



Therapeutic potential of a long-acting form of rhDNase I for the treatment of cystic fibrosis

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ABSTRACT

rhDNase I (recombinant human deoxyribonuclease I, known as Pulmozyme®) has mucolytic action, which is clinically exploited in cystic fibrosis (CF). In the lungs, its efficacy is hampered by both limited stability and presence of inhibitors such as G-actin. Here we synthesized three PEGylated rhDNase I conjugates differing in PEG length and conjugation chemistry, the latter affecting the overall charge of the conjugate. The aim was to preserve as much as possible the activity of the enzyme and to protect it from inhibition and degradation. Of note, derivatization with PEG24mer, the shortest chain, is accomplished through amide bonds, thus reducing the overall positive charge of the conjugate.

The activity of these conjugates was tested on supercoiled DNA plasmids, and then on the rheological properties of DNA-supplemented artificial mucus and of CF patients' mucus. The use of PEG24mer produced the largest loss in DNase activity in an in vitro assay (33.7% vs. 83.8% with mPEG20kDa and 71.4% with mPEG2kDa) but in mucus showed the highest reduction in viscosity (complex viscosity at 1.0 Hz: ≈ 1 Pa*s vs. 2.5–5.0 Pa*s with native enzyme or other conjugates), which is likely to be due to less intense inhibitory interactions with G-actin, both with artificial and CF patients' mucus. In nebulization, mPEG24mer-rhDNase I also showed a more promising aerodynamic profile than rhDNase I (a mass median droplet aerodynamic diameter of 3.69 μ m vs. 4.12 μ m; fraction of fine particles of 72.3% vs. 64.1%), supporting its potential as an enhanced treatment option.

1. Introduction

Cystic fibrosis (CF) is a genetic disorder that impairs chloride ion transport. It is classified as an autosomal recessive exocrinopathy because altered ion mobility leads to dense fluid secretions in the exocrine glands and epithelial linings of the respiratory, gastrointestinal, and reproductive tracts [1]. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7 [2]. Certain CFTR mutations reduce the expression of the ion channel protein or produce a non-functional form, resulting in

increased intracellular chloride levels. This imbalance causes the secretion of dense mucus with low water content, promoting its stagnation and accumulation. This, in turn, leads to airway obstruction and susceptibility to bacterial infections.

In response to infections, neutrophils are recruited as part of the body's defense. Upon neutrophil apoptosis, high-molecular-weight DNA (neutrophil extracellular traps, as described by Wartha et al. [3]) and actin are released, further increasing mucus viscosity by interacting with mucins and mucopolysaccharides.

Gene therapy remains the ultimate goal for CF treatment [4].

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However, this approach is currently limited by the complexity of the CFTR gene and the wide variety of mutations among CF patients [5]. Current therapies focus on corrective modulators that improve CFTR ion permeability and symptom management, particularly targeting respiratory complications.

Recombinant human DNase I (rhDNase I), marketed as Pulmozyme®, is used to treat CF respiratory symptoms. It enhances mucociliary clearance by degrading the DNA released from dead neutrophils, thereby reducing sputum viscosity [6,7]. However, rhDNase I has limitations, for example, approximately 30% of CF patients do not respond to Pulmozyme®, potentially due to insufficient magnesium levels in sputum or high actin concentrations, a natural DNase I inhibitor [8]. Additionally, rhDNase I has a short residence time in the lungs, attributed to absorption into the systemic circulation, enzymatic degradation, and cellular uptake [9].

PEGylation, the covalent attachment of polyethylene glycol (PEG) chains to therapeutic proteins, is a widely used strategy to enhance protein pharmacokinetics, prolong residence time, reduce immunogenicity, and increase stability against proteolytic enzymes, while also improving mucus penetration [10,11]. The effectiveness of PEGylation is evidenced by the clinical success of over 20 approved PEGylated therapeutics [12].

PEGylation of rhDNase I has been proposed as a solution to address these limitations. Previous studies demonstrated that N-terminal conjugation of rhDNase I with 20, 30, or 40 kDa PEG preserved enzymatic activity and extended lung retention in CF mouse models [13–15]. However, long PEG chains (≥ 20 kDa) may hinder mucus penetration due to mucoadhesion, caused by interpenetrating network effects, as observed with nanoparticles [16].

In this study, we investigated whether low molecular weight (MW) PEG conjugates of rhDNase I could achieve better outcomes. A single short PEG chain, such as at the N-terminus, may not provide sufficient protection for rhDNase I due to limited polymer shielding. Therefore, we explored random PEGylation by conjugating multiple short PEG chains to lysine residues using two different chemistries: i) aldehyde PEG, which preserves the charge of the lysine ϵ -amino group through reductive alkylation, ii) acylating PEG-NHS, which forms neutral amide bonds with lysines.

These conjugates were characterized and compared to native Pulmozyme® and the N-terminal PEG20kDa-Nter-rhDNase I conjugate.

2. Materials and methods

2.1. Materials

Pulmozyme® was purchased from Roche (Basilea, Switzerland) and contain 2.5 mg of rhDNase I in 2.5 mL of buffer of approximate molecular weight of 32 kDa (according to manufacturer). Linear 20 kDa methoxy PEG aldehyde (PEG-CHO 20 kDa) was from NOF Corporation (Tokyo, Japan), linear 2 kDa methoxy PEG aldehyde (PEG-CHO 2 kDa) and alpha-methoxy-23(ethylene glycol)-omega-propionic acid succinimidyl ester (MW 1214 Da) were from Iris Biotech GmbH (Markredwitz, Germany). Unless otherwise specified, chemicals and reagents were from Merck (Milan, Italy). Actin was obtained from BioVision, Inc. (Milpitas, CA, USA), ATP from Fisher (Waltham, MA, USA). Mini-PROTEAN TGX precast gels 4–15% for SDS-PAGE were obtained from Bio-Rad (Milan, Italy).

2.2. Synthesis of PEG20kDa-Nter-rhDNase I

PEG-CHO 20 kDa was solubilized in the 10% (v/v) of the final reaction volume using $10\times$ PBS buffer pH 6.2. The polymer solution was added to a solution of rhDNase I (1 mg/mL) in 1 mM CaCl₂, 0.15 M NaCl, pH 6.3, at the molar excess of 20-fold with respect to the protein. After gentle stirring for 30 min at 25 °C, NaCNBH₃ (100-fold molar excess with respect to the protein) was added to the mixture. The reaction was

left to react at 25 °C under stirring for 72 h. The conjugate PEG20kDa-Nter-rhDNase I was then purified by Size Exclusion Chromatography (SEC) on an Äkta FPLC purifier system. The separation was performed using a Superdex 200 Increase 10/300 GL column (30 cm $\times$ 10 mm, GE Healthcare Bio-Sciences AB, Umeå, Sweden) and PBS, 1 mM CaCl₂, pH 6.2, at a flow rate of 0.5 mL/min. The effluent from the column was monitored by recording the absorbance at 280 nm. The fraction corresponding to the conjugate was collected, concentrated and the buffer was exchanged with sodium acetate 10 mM, 1 mM CaCl₂, pH 4.5 using a Vivaspin 15 PES (cut-off 10,000 Da, Sartorius Stedim Lab. Ltd. Stonehouse UK). PEG20kDa-Nter-rhDNase I was then further purified from the excess of PEG by Ion Exchange Chromatography (IEC) using a TSK-GEL SP-5PW column (50 $\times$ 7.5 mm, Tosoh Bioscience Stuttgart, Germany) on a Shimadzu HPLC system (LC-10AT VP, SPD-10AV VP). The elution was carried out at a flow rate of 1 mL/min with a salt gradient (buffer A: sodium acetate 10 mM, pH 4.5, 1 mM CaCl₂; buffer B: sodium acetate 10 mM, NaCl 0.1 M pH 5, 1 mM CaCl₂; gradient B%: 0' 0, 5' 5%, 10' 5%, 65' 100%, 80' 100%, 85' 5%, 90' 0%). The purified conjugate was concentrated, buffer exchanged to 1 mM CaCl₂, 0.15 M NaCl, pH 6.3 with Vivaspin 15 PES. Aliquots of PEG20kDa-Nter-rhDNase I were frozen at -80 °C until needed for following testing. All reagents and buffers were prepared with nuclease-free water.

2.3. Synthesis of PEG2kDa-rhDNase I

To a solution of rhDNase I (1 mg/mL) in 1 mM CaCl₂, 0.15 M NaCl, pH 6.3, 30 equivalents of PEG-CHO (2 kDa) (solubilized in the 10% (v/v) of the final reaction volume using $10\times$ PBS buffer pH 7.4) with respect to the protein were added twice (at time 0 and after 72 h). After each addition of the polymer solution, the reaction was stirred for 30 min at 25 °C and 100 equivalents of NaCNBH₃, with respect to the protein, were added. Overall, the mixture was left to react at 25 °C for 96 h. Then the conjugate was purified by SEC as reported above. PEG2kDa-rhDNase I was collected, concentrated, buffer exchanged to 1 mM CaCl₂, 0.15 M NaCl, pH 6.3 with Vivaspin 15 PES (cut-off 10,000 Da, Sartorius Stedim Lab. Ltd. Stonehouse UK). The conjugate was aliquoted and stored at -80 °C.

2.4. Synthesis of PEG24mer-rhDNase I

To a solution of rhDNase I (1 mg/mL) in 1 mM CaCl₂, 0.15 M NaCl, pH 6.3, a portion of $10\times$ PBS buffer pH 8 was added to reach 10% (v/v) of the final volume and pH was adjusted to 8 using a NaOH solution. PEG24mer-NHS, dissolved in DMSO (0.96% w/v), was added to the protein solution at the PEG:protein molar ratio of 30:1. The reaction was carried out under gentle stirring at 25 °C for 1 h and then the conjugate was purified by SEC as reported above. The conjugate PEG24mer-rhDNase I was collected, concentrated, buffer exchanged to 1 mM CaCl₂, 0.15 M NaCl, pH 6.3 with Vivaspin 15 PES (cut-off 10,000 Da, Sartorius Stedim Lab. Ltd. Stonehouse UK) and stored in aliquots at -80 °C.

2.5. Characterization of conjugates

2.5.1. UV-vis spectroscopy

Protein concentrations were determined spectrophotometrically using a Thermo Scientific Evolution 201 spectrophotometer (Waltham, MA, USA) by measuring the absorbance of proteins and protein conjugates at 280 nm. The protein's extinction coefficient generated by ProtParam (<https://web.expasy.org/protparam/>) was 1.567 mL $\cdot$ mg⁻¹ $\cdot$ cm⁻¹. Extinction coefficient at 280 nm for the conjugated protein was considered unvaried with respect to that of the native protein.

2.5.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The conjugates were analyzed by SDS-PAGE following the Laemmli method [17]. Electrophoresis was performed using gradient 4–15% precast gel (Bio-Rad Laboratories Hercules, CA, USA) stained with Coomassie Brilliant Blue R-250 for protein detection and with barium iodide for PEG detection [18]. The electrophoretic run was carried out using an Electrophoresis Power Plus 300 power supply (Fisher Scientific, MI, IT) at 300 V and 60 mA.

2.5.3. Matrix-assisted laser desorption/ionization time of flight mass spectra (MALDI-TOF MS) analysis

MALDI-MS spectra were acquired on a REFLEX time-of-flight instrument (4800 Plus MALDI TOF/TOF, AB Sciex, Framingham, Massachusetts, USA) equipped with a SCOUT ion source operating in positive linear mode as previously described [19]. Ions generated by a pulsed UV laser beam (nitrogen laser, λ 337 nm) were accelerated to 25 kV. A saturated solution of sinapinic acid in water / ACN (1:1, v/v) + 0.1% TFA (v/v) was used as matrix and mixed with an equal volume of sample solution, and 1–2 μ L were loaded on the plate.

2.5.4. Circular dichroism (CD) spectra analysis

For CD studies, the samples were prepared at a concentration of 0.1 mg/mL in 1 mM CaCl_2 , 0.15 M NaCl, pH 6.3. The analyses were carried out with a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control unit and using a quartz cuvette with an optical path of 1 mm. The spectra were collected at 25 °C, in a wavelength range of 200–250 nm with an average of 3 scans and the data at each wavelength were averaged for 8 s. The CD data were converted to mean residue ellipticity, expressed in $\text{deg. cm}^2 \text{dmol}^{-1}$ by applying the formula $\Theta = \Theta_{\text{obs}} (\text{MRW}) / 10 L [\text{C}]$, where Θ is the observed ellipticity in degrees, MRW is the mean residue weight of the protein, [C] is protein concentration in mg/mL, and L is the optical path length in centimeters.

2.6. Resistance to proteolytic degradation by Glu-C endoproteinase

rhDNase I, PEG20kDa-Nter-rhDNase I, PEG2kDa-rhDNase I and PEG24mer-rhDNase I were incubated with Glu-C endoproteinase (Thermo Fisher Scientific, Waltham, MA, USA) in ammonium bicarbonate pH 8.0, at 37 °C in a thermomixer. Glu-C was added at an enzyme/substrate (E/S) ratio of 1:50 (w/w) and the final concentrations of rhDNase I and Glu-C were 0.2 mg/mL and 4 μ g/mL, respectively. Sample aliquots were taken at predetermined time points and directly analyzed by RP-HPLC on an Agilent 1200 Series HPLC with online UV detection from Agilent Technologies (Santa Clara, CA, USA). RP-HPLC analyses were performed using a Jupiter C18 column (4.6 $\times$ 250 mm; 5 μ m; Phenomenex, USA), eluted with H_2O + 0.05% TFA (eluent A) and ACN + 0.05% TFA (eluent B) at a flow-rate of 1 mL/min (gradient B: 0 min 10%, 25 min 95%, 30 min 10%). The absorbance was read at 226 nm. The area of the peak corresponding to the non-degraded protein at each time point was calculated and plotted respect to the area of a time zero (considered as 100%) corresponding to an equivalent solution of rhDNase I or conjugates without the enzyme.

2.7. Activity assays

2.7.1. Hyperchromicity assay

DNase activity was determined by a modification of the hyperchromicity assay of Kunitz [20] evaluating the change in the absorbance at 260 nm of a solution containing a fixed amount of substrate and increasing amounts of enzyme. All reagents were prepared with nuclease-free water. As substrate, a solution of calf thymus DNA 0.04 mg/mL in sodium acetate buffer (NaAc) 50 mM, pH 5, 5 mM MgCl_2 and 1 mM CaCl_2 , was used. To 0.5 mL of substrate solution pipetted into a quartz cuvette, 10 L of rhDNase I or modified rhDNase I (from 0.4 to 2.4 μ g) were added to the calf thymus DNA solution and gently mixed.

Absorbance increments at 260 nm, as consequence of DNA depolymerization by rhDNase I or conjugates, were recorded for 2 min on a Thermo Scientific Evolution 201 spectrophotometer (Waltham, MA, USA) equipped with a Peltier temperature control unit set at 25 °C. The absorbance increments for each amount of enzyme were plotted against the time and for each curve the slope of the linear portion (dA / dt) of the first ten seconds of analysis was determined with the spectrophotometer software and then used to calculate the enzyme activity. The values of conjugates activities were expressed as relative activity % considering the activity of rhDNase I as 100%.

2.7.2. Plasmid digestion assay

The activities of rhDNase I and its conjugates were assayed by evaluating the disappearance of a supercoiled plasmid DNA of approximately 3000 bp (pGEM-T) in 1% agarose gel electrophoresis. The DNA was stained with ethidium bromide. The DNA plasmid was prepared as reported elsewhere [21]. After electrophoresis, the DNA was visualized under UV light and photographed using a ChemiDoc® imager (Biorad). All reagents and buffers were prepared with nuclease-free water.

Time-dependent activity: rhDNase I and conjugates (0.01 ng/ μ L) were incubated for 15 min in the assay buffer (50 mM NaAc, pH 5, 5 mM MgCl_2 and 1 mM CaCl_2) with pGEM-T plasmid DNA (20 ng/ μ L) at 37 °C. At predetermined time points (2 s, 1, 2, 3, 5, 10 and 15 min) reaction aliquots (10 μ L) were withdrawn, and the digestion was stopped by adding the gel-loading buffer (10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol, and 60 mM EDTA) and then analyzed by agarose gel electrophoresis. Electrophoretic run was performed with TAE 1 $\times$ buffer (40 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.1% (v/v) acetic acid) at a voltage of 90 V.

Metal-dependent activity: rhDNase I and conjugates (0.01 ng/ μ L) were incubated for 3 min in 50 mM NaAc buffer, pH 5, with pGEM-T plasmid DNA (20 ng/ μ L) at 37 °C in the presence or absence of Ca^{2+} and Mg^{2+} . Four different reactions were set up: 1) with 5 mM MgCl_2 and 1 mM CaCl_2 ; 2) with 5 mM MgCl_2 ; 3) with 1 mM CaCl_2 ; 4) without MgCl_2 and CaCl_2 . After stopping the reactions with gel-loading buffer, samples were analyzed using the conditions described above.

2.7.3. Actin inhibition assay

rhDNase I and conjugates (0.2 ng/ μ L) were pre-incubated at 37 °C with increasing amount (0, 80, 200 and 500 μ g/mL) of G-actin from rabbit muscle (BioVision, Inc.) for 20 min in the presence of 0.4 mM ATP, 5 mM Tris-HCl, pH 7.5. Reactions were started by addition of pGEM-T plasmid DNA (20 ng/ μ L) with 5 mM MgCl_2 and 1 mM CaCl_2 . After 20 min of DNA digestion the reaction mixtures were quenched with 10 mM EDTA, mixed with gel-loading buffer (49.75% TAE buffer, 0.25% bromophenol blue and 50% glycerol) and analyzed by 1% agarose gel electrophoresis. Electrophoretic run was performed with TAE 1 $\times$ buffer (40 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.1% (v/v) acetic acid) at a voltage of 150 V. After staining the gel with GelRed, an image was acquired with the molecular imager Azure C400 (azure biosystem).

2.8. Preparation of artificial mucus

CF artificial mucus (CF-AM) was assembled as reported in the literature [22]. For 10 mL of CF-AM, 50 mg of porcine stomach mucin Type II (Merck, Milan, Italy) were dispersed in 3.5 mL of 5 mM Tris-HCl, pH 7.5, prepared with nuclease-free water and stirred for 2 h at room temperature. The mucin suspension was then left at 4 °C for 48 h. In a separate tube, 100 mg of DNA from calf thymus (Merck, Milan, Italy) were dissolved overnight in 6.5 mL of 5 mM Tris-HCl, pH 7.5, at 4 °C to achieve complete solubilization. To the mucin suspension, 50 mg of NaCl, 22 mg of KCl, 50 μ L of egg yolk emulsion (Merck, Milan, Italy), 200 μ L of RPMI 1640 amino acids (Merck, Milan, Italy), 400 μ L of ATP 10 mM (Fisher, Waltham, MA, USA) and G-actin from rabbit muscle (BioVision, Inc.) to reach a final concentration of 80 or 200 μ g/mL (considering 10 mL as

final volume), were added. Finally, the two dispersions of mucins and DNA were mixed and left to equilibrate at 25 °C for 2 h. The obtained pH was around 7.0.

2.9. Effect of rhDNase I and conjugates on CF-AM viscosity

All buffers and reagents were prepared with nuclease-free water. CF-AM was prepared as reported above, without MgCl_2 , by dissolving the components in 5 mM Tris-HCl, pH 7.5, and by adding G-actin from rabbit muscle (BioVision, Inc.) to reach a final concentration of 80 and 200 $\mu\text{g/mL}$. rhDNase I (60 nM) and conjugates (same activity equivalent) were incubated in 150 μL of CF-AM containing actin, at 37 °C and 300 rpm (the enzyme and the conjugates were added in less than 4% (v/v) of the total sample volume). After 10 min, the enzymatic reaction was started by the addition of 5 mM MgCl_2 and the incubation was left to proceed at 37 °C and 300 rpm for 10 min. Viscosimetric tests were then performed using a MCR 92 rotational Rheometer (Anton-Paar, Graz, Austria) equipped with a cone/plate geometry (CP20–2, diameter 20 mm, angle of 2°), controlled at 37 °C. Viscosity was recorded over a shear rate of 1 to 300 s^{-1} ($n = 4$, 13 shear rate values).

In preliminary experiments (not reported) all rhDNase I derivatives were also studied at 1, 3, 5, 7, 10, 15, 20 and 30 nM, showing that CF-AM's viscosity was statistically lower than control (mucus incubated with vehicle 1 mM CaCl_2 , 0.15 M NaCl, pH 6.3) only when 20 or 30 nM enzymes were used, but no significant difference existed among the various rhDNase I derivatives; hence we here present the 60 nM where clear differences can be appreciated.

2.10. Effect of rhDNase I and conjugates on CF-sputum of patients

Sputum from Cystic Fibrosis patients was collected after approval by the Ethics Committee (CET-Liguria 348/2024 - dB id 14,076). Patients were selected based on the following criteria: confirmed diagnosis of Cystic Fibrosis, age > 18 years, able to produce sputum, > 24 h from the last administration of rhDNase I and clinically stable. The patients were all chronically colonized by *Pseudomonas aeruginosa*.

Sputum samples from CF patients were collected in sterile containers. The samples were stored separately at –20 °C until needed. Once thawed, the samples were pooled, homogenized and left for 1 h to allow structure recovering. Mucus samples of 300 μL were incubated with rhDNase I (100 nM) or conjugates (same equivalent activity as native enzyme) for 10 min at 37 °C in a thermomixer without shaking. The solutions of testing molecules were added to mucus reaching 4% (v/v) of the total volume. The control sample was prepared by adding the same volume of vehicle (1 mM CaCl_2 , 0.15 M NaCl, pH 6.3). After incubation, the samples were analyzed rheologically on a Haake MARS III rheometer (Thermo Scientific, Karlsruhe, Germany) operating under shear control, using a 3.5 cm diameter plate/plate geometry and maintaining a 0.1 mm gap. Frequency sweep experiments were carried out at a constant-strain amplitude of 0.05 increasing the frequency from 0.1 to 100 Hz ($n = 3$).

2.11. In vitro aerodynamic assessment by next generation impactor

The aerodynamic assessment of the rhDNase I and PEG24mer-rhDNase I liquid formulations was carried out using a Next Generation Impactor (Copley Scientific Ltd., UK) with eFlow® rapid mesh nebulizer (PARI Pharma GmbH, DE). The amount of protein in the solution was of 0.55 mg/2.5 mL of buffer (1 mM CaCl_2 , 0.15 M NaCl, pH 6.3). The NGI was coupled with a SCP5 vacuum pump (Copley Scientific Ltd., UK) and a Critical Flow Controller (TPK Copley, Copley Scientific Ltd., UK) to set an air flow rate at 15 L/min until the end of the nebulisation indicated by the light signal on the nebulizer.

The protein deposited in each stage of the impactor was collected using the same buffer and quantified by RP-HPLC. The analytical reverse phase (RP) HPLC was performed on an Agilent 1200 Series HPLC with

online UV detection from Agilent Technologies (Santa Clara, CA, USA), using a Jupiter C18 column (4.6 × 250 mm; 5 μm ; Phenomenex, USA) operating at a flow rate of 1 mL/min. The elution was conducted with $\text{H}_2\text{O} + 0.05\%$ TFA (eluent A) and $\text{ACN} + 0.05\%$ TFA (eluent B) (gradient B%: 0 min 30%, 3 min 65%, 3.5 min 65%, 4 min 98%, 7 min 98%, 8.5 min 30%). The UV detector was settled at 226 nm. The amount of protein was calculated using a calibration curve made of solutions of the corresponding compound at known concentrations. The protein deposition allowed for the calculation of different aerodynamic parameters. The emitted dose (ED) was the total amount of protein collected in the NGI. A cumulative distribution curve was obtained by plotting the cumulative percentage of mass lower than a stated diameter (probability scale) versus aerodynamic diameter (log scale). The plot equation allowed for the calculation of the fine particle fraction (FPF%), i.e. the ratio between the mass of protein with an aerodynamic diameter lower than 5 μm and the ED multiplied for 100 and the Mass Median Aerodynamic Diameter (MMAD), corresponding to the midpoint of the distribution curve.

3. Results and discussion

3.1. Synthesis and characterization of PEG20kDa-Nter-rhDNase I, PEG2kDa-rhDNase I and PEG24mer-rhDNase I

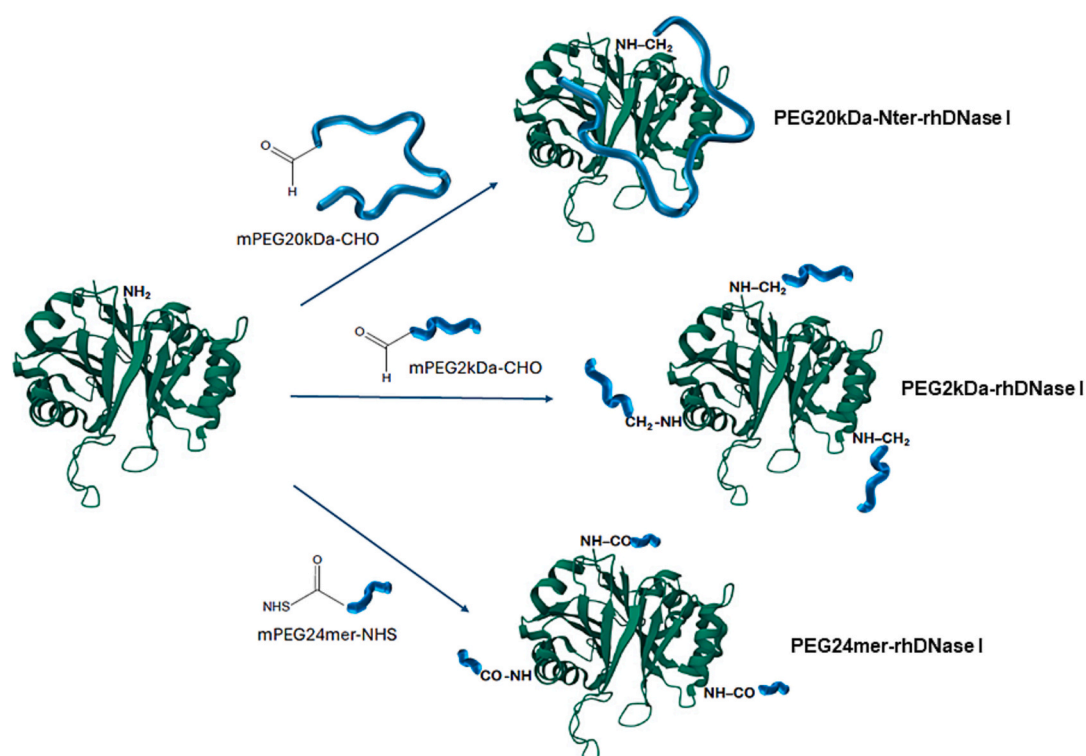
In this study, the synthesis and functional characterization of three PEG-rhDNase I conjugates were investigated. The selected PEGs differed in chain length: a monodisperse PEG of 24 monomers (MW 1214.39 Da), a 2 kDa PEG, and a 20 kDa PEG. Additionally, the conjugation chemistry varied. The bulky PEG20kDa-CHO was site-selectively conjugated to the N-terminal amino group of rhDNase I, while the smaller PEG2kDa-CHO and PEG24mer-NHS were randomly conjugated to the ϵ -amines of lysine residues (Scheme 1).

For PEG20kDa-CHO, N-terminal reductive alkylation of rhDNase I was performed at slightly acidic pH to selectively target the α -amino group, as previously described in the literature [25,26]. In contrast, random conjugation with PEG2kDa-CHO was conducted at pH 7.4 to limit lysine reactivity, preventing excessive PEGylation that could hinder enzyme-DNA interaction. To explore stronger PEGylation conditions, the pH was increased to 8, and a more reactive acylating PEG24mer-NHS was used. This approach allowed for a greater degree of PEGylation, while employing a shorter polymer chain than PEG20kDa.

The rationale behind these approaches was to compare a site-selective monoconjugate (PEG20kDa-Nter-rhDNase I), with a long polymer chain, to two randomly modified conjugates (PEG2kDa-rhDNase I and PEG24mer-rhDNase I), where multiple shorter chains formed a thin PEG coating. The primary goal for all conjugates was to prolong the enzyme's residence time in pulmonary mucus by reducing its sensitivity to proteolytic enzymes and inhibitors (e.g., actin). For conjugates with smaller PEGs, an additional objective was to improve mucus penetration [16].

Beyond polymer chain length and the number of polymer chains per protein, another critical factor influencing conjugate properties is overall charge. Charge can modulate interactions with other molecules, besides the physiological substrate, such as actin and mucopolysaccharides. Conjugates prepared by reductive alkylation retain the same net charge as the native enzyme, as secondary amines are formed during the coupling with PEG-aldehyde. However, PEG24mer-rhDNase I lost one positive charge per conjugated polymer chain due to amide bond formation between the polymer and the protein.

The conjugates were purified from unreacted protein using size exclusion chromatography. A comparison of SEC-FPLC profiles from reaction mixtures (Fig. S1, left panels) and purified conjugates (Fig. S1) confirmed the purity of PEG20kDa-Nter-rhDNase I, PEG2kDa-rhDNase I, and PEG24mer-rhDNase I. In chromatograms of the reaction mixtures (Fig. S1, panels a-c), the unmodified rhDNase I peak was observed at 14.88 mL, while peaks at lower retention times corresponded to the



Scheme 1. Representation of the different approaches of rhDNase I PEGylation by mPEG20kDa-CHO, mPEG2kDa-CHO and mPEG24mer-NHS. The protein structure has been reproduced from PDB (PDB ID 4AWN) [23,24].

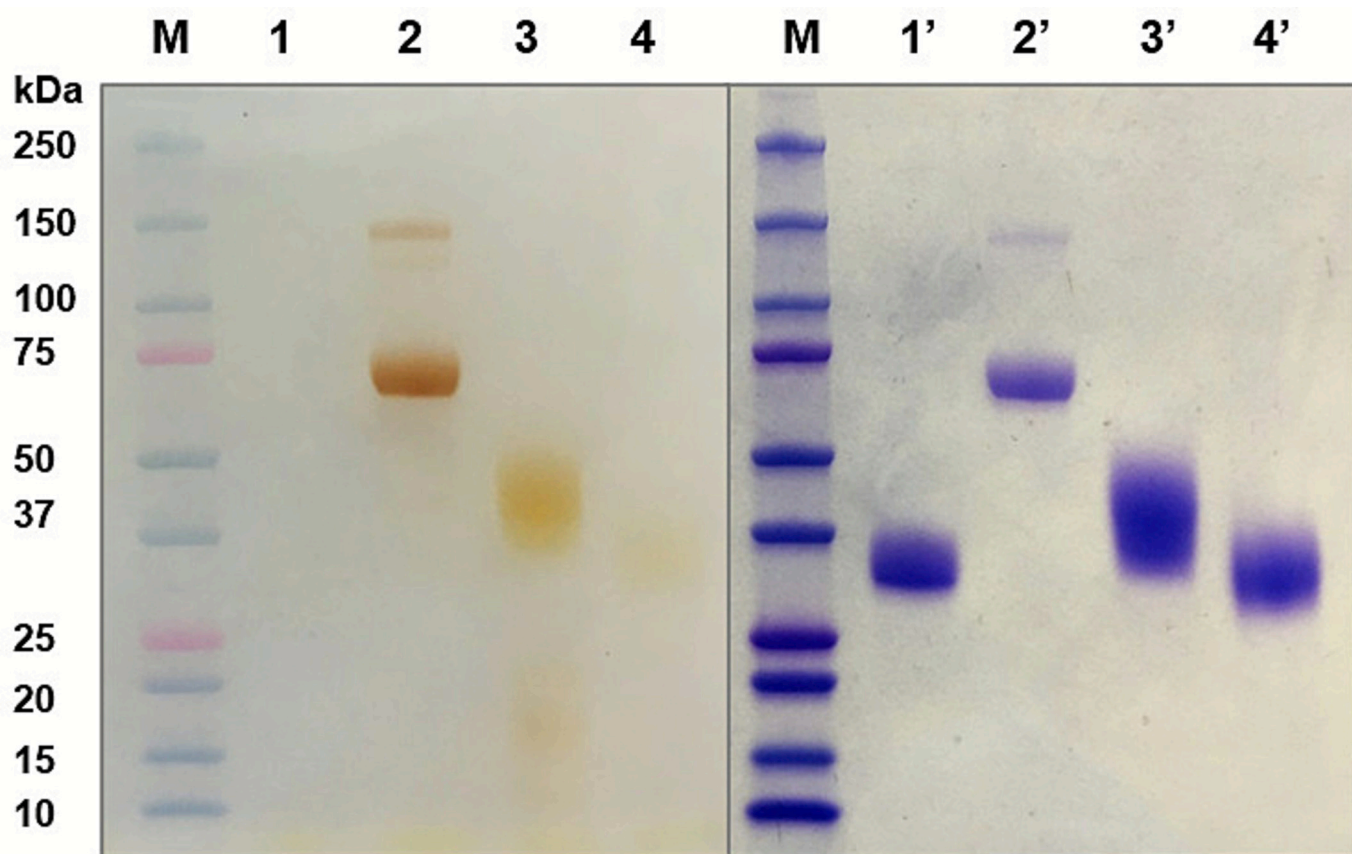


Fig. 1. SDS-PAGE (4–15% gradient gel). Lane 1 and 1': rhDNase I; Lane 2 and 2': PEG20kDa-Nter-rhDNase I; Lane 3 and 3': PEG2kDa-rhDNase I; Lane 4 and 4': PEG24mer-rhDNase I. The markers are in Lane M; The same SDS-PAGE gel was firstly stained by iodine (left panel) for PEG detection and then, after iodine staining removal, the gel was stained with Coomassie (right panel) for protein detection.

conjugates. For PEG20kDa-Nter-rhDNase I, a second purification step using cation exchange chromatography was performed to remove excess unreacted PEG.

PEG20kDa-Nter-rhDNase I, PEG2kDa-rhDNase I, and PEG24mer-rhDNase I were characterized using MALDI-TOF mass spectrometry, SDS-PAGE, and circular dichroism. Mass spectrometry confirmed the formation of the conjugates and provided an estimation of the degree of PEGylation for PEG2kDa-rhDNase I and PEG24mer-rhDNase I (Fig. S2).

The MALDI-TOF spectrum of PEG20kDa-Nter-rhDNase I (Fig. S2b) showed an m/z value of 53,460.8, which corresponded to the sum of the molecular weight of the native protein (32,543.8 Da, Fig. S2a) and one PEG20kDa chain. The spectrum of PEG2kDa-rhDNase I displayed a broad peak centered at 38,446.8 m/z , indicating the attachment of an average of 2.95 PEG2kDa chains (Fig. S2c). For PEG24mer-rhDNase I, the detected m/z value was 35,262.6 Da, corresponding to an average of 2.47 linked PEG24mer chains (Fig. S2d).

The success of PEGylation and the purity of the conjugates were further confirmed by SDS-PAGE analysis. As shown in Fig. 1, the PEGylated products were free of unreacted rhDNase I. A minor content (0.3%) of di-PEGylated rhDNase I was observed in the PEG20kDa-Nter-rhDNase I sample, which became more evident with iodine staining of the SDS-PAGE gel (Fig. 1, right panel). Iodine staining highlights PEG, and thus the di-PEG conjugate appears more prominent due to its double polymer content compared to the monoconjugates.

The apparent hydrodynamic volumes determined by SDS-PAGE were ~ 70 kDa for PEG20kDa-Nter-rhDNase I, ~ 37 kDa for PEG2kDa-rhDNase I, and ~ 35 kDa for PEG24mer-rhDNase I. As expected, PEGylated protein bands in SDS-PAGE showed overestimated molecular weights due to the large hydrodynamic volume of PEG.

In summary, the results from MALDI-TOF and SDS-PAGE analyses confirmed the successful PEGylation of rhDNase I and the effective purification of the conjugates from unreacted protein.

The preservation of a protein's native structure is fundamental to its functionality. Therefore, the secondary structure of rhDNase I in the conjugates was examined using far-UV circular dichroism (CD) spectroscopy. The enzyme features a sandwich-like structure comprising two 6-stranded β -sheets that form a large hydrophobic core, surrounded by helices and loop regions [27]. The CD spectra of the conjugates were superimposable with that of the native rhDNase I (Fig. 2), indicating that no alterations in the protein's secondary structure occurred during the synthesis using the various PEGylation strategies.

Finally, the sensitivity to proteolytic degradation was tested by incubating rhDNase I and conjugates with the proteolytic enzyme Glu-C. All PEGylated conjugates exhibited superior resistance to Glu-C approach compared to native rhDNase I. After 8 h and 30 min, 76.7%

of the rhDNase I has been degraded while conjugates were still present as non-degraded protein in a considerable amount (72% or more) (Fig. S3).

3.2. Evaluation of DNA-hydrolyzing activity of conjugates by Kunitz assay

The activities of rhDNase I and its conjugates were determined using an adapted Kunitz hyperchromicity assay. The *in vitro* biological activity of the conjugates was compared to that of native rhDNase I and expressed as a percentage of relative activity, with the activity of rhDNase I serving as the reference.

The conjugates PEG20kDa-Nter-rhDNase I and PEG2kDa-rhDNase I exhibited slight activity losses, retaining residual activities of 83.8% and 71.4%, respectively (Table 1). In contrast, PEG24mer-rhDNase I showed the lowest activity at 33.7%. This reduction is likely attributable to the loss of positive charges caused by amide bond formation between the enzyme and the polymer, which decreased the conjugate's affinity for negatively charged DNA. This hypothesis aligns with previous findings showing that hyperactive DNase I variants were created by introducing one to six positively charged residues at the DNA-binding interface [28].

Additionally, a sample of rhDNase I was purified via size-exclusion chromatography (SEC) to simulate the purification stress experienced by the conjugates. This step caused only a slight decrease in enzymatic activity, confirming that the loss of activity in the conjugates is primarily due to the PEGylation chemistry.

3.3. DNA-hydrolyzing activity evaluation against supercoiled plasmid DNA

The therapeutic effect of rhDNase I is achieved by reducing mucus viscosity through the degradation of DNA strands into smaller fragments [29]. To validate the results observed in the Kunitz assay, the endonuclease activity of rhDNase I and its conjugates was also assessed using a plasmid digestion assay. The kinetics of DNA degradation were analyzed via agarose gel electrophoresis. rhDNase I and its conjugates were incubated with pGEM-T supercoiled plasmid DNA at 37 °C in the presence of Ca^{2+} and Mg^{2+} , and activity was evaluated at various time points. Supercoiled plasmid DNA was chosen because, compared to linear DNA, it enables discrimination between single- and double-strand cuts. As shown in Fig. 3, rhDNase I and its conjugates followed the same degradation mechanism: they cleave double-stranded DNA by introducing single-strand nicks, as indicated by the formation of circular DNA prior to the release of linear DNA. Indeed, at the earliest reaction time point, all tested compounds exhibited increased intensity of the circular DNA band and decreased intensity of the supercoiled DNA band, both relative to the control. This was followed by the appearance and increase in intensity of the linear DNA band, with subsequent generation of DNA fragments, consistent with previously described DNase I mechanisms [30].

The plasmid digestion assay confirmed that PEG20kDa-Nter-rhDNase I and PEG2kDa-rhDNase I were the most active conjugates. PEG2kDa-rhDNase I exhibited the highest endonuclease activity, closely matching the timing of native rhDNase I. PEG20kDa-Nter-rhDNase I was

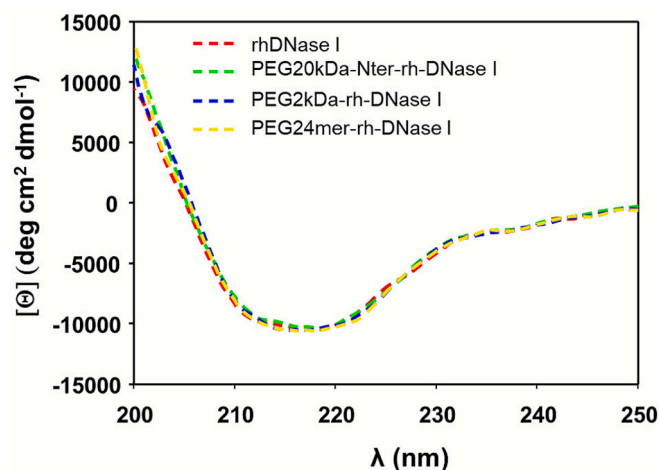


Fig. 2. CD spectra of rhDNase I and conjugates between 200 and 250 nm at 25 °C.

Table 1

Enzymatic activity of native, purified and conjugated rhDNase I calculated by Kunitz hyperchromicity assay. Activities are expressed as % in relation to native rhDNase I.

Compound	Relative Activity (%)
rhDNase I	100 ± 15.6
rhDNase I (after SEC)	95.9 ± 13.4
PEG20kDa-Nter-rhDNase I	83.8 ± 7.9
PEG2kDa-rhDNase I	71.4 ± 16.9
PEG24mer-rhDNase I	33.7 ± 7.2

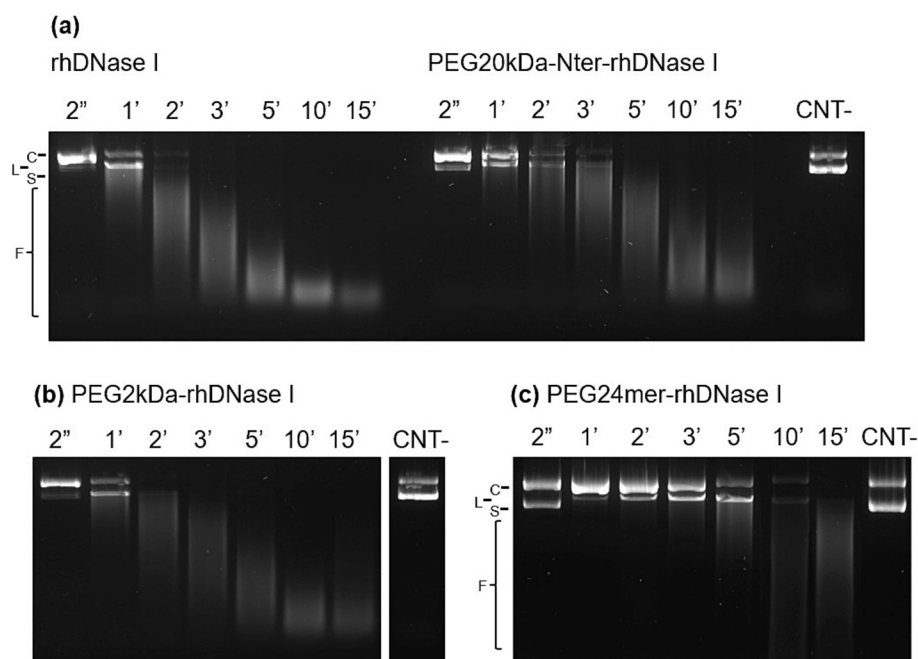


Fig. 3. Agarose gels of endonuclease activity of rhDNase I and conjugates using supercoiled plasmid DNA. pGEM-T plasmid DNA (20 ng/ μ L) was treated with a) rhDNase I, on the left and PEG20kDa-Nter-rhDNase I on the right, b) PEG2kDa-rhDNase I and c) PEG24mer-rhDNase I at a concentration of 0.01 ng/ μ L (rhDNase I equiv.) in 50 mM NaAc buffer, pH 5, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and incubated at 37 °C to up 15 min. Aliquots (10 μ L) were taken after 2 s, 1, 2, 3, 5, 10, and 15 min and mixed with gel-loading buffer to stop the reaction. CNT: control, refers to the plasmid DNA that has not been treated with the enzyme and exhibits supercoiled and circular conformations. Plasmid DNA forms are indicated to the left of the panels as C: circular DNA, L: linear DNA, S: supercoiled DNA, F: degraded fragments.

slightly slower, with complete disappearance of the linear DNA conformation observed after 5 min. PEG24mer-rhDNase I required more than 10 min to degrade the DNA, making it the least active conjugate, in agreement with the Kunitz assay results.

This reduced activity is likely due to the loss of positive charges caused by PEGylation at lysine residues, which diminished the enzyme's binding affinity for DNA, as above suggested.

The dependence of DNA-hydrolyzing activity on calcium and

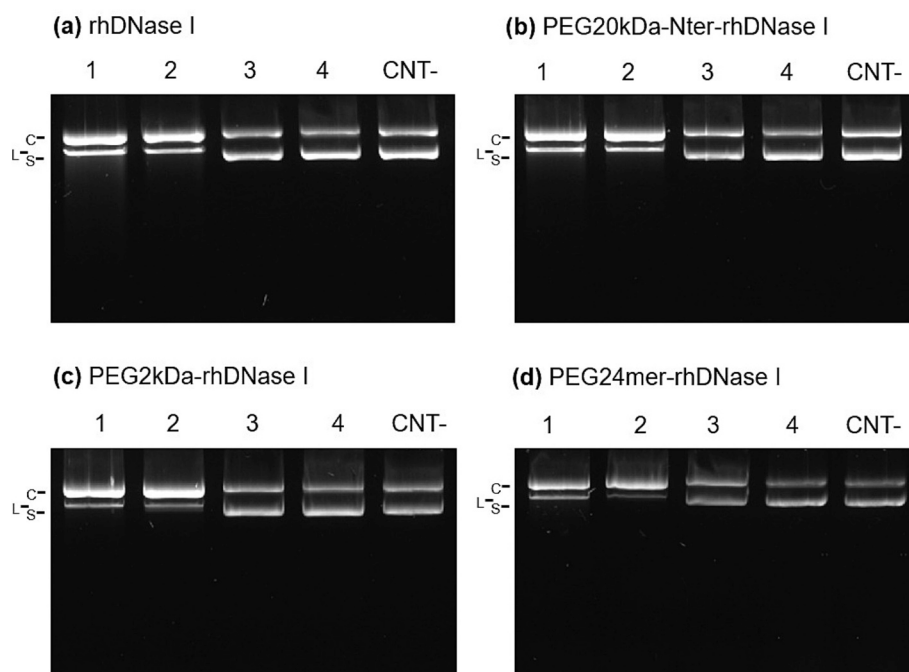


Fig. 4. Agarose gels of endonuclease activity of rhDNase I and conjugates in the presence or absence of $CaCl_2$ and $MgCl_2$. pGEM-T plasmid DNA (20 ng/ μ L) was treated with a) rhDNase I, b) PEG20kDa-Nter-rhDNase I, c) PEG2kDa-rhDNase I and d) PEG24mer-rhDNase I at a concentration of 0.01 ng/ μ L (rhDNase I equiv.) in 50 mM NaAc buffer, pH 5 and incubated at 37 °C for 3 min. Lane 1: reaction in presence of 5 mM $MgCl_2$ and 1 mM $CaCl_2$; Lane 2: reaction in presence of 5 mM $MgCl_2$; Lane 3: reaction in presence of 1 mM $CaCl_2$; Lane 4: reaction in absence of $MgCl_2$ and $CaCl_2$. CNT: control, it refers to plasmid DNA that has not been treated with the enzyme and exhibits supercoiled and circular conformations. Plasmid DNA forms are indicated to the left of the panels as C: circular DNA, L: linear DNA, S: supercoiled DNA.

magnesium cations was evaluated by incubating the compounds with pGEM-T plasmid DNA at 37 °C for 3 min in the presence or absence of CaCl₂ and MgCl₂. As shown in Fig. 4, the presence of both divalent cations was essential for DNase activity, although the reaction still proceeded in the presence of magnesium alone, consistent with findings reported in the literature [31–33].

3.4. DNA-hydrolyzing activity of rhDNase I and conjugates in the presence of actin

Actin is an abundant protein in neutrophils, released into CF sputum upon their lysis, and it serves as a specific, natural inhibitor of DNase I [34]. Alongside DNA, actin contributes to the viscosity of CF sputum [35,36]. It predominantly exists as filamentous polymers (F-actin), which are in equilibrium with the monomeric globular form, G-actin. DNase I binds to monomeric actin, forming a 1:1 complex that gradually depolymerizes F-actin [37,38]. This specific binding tightly inhibits DNase I activity. Actin concentration in CF sputum varies widely among patients, typically ranging from 0.1 to 0.3 mg/mL, though it can reach up to 5 mg/mL in some cases [35,39].

Given actin's role as a natural inhibitor of rhDNase I, its impact on the DNA-hydrolyzing activity of the conjugates was evaluated through a plasmid digestion assay using increasing concentrations of actin (0, 80, 200, and 500 µg/mL). The results were compared to those of the native protein under the same conditions. To promote the depolymerization of F-actin and facilitate the formation of the rhDNase I:G-actin complex, rhDNase I and its conjugates were incubated with actin in the presence of ATP and CaCl₂ [40,41].

As shown in Fig. 5, actin inhibited the activity of both the native protein and the conjugates, albeit to varying extents. At all actin concentrations tested, the accumulation of linear DNA was evident for all compounds. However, PEG24mer-rhDNase I demonstrated the formation of a DNA fragment smear, indicating that its activity persisted. Similarly, PEG20kDa-Nter-rhDNase I began fragmenting linear DNA even at the lowest actin concentration. At 500 µg/mL of actin, PEG24mer-rhDNase I was the only compound that achieved complete degradation of supercoiled DNA.

Overall, native rhDNase I proved the most susceptible to actin inhibition, as a greater amount of circular DNA and residual supercoiled DNA were still detectable when enzyme was incubated with 200 µg/mL of actin. Conversely, PEG20kDa-Nter-rhDNase I and PEG2kDa-rhDNase I exhibited greater DNA-hydrolyzing activity than the native protein under all inhibitory conditions. Despite their lower relative activity in solution compared to rhDNase I, the conjugates were more effective in degrading plasmid DNA in the presence of actin. This enhanced activity is likely due to the PEG's shielding effect, attributed to its high hydrodynamic volume. Interestingly, the higher activity of PEG24mer-

rhDNase I may also be linked to the absence of lysine's positive charges (following PEG conjugation), which could reduce its affinity for negatively charged actin, allowing for a surprising recovery of enzymatic activity under physiological conditions of actin inhibition.

3.5. Effect of rhDNase I and conjugates on the rheology of artificial mucus in presence of actin

Synthetic analogs of mucus always show shear-thinning behavior [42,43], as it is most frequent for macromolecular solutions. CF-AM as prepared by us (i.e. supplemented with free DNA) has a viscosity of 10.2 ± 0.6 Pa·s at 1 s^{-1} shear rate, similarly to our previous studies [21]. In mucus produced by inflamed tissues and mostly in that of all CF patients, cross-linked mucins form very large (usually >1 mm) aggregates, which cause major differences with the rheology of artificial mucus: chiefly, a change from a pronounced viscoelastic (CF mucus) to a predominantly viscous (CF-AM) performance. Additionally, a significant macro- vs. micro-scale discrepancy has been reported in CF but not in artificial mucus [44]. These differences make it virtually impossible to wholly mimic mucus rheology with CF-AM [45,46]. However, CF-AM can provide key comparative information about the effect of DNase activity among the conjugates and the native enzyme: soluble DNA is an important contributor to the viscosity of both artificial and CF mucus, hence the extent of decrease in viscosity after incubation with rhDNase I or its conjugates, irrespective of the degree of heterogeneity and of the overall character of the fluid, would correlate to the effectiveness of DNA degradation.

Here, we have compared the effect of native vs. PEGylated rhDNase I on CF-AM viscosity under conditions of partial inhibition by G-actin: this carries a complex dependency, in that it takes into account DNase activity (reduced by PEGylation), inhibition (possibly reduced by PEGylation) and stability (increased by PEGylation). Due to the use of protease-free water, physiological conditions of pH, temperature etc. and short incubation times, it is unlikely that DNase own degradation/denaturation may play a significant role in our results; therefore, our target narrows to the assessment of which rhDNase I derivative may display the most robust performance in terms of maximal DNA degradation and minimal sensitivity to inhibition.

There is a considerable variability in the reported values of extracellular DNA present in CF sputa, with most reported values around 4 [47] or 3 mg/mL [48]. We here have employed a CF-AM supplemented with 10 mg/mL DNA (according to a recipe used in other studies for a CF therapy [22]), i.e. a concentration higher than that reported for most clinical CF samples, but useful to highlight better both the effects of conjugates on viscosity and the difference in the resistance against G-actin inhibition. In this DNA-rich CF-AM, we have incubated rhDNase I and its conjugates with 80 or 200 µg/mL G-actin for 10 min in order to

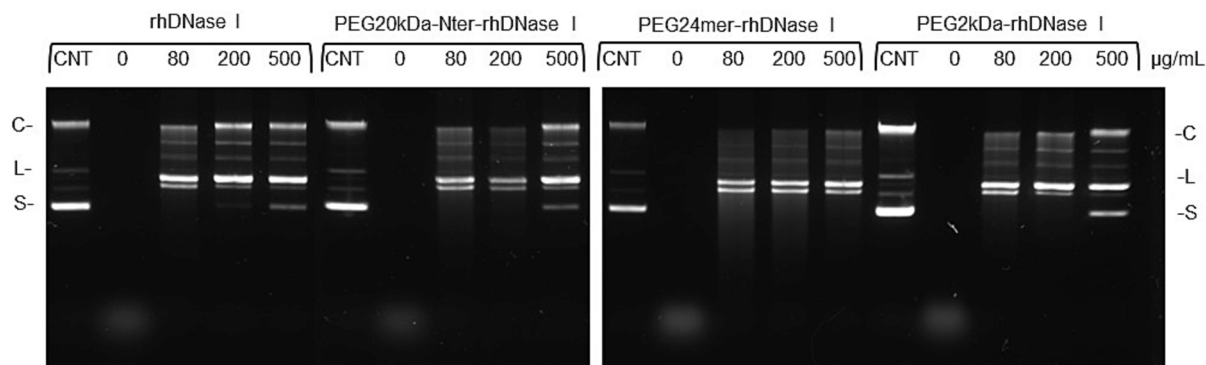


Fig. 5. The endonuclease activity of rhDNase I and conjugates was assessed by measuring migration in agarose gels of pGEM-T plasmid DNA (20 µg/mL) in the presence of rhDNase I and conjugates (0.2 µg/mL rhDNase I, corresponding to 5.4 nM, and equivalent molar concentrations for all conjugates), and increasing amount of G-actin (0, 80, 200 and 500 µg/mL). CNT: control, refers to plasmid DNA that has not been treated with the enzyme and exhibits supercoiled and circular conformations. Plasmid DNA forms are indicated to the right of the image as C: circular DNA, L: linear DNA, S: supercoiled DNA.

achieve the full inhibitory potential, before adding MgCl_2 , which starts DNA degradation by the enzyme (additional 10 min of incubation).

Both native rhDNase I and its conjugates lowered CF-AM's viscosity across a broad shear rate range (37°C , shear rate from 1 to 300 s^{-1}) with $80\text{ }\mu\text{g/mL}$ G-actin, and this was more noticeable for conjugates, and in particular for PEG24mer-rhDNase I (Fig. 6A). Interestingly, the higher concentration of G-actin ($200\text{ }\mu\text{g/mL}$) strongly reduced this effect for all DNases, but not for PEG24mer-rhDNase I, which still showed a markedly lower viscosity.

This finding is more apparent by comparing the results at a single shear rate (Fig. 6B). PEG24mer-rhDNase I, on the contrary, showed both the largest decrease in viscosity (≈ 3.1 – 3.3 -fold less than untreated mucus) and the lowest inhibitory effect of G-actin, with no dependency over the actin concentration.

Moreover, despite the differential effects on viscosity of rhDNase I derivatives, CF-AM's shear-thinning character remained untouched, with the viscosity above 100 s^{-1} about 2.5 orders of magnitude lower than that at 1 s^{-1} . This indicates that the major contributor to CF-AM rheology, i.e. soluble/dispersible mucins, was not affected by DNase action, as expected.

These findings align with the results in Fig. 5 in indicating that PEG24mer-rhDNase I has A) all-in-all the best performance, with the smallest reduction in the overall activity in CF-AM (= lowest viscosity at both actin concentrations); B) the lowest inhibition by G-actin, in that the CF-AM viscosity does not appear to depend on the amount of actin. This feature of PEG24mer-rhDNase I is difficult to rationalize purely on the basis of its PEG chain length: a dose-dependent inhibition by actin was recorded without PEG, with slightly longer (2 kDa) PEG chains and with much longer (20 kDa) ones. We are indeed inclined to ascribe PEG24mer-rhDNase I's behavior to the non-protonatable amide bond connecting enzyme and PEG, as opposed to the protonatable primary amines in the native enzyme or secondary amines in the other two conjugates. The resulting lower surface charge may be the critical factor in the complexation with actin.

3.6. CF patient's mucus rheological behavior after incubation with native and PEGylated rhDNase I derivatives

The effects of rhDNase I and its conjugates on CF patient's mucus were investigated by means of oscillatory rheology. Frequency sweep tests were performed at a constant strain of 0.05 over a frequency range of 0.1 to 100 Hz on both CF mucus and CF mucus treated for 10 min at 37°C with rhDNase I or with its conjugates.

CF mucus is viscoelastic: consistently, its storage modulus G' exceeds

the loss modulus G'' , and their ratio $\tan\delta$ is <0.8 at any frequency (Fig. 7A), which brings it close to the limit of an elastic response. Notably, above a critical frequency (around 60 Hz; see yellow arrow in Fig. 7A, bottom left) $\tan\delta$ drops, i.e. the elastic component becomes more dominant. This hardening at high frequencies indicates that CF mucus became stiffer or more solid-like when subjected to rapid deformations, which probably hints to macroscopic clumping of mucin aggregates.

We hypothesize that DNases act on the soluble ('dispersing') part of the mucus but not on the aggregated ('dispersed'). This action would directly lower the overall viscosity of mucus; indirectly, however, it may also facilitate this shear-induced hardening, by lowering the critical shear necessary for it to happen (see sketch in Fig. 7B).

Our results indicate that, in general, all DNase treatments preserved the shape of the G'/G'' frequency dependency. In particular, at low frequencies both G' and G'' increase gradually, but the plateau in $\tan\delta$ indicates that the overall character of the rheological response remains broadly the same (still viscoelastic bordering elastic, $\tan\delta \approx$ as an average throughout the whole frequency range). Also, a sudden hardening occurs above a critical frequency (yellow arrows in Fig. 7C).

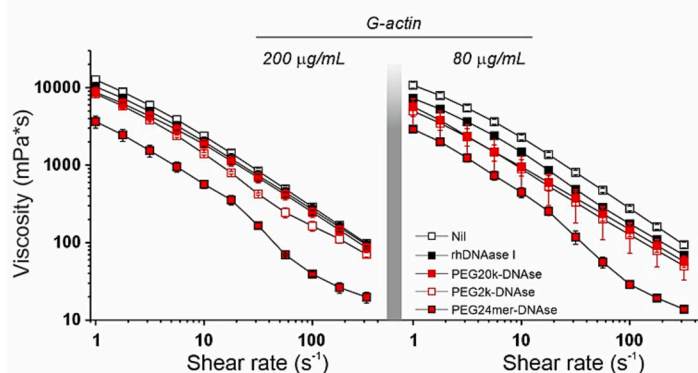
In comparison to untreated mucus, all DNases lowered both moduli (red arrows in Fig. 7C, left) and critical frequency (yellow arrows in Fig. 7C, left). DNA degradation thus made mucus 'softer'/thinner at lower frequencies - such as those typical of breathing (around 0.5 Hz) or ciliary beating (around 10 Hz [47]) - but also easier to harden at higher frequencies. Most importantly for our study, all these effects (both the reduction in G' and in critical frequency) depended on the kind of DNase: native rhDNase I showed the smallest effects, PEG24mer-rhDNase I the largest ones (Fig. 7D, left). The same dependencies can be observed when the rheological results are expressed in terms of complex viscosity (Fig. 7D, right).

In correspondence to the critical frequency, one can calculate an 'average' shear rate of the whole oscillatory motion (i.e., amplitude of the oscillation $\times$ frequency), and it turns out that this parameter perfectly mirrors G' dependency on the kind of rhDNase I derivative (Fig. 7C, right). This suggests that the same physical phenomenon triggered by DNA degradation causes the decrease both in G' and in critical frequency, thereby confirming our hypothesis that the lower viscosity of the fluid phase makes mucus thinner, but also more 'cloggable' at high frequencies.

Of note, G' of the shear-hardened mucus was not affected by the DNase treatment, i.e. depended solely on the features of mucin 'clumps'.

All the key findings from our comparative analysis of the activity of rhDNase I and its derivatives under different conditions and environments are summarized in Table 3.

A | DNase(s) effect on CF-AM viscosity (shear rate depend.)



B | Effect of DNase(s) inhibition on viscosity (1 s^{-1})

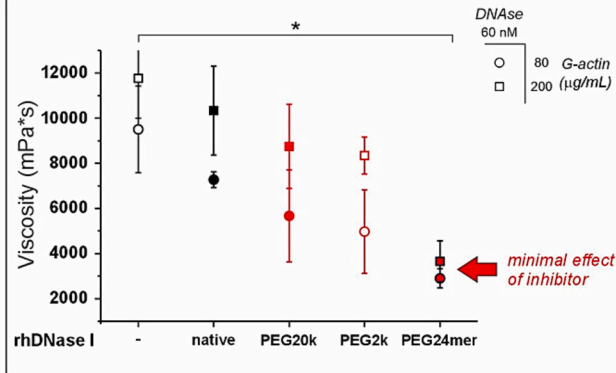


Fig. 6. A) Effect of 60 nM rhDNase I and conjugates (same activity equivalent respect to native enzyme) in CF-AM on the shear viscosity dependency on shear rate. CF-AM was supplemented with 200 (left) or 80 (right) $\mu\text{g/mL}$ of G-actin. All samples showed an essentially identical shear-thinning character, but with significant differences in viscosity. B) Comparison of the effects of rhDNase I (native and conjugates) at a fixed shear rate of 1 s^{-1} ($n = 4$); PEG24mer-rhDNase I was the least sensitive derivative to the inhibiting action of actin (1-way ANOVA test was applied for statistical analysis of the results (viscosity of DNases + actin in CF-AM against the same concentrations of actin in CF-AM) - $*p < 0.05$).

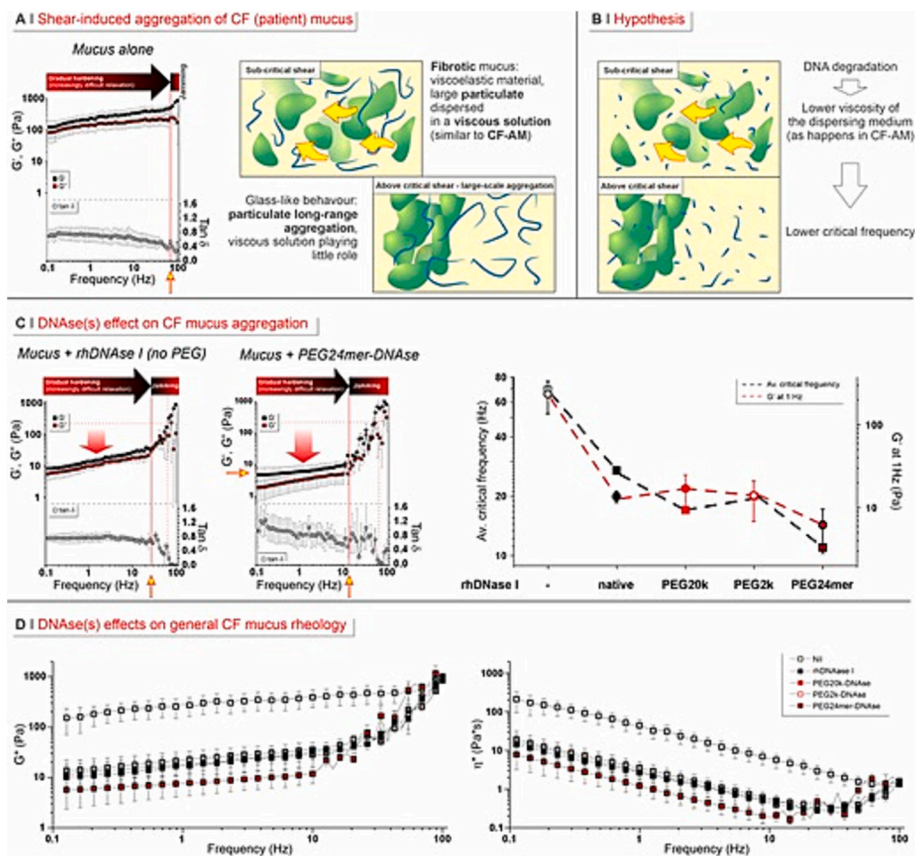


Fig. 7. A) Frequency dependency of storage (G') and loss (G'') modulus (top left), and $\tan\delta$ (bottom left) for untreated CF mucus ($n = 3$). The yellow arrow highlights the critical frequency for hardening. The scheme graphically summarizes the shear-induced aggregation (right). B) Scheme of the possible rhDNase I effect on mucus; the lower viscosity of the dispersing (liquid) medium may ease the phenomena of shear-induced aggregation. C) Left. Frequency dependency of G' , G'' and $\tan\delta$ of CF mucus with 100 nM native rhDNase I (left) and PEG24mer-rhDNase I (centre) ($n = 3$). The horizontal, red pointed line marks the average G'/G'' values of the control (mucus treated with buffer) used as a guide for eyes to assess the reduction (red bold arrows). Right. A critical average shear rate (shear rates oscillate sinusoidally) can be calculated by dividing the oscillation amplitude (0.05) by the critical frequency. The values of G' (1 Hz, i.e. in the $\tan\delta$ plateau) show a perfect agreement with those of this critical shear rate. D) Frequency dependencies of complex modulus (G^* , left) and complex viscosity (η^* , right) for the above described experiments ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. Nebulization and pulmonary deposition by next generation impactor

Pulmozyme® is supplied in single-use ampules containing an aqueous solution of rhDNase I supplemented with CaCl_2 (1 mM) and NaCl (0.15 M). The solution can be administered using a jet nebulizer and compressor or a vibrating mesh nebulizer, which transforms the liquid solution into a fine mist that can be inhaled to reach the lungs.

The nebulization of PEG24mer-rhDNase I was studied and compared to that of rhDNase I using an eFlow mesh nebulizer. The aerodynamic properties of rhDNase I and PEG24mer-rhDNase I were assessed using a Next Generation Impactor (NGI) which allows to separate the particles based on their aerodynamic size, providing data on the respirable fraction and particle size distribution.

In both products analyzed, the emitted protein amount was approximately 290–300 μg , corresponding to about 55% of the loaded dose. This result can be attributed to the nebulizer's tendency to retain more than 1 mL of liquid at the end of the nebulization [49].

The deposition of rhDNase I and PEG24mer-rhDNase I in the NGI revealed that the native protein tends to deposit more than the conjugate in the first stages of the NGI (stages 3 and 4) whereas for PEG24mer-rhDNase I, the deposition is higher in stages 5 and 6, where the smaller particles are captured (Fig. 8). The fine particle mass for the native and conjugated protein was respectively of 185.4 μg and 221.1 μg . Consequently, the conjugate PEG24mer-rhDNase I demonstrated a significantly higher fine particle fraction of 72.3% ($p < 0.001$) and a significantly smaller MMAD of 3.69 μm ($p < 0.01$) compared to free

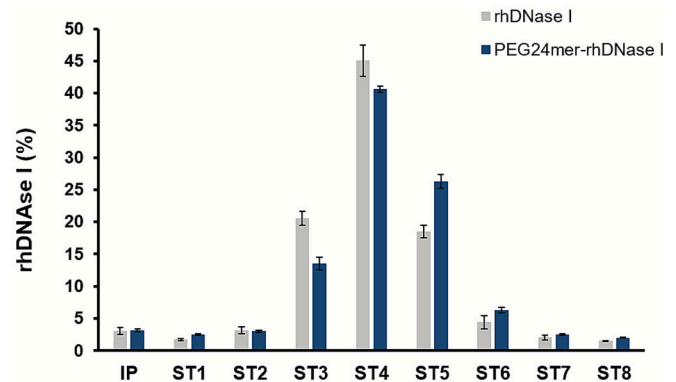


Fig. 8. Aerosol particle size distribution of rhDNase I and PEG24mer-rhDNase I in the NGI. Stage 1 (ST1) $\mu\text{m} \geq 14.1$, Stage 2 (ST2) $\mu\text{m} \geq 8.61$, Stage 3 (ST3) $\mu\text{m} \geq 5.39$, Stage 4 (ST4) $\mu\text{m} \geq 3.30$, Stage 5 (ST5) $\mu\text{m} \geq 2.08$, Stage 6 (ST6) $\mu\text{m} \geq 1.36$, Stage 7 (ST7) $\mu\text{m} \geq 0.98$, Stage 8 (ST8) $\mu\text{m} < 0.98$ ($n = 3 \pm \text{SD}$).

rhDNase I (Table 2). The data obtained are consistent with those reported by Scherer et al. [50] where in addition to characterizing the respirable fraction with different nebulizers, the authors also demonstrated the post-nebulization integrity of the DNase I. This finding is in agreement with the mass balance in our experiments, which was $>98\%$ in both the cases, and the chromatograms did not reveal peaks

Table 2

Nebulization performance of rhDNase I and PEG24mer-rhDNase I formulations (loaded dose 550 µg/2.5 ml) and fine particle fraction lower than 5 µm (n = 3; means ± std. dev); MMAD stays for mass median aerodynamic diameter. Significance was calculated with a two-tailed unpaired t-test with significance level fixed at p-value = 0.05.

Sample	Emitted Dose (µg)	Fine Particle Mass (µg)	Fine Particle Fraction (%)	MMAD (µm)
rhDNase I	289.6 ± 6.6	185.4 ± 1.9	64.1 ± 1.1	4.12 ± 0.10
PEG24mer-rhDNase I	306.0 ± 6.9 ^c	221.1 ± 3.3	72.3 ± 1.0 ^b	3.69 ± 0.05 ^b

^a p < 0.001 versus rhDNase I.

^b p < 0.01 versus rhDNase I.

^c p < 0.05 versus rhDNase I.

attributable to protein degradation.

The result suggests that the polymer facilitates the formation of particles with a reduced surface area, likely due to a surfactant effect. This result is in line with a study investigating the effect of PEGylation of lipid nanoparticles. The aerodynamic assessment through NGI demonstrated that the nebulization is a suitable strategy for the aerosolization of protein, which allowed to achieve a satisfactory ED and drug deposition in the peripheral portion of the lungs. In addition, the modification with small PEG chains allowed a better aerodynamic profile compared with the free rhDNase I, achieving a higher respirable fraction. A further characterization of PEG24mer-rhDNase I was performed by determining its activity after nebulization. The residual enzymatic activity was quantified by Kunitz assay and was found to be retained by 97.4% ± 1.8, suggesting that the nebulization process gently generates a fine mist of the PEGylated protein that is still active.

4. Conclusions

We synthesized three PEG conjugates of rhDNase I by varying the polymer length and the reactive functional group, aiming to prolong the residence time of the enzyme in pulmonary mucus through reduced sensitivity to proteolytic enzymes and/or inhibitors and enhanced mucus penetration. Another key feature of the conjugates is their overall charge, which can modulate interactions with other molecules, such as actin and mucopolysaccharides. The conjugates PEG20kDa-Nter-rhDNase I and PEG2kDa-rhDNase I were prepared by reductive alkylation and designed to maintain the same net charge as the parent enzyme. In contrast, the conjugate PEG24mer-rhDNase I lost a positive charge for each polymer chain coupled.

The most noticeable effect of rhDNase I and of its conjugates is the reduction of viscosity, which we observed both with artificial and CF patients' mucus. We prefer to express the rheological effects in terms of viscosity than G' (which shows a parallel reduction), because we ascribe them to an activity in the dispersing (liquid-like) phase of mucus. The viscosity was reduced more by the PEGylated conjugates than by the native enzyme, but PEG24mer-rhDNase I stood out as by far the most

Table 3

Summary comparison table of the key activity results of rhDNase I and its PEGylated variants.

Compound	Activity on supercoiled DNA plasmid		Activity in mucus	
	- Actin	+ Actin	CF artificial mucus	CF patient mucus
rhDNase I	+++	0/+	+	+
PEG20kDa-Nter-rhDNase I	++	++	++	++
PEG2kDa-rhDNase I	+++	+	++	++
PEG24mer-rhDNase I	0/+	+++	+++	+++

effective derivative, both in its own viscosity-reducing activity and in stabilization against G-actin inhibition. We are inclined to ascribe this to a combination of low hindrance of its shorter PEG chains (thereby minimizing loss of enzymatic activity) and reduced positive charge (less favored complexation with the G-actin) that boost the known advantage of its PEGylated nature (likely reduced proteolytic degradation). Our results thus suggest that PEGylation via acylation with short polymer chains is the most promising strategy to enhance the pharmacological properties of rhDNase I. Additionally, PEG24mer-rhDNase I was successfully nebulized maintaining almost full activity post-nebulization, suggesting that it could be administered in the same way as rhDNase I, with even a better deep lung penetration, making its clinical translation a real possibility.

A final remark is that we have noticed that reduced mucus viscosity – and therefore its easier removal via coughing – may come at the price of a higher likelihood of 'clogging' mucus at higher frequencies/shear. Therefore, a caveat should be issued that extracellular DNA degradation may have a 'Goldilocks' optimum (addressable through the enzyme dose), above which undesired effects may appear. Despite this caveat, the up to 5-fold reduction in mucus viscosity upon DNase PEGylation, as in the case of PEG24mer-rhDNase I, is a remarkable feature together with the reduced sensitivity to G-actin inhibition, which might considerably improve areas where native DNase treatments have shown beneficial effects on sub-sets of CF patients' population, such as in exercise capacity [51].

Abbreviation

rhDNase I	recombinant human deoxyribonuclease I
CF	cystic fibrosis
PEG	polyethylene glycol
CFTR	cystic fibrosis transmembrane conductance regulator
MW	molecular weight
PBS	phosphate-buffered saline
SEC	Size Exclusion Chromatography
IEC	Ion Exchange Chromatography
CD	Circular dichroism
MRW	mean residue weight
CF-AM	CF artificial mucus
CNT	control
NGI	Next Generation Impactor
MMAD	mass median aerodynamic diameter

CRediT authorship contribution statement

Antonella Grigoletto: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giulia Mori:** Writing – review & editing, Formal analysis. **Danila Delfino:** Formal analysis. **Gloria Bizzotto:** Writing – original draft, Methodology, Investigation. **Benedetta Campana:** Formal analysis. **Maria Natalia Calienni:** Formal analysis. **Francesca Buttini:** Validation, Supervision, Methodology, Data curation. **Stefania Glieda:** Formal analysis, Data curation. **Giulia Della Rosa:** Methodology, Formal analysis, Data curation. **Tommaso Tedeschini:** Formal analysis. **Rosaria Casciaro:** Writing – review & editing, Funding acquisition. **Nicola Tirelli:** Writing – review & editing, Validation, Supervision, Data curation. **Riccardo Percudani:** Writing – review & editing, Funding acquisition, Conceptualization. **Gianfranco Pasut:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in

order to proofread the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the published article.

Declaration of competing interest

GP has been scientific advisor for NOF CORPORATION. All other authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.150145>.

Data availability

Data will be made available on request.

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