discontinued
Tofimilast	Pfizer	Discontinued
UK-500,001	Pfizer	Discontinued
GSK256066	GlaxoSmithKline	Discontinued
Ensifentrine	Verona Pharma	Clinical stage
Tanimilast	Chiesi	Clinical stage

351

352   Results obtained from De Savi (de Savi et al., 2014) showed that AstraZeneca molecule

353   had a high plasma protein binding but that the exposure of LPS-challenged rats to a dry

354   powder aerosol of UK-500,001 resulted in inconsistent inhibition of LPS-induced

neutrophilia, despite the significant dose delivered to the lungs. The lack of anti-inflammatory activity in this rat model may be explained by insufficient potency of the molecule which was not compensated by the high doses deposited or as suggested above it may be necessary to have systemic exposure of a PDE4 inhibitor to have an anti-inflammatory activity. Likewise, the administration of GSK266066 did not result in significant inhibition of the lung inflammation when tested in a pre-treatment regime (drug administered 12 h before the challenge). The lack of activity was suggested to be due to the unsuitable window time adopted in the *in vivo* model and with an insufficient availability of the free fraction of the molecule in the target range. However, studies carried out by GSK (Nials et al., 2011) showed that the lipophilic nature of the GSK266066 allowed its retention in the lung tissue up to 40 h, even after the conclusion of the inflammatory event. However, De Savi (de Savi et al., 2014) suggested that it is very likely that the drug is in the lung tissue but not in an 'available' form, perhaps as solid form or in lipid compartments and that the concentration of the free compound which is required for the therapeutic activity in the intracellular cytoplasm -which is where PDE4 is located- was not high enough to exert a pharmacological activity.

Another novel inhaled PDE inhibitor is ensifentrine/RPL554 which has dual inhibitory activity against PDE3 and PDE4 (Abbott-Banner & Page, 2014) that induces both a bronchodilator and an anti-inflammatory activity (Franciosi et al, 2013). Results from a Phase 3 ENHANCE-2 ("Ensifentrine as a Novel Nebulized COPD thErapy") recently carried out showed that an improvement in lung function and a reduction in COPD exacerbation were achieved (www.veronapharma.com).

A second molecule that has produced some promising results was designed by Chiesi. They identified a new PDE4 inhibitor called tanimilast (CHF6001), available as a powder for inhalation administered via NEXThaler® (Chiesi proprietary multi-dose inhaler) which has so far gained positive outcomes in preclinical and clinical studies. It is 10-fold more potent than RFM (IC_{50} = 0.026 nM), with a HARBs ratio of 40.6 (Moretto et al., 2015) and able to equally inhibit both isoforms A and D of the enzyme (Armani et al., 2014).

In vitro assays have showed that CHF6001 is able to induce more potent inhibition of tumour necrosis factor-alfa (TNF-α) production from alveolar macrophages and lung tissue isolated from COPD patients compared to RFM (Lea et al., 2019). In addition, *in*

389 *vitro* it significantly reduced the adhesion of neutrophils to the endothelium and their
390 degranulation, in combination with a reduction of cytokine secretion (Schioppa et al.,
391 2022). *In vivo,* CHF6001 was extremely effective when administered intratracheally to
392 ovalbumin sensitised-rats and intranasally to mice using a cigarette smoke model in both
393 preventive and interventional applications. In the context of the side effects, which is one
394 of the main driving reasons of this investigation, any emesis correlated effect (using
395 ketamine/xylaxine-induced anaesthesia as measures) were recorded at doses 50- to 150-
396 fold higher than the anti-inflammatory ED_{50} in rats. The lack of systemic side effects was
397 confirmed in a ferret model. CHF6001 did not induce emesis up to 10-20 mmol/kg, a dose
398 considerably higher to the dose of GSK256066 that induced emesis (1 mmol/kg) (Villetti
399 et al., 2015). The positive outcomes were corroborated in a randomised, double-blind,
400 placebo-controlled study where CHF6001 was administered via single-dose or multidose
401 dry-powder inhaler. Results confirmed that this molecule has a good safety profile also
402 *in vivo* with no relationship between dose and adverse effects (Mariotti et al., 2018).
403 Overall, the positive outcomes for this molecule appeared to be related to its high potency,
404 prolonged half-life, low permeability and high protein plasma binding which are key in
405 limiting the free fraction of the drug in the systemic circulation hence the occurrence of
406 the systemic side effects (Facchinetti et al., 2021).
407
408 Considering the background provided above, it is now evident that multiple strategies
409 aimed to improve the therapeutic index of this class of drugs have failed. Here the need
410 to pursue the investigation of a different strategy based on the use of a nanoplatform able
411 to encapsulate the only PDE4 inhibitor approved so far, RFM. The well-known anti-
412 inflammatory potency of RFM will be combined with the proven potential of the HSA-
413 NPs. The success and the drawbacks of the HSA-NPs as well as the use of the pulmonary
414 delivery as a lung-selective route of administration will be illustrated in the following
415 paragraphs.
416

417 **1.3   Nanocarriers-mediated drug delivery systems: an overview**
418

419 There are many nanostructures used for drug delivery purposes, each of them differing
420 for shape, size, type of payload and interactions with the biological environment (**Figure**
421 1.5). Those features overall dictate the characteristics, advantages, and disadvantages of
422 each nanocarrier, with specific use in different clinical areas. Detailed classifications of

43

423 the most used nanostructures are reported in several reviews in the literature (Cheng et

424 al., 2021; Edis et al., 2021; Hossen et al., 2019; Mazdaei & Asare-Addo, 2022; Shan et

425 al., 2022) and in book chapters (Pathak & Thassu, 2016) with a specific focus on those

426 used in inhalation (Ibarra-Sánchez et al., 2022; Paranjpe & Müller-Goymann, 2014).
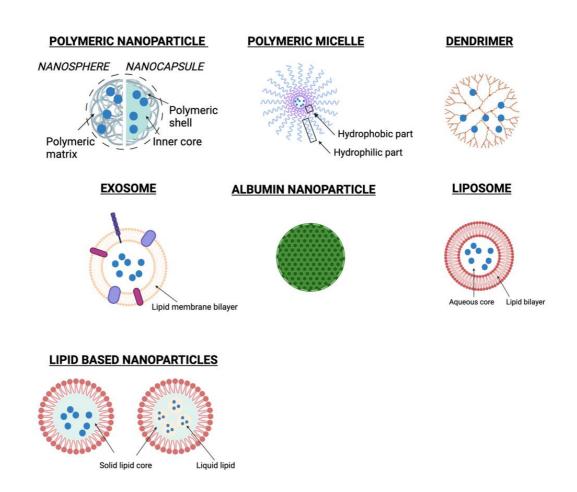
427 *Figure 1.5 Schematic representation of the most common nanocarriers for drug*

428 *delivery.*

429 **1.3.1   Polymeric nanoparticles (PNPs)**

430

431 Polymeric nanoparticles (PNPs) are colloidal systems made up by natural or synthetic

432 polymers. The PNPs include both i) nanospheres, which are nanostructures characterised

433 by dense polymeric matrix containing the drug and ii) nanocapsules, hollow structures

434 with the drug contained within the polymeric matrix (**Figure** 1.5) (Mora-Huertas et al.,

435 2010). The PNPs are characterised by high encapsulation efficiency, biocompatibility,

436 and efficient delivery of macromolecules (Gagliardi et al., 2021; Ibarra-Sánchez et al.,

437 2022). The polymers commonly used for the manufacture of these nanostructures are

44

438   chitosan, poly lactic-*co*-glycolic acid (PLGA), polyglycolide or poly glycolic acid (PGA),

439   polylactic acid (PLA) or polyethylene glycol (PEG).

440   PNPs are widely used as nanocarriers for the delivery of therapeutics via inhalation

441   (Ibarra-Sánchez et al., 2022) (**Table** 1.5). Studies reported the use of PNPs for the

442   delivery of linezolid for the treatment of tuberculosis (Shah et al., 2020) and

443   budesonide/theophylline for the treatment of asthma (Buhecha et al., 2019). The use of

444   PNPs loaded with chemotherapy agents for the treatment of pulmonary cancer is widely

445   reported (Cheng et al., 2021; Gagliardi et al., 2021; Hossen et al., 2019). Nan (Nan, 2019)

446   investigated the co-delivery of cisplatin and doxorubicin in 1,2-distearoyl-sn-glycero-3-

447   phosphoethanolamine-Poly (ethylene glycol) (DSPE-PEG) NPs for lung cancer therapy.

448

449   **1.3.1   Polymeric micelles**

450

451   Polymeric micelles are characterised by a bilayer structure made of a hydrophobic inner

452   core and a hydrophilic outside layer also called 'corona' (Figure 1.5). Due to this bi-

453   functional structure, the encapsulation of poorly soluble water drugs into polymeric

454   micelles can improve the drug's solubility, also protecting the drug from degradation and

455   increasing the time of circulation in the blood stream (Ibarra-Sánchez et al., 2022). Poly

456   N-vinyl pyrrolidone (PVP) and PEG are often used for the corona layer, whereas PLA

457   and polycaprolactone (PLC) are adopted for the hydrophobic core.

458

459   Polymeric micelles have been used for the combined delivery of genes and drugs for the

460   treatment of acute pulmonary inflammation (G. Kim et al., 2018). Moazeni and co-

461   workers (Moazeni et al., 2012) reported the preparation and evaluation of inhalable

462   itraconazole chitosan micelles for the treatment of pulmonary fungal infections.

463   Moreover, the potential of polymeric micelles for the treatment of lung cancer has been

464   investigated by Gill and colleagues (Gill et al., 2011). Paclitaxel-loaded polymeric

465   micelles were fabricated from PEG_{5000}-DSPE and tested over the conventional paclitaxel

466   formulation (Taxol) using a *in vivo* mouse model. Results showed that PNPs provided a

467   better release profile over Taxol as well as increased drug localisation in the lung tissue

468   (Table 1.5).

469

470

1.3.2 Dendrimers

Dendrimers are small synthetic macromolecules with a size from 2 to 10 nm (Sherje et al., 2018). Those tree-like structures are characterised by 3D shape, globular structure, and high branching points. The structure consists of a core of a single atom or molecule from which one or more branches depart from. The branches are formed from repeated units connected by branch connections. The final structure is characterised by radial concentric layers which are key for the properties of this nanocarrier (Figure 1.5). The polymers used in the manufacture of these nanostructures are poly(amidoamine) (PAMAM), poly-L-lysine or polyglycerol.

The use of dendrimers as drug delivery systems for inhalation is not very common due to the potential cytotoxicity and reactivity deriving from the presence of different charges on the dendrimers' surface. However, some successful studies are reported in the literature. Nasr (Nasr et al., 2014) investigated the potential of beclomethasone dipropionate dendrimers as aerosol nanocarriers for pulmonary delivery via nebulisation. Zhong and colleagues (Zhong et al., 2016) investigated the pulmonary delivery of doxorubicin-conjugated PAMAM dendrimers as delivery strategy to reduce cardiac accumulation and improve antitumor activity of doxorubicin in lung metastasis showing successful results (Table 1.5).

Table 1.5 Nanocarriers for drug delivery for respiratory therapy.

Type of nanocarrier	Drug	Clinical applications	Ref
Polymeric NPs (PLGA)	Linezolid	Tuberculosis	(Shah et al., 2020)
Polymeric NPs (PLA)	Budesonide/Theophylline	Asthma/COPD	(Buhecha et al., 2019)
Polymeric NPs (DSPE-PEG/Miglyol® 812)	Cisplatin, doxorubicin	Lung cancer	(Nan, 2019)
Polymeric micelle (Chitosan)	Itraconazole	Pulmonary fungal infections	(Moazeni et al., 2012)
Polymeric micelle (Cholesterol-conjugated polyamidoamine)	Resveratrol/heme oxygenase-1 gene	Acute lung injury	(G. Kim et al., 2018)
Polymeric micelle (PEG$_{5000}$-DSPE)	Paclitaxel	Lung cancer	(Gill et al., 2011)
Dendrimer (PAMAM)	Beclomethasone dipropionate	Asthma	(Nasr et al., 2014)
Dendrimer (PAMAM)	Doxorubicin	Lung cancer	(Zhong et al., 2016)
Exosome	-	Idiopathic pulmonary fibrosis	(Dinh et al., 2020)
Exosome	microRNA molecules	Lung cancer	(Nie et al., 2020)

493

1.3.3 Exosomes

Exosomes are small extracellular vesicles with a size of 40 to 120 nm (Elsharkasy et al., 2020) (Figure 1.5). Cells form exosomes to carry nucleic acids, proteins, lipids, and other metabolites, therefore playing a key role in the cell communication. Due to their origin from cell membranes, they possess a low to non-toxicity and a low clearance rate. An additional advantage is the presence of endogen biomarkers on their surface which increases the interactions of exosomes with the receptor cells membrane.

Exosomes have been used as delivery systems for the treatment of idiopathic pulmonary fibrosis (Dinh et al., 2020) and non-small cell lung cancer (Nie et al., 2020) (Table 1.5).

1.3.4 Albumin-based nanocarriers

When investigating the potential of a nanoplatform for therapeutic administration, it is fundamental to identify the properties that the nanoplatform possess as well as the improvements that it provides compared to 'non-conventional' formulations. HSA-NPs represent an interesting formulation that reached the market in 2005 with the U.S. Food and Drug Administration (FDA) approval of Abraxane®, which is currently used for the treatment of metastatic breast cancer, nonsmall cell lung cancer, metastatic adenocarcinoma of the pancreas, bladder cancer and gastric cancer (**Table** 1.6). Abraxane® is the trade name of nanoparticle albumin-bound (nab™) paclitaxel (nab-paclitaxel). The manufacture of nab-paclitaxel is called nab-technology and is based on an emulsion-evaporation cross-link method (Fu et al., 2009). The first step of this method is based on the dropwise addition of the oil phase (paclitaxel dissolved in chloroform) to the aqueous phase (HSA solution 1% w/v presaturated with 1% chloroform). The final emulsion is then obtained by homogenisation of the crude emulsion at high pressure and by recycling the emulsion through homogenizer. The nanosuspension obtained with this manufacture method possessed a size of ~ 130 nm and a zeta potential of -31 mV which allowed stability in aqueous phase. After manufacture, the NPs suspension is filtered to provide sterility and to remove particles with an unsuitable size. In order to improve the stability of the product, lyophilisation is utilised obtaining a powder which can be easily redisperse in water prior to infusion.

35   Abraxane® replaced the former clinical formulation (Taxol) in which paclitaxel was

36   dissolved in cremophor and ethanol. The use of those cosolvents was fundamental to

37   enable the administration of the drug due to its hydrophobic nature. However, the use of

38   those cosolvents was associated with severe adverse effects which limited its use. Patients

39   experienced acute adverse effects, neutrophilia and hypersensitivity reactions which

40   required preventive treatment with corticosteroids.

41

42   The first advantage provided using Abraxane over Taxol was represented by the reduced

43   ratio of side effects. Indeed, in the newer formulation the drug is present in a non-

44   crystallised amorphous state, and it is noncovalently bound to the albumin molecules. In

45   this form, the drug can easily access the target site in contrast to the former formulation

46   where the cremophor micelles hinder the release of the compound resulting in a lower

47   free drug amount in the blood and a following reduced therapeutic activity. In addition,

48   those micelles also slow the elimination rate from the blood resulting in an increased

49   systemic exposure and higher risk of cosolvents correlated side effects (van Zuylen et al.,

50   2001).

51

52   In addition, Abraxane showed rapid tissue distribution, increased biodistribution volume

53   and higher rate of clearance. The tumour concentration of paclixatel increased when

54   administered encapsulated in HSA-NPs, explained by the increased transcytosis of

55   Abraxane across the endothelial cells compared to Taxol as demonstrated in a mice model

56   of bearing human breast tumour xenograft (Desai et al., 2006). Indeed, albumin is a key

57   element for tumours as they require it for proliferation and sustainment. In addition, the

58   use of Abraxane increased the tolerability of the drug in terms of concentration. Taxol

59   was injected at 0.3 -1.2 mg/mL with an infusion time of 3 – 24 h, whereas Abraxane can

60   be injected over 30 min at 2 – 10 mg/mL (Chen et al., 2015; Ibrahim et al., 2002, 2005;

61   Spada et al., 2021).

62

63   Besides Abraxane, there are other two albumin NPs-based formulations that have been

64   approved as radiopharmaceuticals for clinical use (Table 1.6). Nanocoll is an albumin

65   colloidal NPs formulation used as a diagnostic as an alternative to sulfur colloid NPs. The

66   injectable formulation is obtained via reconstitution with sodium Pertechnetate (^{99m}Tc)

67   (GE Healthcare, 2015). Nanocoll has a small particle size (mean diameter of 30 nm, with

68   most particles with a size < 80 nm) which allows a quicker migration through the

69 lymphatic system with a consistent and suitable retention within the sentinel lymph nodes.
70 Moreover, due to the higher rate of localisation in the bone marrow after intravenous
71 injection, those NPs are more likely usable for bone marrow imagining compared to the
72 sulfur NPs (Thakor et al., 2016). Another albumin-based formulation is Optison which is
73 in the form of albumin microsphere (Table 1.6). It is an injectable suspension of HSA
74 microspheres with perflutren gas as contrast agent. The albumin microspheres have a
75 mean diameter between 3.0 and 4.5 µm with 95% of the microspheres having a size less
76 than 10 µm. This formulation is indicated for use in patients with suboptimal
77 echocardiograms to improve the delineation of the left ventricular endocardial borders
78 (FDA, 2012).
79
80 Extensive reviews about the *in vitro* and *in vivo* investigation of albumin NPs besides the
81 clinical outcomes of various albumin NPs encapsulating a wide range of therapeutic
82 agents are reported in the literature (Elzoghby et al., 2012; Hornok, 2021; Karimi et al.,
83 2016; Sleep, 2015; Spada et al., 2021).

Table 1.6 Albumin-based formulations approved for clinical use.

Trade name	Drug	Type of formulation	Physiochemical properties	Therapeutic indication	Advantages
Abraxane®	Paclitaxel	Nanoparticle albumin-bound (nab™) paclitaxel (nab-paclitaxel)	Size: ca 130 nm Zeta potential: -31 mV	• Metastatic carcinoma • Nonsmall cell lung cancer • Metastatic adenocarcinoma of the pancreas • Bladder cancer • Gastric cancer	• Reduced ration of side effects compared to Taxol (paclitaxel in cremophor and ethanol • Rapid tissue distribution • Increased biodistribution volume • Higher rate of clearance • Increase concentration of paclitaxel in the tumour • Reduced time and frequency of infusion
Nanocoll	Sodium Pertechnetate (^{99m}Tc)	Technetium-99m albumin nanocolloid	Size: mean diameter of 30 nm, with most particles with a size < 80 nm	Radiopharmaceutical (diagnostic use only)	• Small size allows good retention within the sentil lymph nodes • Suitable for bone marrow imaging due to high rate of localisation in the bone marrow
Optison	Perflutren	Albumin microspheres with perflutren	Size: mean diameter between 3 – 4.5 µm	Diagnostic use only	

84

1.3.5 Lipid-based nanoparticles

Despite the positive results obtained, the investigation of the albumin NPs as an inhalable carrier for pulmonary diseases is less explored than other routes of administration as highlighted in the previous paragraph. To our knowledge, the research of inhaled albumin NPs has not yet reached clinical stages and appears to be still preliminary. However, the use of a wide range of other types of nanocarriers has been demonstrated to have potential for pulmonary delivery with advantages compared to 'conventional therapies' (Table 1.5). For example, the lipid nanoparticles (LPNs) have been used in inhaled medicines as described in a comprehensive review highlighting the current trends for drug delivery in respiratory diseases therapeutics which has recently been published (Ibarra-Sánchez et al., 2022).

Indeed, LPNs represent the most successful type of nanocarrier for lung delivery so far developed considering the formulations that have been approved and reached the clinical use. LPNs are characterised by a lipid coating of phospholipids, cholesterol, or surface modified lipids with a therapeutic agent inside the vesicle. Over the decades, studies have been performed to improve their profile and two new categories have been created: solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NCLs) (Figure 1.5). Compared to liposome which are characterised by a lipid bilayer with an aqueous core, SLNs and NCLs have a hydrophobic core which allows the encapsulation of hydrophobic therapeutics. The aerodynamic diameter of LPNs can vary from 100 nm to larger NPs (up to 2.5 µm in the case of liposomes). Due to their main component being represented by physiological lipids, they are considered highly biocompatible. This drug delivery system has been demonstrated to be safe in a murine inhalation model when administered as repeated inhalation exposure at concentration lower than 200 µg deposited dose (Nassimi et al., 2010).

The progress achieved by researchers in the field of LPNs is indeed represented by the first liposomal-based formulation approved by FDA. In 1995 Doxil®, a PEGylated liposome-encapsulated doxorubicin formulation, was approved for the treatment of a wide range of cancers. However, this was for intravenous administration whereas the first true inhaled liposome-based formulation was approved in 2018. This was the case of Arikayce®, Insmed Inc., an inhaled (delivered via nebulisation) liposome amikacin

35 formulation for the treatment of Mycobacterium avium complex infections. The
36 pulmonary administration of the encapsulated amikacin showed increased antibiotic
37 exposure in the lungs compared to the effect obtained from the intravenous administration
38 of the free amikacin (Zhang et al., 2018). When amikacin was delivered by nebulisation
39 to rats in the liposome-encapsulated form, the drug exposure induced in lung tissue,
40 airways and pulmonary macrophages was respectively ~ 42-, 69- and 274- fold higher
41 compared to intravenous amikacin administration which provided very poor distribution
42 of the drug in the same target areas. This clearly described the potential and the
43 improvements that the nanoplatform provides over the conventional free drug
44 formulation in the treatment of the infection caused by the non-tuberculous
45 mycobacterium disease (Zhang et al., 2018).

46

47 The uniqueness of the LPNs is also represented by their chemical nature which allows
48 the encapsulation of a wide range of drugs, both hydrophilic and hydrophobic
49 therapeutics as well as ionisable cationic lipid. In addition, the small size they possess
50 promotes their dissolution into the lung lining surface, whereas their composition makes
51 them highly biodegradable and biocompatible. Extensive reviews highlighted the
52 advantages and disadvantages of the LPNs as well the milestones during the LPNs
53 development towards their success in the clinical use (Li et al., 2022; Rudokas et al.,
54 2016).

55 Despite the preliminary stage at which the investigation of albumin NPs for pulmonary
56 delivery is at, the success gained by different type of nanocarriers mentioned above
57 represents an important milestone for the proof of concept for nanocarrier based
58 therapeutics over conventional formulations via the pulmonary route. The combination
59 of the nanocarriers' benefits over the conventional formulations and of pulmonary
60 delivery over other routes of administration provide the rationale behind the experimental
61 work described in this thesis.

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1.4 Design considerations for albumin nanocarriers for pulmonary delivery: improving lung deposition

1.4.1 Appropriate particle size for lung delivery: design of nanoparticle embedded in microparticles (NEMs)

In the last decade, innovation has happened in the field of pulmonary delivery. The benefits of controlled and targeted delivery to the lungs are clear, but the development of new formulations is complicated by the challenges that must be overcome. The success of an inhalation therapy depends on many factors with the aerodynamic size of the particles being one of the most important of them (**Figure** 1.6). It is now well known that particles with an aerodynamic diameter larger than ~ 5 µm will likely deposit in the conducting region of the airways as the oropharyngeal region. In the respiratory tract particles will be subject to clearance mechanisms and eliminated very efficiently by cough or sneezing. In alternative, particles can also be swallowed and go through the gastrointestinal tract, binding to undesired targets which will result in side effects as previously mentioned in the case of PDE4 inhibitors (Robichaud et al., 2002).

Smaller particles (1 – 5 µm) will deposit predominately in the lower airways and thereby avoid mucocociliary clearance. If macrophages clearance is also avoided, the encapsulated therapeutic will be released and exert the desired pharmacological effect. Particles with diameter less than 1 µm will more likely be exhaled as they would remain in the airstream without being deposited. Physical and aerodynamic size-related mechanisms that dictate the deposition of the particles in the airways will be further discussed in Chapter 6.

With further decrease of the particle size (< 0.5 µm – ultrafine particle), lung deposition may increase (Chalupa et al., 2004; Heyder, 2004; Jaques & Kim, 2000). However, this does not apply to individual NP as such with a size within 100 – 200 nm, which interestingly will more likely be exhaled rather than deposited into the lungs. In addition, due to their high surface area and the strong interparticle cohesive forces they tend to aggregate which reduces their stability and dispersability – hence lack of their successful development as therapeutic inhaled dosage forms (Bailey & Berkland, 2009).

101 It is evident how the need for respirability makes NP design for pulmonary administration
102 challenging and how an advanced formulation strategy is required. Alternative
103 approaches that have been suggested to exploit their benefits by enabling effective
104 pulmonary administration are the so-called Trojan particles and the nanoparticle
105 embedded in microparticles (NEMs). The Trojan particles consists of a large porous
106 carrier in the form of a powder containing NPs and allowing the NPs to be delivered in
107 the lungs. This system possessed both the benefits of the large porous particles and those
108 of the NPs with a resulting ease of flow, good aerosolization and drug delivery potential.
109 The NEMs consists of a nanocomposite system obtained by inclusion of drug-loaded NPs
110 within a microparticle system, using an inert carrier (excipient). This drug delivery system
111 after deposition in the humid lung environment allows the dissolution of the matrix and
112 the further release of the NPs containing the payload (**Figure** 1.6). This approach will be
113 adopted in this thesis and albumin-based NPs converted into mannitol-based NEMs as
114 formulation strategy to improve the respirability of RFM-HSA-NPs..
115
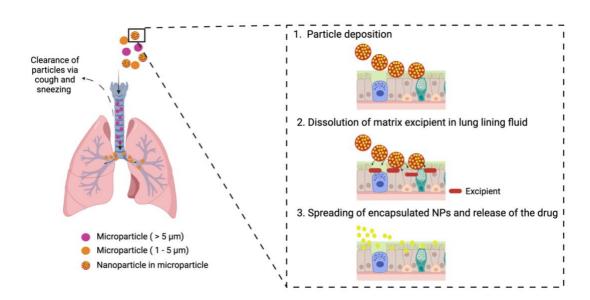
116 *Figure 1.6 Site of deposition of inhaled particles based on the aerodinamic diameter*
117 *and mechanims of NPs delivery through aerosol of nanoparticle embedded*
118 *microparticles.*

119

120

1.4.2 Selecting a nanocarrier based formulation and a suitable inhalation device for pulmonary delivery

In addition to the pharmacological efficacy of the drug itself and the aerodynamic size of the designed formulation, potential NPs must also be compatible with the devices available on the market, especially in view of a potential translation to clinical use (Biddiscombe & Usmani, 2018; Cazzola et al., 2020) . There are four different types of pulmonary inhalation pharmaceutical dosage forms on the market which differ for several factors as the effort needed by the patient, the size of the aerosol obtained, the required drug formulation - if solid (powder) or liquid (solution or suspension). Those are nebulisers, pressurized metered dose inhalers (pMDI), dry powder inhalers (DPI) and soft-mist inhalers (SMIs) (**Table** 1.7).

The above-mentioned approach based on the NEMs represents a valuable formulation strategy to improve the respirability of a NPs-based powder aerosol (Sung et al., 2007). To convert NPs suspension into a dry powder formulation with good aerodynamic characteristics (NEMs system) spray drying is often used. This process involves different steps carefully controlled by specific temperature and pressure values. It starts with the atomisation through a nozzle of a feedstock solution followed by the rapid evaporation of the liquid components of the droplets into solid powder in a heated chamber. The feedstock solution is represented by a mixture in variable ratio of the NPs suspension with an excipient. Commonly used excipients are sugars (lactose, mannitol, trehalose), synthetic polymers (polyethylene glycol), surfactants or amino acids (Alhajj et al., 2021). Mannitol, a polyol, is widely used to develop inhalable spray dried microparticles from NPs and its superiority over trehalose and L-leucine in the spray drying of albumin NPs has been reported (Patel, 2018; Varshosaz et al., 2015).

The potential and the feasibility of the conversion of albumin NPs into NEMs as a delivery strategy to achieve their pulmonary delivery has been reported in the literature. For example, Papay and others (Pápay et al., 2017) investigated the potential as inhaled carrier of a drug powder formulation of bovine serum albumin (BSA)-apigenin-NPs. The NPs were spray dried with leucine and lactose and aerodynamic characterisation was carried out. Both the formulations showed good fine particle fraction (FPF) values which ranged between 58% and 66% for lactose-based and L-leucine formulations respectively suggesting that the particles had good potential to deposit into the lungs. Within the

155 excipients tested, the use of L-leucine resulted in the powder with the most suitable mass
156 median aerodynamic diameter (MMAD) (2.1 µm).

157

158 FPF as well as MMAD and geometric standard deviation (GSD) are some of the
159 descriptors of the aerodynamic particle size distribution of an aerosol. The FPF
160 corresponds to the fraction of the drug mass in the aerosol cloud that has a size lower than
161 the cut-off size (usually considered 5 µm). Despite not corresponding to an exact
162 prediction of the *in vivo* behaviour of the powder, for which full aerodynamic particle
163 size distribution should be considered, FPF is a good parameter to describe the quality of
164 a formulation for inhalation. An interesting review about the advantages and
165 disadvantages of its use in the research and considerations about its application as a
166 predictor of both drug pharmacokinetic and clinical response has been recently published
167 (Newman, 2022). Further details on those parameters and their definitions will be
168 discussed in Chapter 6.
169 In addition, Varshosaz (Varshosaz et al., 2015) studied haloperidol targeted NPs of BSA
170 loaded with doxorubicin. The conversion of the nanocarrier into a NEMs was investigated
171 using three different excipients: trehalose, L-leucine and mannitol. Mannitol-based spray
172 dried powder showed the most favourable aerodynamic characteristics over the three
173 formulations with a MMAD equal to 4.58 µm and an FPF of 66%. A similar formulation
174 strategy was also employed for the pulmonary delivery of rifampicin (Joshi & Prabhakar,
175 2021), a first-line drug for the treatment of tuberculosis. Rifampicin-loaded BSA NPs
176 were first manufactured and then spray dried to obtain a dry powder inhaler formulation
177 which showed promising results in terms of respirability with a MMAD of 3.21 µm and
178 FPF equal to 52% (Joshi & Prabhakar, 2021).

179

180 The lung delivery of pharmaceutical powders obtained via spray-drying can be achieved
181 with two different types of inhalers: DPIs or pMDI (**Table** 1.7 Advantages and
182 disadvanategs of different type of inhaler devices). DPI are breath-actuated devices which
183 delivery efficiency is highly dependent on the inspiration flow rate. Their use does not
184 require coordination of actuation and inhalation hence are easier to use. Those devices
185 also differ for the number of doses than can be loaded and delivered, with i) single or ii)
186 multi-unit dose inhaler using one single gelatine capsule containing the drug of interest,
187 or multiple sealed blisters; iii) multi-dose reservoir with a bulk supply of powder that is
188 individually dosed at each actuation (Bailey & Berkland, 2009). Dry powders are

189 considered very stable, but their formulation is challenging as those micronised particles
190 possess a high surface energy which makes them cohesive with following aggregation
191 and poor flowability when delivered. As a result, there is the need to work on the balance
192 between the interparticulate forces in the bulk powder (Hoppentocht et al., 2014). In this
193 thesis the use of spray-dried rather than micronised particles may represent a promising
194 strategy to improve the stability of the formulation and facilitate its translation to the
195 clinic.
196 pMDIs usually contain a drug dissolved or suspended in a pressure-liquefied propellent.
197 Those devices are portable and compact and offer some advantages as multi-dosing and
198 consisten dosing. Chlorofluorocarbon were originally used in those formulations as
199 propellants; however, after the discovery of their role in the depletion of the ozone layer,
200 they were replaced by hydrofluoroalkane. After dispersion, the propellant evaporates
201 forming an aerosol of dry agglomerated or in situ-precipitated material. pMDIs are more
202 difficult to use as they require coordination between actuation and inhalation to provide
203 a good deposition.
204
205 Besides DPIs and pMDIs, nebulisers are devices that can convert a liquid into an aerosol
206 suitable for patient inhalation. This category can be further divided in jet, ultrasonic and
207 mesh nebuliser. Moreover, the latest technology developed is represented by the SMI
208 (Table 1.7). Mesh nebulisers (both static or vibrating) represents a fairly new category of
209 nebuliser and are operated by battery or electricity, are small and silent. As identified by
210 their name, those devices are characterised by the presence of a piezo element in contact
211 with a mesh which produce vibrations. The vibration of the mesh induces its deformation
212 into the liquid side which is pumped into the mesh holes. The ejected droplets can be then
213 inhaled by the patients. However, the efficiency of this device in terms of aerosol output
214 rate and inhaled fraction, defined as a percentage of nebulised volume loaded in the
215 reservoir, is influenced by the type of the loaded formulations. Viscosity of the
216 formulation can influence the nebulisation resulting in more or less fine droplets.
217 Blockage of the mesh aperture due to precipitation and crystallisation of drug particles
218 has been reported in case of highly concentrated drug suspension (Ari, 2014; Vecellio,
219 2006). On the other side, the device characteristics dictate the droplet size and their
220 distribution. SMIs is the newest type of inhaler, and one example is the Respimat
221 (tiotropium bromide). This is an easy and portable device which does not require

222 propellants. However, it needs to be primed if it has not been used for some days

223 (Pleasants & Hess, 2018).

Table 1.7 Advantages and disadvanategs of different type of inhaler devices

	Advantages	Disadvantages
Nebuliser	Convert liquid into aerosol	
Jet	• Easy to use	• Difficult to clean • Need compressed gas
Ultrasonic	• Easy to use	• Large residual volume • Degradation of heat-sensitive materials
Mesh	• Small and silent • Easy to use • Operated by electricity or power	• Aerosol output rate and inhaled fraction influenced by type of formulation loaded (e.g. viscosity)
Soft mist	• Portable • Multidose • Slow velocity aerosol • Long plume duration	• No breath-actuated • Need to prime if device has not been used in the past days
Metered pressurised dose inhaler (pMDI)	Drug dissolved or suspended in a pressure-liquefied propellent	
	• Portable and compact • Multi-dosing • Consistent dosing	• Coordination between actuation and inhalation required • Use of propellant
Dry powder inhaler (DPI)	Powder, breath-actuated device, delivery efficiency dependent on inspiration flow rate	
	• Coordination of actuation and inhalation not required > easier to use • Single or multi-dose deliverable • Formulation stability	• Dry powder may aggregate > poor flowability when delivered

224

Research into the nebulisation of NPs has most often concerned SLNs. Indeed, this is not surprising considering that as mentioned above those nanocarriers represent the most successful carrier that has reached the market. However, there have been some concerns regarding the negative effects that the nebulisation process may have on the structure of the nanocarriers. In fact, the latter must retain their physiochemical characteristics as well as the pharmacological activity of the payload in order to preserve their pharmaceutical acceptability. Therefore, studies should be performed to determine whether it is possible to produce an aerosol compatible with human use. This was the case of an investigation (Hureaux et al., 2009) which aimed to demonstrate that the encapsulation of paclitaxel in lipid nanocapsules and their nebulisation can potentially represent a promising pulmonary delivery strategy. The first part of this study aimed to determine which nebulisers can produce an aerosol of blank lipid nanocapsule unmodified in terms of size and polydispersity index compared to control. Six nebulisers were used: two jet nebulisers, two ultrasonic and two mesh nebulisers. Results showed that ultrasonic nebulisers were not able to produce the aerosol cloud, whereas jet nebulisers induce an alteration of the original particle distribution probably due to a destruction-rearrangement phenomenon correlated to the stress of the jet effect. In contrast, the mesh nebuliser resulted as the most suitable and it was used to further nebulise the formulation to investigate any changes in the drug payload and its pharmacological activity. Only a small reduction of the payload was reported with 1.65 mg/mL of paclitaxel in the not-nebulised formulation and 1.45 mg/mL in the nebulised volume, with the cytotoxic activity of the nebulised formulation similar to fresh paclitaxel nanocapsules.

In a different study investigating the effect of nebulisation on liposomes, Rudokas (Rudokas et al., 2016) indicated that the nebulisation and the ongoing shear forces impacted their physiochemical properties with resulting fragmentation of the lipid vesicles. Further investigation on liposome and on a possible strategy to overcome the impact of the nebulisation on the vesicle structure was conducted by Elhissi (Elhissi et al., 2007). Improvements in their stability when nebulised was achieved with the use of mesh nebulisers with a bigger pore aperture (Aeroneb Pro - 8 µm) over the air-jet nebuliser (Pari LC Plus) or the use of cholesterol in liposome formulations. Moreover, the effect of nebulisation on polymeric NPs was instead reported by Dailey (Dailey et al., 2003) in which research the use of different type of nebulisers (jet, ultrasonic and piezo-electric crystal nebuliser) influenced the NPs aggregation during the nebulisation.

1.5 Fate of inhaled NPs and microparticles: what happens to pulmonary delivered carriers?

Despite the advantages that the pulmonary route offers, current inhalation therapies are characterised by low availability and short half-life due to clearance mechanisms that take place in the lungs which are usually aimed to protect the lungs from foreign particles entering the body (El-Sherbiny et al., 2015). The type of clearance mechanism that the inhaled particle will undergo depends on the site of deposition. The respiratory tract can be divided into two regions: the upper respiratory tract (including nose, nasal cavity and pharynx) and the lower respiratory tract (larynx, trachea, bronchi, alveoli and lungs). Overall, soluble particles deposited in both the conducting airways and alveolar region will undergo dissolution followed by clearance of their components in solution according to pharmacokinetic principles; whereas insoluble particles will be eliminated by mucociliary escalator if deposited in the conducting airways and phagocytised by alveolar macrophages which will then transport the particles towards the mucociliary escalator (Oberdörster, 1993).

The main clearance mechanism that takes place in the upper region of the respiratory tract is called mucociliary escalator. Cilia and secretory cells are the two main components of this innate mechanism defence. The role of the cilia is to beat in a synchronised manner therefore moving the mucus and the entrapped particles to the pharynx to be swallowed; the secretory cells are responsible for the secretion of the mucus. The airway surface mucus layer is responsible for particle entrapment and overlies a periciliary layer, which allows the cilia to beat rapidly due to its low viscosity. The clearance rate is dependent on both the cilia and the lung lining layer with a faster rate in the central airways and slower with the increase of the airway generation. When particles larger than 6 µm are inhaled, the combined activity of those two cell types results in the particles entrapment in the mucus layer and following elimination via swallowing or coughing due to the cilia movement. The rate of the cilia beating, and the mucus composition may be severely altered in presence of airways diseases, consequently altering the clearance mechanisms of inhaled particles and their deposition (Bustamante-Marin & Ostrowski, 2016; Munkholm & Mortensen, 2014).

68   Inhaled particles smaller enough to escape the mucociliary clearance, cough and sneezing
69   may be eliminated from the lungs via macrophage clearance. The alveolar and the airway
70   macrophages are considered as the first line of cellular host defence. Macrophages are
71   derived from monocytes and are present in large number in the lungs. Based on the
72   anatomical location, four different type of macrophages can be distinguished (Geiser,
73   2010): *pleural macrophages*, are moderately phagocyte and responsible for the influx of
74   polymorphonuclear leukocytes (and consequent interleukin- 6 release) during
75   inflammation; *intravascular macrophages*, rare in humans and rodent, are highly
76   phagocytic and tend to form complexes with the endothelium; *interstitial and surface*
77   (alveolar and airway) *macrophages* are well known for their role in the clearance of
78   particles from the lungs. Interstitial macrophages are key in the release of mediators
79   because of their location in direct contact with the interstitium. Because of their location
80   on the surface of the lungs, surface macrophages are mainly involved in the removal of
81   inhaled particles and in innate immune responses such as chemotaxis. Due to their crucial
82   location on the inner surface of the airways, beneath the surfactant layer and immersed in
83   the lung lining layer, the airway and alveolar macrophages play a primary role in the
84   clearance of particles from the lungs. Usually, macrophages quickly respond to a particle
85   insult, whereas their timing is dependent on the species. The surface macrophages are the
86   cells usually recovered with bronchoalveolar lavage techniques and represent > 97% of
87   recoverable cells from rodents except for guinea pigs, which bronchoalveolar lavage fluid
88   (BALF) was reported to have a resident population of eosinophils (Warheit & Hartsky,
89   1993).
90   The macrophages mediated phagocytosis of micrometre-sized particles appears
91   consistently different from that of nanometre-sized particles. In the context of nanometre-
92   sized carrier, Geiser and others (Geiser et al., 2008) evaluated the deposition of inhaled
93   ultrafine TiO_2 particles (20 nm) in the lungs of rats. They found that the surface
94   macrophages clearance was negligible and sporadic and that at 24 h after inhalation only
95   0.1% of the deposited particles were taken by macrophages, hence concluding that
96   macrophages clearance is negligible in the peripheral lungs where the NPs were
97   hypothetically deposited. However, in contrast, Takeneka and co-workers (Takenaka et
98   al., 2012) showed that after inhalation macrophages engulfed 16-nm Au NPs, concluding
99   that most of the macrophages analysed from the pellet of BALF on day 0 had internalised
100   gold NPs and that this content decreased on day 7 after the inhalation day.
101

102   Considering how the clearance mechanisms can be altered in presence of airways diseases
103   (Gohy et al., 2019; Munkholm & Mortensen, 2014b), it is interesting also to mention an
104   additional study where the cellular uptake of 21-nm Au NPs was evaluated both in a
105   COPD and wild type mice model (Geiser et al., 2013). Particles were mainly found
106   attached on the epithelial surface in form of singlets or small aggregates (< 100 nm) or
107   engulfed by macrophages. At time 0 h after the inhalation, 24% and 58% of the NPs were
108   found in macrophages in the COPD and wild type animal model respectively, whereas
109   the percentage increased in both models at 24 h time point after inhalation. Overall, the
110   NPs uptake by surface macrophages was less efficient in the COPD model compared to
111   the 'healthy' model.
112
113   The time course of the particle uptake mediated by the surface macrophages has been
114   evaluated in healthy humans exposed to a bolus inhalation of radiolabelled sulfur colloid
115   aerosol (0.2 µm colloid size delivered in 6 µm droplets) targeting the central airways
116   (Alexis et al., 2006). Besides radiolabelled particle inhalation, induced sputum was used
117   as technique to examine the particle uptake. Indeed, the use of the induced sputum
118   technique allowed the recovery of cells in the central airways which in the case of healthy
119   patients it is mainly composed of surface macrophages and neutrophils. Results showed
120   that the particle uptake was minimal 40 min after inhalation and maximal (31%) at 100
121   min. Interestingly, the increased macrophage uptake recorded at 100 min corresponded
122   to an increased number and proportion of macrophages, whereas this was not reported for
123   the earlier time point (40 min). This difference was considered a consequence of the time
124   needed to recruit a new population of macrophages after the particle deposition; in
125   addition, they also concluded that consistent particle uptake can only occur in presence
126   of a critical number of macrophages which is higher than the number of macrophages
127   available 40 min after inhalation (Alexis et al., 2006).
128
129   The clearance kinetics reported in humans by Alexis (Alexis et al., 2006) was similar to
130   the kinetics reported by Geiser in Syrian Golden hamster in an earlier study (Geiser et al.,
131   1994). Animals were exposed to an aerosol of 6 µm polystyrene particles and lungs
132   fixated 20 min, 40 min and 24 h after the beginning of the inhalation. They found that 40
133   min after the inhalation, the number of macrophages was increased between 2- and 3-
134   fold whereas the increase was 1.5- and 2.5- fold higher after 24 hours, concluding that
135   the airways macrophages are rapidly (as soon as 40 min) recruited to the site of deposition

64

after the particle exposure. An additional study conducted on hamsters (Geiser et al., 1990) also reported that 15 - 20 min after the end of the aerosol exposure, about 29% of the particles have already been taken by macrophages, whereas overall > 87% of particles were taken after 24 h. The latter study was carried out in hamsters exposed to an aerosol of monodisperse polystyrene microbeads of 5.85 µm diameter and particle deposition was evaluated by fixation of the lungs immediately after aerosol exposure or 24 h after inhalation.

Overall, the retention time of nano and microparticles will depend on the site of deposition and on the cells that the particles will encounter. The retention will be shorter if the particles deposit in the conducting airways due to the high efficiency of the mucociliary clearance and of the cough, whereas the retention time will increase with the increase of the airways generation due to the reduced velocity of the mucus.

1.6 Aims and scope of the thesis

The aim of this thesis was to investigate the potential of HSA-NPs as nanocarriers for lung delivery using RFM, a PDE4 inhibitor, as payload. This included first the development of *in vitro* models suitable to investigate the NPs fate, the release of the payload and the conversion of the NPs into a respirable microparticle formulation for potential clinical translation. In addition, it included the optimisation of *in vitro* and *in vivo* inflammatory models using lipopolysaccharide as trigger agent to test the potential anti-inflammatory activity of RFM as inhaled therapy.

Specific objectives included:
- Manufacture and characterisation of roflumilast -HSA-NPs (RFM-HSA-NPs)
- Conversion of NPs suspension into a respirable powder formulation
- Investigation of the anti-inflammatory activity of inhaled RFM *in vitro* and *in vivo*

1.7 Research questions and objectives

Chapter 2: Can RFM-HSA-NPs be successfully manufactured and release the payload in an *in vitro* model of lung lining fluid?
- Optimise suitable RFM-HSA-NPs manufacture and characterisation methods

169   • Design of relevant *in vitro* models to investigate the NPs degradation and the drug
170   release from the nanocarrier

171

172   **Chapter 3: Can RFM-HSA-NPs be converted into a nanoparticle embedded**
173   **microparticles (NEMs) system for pulmonary delivery?**
174   • Manufacture of a NEM formulation using a spray-drying method
175   • Evaluate the properties and respirability of the obtained spray-dried powder: *in*
176   *vitro* aerodynamic assessment

177

178   **Chapter 4: Does RFM exert an anti-inflammatory activity in an LPS-challenged**
179   **bronchial epithelial cell *in vitro* model?**
180   • Establish and optimise an *in vitro* inflammation model using bronchial epithelial
181   cells and comparing different LPS concentrations and exposure regimes to
182   produce a robust inflammation model
183   • Apply the designed model to test the anti-inflammatory activity of RFM as
184   preventive treatment

185

186   **Chapter 5: Do LPS-challenged guinea pigs represent a valuable *in vivo* model to**
187   **mimic COPD exacerbations and demonstrate anti-inflammatory drug action?**
188   • Optimise an LPS inflammation whole-body chamber model in guinea pigs
189   • Validate the applicability of the model to demonstrate anti-inflammatory drug
190   effects via the oral administration of RFM

191

192   **Chapter 6: What is the potential of inhaled albumin-based NPs for topical lung**
193   **delivery of therapeutics?**
194   • Manufacture and characterise a suspension suitable for *in vivo* lung delivery of
195   RFM to investigate inhaled activity
196   • Perform aerodynamic characterisation of the above-mentioned suspension to
197   validate the quality of the aerosol
198   • Evaluate the anti-inflammatory activity of nebulised RFM in an LPS-challenged
199   guinea pig model
200   • Investigate the impact of nebulisation using a mesh nebuliser on the
201   physiochemical properties of RFM-loaded and blank HSA NP.

CHAPTER 2: Manufacture and characterisation of

roflumilast albumin nanoparticles (RFM-HSA-NPs)

2.1   Introduction

Albumin NPs are a colloidal system that can be produced using simple manufacturing methods and have good physical stability during storage. Due to the high content of charged amino residues, albumin NPs are a good delivery system for both positively and negatively charged molecules. The NPs matrix system allows a sustained delivery when delivered to the target site. Albumin can be obtained from different sources including egg (ovalbumin, OVA), bovine serum (bovine serum albumin, BSA) and human serum (human serum albumin, HSA). Being an endogenous protein, albumin is biocompatible as demonstrated by its use in licensed medicines (Spada et al., 2021).

Serum albumin is the most abundant protein in the bloodstream, and it has important physiological functions as the maintenance of the osmotic pressure and the transport of vitamins, hormones and drugs. Albumin is characterised by three domains (I, II, III), each of them comprised of two subdomains (A and B). The subdomains IIA and IIIA contain two hydrophobic cavities (named Sudlow site I and Sudlow site II) which are the most important binding sites for ligands (Dockal et al., 1999) (**Figure** 2.1).
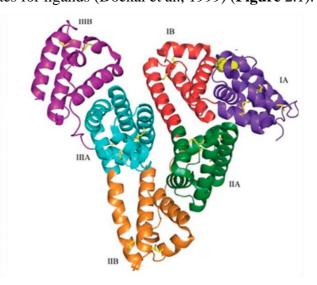
Figure 2.1 Structure of human serum albumin (HSA). Adapted from Spada et al., 2021

HSA is made up of a single chain of 585 amino acids and it is synthetised by the hepatocytes in the liver. It is the most abundant plasma protein, but its majority is stored in the interstitial space. Its biological half-life is of 19 days, despite it can last only 16-18

24   h in circulation. HSA can bind several molecules, both endogenous (as bilirubin, fatty

25   acids, vitamins) and exogenous (molecules as anti-inflammatory or antibiotics drugs),

26   therefore representing a valuable carrier (Spada et al., 2021).

27

28   Regarding the primary structure, BSA contains only 583 amino acids, and its molecular

29   weight differs only by less than 1% compared to HSA's molecular weight. BSA is a

30   globular protein as HSA, and it is negatively and positively charged at neutral and acidic

31   pH respectively. BSA can bind both positively and negatively charged compounds due to

32   the presence of both positive and negatively charged amino acids on its surface. It is a

33   versatile carrier due to its water-solubility, high-loading capability and therefore its

34   ability to bind both hydrophobic and hydrophilic drugs. One difference between those

35   two types of serum albumin is the number of tryptophan residues which are responsible

36   for the fluorescence of the molecule, often used in spectroscopic assays for ligand-binding

37   affinity studies (Ketrat et al., 2020). Due to its low cost, BSA is widely used in research

38   studies as drug carrier. However, its administration in humans is hindered by its

39   immunogenicity linked to its molecular state. The antibody response is driven by the

40   aggregate form and not by the monomer form.

41

42   OVA is extracted from the egg white of which is the main protein. It is composed of a

43   single chain of 386 amino acids, and it has a molecular weight of 42-47 kDa. Its functions

44   are still unknown and due to its immunogenicity, it is often used as antigen rather than as

45   drug carrier for the manufacture of NPs (Spada et al., 2021)

46

47   **2.1.1   Preparation techniques for albumin nanoparticles manufacture**

48

49   Over the years, this topic has been well reviewed concerning the different available

50   techniques but also the optimisation of the process to obtain a reproducible and simple

51   manufacturing method that produces consistent physiochemical particle characteristics.

52   The available mechanisms for manufacture of albumin NPs are desolvation,

53   emulsification, thermal gelation, self-assembly, nano-spray drying and nab-technology.

54   Error! Reference source not found. presents a summary of the techniques and their

55   advantages and disadvantages. Detailed description of the manufacturing methods can be

56   found in several reviews (Elzoghby et al., 2012) (Verma et al., 2018) (Lohcharoenkal et

57   al., 2014) (Tarhini et al., 2017) (Sleep, 2015) (Karimi et al., 2016), (Sundar et al., 2010),

58 original reports (Joshi et al., 2020), (S. H. Lee et al., 2011), (Galisteo-González & Molina-
59 Bolívar, 2014) (Pinto Reis et al., 2006) (Moinard-Checot et al., 2006), (Jahanban-
60 Esfahlan et al., 2016) and book chapters (Elzoghby et al., 2015), (Otagiri & Chuang,
61 2016; Pathak & Thassu, 2016).
62

Table 2.1 Advantages and disadvantages of albumin nanoparticles manufacture methods available in the literature.

Albumin nanoparticle manufacture method	Advantages	Disadvantages	References*
Desolvation	o Easy and highly reproducible o No need of specific device	o Use of organic solvent o Use of crosslinker	(Galisteo-González & Molina-Bolívar, 2014; Jahanban-Esfahlan et al., 2016; Kouchakzadeh et al., 2014; Langer et al., 2003; Sadeghi et al., 2014; Tarhini et al., 2017; von Storp et al., 2012; Weber et al., 2000)
Emulsification	o Easy process	o Residual organic solvent and oil phase o Thermal stabilization only for not heat sensible molecules o Need of surfactant	(Desai, 1999; Lomis et al., 2016a; Müller et al., 1996; Patil, 2003)
Thermal gelation	o Good to obtain albumin nanoscale hydrogels	-	(Yu Shaoyong, Yao Ping, Jiang Ming, 2006)

Self-assembly		-	(Gong et al., 2009)
	o High degree of drug loading		
Nano-spray drying	o Suitable for sensible molecules	o Need of specific device	(Arpagaus, 2018; S. H. Lee et al., 2011)
Nab-technology	o Safe and solvent-free o Suitable for intravenous administered nanoparticles o Polymeric material or surfactant not required	o Need of specific device	(Desai, 2011; Fu et al., 2009; Hawkins et al., 2008)

63   * Selected articles describing the manufacturing methodology and optimisation in detail

1 Of these methods, two of the most widely used, easily reproducible and not requiring

2 specific technology will be explored in more detail: desolvation, which is the method of

3 choice in this experimental work, and the emulsfication process. These two methods will

4 be compared, with an emphasis on the method optimisation regarding reproducibility and

5 particle properties (**Figure** 2.2).

6
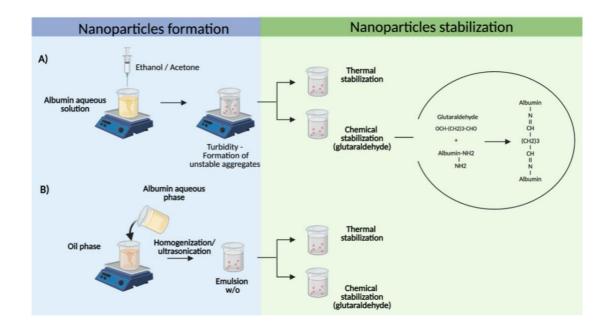
Figure 2.2 Illustration of the main steps in the desolvation (A) and emulsification
(B) manufacturing methods.

7 **2.1.2   Crosslinking techniques**

8

9 Cross-linking is an essential step for NPs preparation, and it influences the drug release

10 as well as the carrier biodegradability. There are some chemical cross-linkers that include

11 the use of molecules like glutaraldehyde, but also glucose, ascorbic acid, tannic acid,

12 citric acid, and sorbitol; alternatively, physical crosslinking methods include heating and

13 ultraviolet (UV) irradiation (Amighi et al., 2020; Niknejad & Mahmoudzadeh, 2015).

14

15 *Glutaraldehyde (GLU)* is the molecule adopted experimentally in this chapter. GLU is

16 included in aqueous solution and various concentration are reported in the literature as

17 effective cross-linking methods. The amount of crosslinker to use is determined by the

18 desired degree of crosslinking of the NP system. The stabilisation is effected by a

19 condensation reaction between the aldehyde group of GLU and the amino moieties of the

20   albumin, forming a Schiff base (D. Zhao et al., 2010). In the case of NPs obtained by the

21   emulsion method, the chemical stabilisation is based on the addition of GLU to the first

22   emulsion or on the resuspension of first emulsion in ether containing formaldehyde or

23   2,3- butadiene. Alternatively, the first emulsion is added dropwise to heated oil phase

24   under constant stirring to evaporate the aqueous phase. Different temperatures are

25   reported in the literature. Weber and co-workers (Weber et al., 2000) performed a

26   systematic study focussing on the relationship between the use of different amount of

27   GLU for cross-linking and the resulting number of available amino groups on the surface

28   of HSA NPs after cross-linking. Amount of GLU from 0 to 200% of the theoretic amount

29   needed to the quantitative cross-linking of the total number of amino groups available in

30   the HSA molecule (equal to 59) were employed. Results showed that the number of

31   available amino groups on the NP surface decreased with GLU percentages from 40 to

32   200%, with the degree of crosslinking of NPs increasing with the increase of the GLU

33   concentration. The use of percentage of GLU lower than 40% produced unstable NPs

34   with an unexpectedly low number of amino groups on the NPs surface, hence concluding

35   that stable NPs will be formed with GLU concentration not lower than 40% of the

36   theoretical amount of GLU to cross-link the total number of amino groups available.

37

38   However, some concerns have arisen about the toxicity of GLU. As an alternative

39   Niknejad and others (Niknejad & Mahmoudzadeh, 2015) evaluated methods including

40   ultraviolet (UV) irradiation in combination or not with glucose. Their data support the

41   idea that a combined UV and glucose technique can be a promising alternative to GLU,

42   producing same size NPs as those produced by GLU crosslinking. The effect of different

43   crosslinking agents in the preparation of BSA NPs was well described by Amighi

44   (Amighi et al., 2020).

45

46   *Heat stabilisation.* As an alternative to the use of a cross-linker, heat stabilisation has also

47   been investigated. The incubation of particles (obtained by desolvation method) for less

48   than 48 hours at 50°C and at 60°C for less than 24 hours did not sufficiently stabilise the

49   colloidal system, confirming that albumin resists cross-linking after being heated at 60°C

50   for ten hours. Heating for two hours at 70°C however showed particles were cross-linked

51   (Weber et al., 2000). Interestingly, in contrast to cross-linking by GLU, the heating does

52   not change the number of amino groups available on the albumin surface. This could be

53   explained by heating process only affecting the internal functional groups of albumin.

54   Heat leads to the formation of amide groups between amino and carboxyl groups of the
55   protein whereas cross-linking involves the reaction of two amino groups of the protein
56   by the aldehyde.

57

58   **2.1.3   Determinants for a controlled size, PDI and zeta potential NPs population**

59

60   The high reproducibility of the desolvation and emulsification processes reflects that the
61   critical processing factors can be controlled to obtain a consistent size, with a narrow
62   particle size distribution and consistent zeta potential. NPs' characteristics obtained by
63   desolvation can be controlled varying GLU concentration, the rate of addition of
64   desolvating agent and its ratio, albumin concentration and pH of albumin aqueous
65   solution (**Table** 2.2). Extensive studies of NPs determinants for controlled size,
66   polydispersity index (PDI) and zeta potential were conducted by J. M. Gallo (Gallo et al.,
67   1984) and B.G. Müller and co-workers (Müller et al., 1996) and by L. Yang (L. Yang et
68   al., 2007).

69

70   *Glutaraldehyde concentration*. Weber (Weber et al., 2000) reported that the GLU
71   concentration and the denaturation time do not affect the size of NPs. Langer (Langer et
72   al., 2003) reported the same results after a study that involved the use of different
73   concentrations of GLU (40, 100 and 200%) of the theoretical amount needed to
74   quantitatively crosslink the available amino groups on the albumin surface. On the other
75   hand, the GLU concentration affects the zeta potential. The increased concentration of
76   GLU reduces the positively charged amino groups and increase the negatively charged
77   carboxyl groups, with a consequent decrease of zeta potential with increasing GLU
78   concentration.

79

80   *Desolvating agent.* The volume of desolvating agent is positively correlated with particle
81   size when the volume is up to 1.5-fold the initial volume of albumin aqueous solution.
82   This effect does not persist with further increase of the ethanol volume. The increase of
83   ethanol volume also positively affects the particle concentration. Langer and co-workers
84   (Langer et al., 2003) confirmed that the amount of desolvating agent controls the particle
85   size, but also that the variability at any given volume was high. Therefore, a controlled
86   addition method was developed, switching from the manual addition to a pump-
87   controlled system consisting of a syringe and a specific gauge needle.

88   *pH of aqueous solution.* It is also widely reported (Langer et al., 2003) how i) the pH

89   strongly influences the size of albumin NPs and ii) particle diameter decreases away from

90   the albumin isoelectric point (pI=4.9). Average particle diameters decreased from 275 nm

91   at pH 7.5 to 180 nm at pH 9. At pI of protein, the net surface charge is zero, therefore

92   there is no electrostatic repulsion and aggregates are formed only by non-specific

93   interactions. At pH below the pI, the protein carries a positive net charge and as the pH

94   deviates more positively from the pI, the net charge becomes more negative as the

95   carboxyl groups of the protein undergo greater deprotonation. The surface charge

96   provides an electrostatic repulsion that results in reduced aggregation and therefore

97   smaller particles (Amighi et al., 2020). Thus, the pH of the aqueous solution also affects

98   the stability of the particles and their zeta potential. For example, increasing the pH value,

99   the zeta potential reduced from - 40 to - 50 mV at pH between 7.2 and 10. Tarhini and

100 colleagues (Tarhini et al., 2018) showed a consistent positive zeta potential (15 mV) for

101 pH equal to 3 and at higher pH in the range 5 to 11 the particles drop to negative values

102 between - 18 and - 23 mV.

103

104 *Albumin concentration.* Albumin concentration also affects the size of NPs. Based on the

105 study performed by Amighi (Amighi et al., 2020), particle size and PDI increase when

106 the albumin concentration increases from 20 to 50 mg/mL and this is explained by the

107 nucleation theory, which can be generally defined as the first step in a self-assembly

108 process that allows a new structure to be formed. A detailed review of the mechanism of

109 nucleation in relation to the growth of NPs in solution is reported in the literature (Thanh

110 et al., 2014). Müller and colleagues (Müller et al., 1996) showed that the smallest particle

111 size when manufacturing albumin nanospheres via an emulsion process was obtained at

112 an albumin concentration of 5 to 20% w/v; no particle sizes below 300 nm cannot be

113 obtained with albumin concentration higher than 20% w/v.

114

115 *Aqueous to non-aqueous phase volume ratio.* It has a consistent and remarkable effect on

116 particles size. It was reported that no particle size below 300 nm can be obtained with an

117 aqueous ratio higher than 9% v/v (Müller et al., 1996).

118

119

120

Table 2.2 Factors affecting size and zeta potential of albumin nanoparticles.

	Effect on	
	Size	**Zeta potential**
Glutaraldehyde (GLU) concentration	No effect on size	Effect on zeta potential – increasing concentration of GLU reduced the positively charged and increased negatively charged groups with a following decrease of zeta potential
Desolvating agent	• Positive correlation when volume of desolvating agent is up to 1.5-fold initial volume albumin solution; • Positive correlation also on particle concentration	-
pH aqueous solution	Particle diameter decreases away from the albumin isoelectric point	Reduction of zeta potential with increasing pH values (7.2 – 10)
Albumin concentration	Size and polydispersity index increase with increasing albumin concentration (from 20 to 50 mg/ml)	-
Aqueous to non-aqueous phase volume ratio	Effect in particle size with no particle size < 300 nm achievable with a ratio > than 9% v/v	-

121

2.1.4 Degradation of albumin nanoparticles

The inclusion of trypsin or other proteases in drug release *in vitro* models is a useful tool to analyse how albumin NPs undergo degradation. This is of particular interest when considering the degradation of the albumin NPs and the drug release in a simulated lung environment. The use of trypsin to investigate albumin-based drug delivery system degradation has been the subject of a number of studies. Wartlick and colleagues (Wartlick et al., 2004) reported the incubation of HSA-NPs with trypsin and proteinase K as method for quantitative evaluation of particle degradation aiming to quantify the matrix-bound drug. Both the enzymes were found to be suitable to obtain fast and complete degradation of the particles within 60 min. They reported that the rate of degradation mediated by those two enzymes was dependent on the degree of cross-linking and that maximum degradation efficiency was obtained with a NPs degree of crosslinking equal to 40%. The effect of trypsin in the release medium was also investigated by Merodio and co-workers who found that the addition of trypsin did not have a significant effect on the release profile of ganciclovir (Merodio et al., 2000); in contrast the release profile of Rose Bengal was dramatically enhanced by the protease activity (W. Lin et al., 2001b) as well as the release of 10-hydroxycamptothecin (HCPT) from BSA-NPs (L. Yang et al., 2007).

Later, Langer and co-workers (Langer et al., 2008) performed a systematic study of the enzymatic degradation of blank albumin NPs in presence of trypsin, proteinase K, protease from bovine pancreas, pancreatin, pepsin and cathepsin B at different pH values. The aim was to study the enzymatic degradation of blank albumin NPs to investigate degradation under the assumption that it is a critical parameter for the release of the embedded drug. Those enzymes were employed also to assess the NPs biodegradability. Results showed that the degradation activity of trypsin was highly dependent on the crosslinking degree, with only 9% of NPs having 100% degree of crosslinking being degraded within 24 h. Specific attention was given to cathepsin B, because of its presence in the lysosomes of cells, which is where drug release from the NP matrix will occur if particles translocate to lysosomes after endocytosis and are therefore liable to degradation by lysosomal enzymes such as cathepsin. The fastest NPs degradation was seen under acidic pH, by pepsin and cathepsin B. Under neutral conditions, degradation was achieved over a longer timeframe of 24 h by trypsin, proteinase K and protease. However, NPs with 100% cross-linking degree were not fully degraded within 24 h by any of the

enzymes (trypsin, proteinase K, protease and cathepsin B), whereas quantitative degradation was possible with lower cross-linking degree NPs by multiple enzymes. It was concluded that trypsin, proteinase K, protease and pepsin were suitable to obtain overall a rapid degradation of albumin NPs and to investigate *in vitro* the release of the embedded drug. It is evident that the choice of the specific enzyme to be used should be also made based on the stability of the drug under acidic or neutral conditions and on the degree of the crosslinking of the NPs. These studies highlight clearly that protease degradation is an essential parameter also for the release of a drug from an inhaled formulation, and that it must be considered in pharmacokinetic studies.

In the context of how proteases affect the degradation of an albumin carrier deposited into the lungs and therefore its drug release rate, an improvement in the understanding of the albumin-based NPs fate after pulmonary administration was recently achieved by Woods and colleagues (Woods et al., 2020). Predominant proteases were quantified in BALF of healthy human volunteers. Four proteases model were defined: (i) trypsin only, where the trypsin concentration adopted mimics the total protease concentration found in BALF (100 µg/mL), (ii) healthy lung protease model, a complex model that combined cathepsin D, cathepsin H, dipeptidyl carboxypeptidase I/ angiotensin-converting enzyme and dipeptidyl peptidase IV, (iii) neutrophil elastase only to model the protease conditions in the lung during inflammation and (iii) inflamed lung protease model, based on the healthy model with the addition of neutrophil elastase. It is interesting to highlight how the presence of the proteases affected the degradation rate outcome in comparison with the incubation of particles in enzyme-free phosphate buffered saline (PSB). Results showed that particles remained intact for over 48 h in PBS, but degradation occurred more rapidly (50% reduction in 10 minutes) in the trypsin only model compared to the healthy model (50% reduction in 150 minutes). The inflamed model showed a more variable degradation profile. These studies illustrate the importance of considering the effects of proteases on the stability of NPs for the delivery to the lungs and to include the protease profile in predictive models.

It is therefore evident how the addition of a protease in a drug release or particle clearance model represents an important factor to make the model more faithful and reliable in terms of the lung simulated environment.

2.1.5   Drug loading into albumin nanoparticles

Albumin is known for its extraordinary binding capacity due to its highly adaptable binding sites. It can bind endogenous molecules, such as non -esterified fatty acids, thyroxine, hemin, and bilirubin (Peters, 1996), and drugs. In general, the loading of a drug in albumin NPs can be achieved either by encapsulation inside the NPs matrix or by interaction with the protein via numerous surface functional groups present on the albumin surface. Water soluble drugs can be loaded by either an incubation or incorporation method. The first is based on the incubation of the drug with the formed and hardened particles; the second on the incorporation of the drug in the albumin solution prior to NPs formation. Alternatively, the drug can be added to GLU solution before NPs formation and cross-linking. In contrast, water insoluble drugs are dissolved into the organic solvent if a desolvation method is used as in the context of this work. **Table** 2.3 summarises drugs that have been loaded in BSA or HSA-NPs according to the literature. The impact of the drug loading method on NPs characteristics was investigated by Merodio and colleagues (Merodio et al., 2000), who characterised three separate formulations where the drug was (a) incubated with the just formed albumin NPs, resulting in surface adsorption and little or no incorporation of the drug into the matrix; (b) added to an aqueous albumin solution; or (c) added to a solution of albumin and GLU prior the addition of desolvating agent and therefore formation of the carrier. They described that the lowest encapsulation efficiency was achieved by adsorption with 12.5 $\pm$ 1.5%, followed by the model c where drug was incubated with albumin and GLU with 20.0 $\pm$ 2.5%. The lower encapsulation efficiency in model c was attributed to a negative effect of GLU on the protein-drug interactions (Das et al., 2005; Merodio et al., 2000). The highest encapsulation efficiency at any specific drug/albumin ratio was achieved by model b with a value of 40.6 $\pm$ 3.3%. In agreement to Merodio, also Lin and colleagues (Lin et al., 2001a) reported loading differences between NPs produced by incubation of the drug with albumin prior to particles formation and by addition of the drug post particle formation for Rose-Bengal albumin NPs.

Merodio and colleagues (Merodio et al., 2000) also investigated the effect of the incubation time of the drug with the albumin NPs, albumin solution or albumin solution with GLU. The ganciclovir loading increased during the first four hours of incubation both in model (a) and (c), remaining constant despite further incubation. In contrast, the

101   drug loading behaviour for NPs formed using model (b) was characterised by a plateau

102   reached after 30 min without a further increasing in the next four hours of incubation.

103   Overall, the drug loading content and encapsulation efficiency can be influenced by

104   factors such as (1) affinity between drug and albumin; (2) drug solubility in water and (3)

105   drug/albumin ratio.

Table 2.3 Examples of drugs that can be encapsulated in albumin NPs described in the literature.

Drug encapsulated	Drug MW (g/mol)	Drug LogP	Manufacture method-crosslinking technique	Drug loading method	Albumin source	Encapsulation Efficiency (%)[†]	Reference
10-hydroxycamptothecin (HCPT)	364.4	0.6**	Emulsion-heat stabilization technique	-	Bovine	57.5	(L. Yang et al., 2007)
Aspirin	180.16	1.2	Desolvation - glutaraldehyde	Incorporation	Bovine	81.1	(Das et al., 2005)
Docetaxel & Quercetin	807.9 302.24	1.6** 1.5**	Desolvation - glutaraldehyde	Dissolution in organic solvent	Bovine	○ DTX-QT-BSA-NPs: 75.18 (DTX), 68.09 (QT) ○ DTX-BSA-NPs: 80.4 (DTX)	(Desale *et al.* 2018)
Ganciclovir sodium salt	277.21	-1.5	Desolvation - glutaraldehyde	Incubation, incorporation, and incubation with albumin and crosslinker	Bovine	12.5, 40.6, 20.0 [††]	(Merodio et al., 2000)

Methotrexate	454.4	-1.8	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl	Dissolution in organic solvent	Human	-	(A. Taheri et al., 2011)
Noscapine hydrochloride	449.9	-	Desolvation - glutaraldehyde	Incorporation	Human	97	(Sebak et al., 2010)
Obidoxime dichloride	359.2	-	Desolvation - glutaraldehyde	Incubation	Human	59.2 (adsorption efficiency)	(Kufleitner et al., 2010)
Paclitaxel	853.9**	2.5**	Desolvation-intramolecular disulfide bonds; Emulsion-solvent evaporation method; Desolvation-glutaraldehyde	Dissolution in organic solvent	Human	85.7; 81.80 – 97.96; 95.3	(Lomis et al., 2016a; Ruan et al., 2018; D. Zhao et al., 2010)
Docetaxel	807.9	1.6**	Self-assembly – Sodium tartrate or gluconate	Dissolution in organic solvent	Human	63.1	(Qu et al., 2019)
Rose Bengal	-	-	Desolvation – glutaraldehyde	Incubation, incorporation	Human*	-	(W. Lin et al., 2001a)

Sorafenib	464.8	4.1**	Desolvation - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl	Dissolution in organic solvent	Human	91.09	(Gao et al., 2019)
Tacrolimus	804.0	2.7**	Nab™-technology	Dissolution in organic solvent	Human	79.3	(Thao et al., 2016)
Colistin	1155.45	-2.4	Double-emulsion (W/ O/ W)	Dissolved in aqueous solution of Span83™ (1% w/v)	Human	98.65	(Scutera et al., 2021)

106 *Albutein

107 ** Values vary from the different sources used (PubChem and DrugBank).

108 [†] If multiple values of encapsulation efficiency are reported in the reference, only the one obtained by the optimal formulation and manufacture

109 method is reported in the table.

110 [††] Values are referred to a ratio of 250 µg ganciclovir/ mg initial protein.

2.1.6   Drug release from albumin nanoparticles

2

3   The method of incorporation of the drug into the carrier - whether being physically

4   entrapped within the cross-linked matrix, adsorbed on the surface, or chemically

5   conjugated to the particles -does not only affect encapsulation efficiency, but also drug

6   release. In general, drug release rate depends on factors like solubility of drug,

7   polymer/drug interactions, water absorption, drug diffusion through NPs matrix,

8   combination of erosion/diffusion process in the carrier (Mohanraj & Chen, 2006). A

9   detailed review of drug release mechanisms in poly (lactic-co-glycolic acid)-based NPs

10 is provided by Fredenberg and colleagues (Fredenberg et al., 2011).

11

12 Desai (Desai, 2011) classified NPs into four different groups depending on binding and

13 loading mechanisms of the drug which can be a) covalently or b) noncovalently linked to

14 a cross-linked albumin matrix; c) noncovalently adsorbed on the surface of crosslinked

15 albumin matrix; d) in a core coated by albumin when the drug is hydrophobic, and a nab-

16 technology (nanoparticle albumin bound technology) method is used. The latter is an

17 additional technique to manufacture albumin NPs. It is usually applied for poorly water-

18 soluble drugs, and it requires the formed drug-albumin NPs to go through a jet under high

19 pressure. **Table** 2.4 describes the correlation between method of drug loading (in presence

20 or absence of a crosslinker) and resulting *in vitro* drug release profiles for experimental

21 albumin NPs formulation reported in the scientific literature.

22

Table 2.4 Evaluation of correlation between drug loading methods in albumin NPs and resulting drug release profiles.

Drug	Drug loading method	Cross-linker	Drug release from NPs	Drug release protocol			Ref
				Method of experiment	**Release medium**	**Trypsin Y/N**	
<td colspan="8" align="center">**Biphasic release: initial burst release followed by sustained release over time**</td>
Docetaxel & quercetin	Incubation in organic solvent	5 µL of 25% GLU	Burst release up to 2 h for both drugs, followed by sustained release for up to 24 hours	Dialysis bag, 37 °C shaking at 100 rpm, 1 mL samples withdrawn periodically and replenished with equal volume of fresh media	PBS, pH 7.4 with 0.5% Tween 80, 24 mL	N	(Desale et al., 2018)
Gabapentin	Dissolved in albumin solution	100 µL of 4% GLU	Initial fast release due to the desorption and diffusion of the drug on the outer surface of the particles; the drug across the albumin matrix is released	Dialysis bag, 37 °C, shaking at 100 rpm; aliquots of 2 mL withdrawn and replaced with fresh medium	PBS, pH 7.4, 50 mL	N	(Wilson et al., 2014)

			slower in a sustained way up to 24h				
Oligonucleotides (PO)	PO-NPB Incubation with albumin solution	GLU: from 0.06 to 12.5 µg/mg	Initial burst (37% of drug released), followed by a continuous and slowed release reaching a cumulative release of 80% after 5 hours	Eppendorf tubes, NPs dispersed in different types of release media, shaking incubator at 37 °C, 60 strokes/min	PBS, pH 7.4 (0.05, 0.15 or 1 M) Citrate buffer 0.19 M (pH 3, 5, 6.5 or 7.4)	N	(Arnedo et al., 2002)
Paclitaxel	Dissolved in organic solvent and later added to albumin solution; three different initial concentration of drug tested (0.5 – 1.0 and 1.5 mg/mL)	None	Initial burst release by the first 12 h (cumulative % of drug released by 12 h inversely proportional to the initial drug concentration); continuous slow increase of drug release by 48 hours.	NPs incubated with PBS at 37 °C, shaking at 120 rpm, aliquots of 0.5 mL withdrawn and replaced with fresh release medium	PBS	N	(Lomis et al., 2016b)
Palladum complex (II)	Addition to drug complex to formed NPs	37.5 µL of 50 % GLU	A percentage of 36.7% of the release within the first 20 h due to the	Dialysis bag, 37 ºC, shaking at 170 rpm for 48 h, 2 mL	PBS, pH 7.4, 40 mL	N	(Karami et al., 2020b)

			adsorbed drug complexes on the NPs surface	release removed and replaced with fresh			
Ganciclovir	A) Addition and incubation (2h) of drug with hardened formed NPs B) Incubation of drug with HSA solution; GLU added to coacervates already formed C) Drug added to HSA solution containing GLU with following incubation	A)1.56 µg/mg HSA B) 1.56 µg/mg HSA C) 1.56 µg/mg HSA	Initial burst release followed by a step of slower release for all three NPs models over 5 days; however, the percentage of drug release over time differs within the three models due to the different fractions of drug on the surface of the NPs (initial burst release) or covalently linked to the protein matrix via GLU. The presence of trypsin slightly increases the amount of drug released for each model.	Dispersed NPs in PBS placed in eppendorf; at predetermined time point supernatant was collected after centrifugation; shaking at 37 °C – 60 strokes/min	PBS, pH 7.4	N and Y (9300 U.I.) bovine pancreas trypsin added to NPs in PBS	(Merodio et al., 2000)

			Sustained release overtime – no burst effect				
Docetaxel	Incubation with ethyl alcohol and mixing with hydrogen phosphate and pre-heated HSA	none	Sustained drug release over 48 hours	Dialysis bag, 37 °C, shaking at 100 rpm, 0.5 mL sample withdrawn and replaced with fresh medium	PBS, with 0.5% v/v Tween 80, 80 mL	N	(Qu et al., 2019)
Tacrolimus	nab-technology	none	Gradual release over 24 hours of tacrolimus content of HSA-NPs	Dialysis bag, 37 °C, 2 mL samples were withdrawn and replaced with fresh medium	PBS, pH 7.4 with 0.1% (v/v) Tween 80	N	(Thao et al., 2016)
Paclitaxel	PTX-PEG-HSA: drug cofused with PEG; mixing of the PTX-PEG solid dispersion with HSA solution PTX-HSA: dissolved in organic solvent	None	Steady and continuous release with no initial burst effect (35% and 53% of PTX released from PTX-HSA and PTX-PEG-HSA respectively over 4 days). Overall, the cumulative PTX released in 10 days is about 80%.	Dialysis bag, 37 ºC, shaking at 100 rpm over 12 days, 1mL aliquots withdrawn and replaced with fresh buffer	PBS, with 0.2% Tween 80, 150 mL	N	(Yin et al., 2015)

Monophasic release							
Phosphodiester oligonucleotides (PO)	PO-NPA: Incubation of PO with pre-formed albumin NPs	GLU: from 0.06 to 12.5 ug/mg	Complete and instantaneous release of almost the whole amount of drug adsorbed on the particles surface	Eppendorf tubes, NPs dispersed in different types of release media, shaking incubator at 37 C, 60 strokes/min	PBS pH 7.4 (0.05, 0.15 or 1 M) Citrate buffer 0.19 M (pH 3, 5, 6.5 or 7.4)	N	(Arnedo et al., 2002)

23

The three most common particles conformations in terms of albumin-drug structure and the resulting three general release profiles are illustrated in **Figure** 2.3. According to these variables, three different drug release profiles can be identified: A) drug release from cross-linked albumin NPs is generally biphasic, with an initial quick release of the drug, followed by a slow-release phase (Arnedo et al., 2002; Desale et al., 2018; Gao et al., 2019; Karami et al., 2020a; Lomis et al., 2016a; Merodio et al., 2000; Wilson et al., 2014). One fraction of the loaded drug is physically bound to the surface of the NP, easily being removed, therefore leading to the initial burst release. Another fraction is incorporated deeper into the NP matrix or covalently linked to albumin with cross-linkers, being removed more slowly, leading to a prolonged sustained release; B) sustained release overtime with no burst effect (Qu et al., 2019; Thao et al., 2016; Yin et al., 2015); C) monophasic burst release when albumin NPs are prepared *via* surface adsorption post-particle production and the drug is only physically adsorbed on the particles (Kufleitner et al., 2010) (Figure 2.3).
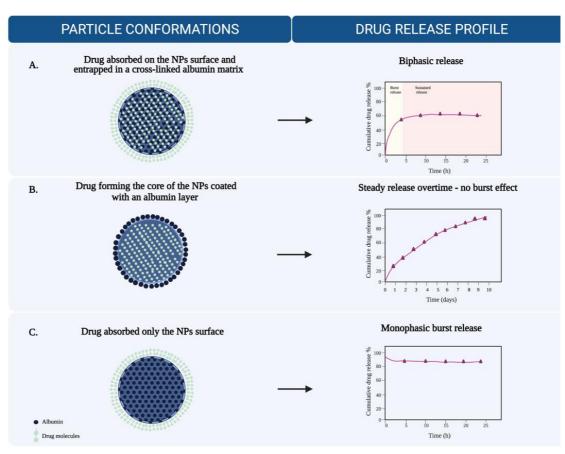
Figure 2.3 Schematic correlation between albumin nanoparticles conformations and drug release profiles.

90

2.2   Aims and objectives

The overall aim of the experiments described in this chapter was to investigate the development of a successful albumin-based drug delivery nanoplatform that can be converted into a respirable formulation. The target product profile for the nanoplatform is that it is able to i) encapsulate RFM; ii) undergo degradation using a clinically relevant model that is characterized by protease concentration comparable to the lung concentration; iv) release RFM in a sustained manner.

Specific objectives included:

1. Design a robust and reproducible lab-based manufacture technique for HSA NPs with small particle size, low polydispersity index and suitable encapsulation efficiency;

2. Use a suite of characterisation techniques to evaluate the physiochemical properties of the NPs;

3. Investigate the degradation rate of the NPs using a simplified *in vitro* model of proteases;

4. Establish and optimise a robust method for quantifying the drug release from the NPs overtime.

2.3   Methods

2.3.1   Manufacture of blank and drug-loaded albumin NPs via desolvation method

Blank and drug-loaded albumin NPs were manufactured using a desolvation method, schematically represented in **Figure** 2.4. Human serum albumin (HSA) (50 mg) was dissolved in 1 mL of Tris buffer HCl 0.01 M pH 8.9 and gently shaken for 10 min (shaker Model R100 Rotatest shaker Luckham) to allow complete dissolution of the powder. An aliquot (25 µl) of sodium hydroxide 1 N was added to the hazy albumin solution under gentle stirring to increase the pH to 9 prior to the ethanol addition. For blank (RFM-free) NPs, ethanol (4 mL) was added dropwise to the albumin solution under stirring (850 rpm, 1 cm magnet) until turbidity appeared and the NPs suspension appearance turned to milk colour. The change of the albumin solution colour from haziness to milky appearance corresponded to NP formation. For drug loaded albumin NP, roflumilast (RFM) (1 mg) was dissolved in the ethanol (4 mL). For both the formulations, controlled addition of

50   ethanol was achieved using a 10 mL syringe (without piston) attached to a 21 G needle.

51   The addition of the total volume of ethanol was performed in 1.25 mL increments at an

52   infusion rate of ca 1.25 mL/min. The suspension was left stirring (without lid) for 15 min.

53   Finally 47 µL of freshly prepared gluteraldheyde 5% w/v in water was added to crosslink

54   and stabilize the NPs. The suspension was left stirring overnight (with a reduced stirring

55   rate ~ 300 - 400 rpm) to allow ethanol evaporation. The obtained final NP suspension had

56   a brown cloudy appearance.

57

58   Residual ethanol, non-encapsulated drug and any undissolved albumin and not cross-

59   linked GLU were removed by ultracentrifugation. Prior to ultracentrifugation, the volume

60   of the NP suspension was increased to 8 mL, by the addition of milliQ water. The NPs

61   were purified by three cycles of centrifugation at 20,000 g for 60, 30 and 30 minutes at

62   20 °C (Optima XE Ultracentrifuge, Beckam Coulter) with redispersion of each pellet to

63   the original volume (8 mL) in double distilled water via ultrasonication (1 min). The pellet

64   obtained from the third cycle of centrifugation was resuspended in 4 mL of water. Each

65   supernatant was collected and stored for further analysis to quantify the albumin and RFM

66   that was not encapsulated.
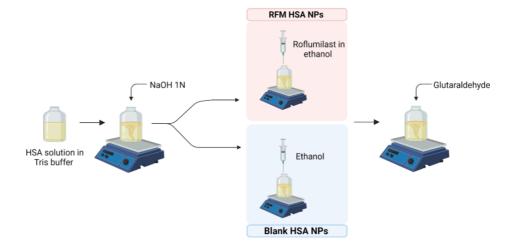
Figure 2.4 Schematic representation of the manufacture process of blank and roflumilast (RFM)-loaded human serum albumin nanoparticles (HSA-NPs).

67

68   **2.3.2   Calculation of degree of cross-linking of HSA NPs**

69

70   The calculation of the NPs degree of crosslinking was carried out based on previously

71   reported methods (Langer et al., 2008; Weber et al., 2000).

72   A molecule of HSA has 60 amino groups with only the Lys- amino group being available
73   to be crosslinked. Considering that GLU is a bifunctional molecule, one molecule of GLU
74   can react with two amino group of the HSA. Therefore, to crosslink the 60 -NH_2 of one
75   molecule of HSA, 30 molecule of GLU will be required. This ratio will result in a degree
76   of crosslinking equal to 100%.

77   Firstly, the number of molecules of HSA available was calculated using the following
78   equation:

79

80   *Equation 2.1 Calculation of number of HSA molecules available*

$$N° \; molecule \; HSA \; = \; n \; mol \; \times \; N_A$$

81

82   With *n mol* being the number of HSA moles obtained as mass of HSA divided by the
83   molecular weight (MW) of HSA and N_A being the Avogadro number (6.023×10^{23}). To
84   calculate the number of GLU molecules required to have 100% crosslinking, the
85   following formula was used:

86

87   *Equation 2.2 Number of GLU molecules*

$$N° \; molecule \; glutaraldehyde \; = \; n° \; molecule \; HSA \; \times \; 30$$

88

89   The number of molecules of GLU available based on the volume used in the manufacture
90   process was calculated using the **Equation** 2.2 to investigate if the degree of cross-linking
91   of the NPs was equal to 100%.

92

93   **2.3.3   Physiochemical characterisation of nanoparticles suspension: Dynamic Light**
94   **Scattering and Nanoparticle Tracking Analysis (NTA)**

95

96   Particle hydrodynamic diameter, polydispersity index (PDI) and zeta potential were
97   determined by Dynamic Light Scattering (DLS) using the Zetasizer Nano Series ZS
98   (Malvern Instruments, UK). The analysis was performed at a scattering angle of 173 °
99   and at 25 °C. For size and zeta potential measurements, NPs were diluted 100-fold with
100   double distilled water. Zeta potential was measured in double distilled water at 37 °C and
101   at a concentration of 0.02 mg/mL.

102

103 The NPs size was also investigated with the Nanoparticles Tracking Analysis (NTA). NPs
104 were diluted 50,000 or 100,000-fold with ultrapure water to achieve a final ideal
105 particles/frame of 20-80. The video data for each measurement were collected for 30 sec
106 and repeated three - four times for each sample.
107
108 **2.3.4 Quantification of encapsulated albumin**
109
110 **2.3.4.1 Yield and albumin quantification via bicinchoninic acid (BCA) protein**
111 **assay**
112 NP yield was calculated using a bicinchoninic acid (BCA) protein assay. The amount of
113 albumin encapsulated was obtained using an indirect method, knowing the total amount
114 of albumin used in the NPs manufacture and the total free amount (not-desolvated)
115 recovered in the supernatants (I and II) obtained from the first 2 purification cycles. If
116 needed, supernatants I and II were diluted with milliQ water to match the concentration
117 range adopted in the calibration curve. The amount of albumin in supernatant III was
118 determined to be negligible in preliminary experiments and therefore not included in the
119 evaluation.
120 A calibration curve was made each time an assay was performed using an HSA standard
121 solution. Albumin (10.2 mg ± 0.11) was dissolved in 5 mL of ultrapure water (2 mg/mL,
122 n=3) and serial dilutions were made in the range 200 – 2000 µg/mL. The blank consisted
123 of ultrapure water without protein. The required volume of the working reagent (WR)
124 was calculated using the following formula:
125
126 *Equation 2.3 Calculation of working reagent required*

$$WR = (\# \text{ standards} + \# \text{ unknown}) \times (\# \text{ replicates}) \times (\text{volume of WR needed})$$

127 The WR reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of reagent
128 B. For microplate measurements, the WR (200 µL) was added to each unknown sample
129 or standards (25 µL). To mix the reagents, the plate was placed on a plate shaker (R100
130 Rotatest shaker, Luckham, England) for 30 seconds. Plate was covered and incubated at
131 37°C for 30 minutes. The plate was cooled at room temperature and absorbance measured
132 at 562 nm using a spectrophotometer (Spark®, Tecan, Switzerland). The average
133 absorbance of the blank was then subtracted from the absorbance of each individual

94

134　　standard and unknown sample. Blank-corrected measurements were plotted against the
135　　concentration (µg/mL). The NP yield was calculated using the equation below:

136　　*Equation 2.4 Calculation of NPs yield*

$$Yield\ (\%) = \frac{Total\ mass\ HSA - free\ mass\ HSA}{Total\ mass\ HSA} \times 100$$

137　　Where *total mass HSA* is the total mass of albumin used in the manufacture process and
138　　*free mass HSA* is the sum of the albumin masses recovered and quantified in the
139　　supernatant I and II during purification.

140

141　　**2.3.5　Bicinchoninic acid (BCA) protein assay validation**

142

143　　Linearity was assessed by the construction of HSA calibration curves across the
144　　concentration range of 200 – 2000 µg/mL (200, 350, 500, 750, 1000, 1500, and 2000
145　　µg/mL) (n=3, measurements in triplicate). The absorbance at 562 nm was plotted against
146　　known concentration to determine the linear equation and the regression coefficient (R^2).
147　　Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the
148　　**Equation** 2.5 where F is a factor of 3.3 or 10 for LOD and LOQ respectively, SD is the
149　　standard deviation of the net absorbance of the lowest concentration (200 µg/mL) and *b*
150　　the slope of the regression line.

151

152　　*Equation 2.5 Limit of detection/quantification formula*

$$LOD/LOQ\ = \frac{F\ \times\ SD}{b}$$

153　　The accuracy was defined as the closeness of agreement between the value accepted as
154　　conventional true and the value observed. It was calculated using the difference between
155　　theoretical (T) and experimental (O) values, taken at three separate concentration values
156　　(200, 750, and 2000 µg/mL which are here defined as low, medium and high) across the
157　　assay in triplicate. Accuracy was calculated using the % relative error (RE) (**Equation**
158　　**2.6, Equation** 2.7).

159

160 *Equation 2.6 Calculatin of accuracy expressed as error*

$$Error (E) = O - T$$

161

162 *Equation 2.7 Calculation of sampling accuracy expressed as % relative error*

$$\% RE = \frac{E}{T} \times 100$$

163 Sampling accuracy was expressed as percentage relative error (% RE) and it was
164 calculated using thirty determinations (ten samplings for three concentrations; 200, 750
165 and 2000 µg/mL) on day 1.
166 Precision is a measure of the closeness of agreement between a series of measurements
167 obtained from multiple sampling of the same sample under a set of prescribed conditions.
168 In this body of work, precision was calculated at two levels: *repeatability* (intra-assay
169 precision) and *intermediate precision*. Repeatability is the precision under the same
170 operating conditions over a short period of time. Three concentrations (low, medium and
171 high; 200, 750 and 2000 µg/mL) were tested on the same day. Results from day 1 are
172 expressed as percentage relative standard deviation (% RSD) (**Equation** 2.8) using nine
173 determinations. Intermediate precision assessed the within-laboratories variations as
174 multiple BCA assays were performed on different days. The samples were tested on day
175 1 and day 3.
176

177 *Equation 2.8 Relative standard deviation (%)*

$$\% RSD = \frac{SD}{mean} \times 100$$

178

179 **2.3.6** ***In vitro* model of lung protease to investigate the stability of albumin NPs**
180

181 The stability and the degradation rate of albumin NPs in a simulated lung environment
182 were investigated using a modified method reported elsewhere (Woods et al., 2020). The
183 evaluation of the proportion of intact NP overtime in the protease model was monitored
184 using two parameters: i) derived count rate (kilo counts per second, kcps) and ii) particle
185 size distribution. The derived count rate has been showed to be proportional to the NP
186 concentration (Tantra et al., 2010). The validation of this relationship was performed
187 preparing a calibration curve of HSA in PBS (0.125, 0.25, 0.5, 1.0 and 2.0 mg/mL).

188   Samples were diluted 1:10 with PBS prior to measurement and analysed at 25°C using

189   the Zetasizer Nano Serie ZS.

190   Trypsin (0.1 mg, 0.25 mg and 0.5 mg) (Type I, ~ 10,000 BAEE units/mg protein) was

191   dissolved in 1 mM of HCl and added to blank NPs (1.6 mg); the resulting mixture was

192   incubated at 37°C under shaking (120 rpm) (Incu-Shake MINI, SciQuip, UK) for 4 hours.

193   Blank NPs in PBS alone at 37°C were used as controls. At allocated time points, aliquots

194   (50 µL) of the enzyme-particles mixture were withdrawn, diluted with PBS (10-fold), and

195   analysed immediately using the Zetasizer Nano Series ZS (Malvern).

196

197   **2.3.7   Quantification of encapsulated roflumilast**

198

199   **2.3.7.1 Roflumilast   quantification   using   a   high-performance   liquid**
200   **chromatography (HPLC) detection method**

201   The amount of encapsulated RFM (encapsulation efficiency; EE) was calculated by

202   measuring the amount of drug not encapsulated recovered in the supernatants I, II and III

203   obtained from the purification process using **Equation** 2.9.

204

205   *Equation 2.9 Encapsulation efficiency (%)*

$$EE\% = \frac{Total\ drug - free\ drug}{Total\ drug} \times 100$$

206   Where *total drug* is the mass of RFM weighed at the beginning of the manufacture

207   process and dissolved in ethanol; *free drug* is the sum of masses of RFM in each of the

208   three supernatants.

209

210   The process to quantify the amount of RFM encapsulated in the NPs was firstly carried

211   out using a method (method 1, **Figure 0.1**) based on the use of acetonitrile and on a

212   centrifugation cycle. An alternative method (method 2, **Figure 0.2)** employed the use of

213   trypsin to obtain a direct quantification of roflumilast using acetonitrile (**Figure 0.3**).

214   However, both the methods resulted in unsatisfactory outcomes and an indirect

215   quantification was considered more reliable and therefore preferred as definitive method.

216   The optimised and final method used in this thesis is represented in **Figure** 2.5. An aliquot

217   of 1 mL of each supernatant resulting from the purification step was placed in a

218   borosilicate glass tube and the total volume evaporated using a Rotary Evaporator (water

219   bath temperature 40°C). The resulting dried sample was reconstituted in a mixture of
220   water-acetonitrile (1 mL, 30:70 v/v), vortexed for 1 min and centrifuged (10,000 rpm x 5
221   min). The resulting supernatant was collected and diluted with the required volume of a
222   mixture of water:acetonitrile (30:70) and the drug present in the samples calculated using
223   a reversed-phase HPLC method described in detail in section 2.3.8. A calibration curve
224   of RFM in the same solvent mixture in the range $0.5 - 150\ \mu g/mL$ was constructed.
225
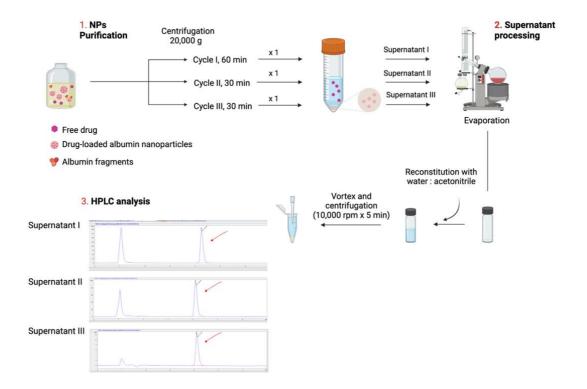
Figure 2.5 Schematic diagram of the indirect method of roflumilast quantification: (1) Separation of free drug and encapsulated roflumilast; (2) processing of non-encapsulated drug; (3) analysis of non-encapsulated drug using an HPLC method.

226

227   **2.3.8   Validation of High-Performance Liquid Chromatography (HPLC) detection**
228   **method**

229

230   The Agilent Technology 1260 Infinity was used for the purpose of this study. The system
231   consisted of a binary solvent system, flow through needle sample and a column thermostat
232   with the analytical column kept at 40°C. The analytes were separated on a YMC Triart
233   C18 column (150 mm x 4.6 mm, 3 µm particle size) with YMC-Triart C18 Guard
234   Cartridge (10 x 4 mm, 12 nm or 3 µm particle size) using isocratic elution. Mobile phase

235 A consisted of 70% of HPLC-grade acetonitrile and mobile phase B of 30% of HPLC-
236 grade water with 0.1% of trifluoroacetic acid (TFA). The flow rate was set to 1 mL/min
237 and the injection volume was 50 µL and the detection wavelength 252 nm (**Table** 2.5).

Table 2.5 Summary of HPLC method for roflumilast detection and quantification.

ROFLUMILAST	
Structure	
Molecular weight	403.207 g/mol
Assay method:	HPLC
Column:	YMC Triart C_{18} 3µm 150-4.6 mm
Mobile phase:	30:70:0.1 H_2O:ACN:TFA Isocratic
Sample diluent:	30:70 H_2O:ACN
Oven Temperature:	40°C
Run time:	7 minutes
Flow rate:	1.0 mL/mL
Autosampler temperature:	Ambient
Injection volume:	50 µL
UV wavelength:	252nm

238

239 Stock solution was prepared in HPLC grade water-acetonitrile mixture (30:70, v/v) at a
240 concentration of 150 µg/mL (n=1) with further dilutions over a concentration range of
241 0.5 – 150 µg/mL.
242 The intra-assay precision (repeatability) was evaluated with ten consecutive injections of
243 150 µg/mL performed on the same day and under the same experimental conditions.
244 Repeatability was represented by standard deviation and relative standard deviation. LOD

245 and LOQ were calculated on the "standard deviation of the response of and the slope"

246 using the Equation 2.5.

247

248 The SD value adopted was equal to the standard deviation of the lowest concentration

249 (0.5 µg/mL) and b is the slope of the regression line.

250 The stability of RFM in the mobile diluent was investigated comparing the area response

251 of the three samples solution (0.5 – 5.0 and 150 µg/mL) analysed on day 1, 2, 3, and 7 to

252 the area response of the same concentration samples obtained on day 0 when the sample

253 was freshly prepared (n=1). Samples were stored in capped volumetric flasks at 4°C

254 during the stability study.

255

256 **2.3.9   Optimisation of a dialysis bag release method for RFM**

257

258 The release of RFM from albumin NP was studied by a dialysis bag method (**Figure** 2.6).

259 Dialysis bags were placed in a small beaker and submerged in 10 mL of release media

260 (PBS 0.01 M + 0.8% Tween 80). The samples were incubated at 37°C under shaking for

261 the duration of the assay. Samples were RFM loaded and blank NPs with trypsin from

262 bovine pancreas (Type I, ˜ 10,000 BAEE units/mg protein) in different trypsin: albumin

263 ratio (1: 100, 1: 2). Mass of HSA used was equal to 3.25 mg and 2 mg when the trypsin:

264 albumin ration was equal to 1:100 and 1: 2 respectively. Controls were i) free RFM

265 solution (0.2 mL in dimethyl sulfoxide (DMSO) or 1 mL in DMSO and release medium);

266 ii) RFM with albumin solution; iii) RFM with blank NPs.

267 The potential enhancement of the RFM-HSA-NPs degradation in the assay mediated by

268 trypsin was investigated via the 'activation' of the enzyme. Trypsin solution was prepared

269 on ice and away from the light. The acid pH of the solution was changed to 7 – 9 via the

270 addition of NaOH.

271 Samples (1 mL) were withdrawn from the release medium (receiver fluid) at

272 predetermined time intervals and the same volume was replaced with fresh medium to

273 maintain sink conditions. RFM was quantified in the samples by HPLC (section 2.3.8)

274 and the cumulative percentage mass of roflumilast release over time was calculated.

275
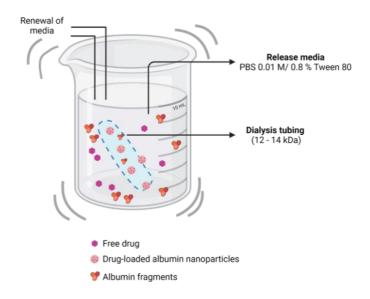
Figure 2.6 In vitro drug release model. Schematic representation of the in vitro drug release study experimental set up.

276

2.4   Results

278

2.4.1   Physiochemical characterisation of NPs via Dynamic Light Scattering (DLS) and Nanoparticles Tracking Analysis (NTA)

281

Blank and drug loaded HSA-NPs were manufactured using a desolvation method. In order to optimise the manufacture process and the physiochemical properties of the NPs, stirrer speed and rate of ethanol addition were investigated. Nine different formulations were manufactured using three stirrer speeds (500, 850 and 1250 rpm) and three different ethanol addition rates (0.25, 1.25 and 2.0 mL/min) (**Table** 2.6). The combination of 850 rpm and 1.25 mL/min resulted in the formulation with the most appropriate physiochemical properties, as small size, low PDI and negative zeta potential (**Table** 2.7). The highest and lowest speeds resulted in sticky formulations and particle size greater than 200 nm.

291 *Table 2.6 Schematic representation of the different combination of stirring rate (rpm)*
292 *and ethanol addition rate (mL/min) tested in the optimisation of the NPs manufacture*
293 *process.*

294

295 Size, PDI and zeta potential of blank and drug-loaded HSA-NPs were characterised using
296 a Dynamic Light Scattering technique. The physiochemical properties of both blank and
297 drug-loaded NPs were very similar, with size in the range of 140 nm, PDI < 0.1 and
298 negative zeta potential (- 42.5 mV and - 36.8 mV for blank and drug loaded NPs
299 respectively) (**Table** 2.7).

300

301 *Table 2.7 Size, polydispersity index (PDI) and zeta potential of blank and drug loaded*
302 *albumin nanoparticles. Data represents mean (SD) of n = 3 − 8 independent*
303 *experiments.*

	Size (nm)	PDI	Zeta potential (mv)
Blank NPs	139.2 (15.7)	0.09 (0.04)	-42.5 (3.23)
RFM-HSA-NPs	147.3 (6.78)	0.05 (0.01)	-36.8 (4.93)

304

305 The DLS measurement performance was evaluated and compared to results obtained
306 from the NTA analysis. In an NTA measurement, the particles' movements are tracked
307 in consecutive video frames (**Figure** 2.7 right panel). The overlapping of NTA and DLS
308 size measurements of drug loaded NPs described particles with good size and in
309 accordance with results from table 2.6 (**Figure** 2.7 left panel).
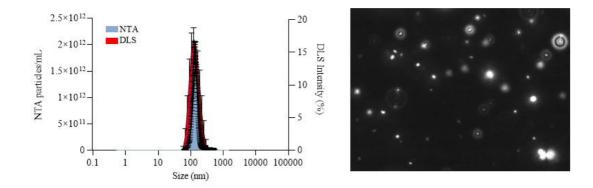
Figure 2.7 Size distribution from NTA and DLS measurements of monodisperse roflumilast albumin NPs (left panel) with an illustrative NTA video frame of one of the samples analysed (right panel). Error bars represents standard deviation (DLS) and standard error (NTA) obtained from three different independent samples (n = 4 measurements for the NTA measurements).

310

311 The degree of crosslinking of the manufactured HSA NPs was calculated using the
312 formula described in section 2.3.2. The number of HSA molecules available based on
313 the mass of HSA employed in the manufacture process resulted equal to 4.53×10^{17}.
314 Based on this value the number of molecules of GLU required to obtained NPs with 100%
315 degree of crosslinking was calculated to be equal to 1.36×10^{19}. Therefore based on the
316 calculated number of molecules of GLU available when using 47 µL of 5% w/v ($1.50 \times$
317 10^{19}), the degree of crosslinking of the HSA-NPs resulted equal to 100%.

318

319 **2.4.2   Evaluation of albumin yield and roflumilast encapsulation into NPs**

320

321 **2.4.2.1   NPs albumin yield and validation of a BCA assay**

322 The albumin entrapment in the RFM-HSA-NPs was calculated using a BCA assay and
323 found to be 73% (n=2). The HSA assay was validated, and results are shown in **Figure**
324 2.8 and in **Table** 2.8. The calibration curves for HSA were constructed by plotting the net
325 absorbance versus the concentration. A linear correlation between absorbance and
326 concentration was found in the range 20 – 2000 µg/mL. LOD and LOQ were calculated
327 and equal to 50.1 µg/mL and 152.3 µg/mL, respectively.

328

329

y=0.0007x + 0.0807
$R^2 = 0.993$
y=0.0007x + 0.0605
$R^2 = 0.9942$
y=0.0007x + 0.0609
$R^2 = 0.993$
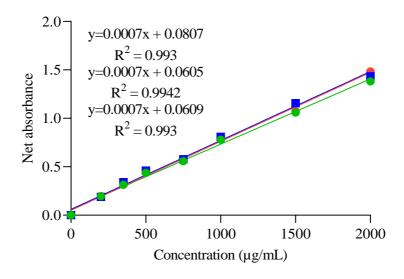
Figure 2.8 Intra-day validation of linearity of human serum albumin (HSA) in the concentration range 200 – 2000 µg/mL. Data represents n = 3, each measurement in triplicate.

The BCA assay was validated in terms of intra-day (repeatability) and inter-day precision. Three concentrations (low, medium and high) were tested on the same day (intra-day) and on two different days (day 1 and day 3) and absorbance measured. Results from day 1, expressed as percentage relative standard deviation (% RSD), were 5.3, 6.2 and 3.7 %, respectively, for low, medium and high concentrations. When tested over two days, the RSD% were equal to 1.4, 5 and 0.2 % for the same concentrations.

Accuracy was calculated using the **Equation** 2.6 and **Equation** 2.7 for the same low, medium and high concentrations. The corresponding RE were 2.5, 2.4 and 1.8 %. The sampling error was also calculated using ten determinations of the three concentrations. The highest RE % were reported for the low and medium concentrations.

***Table 2.8 Validation of BCA assay as method for albumin yield over three
concentrations (low, medium and high). Data represents mean (SD) of n = 3
independent experiments.***

Concentration (µg/mL)	Net absorbance mean (SD)		Precision		Accuracy	Sampling accuracy	LOD	LOQ
	Intra-day	Inter-day	Intra-day (%RSD)	Inter-day (% RSD)	(% relative error)	(% relative error)	(µg/mL)	(µg/mL)
200	0.194 (0.01)	0.192 (0.003)	5.3	1.4	2.5	11.1		
750	0.570 (0.04)	0.591 (0.03)	6.2	5	-2.4	11.2	50.281	152.366
2000	1.443 (0.05)	1.431 (0.003)	3.7	0.2	-1.8	0.8		

347

348 **2.4.2.2 Roflumilast encapsulation into HSA-NPs and validation of an HPLC**
349 **quantification method**

350 The encapsulation efficiency of RFM in HSA-NPs was calculated using the method
351 described in section 2.3.7 and was equal to 30%, with an amount of encapsulated RFM
352 being 19.9 µg for each mg of albumin entrapped, albeit with a high amount of variability
353 between preparations (SD = 12 µg; n=8).

354

355 The HPLC method for RFM quantification was validated for LOD, LOD, precision and
356 RFM was evaluated for stability over 7 days. Firstly, a linear correlation between the
357 concentration and the area under the curve was establish (R^2 = 0.9995) (**Figure** 2.9).

358
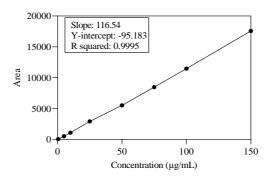
***Figure 2.9 Linear relationship between RFM concentration and area under the
curve in the range 0.5 – 150 µg/mL.***

359 LOD and LOQ were calculated using Equation 2.5 and were equal to 8.65 ng/mL and
360 27.39 ng/mL. Stability was measured for three concentrations of solution (0.5, 50 and 150
361 µg/mL) on day 0, 1, 2, 3 and 7. No significant changes were recorded over the period and
362 the RSD % for the low, medium and high concentrations analysed was equal to 0.99, 0.35

363 and 0.20% (**Table** 2.9). In addition, the precision of the assay was evaluated at the level
364 of repeatability with ten consecutive injections of the highest sample concentration under
365 the same method conditions and the RSD was 0.18%.
366

Table 2.9 Stability of roflumilast in water-acetonitrile solution over 7 days. Data represent n=1indipendent experiment, with measurements in triplicate. Sample solutions were stored in capped volumetric flasks at 4ºC during the length of the stability study.

Concentration (μg/mL)	Day					Sample mean	SD	RSD %
	0	1	2	3	7			
0.5	1.27	1.27	1.27	1.27	1.24	1.26	0.01	0.99
50	48.23	48.07	47.90	47.90	47.74	47.97	0.17	0.35
150	151.58	151.30	152.09	151.46	151.98	151.68	0.30	0.20

367

368 **2.4.3** *In vitro* **model of lung protease to investigate the stability of albumin NPs**
369

370 The degradation of RFM-HSA-NPs mediated by proteases was investigated using the
371 relationship between albumin NP concentration and derived count rate. The NPs used had
372 the same physiochemical properties described previously. A schematic representation of
373 the size and PDI is showed in **Figure** 2.10 **a**. The technique was validated by preparing a
374 calibration curve in the range 0.125 – 2.0 mg/mL. The linear relationship between
375 albumin NP concentration and derived count rate (kcps) was described ($R^2 = 0.9839$)
376 (**Figure** 2.10 **b**).
377
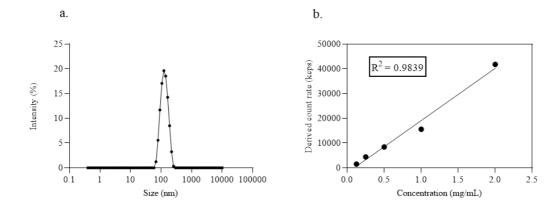
Figure 2.10 Characterisation of HSA NPs for degradation studies. (a) Representative particles size distribution obtained from albumin nanoparticles at 100x dilution in double distilled water at 25°C; (b) Representative linear relationship between derived count rate (kcps) and HSA NPs concentration. Nanoparticles samples are diluted 1:10 with phosphate buffered saline (PBS). Data n=1independent experiment, with measurement in triplicate.

378

379   RFM-HSA-NP were incubated at 37°C with three different concentrations of trypsin (0.1,

380   0.25 and 0.5 mg/mL) and the degradation rate of NP investigated. The effect of increasing

381   trypsin concentration could not be detected as increasing degradation rate when a time

382   window of 4 hours was adopted. At each concentration, the count rate was massively

383   reduced by 1 hour from the starting time of the incubation and remained constant and

384   close to zero until the end of the experimental window. However, a time-dependent

385   degradation profile could be observed in the time frame 0 - 1 h. The investigation of the

386   NP degradation using the same experimental conditions over the shorter period

387   demonstrated that the albumin degradation took place gradually from 0 to 40 min (**Figure**

388   **2.11 D**). Thus, it was concluded that the degradation takes place gradually in the first

389   interval of time and that after 1 hour the NPs were almost completely degraded and

390   fragmented.

391
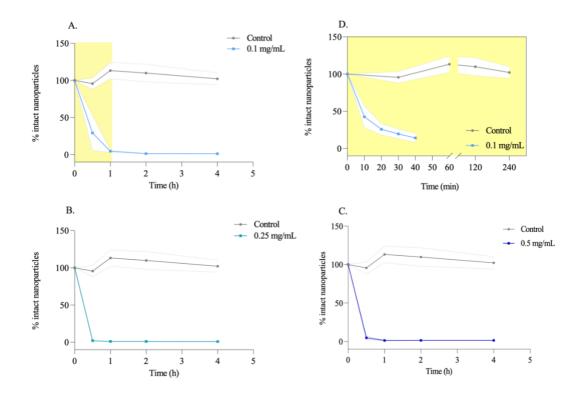
Figure 2.11 Degradation profile of RFM-HSA-NPs following exposure to 0.1 (A), 0.25 (B) and 0.5 mg/mL (C) of trypsin compared to phosphate buffered saline at 37°C (control); (D) Degradation profile of RFM-HSA-NPs following exposure to 0.1 mg/mL of trypsin over 40 minutes only. Degradation is illustrated as reduction of the initial percentage of derived count rate using dynamic light scattering. Dotted lines (··) represent the standard deviation from the mean. Data represents mean ± SD (n = 3 independent experiments).

392

393   The albumin NPs degradation rate was also investigated in terms of change of particle

394   size distribution. The impact of three different trypsin concentrations on the NPs PDI was

395   compared for each time point (0 – 0.5 – 1 – 2 – 4 h) to the control (NPs without trypsin).

396   At time 0, NPs resulted intact as when incubated without trypsin. The quick and consistent

397   reduction of derived count rate showed in Figure 2.11 when NPs were incubated with

398   0.25 and 0.5 mg/mL is in agreement with results reported in **Figure** 2.12. After 30 min,

399   multiple peaks were detected highlighting the presence of several aggregates being

400   formed. The particle population became consistently polydisperse especially after 1 hour

401   with aggregates in the size range of 10 nm, 1000 nm and 10,000 nm. The tracking of

402   particle size changes overtime provided a further look at the sample degradation to

403   complement the evaluation of the derived count rate. Indeed, over the time period from 1

404   to 4 hours ongoing degradative changes were detected to be taking place beyond the initial

405   particle degradation indicated by the constant low derived count rate being reported in

406   Figure 2.11.

407   Incubation with 0.1 mg/mL also resulted in a polydisperse particle population, with

408   multiple peaks at different size highlighting the presence of aggregates and fragments.

409   However, when NPs were incubated with 0.1 mg/mL, these changes in PDI were not

410   observed at the 30 min incubation showing that particle count provides a more sensitive

411   measure of initial NP degradation before the detection of albumin fragments and

412   aggregates becomes apparent.
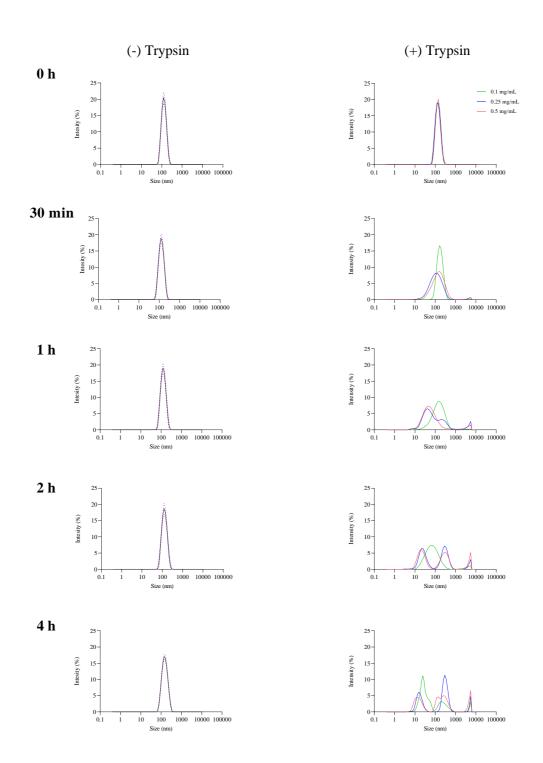
Figure 2.12 Degradation, aggregation and increase in polydispersity index of the RFM-HSA-NPs following exposure to different concentrations of trypsin (0.1, 0.25 and 0.5 mg/mL) at 37•C for up to 4 hours. Data represents mean of n=3 independent experiments and SD (dotted line) for the control and mean only for the positive trypsin samples.

413 **2.4.4 Roflumilast release over time from albumin NPs**

414

415 RFM release from HSA-NPs was assessed over a period of 7 hours. The amount of RFM

416 released from the NPs was calculated using a dialysis bag system combined with a sample

417 preparation method and an HPLC quantification method. A calibration curve of RFM in

418 the release media (PBS 0.01 M/ 0.8% Tween 80) was prepared in the range 0.5 – 20

419 µg/mL and linearity were confirmed ($R^2 = 0.9987$) (n = 1 independent experiment). The

420 chromatogram showed a symmetrical peak at 4.138 min **Figure** 2.13 and a linear

421 relationship between RFM concentration and area under the curve was observed.

422
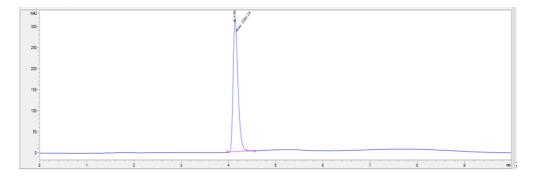
Figure 2.13 Representative chromatogram of RFM (20 μg/mL) in phosphate saline buffer (PBS) 0.01 M/ 0.8% Tween 80.

423

424 The optimisation of the drug release assessment included the evaluation of a suitable drug

425 release media. RFM is poorly soluble in water (0.52 – 0.56 µg/mL at 22°C) (European

426 Medicines Agency, 2010) and therefore different solubility enhancers were tested before

427 choosing Tween 80 as the most suitable. Indeed, the RFM solubility at 37°C in the

428 aforementioned medium (PBS 0.01 M/ 0.8% Tween 80) was experimentally determined

429 to be 25 µg /mL, i.e. the use of 0.8% Tween 80 resulted in a ~ 45x increase of RFM

430 solubility in water at 22°C (European Medicines Agency, 2010). However, the solubility

431 remained considerably low and therefore a volume of release medium of 10 mL was

432 required to ensure the maximum RFM concentration released was always 10x less than

433 the RFM solubility in the same medium.

434

435 The release of RFM loaded in a dialysis bag was first assessed. In order to avoid

436 misleading results induced by the increased solubility of RFM in the medium when

437 dissolved in DMSO, the percentage of DMSO in the system when used was kept below

438   2%, a level that was considered negligible. The mass of RFM loaded in the bag at time 0

439   was calculated to be 16.11 µg using an HPLC method. The cumulative RFM release in

440   terms of percentage and mass increased gradually and peaked at 24 h at ~ 80% (**Figure**

441   2.14).

442

443
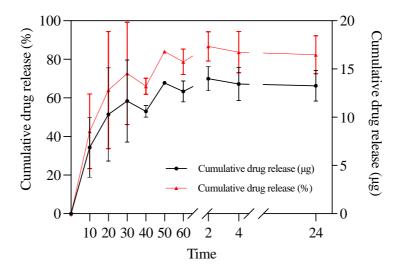
444   *Figure 2.14 In vitro drug release of roflumilast solution in DMSO (100%). A dialysis*

445   *bag with 200 µL of RFM in DMSO was submerged in 10 mL of release media (PBS*

446   *0.01 M/ 0.8% Tween 80). Data represent mean ± SD of n=3 independent experiments.*

447

448   The transfer of RFM in solution (DMSO) was further investigated adjusting the volume

449   of the dialysis bag content. The volume was increased from 0.2 mL to 1 mL via dilution

450   with the release medium. Despite achieving the same percentage of drug release, the

451   profile shape was affected by the total volume in the dialysis bag (**Figure** 2.15). It was

452   hypothesized that the presence of Tween 80 micelles provided a favourable environment

453   and reduced the transfer of RFM through the pore membranes affecting the release

454   profile.

455   Confirmation of bag mass content at time 0 and 24 h was provided by RFM quantification

456   by HPLC. A total amount of 19.90 µg of RFM was loaded in the dialysis bag and after

457   24 h 18.21 µg had transferred to the release medium and a small amount of drug (2.64

458   µg) remained entrapped in the bag (**Table** 2.10).
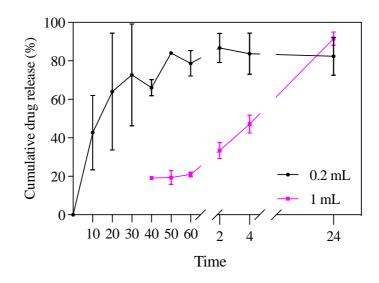
Figure 2.15 Impact of different volumes of dialysis bag content on the free drug release.
(○) A total volume of 0.2 mL of RFM solution in DMSO and (□) RFM solution diluted
in release media (1 mL) in a dialysis bag were submerged in 10 mL of release medium
(PBS 0.01 M/ 0.8% Tween 80). Data represents mean ± SD of n=3 independent
experiments.

Table 2.10 Calculated mass balance in the dialysis bag at time 0 and after 24 h. Data
represent mean of n=3 independent experiments.

RFM mass in the bag at T0	19.90 µg
Mass released at T 24h	18.21 µg
Mass left in the bag at T24h	2.64 µg

The potential formation of complexes between free RFM and albumin in solution was
evaluated by incubating the mixture in presence and absence of trypsin. The presence of
trypsin did not impact the drug release rate as the albumin was free and not in forms of
aggregates or particles. A total percentage of 80% of drug was released both in presence
and absence of trypsin (**Figure** 2.16).
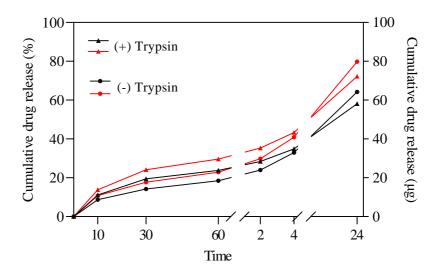
Figure 2.16 Drug release of free RFM and human serum albumin (HSA) solution in presence and absence of trypsin (0.0325 mg) (trypsin:albumin ratio = 1:100). A dialysis bag of a total volume of 1 mL containing 0.9 mL of HSA in Tris HCl buffered and 0.1 mL of RFM in DMSO was submerged in 10 mL of release medium. Aliquots of release medium withdrawn at allocated time point and replaced with fresh medium to maintain sink conditions. Cumulative drug release (%) and mass are represented by red and black lines respectively. Data represent n = 1 independent experiment.

The same experimental conditions were used to investigate the potential for aggregate formation of free RFM when incubated with albumin NPs in presence or absence of trypsin (**Figure** 2.17). The percentage of RFM released from the inner side of the dialysis bag when incubated in presence of albumin NPs was not affected by the presence of trypsin, while the size and PDI of the HSA-NPs at the beginning and at the end of the trypsin incubation showed that the NPs underwent degradation (**Table** 2.11). This confirmed that the albumin NPs in the conditions adopted undergoes degradation when incubating with trypsin but does not affect RFM transfer.
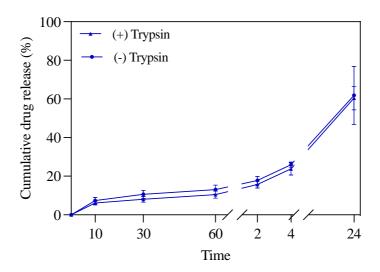
Figure 2.17 Drug release of free RFM incubated with human serum albumin (HSA) NPs in presence and absence of trypsin (0.0325 mg) (trypsin:albumin ratio = 1:100). In each dialysis bag (1 mL) HSA NPs were incubated with free drug in presence or absence of trypsin; each bag was submerged in 10 mL of release media. Aliquots of 1 mL were withdrawn at allocated time point from the receiver chamber and replaced with fresh medium to maintain sink conditions. Data represent mean ± SD of n = 3 independent experiments.

480   An increase in size and PDI from time 0 (124.14 nm; 0.09) to time 24 hours (387.07 nm;
481   0.40) occurred (Table 2.11) and albumin NP degradation was further confirmed by the
482   drop in the derived count rate of the mixture. In terms of percentage of drug release, 60%
483   was detected to transfer with the remaining 40% presumed to be lost to adsorption and
484   interaction with the albumin aggregates.

485

Table 2.11 Size, PDI and derived count rate of NPs at time 0 (- trypsin) and at time 24 h of incubation (NPs left in the dialysis bag). Data represent mean (SD) of n =3 independent experiments.

	Size	Polydispersity index (PDI)	Derived count rate (kcps)
(-) trypsin	124.14 (2.2)	0.09 (0.02)	7572.43 (153.6)
(+) trypsin	387.07 (55.0)	0.40 (0.1)	918.75 (135.4)

486

487 RFM release from albumin NPs degradation was investigated in presence of trypsin both
488 activated and inactivated. The trypsin:albumin ratio was adapted to 1:2. Drug-loaded
489 albumin NPs were incubated with 'activated' and 'inactivated' trypsin and the resulting
490 drug release profiles are shown in **Figure** 2.18. The NP released an initial burst of RFM
491 consistent with the degradation of the particle followed by a steady increase in RFM in
492 the receiver medium reaching a peak percentage at 4 h of about 30% and 15% when
493 'activated' and 'not activated' trypsin was used respectively. In the range between 4 and
494 24 h no further drug release was detected (data not shown).
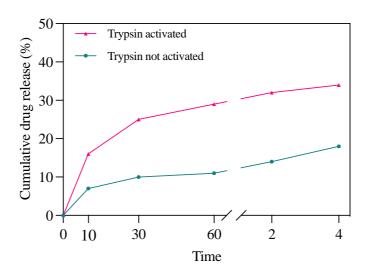
Figure 2.18 Drug release of RFM-HSA-NPs in presence of trypsin activated or not
activated. Trypsin activation was performed changing the pH to 7 - 9 via the addition
of 1 N NaOH. An aliquot of 2 mL of RFM-HSA-NPs was incubated over 24 h with
1 mg of trypsin (trypsin: albumin ratio = 1:2). Data represent n =1 independent
experiment.

495 The particle degradation was corroborated by the changes in size, PDI and derived count
496 rate (**Table** 2.12**)**. Generally, the incubation with trypsin resulted in particles degradation
497 as size and PDI increased consistently, whereas the derived count rate number decreased
498 from time 0 to time 24h. Consistent differences were reported between the use of
499 'activated' or 'not activated' trypsin especially in terms of size and PDI.
500

Table 2.12 Variation of size, PDI and derived count rate after 24 h of incubation with trypsin activated and not activated were recorded using dynamic light scattering. Data represent n =1 independent experiment.

	Size	Polydispersity index (PDI)	Derived count rate (kcps)
T 0	137.23	0.10	23321.0
Trypsin activated			
T 24 h	364.87	0.71	107.47
Trypsin not activated			
T 24 h	205.33	0.45	246.60

501

502   As the 'activated' form of trypsin showed improved activity in the degradation of the

503   HSA-NPs and therefore in enhancing the drug release, it was used for a further confirming

504   experiment using the same protocol. The incubation of RFM-HSA-NPs with 'activated

505   trypsin' induced a sustained release of the drug reaching a maximum of ~ 30% after 4

506   hours of incubation, with negligible release occurred between 4 and 7 h (**Figure** 2.19).
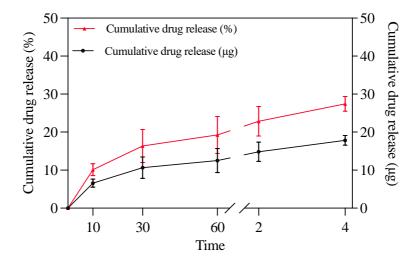
507   *Figure 2.19   Drug release of RFM-HSA-NPs in presence of activated trypsin. An*

508   *aliquot of 2 mL of RFM-HSA-NPs was incubated over 4 h with 1 mg of trypsin (ratio*

509   *trypsin: albumin = 1:2). The activation was performed via change of the pH to 7-9.*

Again, the size, PDI and derived count rate changes confirm the degradative activity of trypsin on the NP (**Table** 2.13). Despite the size and PDI underwent only a small increase, the extensive reduction in the derived count rate demonstrated that the NPs were fully converted into fragments and aggregates.

Table 2.13 Variation of size, PDI and derived count rate after 7 h of incubation with trypsin were recorded using dynamic light scattering.

	Size	Polydispersity index (PDI)	Derived count rate (kcps)
T0	137.23 (-)	0.10 (-)	23321.20 (-)
T 7 h	173.96 (5.31)	0.23 (0.01)	261.53 (47.13)

2.5   Discussion

The NPs designed in this experimental chapter showed good size, PDI and zeta potential, with minor variability throughout the batches manufactured. The evaluation of the optimal manufacture process included the study of different parameters. In agreement with studies reported in the literature (Jahanban-Esfahlan et al., 2016; Langer et al., 2003), the successful formation of NPs with good physiochemical properties was described highly dependent on stirrer speed and ethanol addition rate. For example, it was described that the combination of stirrer speed of 1250 rpm with ethanol addition rate of 2 mL/min or 1.25 mL/min resulted in NPs too polydisperse to carry out a distribution analysis and with presence of large or sedimentary particles.

The investigation of the potential of a nanocarrier system must also include the evaluation of its encapsulation efficiency. The calculation of the total amount of drug that can potentially be loaded in the carrier compared to the total amount of drug used at the beginning of the manufacture process is fundamental to predict the therapeutic potential of the NPs. As for RFM, the use of a poorly water-soluble drug represents a challenge for the EE evaluation. The 'indirect' method is widely used and allows to quantify the EE based on the amount of drug washed out during the purification process. This process was

employed in this experimental chapter and resulted in a high variability of the amount of RFM per mg of HSA (19.9 µg of RFM for each mg of albumin entrapped). The reported SD value equal to 12 µg was considered a result of the lower accuracy of the indirect method of drug quantification.

A valuable alternative for the calculation of the EE is represented by a method that directly determine the amount of drug entrapped in the NPs via the calculation of the amount of drug being released from the nanocarrier. Trypsin, proteinase K, protease and pepsin are reported as adequate enzymes to obtain overall a rapid degradation of albumin nanoparticles and to investigate *in vitro* the release of the embedded drug (Langer et al., 2008).

2.5.1 Investigating the degradation of albumin NPs via the use of trypsin

In this experimental work trypsin was used as key component in two separate assays: the evaluation of the degradation rate of blank NPs in a biorelevant environment and the investigation of the rate of release of RFM from the NPs.

The degradation of HSA-NPs is fundamental for the release of the active ingredient and the following therapeutic activity, but also for the clearance and metabolism of the carrier itself. Despite the critical role that proteases have in the lung health and metabolism in presence of albumin-based carrier, research studies concerning this topic are lacking and not sufficient. However, a recent investigation (Woods et al., 2020) evaluated the total protease concentration in the human lung fluid of volunteers using proteomic analysis and found 14 proteolytic species of proteases, of which the four most abundant were cathepsin D, cathepsin H, dipeptidyl peptidase IV and dipeptidyl carboxypeptidase I/angiotensin-converting enzyme (ACE). From those data four *in vitro* models were developed and the rate of degradation of the HSA-NPs designed in this experimental chapter was described using one of those models reported in the literature (Woods et al., 2020). The NPs were incubated at a concentration relevant to approximate their concentration in the epithelial lung lining fluid at the no observed adverse effect level (NOAEL) reported in pre-clinical study (Woods et al., 2015). The incubation with 0.1 mg/mL of trypsin at 37°C and under shaking induced a slow and full degradation of the NPs from time 0 to 1 h. Those results are in agreement with what reported in this experimental chapter. Although the analysis of the occurred degradation was carried out only via photon correlation spectroscopy, two parameters were used which showed to

570 being complementary. The analysis of the reduction of the derived count rate showed
571 high sensitivity allowing to report a slow and minimal reduction of the total number of
572 particles overtime; in contrast, the changes of the particle size distribution allowed a
573 rather macroscopic analysis which markedly showed the formation of the aggregates.
574 These results further support the interest in HSA-NPs as a controlled release formulation
575 for inhaled and overall provided an informed prediction of the fate of the NPs in a lung
576 simulated environment. They will also inform next studies which will focus on the
577 investigation of the designed HSA-NPs in the disease models reported in the literature
578 (Woods et al., 2020).
579

580 **2.5.2   HSA-NPs provided a sustained release of the encapsulated RFM compared**
581 **to the release of free RFM**
582

583 Results from the investigation of the rate of release of RFM from the NPs have showed
584 that the NPs designed in this experimental thesis can release the drug overtime in a
585 sustained manner. In the *in vitro* assay designed, the amount of RFM released increased
586 from time 0 to 4 h with 20% of cumulative drug released after 60 min and a total of 34%
587 detected at 4 h. The sustained drug release was only described for RFM-HSA-NPs as it
588 is confirmed by the different release profile obtained for free RFM. In this case, a total ~
589 80% of drug was released by 24 h, of which almost 70% was released already after 30
590 min.
591 Interestingly, the release profiles obtained when RFM was dissolved in DMSO only or in
592 DMSO and release medium (containing Tween 80) were considerable different. Although
593 the cumulative percentages release by 24 h were comparable, the kinetic of release
594 appeared slower in presence of Tween 80. Surfactants as Tween 80 are characterized by
595 the critical micelle concentration (CMC) which is defined as the concentration of
596 surfactant above which micelles forms in the given dispersant. Considering that the
597 concentration of Tween 80 adopted exceeded the CMC of Tween 80, the distinct kinetic
598 of release obtained may have been the result of the formation of 'RFM-embedded
599 micelles' which hindered the rate of transport of RFM from the dialysis bag to the receiver
600 medium.
601 In this context, the choice of the release medium often represents a key challenge in the
602 design of an *in vitro* assay investigating the rate of release of the drug from the carrier
603 especially when using a poorly water-soluble drug, as in the case of RFM. A preliminary

investigation (data not shown) showed that PBS 0.01 M/ Tween 80 was the optimal
receiver medium adequate to mimic a biorelevant receiver medium as well as to ensure a
suitable RFM solubility in the medium (Siepmann & Siepmann, 2020). In contrast to this
experimental approach, many studies reported as strategy to overcome the poor water
solubility of the molecules of interest the use of a mixture of PBS and ethanol as receiver
medium. This is the case of a study (Craparo et al., 2022) which investigated the release
of RFM from lipid-polymer hybrid NPs using a dialysis tubing immersed in 30 mL of
PBS/ ethanol (80/20 v/v); similar receiver medium was employed in a study (Fang et al.,
2015) in which the *in vitro* rolipram release was carried out via the use of Franz Cell
having a receptor compartment with 5.5 mL of 30% ethanol in pH 7.4 buffer.

2.5.3 NPs degradation rate is affected by the pH of trypsin solution

The investigation of the rate of drug release from the NPs required as key aspect the
optimisation of the trypsin:albumin ratio as well as of the pH of trypsin solution. Those
variables are fundamental for the effective proteolytic activity of trypsin. The mechanism
of action of the substrate hydrolysis at the active site of trypsin is complex and is based
on a series of biochemical reactions as substrate binding, nucleophilic attack, protonation
and ester hydrolysis (Kaur & Singh, 2022). The rate of hydrolysis mediated by trypsin on
the albumin substrate depends on many factors as temperature, pH, concentration of
substrate and the ratio of enzyme to substrate (Shi et al., 2005). pH affects the hydrolytic
activity of trypsin as it influences the dissociation of the functional groups, which
consequently affects the banding of enzyme and substrate. It was reported that the BSA
hydrolysis is slowest at pH 7 and fastest at pH 9, with a fast reduction with time at pH 10
(Shi et al., 2005). In agreement with these results, in this experimental chapter changing
the pH of the trypsin solution resulted in a modified proteolytic activity and therefore in
a different total amount of drug released. The incubation of RFM-HSA-NPs with
'activated' trypsin induced an almost 2-fold higher total cumulative release by 4 h
compared to 'not activated' trypsin, when the ratio of trypsin:substrate was kept constant.
Therefore, it is evident how changes in the experimental protocol – as the pH of the
enzyme adopted to enhance the NPs degradation- can impact the total amount of drug
released and can result in misleading outcomes if not properly optimised.

637　As mentioned above, the cumulative percentage of RFM released overtime was equal to
638　34%. Some hypotheses can be drawn to investigate the rationale behind the achievement
639　of a total cumulative release overtime not near 100%.

640　Firstly, based on the release profile obtained, the distribution of RFM within the NP
641　matrix and on the NPs surface can be hypothesized. A fraction of RFM may be entrapped
642　in the matrix system and the remaining fraction is covalently and not-covalently bound
643　to the surface of the NPs. One hypothesis is that the amount of drug released detected (34
644　%) only corresponds to the surface-bound drug, whether the other fraction remained
645　attached to the albumin molecules while those were being degraded by trypsin. The
646　formed albumin-drug fragments may be not physically able to be transferred from the
647　dialysis bag to the receiver medium, hence that fraction of drug is not encountered in the
648　total amount of drug released. In order to further confirm or reject this hypothesis, further
649　studies evaluating the exact distribution of the drug within the NPs matrix and on the
650　surface must be carried out.

651

652　Langer and co-workers (Langer et al., 2008) investigated the kinetic of enzymatic
653　degradation of HSA-NPs. The latter was described as highly dependent on the
654　crosslinking degree of the NPs, with only 9% of NPs having 100% degree of crosslinking
655　being fully degraded within 24 h. As the calculated degree of cross-linking of the NPs
656　developed in this experimental chapter was equal to 100%, it can be argued that the cross-
657　linking degree may have hindered the full degradation of the NPs and the achievement of
658　a total cumulative release of 100%. However, in contrast to Langer's study, the
659　evaluation of the changes of size, PDI and derived count rate throughout the assay
660　confirmed that the NPs were fully degraded despite the degree of crosslinking equal to
661　100%. This outcome was also confirmed by the full degradation of blank NPs incubated
662　with RFM in presence and absence of trypsin. As anticipated, the rate of RFM release did
663　not change upon trypsin addition in the dialysis bag as the drug was not embedded in the
664　NPs, but the full occurred degradation of the blank NPs was confirmed by a 4-fold
665　increase in the PDI and a 4-fold increase in the size compared to time 0. However, it is
666　also interesting that the evaluation of size, PDI and derived count rate changes more
667　closely resembles a qualitative assay rather than quantitative. Therefore, further studies
668　could focus on the development of an assay that simultaneously combines the quantitative
669　analysis of drug release (HPLC) with the quantification of the remaining albumin
670　fragments.

671   On the other side, the lack of a full (100%) release of the drug from the nanocarrier may

672   be not relevant if the mass of RFM released is sufficient to achieve a therapeutic activity.

673   Considering the total amount of albumin entrapped in the system and its EE, the total

674   mass of RFM achievable is equal to ~ 727 μg. Taking into account that *in vitro* the amount

675   of drug released overtime was equal to 34%, it can be concluded that the mass of RFM

676   available to produce a therapeutic activity would be equal to ~ 247 μg.

677   The intended aim of the designed NPs system is the investigation of its potential

678   pulmonary delivery. The use of pulmonary delivery often involves a reduced total dose

679   administered compared to oral administration of the same drug (which in the case of RFM

680   is 500 μg daily). This is due to the avoidance of the first pass metabolism and the release

681   of the drug at the target site with a following reduction of the unbounded plasma fraction

682   of the drug and its systemic exposure. Therefore, the EE achieved in this experimental

683   work and the cumulative % released described in the *in vitro* assay provided initial

684   evidence of the potential of a formulation strategy for RFM and can inform further

685   evaluation mainly focussed on the optimisation of RFM content and calculation of the

686   dose achievable when administered to the lungs using a suitable inhalation device.

687

688   Moreover, evidence from the literature also corroborates the successful therapeutic

689   suitability of NPs with EE values and a cumulative drug release from the carrier in an *in*

690   *vitro* assay lower than 100%. Craparo and colleagues (Craparo et al., 2022) investigated

691   the release of RFM from the lipid-polymer hybrid NPs system for lung delivery and found

692   that the drug was slowly released without any burst effect and that the max amount of

693   RFM obtained in the aqueous medium was about 40 % wt of the total amount after 24 h

694   of incubation. Slightly higher (60%) but not near 100% was the amount of rolipram

695   released from a vesicle carrier (Fang et al., 2015). Both the formulations tested (with and

696   without PEG) exhibited an initial burst and were then able to maintain a sustained released

697   overtime (12 h). In this case, the authors commented the lack of total (100%) release was

698   due to the limited volume of receptor compartment in the Franz cell used to investigate

699   the *in vitro* drug release. For cilomilast, similar percentages of drug released were

700   obtained in two different studies investigating the release from phosphatiosomes and from

701   oleic acid and lipid matrix nanocarriers respectively (C. Y. Lin et al., 2019; Liu et al.,

702   2018). Cilomilast was fully and rapidly released from the control (free drug) by 24 h

703   whereas the nanovescicular encapsulation of the drug allowed a sustained and controlled

704   release overtime. However, only 26% of cilomilast was released at 24h. The release

behaviour of the same drug from the NPs was evaluated using Franz diffusion cells, using a receiver medium that contained 30% of ethanol to enhance the drug solubility. Free drug was represented by cilomilast solubilised in 100% DMSO. Interestingly, although the percentage of cilomilast released from the carrier achieved in 24 h was only 14%, the use of the encapsulated drug still represented an advantageous formulation choice due to the sustained released that cannot be achieved with any other formulation strategies.

Although in this experimental work the degradation of the carrier and the following drug release rate are investigated in two distinct *in vitro* experimental models, it is more likely that in a living system the two events will take place simultaneously, with the drug being released while the carrier undergoing degradation. Further studies will focus on the development of a combined *in vitro* assay which employs analytical techniques as the HPLC to accurately quantify the amount of drug released overtime and fluorescence microscopy to monitor the degradation of the HSA-NPs at is happens.

2.6 Conclusion

This chapter describes the development of an albumin based- nanoplatform that can encapsulate and release RFM in a sustained manner after deposition and degradation in the lungs for PDE4 inhibition as a treatment for a lung inflammatory disease. The NPs suspension developed is suitable for its conversion in a respirable form which will be investigated and described in Chapter 3. An easy lab-based and reproducible manufacturing method was developed for the consistent manufacture of the albumin nanocarrier which physiochemical properties were characterised using different techniques. The NPs showed small particle size, uniformity and negative zeta potential. The formulation underwent degradation in an *in vitro* model of lung proteases which employed trypsin as a model enzyme to investigate the NPs fate after degradation. Trypsin was used similarly to establish and optimised a drug release method, which showed that complete RFM release from the HSA-NPs occurred over 4 h. The next step is for the formulation to undergo biological testing to determine whether the encapsulation efficiency and drug release achieved are sufficient to produce therapeutic effects in the lungs. It is estimated that the pulmonary dose required will be much lower than the oral dose currently adopted in clinical use. Development of a respirable dosage

738   form and development of an animal model of lung disease are the next steps to determine

739   whether further optimisation of the NP formulation is needed.

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CHAPTER 3: RFM-HSA-NPs in mannitol-based

microparticles powder for inhalation

3.1 Introduction

Even for drugs characterised by high potency, their effectiveness can be severely hindered by low or very low water solubility, as in the case for the prototype drug used in this work. NPs are a useful platform to deliver those drugs and can improve the systemic or local drug bioavailability allowing a controlled onset of therapeutic action. Indeed, where low-soluble drug cannot dissolve in the lung lining fluid, NPs with their higher surface-to-volume ratio can dissolve much faster than the free drug, enhancing its solubility and facilitating pharmacological activity in the lungs. The use of the pulmonary route of drug delivery to target the lungs or the systemic circulation is a promising method which requires delivery of a pharmaceutical aerosol with particles with suitable aerodynamic size. Particles with an aerodynamic diameter less than 1 µm are more likely to reach the alveolar region but possess low mass and do not deposit efficiently. Although, their low mass hinders their deposition by impaction and sedimentation allowing them to reach the alveoli, deposition by diffusion is slow and they are subject to exhalation and a consequent loss of contribution to the therapeutic dose. A further difficulty for inhaled delivery aerosols composed of sub-micron particles is the high surface area, which enhances the free energy with a resulting possible aggregation between particles (Heyder, 2004; Praphawatvet et al., 2020). These disadvantages of NPs for inhalation can be tackled using an alternative delivery systems called Trojan particles and nanoparticle embedded microparticles (NEMs).

3.1.1 Trojan particles

The development of dry powder for inhalation containing NPs is very challenging to achieve due to aggregation that would take place in the drying phase. Large porous carriers containing NPs called Trojan particles, have been investigated as alternative approach to deliver NPs to the lungs in the form of dry powders (**Table** 3.1). This approach was firstly reported by Tsapis and co-workers (Tsapis et al., 2002), who developed a system based on large porous nanoparticles (LPNPs) with improved

802 aerosolization and physical properties and avoidance of phagocytosis in the lungs. Tsapis
803 and colleagues developed a system of LPNPs composed of
804 dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylethanolamine (DMPE)
805 and lactose using spray drying. The LPNPs possessed both the benefits of the large porous
806 particles (LPPs) (D. A. Edwards et al., 1997) and those of the NPs with a resulting ease
807 of flow and aerosolization and drug delivery potential. LPPs are characterised by low
808 mass density (< 0.4 g/cm^3) and high geometric diameters (>5 µm). Compared to smaller
809 nonporous particles, LPPs have an improved aerosolization efficiency from DPI and have
810 the potential avoidance of macrophage clearance after deposition. NPs have instead a
811 typical geometric size of a couple of hundred nanometers and can remain in the lung
812 lining fluid after deposition for extended periods and release the active ingredient which
813 can exert the pharmacological activity.

814

815 **3.1.2 Nano-embedded microparticles (NEMs)**

816

817 Although Trojan particles possess many advantages for pulmonary delivery, there are
818 some drawbacks correlated to the preservation of the particle aerodynamic properties and
819 integrity during the drying process of the NPs suspension. An alternative approach is
820 represented by the nano-embedded microparticles (NEMs) system which consists of
821 nanocomposite particles obtained by inclusion of drug-loaded NPs within a microparticle
822 system, using an inert carrier (excipient) (**Table** 3.1) (Silva et al., 2014; Ungaro et al.,
823 2012). The specific advantage of this systems is the degradation of the excipient on the
824 pulmonary epithelium after deposition and following release of the NPs. Some of the
825 most used polymers are PLGA and chitosan, whereas mannitol and lactose are widely
826 used excipients. Mannitol is advantageous compared to lactose for its low hygroscopicity,
827 no reducing effect and non-toxicity (Ungaro et al., 2012). Lactose is the only FDA-
828 approved sugar carrier for dry powder aerosol formulations. NEMs are characterised by
829 superior aerodynamic characteristics and pulmonary deposition compared to simply drug
830 encapsulated in NPs; they provide a higher stability over time and a higher deposition of
831 encapsulated APIs in the lungs (Anton et al., 2012; Tsapis et al., 2002).

832

833

Table 3.1 Features of nanoparticles (NPs), large porous particles (LPPs), large porous nanoparticles (LPNPs) and nano-embedded microparticles (NEMs).

Nanoparticles (NPs)	Large porous particles (LPPs)	Large porous nanoparticles (LPNPs) (Trojan system)	Nano-embedded microparticles (NEMs)
Geometric size $\smile$ 100-200 nm	Geometric size $> 5\,\mu m$	Dissolve to produce NPs	Dissolve to produce NPs after reaching deep lung
Potential to remain in the lung lining fluid	Mass density $\leq 0.1\ g/cm^3$	Improved flow and aerosolization properties typical of LPPs	Inert excipients used (mannitol, lactose, trehalose)
Low inertia	Aerosolization efficiency	Long-term residence in situ, prolonged drug release typical of NPs	Improved aerodynamic properties and stability
Potential avoidance of mucociliary and macrophage clearance	Potential avoidance of macrophage clearance		
Inter-particle interactions > potential aggregation	Dissolve to produce molecular components		

834

835 Spray drying is a common technique used to manufacture NEM usually in combination

836 with excipients such as mannitol (Praphawatvet et al., 2020). Alternatives such as wet

837 milling, supercritical fluid extraction or electrospray can be used, but in this body of work,

838 the NEMs were manufactured in two steps: first, the NPs are produced using techniques

839 such as nanoprecipitation or emulsification and then spray-dried with an excipient (Bohr

840 et al., 2014). The resulting dry powder formulations are characterized by greater stability

841 and pulmonary delivery potential compared to NPs. An additional advantage sought by

842 the delivery of an API entrapped in a NEM system over a conventional formulation is the

843 controlled release that can be achieved.

844

845   Spray drying is a very convenient tool to transform a liquid feedstock solution,

846   suspension, or emulsion in dry particles in a highly controllable way. The fluid phase is

847   forced through high pressure nozzles to produce a fine mist from which the aqueous

848   content evaporates, leaving behind a powder (Bailey & Berkland, 2009). This method

849   obtains well-controlled characteristics at the particle and powder level. An extensive

850   review of the impact of excipients and processing parameters on the spray drying of

851   particles for inhalation was recently published by Alhajj and colleagues (Alhajj et al.,

852   2021).

853

854   The final NEMs appearance and morphological structure is dependent on the drying phase

855   of the spray drying process and its kinetics. The Peclet number (*Pe*) (**Equation** 3.1) is a

856   dimensionless mass transport number which gives an idea of the final structure of the

857   NEMs, whether based on a matrix or hollow system. It also allows the understanding of

858   the relationship between the drying process and the resulting particle morphology. The

859   *Pe* is equal to the ratio between two characteristics times: the time required by the solute

860   (single molecule or NP) to diffuse from the edges of the droplet to its centre (R^2 / D, with

861   R^2 being the radius of the droplet and D the diffusion coefficient of the molecule or NP)

862   and the time required for a droplet to dry (τ_d).

863

864   *Equation 3.1 Peclet number*

$$Pe = \frac{R^2}{\tau_d D}$$

865

866   If *Pe* is $\ll 1$, the drying phase will be very slow providing sufficient time to the molecule

867   or NP component within the droplet to redistribute by diffusion, hence obtaining

868   relatively dense dried particles. Vice versa, *Pe* $\gg 1$ corresponds to a quicker drying phase,

869   which will result in the components not having adequate time to move towards the centre

870   hence remaining on the edges of the dried particle (hollow structure). The NPs will then

871   be locked in place due to Van der Waals forces.

872   In other words, the distribution of the NEMs components within the particles is affected

873   by how the solid content of a droplet and the solvent molecules behave during two critical

874   drying phases of the spray drying process: the constant drying rate and the falling drying

875   rate. During the constant drying period, the role of the solid content of the droplet is

876   negligible whereas key role is played by the solvent. Simultaneously molecules of solvent

877   on the surface evaporates while others migrate from the centre of the droplet to its surface.

878   The completion of the solvent molecules evaporation launches the second drying phase –

879   falling drying rate- which is characterized by the particle solidification. How the various

880   components of the particles will redistribute within a droplet during the evaporation

881   process depends on the recession process of the droplet surface. The reduction of the

882   solvent at the particles surface leads to an increase in the local concentration of the solute

883   and results in a concentration gradient between the surface and the centre of the particle.

884   The above mentioned *Pe* can also described this process. A high *Pe* corresponds to a

885   higher surface recession than solute diffusion resulting in surface enrichment which

886   corresponds to hollow particle; in contrast, a low *Pe* will often corresponds to dense

887   particle resulting from a solute diffusion rate higher than the surface recession. There are

888   two different kinetics of drying that can result in two different particle structures: hollow

889   particles are the product of a fast droplet surface recession than solute diffusion which

890   results in surface enrichment; the opposite condition leads to dense particles (**Figure** 3.1).

891   Furthermore, additional morphological changes can take place after the solidification

892   phase (Alhajj et al., 2021; Tsapis et al., 2002).

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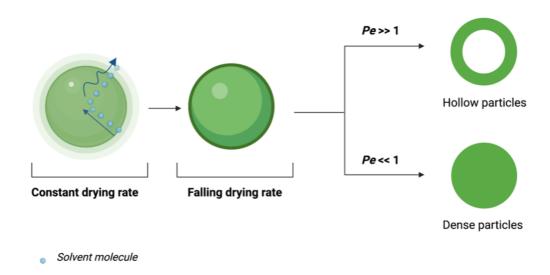
Figure 3.1 Schematic representation of critical phases of spray drying process which regulate the final particle morphology: constant drying phase and falling drying phase. Pe is Peclet number.

894

895   Therefore, based on the drying kinetic, NEMs can have a dense structure, a thick shell or
896   there can be a crust rich in NPs resulting from insufficient time for the NPs to migrate
897   towards the centre of the structure, enclosing the remaining solution. The latter escapes
898   by evaporation from the shell, with the remaining NPs onto the surface of the shell,
899   frequently puncturing it and producing porosity.
900
901   The advantages of those embedded NPs when delivered to the lungs are dependent on the
902   type of excipient used and, on the degradation kinetic of the whole system. Indeed, the
903   microparticles represent a delivery system that due to the high-water solubility of the bulk
904   excipient used (such as mannitol) can be easily dissolved in the lung lining fluid and
905   release the NP system which will disperse and function as designed to dictate the release
906   of the drug. This step is fundamental to maintain the benefits provided by the nanocarrier
907   and control the onset and duration of the therapeutic action. Despite its importance, the
908   investigation of the disintegration rate of the microparticles lacks extensive research.
909   Often researchers evaluate the NEMs disintegration dissolving the system in high volume
910   of water and applying forces such as stirring or mixing. However, this set up does not
911   represent the conditions in the lungs, particularly regarding the lung fluid's composition
912   and its volume. In order to improve the investigation of the NEMs disintegration, an
913   innovative *in vitro* model was designed by Ruge and colleagues (Ruge et al., 2016) who
914   studied the disintegration of a NEMs system after deposition onto a static or dynamic
915   layer of mucus. The study of the microparticle onto a static layer of mucus did not result
916   in release of the NPs from the NEMs system, whereas the individual polymeric NPs were
917   successfully released when forces were applied in the dynamic model (Ruge et al., 2016).
918   Those findings suggest that mechanical forces may be required to overcome the attractive
919   interactions that exists within the NPs. The question that remains unaddressed is to what
920   extent the lung mechanics will enhance the disintegration of the NEMs. An alternative *in*
921   *vitro* model to investigate the disintegration of the microparticles in set up closer to the
922   real lung condition was designed by Torge (Torge et al., 2017). The model was based on
923   the incubation of spray dried powders with different content of mannitol at 37°C in >
924   90% of relative humidity (RH). This innovative model was shown to allow the NEMs
925   disintegration, releasing unchanged nanoparticles.
926
927

3.2 Aims and objectives

The aim of the work in this chapter was to investigate the use of mannitol, a highly water soluble sugar alcohol, as an excipient in a microparticle system composed of 40% w/w RFM-containing HSA-NP and 60% w/w of mannitol. The physiochemical and aerodynamic characterisation of this formulation was carried out focussing on the particle size distribution of the powder, its thermal behaviour, as well as the impact of the spray drying process on the properties of the embedded NPs. The overall aim of these series of experiments was to investigate the potential of RFM-HSA-NPs to be embedded in a respirable formulation, a key step in facilitating their translation to clinical use.

Specific objectives included:

1. Establish a spray-drying protocol to convert the albumin NPs suspension in a nanoparticle embedded microparticles (NEMs) respirable formulation;
2. Characterise the manufacturing process and the obtained powder in terms of yield, particle size distribution and aerosol performance;
3. Investigate the physiochemical spray-dried powder characteristics using dynamic vapour sorption (DVS) and dynamic scanning calorimetry (DSC).

3.3 Methods

3.3.1 Spray-drying conditions of manufacture

For the preparation of HSA-NPs embedded in microparticles, the NPs manufacture process described in Chapter 2 was scaled up and an amount of 600 mg of mannitol was added to the NP suspension (10 mL) and the volume increased to 25 mL with double distilled water. The suspension was then spray dried using a laboratory scale spray dryer according to a previously reported method (Patel et al., 2020). **Table** 3.2 shows the operating conditions for the spray-drying process. The solution was pumped into the spray-dryer and the resultant powder was separated from the airstream using a cyclone separator. The obtained powder was collected, weighed, and stored at room temperature in a desiccator.

Table 3.2 (A) Spray dried feedstock (FS) solution and solvent composition. (B) List of parameters adopted in the spray-drying process.

Excipient name	% w/w in dry product
Feedstock (FS) solution composition	
RFM-HSA NPs	40
Mannitol	60
Total dry % w/v (including active)	100
Solvent composition	
Main solvent	Water
FS total solid content (% w/v)	4
Total volume (mL)	25
FS storage	Ambient

Spray drying conditions	
Feed solution concentration (% w/v)	4
Inlet temperature (°C)	106
Outlet temperature (°C)	65
Feed rate (mL/min)	2
Atomisation flow (L/min)	17
Atomisation pressure (bar)	2.5
Drying air flow (kg/h)	17
Drying air pressure (bar)	2.5
Pulsing nozzle	No
Estimated duration (min)	14
Pump speed (rpm)	4.1

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3.3.2 Physiochemical and aerodynamic characterisation of microparticles

3.3.2.1 Impact of spray-drying on size and polydispersity index (PDI) of embedded NPs

Any changes in the size and polydispersity index of the NPs embedded in microparticles after the spray-drying were evaluated. The spray-dried powder was reconstituted by dispersing 10 mg of the powder in distilled water (2 mL). After centrifugation at 2,000 g for 10 min, 1.5 mL supernatant was removed and changes of the size and zeta potential of the NP after the spray-drying were determined by dynamic light scattering (Zetasizer nano ZSP, Malvern Instruments, UK). Size, PDI and zeta potential were measured at 25°C.

3.3.2.2 Fast Screening Impactor (FSI) studies

Aerosol performance of spray dried formulations was assessed using a Fast Screening Impactor (FSI, Copley Scientific, UK) with a 5 μm cut point insert. Hand-filled sealed aluminium blisters containing 10.76 ± 0.37 mg spray dried powder were dispersed at 60 L/min for 4 seconds using a unit dose dry powder inhaler (Vectura Ltd, UK). Fine particle fraction (FPF % $\leq$ 5 μm) and blister evacuation (% fill mass) were determined gravimetrically (n=3).

3.3.2.3 Particle size distribution

The particles size distribution (D_{10}, D_{50} and D_{90}) of the obtained spray-dried powder was measured using the Hydro MV on the Malvern Mastersizer. The microparticles (9.4 mg) were weighed and dispersed in 10 mL of 0.1% lecithin in iso-octane with sonication for 3 minutes.

3.3.2.4 Dynamic Vapor Sorption (DVS) and differential scanning calorimetry (DSC)

The water sorption/desorption behaviour of the spray dried powder was determined using a DVS Advantage instrument (SMS Ltd, UK). Spray dried powder (~30 mg) was weighed into a sample pan and equilibrated at 0% RH at 25°C until change in mass was less than 0.002%. Incremental 10% step changes in relative humidity from 0 - 90% RH and from 90 - 0% RH were performed at 25°C. Progression to the next step occurred once change in mass was less than 0.002%. Two cycles were performed.

997

Thermal events were measured using differential scanning calorimetry (Discovery DSC, TA instruments). A mass of 3 - 5 mg of the spray dried powder was filled in aluminium DSC pans and hermetically sealed with a sample encapsulation press. Heating rate was 10°C/min from 20-300°C.

3.4 Results

3.4.1 Physiochemical and aerodynamic characterisation of microparticles

A feedstock solution of 40% w/w of RFM-HSA-NPs and 60% w/w of mannitol dissolved in water was spray-dried and the obtained powder was collected and the yield % calculated as ~ 46%. The FPF of the resulting powder measured using a cut-off of 5 μm using a Fast Screening Impactor was 67%, highlighting that a significant portion of the obtained powder had size below 5 μm and could therefore be considered as 'respirable' for potential lung administration. Blister evacuation of the obtained powder from hand-filled sealed aluminium blisters was showed to be of 95% (**Table** 3.3).

The impact of spray-drying on the physiochemical properties of the NPs was evaluated using dynamic light scattering. Spray-drying did not impact the size and PDI of the embedded NPs which remained mostly unchanged except for an increase in the PDI.

Table 3.3 Properties and aerodynamic performance of spray-dried powders composed of 40% (w/w) RFM-HSA-NPs embedded in a mannitol matrix. Values reported are derived from the mean of three consecutive measurements performed on a single batch of spray dried particle (n = 1 independent experiment).

SPRAY DRIED POWDER		
Feedstock solution	40 % w/w RFM-HSA-NPs with mannitol	
Yield (g)	0.4586	
Yield (%)	45.9	
Fine Particle Fraction (%) ± SEM	67.05 ± 1.72	
Blister evacuation (%) ± SEM	94.16 ± 0.67	
	Before spray-drying	**After spray-drying**
Size (nm)	173.1	164.6
PDI	0.043	0.108

1019

1020 **3.4.2   Particles size distribution (PSD)**

1021

1022   Particle size distribution of the obtained spray-dried powder is summarized in **Table** 3.4.

1023   Laser diffraction analysis of the spray-dried powder showed that 50% of the total mass

1024   had a geometric size below 2.21 µm while 90% of the total mass of particles had a size

1025   below 4.53 µm indicating promising characteristics for pulmonary delivery, which were

1026   confirmed by aerodynamic measurements.

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Table 3.4 Particle size distribution (D_{10}, D_{50} and D_{90}) expressed as equivalent volume diameters of the spray died powder measured with using the Hydro MV on the Malvern Mastersizer. Refractive index of the material was 1.480; refractive index of the dispersant is 1.391. Values reported are derived from the mean of six consecutive measurements performed on a single batch of spray dried particles (n =1 independent experiment).

	D_{10}	D_{50}	D_{90}	Below 5 μm (%)
Mean (μm)	0.937	2.21	4.53	92.68
RSD %	0.272	0.254	0.219	0.07

3.4.3 Differential scanning calorimetry (DSC) and dynamic vapour sorption (DVS)

The thermal properties of the NEMs were evaluated using DSC. **Figure** 3.2 shows the thermal traces associated with the spray-dried powder, highlighting the corresponding exothermic or endothermic events. The profile shows a broad endothermic peak located at ∼ 60°C associated with water loss from the system followed by a re-crystallisation event (exothermic peak). Despite not confirmed by a DSC analysis of the pure drug, RFM, the endothermic peak was attributed to the drug itself, with the justification that DSC traces of RFM reported in the literature (Mahmoud et al., 2018) showed a similar endothermic peak (at 69.55°C) and an additional sharp peak at 161.8°C related to its melting point. DSC curves of RFM have also been reported by Viertelhaus and co-workers (Viertelhaus et al., 2013). RFM at room temperature exists in only one crystalline form and a phase transition is reported at 50°C; peak maxima of the phase transition was observed at 51.47 ± 0.05°C (heating) and 48.7 ± 0.9°C (cooling). The melting point peak was reported to be at 159.94 ± 0.11°C. Using different analytical techniques, they also showed that RFM undergoes a reversible phase transition. A value in agreement with the above mentioned is also reported in the patent document of Daxas where RFM melting point is stated at 159.7°C (European Medicines Agency, 2010).

Figure 3.2 Dynamic scanning calorimetry (DSC) thermogram of RF-HSA-NPs (40% w/w) embedded in mannitol microparticles (60% w/w) (NEMs). Two measurements were performed on a single batch of spray dried particles (n =1 independent experiment).

1054   In addition, it is assumed that the crystallisation event is related to the mannitol present
1055   in the formulation. D-mannitol is a material that has different morphological phases
1056   (polymorphism) which produce changes on the melting point. The forms include α with
1057   a melting point of 165.3°C/166 °C, β with a melting point of 166.7°C/ 166.5°C and δ
1058   where a discrepancy was reported regarding the melting point values (either 146.8 –
1059   156.2°C or 150 – 158°C). **Table** 3.5 indicates the different melting points of each
1060   polymorphic forms reported in the literature.
1061

Table 3.5 Melting points of mannitol polymorphic forms reported in the literature.

Mannitol polymorphic forms	Reported melting point peak (°C)	Refs
α	167; 165.6 ± 0.1; 166	(Barreneche et al., 2013; Benetti et al., 2021; Y. Yang et al., 2022)
β	155 and 166; 166.0 ± 0.5; 166.5	
δ	155; 157.1 ± 0.2; 155	

1062

1063 Moreover, the thermogram lacked evidence of the glass transition temperature of
1064 mannitol; hence we could argue that this event is overlayed by the larger water loss peak.
1065 On the other hand, the endothermic event at 161.13°C can be attributed to the melting of
1066 mannitol. Unfortunately, the melting points values reported in the literature for each of
1067 the forms of mannitol were not helpful to understand in which form the mannitol existed
1068 in the spray-dried powder. However, in a study investigating the crystallinity of inhalable
1069 siRNA powder formulation having HSA as dispersion enhancer (Chow et al., 2019), the
1070 DSC trace of raw mannitol showed - as reported in the results of this experimental chapter
1071 - a sharp endothermic peak at around 167°C which corresponds to the melting point of
1072 mannitol in its β form. Indeed, as the material of interest that has been analysed is not a
1073 pure material (mannitol only) but rather a combination at a fixed ratio of mannitol and
1074 RFM-HSA-NPs, we could conclude that the melting event is associated with the mannitol
1075 and the drug both undergoing melting. Based on the qualitative analysis that the DSC
1076 provided and on the presence of endothermic events associated to both drug and mannitol,
1077 the hypothesis is that the spray dried powder existed in form of a crystallised powder.
1078 Further studies based on the comparison of multiple DSC analysis (raw mannitol, raw
1079 roflumilast, raw HSA, HSA-NPs) will help to evaluate the solid-state profile of the
1080 obtained powder.
1081
1082 The study of the spray dried mass behaviour, and whether this was amorphous or
1083 crystalline, in presence of increasing percentages of humidity was performed using a DVS
1084 technique. The adsorption isotherm curve showed the gravimetric changes of the spray
1085 dried powder while receiving increasing amount of water in form of vapour. The typical
1086 DVS isotherm shows the sequence of the increased humidity values (blue, reference) and
1087 the recorded mass changes of the sample of interest (red curve) (**Figure** 3.3). It was
1088 showed that the powder only retained a small portion of vapour. Indeed, the red profile
1089 mostly overlapped the blue profile except for the final section describing that only a small
1090 percentage of water was retained with a consequent increase in the mass. These results
1091 are further explored in **Figure** 3.4 where the hysteresis (i.e. how much water is retained
1092 or expelled from the sample during specific points of the humidity cycle) is reported.
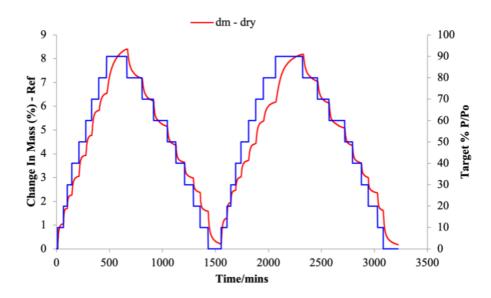
Figure 3.3 Dynamic vapour sorption isotherm of roflumilast albumin nanoparticles embedded in mannitol (NEMs) (blue reference). One measurement was performed on a single batch of spray dried particles (n =1 independent experiment).
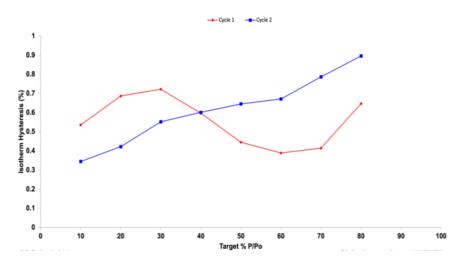
Figure 3.4 Dynamic vapour sorption isotherm (hysteresis) of RFM-HSA-NPs embedded in mannitol (NEMs).

1093

3.5 Discussion

1094

1095

1096 NP can be an effective drug delivery system to treat locally airway diseases, but the
1097 potential of this nanometer size range carrier is hindered by the impact that the
1098 aerosolization may have on the NP's stability and physiochemical properties. A detailed

1099   study of the influence of nebulisation process on NPs suspension was carried out by
1100   Dailey and co-workers (Dailey et al., 2003). Biodegradable NPs were nebulised using jet,
1101   ultrasonic and piezo-electric crystal nebulizer. In agreement with other studies performed
1102   on liposomes (Leung et al., 1996), shear forces and high energy generated during jet
1103   nebulisation and ultrasonic nebulisation respectively were shown to alter the
1104   characteristics of the nanometric system. The NPs were small and normally distributed
1105   before nebulisation, whereas after nebulisation, the suspension had a bimodal distribution
1106   pattern, with signs of aggregation that happened during the nebulisation. Hence, it is
1107   evident that nebulisation can have a negative impact on the physiochemical properties of
1108   the NPs which therefore would not be suitable in this form to be used clinically. On the
1109   other side, the delivery of NPs as dry powder also has drawbacks. Aerodynamic particle
1110   diameter is one of the critical product attributes of the orally delivered inhaled drug
1111   products. NPs would not deposit in the lungs neither by sedimentation or by impaction,
1112   nor substantially by diffusion. In addition, interparticle cohesive forces may lead to
1113   uncontrolled aggregation in the powder and variability in product quality and any
1114   therapeutic effect.
1115

1116   To overcome those disadvantages and being able to deliver therapeutic drugs to the lungs
1117   an alternative formulation strategy has been achieved. Described by Tsapis (Tsapis et al.,
1118   2002), NPs have been shown to successfully be incorporated into microparticles system
1119   and delivered to the airways. An example of the improvements that the incorporation of
1120   a free drug in NPs first and then in microparticles can bring is provided by the study of
1121   Yu and colleagues (Yu et al., 2021). They used colistin and ivacaftor, drugs characterized
1122   by low-water solubility. As strategy to increase the drug solubility and therefore improve
1123   the therapeutic activity of the drugs, they encapsulated the drugs in a NPs formulation
1124   which was then converted to a microparticle. Their studies demonstrated that colistin
1125   acted as a matrix material to bind the NPs of ivacaftor into inhalable microparticles. The
1126   powder formulation obtained via spray-freeze-drying was characterized by high FPF and
1127   superior emitted dose. Importantly, the formulation provided a very significant increase
1128   in dissolution rate compared to the free drug. The superiority for pulmonary delivery of
1129   the NEMs compared to NPs was therefore demonstrated in this study.
1130

1131   The use of the NEM has also been reported in the field of the photodynamic therapy. Elias
1132   Baghadan (Baghdan et al., 2019) developed an inhalable formulation consisting of NPs

1133 loaded with a photosensitizer. The formulation of NPs into microparticles was performed
1134 via spray-drying using mannitol as a bulking agent and resulted in a FPF of 65% and
1135 MMAD of 3.02 µm (Baghdan et al., 2019).

1136

1137 The superiority of the NEMs aerodynamic properties compared to the NP-containing
1138 powders alone and to the raw micronized drug was further demonstrated by Pilcer and
1139 co-workers (Pilcer et al., 2009). Particle dispersion during the inhalation was improved
1140 when the NEMs system was used and the FPF of the formulation increased form 36% for
1141 the raw micronized drug (tobramycin) to about 61% for the most effective NEMs
1142 formulation.

1143

1144 The concept of converting HSA-NPs into microparticles investigated in this thesis has
1145 also been reported elsewhere. In the study conducted by Varshosaz and co-workers
1146 (Varshosaz et al., 2015), haloperidol targeted-BSA NPs were manufactured via
1147 desolvation method and loaded with doxorubicin. The NPs were converted via spray-
1148 drying to microparticles using different inlet temperatures (between 80 and 120°C) and
1149 excipients (mannitol, L-leucine and trehalose). The best results were obtained when HSA-
1150 NPs were spray dried with mannitol at 80°C (inlet temperature), showing a mean
1151 aerodynamic diameter of 4.58 µm and FPF of 66%. Despite the difference in the inlet
1152 temperature used (80 °C in Varshosaz's study (Varshosaz et al., 2015) and 106 °C in this
1153 experimental work), FPF value obtained (66 %) corresponds to the FPF obtained in this
1154 experimental study (67 %). The potential of mannitol as a bulking excipient, described
1155 by Varshosaz and co-workers, was also explored in a similar investigation where three
1156 different excipients – mannitol, leucine and trehalose – were used to evaluate the best
1157 spray dried powder containing HSA-NPs (Patel, 2018). Varshosaz and others also
1158 investigated the morphological structure of the nanocomposites (M_{80} and M_{120}, when
1159 albumin NPs were spray dried with mannitol using 80°C and 120°C respectively as inlet
1160 temperature in the spray drying process). Nanocomposite of mannitol appeared
1161 approximately round and smooth, and this structure was not impacted by the increase in
1162 temperature (from 80 to 120 °C).
1163 Despite any morphological analysis were performed in this experimental work, a previous
1164 scanning electron microscopy analysis of a spray-dried powder incorporating both blank
1165 and RFM-HSA-NPs (Patel, 2018) showed spherical particles for both formulations, with
1166 NP-containing formulation appearing to have a slight rougher surface. Further particle

1167 surface analysis would need to be carried out to better evaluate how the NP influence the
1168 morphology of the final NEM structure.

1169 As mentioned above, the rate of two critical phases in the spray drying process – constant
1170 drying rate and falling drying rate- will highly impact the final structure of the NEMs. In
1171 this context it would be interesting to investigate first the morphology of the pure
1172 mannitol microparticle and then the result of the co-spray dried particles. The distribution
1173 of both blank and RFM-loaded HSA NPs in the mannitol spray-dried matrix (NEMs)
1174 could be achieved using a confocal laser scanning microscopy. This technique allows –
1175 previous labelling of components with fluorescent dyes – to visualize the surface and the
1176 core of the particles. The resulting three-dimensional picture would enable to study the
1177 spatial distribution of the component within a particle.

1178

1179 There are several excipients that can be used to manufacture microparticles via spray
1180 drying and the choice of the most appropriate one for lung administration is mainly based
1181 on the potential toxicological concerns and on the physiochemical properties that relate
1182 to product quality and the desired kinetics of drug release from the particle. When
1183 formulating a NEMs system for lung delivery, it is recommended to achieve low
1184 aggregation of the NPs in the microparticles in order to get rapid disaggregation once
1185 deposited into the lungs. Those features are usually obtained adding an excipient which
1186 provides spatial separation within the individual colloidal NPs ensuring a lower extent of
1187 NP-NP interaction (Ruge et al., 2016).

1188

1189 The interactions between excipient and NP must be 'weak enough' to allow the excipient
1190 dissolution and dissociation from the NPs after deposition, but on the other hand those
1191 interactions need to be strong enough to hold the system together also withstanding the
1192 stress of the spray drying process. Neither the excipient nor the spray drying process
1193 should alter the physiochemical properties of the NPs and the kinetics of drug release
1194 which are designed to be dictated by the NP system.

1195

1196 The investigation of the fate of the administered spray-dried powder is a key step in the
1197 development of an inhalation delivery system as it allows the spreading of the embedded
1198 nanometric system into the target area. The hypothesis is that after deposition into the
1199 lung lining fluid, the NEMs dissociate into multiple primary NPs system which evade
1200 clearing mechanisms and deliver drug. The re-dispersibility of the aggregate system

depends on i) the strength of the NEMs binding forces – which is governed by the chemical characteristics of the excipient and its ability to make bridges with the NPs system – rather than the attractive forces (van der Waals) among the NPs; ii) the degree of microparticle wetting in the medium.

However, it is important to highlight that dissolution of the matrix components in the lung lining fluid is not automatically associated with the disintegration of the NPs and that the understanding of this phenomenon may vary based on the *in vitro* model used and whether it is biorelevant or not. In a study of Ruge and co-workers (Ruge et al., 2016), polymeric (polystyrene) NPs were manufactured and then spray dried with trehalose forming NEMs. The aqueous redispersibility of the system was firstly studied in a model based simply on the redispersion of the NEMs powder in water with following vortexing and Dynamic Light Scattering analysis. Results showed that the NEMs were successfully disintegrated into the single NPs which were efficiently detected by light scattering. However, when a more complex *in vitro* model was used, the NEMs aerosolization onto a static mucus of layer resulted in the NEMs remaining in their microparticulate state. The authors raised the hypothesis that the NEMs were dissolved in the mucus due to the excipient solubility, but the polymeric (polystyrene) NP did not disperse upon NEM disintegration remaining in agglomerates form. This may be due to the interparticulate attractions within the NPs agglomerate such as van-der-Waals or capillary forces. The aqueous re-dispersibility of the NEMs is dependent on the 'excipient bridges' between carrier and NPs, but it is also governed by the degree of particle wetting in an aqueous medium. The latter is influenced by the nano-aggregate surface hydrophilicity and by the shell thickness to particle radius ratio, where particles with thicker shells have less re-dispersibility due to the reduced surface area (Kho & Hadinoto, 2010a).

Regarding disintegration, the methods usually adopted by researchers to investigate the particles wettability such as application of high-intensity forces or ultrasonication do not resemble the conditions that particles are exposed to in the lungs. The need for more complex biorelevant models was addressed by Kho and co-workers in 2010 (Kho & Hadinoto, 2010a). The wettability of hollow spherical silica mannitol-based nanoaggregates was calculated with a method based on a series of dilutions and centrifugations combined with a turbidity measurement, as a function of particle size. This correlation was based on the wetting of particles and their 'excipient bridges' leading

1234   to dissociation of the primary NPs with a concomitant reduction of the aggregate size and

1235   therefore reduction of level of turbidity.

1236

1237   A detailed description of the dispersibility assay was also reported in another research

1238   study (Kho & Hadinoto, 2010b). The same authors also investigated the re-dispersibility

1239   of a nanoaggregate manufactured for the delivery of the antibiotic polycaprolactone using

1240   and testing different ratio of three excipients: mannitol, leucine and lactose (Kho et al.,

1241   2010). They concluded that the re-dispersibility is highly dependent on the excipient

1242   formulation and only the nanoaggregates with lactose-leucine as excipients resulted in

1243   highly re-dispersible formulation. Similar results were obtained in another study where

1244   multiple-excipients formulation involving leucine and lactose produced successful

1245   formulations in terms of morphology and aqueous re-dispersibility (Kho & Hadinoto,

1246   2010b).

1247

1248   In this experimental study, the mannitol-based nanoaggregates were dissolved to release

1249   albumin NP, which were observed to be of consistent size and PDI before and after the

1250   spray-drying, which represents successful re-dispersibility of the nanoaggregates and

1251   indicates that the NPs retained their properties through formulation as NEMs. Further

1252   studies could focus on the redispersability of the designed system in more biorelevant

1253   models which implicate the aerosolization of the NEMs onto a static and dynamic mucus

1254   layer.

1255

1256   Often the *in vitro* models used by researchers to investigate the re-dispersibility of the

1257   nanoaggregates involves the use of shaking, for example as in the quantitative evaluation

1258   of dispersibility performed by Torge (Torge et al., 2017) in presence of excess of

1259   simulated lung fluid. The use of stirring or shaking in *in vitro* models is often claimed to

1260   reproduce the lung dynamicity as they undergo movement associated with breathing

1261   cycles. Besides this, also the motion of ciliary beat should also be considered. The

1262   influence of force that exceeds the cohesion forces between the particles, is in providing

1263   a source of shear stress that will contribute to the disintegration of the particle aggregates

1264   with subsequent spreading of NP on the mucus layer and in the lung lining fluid.

1265

1266   Based on previous studies in the literature which employ mannitol as excipient, we may

1267   anticipate for the system designed in this experimental chapter a good and promising

1268 redispersibility behaviour *in vivo*; however further works are required to fully characterise
1269 the disintegration kinetic of the embedded microparticles in a biorelevant assay, e.g. using
1270 lung simulated fluid. One promising technique to discriminate the performance of the
1271 system may be represented by the isolated lung technique (Beck-Broichsitter et al., 2016).
1272 In addition, further investigation could focus on the *in vivo* distribution of the NEMs in
1273 an LPS-inflammation model. For pre-clinical studies, *in vivo* administration could be
1274 achieved firstly via aerosolization using the Penn Century device. A valuable alternative
1275 for the *in vivo* administration is also represented by the use of a system called
1276 PreciseInhale (Inhalation Sciences, Sweden). The latter allows to generate a dispersed
1277 aerosol and deliver it to an individual specie with minimal loss of drug and an accurate
1278 administration which is constantly monitored via a software. Following this first
1279 preliminary investigation, the adoption of a more complex and reliable method based on
1280 the use of a commercially available Vectura DPI device would be recommended.
1281

1282 ## 3.6 Conclusion
1283

1284 A key step when investigating the potential of a NPs system aimed for inhalation delivery
1285 to the lungs is evaluating its respirability. Hence the main objective of this chapter was
1286 to find a strategy to overcome the issues related to the pulmonary administration of a
1287 delivery system in the nanometre range. A spray-drying manufacture process was used
1288 which, despite being a precedented method, has not been extensively studied for HSA-
1289 NPs embedded in microparticles systems. This chapter presents a successful
1290 manufacturing approach for HSA-NPs in mannitol matrix microparticles. The spray
1291 drying conditions adopted resulted in good process yield and did not impact the
1292 physiochemical properties (size, PDI and zeta potential) of the original NP system. This
1293 is a key aspect when formulating a microparticles system, as its disintegration will release
1294 the NPs which ultimately control the release of the drug. Preliminary aerodynamic
1295 investigation resulted in a promising FPF equal to 67% and PSD analysis also
1296 demonstrated that 90% of the particle population has a geometric diameter below 5 µm.
1297 These data indicate that the microparticles are suitable for delivery and deposition in the
1298 lungs.
1299

1300

1301

CHAPTER 4: Anti-inflammatory activity of roflumilast in an in vitro respiratory epithelial cell assay

4.1   Introduction

BEAS-2B cells are respiratory epithelial cells isolated from normal human bronchial tissue derived from autopsies of non-cancerous individuals, transfected with an adenovirus 12-SV40 virus hybrid and cloned. They have been used as two-dimensional *in vitro* airway model to mimic the epithelium and in the drug development for the screening of the biopharmaceutics of new compounds (Villandre et al., 2022; M. Zhang et al., 2022). In addition, other two cell lines commonly used as 2D-*in vitro* models of the airways are A459 cells, an adenocarcinomic human alveolar basal epithelial cell line and the bronchial adenocarcinoma-derived Calu-3 cell line. Despite their widespread use as starting point in the *in vitro* evaluation of new compounds, a recent review highlighted the limitations that 2D models have compared to 3D models. In 2D cellular models, cells are forced to adapt to an environment that differs from the physiological one. In particular, BEAS-2B fail to express tight junction formation and secrete airway mucins with consequences on their ability to form an effective barrier (Yaqub et al., 2022). Despite the limitations that 2D models may have, BEAS-2B are often utilised in research as model for the airways and in studies to understand the interaction of new drugs with the airway epithelium.

One of the most common *in vitro* models used for COPD involves the exposure of cells to an endotoxin. Endotoxins of gram-negative bacteria can induce potent pathophysiological effects in mammalian cells. The endotoxins are lipopolysaccharide (LPS) composed of an O-specific chain, a core oligosaccharide and a lipid component called lipid A which is responsible for the endotoxic activities. The mechanism of endotoxic activity of LPS involves an active response of the host cells such as endothelial, mononuclear cells or macrophages. The recognition between LPS and host cells results in the secretion of various mediators which activate other cellular inflammatory pathways (Rietschel et al., 1994). In 1990, Wright and colleagues demonstrated that leukocytes respond to LPS by the secretion of cytokines such as TNF-α. The mechanism of action involves the binding of LPS to a glycoprotein found in human serum called

lipopolysaccharide binding protein (LBP) followed by recognition and binding of the complex LPS-LBP to the receptor CD14 anchored to the cell membrane (mCD14) (S. D. Wright et al., 1990).

Although mCD14 is not expressed in endothelial or epithelial cells, Pugin and co-workers demonstrated that the stimulation of those cells by LPS was via a specific mechanism which involved LBP and soluble CD14 (Pugin et al., 1993). Further investigation of the mechanism of action of LPS in BEAS-2B cells showed that in these cells there can be a CD14-indipendent activation and that other proteins may be involved or direct TLR-LPS interactions happens. They concluded that airway epithelial cells respond to LPS in a concentration dependent manner and that the effect is augmented by the presence of serum, supporting the hypothesis that cofactor(s) are necessary to initiate the stimulation (Schulz et al., 2002).

After the confirmation of the involvement of TLR4 in the LPS activation signalling pathway, the same signalling in pulmonary epithelial cells was investigated by Guillott and colleagues (Guillott et al., 2004). They demonstrated: i) the expression of TLR4 and MD-2 in respiratory epithelial cells; ii) the intracellular presence of TLR4; iii) the signalling of LPS in epithelial cells shares some elements with the activation of myeloid cells such as MyD88, IRAK, TRAF6 and MAPK. They demonstrated the expression of TLR4 in human alveolar and bronchial epithelial cells and its intracellular localization of this receptor; in addition, they confirmed that the activation pathway of LPS involves and is dependent on the TLR4 signalling (Guillott et al., 2004).

Therefore, current understanding of the activation pathway for LPS in airway epithelial cells implicates the activation of a complex association of receptors composed by TLR4, MD-2 and CD14 in the case of mononuclear phagocytes.

Besides the LPS inflammation model, another common model for COPD is based on the exposure to cigarette smoke extract (CSE) (Krimmer & Oliver, 2011). The inflammation may differ depending on the number of cigarettes burned - if commercially available or research grade – or by the presence or the absence of filter. As for the LPS, CSE can modulate the release of cytokines and chemokines from the lungs and immune cells. Moreover, as viral infection is an important feature of COPD that can result in exacerbations, the combination of CSE and rhinovirus has also been modelled *in vitro* in

1369   BEAS-2B with a consequent upregulation of interleukin- 8 (IL-8) (Hudy et al., 2010;

1370   Hudy & Proud, 2013).

1371

1372   In the inflammation model designed and adopted in this chapter, the level of specific

1373   extracellular signalling proteins (IL-8, interleukin- 6 (IL-6) and TNF-α) secreted by

1374   bronchial epithelial cells was chosen as a measure of inflammation. In inflammatory lung

1375   diseases, cytokines play a key role sustaining the inflammation and recruiting further cells

1376   into the lungs, orchestrating the chronic inflammation (Chung, 2001). The pivotal role of

1377   cytokines in the pathophysiology of COPD is supported by their increased level found in

1378   the induced sputum of patients, plasma or exhaled breath. More than 50 cytokines have

1379   now been identified to be involved in the molecular pathology of diseases such as COPD.

1380   Clinical studies have demonstrated that TNF-α(Aaron et al., 2001; Keatings et al., 1996),

1381   IL-6(Bhowmik et al., 2000; Bucchioni et al., 2003) and IL-8(Aaron et al., 2001; Barnes,

1382   2008; Barnes et al., 2015; Fischer et al., 2011; Keatings et al., 1996) can be found in the

1383   induced sputum of COPD patients.

1384   According to the literature, TNF-α and IL-6 are also good biomarkers of systemic

1385   inflammation in COPD patients (Karadag et al., 2008). The serum level of those markers

1386   has been reported to be higher in both COPD patients with a stable condition or with

1387   exacerbations phases than in healthy controls, supporting their use as biomarkers of

1388   systemic inflammation in COPD patients. Increased levels of TNF-α, IL-8 and IL-6 were

1389   also reported in bronchioalveolar lavage fluid from 14 healthy smokers compared with

1390   16 healthy non-smokers (Kuschner et al., 1996)

1391   Overall, the role that IL-8, IL-6 and TNF-α play in COPD further corroborate the choice

1392   of using the above-mentioned cytokines as representative COPD markers in the *in vitro*

1393   model of lung inflammation disease designed and optimised in this experimental chapter.

1394

1395   The level of cytokines released by LPS-stimulated BEAS-2B has been reported to vary

1396   in presence or absence of serum. Indeed, multiple cell culture conditions are used in the

1397   literature with a consequent lack of consistency within studies. According to the literature,

1398   cell culture conditions for BEAS-2B include the use of i) serum free bronchial epithelial

1399   cell growth medium (X. Wang et al., 2012) or Dulbecco's Modified Eagle Medium

1400   supplemented with 10% fetal bovine serum (FBS). The latter was used to study heavy

1401   metals induced carcinogenesis (Laulicht et al., 2015; Park et al., 2015), to investigate the

1402   relations between chronic arsenic exposure, reactive oxidative species production and cell

transformation (Carpenter et al., 2011; Chang et al., 2010) or to investigate the protective effect of angiotensin converting enzyme 2 against LPS-induced injury in mice (Ye & Liu, 2020).

Indeed, the exposure of BEAS-2B to serum can alter the response to toxic agents and the cytokine secretion. It is also associated with the cell squamous differentiation.

The design of the *in vitro* model described in this chapter also focussed on the relationship between increasing concentration of serum in the culture medium and concentration of cytokine secreted. This hypothesis was further corroborated by a study carried out by Veranth (Veranth et al., 2008). The hypothesis that the secretion of IL-6 from BEAS-2B can vary based on the cell model used was tested. Three different culturing media were tested: Lechner and Laveck medium (LHC-9), KGM and LHC-9 with FBS. Results showed how the IL-6 secretion can be titrated by increasing amount of serum added to KGM media in absence of particulate matter or in presence of soil dust treatment. The evidence that BEAS-2B used in airways models can be successfully cultured using different conditions, led us to preliminary need of studying the effect of different percentages of serum on the cytokine production.

4.2   Aims and objectives

The overall aim of this series of experiments was to build an *in vitro* inflammation model in bronchial epithelial cells (BEAS-2B) to enable investigation of the anti-inflammatory activity of RFM. LPS-induced inflammation was chosen for this work to enable progression of the research into animal models that are based on the same inducer of inflammation.

Specific objectives included:

1. Optimise BEAS-2B culture conditions, including the amount of FBS used in the growth medium;
2. Optimise the LPS concentration and exposure time that result in a sustained and detectable secretion of cytokines into the culture medium over time;
3. Investigate the anti-inflammatory activity of RFM applied in a pre-treatment protocol for 1.5 h, 3 h or 24 h.
4.

4.3 Methods

4.3.1 Cell culture conditions

Bronchial epithelial cells (BEAS-2B) were grown at 37°C with 5% CO_2 in 75 cm^2 Falcon tissue culture flasks in culture medium (RPMI 1X [+] glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin) which was replaced with fresh medium every three days. Cells were passaged or seeded into 24-well plates when > 70% confluent by washing with Ca^{2+} and Mg^{2+} free PBS before using trypsin 0.25% to dissociate cells from their substrate.

4.3.2 Experimental design

On day 0, cells were seeded in 24-well plate (Greiner CELLSTAR®) in growth medium (0.5 mL) and allowed to adhere to the base of the well overnight; medium was replaced on day 1 and, unless stated otherwise, treatment started on day 2. After each specific treatment, cell supernatant from each well was withdrawn and immediately centrifuged at 1,000 g x 5 min at 4°C to eliminate any cell debris. Supernatants were aliquoted and stored at - 80°C for assay of IL-8, IL-6, or TNF-α level using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (DuoSet, R&D, Biotechne, Minneapolis). Every experiment was performed with three replicate wells for each experimental variable, with the experiment being repeated in duplicate or triplicate. Cells used for those experiments were between passages 29 and 43. The optimal seeding density (day 0) to obtain a confluent monolayer of cells on day 2 was investigated and found to be 2.5 x 10^5 cells/well (data not shown) which was then used as seeding density for every experiment mentioned in this chapter.

4.3.3 Effect of fetal bovine serum concentration on cytokine release

Cells were seeded in 24-well plate at 2.5 x 10^5 cells/well in growth medium (day 0). Medium was replaced with fresh containing a combination of either 2 or 10% FBS on day 1 and day 2. After 72 h in the medium (day 3 in total), cell supernatants were withdrawn and processed as described above (**Figure** 4.1). The impact of different

1469   concentration of FBS on the levels of IL-6, IL-8 and TNF-α release was measured using

1470   a commercial sandwich ELISA kit.

1471
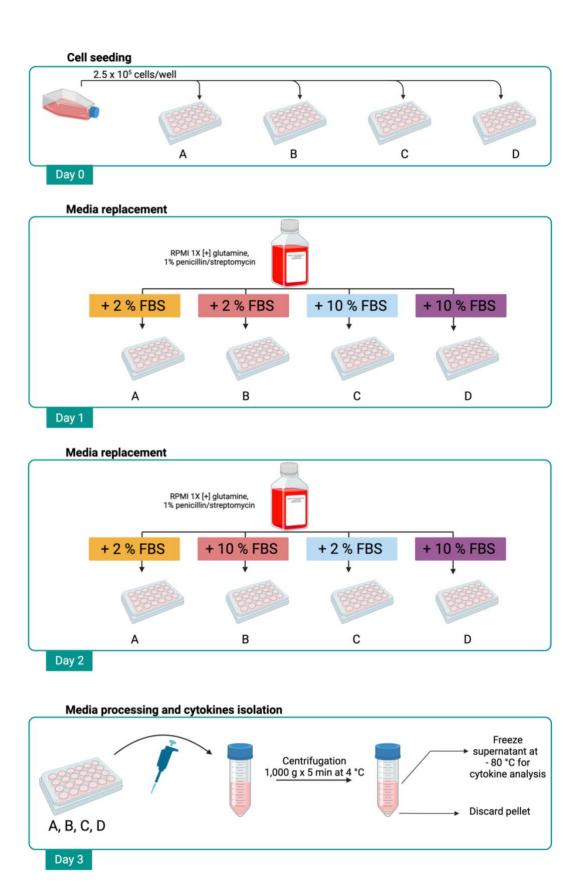
Figure 4.1 Schematic representation of the experimental design adopted in the evaluation of the effect of serum on cytokine secretion.

1472

4.3.4 Optimisation of LPS- induced inflammation model

The LPS inflammation model was optimised in terms of LPS concentration, LPS exposure time and concentration of FBS in culture medium. Based on preliminary results, experiments to evaluate the most suitable concentration and exposure time were performed using 10% FBS in the culture medium throughout.

On day 0, cells were seeded in culture medium, which was replaced with fresh medium on day 1. On day 2, an aliquot of the LPS (O111:B4) stock solution (5 mg/mL) previously prepared was thawed and serial dilutions performed in RPMI 1X [+] glutamine, 10% FBS and 1% penicillin/streptomycin to produce concentrations of 10, 100, 200, 300, 500, 800, 1000 ng/mL. Cells were exposed and incubated with LPS or cell medium (0.5 mL) (negative control) at 37°C. Following 24 h incubation, the medium was withdrawn and processed for assay for cytokine detection as described in paragraph 4.3.2.

The impact of different concentrations of FBS in the cell medium on day 1 and day 2 was investigated when cells were exposed to LPS. On day 0, cells were seeded in growth medium. Medium was replaced on day 1 and on day 2 cells were exposed to 800 ng/mL and 1000 ng/mL (0.5 mL) of LPS in medium with different percentages of FBS (Figure 4.1). Following 24 h incubation, culture medium was withdrawn and processed as described in paragraph 4.3.2.

The effect of LPS incubation time on the IL-6 levels released by BEAS-2B cells was investigated. Cells were seeded on day 0, and medium replaced on day 1. On day 2, cells were incubated with 800 ng/mL (0.5 mL) of LPS for 2, 4, 6, 15, 24 and 48 h. A percentage of 10% of FBS was used in the medium on both days 1 and 2. Control cells were incubated with growth medium only. Following LPS exposure, medium was withdrawn and processed as described in paragraph 4.3.2.

4.3.5 Exposure of LPS-exposed BEAS-2B cells to roflumilast

The anti-inflammatory activity of RFM in the BEAS-2B cell inflammatory model was investigated using a protocol designed to test a preventative effect whereby cells were exposed to the drug for different period of time before LPS was applied for 24 h. Dexamethasone pre-treatment was used as positive control to confirm the ability of the

model to report anti-inflammatory activity. The concentration of LPS used in these drug treatment experiments was 800 ng/mL. To ensure that each well had the same total cell number, a 'modified protocol' was adopted for the drug exposure of 1.5 – 2 or 3 h (**Figure 4.2**).

For RFM and dexamethasone pre-treatment of 24 hours, cells were seeded on day 0 in 24-well plate and cells fed with fresh medium on day 1. On day 2, cells were exposed to the drug dissolved in the culture medium. After 24 h (i.e on day 3 in total) conditioned medium was withdrawn and discarded, and cells exposed to LPS for 24 h. On day 4 the experiment was terminated, and samples processed as described in section 4.3.2. For RFM and dexamethasone pre-treatment of 1.5, 2 or 3 h, the protocol was adapted, and the drug exposure started on day 3 rather than on day 2. After the allocated exposure time, culture medium was withdrawn, and cells were exposed to LPS. On day 4 the experiment was terminated, and samples processed as described in section 4.3.2.

RFM stock solution (0.01 M) in DMSO (100% v/v) was prepared and aliquots frozen. On the day of the experiment, an aliquot was thawed and serial dilutions in growth medium were performed to produce concentration of 0.001, 0.1, 1.0, 100, 1,000 and 10,000 nM. The percentage of DMSO in each sample was calculated to be < 0.1%. Controls samples were represented by control cells exposed to drug- and LPS-free culture medium (negative control) or cells exposed to drug-free culture medium containing 0.1% DMSO, then exposed to LPS (positive control).

A stock solution of dexamethasone (21,600 µg/mL) was prepared in DMSO (100% v/v) and aliquots stored at –80 C°. On day of the experiment serial dilutions were performed to produce dexamethasone concentrations of 0.1, 1.0, 10, 100 µg/mL in culture medium. The percentage of DMSO in each sample was calculated to be < 0.5%. Controls samples were cells exposed to drug- and LPS-free culture medium cells exposed to growth culture medium containing 0.5% DMSO, then exposed to LPS (positive control).

RFM and dexamethasone pre-treatments were evaluated by incubating the cells with the drug at the chosen concentrations for 1.5 h, 3 h and 24 h for RFM and 2 h and 24 h for dexamethasone.

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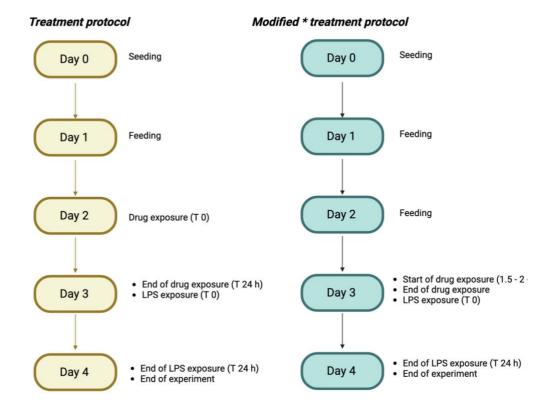
Figure 4.2 Schematic representation of 'treatment protocol' and 'modified treatment protocol' adopted.

1542   **4.3.6   Statistical analysis**

1543

1544   Data were expressed as mean ± SD. Where appropriate, data were analysed using

1545   GraphPad Prism version 9, (GraphPad Software, San Diego, USA). Normal distribution

1546   of data was firstly evaluated and the statistical significance between groups was

1547   determined using parametric tests. Statistical significance was evaluated with unpaired t-

1548   test within two conditions, ordinary one-way or two-way ANOVA multiple comparisons.

1549   Statistically significant differences are represented by asterisks as follow: *, $p \leq 0.05$; **,

1550 $p \leq 0.01$; ***, $p \leq 0.001$. Where stated that a statistical analysis was performed, absence

1551   of asterisks indicates non-statistically significant results.

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4.4   Results
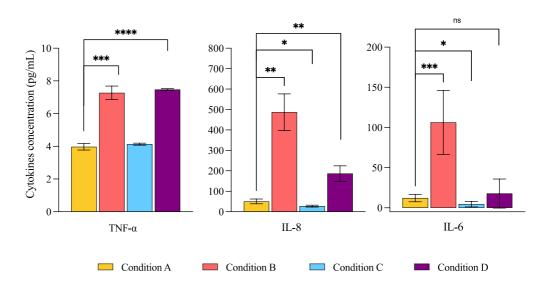
4.4.1   Effect of fetal bovine serum concentration on IL-8 and IL-6 basal release from BEAS-2B cells

The incubation of BEAS-2B cells with different concentration of serum (2% or 10%) on day 1 and 2 after seeding resulted in a basal production of cytokines. The basal release of TNF-α, IL-6 and IL-8 varied according to the concentration of FBS and the duration of exposure (**Figure** 4.3). Overall, there was a consistent trend for the cytokine concentrations released in response to different FBS exposure across the three cytokines evaluated, although the levels of TNF-α detected were considerably lower (between 3.9 and 7.47 pg/mL) compared to IL-8 and IL-6 (IL-8: A) 50.4 pg/mL; B) 486.9 pg/mL; C) 27.7 pg/mL; D) 186.7 pg/mL. IL-6: A) 12.2 pg/mL; B) 106.3 pg/mL; C) 4.6 pg/mL; D) 17.8 pg/mL) (Figure 4.3 a). For both IL-8 and IL-6, the exposure to low (2%) and high (10%) percentages of serum on day 1 and day 2 respectively resulted overall in the highest concentration of secreted cytokines. Lower than the latter but still elevated concentration of cytokines were released when the cells were fed with the highest concentration of serum on both days. In contrast, the exposure to 10% and 2% on day 1 and 2 respectively resulted in the lowest level of cytokines released by the BEAS-2B cells. Results showed that the concentration of TNF-α and IL-8 – but not IL-6 - released in presence of high percentage of serum on day 1 and day 2 were higher and significantly different compared to those released by cells exposed to the lowest percentage of serum on day 1 and 2. In addition, results showed that if cells were exposed to 2% on day 1 , the further exposure on day 2 to 10% of serum significantly 'boosted' the sensitivity of the cells inducing an increased level of cytokine released compared to cells exposed to 2 % on day 2.

Among the four culturing conditions tested, the addition of 10% of serum to the growth medium both on day 1 and day 2 was considered the most appropriate and adopted for the drug treatment exposure experiment. Indeed, when using the optimised seeding density, those percentages of serum can stimulate the cells to secrete cytokine concentrations adequate to be efficiently detectable using commercial ELISA kits and low enough to work as control (no treatment) in the drug treatment protocol.

a.

b.

	% of serum added to growth media			
	Condition A	**Condition B**	**Condition C**	**Condition D**
Feeding Day 1	2 %	2 %	10%	10 %
Feeding Day 2	2 %	10 %	2 %	10 %

Figure 4.3 (a) Effect of fetal bovine serum (2% or 10%) in cell culture medium on secretion level of TNF-α, IL-8 and IL-6. (b) The different regimes for exposing cells to fetal bovine serum. Cells (BEAS-2B) were seeded in 24-well plate on day 0 in growth medium (0.5 mL). On day 1 and 2, cell medium was replaced with fresh medium containing either 2% or 10% of FBS. Twenty-four hours later, conditioned medium was withdrawn, and levels of cytokine detected with commercial sandwich ELISA kit. Statistical significance was evaluated with student t-test. Data represent mean ± SD of one (TNF-α) or two (IL-8 and IL-6) independent experiments (for each experiment, each sample was in triplicate (three wells)).

4.4.2   Effect of LPS on IL-8 and IL-6 production in BEAS-2B

A positive LPS dose-response relationship for IL-8 and IL-6 in BEAS-2B cells was observed (**Figure** 4.4). The exposure of BEAS-2B cells to increasing concentrations (10 – 1000 ng/mL) of LPS induced an increased secretion of both cytokines compared to control. The exposure to the lowest LPS concentration (10 ng/mL) already induced a

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1605 significant increase of IL-8 and IL-6 compared to control as well as when cells were
1606 exposed to 800 and 1000 ng/mL. Higher variability in the IL-6 response to LPS was
1607 observed at the concentration range tested. In both cases, the highest concentrations of
1608 LPS used (800 and 1000 ng/mL) resulted in a robust response from BEAS-2B cells.
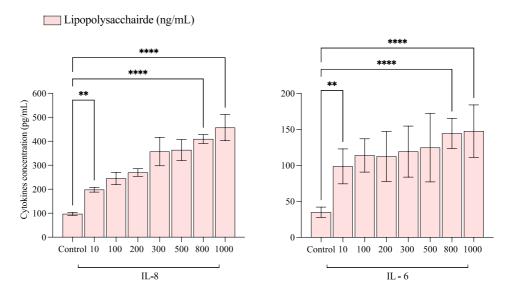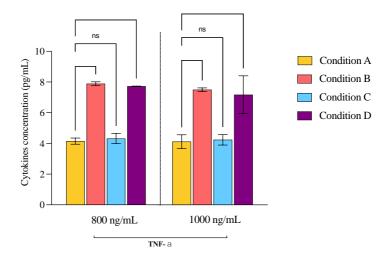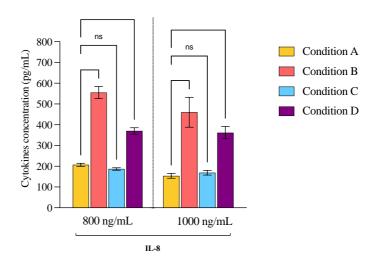
Figure 4.4 Effect of lipopolysaccharide exposure (24 h) on IL-8 and IL-6 production in BEAS-2B. Cells were grown in culture medium for 2 days then exposed to culture medium (control) or LPS (10, 100, 200, 300, 500, 800, 1000 ng/mL) for 24 h. After LPS exposure, medium was withdrawn and assayed for cytokine concentration. Statistical significance was evaluated with ordinary one-way ANOVA multiple comparisons. Data represent mean ± SD of one (IL-8) or two (IL-6) independent experiments, using one or two different passage number (for each experiment, each sample was in triplicate (three wells)).

1609 Following the dose-response study, 800 and 1000 ng/mL LPS were selected for further
1610 investigation. The correlation between LPS doses and serum percentages on day 1 and on
1611 day 2 was evaluated measuring the concentration of IL-8 and TNF-α released by the cells
1612 (**Figure** 4.5). The exposure to 800 and 1000 ng/mL for 24 h using different concentrations
1613 of FBS resulted in the same trend showed in Figure 4.3 (a). The highest level of TNF-α
1614 and IL-8 was detected when cells were exposed to any of the percentages investigated on
1615 day 1 (2 or 10%), but to 10% on day 2. The levels of TNF-α detected were considerably
1616 lower compared to IL-8 concentrations, with values between 4.12 and 8.0 pg/mL, while
1617 IL-8 concentrations (day 1: 10% FBS, day 2: 10% FBS) were in agreement with those
1618 obtained in the LPS dose-response (Figure 4.4). Overall, for both IL-8 and TNF-α the

159

1619 concentration of cytokines released by the cells was similar, regardless of the

1620 concentration of LPS used to stimulate the cells.

1621 A multiple comparison was carried out to investigate if the exposure of BEAS-2B to

1622 percentages of serum different from 2% on both days resulted in significant differences.

1623 For both IL-8 and TNF-α the increase of serum only on day 2 resulted in significant

1624 difference compared to the use of low percentage of serum on both days and vice versa.

1625 Significantly different was also the concentration of cytokines (IL-8 and TNF-α) released

1626 when BEAS-2B were exposed to 10% of FBS on both days compared to 2% of FBS on

1627 both days. In contrast, the increase of FBS percentages on day 2 if on day 1 cells were

1628 exposed to 2% of FBS resulted in not significant differences of cytokines concentrations.

1629 The concentration of 800 ng/mL was considered adequate to sufficiently stimulate the

1630 secretion of the cytokines of interest from BEAS-2B. It was therefore selected as the LPS

1631 dose to be used in the treatment schemes which results are described in paragraphs 4.4.3

1632 and 0.

1633

a.

b.

	% of serum added to growth media			
	Condition A	**Condition B**	**Condition C**	**Condition D**
Feeding Day 1	2 %	2 %	10%	10 %
Feeding Day 2	2 %	10 %	2 %	10 %

1634   *Figure 4.5 (a) Effect of lipopolysaccharide (800 and 1000 ng/mL) on TNF-α and IL-8*

1635   *production in BEAS-2B when cells are incubated with different percentages of fetal*

1636   *bovine serum (2 % or 10%) on day 1 and 2. (b) The different regimes for exposing cells*

1637   *to fetal bovine serum. Statistical significance was evaluated with ordinary one-way*

To optimise the inflammation model, the effect of the duration of LPS exposure was also investigated. The time-dependency of the release of IL-6 induced by LPS (800 ng/mL) was determined. A robust time-dependent relationship was observed between IL-6 secretion and duration of LPS exposure (**Figure** 4.6). The difference between IL-6 concentration in control samples and LPS-challenged samples was significantly different for each time point considered, except for the 2 h exposure (control 27.8 pg/mL vs LPS 193.4 pg/mL, control 31.9 pg/mL vs LPS 349.9 pg/mL at 24 h and 48 h time points, respectively). The concentration of IL-6 detected in the cell supernatant increased from 2 h to 48 h with values of 15.9 pg/mL at 2 h to 349.9 pg/mL at 48 h. According to these results, 24 h was selected as LPS exposure time due to its suitability to induce a robust release of cytokine which modulation by anti-inflammatory drugs will be described in the next paragraph.
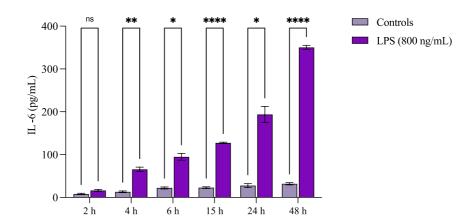
Figure 4.6 Effect of different exposure times to lipopolysaccharide (800 ng/mL) on IL-6 production from BEAS-2B cells. Cells were seeded on day 0 in culture medium which was replaced with fresh medium on day 1; on day 2 cells were exposed to LPS (800 ng/mL) or culture medium (control) for the allocated exposure times. Statistical significance was evaluated with two-way ANOVA multiple comparisons. Data represent mean ± SD of one independent experiment, with measurements in triplicate (three wells).

4.4.3 Anti-inflammatory activity of dexamethasone in an LPS-challenged BEAS-2B model

The exposure of BEAS-2B to a pre-treatment of dexamethasone for 2 or 24 h followed by LPS stimulation resulted in the reduction of the IL-6 levels compared to non-drug treated control (**Figure** 4.7). For the 2 h pre-treatment, the dose-response relationship is less evident with values of 117.5 pg/mL, 73.5 pg/mL, 54.3 pg/mL and 53.0 pg/mL for 0.1, 1.0, 10 and 100 µg/mL, respectively. Interestingly, dexamethasone was less effective in reducing IL-6 secretion when the cells were pre-exposed for 24 h to the same range of concentrations, requiring higher concentrations to achieve the same suppression of IL-6 levels seen at 2 h. i.e. after cells were exposed to 1.0, 10 and 100 µg/mL (positive control: 311.8 pg/mL; 1.0 µg/mL: 173.3 pg/mL; 10 µg/mL: 130.4 pg/mL; 100 µg/mL: 61.6 pg/mL).

For both the pre-treatment regimes, the concentration of IL-6 released from cells exposed to LPS (800 ng/mL) was significant different compared to control (growth media only). The exposure to each dexamethasone concentrations significantly reduced the IL-6 levels compared to positive control when cells were exposed to the 2 h pre-treatment, whereas the treatment with 0.1 µg/mL for 24 h did not significantly reduce the IL-6 levels compared to positive control.

Figure 4.7 Effect of dexamethasone pre-treatment on IL-6 production from LPS-challenged BEAS-2B cells. For the 2 h pre-treatment, cells were seeded on day 0 and medium changed on day 1 and 2. On day 3, cells were exposed to dexamethasone following by exposure to LPS (24 h); for the 24 h pre-treatment, cells were seeded on day 0 and medium changed on day 1. On day 2, cells were exposed to dexamethasone (24 h). On day 3, supernatant was withdrawn, and cells stimulated with LPS (24 h). Statistical significance was evaluated with one-way ANOVA multiple comparisons. Data represent mean ± SD of three independent experiments (using three different passage number) (with measurements in triplicate (three wells)).

1673
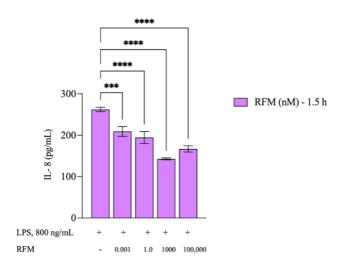
4.4.4 Investigating the anti-inflammatory activity of roflumilast pre-treatment in an LPS-challenged BEAS-2B model

1676

The effect of the PDE-4 inhibitor RFM on the LPS-induced release of IL-8 and IL-6 from BEAS-2B was investigated (**Figure** 4.8 and **Figure** 4.9). LPS strongly stimulated the release of IL-8 which was partially and weakly reduced by RFM. The pre-treatment of RFM for 1.5 h at doses of 0.001 – 1 and 10,000 nM significantly reduced the levels of IL-8 compared to control. In terms of percentage, the reduction was equal to 20%, 25%, 45% and 36% respectively compared to LPS alone (Figure 4.8). However, IL-6 levels were not reduced by any of the concentration of RFM tested, over any of the exposure

times explored (1.5, 3 or 24 h). Interestingly, higher concentrations of IL-6 over control samples were recovered in the supernatant after pre-treatment with 1000 and 10,000 nM (Figure 4.9). A hypothesis is that the 'over release' reported in presence of 1000 and 10,000 nM of RFM may have caused a toxic effect in BEAS-2B as those concentrations were considerably higher than the clinically-relevant plasma concentration of RFM (1 – 2 nM). The latter (free, not bound to plasma proteins) was calculated in a clinical study based on the oral administration of RFM at the clinical dose of 500 µg/mL daily (Bethke et al., 2007).

Levels of TNF-α detected after different pre-treatment exposure time regimens (1.5, 3 and 24 h) with RFM were lower than the lowest point of the recommended standard curve (< 15.6 pg/mL) (data not shown).

a.

b.

Interleukin-8	Roflumilast				
	0	*0.001 nM*	*1 nM*	*1000 nM*	*100,000 nM*
Concentration (pg/mL)	262.07 ± 5.30	208.77 ± 11.75	194.42 ± 14.56	142.70 ± 2.43	166.94 ± 7.54
Reduction from control (%)	-	20 %	25 %	45 %	36 %

Figure 4.8 IL-8 level released by LPS-stimulated BEAS-2B cells in presence or absence of roflumilast treatment. BEAS-2B were pre-treated with growth media (vehicle) or roflumilast (RFM) (0.001, 1, 1000, 10,000 nM) for 1.5 h before being stimulated with LPS (800 ng/mL) for 24 h. Statistical significance was evaluated with one-way ANOVA

multiple comparisons. Data represents mean ± SD of n=1 independent experiment, with

measurements in triplicate (three wells). The results are also expressed as the percentage

inhibition vs vehicle (LPS alone).
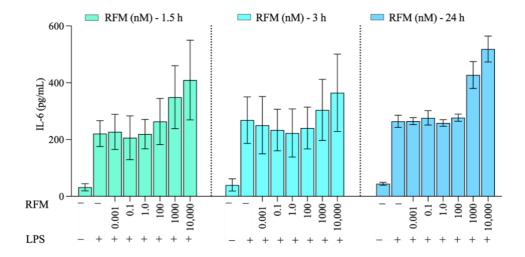
Figure 4.9 Effect of roflumilast pre-treatment (1.5 - 3 - 24 h) on IL-6 production from LPS-challenged BEAS-2B. For the 1.5 and 3 h pre-treatment, cells were seeded on day 0 and media changed on day 1 and 2. On day 3, cells were exposed to dexamethasone following by exposure to LPS (24 h); for the 24 h pre-treatment, cells were seeded on day 0 and media changed on day. On day 2, cells were exposed to dexamethasone (24 h). On day 3, supernatant was withdrawn, and cells stimulated with LPS (24 h). Data represents mean ± SD of one independent (24 h exposure) or two (1.5 and 3 h exposure) independent experiments, using one or two distinct passage number. For every experiment, each measurement was in triplicate (three wells).

4.5   Discussion

In this work, BEAS-2B cells were used to form an *in vitro* model of inflammation in which to investigate the ability of RFM to reducing the levels of IL-8 and IL-6 secretion which were chosen as inflammatory biomarkers. In the context of BEAS-2B cell culturing conditions, it was first necessary to consider the impact that FBS has on the BEAS-2B cell phenotype, as well as on the response to agents such as LPS. The suitability of BEAS-2B cells as experimental model of arsenic toxicity was assessed by Zhao and others (Zhao & Klimecki, 2015). They showed that FBS changes the cell phenotype with consequences such as an increase in the total glycolytic capacity of the cells, increase in the basal

1715   respiration and in the oxygen consumed for ATP production. Earlier studies (Ke' et al.,
1716   1988) highlighted how BEAS-2B cells undergo squamous differentiation in presence of
1717   serum which results in cell senescence and loss of immortality, whereas more recent
1718   studies further investigated the cellular and molecular biological changes that take place
1719   in presence of serum.

1720   A change in the phenotype of the cells when exposed to FBS was also reported by Malm
1721   and co-workers (Malm et al., 2018). In this case, a mechanism called epithelial-
1722   mesenchymal transition was described. They demonstrated that the exposure of BEAS-
1723   2B cells to serum induces the loss of specific markers of epithelial identity and the gain
1724   of markers of mesenchymal cell identity. This process is reversible if serum is removed.
1725   This finding was partially supported by others (Han et al., 2020). They also showed that
1726   BEAS-2B cells shared the expression profile of surface markers with mesenchymal cells,
1727   exhibited osteogenic and adipogenic differential potential, but it was unclear for them
1728   whether those properties were the result of epithelial-mesenchymal transition induced by
1729   transforming growth factor-β 1, which is abundant in serum.

1730

1731   Despite these studies raised concerns about the suitability of BEAS-2B cells as an *in vitro*
1732   epithelial model, many researchers are still using these cells with the addition of serum
1733   to the growth medium to model the airway epithelium successfully. Studies have showed
1734   that BEAS-2B cells can be cultured with Dulbecco's Modified Eagle Medium
1735   supplemented with 10% FBS to study heavy metals induced carcinogenesis (Laulicht et
1736   al., 2015; Park et al., 2015), to investigate the relations between chronic arsenic exposure,
1737   reactive oxidative species production and cell transformation (Carpenter et al., 2011;
1738   Chang et al., 2010) or to investigate the protective effect of angiotensin converting
1739   enzyme 2 against LPS-induced injury in mice (Ye & Liu, 2020). The use of RPMI 1640
1740   in presence of serum is also widely reported and multiple studies have adopted these cells
1741   and these culturing conditions to model the airway epithelium. In a study (Schulz et al.,
1742   2002), BEAS-2B were used as epithelial cells to investigate the molecular pathway
1743   induced by the LPS stimulation, using experimental conditions in agreement to the one
1744   adopted in this experimental chapter. Same culturing conditions were also adopted in two
1745   other studies (M. Wang et al., 2019; Y. Wang et al., 2015) which described the role of
1746   two transient receptor potential protein ion channels in mediating airway tissue injury and
1747   inflammation besides the expression profile and function of miRNAs in acute lung injury.

1748 They concluded that miR-18b may be involved in the process of LPS-induced
1749 inflammation in BEAS-2B.

1750 In this experimental study, specific investigation of any serum induced epithelial
1751 mesenchymal transition or expression of mesenchymal features was not carried out as
1752 this was not the aim of the study. However, the experimental study was conducted based
1753 on the assumption that the cells maintain an epithelial phenotype throughout the whole
1754 experimental window. Indeed, the culturing conditions (growth medium and addition of
1755 serum) used in this experimental study were in agreement and similar to those reported
1756 by others (Schulz et al., 2002; M. Wang et al., 2019; Y. Wang et al., 2015) which
1757 successfully designed airway models.

1758

1759 The percentages of serum at which the cells have been exposed on day 1 and 2 after
1760 seeding impacted the basal level of IL-6 and IL-8. A basal level of cytokine released by
1761 BEAS-2B has been also reported by others (Schulz et al., 2002) who have shown that
1762 despite the basal cytokine expression being low, it can be increased by approximately 10-
1763 fold in presence of serum. The addition of 10% of serum on day 1 and on day 2 of the
1764 culturing protocol was chosen as the final experimental condition because it resulted in a
1765 low basal level of cytokines on day 2 and enabled growth of cells to approach confluency
1766 on the day that drug treatment was applied.

1767 The effect of the exposure of LPS on BEAS-2B in presence of different percentages of
1768 serum was also investigated. The presence of LPS, amplificated the response of BEAS-
1769 2B cells with a 'serum concentration – dependency' comparable to that reported for the
1770 basal level of cytokine release. It is known that LPS sensitizes BEAS-2B cells via a
1771 complex pathway of molecular mechanisms. Schultz and colleagues (Schulz et al., 2002)
1772 investigated the effect of LPS exposure (10 µg/mL) on BEAS-2B cells in presence of
1773 different concentrations of serum (2, 5 or 10%), describing the component of serum
1774 responsible for the sensitization of BEAS-2B cells to LPS. They found that the addition
1775 of soluble CD14 alone to BEAS-2B did not alter the production of IL-8 and IL-6, whereas
1776 the addition of sCD14 in presence of LPS resulted in increased levels in a concentration
1777 dependent manner.

1778

1779 The LPS- stimulation of BEAS-2B resulted in dose and time-dependent release of IL-6
1780 and IL-8. The LPS-challenge of BEAS-2B cells for 24 h with increasing concentrations
1781 of LPS resulted in dose-dependent increased levels of IL-8. The exposure to a constant

1782 concentration for different exposure times resulted in time-dependent increased of IL-6

1783 levels compared to control sample. These findings are in agreement with those of Guillott

1784 and colleagues (Guillott et al., 2004) which showed that the exposure to LPS and in

1785 presence of serum strongly stimulates the release of IL-8 and IL-6 from BEAS-2B cells

1786 in a concentration-dependent manner. Although, the magnitude of the cytokine release

1787 was not comparable. In Guillot's study the stimulation of BEAS-2B for 24 with 100

1788 ng/mL and 1000 ng/mL of LPS resulted in ~ 400 pg/mL and 800 pg/mL respectively for

1789 IL-8 and ~ 300 pg/mL and 900 pg/mL respectively for IL-6; whereas in this experimental

1790 chapter the stimulation of BEAS-2B with the same conditions (LPS, length of exposure)

1791 induced the release of 245.3 pg/mL and 457.2 pg/mL for IL-8 and 113.8 pg/mL and 147.5

1792 pg/mL for IL-6.

1793 Reasons for this discrepancy and the lower magnitude reported in this experimental

1794 chapter may be related to the different number of cells used in the experiments and the

1795 different serotype of LPS (*P. Aeruginosa*) which may have resulted in different responses

1796 (Guillott et al., 2004).

1797

1798 The use of LPS-challenged BEAS-2B cells to model a wide range of inflammatory-based

1799 lung conditions induced by diseases or exposure to environmental toxin or smoke has

1800 been reported previously in the literature (L. Chen et al., 2021; Fuentes-Mattei et al.,

1801 2010; H. H. Jang et al., 2016; Laan et al., 2004; Veranth et al., 2004, 2007). The main

1802 differences within these studies are related to the serotype of LPS used which may impact

1803 the concentration of cytokines detected, the concentration of LPS used and the length of

1804 exposure to LPS. Fuentes-Mattei and colleagues (Fuentes-Mattei et al., 2010) used an

1805 LPS-BEAS-2B model to investigate the immunological markers resulting from exposure

1806 to PM2.5 organic extract from Puerto Rico and found that the stimulation of BEAS-2B

1807 cells for 24 h with 10,000 ng/mL of an unspecified strain of LPS resulted in 942.8 (±

1808 10.9) pg/mL and 1274.1 (± 10.9) pg/mL of IL-6 and IL-8 respectively, whereas

1809 stimulation for 24 h with LPS from *E. Coli* 055:B5 (10 – 1,000 ng/mL) induced the

1810 secretion of IL-6 concentrations from 200 pg/mL to 550 pg/mL and IL-8 from 10,000

1811 pg/mL to 16,000 pg/mL (Schulz et al., 2002).

1812

1813 Beside the use of LPS from *P. Aeruginosa*, many studies report the use of LPS from

1814 *Escherichia Coli*. LPS from *E. Coli* O55:B5 was also adopted by Jang and co-workers

1815 (B.-K. Jang et al., 2020). The stimulation of BEAS-2B cells for 48 h with 1,000 ng/mL

1816   of LPS resulted in increased levels of TNF-α, IL-8 and IL-6 compared to controls (13

1817   pg/mL, 180 pg/mL and 1600 pg/mL, respectively). A time-dependent increase of IL-8

1818   levels when BEAS-2B cells were exposed to LPS from *E. Coli* (1,000 ng/mL, O26:B6)

1819   for 2, 4, 6, 8 and 10 h was also reported by Laan and others (Laan et al., 2004) in a more

1820   complex experiment where LPS treatment was associated to CSE exposure in an

1821   inflammation model.

1822

1823   Increased levels of TNF-α and IL-6 were reported also when BEAS-2B cells were

1824   stimulated by LPS from *E. Coli* but of a different strain (O127:B8) (L. Chen et al., 2021).

1825   They reported the use of LPS at a concentration of 30,000 ng/mL for 24 h which is

1826   considerably higher compared to concentrations used by previously mentioned studies.

1827   Compared to control, the production of TNF-α by BEAS-2B was elevated from 96.7 to

1828   433.6 pg/ml by stimulation with LPS while the IL-6 concentration was reported to be

1829   136.7 and 678.3 pg/mL in absence and presence of LPS, respectively.

1830

1831   Additional uses of BEAS-2B cells to investigate airways diseases and pathological

1832   mechanisms of action are also reported in the literature, i.e. the effects of TNF-α and IL-

1833   1β stimulation (Stecenko et al., 2001) or respiratory syncytial virus and adenovirus (Yoon

1834   et al., 2007).

1835

1836   The ability of the LPS model to respond to an anti-inflammatory intervention was

1837   successfully demonstrated with dexamethasone. Both regimens used (2 and 24 h pre-

1838   treatment) reduced the elevated levels of IL-6 induced by LPS stimulation (800 ng/mL).

1839   The use of dexamethasone as positive control has been reported in other studies that have

1840   investigated the anti-inflammatory potential of new pharmaceutical entities. For example,

1841   the potential anti-inflammatory activity of Salvia plebeian R. Br extract was compared to

1842   dexamethasone activity (10 and 100 µg/mL) in LPS and TNF-α stimulated BEAS-2B

1843   cells (H. H. Jang et al., 2016). The use of the glucocorticoid as positive control is also

1844   reported in a study that aimed to show the potential of cannabidiol in the regulation of

1845   basal and LPS-induced inflammation responses in macrophages, lung epithelial cells and

1846   fibroblast (Muthumalage & Rahman, 2019) and in an additional study where the potential

1847   anti-inflammatory activity of flavonoids in the airways was evaluated (P. Zhang et al.,

1848   2019).

1849

The anti-inflammatory activity of RFM in an airway model using LPS-stimulated BEAS-2B was investigated by studying the ability to suppress increased levels of IL-8 and IL-6 after LPS stimulation using the same protocol that was used with dexamethasone. The pre-treatment for 1.5 h with RFM resulted in a partial and weak reduction of IL-8 with the highest suppression mediated by 1000 nM. The weak suppression of IL-8 was also reported by Edwards and colleagues (M. R. Edwards et al., 2016), who stimulated BEAS-2B with rhinovirus and compared the potential anti-inflammatory activity of a new compound CHF6001 to that of RFM. The suppressive activity of the CHF6001 as a pre-treatment only in reducing the IL-8 levels was 48-fold higher compared to RFM and overall the new compound had the same level of efficacy of a combined RFM pre and post treatment. Furthermore, RFM tested in a concentration range between 0.001 and 1000 nM also exhibited suppressive activity against IL-29, IL-28, RANTES and IP-10 mRNA expression. (M. R. Edwards et al., 2016)

The results obtained in this research are partially in agreement also with a more recent study conducted by Salvator and colleagues (Salvator et al., 2020) who used a different airways model: bronchial explants resected from human lungs instead of bronchial epithelial cell line. Same serotype of LPS as well as similar range of concentrations of LPS were adopted (O111:B4, 1000 ng/mL) in both studies. In addition, RFM was used in a pre-treatment regimen of 1 h exposure to concentrations in the range 0.1 – 1000 nM followed by LPS stimulation for 24 h. Results showed that the LPS-induced release of IL-8 was reduced by 20% at a concentration of 1 nM and this well correlates with our findings (20% IL-8 reduction with 1 nM of RFM). On the other side, in contrast to our findings that described an absence of IL-6 reduction after RFM pre-treatment, Salvator demonstrated that the LPS-induced IL-6 level was reduced by 35 % at 100 nM.

The weak or absent inhibition of LPS-induced IL-8 levels may suggest that the production of IL-8 may not be sensitive to cAMP modulators (Dent et al., 1998; Yoshimura et al., 1997). In addition, Buenestado (Buenestado et al., 2013) showed that the levels of chemokine involved in the recruitment of neutrophils such as IL-8 are not reduced by RFM in a human lung parenchymal explant model.

The RFM pre-treatment schemes did not suppress the increased levels of IL-6 induced by exposure to LPS. This result is in agreement with a study conducted by Lea and co-workers (Lea et al., 2019), which investigated the activity of RFM in alveolar

1884 macrophages from COPD patients and in whole lung tissue explants (Lea et al., 2019).

1885 Macrophages and lung tissue from COPD patients and smoking controls were used in the

1886 method design. RFM (0.000001 – 10 nM) pre-treatment (1h) followed by LPS stimulation

1887 (1000 ng/mL for 24 h) produced a reduction of TNF-α levels in alveolar macrophages

1888 and lung tissue. However, levels of IL-8 and IL-6 in alveolar macrophages or lung

1889 explants were not reduced by RFM (1000 nM).

1890 Besides IL-6 and IL-8, other researchers have conducted studies on the TNF-α

1891 suppression mediated by RFM. It has been widely reported that RFM reduced the LPS-

1892 induced TNF-α levels in a concentration dependent-manner in BEAS-2B and in other

1893 cellular systems as well (Hatzelmann et al., 2010; Salvator et al., 2020). Buenestado and

1894 colleagues (Buenestado et al., 2013) showed that RFM inhibits in a concentration-

1895 dependent manner the increased release of TNF- α from lung macrophages isolated from

1896 resected human lungs and stimulated with LPS for 24 hours (Buenestado et al., 2012).

1897 However, in the airway model used in this thesis, TNF-α levels detected were always

1898 considerably low and often below the calibration range of the commercial ELISA kit

1899 used. For this reason, TNF-α was not utilised an endpoint in the treatment scheme, but

1900 only in the optimisation of the growing conditions and LPS concentration.

1901

1902 ## 4.6   Conclusion

1903

1904 The human bronchial epithelial cell line model was established to investigate whether

1905 RFM can inhibit the inflammation triggered by LPS in a human bronchial cell line

1906 (BEAS-2B) cultured in presence of serum. Initially, the BEAS-2B cells culturing

1907 conditions and how those impact the sensitivity of the cells to LPS-stimulation, including

1908 different exposures to serum as a key component to the medium, were investigated.

1909 Results demonstrated that the serum has an impact on the BEAS-2B cytokine secretion

1910 when cells are exposed to different concentrations for different duration and that

1911 unstimulated cells release basal IL-8, IL-6 and TNF-α at concentrations that are

1912 dependent on the concentration of serum (2 or 10%) to which the cells have been exposed

1913 to on day 1 and 2 after being seeded in 24-well plate. The key finding of this chapter is

1914 represented by the sustain detectable release of IL-6, IL-8 and TNF-α from BEAS-2B

1915 induced by multiple concentrations of LPS (10 – 1000 ng/mL) and exposure time (2 – 48

1916 h), with 800 ng/mL and 24 h being considered the most suitable experimental conditions

1917 to adopt.

1918   The established model was used to investigate pre-treatment with RFM as potential anti-
1919   inflammatory therapy. Dexamethasone was used as a positive control to demonstrate the
1920   ability of the *in vitro* lung inflammation model to respond to drug activity. Two regimens
1921   for pre-treatment of cells (2 and 24 h) were used and resulted in concentration-dependent
1922   reduction of IL-6 and IL-8 over the range of concentrations tested (0.1 – 100 µg/mL).
1923   RFM pre-treatment for 1.5 h at doses of 0.001 – 100,000 nM reduced the levels of IL-8
1924   by 20%, 25%, 45% and 36% compared to untreated control. Thus, RFM was only able to
1925   moderately reduce the levels of chemokines involved in the recruitment of neutrophils,
1926   such as IL-8. There was even less effect on the LPS-induced increase of IL-6 which was
1927   not suppressed at any concentrations or time regimens by RFM.

1928

1929   The research aims for this chapter were achieved as BEAS-2B culture conditions were
1930   optimised and used in an LPS model. A sustained and detectable release of cytokines in
1931   the cell medium overtime was obtained, which was suppressed by dexamethasone and
1932   partially by RFM. Overall, this chapter provide a suitable foundation for the use of this
1933   model in the investigation of the anti-inflammatory activity of other molecules. However,
1934   the model was not suitable to study RFM activity and act as a bioassay to guide
1935   formulation strategy as required in this thesis, i.e. to evaluate the potential of RFM-HSA-
1936   NPs to confer a drug delivery advantage. Due to uncertainty whether this is an *in vitro*
1937   artifact and how the model might be optimised, the development of an *in vivo* model in
1938   which to evaluate RFM activity was preferred.

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CHAPTER 5: A guinea pig model of lung inflammation for evaluation of roflumilast anti-inflammatory activity

5.1 Introduction

The purpose of this experimental chapter was to establish an animal model representing the inflammation associated with COPD. For continuity, the same trigger agent for inducing inflammation, LPS, which was used in Chapter 4 for the *in vitro* respiratory epithelial cell assay was used to establish the animal model. Furthermore, the model was tested for its responsiveness to orally administered RFM to verify whether it had potential as a preclinical model to evaluate the anti-inflammatory effect of RFM administered via the pulmonary route.

Indeed, it is fundamental to use a model that mimics most of the pathophysiological profile of COPD in order to develop effective treatment options. There are several animal models and due to the complex molecular features of COPD, often no model recapitulates all the features of the disease. Therefore, it was of utmost importance to choose the most relevant model and demonstrate its responsiveness to RFM, while also considering the model's feasibility in terms of time, costs, and laboratory equipment available. In this experimental chapter, a model replicating a COPD exacerbation was adopted.

The way an episode of exacerbation in a patient with COPD is defined has not changed hugely over the years. A panel of experts have tried to improve the definition of exacerbation, providing an updated definition and severity classification of exacerbation of COPD (ECOPD) (Celli et al., 2021). The purpose of the meeting was to overcome the shortcomings of the current definition in an attempt to positively improve clinical and healthcare decision. It was in 1821 that the first definition of ECOPD appeared, followed by an update by Anthonisen and colleagues (Anthonisen et al., 1987) which remained almost unchanged over the last 35 years and forms the basis of the current definition of the European Respiratory Society/ American Thoracic Society definition of exacerbation. The latter states that an exacerbation is "an episode of increasing respiratory symptoms, particularly dyspnoea, cough and sputum production and increased sputum purulence" (Wedzicha et al., 2017). More general is the definition adopted by the Global Initiative

1985   for Chronic Obstructive Lung Disease (GOLD) which states that an exacerbation
1986   corresponds to "an acute worsening of respiratory symptoms which results in additional
1987   therapy" (Vestbo et al., 2013).

1988

1989   Despite there being no universal agreement on the definition of exacerbation, it can be
1990   considered as an event characterized by an acute burst of airway inflammation due to
1991   bacteria, viruses, or environmental pollutants. Airway inflammatory markers such as IL-
1992   6 and IL-8 increase during an exacerbation, although not consistently enough to provide
1993   markers for disease severity (Barnes et al., 2015; Celli & Barnes, 2007; Roca et al., 2013;
1994   Segal et al., 2015).
1995   A study focussed on the differences between patients with stable COPD or ECOPD
1996   phases was conducted (Karadag et al., 2008) where the biomarkers of systemic
1997   inflammation in stable (83 patients) and exacerbation phases (20 patients) COPD patients
1998   were compared to healthy controls (30 patients). Levels of TNF-α and IL-6 were higher
1999   in COPD and ECOPD patients than controls, concluding that those are biomarkers of the
2000   systemic inflammatory response in stable COPD patients.
2001   Bronchoalveolar lavage fluid and induced sputum of COPD patients usually have an
2002   increased number of neutrophils and macrophages; increased levels of pro inflammatory
2003   cytokines such as IL-8, IL-6 and TNF-α have been observed in the same specimens
2004   (Chung, 2001).
2005   The importance of biomarkers and their variation over the course of the disease was
2006   highlighted in an extensive review (Barnes et al., 2006). A summary of significance of
2007   the biomarkers in bronchial biopsies, BAL, sputum and exhaled condensate breath was
2008   reported. It was found that the cellular component of BAL in individuals with COPD is
2009   mostly made of macrophages with some neutrophils and T lymphocytes (O'Donnell et
2010   al., 2006). Increased levels of IL-8 were also reported. Sputum IL-8 and TNF-α have
2011   been studied and shown to be increased in the sputum of COPD patients compared to
2012   healthy smokers (Barnes et al., 2006).

2013

2014   Current species used to investigate COPD include rodents, guinea pigs, dogs, and larger
2015   animals such as sheep. A methodological review conducted by Ghorani and others
2016   highlighted that the most used COPD pre-clinical models are based on mice, guinea pigs
2017   and rats which however are considered a poor model due to their resistance to develop
2018   COPD condition hallmarks. Animals can be exposed to different inducers of lung

2019   inflammation such as cigarette smoke, LPS or elastase (Ghorani et al., 2017). This review
2020   also includes an extensive comparison between the different methods used to build COPD
2021   animal models (e.g. number of cigarettes used, length of exposure to trigger agent etc).
2022   An additional comprehensive review of the different pre-clinical models and their
2023   advantages and disadvantages has been published by Tanner and colleagues (Tanner &
2024   Single, 2019). COPD and lung fibrosis pre-clinical models based on rodent species are
2025   summarized in **Table** 5.1. Advantages, disadvantages, and pathophysiological features
2026   expressed by each model are included. It is not uncommon that animals are exposed to
2027   multiple COPD inducers simultaneously; this is the case of the study conducted by Yang
2028   where mice were exposed to cigarette smoke only, LPS only or a combination of both (Y.
2029   Yang et al., 2021). Despite relevant for the understanding of COPD pathophysiology and
2030   related treatments, those combinations will not be covered in this chapter and therefore
2031   were not included in the Table 5.1.

Table 5.1 Rodent models reflecting COPD and related respiratory disorders/ fibrosis described in the literature with their advantages, disadvantages, and corresponding pathophysiological features.

Trigger agent	Route of administration	Animal model (rodent only)	Main pathophysiological features of the model	Advantages	Disadvantages
Lipopolysaccharide (various strains available)	o Intravenous o Pulmonary (Instillation, inhalation)	o Mouse o Rat o Guinea pigs	o Inflammation o Cell influx (macrophages, neutrophils) o Structural changes in the lungs (chronic exposure)	o Short period of time required to induce the inflammation o Very representative of COPD-related exacerbations	o Variability within lot and strain
Elastase	Instillation	o Rat o Mouse	o Lung damage o Loss of alveolar wall	o Short time required to induce the damage o Low cost of reagent o The degree of severity can be easily adjusted varying the	o Narrow dose window o Different mechanism of action of the two elastase enzymes available to be used o Elastase emphysema different from human emphysema

				amount of enzyme administered	o Poor impact of immune cell involvement in this model
Bleomycin (BLM)	o Intravenous o Subcutaneous o Intraperitoneal o Pulmonary (intratracheal or intranasal) o Oropharyngeal	o Mice o Rats o Guinea pigs	o Repeated intratracheal or intraperitoneal administrations may offer nonresolving fibrotic pathology o Acute lung inflammation o Systemic administration induces more homogeneous pattern of fibrosis o Less strong inflammation when administered systemically compared to pulmonary administration	o Ease of drug delivery o Short time to fibrosis evidence	o Lung fibrosis induced by BLM considered not fully representative of idiopathic pulmonary fibrosis o Spontaneous resolution of the fibrosis induced by single-dose BLM o Different timing of the disease development in animal model vs clinical disease o Development of fibrosis limited to Balb/c mice

Cigarette smoke extract	Pulmonary (whole-body of nose-only)	o Mouse o Rat o Guinea pigs	o Pulmonary infiltration of macrophages and neutrophils o Airway fibrosis o Emphysema	o Can induce many COPD features in animals	o High variability of the model outcome due to lack of a standardized protocol for animal exposure o Requires long time exposure
Fluorescin isothiocyanate (FITC)	o Intratracheal	o Mice	o Acute lung injury o Edema and inflammation (with presence of neutrophil) o Fibrosis o Localized pattern of inflammation like bleomycin-induced o Long-term effects (up top 5 months)	o Long-term effects (durable fibrotic response) o Fluorescent imaging	o Absence of consistent results due to difficulties in preparing FITC (different time of particle sonication induce variable particle size which lead to not consistent toxicity)
Silica particles	o Pulmonary route (intratracheal administration, aerosolization)	o Mice o Rats	o Inflammatory response o Lung fibrosis	o Induce similar effects in human and mice o The lung damage persists after the	o Expensive equipment required o Lack of IPF-like lesions

		termination of the silica particles exposure
		o Persistent, toxic inflammatory response
o Oropharyngela aspiration		o Intratracheal model easy and less expensive

References

(Degryse & Lawson, 2011; Ghorani et al., 2017; Jenkins et al., 2017; Jones et al., 2017; Moeller et al., 2008; Moore et al., 2013; Tanner & Single, 2019; Tashiro et al., 2017; Wright et al., 2008)

2032

2033

Instillation of the proteolytic enzyme elastase can induce and maintain an inflammatory response in the rodent lungs with immediate and marked loss of alveolar wall structure. This model consists of instillation of enzymes such as porcine pancreatic elastase, human neutrophilic elastase and papain (elastase activity depending on its purity and source). Despite the time advantage provided by this model (a single dose of elastase results in immediate lung damage), it has a very narrow dose window, and it results in 'artificial' lung tissue damage.

Cigarette-smoke (CS) exposure is another model that can be used. Details of the CS model and its variability within laboratories have been mentioned in Chapter 4. An additional source of variability for CS models is represented by the type of exposure; whether nose-only or whole-body. As later will be mentioned for the LPS model as well, a downside of the whole-body model is the deposition on the animal fur of the CS constituents which can be ingested by the animal and eventually influence the results. The poor control of the dose exposure when a chamber model is used can be overcome with the nose-only exposure, which however can be stressful for some animal species as it requires constraints.

Bleomycin (BLM) is a chemotherapeutic antibiotic that is pro-fibrotic such that pulmonary fibrosis developed after the intravenous administration of the drug to patients with lymphoma (Sleijfer, 2001). BLM can be administered systemically (intravenous, subcutaneous or intraperitoneal administration) or directly to the lungs (intratracheal or oropharyngeal administration). Generally, three or four weeks after a single dose of BLM directly to the lungs, an acute lung damage can be visualized followed by localized inflammation and fibrosis at the site of the agent administration. In contrast, systemic administration induces a more homogeneous fibrosis throughout the lungs, but a lower state of lung inflammation. In this case, the BLM induced fibrosis will be detectable within 4 – 12 weeks from the exposure. The initial site of damage when delivered intravenously is the pulmonary vascular endothelium, followed by the alveolar epithelium.
In terms of dosing regimens used, a wide range is reported in the literature. For single dose exposure, mice can receive from 1.25 U/kg (Hecker et al., 2014) to 4 U/kg (Rojas et al., 2005) usually suspended in 50 – 100 µL of PBS via intratracheal administration. In one of the few studies that has investigated repetitive BLM injury (chronic exposure),

35   mice were exposed to intratracheal administration of BLM (0.04 U) biweekly for a total

36   of 4 months (eight total doses) (Degryse et al., 2010).

37   Chronic pulmonary exposure to BLM induces a more robust fibrosis compared to one

38   single exposure, with an attenuation of the neutrophilic inflammation as the model

39   timeline progresses. The repetitive dosing more closely resembles the recurrent nature of

40   lung injury that occurs in patients with idiopathic pulmonary fibrosis (Degryse & Lawson,

41   2011). Although BLM can be delivered via multiple routes, the intratracheal is the most

42   frequent route used by researchers.

43

44   Besides those models, the administration of fluorescein isothiocyanate (FITC)

45   (Christensen et al., 1999) and silica particles (Lakatos et al., 2006) have also been used

46   to induce lung damage leading to fibrosis. FITC administration induces a localised

47   inflammation pattern similar to that induced by BLM. Interestingly, it conjugates to lung

48   parenchymal proteins, remaining localized to the area of initial injury and allowing the

49   use of immunofluorescence to better investigate the lung damage. However, the main

50   disadvantage of this model is represented by its variability which is correlated to the FITC

51   lot used and to the size of the FITC particulate number or surface characteristics obtained

52   via sonication (Moore et al., 2013; Moore & Hogaboam, 2008). Christensen (Christensen

53   et al., 1999) reported a significant increase in the early mortality for intratracheal

54   inoculation and increase acute toxicity when a FITC preparation protocol that required

55   long sonication time to ensure better dispersion was used. These difficulties are correlated

56   to the variability within studies in different laboratories and different research studies.

57   Finally, silica particles induce a fibrotic damage that more closely resembles the

58   occupational induced silicosis with the presence of fibrotic nodules. The silica particles

59   tend to not being easily cleared from the lungs, hence inducing a persisted stimulus.

60   However, besides the expensive equipment required, the development of the fibrotic

61   nodules can take between 40 and 120 days after exposure.

62

63   Besides the above-mentioned agents used to induce lung damage, LPS can also be used

64   as a COPD model. LPS is a component of the wall of Gram-negative bacteria and its

65   characteristics were described in Chapter 4. Its use in animal models is common and it

66   quickly produces lung damage, which usually persists after the exposure is ceased

67   (Ghorani et al., 2017; Tanner & Single, 2019).

68   Acute (single administration) exposure to LPS induces a lung inflammation characterized

69   by cellular influx whereas chronic exposure is often associated with structural changes of

70   the lungs. This is a valuable model to understand exacerbation-related COPD

71   mechanisms. Indeed, bacterial colonisation of the lower airways is an important factor in

72   determining the degree of airway and systemic inflammation in stable COPD patients and

73   is highly related to the development of exacerbations. Bacterial endotoxins, such as

74   peptidoglycan fragments, activate the immune response exacerbating the lung

75   inflammation. Patients with a high level of bacteria colonisation in the lungs have an

76   increased level of neutrophils and pro inflammatory cytokines in the induced sputum

77   (Celli & Barnes, 2007; Roca et al., 2013; Segal et al., 2015). This makes the use of LPS

78   as inducer in preclinical models of COPD relevant as bacteria isolated from lungs fluids

79   of patients with COPD immediately after exacerbation are mainly Gram-negative (Tanner

80   & Single, 2019).

81

82   The administration of LPS into the airways or systemically is a commonly used model to

83   induce lung inflammation. A narrative review describes the molecular dynamics of LPS-

84   induced lung injury in the rodent model (Domscheit et al., 2020). When delivered via the

85   pulmonary route, LPS damage occurs principally to the alveolar epithelium. Moreover,

86   LPS induces a strong migration of cells into the lung tissue which terminates 72 hours

87   after the challenge followed by secondary fibrosis (Bozinovski et al., 2004). When LPS

88   is administered systemically it acts primarily on the vascular endothelium causing

89   interstitial edema (Domscheit et al., 2020).

90   In pre-clinical models, the exposure to LPS is known to cause severe tissue injury

91   characterized by accumulation of inflammatory cells in the alveolar and interstitial space

92   and alveolar wall thickening (Matute-Bello et al., 2011). The activation of the signalling

93   molecules involved in the LPS-mediated inflammation is very complex and time

94   dependent. It is very likely that multiple signalling molecules are present in the lung tissue

95   at different time points after the LPS challenge. The signalling molecules related to LPS

96   pathophysiological pathways are time-dependent, being activated at different time points

97   during the disease progression (Domscheit et al., 2020b). The kinetics of cell influx after

98   LPS exposure are discussed further in the paragraph 5.5.

99

100   Generally, pulmonary drug administration to pre-clinical COPD models can be achieved

101   using a whole-body exposure system (chamber) or a nose-only exposure system (**Table**

102 5.2). A whole-body chamber model is represented by full or partial immersion of animals

103 in an atmosphere containing the agent. This is advantageous when a large number of

104 animals are used, or a chronic study performed. However, sometimes it is recommended

105 to house animals individually in chambers as they tend to huddle, which can be a source

106 of increased variability in the dose inhaled due to reaction with the animal fur or filtration

107 by it. In addition, if allocated in groups, animals can inhale air which has been exhaled

108 and cleaned by other animals, which is a further mechanism of dose variability.

109 An alternative is represented by nose- or head-only exposure. Such systems are designed

110 to reduce the deposition of the test compound on the animal fur as only the head or the

111 nose/mouth of the animals is exposed to the test compound. However, this model also

112 possesses some downsides and requires trained investigators as well as specific and

113 expensive equipment.

114 To improve the pulmonary exposure of test compound in animals, Wong and others

115 (Wong et al., 2008) developed a single-animal whole-body exposure system for

116 inhalation for use when neither a conventional large whole-body exposure model nor a

117 nose-only were suitable. They combined a series of chambers connected together in a

118 manifold system and exposed mice and rats by inhalation to compounds over a 13-weeks

119 period. They found out that the test compound distribution was uniform, and the

120 compound measured concentrations were 2% within the target concentration, concluding

121 that the method designed was effective when a low amount of test substance is available.

122 Generally, the chamber model is usually the least stressful method for animals as they do

123 not require restraint. However, it is characterized by high variability in terms of dose and

124 concomitant routes of exposure (skin, eyes and oral). In contrast, the nose-only exposure

125 system allows exposure principally to the respiratory tract with less dose variability.

126 However, for some species the restraint may present a source of stress and training is

127 recommend before the exposure to reduce the stress and produce normal physiological

128 states (Phalen et al., 2014).

Table 5.2 Advantages and disadvantages of different exposure methods that can be adopted in the pulmonary exposure of the disease inducing substance in pre-clinical animal models.

Exposure method	Advantages	Disadvantages
Whole-body	o Can allocate large number of animals (allocate single animals is usually recommended) o Long term studies o No restraint for animals > less stress	o Multiple routes of exposure (skin, eye, oral) o Dose variability o Compound of interest may interact with excreta
Nose- or head-only	o More accurate deposition of the product into the respiratory tract	o Stress to some species (acclimatation recommended)
Intratracheal	o High control of deposition of compound of interest	o Unrealistic deposition in the respiratory tract

129

130 Different animal species can be used to model the pathological features of COPD.
131 However, the pre-clinical model designed in this chapter was based on the use of guinea
132 pigs. Guinea pigs possess many similarities to humans, making them a valuable species
133 in the design of COPD models. An extensive review of the use of guinea pigs in studies
134 relevant to asthma and COPD was published in 2008 (Canning & Chou, 2008). Receptor
135 pharmacology in guinea pigs is more similar to that of humans compared to other species
136 and the guinea pig lung anatomy and physiology very closely resembles that of humans.
137 Guinea pigs have goblet cells and mucus glands, with a subepithelial vasculature both
138 between the epithelial and the smooth muscle layer. In both species the trachea,
139 mainstream bronchi and large intrapulmonary bronchi are lined with a pseudo-stratified
140 epithelium. The physiology and the anatomy of the airway smooth muscle of guinea pigs
141 also resembles those of humans. However, the major drawback of the use of guinea pigs
142 in COPD model is represented by the axon reflex (Kroll et al., 1990). This reflex is the
143 response to activation of peripheral terminals of capsaicin sensitive nerves in the airways
144 of rats and guinea pigs, which results in the release of tachykinin which induces many
145 features of asthma such as mucus production, bronchospasm, and inflammatory cell

146 recruitment. However, it has been reported that the way the axon reflex works in guinea
147 pigs and rats is dissimilar to humans. In guinea pigs the axon reflex it is likely to be
148 relevant to responses to experimental challenges whereas it has a limited role in the
149 human airway function.
150

5.2 Aims and objectives

152

153 The overall aim of the work reported in this chapter was to illustrate how an LPS-induced
154 guinea pig model of lung inflammation can be used to model inflammatory lung disease
155 and can be modulated by orally administered RFM. This will provide a platform for the
156 investigation of the pulmonary delivery of RFM in the same preclinical model.
157 Initially the dose-finding studies were performed for LPS-induction of inflammation
158 using a whole-body chamber inhalation model. This was followed by the evaluation of
159 the anti-inflammatory activity of the positive control, orally administered RFM.
160 Specific objectives included:
161   1. Establish a whole-body exposure chamber model for delivery of LPS to guinea
162   pigs nebulised via an Aerogen Pro (ultrasonic mesh nebuliser);
163   2. Investigate the dose-response to LPS using biomarkers including the total and
164   differential count of the cells infiltrated into the lungs as consequence of the
165   endotoxin exposure;
166   3. Confirm the responsiveness of the model by measuring reduction of inflammation
167   by the oral administration of RFM.
168

5.3 Methods

170

5.3.1 Preliminary and qualitative assessment of LPS exposure: intratracheal administration

173

174 Two male Dunkin-Hartley guinea pigs (250–299 g) were exposed to an intratracheal
175 administration of a blue dye in order to investigate the variability often related to this
176 specific way to achieve pulmonary exposure in preclinical studies. Animal received an
177 overdose of sodium pentobarbital (1 g/kg) via an intraperitoneal injection and then they
178 were placed vertically on a bar to facilitate the visualization of the trachea. The
179 administration was carried out using a pyrogen free 1 mL syringe attached to a stain steel

180 gavage needle. This study was aimed to gain qualitative information and therefore was

181 carried out only on two animals. Both animals were intratracheally instilled with 200 µL

182 of Evans Blue (0.02%) above the carina whereas one animal received only the Evans Blue

183 bolus and the second animal received the volume of the dye followed immediately by 1

184 mL of air. The vertical position was retained for one minute after the distribution to allow

185 complete and uniform delivery of the liquid into the lungs.

186

187 **5.3.2   Design of a whole-body chamber LPS-induced inflammation model in guinea**

188   **pigs**

189

190 **5.3.2.1   Preparation of Lipopolysaccharide stock solution**

191 Lipopolysaccharide (LPS) (O111:B4, Sigma L2630) was dissolved in ultrapure water (5

192 mg/mL) under gentle stirring, aliquoted and stored at – 20°C in Eppendorf®, protected

193 from direct light. Immediately before the experiment, an aliquot was thawed and diluted

194 to the desired working concentration in sodium chloride 0.9% w/v.

195

196 **5.3.2.2   Nebuliser setting**

197 LPS was nebulised using a nebuliser Aerogen Pro® (Aerogen Ireland Ltd., S/N 297,

198 volume median diameter with sodium chloride 0.9% 4.44 µm, average flow rate 0.4

199 mL/min (Aerogen ® Pro System Instruction Manual)) attached to an ISO 22 mm t-piece

200 fixed inside the lid of the chamber box and directed towards the inside of the glass box

201 (470 (L) x 200 (H) x 200 (D) mm) (**Figure** 5.1)**.** The nebulisation of sterile sodium

202 chloride 0.9% w/v (control) was performed using a nebuliser Aerogen Pro® (Aerogen

203 Ireland Ltd., S/N 356, volume median diameter 4.82 µm, average flow rate 0.4 mL/min

204 (Aerogen ® Pro System Instruction Manual) with the same set up above mentioned. If

205 stated otherwise, LPS (0.1 mg/mL, 20 mL) was nebulised using an Ultra-Neb 2000

206 ultrasonic nebuliser (Devilbiss, Germany). The nebuliser was connected to the side of

207 same box mentioned above via a connecting tube.

208
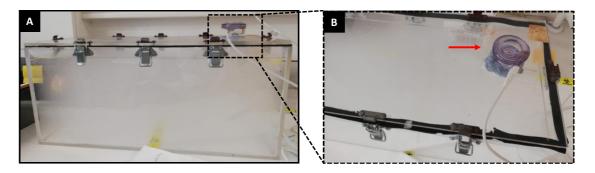
Figure 5.1 Experimental set up used in the design of a whole-body chamber model:
an Aerogen Pro® nebuliser is fitted on the top of a plexiglass chamber.

209

210 **5.3.2.3 LPS dose-response in guinea pigs using a whole-body chamber model**

211 At time 0, male Dunkin-Hartley guinea pigs (250–299 g) were placed individually
212 unanaesthetised and without restraint in the exposure chamber. Animals were allocated
213 to one of three treatment groups which were exposed to i) 0.1 mg/mL, ii) 0.2 mg/mL or
214 iii) 0.5 mg/mL of LPS. The volume of the LPS solution and the length of the exposure
215 were 10 mL and 30 min respectively for all the treatment groups.
216 After the LPS exposure, animals were returned to the housing boxes; four hours later they
217 were exposed to an overdose of sodium pentobarbital (1 g/kg) via an intraperitoneal
218 injection. The trachea was cannulated and bronchoalveolar lavage was performed using
219 5 mL of filtered PBS (pH 7.3 ± 0.2 at 25 °C) which was gently instilled and withdrawn 3
220 times using a 5-mL syringe (**Figure** 5.2). An aliquot of the recovered BAL was
221 immediately centrifuged (4000 rpm), the supernatant was collected and stored at - 80 °C
222 for cytokine evaluation using a commercial sandwich ELISA kit. For differential cell
223 count evaluation, an aliquot of 100 µL of BAL was placed on a microscope slide and
224 immediately processed using a cytocentrifuge (1000 rpm x 1 min). The microscope slides
225 were air-dried and stained using Reastain Quick-Diff following the manufacturer's
226 instructions. Differential cell count was performed using light microscopy. A total of 200
227 cells were counted for each slides using four different field of view and under 400x
228 magnification. The total cell number was calculated by counting a stained (50% v/v
229 Turk's stain) aliquot (10 µL) under a microscope using a haemocytometer.
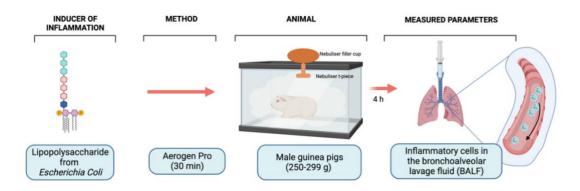
Figure 5.2 Schematic representation of the designed inflammation whole-body chamber model in male Dunkin-Hartley guinea pigs.

230

5.3.3 Oral administration of roflumilast in LPS-exposed guinea pigs

232

5.3.3.1 Preparation of roflumilast suspension for oral administration

For oral administration, RFM was suspended in polyethylene glycol 400 (PEG$_{400}$) by continuous stirring at 70°C. Once fully dissolved, the stock solution was diluted with an equal proportion of 4% w/v methyl cellulose (methocel) until a homogenous milky suspension was obtained.

Methocel 4% w/v was prepared the day before the *in vivo* study. To make a 100 mL preparation of 4% w/v methocel, half of the volume of water needed was refrigerated on ice to cool at $1 - 4$°C; the other half was heated at about 30°C in a glass beaker. The weighed powder was added to the heated methocel and stirred using a glass rod obtaining a white and sticky paste. The ice-cold water was then added, stirred vigorously on a plate. Once the dispersion had reached the temperature at which the methocel becomes water soluble, the powder began to hydrate with following increase of the viscosity. The obtained preparation was allowed to cool at 5°C overnight before used to prepare the drug suspension for *in vivo* administration.

247

5.3.3.2 Oral administration of roflumilast suspension in an LPS-inflammation model

Male Dunkin-Hartley guinea pigs (250–299 g) were exposed to a preventive protocol of RFM administered via the oral route (p.o.). The p.o. administration volume was 1 mL/kg of body weight. Animals were divided in three groups, with four animals in each group: i) *saline challenge:* animals treated with vehicle (PEG$_{400}$ + methocel 4% w/v) and challenged with sterile sodium chloride 0.9%; (ii) *LPS challenge*: animals treated with

189

255 vehicle (PEG$_{400}$ + methocel 4% w/v) and challenged with LPS; (iii) *drug-treated*: animals

256 treated with RFM at a dose of 0.5 mg/kg and challenged with LPS.

257 RFM or vehicle were administered p.o. at time 0 to conscious animals. One hour later,

258 animals were placed in the plexiglass chamber and exposed to nebulised LPS from *E.*

259 *Coli*. LPS (0.1 mg/mL, 20 mL) using a Devilbiss nebuliser. At the end of the LPS

260 exposure, animals were placed in their housing boxes; four hours later they were exposed

261 to an overdose of sodium pentobarbital (1g/kg) via an intraperitoneal injection. The

262 trachea was cannulated and bronchoalveolar lavage was performed using 5 mL of filtered

263 phosphate buffer solution that was gently instilled and withdrawn 3 times using a 5 mL

264 syringe. Bronchoalveolar lavage fluid was processed as mentioned in paragraph 5.3.2.3.

265

266 All experimental procedures and conditions were reviewed and approved by the ethics

267 committee of King's College London and conducted in accordance with the United

268 Kingdom Animal Scientific Procedures Act, 1986. Procedures were conducted under the

269 Project Licence PEF229300 and the Personal Licence no. I8232C275. The Project

270 Licence holder is Prof. Clive Page and the Protocol adopted is no. 2 (Studies of lung

271 pathophysiology) which has a moderate severity category. The author thanks Dr Sandra

272 Rudman for having conducted some of the experimental procedures described as well as

273 for her support during those.

274

275 ### 5.3.3.3 Statistical analysis

276 Data were expressed as mean ± SD. Where appropriate, data were analysed using

277 GraphPad Prism version 9, (GraphPad Software, San Diego, USA). Normal distribution

278 of data was firstly evaluated and the statistical significance between groups was

279 determined using one-way ANOVA with Šídák's, multiple comparison tests where

280 appropriate. Statistically significant differences are represented by asterisks as follow: *,

281 $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Where stated that a statistical analysis was

282 performed, absence of asterisks indicates non-statistically significant results.

283

284 ## 5.4 Results

285

286 ### 5.4.1 Lung distribution of a dye after intratracheal administration

287

288 The intratracheal route is often reported in studies in which the lungs are the target organ
289 for both the development of the inflammatory model and for the investigation of the anti-
290 inflammatory treatment. This assessment was carried out to determine the pattern of
291 distribution of a liquid administered to the lungs via the intratracheal route and how this
292 is affected by a minor protocol modification. **Figure** 5.3 describes the different patter of
293 distribution if only the liquid was instilled (Figure 5.3 A, B) or if the administration of
294 the liquid was immediately followed by 1 mL of air (Figure 5.3 C, D). The absence of air
295 resulted in a highly localized dye with accumulation mainly in the trachea and a patchy
296 and uneven distribution; in contrast the addition of air after the delivery of the volume of
297 dye had a considerable positive impact on the distribution. The colour was well
298 distributed into the airway and Figure 5.3 C and D show how the colour, despite being
299 mainly located in the lower lobes, is well distributed in the alveolar structure.
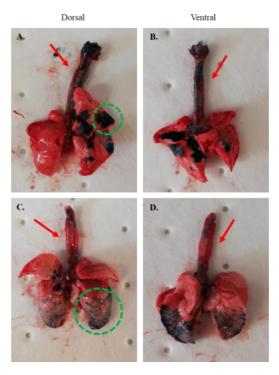
Figure 5.3 Representative pictures (dorsal and ventral views) of guinea pig lungs showing how the specific technique of intratracheal administration used can impact the distribution of an instilled solution. (A, B) Administration of 200 µL of Evans Blue (0.02%) only; (C, D) Administration of 200 µL of Evans Blue (0.02%) followed by 1 mL of air bolus. The experimental procedure was carried out on two animals receiving the intratracheal administration using the two different technique. Pictures were taken from two different animals, one per technique used.

300

5.4.2 LPS-induced lung inflammation in guinea pigs

To determine the potential of nebulised LPS to induce an inflammation in the lungs of guinea pigs, the total and differential cell number infiltrated into the lungs of the animals after the exposure to LPS were determined (**Figure** 5.4). In this experimental study, the outcome was measured 4 hours after the exposure to LPS. At this time point the neutrophils influx has already started and, despite possibly not being at their peak concentration (Domscheit et al., 2020b), inflammatory cells were significantly present.

Figure 5.4 Representative morphology of cells recoverable from the BAL of guinea pigs.

The total and differential cells collected in BALF four hours after the exposure to LPS are shown in **Figure** 5.5. The exposure to increasing LPS doses resulted in 175.0 x 10^4 cells/mL, 324.25 x 10^4 cells/mL and 350.25 10^4 cells/mL for 0.1, 0.2 and 0.5 mg/mL LPS respectively. The recovered total number of cells was similar when animals were exposed to the two highest doses of LPS. The number of eosinophils did not change when animals were exposed to increasing concentrations of LPS, nor was there clear relationship between number of macrophages and dose of LPS as the dose 0.2 mg/mL corresponded to the lowest number of macrophages with the lowest and the highest doses resulting in

321   similar number of macrophages. Compared to eosinophils and macrophages, the number
322   of neutrophils was highly impacted by the LPS exposure and by the different
323   concentrations tested. The neutrophils recovered in the BALF increased from 128.8 x 10^4
324   cells/mL to 291.75 x 10^4 cells/mL and 294.75 x 10^4 cells/mL with increasing LPS dose.
325   Indeed, differential cell count analysis highlighted that the increased total cell number
326   was mainly determined by neutrophils with lesser impact of the smaller numbers of
327   macrophages and eosinophils.
328   The dose of 0.2 mg/mL was considered optimal for these experimental studies as it
329   resulted in a clearly detectable influx of inflammatory cells. Therefore, it was adopted as
330   LPS dose for nebulisation in the following *in vivo* experimental studies.
331
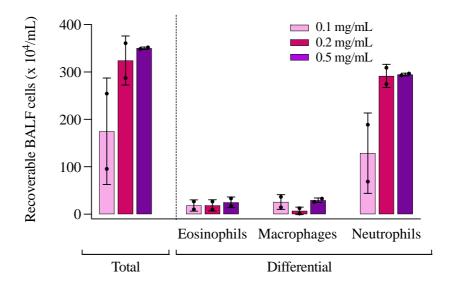
332   *Figure 5.5 Total and differential cell count from bronchoalveolar lavage fluid (BALF)*
333   *of guinea pigs exposed to increasing concentration of lipopolysaccharide from E. Coli.*
334   *Cells were recovered four hours after the exposure to LPS. Cell populations were*
335   *identified on air-dried cytocentrifuged smears after staining with Diff-Quick stain.*
336   *Differential cell counts were performed on 200 total cells using four different fields of*
337   *view. Data represent mean ± SD of n =1 independent experiment, (n =2 animals/group).*

338   The impact of LPS on the levels of TNF-α in the BALF was investigated using a
339   commercial ELISA kit. The concentrations of TNF-α detected were 1.6 x 10^4 pg/mL,
340   1.47 x 10^4 pg/mL and 1.78 x 10^4 pg/mL when animals were exposed to 0.1, 0.2 and 0.5
341   mg/mL LPS, respectively (**Figure** 5.6). The concentration of TNF-α did not vary
342   accordingly to the increasing concentrations of LPS to which the animals were exposed.
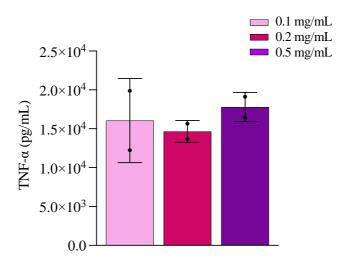
Figure 5.6 Levels of human tumour necrosis factor alfa (TNF-α) in bronchoalveolar lavage fluid (BALF) from guinea pigs four hours after the exposure to increasing doses of Lipopolysaccharide (LPS). Cytokine levels were detected using a commercial ELISA kit. Data represents mean ± SD of n=1 independent experiment, (n=2 animals/group).

343

5.4.3 Anti-inflammatory activity of orally administered roflumilast in LPS-exposed guinea pigs

346

347 To evaluate the anti-inflammatory activity of orally administered RFM in LPS-exposed
348 guinea pigs, the characteristics of the cell population infiltrated into the lugs after four
349 hours from the LPS nebulisation was studied. Evaluation of leukocytes included a total
350 white blood cell count and a differential count, with absolute numbers of each type of
351 leukocytes. Representative guinea pigs BAL cytospin preparations showing the different
352 cell population infiltrated in the lungs can be visually recognised based on the type of
353 treatment or challenge received (**Figure** 5.7). In the cytospin of saline-challenge guinea
354 pigs (Figure 5.7 A) a predominance of macrophages can be described. Macrophages
355 possess a large size and high cytoplasm to nuclear ratio. A prevalence of neutrophils can
356 be highlighted in Figure 5.7 B, whereas neutrophils are identifiable due to their segmented
357 nuclear morphology. The reduced ratio of neutrophils in the Figure 5.7 C makes easier to
358 identify also eosinophils (with less segmented nuclei and round, red cytoplasmatic
359 granules) and lymphocytes (with their little cytoplasm) (Siegel & Walton, 2020).

360
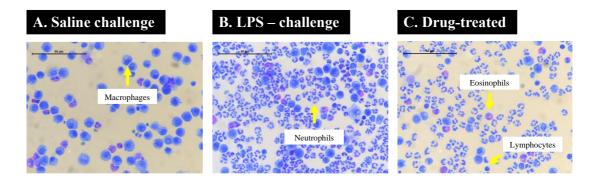
Figure 5.7 Representative guinea pig BAL cytospin preparations. a) Predominance of alveolar macrophages in BAL of healthy guinea pigs exposed to saline challenge and treated with vehicle; the photomicrographs also show a number of eosinophils (red staining); b) BAL neutrophils predominance in guinea pigs challenge with 0.2 mg/mL, 10 mL of LPS and treated with vehicle; c) Reduced number of neutrophils in combination with a small number of eosinophils, macrophages and lymphocytes in drug-treated guinea pigs' BAL.

The exposure to nebulised LPS induced a significant increase of the total cells recovered in the BALF compared to the number of cells in the lungs after exposure to nebulised saline (**Figure** 5.8). The oral treatment with roflumilast at a dose of 0.5 mg/kg significantly reduced the total number of cells, highlighting the anti-inflammatory activity of roflumilast in the model of interest. The exposure to LPS induced a very significant increase in the number of neutrophils compared to saline-challenge, highlighting the efficacy of LPS to initiate a state of inflammation in the lungs of the animals. In contrast, the exposure to LPS had no impact on the number of both eosinophils and macrophages. Roflumilast pre-treatment significantly reduced the number of eosinophils (LPS-challenge vs drug-treated: 89.18 x 10^4 cells/mL vs 16.96 x 10^4 cells/mL) and neutrophils (LPS-challenge vs drug-treated: 841.15 x 10^4 cells/mL vs 224.84 x 10^4 cells/mL) recovered in the BALF 4 hours after the LPS challenge, demonstrating the anti-inflammatory activity of RFM when delivered via the oral route in the model of lung inflammation that was established.

In summary, exposure to LPS resulted in an increased number of neutrophils that infiltrated the lungs and this inflammatory response was effectively reduced by treatment with RFM. This demonstrates both the potential of the LPS as inflammation inducer and confirms the activity of roflumilast as a drug with anti-inflammatory activity when delivered via the oral route in the preclinical model.

381
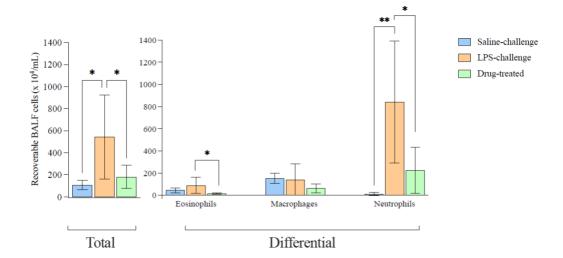
Figure 5.8 Oral administration of free RFM. Total and differential count of cells recovered from bronchoalveolar lavage fluid of guinea pigs. Cell populations were identified on air-dried cytocentrifuged smears after staining with Diff-Quick stain. Differential cell counts were performed on 200 total cells using four different fields of view. Data represent mean ± SD of n = 2 independent experiments, (n =4-8 animals/ group). One-way ANOVA followed by Sidak's multiple comparison test was used to examine differences between groups. Significant differences are shown as: *, p = ≤ 0.05; **, p = ≤ 0.01; no bar = not significant.

382

5.5 Discussion

384

In this work, guinea pigs and LPS were used to design and optimise a model that replicates the conditions and related markers of a COPD exacerbation. The final aim was to obtain a robust inflammation in the lungs detected mainly via the total white blood cell and the differential count and to confirm that the oral administration of RFM in the same model resulted in significant reduction of the induced inflammation. The model was designed and optimised in terms of i) LPS dose and ii) how the animals were exposed to LPS – whole-body chamber and inhalation exposure.

392

As mentioned in the introduction section of this current chapter, LPS represents only one of the possible agents that can be used to induce a model that resemble most of the COPD and the related exacerbations features. The use of LPS in a whole-body chamber model is well-known as well as the molecular pathway related to its cascade activation. The

cellular response to LPS is associated with the activation of the TLR4 receptor and corresponding pathway. A detailed review about LPS and its molecular pathway activation was written by Lu and others (Lu et al., 2008). LPS is one of the most studied immunostimulatory compound and as mentioned elsewhere is a component of the outer membrane of Gram-negative bacteria. The activation of the LPS-related cellular signalling involves multiple molecules: LPS binding protein (LBP), CD14, MD-2 and toll-like receptor 4 (TLR4). The association between LPS and CD14 is mediated by LBP. CD14, also present in a soluble form, mediates the binding of LPS to the complex TLR-4/MD-2 whereas MD-2 is a soluble protein that can directly form complexes with LPS. Upon LPS recognition, TLR4 undergoes oligomerization with following recruitment of adaptors using the domains of its receptor (TIR). There are five different TIR domains-containing adaptor proteins (MyD88, TIRAP, TRIF and TRAM) and they are all necessary for the TLR functioning. It has been demonstrated that the TLR4 signalling correlated to the activation of pro-inflammatory cytokines is a MyD88 - dependent pathway. Upon LPS recognition and activation of the TLR4 pathway, several death domain-containing kinases are activated and responsible for the transcription of proinflammatory cytokines (Lu et al., 2008).

The experimental preclinical model developed in this chapter successfully resulted in a measurable lung inflammation using 4 hours as total time window after LPS exposure. Other studies have investigated the kinetic of cells influx looking at how the concentration of pro inflammatory cells was changing over time in a longer period. Toward and Broadley (Toward & Broadley, 2000) evaluated the relation between aerosolized LPS exposure and extent of leukocyte infiltration at 0.5 h, 1 h, 2 h, 4 h, 24 h and 48 h after the LPS exposure. Male Dunkin-Hartley guinea pigs were exposed for 1 h to aerosolized LPS (*E. Coli,* O26:B6) (30 µg/mL) or vehicle (0.9% w/v saline) in a sealed chamber (620 x 300 x 420 mm) using a Wright nebulizer (flow rate 0.5 mL/min). They showed that after the above mentioned LPS exposure (concentration and length) an increased number of neutrophils was detectable already after 0.5 h ($\sim 8 \times 10^6$ cells/mL). The neutrophils concentration increased throughout, peaking at 24 h ($\sim 35 \times 10^6$ cells/mL) and decreasing at 48 h ($\sim 15 \times 10^6$ cells/mL). Macrophages increased up to 48 h after the LPS challenge, with a minor increase in the number of eosinophils (Toward & Broadley, 2000). Our findings are in agreement with those of Toward and Broadley as after 4 hours from the LPS exposure a measurable and significant change in the total cell count was detected.

However, the exact concentrations of neutrophils at 4 hours in the two studies are not in agreement. The number of neutrophils recovered in our study was between 3 x 10^6 cells/mL and 8 x 10^6 cells/mL, whereas ~ 30 x 10^6 cells/mL neutrophils were recovered in Toward and Broadley's study. In addition, compared to the study of Toward and Broadley, our method adopted a smaller chamber (470 mm (L) x 200 mm (H) x 200 mm). These results are interesting as we would have expected an opposite outcome with higher concentration of LPS and smaller chamber resulting in increased air concentration of LPS and stronger neutrophilia. This outcome may be attributed to the different strain of LPS use within studies, nebuliser and resulting particle size as well as the specific setting of the chamber used.

Although 0.9 mg of LPS results in a peak of neutrophils at 24 h, it is possible that the combination of a higher dose of LPS (2 mg) and a smaller chamber result in an earlier detectable peak of neutrophils. This hypothesis was not explored, but the use of 4 h was determined to be a suitable time point at which there was a robust increase in the number of neutrophils in the lungs in response to LPS in the model that was established and was suitable as tool to investigate the anti-inflammatory activity of a drug. In contrast the lack of significant changes in the macrophages number in our results (saline challenged vs LPS challenged) corresponded to little changes in macrophage numbers reported previously at 4 h compared to 48 (Toward & Broadley, 2000).

Another study that evaluated the effect of the time elapsed from 0 h to 72 h after LPS intranasal instillation was conducted by Lee and others (S.-Y. Lee et al., 2018). Female BALB/c mice were exposed to 20 µg/20 µL of LPS (strain not specified) via intranasal instillation and biomarkers of COPD severity including the total white blood cell and neutrophils count in the BAL were measured at time 0, 3 h, 24 h, 48 h and 72 h. They found that the total number of cells increased over time, becoming significant different 48 h after the LPS exposure. Despite an increase in the number of eosinophils overtime, only the change in the neutrophil number was statistically significant at 48 h and 72 h post-challenge. They also investigated how the level of pro-inflammatory cytokines changed overtime and found that they all increased overtime from 3 to 72 h after the LPS instillation. The changes recorded in the IL-8 levels were the largest compared to TNF-α and others and its expression was dramatically increased at 72 h. On the other hand, the levels of TNF-α were increased at 3 h after the challenge compared to control, followed by a decrease at 24 h and a further and robust increase at 48 h and 72 h after the challenge.

465   These data are in agreement with the TNF-α kinetic described by others (Bozinovski et

466   al., 2004). Mice were exposed to intranasal instillation of 10 µg/mice of LPS (*E. Coli*,

467   O26:B6) at time 0 with TNF-α levels detected at 0, 5 h and 24 h. The TFN-α levels

468   increased sharply from 0 to 5 h (5 ng/mL) with a following decrease overtime until 24 h

469   post-challenge. In both studies an early increase was reported which is in agreement with

470   the fact that the release of this cytokine represents an early event in response to LPS

471   stimulation. Moreover, in both studies a decrease at 24 h was described.

472   These data suggest that although the time point used in this experimental chapter was

473   demonstrated to be suitable to detect a significant change in the neutrophils number, it

474   may not be suitable to detect changes in the level of TNF-α and a longer period of time

475   including earlier and later time points might be required to detect increases of its level.

476   Bozinovski and co-workers (Bozinovski et al., 2004) also investigate the kinetic of

477   neutrophilia overtime. Evident increase of neutrophils number was detected as early as 2

478   h post-challenge with a robust increase 6 h after instillation of LPS, and the highest

479   concentration reached after 24 h (1.3 x 10^6 cells/mL); 48 h after the challenge, the

480   concentration of neutrophils declined and completely resolved by 72 h (Bozinovski et al.,

481   2004). It was also reported that the number of macrophages steadily increased from 6 h

482   to 48 h post challenge, with a following robust further increase at 72 h post-challenge.

483   This latter increase was in contrast with the kinetic reported for the neutrophils and it was

484   considered associated to the *in situ* replication of the macrophages.

485   Although experimental conditions may vary within studies (mode of LPS exposure, LPS

486   concentration etc.), it is evident how after a LPS challenge the influx of inflammatory

487   cells is always described. The neutrophils mobilisation and release of pro-inflammatory

488   cytokines is an early event and 4 hours represents a suitable time point at which the

489   neutrophils activation has already started. In some studies that considered a wider time

490   window, the neutrophils concentration was showed to peak 24 h after the challenge with

491   a further decline at 48 h. Additional studies are required to further elucidate the kinetic in

492   the model used in this experimental chapter and those will consider a longer time range

493   with multiple time points analysed.

494

495   The design and optimisation of the LPS model in this experimental chapter considered

496   variables as animal welfare, severity of the procedure (3R) as well as the dose of the

497   trigger agent the animals had to be exposed to. The animals should receive a dose that yet

498   produces a measurable change in the endpoint or marker of interest, despite not causing

499   side effects that may hinder the detection of the pharmacological effect or bring to

500   misinterpretation of the results or cause unnecessary and suffering events to the specie.

501

502   In a dose-response and kinetic study carried out by Larsson (Larsson et al., 2000),

503   C57BL/6JBom mice were exposed to different nebuliser concentration of LPS (range of

504   0.001 to 1 mg/mL) (*E. Coli*, O128:B12) using a nose-only Battelle exposure chamber.

505   The study showed that the increase of the LPS concentration resulted in an increased

506   accumulation of leukocytes recovered 16 h after the LPS exposure. Despite the different

507   species used, exposure mode and the time window adopted (16 h vs 4 h), the LPS dose-

508   dependency detected in this experimental chapter is in agreement with the finding of

509   Larsson and others. In this context, it is important to highlight that a systematic

510   comparison of LPS doses and corresponding outcomes used in preclinical models and

511   available in the literature is often challenging. There is a lack of consistency in how the

512   LPS concentration is reported. This can be described as the value of the concentration

513   (mg/mL) loaded in the nebuliser reservoir or as the dose administered directly to the lung

514   if an intratracheal administration is performed. In other studies, the delivered dose

515   (mg/kg) is reported with a theoretical estimation of the lung deposition equal to 20 %.

516   In the same study, the kinetics of the inflammatory response were studied using a low

517   (0.1 mg/mL) and high dose (1 mg/mL) of LPS. The accumulation of neutrophils and

518   eosinophils after the low and high dose exposure did not differ significantly during the

519   first 12 h. At high dose, the total neutrophils and eosinophils number further increased

520   and peaked at 24 h, whereas the inflammation peaked and resolved 12 h after the

521   challenge for the low-dose group. This demonstrated an early cell influx at the low dose

522   and sustained cell influx overtime for the high dose. The number of cells returned to pre-

523   challenge levels 48 h after the challenge.

524

525   In this experimental chapter the LPS dose-dependency was described using three different

526   doses of LPS (0.1 – 0.2 – 0.5 mg/mL). In agreement with other scientific studies, results

527   shows that the exposure to increasing concentration of LPS results in increased number

528   of leukocytes recovered. However, it would be beneficial to newly carry out the

529   experiment increasing the number of subjects in each group. Indeed, the small number of

530   animals in each group (n = 2) did not allow to evaluate whether the data follows a normal

531   distribution or not. Therefore, the statistical difference within groups was not

532   investigated.

533   Larsson and co-workers showed that the LPS-induced inflammation was also correlated
534   to an increased level of pro-inflammatory cytokines when the profile of cytokines in the
535   lung tissue was evaluated after low and high dose. The low dose induced a transient and
536   early (2 h after the challenge) onset for the pro-inflammatory chemokine analysed; in
537   contrast the high dose induced a delayed but more sustained release of the same cytokines
538   (Larsson et al., 2000). The expression of TNF-α and other cytokine was strongly
539   exhibited in the late phase (20 h) of the inflammation process after high- dose exposure.
540   The key role of TNF-α in the recruitment of the neutrophils in the lungs is discussed and
541   demonstrated by Kips and co-workers, who showed that its inhibition in rats exposed to
542   LPS reduced the neutrophil influx (Kips et al., 1992). Multiple rats were placed in a
543   whole-body chamber and exposed to aerosolized LPS (0.001 mg/mL and 0.01 mg/mL)
544   for 30 min. Lungs were lavaged 1.5 h after the endotoxin exposure and concentrations
545   were detected. The exposure to the low and high dose of LPS resulted in 17.9 ± 6.9 and
546   80.5 ± 7.8 U/mL, respectively, with no TNF-α detected when the rodents were exposed
547   to saline. Interestingly, the exposure to a pre-treatment with anti-TNF antibodies resulted
548   in partial inhibition of the neutrophil influx, indicating that TNF-α is a mediator of the
549   endotoxin-induced inflammation (Kips et al., 1992)
550
551   As previously mentioned, two of the possible routes to achieve pulmonary exposure of
552   LPS in preclinical models are intratracheal instillation and inhalation. More generally, the
553   use of intratracheal instillation when administering an endotoxin to establish an
554   inflammation model or a drug to treat the inflammation represents a source of variability
555   especially when comparing findings obtained from different studies and carried out in
556   different laboratories. One source of variability is represented by the use of a fixed
557   volume of air immediately after the instillation of the agent of interest. Results reported
558   in this experimental chapter showed that this minor protocol modification consistently
559   impacted the final distribution of the substance of interest. The use of 1 mL air allows a
560   more uniform distribution of the instilled volume, which otherwise deposit in an uneven
561   pattern throughout the lungs. Those results highlighted how the use of the intratracheal
562   administration increases the variability of the outcome, concluding that the inhalation is
563   a superior route of administration for LPS in the designed inflammation model.
564   The variability of lung response after exposure to LPS via intratracheal instillation (5
565   mg/kg) and nose-only inhalation of an aqueous LPS aerosol (concentration 100 mg
566   LPS/m^3) for 6 h was investigated in another study (F. Liu et al., 2013). It is not surprising

that the inhalation route provided a more uniform type of injury, whereas the dose needed to induce lung injury was lower when using inhalation compared to intratracheal instillation. The total cell count and cytodifferentiation in BAL were measured alongside other endpoints at 1, 3, 7, and 14 days after intratracheal bolus instillation or after inhalation of LPS. The total cell count and polymorphonuclear neutrophils differ according to the method (bolus intratracheal or inhalation) not in the LPS - dosed animals, but rather in the control - dosed animals. A difference was noticeable for the control groups, with groups receiving vehicle via intratracheal administration showing a higher number of infiltrated cells (total and differential) compared to inhalation, highlighting an inflammation induced by the instillation method itself compared to inhalation. Hence concluding that the inhalation method appeared superior compared to the intratracheal method. In addition, the study demonstrated that the same quantitative outcome was achievable by using a lower dose by inhalation compared to intratracheal administration. Indeed, the same conclusion was drawn by Smith and others in two different studies (Foster et al., 2003; Smith et al., 2011) where LPS was administered via intratracheal route or inhalation.

Indeed, when investigating the benefits of the pulmonary route in pre-clinical models, intratracheal instillation is one of the most well-known and used methods. However, the use of an excessive volume of substance of interest (either endotoxin or therapeutic drug) may affect the results and lead to misinterpretation and cause a lung damage itself. It could result in uneven lung distribution and responses caused by non-homogeneous distribution of the substance of interest. In addition, the administration of high volume may alter the lung clearance mechanisms and impact any kinetic study of the instilled material, making the pulmonary delivery via instillation considerably less controlled than the inhalation.

The exposure to an inhaled material requires an estimation of the dose used. Indeed, in preclinical as in clinical models only a percentage of the total dose delivered will impact the lungs of the subject and be available to exert a therapeutic effect. For example, Larsson and co-workers (Larsson et al., 2000) assumed that only 20% of the delivered dose via inhalation (nose-only system) was deposited into mice lungs. Therefore, they calculated that the lung burden provided by the concentrations of 0.1 mg/mL and 1 mg/mL was of 0.02 and 0.2 mg/mouse respectively. An alternative way to estimate the

601   delivered dose is using the equation developed by a working group of the Association of

602   Inhalation Toxicologists. The equation (**Equation** 5.1) defines the delivered dose (DD)

603   and takes into account the concentration of substance in the air (mg/L), the respiratory

604   minute volume or the volume of air inhaled in one minute (RMV) (L/min), the duration

605   of exposure (D) (min), the proportion by weight of particles that are inhalable by the test

606   species i.e. inhalable fraction (IF) and the body weight (BW).

607

608   *Equation 5.1 Calculation of the delivered dose achievable via inhalation*

$$DD \ = \frac{C \ x \ RMV \ x \ D \ (x \ IF)}{BW}$$

609

610   The RMV for different species (mice, rats, dogs and non-human primates) can be

611   calculated using the formula below (**Equation** 5.2):

612

613   *Equation 5.2 Calculation of respiratory minute volume*

$$RMV \ (L/min) \ = \ 0.608 \ x \ BW(kg)^{0.852}$$

614

615   where BW does correspond to specie bodyweight expressed in kg.

616

617   This equation represents an extremely valuable tool to estimate the dose at which the

618   specie is exposed which usually differ from the theoretical dose. This is the case of a

619   study conducted by Liu (F. Liu et al., 2013). Rats were exposed to an LPS intratracheal

620   dose equal to 5 mg/kg. However, the lung burden or thoracic dose calculated using the

621   Equation 5.1 resulted of 2.6 mg/kg.

622   In addition to the use of this equation which enables to have an estimation of the delivered

623   dose, an ideal inhalation system in preclinical studies should be characterized by

624   controlled temperature and humidity of the nebulised solution. The integrity and stability

625   of the aerosol generation and exposure should be monitored real time as well as the total

626   mass of the API of interest at which the animal species is exposed to. Usually this is

627   allowed by the gravimetric determination of the inhaled molecule via filters located at the

628   breathing zone areas.

629

630 The inhalation system built in this experimental chapter was less controllable as not
631 coupled with a real time analysis of the breathing zone area of the guinea pigs. Despite it
632 was unlikely to have an estimation of the deposited dose of LPS, successful deposition of
633 the endotoxin particles was confirmed by the increased inflammatory cells recovered in
634 the BALF. The Equation 5.1 will be used in Chapter 6 to evaluate the deposited dose of
635 inhaled RFM.

636

637 The anti-inflammatory activity of RFM in COPD models is well-known. In 2005, orally
638 administered RFM was shown to partially improve lung inflammation and completely
639 prevent the parenchymal destruction induced by CS (Martorana et al., 2005). Indeed,
640 these therapeutic actions of RFM were evaluated in an acute and chronic mice model.
641 The anti-inflammatory activity of orally administered RFM was also described in Brown
642 Norway rats as RFM inhibited eosinophilia and LPS-induced circulating TNF-α
643 (Bundschuh et al., 2001). The activity of RFM in attenuating the pulmonary inflammation
644 has also been demonstrated clinically. Healthy subjects were challenged with segmental
645 endotoxin and treated for 28 days with placebo or orally RFM (500 µg). After 29 days a
646 baseline BALF was performed followed by exposure to segmental endotoxin challenge
647 (4 mg/kg). After 24 h, cells in the lungs were collected and counted. RFM was showed to
648 reduce by 39% and 74% the number of eosinophils and neutrophils respectively compared
649 to placebo. The number of macrophages and lymphocytes showed unchanged if subjects
650 received placebo or RFM (Hohlfeld et al., 2008).
651 The model established in this chapter was able to demonstrate similar anti-inflammatory
652 activity of RFM, where a single oral dose was shown to significantly reduce the lung
653 inflammation expressed as neutrophilia in the lungs of LPS challenged guinea pigs.

654

655 **5.6 Conclusion**

656

657 A preclinical model was established which mimics the lung inflammation associated with
658 COPD exacerbations. A dose-dependency was described with increasing LPS doses (0.1,
659 0.2 and 0.5 mg/mL) resulting in increased total and differential cell count recovered from
660 the BAL. The responses measured for the biomarkers selected to investigate the
661 modulation of the inflammation were comparable with those reported in the literature.
662 The results illustrated that a single exposure via nebulisation to LPS (nebuliser
663 concentration of 0.2 mg/mL) can elicit an acute and significant increase of cell infiltration

664 into the airway, making this model useful for studying inflammation. The model has ease

665 of use and the cost effectiveness, especially when compared to the laboratory equipment

666 that a nose-only system or a chronic exposure to CS would require. The main limitations,

667 which adds to the intrinsic variability of LPS response in preclinical models, concern the

668 calculation of the aerosol concentration and the guinea pig lung function monitoring

669 which was not performed. The modulation of inflammation by orally administered RFM

670 (0.5 mg/kg) was demonstrated. The LPS-induced neutrophilia was reduced by a

671 preventive protocol of RFM in the designed model, highlighting its suitability to

672 investigate the exposure of the same drug by the pulmonary route. This response will

673 serve in Chapter 6 to investigate the potential of inhalation to deliver RFM effectively for

674 an anti-inflammatory action and to test formulation strategies. In summary, this model

675 was shown to be fit for purpose to investigate the anti-inflammatory activity of

676 therapeutic agents and be used to study their administration by pulmonary delivery.

677

CHAPTER 6: In vivo anti-inflammatory activity of

nebulised roflumilast

6.1 Introduction

The design of a formulation for lung delivery as opposed to oral or intravenous administration comes with particular challenges. The deposition of a formulation in the lungs and local therapeutic activity depends on a complex series of factors, including particle properties (density, aerodynamic diameter), respiratory physiology, fluid dynamics, the physical deposition mechanisms and type of inhalation device employed (Hofmann, 2011).

Despite the airway system and the particle deposition being dependent on multiple factors, three main mechanisms dictate particle deposition in the lungs: impaction, sedimentation, and diffusion (Carvalho, Peters, et al., 2011; Darquenne, 2012; Heyder, 2004; Hofmann, 2011). To a lesser extent interception and electrostatic precipitation are also relevant, particularly for elongated fibrous particle or chain-like aggregates and for electrically charged particles, respectively.

Diffusion is the mechanism that dictates the deposition of particles smaller than 0.5 µm, albeit these carry little mass and are less relevant in terms of pharmaceutical aerosols. Deposition is correlated to particle size and to respiratory rate, and increases when longer distances are travelled by particles with smaller size under the influence of increasing respiratory cycle period. The total diffusional deposition decreases with increasing particles size up to 1 µm.

Particles larger than 0.1 µm deposit by diffusion but also by sedimentation, which is under the influence of gravity, which increases with increasing particle mass, particle density and respiratory cycle period, whereas particles with a diameter >1 µm deposit by both sedimentation and by impaction, producing the typical U shape of the total deposition curve (**Figure 6.1**).
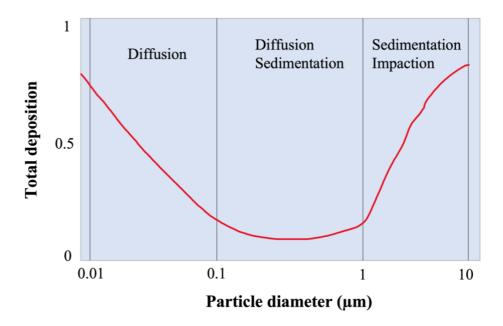
Figure 6.1 Schematic representation of relationship between particle deposition mechanisms in the lungs and particle aerodynamic diameter.

706 Impaction plays an important role in the deposition of particles in the tracheobronchial
707 region – where the air speed is high, and the flow is turbulent - whereas the gravitation
708 sedimentation predominates in the smaller bronchi and bronchioles. In the alveolar region
709 particles deposit by both sedimentation and diffusion (Heyder, 2004; Labiris & Dolovich,
710 2003). A schematic representation of the mechanisms of particle deposition in the
711 relationship with the particle size is displayed in **Figure** 6.2.
712
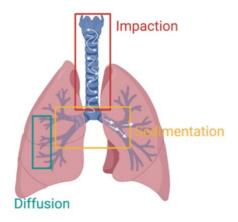
713 *Figure 6.2 Schematic representation of three main mechanisms of particles deposition*
714 *in the lungs based on the particle aerodynamic diameter: impaction, sedimentation and*

715 *diffusion. The figure also schematically represents the turbulent flow (oro-tracheal*
716 *region) and the laminar flow (respiratory airways).*

717 Inertial impaction is the predominant deposition mechanism for particles with an
718 aerodynamic diameter >5 µm as particles have enough momentum to keep their trajectory
719 despite changes in the airflow and are not able to follow airstream direction changes
720 resulting in impaction of the particles on the walls of the airways, especially in the upper
721 airways and at the bifurcations. The probability of a particle depositing by impaction is
722 described by the Stokes number (**Equation** 6.1) – the higher the Stokes number the higher
723 the probability of impaction into the airways.

724

725 *Equation 6.1 Stokes' number equation*

$$Stk = \frac{\rho \times d^2 \times V}{18 \times \eta \times R}$$

726 Where ρ is the particle density, d^2 is the particle diameter, V is the air velocity, η is the
727 air viscosity and R the airway radius. Larger particles are transported by inertial impaction
728 which increases with particle size, particle density and airflow rate as well (Heyder,
729 2004).

730

731 The gravitational sedimentation is correlated to the gravity. It mainly occurs in the small
732 airways or in the alveolar cavities where the space is restricted. The settling velocity is
733 expressed by **Equation** 6.2 and increases with particle size and residence time, hence
734 breathing manoeuvres such as breath-holding increases the probability of particle
735 deposition by this mechanism.

736

737 *Equation 6.2 Settling velocity*

$$Vs = \frac{\rho \times d^2}{18\mu} \times g$$

738 With g being the gravitational acceleration.
739

740   In contrast, smaller particles ($< 0.5\ \mu m$) are characterized by Brownian motion (motions

741   of particles due to their collision with gas molecules) and will more likely deposit into

742   the lungs by diffusion (Williams et al., 2011). Opposite to impaction and sedimentation,

743   the deposition by diffusion is inversely related to particle size (Darquenne, 2012;

744   Williams et al., 2011).

745

746   The aerodynamic diameter is determined mainly by measuring deposition by impaction.

747   It is defined as the diameter (d_{ae}) of a sphere with density of 1 g/cm^3 and with the same

748   velocity in the air as the particle of interest. It is dependent on the actual diameter of the

749   sphere (d) and the spherical particle density (ρ) by the following equation (**Equation** 6.3):

750

751   *Equation 6.3 Aerodynamic particle diameter (d_{ae})*

$$d\mathrm{ae} = d \times \sqrt{\frac{\rho}{\rho 0}}$$

752

753   One the most useful and used parameters to describe the particle size of an aerosol is the

754   mass aerodynamic diameter (MMAD). It divides the particle population in two, with 50%

755   of the particle population being smaller and 50% larger of the stated value. The geometric

756   standard deviation (GSD) describes the dispersity of the particle size distribution around

757   the MMAD value. It can be summarized that particles with a MMAD between $5 - 10\ \mu m$

758   are mainly deposited in the large conducting airways and oropharyngeal region, whereas

759   smaller particles with a MMAD between 1 and 5 μm will likely deposit in the small

760   airways, with $> 50\%$ of the particle with aerodynamic diameters of 3 μm deposited in the

761   alveolar region. Smaller particles ($< 3\ \mu m$) have a higher chance (80%) to deposit into

762   the lower airways (of which 50 - 60% in the alveoli) (Labiris & Dolovich, 2003).

763

764   Parameters as the MMAD, the GSD and the FPF - defined as the amount of the deposited

765   dose that lies below the 5 μm range - are key to assess the likely distribution pattern of

766   an aerosol in the human respiratory tract. One of the most used and well-known *in vitro*

767   tests to provide those metrics is the deposition in a multistage cascade impactor, called

768   Next Generation Impactor (NGI) (Marple Virgil A., 2003). Combined to a vacuum pump

769   set at a clinically relevant flow rate recommended by the Pharmacopeia (General

770   Chapters_ 601_ Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder

771   Inhalers. United Stated Pharmacopeia (USP) 35, 2012) and matched to the inhalation

772   device of interest, this equipment can fractionate the aerosol based on the aerodynamic

773   diameter. Particles with sufficient inertia, will deviate from the air stream, go through

774   several nozzles with specific particle size cut-off and impact on plates (also called stages).

775   The presence of multiple nozzles, each with a specific cut-off size, will allow the particles

776   to impact and deposit on specific stages according to their aerodynamic diameter,

777   providing a particle distribution. Importantly, the flow rate through the impactor

778   determines the cut-off diameters which are function of nozzle geometry, particle density

779   and air velocity in the nozzle (de Boer et al., 2002). There are eight stages in the NGI and

780   the measurement of mass deposited in each stage allows metrics to be calculated from the

781   cumulative mass-weighted aerodynamic particle size distribution (Marple et al., 2004;

782   Marple Virgil A., 2003).

783   This equipment is a valuable tool for measuring particle size distribution and providing

784   an estimation of regional particle deposition in the lungs, despite the NGI not providing

785   an exact replica of lungs' sections. In addition, the measurements provided by the NGI

786   represent only a single approach that must be combined with other *in vitro* investigations.

787   Indeed, the cascade impactor has some limitations: i) in contrast with the human

788   inspiratory profile which has a varying flow rate, the NGI operates at constant flow in

789   order to preserve the effective cut-off diameter; ii) temperature and humidity of human

790   lungs are not replicated in the apparatus (Mitchell et al., 2007).

791

792   A detailed prediction of the aerosol deposition is extremely complex because it depends

793   on many factors including the airway calibre and the lung anatomy. In particular it should

794   be considered that: i) the presence of a lung disease, such as COPD or cystic fibrosis is

795   usually associated with alteration of normal physiology and structure hence the need to

796   consider them in the prediction of particle deposition; ii) often a particle deposition

797   prediction takes into account the physiology and structure of the human lung whereas *in*

798   *vivo* models often use rodents (as in this thesis) as the first species to investigate the

799   therapeutic potential of inhaled medicines.

800   Inflammatory lung conditions, such as COPD are characterized by obstruction of the

801   airways, increase production of mucus and a general and consistent alteration in the lung

802   architecture (Barnes et al., 2015). The lungs of COPD patients are characterized by

803   narrowing of the bronchioles (bronchiolitis) or enlargement of distal airspace

804   (emphysema), and these modifications may alter the normal deposition pattern (Labiris

805   & Dolovich, 2003). When designing a drug delivery system for the lungs of patients with

806   similar conditions, the deposition pattern in presence of those alteration is a key aspect as

807   there is a possibility that the drug delivered will not deposit in the obstructed region which

808   can also be the target region. Laube (Laube et al., 1989) studied the lung distribution of

809   0.9% saline labelled with ^{99m}Tc sulphur colloid in healthy subjects and patients with CF.

810   Subjects were exposed to a polydisperse radiolabelled aerosol (MMAD = 1.12 µm) and

811   the distribution of aerosol was uniform in the healthy subjects, whereas aerosol

812   distribution in the lungs of CF patients appeared non uniform with regions of higher,

813   lower, or no deposition. On the other side, Jakobsson (Jakobsson et al., 2018) studied the

814   particle deposition pattern in healthy and COPD subjects exposed to a nanometer size

815   range aerosol and reported an altered deposition of inhaled NPs between the groups.

816   Healthy never-smoking subjects, asymptomatic active and former smokers and subjects

817   with COPD were exposed to an inhaled aerosol of monodisperse polystyrene latex

818   nanospheres (50 and 100 nm) and lung deposition was measured after a 10 s breath-hold;

819   results showed no difference between healthy never-smokers and former smokers but

820   subjects with emphysema had a lower deposition fraction mainly due to the emphysema

821   and to the alterations of the lung morphology. There are, however, discrepancies within

822   the lung deposition studies of NPs reported in the literature (especially important in the

823   matter of pollution and environmental studies) as different methodologies are used or

824   different degree of lung inflation or residence time of the aerosol are considered.

825

826   The weak link between preclinical models and clinical studies in terms of prediction of

827   particle deposition, translocation and response is related to the differences of airway

828   morphology and breathing parameters between human and the rodent species which are

829   often used as pre-clinical models. As particles that are inhalable for humans may not be

830   inhalable to rodents, experimental adjustments may be necessary. In addition, rodents and

831   rabbits are compulsive nose breathing species, making the nasal route rather than oral

832   inhalation the means of aerosol administration. Nasal filtration in rodents influences the

833   total amount of particles available for lung deposition and since particle clearance in the

834   nasal region can be rapid, longer exposure period may be required to increase the

835   delivered dose.

836

837   In addition, for nasal breathing the inhalability is correlated and dependent on the size

838   and orientation of the nasal opening hence it can be determined when comparing animals

839   and human deposition studies. Another factor is the minute ventilation which is used to

840   estimate the delivered dose.

841   A critical aspect in terms of differences in particle deposition is the geometry of the lungs,

842   which is dissimilar between animals and humans. Detailed nasal geometry in Fischer 344

843   rats has been described in the literature (Kimbell et al., 1993). Raabe and colleagues

844   (Raabe et al., 1988) investigated the deposition pattern of aerosol particles with an

845   aerodynamic diameter up to 10 µm in multiple species including mice, guinea pigs,

846   hamsters and rabbits. Animals were exposed to monodisperse aerosol of fused

847   aluminosilicate particles labelled with radioactive [169]Yb via a modified vibrating liquid

848   stream generator for 45 minutes. Results showed that particles with MMAD > 3 µm

849   mainly deposited in the nasal-pharyngeal region whereas particles with MMAD > 10 µm

850   completely deposited in the nasal-pharyngeal and laryngeal region with no deposition in

851   the pulmonary tract.

852   The advantages of combining the newest tools of the nuclear medicine to allow a more

853   detailed evaluation of particle deposition with the use of a polydisperse aerosol population

854   which more closely resembles the currently pharmaceutical aerosol used were explored

855   by Kuehl in 2012 (Kuehl et al., 2012). Aerosol deposition pattern was investigated as a

856   function of particle size in rats and mice using radiolabelling techniques applied to a

857   polydisperse aerosol of radio labelled particles with MMAD of 0.5, 1.0, 3.0 and 5.0 µm,

858   which were nebulised via air jet nebuliser to four mice and four rats using a nose-only

859   exposure system in combination with SPECT/CT imaging. Unsurprisingly, results

860   showed that the deposition fraction decreases with the increasing particle size with higher

861   peripheral deposition for smaller particles. Smaller particles showed an increase

862   deposition in the lungs and in the peripheral lungs compared to bigger particles.

863   A similar and very extensive study was conducted by Asgharian (Asgharian et al., 2003)

864   which exposed fifty-five female Long-Evans rats to a monodisperse radiolabelled aerosol

865   with MMAD in the range 0.9 to 4.2 µm. Deposition data revealed that the particle

866   deposition fraction, defined as the amount of material deposited in the tissue of interest

867   divided by the total amount of material inhaled, decreased with increasing particle size

868   due to a reduction in respirability correlated with an increase in particle size.

869

870   This chapter aims to explore the therapeutic potential of an inhalable NPs-based

871   formulation encapsulating RFM using a model of lung inflammation in guinea pigs. The

872   experimental methods and formulations adopted have been described in the previous
873   chapters, in which the suitability of those methods was established.

874   The choice of the inhalation device used for the pulmonary administration to guinea pigs
875   was based on the need to aerosolise a liquid formulation, which is the approach typically
876   used in early development. The device employed in this investigation was a mesh
877   nebuliser (Aerogen Pro), which is commonly used in hospital settings to administer
878   medical aerosols to patients. Generally, a nebuliser is a device that can convert a liquid
879   into a droplet aerosol suitable for inhalation. There are three types of nebulisers: air-jet,
880   ultrasonic and mesh nebuliser (Ari, 2014). Jet nebuliser required large (2 to 10 L/min)
881   volume of pressurized gas to draw medication up through a capillary tube; however, they
882   can be difficult to use due to the need of compressed gas and additional tubing. In the
883   ultrasonic nebuliser the aerosol is produced due to the vibration of a piezo electric crystal,
884   and they can be large-volume or small-volume. Downsides of those devices include large
885   residual volume and degradation of heat-sensitive material. On the other hand, mesh
886   nebuliser represents newer technology with a mechanism of aerosolization based on the
887   vibration of a mesh with multiple apertures with a specific diameter (in the case of the
888   device used in this work, the aperture range was between 3.5 and 5 µm).

889   In contrast to ultrasonic and jet nebuliser, the mesh nebuliser employs single-pass
890   technology, avoiding recirculation of the droplets, avoiding heating and reducing the
891   extent of shear forces while in the reservoir. These characteristics have made them
892   suitable also for delivery of labile molecules such as proteins and mAbs (Matthews et al.,
893   2020).

894

895   The aerosol delivery of NPs to the lungs represents an interest topic of the nanomedicine
896   as it includes both the advantages of the pulmonary route and those of the nanocarriers
897   (N. Osman et al., 2018; N. M. Osman et al., 2020). The administration of a therapeutic
898   directly to the lungs allows to bypass the gastrointestinal tract and deliver a higher dose
899   to the target site while using a smaller total dose compared to other routes of
900   administration. The advantages of delivering drug in a nanocarrier, include a longer
901   retention at the site of action, the possibility of sustained release and a reduction of the
902   systemic side effects, the option to improve drug solubility due to the encapsulation of
903   the drug in the nanocarrier, and the avoidance of the clearance mechanisms which may
904   further prolong the residence time.

905 Rather than immediately assess the delivery of a nanocarrier to the lungs based on the
906 NPs in microparticle (NEMs), preliminary experiments focussed on a formulation
907 strategy based on the delivery via a simple nebulisation of drug *in vivo* and the
908 nanoparticle suspension *in vitro*.

910 To our knowledge, there are no currently clinical trials investigating therapies based on
911 inhalable albumin NPs and pre-clinical studies investigating the pulmonary delivery in
912 rodents species are very few, usually employing basic strategies to deliver the nanocarrier
913 to the lungs such as the intratracheal route (often achieved via the use of a powder
914 insufflator called PennCentury). A detailed overview of the downsides of the intratracheal
915 route of administration were highlighted in Chapter 5.

917 In contrast, liposome delivery via the pulmonary route has gained considerable success
918 in pre-clinical and clinical studies (Carvalho, Carvalho, et al., 2011). A liposomal
919 amikacin suspension, Arikayce® for the treatment of the Mycobacterium avium complex
920 lung disease via nebulisation has been approved by the EMA and the FDA (Arikayce
921 Liposomal - Product Information; Khan & Chaudary, 2020). Various studies have also
922 successfully demonstrated *in vivo* the potential of inhalable liposome compared to other
923 routes of administration or formulation. An example is provided by one of the studies
924 conducted by Koshkina (Koshkina et al., 1999) where three different strains of mice
925 (C57BL/6, Swiss nu/nu and BALC/c mice) were exposed to inhaled camptothecin
926 formulated in dilauroylphosphatidylcholine liposomes via Aerotech II nebuliser (MMAD
927 of 1.6 µm). The drug deposition in the lungs was demonstrated to be higher when the
928 drug was administered in liposomes via inhalation compared to intramuscular
929 administration of camptothecin (control). Interestingly, prior to this study the
930 intramuscular administration of camptothecin was considered the most effective route of
931 administration in immunodeficient mice in cancer models, whereas this study concluded
932 that the aerosol was more advantageous. In addition, they showed that the drug
933 concentrations achieved at 30 min following inhalation in the aerosol-treated mice were
934 greater compared to those in the non-aerosol-treated mice. The same liposome-based
935 formulation was adopted in a different study (Koshkina et al., 2001) to encapsulate
936 paclitaxel as treatment for a murine renal carcinoma pulmonary metastases model. The
937 liposomes were nebulised with an Aero-Mist jet nebuliser and the therapeutic efficacy
938 compared to intravenous administration (control). Results showed higher concentration

939 of paclitaxel in the lungs when administered via inhalation compared to control
940 (intravenous administration), with a slower clearance of the drug from the lung as well.
941

6.2   Aims and objectives

The overall aim of this series of experiments was to investigate the anti-inflammatory activity of RFM delivered by the pulmonary route as a precursor to optimising this therapeutic effect by formulating as RFM-HSA-NPs. The first experimental step carried out was based on the formulation and characterisation of a RFM suspension in a biocompatible vehicle of saline, Tween® 80 and DMSO and its nebulisation to reduce the lung inflammation in LPS-challenge guinea pigs. The practicality of NPs suspension administration via nebulisation was also investigated *in vitro* by determining the impact of the nebulisation on the physiochemical characteristics of the nanocarrier.

Specific objectives included:

1. Formulate a RFM suspension and evaluate of particle size and geometric diameter using Morphologi 4;
2. Aerodynamic assessment of the nebulised roflumilast suspension using the Next Generation Impactor;
3. Calculate the RFM delivered dose (DD) to guinea pigs during the nebulisation cycle;
4. Evaluate the effect of nebulised RFM on lung inflammation using the guinea pigs LPS model;
5. Investigate the physiochemical stability of NP to nebulisation using Dynamic Light Scattering.

6.3   Methods

6.3.1   Manufacture and characterisation of roflumilast suspension prior to *in vivo* administration

6.3.1.1   Manufacture of roflumilast suspension

RPM suspensions (100 and 500 µg/mL) were prepared by suspending the drug powder in a vehicle of sodium chloride 0.9% / 0.2% Tween 80 and 0.05% DMSO (Nials et al., 2011; Villetti et al., 2015). Firstly, RFM was weighed and suspended in the appropriate volume

973 of sodium chloride 0.9%/ 0.2% Tween 80 by a sonication process (10 minutes). Then an
974 aliquot of DMSO corresponding to 0.05% of the total volume was added and the
975 suspension was probe sonicated for 30 minutes (amplitude 30 mA).

976

977 The development and characterisation process of RFM suspensions prior to *in vivo*
978 administration is illustrated in **Figure** 6.3.

979

980
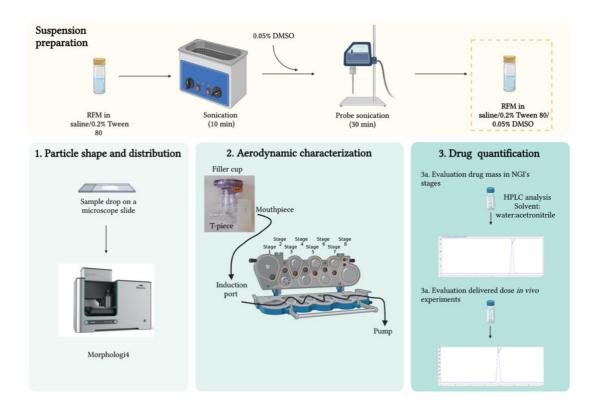
Figure 6.3 Schematic representation of manufacture, characterisation, and aerodynamic assessment of nebulised aerosols of roflumilast suspension prior to in vivo administration. After the preparation, the size and the shape of the particles were evaluated using Morphologi4, the aerodynamic characteristics evaluated with the Next Generation Impactor (NGI).

981

6.3.1.2 Ensuring dose homogeneity, investigating particle shape and geometric size distribution using Malvern Morphologi 4 system and drug concentration

984 Size and shape of the drug solid particles in the RFM suspension (500 µg/mL) prepared
985 as described above were investigated. This analysis was carried out using a Malvern
986 Morphologi 4 system (Malvern, UK) particle characterisation device. For each analysis,

987   an aliquot (10 µL) was placed on a microscope glass slide and covered with a cover slip.

988   The area of analysis (1.779 mm^2) as well as the volume used was kept constant. The

989   analysis was carried out at 50x magnification (0.5 – 50 µm). Particles with elongated

990   shape resulting from sample impurities and aggregates of particles (diameter 31 – 50 µm)

991   were excluded from the analysis**.** Stock solutions were prepared in triplicate and analysed

992   in triplicate.

993

994   Furthermore, an assessment of the dose homogeneity within samples was carried out by

995   investigating the concentration of RFM in each sample using the HPLC quantification

996   method mentioned in paragraph 2.3.8. This ensured that the drug concentration achieved

997   in each sample was consistent over time as multiple stock solutions were manufactured

998   during the *in vivo* experiment. RFM stock solutions (100 and 500 µg/mL) were prepared,

999   and dilutions were carried out to produce suspensions of range 50, 100, 200, 300, 400 and

1000   500 µg/mL. Sodium chloride 0.9% + 0.2% Tween 80 was used as diluent. Prior to HPLC

1001   analysis, an aliquot (100 µL) of each suspension (100 µg/mL and 500 µg/mL) was mixed

1002   with an aliquot (900 µL) of HPLC grade acetonitrile. The samples were vortexed and the

1003   amount of drug in the sample calculated using the HPLC method described in section

1004   2.3.8.

1005

1006   **6.3.1.3   Aerodynamic characterisation of nebulised roflumilast using the Next**

1007   **Generation Impactor (NGI)**

1008   **6.3.1.3.1   NGI settings**

1009   The aerodynamic characteristics of nebulised RFM suspension (100 and 500 µg/mL)

1010   were evaluated using the Next Generator Impactor (NGI) attached to an Aerogen

1011   Nebuliser Pro (Aerogen Ireland Ltd., S/N 356, volume median diameter with saline 0.9%

1012   4.82 µm, average flow rate 0.38 mL/min) via a mouthpiece. The nebuliser was attached

1013   to an ISO 15 mm t-piece which was directed towards the induction port with a mouthpiece

1014   and the NGI system attached to a vacuum source. The experimental set-up (apart from

1015   the vacuum pump) is illustrated in **Figure** 6.4. The assembled impactor coupled with the

1016   induction port and the mouthpiece were refrigerated at 5°C in a cold room for 90 min

1017   prior to use in order to control the evaporation of droplets produced during the

1018   nebulisation (United Stated Pharmacopeia (USP) 35, 2012). The system was used within

1019   5 min from the impactor removal from the refrigerator and the nebulisation of 2 or 4 mL

1020   RFM suspension was completed within 10 - 12 minutes of removal of the system from

1021   the refrigerator. The NGI was coupled with a vacuum pump (Gast, Benton Harbor, USA)

1022   and the airflow was adjusted prior to refrigeration to 15 L/min using a flow meter attached

1023   to the induction port. The flow rate value was used as this value is a good approximation

1024   of the mid-inhalation flow rate achievable by a healthy adult breathing at 500 mL tidal

1025   volume (United Stated Pharmacopeia (USP) 35, 2012).

1026
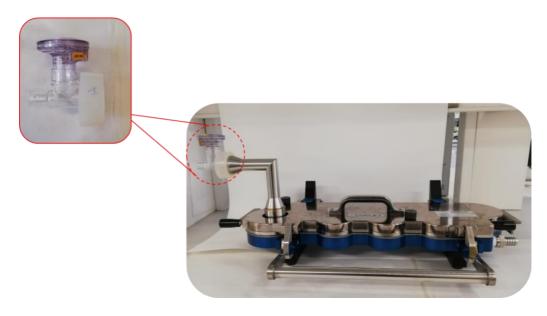
Figure 6.4 Experimental set-up for Next Generation Impactor (NGI) testing. (From left to right of picture) Aerogen® Pro nebuliser (Aerogen Ireland Ltd, S/N 356, volume median diameter with saline 0.9% 4.82 µm, average flow rate 0.38 mL/min) attached to an ISO 15 mm t-piece directed toward the induction port through a mouthpiece adapter (in the focused section).

1027

1028   **6.3.1.3.2 Evaluation of the respirability of nebulised roflumilast suspension**

1029   The volume of RFM suspension to be nebulised was selected in order to ensure good drug

1030   detection in each stage of the cascade impactor whilst avoiding the streaking phenomena

1031   in the system stages. This was investigated by nebulisation of different volumes (2, 3 and

1032   5 mL) of the RFM suspension (100 µg/mL).

1033

1034   For each determination of the aerodynamic particle size distribution produced by

1035   nebulisation of the RFM suspension a fixed aliquot (2 mL or 4 mL for 500 and 100

1036   µg/mL, respectively) was added into the nebuliser reservoir and nebulised to dryness. To

1037   ensure a constant flow throughout the system, vacuum pump was activated 1 min prior to

1038   the nebulisation. After achieving nebulisation to dryness, vacuum pump and nebuliser

1039 were switched off. The amount of drug deposited onto each stage was calculated using a
1040 previously validated HPLC method (section 2.3.8). Complete recovery of the deposited
1041 drug was achieved by rinsing each stage with a fixed volume of HPLC water-acetonitrile
1042 (ratio 30:70). The mouthpiece was washed with 1 mL, induction port and stages 1 to 5
1043 with 5 mL and t-piece and stages 6 to 8 with 3 mL. These volumes were selected to
1044 provide a concentration of RFM that was readily quantified (data not shown). The
1045 suitability of the method used to recover the drug deposited in each section of the NGI
1046 was evaluated via nebulisation of 2 mL of saline/ 0.2 % Tween 80 + 0.05% DMSO
1047 (control) with following addition to each section of the impactor of a fixed volume of a
1048 known concentration of a RFM stock solution in water:acetonitrile (30:70) (50 µg/mL).
1049 The amount of drug in each of those sample was calculated as described previously.
1050
1051 The linearity of the RFM concentration curve in water:acetonitrile (30:70) was
1052 determined in the range 0.05 - 150 µg/mL by HPLC. To increase sensitivity, two
1053 calibration curves were built: a) 0.05 - 4.0 µg/mL; b) 5 - 150 µg/mL. Working dilutions
1054 were obtained from stock solutions of RFM in water:acetonitrile (30:70) (50 µg/mL and
1055 500 µg/mL for the low range and high range concentrations).
1056
1057 The aerodynamic parameters calculated from the NGI assessment were FPF, fine particle
1058 dose (FPD), MMAD and GSD (**Table** 6.1**).**
1059

Table 6.1 Definitions of the most used parameters for aerosol characterisation.

- **FINE PARTICLE FRACTION (FPF):** $\dfrac{\textit{Fine particle dose}}{\textit{Total emitted dose}}$

- **FINE PARTICLE DOSE (FPD):** *Mass of particles less than 5 µm in size within the total emitted dose*

- **MASS MEDIAN AERODINAMYC DIAMETER (MMAD):** *Diameter at which 50% of the particles of an aerosol by mass are larger and 50% smaller*

- *GEOMETRIC STANDARD DEVIATION (GSD): standard deviation of the logarithm of particle diameter*

1060
1061

6.3.2   Exposure of LPS-challenged guinea pigs to a pre-treatment of roflumilast via nebulisation using a whole-body chamber model

6.3.2.1   Calculation of *in vivo* delivered dose (DD) of roflumilast

Guinea pigs were exposed to nebulised LPS and RFM via the use of a whole-body chamber model. As described in Chapter 5, an estimation of the dose of drug that animals have been exposed to is required. Hence, the delivered dose (DD) of nebulised roflumilast was calculated using Equation 5.1 and Equation 5.2.

6.3.2.2   Nebulised roflumilast administered to LPS-challenged guinea pigs

Unanaesthetised Male Dunkin-Hartley guinea pigs (250 – 299 g) were placed individually and without restraint in the exposure chamber (47 cm (L) x 20 cm (H) x 20 cm (D)). In order to achieve the target delivered dose, the RFM stock solution of 500 µg/mL was used rather than the 100 µg/mL. The nebuliser filler cup was loaded with two aliquots of 2 mL each, for a total volume of 4 mL. The first aliquot was nebulised first followed immediately by the second aliquot.

RFM and vehicle (control) were nebulised using Aerogen Pro® (Aerogen Ireland Ltd., S/N 356, volume median diameter with saline 0.9% 4.82 µm, average flow rate 0.38 mL/min) attached to an ISO 22 mm t-piece fixed inside the lid of the chamber box and directed towards the inside of the glass box (470 (L) x 200 (H) x 200 (D) mm). LPS was nebulised following the same nebulisation settings mentioned in paragraph 5.3.2.2.

Subjects were divided in two groups which were exposed at time 0 to i) nebulised RFM (calculated delivered dose: 50 µg/kg); ii) nebulised sodium chloride 0.9%/ 0.2% Tween 80 + 0.05% DMSO (control). The length of the total drug/vehicle exposure was 20 min. One hour after the drug exposure, animals were again placed in the exposure chamber and exposed to nebulised LPS (0.2 mg/mL, 10 mL). Four hours after the inflammatory challenge, animals were euthanized with an overdose of pentobarbital (1 g/kg) via an intraperitoneal injection. The trachea was cannulated and bronchoalveolar lavage was performed using 5 mL of filtered PBS that was gently instilled and withdrawn 3 times to flush the lungs using a 5 mL syringe. Bronchoalveolar lavage fluid was processed and analysed for cytokine and cell content as mentioned in paragraph 5.3.2.3.

All experimental procedures and conditions were reviewed and approved by the ethics committee of King's College London and conducted in accordance with the United Kingdom Animal Scientific Procedures Act, 1986. Further details are mentioned in section 5.3.3.2.

6.3.3 Physiochemical characterisation of nebulised NPs using the Dynamic Light Scattering

The impact of the nebulisation on the size, PDI and zeta potential of blank and drug loaded HSA-NPs was investigated. An aliquot (0.4 mL) was placed in the filler cup of a Aerogen Pro (S/N 356) attached to an ISO 15 mm t-piece which was tightly connected a 15 mL falcon tube. The nebulisation was started, and the emitted aerosol collected as a condensate in a 15 mL tube after the nebulisation. For size and PDI evaluation, an aliquot of 100 µL of the condensate fluid was collected and diluted 100-fold with double distilled water to match the dilution adopted for NPs analysis pre-nebulisation (paragraph 2.3.3). For zeta potential measurement, an aliquot of 5 µL was collected form the condensate fluid and diluted 200-fold with double distilled water. Size, polydispersity index and zeta potential were measured using the Dynamic Light Scattering (using a method described in paragraph 2.3.3).

6.4 Results

6.4.1 Optimisation of roflumilast suspension prior to *in vivo* administration: particle size, geometric diameter, and aerodynamic assessment

6.4.1.1 Particle shape and geometric size distribution of roflumilast suspension

The particle shape and geometric size distribution of RFM suspension prior to *in vivo* administration was studied by Morphologi 4, using the circular equivalent diameter which is the diameter of a circle calculated from the equivalent area of the measured particles, and the average D_{10}, D_{50} and D_{90} values.

The aspect ratio of the drug particles in the RFM suspension (500 µg/mL) appears with irregular symmetry with some particles having an elongated aspect, whether others are smaller and rounder. Moreover, most of the particles have rougher outlines (**Figure** 6.5**).**

1128   The investigation of the particle morphology of raw RFM was not performed but further
1129   studies may investigate this aspect to provide evidence for the evaluation of the effect of
1130   sonication (time and amplitude) performed during the suspension preparation on the
1131   particle aspect form.

1132
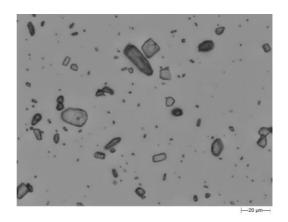
1133   *Figure 6.5 Representative particles images of 500 µg/mL RFM suspensions using*
1134   *Malvern Instrument (50x). Scale bar is 20 µm.*

1135   The geometric particle size was also evaluated and provided useful information regarding
1136   the quality of the formulated suspension prior to its nebulisation in a preclinical model.
1137   Results showed that 10%, 50% and 90% of the sample is smaller than 6 µm, 13.2 µm and
1138   24.6 µm respectively. Therefore, the particles in the suspension are in a size range
1139   between 6 and 24.6 µm, although the obtained standard deviation of D_{90} value is equal to
1140   12.0 highlighting variability within measurements (**Table** 6.2). Unfortunately, any
1141   information about the raw material (roflumilast) and whether this was micronized or not
1142   was provided by the supplier (Sigma).

1143

Table 6.2 Volume distribution statistics over 9 measurements for the RFM suspensions (500 µg/mL) calculated using Morphologi 4 Malvern Instrument. Values are reported as mean and standard deviation (SD) of n=3 independent experiments, measurements performed in triplicate.

	D [v, 0.1]	D [v, 0.5]	D [v, 0.9]
Mean	6.0	13.2	24.6
SD	2.15	3.83	12.0

1144

The mesh aperture pores of the Aerogen Pro® nebuliser adopted for the RFM nebulisation have a size between 3 – 5.5 µm. Based on the described geometric particle size, the nebulisation of the suspension will likely result in loss of a fraction of the total mass of RFM available which may be retained on the mesh. However, the aerodynamic assessment of the RFM suspension will provide further details on its respirability. It will also serve to estimate the delivered dose at which the species will be exposed to in the preclinical model.

6.4.1.2 Aerodynamic assessment of roflumilast suspension using the Next Generation Impactor (NGI)

The aerodynamic assessment of RFM in suspension was investigated using the NGI. The detection of the drug concentration in each stage of the cascade impactor was carried out using the method described in paragraph 6.3.1.3 combined with an HPLC method. For a more accurate detection of small RFM concentrations deposited in some of the stages, calibration curves were constructed in the ranges 0.05 – 4.0 µg/mL and then over the range 5.0 – 150 µg/mL ($R^2 = 0.9998$ and $R^2 = 0.9974$ respectively).

The method validation included the evaluation of the volume of RFM suspension that provided good quantification of RFM without overloading the impactor stages with the volume of liquid deposited (**Figure** 6.6). This analysis was carried out using the suspension of 100 µg/mL. The visual inspection of the impactor stages after the nebulisation cycle did not reveal any overloading. In addition, the RFM recovered from each stage of the impactor was not affected by different volumes used, providing another indication that overloading or streaking was not occurring.
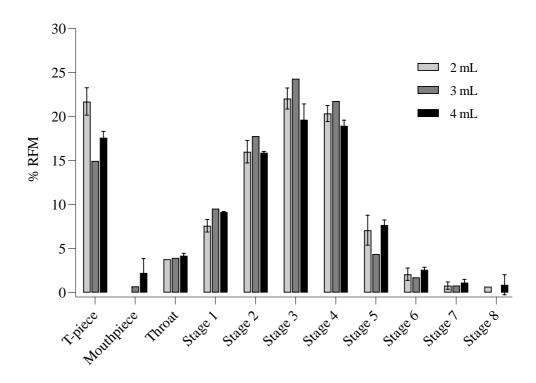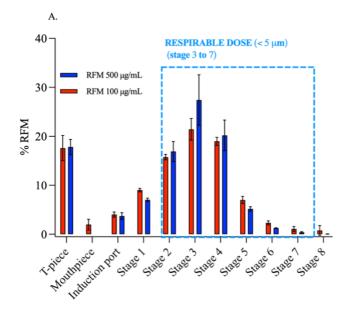
Figure 6.6 Evaluation of streaking phenomena. Roflumilast (%) recovery after the nebulisation of different volumes (2, 3, and 4 mL) of 100 µg/mL roflumilast suspension. Results are shown as percentage of the total mass recovered from each compartment, represented as mean ± SD of 1-2 independent experiments for each volume considered.

1169   RFM suspensions were recovered in terms of percentage of drug deposited on each NGI
1170   stage when both the suspensions were separately nebulised at 15 L/min. The nebulisation
1171   of both the suspensions concentration resulted in ~ 17% loss of drug in the t-piece, with
1172   the highest amount of drug deposited in the stage 2 – 3 and 4 (**Figure** 6.7 **A**). The cut-off
1173   diameters of the NGI under its operating conditions are shown in **Figure** 6.7 **B**. The
1174   stages of interest when estimating the respirable dose are stages 3 to 7. With a cut off size
1175   from 5.39 µm to 0.98 µm, the nebulisation of 100 µg/mL resulted in ~ 22% - 19% - 7% -
1176   3% - 1% and 0.8% of drug deposited in stages 3 - 4 - 5 - 6 and 7 respectively. Nebulisation
1177   of the higher drug concentration resulted in the same % of mass recovered deposition
1178   trend, with percentages of RFM deposited in stage 3 of 28%, stage 4 of 21%, stage 5 of
1179   6%, stage 7 of 0.4% and stage 8 of 0.07%.

1180

Figure 6.7 A) Roflumilast mass (%) stage deposition from NGI when 100 μg/mL (red)
and 500 μg/mL (blue) suspensions are nebulised at 15 L/min using an Aerogen Pro;
(B) Cut-off sizes of Next Generation Impactor system at 15 L/min. Data is represented
as mean ± SD of five independent experiments. The percentage of the mass fraction in
each stage has been calculated considering as total mass the sum of masses recovered
in each part of the apparatus (t-piece, mouthpiece, throat and stages).

In terms of mass distribution across the stages of the NGI, the nebulisation of 4 mL of
500 μg/mL (calculated concentration of RFM added to the nebuliser filler cup (mean ±
SD): 878.23 μg/mL ± 116.17) resulted in the following values of mass recovered from t-
piece to stage 8: 37.3 μg, 8.0 μg, 14.9 μg, 36.2 μg, 57.4 μg, 43.1 μg, 11.1 μg, 2.7 μg, 0.9
μg and 0.125 μg (**Table** 6.3).

1196 *Table 6.3 Mass distribution across NGI stages recovered after the nebulisation of a*
1197 *volume of 4 mL of RFM suspension (500 µg/mL). Data represent mean ± SD of five*
1198 *independent experiments.*

NGI stage	Mass (µg) recovered
T-piece	37.3 ± 5.60
Induction port	8.0 ± 2.79
Stage 1	14.9 ± 3.07
Stage 2	36.2 ± 9.59
Stage 3	57.4 ± 14.22
Stage 4	43.1 ± 11.91
Stage 5	11.1 ± 2.91
Stage 6	2.7 ± 0.66
Stage 7	0.99 ± 0.44
Stage 8	0.125 ± 0.07

1199

1200 The aerodynamic parameters calculated from the NGI assessment were FPF, FPD,
1201 MMAD and GSD. Considering the FPF as the fraction or percentage of particle having
1202 an aerodynamic diameter less than 5 µm and therefore being suitable for lung deposition,
1203 the nebulisation of RFM suspension resulted in a FPF of 35% and 30.27% when the lower
1204 and higher drug concentrations suspensions were nebulised. In terms of mass of RFM
1205 that may deposit into the lung, a mass of 18.47 µg and 57.08 µg (FPD) was recovered for
1206 lower and higher drug suspension respectively. Besides this, the MMAD values
1207 calculated showed that 50% of the particles of the obtained aerosol have a size smaller
1208 than 6.42 µm and 6.59 µm for the 100 and 500 µg/mL drug suspension (Table 6.4).
1209 Although the suspension included particles with geometric size clearly greater than those
1210 that would pass through the mesh, there were more particles in the size range that would.
1211 The aerodynamic particle size may have differed from the geometric due to the non-
1212 spherical nature of the suspended particles and shear forces that operate during
1213 nebulisation. The evaluation of the geometric particle size of nebulised particles
1214 recovered from the NGI was not performed but it could inform the impact of the

1215 nebulisation on the physical particle size distribution, and it can represent the focus of
1216 future studies.

1217

1218 The GSD is a measurement of the spread of the size in the population. The values were
1219 1.95 and 1.76 for 100 µg/mL and 500 µg/mL (**Table** 6.4) showing a relatively tight
1220 aerodynamic particle size distribution after nebulisation.

1221

Table 6.4 Aerosol performance parameters of two different concentrations of RFM
suspensions in 0.9% saline/0.2% Tween 80 + 0.05% DMSO when nebulised with
Aerogen Pro. Data represents mean (SD) of five independent experiments for 100
µg/mL (4 mL); four independent experiments for 500 µg/mL (2 mL).

	100 µg/mL	500 µg/mL
FPF (%)	35.00 (2.7)	30.27 (3.0)
FPD (µg)	18.47 (3.36)	57.08 (6.43)
MMAD (µm)	6.42 (0.18)	6.59 (0.18)
GSD	1.95	1.76

1222 Total emitted dose used calculated as mass recovered from the mouthpiece, induction
1223 port and stages of the NGI (t-piece excluded as that mass could be considered as lost and
1224 it will not be available for lung deposition).

1225

1226 Nebulisation of 4 mL of 100 µg/mL resulted in the recovery of 65.05 µg of RFM while
1227 the nebulisation of 2 mL of 500 µg/mL resulted in the recovery throughout the impactor
1228 (from t-piece to stage 8) of ~ 212 µg of drug. When the RFM high dose suspension was
1229 nebulised, the mass of RFM deposited in stages 3 to 7 and therefore that can be considered
1230 as respirable was equal to 115 µg (**Table** 6.5).The latter information is key when
1231 estimating the total mass of drug that will deposit in the lungs of the specie treated. These
1232 results will be used for the calculation of the DD described in the next paragraph.

Table 6.5 Evaluation of calculated masses available over different stages of the NGI after nebulisation. Data represent mean and (SD) for n=5 independent experiments for both concentrations.

	100 µg/mL	500 µg/mL
Total volume nebulised (mL)	4	2
Total mass recovered from t-piece to S8	65.05 (14.63)	211.7 (38.79) *
Mass recovered from S3 to S7 (respirable fraction/dose)	33.21 (8.09)	115.15 (21.47)

1233   ***Mass deposited in the mouthpiece not recoverable.***

1234

1235   **6.4.2   Pulmonary administration of roflumilast to LPS-challenged guinea pigs**
1236   **using a whole-body chamber model**

1237

1238   **6.4.2.1   Calculation of *in vivo* delivered dose (DD)**

1239   The calculation of the dose of nebulised RFM to which the guinea pigs were exposed was

1240   calculated using the Equation 5.1 and Equation 5.2. This estimation was carried out using

1241   the data obtained from the aerodynamic assessment of the nebulised drug suspension. As

1242   schematically represented in **Figure** 6.8, the delivered dose (and more precisely the

1243   concentration of drug in the air, C) was calculated as the total mass of RFM recovered

1244   from stage 3 to stage 7 – and equal to 115.15 µg – divided by the total volume of the

1245   plexiglass chamber used as whole-body chamber (18.8 L).

1246
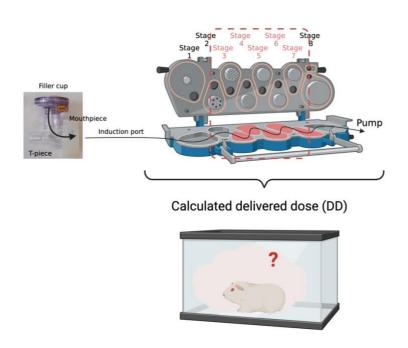
Figure 6.8 Estimation of the delivered dose (DD). Schematic representation of the calculation of the concentration of substance in the air (C).

The average animal weight was (0.316 kg ± 0.008), calculated from the weight of each animal on the day of the experiment. The duration of each nebulisation cycle was equal to 5.5 min. The calculation of the delivered dose when taking into consideration two cycles of nebulisation (two aliquots, 2 mL each) resulted in a total delivered dose of 48.6 µg/Kg, approximated to 50 µg/kg. A dataset used to calculate DD is reported in **Table** 6.6 and a summary of the derivation of aerosol parameter used to calculate delivered dose is described in **Table** 6.7.

Table 6.6 Dataset of values used to calculate the delivered dose (DD).

TABLE	
Respirable mass presented to each animal (mg)	0.115
Volume whole-body exposure chamber (L)	18.8
C (mg/L)	0.006125
RMW (L/min)	0.228
D (min)	5.5
Average body weight (kg)	0.316

1256

Table 6.7 Summary of details of the in vivo administration of RFM suspension using the nebulisation as route of administration.

Stock solution nebulised (µg/mL)	Time of nebulisation (min)	Total calculated delivered dose* (µg/kg)
500	**5.5/nebulisation cycle**	**50 µg/kg****

1257   *Amount per unit of body weight that is presented to the animal

1258   **Exact calculated value is 48.58 µg/kg

1259

1260

1261 **6.4.2.2 Pulmonary delivery of roflumilast in LPS-challenged guinea pigs**

1262 The anti-inflammatory activity of RFM suspension when delivered to the lungs via

1263 nebulisation was investigated. The exposure to nebulised vehicle followed by LPS,

1264 induced an increased level of total cells in the BALF which was mainly represented by

1265 an increased level of neutrophils. Indeed, the number of the other white blood cells could

1266 be considerable negligible. However, the condition of lung inflammation induced by LPS

1267 was not fully or significantly reduced by a single prior pulmonary administration of RFM

1268 at a dose of 50 µg/kg (**Figure** 6.9).

1269

Figure 6.9 Nebulisation of RFM suspension (saline + 0.2% Tween 80 + 0.05% DMSO) in LPS-challenged guinea pigs. Total and differential count of cells recovered from bronchoalveolar lavage fluid (BALF) of guinea pigs. Groups represent vehicle: animals treated with vehicle (saline/0.2% Tween 80 + 0.05% DMSO) and challenged with LPS; drug-treated: animals treated with two consecutive aliquots (2 mL each) of nebulised RFM suspension and challenged with LPS. Data represents mean ± SD of one independent experiment (3 animals/group).

Similar outcomes were achieved in test and control groups when evaluating the level of TNF-α in the BAL of guinea pigs. As observed for the number of white blood cell recovered in the BAL, the exposure of guinea pigs to an aerosol of RFM did not affect the level of TNF-α detected in the control sample (vehicle) **Figure** 6.10.
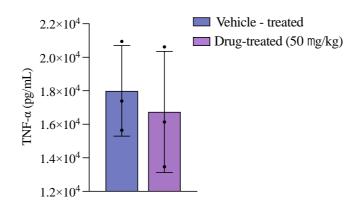
Figure 6.10 Levels of human tumour necrosis factor alfa (TNF-alfa) in bronchoalveolar lavage fluid (BALF) of RFM treated LPS-challenged guinea pigs. Animals were treated with nebulised vehicle (saline/0.2% Tween 80 + 0.05% DMSO) or drug (50 µg/kg). After 1 h animals have been exposed to LPS (0.2 mg/mL) and four hours after animals have been euthanized and lungs lavaged. Cytokine levels were detected using a commercial ELISA kit. Data represents mean ± SD of one independent experiment (3 animals/group).

1276

1277   **6.4.3   Impact of nebulisation on the HSA-NPs physiochemical characteristics**

1278

1279   The impact of nebulisation on the physiochemical properties of the blank and drug-loaded

1280   HSA-NPs were investigated. The nebulisation induced a minimal decrease in the size,

1281   PDI and zeta potential values for both blank and drug-loaded NPs. Derived count rate

1282   remained overall constant compared to pre-nebulisation measurement, with only a

1283   negligible increase of the derived count rate of the blank NPs (pre-nebulisation:

1284   130220.40 kcps; post-nebulisation: 151824.17 kcps) (**Table** 6.8).

1285

1286

Table 6.8 Size, polydispersity index (PDI) and zeta potential of blank and drug loaded nanoparticles (NPs) prior and after nebulisation with Aerogen Pro®. A 15 mL falcon tube was connected with tape to the inlet of a 15 mm ISO t-piece. A fixed volume of NPs (0.4 mL) was nebulised for 1 min and the condensation fluid in the falcon tube was recovered and diluted with double distilled water. Physiochemical properties were evaluated with Dynamic Light Scattering. Data represent mean ± SD of 1 – 4 independent experiments performed on one single batch of nanoparticles.

		PRE-NEBULISATION				POST-NEBULISATION			
		Size (nm)	PDI	Derived count rate (kcps)	Zeta potential (mV)	Size (nm)	PDI	Derived count rate (kcps)	Zeta potential (mV)
Blank NPs	Average	180.67	0.08	130220.40	-32.09	173.17	0.06	151824.17	-31.75
	SD	-	-	-	0.49	0.67	0.01	-	7.24
Drug-loaded NPs	Average	170.20	0.04	165822.90	-33.93	170.30	0.03	168834.63	-32.18
	SD	-	-	-	1.33	3.51	0.01	-	0.59

1287

6.5   Discussion

1288
1289

1290 The aim of this experimental chapter was to investigate the anti-inflammatory effect of
1291 inhaled RFM and the hypothesis that delivery of RFM formulated as inhaled albumin NPs
1292 can enhance this activity. The discussion of the results obtained will be divided in two
1293 sections. Initially the effect of the nebulisation on the HSA NPs was investigated. Indeed,
1294 the nebulisation was used as methodology to deliver RFM *in vivo* first and to evaluate *in*
1295 *vitro* the potential delivery of the blank and drug-loaded HSA NPs afterwards. The second
1296 part will discuss the absence of a significant reduction in the number of inflammatory
1297 cells recovered in the BAL after exposure to nebulised RFM in an LPS-challenged guinea
1298 pigs model.

1299

6.5.1   *In vitro* nebulisation of blank and RFM-HSA-NPs

1300
1301

1302 The nebulisation of blank and drug-loaded NPs described in paragraph 6.3.3 showed that
1303 the physiochemical properties investigated (size, PDI and zeta potential) remain
1304 unchanged after a cycle of nebulisation using a mesh nebuliser (Aerogen Pro®). As those
1305 features are key for the benefits provided by the NPs, it can be assumed that their

1306	properties are retained and that they would be delivered to the lungs as an intact
1307	nanocarrier platform.
1308	Only a small increase of the derived count rate of blank NPs after the nebulisation was
1309	detected, whereas the derived count rate of RFM-HSA-NPs remained unchanged. It is
1310	very likely that the ~ 15% increase in the count rate of blank NPs described depends on
1311	to the NPs being more concentrated after nebulisation due to a processing issue when
1312	collected from the tube. Therefore, those results showed that the nebulisation does not
1313	impact the physiochemical properties of the NPs, nor their concentration. However, in
1314	contrast with our results Aeroneb Pro® has been reported to fragment latex spheres into
1315	smaller particles in combination with uncontrolled aggregation after nebulisation (Najlah
1316	et al., 2014). As the count rate has been demonstrated to be proportional to particle
1317	concentration (Tantra et al., 2010), it can be hypothesized that the aggregation and
1318	fragmentation of nebulised HSA-NPs would result in a consistent reduction of the derived
1319	count rate. In fact, although the different experimental settings adopted and the NPs not
1320	being nebulised, this relationship was described in Chapter 2 (paragraph 2.4.3) where the
1321	occurred trypsin-mediated degradation induced aggregation and fragmentation of the
1322	NPs, which corresponded to a decrease of the derived count rate. Therefore, further
1323	confirming that the nebulisation did not impact the physiochemical properties of the NPs
1324	which did not undergo fragmentation or aggregation.
1325	
1326	Interestingly, a study investigating the impact of nebulisation on NPs characteristics was
1327	conducted by Dailey (Ann Dailey et al., 2003). NPs made of four different polymers were
1328	manufactured and characterised before nebulisation using a jet, ultrasonic or piezo-
1329	electric crystal nebulisers. Results showed that the technique of aerosol generation had
1330	an impact on the NPs aggregation and the latter was dependent on the hydrophobicity of
1331	the particle surface. The nebulisation of NPs with high surface hydrophobicity showed
1332	high level of aggregation, which was reduced when using ultrasonic nebuliser or NPs
1333	with a more hydrophilic surface.
1334	
1335	The suitability of a mesh nebuliser to deliver labile drugs such as proteins or mAbs
1336	without degradation has been demonstrated (Cunningham & Tanner, 2020; Forde et al.,
1337	2019; Respaud et al., 2014), hence further corroborating that the physiochemical
1338	properties of the HSA NPs are likely to remain unchanged and the NPs structure
1339	unmodified.

6.5.2   Effect of inhaled RFM on lung inflammation *in vivo*

To investigate the activity of RFM delivered to the lungs the intratracheal administration was originally chosen as optimal technique. As discussed in Chapter 5, the instillation directly above the carina represents a convenient approach which does not require specific laboratory devices and does provide straightforward results. Although it comes with some disadvantages, it allows complete and controlled delivery of the total intended volume above the carina, without the challenges correlated to the nebulisation process using a whole-body chamber model. Therefore, the intratracheal instillation represented the preliminary chosen approach to investigate whether RFM in the designed model could exert a therapeutic anti-inflammatory activity.

As discussed in Chapter 2, RFM is characterized by a poor water solubility ($0.52 - 0.56$ µg/mL at 22 °C) (European Medicines Agency, 2010) which represents a challenge for its *in vivo* administration. In contrast, RFM is highly soluble in DMSO (~ 20,000 µg/mL) (Cayman Chemical, n.d.). However, due to the well-known toxicity of DMSO in high concentrations and the side effects that causes *in vivo* (Rubin, 1975), the instillation of solutions of RFM in 100% DMSO was not considered a suitable option. It was also not adequate to prepare a SS in 100% DMSO and dilute it with an aqueous based solvent. Based on the average weight animal and the target required dose, the percentage of DMSO in the final suspension would have been too high for *in vivo* administration, perhaps causing side effects.

Therefore, the adopted formulation strategy which was described in this chapter was based on the use of a saline/0.2% Tween 80 with the addition of a small percentage of DMSO equal to 0.05% v/v. This value lies in the wide range of percentages of DMSO used in preclinical studies and reported in the literature (from 20% to 0.01 % v/v) (Fung et al., 2011; Ito et al., 2002). On the other side, the use of saline and 0.2% Tween 80 as vehicle for the intratracheal instillation of poorly water-soluble molecules was also reported in research studies which investigated the anti-inflammatory activity *in vivo* of two test compounds (GSK256066 and CHF6001) as new PDE-4 inhibitors (Nials et al., 2011; Tralau-Stewart et al., 2011; Villetti et al., 2015).

The two preliminary target doses were 30 and 60 µg/kg and the instillation volume equal to 200 µL. Those doses were obtained via dilution from one of the three SS freshly

1373   prepared (50, 100 and 200 µg/mL) based on the body weight of the specie on the day of

1374   the intratracheal instillation.

1375

1376   As the preclinical study of RFM required multiple days and the suspensions were

1377   prepared on different days, the consistency of the preparation method and of the obtained

1378   final RFM suspension was fundamental to minimize the inter-specie variability. The

1379   evaluation of the geometric particle size showed that the particle possessed similar

1380   characteristics to those described for the highest concentration (500 µg/mL) in paragraph

1381   6.4.1.1 (data not shown). As the intratracheal instillation provides the controlled exposure

1382   of the lungs (or sections of them) to the volume of interest, the geometric particle size of

1383   the RFM suspension had a lower impact overall compared to when administered via

1384   nebulisation.

1385

1386   Although intratracheal instillation was chosen as the original intended administration

1387   approach to achieve RFM lung exposure in guinea pigs, the newly laboratory availability

1388   of two different mesh nebulisers allowed to adopt a more complex and sensible lung

1389   exposure model based on the combined use of nebulisation and whole-body exposure

1390   chamber.

1391   The calculation of the target delivered dose was carried out applying the equation

1392   designed by the Association of Inhalation Toxicologists (Alexander et al., 2008)

1393   discussed in Chapter 5. In order to achieve the target dose, a more concentrated RFM

1394   suspension (500 µg/mL) was formulated and characterized. The geometric particle

1395   distribution analysis of RFM suspension revealed that 50% of the particles had a

1396   geometric size below 13.2 µm (D_{50}) and therefore bigger than the mesh aperture pore size

1397   (3 – 5.5 µm), whether 10% had a geometric size below 6 µm (D_{10}). Those results

1398   anticipated that the nebulisation would have resulted in the loss of the fraction of RFM

1399   with an inappropriate size to pass through the mesh; however, on the other hand those

1400   results showed that a fraction of the evaluated suspension had a geometric size compatible

1401   with the pore mesh aperture, providing sufficient evidence of its nebulisation via mesh

1402   nebuliser and its potential deposition in the lungs.

1403

1404   In fact, the aerodynamic assessment confirmed that the nebulisation of 2 mL of RFM (500

1405   µg/mL) resulted in the successful deposition of the drug in each stage of the impactor and

1406   this was validated via complete drug recovery and HPLC quantification. The respirable

1407 dose calculated as the total mass recovered after nebulisation from stages 3 to 7 of the
1408 NGI was equal to 115.5 µg. The *in vitro* aerodynamic assessment showed that RFM had
1409 the potential to deposit in the lung of the specie and that the fraction with a suitable
1410 aerodynamic size was sufficient to induce a therapeutic effect. Confirmation of the
1411 potential deposition of nebulised RFM in the lungs was provided also by an MMAD equal
1412 to ~ 6 µm.

1413

1414 In this experimental chapter the adopted delivered dose was equal to 50 µg/kg. The
1415 selection of this dose was performed based on two aspects. Limited results exist on the
1416 efficacy of inhaled roflumilast and few studies have been published in this field.
1417 However, an interesting research (Chapman et al., 2007) investigated the effect of RFM
1418 delivered to Brown Norway rats intratracheally or via nose-only inhalation in an
1419 ovalbumin model. Rats were exposed to intratracheal administration (20 - 60 - 200 µg/kg)
1420 5 h before or 24 h after the antigen challenge. Both treatment protocols resulted in the
1421 reduction of the total number of inflammatory cells in the BAL, with effects also on
1422 eosinophils and neutrophils number (except for the lowest dose – 20 µg/kg). The ED_{50} to
1423 inhibit the increase in total cells was 50 µg/kg. A partial reduction of the inflammatory
1424 cells in the BAL was also reported with a different dose regime (intratracheal
1425 administration 600 µg/kg, 24 h after the antigen challenge).
1426 Similar results were described for the nose-only delivery of RFM which inhibited the
1427 increase in total cells, neutrophils and eosinophils. The estimated pulmonary deposition
1428 ED_{50} was calculated from the particle size distribution and equal to 10 µg/kg. In terms of
1429 formulation administered, intratracheally administered RFM was formulated as
1430 micronized powder admixed with lactose into a drug powder microspray needle (Penn
1431 Century), whereas the nose-only exposure was based on a micronized roflumilast with
1432 lactose.
1433 Doses in the same range were also tested in the investigation of the anti-inflammatory
1434 activity of a test compound developed as PDE-4 inhibitor (GSK256066). GSK256066
1435 was administered intratracheally to LPS-challenged rats as aqueous suspension (instilled
1436 volume: 200 µL) at doses 0.1 – 100 µg/kg. The test compound induced a dose-dependent
1437 inhibition of the LPS-induced neutrophilia with statistical significance detected at 3 µg/kg
1438 and maximal (72%) inhibition of lung neutrophilia at 30 µg/kg. As GSK256066 is a more
1439 potent drug than RFM, the described effective doses were lower compared to those of
1440 RFM (Nials et al., 2011; Tralau-Stewart et al., 2011).

1441 Although being in agreement with doses reported in the literature, the target dose
1442 employed in this experimental chapter (50 µg/kg) did not significantly reduce the lung
1443 influx of neutrophils induced by the validated LPS exposure, unlike when delivered orally
1444 (0.5 mg/mL).

1445

1446 Despite the lack of inhalable HSA-NPs' investigations, the benefits that the pulmonary
1447 delivery of a drug using nanoplatform provides over the oral and intravenous
1448 administration has been extensively demonstrated in literature studies for other type of
1449 nanocarriers. Therefore, the aim of this study was to further investigate if the inhaled
1450 delivery of RFM provides benefits over its oral administration, but most importantly if
1451 lower or comparable doses of inhaled RFM-HSA-NPs can exert an anti-inflammatory
1452 effect equivalent to the effect induced by inhalation of RFM. It is clear how this
1453 comparison would have been sensible only with RFM-HSA-NPs doses in the same range
1454 of those used for the administration of inhaled RFM.

1455

1456 Therefore, the second key factor considered in the evaluation of the target dose of inhaled
1457 RFM was correlated to the dose of RFM achievable when delivered as RFM-HSA-NPs
1458 in a whole-body chamber. As mentioned in Chapter 5, the calculation of the estimated
1459 delivered dose can be achieved via the application of an equation that considers several
1460 factors. Firstly, to calculate the concentration of RFM in the air (C), yield and mass of
1461 RFM encapsulated per mg of HSA should be considered. The yield described for RFM-
1462 HSA-NPs is equal to 73% which corresponds to ~ 9 mg/mL of total entrapped albumin
1463 (section 2.4.2.1). The amount of RFM encapsulated for each mg of albumin is equal to
1464 19.9 µg (section 2.4.2.2). Assuming the use of the same plexiglass box (18.8 L), the
1465 nebulisation of 2 mL of RFM-HSA-NPs would produce a RFM concentration in the air
1466 (box) of 0.02 mg/mL. Considering the average body weight equal to 0.316 kg, the RMW
1467 would be equal to 0.228 L/min. Based on the average flow rate of the Aerogen Pro (0.4
1468 mL/min) the duration of the nebulisation of 2 mL of NPs suspension would last 5.5 min.
1469 The resulting delivered dose would be equal to ~ 80 µg/mL which eventually could be
1470 doubled if two aliquots of 2 mL were nebulised. However, the Equation 5.1 also includes
1471 a factor called inhalable fraction (IF) whereas incorporation of this parameter is not
1472 essential provided that the aerosol has reasonable respirability for the intended specie. In
1473 fact, this value was not included in the calculation of the DD of inhaled RFM as the
1474 concentration in the air already considered only the respirable fraction (obtained via

1475   aerodynamic particle distribution – stages 3 to 7). However, as the aerodynamic
1476   distribution of RFM-HSA-NPs was not performed in this experiment study, it is likely
1477   that the achievable target dose would be lower than 80 µg/mL (2 mL) or 160 µg/mL (4
1478   mL). This estimation further rationalise the target dose adopted in the evaluation of the
1479   anti-inflammatory activity of RFM (50 µg/kg) in this chapter as it allows the future
1480   assessment of the potential improved therapeutic activity of RFM in a nanocarrier using
1481   as positive control the administration of inhaled RFM and comparable doses.

1482

1483   Used in both *in vitro* and *in vivo* experiments, roflumilast showed to reduce the LPS-
1484   induced IL-8 increase in BEAS-2B (dose: 0.001 – 100,000 nM - Chapter 4) and
1485   neutrophilia in BAL of orally administered guinea pigs (dose: 0.5 mg/kg - Chapter 5)
1486   hence suggesting that it may be able to exert an anti-inflammatory activity when delivered
1487   via inhalation in a preclinical model. Inhalation of RFM in 100% DMSO at dose of 500
1488   µg/mL for 15 min once day for five days was described to reduce the ovalbumin-induced
1489   lung inflammation in mice (Murad et al., 2017). However, in this experimental chapter
1490   the limitations correlated to the application of the nose-breathing preclinical models
1491   besides others may have restricted the deposited dose. Nasal filtration in rodents
1492   influences the total amount of particles available for lung deposition and longer exposure
1493   period may be required to increase the delivered dose.

1494

1495   The target dose investigated in this experimental chapter was in agreement with some of
1496   the doses used in previous studies which successfully showed RFM anti-inflammatory
1497   activity. It is likely that the lack of significant reduction of the LPS-induced neutrophilia
1498   described in this Chapter was the result of an insufficient dose of RFM that is also
1499   characterized by very low solubility in the lung lining fluid. This may have hindered its
1500   dissolution and binding to the PDE4 intracellular enzyme. In this context, the
1501   encapsulation of RFM in NPs will induce a much faster dissolution of the drug due to
1502   their higher surface area-to-volume ratio, thus overcoming the low solubility issue (Bohr
1503   et al., 2014) and increasing the drug availability. Future studies investigating the *in vivo*
1504   administration of inhaled RFM-HSA-NPs may confirm this hypothesis.

1505

1506   Although the potential of RFM in reducing the neutrophilia was reported by Chapman
1507   (Chapman et al., 2007) at doses of 60 – 200 and 600 µg/kg, it is also relevant to mention
1508   that the *in vivo* models adopted within studies were different (antigen vs LPS challenge)

1509 and the raw powder used was micronized. In addition, in this study milling of the RFM
1510 raw powder could represent an approach to improve the drug availability via optimisation
1511 of the geometric size of the particles, thus also improving i) their dissolution in the lung
1512 lining fluid; ii) the aerodynamic assessment of the RFM suspension via improvement of
1513 the percentage of particles liable to go through the mesh pores.
1514 Future studies will further investigate the dose-response of inhaled RFM using a
1515 combined approach that improves the geometric and aerodynamic particle size of the
1516 suspension besides the application of a bigger population of species in the same LPS
1517 model.
1518

6.6 Conclusion

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1521 This experimental chapter aimed to the investigation of the potential anti-inflammatory
1522 activity of nebulised RFM as a precursor to optimising this therapeutic effect by the
1523 encapsulation of RFM in HSA-NPs. A RFM suspension for nebulisation was formulated
1524 that when nebulised using a mesh nebuliser resulted in its deposition across all the stages
1525 of a cascade impactor, depositing a respirable mass of 115 µg. The RFM suspension
1526 resulted was employed for the evaluation of the anti-inflammatory activity when
1527 nebulised to the LPS-challenged guinea pigs model of inflammation described in the
1528 previous chapter.
1529 Nebulised RFM did not induce a significant reduction of the number of inflammatory
1530 cells infiltrated into the lungs at the dose (delivered dose: 50 µg/kg) and protocol adopted.
1531 It is unclear whether the lack of a substantial therapeutic activity has a biochemical or
1532 pharmacological basis or is due to insufficient delivered dose. Although the lack of
1533 significant anti-inflammatory activity at the dose and protocol adopted, overall the
1534 outlined approach proved to be useful for the investigation of the potential lung delivery
1535 of RFM and in future studies of RFM-HSA-NPs. Furthermore, the potential of HSA-NPs
1536 as carrier for pulmonary delivery was corroborated by the lack of changes in their
1537 physiochemical properties that was reported for the blank and RFM-HSA-NPs after
1538 nebulisation. Overall those results highlighted the importance of the demonstration of the
1539 potential of inhaled RFM first before an optimisation using a formulation approach such
1540 as RFM-HSA-NPs can be undertaken.
1541 The data provided in this chapter provided initial evidence that demonstrating the
1542 effectiveness of RFM via the inhaled route is essential before extending studies to the

1543   benefits that HSA-NPs may provide compared to the delivery of this drug in a non-carrier

1544   system.

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7.1   Original premise

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1579   Pulmonary delivery of therapeutics has gained increasing interest over the past decades

1580   due to the advantages that it provides over other routes of administration. The lungs

1581   possess a large surface area which represents an excellent target for local therapeutic

1582   activity and absorption for systemic delivery (Patton & Byron, 2007). Delivery to the

1583   lungs avoids first pass metabolism which can result in the reduction of the total dose

1584   available. Moreover, lung delivery of therapeutics allows to use a reduced dose compared

1585   to other routes of administration, resulting in lower side effects and higher efficacy

1586   (Gulati et al., 2021; Praphawatvet et al., 2020). However, often the delivery to the lungs

1587   comes with challenges if the drug has a low solubility or if it is liable to degradation or

1588   losses by the multiple lung clearance mechanisms.

1589   The encapsulation of the API in a nanocarrier via an engineered formulation and modified

1590   release represents an extremely useful delivery strategy that allows to overcome the

1591   challenges associated with the delivery or pharmacokinetic of a drug (Abdelaziz et al.,

1592   2018; Anderson et al., 2020). The encapsulation of a drug in a NPs-based formulation

1593   allows for example i) to modify the release of the drug overtime; ii) to obtain the precise

1594   targeting of cells or organs of interest by functionalised nanocarriers; iii) to protect the

1595   payload from degradation.

1596

1597   The commercialisation of paclitaxel HSA-NPs for intravenous administration (Abraxane)

1598   for the treatment of various type of cancers in 2005 has made the potential of this carrier

1599   for clinical use clear (Hornok, 2021; Spada et al., 2021). Despite the successful and

1600   extensive investigation of albumin NPs for intravenous administration for cancer therapy

1601   (Hassanin & Elzoghby, 2020; Kunde & Wairkar, 2022), the use of albumin NPs for

1602   pulmonary delivery has not been completely or extensively explored yet. It was

1603   hypothesised that NP formulation might be a strategy to improve the therapeutic profile

1604   of the PDE inhibitors (Crocetti et al., 2022), the need for which was described in

1605   paragraph 1.2.4. The pulmonary delivery of PDE inhibitors to treat COPD associated

1606   exacerbations represents an unmet goal.

1607   A strategy was developed in this thesis to first encapsulate RFM in HSA-NPs, then

1608   explore their delivery via the pulmonary route. This may allow to overcome both the

1609 unfavourable therapeutic profile of this class of drugs while RFM being a drug prototype

1610 that will allow the investigation of the potential use of this carrier as a PK-modifying

1611 formulation for inhalation.

1612

7.2 Drug-loaded albumin NPs for lung delivery

1614

1615 The aims of this thesis were achieved by optimising an RFM-HSA-NPs manufacture

1616 method first and by characterising the obtained NPs via the design of *in vitro* models to

1617 investigate the nanocarrier degradation in a simulated lung environment as well as the

1618 release of the payload overtime. RFM-HSA-NPs were converted into a respirable form

1619 based on NPs-embedded-microparticles (NEMs) as key 'feasibility' step to facilitate their

1620 translation to clinical use. Moreover, before RFM-HSA-NPs can be established as

1621 clinically acceptable enabling formulation for pulmonary drug delivery purposes, the

1622 potential anti-inflammatory activity of RFM when delivered to the lungs was investigated

1623 via the design of an LPS-challenged BEAS-2B *in vitro* model first and an LPS-challenged

1624 guinea pig *in vivo* model later. The key findings are outlined below in relation to the

1625 experimental chapter within this thesis.

1626

1627 In **Chapter 2**, the main research question centred around the potential of the successful

1628 manufacture of RFM-HSA-NPs as good nanocarrier being able to be RFM-loaded,

1629 undergo degradation and efficiently release the payload. The data clearly demonstrated

1630 that the manufacture method parameters adopted resulted in the production of NPs with

1631 a consistent size of ~140 nm, PDI < 0.1 and negative zeta potential which prevents

1632 aggregation. As the proteases play an important role in the pathogenesis of lung disease

1633 as well as in the function of healthy lungs (Abboud & Vimalanathan, 2008; Fischer et al.,

1634 2011; Greene & McElvaney, 2009; Pandey et al., 2017; Stockley, 1999), the use of trypsin

1635 at a total lung concentration of 0.1 mg/mL (Woods et al., 2020) in an *in vitro* model

1636 represented an informative approach to predict and investigate the HSA-NPs degradation

1637 in the lungs. The degradation of the HSA-NPs occurred gradually in the first interval of

1638 time and after 1 hour the NPs were almost completely degraded and fragmented. These

1639 results are in agreement with others previously reported in the literature (Woods et al.,

1640 2020). Interestingly, we employed as parameters to monitor the NPs degradation the PDI

1641 and the derived count rate, with the latter providing a more sensitive measure of initial

1642 NP degradation before the detection of albumin fragments and aggregates becomes

1643   apparent via the alteration of the PDI value. Furthermore, the novelty of this model is

1644   represented by the general lack in the literature of accepted simulant models to represent

1645   the protease activity in the lungs.

1646

1647   The results highlighted in Chapter 2 also confirmed the potential of the HSA-NPs

1648   manufactured via the method adopted to successfully release the payload without which

1649   no therapeutic action would occur. Considering that RFM is a very poorly water-soluble

1650   drug (European Medicines Agency, 2010), the investigation of the rate of release of the

1651   payload from the NPs while still employing a biorelevant model represented an

1652   experimental challenge. Results showed the importance of the optimisation of the *in vitro*

1653   drug release model in terms of release medium adopted, concentration and pH of trypsin

1654   solution as well as ratio of trypsin: substrate. The superiority of HSA-NPs over not-

1655   encapsulated RFM to achieve a sustained release overtime was clearly demonstrated. The

1656   release kinetics in the window time investigated (0 to 4 h) was in agreement with the

1657   degradation rate of the nanocarrier, with most of the drug being released by 60 min

1658   followed by a slower release until 4 h. Although there are differences in the model and

1659   protocol adopted, these results do not concord with the report by Langer (Langer et al.,

1660   2008) that HSA-NPs with a 100% degree of cross-linking (as those designed in this thesis)

1661   showed only a 9% degradation within 24 h.

1662   Overall, the research objectives of **Chapter 2** were achieved, and enabled further work

1663   in **Chapter 3** to pursue the manufacture and characterisation of RFM-HSA-NPs

1664   embedded in microparticles as a key feasibility assessment to facilitate their translation

1665   to clinical use by demonstrating a marketable pharmaceutical formulation.

1666

1667   The key objective in **Chapter 3** was to investigate whether the designed RFM-HSA-NPs

1668   could be converted successfully in a formulation that could be manufactured as a stable

1669   dosage form suitable for clinical use. The aerodynamic diameter highly influences the

1670   deposition in the lungs of inhaled material. The manufacture of NPs embedded in

1671   microparticles (Tsapis et al., 2002) is a widely known formulation strategy to improve

1672   the respirability of NPs which was achieved successfully in this experimental work. The

1673   obtained spray-dried powder possessed good aerodynamic characteristics for pulmonary

1674   delivery with a FPF equal to 67% and a geometric particles size (D_{50}) equal to 2.21 µm.

1675

After deposition into the lungs, the NEMs must disintegrate to release the embedded NPs. Results showed that mannitol-based nanoaggregates were dissolved rapidly to release HSA-NPs although further studies employing a more biorelevant model should be carried out to more precisely investigate the rate of degradation (Ruge et al., 2016).

In the context of investigating formulation strategies to achieve pulmonary delivery of RFM-HSA-NPs, **Chapter 6** also focussed on the impact of the nebulisation on the physiochemical properties of blank and drug-loaded NPs. Indeed, pulmonary delivery can be obtained with different inhalation devices in which the APIs can be loaded in different type of formulations. Besides the investigation of NEMs as spray-dried powder, the nebulisation of RFM-HSA-NPs with a mesh nebuliser (Aerogen Pro) was carried out. Interestingly, in contrast with an expected reduced derived count rate due to the loss of NPs during the nebulisation, results described an unchanged formulation. Size, PDI and zeta potential remained overall constant, indicating the preservation of the NPs' key properties after aerosolization by mesh nebuliser.

Overall, the ultimate objective of this thesis was to investigate the anti-inflammatory activity of RFM delivered by the pulmonary route as a precursor to optimising this therapeutic effect by formulating as RFM-HSA-NPs. This was achieved firstly in **Chapter 4** by investigating whether RFM can exert an anti-inflammatory activity in an LPS-challenged bronchial epithelial (BEAS-2B) *in vitro* model. The two following chapters focussed i) on the design of an LPS-challenged guinea pig model employed to confirm the suitability of the model itself via the anti-inflammatory activity of orally administered RFM (**Chapter 5**); ii) on the *in vivo* evaluation of the effect of nebulised RFM afterwards (**Chapter 6**). The continuity throughout this part of the research was provided by the use of the same trigger agent (LPS O111:B4 from *E. Coli*) for inducing inflammation. LPS was selected as a clinically relevant inducer of inflammation in COPD exacerbation. Endpoints and outcomes used to measure the extent of the lung inflammation were also designed to provide consistency and add to value of those chapters.

In **Chapter 4**, the objectives were accomplished, with the caveat that RFM significantly reduced only the levels of IL-8 released by LPS-challenged BEAS-2B in a pre-treatment protocol (1.5 h), whereas the levels of IL-6 were not impacted by any of the protocols

1710 adopted (pre-treatment: 1.5 h, 3 h and 24 h). The sensitivity of BEAS-2B to serum and

1711 LPS (at different concentrations and exposure times) in releasing cytokines has been

1712 identified in other studies (Guillott et al., 2004; Schulz et al., 2002). Our findings

1713 regarding the weak reduction of IL-8 levels achieved by RFM have also been seen with

1714 some studies (M. R. Edwards et al., 2016; Salvator et al., 2020). In contrast, IL-6 level

1715 was reported to be reduced by RFM treatment in bronchial explants (Salvator et al., 2020),

1716 but not in alveolar macrophages (Lea et al., 2019).

1717

1718 Results described in **Chapter 5** showed that LPS challenged guinea pigs represented a

1719 valuable *in vivo* model to mimic COPD exacerbations. Among the *in vivo* COPD models

1720 applied by others, the whole-body exposure model is a useful approach to achieve lung

1721 exposure in the specie. Moreover, the applicability of the model in the field of RFM lung

1722 delivery research was confirmed by the reduced neutrophilia induced by a pre-treatment

1723 of orally administered RFM (0.5 mg/kg).

1724

1725 Finally, the data presented in **Chapter 6** found that 50 µg/kg RFM delivered to the lungs

1726 in an LPS model does not induce a significant anti-inflammatory effect. Although it is

1727 difficult to accurately verify the delivered dose, the aerosol of RFM produced by

1728 nebulisation possessed good aerodynamic characteristics and had the properties required

1729 to deposit into the lungs. This was demonstrated by the aerodynamic assessment of RFM

1730 and generation of a respirable dose equal to 115 µg. Considering the data presented in

1731 this Chapter and the lack of success over the past decade in the investigation of new

1732 inhaled PDE inhibitors reported in the literature, it can be argued that perhaps PDE4 can

1733 provide an anti-inflammatory activity only when delivered systemically. On the other

1734 hand, it can be argued that the unchanged levels of IL-6 in BEAS-2B *in vitro* model after

1735 RFM pre-treatment anticipated the lack of anti-inflammatory activity of nebulised RFM

1736 *in vivo.* On the other hand, it should also be considered that the poor water-solubility of

1737 RFM may have hindered its dissolution in the lung lining fluid with a consequent lack of

1738 PDE4 inhibition. Although different limitations and ethical considerations hindered the

1739 *in vivo* testing of the RFM-HSA-NPs and the evaluation of its potential in improving the

1740 RFM solubility, it can be argued that a slow dissolving suspension would work as well as

1741 a nanocarrier that enhances the drug solubilisation. Clearly further investigation is

1742 required to confirm this hypothesis, and it is interesting how the sustained release

1743 enhanced by the encapsulation of RFM in HSA-NPs described in Chapter 2 was well

1744 resembled by the release of RFM suspended in Tween 80 and DMSO.

1745

7.3   Contribution to knowledge

1747

1748 In the existing literature there is a lack of studies concerning the potential, degradation,

1749 and *in vivo* testing of HSA-NPs for pulmonary delivery of anti-inflammatory drugs.

1750 Despite the extensive research of HSA-NPs for intravenous administration over the years

1751 driven by the approval of Abraxane, the inhalation route has a wide range of challenges

1752 in terms of formulation, administration and methods for pre-clinical formulation

1753 development.

1754

1755 Whilst the manufacture method of RFM-HSA-NPs is widely known and used in the

1756 literature as well as the drug release assay employed to test the kinetic release of RFM

1757 (dialysis bag), the protocol required adaptations to overcome the challenges of RFM itself

1758 and provides a contribution to the field that may be informative for other studies

1759 employing drugs with similar solubility profile. Moreover, the use of trypsin in the *in*

1760 *vitro* degradation assay which utilised PDI and derived count rate as parameters is one of

1761 the few presented in the literature investigating the degradation of the HSA-NPs. The

1762 simultaneous quantification of the drug release which may be performed in future studies

1763 would represent a more comprehensive approach to characterising formulation

1764 breakdown and drug release than is currently performed according to the literature.

1765

1766 This work also represents the first attempt at improving the therapeutic window of a PDE4

1767 inhibitor via the use of HSA-NPs as drug delivery system generally, but more specifically

1768 for pulmonary delivery. Despite some experimental limitations and the need for key

1769 additional studies, the thesis makes several contributions to identifying the challenges and

1770 providing tools that can be used to evaluate the potential of RFM for pulmonary delivery

1771 and the contribution of HSA-NPs as a formulation platform.

1772

7.4   Experimental limitations

1774

1775 In **Chapter 4** and in **Chapter 6**, the main limitation was correlated to the unexpected lack

1776 of RFM activity in the bioassay which hindered the establishment of a baseline effect.

1777   This subsequently prevented the investigation of any possible enhancement that the NP

1778   formulation may produce compared to not-encapsulated RFM.

1779   Based on the nanocarrier's EE achieved in **Chapter 2**, the estimated delivered dose of

1780   RFM as inhaled RFM-HSA-NPs could be adequate to induce an anti-inflammatory effect

1781   *in vivo* in the LPS-challenged guinea pig model designed. However, the lack of

1782   experimental results in this context did not allow to confirm this hypothesis, hindering

1783   any further optimisation of the formulation (i.e., albumin – drug ratio) which may have

1784   benefited the achievement of a higher target dose.

1785   Moreover, in Chapter 2 the discussion of the results of the RFM-HSA-NPs kinetic of

1786   release may have benefited from the evaluation of the exact distribution of the drug in the

1787   NPs, if mainly in the matrix or whether on the surface.

1788   The main issue in the delivery of the spray dried microparticles which development was

1789   described in **Chapter 3** may be the excipient burden which could limit the maximum

1790   deliverable dose and subsequently the viability of the delivery concept.

1791   The designed whole-body LPS model (**Chapter 5** and **6**) was informative for the

1792   preliminary objectives of this thesis. However, the use of a more advanced nose-only

1793   aerosol exposure system would have overall benefited the study. Its use would have

1794   ensured i) exposure mostly to the respiratory tract; ii) use of less material. The possibility

1795   to acquire the breathing data of the species would have improved the accuracy of the

1796   estimation of the inhalation doses. In addition, the possibility to have an unbiased sample

1797   from the species breathing zone would have been key to determine the sampling

1798   efficiency of the subject. Besides the typical nose-only exposure system that can also

1799   allocate multiple species at the same time due to multiple exposure tubes (Phalen et al.,

1800   2014), the PreciseInhale® also allows to achieve precise lung deposition of the powder of

1801   interest to a single animal at a time. The use of the PreciseInhale® combined with the

1802   nose-only exposure model would allow reducing the variability of exposure between

1803   species; in addition, the active monitoring of aerosol concentration and individual

1804   breathing parameters would further ensure high precision of dosing.

1805   The main limitation of **Chapter 6** was correlated to the lack of a dose-response study for

1806   RFM suspension and for the RFM-HSA-NPs. Although preliminary results in this work

1807   showed that lung inflammation in species exposed to LPS is not reduced by nebulised 50

1808   µg/kg RFM, a follow up experiment with two additional higher doses (100 and 200

1809   µg/kg) would have been significant in drafting a more accurate conclusion. In the first

1810   case, the main challenge was the almost complete insolubility of RFM in aqueous medium

1811   which hindered the achievement of the desired dose in a biocompatible vehicle. Indeed,

1812   the lack of time also did not allow the investigation of alternative formulation strategies

1813   such as the use of a micronized powder coupled with a different inhalation device. Finally,

1814   resources and ethical constraints limited the use of larger testing groups *in vivo* which

1815   would have improved the understanding of the final results.

1816

1817   7.5   Future work

1818   Extensive further experiments should be performed to elucidate the unclear lack of anti-

1819   inflammatory activity reported in this thesis for nebulised RFM. We have hypothesised

1820   that the lack of pharmacological activity in the *in vivo* lung inflammation model could be

1821   due to the unsuitability of the formulation used (RFM suspension in saline/ Tween 80/

1822   DMSO) which may have resulted in crystallised RFM not available for absorption and

1823   therefore in undissolved particulate matter due to the poor water solubility of RFM (R.

1824   M. Jones & Neef, 2012). An additional hypothesis is that Tween 80 present above the

1825   CMC in the vehicle used for *in vivo* lung delivery of RFM may have entrapped RFM

1826   reducing its cells uptake in the lungs. Similarly, the accumulation of paclitaxel in human

1827   erythrocytes was severely altered by paclitaxel being trapped in Cremophor micelles

1828   when used above the CMC (van Zuylen et al., 2001). Future *in vitro* and *in vivo*

1829   investigation of the cellular distribution of RFM in inflammatory cells expressing PDE4

1830   and lung tissue would elucidate whether the entrapping of RFM in Tween 80 micelles

1831   hindered RFM from binding the PDE4 enzyme and express anti-inflammatory activity.

1832   On the other hand, the use of a different formulation as micronised RFM powder rather

1833   than RFM in suspension may also help to elucidate whether the lack of anti-inflammatory

1834   activity in the model adopted was due to a formulation or pharmacological reason.

1835   An additional hypothesis is that RFM once deposited into the lungs does not undergo

1836   sufficient metabolization by CYP 3A4 and 1A2 which cell-specific localisation in the

1837   lung is still largely unknown (Hukkanen et al., 2001). The expression of the cytochrome

1838   3A4 and 1A2 in the lungs should therefore be investigated via Western blot or reverse

1839   transcription polymerase chain reaction. However, despite the N-oxide metabolite also

1840   accounts for the total therapeutic activity, the parent molecule, RFM, can exert a

1841   pharmacological effect even if not metabolised. Therefore, the above-mentioned

1842   hypothesis does not fully explain the lack of anti-inflammatory activity reported in this

1843   thesis.

1844 Despite the target dose adopted (50 µg/kg) was in agreement with other studies in which
1845 *in vivo* anti-inflammatory activity of pulmonary delivered RFM was described (Chapman
1846 et al., 2007), further experiments should investigate the effect of a higher target dose
1847 (single administration) or multiple administrations in the same LPS model designed in
1848 Chapter 5.

1849 Meanwhile, besides the investigation of the reasons for the lack of the effect of RFM
1850 itself, it should be highlighted that in this thesis RFM served also as a prototype.
1851 Therefore, the investigation of the *in vivo* biodistribution of inhaled blank HSA-NPs may
1852 further corroborate the proof of concept for the formulation and its potential for clinical
1853 use. The use of the LPS guinea pig model designed in this thesis could be combined to
1854 imagining technique. In addition, the encapsulation of a different drug – perhaps with a
1855 less complex solubility profile – into the HSA-NPs would expedite the understanding of
1856 the potential of pulmonary delivered HSA-NPs, allowing the *in vivo* testing of the NPs.

1857 Finally, the investigation of the exact distribution of RFM in the HSA-NPs would enhance
1858 the understanding of the potential of this carrier. This could be conducted with the
1859 formulation of fluorescent RFM- HSA-NPs.

1860 Moreover, in view of the optimisation of the respirability of the HSA-NPs, full
1861 aerodynamic assessment of the NEMs should be carried out besides the evaluation of
1862 their disintegration in a more biorelevant assay. It would also be key to evaluate the
1863 simultaneous release kinetic of the drug from the drug delivery system adopting the same
1864 assay.

1865 ## 7.6   Conclusion
1866
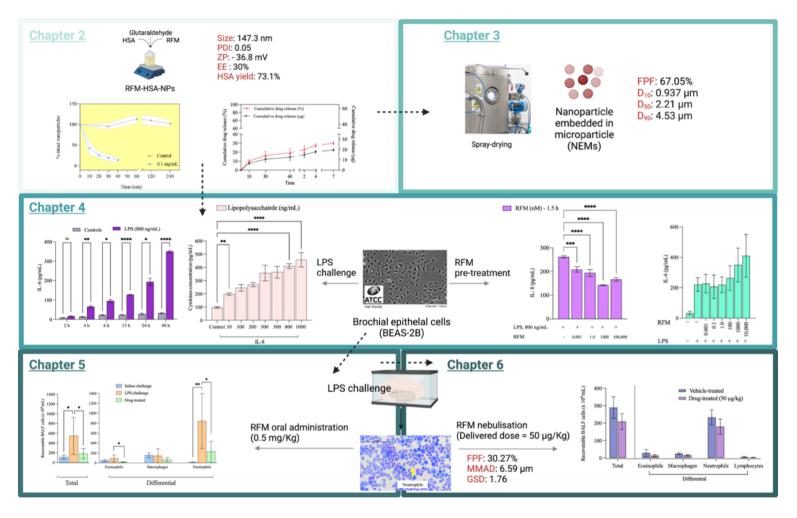
1867 The data presented in this work demonstrate how the use of HSA-NPs for pulmonary
1868 administration of anti-inflammatory drugs should be further pursued. This drug delivery
1869 system has been showed to encapsulate a hydrophobic drug and to undergo degradation
1870 in a lung simulated environment. The encapsulation of RFM in HSA-NPs resulted in a
1871 modified kinetic of release of the drug, providing a mechanism that would prolong drug
1872 retention in the lungs.
1873 The RFM-HSA-NPs were successfully processed into NP embedded microparticles
1874 (NEMs) providing a respirable powder dosage form, which combined with the use of
1875 suitable inhalation manoeuvres, has good potential as a product for clinical use.

1876   Additionally, this thesis established an *in vitro* respiratory epithelial model and an *in vivo*

1877   LPS model of inflammation, in which the effects of anti-inflammatory positive controls

1878   (dexamethasone and orally delivered RFM, respectively) were clearly seen.

1879   Unexpectedly, the effect of RFM administered to the cell culture model and delivered as

1880   an aerosol to the *in vivo* model, resulted in little or no anti-inflammatory activity. This

1881   was a fundamental step upon which plans to proceeding into the optimisation of the

1882   formulation strategy foundered. Although results showed that RFM may not be able to

1883   exert an anti-inflammatory activity when delivered locally to the lungs, further studies

1884   are required to elucidate the mechanisms behind this lack of therapeutic activity. Overall,

1885   the work presented in this thesis, provides a solid foundation for future research in the

1886   field of pulmonary delivery and in the application of HSA-NPs as PK-modifying inhaled

1887   drug delivery system.

1

2 *Figure 7.1General abstract showing main outcomes of each experimental chapter*

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Supplementary data

1340 **Supplementary data**

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1342 **Figure 0.1** *Schematic representation of the method (method 1) adopted for the direct*
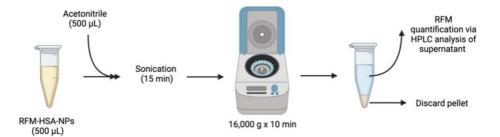1343 *quantification of the roflumilast encapsulated in HSA-NPs.*

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1345 **Figure 0.2** *Schematic representation of the method (method 2) adopted for the direct*
1346 *quantification of the roflumilast encapsulated in HSA-NPs.*

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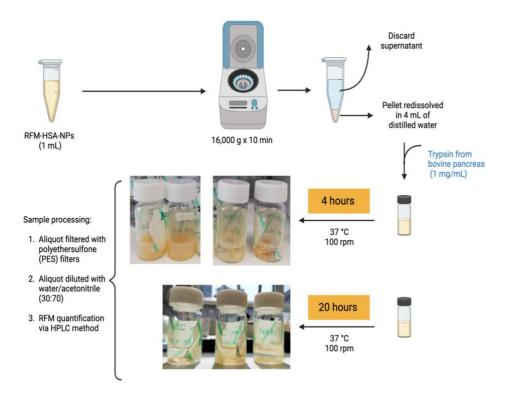1351   **Figure 0.3** *Representative HPLC chromatograms of RFM in water:acetonitrile (30:70)*

1352   *(samples obtained via method 1 and method 2).*

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