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# Enhanced properties of discrete pulmonary deoxyribonuclease I (DNaseI) loaded PLGA nanoparticles during encapsulation and activity determination

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ABSTRACT

In the present work, DNaseI loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) for pulmonary delivery were prepared using emulsion solvent evaporation. The effects of the various formulation and experimental variables on the size and morphological characteristics of the particles as well as on the encapsulation efficiency were investigated. The stability of the encapsulated DNaseI was evaluated and the respirable fraction was determined. Cytotoxicity of the NPs was evaluated on lung epithelial cells. The results showed that by using leucine and dipalmito-phosphatidyl-choline (DPPC), discrete NPs with 76% retained biological activity were prepared. A high respirable fraction (particles below 6 μm) reaching 71.3% was achieved after nebulization of the NP suspension. The results revealed the suitability of the prepared particles for pulmonary delivery and highlighted the role of excipients in the stabilization of DNaseI against the stresses encountered during preparation.



## 1. Introduction

Cystic fibrosis (CF) is a common autosomal recessive disorder in the Caucasian population, affecting 1 in 2000 of live births (Sheppard and Nicholson, 2002). It affects the epithelial lining of major organs, particularly the lungs, with the majority of mortalities occurring due to pulmonary complications (Gómez and Prince, 2007). The pulmonary disease is characterized by retention of airway secretion, with the production of viscous sputum, often leading to recurrent respiratory infections, inflammation and lung damage. The viscoelastic sputum in CF patients is made of neutrophils-derived DNA (Rubin, 2007). DNaseI is a hydrophilic protein acting locally by depolymerising the extracellular DNA reducing the viscoelasticity of purulent airway secretions and improving the lung clearance indices (Henke and Ratjen, 2007). Presently, naked DNaseI, the only approved available medication for the disease, is delivered as a solution by nebulization. However, this treatment is limited as certain type of nebulizers may compromise enzyme activity. The high frequency of administration (2–3 times daily) and side effects such as hoarseness and laryngitis associated with the administration of naked enzyme constitute also major drawbacks of the current treatment (Gonda, 1996; Garcia-Contreras and Hickey, 2002; Jones et al., 2006; Henke and Ratjen, 2007).

The encapsulation of DNaseI in PLGA-NPs to be delivered as a suspension by nebulization could convey an enormous advantage in terms of improvement in clinical efficacy, treatment cost and patient compliance. Polymeric NPs offer additional degrees of delivery system manipulation, providing sustained release with a reduced frequency of administration and improvement of enzyme stability with an efficient lung deposition and reduced uptake by the alveolar macrophages (Sung et al., 2007; Yang et al., 2008). Moreover, studies using inhaled NPs dispersed in aqueous droplets reported mucus clearance possibly due to rapid displacement of particles to the airway epithelium via surface energetics (Schurch et al., 1990).

Because of their hydrophilic character, $w_1/o/w_2$ emulsification method is the most extensively used for the encapsulation of proteins into PLGA. However, such method exposes these fragile molecules to various stresses: large aqueous/organic interfaces, high centrifugation speeds affecting loosely bound surface protein, exposure to the water–ice interface during freezing and the dehydration during lyophilization. These stresses may in some cases cause irreversible protein inactivation (Kim et al., 2002; Ruan et al., 2002). Severe drops in biological activities as the result of exposure to w/o interfaces had been reported for lysozyme, rh interferon-

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γ, tetanus toxoid and chymotrypsin (Pérez and Griebenow, 2001; Pérez et al., 2003). DNaseI is a labile molecule with a great susceptibility to denaturation. It is also physically unstable upon storage within aqueous solutions at room temperature and is susceptible to glycation in the solid state (Jones et al., 2006).

NP suspension must be physically and chemically stable to reach clinical relevance. Moreover, concerns regarding the safety and clearance of the polymers and excipients from the lungs constitute one of the major reasons for the delay of commercialization of controlled pulmonary drug delivery systems. It is believed that exposure of lung epithelial cells to foreign particulate matter can cause disruption of the epithelial tight junctions and formation of intercellular channels for the passage of the molecules from airway lumen to blood (Sivadas et al., 2008).

In this work, efforts were done to develop DNaseI loaded PLGA-NPs avoiding the exposure of the protein to any substantial stress while keeping its activity at maximum and minimizing lung cell toxicity. Various strategies were adopted concerning formulation and manufacturing parameters. The physico-chemical characteristics as well as biological activity and biocompatibility of the prepared NPs, were studied. We also focused on finding an adequate method for the determination of the encapsulated enzyme activity.

## 2. Materials and methods

### 2.1. Materials

DNaseI lyophilized powder from bovine pancreas was obtained from Roche, Germany. PLGA average molecular weight 58.8 kDa from PolySciences, Inc., UK. 1,2-Dipalmitoyl-$s_n$-glycerol-3-phosphocholine (DPPC) was purchased from Genzyme, Liestal, Switzerland. Hydroxypropyl-β-cyclodextrin (HP-β-CD), Mw 1447, degree of substitution 5.4%, from Cargill Inc., Japan. Calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), salmon sperm DNA sodium salt, Trizma base (TRIS), ethidium bromide, poly(vinyl alcohol) (PVA) (87–89% hydrolysed, 13–23 kDa), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyethyleneimine (PEI) from Sigma–Aldrich, UK. Trehalose, leucine, Fluka (Switzerland). Sodium hydroxide (NaOH), Fisher Co., UK. Sodium dodecyl sulfate (SDS), EDTA, BDH, UK. Dichloromethane (DCM) from BDH Laboratory Supplies. Bicinchoninic acid (Micro BCA™) protein assay kit was supplied by Pierce, Rockford, IL, USA. All other reagents were of analytical grade.

### 2.2. Preparation of DNaseI loaded PLGA NPs

Particles were prepared by the modified $w_1/o/w_2$ double emulsion method (Freitas et al., 2005) at different phase volume ratios (PVRs). DNaseI (2.5 mg) was gently dissolved in an aqueous solution of PVA containing 5 mM $Ca^{2+}$, 2 mM $Mg^{2+}$ and (1:1) drug–HP-β-CD weight ratio. This inner aqueous phase was emulsified with DCM solution of PLGA ± surfactant by homogenization (Ultra-Turrax homogenizer, IKA-Werke, Staufen, Germany). The resulting $w_1/o$ emulsion was subsequently homogenized (Silverson homogenizer, LR4T, Silverson, Chesham, UK) in 45 mL of an aqueous PVA solution. Solvent evaporation was then done on a magnetic stirrer plate for 4 h at ambient temperature and pressure. The formed particles were separated by centrifugation (20,000 rpm, 25 min, 10 °C; Beckman J2-21 High speed centrifuge), washed with double distilled water to remove any residual PVA or unencapsulated DNaseI until no DNaseI was detected in the supernatant (three times). The particles were then resuspended in 2 mL of water containing trehalose as cryoprotectant. The particle suspensions were freeze dried (Virtis, UK) at −80 °C and $6 \times 10^{-4}$ mbar for 48 h to obtain free flowing powders. The experiments were divided into 4 groups as shown in Table 1.

### 2.3. NP characterization

The hydrodynamic diameters (*z*-average) of NPs and the polydispersity index (PI) were determined in triplicate in double distilled and filtered water (0.2 μm Whatman filters) by photon correlation spectroscopy (PCS; Malvern ZetaSizer, Malvern Instruments, UK) at a scattering angle of 90° at 25 °C. The zeta potential (ζ in mV) of particles dispersed in a 10 mM potassium chloride solution was assessed using the same equipment. The morphology of the NPs was examined by TEM without staining. The TEM pictures were captured using an FEI-Philips, BioTwin CM120 with a Lab 6 emitter and 120 kV accelerating potential. The surface morphology was examined and photographed using a scanning electron microscope (SEM, Phillips/FEI XL30 SEM) (Florindo et al., 2008).

### 2.4. Quantitation of DNaseI in the prepared particles

The amount of encapsulated DNaseI was determined by dissolving 5 mg of NPs in 1 mL of 0.1 M NaOH containing 0.5% (w/v) SDS after stirring at 37 °C overnight. A set of protein calibration solutions of DNaseI between 5 and 40 μg/mL was prepared. Micro BCA™ was used as per the manufacturer's instructions in 96 well plates. The absorbance in each well was measured at 562 nm using a Dynex MRX plate reader (Richfield), and the concentration of the samples was calculated. From the results, the encapsulation efficiency % (EE) and the percentage (w/w) of total protein entrapped per weight of polymer (proteins loading capacity) was determined as described elsewhere (Florindo et al., 2008).

### 2.5. Determination of enzyme activity by radial enzyme diffusion (RED) assay

The radial enzyme diffusion assay, modified from the methods of Nadano et al. (1993), was conducted. An agarose mixture consisting of 50 mL of 0.8% (w/v) melted agarose in reaction buffer (20 mM TRIS HCl, 2 mM $CaCl_2$, 20 mM $MgCl_2$, pH 7.3), containing 2.7 mL salmon sperm DNA (1%, w/v) and 10 μL ethidium bromide (10 mg/mL) was prepared. The agarose mixture (2 mL) was pipetted into each well of a 12-well tissue culture plate. After solidification, circular well (0.2 mm) was incised in the centre of each circular gel. Samples were prepared by adding 10 mg of the particles to 200 μL of the reaction buffer and were incubated at 37 °C with shaking for 2 h. The suspensions were centrifuged (IEC Micromax eppendorf centrifuge, UK) at 15,000 rpm for 5 min. A volume of 3 μL of the supernatant was dispensed into each circular well after determination of its DNaseI content using Micro BCA™. The plates were covered with the lid and were incubated at 37 °C for 5 h. The reaction was stopped by adding an overlay solution of 0.5 M EDTA. DNaseI activity was observed using an ultraviolet transilluminator at 312 nm. Well defined dark circles visible after illumination with UV light (312 nm) were formed. The increase in diameter of the dark zone ($mm^2$) created by the hydrolysed DNA correlates linearly with the amount of active DNaseI dispensed. The samples were assayed to quantify DNaseI activity (area of inhibition in $mm^2$) against a series of calibrated standards of DNaseI (2.5–12.5 μg/mL) prepared in reaction buffer. The recovered activities were calculated as % of that of the aqueous solutions of lyophilized protein supplied by Roche (considered as 100% activity and served as positive control). A negative control consisted of blank NPs of same composition treated in the same way.

**Table 1**
Formulation and characterization of DNaseI loaded PLGA-NPs.

| Group | Formula | $V_1$[a] | PLGA (%, w/v) | $w_1$:o:$w_2$ volume ratio | Surfactant (%, w/w) | External phase excipient[b] | Homogenization[c] | *z*-Average nm (s.d.) | PI (s.d.) | EE% (s.d.) | Drug loading[d] % (s.d.) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | A1 | 1 | 2.5 | 1:4:45 | – | | 1 | 200.7(4) | 0.319(0.215) | 34(3.2) | 0.94(0.16) |
| No | A2 | 1 | 5 | 1:2:45 | – | – | 1 | 190.4(4.4) | 0.215(0.06) | 67(3.5) | 1.72(0.18) |
| surfactant | A3 | 0.5 | 2.5 | 0.5:4:45 | – | – | 1 | 292.9(4.9) | 0.203(0.024) | 45(2.3) | 1.22(0.12) |
| | A4 | 0.5 | 5 | 0.5:2:45 | – | – | 1 | Microparticles (MPs) were obtained | | | |
| II | B1 | 1 | 2.5 | 1:4:45 | P (2) | – | 1 | 193.1(8.5) | 0.15(0.037) | 25(1.7) | 0.64(0.08) |
| Pluronic (P) | B2 | 1 | 5 | 1:2:45 | P (2) | – | 1 | 202.0(3.1) | 0.13(0.03) | 39.5(2.1) | 0.98(0.05) |
| | B3 | 0.5 | 2.5 | 0.5:4:45 | P (2) | – | 1 | 201.2(6.6) | 0.14(0.011) | 41.9(3.6) | 1.04(0.09) |
| | B4 | 0.5 | 5 | 0.5:2:45 | P (2) | – | 1 | 217.5 (4.1) | 0.15(0.041) | 37.3(1.2) | 0.93(0.03) |
| III | C1 | 1 | 2.5 | 1:4:45 | D (10) | – | 1 | 299.0 (5.2) | 0.24 (0.120) | 33.3(6.8) | 0.83(0.17) |
| DPPC (D) | C2 | 1 | 5 | 1:2:45 | D (10) | – | 1 | 337.0 (5.2) | 0.38 (0.080) | 36.5(7.8) | 0.91(0.19) |
| | C3 | 0.5 | 2.5 | 0.5:4:45 | D (10) | – | 1 | 319.0 (4.2) | 0.35(0.055) | 47.9(3.2) | 1.20(0.08) |
| | C4 | 0.5 | 5 | 0.5:2:45 | D (10) | – | 1 | 349.0 (7.5) | 0.41(0.04) | 73.4(3.2) | 1.80(0.23) |
| | C5 | 0.5 | 2.5 | 0.5:4:45 | D (20) | – | 1 | 245.4(4.3) | 0.2(0.050) | 41.2(3.1) | 1.03(0.07) |
| IV | D1 | 0.5 | 2.5 | 0.5:4:45 | D (10) | Leu | 1 | 247.9(6.2) | 0.26(0.008) | 65(3.9) | 1.63(0.09) |
| DPPC (D) | D2 | 0.5 | 5 | 0.5:2:45 | D (10) | Leu | 1 | 255.8(2.33) | 0.14(0.016) | 76(4.3) | 1.90(0.10) |
| and leucine | D3 | 0.5 | 5 | 0.5:2:45 | D (20) | Leu | 1 | 195.3(3.49) | 0.14(0.005) | 46(6.5) | 0.90(0.16) |
| | D4 | 0.5 | 5 | 0.5:2:45 | D (10) | Leu, NaCl | 1 | 239.0 (2) | 0.15 (0.014) | 87.5(3.9) | 2.19(0.09) |
| | D5 | 0.5 | 5 | 0.5:2:45 | D (10) | Leu | 2 | 257.0(2.83) | 0.15(0.100) | 75.8(4.2) | 1.88(0.10) |

[a] Internal phase volume.
[b] Leucine concentration is 0.01% (w/v) and NaCl is 0.9% (w/v).
[c] 1 denotes: 24,000 rpm for 4 min for $w_1/o$ emulsion and 10,000 rpm for 10 min for $w_1/o/w_2$ emulsion. 2 denotes: 20,000 rpm for $w_1/o$ emulsion for 2 min and 7000 rpm for 5 min for $w_1/o/w_2$ emulsion.
[d] Calculated based on polymer weight.

### 2.6. Determination of aerodynamic diameter after nebulization

The aerodynamic diameters and the size distribution of aerosol droplets during nebulization with a PARI LC PLUS® jet nebulizer was analyzed using a Malvern 2600C laser diffraction size analyzer with 63 mm lens. A volume of 2 mL of a 12.5 mg/mL suspensions of NPs in physiological saline was added to the jet nebulizer attached to a TurboBoy® compressor and clamped at a distance of 2.5 cm from the centre of the laser beam and adjusted to permit the aerosol cloud to cross the beam at a distance of 2.5 cm from the lens of the instrument. The size of the aerosol droplets was expressed as the volume mean diameter (VMD). The fine particle fraction (FPF) was evaluated as the volume fraction of the aerosol falling below 6.02 μm (De Boer et al., 2002). The residue of the nebulizer bowl was determined gravimetrically and the amount of DNaseI remaining was determined using the Micro BCA® protein assay.

### 2.7. In vitro DNaseI release

An amount of 10 mg from the selected formulation was suspended in 1 mL PBS (pH = 7.4) containing 5 mM SDS and 0.02% sodium azide as preservative in LoBind Eppendorf tubes®. The suspensions were incubated at 37 °C in a shaker incubator rotating at 100 rpm. At specified time intervals, the eppendorf tubes were centrifuged (IEC Micromax eppendorf centrifuge, UK) at 5000 rpm for 10 min and 350 μL of the release medium was withdrawn and replaced with fresh PBS. The amount of DNaseI released was measured using a Micro BCA® protein assay.

### 2.8. Cytotoxicity evaluation by MTT assay

Cytotoxicity of selected formulation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in lung epithelial cells (A549). The A549 cells were maintained in F-12 Ham supplemented with 10% fetal bovine serum (FBS) and Streptomycin/Penicillin antibiotics (100 μg/mL) in a humidified air atmosphere (5% $CO_2$, 95% RH, 37 °C) as described elsewhere (Florindo et al., 2008). Cell viability was determined as a percentage of the negative control (untreated cells). PEI was used as a positive control.

### 2.9. Statistical analysis

Three batches of NPs were prepared for each formulation. Blank formulations were prepared along with each formula. All measurements were performed in triplicate and data were shown as the mean with s.d. For selected evaluation tests, the means of all tested formulations were compared with each other by means of a one-way ANOVA with the Student–Newman–Keuls multiple comparison test. The statistical significance level ($P$) was set at $\leq 0.05$.

## 3. Results and discussion

Pulmonary delivery of a protein encapsulated in PLGA-NP carrier system imposes both stability and biocompatibility issues. Excipients were added in the internal phase to increase the enzyme stability and efficacy. These include: $CaCl_2$ to stabilize the enzyme against heat and shear stresses, minute amounts of $Mg^{2+}$ as an essential requirement for enzyme activity and HP-β-CD to stabilize the protein and facilitate its refolding (Pan and Lazarus, 1999; Kang and Singh, 2003). A preliminary optimization experiment was first conducted in order to choose the formulation and fabrication parameters (see supplementary data).

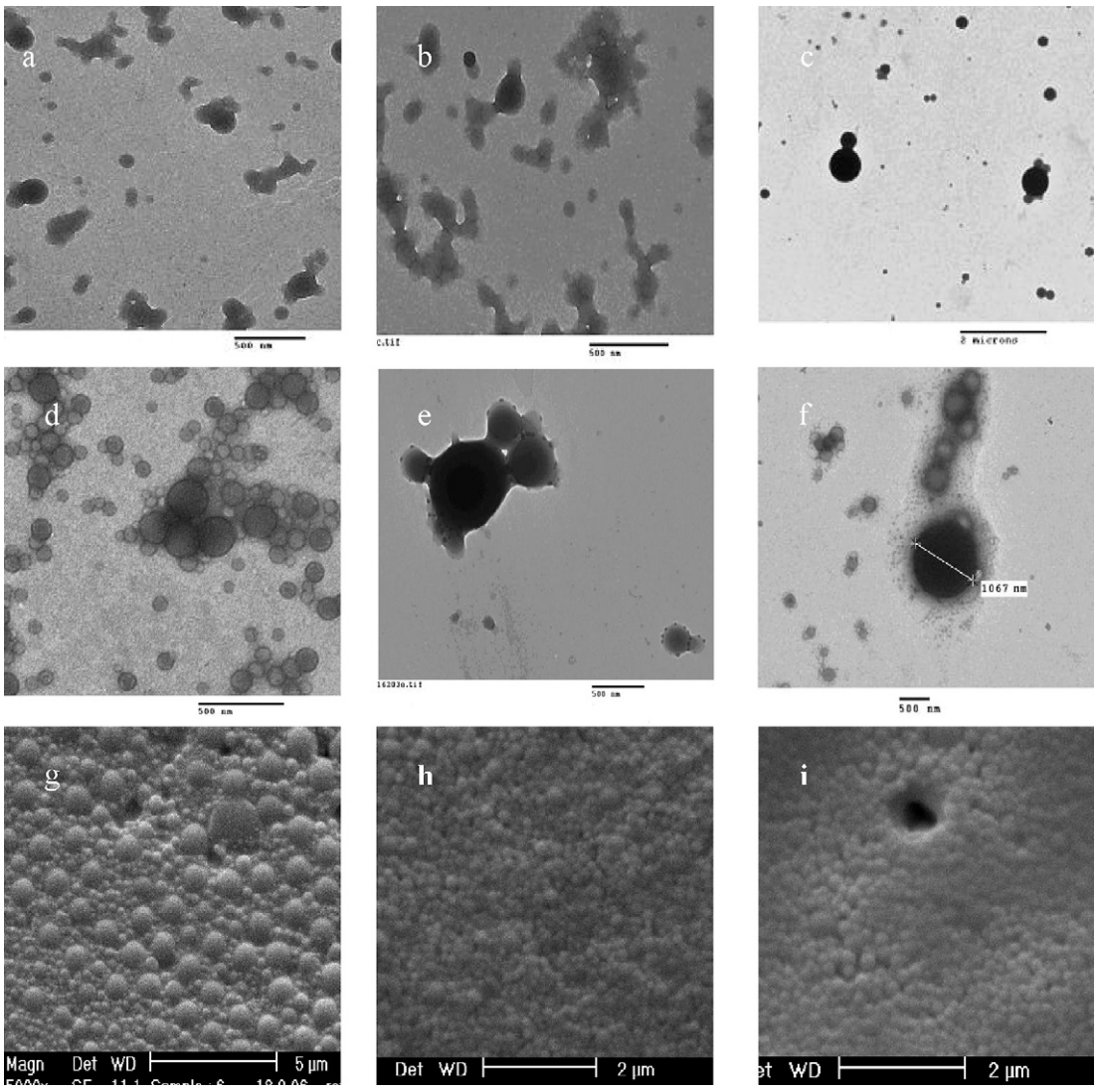


**Fig. 1.** TEM of freeze dried formulae (a) A2, (b) B2, (c) C3 and (d) D2. TEM during preparation of C3 after (e) 1 h, (f) 3 h after secondary emulsification step and SEM of formulae (g) C3, (h) D1 and (i) D2.

### 3.1. Particle size, EE, morphology and zeta potential of NPs

Surfactant free particles offer the potential of eliminating side effects resulting from the particle surface located surfactant on the human body (De Boer et al., 2001). Hence group I of DNaseI loaded PLGA particles was prepared without surfactant in the oil phase. NPs were obtained at all the used PVR except 0.5:2:45, where large MPs were produced due to the increased viscosity of the drug/matrix dispersion. Formulae A1 and A3, produced NPs with respective *z*-average of 200 and 292 nm showing a particle size increase with decrease in internal phase volume ($V_1$) or in other sense with decrease of volume fraction between drug/matrix dispersion and aqueous continuous phase. Previous reports about the impact of the volume ratio between drug/matrix dispersion and the continuous phase on the size of the resulting particles were conflicting. Some studies reported a reduction in the mean particle size, whereas others reported no significant effect (Jeffery et al., 1993; Sansdrap and Moes, 1998; Lamprecht et al., 1999). Although formula A2 was prepared with 5% (w/v) PLGA, yet it exhibited a smaller *z*-average than formula A1 prepared with 2.5% (w/v) polymer probably due to the lower content of DCM requiring less time in evaporation and hence decreasing aggregation possibilities for NPs. The PI of this group was generally high especially in case of formula A1. It can also be seen that the highest EE in this group was seen with formula A2 (67%) prepared at PVR 1:2:45. Unpredictably, this formula with the smallest particle size, showed the highest drug loading, 1.72% (w/w) of polymer. This indicates that, in absence of a surfactant, the increase in polymer concentration and decrease in the internal phase volume favoured the enzyme encapsulation. The longer time taken for evaporation of larger solvent volume might have facilitated protein loss in the aqueous external phase. This observation was in accordance with previous works, although not exclusive as an opposite observation was reported by others (Pean et al., 1998; Sansdrap and Moes, 1998; Lamprecht et al., 1999). The TEM of NPs in this group (exemplified by formula A2, Fig. 1a), shows spherical aggregated NPs. Although not fused in TEM, the NPs showed great difficulty for resuspension after freeze drying. Hence surfactant addition was considered.

Surfactants are known to limit the aggregation tendency of the NPs. Among the non-ionic surfactants, pluronics had been extensively studied (Wei et al., 2007). In our work pluronic L-44NF was added to the oil phase of formulae B1–B4. The addition of Pluronic leads to the production of NPs even at the PVR 0.5:2:45, a slight change in the particle size of the NPs prepared at the other phase volume ratios and a great reduction in the PI of all formulae reflecting a narrower size distribution compared to group I. However, the

addition of pluronic had a negative effect on the EE of DNaseI as evidenced by comparing B1 (25%), B2 (39.5%) and B3 (41.9) to A1 (34%), A2 (67%) and A3 (45%) respectively. This finding was in agreement with previous results and was attributed to a kind of competition between the surfactant and the protein, known also by its amphiphilic nature (Wei et al., 2007). This competition might have led to the displacement of DNaseI molecules from water/organic solvent interface decreasing its amount within the primary emulsion. Finally, the freeze dried NPs were easily redispersed. The TEM, Fig. 1b, shows spherical NPs with very slight aggregation.

The improvement of NP characteristics by the use of pluronic L-44NF encouraged us to replace it by DPPC (group III). This biocompatible surfactant constitutes the primary component of the lung absorptive surface and is therefore poorly recognised by scavenger receptors on airway phagocytes (Evora et al., 1998; Dellamary et al., 2004). NPs prepared with 10% DPPC demonstrated relatively bigger size and higher PI than their counterpart formulae prepared without surfactant or those containing pluronic. The increase in the polymer concentration increased the particle size as seen by comparing C2 and C4 to C1 and C3 respectively (Table 1). The effect of the PVR on the EE was very obvious when comparing formula C4 with C2 both prepared with 5% polymer concentration but with respective EE of 73.4 and 36.5%. Furthermore, the EE was higher in case of DPPC emulsified NPs than in case of pluronic as evidenced by comparing C1, C3 and C4 with respective EE (33.3, 47.9 and 73.4%) to B1, B3 and B4 with EE of (25, 41.9 and 37.3%) respectively. On the other hand, no significant difference was found between EE of formulae C2 and B2. The TEM (Fig. 1c) and SEM (Fig. 1g) confirmed the results of the particle size determined by the PCS. A proportion of the particles appeared with an average diameter of 1 μm. For a deeper insight in the process and the formulation parameters affecting it, TEM was taken after each stage of preparation: $w_1/o$, $w_1/o/w_2$ and NP solidification. NPs were firstly seen but by the end of the process some MPs were formed. This was thought to result from the insufficiency of the amount of emulsifier used, especially that the particle size determination carried out at the various manufacture stages showed NP aggregation after the second emulsification step (TEM, Fig. 1e and f). At this point, two strategies were taken. The first involved increasing the DPPC concentration as shown in formula C5 and the second strategy was based on the use of an anti-aggregating agent in the external phase (group IV). The increase in DPPC concentration decreased both the particle size and the EE as evidenced by comparing C5 (245.4 nm/41.2%) to C3 (319 nm/47.9%). This decrease in EE might be due to association of drug molecules with excess surfactant molecules and their leaking from the particulate carriers. The relatively higher *z*-average of C5 and C3 compared to both A3 and B3 prepared without surfactant or with pluronic L-44NF respectively could be attributed to the large size of the alkyl chain of the DPPC molecule (supplement).

In group IV formulae, it is obvious that the addition of leucine in the external phase of this group of NPs not only decreased the size of the NPs but also greatly improved the PI. This was evidenced by comparing formulae D1 and D2 with their counterpart free from leucine (formulae C3 and C4). Leucine, by virtue of its surface active properties, acted as anti-aggregating agent. Furthermore, an improvement in the EE was also seen by comparing D1 and D2, with respective EE 65 and 75% to their analogues (47.9 and 73% respectively). This might be due to leucine hydrophobic character which could have formed a layer around the particles decreasing the diffusion of the water soluble protein. Moreover, a possible intermolecular hydrogen bonding between protein and leucine functional groups (amino and hydroxyl) might have enhanced protein encapsulation. Increasing PLGA concentration from 2.5 to 5% had no effect as D2 and D1 had very close *z*-average and even though D2 had a lower PI than D1. However, increasing DPPC concentration to 20% (formula D3) produced NPs with particle size and PI smaller than D2. However, its lower EE (46%) discouraged the use of DPPC in its high concentration. TEM, presented in Fig. 1d, shows discrete spherical NPs. Fig. 1h and i illustrates the SEM of formulae D1 and D2. It is worthy to note that leucine effect was extremely obvious after freeze drying confirming its strong anti-aggregating effect. To decrease the diffusion of the water soluble protein, 0.9% (w/v) of NaCl was added in the external phase. This caused a considerable increase in the EE where formula D4 showed an EE of 87.5% compared to 76% in its counterpart prepared without NaCl (D2). This could be explained by an increase in the osmotic pressure in the external phase, decreasing protein diffusion. Moreover, a reduction in the homogenization speed and time to 20,000 rpm for 2 min in case of the $w_1/o$ emulsion (instead of 24,000 for 4 min) and 7000 for 5 min in the second emulsion (instead of 10,000 for 10 min) did not cause any noticeable change in the particle size of the produced particles as shown in formula D5. Previous trials to use such lower speeds and times were unsuccessful in the preparation of groups I, II and III. Larger particles with unaccepted high PI were obtained.

Although NPs are an attractive delivery system to the RT, yet, their delivery to the lung is severely limited because of their low inertia and possible exhalation (Hinds, 1998). Therefore, we intended to deliver our prepared NPs as an aqueous colloidal dispersion by nebulization incorporating the NPs into the respirable percentage of aerosolized droplets (Yang et al., 2008). It was therefore of prime importance to determine their zeta potential which allows the predictions of the storage stability of colloidal dispersions. The higher the absolute values of the zeta potential, the larger the amount of charge on their surface producing stronger repellent interaction among the NPs and hence better stability. Surface electrostatic charge is an important factor influencing the deposition of inhaled NPs and charged NPs proved to have higher deposition efficiencies as compared to neutrally charged NPs (Yang et al., 2008). $\zeta$ for formula D5 showing optimum characteristics was found to be −37 mV (±2.5) i.e. lower than −30 mV, the typical threshold value for flocculation (Straub et al., 2005). This high negative surface charge can be attributed to the presence of carboxyl end groups from PLGA and hydroxyl groups from the PVA located near the surface added to the ionization of the phosphatic group of the lipid DPPC.

### 3.2. Biological activity of the enzyme

For successful delivery of the protein from PLGA particles, enzyme activity and stability should be assured (Castellanos et al., 2002). The most extensively used method for the evaluation of the enzyme activity is the KUNITZ method which is basically a colorimetric method (Jones et al., 2006). Using this method for clear DNaseI solution would be easy. However, the turbidity resulting from the used excipients represented one of the major limitations to this method and procedures adopted to render them clear could affect the enzyme activity. In addition, extracting the protein from the particles to determine its activity requires the use of an organic solvent. Exposure to such an organic–aqueous interface could induce aggregation which could not be distinguished from the aggregation resulting from the preparation of the particles. Accordingly, RED was adopted with some modifications (supplementary data). The data presented in Fig. 2 reveal that encapsulation of the enzyme in the PLGA-NPs of formula A2 containing PVA, $CaCl_2$, HP-β-CD and trehalose helped in preserving 46% of its activity in spite of the severe stresses encountered during particle fabrication. Previous reports demonstrated that the non-ionic surfactant, PVA successfully decreased the aggregation of lysozyme and that sugars can prevent dehydration induced protein denaturation (Van de Weert et al., 2000; Castellanos et al., 2002). Formulae B2 and C2 showed significant increases ($P \leq 0.01$) in remaining activity than A2. Both pluronic and DPPC caused an average increase of 13% in the

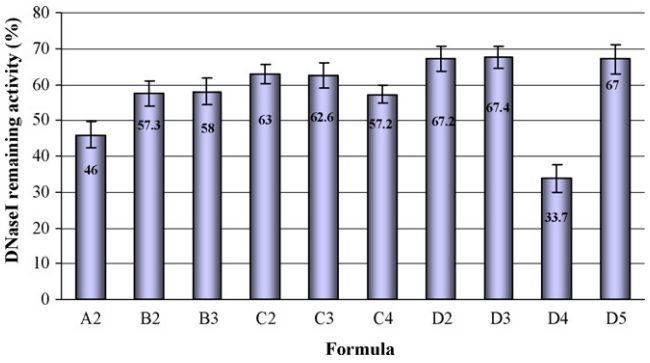


**Fig. 2.** Remaining activity of DNaseI in PLGA NPs as determined by RED.

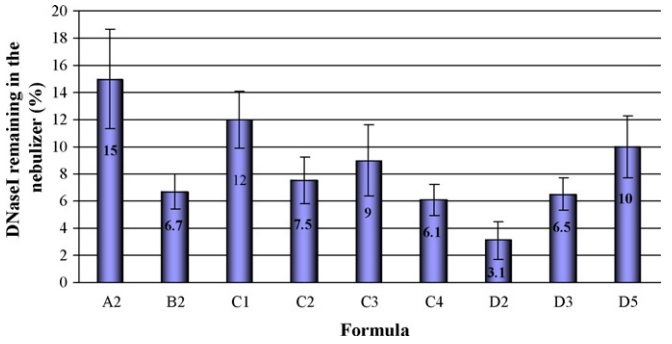


**Fig. 3.** DNaseI remaining in the device after nebulization of a 25 mg/2 mL NP suspension.

recovered activity owing to their surface active properties. Surface active compounds had been found to prevent interface-induced aggregation of carbonic anhydrase, tetanus toxoid and lysozyme. Moreover, pluronic had been shown to improve the refolding and recovery of some proteins (Yazdanparast and Khodagholi, 2006). Formulae B2 and B3, prepared with different volumes of DCM, showed similar DNaseI activities. A similar observation was noticed for C2 and C3. Although, we expected that the presence of leucine would increase the activity of DNaseI since previous studies had reported the ability of some amino acids to improve the recovery and refolding of some proteins (Sabulal and Kishore, 1997). However, formula D2 showed a slight non significant increase in the activity compared to C4. This is probably because leucine was added in the external phase and it could not protect the enzyme from denaturation resulting from the exposure to the aqueous/organic interface. Formula D4 containing NaCl had lower activity than D2. The high ionic strength of the solution, imparted by the addition of this electrolyte which remained in contact with the particles for about 4 h was probably responsible of such effect. Reduction of the homogenization speed in formula D5 did not affect significantly the enzyme activity at $P \geq 0.05$ demonstrating that the procedure did not impose any substantial stress on the enzyme activity. Increasing the DPPC concentration to 20% did not affect DNaseI activity as shown by comparing formulae D3 with 67.4% activity to D2 (67.2%).

### 3.3. Determination of the aerodynamic diameter after nebulization

Laser diffraction is well established for size analysis of nebulized droplets and has shown good correlation with pulmonary deposition finding (Pilcer et al., 2008). Satisfactory agreement was found between the size distribution of rh DNase aerosols obtained by laser diffraction and an inertial sizing method using the cascade impactor (Cipolla et al., 1994a,b). Since, the method is easy, simple and fast, it was used for the rapid determination of the aerosolization properties of the prepared DNaseI–NP aqueous suspensions. To study the effect of various formulation variables on nebulization properties of NP suspensions, some formulae were selected. The selected formulae namely, A2, B2, C1, C2, C3, C4, D2, D3 and D5, had VMD of the aerosol droplets ranging from 4.22 to 5.75 μm, as shown in Table 2, signifying suitability for pulmonary administration. The FPF varied from 54.7% for formula A2 to 71.3% with formula D2. For all formulae, the nebulization time required to deliver the whole dose (25 mg/2 mL) was relatively short ranging from 4 to 10 min. The residue of the nebulizer bowl was determined gravimetrically and the results, shown in Table 2, reveal that more than 85% of the dose was delivered from the device. Formulae B2, D2 and D3 delivered higher proportion of the dose than the others. Results of the quantification of the amount of DNaseI remaining in the nebulizer, shown in the same table, were in accordance with the previous observation. As seen in Fig. 3, with all formulae (except the control A2), the remaining protein in the device after nebulization did not exceed 11% and only 3% of the DNaseI dose remained in the device achieving a nebulizer efficiency (fraction of the dose initially placed in the nebulizer that will reach the mouthpiece as a part of the aerosol cloud) of 97% in case of formula D2. Upon rehydration of the NPs in saline, no free DNaseI was detected until end of nebulization. Laser diffraction data were collected every 2 min until end of the suspension because of possible changes which can occur in the concentration of the suspension during the nebulization process which can in turn affect the VMD and the FPF (Phipps and Gonda, 1994). Fig. 4 shows that the FPF of all the formulae was almost constant over time suggesting stability of nebulization. It is imperative to validate the reproducibility of performance during repeated cycles of aerosolization and cleaning of a reusable jet nebulizer system. Standard deviation values were always low and did not exceed 5.5% as shown in Table 2. Since the preferred site of action of inhaled DNaseI was presumed to be in the airways, it was postulated that the dose of DNaseI delivered in the size fraction 1–6 μm was the parameter important for therapy (Gonda, 1996). In addition, earlier studies showed that for the nebulization of the rh DNase solution of the marketed product, the aerosol delivery systems tested pro-

**Table 2**
Laser diffraction data after nebulization using a PARI Jet nebulizer.

| Formula | D[4,3][a] | Span | FPF | Nebulization time (min) | % delivered dose[b] (s.d.) |
|---|---|---|---|---|---|
| A2 | 5.75 | 2.05 | 54.7 | 8 | 84.3(2.3) |
| B2 | 4.81 | 2.08 | 65.3 | 4 | 92.2 (2.4) |
| C1 | 5.77 | 1.93 | 56.5 | 8 | 86.4(5.5) |
| C2 | 4.67 | 2.3 | 67.9 | 10 | 91(3.1) |
| C3 | 4.79 | 2.29 | 66.9 | 8 | 90(2.5) |
| C4 | 4.81 | 2.23 | 66.5 | 4 | 91.3(3.4) |
| D2 | 4.42 | 1.81 | 71.3 | 6 | 94.1(3.5) |
| D3 | 4.22 | 2.18 | 69.3 | 6 | 93(1.6) |
| D5 | 4.31 | 2.05 | 71.0 | 6 | 92.8(4.6) |

[a] Volume mean diameter.
[b] Determined gravimetrically.

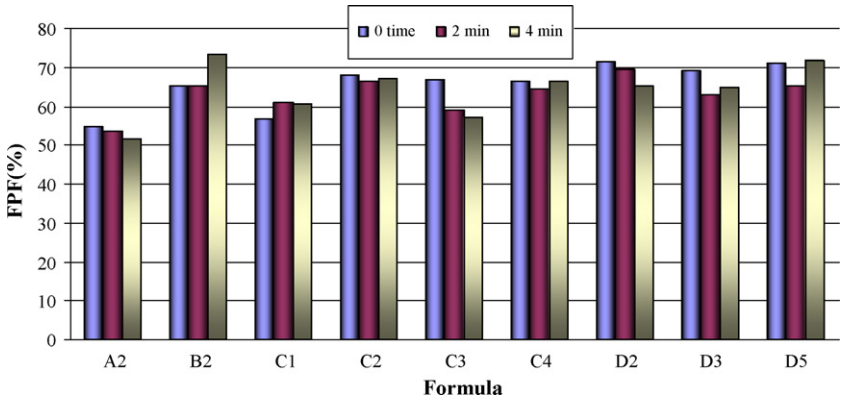


**Fig. 4.** FPF obtained at various times during nebulization of a 25 mg/mL.

**Table 3**
DNaseI remaining activity at the various stages of PLGA-NP preparation by emulsion solvent evaporation.

| Stage | Recovered activity % (s.d.) |
|---|---|
| Original | 100 |
| $w_1/o$ emulsion | 90 (1.5) |
| $w_1/o/w_2$ emulsion | 85(2) |
| Following solvent removal | 75 (4) |
| Following centrifugation and washing | 72 (3.6) |
| Following lyophilization | 67 (4) |

duced respirable fractions from 43 to 81% (Cipolla et al., 1994a,b). In our study, all tested formulae showed a VMD below 6 μm and the FPF was higher than 54% reaching a maximum of 71.3% with formula D2. This formula showed a nebulizer efficiency of 97% and was considerably higher than the efficiency previously reported with rh-DNase solutions (22–62%) (Cipolla et al., 1994a,b). Accordingly, the procedure adopted in this work gave DNaseI NPs with satisfactory nebulizing efficiency.

The enzyme activity of the NPs of formula D5 with the best characteristics was tracked to point out the steps affecting most of the enzyme activity. Table 3 shows that around 10% of the activity is lost during the formation of $w_1/o$ emulsion where a high shear (20,000 rpm) and high aqueous–organic interfaces were present. During the formation the $w_1/o/w_2$ emulsion, 7000 rpm for 5 min with the aqueous–organic interfaces leads to the loss of another 5% of the activity. For the ESE, the 3 centrifugation and washing cycles probably affected the activity of the enzyme and 3% of the activity was lost after this step. Leaving the solvent to evaporate at ambient temperature and pressure for 4 h, represent a stress which decreased the enzyme activity by about 10%. The creation of ice–liquid interface was another hazard encountered during freezing and lyophilization where 5% of the activity was lost.

### 3.4. In vitro DNaseI release

The release profile of DNaseI from D5 is illustrated in Fig. 5. A burst release of 49.4% (w/w) was seen within 6 h, after which, a sustained release profile was noticed during the 2 weeks sampling time releasing 82%. The initial burst release seen from DNaseI NPs reflects the early diffusion process of release of unaggregated protein through the porous channels. The interconnected pores are the major pathways for water escape during solidification and lyophilization. In this work the amount of DNaseI released in the first 6 h was very high (42.4%). That could probably suggest the presence of very small pores on the surface of the NPs. Although, they were not be detected by TEM or SEM due to the small NP size and the limited magnification power of the SEM, yet, their presence and possible interconnection could be assumed. Another possible explanation of the fast release from the PLGA NPs is possibly the use of DPPC in the manufacture of the particles as previously suggested by others (Tomoda and Makino, 2007). Incomplete release was frequently encountered with many proteins entrapped in PLGA particles principally attributed to both aggregation and non-specific adsorption occurring within the particles. On the other hand, some investigators reported a complete release of the encapsulated protein after affording proper stabilization strategy.

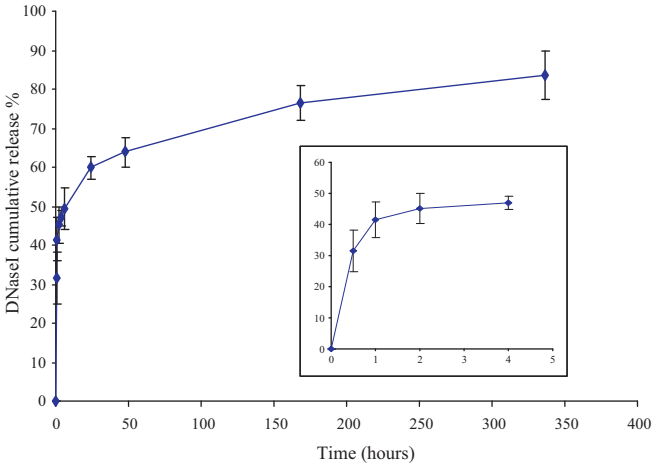


**Fig. 5.** Release of DNaseI from PLGA-NPs.

### 3.5. In vitro cytotoxicity

NPs represent an emerging class of functional materials defined by size dependent properties. Their features such as high adsorption capacity, hydrophobicity, surface charge, optical and magnetic properties or catalytic activity can affect their safety on the tissues. Hence MTT assay was conducted on lung epithelial cells. Fig. 6 shows that the average cell viabilities in the presence of the DNaseI NPs ranged from 91 to 108% and was significantly higher ($P \leq 0.001$) than the values obtained with the positive control PEI at all studied NP concentrations. No significant differences were found between the negative control, formula D5 and its blank at $P \leq 0.05$ demonstrating the safety of the prepared NPs on the lung epithelial cells.

DNaseI is presently administered in a dose of 2.5 mg/mL. That is why achieving a high protein loading was one of the present study objectives. A maximum EE of (75.8 ± 4.2) was achieved in formula D5 with an enzyme loading of 1.88% based on polymer weight. With the high enzymatic activity retained after encapsulation and the high nebulization efficiency achieved with this formula, this could represent a good prophylactic option for CF patients presenting

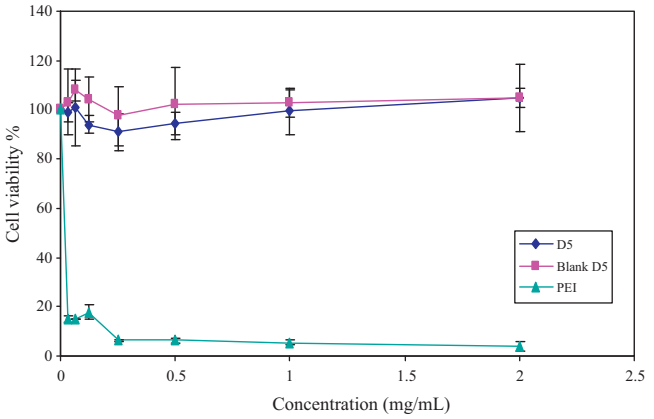


**Fig. 6.** A549 cell viability measured by MTT cytotoxicity assay after exposure to increasing concentrations of the DNaseI loaded PLGA-NPs.

better properties than the solution (Cipolla et al., 1994a,b). However, this would necessitate the administration of a large amount of powder. Therefore, DNaseI lyophilized powder supplied in a higher activity (14,200 units/mg) was used in particle preparation. NPs with similar particle size, morphology and EE were obtained. The determined recovered activity was 76% ± 2.7 achieving thus more than 8 fold increase in the activity compared to the previously used grade with an activity of about 2000 units/mg. Accordingly, this method could be a good approach to prepare stable DNaseI NPs with optimum properties to achieve a potential therapeutic application. Future work will be taken to ensure the clinical relevance of this hypothesis.

## 4. Conclusion

The integration of nanotechnology and pulmonary delivery of drug aerosols represents a new exciting frontier for pharmaceutical dosage form design. However, the challenges imposed are numerous. So this work has discussed the various formulation strategies used in order to stabilize the PLGA loaded DNaseI–NPs for pulmonary delivery, with potential application for indications where long durations of delivery by inhalation are required. The results showed that formulation variables notably leucine and DPPC greatly improved size and morphological properties of particles. The activity of the enzyme was affected by the formulation factors. The obtained results revealed the suitability of the prepared particles for pulmonary delivery and highlighted the role of excipients in the stabilization of DNaseI against the various stresses encountered during preparation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.02.013.

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