

Université catholique de Louvain
Louvain Drug Research Institute
Advanced Drug Delivery and Biomaterials

**DEVELOPMENT OF A LONG-ACTING VERSION OF
RECOMBINANT HUMAN DEOXYRIBONUCLEASE I FOR THE
TREATMENT OF CYSTIC FIBROSIS LUNG DISEASE**

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Thesis presented in fulfilment of the requirements for the degree of
Doctor of Philosophy in Biomedical and Pharmaceutical Sciences

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2018

It always seems impossible until it is done.

- Nelson Mandela –

REMERCIEMENTS

Je voudrais remercier ma promotrice de thèse, Madame la Professeure Rita Vanbever, de m'avoir donné l'opportunité de réaliser ma thèse de doctorat au sein de son groupe de recherche. Merci pour son soutien scientifique et la confiance qu'elle m'a accordée.

Je souhaiterais également remercier ma co-promotrice de thèse, Madame la Professeure Teresinha Leal, pour ses précieux conseils et nos échanges scientifiques sur mon projet.

Je serai éternellement reconnaissante envers Dr. Falguni Gadkari, mon maitre de stage aux Etats-Unis pour m'avoir initiée au monde de la Recherche pharmaceutique et donné l'envie de poursuivre mes études dans cette voie. Merci pour les connaissances scientifiques que tu m'as transmises, ton soutien inconditionnel et ton amitié qui perdure depuis déjà 6 ans.

J'aimerais également remercier Madame la Professeure Véronique Prémat et mes collègues du groupe Advanced Drug Delivery and Biomaterials (ADDB) pour leur accueil, leur aide et l'environnement de travail amical. Je suis particulièrement reconnaissante envers : mon ami Bernard pour son soutien inconditionnel, son aide précieuse et les cours de Spinning que l'on a partagés ; mes anciens collègues Harshad, Danielle, Maria et Cristina pour leur aide et amitié ; mes amies Laure et Spela pour tous les bons moments que nous avons partagés au sein du laboratoire et en dehors ; et John pour son soutien et sa bienveillance. J'aimerais aussi remercier Murielle pour la personne qu'elle est, pour les soirées passées toutes les deux au sein du laboratoire et son aide précieuse tout au long de ma thèse. Un grand merci à Kevin et Nathalie pour leur gentillesse et leur aide. Je souhaiterais remercier Anne, Ana et Gaëlle pour leurs encouragements et nos discussions constructives. Un merci tout particulier à Chiara d'avoir partagé ces années de thèse avec moi. Enfin, merci à tous les membres ADDB : Dario, Alessandra, Nikos, Kifah, Pallavi, Neha, Loïc, Pauline, Fabienne, Thibaut, Sohaib, Xiao, Ariane et Yasmine.

Je tiens à remercier Sabrina, Mathilde et Barbara du groupe Louvain Centre for Toxicology and Applied Pharmacology (LTAP) pour leur aide précieuse. Merci à Audrey pour les bons moments que l'on a partagés à Paris, ce fût un réel plaisir d'assister à ces deux conférences sur la mucoviscidose avec toi.

Je souhaiterais également remercier d'autres membres de l'Université catholique de Louvain: Paméla Cheou pour son aide pour les ELISA ; Hervé Degand et Dr. Didier Vertommen pour les analyses MALDI-TOF MS et Philippine Debeyne pour son aide quant à la quantification des ions.

Un grand merci à Anne-Sophie Aubriot, Nathalie Bauwens et David Kinoo pour leur implication dans l'évaluation clinique ainsi que Dr. François Vermeulen pour son aide inestimable.

Je voudrais également remercier Dr. Mireille Dumoulin pour sa collaboration sur ce projet.

J'apprécie Jordane, Camille et Cécile, mes stagiaires, pour leur précieuse contribution à cette thèse.

Je souhaite exprimer ma gratitude aux membres de mon comité d'encadrement : Professeurs Françoise Van Bambeke, Patrice Soumillion et Patrick Lebecque pour leur suivi, leurs suggestions et leurs conseils.

Je souhaite également remercier les membres externes de mon jury de thèse, Professeur Ben Forbes (King's College London) et Dr. Vincent Raussens (Université Libre de Bruxelles - ULB), pour avoir évalué ce travail avec grand intérêt et pour nos discussions intéressantes et constructives.

Je suis reconnaissante envers l'Association Belge de Lutte contre la Mucoviscidose, les Fonds Spéciaux de Recherche de l'Université catholique de Louvain et les Laboratoires SMB pour leur soutien financier. Je tiens particulièrement à remercier Dr. Francis Vanderbist et Dr. Thami Sebti pour leur aide et l'intérêt qu'ils ont eu envers ce travail.

Un grand merci à mes chers amis « bostoniens » : Julien, Quentin, Fanny et Mélanie pour l'incroyable année que nous avons passée ensemble, pour leur soutien en tant qu'anciens doctorants ou doctorants actuels. Je souhaite également remercier Morgan et Bastoon de m'accueillir chaleureusement le week-end ; Yasmina, Séverine H., Sarah, Mathilde, Fred, Greg, Yoann et tous mes amis « parisiens » pour leur amitié et leur soutien pendant ma thèse ; Antoine et Mylène, mes amis de toujours ; Charlotte, Benjamin et Coralie ainsi que l'équipe de Touch du Boitsfort Rugby Club pour tous les bons moments partagés à Bruxelles et sur le terrain. Enfin, merci à Séverine R. de m'avoir donné l'opportunité d'être coach chez UCL Sport.

Je voudrais également remercier ma belle-famille pour son soutien. Vous m'avez mise à l'aise dès le premier jour et accueillie chaleureusement dans la famille Marchand-Guerra.

Je serai éternellement reconnaissante envers ma famille, particulièrement ma Maman, mon Papa et mon petit frère. Merci pour votre soutien inconditionnel, vos encouragements et votre amour. Je ne serais pas la personne que je suis sans vous. Ces dernières années n'ont pas été faciles pour nous tous, mais nous en sommes sortis plus forts. Merci pour votre aide de chaque instant et d'être aussi attentionnés.

Enfin et surtout, je souhaite remercier mon Amour, mon âme-sœur, mon meilleur ami et mon confident Cédric de m'aimer sans condition. Ces derniers mois n'ont pas été « un long fleuve tranquille » mais tu as été d'un soutien infailible. Tu as toujours cru en moi (bien plus que je ne crois moi-même en moi). Merci d'être Toi ! Je t'aime plus que tout au monde et je ne serais pas arrivée ici sans toi. Ton amour et ta patience sont le ciment de cette thèse de doctorat.

Je tiens à dédier cette thèse à mon bien-aimé et regretté Papi Loulou. J'avais sept ans quand tu as rejoint les anges. J'aurais aimé partager cette thèse avec toi, je sais que tu aurais été très fier. Tu me manques.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Professor Rita Vanbever, for the opportunity she gave me to perform my PhD thesis in her research group, for her scientific support and the trust she conferred on me.

I also wish to thank my co-supervisor, Professor Teresinha Leal, for her precious advices and scientific discussion about the research.

I will forever be thankful to Dr. Falguni Gadkari, my former undergraduate diploma supervisor. Thank you for having me introduced to the world of pharmaceutical research, for giving me the urge to continue in this field. I am also grateful for all the scientific knowledges you taught me, your support and friendship.

I would also thank Professor Véronique Pr  at and my colleagues of Advanced Drug Delivery and Biomaterials (ADDB) group for their welcome, their help and the friendly and inspiring environment. I am particularly grateful to: my friend Bernard for his unconditional support, his precious help and the spinning classes we shared; to my former colleagues Harshad, Danielle, Maria and Cristina for their help and friendship; to my friends Laure and Spela for the great moments we shared in and outside of the laboratory and to John for his valuable support and kindness. I would also thank Murielle for being such a nice and attentive person, for the late evenings we shared in the laboratory and her help during my PhD. Many thanks to K  vin and Nathalie for their kindness and help. I also appreciated the encouragement and inspiring discussions from Anne, Ga  lle and Ana. Many thanks to Chiara for the mutual support during our respective PhD. I am also thankful to my all lab mates and ADDB members: Dario and Alessandra (my PhD-generation in ADDB), Nikos, Kifah, Pallavi, Neha, Lo  c, Pauline, Fabienne, Thibaut, Sohaib, Xiao, Ariane and Yasmine.

I would like to express my thanks to Sabrina, Mathilde and Barbara from Louvain Centre for Toxicology and Applied Pharmacology (LTAP) for their valuable help. Many thanks to Audrey for the great time we spent together in Paris, it was a real pleasure to share these two Cystic Fibrosis meetings with you.

I also wish to thank other members of the Université catholique de Louvain: Paméla Cheou for her generous help with the ELISA; Hervé Degand and Dr. Didier Vertommen for the MALDI-TOF MS analyses and Philippine Debeyne for her assistance in ions quantification.

Many thanks to Anne-Sophie Aubriot, Nathalie Bauwens and David Kinoo for their involvement in the collection of samples for my clinical evaluation as well as Dr. François Vermeulen for his invaluable help.

I would also like to thank Dr. Mireille Dumoulin for her collaboration on this project.

I appreciate Jordane, Camille and Cécile, my former students, for their valuable contribution on this thesis.

I wish to express my gratitude to the members of my supervision committee: Professors Françoise Van Bambeke, Patrice Soumillion and Patrick Lebecque for their follow up, suggestions and advices.

I wish also to thank the external members of my jury, Professor Ben Forbes (King's College London) and Dr. Vincent Raussens (Université Libre de Bruxelles - ULB), for evaluating this work with their external point of view and for our interesting discussion.

I am grateful to the Belgian Cystic Fibrosis Association, Special Funds for Research of the Université catholique de Louvain and Laboratoires SMB for their financial support. I would like to particularly thank Dr. Francis Vanderbist and Dr. Thami Sebti for the help and inspiring discussions about this work.

Many thanks to my dear “Bostonian” friends: Julien, Quentin, Fanny and Mélanie for the amazing year we spent together, for their understanding and support as former or current PhD students. I wish also to thank Morgan and Bastoon for being my weekend-roommates; Yasmina, Séverine H., Sarah, Mathilde, Fred, Greg, Yoann and all my “Parisian” friends for their friendship and their support during my PhD; Antoine and Mylène, my forever friends; Charlotte, Benjamin and Coralie as well as the Boitsfort Touch Rugby team for the great times in Brussels and on the field. Thank you to Séverine R. for giving me the chance to be coach at UCL Sport.

I would like to express my gratitude to my step family for the support during these years. You have made me comfortable from the first day and I feel like I am part of the Marchand-Guerra family.

I will forever be thankful to my family, especially my Mom, Dad and brother. Thank you for your infinite support, encouragement and love. I would not be the woman I am without you. These past years have not been an easy ride for all of us but we have been through it. I will always be grateful for the help and care you provide to me.

Lastly and most importantly, I wish to thank my love, soul mate, best friend and confidant Cédric for loving me unconditionally. These past months were not 'a long quiet river' but you have been an unconditional supporter. You have always believed in me (and more than myself). Thank you so much for being you! I love you more than anything and would not have made it without you. Your love and patience are the backbone of my PhD thesis.

I dedicate this thesis to my beloved Papi Loulou, I was only seven years old when you joined the angels. I would have liked to share it with you, I know you would have been proud. I miss you.

ABBREVIATIONS

<i>A. xylosoxidans</i>	<i>Achromobacter xylosoxidans</i>
AAT	Alpha-1 anti-trypsin
ACK	Ammonium-chloride-potassium
AEC	Anion-exchange chromatography
AM	Alveolar macrophages
ANS	8-anilinonaphthalene-1-sulfonate
ASL	Airway surface liquid
ATP	Adenosine triphosphate
AUC	Area under plasma concentrations-time curve
<i>B. cepacia</i>	<i>Burkholderia cepacia</i>
BAL	Bronchoalveolar lavage
BFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine ligand
CD	Circular dichroism
CF	Cystic fibrosis
CFBD	Cystic fibrosis-related bone disease
CFLD	Cystic fibrosis-associated liver disease
CFRD	Cystic fibrosis-related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator
DPI	Dry powder inhaler
EC50	Effective concentration at 50%
EDTA	Ethylenediaminetetraacetic
ELF	Epithelial lining fluid
ENaC	Transepithelial Na ⁺ channel
Fab'	Monoclonal antibody antigen-binding fragment
F-actin	Filamentous actin
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and drug administration
FEV ₁	Forced expiratory volume in one second
FITC	Fluorescein isothiocyanate
FN	Fibronectin
G'	Elastic modulus
G''	viscous modulus
G-actin	Globular actin
GdmCl	Guanidinium chloride
GH	Growth hormone

ABBREVIATIONS

GLP-1	Glucagon-like peptide-1s
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFCWO	High frequency chest wall oscillation
hGH	Human growth hormone
HRP	Horse radish peroxidase
I	Intermediate state
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MDR-PA	<i>Multidrug-resistant pseudomonas aeruginosa</i>
Met-G-CSF	Methionyl-granulocyte colony stimulating factor
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MRSA	<i>Methicillin-resistant staphylococcus aureus</i>
MW	Molecular weight
N	Native state
NAL	Nasal lavage
NBD	Nucleotide-binding domain
NE	Neutrophil elastase
NET	Neutrophil cellular trap
NM-BAPTA	5-nitro-5'-methyl-(1,2-bis(o-aminophenoxy)ethan-N,N,N',N'-tetraacetic acid
N-ter	N-terminal
<i>P. aeruginosa</i> , PA	<i>Pseudomonas aeruginosa</i>
Pa	Pascal
PBS	Phosphate-buffered saline
PCL	Periciliary sol layer
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEG20	Linear 20 kda polyethylene glycol
PEG30	Linear 30 kda polyethylene glycol
PEG40	Two-arm 40 kda polyethylene glycol
PI	Pancreatic insufficiency
PKA	Protein kinase A
PS	Pancreatic sufficient
PSC	Premature stop codon
R	Regulation
rhDNase	Recombinant human deoxyribonuclease I
rhG-CSF	Recombinant human granulocyte-colony stimulating factor
RITC	Rhodamine isothiocyanate

<i>S. aureus</i> , SA	<i>Staphylococcus aureus</i>
<i>S. maltophilia</i>	<i>Stenotrophomonas maltophilia</i>
sCT	Salmon calcitonine
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEM	Standard error of the mean
TFA	Trifluoroacetic acid
T _m	Temperature of mid-transition
TMD	Transmembrane domain
TNF	Tumor necrosis factor
Tris	Trishydroxymethylaminomethane
U	Unfolded state
WT	Wild type
β-ENaC	β-epithelial Na ⁺ channel–overexpressing

ABSTRACT

Cystic fibrosis (CF) is a life-threatening disease affecting many organs and especially the respiratory tract. About 70,000 individuals suffer from this genetic disease worldwide [1]. CF is due to a defect in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. In patients with CF, an impaired or missing CFTR protein induces the production of dehydrated and thick mucus, thereby resulting in a vicious cycle of infection, inflammation and obstruction of the lungs [2]. The accumulation of sputum compromises the lung function. Pulmonary failure then leads to death of almost all patients.

Current therapies for the symptomatic treatment of CF lung disease include physiotherapy, antibiotics as well as inhaled mucolytics [3]. Recombinant human deoxyribonuclease I (rhDNase) is the mucolytic agent most widely used. Its administration by inhalation allows to fluidize the mucus by cleaving the extracellular DNA contained in it [4]. However, the efficacy of rhDNase is compromised by its rapid clearance from the lungs [5]. Accordingly, rhDNase requires a daily administration, which contributes to the high therapy burden and reduces the patient compliance [6].

The goal of this thesis was to address the limitation of this treatment by developing a long-acting PEGylated version of rhDNase with a preserved enzymatic activity. The novelty of this project was to use PEGylation as a modification process for the pulmonary delivery of rhDNase.

Site-specific PEGylation of rhDNase was achieved using linear 20 kDa, linear 30 kDa or two-arm 40 kDa polyethylene glycol (PEG) propionaldehydes. The effects of PEGylation on the stability of rhDNase were investigated using circular dichroism and fluorescence spectroscopy. A clinical evaluation in CF sputa was conducted and involved the measurement of the rheological properties of the sputa following addition of rhDNase or rhDNase conjugated to 40 kDa PEG. *In vivo* lung disposition studies were carried out in healthy mice. Short-term and long-term therapeutic efficacies of rhDNase conjugated to 30 kDa or 40 kDa PEG to treat CF lung disease was assessed in transepithelial Na⁺ channel–overexpressing (β -ENaC) mice.

Mono-PEGylation of rhDNase on its N-terminal residue was associated with a full preservation of its enzymatic activity and its conformation whatever the PEG size. Chemical-induced stress

highlighted that PEG induced the destabilization of the protein conformation and that this effect increased with the PEG size. PEGylation did not alter rhDNase thermal stability and significantly reduced the formation of rhDNase aggregates. rhDNase and rhDNase PEGylated with 40 kDa PEG induced a similar decrease of elastic and viscous moduli of CF sputa, independently of the components such as DNA, actin and ions contained in the samples. *In vivo* disposition studies in healthy mice showed that PEGylation of rhDNase sustained the presence of the enzyme within the lungs for more than 15 days whereas the unconjugated protein was cleared within 24 hours. The larger the PEG size, the more prolonged the retention of the enzyme in the lungs. A short-term therapeutic efficacy study highlighted that one dose of rhDNase conjugated to 30 kDa or 40 kDa PEG presented a similar activity on DNA hydrolysis in bronchoalveolar lavage as 5 daily doses of rhDNase. In addition, a long-term therapeutic efficacy study showed that a weekly administration of rhDNase PEGylated with either 30 kDa or 40 kDa PEG was efficacious to decrease DNA content in bronchoalveolar lavage and did not result in any apparent accumulation of both conjugates in the lungs after 6 weeks. Finally, PEGylation of rhDNase preserved the protein stability to nebulization.

The sustained presence of PEGylated rhDNase in the lungs and its therapeutic efficacy present interesting perspectives for the development of a long-acting rhDNase with reduced therapy burden.

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FOREWORD AND AIM OF THE THESIS

Cystic Fibrosis (CF) is a lethal genetic disease caused by the mutation of the gene coding for the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein [7,8]. CF is a multi-systemic disease that predominantly affects the airway epithelium [9]. To date, more than 2,000 mutations in *CFTR* have been identified [10,11]. Therefore, a pharmacological approach tailored to treat each patient mutation is considered a promising strategy [12,13]. Very intense researches have identified therapies for specific mutations. Ivacaftor (Kalydeco®), a potent CFTR channel potentiator, is indicated for patients suffering from 38 function mutations [14], with the most frequent being G551D [15]. More recently, Orkambi®, which is a combination of Lumacaftor (CFTR channel corrector) and Ivacaftor, has been approved for patients with CF who have two copies of the F508del mutation [14]. However, the applications of Kalydeco® and Orkambi® are restricted by the percentage of patients suffering from the targeted mutations: only 3.9% of European patients have at least one mutation of class III and ~50% display F508del homozygote gene mutation [16]. By contrast, the symptomatic treatment of CF presents a positive impact on survival and involves the whole population affected by the disease, irrespectively of the class of mutations. Since morbi-mortality in CF is related to bronchial obstruction by a hyperviscous mucus, efforts to optimize the symptomatic treatment of the disease at the respiratory level remains valid. In particular, recombinant human deoxyribonuclease I (rhDNase) is used as a mucolytic agent for the symptomatic treatment of CF lung disease. rhDNase cleaves the extracellular DNA released from apoptosis of infiltrating cells and thus allows to fluidize the mucus [4]. In 1999, 58% of patients (> 6 years old) with CF in the USA were taking rhDNase as mucolytic agent. In 2016, they were 88% [17]. Thus, rhDNase became the reference mucolytic agent in CF and is now taken by almost all patients.

However, the main limitation of rhDNase is its short residence time within the respiratory tract following inhalation since the protein is eliminated from the lungs within few hours [5]. Accordingly, rhDNase treatment implies a daily administration which results in low patient compliance [6] due to the time burden (more than 15 min are required for the set-up, inhalation, cleaning and disinfection of the nebulizer).

In this context, the aim of this thesis was to develop a long-acting version of rhDNase for CF lung disease treatment. The main hypothesis involved the use of PEGylation as an approach to sustain the local residency of rhDNase following delivery to the lungs. The PEGylated rhDNase could be taken at longer intervals of time than the current daily administration and thus could lead to reduced treatment burden.

PEGylation has proven its efficacy following injection and many PEGylated proteins are currently commercialized for subcutaneous, intravenous or intramuscular delivery [18]. PEGylation has also been evaluated as a modification process for pulmonary delivery of proteins intended to reach the bloodstream [19–23]. Recently, our research team showed that PEGylation significantly sustained the residence time within the lungs of antibody fragments following intratracheal instillation of mice [24]. Therefore, PEGylation could be applied to rhDNase to sustain its presence within the lungs following pulmonary delivery.

Our main hypothesis was tested through five specific aims:

Aim 1: To PEGylate rhDNase with preservation of its enzymatic activity. Three high molecular weight PEGs were used for PEGylation: linear 20 kDa, linear 30 kDa and two-arm 40 kDa PEGs. These PEG size and architecture were selected based on large molecular weight PEGs already used clinically [25–27]. The hypothesis behind the sustained activity of the PEGylated rhDNase was related to the PEG attachment site. It was chosen at the opposite side of rhDNase site of action to avoid loss of activity.

Aim 2: To evaluate the stability of PEGylated rhDNase toward stresses-induced unfolding. The conformation and physical properties of proteins might be modified through PEGylation due to the nature of interactions involved between molecules. Indeed, attachment of a highly hydrated PEG would confer favorable properties such as a reduced aggregation tendency [28].

Aim 3: To evaluate the impact of PEGylation on the mucolytic activity of rhDNase in CF sputa. The hypothesis of this specific aim was based on the fact that if the PEGylation does not affect rhDNase activity on pure DNA solution, PEGylated rhDNase might offer a higher mucolytic activity than the non-conjugated protein in sputa from patients with CF due to rhDNase protection towards proteolysis provided by PEG.

Aim 4: To evaluate the *in vivo* residence time of PEGylated rhDNase following pulmonary delivery in healthy mice. We hypothesized that the properties conferred by the PEG such as enhanced adhesiveness to mucus, improved proteolytic resistance and reduced endocytosis by alveolar macrophages and epithelial cells would allow to prolong the presence of rhDNase within the lungs.

Aim 5: To determine the *in vivo* efficacy of rhDNase conjugated to 30 kDa and 40 kDa PEG in a murine model of CF pulmonary disease. An increased residence time within the lungs should result in an improved therapeutic efficacy or would lead to a sustained efficacy with reduced administration frequency.

This thesis has been divided into six chapters. Chapter I reviews the CF disease – especially the lung-related disorder and its treatments – and the use of PEGylation as a tool for improving the pulmonary delivery of biopharmaceuticals. Chapters II to V present the findings related to the five specific aims described above. Chapter V reports results of aims 4 and 5 and also investigates the stability of PEGylated rhDNase toward nebulization. Finally, chapter VI summarizes the mains achievements of the thesis and its perspectives.

CHAPTER I. INTRODUCTION

I. CYSTIC FIBROSIS LUNG DISEASE AND THERAPY STRATEGIES

Cystic Fibrosis (CF) is a multi-systemic genetic disorder which affects the exocrine glands [29]. It is caused by a mutation in a gene that encodes the CF transmembrane conductance regulator (CFTR) protein, which functions mainly as a cyclic adenosine monophosphate (cAMP)-regulated chloride channel. A defect of the *CFTR* gene results in an impaired, reduced or absent protein at the epithelial membrane of many organs, thus leading to a defective transepithelial ion transport mechanisms [30]. CF is the most common fatal autosomal recessive condition in the Caucasian population, affecting about one in 2,500 births [31]. Despite the fact that CF is a multi-organ disorder, it is classically diagnosed by symptoms of acute/chronic respiratory disease, with persistent pulmonary infections and airway mucus plugging resulting in failure to expectorate [32]. CF is a rare disease that still remains incurable with a median predicted survival age of 47.7 years for infants born in 2016 [17].

I.1. HISTORICAL OVERVIEW

The first clear description of the *fibrocystic disease of the pancreas* was made in 1938, based on the review of one thousand biopsies, by Dorothy Andersen, a pathologist at the New York Hospital. She firmly established that the diagnosis of CF of the pancreas was not related to celiac disease. Autopsy studies conducted on malnourished infants distinguished a disease of mucus plugging the glandular ducts, damage of the pancreas tissue and the association with meconium ileus, further named as 'CF of the pancreas' [33]. In the 1940s, the presence of thick and sticky mucus clogging the ducts of mucus glands allowed the alternative name of 'mucoviscidosis' [34]. During the same decade, CF was recognized as a genetic disease transmitted in an autosomal recessive pattern [35]. Antibiotics were included in the treatment of respiratory failure since lung disease was recognized as one of the major symptoms of CF [36]. Later, Darling and co-workers discovered that the sweat of patients with CF contained abnormal high concentrations of chloride and sodium ions [37,38]. In early 1960s, Gibson and Cooke allowed progress in diagnosis with development of the pilocarpine sweat test, measuring the electrolytes concentration that is excreted in sweat of individuals with CF [39]. During the following decades, Knowles et al. described the alteration of chloride and sodium transport across the airway epithelia [40]. Also, identification of the gene responsible of CF

allowed to clone it [7,8] and progress was made in the understanding of the CFTR protein defect. To date, findings through basic scientific research have allowed for an improvement in the health of people with CF with the development of therapeutic agents such as recombinant human deoxyribonuclease I (Pulmozyme®, Roche), inhaled tobramycin (Tobi®, Novartis) as well as Ivacaftor and Lumacaftor/Ivacaftor (Kalydeco® and Orkambi®, Vertex Pharmaceuticals) [14]. Promising strategies currently being developed concern CFTR modulation [36]. However, drug targeting the symptomatic disease is of great interest since all patients with CF, irrespective of the class of CFTR mutation, can benefit from it.

I.2. CFTR DEFECTS AND MUTATIONS

I.2.1. CFTR gene

The *CFTR* gene, encompassing approximately 180,000 base pairs, is located on the long arm of chromosome 7 (gene ID: 1080, NCBI) and codes for a 1,480-amino acid membrane protein, named CFTR protein [41,42]. To date, more than 2,000 mutations have been identified [10,11] (although not all cause CF disease [43]) and only the functional importance of a small number is known. The inheritance of CF implies that a person with CF displays two defective copies of the gene. Individuals with one mutated *CFTR* allele do not suffer from CF and are termed carriers. Two heterozygous carriers of a pathogenic *CFTR* gene mutation display, with each pregnancy, a 25% risk of having a homozygous new-born child with CF. A patient with CF can carry in each allele an identical or two different CF-causing mutations (heterozygous compounds) [1].

I.2.2. CFTR protein

CFTR protein is a selective channel that allows transport of ions, especially chloride and bicarbonate, across the apical membrane of epithelial cells [44,45]. CFTR has also been reported to influence the expression of potential modifiers of the CF phenotype such as proteins involved in inflammatory responses, maturational processing, ion transport as well as cell signaling [46–50]. It is expressed in many epithelial cells including sweat duct, airway, pancreatic duct, intestine, biliary tree and vas deferens [51]. CFTR, which is a 168 kDa glycosylated protein (**Figure 1**), belongs to the adenosine triphosphate (ATP)-binding cassette superfamily of transporters [52] and includes two transmembrane domains (TMD), two

nucleotide-binding domains (NBD) and a charged cytoplasmic regulation (R) domain (**Figure 1**) [42]. The activation of CFTR is based on the phosphorylation of its R domain mediated by protein kinase A (PKA) [52,53]. It is also linked to ATP hydrolysis at both NBDs [54,55] since R domain phosphorylation induces a conformational change of the TMDs, promoting ATP-binding to the NBDs [56]. In a single open-close gating cycle, CFTR channel hydrolyzes one ATP molecule at NBD1 to allow the channel to open and then binds and hydrolyzes a second ATP molecule at NBD2 to allow the channel to close [52].

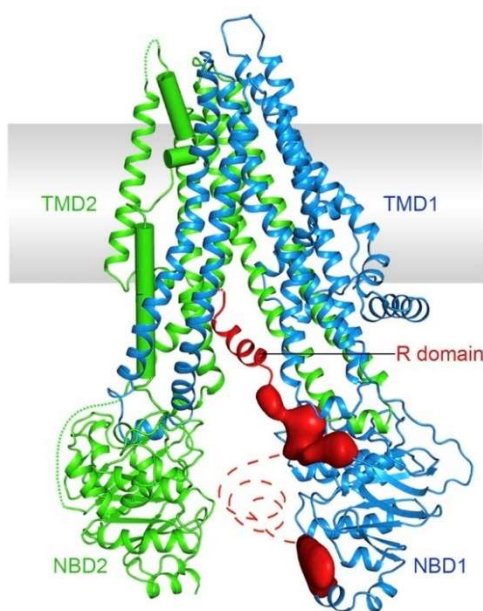


Figure 1: Structure of the CFTR protein, divided into five domains: two Transmembrane Domains (TMDs); two Nucleotide Binding Domains (NBDs) and one central Regulatory (R) domain (adapted from [57]).

CFTR protein synthesis follows the usual protein biosynthesis process, from gene transcription to cell membrane expression. Transcription of the DNA code into messenger RNA (mRNA) initiates the journey of CFTR (**Figure 2**). The mRNA strand passes through the nuclear pore into the cytoplasm to begin the translation process. Translation of the mRNA sequence within the endoplasmic reticulum allows the assembly of a polypeptide chain of amino acids, further leading to protein maturation. Cytoplasmic transfer of the immature CFTR channel to the Golgi apparatus enables pursuing the folding of the 3D structure of the protein. The mature CFTR complex is then packaged into vesicles for its trafficking to the apical membrane. Finally, the vesicle carrying the protein fuses with the membrane, allowing CFTR expression in the cell membrane [42].

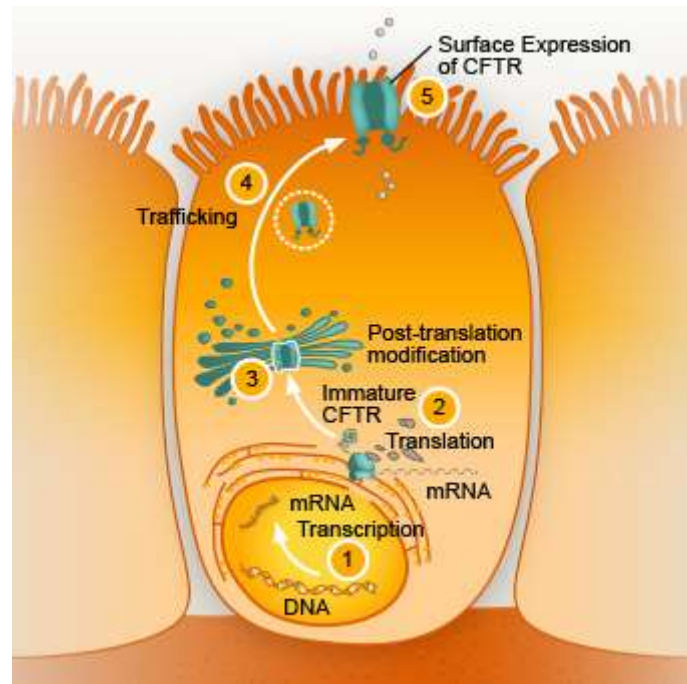


Figure 2: Process of CFTR protein synthesis, from gene transcription to cell membrane expression [58].

1.2.3. Classes of CFTR mutations

The mutation affecting *CFTR* can be assigned to one of the five steps of synthesis (**Figure 2**). The most common mutations, representative of *CFTR* defects affecting about 80% of patients, can be grouped into classes according to the mechanism involved in the disruption of the protein synthesis, circulation or function [59–61]. These classes of mutation are described below and in **Figure 3**. It is worth mentioning that the classification presented below is simplified. In fact, a single mutation can cause combinatorial defects in the CFTR channel biology, leading to the phenotypic complexity of CFTR mutants [62].

- Class I mutations are characterized by the absence of CFTR channel since the introduction of stop codons or splicing defects impede the protein synthesis [36,63]. Instability in mRNA (resulting in no detectable protein) or in CFTR protein (rapidly degraded or devoid of function) implies no CFTR in the cell membrane [59,64].
- Class II mutations block the processing of the protein, resulting in an absence of a functional protein at the plasma membrane [63,64]. The immature form of CFTR is subsequently degraded by the ubiquitin-proteasome pathway [36], a system that asserts quality control over maturing CFTR polypeptide [42].

- Class III mutations produce a fully processed CFTR protein that is expressed in the plasma membrane but does not function as a chloride channel due to regulation impairment [36,63,64].
- Class IV mutations are also characterized by a synthesized and fully processed CFTR protein, correctly expressed at the plasma membrane [36,63]. However the conductance of chloride ions and/or gating properties of the channel are altered due to mutations in the TMDs [36,59,64].
- Class V mutations result in a partly defective CFTR synthesis or processing [36] caused by promoter mutations, abnormal splicing or inefficient protein maturation [64].
- Class VI mutations reduce the membrane stability of the well synthesized CFTR channel [63,65]. Also, the conductance of ions other than chloride can be impacted [36].

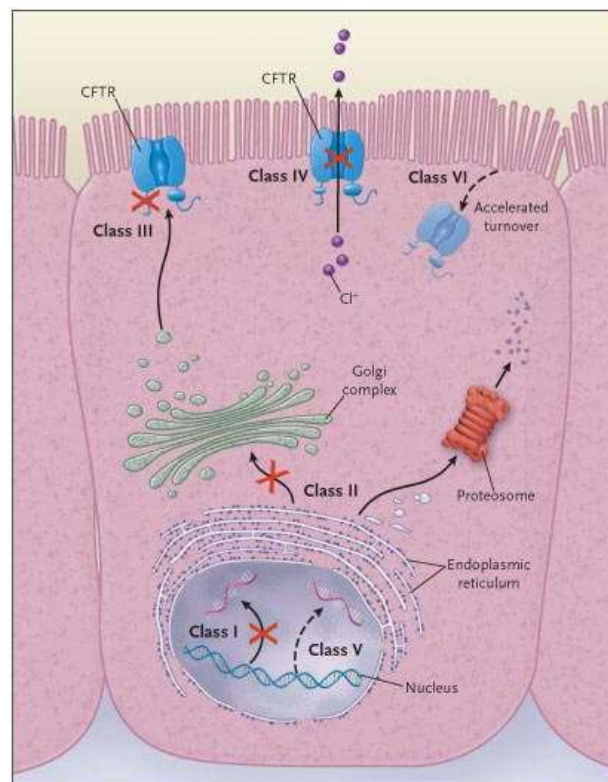


Figure 3: The six categories of CFTR mutations [41]. Classes I and II mutations lead to alteration of the protein synthesis. Class III is characterized by a defective regulation of the channel. A defective conductance of chloride is observed for Class IV. Class V induces impaired production or processing of the protein. In Class VI, the conductance of other ions than chloride is defective.

In case of CF, one third of the *CFTR* mutations are localized in the intracellular part of the protein. These CF-causing mutations mostly occur in NBD1 [66] while few occur in NBD2 [67]. Among the six classes reported above, only five *CFTR* mutations (F508del, G551D, W1282X, G542X and N1303K) occur with a frequency of more than 1% (**Table 1**) [42]. The most frequent mutation worldwide, corresponding to a deletion of phenylalanine in position 508 (F508del), belongs to class II mutations and is present in approximately two-thirds of patients with CF [30,63,68]. In addition to the lack of trafficking, F508del-*CFTR* can present other defects. If F508del-*CFTR* is expressed at the apical membrane, it presents a defective channel opening, therefore operating as a class III *CFTR* mutation [59,69]. The F508del mutation can also decrease the stability of *CFTR* at the membrane [70]. In this particular case, F508del-*CFTR* residence time is reduced, which allow to classify it as a class VI mutation.

The severity of the disease has been related to the class mutation(s) the patient with CF suffers from. Classes I to III have been reported as inducing severe CF phenotypes (**Table 1**) whereas milder CF phenotypes have been associated with classes IV and V mutations [36,71]. Although they have been investigated in few patients, most class VI mutants should be considered as severe [72].

Table 1: Simplified classification of the CF-causing mutations with worldwide prevalence > 1% in individuals with CF [7,42,65,67,68].

Mutation	Frequency	Class mutation	Characteristics	Functional incidence	Phenotype severity
F508del	66 %	Class II	Deletion of phenylalanine in position 508 - CF-causing NBD1 mutant	Defective CFTR processing associated with no Cl ⁻ transport	Severe
G542X	2.4 %	Class I	Glycine to Stop at residue 542 - CF-causing NBD1 mutant	Translational defect and truncated CFTR protein – no Cl ⁻ transport	Severe
G551D	1.6 %	Class III	Glycine to aspartate substitution at residue 551 - CF-causing NBD1 mutant	Defective CFTR regulation associated with failure channel opening – no Cl ⁻ transport	Severe
N1303K	1.3 %	Class III	Asparagine to lysine at residue 1303 - CF-causing NBD2 mutant	Defective CFTR processing – no Cl ⁻ transport	Severe
W1282X	1.2 %	Class I	Tryptophan to stop at 1282 - CF-causing NBD2 mutant	Premature cessation of translation – no CFTR – no Cl ⁻ transport	Severe

I.3. CLINICAL MANIFESTATIONS IN CF

Clinical presentation of the condition of patients with CF is complex: diverse symptoms often affect organs with varying severity. CF mainly affects the lungs, the pancreas and intestines, the endocrine system as well as the liver and sex organs.

I.3.1. Gastro-intestinal disorder

The thickened secretions characteristic of CF might obstruct tubes and ducts in the pancreas [73], resulting in poor influx of the digestive enzymes into the small intestine. Since digestive enzymes are involved in the disintegration of food, their absence leads to impaired absorption of fats and proteins by the intestines [74].

Around 17% of newborn children with CF are affected by meconium ileus [17,75], an obstructive bowel condition caused by abnormally thick and impacted meconium [73]. A great majority of infants with CF also develop pancreatic insufficiency (PI), which can be present at birth or develop during the first years of life [73]. In 2016, about 90% of patients with CF suffered from PI [17]. PI is characterized by bulky stools, flatulence, abdominal bloating and poor weight gain. A default in pancreas function also induces steatorrhea (excess fat in feces), fat-soluble-vitamin deficiency (A, D, E and K [76]) and malnutrition [74]. Since controlling these symptoms is crucial for the normal growth of patients with gastro-intestinal disorder, attention to nutrition is also an integral part of CF care. Therefore, adequate caloric intake, correction of fat-soluble-vitamin deficiency as well as pancreatic enzyme replacement therapy ([17]) represent all the fundamental components for the gastro-intestinal disease management [76].

Approximately 10% of patients are pancreatic sufficient (PS) and present milder forms of CF [42]. PS patients also have evidence of pancreatic damage but, unlike PI individuals, retain sufficient endogenous exocrine pancreatic function to sustain normal digestion [77]. Reduction of the exocrine pancreatic capacity combined with an increased risk of pancreatitis (inflammation of the pancreas) has been reported in PS patients [78]. In 2016, 84.8% of patients displaying mutation classes IV and V presented pancreatitis complications compared to 0.4% of individuals with mutation classes I to III [17], thus confirming that exocrine pancreatic status is directly linked to the genotype [79].

I.3.2. Endocrine disorders

Multiple endocrine disorders can be developed in patients with CF. Diabetes, linear growth abnormality as well as male hypogonadism and bone disease represent the most frequent endocrine complications in CF.

CF-related diabetes (CFRD) is an important and highly prevalent complication in individuals with CF. In 2016, 21.5% of patients were affected by CFRD [17]. Pathogenesis is directly connected to *CFTR* gene defect, with complications mainly observed in patients displaying severe *CFTR* mutations associated with exocrine PI [80–82]. The CF Foundation reported in 2016 that 25.2% of patients with mutations classes I to III presented CFRD complications, compared to 7.0% for individuals with mutations classes IV and V [17]. Preserved levels of insulin sensitivity are usually detectable in people with CF, unless patients are particularly ill or have severe chronic inflammation [83,84]. However, as for both type 1 and type 2 diabetes, hyperglycemia (i.e. elevated blood glucose) is characteristic of CFRD. Also, patients with CF have abnormal insulin production, which declines gradually [85]. The main complications of CFRD are worsened lung disease, weight loss and increased mortality [86–89]. Symptoms of other forms of diabetes including retinopathy (vision impairment due to retinal damage) and nephropathy (damage of the kidney caused by hyperglycemia) might also be observed as CFRD complications, although their prevalence is less than for type 1 and type 2 diabetes [90]. Long-acting insulin injections associated with extra short-acting insulin intake during meals are characteristic of the typical insulin regimen of patients with CFRD [85].

Linear growth abnormality is a very common issue in CF. A reduced weight at birth of infants with CF was already reported in the 1950s [91,92]. In the 1990s, Lai and co-workers highlighted a prevalence in short stature in approximately 20% of children with CF [93], with several further studies reporting reduced linear growth in CF [94–97]. The most important factors involved in growth failure are fat and micronutrient malabsorption, chronic inflammation, lung disease as well as corticosteroid treatment [85]. Impaired growth hormone (GH) secretion has also been reported in pediatric patients with CF [97]. Finally, delayed puberty and parent height below average values might contribute to the reduced growth observed in patients with CF [98]. Therefore, nutritional support and pharmacological therapy such as recombinant human GH should be adapted for the good management of linear growth

abnormality in children with CF. Also, corticosteroid medications should be reduced to the lowest dose to achieve therapeutic goals [85,99].

Male hypogonadism may affect men with CF. Hypogonadism refers to a decreased functional activity of the gonads which produce hormones and gametes [100]. Reduced levels of testosterone characterize male hypogonadism with 20% to 53% of men with CF showing testosterone concentrations of < 10 nmol/L (normal range between 10 and 30 nmol/L) [101,102]. This hormone is involved in masculine growth and development during puberty, and men with CF affected with this medical condition may suffer from reduced bone mass or bone mineral density [101,103]. Testosterone therapy might be considered but patients must be warned of its adverse effects such as spermatogenesis decrease and the negative impact on reproductive potential [85].

CF-related bone disease (CFBD) has emerged as a common complication associated with the improvement in patient survival rate [17]. CFBD, including osteopenia (slightly less bone mineral density than normal condition), osteoporosis (low bone mineral density) and bone fracture, presents an increased prevalence with age [104]. In 2016, the CF Foundation reported osteopenia complications in 21% of patients over 18 years whereas only 1.5% of infants presented this clinical issue. Similar tendencies were observed for other CF-associated bone conditions, except for bone fracture [17]. The causes of CFBD are multi-factorial [105]. Although King and co-workers associated reduced bone density with F508del-CFTR mutant [106], Aris et al. later reviewed that CFBD is independent of the CFTR class mutation [104], further supported by the latest Patient registry annual data report of the CF Foundation. In 2016, bone complications were similarly reported for all CFTR class mutations [17]. Genetics, malnutrition, deficiencies in calcium and vitamin D, corticosteroid therapy, inflammation, diabetes, sex hormones insufficiency, growth issue and lack of exercise are established as risk factors for CFBD [104,105,107]. Treatment of CFBD targets both preventive and curative management. Supplementation of vitamin D and calcium associated with optimal nutrient intake, exercise programs as well as management of other disease states, such as endocrine and pulmonary conditions, further enhance the bone health [104,107,108]. Bisphosphonates may improve bone mineral density in patients with CF [109] but potential safety issues such as development of cirrhosis reduce their use [104].

I.3.3. Hepatobiliary disease

CF-associated liver disease (CFLD) is one of the common symptoms of CF. It is also considered as the third lethal cause in CF. According to the CF Foundation, 2.7% of patients died from liver disease or failure in 2016 [17]. CFLD affects 30% of patients with development of symptoms occurring mostly during puberty [110]. The prevalence of CFLD is associated with the severity of the disease, with patients harboring complete loss of CFTR protein function being the most affected [111]. In 2016, the CF Foundation reported that liver disease was more prevalent among individuals with CFTR mutation classes I to III with 11.2% of patients presenting hepatobiliary complications compared to 2.6% of patients displaying CFTR mutation classes IV and V [17]. However the liver pathogenesis is not directly related to a *CFTR* gene defect but might be connected to the symptoms of the gastro-intestinal disorder, such as deficiencies of essential fatty acids and malnutrition [112]. In addition, patients with history of meconium ileus and pancreatic insufficiency are predictive of liver disease [113]. Finally, insulin resistance or oxidative stress may cause the onset and development of liver disease [112]. An abnormal CFTR protein in the biliary system may induce impaired secretions and the deposition of thick and alkalinity-reduced bile in ducts [110]. Common clinical symptoms of CFLD are gall stones, hepatic steatosis and cirrhosis [110,114]. Gall stones result from increased bile viscosity associated with a reduction of the gallbladder dimension [110]. Hepatic steatosis (accumulation of fatty acids in the liver [115]) occurs with a prevalence of 23–75% in patients with CF [112,116]. Cirrhosis is the irreversible stage of damage and leads to liver failure. Cirrhosis is characterized by jaundice, by disorder in the coagulation process as well as by portal hypertension [110]. CF care of the hepatobiliary system employs ursodeoxycholic acid, a bile acid aiming at improving liver biochemistry, hepatic excretory function as well as liver histology and essential fatty acid status [117]. However, its efficacy has not been demonstrated in the prevention of liver cirrhosis [110].

I.3.4. Reproduction

CFTR gene mutations affect both male and female fertility. However, not all *CFTR* mutations appear to cause infertility [118].

In the case of men with CF, the vas deferens is very sensitive to CFTR dysfunction. Congenital bilateral absence of the vas deferens [119] in 95% of men with CF implies azoospermia (no

sperm in the semen, [120]) and infertility [121]. However, this disorder does not only affect men with two defective copies of the *CFTR* gene but also individuals with only one mutated allele and no clinical signs of CF [119,122]. Treatment of infertility of men with CF is impossible since there is no chance to correct an absence of the vas deferens. However, since sperm production in the testicles is normal in 90% of men with CF, fatherhood is attainable through assisted reproductive technology [123].

Women with CF display thick cervical mucus [124] and may suffer from delayed puberty and amenorrhea (absence of menstrual period) [125–127]. Also, although they could face some ovulation issues due to poor nutritional status and severe lung disease [128], the majority of women with CF do not have compromised fertility [128].

I.3.5. Pulmonary disease

Amongst the clinical conditions, CF severity and patient survival time are determined by the degree of changes in the respiratory system. Pulmonary involvement occurs in almost all patients and is referred to as the first cause of death. The latest Patient registry annual data report of the CF Foundation reported that respiratory failure leads to 66.8% of the deaths in 2016 [17]. Therefore, lung function is an important clinical indicator of the health of individuals with CF.

CF-associated lung phenotype tends to be more susceptible to *CFTR*-independent factors than other CF-associated organ conditions presented above. Environmental and treatment factors are considered to play an important role in lung function [63], resulting in a great level of variability in the pulmonary disease severity [114,129]. Nevertheless, a marked genotype/phenotype relationship has been noticed for the lung disease severity. More severe lung disease was reported in individuals with class I to III mutations than in patients affected by classes IV and V [114,130].

The phenotype of CF lung disease develops during life. At birth, the lungs of infants with CF are in a pro-inflammatory state, as demonstrated by Tirouvanziam and co-workers in their investigation of airway grafts from human fetus with CF [131]. Inflammation is therefore a primary defect of CF lung disease, well before the acquisition of infection. Early in childhood, patients with CF acquire symptomatic common viral infections [132], which are easily cleared

with vigorous antibiotics [51]. Later, persistent respiratory infections occur, resulting from trapping of inhaled bacteria in the dehydrated mucus and from slower clearance of the pulmonary secretions [133]. Lung deterioration associated with permanent colonization of the airways is a consequence of impaired chloride secretion, hyper-absorption of sodium as well as reduced water content of the mucus induced by CFTR protein defect [51,132,133]. The lungs of individuals with CF are also characterized by a chronic non-resolving inflammation [134]. Vicious cycles of obstruction, infection and inflammation ultimately result in pulmonary insufficiency and eventually in respiratory failure [135]. Care management of CF lung disease includes physiotherapy (mechanical airway clearance), mucolytic agents, bronchodilators, antibiotics, as well as a variety of novel treatments. More details about the pathophysiology of CF lung disease as well as a review of the related therapy strategies are given in the next sections.

I.4. PATHOPHYSIOLOGY OF THE CF LUNG DISEASE

The cascade of the pathophysiology of CF lung disease is illustrated in **Figure 4**. Defects in the *CFTR* gene result in an absent or defective CFTR protein at the apical membrane of epithelial cells. Impaired ion transport then leads to depletion of airway surface liquid (ASL) which is associated with cilia beating. Ciliary collapse affects the self-cleaning mechanism of the lungs, leading to a vicious cycle of obstruction, infection and inflammation of the airways [30].

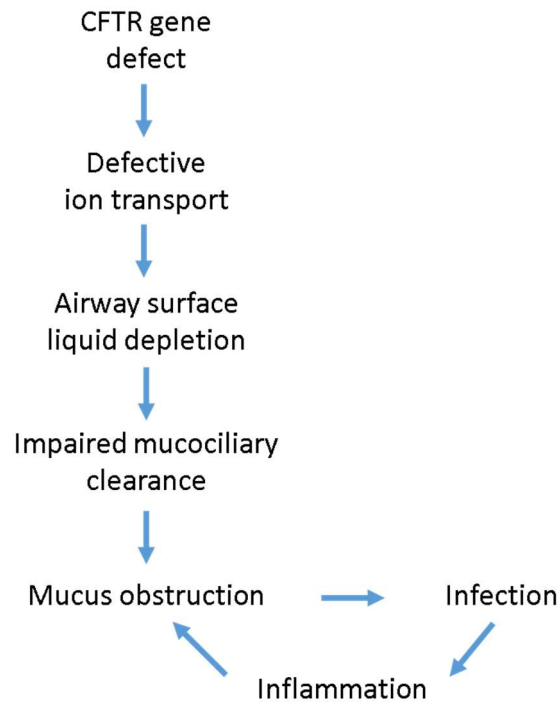


Figure 4: Cascade of the pathophysiology of CF lung disease (adapted from [30]).

I.4.1. Role of CFTR protein in the lungs

Fluid homeostasis on the epithelium surface is driven by CFTR protein. In normal airways, CFTR functions as a chloride channel and regulates sodium influx and hydration of the ASL (**Figure 5A**). In the case of CF, defects of the CFTR channel causes impaired chloride ion efflux from the epithelial cells into the respiratory mucus as well as an increased sodium influx in the other direction through the epithelial Na^+ channel (ENaC) (**Figure 5B**). Consequently, a net increase of water absorption into the cells is observed, which induces a reduction of the epithelial lining fluid (ELF), thereby leading to enhanced mucus viscosity and elasticity [136]. Sticky mucus then impairs the cilia beating, which is responsible for removal of pathogens, thus providing a favorable environment for bacterial infection and related inflammation [137,138].

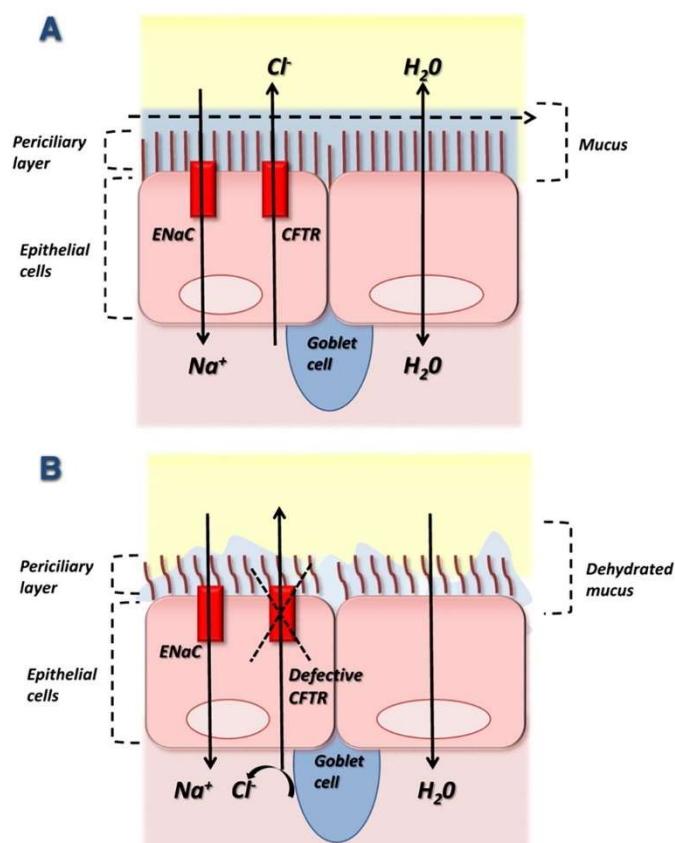


Figure 5: Impact of the CF disease on CFTR channel function. **A.** Cl^- secretion and Na^+ absorption control hydration of the epithelial lining fluids in healthy individuals. **B.** Absence or dysfunction of CFTR protein leads to impaired Cl^- transport, enhanced Na^+ absorption through the epithelial Na^+ -channel (ENaC), resulting in dehydration of the airway surface fluid layer and poor mucociliary clearance (adapted from [139]).

I.4.2. Chronic bacterial infections and inflammations

The primary source of morbidity and mortality in CF is caused by the progressive destruction of the airways. This decline results from chronic inflammation arising from the microbial colonization of the lungs [137].

During early stages of life, CF lungs are frequently infected with *Staphylococcus aureus* (*S. aureus*; SA) and/or *Haemophilus influenzae* (*H. influenzae*) [17,73]. Older individuals, such as teenagers and young adults, then predominantly suffer from colonization of *Pseudomonas aeruginosa* (*P. aeruginosa*; PA) but also from other opportunistic gram negative organisms [140,141]. The related chronic infection affects the likely course of the medical condition since *P. aeruginosa* has been proven to drive function decline, resulting in increased morbidity [142–145]. Also, persistent infections lead to generation and secretion of several

cytokines such as interleukin (IL)-6 and IL-8 [146,147], thereby recruiting polymorphonuclear cells into the airways [148]. In CF lungs, neutrophils represent about 70% of the inflammatory cell population, whereas only 1% of neutrophils are present in healthy lungs [149]. Release of toxins and elastases from *P. aeruginosa* amplifies the course of infection and inflammation. These substances and enzymes cleave markers on the surface of neutrophils, resulting in the release of neutrophil-derived proteases that exacerbate injury of viable polymorphonuclears in the lungs [150]. However, thanks to the use of therapies intended to eradicate the initial acquisition of *P. aeruginosa* [151], its prevalence has decreased since 1990 [17].

CF airways can be infected by other pathogens such as *Methicillin-resistant Staphylococcus aureus* (MRSA), *Stenotrophomonas maltophilia* (*S. maltophilia*), *multidrug-resistant P. aeruginosa* (MDR-PA), *Achromobacter* and *Burkholderia cepacia* (*B. cepacia*) complex [17,152]. The prevalence of the main bacteria found in CF lungs is reported in **Table 2**.

Table 2: Prevalence of bacteria found in CF lungs (adapted from [114]).

Bacteria	% of patients infected
<i>Staphylococcus aureus</i>	70.6
<i>Pseudomonas aeruginosa</i>	47.5
MRSA	26.0
<i>Haemophilus influenzae</i>	15.5
<i>Stenotrophomonas maltophilia</i>	13.6
MDR-PA	9.2
<i>Achromobacter</i>	6.1
<i>Burkholderia cepacia</i> complex	2.6

Drug resistance is an inevitable problem in CF-related infection, associated with the difficulty to eradicate pathogens responsible for chronic infections. In cases of *S. aureus*, MRSA is defined as resistance to the antibiotic methicillin. Over the last several years, the prevalence of MRSA infection has increased, especially in individuals between 10 and 30 years of age. In 2016, 26% of patients with CF were colonized with MRSA whereas only 6.1% were affected in 2000 [17,114]. High variations exist between CF care centers, with low concerns in Europe [153], and with prevalence ranging from 0 to 50% in the USA in 2013 [114]. MRSA is associated with poor lung function [154], it is often resistant to commonly used antibiotics and may be difficult to control [155]. There are no current recommendations or guidelines specific for MRSA treatment in CF [153]. In case of *P. aeruginosa*, MDR refers to resistance to all agents

in two or more classes of standard antibiotics (penicillins, cephalosporins, fluoroquinolones, aminoglycosides, etc.), hence being referred to as multidrug resistant [114,156,157]. MDR-PA has been associated with an increased number of exacerbations, accelerated rate of lung function decline as well as increased mortality [158]. Among all bacterial infections occurring in CF, MDR-PA is one of the rare pathogens appearing during adulthood [114].

Stenotrophomonas maltophilia is a gram-negative pathogen that colonizes patients with CF with a prevalence plateauing since 2005 [17]. It is not clear if *S. maltophilia* causes simple infection or if it is implicated in lung function decline. Also, the effectiveness of antibiotics therapy against this specific pathogen has not yet been demonstrated [159].

Achromobacter refers to gram-negative bacilli [160], with the most prevalent being *Achromobacter xylosoxidans* (*A. xylosoxidans*) [17]. *A. xylosoxidans* is found in 2 to 19.7% of patients with CF [161–163]. Chronic infection with this multidrug-resistant microorganism causes increased lung inflammation as well as reduced lung function [163,164]. Effective treatment can be challenging due to the resistance pattern of *A. xylosoxidans* [160]. In non-CF individuals, achromobacterial lung infections respond well to antibiotics such as penicillin and cephalosporins [160].

Burkholderia cepacia complex is a complex of at least nine different species [73,165]. Infection with *B. cepacia complex* may result in a rapid lung function decline and an increased mortality in patients with CF [166]. Its low prevalence has plateaued since 1990 and first infection occurs in adulthood, as it is the case for MDR-PA [17,114].

1.4.3. Impaired mechanisms of pathogen elimination

The respiratory tract is permanently exposed to inhaled particles, dusts and pathogens, and ASL represents the first line of defense to clear the airways without any potentially harmful inflammatory reaction [167,168]. ASL is a thin layer that covers the airway epithelium on its apical surface and is mandatory for effective mucociliary clearance [169]. It is composed of a mucus gel and a periciliary sol layer (PCL) (**Figure 5A**). The mucus gel is a slippery and watery substance, consisting of 97% water, with mucins, DNA, lipids, ions, proteins, cells and cellular debris accounting for the remaining 3% of the gel [170,171]. The diversity of the mucus polymers allows entrapment of a wide variety of particles, like bacteria, for further clearance

from the airways [172]. The PCL is an aqueous solution with a height of approximately 7 μm , characterized by a relatively low viscosity enabling effective ciliary beating [167]. In cases of CF, CFTR dysfunction impedes chloride transport into the mucus, which leads to dehydration of the PCL and thus the ASL. Loss of the regulatory effect of CFTR induces an increased transepithelial sodium absorption which worsens the dehydration phenomenon. Also, mucus dehydration increases its viscoelasticity and thus decreases its transportability. Therefore, cilia are unable to function properly, resulting in impaired mucociliary clearance and mucus stasis [138,139,167]. Mucus accumulation leads to the formation of adherent plaques on the airway epithelium [173], creating a favorable environment for pathogen persistence. In addition, high concentrations of polymeric macromolecules narrow the pores of the mucus from 500 nm to approximately 150 nm [174], resulting in poor neutrophil migration intended to clear the pathogens [175].

In healthy subjects, other important units such as leukocytes supplement the immune response by neutralizing the infectious agents that have escaped the mucociliary clearance [168]. Neutrophils are the major cellular components of the innate immune system [176], phagocytosing the pathogens in order to keep an infection under control until the development of adaptive immunological responses [167]. During an inflammatory response, recruitment of neutrophils occurs via several factors such as chemoattractants (including IL-8 [176]) and pro-inflammatory cytokines [167,177]. Following neutrophil migration to the site of infection, phagocytosis can begin [167,177]. After the successful process of recognition, ingestion, killing and degradation of ingested pathogens, neutrophils are removed by apoptosis [178], resolving the inflammatory process and thus avoiding further tissue damage [167]. In the case of CF, the airways are characterized by a neutrophil-dominated inflammatory process [179]. However, the overwhelming bacterial load and the mucus characteristics lead to neutrophil phagocytosis with reduced efficiency [177]. Also, the inability of neutrophils to migrate induces their accumulation, which may result in self-injury and the release of oxidants and proteases [177] such as serine proteases (including neutrophil elastase; NE) and matrix metalloproteinases (MMPs; including collagenase (MMP-8) and gelatinase (MMP-9) [180,181]. NE degrades structural proteins such as elastin and fibronectin, leading to the damage of the lung architecture [176]. It also stimulates IL-8 release, thus recruiting even more neutrophils into the lungs and perpetuates the cycle of inflammation by

further release of NE [182]. MMP-8 and MMP-9 may amplify lung inflammation and promote injury and airway-remodeling processes [183]. In normal lungs, the airways are protected from the damaging effects of proteases, especially NE, mainly by alpha-1 anti-trypsin (AAT) [176]. However, the elevated NE levels present in CF lungs [176] overwhelm AAT activity, since AAT is proteolytically degraded by NE at molar ratios exceeding 1:1 in favor of NE [184].

In response to inflammatory stimuli, neutrophils can also contribute to CF pathology through a cell death mechanism known as neutrophil extracellular trap (NET). After pathogen contact or prolonged activation, neutrophils release DNA fibers decorated with proteins (such as NE), forming NETs that disarm and kill bacteria present in the airways [185]. Dramatic morphological changes of neutrophils (flattening and membrane protrusions) allow the formation of NETs, mediated by different signaling pathways such as IL-8 or lipopolysaccharide [185]. On a regular basis, NETs function as antimicrobial units and restrict infections due to their ability to entrap almost all types of pathogens (Gram-positive and Gram-negative bacteria, yeasts, viruses and protozoan parasites) [186]. However, in cases of CF, their accumulation and death (NETosis) lead to pathological consequences such as airway obstruction (due to the presence of extracellular DNA derived from NETs) and destruction of tissues by their components such as NE [176,187–189].

As mentioned above, in CF, degeneration of neutrophils following their recruitment to the site of infection induces the release of DNA and of filamentous actin (F-actin) [190]. In normal conditions, F-actin is involved in the structural integrity of the cells [191]. However, in CF, the copolymerization of extracellular DNA and F-actin increases the viscosity and tenacity of the mucus [192], thus enhancing the thickness of the secretions containing pathogens and reducing their clearance [171].

I.5. THERAPEUTIC STRATEGIES FOR CF LUNG DISEASE

Conventional pulmonary treatments of CF target the disease symptoms such as mucus plugging and infection. These continuous therapies represent a major component of the CF treatment regimen [17]. However, more recent research has led to the emergence of therapies targeting the basic defect of CFTR, several of which are in clinical trials, or have progressed to approved treatments [14]. Therefore, medications used to treat CF lung disease

include strategies targeting specific CFTR mutations, mucolytic agents, airway surface rehydration therapies, antibiotics as well as anti-inflammatory agents [14,43,193,194]. Physiotherapy and exercise are also recommended for airway clearance [195] as well as nutrition since a strong correlation exists between nutritional status and lung function [73]. Lung transplantation represents the final option for patients with end stage lung disease, but the limited numbers of lungs available for transplant constitutes a non-negligible limitation.

I.5.1. Strategies targeting specific *CFTR* mutations

Progress in the mapping of the human genome as well as advances in molecular technology allowed for the initiation of targeted therapies research [65]. The well-defined genetic defect in CF has led to the development of strategies targeting specific mutations to improve CFTR function. Potentiators, correctors and production correctors constitute the three main categories of strategies targeting specific *CFTR* mutations.

I.5.1.1. *CFTR* potentiators

CFTR potentiators refer to compounds that increase the activity of defective CFTR at the cell surface (Figure 6), such as CFTR mutants expressed in the apical membrane. CFTR potentiators target gating or conductance defects that can be observed in Class III and IV mutations [42].

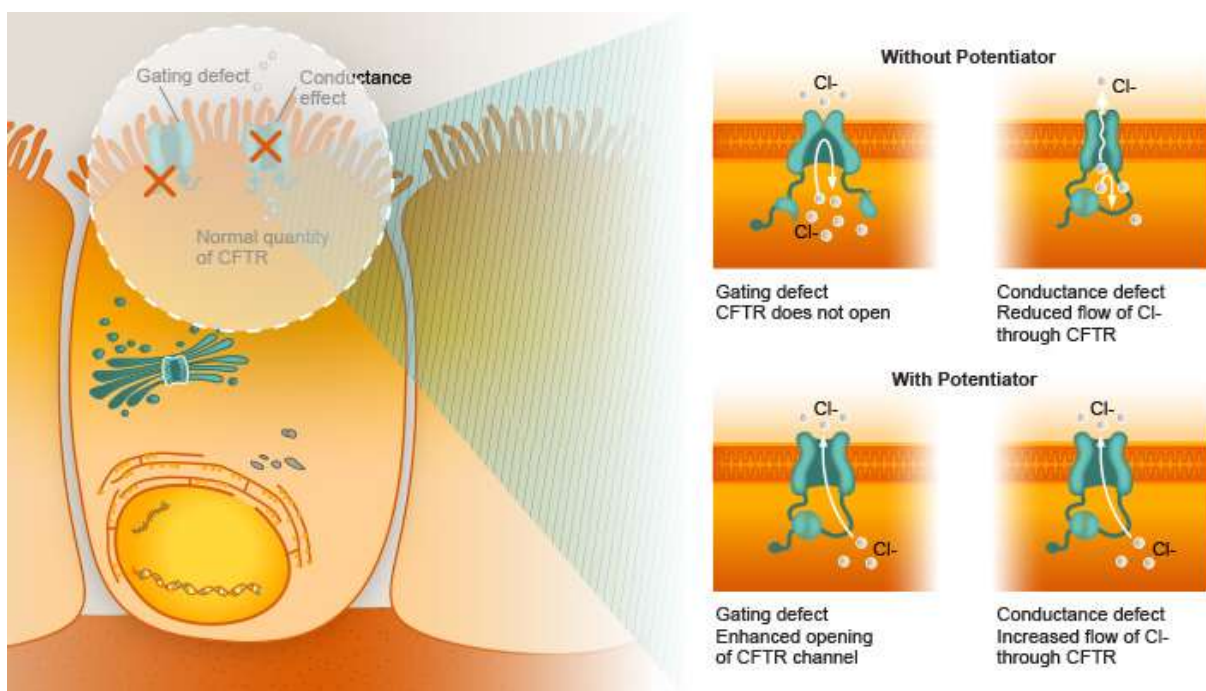


Figure 6: Role of CFTR potentiator on the opening or conductance of the CFTR channel [196].

Intensive research has led to the development of Ivacaftor, an oral medication that promotes opening of the chloride channel on the cell surface. In 2009, Van Goor and co-workers demonstrated that Ivacaftor significantly improved chloride transport, diminished exaggerated sodium absorption and increased cilia beating in bronchial epithelial cultures carrying the G551D mutation on one allele [197]. Ivacaftor has then been approved in 2008, under the name of Kalydeco® (Vertex pharmaceuticals, Boston, MA, USA). This dihydroquinoline carboxamide compound was first available to patients with CF with at least one mutation of G551D [42]. Although such class III mutations are individually rare (**Table 1**), Ivacaftor therapy was later approved for patients displaying a copy of other non-G551D CFTR gating mutations [65]. In 2017, 23 additional *CFTR* mutations, resulting in partially functioning CFTR protein at the cell surface, were added to the list of Ivacaftor-responsive mutations. That same year, five splice mutations, characterized as abnormalities in the *CFTR* DNA code resulting in an altered and ineffective CFTR protein, were also included in the mutations eligible for treatment with Ivacaftor. To date, Ivacaftor is approved for 38 *CFTR* mutations affecting people aged two and older [14]. However, although long-term benefits remain uncertain, improvement in lung function and weight gain have been observed in advanced CF lung disease [198–200].

1.5.1.2. *CFTR* correctors

CFTR correctors are employed to overcome defective protein intracellular processing resulting in the production of misfolded CFTR. These compounds improve the trafficking of CFTR mutants from class II mutation and increase their expression in the plasma membrane (**Figure 7**) [65].

Van Goor et al. evaluated the *in vitro* efficacy of a CFTR corrector named Lumacaftor [201]. They highlighted that F508del CFTR corrected with this small molecule compound acquired biochemical and functional characteristics similar to a normal channel. They also demonstrated that Lumacaftor improved CFTR processing and chloride secretions in bronchial epithelial cells derived from individuals with CF presenting two copies of the F508del mutation.

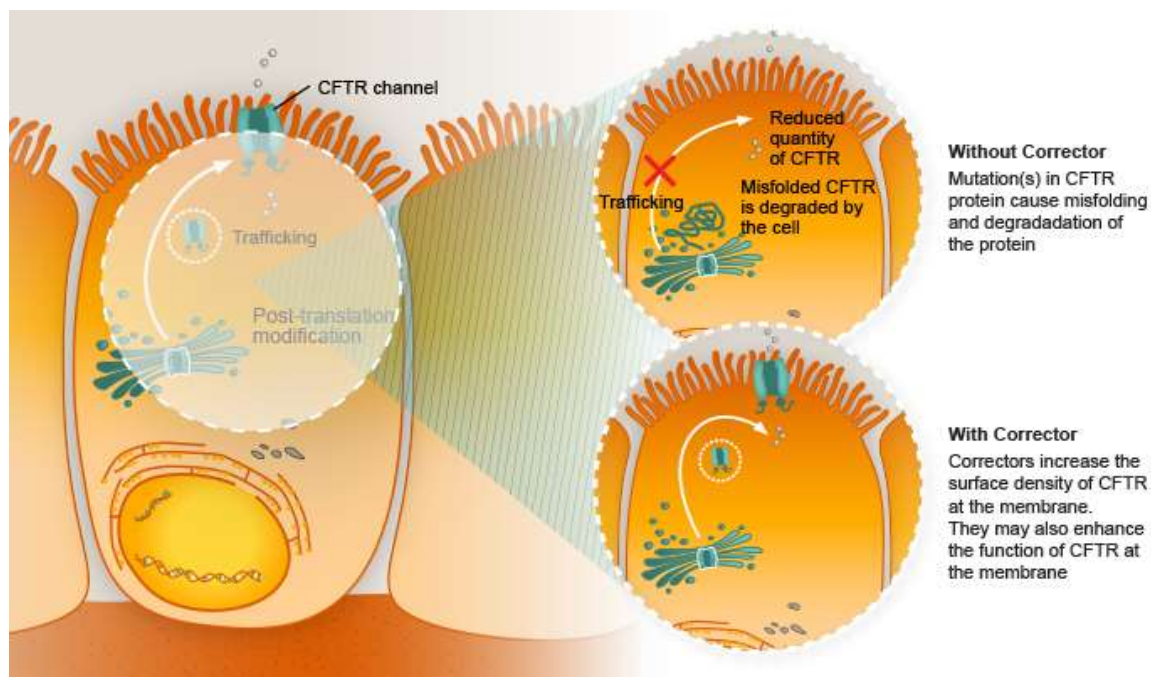


Figure 7: CFTR corrector role in CFTR protein trafficking to the membrane [196].

In the case of F508del-CFTR operating as a class III mutation, CFTR potentiator compounds, such as Ivacaftor, became a co-therapeutic option. In 2011, Rogan et al. reported that potentiator compounds could increase the F508del-CFTR-dependent ion transport [42]. The positive impact of the investigations has permitted to consider the combination of therapies to enhance mutant CFTR function.

Orkambi® is the commercial name of Lumacaftor and Ivacaftor combination, developed by Vertex pharmaceuticals. Conceptually, Lumacaftor allows fixing the defective trafficking of the CFTR protein, thus correcting its proper expression in the apical membrane of epithelial cells. In turn, Ivacaftor potentiates the channel opening function at the cell surface. Phase III studies demonstrated the clinical efficacy of the Lumacaftor and Ivacaftor combination in patients homozygous for F508del [202]. However, this combination therapy did not improve lung function in patients with CF who were heterozygous for the F508del mutation [203,204]. The fact that F508del possesses properties of a class VI mutation might partly explain why Lumacaftor and Ivacaftor combination led to poor results in patients heterozygous for F508del. To date, Orkambi® is approved for oral administration for people with CF aged six and older, displaying a homozygous F508del gene mutation [14].

Another combination therapy of Ivacaftor and a CFTR corrector (Tezacaftor), that shares structural feature with Lumacaftor, has recently been approved for clinical use under the name Symdeko®. Symdeko®, developed by Vertex pharmaceuticals is commercially available for patients aged twelve years and older displaying two copies of the F508del mutation and in patients with one F508del mutation copy and a second copy of a *CFTR* mutation predicted to retain residual function, such as class IV mutations. Symdeko® is currently in a phase III clinical trial for children aged six through eleven years [14].

Other CFTR potentiators, correctors and combinations are in the drug development pipeline for CF from preclinical studies up to phase II clinical trials and can be viewed on the CF Foundation website [14].

1.5.1.3. CFTR Production correctors

Production correctors, also named as read-through agents, promote transcription of CFTR in class I mutations [65]. Despite the presence of a premature stop codon (PSC) in the mRNA sequence, production correctors permit the production of a full-length CFTR protein, further transported to the Golgi apparatus for post-translational modification (**Figure 8**). Ataluren, which is an orally available agent marketed in Europe for the nonsense mutation Duchenne muscular dystrophy, has recently failed to significantly improve respiratory function in class I CFTR mutation, according to results of a phase III clinical trial [205]. To the best of our knowledge, no other CFTR production correctors are currently under investigation for CF lung disease.

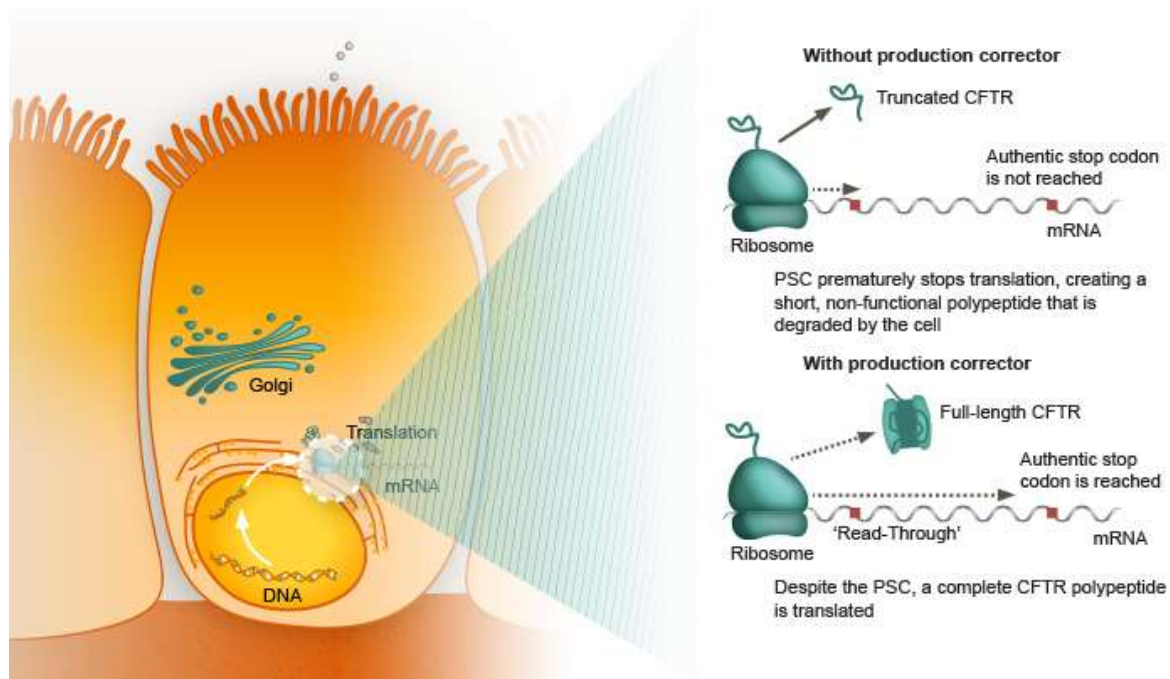


Figure 8: Role of CFTR production corrector on the translation of the mRNA sequence [196]. PSC refers as Premature Stop Codon.

1.5.2. Symptomatic treatment of CF lung disease

1.5.2.1. Mucolytic agents

The mucociliary clearance is one of the main concerns in CF lung disease. The use of mucolytic agents facilitates expectoration of pulmonary secretions, reduces infection frequency and diminishes airway damage caused by inflammation [206].

In 1990, Shak and co-workers demonstrated that recombinant human deoxyribonuclease I (rhDNase) reduced viscoelasticity of CF secretions by hydrolyzing extracellular DNA [4]. To date, rhDNase is the referent mucolytic agent prescribed in patients with mild to severe lung disease and is commercially available under the name Pulmozyme® (Roche, Basel, Switzerland) [207]. rhDNase is recommended for all patients, irrespective of CFTR mutations [17]. This inhaled medicine also allows for improved lung function and reduced pulmonary exacerbations [208]. In 2016, 87.5% of patients aged six years and older underwent rhDNase treatment in the United States. rhDNase was also prescribed to 41.5% of infants below three years and to 70.7% of children between three and five years of age [17]. However, several

studies demonstrated that rhDNase treatment displayed different clinical outcomes among individuals with CF [209–211]. The large inter-individual variability has led to the conclusion that about 30% of patients do not benefit from rhDNase therapy [190]. The reason behind the failure of the treatment is unclear, although Sanders et al. suggested that the concentration of magnesium ions in CF sputa might be involved in resistance to rhDNase therapy. The authors highlighted that clinical non-responders presented lower levels of magnesium ions in their pulmonary secretions compared to clinical responders. They speculated that oral intake of magnesium could mediate actin polymerization, decrease the content of globular actin - a potent inhibitor of rhDNase - in CF sputa and thereby increase rhDNase activity in clinical non-responders [190]. A complete overview of rhDNase is provided in the **I.6 section** on page 33.

The mode of action of N-acetyl-L-cysteine is thought promoting breaks in the disulfide bridges between proteins, especially the mucins present in the mucus [212,213]. Short- and long-term clinical trials were conducted on oral or inhaled N-acetyl-L-cysteine and showed none or few beneficial effects on CF lung function [214–216]. Therefore, no consistent evidence supported the use of N-acetyl-L-cysteine in CF.

1.5.2.2. Airway surface rehydration strategies

Inadequate ASL is considered as one of the main factors in the development of CF lung disease. Therefore, one treatment approach is to increase the fluid layer with an inhaled osmotic agent [217].

Hypertonic saline was first investigated for its beneficial effect on mucociliary transport [218]. Cough induction and mucus hydration were suggested to be the underlying mechanisms associated to hypertonic saline treatment [218]. However, Donaldson et al. suggested that hypertonic saline also increased the hydration of ASL, leading to improved lung function in the short-term [219]. A phase III clinical trial conducted in Australia highlighted beneficial effects of hypertonic saline on pulmonary health in people with CF aged six years and older. This multicenter study showed a relatively modest improvement in lung function but an interesting reduction in pulmonary exacerbations [220]. Hypertonic saline is approved for aerosolization [14] and is commonly used with an associated bronchodilator as it may cause bronchoconstriction [43]. The beneficial effects of hypertonic saline, combined with its low

cost and its few adverse events, may lead to its wide use in CF. However, the net benefits of hypertonic saline treatment are well below those of rhDNase [3].

Inhaled powdered mannitol, commonly referred to as Bronchitol® (Pharmaxis, New South Wales, Australia) is being tested as an alternative to hypertonic saline. Mannitol is a polyol (sugar alcohol) used as an osmotic agent for inducing rehydration of ASL and, hence, clearance of mucus [43]. In 2012, a phase III study in people with CF aged six years and older was deemed successful, leading to Bronchitol® approval for CF treatment in Australia and in the United Kingdom [14]. The product, delivered as a twice daily administration, is also approved for treatment of adults with CF throughout the European Union [221]. Improved lung function and reduction of pulmonary exacerbations were the main outcomes of the phase III study mentioned above [222]. More recently, a second phase III clinical trial, designed for future approval in the United States, was exclusively conducted in adults and demonstrated the safety and effectiveness of Bronchitol® [223]. A submission to the Food and Drug Administration (FDA) is expected in 2018.

Spyryx Biosciences, Inc. (Durham, NC, USA) is developing SPX-101, a peptide intended to block ENaC hyperactivation in CF [224]. SPX-101 allows mimicking of the natural regulation of ENaC activity by internalizing the channel from the membrane. Preclinical data highlighted an improved mucus transport due to regulation of airway hydration [225]. A phase II clinical trial is currently investigating the efficacy and safety of inhaled SPX-101 in patients with CF [226].

1.5.2.3. Antibiotics

Conventional antibacterial agents are used in four different contexts for the treatment of CF lung disease: prophylaxis, early infection eradication, chronic infection therapy and exacerbation reduction [43]. The use of effective antibiotics for prevention, eradication and control is part of the decreased respiratory morbidity and increased survival observed in patients over the decades [114,227]. Lack of antibiotic treatment during childhood may cause early infection and inflammation, further leading to the establishment of, and later fatal respiratory failure. **Table 3** summarizes the different FDA approved antibiotics for CF lung disease therapy as well as the medications currently investigated in phase III clinical trials.

Prophylactic treatment could be employed to reduce the prevalence of *S. aureus* infection observed at early stage of life. In 1994, Weaver and co-workers highlighted that the continuous use of the anti-staphylococcal antibiotic Flucloxacillin (a beta-lactam antibiotic of the penicillin class) allowed to reduce *S. aureus* infection incidence as well as hospitalization rate in infants under two years of age [228]. However, this clinical trial failed to show an associated improvement in lung function. In 2002, Stutman et al. highlighted that prophylaxis with Cephalexin (a cephalosporin class antibiotic) successfully delayed the acquisition of *S. aureus* in infants with CF but also enhanced *P. aeruginosa* colonization [229]. Limited evidence of pulmonary function improvement was also reported in other investigations on anti-staphylococcal antibiotics [230]. Due to controversial outcomes, in 2007 the United States guidelines did not recommend the prophylactic use of oral anti-staphylococcal antibiotics in individuals with CF [3]. More recently, the CF Foundation neither recommended the use of prophylactic antibiotic therapy against *P. aeruginosa* [151] since the clinical trial on the use of Colistin (belonging to polymyxin antibiotics class) and Ciprofloxacin (a fluoroquinolone class antibiotic) as prophylactic antibacterial agents did not clearly demonstrate the evidence of reduced risk of initial or chronic *P. aeruginosa* infection in patients with CF [231]. To the best of our knowledge, no additional clinical trials are currently investigating prophylactic therapies for CF lung bacterial infections.

Patients with *P. aeruginosa* colonization have a two- to three-fold increased risk of death over an eight year period compared to individuals without *P. aeruginosa* colonization [145]. Therefore oral, inhaled and/or intravenous antibiotics are prescribed and eradication is successful at 73% to 85% in case of new *P. aeruginosa* infection [232–234]. Eradication strategies towards *P. aeruginosa* vary between countries, with inhaled Tobramycin (a aminoglycoside class antibiotic) preferably used in the United States [151] while in Europe, the use of a combination of oral Ciprofloxacin and nebulized Colistin is recommended [235,236]. To date, a clinical trial in the United Kingdom is currently investigating the benefits of intravenous Ceftazidime (from cephalosporin antibiotics class) with Tobramycin *versus* oral Ciprofloxacin; both treatment candidates being administered with nebulized Colistin [237].

Once bacterial infections become chronic, emphasis is directed on control of the inflammatory response. Intravenous, oral and inhaled antibiotics are used to control infection [238]. Antibiotics delivered by intravenous route allow for a reduction in pulmonary inflammation

but present short-lived effects. Also, their benefits are lost once the intravenous therapy is discontinued [239]. Oral antibacterial agents do not present clinical evidence of efficacy on the bacterial burden observed in CF lungs. In addition, their frequency of administration may lead to rapid development of antimicrobial resistance [238]. Therefore, nebulization of antibiotics intended to suppress chronic bacterial infection represents the best option. This route of delivery allows to achieve high drug concentrations with low systemic toxicity [240], thereby improving lung function [241,242]. Patients with chronic *P. aeruginosa* colonization are typically prescribed Tobramycin, Colistin and Aztreonam (a monobactam class antibiotic) [243], although the use of Colistin has decreased in recent years [17].

Table 3: Conventional antibiotics used for CF lung disease [3,14]

Drug	Bacteria and context	Route of administration	Patients	Characteristics	Pipeline
Azithromycin	<i>P. aeruginosa</i> chronic infection and/or Pulmonary exacerbations	Oral	Over 6 years	- Improved lung function, weight gain and decreased respiratory exacerbations [438–440] - Decreases exacerbations in young patients not infected with PA [441]	FDA approval in 1996
Aztreonam (Cayston®)	<i>P. aeruginosa</i> chronic infection	Nebulization	Over 6 years	- Improved lung function, reduction in bacterial burden, weight gain [442] - Reduction in pulmonary exacerbations [443]	FDA approval in 2010
Colistin	<i>P. aeruginosa</i> chronic infection	Nebulization or inhalation	Over 2 years	Reduced bacterial content in CF sputum and increased FEV₁ [444]	Approved
Inhaled tobramycin (TOBI® or BETHKI®)	<i>P. aeruginosa</i> chronic infection	Nebulization	Over 6 years	- Solution - Improved lung function and quality of life, reduces hospitalization rate [445–448]	FDA approval in 1997 (TOBI®) and 2012 (BETHKI®)
TOBI® PodHaler™	<i>P. aeruginosa</i> chronic infection	Inhalation	Over 6 years	- Pocket-sized inhaler - Powder - Reduced administration time (5.6 min versus 19.7 min for TOBI®) [242] - Better adherence compared to TOBI® [449]	FDA approval in 2013
Vancomycin (AeroVanc™)	MRSA infection	Inhalation	Over 6 years	Reduced MRSA density and improved pulmonary function [450]	Phase III

1.5.2.4. Anti-inflammatory agents

As inflammation plays a pivotal role in the progression of CF lung disease, anti-inflammatory medications such as Ibuprofen can be prescribed. In 1994, a four-year clinical trial demonstrated that Ibuprofen (i.e. isobutylphenylpropionic acid) lessened lung function decline, especially in patients with CF below the age of 13 when they were enrolled in the study [244]. A two-year multicenter trial also showed beneficial effects on lung function and safety of Ibuprofen [245], consolidated by an analysis of observational data from the CF Foundation [246]. Despite these positive outcomes, challenges associated with obtaining a pharmacokinetic study for appropriate dosing and concerns about adverse effects led to poor prescription of Ibuprofen in CF [247]. Later, the CF Foundation pulmonary guidelines committee highlighted insufficient evidence to recommend the use of Ibuprofen for adults with CF [3]. Consequently, in 2016, the CF Foundation reported that no children were under Ibuprofen therapy and that less than 2% of adults used it [17].

1.5.2.5. Physiotherapy, exercises and nutrition

The CF Foundation pulmonary guidelines recommend airway clearance for all individuals with CF [195]. Airway clearance techniques include postural drainage, positive expiratory pressure devices, forced expiration, high frequency chest wall oscillation (HFCWO) and exercise [17,73]. Convenience, ease of administration and patient satisfaction are the main driving forces for the choice of airway clearance method. In 2016, 74.8% of patients with CF older than two years preferred HFCWO [17]. Exercise is also beneficial for airway clearance as well as for the overall health of the patients [73,195]. The last update of the CF Foundation reported that more than 40% of individuals with CF between 10 and 40 years of age used exercise as a primary or secondary airway clearance technique [17].

A strong correlation exists between nutritional status and pulmonary function, as reported by the 2016 Patient annual data report of the CF Foundation [17]. Therefore, attention to nutritional wellbeing should be considered as a key parameter for CF lung health.

1.5.2.6. Lung transplantation

Lung transplantation is the final therapeutic option for patients with end-stage CF lung disease. Overall, 265 individuals received a lung transplant in 2016, with 6.2% transplants referring to pediatric patients [17]. In Europe, transplantation is unusual in children with CF. However, in cases of maximum medical therapy failure, children with a projected life expectancy of less than two years can be considered for a lung transplant [73]. Post-transplant, more than 70% of adults survive for five years [248]. Liou and co-workers showed that children with a five-year predicted survival of less than 50% had no survival improvement from lung transplantation and that pediatric patients with a five-year predicted survival between 50% and 90% had decreased survival following lung transplantation [249]. Therefore, lung transplantation in CF may improve the survival of adults but should be carefully considered for children. Finally, age, sex, lung infection and colonization as well as rate of lung function decline are all parameters that affect the decision of undergoing lung transplantation [73].

1.6. RECOMBINANT HUMAN DEOXYRIBONUCLEASE I

Human deoxyribonuclease I is an endonuclease catalyzing the hydrolysis of extracellular DNA [250]. This 260-amino residue enzyme is secreted by the pancreas as well as by the parotid glands. Its presence has also been reported in blood, urine and other tissues [5,251]. Human deoxyribonuclease I nonspecifically cleaves the phosphodiester bonds of double-stranded DNA into fragments [5]. Recombinant human deoxyribonuclease I (rhDNase) is a monomeric 30 kDa glycoprotein, with identical amino acid sequence to that of the native enzyme purified from urine [5]. rhDNase is prescribed in the treatment of CF lung disease [2].

1.6.1. Historical overview

1.6.1.1. Bovine pancreatic deoxyribonuclease

In the 1950s and 1960s, intensive research demonstrated that bovine pancreatic deoxyribonuclease significantly reduced the sputum viscoelasticity associated with lung disease, by cleaving extracellular DNA contained in it [252,253]. However, significant

immunological complications and adverse effects such as bronchospasm prevented its clinical use [253,254].

1.6.1.2. Recombinant human deoxyribonuclease I

In the 1990s, Shak et al. developed a recombinant version of the human deoxyribonuclease I (rhDNase) [4]. The authors reported that rhDNase had similar mucolytic effects to bovine deoxyribonuclease, transforming thick and purulent sputum into a flowing liquid. A short-term clinical evaluation of nebulized rhDNase in the treatment of CF lung disease highlighted that rhDNase significantly improved lung function by cleaving DNA contained in purulent secretions [255]. Also, no significant adverse effects were observed following nebulization of the protein [255,256]. A large short-term investigation in pediatric and adult patients with CF showed lung function improvement, safety as well as perception of well-being [257]. rhDNase received FDA approval for treatment of CF in 1993 [14].

1.6.2. Formulation

rhDNase is produced in genetically engineered cells of Chinese hamster ovary with DNA encoding for the native human protein [4]. rhDNase is manufactured by Genentech, Inc. (South San Francisco, CA, USA) and commercialized under the branded name Pulmozyme® by Roche (Basel, Switzerland). rhDNase is formulated as a sterile aqueous solution containing 1.0 mg/mL of rhDNase, 0.15 mg/mL of calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), and 8.77 mg/mL of sodium chloride (NaCl). The pH of the solution is 6.3 [5].

1.6.3. Pharmacology and clinical use

In the early 1990s, a long-term study involved patients with moderate and stable CF lung disease. rhDNase-treated children and adults showed significant decrease in respiratory exacerbations and hospitalization rate and required fewer antibiotics compared to the placebo group. This long-term clinical evaluation was also associated with rhDNase tolerability [208]. McCoy and co-workers then evaluated the efficacy of rhDNase in patients with advanced CF lung disease [258]. Over the 12-week study period, rhDNase treatment was shown to be safe, well tolerated and significantly improved lung function. More recently, analysis of the Epidemiologic Study of CF highlighted a reduction of lung function decline in patients treated with rhDNase for two years [259]. The observed clinical benefits of rhDNase

on CF lung disease have led the CF Foundation to recommend this therapy to patients aged six years and older with moderate to severe lung disease [3]. Intensive investigations suggested that rhDNase may present beneficial effects if prescribed during the early course of the disease [259,260]. Therefore, the CF Foundation also recommends to prescribe rhDNase to patients with milder lung disease to improve their lung function and reduce exacerbations [3].

rhDNase is prescribed to the majority of individuals with CF [17], in association with standard therapies for CF lung disease (see Therapeutic strategies for CF lung disease). The recommended daily dose is 2.5 mg of rhDNase and some patients over the age of 21 might benefit from double the daily dosage to reduce the exacerbation risk [207]. rhDNase is delivered to the lungs through nebulization with recommended systems.

Deposition of a drug in the airways is driven by the particle size of the aerosol [261]. Homogeneous lung deposition of rhDNase is achieved for particle size ranging between 1 and 6 μm [262]. Two types of nebulization systems (jet and vibrating membrane nebulizers) are commercially available for pulmonary delivery of rhDNase. Jet nebulizers produce aerosols in the desired respirable range of 1-6 μm , with a mass median aerodynamic diameter of 4 to 6 μm [263]. The operation requires a compressed air source delivered through a jet, which causes a region of negative pressure in the system. The medication solution (or suspension) enters the gas stream and is sheared into an unstable liquid film, then broken into droplets due to surface tension forces [264]. The physical stresses induced by high shear rate (creation of a large air-water interface, high pressure and/or turbulence) might impact the integrity of the protein molecule. However, in case of rhDNase, no apparent degradation of the protein structure or enzymatic activity has been reported [262]. Jet nebulizers were the first systems approved by the FDA for rhDNase delivery [208]. However, they are time consuming, bulky, noisy and require a power source [265]. Significant advances in nebulizer technology have occurred since the original approval of rhDNase treatment in CF. Vibrating membrane nebulizers such as the eFlow® (PARI Pharma, Munich, Germany) provide a more rapid and efficient delivery of rhDNase particles in the respirable range. The technology of the vibrating perforated membrane preserves the protein structure integrity and allows to create an aerosol with a particle size of 4 μm , comparable to the jet nebulizer systems [265]. Therefore, vibrating membrane nebulizers are considered the most effective, compact, quiet and rapid

delivery systems for rhDNase. Considering that patients with CF often take several daily aerosol medications, nebulization of rhDNase with systems such as the eFlow® might reduce the time burden and thus might increase patient compliance.

I.6.4. Limitations and potential strategies

Despite its beneficial outcomes for patients with CF, rhDNase therapy presents limitations. The main limitation of rhDNase concerns its clearance from the lungs. Lazarus and Wagener reported a rapid elimination of the protein following inhalation [5]. Immediately after delivery, a concentration of 3 µg/mL of rhDNase was measured in CF sputum. Two hours post-delivery, only 0.6 µg/mL of rhDNase were recovered from the CF samples. These results support the requirement to deliver the protein once to twice daily.

rhDNase therapy also suffers from other limitations related to the following biochemical parameters:

- Globular actin (G-actin), is referred as a potent inhibitor of rhDNase [266]. An equilibrium exists between G- and F-actin forms and is influenced by the concentrations of metal ions such as calcium, magnesium and potassium [267]. High concentrations of F-actin have been reported in CF lung secretions [268], potentially depolymerizing from filamentous to globular actin.
- rhDNase stability and activity are dependent on the presence of divalent cations such as calcium and magnesium [269–271]. Therefore, a deficient ion microenvironment might impact the protein efficiency, as suggested by Sanders et al [190].

Finally, adherence to rhDNase therapy might be reduced due to the chronic condition of CF. Impaired adherence to medication may be associated to complications, increased risk of infections and thereby greater health service need and cost. Overall, recent investigation reported that treatment adherence for chronic disease is approximately 50%. Concerning rhDNase, mean adherence was estimated to reach 59% but overestimation may have occurred since patient refill was recorded while it did not account for respect of posology [6].

Therefore, strategies have emerged to circumvent the limitations of rhDNase. They focus on the development of hyperactive and actin-resistant variants.

Pan and Lazarus engineered variants with a hyperactivity for DNA hydrolysis [272]. The rationale was based on introducing positively charged residues (such as arginine and lysine) on rhDNase at its DNA binding interface. Formation of salt bridges with phosphates on the DNA backbone allowed to increase rhDNase binding affinity for DNA. The authors demonstrated that the introduction of positive charges in rhDNase resulted in an *in vitro* DNA cleavage activity up to 35-fold higher in the modified than in the non-modified protein.

The introduction of charged, aliphatic or aromatic residues on the central hydrophobic actin binding interface of rhDNase allows the formation of actin-resistant variants [273]. Protalix (Karmiel, Israel) is currently developing alidornase alfa (PRX-110), a chemically modified actin-resistant rhDNase [274]. A recent phase II clinical trial succeeded in demonstrating the safety and tolerability of alidornase alfa. Also, a reduction in DNA content and sputum viscoelasticity were recorded and correlated with the improved lung function observed in the patients enrolled in the study [275].

Combinations of hyperactivity and actin-resistance properties have led to new engineered candidates for CF therapy [276]. The data obtained using CF sputum highlighted that the level of hyperactivity was inversely proportional to the DNA concentration and length [276]. Therefore, considering that CF sputum contains high concentrations of DNA [268], the beneficial effect of hyperactive variants over non-modified rhDNase might be limited.

Other strategies could be considered to overcome the limitations of rhDNase. Covalent attachment of polyethylene glycol (PEG) chains to proteins, named as PEGylation, is a well-established strategy for improving pharmacokinetic properties as well as therapeutic efficacy [277]. PEGylation allows for a reduced clearance, prevents proteolytic degradation while also presenting other positive effects on proteins. PEGylation of rhDNase might prolong its presence within the lungs and thus overcome the main drawback of rhDNase therapy, i.e. its rapid elimination.

II. PEGYLATION, AN APPROACH FOR IMPROVING THE PULMONARY DELIVERY OF BIOPHARMACEUTICALS

Adapted from: *PEGylation, an approach for improving the pulmonary delivery of biopharmaceuticals*, Current Opinion in Colloid & Interface Science, 2017
Marie-Julie Guichard, Teresinha Leal and Rita Vanbever

II.1. INTRODUCTION

Biopharmaceuticals are biological products intended for medicinal purposes that include peptides, proteins, antibodies, antibody fragments, enzymes and nucleic acids. This class of pharmaceuticals has grown since the first product, insulin, was commercialized in 1982 [278]. To date, more than 200 biopharmaceuticals are already approved in the United States or in Europe for the treatment of various diseases [279]. Biopharmaceuticals have been widely delivered by injection and more recently, their pulmonary administration has been investigated [280].

Pulmonary delivery is an attractive route of drug administration and it represents an alternative to parenteral delivery. Pulmonary administration can be used for local or systemic drug delivery and it presents several advantages [240,281]. Inhalation of drugs using portable inhalers is convenient for the patients. Inhalation aerosols are extremely efficient to treat respiratory diseases because they allow a targeted therapy with low delivered doses and high drug concentrations in the tissue of interest. The low local dosing reduces systemic side effects. The large surface area of the alveoli, the low thickness of the alveolar epithelium and the high vascularization can result in rapid systemic drug absorption. As a non-invasive route of drug administration to the systemic circulation, pulmonary drug delivery avoids the hepatic first-pass metabolism, the degradation of the biopharmaceutical in the gastro-intestinal tract and may result in higher patient compliance since it does not induce pain contrary to injection [282]. However, clearance mechanisms such as mucociliary clearance, alveolar macrophages uptake and proteolytic breakdown may lead to rapid protein elimination [283–285].

Several techniques have been used to circumvent the rapid elimination of biopharmaceuticals from the lungs. Nanoparticles and liposomes have been well described but those technologies

do not permit to deliver high doses of proteins and their preparation can be stressful for the biopharmaceuticals [286,287]. PEGylation is a well-established approach to improve the therapeutic value of proteins since conjugation to polyethylene glycol (PEG; **Figure 9**) improves the stability of the protein and prolongs its retention in intact form in the body. PEGylation involves the covalent attachment of one or several PEG chains to a molecule [288]. PEG is a polymer composed of repeated ethylene glycol units (**Figure 9**). It is soluble in water and organic solvents, non-toxic and approved by the Food and Drug Administration in food and cosmetics, as an excipient in drug formulation and for drug modification [289]. PEGylation has been designed to increase the size and confer a shielding effect to the conjugated molecule, leading to optimization of its pharmacokinetic properties. The increase in molecular size and the steric hindrance prolong the protein residency in the body due to reduced renal clearance, enhanced proteolytic resistance and reduced recognition by specific antibodies. The main advantages that PEG conjugation offers to molecules is an increased half-life, a reduced immunogenicity and an improved physical stability [290]. Initially, PEGylation of proteins has been evaluated for improved performance following injection and twelve PEG-protein conjugates have already been marketed since 1990s [18]. To date, many more PEGylated biopharmaceuticals are currently in preclinical or clinical development and pulmonary delivery is considered as an administration route for some of them [291].

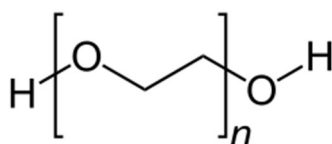


Figure 9: Structure of polyethylene glycol (PEG), with n being the number of ethylene glycol unit.

Several coupling chemistries have been described for PEGylation of biopharmaceuticals. A variety of mono-functionalized PEG reagents have been used, including linear and branched polymers with molecular weight (MW) up to 40 kDa [18]. The nature of the functional group attached to one end of a PEG chain allows reaction with different amino acids, such as the N-terminal residue, lysine and cysteine [292]. Random and site-directed PEGylation strategies using acylating, alkylating and thiol modifying PEG reagents have been used for the PEGylated biopharmaceuticals already on the market [18]. Acylating PEG reagents react with amines such

as the N-terminus to form an amide linkage. Coupling a protein with an alkylating PEG reagent yields a stable amine linkage and aldehyde-functionalized PEG may be used for site-selective PEGylation of the N-terminal amino acid residue of proteins. Finally, PEG maleimide is used for modification of thiol groups such as of cysteines. However, a limited number of 'free' cysteine residues is usually available in proteins. Therefore, a cysteine might be added by protein engineering, allowing site-specific PEGylation.

Protein conjugation to PEG increases its half-life but may cause an interference with the biological activity of the biopharmaceutical. Therefore, it is essential to consider site-specific PEGylation instead of random-site attachment of PEG. If the conjugation is non-specific, the PEG may interfere with the active site of the biopharmaceutical leading to reduced biological activity [290]. Thus, the optimum number and size of the PEG to be covalently attached as well as the site of PEGylation must be well defined to obtain an optimal balance between the biological activity and the pharmacokinetics of the conjugate. Yet, the pharmacokinetic advantages offered by PEGylation largely exceed the drawback of reduced activity. In this review, we focus on PEGylation as a promising approach for the pulmonary delivery of biopharmaceuticals.

II.2. PULMONARY ADMINISTRATION OF PEGYLATED BIOPHARMACEUTICALS FOR LOCAL DELIVERY

II.2.1. Sustained residence within the lungs

Although only one protein (recombinant human deoxyribonuclease I) for inhalation is currently available on the market, more and more are developed for a local administration to the lungs to treat various respiratory diseases [280]. The rapid elimination of proteins from the lungs implies the requirement to deliver these biopharmaceuticals once to twice daily and this high treatment burden may lead to low patient compliance [281,293,294]. PEGylated versions of biopharmaceuticals have been investigated for pulmonary delivery and have been shown to sustain protein residency within the lungs. Therefore, PEGylation presents the potential to reduce the frequency of the pulmonary administration of therapeutic proteins, and thereby to improve patient compliance and therapeutic outcome of inhaled medications. It is likely that as inhaled proteins will become more broadly available, more attention will be given to their sustained presence within the lungs and to protein PEGylation. Cantin et al.

reported that PEGylation of recombinant human alpha1-antitrypsine with a 20 kDa PEG led to persistence of the conjugate in autoradiograms of bronchoalveolar lavage (BAL) after intranasal instillation in mice for at least 48 h whereas the non-PEGylated counterpart was cleared within 24 h [295]. Plasma-derived alpha1-antitrypsine is commonly delivered intravenously for the treatment of hereditary emphysema and may be useful in other respiratory diseases. Cantin et al. demonstrated that recombinant human alpha1-antitrypsine conjugated to 20 kDa PEG protected mice against human leukocyte elastase-induced lung hemorrhage and that the protection conferred by the conjugated protein was sustained for 72 h, contrary to the non-conjugated counterpart. Anti-interleukin (IL) 17A and anti-IL13 antibody fragments specifically inhibit IL17A and IL13, two cytokines involved in the generation and persistence of airway inflammation in asthma. However, these antibody fragments and the full-length antibody are cleared from the respiratory tract within 24 h [24]. Koussoroplis et al. showed that mono-PEGylation of anti-IL17A F(ab')₂ and anti-IL13 Fab' with two-armed 40 kDa PEG sustained the presence of these fragments in the murine lungs after intranasal administration. Indeed, 40% of the dose initially deposited in the lungs remained in BAL and lung homogenate 48 h post-delivery. Moreover, the authors demonstrated that anti-IL17A F(ab')₂ conjugation to 40 kDa PEG enhanced the retention of the antibody fragment in a murine model of house dust mite-induced lung inflammation and improved its therapeutic efficacy in reducing cellular infiltration and inflammation markers [24]. Interferon (IFN)_{α2b} (IntronA®), IFN_{α2b} conjugated to linear 12 kDa PEG (Pegintron®) and IFN_{α2a} conjugated to two-armed 40 kDa PEG (Pegasys®) are approved for the treatment of chronic hepatitis B or C by subcutaneous injection. The kinetics of the lung disposition of these proteins were evaluated after intratracheal instillation in rats [23]. 12 kDa PEG-IFN_{α2b} was better retained in the lungs than IFN_{α2b} and 40 kDa PEG-IFN_{α2a}. Eight hours after delivery, 54% of the 12 kDa PEG-IFN_{α2b} dose were recovered in the lungs of rats compared to 37% for 40 kDa PEG-IFN_{α2a} and 19% for IFN_{α2b}. Thirty hours after delivery, negligible amounts of the three proteins were recovered from the lungs. The authors hypothesized that the more rapid elimination of the 40 kDa PEG conjugate over the 12 kDa PEG conjugate was due to higher mucociliary clearance of large constructs. However, IFN_{α2a} and IFN_{α2b} are two different molecules and they might present different stabilities in the lungs *in vivo*. Therefore, this study might not allow to conclude on the impact of the PEG MW on PEGylated protein residency in the lungs.

The increase in the pulmonary retention of the above biopharmaceuticals by PEGylation was demonstrated in mice or in rats. Freches et al. compared the effect of protein PEGylation on the local residence time of an anti-IL17A Fab' antibody fragment after pulmonary administration in the mouse, rat and rabbit [296]. The architecture and morphology of the airways and alveolar regions, as well as the cell types found in the lung tissue greatly differ between animal species. The respiratory tract in mice is primitive and asymmetric with a limited number of airway generations (< 15 generations) and no respiratory bronchioles. By contrast, the human respiratory tract is highly complex, symmetric and presents a large number of airway generations (23 generations) as well as respiratory bronchioles [296,297]. The respiratory tract gets more complex in the rat and then in the rabbit than it is in the mouse. The unconjugated Fab' was cleared from the lungs within 24 h following intratracheal instillation in the three animal species, while large quantities of the PEGylated Fab' remained present up to 48 h (**Figure 10**). No significant differences in clearance were noted between the mouse, rat and rabbit although there was a tendency of longer residence time in higher species.

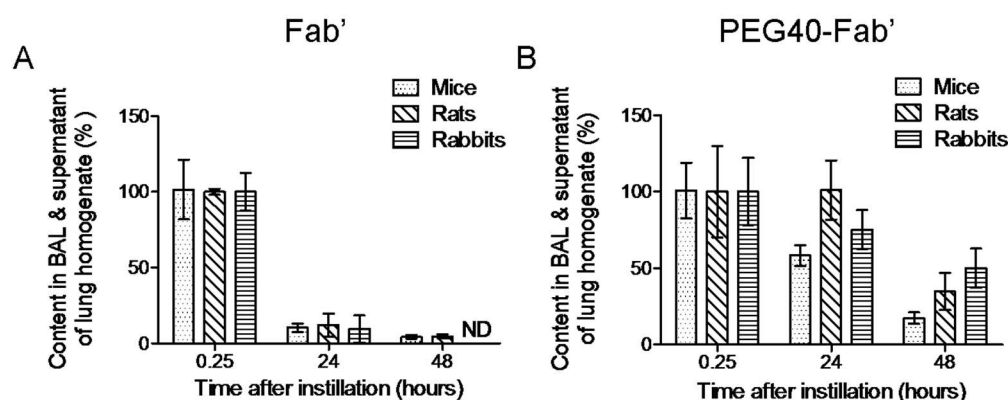


Figure 10: Quantities of anti-IL-17A Fab' and PEG40-Fab' antibodies recovered from bronchoalveolar lavage (BAL) and supernatant of lung homogenate at different times following pulmonary delivery in mice, rats and rabbits (mean $\pm$ SEM, ND = not done) [296]. Data collected showed a sustained presence of PEG40-Fab' for at least 48h whereas Fab' was mostly cleared within a day.

As demonstrated by the data on IFN $_{\alpha 2a}$ and on the antibody fragments, Fab' and F(ab') $_2$, the nature of the protein has a strong impact on the residence time of 40 kDa PEG-protein conjugates in the lungs. While 40 kDa PEG-IFN $_{\alpha 2a}$ was present in the lungs for 8 h, 40 kDa PEG-Fab' and 40 kDa PEG-F(ab') $_2$ remained present up to 48 h. Cytokines have much shorter half-

lives *in vivo* than antibodies and antibody fragments and the short residence times of their PEGylated conjugates in the lungs likely reflect their short biological stability *in vivo* [11].

II.2.2. Mechanisms involved in the sustained retention within the lungs

Several mechanisms may explain the sustained retention within the lungs of the PEGylated biopharmaceuticals: an increased molecular size, an enhanced adhesiveness to mucus, an improved protein resistance towards proteolysis and a protection against endocytosis by alveolar macrophages [20]. The experimental evidence for each of these mechanisms is described below.

II.2.2.1. Impact of molecular size

Gursahani et al. studied the residence time of PEG alone in the lungs and established a correlation with its size [298]. Linear PEG chains labelled with fluorescein isothiocyanate (FITC) and presenting a MW varying from 0.55 kDa to 20 kDa were investigated. The authors showed that the larger the PEG chain, the longer its retention in rat lungs (**Figure 11**). Therefore, the molecular size is an important determinant of the residence time of the polymer in the lungs and this correlation has also been well established using proteins of different MW [240].

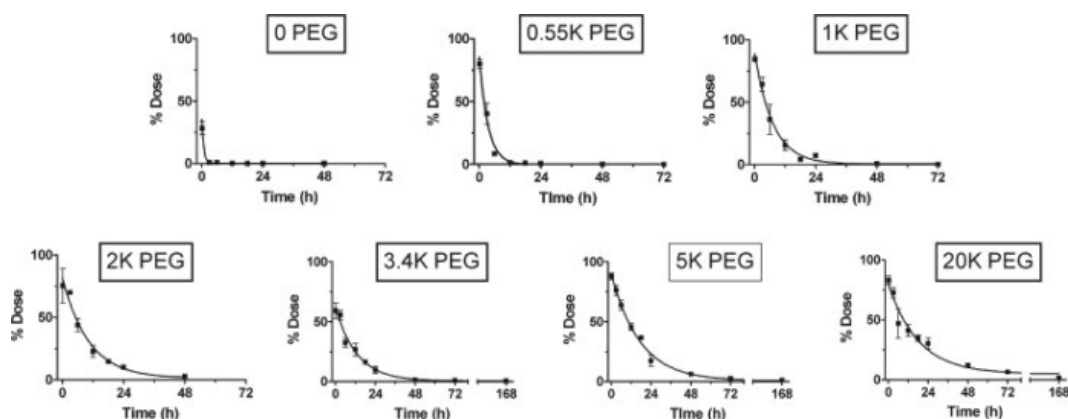


Figure 11: Elimination of PEG–FITC from BAL following intratracheal administration in rats [298]. PEGs of different sizes were evaluated and a correlation was established between the chain length and the sustained presence within the lungs.

However, the nature of the molecule also plays a significant role in residence time. Koussoroplis et al. showed that even though full-length anti-IL17A antibody and 40 kDa PEG-F(ab')₂ were of similar molecular size (150 kDa and 140 kDa, respectively), they presented different retention times within the lungs. In fact, 48 h post-delivery, the full-length antibody was almost cleared whereas 40% of the dose of the PEGylated F(ab')₂ fragment was still recovered [24]. The same authors evaluated the retention of 70 kDa dextran compared with 40 kDa PEG. These two macromolecules have a same hydrodynamic size of 11 nm. Less than 10% of the 70 kDa dextran dose was recovered from the lungs at 48 h whereas 62% of 40 kDa PEG were still remaining [24]. Consequently, the molecular size is not the only property which determines the residence time of molecules in the lungs but the nature of the compound delivered is also a major factor.

II.2.2.2. Increased adhesiveness to mucus

PEG has been described as a mucoadhesive neutral polymer [299,300]. Conjugation of PEG to polyacrylic acid increased the mucoadhesive properties of the polymer films. PEG chains with MW from 200 Da to 1000 Da were evaluated and analysis showed that the higher the PEG percentage in the film, the higher its mucoadhesion [301]. However, no clear correlation between the PEG size and mucoadhesion was apparent. Hassan et al. measured mucoadhesion of PEG through viscosity measurements of PEG-mucin mixtures. They reported that 4 kDa PEG was mucoadhesive and that its adhesiveness increased with its concentration [302]. Four kDa PEG exhibited similar adhesiveness as 70 kDa dextran and bovine serum albumin. Park and Robinson showed that increasing PEG size from 8 kDa to 100 kDa increased its bioadhesive character [303]. The linearity and the high flexibility of the PEG chain favor PEG diffusion within the mucus and its entanglement with mucin fibers. The two lone electron pairs of oxygen in PEG repeat unit then form hydrogen bonding with mucin.

The mucoadhesion property of PEG might be involved in the sustained retention of PEG-protein conjugates in the lungs. Koussoroplis et al. highlighted that the longer residence time of 40 kDa PEG over 70 kDa dextran in the lungs correlated with its enhanced adhesiveness to mucus in rheology studies of polymer-mucin mixtures [24]. The adhesion of PEG-protein conjugates to mucus was also suggested by the increase in recovery of 40 kDa PEG-F(ab')₂ from the respiratory tract after addition of triton, a detergent able to break down the mucus

gel, to the lavage solution. In fact, the recovery rate increased from 24% to 59% after addition of triton [24]. In line with this, Patil et al conducted fluorescence recovery after photobleaching experiments of anti-IL-17A Fab', 20 kDa PEG-anti-IL-17A Fab' and 40 kDa PEG-anti-IL-17A Fab' in normal human respiratory mucus and showed significant binding of the PEGylated Fab' fragments to the mucus [304].

II.2.2.3. Increased protein resistance against proteolysis

PEG attachment on biopharmaceuticals has been shown to protect them from proteolytic breakdown. Youn et al. reported that the conjugation of glucagon-like peptide-1 to 2 kDa PEG largely enhanced the stability of the peptide to dipeptidyl peptidase IV [305]. Zhang et al. evaluated the stability of multi-PEGylated fibronectin to α -chymotrypsin [306]. Conjugation to 2 kDa, 5 kDa or 10 kDa PEGs conferred increased resistance to proteolysis. The steric hindrance created by the PEG chains protected the protein from proteolysis and the higher the MW of the polymer, the better the resistance to the protease. Therefore, increased protein resistance against proteolysis could be one of the mechanisms involved in the sustained retention of PEGylated biopharmaceuticals in the lungs following pulmonary administration.

Resistance to lung proteases was evaluated for salmon calcitonin conjugated to PEG of different sizes (1 kDa, 2 kDa and 5 kDa) [21]. The authors showed that unmodified salmon calcitonin (sCT) was degraded in rat lung homogenates unlike the PEGylated versions (**Figure 12**). All the conjugates were more resistant to proteolytic breakdown than unconjugated salmon calcitonin and the higher the MW of the PEG attached to the hormone, the greater the resistance. Lee et al obtained similar results with glucagon-like peptide-1 conjugated to 2 kDa, 5 kDa or 10 kDa PEG [22]. The half-life of the peptide in rat lung homogenate increased from 9 min for the unconjugated peptide, to 98, 123 and 192 min for the 2 kDa, 5 kDa and 10 kDa PEG conjugates, respectively. To evaluate if PEG conferred increased resistance against proteolysis in the lungs to an anti-IL13 Fab', Koussoroplis et al. similarly incubated non-PEGylated and 40 kDa PEG-Fab' in BAL and lung homogenates of mice for 48 h and measured Fab' content over time [24]. The concentrations of the anti-IL-13 Fab' and PEG40-Fab' in biological fluids did not decrease over the two days, indicating no proteolytic breakdown. Therefore, the unconjugated Fab' antibody fragment was already resistant to proteases and PEGylation did not alter this property.

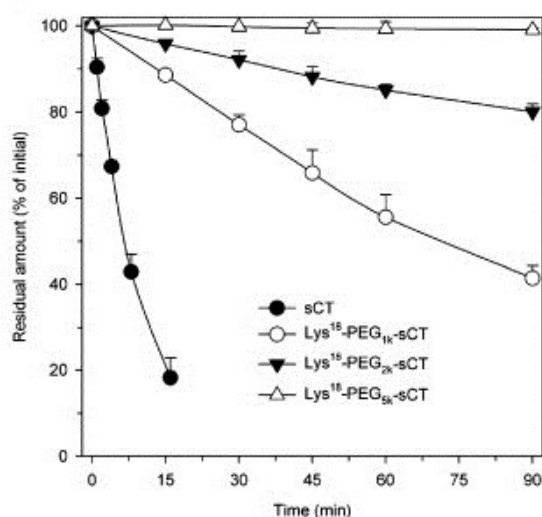


Figure 12: Evaluation of the degradation profiles of sCT and PEGylated sCT incubated in rat lung homogenates (pH 7.4, mean $\pm$ SD) [21]. Data collected showed an improved stability of the hormone after coupling to PEG: the larger the polymer, the higher the proteolytic resistance of sCT.

II.2.2.4. Protection against endocytosis by alveolar macrophages and epithelial cells

Alveolar macrophages represent one of the clearance pathways of biopharmaceuticals in the respiratory tract [285]. Gursahani et al. observed that PEG-FITC of various sizes were taken up by alveolar macrophages [298]. The uptake was minimal and short-lived for small PEG chains (≤ 1 kDa). However, approximately 3% of the delivered dose was recovered in the cellular fraction of BAL in case of 2-3.4 kDa PEG. Most of the cells contained in BAL are alveolar macrophages. However, this amount of PEG recovered in the cell pellet of BAL decreased over 7 days. The uptake in alveolar macrophages increased up to 10% of the delivered dose for 5-10 kDa PEG and this amount was not cleared from alveolar macrophages after 7 days. Forty kDa PEG-Fab' was also taken up by alveolar macrophages but to a lower extent than the non-PEGylated antibody fragment [24]. Twenty-four hours after delivery, almost 100% of alveolar macrophages had internalized Fab' whereas 87% had taken up 40 kDa PEG-Fab'. Because higher quantities of 40 kDa PEG-Fab' remained for longer times in the airway lumen of mice compared with the unconjugated Fab', alveolar macrophages were exposed to higher concentrations of 40 kDa PEG-Fab' for longer times, compared with Fab'. Even though this higher exposure, the uptake of 40 kDa PEG-Fab' was lower than that of Fab'. Therefore, PEGylation partly prevented the endocytosis of the protein. McLeod et al. measured the

quantity of IFN α constructs (PEGylated or not) in the cell pellet obtained by BAL [23]. Less than 1% of the molecules delivered was recovered in the cell pellet, suggesting no uptake by alveolar macrophages.

The duration of exposure of the macromolecules to alveolar macrophages can partly explain the differences in uptake between the proteins and between the free PEGs of varying MW. Uptake by alveolar macrophages is a passive endocytosis process: phagocytes slowly take up the surrounding epithelial lining fluid in an uninterrupted manner as well as the macromolecules present in this fluid. The longer the macrophages exposure to the macromolecule, the larger the uptake by endocytosis. This is verified when comparing the time of residence of IFN α (8 h), antibody fragments (24 h) and free PEG chains in the lungs. However, it is not verified for PEGylated vs non-PEGylated Fab' since 40 kDa PEG-Fab' remained for longer times in the lungs but was less taken up. Endocytosis can occur from the fluid-phase, without binding to the plasma membrane, but it can also occur by non-specific adsorptive pinocytosis, through cell membrane binding, but with no specific receptors. Recognition of specific sites on the plasma membrane (receptor-mediated endocytosis) is unlikely because alveolar macrophages have no receptor for antibody fragments and for PEG. Hence, the decreased uptake of the 40 kDa PEG-Fab' might be due to a decreased rate of adsorptive endocytosis. Positive charge and hydrophobicity are both determinants of adsorptive endocytosis of proteins and the PEG chain might increase the hydrophilicity of the protein as well as might hide the cationic surface charges of the Fab' [307].

Patil et al conducted transport experiments of anti-IL-17A Fab', 20 kDa PEG-anti-IL-17A Fab' and 40 kDa PEG-anti-IL-17A Fab on monolayers of Calu-3 epithelial respiratory cells cultured at an air-interface [304]. They highlighted that PEGylation sustained the presence of the Fab' fragment at the apical side of the cell monolayer and decreased its cell uptake and transepithelial transport. These observations might be explained by the adhesion of PEGylated Fab' fragments to the mucin produced by the cells [308]. The decrease in transepithelial transport might also come from the decreased paracellular diffusion of the enlarged conjugated proteins and from the decreased transcytosis reflected in the decreased cell uptake. While 40 kDa PEG had a significant effect on apical side residency, cell uptake and transepithelial transport, 20 kDa PEG unexpectedly had only an effect on apical side residency and transepithelial transport but not on cell uptake.

II.3. PULMONARY ADMINISTRATION OF PEGYLATED BIOPHARMACEUTICALS FOR SYSTEMIC DELIVERY

Inhalation aerosols can also be used for the delivery of biopharmaceuticals to the systemic circulation [240]. The intrinsic structure of the lungs and their high vascularization allow the molecules to readily cross the alveolar epithelium and then the capillary endothelium, resulting in their transport into the bloodstream. However, biopharmaceuticals are prone to proteolysis and other clearance mechanisms either in the respiratory tract or in the systemic circulation [284]. Therefore, PEGylation might represent an attractive approach for modification of biopharmaceuticals delivered to the systemic circulation *via* the lungs. PEGylation could simultaneously improve protein systemic absorption from the lungs and prolong their plasma half-life. However, PEGylation increases the molecular size of the biopharmaceutical and thereby slow down its transport across the pulmonary tissue towards the bloodstream. This prolonged presence within the lungs might increase the exposure of the biopharmaceutical to clearance pathways in the respiratory tract. Hence, it might eventually lead to a lower systemic absorption of the macromolecules.

Niven et al assessed the systemic absorption of PEGylated versions of recombinant human granulocyte-colony stimulating factor (rhG-CSF) after pulmonary delivery in the rat. PEGylation of the cytokine with either one linear 6 kDa or 12 kDa PEG chain decreased its bioavailability following intratracheal instillation [19]. The bioavailability of rhG-CSF decreased from 16% to 4.5% and 1.2% for 6 kDa and 12 kDa PEG-rhG-CSF, respectively. In addition, the time to maximal plasma concentration increased from 160 min for rhG-CSF to 270 min and 375 min for 6 kDa and 12 kDa PEG-rhG-CSF, respectively. 5 kDa PEG-insulin delivered by intratracheal administration to dogs induced little serum glucose reduction, suggesting poor absorption from the lungs [20]. However, conjugation of insulin to 2 kDa PEG increased the area under plasma concentrations-time curves (AUC) 5-fold following inhalation in the dog. Insulin randomly PEGylated with 750 Da PEG sustained serum insulin rise and serum glucose drop following inhalation in the dog. McLeod et al. assessed the systemic absorption of IFN $_{\alpha 2b}$, 12 kDa PEG-IFN $_{\alpha 2b}$ and 40 kDa PEG-IFN $_{\alpha 2a}$ after intratracheal instillation in the rat [23]. PEGylation largely decreased the systemic absorption of the cytokine, with a bioavailability of 15% for native IFN $_{\alpha 2b}$ and of 5.5% and < 0.4% for 12 kDa PEG-IFN $_{\alpha 2b}$ and 40 kDa PEG-IFN $_{\alpha 2a}$,

respectively. However, the longer plasma half-life of 12 kDa PEG-IFN $_{\alpha 2b}$ and 40 kDa PEG-IFN $_{\alpha 2a}$ compared with IFN $_{\alpha 2b}$ resulted in an 8-fold increase in the AUC.

Youn et al. showed that native salmon calcitonin was rapidly absorbed from the lungs following intratracheal instillation in rats and eliminated from the systemic circulation [21]. Conjugation of salmon calcitonin to 2 kDa or 5 kDa PEG increased the AUC 4- and 7-fold, respectively, delayed the time to maximal plasma concentration from 15 min to 22 and 25 min, respectively, and prolonged the plasma half-life from 35 min to 101 min and 119 min, respectively. Lee et al. reported similar results with glucagon-like peptide-1 [22]. Attachment of 2 kDa, 5 kDa or 10 kDa PEG on the peptide increased the AUC 3-, 8- and 11-fold, respectively, following intratracheal instillation in rats, delayed the time to maximal plasma concentration from 10 min to 12, 44 and 40 min, respectively, and prolonged the plasma half-life from 9 min to 23 min, 42 min and 82 min, respectively.

Overall, different principles can be learned from these results. PEGylation reduces and delays absorption of biopharmaceuticals from the lungs into the bloodstream. The AUC is generally increased. However, the increased AUC does not reflect an enhanced bioavailability but rather a prolonged plasma half-life of the PEGylated protein. The delayed time to maximal plasma concentration reflects a slowed absorption from the lungs and a prolonged presence of the PEG-protein conjugate within the lungs. The larger the PEG size, the larger these effects. This prolonged presence within the lungs likely increased the exposure of the biopharmaceutical to local elimination pathways and this prolonged exposure eventually decreased systemic absorption.

II.4. BIOLOGICAL ACTIVITY AND PHYSICAL STABILITY

PEGylation is a well-established approach to improve the pharmacokinetic properties of proteins. However, PEGylation might result in a partial loss of the biological activity of the biopharmaceutical. The site on the macromolecule used for PEGylation has to be carefully selected. In order to avoid a reduction in biological activity, the PEGylation site should be distant to the active site. Indeed, PEG confers a steric hindrance and may mask the active site of the molecule. Yamamoto et al. showed that site-specific PEGylation of tumor necrosis factor

resulted in a 20% decrease in activity of the cytokine while random PEGylation led to a 90% activity loss [309].

The size and the architecture (linear or multi-armed) of PEG play a major role in the preservation of the activity of the biopharmaceutical [280,310]. Youn et al. specifically PEGylated salmon calcitonin on its Lys¹⁸-amine with a linear 1 kDa, 2 kDa or 5 kDa PEG using an Fmoc (9-fluorenylmethoxycarbonyl) protection/deprotection strategy [21]. The derivatives retained 84, 81 and 30% of salmon calcitonin activity, respectively, indicating that the higher the PEG length, the higher the steric hindrance and the larger the loss of the peptide activity. Site-specific PEGylation of human growth hormone (hGH) was achieved by replacement of three specific amino acids by azido-bearing unnatural amino acid and by using click chemistry for PEGylation of these mutated sites [311]. The three unnatural amino acids were distant from hGH receptor-binding domains. The affinity of each mono-PEGylated hGH isomer for its receptor decreased as the length of the PEG chain increased. 1.7- to 35-fold lower affinities were observed for linear PEG chain ranging from 5 kDa to 20 kDa. However, a 508-fold lower activity was observed for two-armed 40 kDa PEG [311]. Mono-PEGylation led to similar biological activities as di-PEGylation at the same total PEG weight. Tri-PEGylated hGH with 5 kDa PEG presented similar biological activity as di-PEGylated hGH with 20 kDa PEG and as mono-PEGylated hGH with two-armed 40 kDa PEG. Therefore, the covalent binding of a high MW PEG or of a high number of PEG chains might mask the active site of the biopharmaceutical and affect its activity.

Mono-PEGylation of recombinant human deoxyribonuclease I (rhDNase) on its N-terminal leucine residue using linear 20 kDa, 30 kDa or two-arm 40 kDa PEG aldehyde fully preserved the enzymatic activity of the native enzyme [312]. The N-terminus of the enzyme is on its surface and this localization allowed a good accessibility to PEG. In addition, the N-terminus is on the opposite side of the active site, which avoided the loss of rhDNase enzyme activity. A long-acting PEGylated version of rhDNase would allow to lower its administration frequency, alleviating the treatment burden of patients with CF. The PEGs used for conjugation to rhDNase (two linear and a two-arm high MW PEGs) were chosen based on those used in the clinic. Although the *in vivo* disposition of a PEGylated protein following its pulmonary delivery is assumed to be different from injection, it was hypothesized that the higher the PEG MW, the more sustained the protein presence in the lungs, as already demonstrated for the

retention of PEG-FITC following intratracheal administration in rats (**Figure 11**). The choice of a two-arm 40 kDa PEG was directed by the increased surface shielding effect it would confer, compared to a linear PEG [313].

Interestingly, conjugation of full-length anti-sialoadhesin antibodies to linear 5 kDa or 20 kDa PEG largely enhanced their inhibitory effect [314]. Similarly, the biological activity of an anti-IL17A Fab' antibody fragment was greatly enhanced after conjugation to 2-armed 40 kDa PEG [296]. The cell-based bioassay indicated an effective half-maximal inhibitory concentration of 1.7 µg/mL for the unconjugated antibody fragment and of 0.2 µg/mL for the 40 kDa PEG-Fab', highlighting an 8.5-fold improved biological activity. Similar results were shown by Chan et al. for trastuzumab Fab' PEGylated with 10 kDa PEG. Site-specific conjugation on the hinge region slightly enhanced the anti-proliferative activity of the antibody fragment since the half-maximal inhibitory concentration was 0.7 µg/mL and 0.4 µg/mL for the unconjugated Fab' and 10 kDa PEG-Fab', respectively [315]. These antibodies and antibody fragments bind either a membrane receptor or, a cytokine. The antibody-receptor or antibody-cytokine binding partly prevented the ligand to attach to its receptor or the cytokine to bind to its receptor. It is likely that this inhibition effect was enhanced due to the steric hindrance of PEG.

In view of these findings, the biological activity of a protein is not systematically decreased after conjugation to PEG. It can be fully preserved by selecting the PEGylation site away from the active site [312]. It can even be improved as demonstrated by Ducreux et al. and Freches et al. [296]. Therefore, the site of PEGylation is a critical factor to carefully consider in the first place. Then, the degree and the size of the PEG must be taken into account to preserve the biological activity of the macromolecule as much as possible. It should be noted that, in spite of a reduced activity, several PEG-protein conjugates for injection are commercially available because of their tremendous increase in serum half-life. For instance, Pegasys® (40 kDa PEG-IFN α 2a) only retains 7% of the native interferon activity and is on the market since 2002 [277].

The resilience of biopharmaceuticals to aerosolization is another parameter that will need to be considered when developing a PEGylated product for inhalation. Nebulization of proteins might cause their aggregation since the biomolecules are exposed to a large air-liquid interface where they can denature and aggregate. Several studies have shown that the stability of biopharmaceuticals generally increases following PEGylation, thereby leading to a

reduction or elimination of protein aggregation and to a preserved protein structure. For instance, PEGylation of insulin significantly improved the protein stability following an accelerated shake test [316]. Also, a Fab' antibody fragment conjugated to two moieties of 30 kDa PEG presented higher resistance to protein aggregation than the unconjugated Fab' when exposed to orbital agitation [317]. The hypothesis behind the improved stability of PEGylated products is that PEG attachment creates a steric hindrance to the association of proteins. Indeed, PEGylation prevents intermolecular protein-protein interactions, which can otherwise result in protein aggregation, as previously reported [28,317,318].

II.5. SAFETY

PEG is generally considered to have low toxicity whatever its MW and route of administration [289]. Small MW PEGs (< 10kDa) are common excipients in oral, intravenous, nasal and inhalation formulations. In PEGylated biopharmaceuticals, larger PEGs may be used (up to 40 kDa) and a case-by-case safety evaluation is undertaken for these products during development [319]. Preclinical toxicology studies have focused either on PEG alone or on PEGylated biopharmaceuticals.

II.5.1. Systemic toxicity

The toxicity of free large PEG of different sizes was studied after intravenous injection in rats [320]. Rats were administered 100 mg/kg of linear 10 kDa, 20 kDa or 40 kDa PEG for 3 months. To adjust for PEG MW, 10 kDa PEG was delivered daily, 20 kDa PEG every other day and PEG40 twice weekly. The PEGs were well tolerated and produced no systemic toxicity. The tissue distribution of the PEG and the appearance of cytoplasmic vacuolation were markedly influenced by the MW of the PEG. In 10 kDa PEG- and 20 kDa PEG-treated groups, PEG was mostly found in the renal tubule epithelium and in alveolar macrophages and hepatic Kupffer cells. No cellular vacuolation was present. Because PEG is non-biodegradable, its primary clearance mechanism is renal excretion of the intact molecule. Therefore, the renal presence of 10 kDa and 20 kDa PEG likely results from high local tissue concentration of PEG. In contrast, 40 kDa PEG was found in splenic macrophages, renal interstitial macrophages and choroid plexus epithelial cells. Cytoplasmic vacuolation was frequently associated with these localizations. The alteration in tissue distribution observed with an increase in PEG size is

probably related to the marked reduction in the ability of > 30 kDa PEG to be filtered through the renal glomerulus and to a prolonged plasma half-life. Macrophages have an important role in removing foreign material from the local tissue environment and cytoplasmic vacuolation is probably an adaptive response to an increased demand for PEG clearance.

Ivens et al. reviewed the preclinical safety data of the PEGylated biopharmaceuticals delivered by injection currently on the market and they discussed the impact of the PEG molecule on the overall safety [319]. Adverse effects observed in preclinical studies were usually related to the intended pharmacological action of the active drug, rather than to the PEG moiety. Cellular vacuolation in certain tissues and cell types has been observed for approximately half of the approved PEGylated drugs. Vacuolation was seen most frequently in macrophages. No functional changes related to PEG for organs and tissues where cellular vacuolation was seen have been reported. Many factors might impact the occurrence and degree of cellular vacuolation including the drug dose, the dose frequency and duration of treatment, the overall MW of the drug and the MW of the PEG itself, the pathways of drug clearance. Vacuolation was absent below a certain dose of PEG per month (0.4 $\mu\text{mol/kg/month}$) and it has been observed for PEG MW of at least 30 kDa PEG. Some of the vacuolations were reversible within the recovery time of the study and some others were not. Recovery probably depends on PEG removal mechanisms and on cell turnover in tissues.

Immunogenicity is a major concern for biopharmaceuticals because specific antibodies can reduce efficacy and can cause hypersensitivity reactions. PEG has been considered as a non-antigenic, non-immunogenic component and, in some cases, it has been used to reduce protein immunogenicity. Davis et al. were the first to demonstrate that PEGylation eliminated the immunogenicity of bovine adenosine deaminase in mice [321]. More recently, PEGylation of hGH was shown to reduce the production of hGH-specific immunoglobulin (Ig) G after injection in rats. Multi-PEGylation was more efficacious to reduce immunogenicity than mono-PEGylation and a larger PEG than a smaller one [311]. However, a number of reports have documented the presence of anti-PEG antibodies, such as IgG and IgM, in preclinical models following repeated injections of PEGylated biopharmaceuticals [322–324]. In addition, a fraction of the population treated with PEG-uricase (Krystexxa®) and PEG-asparaginase (Onscapar®) loses treatment efficacy because anti-PEG antibodies induce rapid blood clearance of the proteins [319]. Because uricase is from porcine origin and asparaginase is a

bacterial enzyme, the protein parts of these PEGylated biopharmaceuticals are highly immunogenic. The occurrence of anti-PEG antibodies might be favored by the immunogenicity of the protein part. However, the PEG MW, the linker-type and the degree of PEGylation might also be important parameters involved in the production of anti-PEG antibodies. Anti-PEG antibodies were also found in healthy blood donors who have not received PEGylated biopharmaceuticals [323]. Everyday use compounds such as cosmetics, food or household chemicals contain PEGs and this might explain the occurrence of anti-PEG antibodies in a subset of the population.

II.5.2. Local toxicity

Small MW PEGs are excipients included in inhalation products on the market. For instance, sodium cromoglycate (Lomudal®), an anti-allergy drug, is formulated in pressurized metered dose inhaler using 600 Da PEG as an excipient. However, data on the PEG quantities in the formulation are not available, [325] while they are certainly crucial for PEG safety in the lungs. Moreover, larger MW PEGs are retained for longer periods in the lungs than small MW PEGs and this retention might cause PEG accumulation in the lungs over chronic use of inhaled PEGylated pharmaceuticals [298]. Also, alveolar macrophages might take up the PEG and accumulate it.

One study assessed the toxicity of 3.35 kDa PEG after aerosol exposure in rats [326]. The animals were exposed 6 h daily, 5 days a week and for eleven days. The data collected showed no mortality and no clinical signs of toxicity. The lungs were the only organ showing histological lesions after exposure to the polymer. The reported lesions were generally minimal to mild in severity and consisted in alveolar histiocytosis with foamy vacuolated macrophages. No necrosis or cellular debris were recovered from the lungs. The authors suggested that the presence of an increased number of macrophages in the lungs after their exposure to PEG was a normal response to the introduction of any compound or any dust. Accordingly, Forbes et al. reported that the presence of foamy macrophages with no mark of lung inflammation is an adaptive and non-adverse effect [327]. Although 3.35 kDa PEG is larger than the one used in inhalation products, there still remains a need to verify the safety and time for complete elimination of large (20 to 40 kDa) PEG molecules from the lungs.

II.6. CONCLUSION

PEGylation of therapeutic proteins represents an attractive approach for sustaining their residence time in the lungs and thereby decreasing the daily burden of inhalation therapies. The approach appears applicable to any protein although the magnitude of its effect varies with the protein. The approach also appears applicable to any respiratory disease although the treatment of chronic lung diseases such as cystic fibrosis, asthma and chronic obstructive pulmonary disease might benefit more from long-acting inhaled medications than the treatment of acute lung diseases such as respiratory infections. Mechanisms involved in the pulmonary retention of PEG-protein conjugates might include increased molecular size, mucoadhesion, enhanced proteolytic resistance and escape from the uptake by alveolar macrophages.

Pulmonary delivery of PEGylated peptides and proteins is also interesting as a non-invasive route of administration of long-acting biopharmaceuticals to the bloodstream. However, PEGylation decreases the systemic absorption of the compound, especially when the PEG size is large.

PEG presents low toxicity whatever its MW and route of administration. Adverse effects seen with PEG-protein conjugates administered by injection are mostly related to the active part of the drug molecule and not to PEG. Vacuolation in macrophages from different tissues frequently appears following injection of PEGylated biopharmaceuticals but it has not been associated with changes in organ function [18]. Macrophages presenting a granular or vacuolated cytoplasmic appearance are considered as a normal alveolar macrophages response following exposure to various pathogens or compounds. Vacuolation is caused by an increase in number and size of alveolar macrophages. It can be transient or irreversible and the presence of such 'foamy' and enlarged macrophages with no mark of other changes (such as cellular distortion, necrosis or inflammation) should be considered as an adaptive and non-adverse effect [319,327]. Safety studies following pulmonary delivery of PEGylated proteins are awaited. They should check for the occurrence of vacuolation in alveolar macrophages and pulmonary inflammation.

PEGylated proteins could be delivered to the lungs using nebulizers, dry powder inhalers or non-pressurized metered dose inhalers. However, dry powder inhalers or non-pressurized metered dose inhalers could be advantageous because they considerably lower the time required by the patient for the administration compared with nebulizers. While non-pressurized metered dose inhalers can deliver a simple solution of the protein, an inhalation dry powder will require a complete formulation development and the use of excipients that will potentially demand safety evaluation as well. Each inhaler device will imply a different timing of application of physical stresses to the protein and hence a different timing for the verification of the protein integrity. The formulation of an inhalation dry powder will require the process of spray-drying, a stressful method for the protein with the formation of huge air-water interface where the protein might aggregate. By contrast, the physical stresses applied to the protein will directly occur during administration using nebulizers and non-pressurized metered dose inhalers: creation of a large air-water interface during aerosolization, high pressure and/or turbulence.

**CHAPTER II. PRODUCTION AND CHARACTERIZATION OF A
PEGYLATED DERIVATIVE OF RECOMBINANT HUMAN
DEOXYRIBONUCLEASE I FOR CYSTIC FIBROSIS THERAPY**

PRODUCTION AND CHARACTERIZATION OF A PEGYLATED DERIVATIVE OF RECOMBINANT HUMAN DEOXYRIBONUCLEASE I FOR CYSTIC FIBROSIS THERAPY

Adapted from: Production and characterization of a PEGylated derivative of recombinant human deoxyribonuclease I for cystic fibrosis therapy, International Journal of Pharmaceutics 524 (2017) 156-167

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Personal contribution: all experiments except from the MALDI-TOF analysis

ABSTRACT

Recombinant human deoxyribonuclease I (rhDNase) is the mucolytic agent most widely used for the treatment of respiratory disease in cystic fibrosis. However, rhDNase is rapidly cleared from the lungs which implies a high dosing frequency and limited patient adherence. The aim of this study was to produce a long-acting PEGylated derivative of rhDNase presenting a preserved enzymatic activity. Site-specific PEGylation on the N-terminal (N-ter) leucine residue of rhDNase was achieved by reductive alkylation at acidic pH using linear 20 kDa, linear 30 kDa or two-arm 40 kDa polyethylene glycol (PEG) propionaldehydes. Yields of mono-PEGylated products ranged between 45% and 61%. Conjugation to PEG fully preserved the secondary structure and the *in vitro* enzymatic activity of the native protein. These properties offer interesting perspectives for *in vivo* inhalation studies of the PEGylated enzyme.

I. INTRODUCTION

Cystic Fibrosis (CF) is a multi-systemic inherited disease that mainly affects the airway epithelium [9]. Thick mucus secretions obstruct the airways and make patients with CF particularly vulnerable to infections by pathogens. The pulmonary phenotype determines the prognosis and will prove fatal for almost all patients. With a better understanding of the pathophysiology of the disease and with early treatment, life expectancy has steadily improved over the past decades [114]. Although the average age of survival reached 39.3 years in 2014 in the USA, the quality of life of patients remains affected by the high daily burden of the symptomatic treatment. Among others, the treatment includes respiratory physiotherapy, inhaled mucolytics and bronchodilators, pulmonary and systemic administration of antibiotics and supplementation of pancreatic enzymes and liposoluble vitamins [3].

Dornase alfa (Pulmozyme®) or recombinant human deoxyribonuclease I (rhDNase) is the mucolytic agent most widely used in the symptomatic treatment of CF. Respiratory secretions of patients contain a considerable number of neutrophils and their apoptosis leads to local accumulation of DNA. rhDNase is a 30 kDa glycoprotein which cleaves the DNA in respiratory secretions of patients and thins them [4]. Its daily administration by inhalation can break the vicious cycle of obstruction, inflammation and recurrent respiratory infections [2].

However, the current treatment with rhDNase presents limitations. First, rhDNase is cleared within a few hours from the lungs: when the dose of 2.5 mg is inhaled, a concentration of 3 µg/mL is measured in sputum immediately after inhalation and it is reduced to 0.6 µg/mL after two hours [5]. This rapid elimination of rhDNase implies the requirement to deliver the protein once to twice daily. Secondly, the time required for nebulization of the dose is long since it takes more than 10 min with the PARI LC-Plus® nebulizer [328]. Moreover, an additional time of 5 min is required to set-up, breakdown, clean and disinfect the nebulizer. Most CF patients take several aerosol drugs every day and this imposes a significant time burden and may lead to reduced adherence. Although new nebulizers such as the eFlow® limit the treatment burden by reducing the nebulization time to 3 min [265], the daily burden of rhDNase administration remains a major hurdle to good patient adherence [6].

The covalent attachment of PEG chains to therapeutic proteins, named PEGylation, is a common approach to increase serum protein half-life following injection [277]. PEGylation of proteins prolongs their residence in the body due to reduced renal clearance, enhanced proteolytic resistance and reduced recognition by specific antibodies. The prolongation of serum protein half-life directly depends on the PEG size: the larger the PEG molecular weight, the longer the serum protein half-life following injection [329]. Conjugation of PEG to proteins has also been shown to increase their local residence time following pulmonary delivery in preclinical models. Cantin *et al.* showed that mono-PEGylation of α 1-proteinase inhibitor with linear 20 kDa PEG sustained the presence of the molecule in bronchoalveolar lavage in mice for at least 48 h whereas the unconjugated protein was more rapidly cleared [295]. Koussoroplis *et al.* reported similar results for anti-IL17A F(ab')₂ and Fab' antibody fragments conjugated to a two-arm 40 kDa PEG and demonstrated that the prolonged pulmonary residency of the anti-IL17A PEG40-F(ab')₂ translated into an improved efficacy in reducing lung inflammation in a murine model of house dust mite-induced lung inflammation [24]. Freches *et al.* confirmed the sustained-release approach in rats and rabbits [296]. However, the impact of the PEG size on the residence time of PEGylated proteins in the lungs has not been thoroughly studied. Mucoadhesion, escape from alveolar macrophages, increased molecular size and enhanced proteolytic resistance might be mechanisms involved in the pulmonary retention of proteins provided by PEG [19,21,24].

The goal of this research was to produce a long-acting mono-PEGylated derivative of rhDNase that presents a preserved enzymatic activity. The sustained presence of a PEGylated version of rhDNase in the lungs could reduce rhDNase administration frequency and thereby rhDNase therapy burden. A single PEG chain of 20 kDa, 30 kDa or 40 kDa was attached on the N-terminal amino acid of rhDNase by reductive alkylation at acidic pH. The generated products were purified and characterized for their degree and site of PEGylation, secondary structure and enzymatic activity *in vitro*.

II. MATERIAL AND METHODS

II.1. MATERIAL

Pulmozyme[®] was from Genentech, Inc. (South San Francisco, CA, USA). Linear 20 kDa and 30 kDa methoxy PEG (PEG20 and PEG30, respectively) propionaldehyde and two-arm 40 kDa methoxy PEG (PEG40) propionaldehyde were obtained from NOF Corporation (Tokyo, Japan). Unless otherwise stated, chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA).

II.2. PRODUCTION OF PEGYLATED RHDNASE

rhDNase (1 mg/mL or 33 μ M) was dialyzed against 1 mM CaCl_2 , 0.1 M CH_3COONa at pH 5 or pH 5.5 (PEG20 and PEG30) or at pH 5.5 or pH 6 (PEG40), at 4 °C during 6 h (Spectra/Por molecular porous membrane tubing MWCO 6-8 kDa, Spectrum). Dialyzed rhDNase was then added to a vial containing methoxy PEG propionaldehyde powder at a [PEG]:[protein] molar ratio of 8:1 or 16:1. PEG instantly dissolved in the rhDNase solution. Sodium cyanoborohydride (19.6 μ L of a 173 mM solution in water per mg of rhDNase) was then added to the reaction mixture. The reaction was continued under stirring at room temperature for 48 h or 96 h.

Sodium cyanoborohydride is a mild reducing agent, especially used to convert imines to amines. It is the preferred reducing agent used in reductive alkylation, in which aldehydes are treated with amines. At pH 6-8, the more basic imines are protonated and reduced faster than the aldehydes [330]. This selectivity allowed for a convenient reductive alkylation procedure of rhDNase.

II.3. PURIFICATION OF PEGYLATED RHDNASE

The PEGylated products were purified by anion-exchange chromatography (AEC) on a Resource Q, 1 mL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The reaction mixture was dialyzed against 20 mM $\text{N}(\text{CH}_2\text{CH}_2\text{OH})_3$, 5 mM NaCl, 1 mM CaCl_2 , pH 7.5 overnight (Spectra/Por molecular porous membrane tubing MWCO 6-8 kDa, Spectrum) prior to

purification of PEG-conjugates and loaded on the AEC column. The chromatography was performed on an ÄKTA™ purifier 10 system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The samples were filtered through a polyvinylidene fluoride (PVDF) syringe-tip of 0.2 µm pore size prior to loading. A salt gradient elution was used. Buffer A was 20 mM N(CH₂CH₂OH)₃, 5 mM NaCl, 1 mM CaCl₂, pH 7.5 and buffer B was 20 mM N(CH₂CH₂OH)₃, 250 mM NaCl, 1 mM CaCl₂, pH 7.5. Absorbance at 280 nm was monitored where PEG has no signal contribution. Data were recorded by UNICORN 5.2 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Product yields were calculated by the comparison of the relative area under the peak of each molecular species. The collected fractions containing the PEGylated species were gathered, concentrated using Vivaspin 15R sample concentrator (10,000 molecular weight cut-off, Sartorius) and stored in 150 mM NaCl, 1 mM CaCl₂ at 4°C as marketed rhDNase.

II.4. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The extent of rhDNase PEGylation was evaluated by SDS-PAGE. SDS-PAGE of the AEC collected fractions was performed using a gradient 4-20% Mini-PROTEAN®TGX™ precast gel (Bio-Rad, Hercules, CA, USA) along with custom made buffer containing 0.025 M tris(hydroxymethyl)aminomethane, 0.192 M glycine and 0.1% sodium dodecyl sulfate, pH 8.7. Two µg of protein (66 pmol of rhDNase or PEG-rhDNase; without counting the PEG moiety in PEG-rhDNase mass) were loaded in each well and the gels were run at 120 V for 90 min. The protein bands were visualized by staining with GelCode® Blue Stain Reagent (Thermo Fisher Scientific, Rockford, IL, USA). PEG was specifically stained with a barium iodide solution based on Kurfürst's method [331]. The gel was soaked in 0.1 M HClO₄ for 30 min and then immersed in a 5% BaCl₂ in 1 M HCl solution for 10 min. Then, iodine solution (1.3% I₂ + 4% KI) was added and incubated for 5 min. Finally, the gel was left to destain in 0.05 M HCl for 30 min.

II.5. MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTRA (MALDI-TOF MS) ANALYSIS

Two analyses were performed for determination of the degree and site of PEGylation of rhDNase: intact protein and digested protein analyses.

II.5.1. Intact proteins

Samples of 0.5 mg/mL (17 μ M of rhDNase or PEG-rhDNase; without counting the PEG moiety in PEG-rhDNase mass) were vortexed at room temperature for 5 min. Subsequently, they were diluted 10 x in 0.1% (v/v) trifluoroacetic acid (TFA), 2% (v/v) acetonitrile. One μ L of 10 mg/mL alpha-cyano-4-hydroxycinnamic acid (alpha-cyano MALDI matrix, Protea Biosciences, Morgantown, WV, USA) solution in 0.1% (v/v) TFA, 70% (v/v) acetonitrile was added to 1 μ L of sample and mixed. One μ L of this solution was then placed on an MALDI plate Opti-TOF™ 384 Well Insert (Applied Biosystems, Foster City, CA, USA) and dried. MALDI-TOF MS spectra were acquired with linear high mass positive acquisition method using an Applied Biosystems 4800 MALDI TOF/TOF™ Analyzer spectrometer with a 200 Hz solid state laser operating at λ = 355 nm. A 6000 laser intensity and 4000 laser shots by spot, in a range of m/z between 10 kDa and 200 kDa, were used to obtain spectra. External mass calibration was performed using 50 pmol of phosphorylase B rabbit muscle (phosphorylase B, Protea Biosciences, Morgantown, WV, USA) spotted around samples on the MALDI plate. Data were collected with the Applied Biosystems 4000 Series Explorer™ software.

II.5.2. Digested proteins

The proteins were first precipitated and then digested.

Precipitation: 10 μ L of protein sample (5 μ g or 0.17 nmol of rhDNase or PEG-rhDNase; without counting the PEG moiety in PEG-rhDNase mass) were precipitated with chloroform/methanol [332]. Methanol and chloroform were stored at – 20°C then in water on ice for 1 h before the procedure. Protein samples were transferred into a 1.5 mL LoBind Eppendorf tube® and placed on ice. Cold water was added to reach 100 μ L. Hundred μ L of chloroform and 400 μ L of methanol were added and vortexed for 30 s. Samples were kept on ice for 15 min. 300 μ L of cold water were then added and vortexed for 30 s. Emulsions were placed on ice for 5 min

and centrifuged at 4°C, 15 min at 20,000 *g* using model 5417C Eppendorf centrifuge. Upper phase was removed and 800 µL of methanol were added. The solutions were centrifuged at 4° C and 20,000 *g* for 15 min. The supernatant was removed and the pellets were dried in a SC 200 Savant Speed Vac Concentrator and stored at – 20°C.

Digestion: Protein pellets were solubilized in 20 µL of 50 mM triethylammonium bicarbonate, pH 8.0. Proteins were then reduced by incubation at 60 °C with 25 mM tris(2-carboxyethyl)phosphine for 1 h. They were alkylated with 200 mM methyl-methanethiosulfonate at room temperature in the dark for 15 min. The reduced and alkylated proteins were digested at 37 °C using 1 µg of sequencing grade modified V8 (Promega, Madison, WI, USA) for 16 h. Then supernatants containing peptides were vacuum dried in a SC 200 Savant Speed Vac Concentrator. Peptides were solubilised in 20 µL of 0.1 % TFA, desalted and concentrated with C18 ZipTip® (Merck Millipore, Merck KGaA, Darmstadt, Germany), according to manufacturer's protocol.

MS and MS/MS analysis: 2 µL of 10 mg/mL of alpha-cyano-4-hydroxycinnamic acid (alpha-cyano MALDI matrix) solution in 50 % (v/v) acetonitrile, 0.1 % (v/v) TFA were mixed with 2 µL of each ZipTip concentrated peptide solution. One µl of this solution was placed on an Applied Biosystems MALDI plate Opti-TOF™ 384 Well Insert (Foster City, USA). MALDI-TOF MS and MS/MS spectra were acquired using an Applied Biosystems 4800 MALDI TOF/TOF™ Analyzer spectrometer using a 200 Hz solid state laser operating at $\lambda = 355$ nm. MALDI-TOF MS spectra were obtained with a 3200 laser intensity and 2000 laser shots by spot in a range of *m/z* between 800 Da and 6 kDa. Internal calibration was performed using some specific proteolytic peptides from digested protease. MS/MS spectra were obtained by selecting 25 most intense precursor ions by spot and in using 4200 laser intensity and 2000 laser shots by precursor. The automatically selected precursors were performed at a collision energy of 1 kV with collision gas air at a pressure of about 1×10^6 Torr. Data were collected with the Applied Biosystems 4000 Series Explorer™ software.

II.5.3. Data analysis

MALDI-TOF MS and MS/MS queries were performed using the Applied Biosystems GPS Explorer™ 3.6 software working with the Ltd MASCOT® Database search engine v2.1 (Matrix Science, Boston, MA, USA). A local database containing protein sequences from rhDNase was used. 200 ppm precursor tolerance for MALDI-TOF MS spectra and 0.2 Da fragment tolerance for MS/MS spectra was allowed. The selected charge state of + 1, one trypsin miscleavage and variable modifications consisting of methionine oxidation and MMTS modified cysteine were applied. For protein direct identification with MASCOT, protein scores greater than 40 were considered significant ($p < 0.05$).

II.6. CIRCULAR DICHROISM (CD) SPECTRA ANALYSIS FOR STRUCTURAL CHARACTERIZATION

Before analysis, the samples (200 µg/mL or 6.7 µM of rhDNase; without counting the PEG moiety in PEG-rhDNase mass) were extensively dialyzed against water at 4°C. CD measurements were performed with a Chirascan™-plus CD Spectrometer (Applied Photophysics) using a 1.0 mm path-length suprasil quartz cell (Hellma Analytics, Kruibeke, Belgium) at 25°C. Circular dichroism data were collected from 180 to 300 nm with a spectral bandwidth set to 1 nm and a current time per point of 1 s. The CD signal was baseline-corrected by subtraction of the background for the spectrum obtained with water. The observed ellipticity was reported in millidegrees.

II.7. COLORIMETRIC DETERMINATION OF RHDNASE ACTIVITY

This assay was adapted from Sinicropi's paper [333]. A solution of 2 mg/mL of DNA isolated from salmon testes (Sigma-Aldrich) was prepared in Buffer A (25 mM HEPES, 1 mM EDTA, pH 7.5) and mixed for 3-4 days at room temperature until the solution was homogenous. The solution was stored at 4°C. A 0.4% solution of Methyl Green (Sigma-Aldrich) was prepared in Buffer B (20 mM acetate-NaOH, pH 4.2). The solution was extracted with chloroform to remove traces of crystal violet until the organic layer was colorless. The upper aqueous layer was separated, stirred in the hood for 2-3 h to evaporate the excess chloroform and the solution was stored at 4°C.

The DNA Methyl Green substrate was prepared by gently mixing 77% (v/v) of 2 mg/mL DNA, 4.6% of 0.4% Methyl Green and 18.4% of Buffer C (25 mM HEPES, 4 mM CaCl₂, 4 mM MgCl₂, 0.1% bovine serum albumin - BSA, 0.01% thimerosal, 0.05% Tween20, pH 7.5). The solution was stored at 4°C and incubated at room temperature overnight before the assay.

Samples (17 serial 1.67-fold dilutions of rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase) were prepared in Buffer C. Each well of a 96 microplate was filled with 100 µL of samples and 100 µL of DNA-Methyl Green substrate. The plate was sealed and incubated for 6 h at 37°C. The absorbance at 650 nm was measured at the end of the incubation.

III. RESULTS AND DISCUSSION

III.1. PRODUCTION OF PEGYLATED rhDNase

In the present study, mono-PEGylation of rhDNase was pursued in order to maintain its activity. PEG is a non-toxic agent approved by regulatory authorities for modification of biopharmaceuticals [334]. PEG is a linear or branched polyether that can be activated at one of its termini for coupling to a protein. The functional group of the activated PEG is chosen based on the coupling reaction to use and depends on the functional group of the amino acid to PEGylate. For proteins, several typical functional groups and amino acids can be listed: the amino group of lysine, the thiol group of cysteine, the imidazole group of histidine, the carboxylic acid group of glutamic acid or the N-terminal amino group [288]. Two types of PEGylation have been developed [288]; random PEGylation resulting in multi-PEGylated products and a mix of conjugates, and site-specific PEGylation targeting position(s) to PEGylate on a molecule. Random PEGylation may lead to a loss of biological activity and a difficulty to purify the molecules. This is not the case for site-specific PEGylation chemistries. PEG propionaldehyde is utilized for PEG coupling to protein amino groups. It maintains the charge of the protein in the conjugate by forming secondary amines [335]. PEG propionaldehyde is easy to prepare. It can be used for random PEGylation chemistry by attachment on several protein amino groups as well as for site-specific PEGylation chemistry by selective attachment on the protein N-ter amino group [288].

In our study, rhDNase was selectively PEGylated on the N-terminal leucine residue by reductive alkylation at acidic pH using PEG propionaldehyde. The reaction occurs through a nucleophilic addition of the available electrons of the protein amino group to the electrophilic carbonyl group of the PEG aldehyde. Alkylation at slightly acidic pH takes advantage of the difference between the pKa values of the alpha-amino group of the N-terminal amino acid residue (pKa 7.8) and the epsilon-amino groups of the lysine residues (pKa 10.1), and allows selective PEGylation on the N-terminal [336,337]. Moreover, the presence of the N-terminal on rhDNase surface allows good accessibility to PEG (**Figure 13**) [338]. The N-ter is also on the opposite side of the active site (**Figure 13**), potentially avoiding loss of rhDNase enzyme

activity. This PEGylation chemistry has already been used for modification of biomolecules such as methionyl-granulocyte colony stimulating factor (Neulasta®) and epidermal growth factor [334,337].

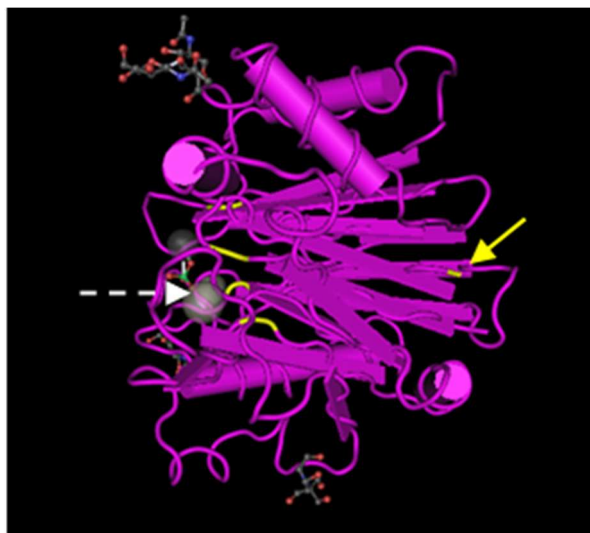


Figure 13: 3D structure of rhDNase (4AWN from the Protein Data Bank). The amino acids of the active site (His-134, Asn-170, Glu-39, His-252, Asp-168 and Asp-251; dotted line arrow) as well as the N-terminal leucine (at the surface of the protein; full line arrow) are indicated. Calcium (grey balls) and magnesium (black ball) ions are represented as well as N-acetyl-D-glucosamine (skeletal organic structure on top), 2-[bis-(2-hydroxy-ethyl)-amino]-2-hydroxymethyl-propane-1,3-diol (skeletal organic structure on bottom) and phosphate ion (linked to magnesium ion) [338].

Different PEG sizes and architectures were used: linear 20 kDa, linear 30 kDa and two-arm 40 kDa PEGs. These PEG sizes and architectures were selected based on PEGs already used clinically with the objective to obtain a maximum effect of PEG on extension of rhDNase residency within the lungs. A two-arm PEG chain presents an increased surface shielding effect compared with a linear PEG [313].

We evaluated a series of factors that affect the yield and the degree of PEGylation including reaction time, [PEG]:[rhDNase] molar ratio and pH (**Table 4**). These assays were performed at room temperature.

The effect of incubation time was first evaluated followed by [PEG]:[rhDNase] molar ratio. The reaction times of 48 h and 96 h were tested. Yields of mono-PEGylation increased with reaction time and reached 65% for PEG20, 59% for PEG30 and 73% for PEG40 at pH 5.5 and at a [PEG]:[rhDNase] molar ratio of 16:1 after 96 h. However, these conditions resulted in di-

PEGylation which was approximately 20% for PEG20 and PEG30. Di-PEGylation was lower (~9%) for PEG40 than for other PEGs due to the higher steric hindrance of branched PEG (**Table 4**) [339]. [PEG]:[rhDNase] molar ratios of 8:1 and 16:1 were also compared for all PEGs tested and the yield of mono-PEGylation and di-PEGylation increased with [PEG]:[rhDNase] molar ratio (**Table 4**).

Two pH conditions were then evaluated for each PEG; pH 5 and 5.5 for PEG20 and PEG30, and pH 5.5 and 6 for PEG40. These pH values were selected based on the pKa value of the N-terminal alpha residue (pKa 7.8) relative to the pKa value of lysine amino groups (pKa 10.1). Indeed, at pH between 5 to 6, there is 1000- to 100- times more NH_3^+ than NH_2 forms for the alpha-amino group while there is 100,000- to 10,000- times more NH_3^+ than NH_2 forms for Epsilon-amino groups [340]. Nucleophilic attack of the alpha-amino group on the PEG aldehyde is then favored at acidic pH. However, the pH of the reaction should not be too low to avoid degradation of the protein and a too low reactivity of the alpha-amino group. Decreasing the pH from 5.5 to 5.0 resulted in lower percentages of di-PEGylated proteins for PEG20 and PEG30, suggesting a better selectivity of the N-terminal leucine instead of lysine residues (**Table 4**). Decreasing the pH also reduced the yield of the mono-PEGylated products. pH 5 was selected as the most suitable pH for reaction with PEG20 and PEG30 because of its better selectivity for PEGylation on the N-terminal site. The steric hindrance of PEG40 allowed to PEGylate rhDNase at slightly higher pH such as pH 6. The increase of pH from 5.5 to 6.0 did not drastically affect neither the percentage of di-PEGylated product nor the mono-PEGylation yield so that pH 5.5 was kept for the reaction with PEG40 (**Table 4**).

Table 4: Optimization of the PEGylation conditions of rhDNase with PEG20, PEG30 or PEG40^a.

Compound	Conditions			% 1xPEG-rhDNase ^b	% 2xPEG-rhDNase ^b
	Time	Ratio	pH		
PEG20-rhDNase	48 h	1:8	5	18	5
	96 h		5	27	8
	48 h	1:16	5	48	9
	96 h		5	45	13
	48 h		5.5	53	17
	96 h		5.5	65	21
PEG30-rhDNase	48 h	1:8	5	25	8
	96 h		5	31	8
	48 h	1:16	5	41	11
	96 h		5	48	14
	48 h		5.5	42	14
	96 h		5.5	59	22
PEG40-rhDNase	48 h	1:8	5.5	36	4
	96 h		5.5	61	6
	48 h		6	46	3
	96 h		6	56	4
	48 h	1:16	5.5	68	9
	96 h		5.5	73	9

(a) The reaction conditions not mentioned in the table were 1 mg/mL (33 μ M) of rhDNase and room temperature. Each reaction was carried out at least twice and the percentages of PEGylated products are average values. (b) Percentages of 1xPEG-rhDNase and 2xPEG-rhDNase were calculated by the measurement of the relative area under the peak of each molecular species in anion-exchange chromatograms.

Mono-PEGylated rhDNases were purified from the reaction mixture by anion-exchange chromatography (AEC). Chromatograms showed two double peaks for each PEG used (**Figure 14** and *Supplementary Figures S1 & S2*). The elution of rhDNase as a double peak likely results from the glycosylation heterogeneity of rhDNase [341]. Fractions from the chromatography were collected and run on SDS-PAGE to allow identification of the peaks and to select AEC fractions pure of mono-PEGylated rhDNase [339]. Since the migration of the PEGylated protein through the pores of the SDS-PAGE gel was slowed by the large, heavily hydrated and uncharged polymer, the corresponding bands were retained at higher molecular weights than expected. However, SDS-PAGE analysis allowed to identify the AEC peaks and showed the degree of PEGylation (**Figure 14B**) [342]. The first eluted peak corresponded to the mono-PEGylated product whereas the second one was native rhDNase. A peak shoulder on the left

side of the mono-PEGylated protein peak was observed and related to di-PEGylation (**Figure 14A**). Figures for PEGylation with PEG20 and PEG30 are provided in *Supplementary material (Figures S1 & S2)*.

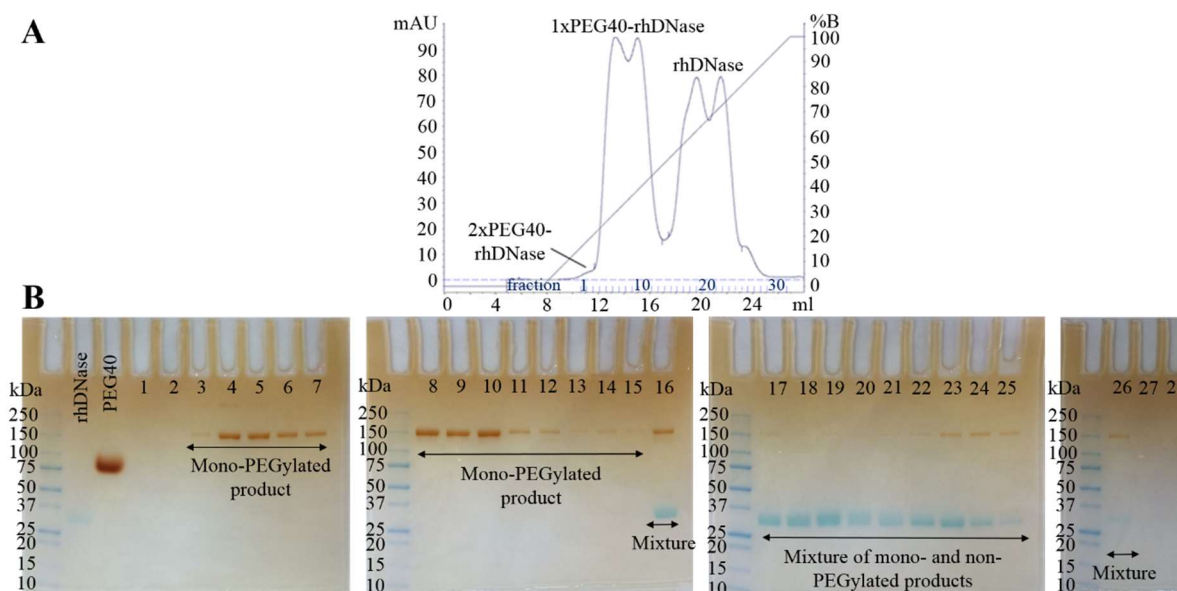


Figure 14: Characterization of the PEGylation reaction of rhDNase with PEG40 propionaldehyde. The reaction conditions were 1 mg/mL (33 μ M) of rhDNase, 96 h reaction time, 8:1 [PEG]:[rhDNase] molar ratio, pH 5.5 and room temperature. **A.** Anion-exchange chromatogram of the PEGylation reaction mixture showing, from left to right, 2xPEG40-rhDNase (shoulder), 1xPEG40-rhDNase (first double peak) and unmodified rhDNase (second double peak). **B.** SDS-PAGE of anion-exchange chromatography fractions. Blue bands represent unconjugated rhDNase and brown bands at 150 kDa represent mono-PEGylated rhDNase. The brown band at ~75 kDa corresponds to 40 kDa PEG alone used as a control. Fractions 3 to 15 contain purified 1xPEG40-rhDNase.

To conclude, the PEGylation reaction yielded mono-PEGylated products but also di-PEGylated proteins with a percentage increasing with reaction time, [PEG]:[rhDNase] molar ratio and pH (**Table 4**). We considered that less than 15% of di-PEGylated rhDNase permitted purification of mono-PEGylated products from the reaction mixture with negligible overlap of peaks in AEC. On this basis, we retained the following PEGylation conditions: 16:1 molar ratio [PEG]:[rhDNase], 96 h, room temperature, pH 5 for PEG20 and PEG30 and 8:1 molar ratio [PEG]:[rhDNase], 96 h, room temperature, pH 5.5 for PEG40. After reaction, 45% 1xPEG20-rhDNase and 13% di-PEGylated protein were obtained for PEG20, 48% 1xPEG30-rhDNase and 14% di-PEGylated protein for PEG30 and 61% 1xPEG40-rhDNase and 6% di-PEGylated protein for PEG40 (**Table 4**). Although a 16:1 molar ratio [PEG]:[rhDNase] yielded $\geq 68\%$ 1xPEG40-

rhDNase with < 15% di-PEGylated protein, we selected the 8:1 molar ratio [PEG]:[rhDNase] for PEG40 because this molar ratio saves the PEG reagent.

Following purification, the purity of each mono-PEGylated rhDNase was assessed by SDS-PAGE with iodine staining. Considering the detection sensitivity of this staining (7 ng; [343]), we considered that the purity of PEGylated rhDNases was > 99.5% (purity of PEG20-rhDNase; based on the quantity of PEG deposited).

The long reaction time and the high [PEG]:[rhDNase] molar ratio of the PEGylation reaction likely resulted from the low number of uncharged N-ter alpha residues at the pH of the reaction as well as from a reaction performed in very dilute protein solution. The concentration of 1 mg/mL of the rhDNase solution was imposed by the use of Pulmozyme®. The use of a higher rhDNase concentration should lead to a more efficient PEGylation reaction [339]. In the same reaction conditions, PEG40 led to a higher yield of mono-PEGylated rhDNase and to a lower percentage of di-PEGylated product than PEG20 or PEG30 (**Table 4**). The steric hindrance created by the 'Y' shape of PEG40 probably led to a more selective mono-PEGylation since large and branched PEGs were not able to access the lysine residues of rhDNase once a PEG chain is attached to the N-ter alpha residue [339].

III.1 PHYSICO-CHEMICAL CHARACTERIZATION OF PEGYLATED RHDNASES

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to identify the degree and site of PEGylation of the rhDNase compounds prepared under the retained reaction conditions [344]. The spectrum of rhDNase appeared to have one main peak at 32.2 kDa, close to its expected molecular mass and corresponding to the protein ionized once (**Figure 15A**). The spectrum of PEG20-rhDNase (*Supplementary Figure S3A*) showed a main peak at 53.9 kDa equivalent to the molecular mass of rhDNase with a shift of + 20 kDa related to the addition of PEG20. The same observations could be made for PEG30-rhDNase and PEG40-rhDNase with molecular masses of 65.1 kDa and 75.8 kDa, respectively (*Supplementary Figure S3B* and **Figure 15B**). For rhDNase, peaks at 16.1 kDa and 65.2 kDa were also present and were related to rhDNase ionized twice and rhDNase dimer, respectively. For each PEGylated protein, a peak corresponding to its twice ionized form was also observed. The spectrum of PEG20-rhDNase showed a peak at 107.7 kDa associated with its dimer form.

The difference in molecular mass between the native protein and PEGylated products is close to the molecular mass of the PEG used for PEGylation and corresponds to one PEG chain. This confirms that, in the conditions selected above, mono-PEGylation of rhDNase occurred [340]. The difference between the measured and theoretical molecular weights of the conjugates is due to the polydispersity of the polymer and was previously reported by Kinstler and co-workers for the PEGylation of rhG-CSF [336]. No information on the homogeneity of the methoxy PEG propionaldehydes used for coupling with rhDNase was provided by the manufacturer. However, the low polydispersity of the PEGs used for reaction with rhDNase was confirmed by MALDI-TOF MS analysis (**Figure 15** and *Supplementary Figure S3*), also supported by the literature. Indeed, Roberts et al. reported polydispersity in the range of 1.01 for low molecular weight PEGs (< 5 kDa) to 1.1 for high molecular weight PEGs (> 50 kDa) [288]. Finally, despite the difficulty to firmly determine the molecular weight of the conjugates, the results generated are in good agreement with mono-PEGylation.

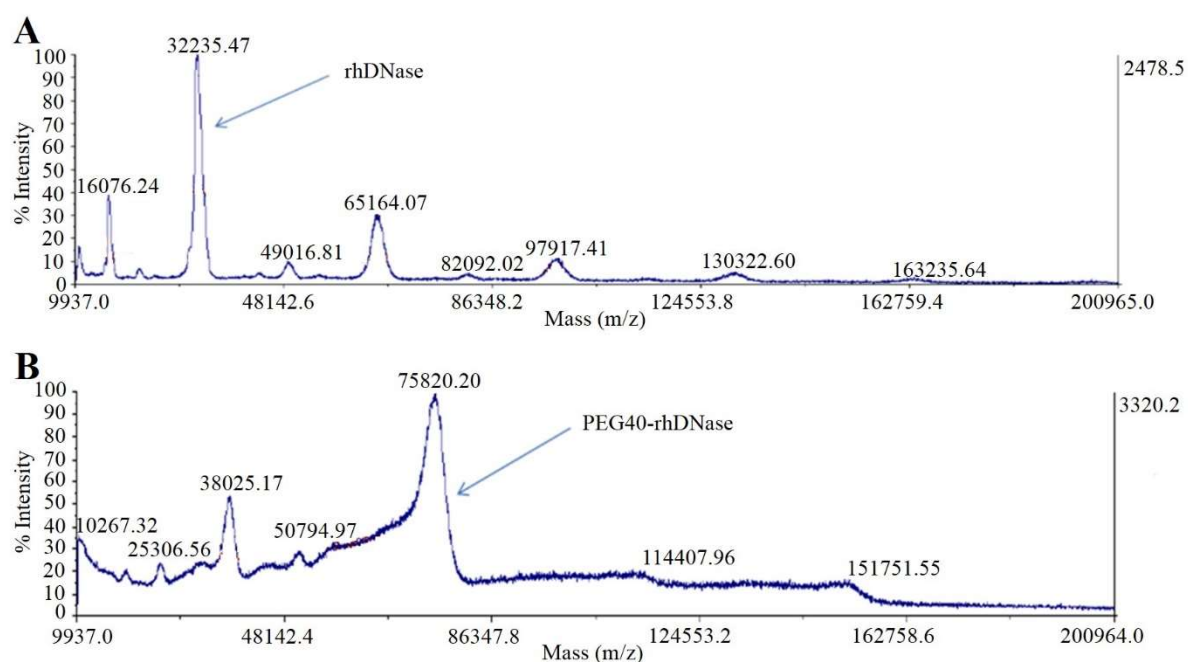


Figure 15: MALDI-TOF spectra of **A.** rhDNase prior to PEGylation, **B.** rhDNase following coupling to 40 kDa PEG. The figures showed that one PEG molecule was linked to each rhDNase. rhDNase showed a main peak at 32.2 kDa and peaks at 16.1 kDa and at 65.2 kDa associated with twice ionized and dimer forms. PEG40-rhDNase presented a main peak at 75.8 kDa and another peak at 38.0 kDa related to its twice ionized form.

A digested protein MALDI-TOF MS analysis using V8 protease was conducted on rhDNase and PEGylated proteins to confirm PEG attachment on the N-ter residue. The analysis led to generation of three peptides: the N-ter peptide at $m/z=1451.73$, a peptide containing a lysine residue at $m/z=4416.86$ and another peptide that did not contain a lysine residue at $m/z=1577.76$ (**Figure 16** and *Supplementary Figures S5 and S6*). We normalized the intensity of the N-ter and lysine peptides with the intensity of the peptide at $m/z=1577.76$ in order to estimate the percentages of non-modified peptides after PEGylation. The peak of the N-ter peptide resulting from rhDNase digestion almost vanished upon PEGylation with PEG40 (normalized intensity of 5% remaining) and decreased upon PEGylation with PEG20 and PEG30 (normalized intensity of 10% remaining for PEG20 and 24% remaining for PEG30; **Figure 16B**, **Figure 16C** and *Supplementary Figure S4*). The other peaks beyond $m/z=1451.73$ in the peptide spectrum correspond to the same peptide with a different isotopic composition. The peak at $m/z=1451.73$ is on the left of the spectrum and is related to monoisotopic composition of the N-ter peptide (carbon 12 only). This decrease in N-ter peptide peak intensity for PEGylated proteins underscores that the N-ter rhDNase peptide reacted with PEG. However, the absence of the total loss of the N-ter peptide peak suggests that unconjugated protein might remain after purification or that mono-PEGylation also occurred on lysine residues of rhDNase. These observations are dependent upon the PEG utilized. Purification of PEG40-rhDNase was easily achieved since the peaks of conjugate and native protein were well separated (**Figure 14A**). For PEG20-rhDNase and PEG30-rhDNase, the less efficient separation might lead to the presence of the native protein in the final product (*Supplementary Figure S4* and **Figure 16B**). The normalized intensity of the lysine peptide obtained after digestion with V8 protease did not vary after PEGylation with PEG40 but decreased for PEG20-rhDNase and PEG30-rhDNase suggesting that mono-PEGylation could also have occurred on the lysine residue for these PEG sizes (*Supplementary Figure S5*). However, rhDNase contains 6 lysine residues well dispersed on its 3D structure. Considering the reaction conditions presented above and the Henderson-Hasselbalch equation, the proportion of unprotonated N-ter amine is 100-fold superior to the proportion of unprotonated lysine amine. However, the unprotonated amine form of a lysine is more nucleophilic than that of the N-ter leucine residue [288]. Therefore, it can be suggested that more than 6% of the PEGylation reaction could have occurred on the lysine

residues (>1% per lysine residue). Taking into account the low sensitivity of the mass spectrometry analysis, the lysine modification could not have been detected.

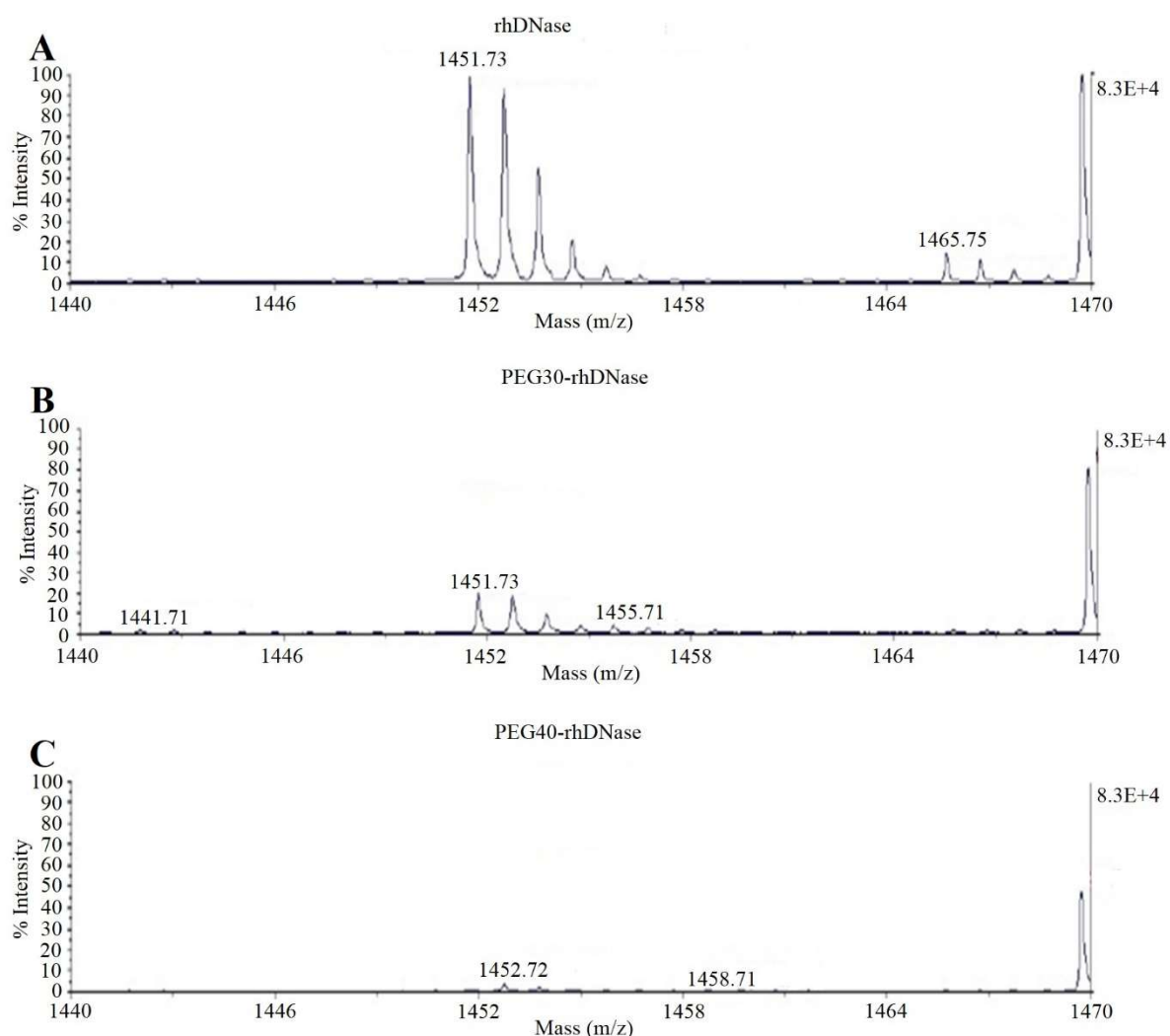


Figure 16: MALDI-TOF spectra of the N-ter peptide **A.** rhDNase prior to PEGylation, **B.** rhDNase following coupling to 30 kDa PEG and **C.** rhDNase following coupling to 40kDa PEG. The figures showed extinction or decrease of N-ter peaks confirming PEGylation on this residue.

PEGylation preserved the secondary structure of rhDNase (**Figure 17**). It is a crucial point since the protein functionality is intimately related to the preservation of its native structure [334]. Plesner et al. showed that PEGylated BSA maintained the protein secondary structure. The far UV scans were similar to the native BSA and no change were seen between the different PEGs used [345]. Also, Dhalluin et al. pointed out that PEG40-INF α_{2a} showed identical CD spectrum as native INF α_{2a} [28].

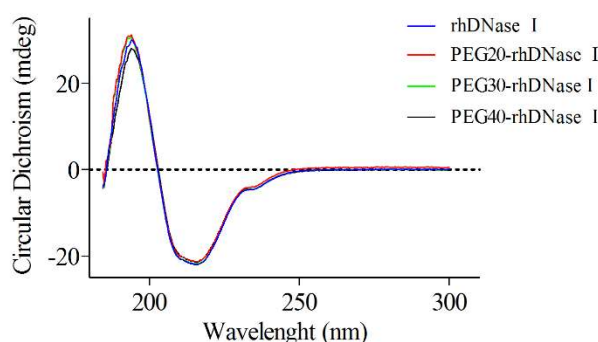


Figure 17: CD spectra of native rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase (6.7 μ M of rhDNase or PEG-rhDNase; the PEG was not counted in the concentration of the samples. Therefore, all rhDNase constructs were analyzed with the same number of moles of rhDNase). Structural analysis showed no modification of the secondary structure following addition of PEGs.

III.2 *IN VITRO* ENZYMATIC ACTIVITY OF THE PEGYLATED RHDNASES

An enzymatic activity assay was conducted to check whether PEGylated rhDNases lost any biological functionality (**Figure 18** and *Supplementary Figure S7*) [340]. To estimate the *in vitro* biological activity of the modified rhDNase *versus* the native protein, a methyl green assay was used. It showed that the enzymatic activity of PEGylated rhDNase was identical to that of the native protein, in line with our hypothesis (**Figure 13**). This assay measures the discoloration of a DNA-methyl green complex after addition of rhDNase and DNA cleavage [333]. All PEGylated proteins showed identical enzymatic activity as native rhDNase (**Figure 18** and *Supplementary Figure S7*).

Preserving the biological activity of proteins following PEGylation is challenging and several PEG-protein conjugates on the market present a partially lost biological activity. Pegasys[®] (PEG40-IFN α_{2a}) only retains 7% of the native interferon activity [346]. PEGylated adenosine deaminase (Adagen[®]) only retains 28% of the native protein activity [321]. However, in spite of their reduced activity, these PEGylated biopharmaceuticals are commercially available because of their tremendous increase in serum half-life. Therefore, maintaining the activity of rhDNase following PEGylation is an achievement and a favorable feature in considering the development of a future long-acting mucolytic product.

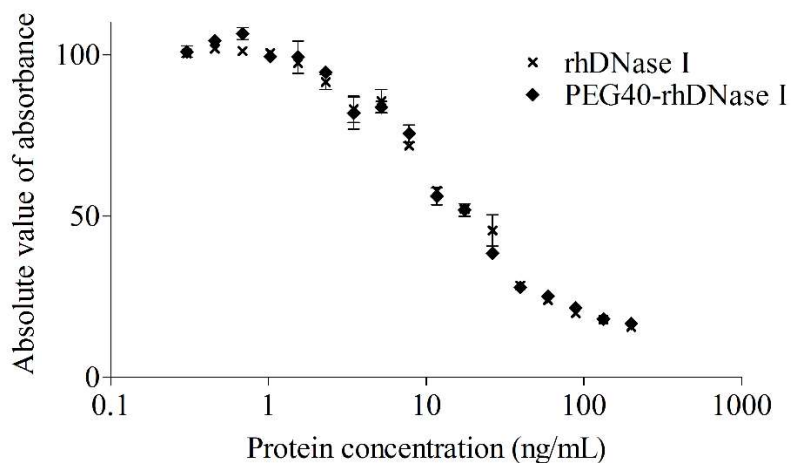


Figure 18: Hydrolysis of DNA by rhDNase and PEG40-rhDNase at increasing concentrations of the rhDNase moiety of the enzymes (n=3). The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore analyzed at the same molarities. DNA was complexed with methyl green and DNA hydrolysis produced unbound methyl green and a decrease of the absorbance of the solution at 650 nm after 6h of incubation at 37°C. The effective concentration at 50% was 14.8 ± 2.1 (mean $\pm$ SEM) and 14.3 ± 2.3 ng/mL for rhDNase and PEG40-rhDNase, respectively. The data were compared by two way-ANOVA with no difference seen between PEGylated rhDNase and native rhDNase.

IV. CONCLUSION

In conclusion, mono-PEGylation of rhDNase using linear 20 kDa PEG, linear 30 kDa PEG and two-arm 40 kDa PEG chain was achieved. The reaction conditions retained for the coupling were: 16:1 molar ratio [PEG]:[rhDNase], 96 h, room temperature, pH 5 for PEG20 and PEG30 and 8:1 molar ratio [PEG]:[rhDNase], 96 h, room temperature, pH 5.5 for PEG40. Mono-PEGylation mainly occurred at the N-ter residue of rhDNase and yields of the modified proteins reached 45% for PEG20, 48% for PEG30 and 61% for PEG40 after purification by anion-exchange chromatography. The PEGylated rhDNases preserved the secondary structure and the full biological activity of the native protein. These characteristics are an incentive for further studies. Accordingly, the mucolytic activity of the compounds on CF sputa is currently evaluated. In addition, an *in vivo* disposition study presently assesses the time of residence of PEGylated rhDNases in the murine lungs, compared with the unconjugated protein. This will highlight the prolonged lung retention of rhDNase provided by PEG of different sizes and might pave the way towards the development of a long-acting rhDNase with reduced therapy burden.

**CHAPTER III. IMPACT OF PEGYLATION ON THE STABILITY OF
RECOMBINANT HUMAN DEOXYRIBONUCLEASE I**

IMPACT OF PEGYLATION ON THE STABILITY OF RECOMBINANT HUMAN DEOXYRIBONUCLEASE I

Marie-Julie Guichard, Teresinha Leal, Mireille Dumoulin and Rita Vanbever
Personal contribution: all experiments except those of rhDNase conjugated to linear 20 kDa
and 30 kDa PEGs

ABSTRACT

The effects of PEGylation on the stability of recombinant human deoxyribonuclease I (rhDNase) were investigated using circular dichroism and fluorescence spectroscopies. Mono-PEGylation of rhDNase was achieved with polyethylene glycols (PEG)s of varying size: linear 20 kDa, linear 30 kDa or two-arm 40 kDa PEG. The PEGylation of rhDNase resulted in the destabilization of the protein upon guanidinium chloride (GdmCl)-induced unfolding. Indeed, the extent of destabilization depended on the PEG molecular weight: the bigger the PEG, the more destabilized the protein. The thermal denaturation of rhDNase and rhDNase conjugated with two-arm 40 kDa PEG were also investigated. The results showed that PEGylation had no significant effects on the temperature of mid-transition of the enzyme. However, the 40 kDa PEG conjugation significantly reduced the formation of aggregates while exposing the protein to high temperatures. The data presented constitute a preliminary investigation. Complementary studies will be conducted to firmly confirm the outcomes of this paper.

I. INTRODUCTION

PEGylation is the process of covalent attachment of polyethylene glycol (PEG) to a protein [288]. It is used to prolong the *in vivo* residence time, thus resulting in improved pharmaceutical properties. However, the PEGylation of a protein can have some drawbacks as it may perturb its structure, stability and activity [321,346–348]. While several studies demonstrated that PEG attachment did not alter either the secondary or the tertiary structure of proteins [318,334,345,349], others related a significant destabilization upon PEGylation [350]. Consequently, it is not possible to predict the effects of this modification process on proteins. Human deoxyribonuclease I (rhDNase, commercialized as Pulmozyme® by Roche, Basel, Switzerland) is the reference medication for the symptomatic treatment of cystic fibrosis (CF) lung disease. The role of this 30 kDa glycoprotein is to cleave the DNA phosphodiester bonds contained in the respiratory secretions [4]. Due to its rapid clearance from the lungs, rhDNase is daily administered by inhalation to patients with CF [2]. In order to increase the residence of the enzyme after its administration, we have recently developed PEGylated versions of rhDNase. We have demonstrated that a single PEG chain of 20 kDa, 30 kDa or 40 kDa, attached on the N-terminal amino acid residue of rhDNase, does not affect the secondary structure and the enzymatic activity of the protein [312]. These results do not, however, rule out a possible effect on the stability of rhDNase, which is a fundamental parameter for further pharmaceutical development of the PEGylated protein. The objective of the present study was to compare the guanidinium chloride (GdmCl)- and temperature-induced unfolding properties of rhDNase and mono-PEGylated rhDNase with 20 kDa, 30 kDa or 40 kDa PEG (further termed as PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase, respectively). The changes in the structure of the proteins upon unfolding were monitored by intrinsic fluorescence, 8-anilinonaphthalene-1-sulfonate (ANS)-binding fluorescence and circular dichroism (CD) in the far UV region. The results obtained indicated that PEGylation destabilized rhDNase upon GdmCl-induced denaturation but it did not significantly affect the temperature of mid-denaturation of the enzyme. In addition, these preliminary results suggested that the PEGylated rhDNase was less prone to aggregation upon thermal denaturation than the unmodified protein. These preliminary results contribute to the better understanding of the effect of PEGylation on the stability of pharmaceutical proteins.

II. MATERIAL AND METHODS

II.1. MATERIAL

Pulmozyme® was from Genentech, Inc. (South San Francisco, CA, USA). Linear 20 kDa and 30 kDa methoxy PEG propionaldehydes and two-arm 40 kDa methoxy PEG propionaldehyde were obtained from NOF Corporation (Tokyo, Japan).

The N-terminal site-specific mono-PEGylation of rhDNase was carried out as previously described [312].

Ultrapure GdmCl and ANS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise stated, other chemicals and reagents were also from Sigma-Aldrich.

II.2. GUANIDINIUM CHLORIDE (GDMCL)-INDUCED UNFOLDING

rhDNase and PEGylated rhDNases (0.125 mg/mL or 4.2 μ M of rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass) were incubated overnight at 25°C in 150 mM NaCl, 1 mM CaCl₂ containing different GdmCl concentrations ranging from 0 to 5.1 M. The concentrations of rhDNase and PEGylated rhDNases were determined from absorbance measurement at 280 nm, where the PEG has no signal contribution. The concentration in GdmCl in each sample was determined from refractive index measurements [351] using a R5000 hand-held refractometer (Atago, Tokyo, Japan). Intrinsic fluorescence, ANS-binding fluorescence and far UV-CD spectra were recorded using the parameters described below. The unfolding curves were analyzed on the basis of a two-state model ($N \rightleftharpoons U$) or a three-state model ($N \rightleftharpoons I \rightleftharpoons U$), according to Dumoulin *et al.* [352] and Montagner *et al.* [353], respectively.

II.3. THERMAL UNFOLDING OF RHDNASE AND PEGYLATED RHDNASE

The protein concentration was 0.125 mg/mL (4.2 μ M in rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass) in 150 mM NaCl, 1 mM CaCl₂. The temperature was linearly increased from 25°C to 95-97°C at a rate of 0.5°C/min and the actual

temperature of the sample was monitored using a thermocouple. The reversibility of the unfolding reaction was monitored by decreasing the temperature from 95°C or 97°C to 25°C also at 0.5°C/min. The unfolding transitions were monitored by fluorescence and far UV-CD as described below. A two-state model ($N \rightleftharpoons U$) was assumed for the thermal unfolding reaction [354]. The temperature of mid-transition (T_m) was determined for each protein.

II.4. FLUORESCENCE SPECTROSCOPY

All fluorescence measurements were carried out using a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA) equipped with a 4-cells Peltier cell-holder and 1 cm path length quartz cells.

Intrinsic fluorescence emission spectra were recorded at 25°C in presence of different GdmCl concentrations (ranging from 0 to 5.1 M). Fluorescence spectra were also recorded at 95°C or 97°C in the absence of GdmCl. The excitation wavelength was 280 nm or 295 nm and emission spectra were recorded between 305 nm and 460 nm. The excitation and emission slit widths were both 5 nm and the high voltage on the photomultiplier was set at 750 V. The scan rate was 600 nm/min. For each sample, five spectra were accumulated, averaged and corrected from the signal recorded for the solvent alone. The unfolding transitions were monitored at 336.9 nm. The unfolding transitions were also obtained by monitoring the changes in the wavelength of maximum fluorescence, which was determined as previously described [352].

The following parameters were used to monitor the thermal unfolding. The excitation wavelength was set at 295 nm and the emission of fluorescence was monitored at 342 nm. The excitation and emission slit widths were both 5 nm. The high voltage on the photomultiplier was set at 750 V.

ANS-binding fluorescence was used to monitor the exposition of hydrophobic patches to the solvent. A concentrated ANS solution was prepared in 150 mM NaCl, 1 mM CaCl₂. Its concentration was determined at 350 nm using the molar extinction coefficient of 4950 M⁻¹.cm⁻¹. ANS was added to rhDNase or PEG40-rhDNase (0.125 mg/mL or 4.2 μM in rhDNase,

without counting the PEG moiety in the PEG-rhDNase mass) at a [ANS]:[protein] molar ratio of 10:1.

ANS-binding fluorescence measurements in the presence of different GdmCl concentrations were performed using the same samples as those used for intrinsic fluorescence measurement. The excitation was performed at 350 nm and emission spectra were recorded between 420 nm and 600 nm. The fluorescence spectra were corrected by subtracting the background fluorescence of ANS alone. The fluorescence intensity at 480 nm was used to monitor the denaturation of the proteins. High voltage of 950 V was applied to the photomultiplier.

For the thermal unfolding, the wavelengths of excitation and emission were 350 nm and 480 nm, respectively. The excitation and emission slit widths were both 5 nm. The voltage applied to the photomultiplier was 950 V. The signal observed with the protein sample was corrected from the contribution of ANS alone.

II.5. CIRCULAR DICHROISM (CD)

CD spectra in the far-UV region (190 nm to 250 nm) were recorded on a J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan) equipped with a four-cell Peltier holder. The spectra were measured using a 1 mm precision Quartz Suprasil cell (Hellma Analytics, Müllheim, Germany). The scan rate was 50 nm/min and the integration time was 2 s. The proteins were diluted in 150 mM NaCl, 1 mM CaCl₂ to a concentration of 0.125 mg/mL (or 4.2 μ M in rhDNase, without counting the PEG moiety in the PEG-rhDNase mass). Six spectra were accumulated and corrected from the signal of the solvent.

GdmCl unfolding curves were recorded at fixed wavelengths of 217 nm and 222 nm, using a 1 nm bandwidth. The proteins were diluted to a final concentration of 0.125 mg/mL (or 4.2 μ M in rhDNase, without counting the PEG moiety in the PEG-rhDNase mass) in 150 mM NaCl, 1 mM CaCl₂. At all denaturant concentrations, at least 30 data points were acquired with a reading frequency of 1/15 s⁻¹ and a 2 s integration time, then averaged. The resulting values were corrected for the contribution of the solvent.

Thermal unfolding transitions were monitored using the CD signal at 217 nm and 222 nm, with a protein concentration of 0.125 mg/mL (or 4.2 μ M in rhDNase, without counting the PEG moiety in the PEG-rhDNase mass; in 150 mM NaCl, 1 mM CaCl₂) and a 1 mm precision Quartz Suprasil cell. The temperature was increased from 25°C to 95°C or 97°C at a rate of 0.5°C/min. The reversibility was assayed by cooling the sample down to 25°C at a rate of 0.5°C/min. Data were acquired every 30 s with a 2 s integration time and a 1 nm bandwidth.

III. RESULTS

III.1. EFFECTS OF PEGYLATION ON THE STRUCTURE OF rHDNASE

The secondary and tertiary structures of PEGylated conjugates were analyzed by far UV-CD and intrinsic fluorescence, respectively, and compared to those of rHDNase. The far UV-CD spectra of the three PEGylated proteins were superimposed to that of the unmodified protein (data not shown). Thus, in agreement with the results previously published, mono-PEGylation of rHDNase with the three PEG did not affect the secondary structure of the enzyme [312]. The tryptophan fluorescence spectra of the three PEGylated proteins were also similar (**Figure 19**). Consequently, neither the rHDNase secondary structure, nor its tertiary structure were altered by PEGylation. These observations are in agreement with the fact that none of the PEG attachment affected the enzymatic activity of rHDNase [312].

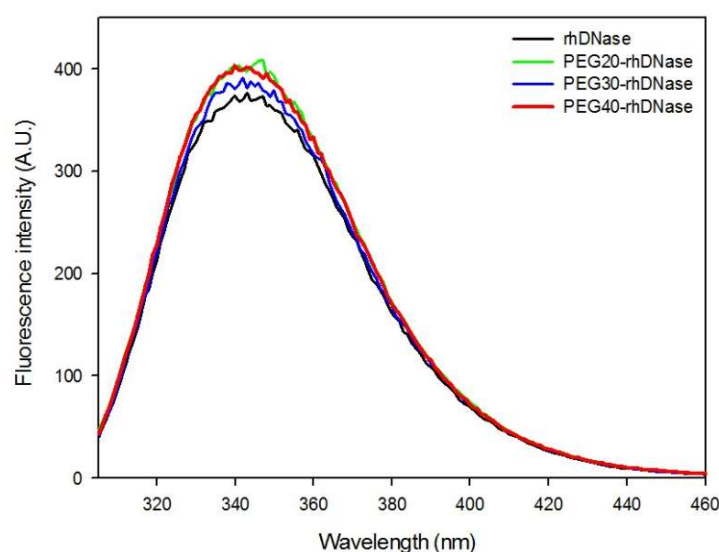


Figure 19: Tryptophan fluorescence spectrum of rHDNase (black line), PEG20-rHDNase (green line), PEG30-rHDNase (blue line) and PEG40-rHDNase (red line). The proteins were diluted in 150 mM NaCl, 1 mM CaCl₂ at a final concentration of 0.125 mg/mL (or 4.2 μ M in rHDNase, without counting the PEG moiety in the PEG-rHDNase mass). The spectra were recorded at 25°C.

III.2. EFFECTS OF PEGYLATION ON GDMCl-INDUCED UNFOLDING OF RHDNASE

The GdmCl-induced unfolding of rhDNase was first investigated by recording intrinsic fluorescence and far UV-CD spectra as function of the concentration in denaturant (**Figure 20**). As the concentration in denaturant increased, the fluorescence intensity decreased and the wavelength of the maximum fluorescence (λ_{max}) was shifted to higher wavelengths (i.e. from 342 to 360 nm, **Figure 20A**). These observations indicated that, at least, one of the tryptophan residues became exposed to the solvent, and thus that the protein unfolded. The spectra of rhDNase samples containing GdmCl concentrations higher than 2.7 M did not significantly differ from each other (**Figure 20A**). This suggested that the protein was fully denatured at this concentration in denaturant. The spectra of rhDNase recorded in the absence and in the presence of 5.1 M GdmCl significantly differed in the region of 330 nm to 340 nm, with the largest difference between the native and unfolded states being at 336.2 nm (**Figure 20B**). Therefore, the unfolding transition could be monitored at this specific wavelength. However, no data were available at 336.2 nm. Therefore, the transition was monitored at 336.9 nm.

The far UV-CD spectra of rhDNase recorded at increasing concentrations of GdmCl showed a major decrease in the signal of helical and β -sheet contents starting at 2.7 M (**Figure 20C**). The spectra of rhDNase recorded in the absence and in the presence of 5.1 M GdmCl differed the most at 217 nm (**Figure 20C**). Therefore, the unfolding transition was monitored at this wavelength. The transition of rhDNase in far UV-CD was also followed at 222 nm since both wavelengths are indicative of β -sheets and α -helices.

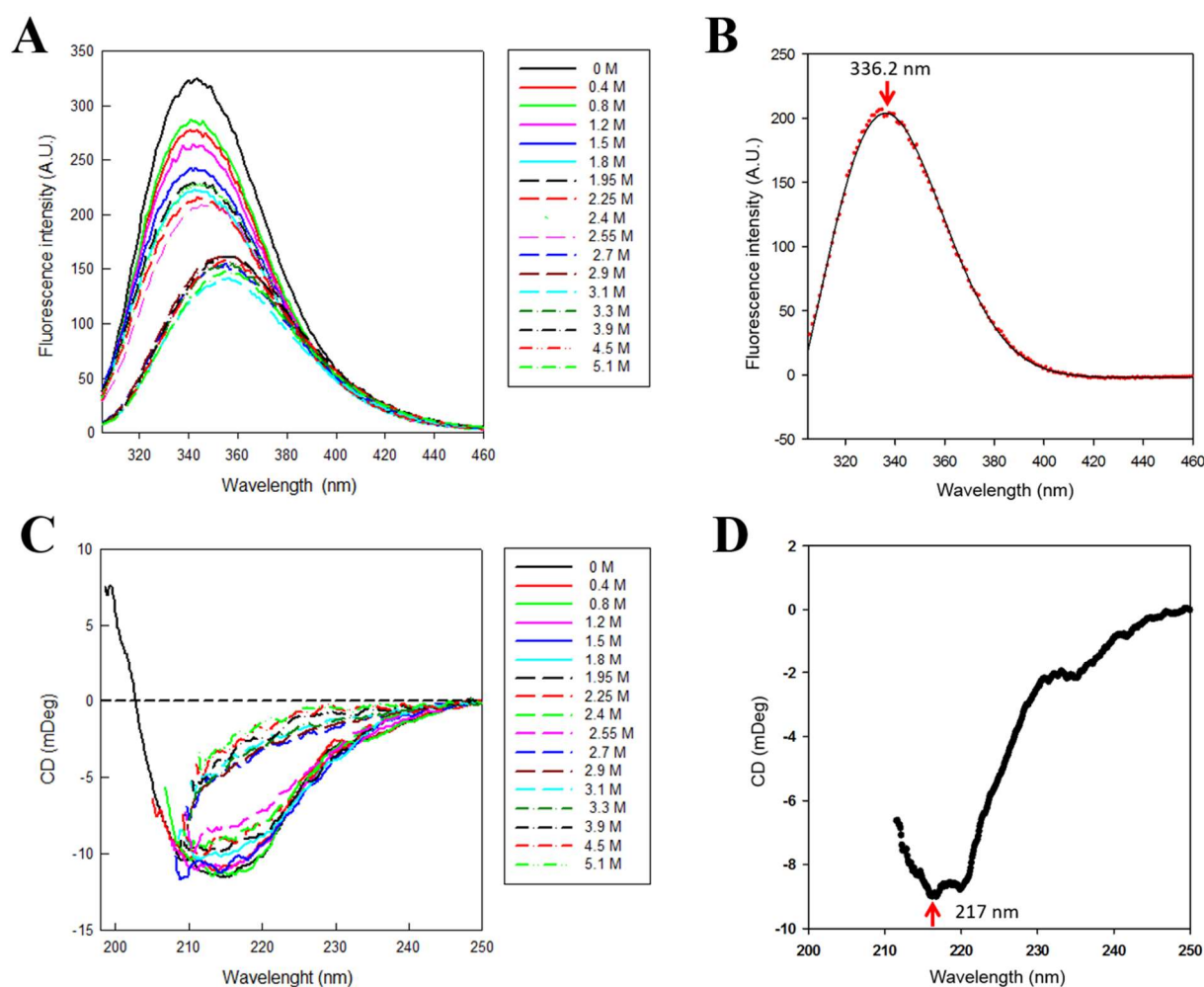


Figure 20: Fluorescence (A) and far UV-CD (B) spectra of rhDNase (at 0.125 mg/mL or 4.2 μM) at different concentrations in GdmCl. C. and D. represent the difference spectra between the native (0 M GdmCl) and unfolded rhDNase (5.1 M GdmCl) in terms of fluorescence intensity and CD, respectively. The largest difference between the native and unfolded states is at 336.2 nm for the intrinsic fluorescence (C). For far UV-CD, the maximal difference is at 217 nm (D).

Two transitions between the native and unfolded states of rhDNase are observed in **Figure 21**, indicating that the denaturation of both the secondary and tertiary structures of rhDNase occurred via a three-state model. A first transition occurred at denaturant concentrations comprised between 1.5 and 2.5 M. A second transition was observed at higher concentrations of GdmCl. The transition monitored by intrinsic fluorescence and far UV-CD spectra were well superimposed, indicating that the secondary and tertiary structures unfolded cooperatively.

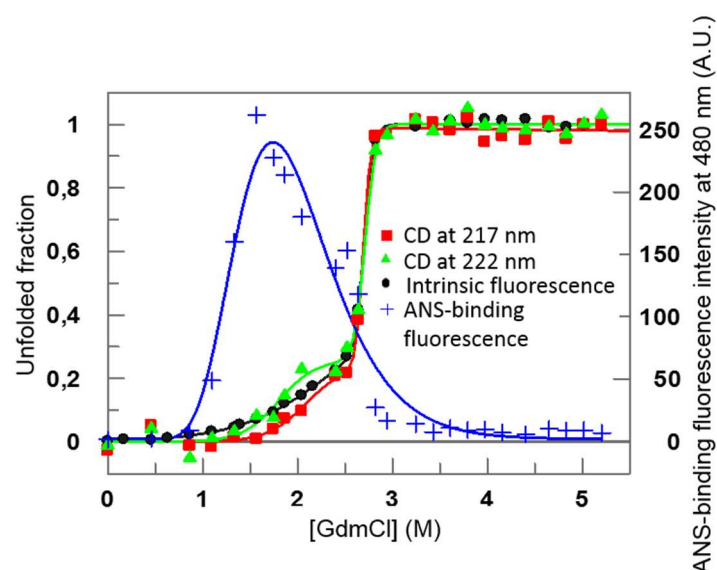


Figure 21: GdmCl-induced unfolding transition of rhDNase (at 0.125 mg/mL or 4.2 μ M). Normalized transitions (from transitions monitored by the changes in fluorescence intensity at 336.9 nm and by far UV-CD signal at 217 nm and 222 nm). The transition monitored by ANS-binding fluorescence is also shown. Intrinsic fluorescence and far UV-CD data were analyzed based on a three-state model. Solid lines represent least-squares fit analyses of the experimental data.

The GdmCl-induced unfolding transitions obtained for PEG40-rhDNase were similar to those of the unmodified enzyme: two transitions were observed for the unfolding of the secondary and tertiary structures (**Figure 22**). Therefore, the PEGylation of rhDNase with a large 40 kDa PEG chain did not modify the unfolding pathway of the protein. However, the transitions monitored for PEG40-rhDNase suggested that the amount of the intermediate species was higher than for rhDNase. Nevertheless, this result should be carefully interpreted. Additional experiments will be conducted.

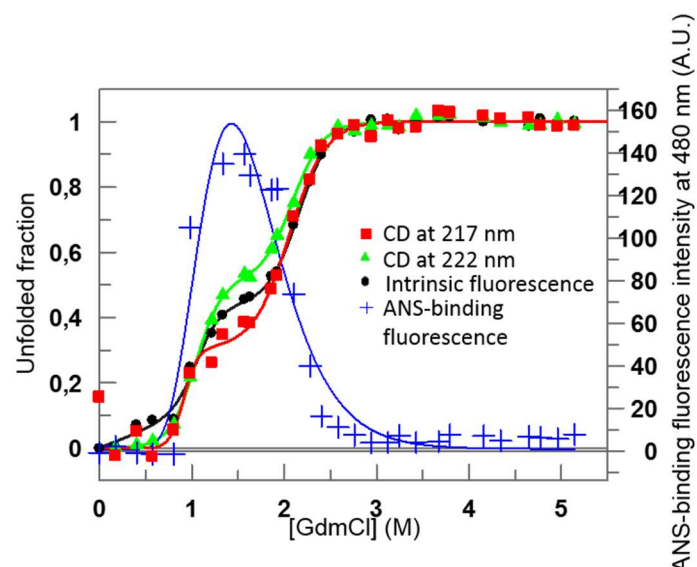


Figure 22: GdmCl-induced unfolding transition of PEG40-rhDNase (at 0.125 mg/mL or 4.2 μ M in rhDNase, without counting the PEG moiety). Normalized transitions (from transition monitored by the changes in fluorescence intensity at 336.9 nm and far UV-CD signal at 217 nm and 222 nm). The transition monitored by ANS-binding fluorescence is also shown. Intrinsic fluorescence and far UV-CD data were analyzed based on a three-state model. Solid lines represent least-squares fit analyses of the experimental data.

GdmCl-induced unfolding of rhDNase and PEG40-rhDNase were also monitored by ANS-binding fluorescence (**Figure 21** and **Figure 22**). ANS is a fluorescent dye that binds to exposed hydrophobic surfaces of proteins [355], and its fluorescence properties are modified upon binding (i.e. there a shift of the wavelength of maximum fluorescence from ~ 530 nm to 480 nm and a drastic increase in its fluorescence intensity). An increase in fluorescence intensity was observed centered at 1.8 M GdmCl for rhDNase (**Figure 21**) and at 1.2 M GdmCl for PEG40-rhDNase (**Figure 22**). The binding of ANS seemed to essentially occur at GdmCl concentrations corresponding to the first transition observed by intrinsic fluorescence and far UV-CD for both proteins. Therefore, at these concentrations of denaturant, both rhDNase and PEG40-rhDNase populated an intermediate species, which exposed hydrophobic patches to the solvent. Regarding rhDNase (**Figure 21**), the ANS peak was not centered with the plateau of the first transition, potentially explained by the fact that some data points were removed from the analysis due to their unexpected values.

In order to determine if the extent of destabilization varied with the PEG size, the unfolding of PEG20-rhDNase and PEG30-rhDNase were monitored by intrinsic fluorescence. In this evaluation, changes in wavelength corresponding to the maximum fluorescence intensity

(lambda max) were recorded for all the four rhDNase constructs. Both PEG20-rhDNase and PEG30-rhDNase curves displayed a single transition, highlighting an apparent cooperative unfolding of the tertiary structure (**Figure 23**). Therefore, a two-state model was used for the fit of the transitions of PEG20-rhDNase and PEG30-rhDNase. However, the unfolding transitions of both PEG20-rhDNase and PEG30-rhDNase were analyzed at a lower number of GdmCl concentrations, thereby certainly missing the first transition observed for rhDNase and PEG40-rhDNase (**Figure 23**). Nevertheless, considering rhDNase and its PEGylated counterparts, the overlay of all the transitions clearly indicated that the bigger the PEG, the more destabilized the protein. This qualitative analysis also suggested that the two-arm structure of 40 kDa PEG induced the highest destabilization of rhDNase whereas both linear 20 kDa and 30 kDa PEGs would destabilize the protein structure in a similar way.

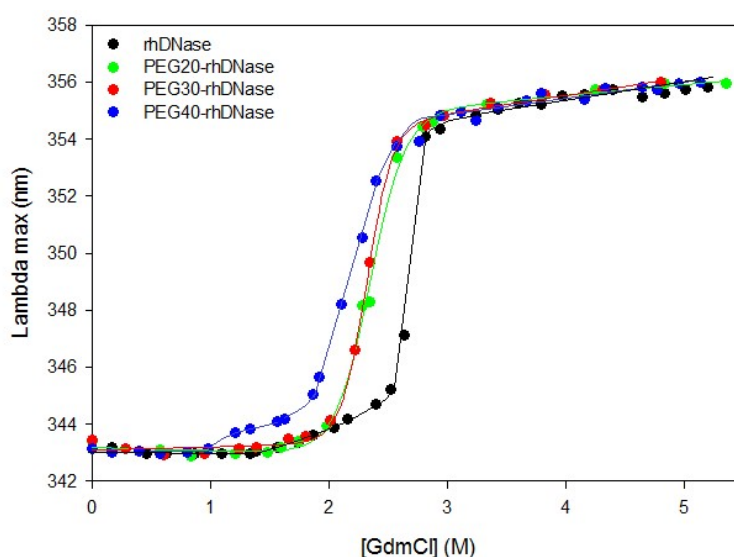


Figure 23: GdmCl-induced unfolding transitions monitored by the changes in wavelength at the maximum fluorescence intensity (lambda max) of rhDNase (black dots), PEG20-rhDNase (green dots), PEG30-rhDNase (red dots) and PEG40-rhDNase (blue dots). All rhDNase constructs were analyzed at a concentration of 0.125 mg/mL or 4.2 μ M in rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass. Data were analyzed based on a two-state model (PEG20-rhDNase and PEG30-rhDNase) or three-state model (rhDNase and PEG40-rhDNase). Solid lines represent least-squares fit analyses of the experimental data according to a two-state or three-state unfolding mechanism.

III.3. EFFECT OF PEGYLATION ON TEMPERATURE-INDUCED UNFOLDING OF rhDNASE

The thermal unfolding of rhDNase was also monitored by intrinsic fluorescence, ANS-binding fluorescence and far UV-CD (**Figure 24A** and **B**). A comparison of the intrinsic fluorescence spectra of the protein recorded at 25°C, 97°C and after cooling the sample back to 25°C clearly indicated that the thermal unfolding process was not fully reversible. The spectra obtained at 25°C before and after heating the protein to 97°C were not superimposable (**Figure 24A**). Moreover, the far UV-CD spectrum recorded after the cooling of the sample to 25°C was similar to that of 97°C (**Figure 24B**). The thermal unfolding of both the secondary and tertiary structures of rhDNase was therefore not fully reversible. Comparing the intrinsic fluorescence spectrum of rhDNase recorded at 97°C with that of rhDNase exposed to 3.1 M GdmCl (characteristic of an unfolded protein), we suggested that rhDNase exposed to 97°C completely lost its tertiary structure (almost no residual signal; **Figure 24A**). On the opposite, the far UV-CD spectrum of rhDNase obtained at 97°C was not characteristic of a denatured protein, contrary to that observed in the presence of 3.1 M GdmCl (**Figure 8B**). The signal observed in the region between 208 nm and 230 nm was expected to decrease (in absolute value) upon unfolding. However, we observed a signal characteristic of an increase in its secondary structures. Consequently, we assumed the formation of an intermediate state or of an aggregated species of rhDNase at 97°C (**Figure 24B**).

PEG40-rhDNase presented a different behavior when exposed to thermal denaturation (**Figure 24C** and **D**). Following the thermal unfolding process, PEG40-rhDNase retained a certain part of its native secondary structure (**Figure 24D**). However, a slight decrease (in absolute value) in its signal was observed upon unfolding (**Figure 24D**), suggesting that different phenomena occurred for the denaturation of the secondary structures of rhDNase and PEG40-rhDNase. Comparing the results of rhDNase and PEG40-rhDNase obtained at 25°C before and after heating, we assumed that rhDNase PEGylated with a single 40 kDa PEG showed a higher reversibility of the thermal unfolding than the free protein. Indeed, the fluorescence and far UV-CD spectra of PEG40-rhDNase obtained after cooling the sample to 25°C were closer to the spectra of its native state, compared to those of rhDNase (**Figure 24**).

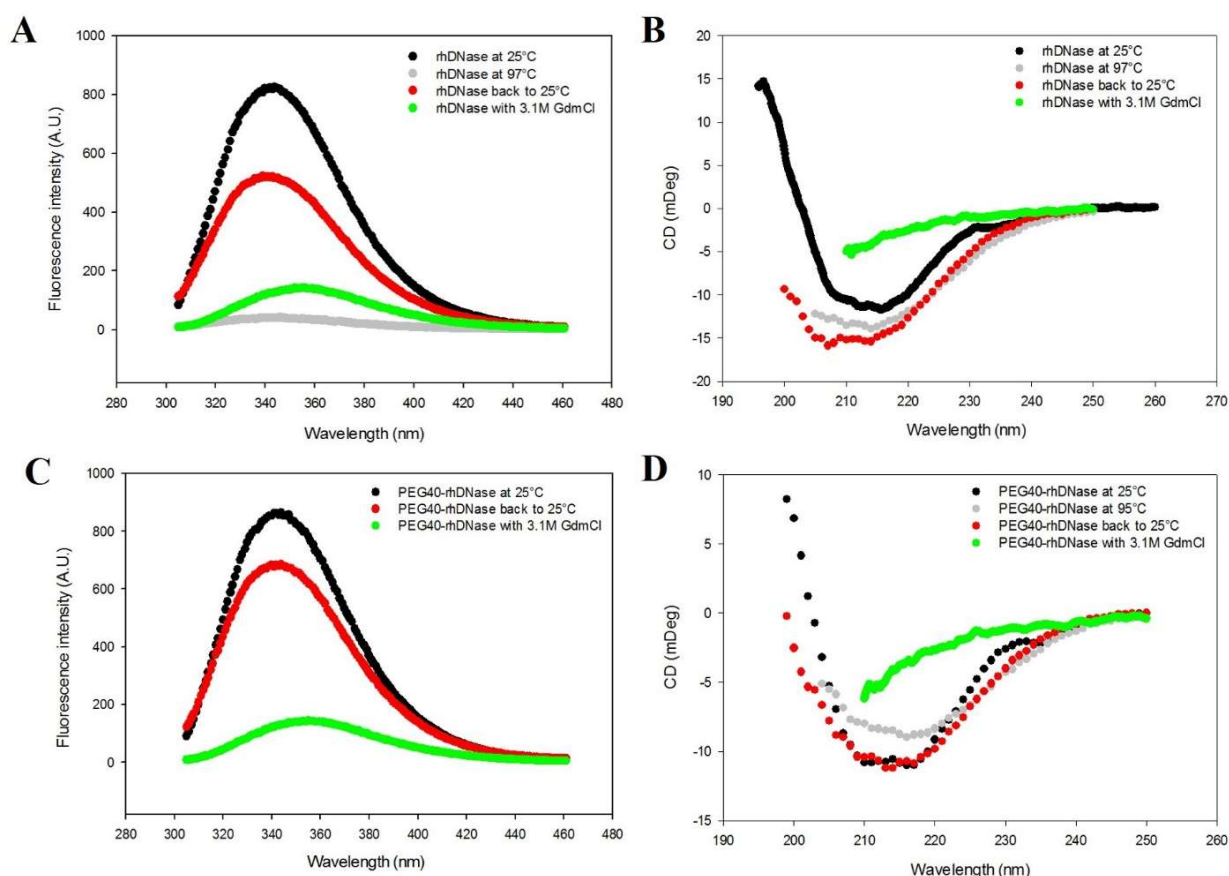


Figure 24: Influence of the temperature on rhDNase and PEG40-rhDNase: fluorescence spectra (**A** and **C**) and far UV-CD spectra (**B** and **D**). Both proteins were analyzed at a concentration of 0.125 mg/mL or 4.2 μ M in rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass. Spectra were compared to the signals obtained in presence of 3.1 M GdmCl.

rhDNase and PEG40-rhDNase were stable to the same extent upon thermal denaturation. Monitoring of the unfolding by intrinsic fluorescence highlighted steep pre-and post-transition baselines for both proteins, compared to the change in signal observed upon the unfolding transition (i.e. temperatures comprised between 55–65°C) (**Figure 25A**). A single transition was observed, suggesting that the tertiary structures of both rhDNase and PEG40-rhDNase unfolded via a two-state process. However, as shown on **Figure 24A** and **C**, the transitions were not fully reversible. Therefore, only the apparent temperature of mid-denaturation ($T_{m, app}$) was derived from these experiments. The apparent T_m were similar for rhDNase and PEG40-rhDNase, with values of 61.7°C and 61.9°C, respectively (**Table 5**).

The unfolding transitions of rhDNase monitored by far UV-CD at 217 nm and 222 nm both displayed a single transition (**Figure 25B**). However, as underline above, these transitions

corresponded to an increase in the secondary structure content, and thus did not describe the transition from N to U. Rather, they were likely to monitor a transition from native to an intermediate state or to an aggregated species (see below). Fitting the transitions of rhDNase to an apparent two-state model allowed the determination of the apparent T_m of 62.6°C and 62.1°C when monitored at 217 and 222 nm, respectively (**Table 5**). The normalized transitions monitored by both far UV-CD and intrinsic fluorescence superimposed, suggesting that they monitored the same process whatever this later was (**Figure 26A**).

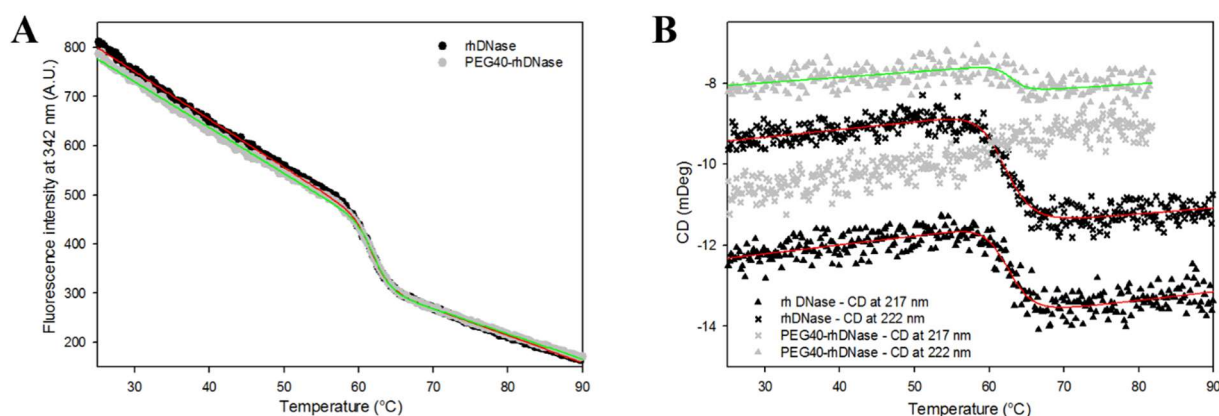


Figure 25: Comparison of the thermal unfolding of rhDNase and PEG40-rhDNase analyzed at a concentration of 0.125 mg/mL or 4.2 μ M in rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass. **A.** Comparison of the unfolding transitions monitored by intrinsic fluorescence. **B.** Comparison of the unfolding transitions monitored by far UV-CD at 217 nm (crosses) and 222 nm (triangles). Solid lines represent least-squares fit analyses of the experimental data according to a two-state unfolding mechanism.

The far UV-CD unfolding transitions of PEG40-rhDNase obtained at 217 nm and 222 nm displayed different outcomes. No clear transition was observed at 217 nm since the changes in the intensity at this wavelength were rather linear (**Figure 25B**). Regarding the data obtained at 222 nm, a discrete single transition was observed around 60°C (**Figure 25B**). Similarly to rhDNase, the shape of the transition of PEG40-rhDNase at 222 nm suggested an increase in the secondary structure content (**Figure 25B**). Therefore, this transition did not describe the transition from N to U. The adjustment of the experimental data to an apparent two-state unfolding process, allowed to determine an apparent T_m of 63.0°C for PEG40-rhDNase (**Table 5**). Given the small changes in the far UV-CD signal at 222 nm for PEG40-rhDNase (**Figure 25B**), the corresponding normalized transition was extremely noisy and no clear transition was visualized (**Figure 26B**).

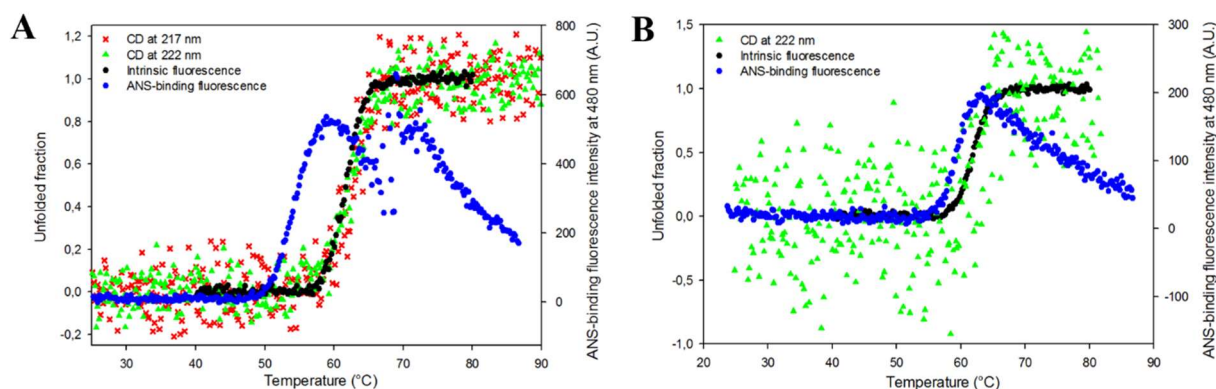


Figure 26: Thermal unfolding transition of (A) rhDNase and (B) PEG40-rhDNase analyzed at a concentration of 0.125 mg/mL or 4.2 μ M in rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass. The normalized transitions were monitored by intrinsic fluorescence (black points) and far UV-CD at 217 nm (crosses) and at 222 nm (triangles). Data were analyzed based on a two-state model. The thermal unfolding transitions of rhDNase and PEG40-rhDNase were also monitored by ANS-binding fluorescence at 480 nm (blue points).

Table 5: Apparent temperature of mid-denaturation (T_m) derived from the thermal unfolding transition of rhDNase and PEG40-rhDNase based on fluorescence intensity and far UV-CD measurements (mean $\pm$ SD). Both proteins were analyzed at a concentration of 0.125 mg/mL or 4.2 μ M in rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass. The transitions were analyzed based on a two-state transition. N/A, not applicable.

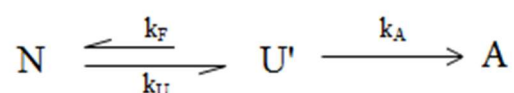
Protein	Fluorescence intensity	CD at 217 nm	CD at 222 nm
rhDNase	$61.7 \pm 0.1^\circ\text{C}$	$62.6 \pm 0.3^\circ\text{C}$	$62.1 \pm 0.3^\circ\text{C}$
PEG40-rhDNase	$61.9 \pm 0.1^\circ\text{C}$	N/A	$63.0 \pm 0.6^\circ\text{C}$

ANS-binding fluorescence indicated that both rhDNase and PEG40-rhDNase populated an intermediate species that exposed hydrophobic patches (**Figure 26**). Surprisingly, this intermediate species started to be populated at temperatures lower than those for which changes in fluorescence and far UV-CD were observed. At 90°C, the ANS-binding fluorescence did not go back to zero (**Figure 26**), while unfolded species normally do not bind ANS. Therefore, we suggested that some aggregates binding ANS were formed upon thermal unfolding. Regarding the ANS-binding fluorescence of rhDNase, the transition exhibited an unusual profile (**Figure 26A**). The reasons behind were not fully understood and the experiment will be repeated.

In order to test the hypothesis of the formation of aggregates binding ANS, the ANS-binding fluorescence spectra of the two proteins back to 25°C were recorded (**Figure 27**). The results

obtained showed a significant binding of ANS, suggesting that an intermediate or aggregates species (for which the thermal unfolding was not fully reversible) was populated for both proteins. rhDNase and PEG40-rhDNase samples were therefore centrifuged (at 17,000 g for 30 min, at 15°C) in order to remove the large (and insoluble) aggregates eventually formed. Then, ANS-binding fluorescence spectra of both supernatants were recorded (**Figure 27**). Considering rhDNase, the fluorescence signal was drastically reduced (**Figure 27A**). However, no reduction in signal for the supernatant of PEG40-rhDNase was observed (**Figure 27B**). This finding clearly indicated that large aggregates exposing hydrophobic patches were formed upon thermal unfolding of rhDNase. In order to remove potential small (and soluble) aggregates, rhDNase and PEG40-rhDNase supernatants were then ultra-centrifuged (at 360,000 g for 30 min, at 15°C). No further changes in the ANS-binding spectra were observed for both proteins (**Figure 27**), indicating that the ANS-binding fluorescence observed was not associated with the formation of soluble aggregates. In the case of PEG40-rhDNase, we hypothesized that the large signal observed following ultra-centrifugation might be due to the irreversible formation of an intermediate species or soluble aggregates that cannot be separated by the ultra-centrifugation conditions we used.

Considering all these data, we proposed that the thermal unfolding of both rhDNase and PEG40-rhDNase occurred via the following model:



with U' being an unfolded state most likely different from that observed with high concentrations of GdmCl, A being insoluble aggregates and k_F , k_U and k_A corresponding to the folding, unfolding and aggregation rate constants, respectively.

Although PEG attachment to rhDNase did not modify the equilibrium between N and U states (i.e. rhDNase and PEG40-rhDNase displayed a similar apparent T_m), the rate constant of aggregation was decreased by PEGylation of the enzyme. Further experiments should be carried out to confirm this model. In particular, it would be interesting to monitor the thermal unfolding using complementary techniques such as Fourier Transform Infrared Spectroscopy and Differential Scanning Calorimetry. In addition, it would be interesting to more precisely quantify the aggregation phenomenon and the extent of reversibility.

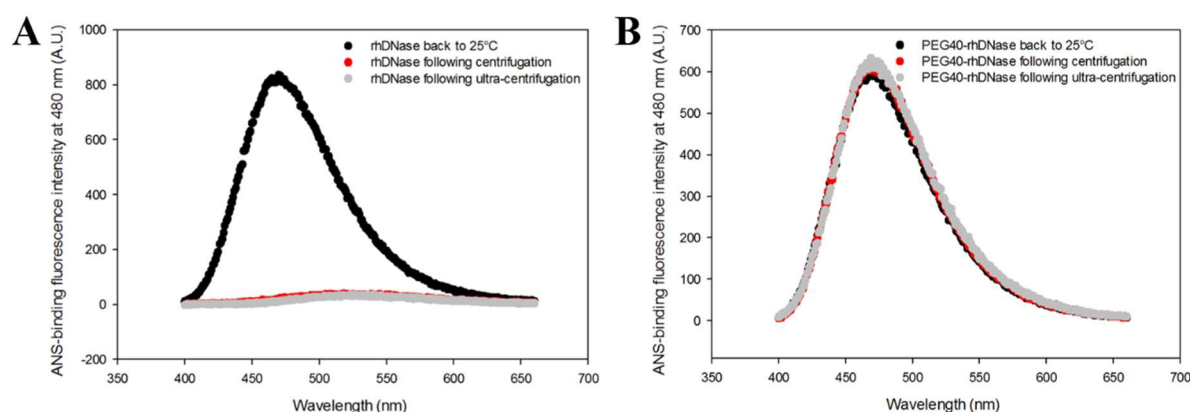


Figure 27: Evaluation of the presence of aggregates induced by thermal denaturation of (A) rhDNase and (B) PEG40-rhDNase monitored by ANS-binding fluorescence. ANS-binding fluorescence intensity was monitored back to 25°C (black points), then after centrifugation (red points; at 17,000 g for 30 min, at 15°C) and ultra-centrifugation (grey points; at 360,000 g for 30 min, at 15°C) in order to statute on the formation of aggregates for both proteins. Both proteins were analyzed at a concentration of 0.125 mg/mL or 4.2 μ M in rhDNase or PEG-rhDNase, without counting the PEG moiety in the PEG-rhDNase mass.

IV. DISCUSSION

The stability properties of rhDNase and PEG40-rhDNase were evaluated under GdmCl- and temperature-induced unfolding conditions. Analysis of intrinsic fluorescence and far UV-CD spectra for both proteins obtained during GdmCl-induced unfolding followed a three-state model (**Figure 21** and **Figure 22**), characteristic of the formation of an intermediate species. However, in the case of temperature-induced unfolding, the presence of the partially folded species and/or aggregates was only noticeable with the use of ANS (**Figure 26**).

IV.1. GDMCL-INDUCED DENATURATION

GdmCl-induced unfolding was monitored by spectroscopic techniques to determine the effects of PEGylation on the stability of rhDNase. First of all, the transition curves of rhDNase exposed to various GdmCl concentrations were monitored by intrinsic fluorescence and far UV-CD. Both data highlighted that the denaturation of rhDNase followed a three-state model with superposition of the transition curves, thereby indicating that its secondary and tertiary structures were simultaneously denatured. The GdmCl-induced unfolding of the protein was also monitored by ANS-binding fluorescence which allowed to monitor the exposition of hydrophobic patches. A peak of ANS-binding fluorescence was observed at GdmCl concentrations close to those corresponding to the first transition observed by intrinsic fluorescence and far UV-CD. This observation confirmed that an intermediate species was present at the concentrations in denaturant higher than 1 M. Thus, the transition could be schematized as $N \rightleftharpoons I \rightleftharpoons U$. However, it should be noted that the reversibility of the GdmCl-induced unfolding remains to be established. The unfolding transitions of PEG40-rhDNase monitored by intrinsic fluorescence and far UV-CD showed a similar profile as that of the unmodified protein, i.e. two transitions for its secondary and tertiary structures unfolding. PEG40-rhDNase also showed ANS-binding at GdmCl concentrations corresponding to the first transition observed by intrinsic fluorescence and far UV-CD. Comparison of the unfolding transitions obtained from fluorescence measurements of rhDNase and its three PEGylated counterparts showed that PEGylation destabilized rhDNase exposed to various GdmCl concentrations, with the bigger the PEG, the higher the destabilization. Moreover, this analysis highlighted that the structure of the PEG impacted the stability of rhDNase. The branched form of 40 kDa PEG induced a higher destabilization than both 20 kDa and 30 kDa linear PEGs.

To our best knowledge, no investigation has already evaluated the influence of PEG structure on protein stability toward GdmCl-induced unfolding. Based on the outcomes of this paper, we suggest that the structure of the PEG may be a critical point in the stability of rhDNase.

Contrary to our findings, Hadadian et al. demonstrated that human basic fibroblast growth factor (BFGF) PEGylated with 10 kDa PEG shows higher stability to GdmCl-induced denaturation than the free counterpart [356]. BFGF was totally denatured when exposed to 0.8 M GdmCl whereas 10 kDa PEG-BFGF needed 1.2 M GdmCl to be denatured.

IV.2. TEMPERATURE-INDUCED DENATURATION

rhDNase and PEG40-rhDNase displayed a similar thermal stability with an apparent T_m around 62°C. In both cases, the thermal unfolding was not fully reversible. Dhalluin and co-workers demonstrated that interferon α_2a (IFN $_{\alpha_2a}$) and its 40 kDa PEG-conjugate both unfolded through an irreversible process when exposed to elevated temperatures [28]. The irreversibility of the thermal denaturation was also reported by Natalello et al. for their PEGylated version of methionyl-granulocyte colony stimulating factor (Met-G-CSF) with 20 kDa PEG [334]. The far UV-CD response obtained at 25°C after the thermal exposition of their PEG-protein conjugate was similar to that of 95°C, and comparable to Met-G-CSF. Plesner and co-workers demonstrated that both recombinant human factor VIIa and its 40 kDa PEG-counterpart displayed similar T_m , with values of $60.3 \pm 0.2^\circ\text{C}$ and $60.7 \pm 0.2^\circ\text{C}$, respectively [318]. Our findings are also in agreement with results recorded for a monoclonal antibody antigen-binding fragment (Fab') di-PEGylated with two 30 kDa PEG chains which exhibited similar thermal stability as the free Fab' [317]. However, several other studies demonstrated that PEGylation conferred increased thermal stability to proteins. Dhalluin et al., in their evaluation of PEGylated IFN $_{\alpha_2a}$, highlighted that 40 kDa PEG conjugation conferred a slightly increased T_m ($67.4 \pm 0.3^\circ\text{C}$ versus $66.1 \pm 0.3^\circ\text{C}$ for non-conjugated IFN $_{\alpha_2a}$) [28]. Also, PEGylation improved the thermal stability of trypsin, with T_m increasing from 48.7°C to 55.7°C for trypsin and its 20 kDa PEG-conjugate, respectively [357]. Natalello et al. reported that attachment of a 20 kDa PEG to Met-G-CSF resulted in a higher resistance to temperature than the non-PEGylated glycoprotein [334]. In the same manner, other researchers demonstrated an improved thermal stability of their PEGylated proteins [358,359]. Therefore, a general

conclusion on the effect of PEGylation on the protein thermal behavior cannot be established but PEG conjugation does not seem to negatively impact the protein stability.

Extrinsic fluorescence of ANS was then used to further elucidate the effects of PEGylation on the thermal stability of rhDNase. The binding of ANS suggested that a significant hydrophobic surface area became accessible to the solvent upon thermal unfolding. Data obtained also showed that rhDNase and PEG40-rhDNase had a significantly different behavior to aggregation. PEG attachment allowed to reduce the formation of aggregated rhDNase. Similar data were collected by Natalello et al. in their evaluation of the thermal stability of Met-G-CSF coupled to 20 kDa PEG [334]. The authors showed that PEGylation suppressed the aggregation of the protein exposed to temperatures up to 95°C. In a similar manner, Dhalluin and co-workers demonstrated that conjugation of 40 kDa PEG to IFN $_{\alpha 2a}$ led to a reduced aggregation tendency of the protein [28]. Also, Plesner et al. reported that recombinant human factor VIIa glycoPEGylated with either 10 kDa or 40 kDa PEG delayed the thermally induced aggregation [318]. However, PEG conjugation can also lead to different stability profiles. Indeed, PEGylation of recombinant human G-CSF (rhG-CSF) with 20 kDa PEG showed lower stability than the unconjugated protein [360]. The authors reported that aggregation was rapidly observed at 37°C for 20 kDa PEG-rhG-CSF whereas it was delayed for rhG-CSF. Similarly, PEGylation of insulin with 5 kDa PEG did not protect the protein from aggregation [361].

Aggregation can strongly affect the bioavailability, immunogenicity and pharmacokinetics of proteins [362]. The protection towards aggregation conferred by 40 kDa PEG attachment to rhDNase could be related to the steric hindrance created by the polymer moiety [317,318]. Similar explanations were suggested by Dhalluin et al. for the increased thermal stability of 40 kDa PEG-IFN $_{\alpha 2a}$. The PEG being a highly hydrated polymer, it can confer favorable properties to the protein such as a reduced aggregation tendency [28]. Given the fact that PEGylated rhDNase reduced the aggregation behavior but not the thermally induced unfolding, we suggested that PEGylation may affect the aggregation properties of rhDNase but not through improved thermal stability [316,318].

V. CONCLUSION

Recombinant human deoxyribonuclease I was mono-PEGylated with 20 kDa, 30 kDa or 40 kDa PEG. The impact of PEGylation on its stability was evaluated with fluorescence spectroscopy and circular dichroism based on GdmCl- and temperature-induced unfolding. Considering the GdmCl-induced unfolding of rhDNase and its PEGylated derivatives, the fluorescence intensity measurements suggested that 20 kDa, 30 kDa or 40 kDa PEG conjugation to the protein led to its destabilization, with the bigger the PEG, the lower the stability. However, data obtained for temperature-induced unfolding showed that PEGylation with 40 kDa PEG did not affect the stability of rhDNase. More importantly, PEG attachment reduced rhDNase aggregation. Finally, the effect of PEGylation on a protein stability cannot be generalized and may depend on the molecule nature and the length of the PEG polymer conjugated to it.

**CHAPTER IV. IMPACT OF PEGYLATION ON THE MUCOLYTIC ACTIVITY
OF RECOMBINANT HUMAN DEOXYRIBONUCLEASE I IN CYSTIC
FIBROSIS SPUTUM**

IMPACT OF PEGYLATION ON THE MUCOLYTIC ACTIVITY OF RECOMBINANT HUMAN DEOXYRIBONUCLEASE I IN CYSTIC FIBROSIS SPUTUM

Adapted from: Impact of PEGylation on the mucolytic activity of recombinant human deoxyribonuclease I in cystic fibrosis sputum, submitted to Clinical Science (March 2018)

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Personal contribution: all experiments except collection of sputum from patients with cystic fibrosis

ABSTRACT

Highly viscous mucus and its impaired clearance characterize the lungs of patients with cystic fibrosis (CF). Pulmonary secretions of patients with CF display increased concentrations of high molecular weight components such as DNA and actin. Recombinant human deoxyribonuclease I (rhDNase) delivered by inhalation cleaves DNA filaments contained in respiratory secretions and thins them. However, rapid clearance of rhDNase from the lungs implies a daily administration and thereby a high therapy burden and a reduced patient compliance. A PEGylated version of rhDNase could sustain the presence of the protein within the lungs and reduce its administration frequency. Here, we evaluated the enzymatic activity of rhDNase conjugated to a two-arm 40 kDa polyethylene glycol (PEG) in CF sputa. Rheology data indicated that both rhDNase and PEG40-rhDNase presented similar mucolytic activity in CF sputa, independently of the purulence of the sputum samples as well as of their DNA, actin and ions contents. The macroscopic appearance of the samples correlated with the DNA content of the sputa: the more purulent the sample, the higher the DNA concentration. Finally, quantification of rhDNase and PEG40-rhDNase in CF sputa suggests that PEGylation largely increases the stability of rhDNase in CF respiratory secretions. These results are considered positive and provide support to the continuation of the research on a long-acting version of rhDNase to treat CF lung disease.

I. INTRODUCTION

Cystic fibrosis (CF) is an inherited disease caused by an autosomal recessive defect in the gene coding for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, a chloride channel. The morbidity and mortality in CF is mainly due to the pulmonary phenotype of the disease. Clinically, the effects of CF in the lungs are cough, expectoration of airway secretions, recurrent lung infections and rapid loss of pulmonary function [41,51].

The airway mucus is a thin and mobile gel that covers the respiratory tissue surface. It is mainly composed of water and biopolymers, such as the mucin, a heavily glycosylated protein [171]. The mucus presents a complex physical behavior. It is described as a viscoelastic gel since it possesses both viscosity and elasticity properties [363]. Healthy mucus is characterized by low viscosity and elasticity and is easily transported by ciliary beating. Effective mucus clearance is essential for lung health, and airway disease can be a consequence of an impaired clearance. Pathologic mucus has higher viscosity and elasticity than normal mucus and is more difficult to clear. The conversion from healthy mobile mucus to diseased mucus can result from many mechanisms such as dehydration, abnormal transepithelial ion transport, increased production of mucin or lysis of inflammatory cells [171]. In case of CF, apoptosis of neutrophils and/or neutrophil extracellular traps death (NETosis) increase the extracellular DNA and filamentous actin (F-actin) contents [187,364]; thereby contributing to the 10- to 100-fold increase of mucus viscoelasticity [192]. Consequently, the mucus becomes thick and impaired clearance as well as bacterial growth characterize the lungs of patients with CF [171]. The presence in CF sputa of components such as DNA, proteins, lipids and cations may influence the viscoelastic properties of the respiratory secretions [365–367].

The pathologic CF mucus has led to the development of therapeutic medications intended to decrease its stiffness. Inhaled recombinant human deoxyribonuclease I (rhDNase) has proven its mucolytic activity in CF lung disease for many years [4]. Shak et al. demonstrated that rhDNase transformed CF sputum from a non-flowing viscous substance to a liquid within minutes [4]. Therefore, DNA hydrolysis by rhDNase in CF respiratory secretions leads to reduced sputum viscosity. Nevertheless, symptomatic rhDNase treatment presents two major limitations: the protein is inhibited by globular actin (G-actin) [273] and it is rapidly cleared from the lungs [5]. When the daily dose of rhDNase of 2.5 mg is inhaled, a concentration of 3

µg/mL is measured in sputum immediately after inhalation and it is reduced to 0.6 µg/mL after two hours [5]. However, the effects of rhDNase on sputum rheology persist beyond 12 hours [368].

Recently, our laboratory has developed a PEGylated version of rhDNase [312]. Conjugation of proteins to PEG, named PEGylation, is a process largely used by the pharmaceutical industry to increase the serum half-life of proteins delivered by injection [277]. PEGylation has also been shown to prolong protein residence time in the lungs following pulmonary delivery in animal models [24,295,296]. For instance, PEGylation of an anti-IL17A Fab' antibody fragment with a two-arm 40 kDa PEG chain sustained the presence of the protein in rodent lungs for more than 48 h whereas the unconjugated protein was cleared within 24 h [296]. In the same way, PEGylation of rhDNase could sustain the presence of the enzyme within the lungs following inhalation. The sustained presence of a PEGylated version of rhDNase in the lungs could space rhDNase dosing and improve patient compliance [6].

A drawback of protein PEGylation is that it can reduce the biological activity of the protein. As an example, PEGylation of interferon (IFN)_{α2a} drastically altered its activity [277]. Indeed, 40 kDa PEG-IFN_{α2a} only retained 7% of the unconjugated interferon activity *in vitro*. However, the large increase in 40 kDa PEG-IFN_{α2a} *in vivo* serum half-life led to its clinical approval under the name Pegasys®, despite its reduced biological activity *in vitro*, in 2002. In a similar manner, PegINTRON® and Neulasta® present significant loss in biological activity [369,370]. We have previously demonstrated that the covalent attachment of a large PEG chain (linear 20 kDa, linear 30 kDa or two-arm 40kDa) on the N-terminal residue of rhDNase fully preserved its mucolytic activity on DNA solution *in vitro* [312]. However, analysis of PEGylated rhDNase mucolytic activity in CF sputa is more relevant and might provide additional information. In fact, PEGylation of rhDNase could improve its *ex vivo* enzymatic activity in CF sputa since PEG attachment creates a steric hindrance that could protect the protein towards proteolysis [371] and towards inhibition by actin [266].

The activity of rhDNase has already been analyzed in CF sputa, with nevertheless no assessment of a potential link with the macroscopic appearance of the samples [4,190,372]. However, some investigations assessed the relationship between purulence and viscosity of CF sputa. Picot et al. showed that the macroscopic appearance of CF sputa correlated with

their DNA content, but the authors did not report any significant relationship between purulence and viscosity [373]. Later, another investigation suggested that the viscoelasticity properties of CF sputa were dependent on the purulence of the samples [374]. However, no statistical evidence allowed to firmly conclude. More recently, Serisier and co-workers highlighted that significant differences in CF sputum rheology were seen between samples, according to their degree of purulence [375].

Therefore, the goal of this research was to determine the mucolytic activity of rhDNase conjugated to a two-arm 40 kDa PEG (PEG40-rhDNase) in sputa from patients with CF. The rheological properties (elastic and viscous moduli) of CF sputa were measured following addition of rhDNase or of PEG40-rhDNase. The samples were categorized based on macroscopic appearance to evaluate if the *ex vivo* mucolytic activity of rhDNase would depend on the purulence of the CF sputa. DNA and G-actin were quantified by fluorescence labelling and correlated to the visual inspection of samples (muco-purulent or purulent sputum) and to rhDNase and PEG40-rhDNase activities. Ion contents, such as calcium, magnesium and potassium, were measured in CF sputa and correlated with the purulence, with G-actin concentration and with the enzymatic activity of rhDNase and PEG40-rhDNase as well. Finally, non-PEGylated and PEGylated protein contents were measured in CF biological samples following the rheology assay to evaluate their resistance to degradation in a relevant biological medium.

II. MATERIAL AND METHODS

II.1 MATERIAL

rhDNase (Pulmozyme®) was from Genentech, Inc. (South San Francisco, CA, USA). Two-arm 40 kDa methoxy PEG (PEG40) propionaldehyde was obtained from NOF Corporation (Tokyo, Japan). Unless otherwise stated, chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA). The N-terminal site-specific mono-PEGylation of rhDNase was conducted as previously described [312].

II.2 COLLECTION OF SPUTUM FROM PATIENTS WITH CF

CF sputa were collected by expectoration during physiotherapy. Patients over 18 and below 60 years able to expectorate at least 2 mL of sputum were included. 78% of patients with CF are treated with Pulmozyme® in Belgium (32) and only patients under Pulmozyme® provided sputum. Twenty-four hours pre-collection of sputum, patients were asked to stop their rhDNase treatment. Sputa were immediately frozen at -80°C after expectoration. Patients under oral corticosteroid treatment, with a forced expiratory volume in one second (FEV₁) < 40% of the predicted value and pregnant women were excluded from the study. Patients with FEV₁ < 40% were not evaluated since they are not concerned by Pulmozyme®. Indeed, in Belgium, reimbursement of Pulmozyme® is only for patients with FEV₁ > 40% since adverse effects such as hemoptysis are more frequent in patients with FEV₁ < 40%. The clinical characteristics of the patients enrolled in this evaluation are summarized in **Table 6**. The experimental protocol was approved by the Ethics Committee of the Université catholique de Louvain (UCL; registration number: B403201422928).

Table 6: Characteristics of patients with CF enrolled in the clinical evaluation. Data presented are mean $\pm$ SD.

	Patients
Age (years)	33 $\pm$ 10
BMI ^a	22.1 $\pm$ 3.0
Genotypes	50% with F508del/F508del 20% with F508del/A455E 10% with F508del/M1137R 10% with 711+1G>T/711+1G>T 10% with F508del/N1303K
FEV ₁ ^b (% predicted)	53.7 $\pm$ 12.1
FVC ^c (% predicted)	78.6 $\pm$ 15.2

^aBMI: body mass index

^bFEV₁: forced expiratory volume in 1 s

^cFVC: forced vital capacity

II.3 CHARACTERIZATION OF SPUTUM

Sputa were sorted according to macroscopic appearance into two categories: purulent for samples uniformly purulent whether green or yellow and mucopurulent for samples in which purulent and mucoid (no pus) parts were mixed [375].

II.4 DETERMINATION OF THE STRAIN RANGE FOR MEASUREMENT

The principle of the rheology measurements is detailed in *The Rheology Handbook* [376].

The range of strains associated with non-destructive conditions of the sputum was determined using the rheometer MCR102 (Anton-Paar, Graz, Austria). CF sputa were thawed at room temperature. Six hundred milligrams of sputum were loaded on the rheometer and incubated during 30 min at 37°C to allow relaxation of the sample [377]. The assay was performed using a 50 mm stain cone plate with a 1° angle (CP50-1°; Anton-Paar). Shear strains between 0.01% to 1000% were applied [363]. The results were recorded with RheoCompass™ software (Anton-Paar).

II.5 RHEOLOGY MEASUREMENT

CF sputa were thawed at room temperature. Six hundred milligrams of sample were gently mixed with 30 μ L of rhDNase or PEGylated rhDNase (at 150 μ g/mL or 5 μ M in rhDNase moiety, in 1 mM CaCl_2 , 150 mM NaCl) to reach a final concentration of 10 μ g/mL (0.33 μ M) in rhDNase in the sputum. The PEG was not counted in the mass of PEG-rhDNase. Therefore, the different sputum samples were exposed to the same molarity of the rhDNase enzymes, that is, 0.33 μ M. Control samples were made of sputum containing 30 μ L of rhDNase vehicle, without rhDNase. rhDNase, PEG40-rhDNase and vehicle treatments were applied to aliquots of the same sputum sample, which allowed to perform paired statistical tests. Rheology assays were performed using the rheometer MCR102 and a 50 mm stain cone plate with a 1° angle (CP50-1°). Treated sputum was loaded on the rheometer and incubated at 37°C for 60 min. Strains between 0.01% and 10% were applied at a constant frequency of 10 Hz. Elastic and viscous moduli were recorded with RheoCompass™ software.

II.6 RHDNASE AND PEG40-RHDNASE CONTENT IN CF SPUTUM

After rheology measurements, sputa were gently collected from the rheometer plate. rhDNase and PEG40-rhDNase were then quantified by ELISA in these collected sputa. A 96-well plate was coated using rabbit polyclonal antibody to human rhDNase (Bio-connect, Huissen, The Netherlands) in 1% NaHCO_3 solution, pH 9.6 at 4°C overnight and blocked with 1% proteins from milk powder in PBS-Tween 0.1% at 37°C for 1 h. Sputum samples were diluted at 1:1 in phosphate buffer saline (PBS), vortexed for 2 min and centrifuged at 4500 g, 4°C for 10 min. Supernatant of samples as well as standards (rhDNase or PEG40-rhDNase) were incubated at 37°C for 1 h. Dilutions were done in PBS-Tween 0.1% + 0.1% bovine serum albumin (BSA). Biotin-conjugated antibody (same antibody as the one used for coating) and streptavidin-horseradish peroxidase were used for development. Revelation was conducted using Tetramethylbenzidine solution (Thermo Fisher Scientific, Rockford, IL, USA) and stopped with 2 M H_2SO_4 . The ELISA plate was read at 450 nm (Multiskan EX, Thermo Fisher Scientific, Rockford, IL, USA). The sensitivity of the ELISA assay being reduced for PEG40-rhDNase, the analyses were performed with the corresponding rhDNase construct to avoid any potential

underestimation of the protein quantity in each sample containing the PEGylated rhDNase. The related calibration curves are given in *Supplementary figure S8*.

II.7 DNA QUANTIFICATION

DNA contained in CF sputa was quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, Rockford, IL, USA). The protocol from the manufacturer was slightly adapted [378]. Twenty mg of sputum were diluted 10-fold in 0.1 M dithiothreitol (DTT). The mixture was vortexed until complete dissolution of the sample. Then, 5 µL of the diluted mucus were added to 100 µL of 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM trishydroxymethylaminomethane (Tris) buffer at pH 8.0. 8.4 µL of the previous solution were diluted in 492 µL of TE (Tris-EDTA) 1x buffer. 500 µL of PicoGreen® reagent were added. An aliquot of 200 µL was incubated 5 min in the dark in a black 96-well plate. Fluorescence was measured with a SpectraMax® M3 multi-mode microplate reader (Molecular Devices, Sunnyval, USA) with 485 nm excitation and 530 nm emission filters.

II.8 *IN VITRO* ENZYMATIC ACTIVITY OF RHDNASE IN PRESENCE OF ACTIN

Rheology assays were performed on a salmon testes DNA solution at 6 mg/mL in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.5. Actin solution at 320 µg/mL was prepared in 4 mM CaCl₂, 2 mM MgCl₂, 0.2% BSA, 28 mM KCl solution at pH 7.5. 250 µL of DNA and 125 µL of actin (or actin vehicle, for the condition without actin) were incubated at 37°C for 30 min. Then, rhDNase solution was added to reach a final concentration of 1 µg/mL (33 nM) rhDNase (125 µL; final volume of 500 µL). Viscosity measurements were performed during 30 min at 37°C using the Modular Compact Rheometer MCR102. A 50 mm stain cone plate with a 0.5° angle was used. A solvent trap containing distilled water prevented dehydration of samples during rheological analysis. The experiments were performed in rotational mode at 1000 rpm. The results were recorded by RheoCompass™ software.

II.9 ACTIN QUANTIFICATION

F- and G-actin contained in sputa were quantified using rhodamine-labelled phalloidin fluorescence labeling [379]. The sputum was diluted 20-fold in a polymerizing buffer (150 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM Na_2ATP , 1 mM DTT, 2 mM Tris at pH 7.6) during 30 min at 37°C to allow the polymerization of G-actin [190]. Quantification of F-actin was conducted by replacing the polymerizing buffer by a solution containing 0.2 mM $CaCl_2$, 0.2 mM Na_2ATP , 1 mM DTT, 2 mM Tris at pH 7.6. One μL of 6.6 μM rhodamine-labelled phalloidin (ThermoFisher Scientific, Rockford, IL, USA) was added to 25 μL of the diluted sample in a black 384-well plate and incubated 1 h on ice in the dark. The rhodamine-labelled phalloidin was extracted overnight with methanol at 4°C in the dark. The fluorescence enhancement was then measured with a SpectraMax® M3 multi-mode microplate reader (Molecular Devices, Sunnyval, USA) with 550 nm excitation and 580 nm emission filters. A standard curve of F-actin was prepared by dissolving 1 to 10 $\mu g/mL$ actin from rabbit muscle in polymerizing buffer and incubating at 37°C for 30 min.

II.10 QUANTIFICATION OF DRY MASS OF CF SPUTA

The solid content of sputa was measured by aliquoting between 100 and 200 μg of sputum on a pre-weighted aluminium foil piece. The samples were placed in an 80°C oven overnight. The final mass of the dried foil and sample was recorded, and the percentage of dry mass was calculated. The protocol was adapted from [380].

II.11 ION QUANTIFICATION

Calcium, magnesium and potassium ions were quantified in CF sputa.

Calcium and magnesium: Samples were prepared by dissolving at least 70 mg of CF sputa in 7 times their mass in water. Samples were then vortexed and centrifuged to separate the solid parts from the supernatant containing Ca^{2+} and Mg^{2+} . The concentrations measurements were performed using an automatic analyzer (Cobas® 8000, Roche Diagnostics, Roche, Basel, Switzerland). Ca^{2+} were assayed with the 5-nitro-5'-methyl-(1,2-bis(o-aminophenoxy)ethan-

N,N,N',N'-tetraacetic acid (NM-BAPTA) method. Mg^{2+} concentration was determined with the use of the xylydyl blue reaction according to the manufacturer's instructions.

Potassium: Samples were prepared by dissolving at least 70 mg of CF sputa in 10 times their mass in water. Samples were then vortexed and centrifuged to separate the solid parts from the supernatant containing K^+ . Lithium was used as an internal standard. Standard Calib 140/5 electrolytes for *in vitro* diagnostics use (Instrumentation Laboratory, Bedford, MA, USA) was used as a single standard for calibration. A volume of 20 μ L of supernatant was then diluted with 2 mL of lithium sulfate. The analyses were performed in duplicate with a flame photometer (Model 420 Flame Photometer, Sherwood Scientific Ltd, Cambridge, UK) operated in the atomic emission mode, using a propane flame. The concentrations were expressed in mM.

II.12 DATA ANALYSIS

All results are presented as mean values $\pm$ standard deviation (SD). Friedman tests with Dunn's multiple comparison test, or Wilcoxon matched pairs test were employed for the statistical analysis of the viscoelasticity measurements of CF sputa (**Figure 28**; n=17 and **Figure 29**; n=5 to 12). Mann-Whitney tests were used for the quantification of rhDNase and PEG40-rhDNase in CF sputa (**Figure 30**; n=11 to 13) as well as for the DNA, actin and dry mass contents of purulent and muco-purulent sputa (**Figure 31** and **Figure 33**; n=24, **Figure 35**; n=11). Statistical analysis of viscosity measurements in presence of actin was also performed with Mann-Whitney test (**Figure 32**; n=5). Twenty-three samples were quantified in terms of ions and analyzed with Kruskal-Wallis test with Dunn's post-test (**Table 7**). The data presented in **Figure 34** were analyzed by Spearman correlation.

All these tests were performed using the GraphPad Prism software (CA, USA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

III. RESULTS AND DISCUSSION

III.1 RHEOLOGY MEASUREMENTS

Characterization of sputa focuses on two properties: elastic modulus (G'), which measures the tendency for the gel to recover its original shape following stress-induced deformation, and viscous modulus (G''), which represents the resistance of the gel to flow [363]. Non-destructive conditions were required to preserve the structure of the sputa [377,381]. Therefore, before analyzing the rheological properties of the samples, we determined the linear viscoelastic region of CF sputum in terms of strain, that is, the region of small deformations in which the viscoelastic parameters remain constant [382]. Both elastic and viscous moduli were steady between 0.01% and 1% of strain (*Supplementary figure S9*). Consequently, in our experiments, CF sputum was only subject to small oscillations to minimize its deformation and thus reflect the mucus rheology in its native state in the lungs [363]. A constant frequency of 10 Hz was applied to mimic the beat frequency of the cilia motion [383].

The effects of rhDNase and PEG40-rhDNase on the viscoelasticity of CF sputa were then analyzed. Seventeen CF sputa were included in the rheology analysis. The elastic and viscous moduli of the samples after addition of PEG40-rhDNase were similar to the moduli obtained after the addition of the unconjugated protein (**Figure 28**). As previously reported by Lai et al. [363], CF sputum was more elastic than viscous under oscillatory and controlled shear strain. This was the case for all the three conditions tested: control, rhDNase and PEG40-rhDNase (**Figure 28**). The mean decrease in sputum elasticity was 64% for rhDNase and 68% for PEG40-rhDNase. Similarly, sputum viscosity decreased by 66% and 68% following addition of rhDNase and PEG40-rhDNase, respectively. Our results are in good agreement with data reported by Sanders et al. [190]. Sanders et al. showed that sputum elasticity decreased by 32% and sputum viscosity by 17% following a 20 min incubation with rhDNase at a final concentration of 10 $\mu\text{g/mL}$ (0.33 μM) in case of clinical rhDNase-responders. In our study, samples were incubated for 60 min with rhDNase before rheology measurement (three-times longer than in Sanders et al.) and could thus explain the better activity we observed. Clinical responders to rhDNase therapy and clinical non-responders can be distinguished among patients with CF

[384]. Thirty percent of patients with CF are qualified as clinical non-responders since their sputa are not degraded by rhDNase whereas sputa from clinical responders are extensively degraded by rhDNase [190]. In our study, only patients taking rhDNase and considered as responders provided sputa.

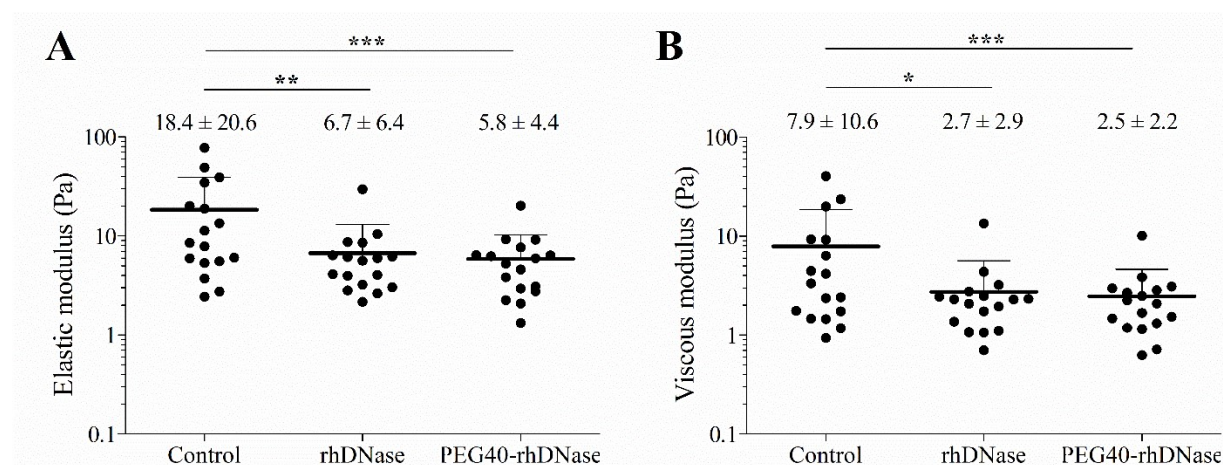


Figure 28: Effect of rhDNase and PEG40-rhDNase (0.33 μ M in rhDNase or PEG-rhDNase) on the viscoelasticity of CF sputa (n=17, mean $\pm$ SD), **A.** Elastic modulus. **B.** Viscous modulus, both expressed in Pascal (Pa; $\text{kg}\cdot\text{m}^{-1}\cdot\text{s}^{-2}$). Both proteins were incubated with the sample at 37°C for 60 min and showed similar enzymatic activity on the samples. rhDNase and PEG40-rhDNase groups were compared to the control group (Friedman test with Dunn's multiple comparison test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Our sputa were then categorized to correlate the enzymatic activity of both enzymes with the macroscopic appearance of the samples. To the best of our knowledge, our investigation is the first to integrate the purulence parameter in the analysis of rhDNase mucolytic activity in CF sputa. Among the seventeen samples, twelve were purulent and five were muco-purulent. Although not confirmed by the statistical analysis, a trend was observed between purulence and viscoelasticity of the sputum: the more purulent, the more elastic and the more viscous the sputum (**Figure 29A** and **B**). rhDNase and PEG40-rhDNase showed similar activity whether sputa were purulent or muco-purulent (**Figure 29C** and **D**). Overall, our rheological results showed that PEGylation preserved the mucolytic activity of rhDNase *ex vivo*. A large variation amongst samples was observed, potentially explained by variations in water, DNA, actin and glycoprotein content in CF sputa [385].

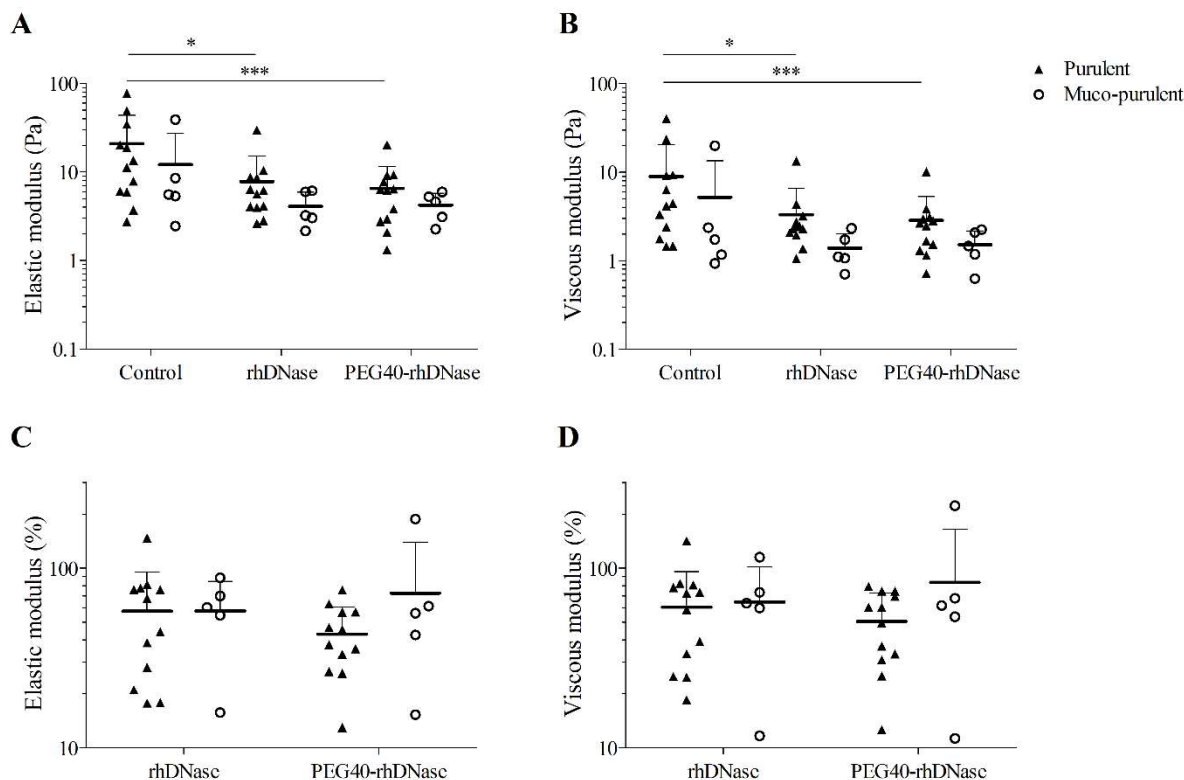


Figure 29: Effect of rhDNase and PEG40-rhDNase (0.33 μ M in rhDNase or PEG-rhDNase) on the viscoelasticity of CF sputa based on the macroscopic appearance of the samples (mean $\pm$ SD). Both proteins were incubated with the samples at 37°C for 60 min. In **A** and **B**, absolute values of elastic and viscous moduli are shown. rhDNase and PEG40-rhDNase groups were compared to the control group and the moduli of purulent samples significantly decreased after treatment with rhDNase or PEG40-rhDNase (Friedman test with Dunn's multiple comparison test, * $p < 0.05$, and *** $p < 0.001$). In **C** and **D**, elastic and viscous moduli are expressed as % of the control samples. The data were compared Wilcoxon matched pairs test with no difference seen between rhDNase and PEG40-rhDNase treatments on purulent ($n=12$) and mucopurulent ($n=5$) samples.

rhDNase or PEG40-rhDNase contained in CF sputa after rheology measurement were quantified (**Figure 30**). This analysis allowed us to evaluate the degradation of the proteins after incubation in the fluid. rhDNase and PEG40-rhDNase were initially added to CF sputum at a concentration of 10 μ g/mL (0.33 μ M in rhDNase, without counting the PEG moiety in the mass of the PEG-rhDNase). Following incubation and rheology, 27 ng/mL and 658 ng/mL of rhDNase and PEG40-rhDNase were measured in CF sputa, respectively. Therefore, 6.6% of the initial dose of PEG40-rhDNase remained after incubation with the sample whereas only 0.3% of rhDNase was measured, suggesting a higher stability of PEG40-rhDNase than the unconjugated protein. Therefore, these results suggested that PEGylation increased the resistance of rhDNase towards proteolysis [277].

DNA accounts for 0.02% of the mucus by mass [363]. Therefore, the DNA content in CF sputa was three-fold higher than in normal mucus. The aspect of the sputa correlated with the determined DNA concentration of each sample: the higher the concentration of DNA, the more purulent the sample. However, no correlation could be established between the DNA content and the elastic and viscous moduli of the samples (*Supplementary figure S10A*). The ability of rhDNase and PEG40-rhDNase to reduce G' and G'' of CF sputa reported above (**Figure 28**) confirms that DNA is a component responsible for the considerable viscoelasticity of diseased sputum, as previously reported by Shak and co-workers [4]. However, the lack of correlation between DNA content and sputum viscoelasticity (*Supplementary figure S10A*) indicates that DNA is not the only parameter involved [373].

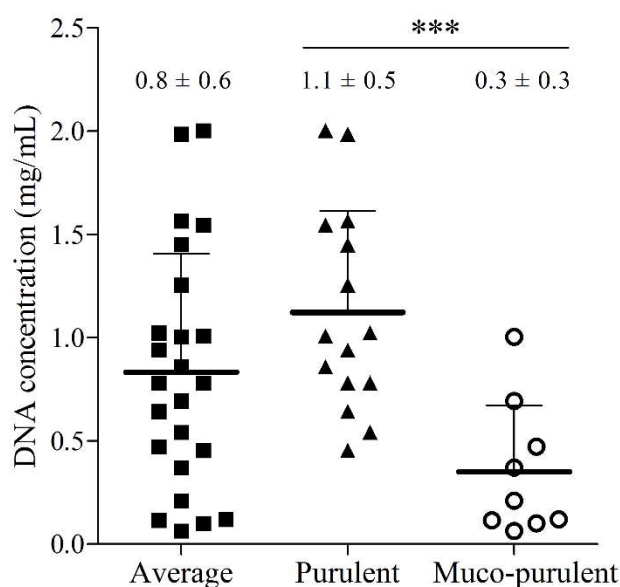


Figure 31: Quantification of extracellular DNA in CF sputa (n=24, mean $\pm$ SD). A correlation is visible between the visual aspect of the sample and the concentration of DNA. DNA content in purulent and muco-purulent samples showed significant difference (Mann-Whitney test, *** $p < 0.001$).

III.3 ACTIN QUANTIFICATION

Globular actin (G-actin) is a potent inhibitor of rhDNase [266]. We might expect an improved enzymatic activity of PEG40-rhDNase in the presence of actin compared with the unconjugated protein. In fact, PEG is known to prevent the approach of any other proteins, as

G-actin and proteases, near the PEGylated protein due to the flexibility and hydration of the polymer and the steric hindrance it creates [277]. However, PEG40-rhDNase was inhibited by actin to the same extent as rhDNase was (**Figure 32**), indicating that the actin-binding and PEGylation sites (N-terminus, [312]) were not sufficiently close to each other (**Figure 32C**). G-actin and rhDNase form a stoichiometric 1:1 complex with high affinity (dissociation constant of 30 nM [386]) and PEG might not prevent their association unless it is present within the binding interface.

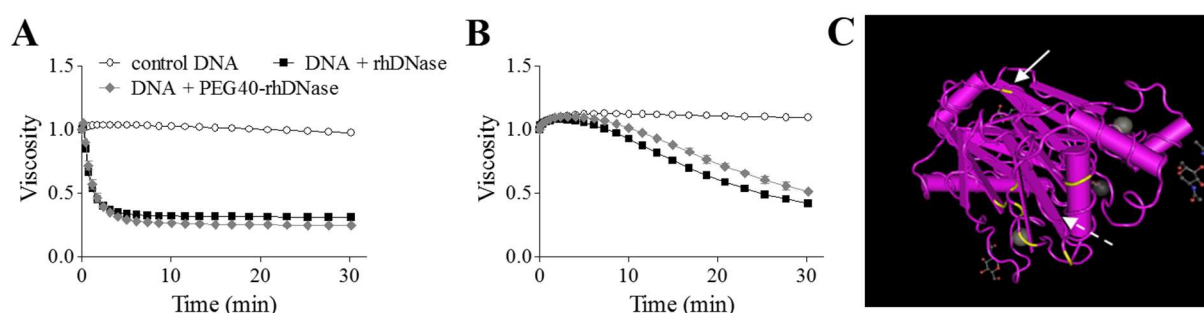


Figure 32: Viscosity analysis of DNA (**A**) and DNA containing actin (**B**) solutions following addition of rhDNase or PEG40-rhDNase (at 33 nM in rhDNase or PEG-rhDNase; $n=5$, data were normalized to the time zero DNA viscosity for each protein). A kinetic evaluation of the enzymatic activity of each protein was recorded over 30 min and showed to be identical for both proteins in absence or presence of actin (Mann-Whitney test, $p > 0.05$). Actin inhibition of rhDNase and PEG40-rhDNase is illustrated in **B**. **C**. 3D structure of rhDNase (4AWN from the Protein Data Bank). The N-terminal leucine (at the surface of the protein; full line arrow) and the amino acids of rhDNase interacting with globular actin (at the surface of the protein; dotted line arrow) are indicated. Calcium (grey balls) and magnesium (black ball) ions are represented as well as N-acetyl- β -glucosamine (skeletal organic structure on the right), 2-[bis-(2-hydroxy-ethyl)-amino]-2-hydroxymethyl-propane-1,3-diol (skeletal organic structure on bottom) and phosphate ion (linked to magnesium ion) [338].

Because G-actin inhibited both rhDNase and PEG40-rhDNase *in vitro*, we quantified it in CF sputa in order to see whether mucolytic activities inversely correlated with G-actin content. The specific binding of rhodamine-labelled phalloidin to F-actin was used to measure the content of the different forms of actin in the sputa. The 24 CF sputa contained on average 94 ± 83 $\mu\text{g/mL}$ of G-actin and 173 ± 110 $\mu\text{g/mL}$ of F-actin (**Figure 33**). Overall, all samples contained similar quantities of total actin (G+F actin; **Figure 33**). The quantity of G-actin in the sputa correlated with their purulence (**Figure 33**), with the more purulent the sample, the higher the G-actin content. Also, the F-actin correlated with the elastic and viscous moduli of samples (*Supplementary figure S10B*), as expected [387]. However, no correlation was established between the concentration of G-actin (from 0 to 132 $\mu\text{g/mL}$) and the activity of

rhDNase, highlighting that rhDNase and PEGylated rhDNase were active to the same extent whatever the content of G-actin in the sputa (**Figure 34**).

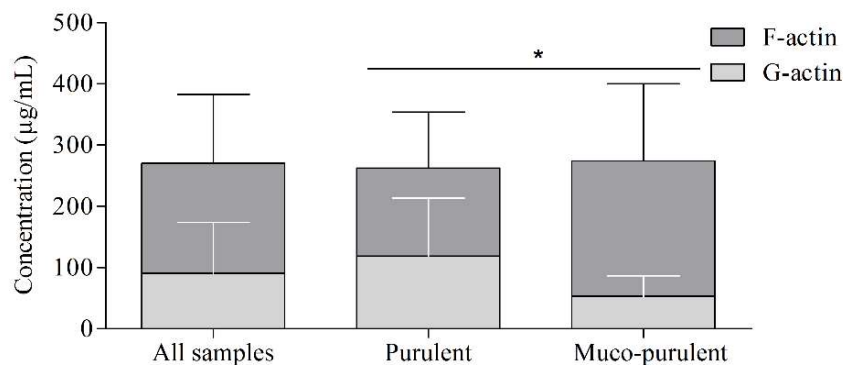


Figure 33: G-actin and F-actin quantification in CF sputa (n=24, mean $\pm$ SD). The more purulent the sample, the higher the concentration of G-actin (Mann-Whitney test, * $p < 0.05$).

Considering *in vitro* results presented above (**Figure 32**), rhDNase enzymatic activity *ex vivo* could have depended on the G-actin content. *In vitro* experiments highlighted a slowdown of rhDNase activity in presence of actin. However, 30 min following incubation with DNA and actin, rhDNase compounds had decreased the viscosity of DNA solution to almost the same extent as in the absence of actin (**Figure 32**). Therefore, measurement of rhDNase activity 1 h following incubation in CF sputa might have similarly resulted in maximum *ex vivo* activity, thereby masking any G-actin inhibition.

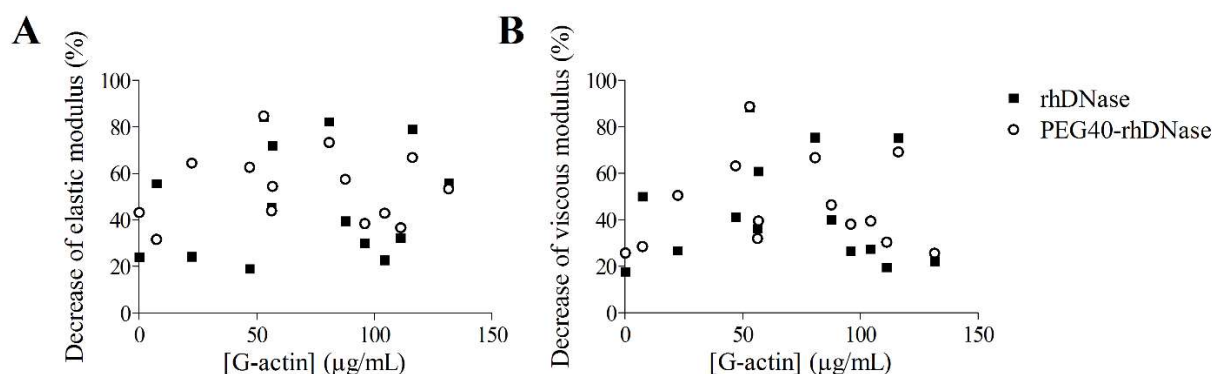


Figure 34: Influence of G-actin content on the activity of rhDNase and PEG40-rhDNase (at 0.33 μ M in rhDNase; both proteins were incubated for 60 min in the samples) in CF sputa. Elastic (**A**) and viscous (**B**) moduli. No correlation was established between G-actin concentration and decrease of elastic or viscous modulus following addition of rhDNase or PEG40-rhDNase (Spearman test, $p > 0.05$).

III.4 QUANTIFICATION OF DRY MASS OF CF SPUTA

The mucus solids concentration has been associated with the severity of many lung diseases such as CF [171,364,388]. Therefore, the dry mass of our sputum samples was quantified and compared to the visual aspect of the sputa (**Figure 35**).

This dry mass quantification analysis included 11 samples. Eight were purulent and three were muco-purulent. The content of dry mass in the samples was on average 7.1% (values between 3.9% and 11.6%). Our results were in agreement with data of David and co-workers. In their quantification of the sputum solids concentration from patients with CF, the authors highlighted an average percentage of 7.0 [380]. In our evaluation, we demonstrated that the aspect of the CF sputa correlated with the percentage of dry mass of each sample: the more purulent the sample, the higher the content in dry mass (**Figure 35**).

The concentration of mucins has been reported as the main driver of sputum biophysical properties [380]. It has been also suggested that mucus percentage solids correlated with mucin concentration and that the hydration of mucus dramatically affects its elastic and viscous properties [171,389]. Consequently, we evaluated the dependence of the sputum dry mass on its viscoelastic properties (*Supplementary figure S11*). However, our results showed no correlation between the % dry mass of the CF sputa and their elastic and viscous moduli. Finally, we highlighted that both rhDNase and PEG40-rhDNase were active whatever the % dry mass of the samples (*Supplementary figure S12*).

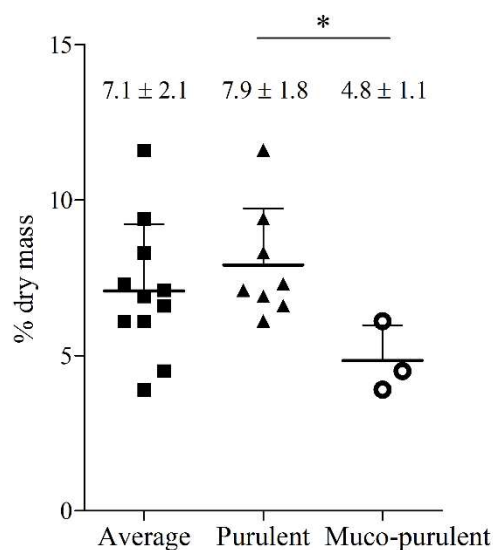


Figure 35: Quantification of dry mass of CF sputa (n=11, mean $\pm$ SD). The percentage of dry mass content in purulent and muco-purulent samples showed significant difference (Mann-Whitney test, *** $p < 0.001$).

III.5 IONS QUANTIFICATION

Divalent metal ions such as Ca^{2+} and Mg^{2+} are required for the activity of rhDNase [390]. Therefore, CF sputa were analyzed for ions content (Ca^{2+} , Mg^{2+} and K^+). Mean values, also taking into account the purulence of samples, are shown in **Table 7**. In 1984, Kilbourn already analyzed the composition of sputa from patients with CF and reported that, over a total of 12 patients, samples contained 1.1 ± 0.2 mM of Ca^{2+} , 0.14 ± 0.01 mM of Mg^{2+} and 28 ± 2 mM of K^+ [391]. More recently, Sanders et al. quantified Ca^{2+} , Mg^{2+} and K^+ in CF sputa from clinical responders and clinical non-responders to rhDNase treatment [190]. Samples from clinical responders contained 3.0 mM of Ca^{2+} , 2.0 mM of Mg^{2+} and 22 mM of K^+ . Clinical non-responders sputa were also characterized by a Ca^{2+} concentration of 3.0 mM but presented lower Mg^{2+} and K^+ contents with concentrations of 1.3 mM and 18 mM, respectively. Except for the one-order of magnitude increase in magnesium concentration, our data were in good agreement with the values obtained by Kilbourn. Compared to Sanders et al. data, our results showed similar concentrations of Mg^{2+} and K^+ and lower content of Ca^{2+} . We sorted the samples based on their visual aspect (**Table 7**) and showed that Mg^{2+} , Ca^{2+} and K^+ concentrations were not linked to the purulence of the sputa.

Table 7: Quantities of Ca^{2+} , Mg^{2+} and K^+ found in CF sputa. Similar concentrations were measured in purulent (n=14) and muco-purulent (n=8) samples. Data presented are mean $\pm$ SD.

Purulence	$[\text{Ca}^{2+}]$ (mM)	$[\text{Mg}^{2+}]$ (mM)	$[\text{K}^+]$ (mM)
Purulent	1.4 ± 0.8	2.2 ± 0.8	24 ± 5
Muco-purulent	1.8 ± 0.7	1.9 ± 0.6	26 ± 9
Average	1.5 ± 0.7	2.1 ± 0.7	24 ± 7

III.6 RELATIONSHIPS BETWEEN THE DIFFERENT SPUTUM PARAMETERS

We demonstrated that the more purulent the sputum, the higher the DNA and G-actin contents (**Figure 31** and **Figure 33**) as well as the higher the % dry mass (**Figure 35**). Also, we highlighted that the quantity of F-actin correlated with the elastic and viscous moduli of the samples (*Supplementary figure S10B*). However, our results did not show any significant correlation between the viscoelastic properties of the CF sputa and the concentration of DNA (*Supplementary figure S10A*). Moreover, we demonstrated that rhDNase and PEG40-rhDNase were active whatever the G-actin content (**Figure 34**).

Further, we assessed the possible link between rhDNase or PEG40-rhDNase enzymatic activity and ions contents in sputa.

Ca^{2+} and Mg^{2+} are involved in the stability and activity of rhDNase [270,390]. Therefore, concentrations of Ca^{2+} and Mg^{2+} were compared to the enzymatic activity of rhDNase (*Supplementary figure S13*). The activity of rhDNase on the elastic modulus of the sputa inversely correlated with the content of Ca^{2+} . However, the correlation was poor since the coefficient of determination (R^2) was only 0.29. The activity of PEG40-rhDNase on the elastic modulus of CF sputa did not depend on Ca^{2+} concentration. Also, the activity of both proteins on the viscous modulus were independent of the concentrations of calcium and magnesium ions in CF sputa. Therefore, rhDNase and PEG40-rhDNase were overall active whatever these metal ion contents in sputa. Sanders et al., in their evaluation of the role of magnesium ions in the failure of rhDNase therapy in patients with CF, showed that the enzymatic activity of rhDNase correlated with the Mg^{2+} content in CF sputa [190]. The authors defined three groups based on magnesium concentration: sputa containing $[\text{Mg}^{2+}] > 1.7$ mM were strongly degraded by rhDNase, sputa containing $1.4 \text{ mM} < [\text{Mg}^{2+}] < 1.7$ mM were weakly degraded by

rhDNase and sputa containing $[Mg^{2+}] < 1.4$ mM were not degraded by rhDNase. However, we did not observe such a relationship, although the Mg^{2+} concentration in our samples also varied from 1.0 to 2.8 mM (*Supplementary figure S13*). Overall, our results highlighted that both rhDNase and PEG40-rhDNase were active whatever the Mg^{2+} concentration in CF sputa.

Moreover, the presence of metal ions in CF sputa such as magnesium and potassium promotes the formation of F-actin to the detriment of the globular form of actin (G-actin) [267]. Our results demonstrated that no correlation could be established between the contents of Mg^{2+} and K^+ and G-actin concentration (*Supplementary figure S14*). Sanders et al. suggested that rhDNase activity in CF sputa was indirectly linked to the concentration of Mg^{2+} through the conformation of actin under the globular or filamentous form [190]. Our results showed that G-actin concentration did not significantly correlate with the magnesium ions content found in CF sputa (*Supplementary figure S14A*), nor with rhDNase activity (**Figure 34**). Sanders et al. reported that both magnesium and potassium ions promote the polymerization of G-actin into F-actin starting at concentrations of 0.4 mM and 10 mM, respectively [190]. Regarding the content of both ions found in our CF sputum samples (2.1 mM for Mg^{2+} and 24 mM for K^+), our data are in good agreement with the fact that, at such Mg^{2+} and K^+ concentrations, more F-actin than G-actin is present in CF sputa (**Figure 33**). However, purulent and muco-purulent samples contained similar Mg^{2+} and K^+ concentrations but different G-actin content, suggesting that Mg^{2+} and K^+ ions might not be the only parameters that influence the polymerization of G-actin *in vivo*.

Finally, taking together all data collected for rhDNase and PEG40-rhDNase activities in CF sputum, the ability of both proteins to degrade the sputum was not dictated by the concentration of any parameter measured (DNA, G-actin, Ca^{2+} , Mg^{2+} and K^+).

IV. CONCLUSION

Since rheological methods can directly assess the efficacy of mucolytic agents, PEG40-rhDNase and rhDNase were evaluated in terms of enzymatic activity in CF sputa in order to predict the therapeutic value of the PEGylated protein. This clinical evaluation indicated that PEGylation preserved the mucolytic activity of rhDNase since both PEG40-rhDNase and the unconjugated enzyme induced a similar decrease of elastic and viscous moduli of the sputa. Concentrations of rhDNase remaining in CF sputa following 1 h incubation at 37°C and rheology indicated that PEGylation increased rhDNase stability in respiratory secretions. DNA and G-actin quantities found in CF sputa were linked to the visual inspection of samples: the more purulent the sputum, the higher the DNA and G-actin concentrations. Overall, both rhDNase and PEG40-rhDNase were active whatever the purulence and the DNA content of the CF sputum. Similarly, metal ions concentrations did not interfere with the activity of rhDNase, PEGylated or not. *In vitro* analysis showed that rhDNase and PEG40-rhDNase were partially inhibited by G-actin, but *ex vivo* evaluation of their mucolytic activity in CF sputa did not show a relationship between activity and G-actin content.

To sum up, PEGylation of rhDNase with a two-arm 40 kDa PEG did not alter the protein activity in CF sputa *ex vivo*. In view of the significant loss in biological activity of several PEGylated proteins on the market [277], this is a positive outcome which supports the further development of a PEGylated version of rhDNase. The next steps of this research will involve the assessment of PEG40-rhDNase residence time in the murine lungs and the analysis of its therapeutic efficacy in an animal model of CF-lung disease, in comparison with un-conjugated rhDNase.

**CHAPTER V. PEGYLATION OF RECOMBINANT HUMAN
DEOXYRIBONUCLEASE I PROVIDES A LONG-ACTING VERSION OF THE
MUCOLYTIC FOR PATIENTS WITH CYSTIC FIBROSIS**

PEGYLATION OF RECOMBINANT HUMAN DEOXYRIBONUCLEASE I PROVIDES A LONG-ACTING VERSION OF THE MUCOLYTIC FOR PATIENTS WITH CYSTIC FIBROSIS

Adapted from: PEGylation of recombinant human deoxyribonuclease I provides a long-acting version of the mucolytic for patients with cystic fibrosis, to be submitted

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Personal contribution: all experiments except from IL-6, TNF α and IL-10 quantification

ABSTRACT

Recombinant human deoxyribonuclease I (rhDNase) is delivered by inhalation to the respiratory tract of patients with cystic fibrosis (CF). However, rhDNase is rapidly cleared from the lungs, which implies a daily administration and a high treatment burden. The aim of this study was to investigate the benefits of rhDNase conjugation to polyethylene glycol (PEG), termed PEGylation, on the local residence time of rhDNase in the lungs. PEGylation of rhDNase with 20 kDa, 30 kDa or 40 kDa PEG sustained the presence of the enzyme within the lungs of healthy mice for more than 15 days whereas rhDNase was cleared within 24 h. The larger the PEG, the longer the residence time. The prolonged residence time of PEG40-rhDNase was associated with the preservation of its mucolytic activity up to 20 days within the lungs. The therapeutic efficacy of rhDNase conjugated to 30 kDa and 40 kDa PEGs was investigated in β -transepithelial Na⁺ channel-overexpressing (β -ENaC) mice, a genetically engineered murine model of CF. A single dose of both PEGylated rhDNases showed similar DNA hydrolysis in the lumen of respiratory airways to five daily doses of rhDNase. In addition, a weekly administration of rhDNase PEGylated with either 30 kDa or 40 kDa PEG was efficacious to clear DNA from the respiratory tract and did not result in any apparent accumulation of both conjugates in the lungs over 6 weeks. Finally, PEGylation of rhDNase did not alter the protein stability to nebulization, highlighting that nebulization could be used as a delivery method for potential future clinical application. The sustained presence of rhDNase provided by large PEGs attachment and its therapeutic efficacy offer interesting perspectives for the development of a long-acting rhDNase with reduced therapy burden.

I. INTRODUCTION

Cystic fibrosis (CF) is an autosomal genetic recessive disease which affects multiple organs such as the gastro-intestinal tract, the pancreas and the airway epithelium. However, the majority of the pathology is due to the respiratory function failure. Pulmonary secretions obstruct the respiratory tract of patients with CF [392], which translates in an impaired ability to mucus clearance. The pulmonary secretions contain high quantities of proteins, proteases and extracellular DNA [180,388], resulting from the death of neutrophils [177].

Recombinant human deoxyribonuclease I (rhDNase), delivered by inhalation, allows to fluidize the mucus of patients with CF by hydrolyzing the DNA polymers contained in it [4]. Although, it is directly administered to the site of action, rhDNase is rapidly cleared from the lungs (remaining less than 24 h in the pulmonary tract). Consequently, a daily administration is necessary [5], which results in high treatment burden and may decrease the patient adherence. Therefore, rhDNase would benefit from a method that sustains its presence within the lungs.

PEGylation is a common process used to improve the therapeutic value of a protein. Indeed, conjugation to polyethylene glycol (PEG) chains prolongs the residence time of proteins in the lungs. Adhesiveness to mucus, improved proteolytic resistance, reduced endocytosis by respiratory cells and reduced transport across lung epithelium represent most of the mechanisms that may explain the sustained retention within the lungs of PEGylated proteins, as previously discussed in the introduction (**section II.2.2**).

rhDNase has been mono-PEGylated on its N-terminus with a linear chain of 20 kDa PEG, a linear chain of 30 kDa PEG or a two-arm 40 kDa PEG [312]. Our group previously demonstrated that PEGylated versions of rhDNase retain the full biological activity of the protein *in vitro* [312] and in CF sputa *ex vivo* (**Chapter IV**).

The goals of this research article were to demonstrate the sustained presence within the lungs of PEGylated rhDNases and to evaluate the therapeutic efficacy of rhDNase conjugated to 30 kDa or 40 kDa PEG in a genetically engineered murine model of CF. Transgenic mice with overexpression of the β -subunit of the amiloride-sensitive transepithelial Na⁺ channel (β -ENaC) develop mucus hypersecretion, airway obstruction as well as chronic inflammation and

thus present CF-like lung disease [393]. Therefore, β -ENaC transgenic mouse represented an interesting preclinical model to evaluate the therapeutic efficacy of rhDNase constructs. Finally, since the clinical efficacy of PEGylated rhDNase will not only depend on its ability to sustain its presence within the lungs, we evaluated the aptitude of the PEG-conjugates to retain the enzymatic activity of rhDNase following jet nebulization.

II. MATERIAL AND METHODS

II.1. MATERIAL

Pulmozyme® was from Genentech, Inc. (South San Francisco, CA, USA).

Linear 20 kDa and 30 kDa methoxy PEG (PEG20 and PEG30, respectively) propionaldehydes and two-arm 40 kDa methoxy PEG (PEG40) propionaldehyde were obtained from NOF Corporation (Tokyo, Japan). Unless otherwise stated, chemicals and reagents were from Sigma-Aldrich (St. Louis, MO, USA).

The N-terminal site-specific mono-PEGylation was conducted as previously described [312].

II.2. ANIMALS

Specific-pathogen-free female NMRI mice (7 to 12 weeks old; Elevage Janvier, Le Genets-St-Isle, France) were used for the *in vivo* disposition experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Université catholique de Louvain (UCL; Permits number: 2012/UCL/MD/006 and 2016/UCL/MD/019).

Transgenic mice overexpressing the β -subunit of the amiloride-sensitive transepithelial Na⁺ channel (β -ENaC) and their wild type (WT) littermate controls were used in the therapeutic efficacy experiments. The mouse model was built in the C57Bl6 genetic background [394] and a colony was bred and housed at the animal care facility of the UCL following recommendations of the Federation of European Laboratory Animal Science Associations (FELASA, [395]), with free access to food and water. Offspring were genotyped by polymerase chain reaction (PCR) of genomic DNA at 21 days of age. Experiments were performed in sex-matched mice aged from 9 to 16 weeks. The studies and procedures were approved by the ethics committee for animal research of the UCL (2017/UCL/MD/015) and in agreement with the European Community regulations for animal use in research (CEE n° 86/609).

All studies were performed under anaesthesia and all efforts were made to minimize animal discomfort.

II.3. KINETICS OF *IN VIVO* DISPOSITION IN THE RESPIRATORY TRACT

II.3.1. Intratracheal instillation

Female NMRI mice were anaesthetized using ketamine/xylazine (90/10 mg/kg) intraperitoneal injection. Biotinylated rhDNase, PEG20-rhDNase, PEG30-rhDNase, PEG40-rhDNase or PEG40-rhodamine isothiocyanate (PEG40-RITC; Nanocs, New York, NY, USA) were administered intratracheally. rhDNase was biotin-labeled using EZ-Link Sulfo-NHS-LC-Biotin reagent (Thermo Fisher Scientific, Rockford, IL, USA). Five µg of biotinylated protein (the PEG moiety was not counted in the mass delivered. Therefore, the different mouse groups received the same number of moles of rhDNase, that is, 0.17 nmol) or 80 µg of PEG40-RITC in 25 µL phosphate-buffered saline (PBS) were then delivered to the mice. During the administration, the mouse was maintained in a 45° angle position and the protein or PEG40-RITC solution was instilled via the mouth in the trachea of the mouse. An insufflation of 200 µL air bolus was then applied to push the solution to the distal tract [396].

II.3.2. Sample collection

At various pre-determined times (immediately, 4 h, 24 h, 3 days, 7 days, 10 days, 20 days and 1 month) following protein administration, serum was collected by orbital bleeding. The mice were sacrificed by cervical dislocation. A nasal lavage (NAL) was performed by cannulating the trachea towards the nasal cavities and instilling 1 mL Hanks' balanced salt solution (HBSS; Gibco™, Life Technologies, Gent, Belgium) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The fluid emerging from the nostrils was collected. A bronchoalveolar lavage (BAL) was then performed. One mL of HBSS containing protease inhibitor was injected into the trachea, left for 10 to 30 s, followed by withdrawal and re-injection of twice 0.5 mL of the fluid. Then, all the BAL liquid was removed from the lungs. Afterwards, the lungs were removed and ground to release the proteins using a tissue grinder (Polytron; Kinematica, Luzern, Switzerland). For all the proteins, tissue processing was carried out using Triton® X-100 (Merck Millipore, Darmstadt, Germany) diluted at 1:1000 in HBSS containing protease inhibitor. Triton allows to increase the recovery of proteins conjugated to large PEG chains from the biological samples [24]. The lungs were grounded in 0.2 mL of HBSS containing protease inhibitors and Triton® then 0.8 mL of the same HBSS solution was added.

The tubs were rinsed twice with 1 mL HBSS containing protease inhibitors and Triton®. A total of 3 mL was collected for the lung homogenate. Sera, BAL and tissue homogenate samples were then centrifuged at 4500 g at 4 °C for 10 min. The supernatants were stored at -20 °C until they were assayed for rhDNase content by ELISA.

Similar method was followed for *in vivo* disposition evaluation of PEG40-RITC. Mice were sacrificed immediately, 24 h, 3 days, 7 days, 10 days, 15 days and 20 days following polymer administration. Sera, NAL, BAL and lungs were collected. NAL and BAL were performed using HBSS without protease inhibitor cocktail and lungs were grinded in HBSS containing 0.1% Triton®. Cells pellets were collected after centrifugation of BAL for quantification of PEG in macrophages. Cell pellets were re-suspended in 1 mL PBS to proceed with total cell counts. Ten µL were mixed with 10 µL trypan blue solution and counted using TC20™ automated cell counter (Bio-Rad, Hercules, CA, USA). Centrifugation of the lavage at 4500 g 10 min at 4°C was then performed. The cells were suspended in 500 µL of ammonium-chloride-potassium (ACK) lysing buffer (Lonza, Basel, Switzerland) and stored at -20 °C until they were assayed for PEG content by ELISA.

II.3.3. Quantification of rhDNase constructs

Biotinylated rhDNase constructs were quantified by ELISA. Briefly, a 96-well plate was coated using streptavidin (5 µg/mL) in 1% NaHCO₃ solution, pH 9.6 at 4°C overnight and blocked with 1% proteins from milk powder in PBS-Tween 0.1% at 37°C for 1 h. Sera, NAL, BAL and lung supernatant samples as well as standards (biotinylated rhDNase, PEG20-rhDNase, PEG30-rhDNase or PEG40-rhDNase) were incubated at 37°C for 1 h. Dilutions were done in PBS-Tween 0.1% + 0.1% bovine serum albumin (BSA). A first rabbit polyclonal antibody to human rhDNase (Bio-connect, Huissen, The Netherlands) and a goat anti-rabbit IgG conjugated to horse radish peroxidase (HRP; Abcam, Cambridge, UK) were used for development. Revelation was conducted using Tetramethylbenzidine solution (Thermo Fisher Scientific, Rockford, IL, USA) and stopped with 2 M H₂SO₄. The ELISA plate was read at 450 nm (Multiskan EX, Thermo Fisher Scientific, Rockford, IL, USA).

It is also relevant to notice that the degrees of biotin labelling could be different between rhDNase and its PEGylated conjugates. N-Hydroxysuccinimide (NHS)-biotin was used for

coupling to amines and rhDNase presented free N-ter amine residue whereas the PEGylated enzymes did not (**Chapter II**). Therefore, the degree of labelling of rhDNase could be higher than that of its PEGylated counterparts. ELISA assays were performed with the corresponding biotinylated rhDNase construct to avoid any potential underestimation of the protein quantity in each sample containing PEGylated rhDNase.

Biotinylated rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase lower limits of detection were 0.06 µg, 0.6 µg, 0.8 µg and 0.8 µg, respectively. All analyses were performed in duplicate.

II.3.4. Quantification of PEG40-RITC

Fluorescence. The fate of fluorescent PEG40-RITC in the sera, NAL, BAL and lung supernatant of NMRI mice was measured with a SpectraMax® M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) with 553 nm excitation and 627 nm emission filters. PEG40-RITC lower limit of detection was 0.4 µg.

ELISA. The amount of PEG40-RITC in BAL cell pellet of NMRI mice was quantified using PEGylated protein ELISA kit according to the manufacturer protocol (Enzo Life Science, Farmingdale, NY, USA). PEG40-RITC lower limit of detection was 3.2 ng.

II.4. RHEOLOGY

Rheology assays were performed on a salmon testes DNA solution at 6 mg/mL in 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.5. DNA solution was diluted to 0.75 mg/mL with a 25 mM HEPES, 4 mM CaCl₂, 2 or 4 mM MgCl₂, 0.2% bovine serum albumin, 28 mM KCl solution at pH 7.5 and incubated at 37°C for 30 min. Then, BAL or lung homogenate of NMRI mice containing biotinylated rhDNase or biotinylated PEGylated rhDNase was added to reach a final concentration of 0.25 µg/mL (8.3 µM) of rhDNase (final volume of 500 µL; the PEG was not counted in the mass of the PEG-rhDNase). Viscosity measurements were performed during 30 to 60 min at 37°C using a Modular Compact Rheometer MCR102 (Anton Paar, Gent, Belgium). A 50 mm stain cone plate with a 0.5° angle (Anton Paar, Gent, Belgium) was used. A solvent trap containing distilled water prevented dehydration of samples during

rheological analysis. The experiments were performed in rotational mode at 1000 rpm. The results were recorded by RheoCompass™ software (Anton Paar, Gent, Belgium).

II.5. THERAPEUTIC EFFICACY

II.5.1. Intratracheal instillation

β -ENaC and WT mice were anaesthetized using ketamine/xylazine (90/10 mg/kg) intraperitoneal injection. Ten μ g of rhDNase, PEG30-rhDNase or PEG40-rhDNase in 25 μ L PBS were delivered to β -ENaC and WT mice. The PEG moiety was not counted in the mass delivered. Therefore, the different mouse groups received the same number of moles of rhDNase, that is, 0.33 nmol. During the administration, the mouse was maintained in a 45° angle position and the protein solution was instilled via the mouth in the trachea of the mouse. An insufflation of 200 μ L air bolus was then done to push the solution to the distal tract [396].

Short-term study. One dose of PEG30-rhDNase or PEG40-rhDNase was delivered to mice at day 1 and compared with 5 doses (1 dose/day during 5 days) of rhDNase. Mice were anaesthetized every day and 25 μ L PBS were administered to the PEG30-rhDNase and PEG40-rhDNase groups at days 2 to 5. Mice were sacrificed at day 6 and after blood collection.

Long-term study. One dose of PEG30-rhDNase or PEG40-rhDNase was delivered to β -ENaC and WT mice once a week during 5 weeks. Mice were sacrificed 7 days following the last dose and after blood collection.

II.5.2. Sample collection

The mice were sacrificed by cervical dislocation. A BAL was performed followed by resection of the lungs. The method displayed in section II.3.2 was followed. Cells pellets were collected after centrifugation of BAL for cellular markers. BAL and lung supernatant were then frozen at -20°C for future analyses.

II.5.3. Quantification of pro-inflammatory cytokine markers in BAL

Pro-inflammatory mouse cytokines interleukin (IL)-6, tumor necrosis factor (TNF) $_{\alpha}$ and chemokine ligand (CCL) $_2$ were quantified by ELISA.

IL-6 and TNF α . Briefly, a 96-well plate was coated using anti-mouse IL-6 monoclonal antibody (2.4 mg/mL) or anti-mouse TNF α purified clone (0.5 mg/mL; eBioscience, San Diego, CA, USA) in 40 mM glycine solution, pH 9.4 at 4°C overnight and blocked with 10% fetal calf serum (FCS) in PBS at 37°C for 1 h. BAL samples as well as standards (mouse IL-6 or mouse TNF α) were incubated at 37°C for 2 h. anti-mouse IL-6 biotin conjugated monoclonal antibody or anti-mouse TNF α biotin conjugated clone (eBioscience) was added and the plate was incubated at 37°C for 90 min. Avidine-HRP (Biolegend, San Diego, CA, USA) was used for development (37°C, 30 min). Revelation was conducted using Tetramethylbenzidine solution and stopped with 2 M H₂SO₄. The ELISA plate was read at 450 nm.

CCL₂. was assessed using standard sandwich linked-immunosorbent assay-ELISA (Mouse CCL₂/JE/MCP-1 DuoSet ELISA; Biotechne, Minneapolis, MN, USA).

IL-6, TNF α and CCL₂ lower limits of detection were 0.8, 8.9 and 0.6 pg/mL, respectively. All biochemical analyses were performed in duplicate.

II.5.4. Quantification of IL-10 (anti-inflammatory cytokine) in BAL

Anti-inflammatory IL-10 cytokine was monitored in BAL fluid by ELISA.

A 96-well plate was coated using anti-mouse IL-10 monoclonal antibody (0.1 mg/mL) in 40 mM glycine solution, pH 9.4 at 4°C overnight and blocked with 10% FCS in PBS at 37°C for 1 h. BAL samples as well as standards (mouse IL-10) were incubated at 37°C for 2 h. Anti-mouse IL-10 biotin antibody (Biolegend, San Diego, CA, USA) was added and the plate was incubated at 37°C for 90 min. Avidine-HRP (Biolegend, San Diego, CA, USA) was used for development (37°C, 30 min). Revelation was conducted using Tetramethylbenzidine solution and stopped with 2 M H₂SO₄. The ELISA plate was read at 450 nm.

The lower limit of detection was 40.6 pg/mL. The biochemical analysis was performed in duplicate.

II.5.5. Quantification of cellular markers in BAL

100 μ L of ACK lysing buffer were added in each BAL cells pellet. Samples were then incubated at 4°C for 10 min. 500 μ L of 10% fetal bovine serum (FBS; Gibco™, Life Technologies, Gent,

Belgium) was then added and samples were centrifuged at 260 g, 4°C for 10 min. The supernatant was discarded and cell pellets were re-suspended in 500 µL of 10% FBS to proceed with total cell counts. Ten µL were mixed with 10 µL trypan blue solution and counted using TC20™ automated cell counter (Bio-Rad, Hercules, CA, USA). Cytospin™ (Thermo Fisher Scientific, Rockford, IL, USA) was then performed to get 30,000 cells on Superfrost plus® microscope slides (Thermo Fisher Scientific, Rockford, IL, USA). Fixation of cells was then carried using staining set Diff-Quik® (Medion Diagnostics, Düringen, Switzerland). Macrophages, neutrophils, lymphocytes and eosinophils were then counted using Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany).

II.5.6. DNA quantification in BAL

DNA contained in BAL was quantified using a Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, Rockford, IL, USA). The protocol was adapted from the manufacturer method [378]. Briefly, samples and standards were diluted in 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM trishydroxymethylaminomethane (Tris) buffer at pH 8.0 and the same volume of PicoGreen® reagent (diluted 1:200 in Tris-EDTA buffer) was added. Samples and standards were incubated in darkness for 5 min in a black 96-well plate. Fluorescence was measured with a SpectraMax® M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) with 485 nm excitation and 530 nm emission filters.

II.5.7. Quantification of rhDNase constructs in BAL and lung supernatant

A 96-well plate was coated using first rabbit polyclonal antibody to human rhDNase (5 µg/mL; Bio-connect, Huissen, The Netherlands) in 1% NaHCO₃ solution, pH 9.6 at 4°C overnight and blocked with 1 to 5% proteins from milk powder in PBS-Tween 0.1% at 37°C for 1 h. BAL and lung supernatant samples as well as standards (rhDNase, PEG30-rhDNase or PEG40-rhDNase) were incubated at 37°C for 1 h. Dilutions were done in PBS-Tween 0.1% + 0.1% BSA. Biotin-conjugated antibody (same antibody as the one used for coating) and streptavidin-HRP were used for development. Revelation was conducted using Tetramethylbenzidine solution and stopped with 2 M H₂SO₄. The ELISA plate was read at 450 nm. The sensitivity of the ELISA assay being reduced for PEGylated rhDNase, the analyses were performed with the corresponding rhDNase construct to avoid any potential underestimation of the protein quantity in each sample containing the PEGylated rhDNase.

In fluids where rhDNase, PEG30-rhDNase and PEG40-rhDNase were detectable, the lower limits of detection were 198 ng/mL, 643 ng/mL and 1330 ng/mL, respectively. All analyses were performed in duplicate.

II.6. NEBULIZATION OF rhDNase AND PEGYLATED CONJUGATES

rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase were nebulized using an air-jet nebulizer (Sidestream®, Teleflex Medical, Wayne, PA, USA with Respironics Prota-neb®, Philips, Amsterdam, The Netherlands). One mL of rhDNase or PEGylated rhDNase at 0.15 mg/mL (5 μ M of rhDNase; the PEG moiety was not counted in the concentration of PEG-rhDNase) in 150 mM NaCl and 1 mM dihydrate CaCl₂ was placed in the nebulizer chamber and allowed to nebulize for 15 min. The droplets generated were then collected using 1.5 mL Eppendorf® tubes and analyzed using a colorimetric assay and size exclusion chromatography (SEC).

II.6.1. Colorimetric determination of rhDNase activity

This assay was adapted from Sinicropi's paper [333]. A solution of 2 mg/mL of DNA isolated from salmon testes (Sigma-Aldrich) was prepared in Buffer A (25 mM HEPES, 1 mM EDTA, pH 7.5) and mixed for 3–4 days at room temperature until the solution was homogenous. The solution was stored at 4°C. A 0.4% solution of Methyl Green (Sigma-Aldrich) was prepared in Buffer B (20 mM acetate-NaOH, pH 4.2). The solution was extracted with chloroform to remove traces of crystal violet until the organic layer was colorless. The upper aqueous layer was separated, stirred in the hood for 2–3 h to evaporate the excess chloroform and the solution was stored at 4°C. The DNA Methyl Green substrate was prepared by gently mixing 77% (v/v) of 2 mg/mL DNA, 4.6% of 0.4% Methyl Green and 18.4% of Buffer C (25 mM HEPES, 4 mM CaCl₂, 4 mM MgCl₂, 0.1% BSA, 0.01% thimerosal, 0.05% Tween20, pH 7.5). The solution was stored at 4°C and incubated at room temperature overnight before the assay. Samples (9 serial 2-fold dilutions of rhDNase, PEG20-rhDNase, PEG30-rhDNase or PEG40-rhDNase) were prepared in Buffer C. Each well of a 96-well plate was filled with 100 μ L of samples and 100 μ L of DNA-Methyl Green substrate. The plate was sealed and incubated for 6 h at 37°C. The absorbance at 650 nm was measured at the end of the incubation.

II.6.2. Size exclusion chromatography (SEC)

SEC was performed on an ÄKTA™ purifier 10 system (GE Healthcare Bio-Sciences, Uppsala, Sweden) using a Superose 6 Increase 10/300 GL column (GE Healthcare Bio-Sciences, Uppsala, Sweden). A pre-filtrated and degassed solution of 150 mM NaCl, 1 mM CaCl₂ was used for elution. Each sample was analyzed twice and absorbance was measured at 280 nm (aromatic ring absorption) and 215 nm (peptide bond absorption).

II.7. DATA ANALYSIS

All results are presented as mean values $\pm$ standard error of the mean (SEM). ANOVA tests with Bonferroni post-test or Dunnett's multiple comparison test were used for the statistical analysis of the quantity of rhDNase constructs recovered in the lungs of mice (**Figure 36**, **Figure 41** and **Figure 44**), for the statistical analysis of the rheology measurements of rhDNase and PEGylated rhDNases activities in lung supernatant and BAL (**Figure 37** and **Figure 38**), as well as for the quantification of DNA (**Figure 40** and **Figure 43**). Unpaired t-tests were employed for comparing the enzymatic activity of rhDNase constructs following nebulization (**Table 8**). All tests were performed using the GraphPad Prism software (CA, USA). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

III. RESULTS AND DISCUSSION

III.1. KINETICS OF *IN VIVO* DISPOSITION IN THE RESPIRATORY TRACT

This study was conducted with biotinylated rhDNase constructs to allow easy quantification of the rhDNase moiety of all the proteins following their intratracheal instillation in NMRI mice. Biotin is a hydrophobic compound that may impact the behavior of rhDNase. Consequently, we assessed the enzymatic activity of biotinylated PEG40-rhDNase on pure DNA solution and compared it to that of the non-biotinylated counterpart (*Supplementary Figure S15*). The viscosity results highlighted that biotin conjugation did not affect the DNA hydrolysis mediated by PEG40-rhDNase. Considering that PEGylation preserved the *in vitro* and *ex vivo* enzymatic activities of rhDNase (**Chapters II and IV**), we assumed that none of the enzymes activity would be impacted by biotinylation.

III.1.1. Quantification of rhDNase and PEGylated conjugates

To study the impact of PEGylation and of the PEG length on the residence time of rhDNase in the lungs, we delivered PEG20-rhDNase, PEG30-rhDNase, PEG40-rhDNase or the non-conjugated enzyme to the respiratory tract of mice by intratracheal instillation. We then measured rhDNase content in sera, NAL, BAL and lung homogenate at different time points after delivery.

The nasal cavity did not retain any of the proteins after 24 h (*Supplementary Figure S16*). The lungs retained the PEGylated rhDNases better than the nasal cavity (**Figure 36**). Immediately after delivery to the lungs, all the proteins were found in BAL (**Figure 36A**) rather than in lung homogenate (**Figure 36B**). Four hours post-delivery, the BAL contained more than 40% of the administered dose of each PEGylated conjugates (66% for PEG20-rhDNase, 60% for PEG30-rhDNase and 42% for PEG40-rhDNase) whereas 88% of the administered dose of rhDNase were already cleared from the respiratory tract (**Figure 36** and *Supplementary Figure S16*). At the same time point, the lungs retained 16% of PEG40-rhDNase, 9% of PEG30-rhDNase and 3% of PEG20-rhDNase (**Figure 36B**). These findings highlighted that the size of the PEG might dictate the binding rate of rhDNase to the mucus or pulmonary tissue, with the bigger the PEG, the higher the amount of PEGylated rhDNase in lung homogenates. Twenty-four hours

post-delivery, the total respiratory tract content of rhDNase had decreased to 0.1% of the administered dose, highlighting that the protein was cleared within a day. Overall, rhDNase mostly remained in the lung lumen of healthy mice and did not enter the pulmonary tissue. Regarding its PEGylated conjugates, the BAL content at 24 h decreased to 12% for PEG20-rhDNase, 2% for PEG30-rhDNase and 7% for PEG40-rhDNase whereas their lung tissue contents remained similar to that of the 4 h time point. Later on, the proteins were solely found in lung homogenates. Fifteen days post-delivery, 38% of PEG20-rhDNase, 33% of PEG30-rhDNase and 63% of PEG40-rhDNase remained present in lung homogenates. Therefore, conjugation of rhDNase to 20 kDa, 30 kDa PEG or 40 kDa PEG sustained the presence of the enzyme within the lungs for more than 15 days. Twenty days post-delivery, PEG20-rhDNase and PEG30-rhDNase were not detected in the lungs since the doses recovered were below the background of control mice. However, 62% of PEG40-rhDNase were still recovered in the lung homogenates at the same time point. One month post-delivery, PEG40-rhDNase was cleared from the lungs.

The site of action of rhDNase (and consequently of PEG-rhDNase) is the mucus in which the extracellular DNA to cleave is present [4]. However, based on the results obtained for our *in vivo* disposition study (**Figure 36**), we might wonder if the PEGylated conjugates remained in the mucus or penetrated the lung tissue. The procedure followed to recover rhDNase constructs from the lungs did not permit to dissociate the amount contained in the mucus from that of the lung tissue. However, our group recently demonstrated using confocal scanning microscopy that PEGylated rhDNases did not penetrate the lung epithelium (data not shown). Consequently, we assume that the quantities of all the three conjugates recovered from the lung homogenates were anchored in the mucus rather than in the lung tissue.

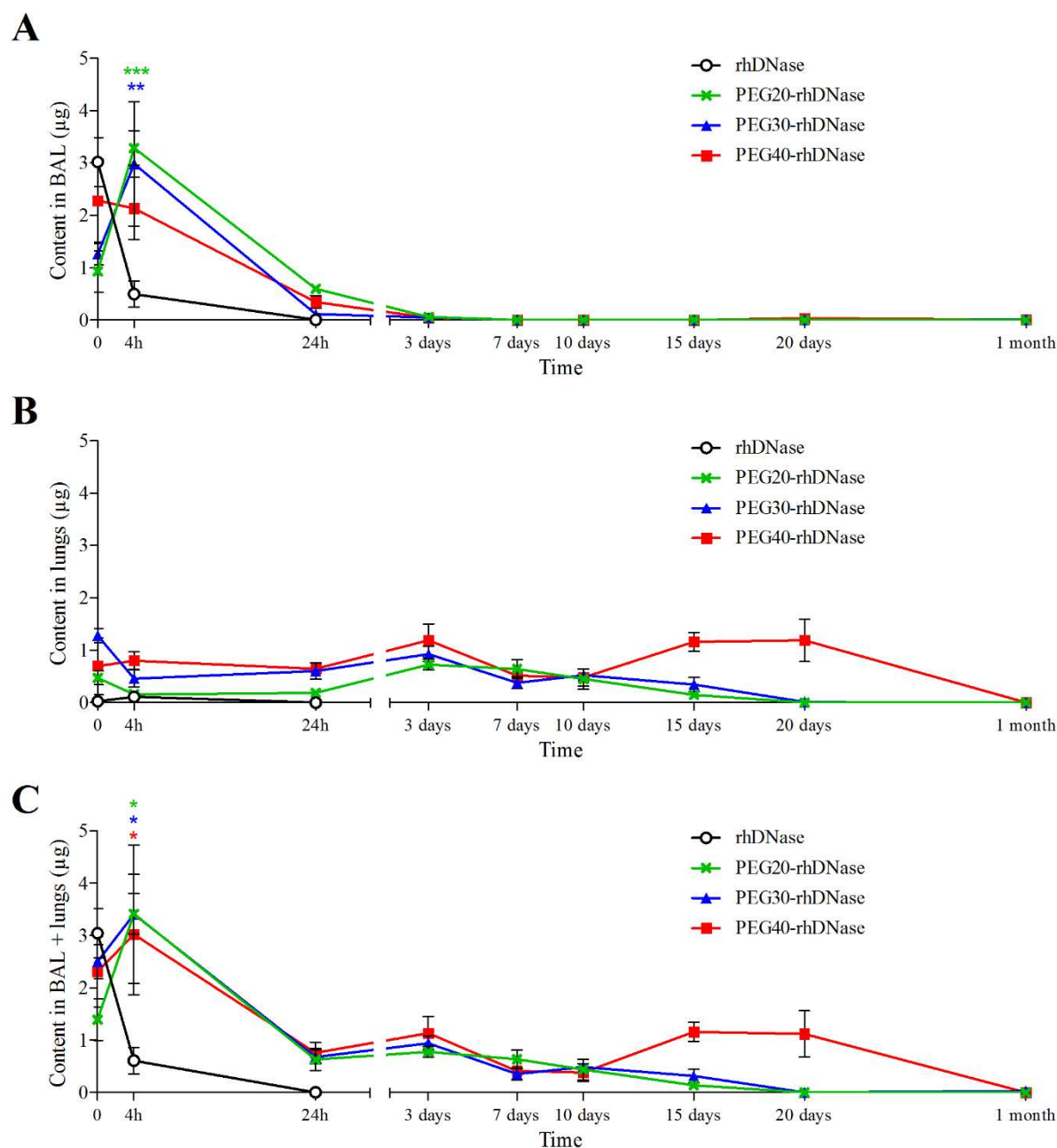


Figure 36: Quantities of biotinylated rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase recovered from the respiratory tract (**A.** bronchoalveolar lavage (BAL), **B.** supernatant of lung homogenate (lungs) and **C.** BAL + lungs) of NMRI mice at different times following the intratracheal delivery of a 5 µg (0.17 nmol) rhDNase dose. PEG was not counted in the mass delivered and recovered from the respiratory tract. Therefore, the different mouse groups received the same number of moles of rhDNase compounds. The amounts of rhDNase recovered from BAL and lungs are expressed in µg of biotinylated rhDNase. Due to the sensitivity difference between ELISA assays, the background of each rhDNase construct was subtracted for each time point. The PEGylated rhDNase groups were compared to the rhDNase group at 0, 4 h and 1 day time points (two-way ANOVA, Bonferroni post-test). The data represent the mean values (± SEM) of 5 to 10 mice per time point.

Considering that rhDNase PEGylated with either linear 20 kDa or 30 kDa PEG showed similar retention within the lungs, PEG molecular weight did not appear to strongly affect the residence time of PEGylated rhDNase. However, the content of PEG20-rhDNase remaining active after 10 days in the lungs might be overestimated, as described later. Two-arm 40 kDa PEG attachment to rhDNase allowed its sustained residence time up to 20 days, with a significantly higher dose recovered at 20 days compared to linear 20 kDa and 30 kDa PEG conjugates. Similarly, Patil and co-workers showed that the conjugation of a two-arm 40 kDa PEG chain to anti-IL17A Fab' fragment better prolonged the residence time of the antibody than a linear 20 kDa PEG chain [304].

PEG being described as a mucoadhesive polymer [299,300], we speculated that branched PEG permitted stronger entrapment in mucus gel, thereby better enhancing rhDNase residence time within the lungs of mice. However, Patil and co-workers demonstrated that anti-IL17A Fab' fragment conjugated to either linear 20 kDa or two-arm 40 kDa PEG showed similar binding to respiratory mucus [304]. Patil et al. also demonstrated that PEG conjugation to anti-IL17A Fab' decreased the uptake by epithelial cells, where the bigger the PEG, the lower the protein uptake [304]. Therefore, we assume that the superiority in the retention time of PEG40-rhDNase might result from a reduced uptake by epithelial cells, compared to PEG20-rhDNase and PEG30-rhDNase.

Koussoroplis et al. showed that the conjugation of a two-arm 40 kDa PEG chain to anti-IL17A F(ab')₂ and anti-IL13 Fab' antibody fragments greatly prolonged their presence in the lungs of mice [24]. The content of PEGylated antibody fragments within the lungs plateaued up to 4 h post-delivery, whereas clearance of non-conjugated proteins started immediately after administration. Forty-eight hours post-delivery, anti-IL17A F(ab')₂ and anti-IL13 Fab' contents in the lungs decreased to 10% and 14% of the dose initially deposited, respectively. However, this value was 40% for both PEG40-F(ab')₂ and PEG40-Fab'. Mcleod et al. showed that IFN_{α2b} PEGylated with a 12 kDa PEG chain and IFN_{α2a} conjugated to two-arm 40 kDa PEG presented an enhanced retention in the lungs compared with the native protein [23]. 54% and 37% of the delivered dose of PEG12-IFN_{α2b} and PEG40-IFN_{α2a} remained in the lungs 8 h after delivery whereas this value was 19% for the native IFN_{α2b} [23]. More recently, Patil and co-workers delivered anti-IL13 Fab' and PEGylated anti-IL17A Fab' to mice through central airway or deep

lungs deposition [304]. PEGylation of anti-IL13 with two-arm 40 kDa PEG did not significantly sustain the presence of the protein within the lungs following deep lung delivery whereas it greatly increased its residence time after deposition to the central airways. The authors also highlighted that the molecular weight of PEG (linear 20 kDa and two-arm 40 kDa) had a moderate influence on the presence of anti-IL17A Fab' fragment within the lungs. Non-conjugated anti-IL17A Fab' was cleared within 24 h, whereas the clearance of both PEG20-Fab' and PEG40-Fab' was gradual till 48 h.

It is well known that the higher the molecular weight of the protein, the longer the residence time in the lungs [397]. However, a large variability exists between proteins, as demonstrated below. Comparing the residence time of rhDNase (30 kDa) with that of non-conjugated anti-IL13 Fab' and anti-IL17A F(ab')₂ (47 kDa and 100 kDa, respectively [24]), the protein molecular size appeared to contribute to its residence time within the lungs following pulmonary administration. Four hours post-delivery, the lungs content of rhDNase was 12% of the administered dose whereas 50% and 70% of anti-IL13 Fab' and anti-IL17A F(ab')₂ remained in the respiratory tract of mice, respectively. Therefore, the higher the protein molecular weight, the longer its local retention. However, considering IFN_{α2b} (19 kDa), 19% of the initial deposited dose was recovered 8 h following intratracheal delivery [23]. IFN_{α2b} was therefore less rapidly eliminated than larger proteins such as rhDNase. Consequently, the molecular size is not the only property which determines the protein residence time within the lungs but the nature of the compound delivered is also a major factor; as previously reported [371]. Moreover, the PEGylated versions of rhDNase, anti-IL13 Fab' and anti-IL17A F(ab')₂ presented different molecular sizes, which did not correlate with their retention time within the lungs [24]. Also, considering PEG30-rhDNase and PEG40-IFN_{α2a}, both conjugates were of similar size (60 kDa) but PEG30-rhDNase remained three-times longer in the lungs [23]. Patil and co-workers highlighted that the site of delivery of anti-IL17A Fab' fragments (PEGylated or not) had a large impact on their residence time within the respiratory tract [304]. Deep lungs deposition allowed to prolong the residence time of both anti-IL13 Fab' and anti-IL17A Fab' constructs over central airway deposition [304]. Consequently, the site of delivery influences the presence of proteins within the lungs. These findings then support that several parameters, including the nature of the protein, the site of delivery and the PEG muco-adhesive property, impact the residence time of proteins (PEGylated or not).

Reduced transport across the lung epithelium has also been reported as a parameter leading to the prolonged residence of PEGylated proteins within the respiratory tract [19,20,23]. Therefore, we evaluated the fate of rhDNase constructs in the blood of instilled mice. PEGylation of rhDNase with large PEG chains (up to 40 kDa) did not increase the systemic exposure to the enzyme (data not shown) since the doses recovered were below the background of control mice. Our results are in good agreement with previous rhDNase inhalation studies in rats [398]. The authors demonstrated that a low percentage of rhDNase was absorbed in the systemic circulation following pulmonary delivery (< 15%). Similarly, Mcleod and co-workers highlighted that PEGylation of IFN α largely decreased the systemic absorption of the cytokine from the lungs in rats, with a bioavailability of 15% for native IFN α and of 5.5% and < 0.4% for its PEG12- and PEG40- derivatives, respectively [23].

Overall, comparing the local residence time of all PEGylated proteins presented above, PEG attachment to rhDNase resulted in an impressive prolongation of its residence time (for more than 15 days) within the lungs following pulmonary delivery. To our best knowledge, our investigation is the first to demonstrate a residence time of more than 15 days for a PEGylated protein.

We also recorded the weight of the mice to have a first assessment of the PEGylated rhDNases safety. The data collected showed no significant loss of weight over one month for all conjugates (*Supplementary Figure S17*). Therefore, no obvious toxicity was evident for all the PEGylated rhDNases.

III.1.2. Rheology of PEGylated rhDNases contained in lung supernatant samples

To confirm that PEGylated conjugates were still active in the lungs overtime, we assessed DNA degradation by the proteins contained in lung supernatant (**Figure 37**). Data obtained at t=0 showed that PEG40-rhDNase is less active than the two other PEGylated conjugates (**Figure 37A**). To our best knowledge, only one investigation compared the activity of linear *versus* branched PEG-conjugates. Vugmeyster et al. demonstrated that two-arm 40 kDa PEG-TNF α nanobody presented 13% lower activity than the linear 40 kDa PEG-conjugate [310]. The authors also proposed several theoretical conformations adopted by different PEG structures. They suggested that *in vivo* PEG wrapped around the protein, creating a protective shell.

Consequently, we hypothesize that this compact conformation could have been adopted for all PEGylated rhDNases *in vivo* but that the ‘umbrella-like’ structure of the two-arm 40 kDa PEG might have partially hide rhDNase active site, contrary to linear 20 kDa and 30 kDa PEGs. Another explanation could result from the extraction process of the rhDNase constructs contained in the lungs. Tissue crushing might have impaired the protein activity, with PEG40-rhDNase being the more impacted.

We also assessed DNA degradation by rhDNase and PEGylated rhDNases contained in BAL samples at t=0. The results obtained showed better activity of all rhDNase conjugates in BAL than in lung supernatant. However, BAL findings are in good agreement with those of lung supernatant, highlighting that rhDNase activity was reduced by 40 kDa PEG attachment (**Figure 38**). We assume that the difference in activity observed in BAL and lung supernatant could result from the composition of each fluid. BAL mainly contains alveolar macrophages (AM) and components of the epithelial lining fluid while lung supernatant is obtained from tissue crushing. This method allows the release of proteins and proteases, which degrade rhDNase. The presence of such molecules could potentially impede the binding between rhDNase and DNA polymers, thereby slowing down rhDNase enzymatic activity.

PEG20-rhDNase and PEG30-rhDNase partially lost their activity during residence in the lungs and this loss increased with time (**Figure 37B and C**). However, PEG30-rhDNase better retained its activity over 10 days than PEG20-rhDNase, thereby confirming that the content of active PEG20-rhDNase was overestimated by ELISA. Therefore, the longer the linear PEG, the more retained the activity. After 20 days in the lungs, PEG40-rhDNase remained as active as at t=0 (**Figure 37D**). The steric hindrance created by the two-arm 40 kDa PEG, compared to linear PEGs [310], might explain the sustained activity of rhDNase up to 20 days of residence in the lungs since it would allow to protect the protein from degradation.

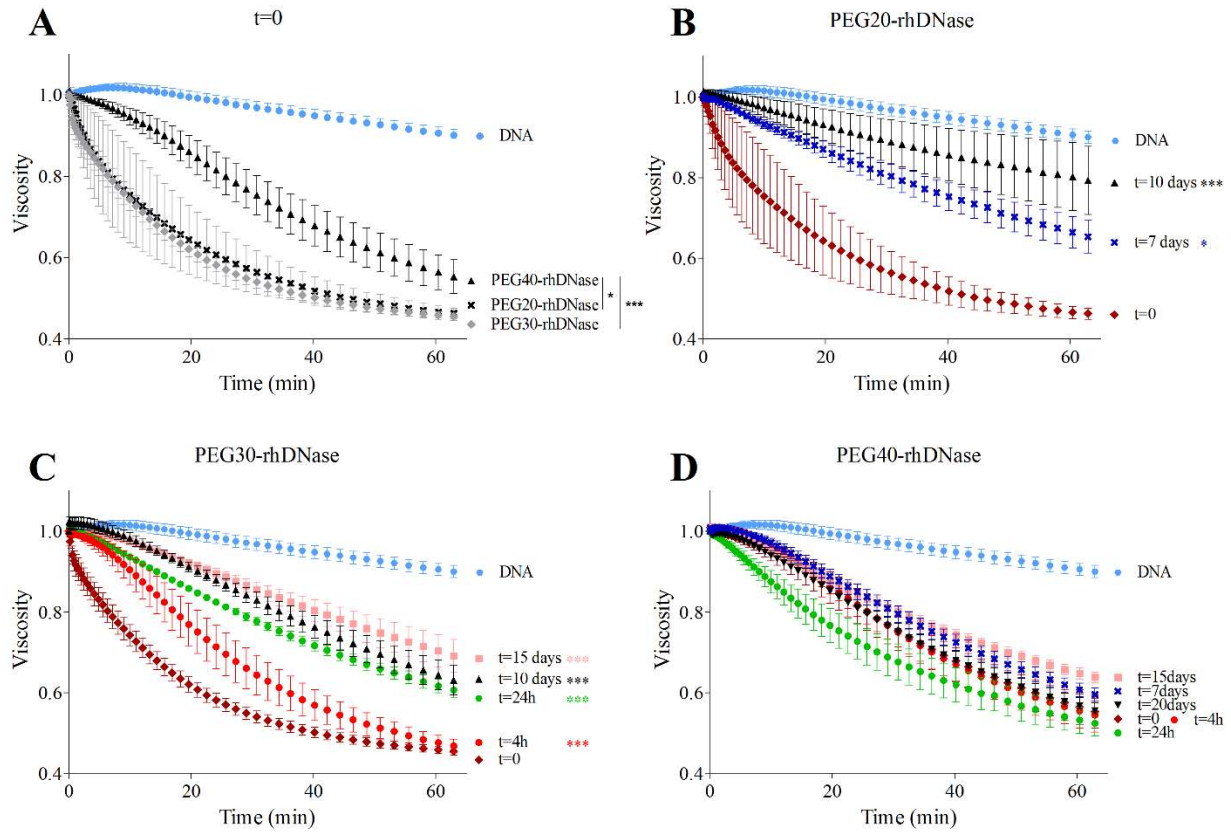


Figure 37: Activity of all biotinylated rhDNases at t=0 (A), PEG20-rhDNase (B), PEG30-rhDNase (C) and PEG40-rhDNase (D) contained in lung supernatant of NMRI mice intratracheally instilled and sacrificed at different time points (n=3 to 10, data were normalized to the time zero DNA for each time point). The concentrations of biotinylated PEGylated rhDNases were normalized to 0.25 $\mu\text{g/mL}$ (8.3 μM) of rhDNase and DNA concentration was of 0.75 mg/mL. PEG was not counted in the concentration of rhDNase used for the rheology assay. Therefore, the different analyses were conducted with the same number of moles of rhDNase. For each rhDNase construct, data were compared to t=0 (two-way ANOVA, Bonferroni post-test, *** $p < 0.001$, * $p < 0.05$).

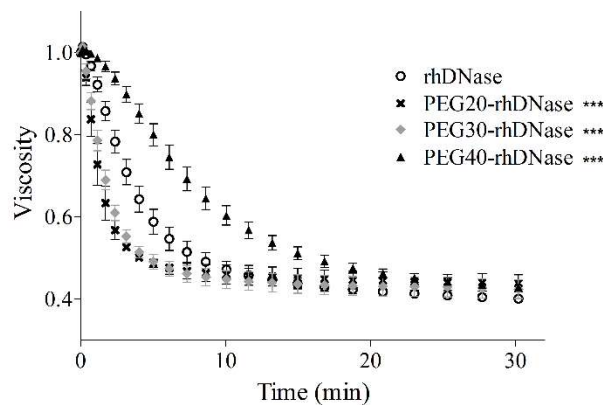


Figure 38: Activity of all biotinylated rhDNases contained in BAL of NMRI mice intratracheally instilled and sacrificed at t=0 (n=3 to 5, data were normalized to the time zero DNA for each time point). The concentrations of free or PEGylated rhDNase were normalized to 0.25 $\mu\text{g/mL}$ (8.3 μM) of rhDNase and DNA concentration was of 0.75 mg/mL. PEG was not counted in the concentration of rhDNase used for the rheology assay. Therefore, the different analyses were conducted with the same number of moles of rhDNase. Data were compared to rhDNase group (two-way ANOVA, Bonferroni post-test, *** $p < 0.001$).

III.2. THERAPEUTIC EFFICACY

III.2.1. Short-term evaluation

We evaluated the ability of conjugated versus non-conjugated rhDNase to reduce DNA content and to resolve pulmonary inflammation in β -ENaC mice. β -ENaC are transgenic mice with airway-specific overexpression of the transepithelial Na^+ channel (ENaC). They mimic CF ion transport deficiency in the lungs. Previous researches showed that β -ENaC mice were characterized by a chronic airway inflammation [393]. We first confirmed that this transgenic model presented a chronic neutrophil inflammation in the lungs (*Supplementary Table S1*). The goal of our short-term therapeutic efficacy study was to compare the *in vivo* activity of five daily doses of rhDNase and a single dose of PEGylated conjugates (**Figure 39**). The results obtained for the disposition study showed that the free protein was cleared from the lungs within 24 h whereas PEG30-rhDNase and PEG40-rhDNase remained for, at least, 15 days (**Figure 36**). Based on these data, we evaluated the therapeutic efficacy 5 days after the delivery of one dose of PEG30-rhDNase or PEG40-rhDNase and compared it to the therapeutic efficacy of rhDNase delivered every day during the same period of time. DNA, cytokines and cellular markers of inflammation were measured in BAL. rhDNase, PEG30-rhDNase and PEG40-rhDNase contents were assessed in BAL and lung homogenate.

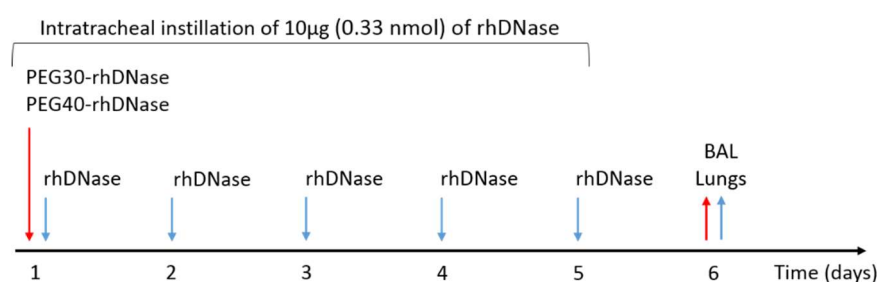


Figure 39: Design of the short-term therapeutic efficacy study in WT and β -ENaC mice. A single dose of PEG30-rhDNase or PEG40-rhDNase was compared with 5 daily doses of rhDNase. Ten μg (0.33 nmol) of rhDNase constructs were administered to WT and β -ENaC mice. The PEG moiety was not counted in the dose delivered so the different mouse groups received the same number of moles of rhDNase.

Quantification of DNA in β -ENaC mice showed activity of all rhDNase constructs (**Figure 40**). 328 ng/mL of DNA was found in PBS group whereas this concentration decreased to 117 ng/mL, 150 ng/mL and 161 ng/mL for rhDNase, PEG30-rhDNase and PEG40-rhDNase groups,

respectively. Thus, similar DNA concentrations were measured in all protein groups. Therefore, one dose of PEG30-rhDNase or PEG40-rhDNase and 5 daily doses of rhDNase showed similar activity on DNA hydrolysis in BAL. Contrary to β -ENaC mice, WT mice contained very low quantities of DNA (less than 80 ng/mL for all the groups). Consequently, no significant difference was observed between control and rhDNase constructs groups (**Figure 40**).

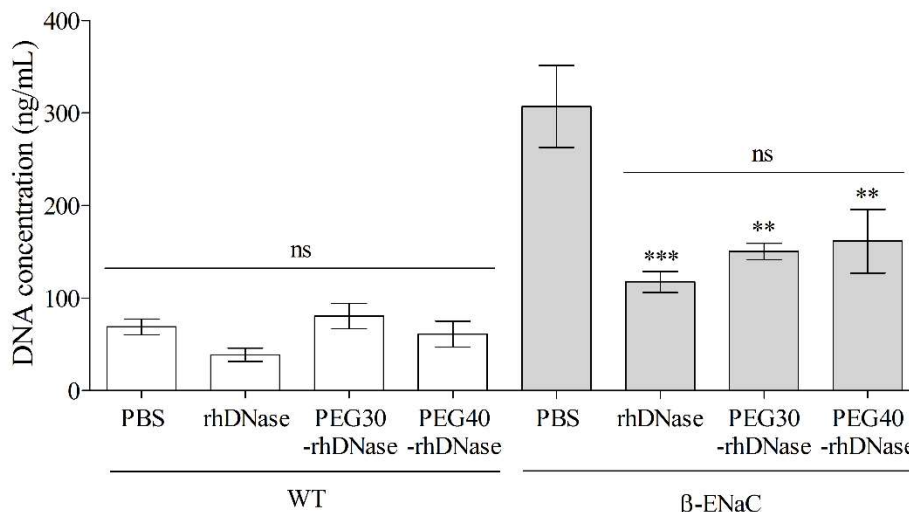


Figure 40: DNA content in BAL of WT and β -ENaC mice following intratracheal delivery of 10 μ g (0.33 nmol) of rhDNase (5 daily doses and sacrifice 24 h following the last dose), PEG30-rhDNase (single dose at day 1 and sacrifice at day 6) or PEG40-rhDNase (single dose at day 1 and sacrifice at day 6), (n=3 to 7). The PEG moiety was not counted in the dose delivered so the different mouse groups received the same number of moles of rhDNase, that was 0.33 nmol. The mice were sacrificed 24 h following the last dose of rhDNase or 5 days following the single dose of PEGylated rhDNases. Data were compared to PBS groups (one-way ANOVA, Dunnett's Multiple Comparison Test). Data expressed as mean values ($\pm$ SEM).

Quantification of pro-inflammatory (IL-6, TNF α , CCL $_2$) cytokine markers in β -ENaC mice showed no significant difference between groups and almost no inflammatory cytokines were measured in WT mice (*Supplementary figure S18A, B and C*). However, rhDNase-treated group showed anti-inflammatory IL-10 cytokine induction compared to the PBS group (*Supplementary figure S18D*).

In addition, no significant difference between groups were observed for quantification of cellular markers (*Supplementary figure S19*). Overall, no activity of rhDNase on inflammatory cells recruitment was observed in β -ENaC mice. Also, data obtained for WT mice showed no neutrophilic inflammation induced by rhDNase.

It has been previously suggested that rhDNase presented indirect anti-inflammatory properties. A long-term clinical trial (over 3 years) reported that the percentage of neutrophils in BAL fluid significantly increased in untreated patients with CF while it remained constant in rhDNase-treated group [399]. The authors then speculated that rhDNase had a positive influence on airway inflammation. Nevertheless, a more recent short-term evaluation (18 months) of rhDNase treatment in patients with early CF lung disease highlighted a significant activity on DNA hydrolysis contained in BAL samples but no effect on reduction of the percentage of neutrophils [400]. Our results are in good agreement with this clinical trial, with effect on DNA concentration and no ability to reduce the neutrophils content in BAL. Taking together all the data reported above, we believe that a long-term therapeutic efficacy may allow to statute on a potential indirect anti-inflammatory effect of PEGylated rhDNase.

rhDNase, PEG30-rhDNase and PEG40-rhDNase were quantified in BAL and lung supernatant of WT and β -ENaC mice of the short-term study (**Figure 41**). This analysis allowed us to evaluate the elimination of the proteins after instillation of 5 daily doses (rhDNase) or of a single dose (PEG30-rhDNase and PEG40-rhDNase). Overall, higher quantities were measured in β -ENaC mice compared to their WT littermates. Twenty-four hours following the last rhDNase dose, 66%, 39% and 100% of rhDNase, PEG30-rhDNase and PEG40-rhDNase were recovered in β -ENaC mice, respectively. WT mice contained 39% of rhDNase whereas 18% of PEG30-rhDNase and 69% of PEG40-rhDNase were measured in their respiratory tract. It is worth mentioning that the content of rhDNase recovered in both β -ENaC and WT mice is expressed as a fraction of the last dose of the protein delivered. The contents of rhDNase in both animal species were not significantly different from the content of PEG30-rhDNase, confirming that a single dose of PEG30-rhDNase presented a similar therapeutic efficacy to 5 daily doses of rhDNase. However, considering PEG40-rhDNase, a significant higher quantity was recovered in β -ENaC mice. Also, comparing the contents of PEG30-rhDNase and PEG40-rhDNase in both WT and β -ENaC mice, the larger the PEG attached to rhDNase, the higher the content recovered in BAL and lung supernatant 5 days after instillation. These findings suggest a higher stability of PEG40-rhDNase than PEG30-rhDNase in the respiratory tract of diseased animals such as β -ENaC mice.

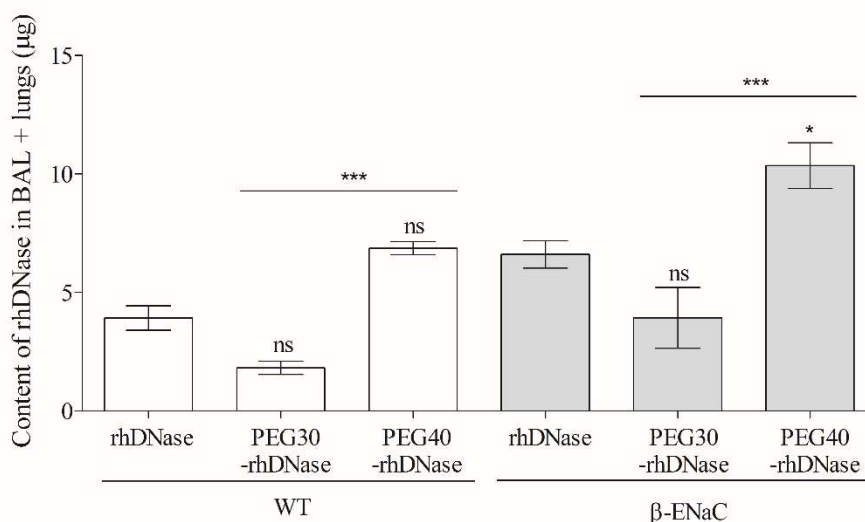


Figure 41: Quantities of rhDNase (5 daily doses and sacrifice 24 h following the last dose), PEG30-rhDNase (single dose at day 1 and sacrifice at day 6) and PEG40-rhDNase (single dose at day 1 and sacrifice at day 6) recovered from the respiratory tract of WT and β -ENaC mice following the intratracheal delivery of a 10 μ g (0.33 nmol) rhDNase dose. PEG moiety was not counted in the dose delivered. The amounts of rhDNase recovered from bronchoalveolar lavage (BAL) and supernatant of lung homogenate (lungs) are expressed in μ g of protein. The PEGylated rhDNase groups were compared to the rhDNase group and to each other (one-way ANOVA, Bonferroni post-test). The data represent the mean values ($\pm$ SEM) of 4 to 6 mice group.

In our *in vivo* disposition study, we demonstrated that rhDNase was cleared from the lungs within 24 h (**Figure 36**). Therefore, we assumed that rhDNase would also be cleared within 24 h in the respiratory tract of WT and β -ENaC mice. However, the results obtained for the therapeutic efficacy study highlighted that 4 to 7 μ g of rhDNase were recovered in the lungs of WT and β -ENaC mice, respectively (**Figure 41**). Several points might explain the difference observed between the two studies. First of all, the doses delivered to healthy and WT or β -ENaC mice were significantly different. In the therapeutic efficacy study, the animals received twice the rhDNase dose delivered during the *in vivo* disposition study (10 μ g *versus* 5 μ g). Consequently, this could have impacted the content of rhDNase recovered in each investigation. Secondly, rhDNase dose was repetitively delivered to WT and β -ENaC mice, which could have resulted in protein accumulation in the lungs. Considering the results obtained for PEG30-rhDNase and PEG40-rhDNase, the *in vivo* disposition study showed similar content for both conjugates 3 days and 7 days post-delivery (**Figure 36**) whereas in our investigation of their therapeutic efficacy, a significant higher quantity was recovered for PEG40-rhDNase than for PEG30-rhDNase 5 days post-delivery (**Figure 41**). Although we

expected similar outcomes for PEGylated rhDNases in both investigations, the findings of the therapeutic efficacy study are consistent with the suggestion that two-arm 40 kDa PEG allows a lower rhDNase uptake by epithelial cells than linear 30 kDa, thereby resulting in higher content recovered in the lungs of WT and β -ENaC mice.

Considering our results obtained for both *in vivo* disposition and therapeutic efficacy studies, biotinylation of rhDNase constructs did not appear to impact the pharmacokinetics of the proteins. Indeed, PEGylation allowed to prolong the residence time of both free and biotin-bound rhDNase. Previous investigations supported our finding. Koussoroplis et al. first demonstrated that the presence of biotinylated anti-IL17A Fab' fragment conjugated with a 40 kDa PEG was sustained for more than 48 h in the lungs of healthy mice [24]. Later, Patil and co-workers confirmed the prolonged residence time of the non-biotinylated counterpart in the pulmonary tissue of instilled mice [304]. Similarly, Freches and colleagues highlighted a presence of 48 h of non-biotinylated PEG40-anti-IL17A Fab' in species larger than the mouse [296]. Consequently, biotinylated or non-biotinylated proteins could either be considered for studying their *in vivo* disposition following pulmonary delivery.

The weight of mice was followed every day of the study to evaluate the safety of rhDNase, PEG30-rhDNase and PEG40-rhDNase (*Supplementary figure S20*). Data obtained showed that all groups, including the PBS control groups, lost between 4% and 6% of their initial weight. T-test analysis between day 1 and day 6 showed that this loss of weight was not significant for all the mice groups. Therefore, no obvious toxicity was observed for the therapeutic efficacy study of rhDNase, PEG30-rhDNase and PEG40-rhDNase. However, all the mice, WT and β -ENaC, appear to suffer from the everyday manipulation.

III.2.2. Long-term evaluation

Based on the results obtained for the short-term therapeutic efficacy study presented above, we weekly administered a dose of PEG30-rhDNase or PEG40-rhDNase (10 μ g or 0.33 nmol rhDNase dose, without counting the PEG moiety in the PEG-rhDNase mass) for 5 weeks and we evaluated their ability to decrease DNA content in the respiratory tract and to resolve pulmonary inflammation in β -ENaC mice. DNA, cytokines and cellular markers of inflammation

were measured in BAL 7 days after the last dose. PEG30-rhDNase and PEG40-rhDNase were also quantified in BAL and lung homogenate at the end of the study.

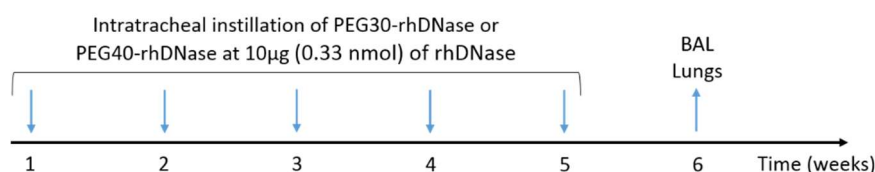


Figure 42: Design of the long-term therapeutic efficacy study in WT and β -ENaC mice. Ten μ g (0.33 nmol) of PEG30-rhDNase or PEG40-rhDNase were weekly administered during 5 weeks and the mice were sacrificed 7 days following the last dose. The PEG moiety was not counted in the dose delivered so the different mouse groups received the same number of moles of rhDNase (0.33 nmol).

Quantification of DNA in β -ENaC mice showed activity of both PEGylated rhDNases (**Figure 43**). 490 ng/mL of DNA was found in PBS group whereas this concentration decreased to 250 ng/mL and 190 ng/mL for PEG30-rhDNase and PEG40-rhDNase groups, respectively. Considering the results obtained for the short-term therapeutic efficacy study (**Figure 40**), higher DNA concentration was recovered in this long-term evaluation for PEG30-rhDNase whereas similar DNA content was found for PEG40-rhDNase. Therefore, we assumed that PEG40-rhDNase presented a better DNA hydrolysis activity than PEG30-rhDNase. However, the statistical analysis of the DNA contents remaining after the long-term study did not demonstrate any difference for both rhDNase conjugates. Overall, the results highlighted that one weekly dose of PEG30-rhDNase and PEG40-rhDNase showed similar activity on DNA hydrolysis in BAL.

Contrary to β -ENaC mice, WT mice contained very low quantities of DNA (less than 90 ng/mL for all the groups; **Figure 43**). These results confirmed the outcomes of the short-term study, in which no significant difference was observed between control and rhDNase constructs groups of WT mice.

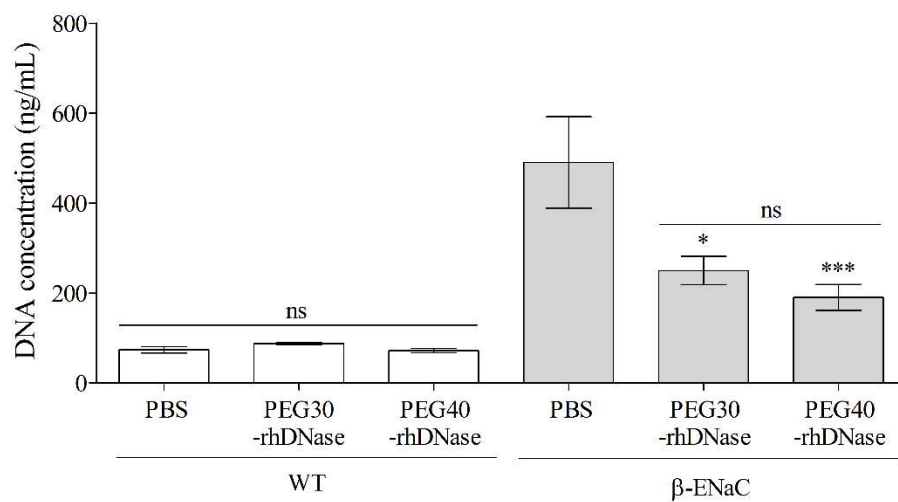


Figure 43: DNA content in WT and β -ENaC mice following intratracheal delivery of five weekly doses of 10 μ g (0.33 nmol) of PEG30-rhDNase or PEG40-rhDNase ($n=4$ to 5). PEG moiety was not counted in the dose delivered so the different mouse groups received the same number of moles of rhDNase, that was 0.33 nmol. The mice were sacrificed 7 days following the last dose of PEG30-rhDNase or PEG40-rhDNase. Data were compared to PBS groups (one-way ANOVA, Dunnett's Multiple Comparison Test). Data expressed as mean values ($\pm$ SEM).

Quantification of pro-inflammatory (IL-6, CCL₂) cytokine markers in β -ENaC mice showed no significant difference between groups (*Supplementary figure S21*). Moreover, no TNF $_{\alpha}$ cytokine was measured in WT mice (data not shown). Similarly, the results obtained did not highlighted any anti-inflammatory IL-10 cytokine induction for all the groups (data not shown).

In addition, no significant difference between groups were observed for quantification of cellular markers (*Supplementary figure S22*). Overall, data obtained for WT mice showed no neutrophilic inflammation induced by rhDNase (*Supplementary figure S22A*). However, considering β -ENaC mice, an apparent decrease of the neutrophils contents in mice treated with either PEG30-rhDNase or PEG40-rhDNase was observed (*Supplementary figure S22B*). Indeed, 25.4% of the total cells recovered from the BAL of the control group were neutrophils whereas only 18.5% and 8.8% were present in PEG30-rhDNase and PEG40-rhDNase groups, respectively. These results therefore suggest a potential indirect anti-inflammatory effect of PEGylated rhDNase, as previously reported by the 3-year clinical trial in patients with CF, mentioned above [399]. Nevertheless, we assume that a therapeutic efficacy study in β -ENaC mice for more than 6 weeks (duration of our long-term evaluation) would allow to further

confirm the apparent effect of rhDNase constructs on the reduction of neutrophils recruitment in CF lung disease.

PEG30-rhDNase and PEG40-rhDNase were quantified in BAL and lung supernatant of WT and β -ENaC mice 7 days following delivery of the last weekly dose (**Figure 44**). Overall, similar quantities were measured in WT and β -ENaC mice. Comparing with the results obtained for the short-term evaluation, higher quantities of PEG30-rhDNase were recovered in both animal species. This could be explained by the fact that repeated doses of the conjugate were delivered in this long-term study whereas a single dose was delivered in the short-term evaluation. Considering PEG40-rhDNase, slightly lower contents were recovered in the long-term study. Overall, the quantities of both PEG30-rhDNase and PEG40-rhDNase recovered in this 6 weeks evaluation were of the same order of magnitude as that of the 6 days experiment. Consequently, we conclude that a chronic administration of both PEGylated rhDNases did not translate into any apparent accumulation of these conjugates in the lungs of instilled mice.

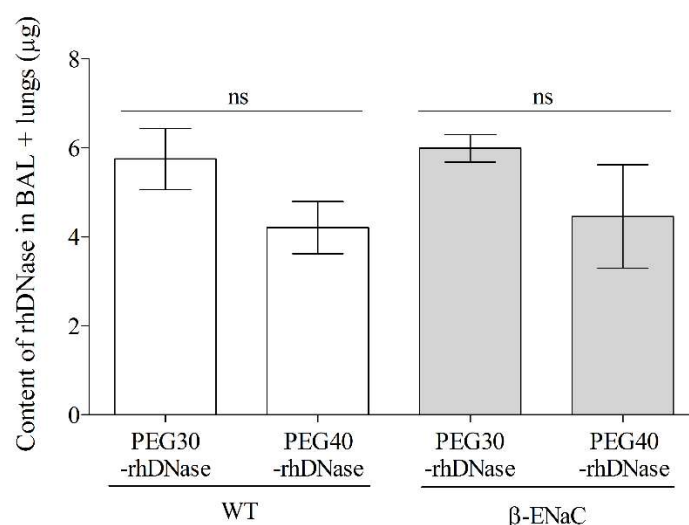


Figure 44: Quantities of PEG30-rhDNase and PEG40-rhDNase recovered from the respiratory tract of WT and β -ENaC mice following the intratracheal delivery of five weekly doses of 10 μ g (0.33 nmol) of rhDNase (n=4 to 5). PEG moiety was not counted in the dose delivered so the different mouse groups received the same number of moles of rhDNase, that was 0.33 nmol. The amounts of rhDNase recovered from bronchoalveolar lavage (BAL) and supernatant of lung homogenate (lungs) are expressed in μ g of protein. The different groups were compared to each other (one-way ANOVA, Bonferroni post-test). The data represent the mean values ($\pm$ SEM) of 4 to 5 mice group.

The weight of mice was followed throughout the study to evaluate the safety of PEG30-rhDNase and PEG40-rhDNase (*Supplementary figure S23*). Data obtained showed that all groups gained between 2% and 11% of their initial weight at the end of the 6 weeks experiment. Therefore, no toxicity was observed for the long-term therapeutic efficacy study of PEG30-rhDNase and PEG40-rhDNase and the mice did not appear to suffer from the weekly manipulation.

III.3. QUANTIFICATION OF PEG40-RITC

We analyzed the fate of PEG40-RITC in the lungs following intratracheal instillation in NMRI mice. PEG is non-biodegradable and might potentially accumulate in the lungs. The fate of the PEG in the respiratory tract of mice was studied by quantification of the PEG40-RITC fluorescence in nasal lavage, BAL and lung supernatant at pre-determined times after pulmonary administration. Uptake of PEG40-RITC by alveolar macrophages (AM) was also studied.

PEG40-RITC was cleared from the nasal cavity within 24 h (*Supplementary Figure S24*). **Figure 45** presents the content of PEG40-RITC in the lungs and AM over time. The lungs retained the PEGylated rhDNase better than free PEG. Twenty days post-delivery, PEG40-RITC was cleared from the lungs since the dose recovered was below the background of control mice (**Figure 45A**). At the same time point, more than 50% of PEG40-rhDNase remained present in the lungs. Therefore, conjugation of rhDNase to 40 kDa PEG sustained the presence of the enzyme within the lungs for longer period of time than the PEG alone.

Uptake of PEG40-RITC by AM showed that endocytosis was maximal 3 days post-delivery (**Figure 45B**). However, it represented only 1.6% of the delivered dose and the dose fraction taken up was progressively cleared from AM. This finding further supports that two-arm 40 kDa PEG conjugation to rhDNase would allow to reduce its uptake by cells.

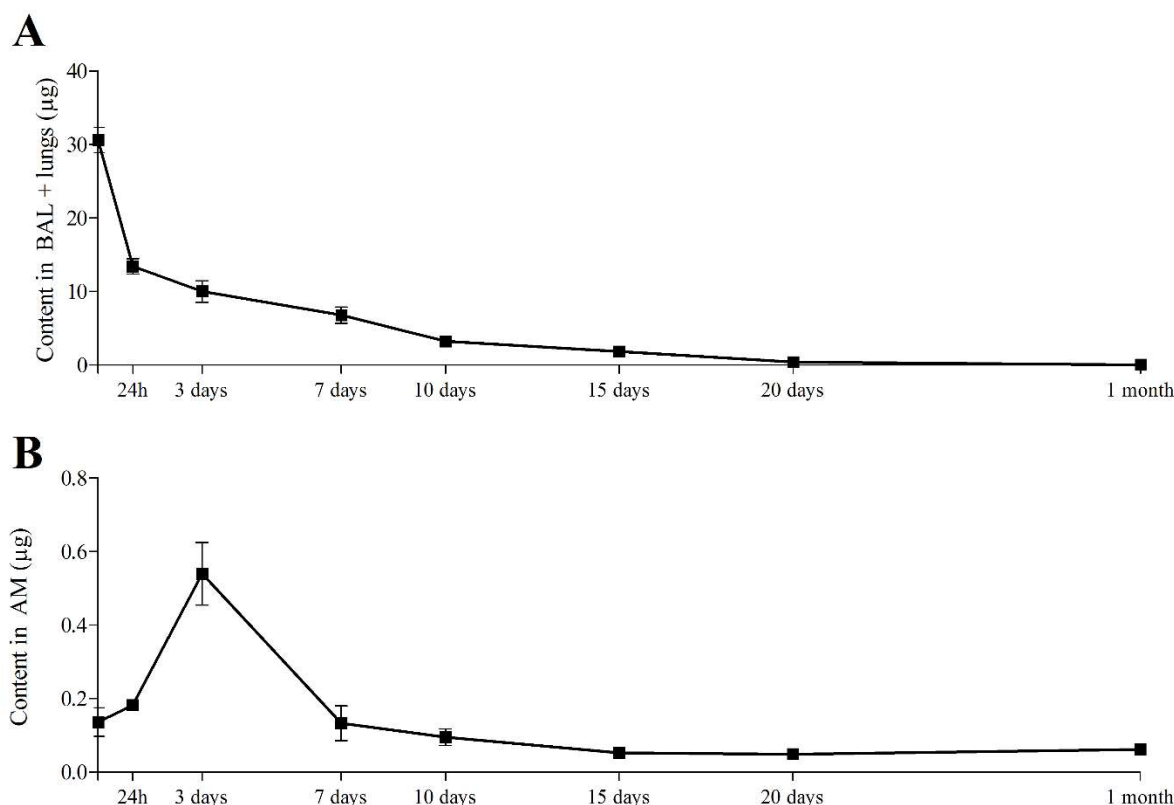


Figure 45: Quantities of PEG40-RITC recovered from the respiratory tract (A) and AM (B) of NMRI mice at different times following the intratracheal delivery of 80 µg of PEG (n=5, mean ± SEM). The background was subtracted for each time point. The lungs retained PEG40-RITC for more than 15 days. Only 0.1% of the administered dose was recovered in AM after 15 days. The PEG was not detectable after 20 days in the lungs.

III.4. STABILITY TO NEBULIZATION

Since rhDNase is daily delivered to the lungs of patients with CF by inhalation, we evaluated the stability of PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase to nebulization. Air-jet nebulizers were the first devices approved by the Food and Drug Administration for pulmonary delivery of rhDNase [208]. Such nebulizers, powered by compressed air, create droplets of drug formulated in aqueous solution or suspension [401]. Therefore, the process used to generate the droplets results in exposure of the protein to stresses such as high shear rates and air-water interfaces [265]. Thus, nebulization might cause protein denaturation and aggregation. Droplets that are not sufficiently small impact the jet breeze wall and return to the solution, so that the protein may undergo several nebulization cycles.

The activities of rhDNase and PEGylated conjugates after nebulization with air-jet nebulizer were then determined using a methyl green assay. Results obtained highlighted that the four

rhDNases retained the full activity following nebulization (**Table 8**). The effective concentration at 50% (EC50) varied between rhDNase constructs since a variability existed between two independent assays. Although all rhDNases were tested at the same set of concentrations, the range of EC50 estimates was large [402]. EC50 corresponds to the midway between the estimates of the top and bottom of the curve, which may vary between assays. Consequently, for comparison of EC50 between two samples, analysis on the same plate was mandatory. The potential aggregation of PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase was monitored by SEC and compared to data obtained for rhDNase. SEC analysis prior to nebulization showed peak of rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase at retention volumes of 17.9 mL, 15.1 mL, 14.2 mL and 13.6 mL, respectively (**Figure 46A, C, E and G**). After nebulization, no additional peak (with lower retention volume than that of non-nebulized rhDNases) was observed on SEC chromatograms for all proteins (**Figure 46B, D, F and H**). Therefore, no aggregates were formed. No physical degradation of rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase was observed following nebulization.

The results obtained for PEGylated conjugates following nebulization are in good agreement with the statement that PEGylation generally preserves the protein structure and reduces or eliminates protein aggregation [371]. In case of rhDNase, its stability throughout nebulization has already been demonstrated since it is administered to patients with CF using this delivery method. The goal of our evaluation of PEGylated rhDNase behavior following nebulization was to firmly confirm that the PEGylated protein could be delivered to patients with CF using the same administration method as for rhDNase.

Table 8: Evaluation of the stability of rhDNase and PEGylated conjugates to nebulization. All the rhDNase constructs were nebulized at a concentration of 0.15 mg/mL in rhDNase (5 μ M; the PEG moiety was not counted in the concentration of PEG-rhDNase) in 150 mM NaCl and 1 mM dihydrate CaCl₂. Hydrolysis of DNA by nebulized rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase was assessed at increasing concentrations of the enzymes (n=3). DNA was complexed with methyl green and DNA hydrolysis produced unbound methyl green and a decrease of the absorbance of the solution at 650 nm after 6 h of incubation at 37°C. The effective concentrations at 50% (EC50) before and after nebulization are reported below (mean $\pm$ SEM). The data were compared by unpaired t-test with no difference seen for all proteins.

Protein	EC50 before nebulization (ng/mL)	EC50 after nebulization (ng/mL)
rhDNase	16.0 $\pm$ 2.6	16.6 $\pm$ 2.4
PEG20-rhDNase	28.2 $\pm$ 3.7	26.8 $\pm$ 3.2
PEG30-rhDNase	36.5 $\pm$ 10.5	37.5 $\pm$ 11.4
PEG40-rhDNase	23.6 $\pm$ 2.0	22.6 $\pm$ 1.9

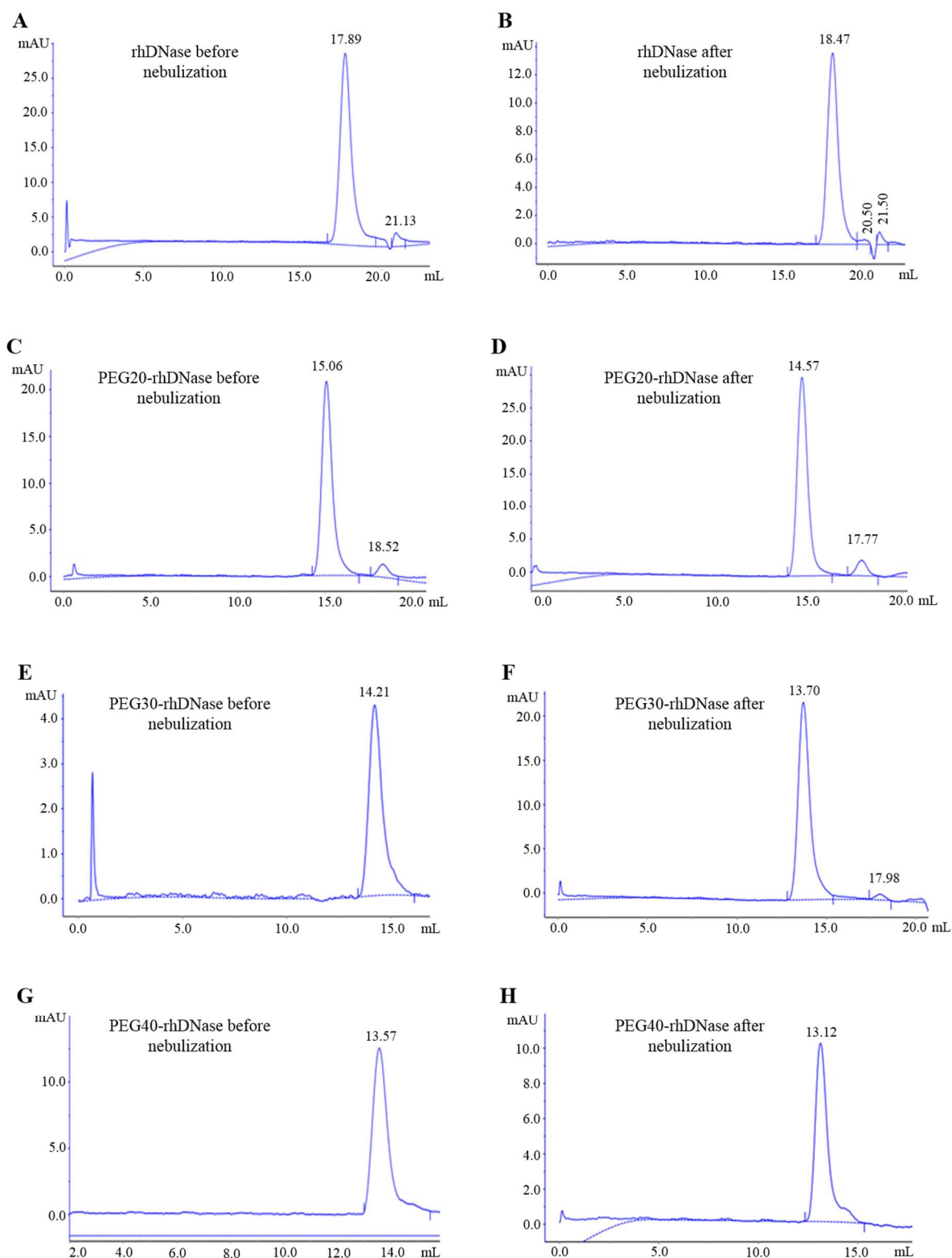


Figure 46: Size-exclusion chromatography (SEC) analysis of aggregates formation following nebulization of rhDNase (**A.** before stress and **B.** after stress), PEG20-rhDNase (**C.** before stress and **D.** after stress), PEG30-rhDNase (**E.** before stress and **F.** after stress) and PEG40-rhDNase (**G.** before stress and **H.** after stress). All the rhDNase constructs were nebulized at a concentration of 0.15 mg/mL in rhDNase (5 μ M; the PEG moiety was not counted in the concentration) in 150 mM NaCl and 1 mM dihydrate CaCl_2 . All the proteins showed a similar chromatogram and no aggregates were formed.

IV. CONCLUSION

PEGylation of rhDNase allowed to sustain its presence within the lungs for more than 15 days while the non-conjugated protein was cleared within a day. The effect was dependent on the PEG size: the bigger the PEG, the more prolonged rhDNase residency within the lungs. In addition, PEG20-rhDNase and PEG30-rhDNase showed progressive loss of their enzymatic activity whereas PEG40-rhDNase remained active after 20 days of residence in the respiratory tract of healthy mice. We further demonstrated that one dose of PEG30-rhDNase or PEG40-rhDNase and five daily doses of rhDNase showed similar DNA hydrolysis in β -ENaC mice. Furthermore, a weekly administration of both PEGylated rhDNases confirmed their efficacy to clear DNA from the respiratory tract and did not result in any apparent accumulation of the conjugates in the lungs over 6 weeks. These findings allow to firmly conclude on the reduced dosing frequency that PEGylated rhDNase could provide to patients with CF lung disease. Finally, PEGylated rhDNases showed a stability to nebulization comparable to that of rhDNase, highlighting that nebulization could be used as a delivery method for potential future clinical study of PEGylated rhDNase.

CHAPTER VI. DISCUSSION AND CONCLUSION

I. MAIN ACHIEVEMENTS OF THE THESIS

Morbi-mortality in CF is related to bronchial obstruction by a hyperviscous mucus. rhDNase is the reference mucolytic agent for the treatment of CF lung disease. However, rhDNase is rapidly cleared from the lungs, which denotes its daily administration and thus leads to high treatment burden. The present project aimed at developing a new version of rhDNase conferring a sustained presence within the lungs and an improved activity through the PEGylation of the enzyme.

rhDNase was mono-PEGylated on its N-terminal leucine residue by reductive alkylation at acidic pH using aldehyde-functionalized PEGs of varying size: linear 20 kDa, linear 30 kDa and two-arm 40 kDa polymers. These polymers were selected from large PEGs already used clinically [25–27]. The enzymatic activity of the PEGylated conjugates was analyzed *in vitro* on DNA solution and showed to be identical to that of the non-conjugated protein. The PEGylated rhDNases were also analyzed for their degree and site of PEGylation as well as for their secondary and tertiary structures. Results obtained confirmed that rhDNase was mono-PEGylated mainly on its N-terminal residue and that its conformation was not altered after PEGylation.

rhDNase and its PEGylated counterparts were exposed to stresses, such as a chemical denaturant and elevated temperatures, to assess the impact of PEGylation on the stability of the protein. Exposure to guanidinium chloride highlighted a destabilization of the rhDNase structure by PEGylation. The destabilization was dependent on the PEG size: the bigger the PEG, the lower the stability. By contrast, thermal denaturation demonstrated that rhDNase and the 40 kDa PEG-conjugate were stable to the same extent to high temperatures. This preliminary evaluation also showed that 40 kDa PEG attachment on rhDNase reduced the formation of protein aggregates while exposing the protein to high temperatures.

The clinical evaluation of rhDNase in CF sputa indicated that rhDNase PEGylated with 40 kDa PEG and the non-conjugated enzyme induced a similar decrease of elastic and viscous moduli of CF sputa. This confirmed that PEGylation of rhDNase preserved the *ex vivo* enzymatic activity of the protein, independently of DNA, G-actin and ions contained in the sputa. In addition, results highlighted that the more purulent the sputum, the higher the DNA and G-

actin concentrations. Finally, PEGylation greatly increased the stability of rhDNase in CF respiratory secretions.

In vivo disposition studies demonstrated that all PEGylated products sustained their residence within the lungs for more than 15 days while rhDNase was cleared within a day. The larger the PEG size, the more prolonged the retention of the enzyme in the lungs. Conjugation to 40 kDa PEG led to the preservation of the *in vivo* protein enzymatic activity in the lungs overtime.

A short-term therapeutic efficacy study in β -ENaC mice demonstrated similar enzymatic activity on DNA hydrolysis of one dose of PEGylated rhDNase and 5 daily doses of the non-conjugated protein. This study also indicated that 40 kDa PEG conferred a more prolonged residency in the lungs to rhDNase over 30 kDa PEG. However, no direct anti-inflammatory effects of all rhDNase versions were observed in this study. In addition, in a more prolonged therapeutic efficacy study over 6 weeks, a weekly administration of rhDNase PEGylated with either 30 kDa or 40 kDa PEG confirmed its efficacy on DNA hydrolysis in the bronchoalveolar lavage and did not result in any apparent accumulation of both conjugates in the lungs.

Finally, nebulization of PEGylated rhDNases did not induce loss of enzymatic activity or aggregation of the protein.

Therefore, the main contribution of this thesis lies within the impressive prolongation of rhDNase residence time within the lungs following pulmonary delivery, thanks to the use of PEGylation as a modification process. In the long-term, the impact of this study would be a reduced therapy burden and an improved quality of life, associated with a better compliance for patients with CF.

II. DISCUSSION OF THE WORK

II.1. SYNTHESIS OF PEGYLATED rhDNASES WITH SUSTAINED ENZYMATIC ACTIVITY

PEGylation of rhDNase was achieved with high molecular weight PEGs: 20 kDa, 30 kDa and 40 kDa. The objective was to obtain a maximum PEG effect on a) extension of rhDNase pulmonary residency and b) on protection towards the approach of local proteases as well as of G-actin, which is a potent inhibitor of rhDNase. These PEG molecular weights were also chosen to avoid any PEG accumulation in the body. Indeed, following intravenous delivery, PEG is eliminated unchanged in the urine as long as its molecular weight is less than 40 kDa [403].

Following the PEGylation reaction, un-reacted rhDNase, mono- and di-PEGylated products were present and observed during the anion-exchange chromatography process. Prolonged contact (i.e., 96 h reaction) between PEG, rhDNase and the reducing agent might explain the conjugations obtained.

The yields of mono-PEGylation were 45%, 48% and 61% for PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase, respectively. Lee et al. achieved mono-PEGylation of Interferon $\alpha 2a$ with yields ranging from 50% to 65%, depending on the molecular weight of the linear PEG aldehyde used for conjugation [404]. The authors highlighted that lower yields were achieved by increasing the PEG length. Moosmann and co-workers later confirmed that the longer the linear PEG chain, the lower the yield of PEGylated lysozyme [405]. However, our results obtained for linear 20 kDa and linear 30 kDa PEG conjugation to rhDNase did not support this finding. Indeed, statistical analysis of the yields of both rhDNase conjugates showed no difference (data not shown). However, the yield of mono-PEGylated rhDNase with two-arm 40 kDa PEG was significantly higher than that of both linear polymers. We therefore speculated that PEGs with various structures might present different reactivity toward protein attachment. Regarding the structure of both linear and two-arm PEG aldehydes used for the PEGylation of rhDNase (**Figure 47**), the presence of a carbamate in the structure of the 40 kDa PEG aldehyde (**Figure 47B**) might have an inductive effect on the polymer reactivity. However, the carbamate is located at four covalent bonds of the aldehyde function. Consequently, the most convincing argument might result from the branched form of the 40 kDa PEG. Indeed, it

would create a shielding effect on the protein surface [406], thus preventing a second PEG attachment.



Figure 47: Structure of (A) linear 20 kDa and 30 kDa PEG aldehydes and (B) two-arm 40 kDa PEG aldehyde used for PEGylation of rhDNase (from <https://www.nofamerica.com>)

Moosman et al. analyzed the PEGylation process involving PEG aldehydes and proposed a reaction kinetic model for the formation of mono-, di- and multi-PEGylated products [405]. The model assumes that PEG and protein first react to form a mono-PEGylated conjugate, then react with a second PEG aldehyde to form a di-PEGylated product, and so on. PEGylation of rhDNase with two-arm 40 kDa PEG showed lower di-PEGylation than the two other PEG conjugations. This might also have resulted from the shielding effect of the branched form of the PEG, which would have limited the accessibility of another PEG molecule, as assumed by the model presented.

II.2. SUSTAINED AND REDUCED STABILITY OF PEGYLATED CONJUGATES TOWARD STRESS-INDUCED DENATURATION

In the evaluation of rhDNase stability towards GdmCl-induced unfolding, we showed that destabilization of rhDNase structure by PEGylation was dependent on the PEG size: the bigger the PEG, the lower the stability. It has been reported that PEG attachment can modify the solvent accessible surface area of proteins [347]. A decrease in solvent accessible surface area results in a more compact and less dynamic folded conformation [347]. In addition, water molecules stabilize the protein structure in participating in hydrogen bonds [407]. Therefore, we assume that large PEGs of varying size and form (i.e. linear 20 kDa, linear 30 kDa and two-arm 40 kDa PEGs) may lead to different levels of solvent accessible surface area, thereby resulting in decreased stability toward GdmCl-induced unfolding.

Transitions from native to denatured state of rhDNase and 40 kDa PEG-conjugate showed different preliminary outcomes for GdmCl-stress induced unfolding and thermal denaturation. Other investigations have also shown different protein unfolding profiles following chemical and thermal denaturation. Anumalla and co-workers reported that ribonuclease A and α -lactalbumin showed increased thermal stability following glutamate addition but displayed different behaviors when exposed to GdmCl [408]. Moreover, the stability of proteins relies upon the balance between electrostatic, hydrophobic, Van der Waals and hydrogen bonding interactions [409]. It has been reported that the ionic nature of GdmCl can result in disruption of the protein electrostatic interactions [410]. Rupture in hydrogen bonds and hydrophobic interactions are considered to contribute to the unfolding reaction induced by temperature elevation [411,412]. Therefore, the nature of interactions involved in GdmCl-induced unfolding and thermal denaturation may explain why rhDNase and PEG40-rhDNase are not equally sensitive to both stresses.

An increased stability is beneficial for product development and storage, where proteins can be subjected to several denaturing stresses [413,414]. As previously mentioned, exposure of rhDNase and its PEGylated counterparts to GdmCl highlighted rhDNase destabilization induced by large PEG chain attachment. However, we evaluated the enzymatic activity of rhDNase conjugated to 20 kDa, 30 kDa and 40 kDa PEGs up to one year of storage at 4°C (data not shown), corresponding to the storage temperature of commercialized rhDNase [207]. All PEGylated rhDNases preserved their activity over one year, thereby highlighting that their stability was sustained during this period of time. Consequently, we assume that development and storage of the PEGylated rhDNase would not encounter any issues.

II.3. PRESERVED RHDNASE MUCOLYTIC ACTIVITY IN CF SPUTA FOLLOWING PEGYLATION

Although rhDNase activity in CF sputa has already been evaluated [190], the investigation presented in **Chapter IV** is the first to integrate the purulence parameter. We demonstrated a similar enzymatic activity of rhDNase on purulent and muco-purulent CF sputa. In the 1990s, clinical investigations reported the efficacy of rhDNase therapy in patients with moderate and stable lung disease as well as in individuals with CF suffering from a severe phenotype [208,258]. With purulence of the pulmonary secretions being an indicator of the severity of the disease, our clinical investigation allowed to support the outcomes of the clinical trials

presented above. Also, our results confirmed the recommendations associated with the use of rhDNase therapy in the management of CF lung disease, that are its chronic use in individuals with CF, 6 years of age and older, with mild to severe lung disease [3].

The enzymatic activity of PEGylated rhDNases has been analyzed *in vitro* in the absence and in the presence of G-actin and was shown to be identical to that of the free protein. We expected an improved enzymatic activity of the PEGylated conjugates in the presence of G-actin compared to rhDNase. However, the PEGylated products were inhibited by G-actin to the same extent as rhDNase, indicating that the actin-binding site and N-terminus were not sufficiently close to each other.

Ex vivo evaluation of CF sputa highlighted that no correlation was established between the concentration of G-actin (from 0 to 132 µg/mL) and the activity of rhDNase, PEGylated or not. Considering *in vitro* results presented above, rhDNase enzymatic activity *ex vivo* should have depended on the G-actin content, with the lower the G-actin concentration in CF sputa, the higher the DNA hydrolysis. However, decrease in sputum viscoelasticity following the addition of rhDNase or of the PEGylated conjugate did not differ between samples with low and high actin concentration. In our *ex vivo* evaluation, rhDNase activity was measured one hour following incubation in CF sputa, containing various G-actin concentrations. *In vitro* results were obtained over 30 min following the addition of rhDNase to a solution containing DNA and actin. Since *in vitro* experiments highlighted a partial actin inhibition of rhDNase, measurement of rhDNase activity up to one hour might have resulted in maximum *in vitro* activity. Therefore, we hypothesize that rhDNase activity in CF sputa might have reached its maximal activity, thereby masking any G-actin inhibition.

The higher quantity of rhDNase conjugated with 40 kDa PEG measured in CF sputa over free rhDNase suggested that PEGylation increased the resistance of rhDNase toward degradation in respiratory secretions. Following a daily administration of rhDNase, Lazarus and Wagener reported a concentration of 3 µg/mL measured in CF sputum immediately after delivery [5]. Two hours post-delivery, only 0.6 µg/mL of rhDNase remained in the samples, accounting for 20% of the deposited dose. In our investigation, after incubation in CF sputa, only 0.3% and 6.6% of the initial doses of rhDNase and PEGylated rhDNase were measured, respectively. Therefore, we observed a net loss of both proteins. Considering that rhDNase and its

conjugated counterpart were in contact with proteases of CF sputa for a long period of time (i.e. including one hour incubation in CF sputa, rheology measurement, sample collection, freezing, thawing and extraction), we suggest that the extended incubation time could have caused the protein loss. We also hypothesize that the extraction process employed for further rhDNase quantification could have impacted the quantities recovered.

II.4. EXTENDED LUNG RETENTION OF PEGYLATED rhDNASES

This study showed that conjugation of large PEG chains to rhDNase significantly increased its residence within the lungs compared to the non-conjugated protein. We hypothesized that decreased uptake by alveolar macrophages and epithelial cells, enhanced resistance towards proteolysis, reduced transport across the lung epithelium and adhesiveness to mucus [304,371] could explain the extended lung retention. Protein uptake by alveolar macrophages was not investigated and thus did not allow to statute on this hypothetic mechanism. Patil et al. highlighted that PEGylation of anti-IL17A and anti-IL13 Fab' fragments reduced the *in vitro* uptake of the constructs by epithelial cells [304]. The authors also showed that the effects depended on the PEG size, where the bigger the PEG, the lower the uptake by the cells. Consequently, we hypothesize that a reduced uptake by epithelial cells would contribute to the extended lung retention of PEGylated rhDNases. Protein stability towards proteases contained in BAL and lung supernatant of healthy mice was evaluated (data not shown). However, controversial results did not permit to firmly conclude on the enhanced resistance towards proteolysis conferred by PEG attachment to rhDNase. We are currently exposing rhDNase constructs to pure serine proteases found in CF lungs [148]. Ongoing data suggest that the presence of 1 mM CaCl_2 in rhDNase buffer inhibits the protease, which confers a protection towards proteolysis (data not shown). rhDNase is poorly transported across the lung epithelium [398]. In addition, we demonstrated that PEGylated rhDNases did not impact its systemic exposure (data not shown). Therefore, the sustained retention of PEGylated rhDNases was not dictated by a reduced absorption from the lungs into the bloodstream. rhDNase PEGylated with two-arm 40 kDa PEG significantly improved the residence time of the protein within the lungs over both linear 20 kDa and 30 kDa PEG chain attachment. Considering that PEG is mucoadhesive [299,300], we assume that large PEGs would permit

strong entrapment with cell-anchored mucins [174,415], thereby contributing to sustained retention time of rhDNase in the lungs.

PEGylation is one of the delivery strategies available for sustained drug release in the lungs following pulmonary delivery. Carriers have also been evaluated for prolonged residence time and progressive release of drugs within the lungs. Liu et al. instilled liposomes containing insulin into rat lungs and reported a sustained release of 6 h [416]. Edwards and co-workers investigated the release of testosterone loaded in porous particles [417]. Testosterone encapsulation allowed to sustain its presence in blood for 12 h to 24 h following pulmonary delivery in rats. Gill et al. intratracheally administered PEG-lipid micelles of paclitaxel in rats. They showed that the micelles better retained paclitaxel within the lungs *in vivo* than free paclitaxel in solution [418]. However, 12 h post-delivery, 54% of the dose of paclitaxel loaded micelles were cleared from the lungs compared to levels obtained at one hour. Li and co-workers delivered solid-lipid nanoparticles containing thymopentin to the lungs of rats. The authors showed a sustained thymopentin plasma concentration over 48 h, whereas free thymopentin was cleared within 10 h [419]. However, less than 10% of the maximal dose were still present at 48 h. Kaminska et al. evaluated the sustained residence of doxorubicin-conjugated dendrimer following pulmonary delivery to rats [420]. Free doxorubicin was rapidly eliminated from the lungs. The authors showed that 60% of the intratracheal dose of the dendrimer was cleared within a day and that only 15% remained after a week. Comparing the residence time of the encapsulated drugs presented in these studies with that of PEGylated rhDNase, PEG attachment to rhDNase greatly improved its presence within the lungs. Fifteen days post-delivery, 33% to 63% of the conjugates remained in the respiratory tract of instilled mice. Although the encapsulation strategies allow to control the drug release, they would not be as efficient as PEGylation for rhDNase. In addition, encapsulation of quantities as high as 2.5 mg (daily dose of rhDNase inhaled by patients with CF [207]) would be challenging considering the quantity of excipients that would be required (1% encapsulated for 99% of excipients).

A 3-year clinical trial suggested that rhDNase presented indirect anti-inflammatory properties [399,421]. Consequently, we evaluated the ability of rhDNase and its conjugates with 30 kDa and 40 kDa PEGs to resolve the pulmonary inflammation observed in β -ENaC mice over 6 days. However, no effect of all rhDNases on inflammatory cellular markers was observed. Zhou, Mall

and co-workers showed an anti-inflammatory response of amiloride in the early stages of life of β -ENaC mice, when the pulmonary phenotype was not yet well established [422]. Also, the authors evaluated the amiloride treatment over 13 to 14 days on homozygous mice for over-expression of the β -ENaC subunit. In our investigation, rhDNase therapy was conducted during 5 days in older heterozygous mice. Therefore, all the differences observed between these two studies might explain the absence of rhDNase effect on pulmonary inflammation in β -ENaC mice. We then evaluated the ability of both 30 kDa and 40 kDa PEG conjugates to resolve pulmonary inflammation in β -ENaC mice over 6 weeks. An apparent decrease of the neutrophils contents in mice treated with PEGylated rhDNases was observed. Therefore, we believe that a more prolonged therapeutic efficacy (several months) might allow to firmly statute on a potential indirect anti-inflammatory effect of PEGylated rhDNase.

III. CONCLUSION

The present thesis demonstrates that following PEGylation of rhDNase with high molecular weight PEGs, rhDNase sustains its enzymatic activity both *in vitro* on pure DNA solution and *ex vivo* in CF sputa. It also shows that PEGylation preserves rhDNase stability towards thermal denaturation, while destabilizing the protein when exposed to a denaturing agent. Furthermore, this work highlights that PEG attachment on rhDNase greatly prolongs its pulmonary residence time after administration to the lungs. Finally, our investigation suggests that rhDNase PEGylated with linear 30 kDa or two-arm 40 kDa PEG has a superior therapeutic potency in a CF lung inflammation model than the unconjugated protein, due to its improved local presence as well as its potential stability towards proteolysis.

The aim of the project was to develop a long-acting version of rhDNase for CF lung disease treatment. In the long-term, the development of a PEGylated rhDNase would allow to lower the administration frequency and thus improve the patient compliance. At this time of the project development, if we would have to choose one of the three conjugates for further evaluation, our reasoning would be based on the following points:

- Considering the *in vivo* residence time, rhDNase conjugated with two-arm 40 kDa PEG would be the best option since it remains active for 20 days in the lungs. As previously mentioned, the ultimate goal would be to take rhDNase at longer intervals of time than the current daily administration. Therefore, the longer the retention time within the lungs, the more interesting the rhDNase conjugate for further development.
- Hemoptysis (cough containing blood) has been reported as a pulmonary complication of CF. Although rhDNase has not been identified as a risk factor for hemoptysis [423–425], some patients experienced this complication following administration of rhDNase [426], independently of the severity of the lung disease. Therefore, any clinician would not prescribe a PEGylated version of rhDNase with sustained activity and residence time for more than a week.
- Consequently, rhDNase conjugated with linear 30 kDa PEG would be to consider for the further development. Its residence time in the lungs of healthy mice is shorter than that of rhDNase PEGylated with two-arm 40 kDa PEG (15 days). Also, its therapeutic

potency is similar to that of 40 kDa PEG conjugate and does not accumulate in the lungs following chronic administration.

- No decision could be made regarding the development of rhDNase conjugated with linear 20 kDa PEG since its evaluation in the β -ENaC mouse (murine model of CF pulmonary disease) has not been conducted.

Finally, the extrapolation of all the findings presented above would allow to consider rhDNase PEGylated with linear 30 kDa PEG for a weekly administration in patients with CF. rhDNase conjugated to 20 kDa PEG might also be considered providing that results on therapeutic efficacy studies are obtained.

IV. PERSPECTIVES

The use of PEGylation as a modification process to sustain the presence of rhDNase in the lungs is a promising strategy that deserves further investigation. Additional efforts should be made to a) address the impact of PEGylation with a linear 40 kDa PEG on rhDNase stability, activity and residence time, b) assess the tolerability of PEGylated rhDNase in animal species larger than the mouse, and c) develop a PEGylated version of an actin-resistant rhDNase.

IV.1. IMPACT OF PEGYLATION WITH A LINEAR 40 kDa PEG ON RHDNASE STABILITY, ACTIVITY AND RESIDENCE TIME

In our work, we investigated the stability of rhDNase and its PEGylated counterparts towards GdmCl-stress induced denaturation. Our findings highlighted that PEG affected rhDNase stability and that the higher the PEG molecular weight, the lower the stability. However, linear 20 kDa and 30 kDa PEGs were compared to a two-arm 40 kDa PEG. Therefore, it would be interesting to evaluate the stability of a linear 40 kDa PEG in order to firmly confirm the relationship between PEG molecular weight and architecture and GdmCl-stress induced denaturation. To our best knowledge, only one investigation has already evaluated the influence of PEG structure on protein stability [427].

The chemistry to attach linear 40 kDa PEG to rhDNase would be the same as the one employed for rhDNase modification described in this work.

Determination of *in vitro* enzymatic activity of linear 40 kDa PEG-rhDNase conjugate as well as its *in vivo* residence time in mice could also be interesting. These results would thus complement the evaluation of the influence of PEG structure on stability of rhDNase.

IV.2. TOLERABILITY OF PEGYLATED RHDNASE IN ANIMAL SPECIES LARGER THAN THE MOUSE

In order to proceed to clinical trials, it is mandatory to evaluate the tolerability of the lungs to the delivery of PEGylated rhDNase. Therefore, a full toxicological program should be considered, involving acute inhalation testing and chronic toxicity assessment [428]. The main objective of acute inhalation would be to determine the pathology resulting from different dose levels. Biochemical and cellular markers would be monitored. Histological examination

of the lung parenchyma should also be performed to firmly conclude on the safety of acute lung exposure to PEGylated rhDNase. Adverse effects resulting from repeated exposure as well as maximal tolerable dose would be evaluated. A chronic evaluation would allow to determine the long-term effects of PEGylated rhDNase, which may be extrapolated to the safety in humans.

Toxicity of the polymer alone could also be investigated although PEG is generally considered to have low toxicity, independently of its molecular weight and route of administration [289]. Large molecular weight PEGs are retained in the lungs for longer period of time than small molecular weight PEGs [371]. Therefore, considering chronic use of PEGylated proteins, PEG retention might result in its accumulation in the lungs [298], potentially leading to local toxicity. To our best knowledge, only Klönne and co-workers evaluated PEG toxicity following aerosolization in rats but their investigation concerned small PEGs of 3.35 kDa [326]. Consequently, there is a need to verify the safety of large PEGs such as those used for rhDNase modification.

As the architecture and morphology of the airways differs between animal species [297], it could be interesting to conduct the toxicity study in different preclinical models. Also, guidelines recommend to evaluate the safety in one rodent and one non-rodent species [429]. Therefore, rat and dogs could be chosen [430]. Although rats are not similar to humans, they have been widely used for pulmonary delivery since relatively small amounts of test material are required [431]. Dogs are used to complement and verify findings in rodents [432].

The method of delivery should also be considered, with direct administration or passive inhalation being used for pulmonary delivery [433]. Direct administration procedure employs a stainless steel tube introduced in the trachea. In case of large animals such as dogs, surgery may be required for accommodation of the device. Therefore, passive inhalation would be more suitable and convenient to deliver PEGylated rhDNase to the lungs. Furthermore, passive inhalation in conscious animals is considered as the most relevant method for extrapolation to therapy in humans [433]. Whole body exposure, face-mask as well as nose-only methods are commonly used as inhalation systems [434]. The major disadvantage of whole body exposure is the possibility of protein absorption by other routes of delivery, such as oral and percutaneous [435]. Consequently, head-only and nose-only would be preferred,

even if these systems are associated with increased stress for the animals [434]. Generally, the nose-only system is used for delivery of inhalable candidates to rats, while a face-mask is employed for dog exposure [436].

Mucociliary clearance differs between animal species, with fast elimination in rodents compared to larger mammals [297]. Therefore, variable protein clearance might be expected in rats and dogs. Consequently, a toxicokinetic study should be conducted in both animal species to a) evaluate the toxicity of PEGylated rhDNase and PEG alone and b) confirm the sustained-release of PEG-conjugates observed in mice. Quantification of both polymer and protein parts of PEGylated rhDNase in BAL and pulmonary tissue could provide information of their respective clearance in the lungs.

To date, Pulmozyme® is used as starting material for the production of PEGylated rhDNase. However, considering the large quantities of PEGylated rhDNase that tolerability studies would require, in-house production of the protein would be needed. rhDNase could be expressed in cells such as that of Chinese hamster ovary already used for production of Pulmozyme® [4], then mono-PEGylated following the procedure described in this thesis.

IV.3. DEVELOPMENT OF A PEGYLATED VERSION OF AN ACTIN-RESISTANT RHDNASE

In this study, PEGylation of rhDNase on its N-terminal residue did not allow to circumvent the inhibition by G-actin. Therefore, we hypothesize that PEG attachment within the actin binding site on rhDNase would protect it from G-actin inhibition. However, the mutation of an amino acid into a cysteine for site-selective thiol PEGylation using a PEG maleimide is not applicable. Indeed, the rhDNase sequence already contains four cysteines, all located on the protein surface (**Figure 48**), thereby potentially resulting in multi-PEGylation.

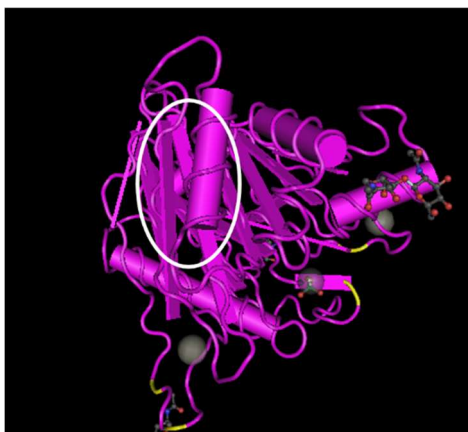


Figure 48: 3D structure of rhDNase (from the Protein Data Bank). The cysteines (four amino acids) are indicated in yellow. The actin-binding site is indicated by the white circle.

Moreover, models of deoxyribonuclease I, actin and DNA complex revealed adjacent binding sites for actin and DNA (**Figure 49**). Therefore, PEGylation of rhDNase on actin-binding site would lead to reduced or inhibited DNA hydrolysis.

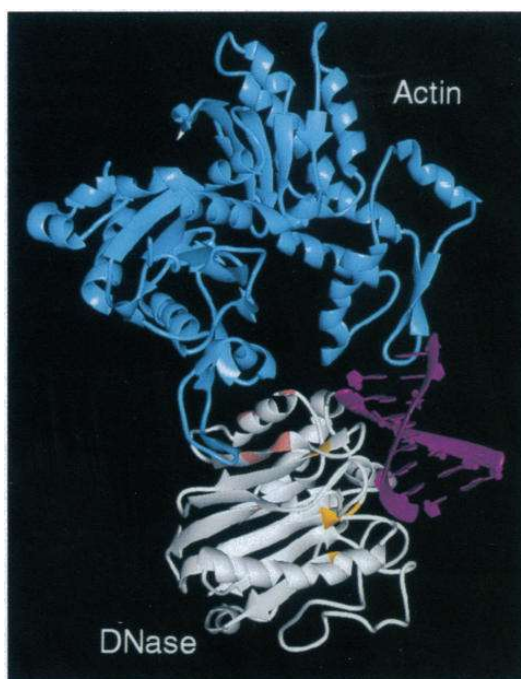


Figure 49: Model of deoxyribonuclease I (DNase; in white) complexed with actin (in blue) and DNA (in purple) shows adjacent binding sites for actin and DNA [273].

Consequently, an alternative protein modification should be considered and would concern the development of a PEGylated and actin-resistant rhDNase. In this thesis, we demonstrated that PEGylation of rhDNase with large PEGs preserved its enzymatic activity and sustained its presence within the lungs for more than 15 days. In 1996, Ulmer et al. engineered actin-resistant variants by the introduction of charged, aliphatic or aromatic residues on the actin-binding site of rhDNase. The authors demonstrated that these rhDNase versions retained enzymatic activity and were no more inhibited by actin [273]. Therefore, we hypothesize that PEGylated and actin-resistant mutants of rhDNase would benefit from the same properties.

First, site-directed mutagenesis [273] would be carried out as described by Kunkel and co-workers [437]. Then, DNA variants would be expressed in cells such as that of Chinese hamster ovary which are used for the production of rhDNase [4]. *In vitro* enzymatic activity and resistance to actin of the rhDNase mutants should be verified before PEGylation. Mono-PEGylation with large PEGs on the N-terminal residue of rhDNase would then be performed. The reaction would follow the procedure described in this thesis. Reductive alkylation presents the advantage of simplicity and is performed under mild conditions that do not disrupt the protein. Finally, the *in vitro* biological activity of PEGylated and actin-resistant rhDNases should be assessed before proceeding to *in vivo* therapeutic efficacy in β -ENaC mice.

SUPPLEMENTARY MATERIAL

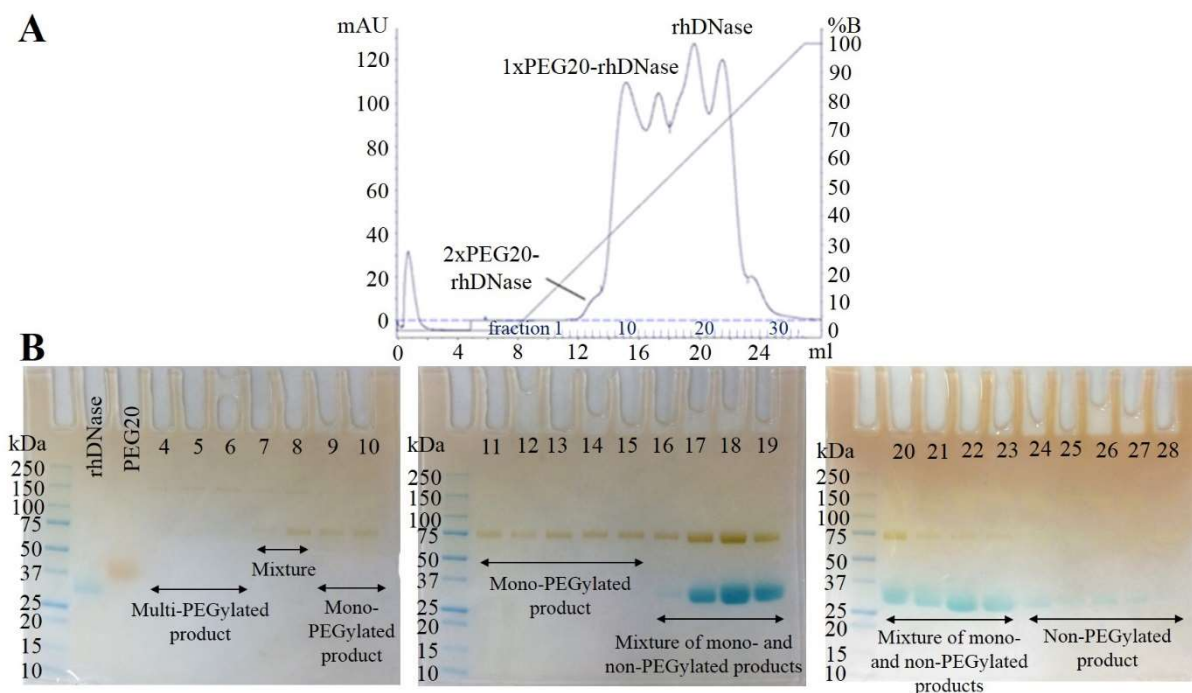


Figure S1: Characterization of the PEGylation reaction of rhDNase with PEG20 propionaldehyde. The reaction conditions were 1mg/mL (33 μ M) of rhDNase, 96h reaction time, 16:1 [PEG]:[rhDNase] molar ratio, pH 5 and room temperature. **A.** Anion-exchange chromatogram of the PEGylation reaction mixture showing, from left to right, 2xPEG20-rhDNase (shoulder), 1xPEG20-rhDNase (first double peak) and unmodified rhDNase (second double peak). **B.** SDS-PAGE of anion-exchange chromatography fractions. Blue bands represent unconjugated rhDNase and brown bands at 75 kDa represent mono-PEGylated rhDNase. The brown band at ~37 kDa corresponds to 20 kDa PEG alone used as a control. Fractions 9 to 15 contain purified 1xPEG20-rhDNase.

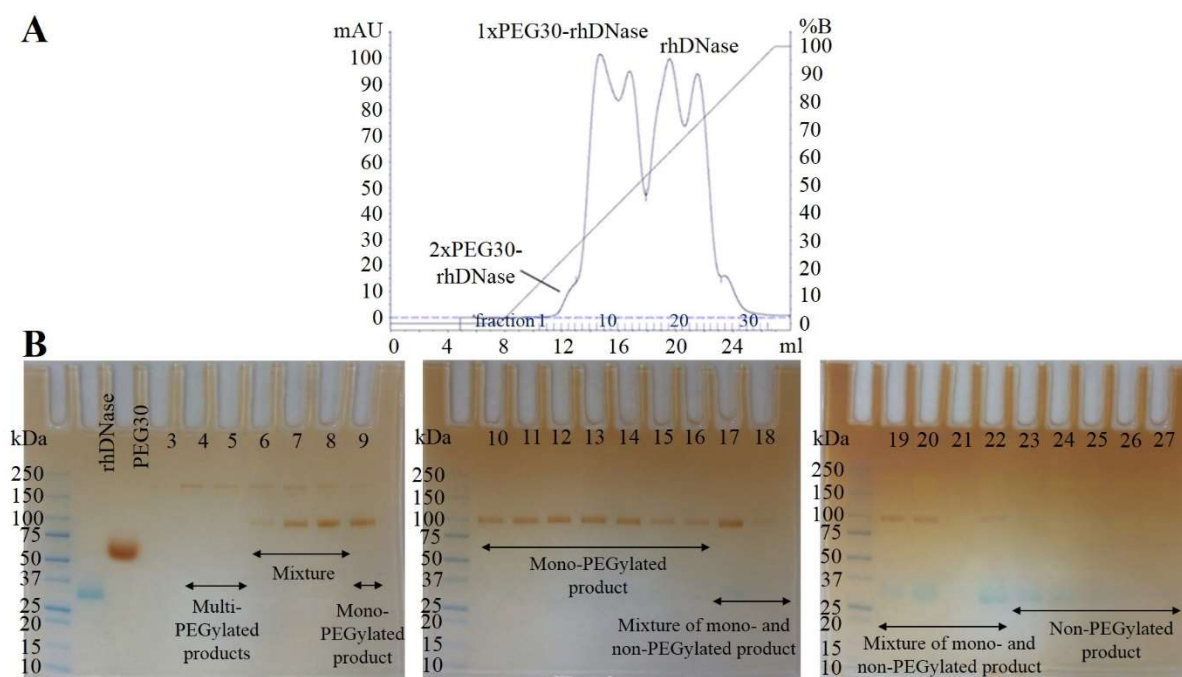


Figure S2: Characterization of the PEGylation reaction of rhDNase with PEG30 propionaldehyde. The reaction conditions were 1mg/mL (33 μ M) of rhDNase, 96h reaction time, 16:1 [PEG]:[rhDNase] molar ratio, pH 5 and room temperature. **A.** Anion-exchange chromatogram of the PEGylation reaction mixture showing, from left to right, 2xPEG30-rhDNase (shoulder), 1xPEG30-rhDNase (first double peak) and unmodified rhDNase (second double peak). **B.** SDS-PAGE of anion-exchange chromatography fractions. Blue bands represent unconjugated rhDNase and brown bands at 100 kDa represent mono-PEGylated rhDNase. The brown band between 50 and 75 kDa corresponds to 30 kDa PEG alone used as a control. Fractions 9 to 16 contain purified 1xPEG30-rhDNase.

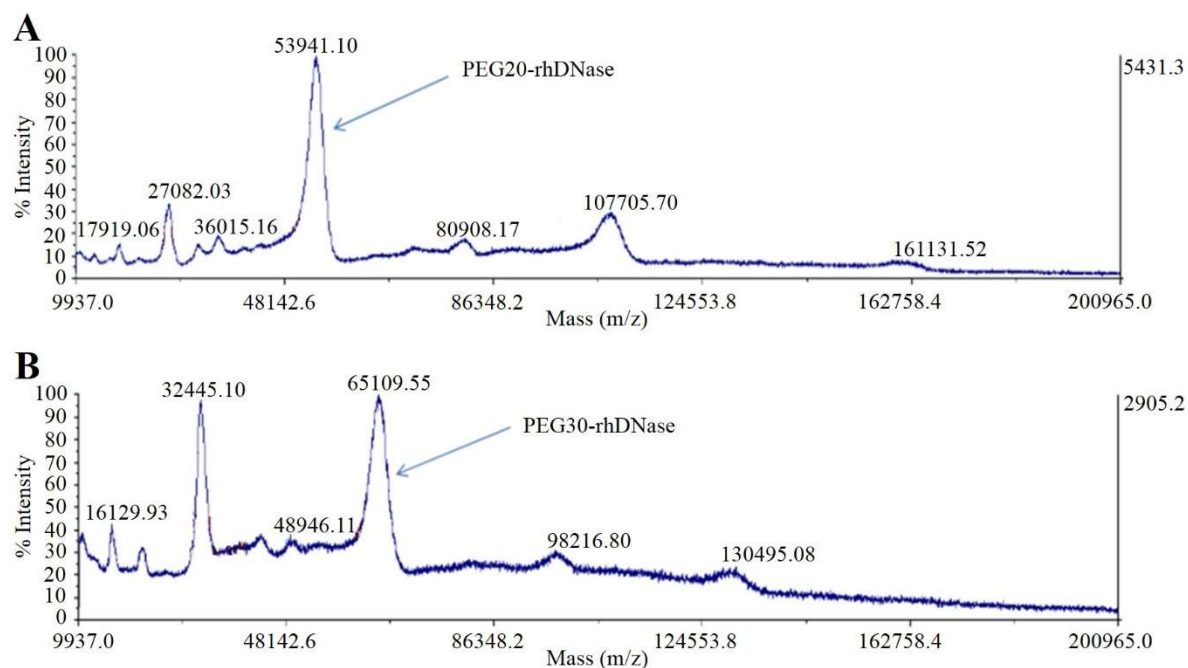


Figure S3: MALDI-TOF spectra of **A.** rhDNase following coupling to 20 kDa PEG, **B.** rhDNase following coupling to 30 kDa PEG. The figures showed that one PEG molecule was linked to each rhDNase. PEG20-rhDNase showed a main peak at 53.9 kDa and peaks at 27.1 kDa and at 107.7 kDa associated with twice ionized and dimer forms. PEG30-rhDNase presented a main peak at 65.1 kDa and another peak at 32.4 kDa related to its twice ionized form.

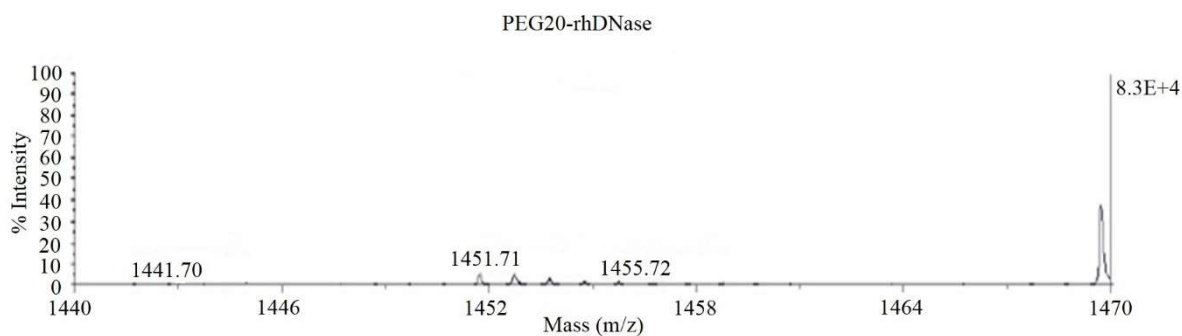


Figure S4: MALDI-TOF spectra of the N-ter peptide of rhDNase following coupling to 20 kDa PEG. The figure showed extinction of the N-ter peaks confirming PEGylation on this residue.

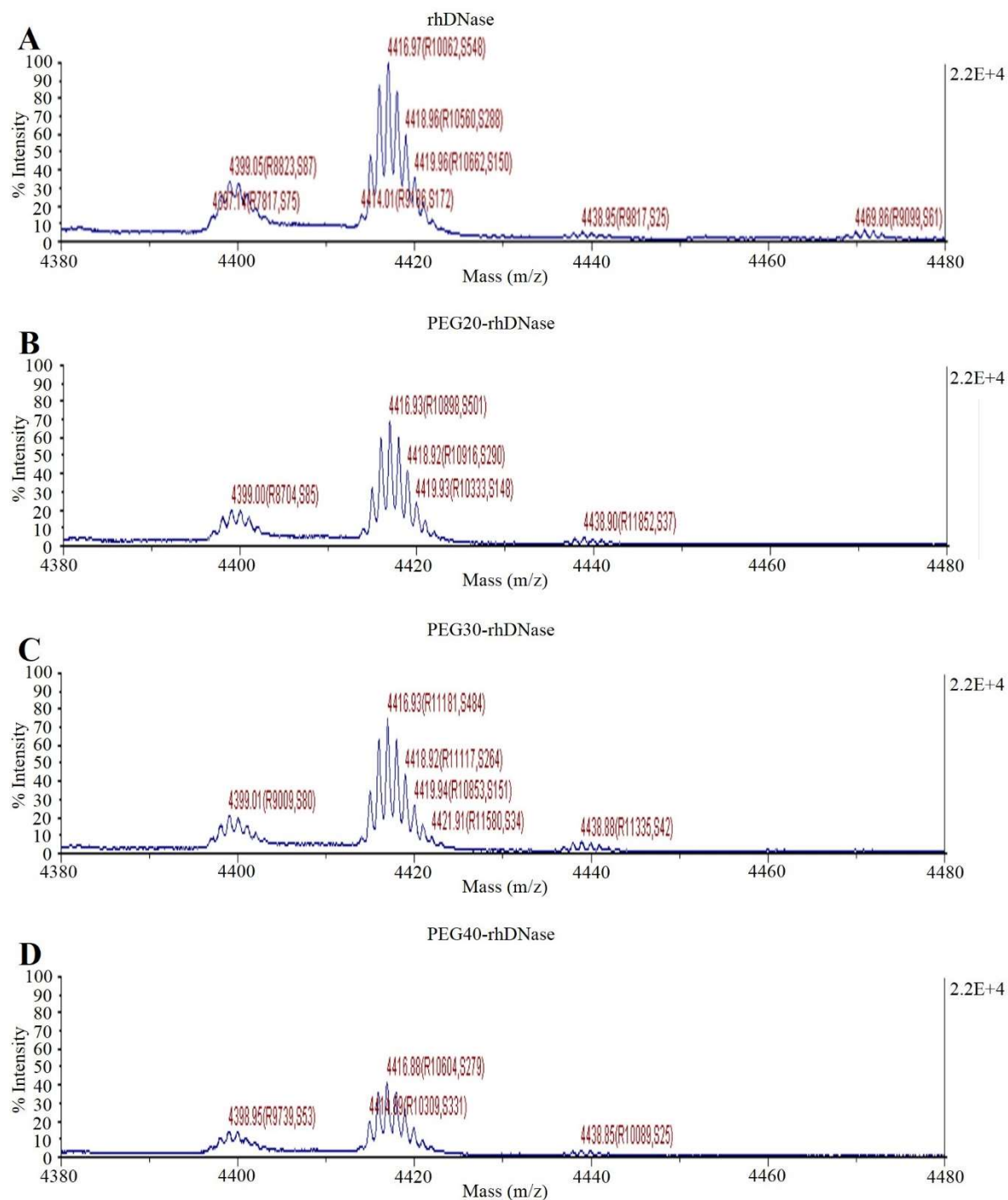


Figure S5: MALDI-TOF spectra of the peptide with $m/z=4416.86$. **A.** rhDNase prior to PEGylation, **B.** rhDNase following coupling to 20 kDa PEG, **C.** rhDNase following coupling to 30 kDa PEG and **D.** rhDNase following coupling to 40 kDa PEG. The figures showed no extinction of the peak for PEGylation with PEG40 and a decrease by half for PEG20-rhDNase and PEG30-rhDNase.

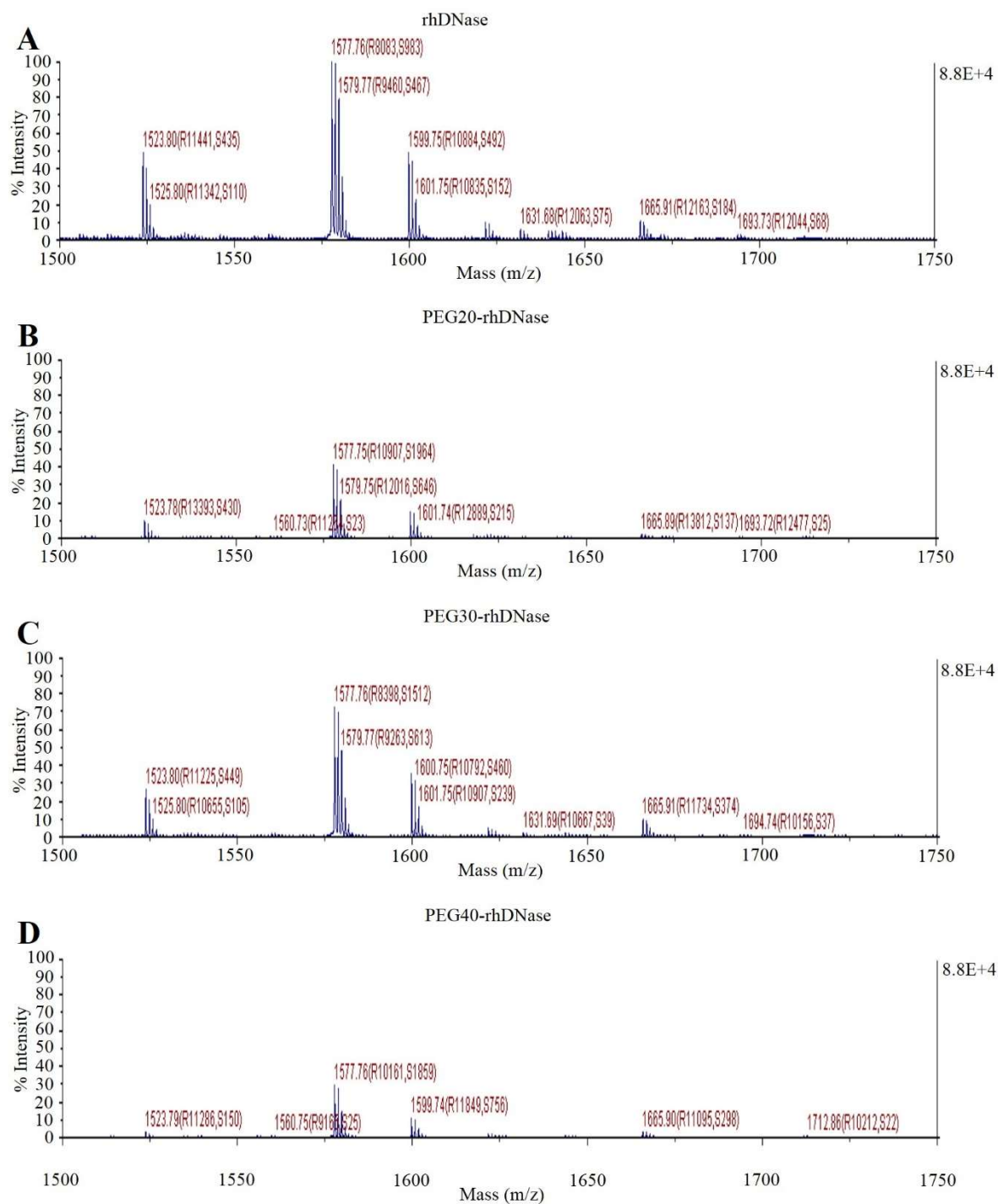


Figure S6: MALDI-TOF spectra of the peptide with $m/z=1577.76$. **A.** rhDNase prior to PEGylation, **B.** rhDNase following coupling to 20 kDa PEG, **C.** rhDNase following coupling to 30 kDa PEG and **D.** rhDNase following coupling to 40 kDa PEG. The figures showed no extinction of the peaks confirming that PEGylation did not occur on this residue.

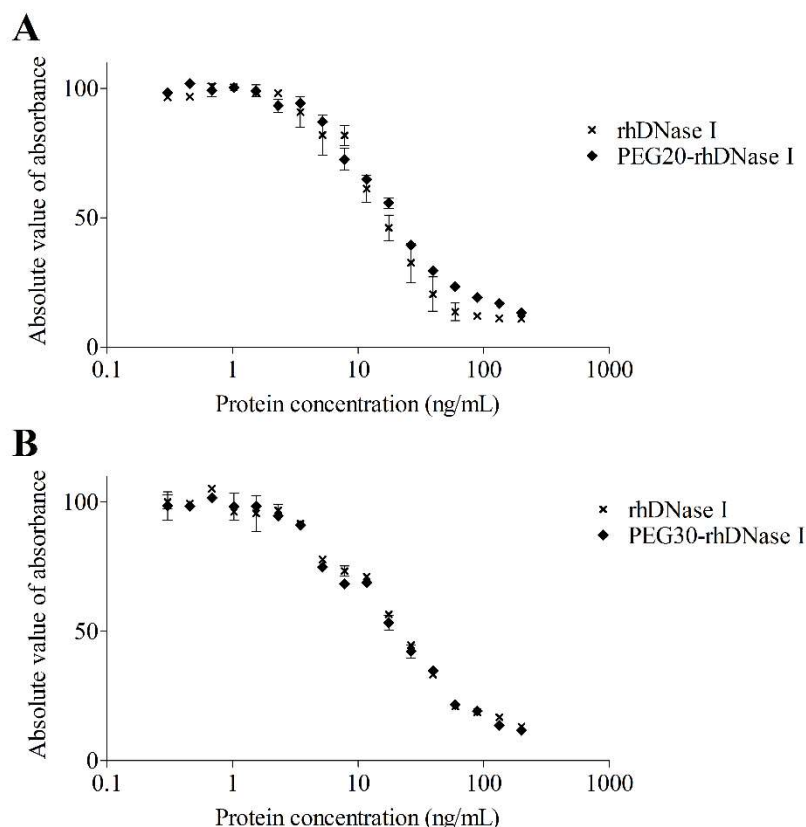


Figure S7: Hydrolysis of DNA by rhDNase and PEGylated rhDNase at increasing concentrations of the rhDNase moiety of the enzymes ($n=3$). The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore analyzed at the same molarities. DNA was complexed with methyl green and DNA hydrolysis produced unbound methyl green and a decrease of the absorbance of the solution at 650 nm after 6h of incubation at 37°C. **A.** The effective concentration at 50% was 17.7 ± 3.5 (mean $\pm$ SEM) and 19.8 ± 3.2 ng/mL for rhDNase and PEG20-rhDNase, respectively. **B.** The effective concentration at 50% was 19.8 ± 3.2 and 19.2 ± 3.3 ng/mL for rhDNase and PEG30-rhDNase, respectively. The enzymatic activity of PEGylated rhDNase was compared to native rhDNase on separated days, which explains the variability in the effective concentration at 50% of rhDNase in each test. The data were compared by two way-ANOVA and no difference was seen between each PEGylated rhDNase and native rhDNase.

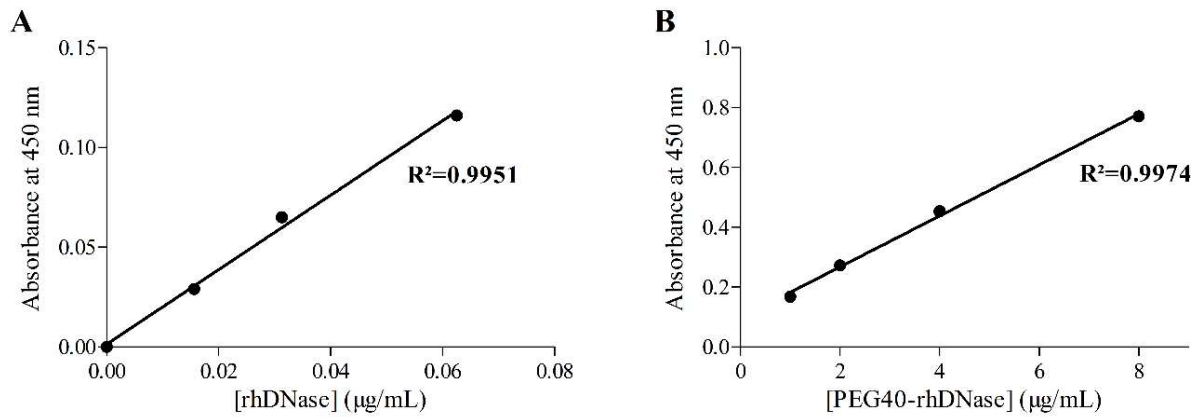


Figure S8: Calibration curves of rhDNase (A) and PEG40-rhDNase (B) for quantification of their content in CF sputum. The X axis represents the concentration in rhDNase of each enzyme. A reduced sensitivity of the ELISA assay was observed for PEG40-rhDNase.

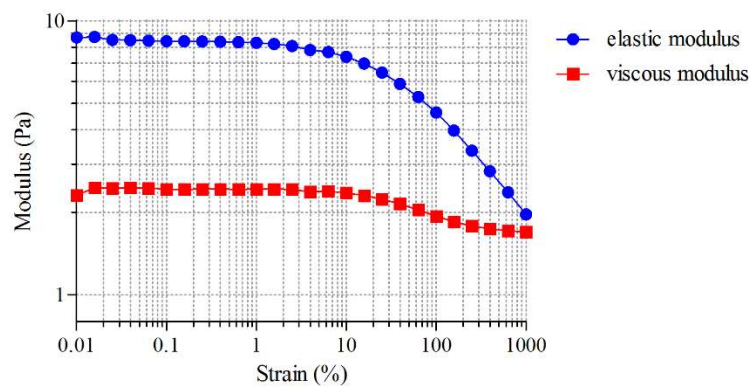


Figure S9: Evaluation of the range of non-destructive strain in CF sputum. Linear viscoelastic region was observed between 0.01% and 1% of shear strain. Therefore, this range of strains was selected for rheology experiments.

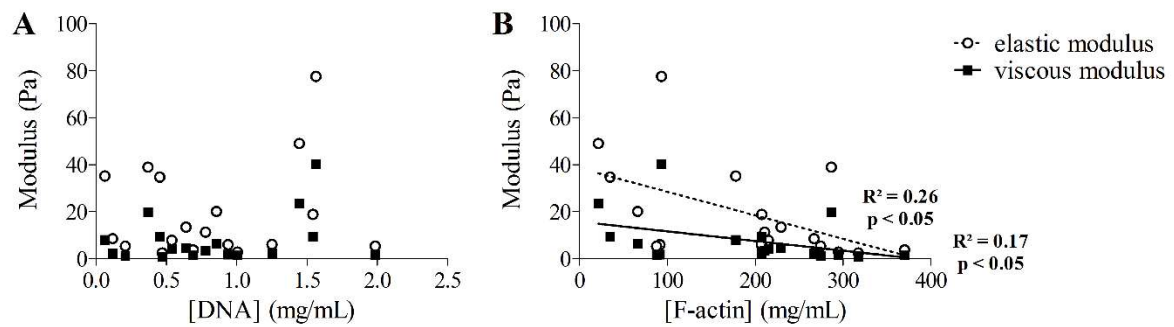


Figure S10: Influence of DNA (A) and F-actin (B) concentration of CF sputa on elastic and viscous moduli of the samples. A significant correlation was only verified for F-actin concentration and elastic and viscous moduli of the samples (B, Spearman test, $p < 0.05$). No correlation was established between DNA concentration and elastic and viscous moduli of the samples (Spearman test, $p > 0.05$).

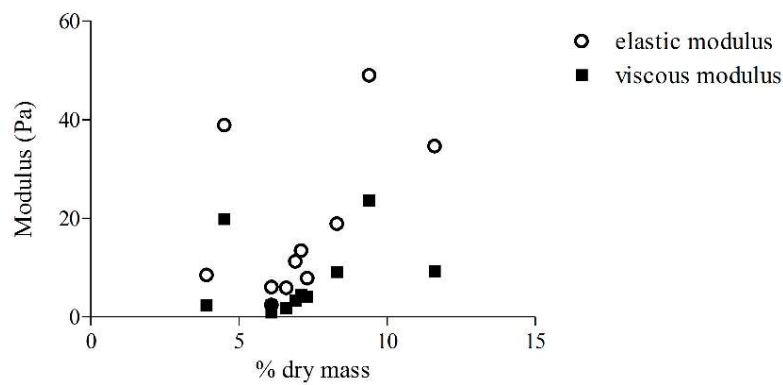


Figure S11: Influence of % dry mass of CF sputa on elastic and viscous moduli of the samples. No correlation was established (Spearman test, $p > 0.05$).

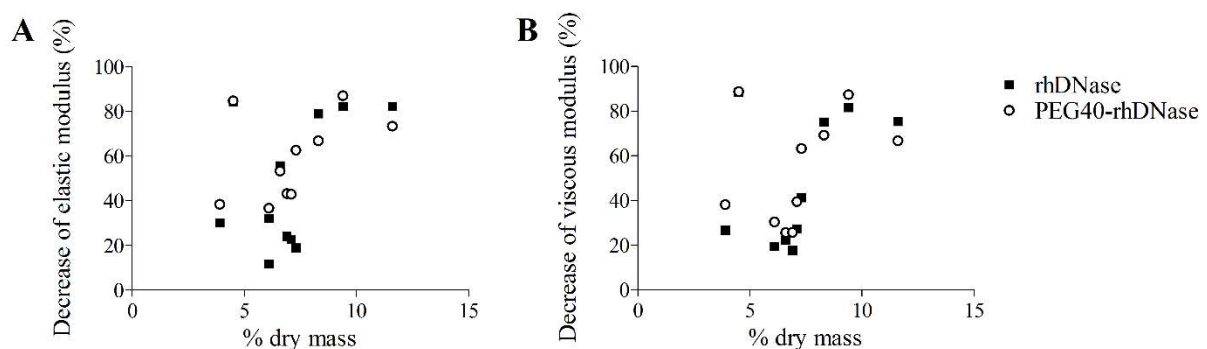


Figure S12: Influence of % dry mass of CF sputa on the mucolytic activity of rhDNase and PEG40-rhDNase (at 0.33 μM in rhDNase; both proteins were incubated for 60 min in the samples). No correlation was established between % dry mass and decrease of elastic (A) or viscous modulus (B) following addition of rhDNase or PEG40-rhDNase (Spearman test, $p > 0.05$).

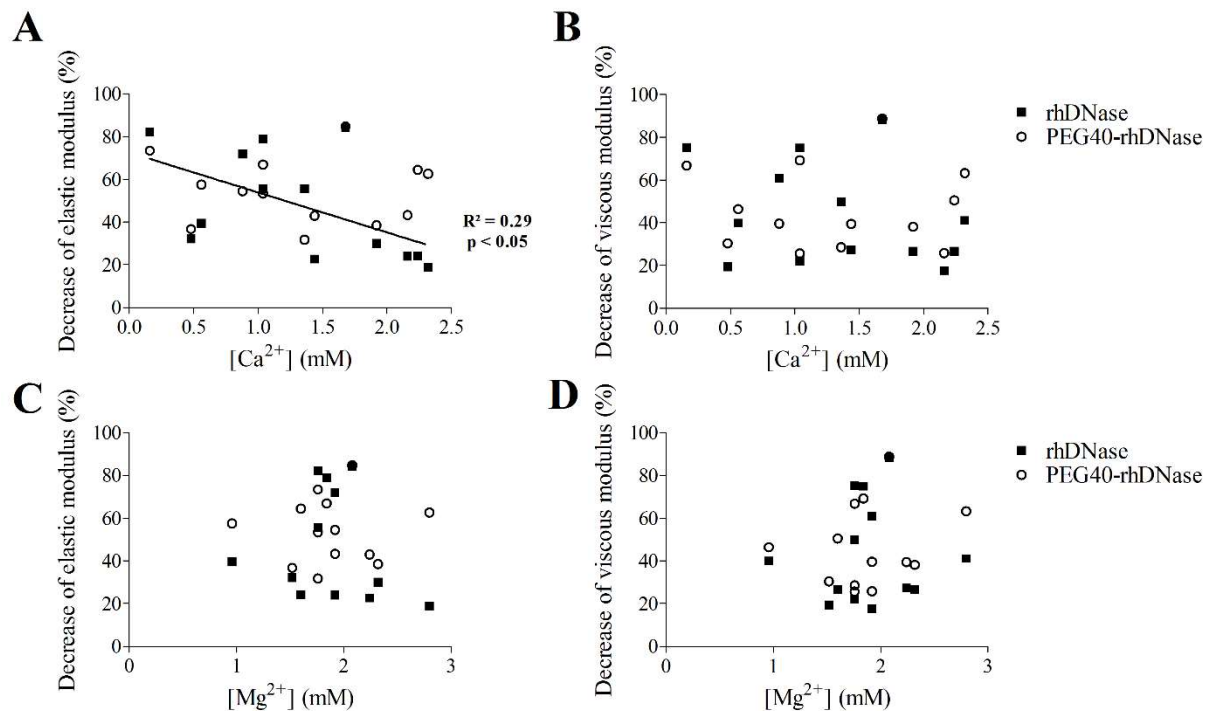


Figure S13: Influence of Ca²⁺ (A and B) and Mg²⁺ (C and D) contents in CF sputa on the activity of rhDNase and PEG40-rhDNase (at 0.33 μ M in rhDNase; both proteins were incubated for 60 min in the samples). A significant correlation was only verified for calcium ions concentration and the activity of rhDNase on the elastic part of the samples (A, Spearman test, $p < 0.05$). No correlation was established between Ca²⁺ and decrease of elastic modulus nor between Mg²⁺ and decrease of elastic or viscous modulus following addition of rhDNase or PEG40-rhDNase (Spearman test, $p > 0.05$).

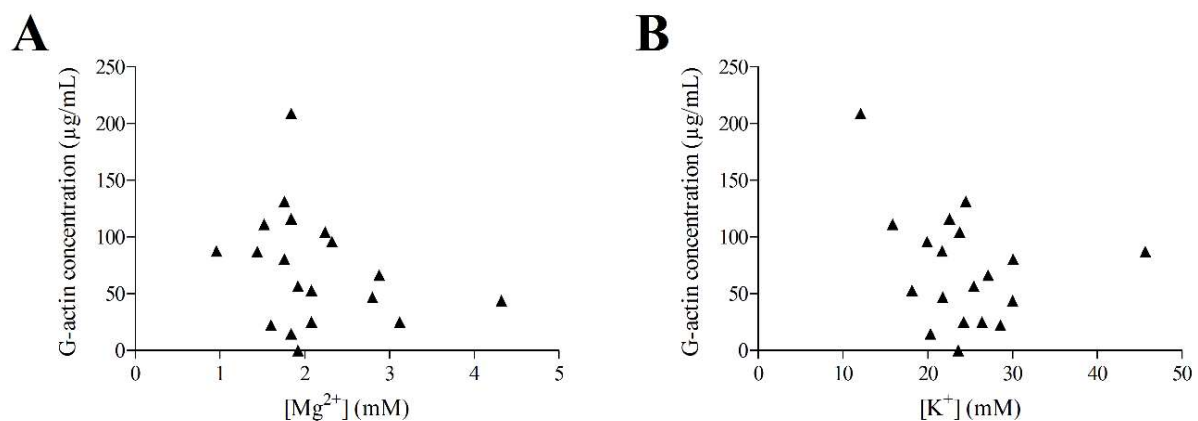


Figure S14: Influence of Mg²⁺ (A) and K⁺ (B) contents on G-actin concentration in CF sputa. No correlation was established for both ions (Spearman test, $p > 0.05$).

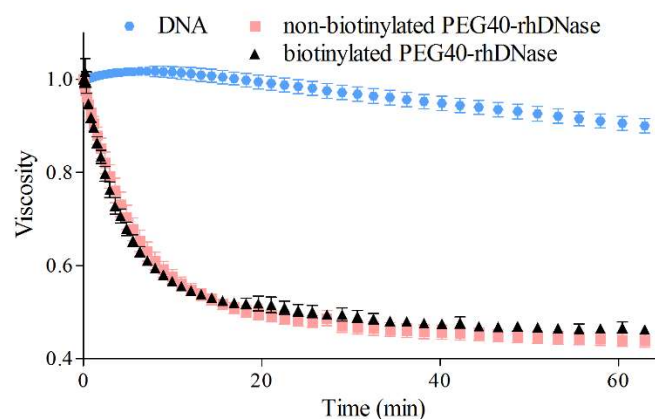


Figure S15: Viscosity analysis of DNA solution following addition of non-biotinylated PEG40-rhDNase or biotinylated PEG40-rhDNase (n=3, data were normalized to the time zero DNA viscosity for each protein). The concentrations of non-biotinylated and biotinylated PEG40-rhDNase were normalized to 0.25 $\mu\text{g/mL}$ (8.3 μM) of rhDNase and DNA concentration was of 0.75 mg/mL. PEG was not counted in the concentration of rhDNase used for the rheology assay. Therefore, the different analyses were conducted with the same number of moles of rhDNase. A kinetic evaluation of the enzymatic activity of each protein was recorded over 60 min and showed to be identical for both proteins in absence or presence of biotin (Mann-Whitney test, $p > 0.05$).

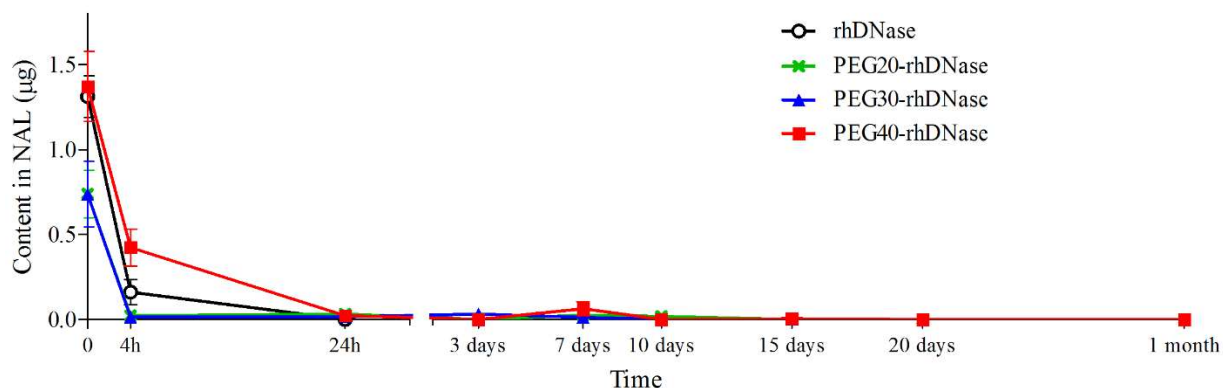


Figure S16: Quantities of biotinylated rhDNase, PEG20-rhDNase, PEG30-rhDNase and PEG40-rhDNase recovered from the nasal lavage (NAL) of NMRI mice at different times following the intratracheal delivery of a 5 μg (0.17 nmol) rhDNase dose (without counting the PEG moiety in the dose). The amount of rhDNase is expressed in μg of protein. Data expressed as mean values ($\pm$ SEM) of 5 to 10 mice per time point.

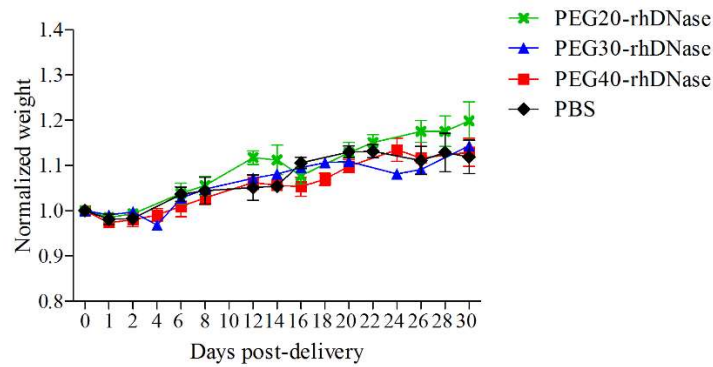


Figure S17: Normalized body weights of NMRI mice receiving 5 μ g (0.17 nmol of rhDNase, without counting the PEG moiety) of biotinylated PEGylated rhDNase intratracheally. Mean $\pm$ SEM are shown, n=3 (PBS) to n=5 (PEGylated proteins) mice per group.

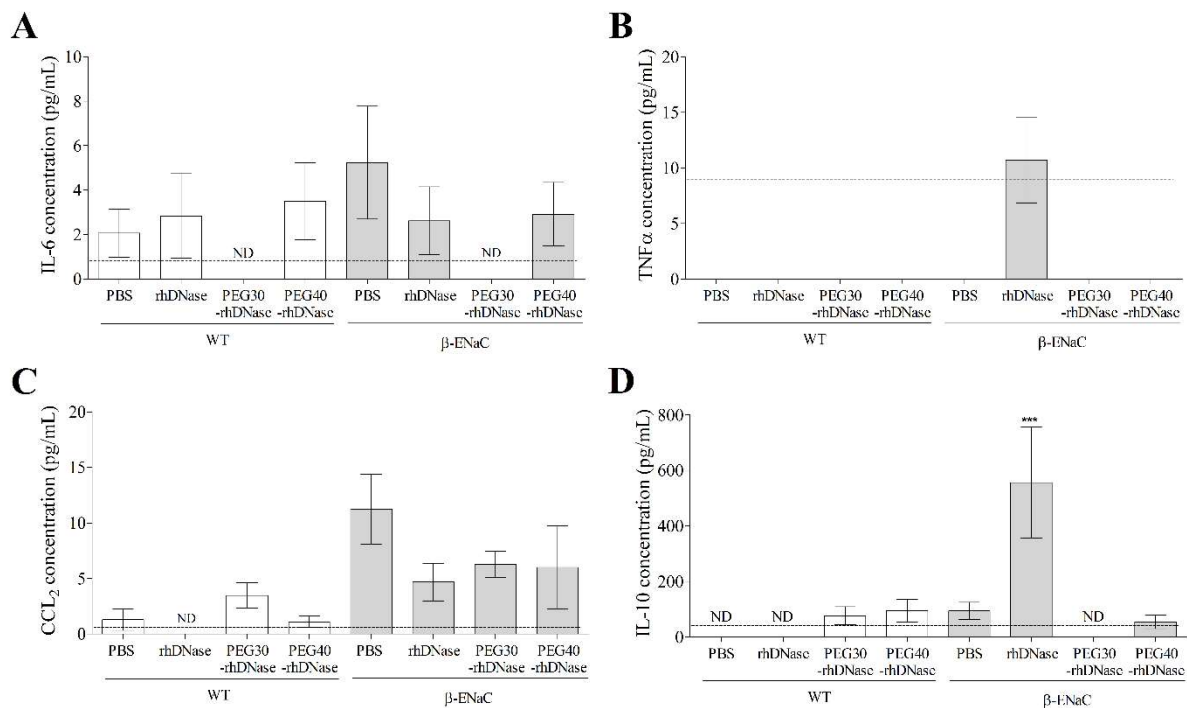


Figure S18: Pro-inflammatory cytokines (A. IL-6, B. TNF α , C. CCL $_2$) and anti-inflammatory cytokine (D. IL-10) content in WT and β -ENaC mice following intratracheal delivery of 10 μ g (0.33 nmol) of rhDNase (5 daily doses and sacrifice 24 h following the last dose), PEG30-rhDNase (single dose at day 1 and sacrifice at day 6) or PEG40-rhDNase (single dose at day 1 and sacrifice at day 6) (n=4 to 7, mean $\pm$ SEM, backgrounds represented with dotted lines). The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore administered at the same molarities. Data were compared to PBS groups and showed no significant difference, except for IL-10 data of β -ENaC mice receiving rhDNase (one-way ANOVA, Dunnett's multiple comparison test). ND = not detected.

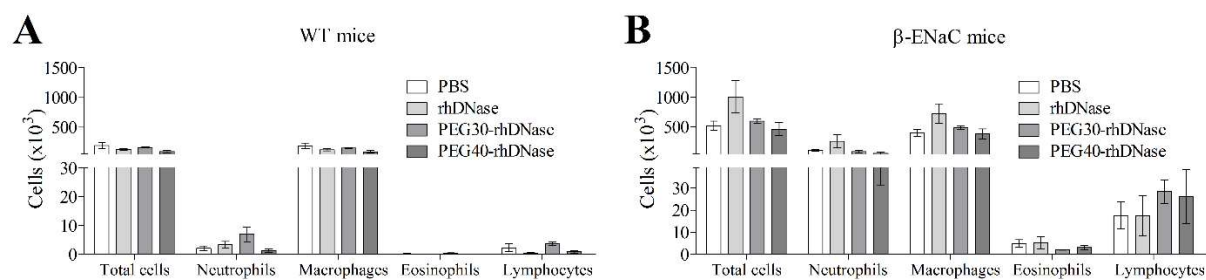


Figure S19: Cellular markers found in bronchoalveolar lavages (BAL) of WT (A) and β -ENaC (B) mice following intratracheal delivery of 10 μ g (0.33 nmol) of rhDNase (5 daily doses and sacrifice 24 h following the last dose), PEG30-rhDNase (single dose at day 1 and sacrifice at day 6) or PEG40-rhDNase (single dose at day 1 and sacrifice at day 6) ($n=4$ to 7, mean $\pm$ SEM). The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore administered at the same molarities. Data were compared to PBS groups and showed no significant difference (one-way ANOVA, Dunnett's multiple comparison test).

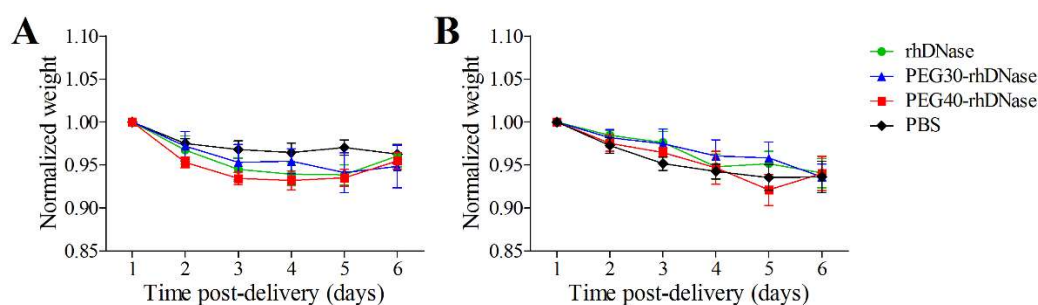


Figure S20: Normalized body weights of WT (A) and β -ENaC (B) mice receiving 10 μ g (0.33 nmol) of rhDNase (5 daily doses and sacrifice 24 h following the last dose), PEG30-rhDNase (single dose at day 1 and sacrifice at day 6) or PEG40-rhDNase (single dose at day 1 and sacrifice at day 6) intratracheally. The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore administered at the same molarities. Mean $\pm$ SEM are shown, $n=4$ to 9 mice per group.

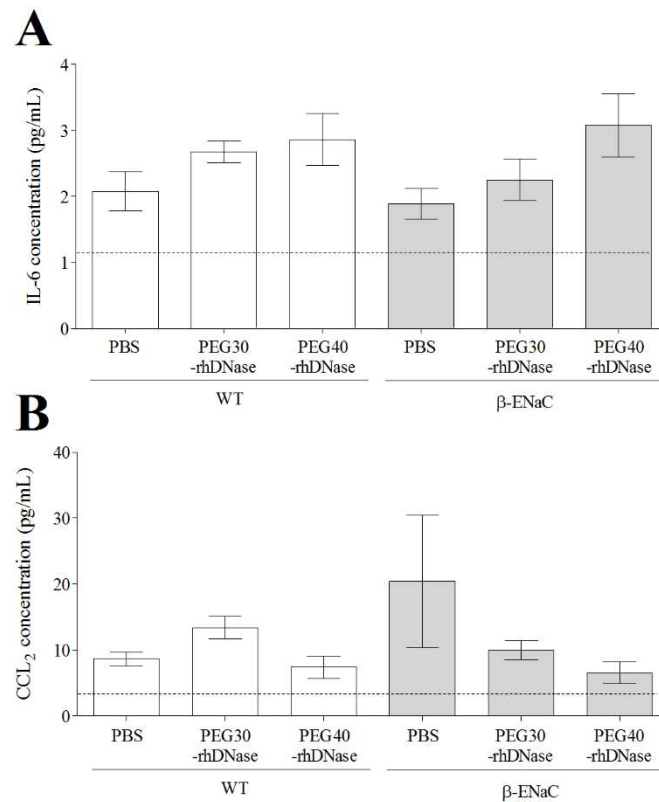


Figure S21: Pro-inflammatory cytokines (**A**. IL-6, **B**. CCL₂) content in WT and β-ENaC mice following intratracheal delivery of 5 weekly doses of 10 μg (0.33 nmol) of PEG30-rhDNase or PEG40-rhDNase and sacrifice of mice 7 days following the last dose (n=4 to 5, mean ± SEM, backgrounds represented with dotted lines). The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore administered at the same molarities. Data were compared to PBS groups and showed no significant difference (one-way ANOVA, Dunnett's multiple comparison test). ND = not detected.

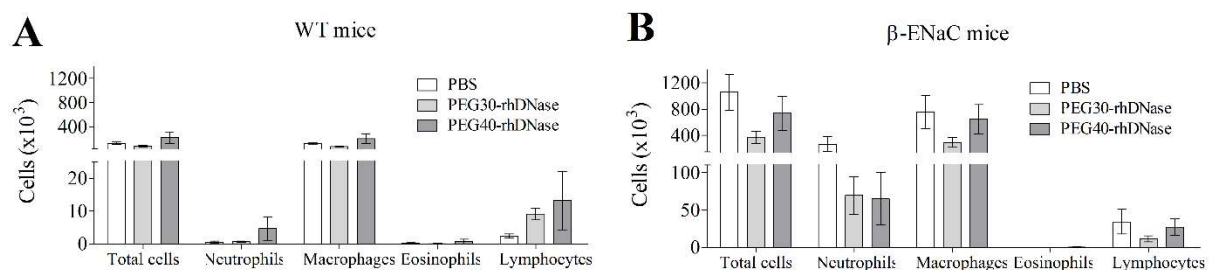


Figure S22: Cellular markers found in bronchoalveolar lavages (BAL) of WT (**A**) and β-ENaC (**B**) mice following intratracheal delivery of 5 weekly doses of 10 μg (0.33 nmol) of PEG30-rhDNase or PEG40-rhDNase and sacrifice of mice 7 days following the last dose (n=4 to 5, mean ± SEM).). The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore administered at the same molarities. Data were compared to PBS groups and showed no significant difference (one-way ANOVA, Dunnett's multiple comparison test).

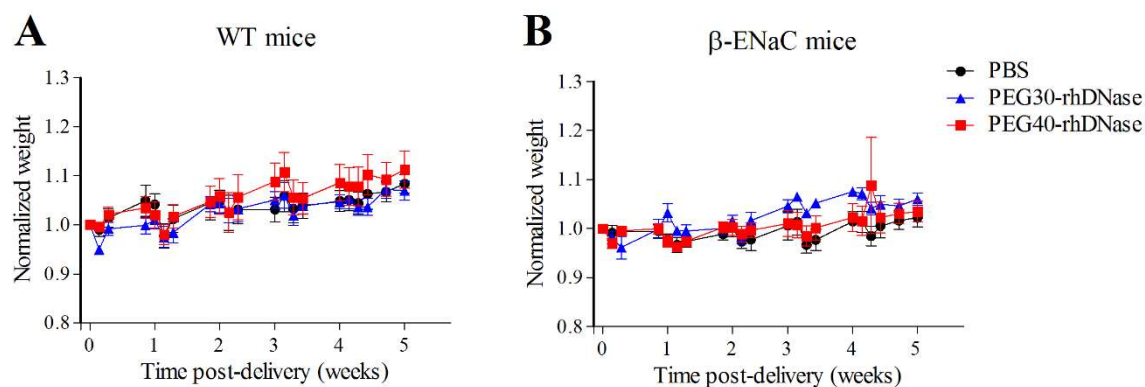


Figure S23: Normalized body weights of WT (A) and β-ENaC (B) mice receiving 5 weekly doses of 10 μg (0.33 nmol) of PEG30-rhDNase or PEG40-rhDNase intratracheally and sacrifice of mice 7 days following the last dose. The PEG was not counted in the concentration of the PEG-rhDNase samples and the rhDNase constructs were therefore administered at the same molarities. Mean ± SEM are shown, n=4 to 5 mice per group.

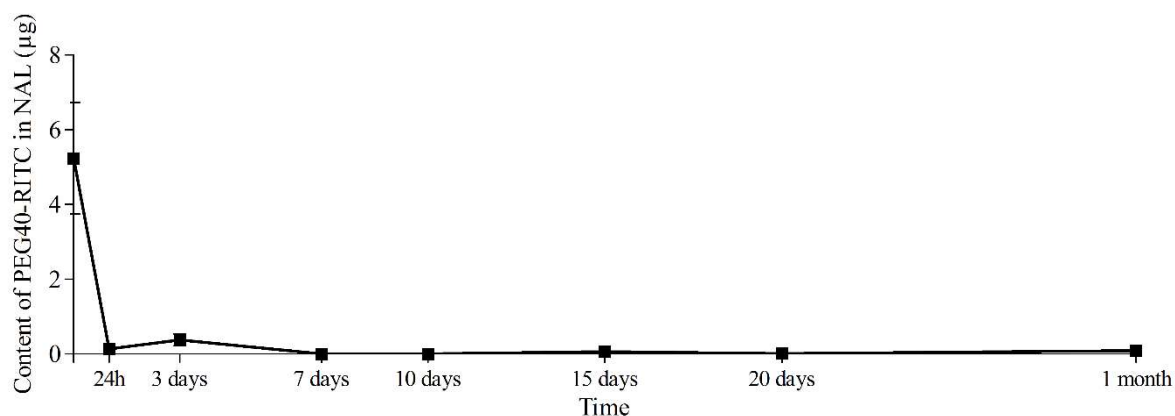


Figure S24: Quantities of PEG40-RITC recovered from the NAL of NMRI mice at different times following the intratracheal delivery of a 80 μg PEG dose. Data expressed as mean values (± SEM) of 5 mice per time point.

Table S1: Evaluation of the content of neutrophils and macrophages in BAL of WT and β-ENaC mice (mean ± SEM, n=7 to 8). β-ENaC mice present a chronic inflammation in the lungs compared to their WT littermates.

Group	Neutrophils (x10 ³)	Macrophages (x10 ³)
WT	9 ± 6	595 ± 206
β-ENaC	549 ± 79	1372 ± 182

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