



Inhalable DNase I microparticles engineered with biologically active excipients



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ABSTRACT

Highly viscous mucus poses a big challenge for the delivery of particulates carrying therapeutics to patients with cystic fibrosis. In this study, surface modifying DNase I loaded particles using different excipients to achieve better lung deposition, higher enzyme stability or better biological activity had been exploited. For the purpose, controlled release microparticles (MP) were prepared by co-spray drying DNase I with the polymer poly-lactic-co-glycolic acid (PLGA) and the biocompatible lipid surfactant 1,2-dipalmitoyl-S_n-phosphatidyl choline (DPPC) using various hydrophilic excipients. The effect of the included modifiers on the particle morphology, size, zeta potential as well as enzyme encapsulation efficiency, biological activity and release had been evaluated. Powder aerosolisation performance and particle phagocytosis by murine macrophages were also investigated. The results showed that more than 80% of enzyme activity was recovered after MP preparation and that selected surface modifiers greatly increased the enzyme encapsulation efficiency. The particle morphology was greatly modified altering in turn the powders inhalation indices where dextran, ovalbumin and chitosan hydrochloride increased considerably the respirable fraction compared to the normal hydrophilic carriers lactose and PVP. Despite of the improved aerosolisation caused by chitosan hydrochloride, yet retardation of chitosan coated particles in artificial mucus samples discouraged its application. On the other hand, dextran and polyanions enhanced DNase I effect in reducing cystic fibrosis mucus viscosity. DPPC proved good ability to reduce particles phagocytic uptake even in the presence of the selected adjuvants. The prepared MP systems were biocompatible with lung epithelial cells. To conclude, controlled release DNase I loaded PLGA-MP with high inhalation indices and enhanced mucolytic activity on CF sputum could be obtained by surface modifying the particles with PGA or dextran.

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1. Introduction

Cystic fibrosis (CF) is the most common autosomal, recessive, life-span shortening disease in Caucasians. Development of chronic pulmonary diseases is the main cause of mortality in CF patients [1]. Currently, DNase I (Mw 33 KDa) is delivered as a nebulising solution. The mucolytic agent acts locally to cleave undesirable neutrophils-derived DNA which, together with bundles of F-actin, is responsible for the presence of viscoelastic sputum in CF patients. However, the compromised enzyme activity especially with

ultrasonic nebulizers, the high dosage frequency with susceptibility to local side effects as well as the tremendous financial burden are all drawbacks of current treatment [2,3]. More efforts are still needed to produce stable and potent formulations for treatment of CF.

Previous investigators had pointed out that mucus decreases particles diffusion impeding the efficiency of particulate based drug and gene therapy to the lungs especially for CF patients [4]. They owed this delay in particle through the mucus, not only to the particle size, but also to particle surface hydrophobicity. For instance, they found that small nanoparticles (200 nm) showed better diffusion than larger ones (500 nm) and this diffusion was improved after coating with a hydrophilic polymer [4,5]. However, particles measuring less than 1 µm are exhaled during normal tidal breathing while those more than 6 µm are deposited in the upper

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airways [6]. Because small MP show better deposition in the respiratory tract in addition to the higher stability of macromolecules in the dry state, we aimed at engineering surface modified MP with the ability to cross mucus, depending on their biological effect rather than their size. For the purpose, hydrophilic surface modifiers, with various surface charges, were used with DNase I to prepare dry MP by spray drying (SD). This fast and economic technique offers, among other advantages, the potential of modulating the physicochemical characteristics of MP permitting drug release modification, muco-adhesion, stabilization and dispersibility enhancement [7–13]. However, using this method, it had been previously found that a 40% of DNase I biological activity was lost [12]. Other investigators demonstrated that temperatures higher than 90 °C irreversibly denatured DNase I and the extent of denaturation increased with the increase in temperature [13]. The use of lactose as carrier did not protect the enzyme and addition of trehalose and Tween protected it against the thermally induced aggregation but caused severe particles aggregation [14]. In another study, the incorporation of sodium chloride in the formulation improved its aerosolisation properties yet the effect of salt on the activity of DNase I was not investigated [15]. It is clear that despite the superior flexibility the spray drying can offer from formulation point of view, it is important that the design of the formulation takes into consideration balancing the formulation stability and its biological potency.

Excipients playing dual role in the formulation are always preferred especially if one of their effects is to enhance the biological activity of the drug. In this respect, literature survey revealed that *ex vivo* addition of poly-aspartate or poly-glutamate to DNA and F-actin containing bundles of CF sputum disperses and lowers the elastic moduli of these samples. Moreover, the addition of poly-aspartate to sputum samples had been found to decrease the bacterial growth [16]. As an oligosaccharide, dextran demonstrated therapeutic potential in treatment of CF *in vitro* and in animal models by causing a reduction in mucus cross-linkage bonding, leading to reduced mucus viscoelastic modulus [17–19]. It also interferes with bacterial adhesion to epithelial cells, preventing *Pseudomonas aeruginosa* pneumonia and death in neonatal mice [20]. The polysaccharide chitosan (CS) had also been found to affect pulmonary deposition by modifying the particle surface and counteracting the mucociliary clearance mechanism, providing sustained drug release properties [9]. Inclusion of phospholipids, which constitutes 80–90% of the major components of lung surfactants, at the surface of the inhaled particles was found to decrease significantly their phagocytic uptake due to overall reduction in opsonin adsorption [21,22]. In addition, phospholipids were found to improve the properties of the respirable fine particle fraction of the inhaled particles [8].

The aim of this study is to rationally engineer bioactive multi-component micro-particulate system for treatment of CF via pulmonary route. MP were prepared using spray drying of a modified double emulsion. We hypothesize that (i) the formulation methodology with the selected enzyme stabilizers (divalent ions, ovalbumin and hydroxyl propyl- β -cyclodextrin) should preserve the biological activity of DNase I and (ii) imparting the hydrophilic properties to MP by inclusion of hydrophilic excipients having potential in reduction of mucus viscoelasticity with the mucolytic DNase I enzyme will work synergistically to improve penetrability of the MP within the thick mucus; and finally (iii) preparation of controlled release MP would widen the therapeutic benefit of the enzyme, decreasing dose frequency with subsequent decrease in local side effects. Testing the effect of the chosen surface or activity modifiers on the aerosolisation performance and on particles phagocytic uptake was also considered in this work.

2. Materials and methods

2.1. Materials

DNase I lyophilized powder from bovine pancreas was obtained from Roche, Germany. PLGA (Mw: 7 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%, Cargill Inc., Japan. Polyglutamic acid (PGA: 200–500 KDa), Wako chemicals, Japan. Calcium chloride (CaCl₂), magnesium chloride (MgCl₂), sodium chloride (NaCl), Salmon sperms 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), polyaspartic acid sodium salt (PAA), polyethyleneimine (PEI), dextran sulphate (DEX, Mw 5000), dextran-FITC, lactose, polyvinyl pyrrolidone (PVP K-30), ovalbumin (OVA) and mucin type II were obtained from Sigma Aldrich, U.K. Trehalose, leucine, chitosan low molecular weight were obtained from Fluka (Switzerland). Sodium hydroxide (NaOH), Fisher Co. UK. Sodium dodecyl sulphate (SDS), EDTA, Dichloromethane (DCM) from BDH Laboratory Supplies, UK. Bicinchoninic acid (Micro BCA™) protein assay kit was from Pierce, Rockford, IL, USA. Chitosan hydrochloride and polyglutamic acid sodium salt were synthesized in our lab from the corresponding base and acid respectively using spray drying [23].

2.2. Microparticles preparation

2.2.1. Controlled release microparticles (CR-MP)

CR-MP were prepared using a modified emulsion spray drying method (ESD). An amount of 20 mg of DNase I was gently dissolved in 1 mL of an aqueous solution of 5% w/v PVA containing 5 mM CaCl₂, 2 mM MgCl₂ and 20 mg of HP- β -CD. This aqueous phase was homogenized in 4 mL of DCM containing 5% PLGA 50:50 (7 KDa) and 0.25% W/V of DPPC for 2 min at 20,000 rpm (*Ultraturrax*® T25 homogenizer, Germany) forming w₁/o emulsion which was subsequently added dropwise into 30 mL of an external aqueous phase (w₂) of PVA (0.25% w/v) and homogenized (*Silverson*® homogenizer, Chesham, UK) at 5000 rpm for 10 min. Various concentrations of leucine and/or 0.1% W/V of surface modifiers (lactose, PVP K-30, OVA, CS-HCl, PGA, PAA and DEX) were added to the external aqueous phase. The resulting double-emulsion was spray-dried using a mini spray dryer (*Buchi, B-190, Switzerland*) equipped with a high-performance cyclone at an air flow rate of 800 L/h. The spray drying process conditions were optimized and the final selected parameters were: Inlet temperature: 65–70(°C), aspiration: 75% and pump rate: 7 mL/min. The spray dried powders (SDP) were collected and stored in vacuum desiccators, at room temperature, for further analysis.

2.2.2. Immediate release DNase I microparticles (IR-MP)

A solution containing 5 mM CaCl₂, 2 mM MgCl₂, 10 mM NaCl and 0.4% w/v of HP- β -CD in the presence or absence of 0.4% w/v of the activity enhancer in water (dextran or PGA) was first prepared. DNase I, (0.4% w/v), was gently dissolved in 10 mL of the solution followed by addition of an equal concentration of leucine. The solutions were spray dried using the same parameters as CR-MP. These MP were only used in biological evaluation of activity enhancers.

2.3. Evaluation of the spray dried powders (SDP)

2.3.1. Spray drying yield

SDP yields were quantified as percent of initially added amounts.

2.3.2. Determination of the angle of repose

The fixed height cone method was adopted and the angle of repose was calculated as follows:

$$\tan \theta = 2h/D. \quad (1)$$

where: θ is the angle of repose, h is the height of the formed cone and D is the diameter of the base of the formed cone [24,25].

2.3.3. Thermogravimetric analysis (TGA)

Water content of SDP was determined using a Pyris-6-TGA (Perkin–Elmer, USA). A sample of 2.5–4.5 mg of SDP was heated from 30 to 150 °C, at a heating rate of 10 °C/min. Percentage weight change of the sample was measured and water content was calculated as % of initial powder weight.

2.3.4. X-ray diffraction (XRPD)

XRP diffractometer (Philips PW 3710, USA), running at 45 kV, 30 mA, and scanning from angles of 5–45°, was used.

2.3.5. Particle size determination

2.3.5.1. Wet dispersion method. Approximately 2 mg of SDP was dispersed in 1 mL of cyclohexane. The volume mean diameter (VMD), D [3,4], and the span was determined using laser diffraction particle size analyzer (Malvern Mastersizer S, UK) as previously described elsewhere [10].

2.3.5.2. Dry dispersion method. Approximately 5 mg of some SDP formulae were dispersed using RODOS dry feeder of laser diffractometer (Sympatec HELOS, Clausthal Zellerfeld, Germany) at various compressed air pressures (0.2, 0.3, 0.5 and 1 bar) to evaluate the effect of leucine on the dispersion properties of the powders. The particle size analysis was performed by WINDOX 5 software.

2.3.6. Particle morphology

The surface morphology of MP was examined by scanning electron microscopy (SEM) (Cambridge Instruments, Cambridge, UK) as previously described [26].

2.3.7. Zeta potential (ζ) determination

Determination of ζ of a MP suspension was performed in a capillary cell using Zetasizer (Malvern Instruments-1000, UK) equipped with a 4 mW He–Ne laser at a wavelength of 633 nm at 25 °C as described previously [26].

2.3.8. Quantification of the protein in the prepared particles

Micro-BCA Protein assay™ was performed as per manufacturer's instructions. Determination of protein encapsulation efficiency (EE %) was carried out after DNase I extraction using a modified alkaline hydrolysis extraction method as previously described [27].

2.3.9. Biological activity of the enzyme

2.3.9.1. Agarose gel electrophoresis. Accurately weighed amounts (10 mg) of SDP were suspended in 200 μ l of TRIS buffer (pH 7.4 \pm 0.2) in eppendorf tubes and incubated for 2 h in a shaker incubator (Jencons, England) and the procedure was completed as previously described elsewhere [28].

2.3.9.2. Radial enzyme diffusion (RED) assay. In order to quantify the enzymatic activity of DNase I, radial enzyme diffusion assay, modified from the methods of Nadano et al. was conducted [29]. A volume of 2 μ l of the DNase I solution extracted from the MP was used as previously described [28].

2.3.9.3. Activity in artificial mucus. An amount of 1 mg of SDP was added to 200 μ l of a solution containing 5% mg/mL of salmon sperms DNA and 20 mg/mL of mucin in water. Samples were placed in the shaker incubator at 37 °C and 50 rpm. At definite time intervals, aliquots of incubated solutions were withdrawn and diluted with DNase I reaction buffer (20 mM TRIS HCl, 2 mM CaCl₂, 20 mM MgCl₂, pH 7.3). The reaction was then stopped by addition of 20 mM EDTA diluted with DNA loading buffer. A volume of 20 μ l of each solution was then applied to a 0.8% agarose gel in TAE buffer containing 0.5 μ g/mL ethidium bromide. Electrophoresis was carried out at constant voltage of 70 V for 90 min in TAE buffer. DNA bands were visualized under a UV transilluminator (Bio-RAD, USA).

2.3.9.4. Diffusion assay. To test the biological activity of the enzyme and excipients, an assay based on recording the diffusion of fluorescent nanospheres in sputum samples collected from CF patients was used as reported by Broughton-Head et al. with some modifications [30]. The tests were carried in two 96-well plates placed over each other. The upper plate was a multiscreen filtration plate with 0.8 μ m pore size, mixed cellulose ester membrane. The lower plate (Nunc® black plate), was filled with 370 μ l of PBS and was placed directly so as to receive the filtrate from the upper one. Sputum samples were collected from adult CF patients attending outpatient clinics at the National Lung and Heart Institute, London, UK. Samples were spontaneously expectorated and stored at –80C prior to use in experiments. For the test, the CF sputum was first homogenized with 10 strokes in 10 mL syringe. The samples were then mixed with 200 nm carboxylate modified FITC nanospheres in a volume concentration of 10% prior to addition of 100 μ l of the sample solution in each well of the upper plate which was then covered by its lid. The plates were placed for 1 h at 37 °C in a shaker incubator rotating at 50 rpm. The fluorescence (excitation 485 nm and emission 535 nm) in the lower plate was measured on a plate reader (Victor 1420 multilabel counter, UK). Where indicated, sputum samples were pretreated with either reference DNase I in reaction buffer (positive control), different immediate release formulae or their blanks in 20 mM Tris buffer (pH 7.3) to a final concentration of DNase I of 2.9 μ g/mL for 1 h. The samples were loaded in the upper plate and the test was completed as previously. The diffusion of the nanospheres in the test solutions was calculated as percentage of the diffusion in water (100% diffusion). A negative control treated with the same volume of reaction buffer containing no drug was also done.

2.3.10. In vitro aerodynamic deposition

Aerosolization properties of SDP were tested using a breath-activated inhaler device (Aerolizer®, Novartis) attached to a twin stage glass impinger (TSI) (Copley, Nottingham, UK) operated at air flow rate of 60 L/min for 2 \times 5 s aspirations. Volumes of 7 and 30 mL of 0.1 M NaOH containing 0.5% w/v SDS were introduced into stage 1 and 2 respectively and 25 mg of each SDP were loaded in HPMC No 3 capsules. Volumes of 10, 50 and 100 mL of collecting solvents were used to collect powders remaining in capsule/mouthpiece stage 1 and stage 2 respectively and drug content was determined as explained in Section 2.3.8. Fine particle fraction (FPF), corresponding to stage 2 deposition, emitted fraction (EF), respirable particle fraction (RP) and effective inhalation index (EI) were calculated as previously described elsewhere using equations (2)–(4) [31].

$$EF\% = \left[\frac{(m_{\text{full}} - m_{\text{empty}})}{m_{\text{MP}}} \right] \times 100 \quad (2)$$

$$RP\% = \left[\frac{(St2)}{EF} \right] \times 100 \quad (3)$$

$$EI\% = (EF \times St2)^{1/2} \quad (4)$$

Where m_{full} and m_{empty} are the masses of the capsule before and after simulating the inhalation respectively and m_{MP} is the powder mass of MP. EF is the fraction (%) emitted from the inhalation system, and St2 is the fraction % distributed to stage 2.

2.3.11. In vitro DNase I release

Accurately weighed amount of particles (10 mg) were suspended in PBS (pH = 7.4) with 0.02% sodium azide as preservative in LoBind Eppendorf® tubes. The test was done using Micro-BCA™ protein assay as previously described [26].

2.3.12. Cytotoxicity evaluation by MTT assay

Cytotoxicities of selected formulations were determined assay in lung epithelial cells (A549) by MTT assay. A549 cells were maintained in F-12 Ham supplemented with 10% foetal bovine serum (FBS), 100 µg/mL of streptomycin and 100Units/mL of penicillin in a humidified air atmosphere (5% CO₂, 95% RH, 37 °C) as described elsewhere [32]. Cell viability was determined as a percentage of the negative control (untreated cells) and PEI was used as a positive control.

2.3.13. Phagocytic uptake study

2.3.13.1. Preparation of FITC-labelled DNase I loaded PLGA microparticles. Dextran-FITC was incorporated in the aqueous internal phase and the double emulsion was rapidly spray dried using the previous experimental conditions. Formulae F2 (free from surface modification) and F7, F8, F9 surface modified with DEX, PGA and PAA respectively were used in this study.

2.3.13.2. Microparticles phagocytic uptake by mice macrophages

J774A.1. Murine monocyte macrophages (J774.1) were used to study the phagocytic uptake of MP. Cells were maintained in complete medium consisting of DMEM supplemented with 2 mM-glutamine, 10% foetal bovine serum, 100 µg/mL of streptomycin and 100Units/mL of penicillin and incubated at 37 °C and 5% CO₂. For uptake experiments, cells were seeded in 24 wells plate (5×10^4 cells/200 µl/well) on 35 mm glass cover slips coated with poly-D-lysine, incubated for 24 h and then washed with DMEM-L-glutamine. The cells were then incubated with the particles (2 mg/mL in 500 µl DMEM-L-glutamine) for 30 min. The medium

was then aspirated and the cells washed 3 times with DMEM and fixed with 4% paraformaldehyde solution for 15 min after which they were washed 3 times with PBS. For nuclear staining, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, RNase treated (100 µg/mL in PBS) for 20 min at 37 °C, and incubated with propidium iodide (1 µg/mL) in PBS for 1–5 min, then rinsed three times with PBS. For microscopical examination, the cover slips were mounted with aqueous polyvinyl alcohol Citifluor reagent mixed with AF100 antifade reagent (1:10). Slides were examined under the confocal microscope (Zeiss LSM 510 Meta, Germany). The lasers used were 30 mW argon laser (488 nm for green channel) and 1 mW 543 nm HeNe laser (for red channel). The emission was collected using a band pass filter between 505 and 530 nm for green, 560 nm long pass filter for propidium iodide.

2.3.14. Statistical analysis

Three batches 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. The mean 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 ≤0.05.

3. Results and discussion

3.1. Optimization experiment

A preliminary optimization study to select the most appropriate spray drying and formulation parameters for the emulsion was first performed [29]. It was reported that a 10 °C increase in DNase I denaturation temperature (67.4 °C) can be tolerated by the use of calcium ions due to stabilization of the enzyme two disulphide bonds [3,12,13]. Based on these data and using formulations parameters selected from a previous work [29], setting the inlet temperature of 95 °C resulted in an outlet temperature ranging from 59 to 65 °C depending on the pump rate and the aspiration level settings. The obtained powders had a rubbery texture with a large proportion attached to the cyclone wall in spite of the low water content (<4%) as revealed by TGA. This was attributed to the outlet temperature which exceeded the polymer glass transition temperature (T_g) [10,33,34]. By decreasing the inlet temperature to 70 °C, the outlet temperature ranged from 40 to 42 °C and the rubbery nature of the powders decreased. However, the yield values did not exceed 34%, in spite of the use of a high performance cyclone. Moreover, the obtained powders were of cohesive nature

Table 1
Characterization of PLGA spray dried powder formulae.

Formula	Surface/activity modifier	Leucine conc ^a (W/W)	Yield%	Water content (%)	ζ (mV)	EE (%)	VMD	Span	Remaining activity
F1	–	–	34 (4.6)	2.5(0.01)	–7.4 (0.5)	63(3.8)	6.2 (1.2)	1.9 (0.03)	78 (3.5)
L1	–	–	34 (5.6)	NA	NA	NA	6.5 (0.5)	1.6 (0.5)	NA
L2	–	15	51.3 (5.5)	NA	NA	NA	4.64 (0.4)	1.5 (0.4)	NA
L3	–	30	74 (6.0)	NA	NA	NA	3.5 (0.6)	1.9 (0.3)	NA
L4	–	60	72 (5.2)	NA	NA	NA	2.5 (0.3)	2.3 (0.2)	NA
F2	–	30	69 (3.2)	1.2(0.01)	–8.3 (0.9)	71(4.8)	5.7 (1.2)	1.7 (0.2)	81 (4.0)
F3	Lactose	30	71 (5.7)	3.5(0.23)	–6.5 (0.3)	93.2(2.2)	6.7 (1.6)	1.4 (0.2)	80 (2.6)
F4	CS-HCl	30	72 (3.5)	4.0(0.50)	7.2 (0.9)	90 (5.6)	6.4 (0.7)	2.3 (0.2)	83 (4.2)
F5	PVP	30	73 (4.5)	2.3(0.02)	–12.3 (1.2)	94.8(1.1)	5.4 (2.0)	1.7 (0.2)	84.6 (2)
F6	OVA	30	72 (4.3)	2.3(0.02)	–8.2 (0.7)	76 (4.7)	7.7(0.9)	1.2 (0.2)	83 (2.4)
F7	DEX	30	71 (6.7)	3.0(0.06)	–6.2 (0.1)	88 (4.3)	5.6 (2.1)	1.2 (0.1)	82 (3.4)
F8	PGA	30	74 (5.1)	3.2(0.15)	–31 (0.8)	89 (3.3)	5.8 (0.4)	1.3 (0.2)	80 (2.8)
F9	PAA	30	71 (4.5)	3.0(0.18)	–19.4 (0.6)	87 (5.2)	4.7 (1.0)	1.7 (0.3)	81 (4.4)

Values are mean of 3 values (SD), NA: test not applicable as they blank formulae (no DNase I).

^a calculated as w/w of PLGA used.

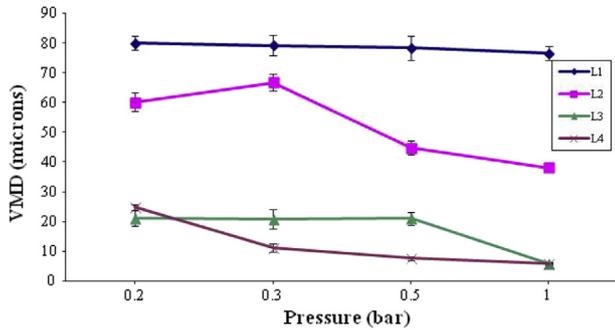


Fig. 1. Effect of dispersion pressure on the particle volume mean diameter (VMD) of spray dried powder (SDP) of PLGA-MP determined by the dry method (HELOS laser diffraction-RODOS feeder).

exhibiting poor flowability, where the angle of repose values ranged from 45 to 49 °C. X-ray diffractogram of the optimized formula F1, (Fig. S1, Supplement), is characterized by the absence of intensity peaks indicating an amorphous nature ensuring that the process conditions optimized the speed of solvent removal, allowing amorphous powders production and crystal formation prevention. The particle size volume distribution of F1, (Fig. S2, supplement), shows a unimodal particle size distribution with no detectable aggregation and the measured VMD (6.2 μm), shown in Table 1, indicated suitability of the size for delivery to the pulmonary airways. Although the chosen parameters were able to produce dry particles with suitable size, yet the poor flowability of such cohesive powder will eventually lead to poor aerosolization properties and subsequent unsuitability for direct delivery to the lungs.

3.2. Optimization of dispersibility enhancer

Due to the potential of various amino acids as dispersing aid [35,36], leucine was added to the blank of the optimized formula (F1) in various concentrations as seen in Table 1. The addition of leucine caused a significant increase in the yield depending on its amount, reaching a maximum of about 40% increase in L3, containing 30% of leucine calculated based on polymer weight as shown in Fig.S3, Supplement. Further increase in leucine concentration caused did not significantly decrease the obtained yield. Needless to say that the flow properties of the powder improved as could be depicted from the same figure. In fact, dry powders delivered by inhalers do not behave as individual particles but rather as aggregates depending on the dispersion pressure. Moreover, particle size measurement in suspension does not reflect the dispersion properties of the dry powder, although it can be used for routine quality control. Therefore, the dry method was applied and the 'RODOS' dry powder feeder was used to disperse SDP at 4 different dispersing forces in order to evaluate the effect of leucine on the powder dispersion (Fig. S4–S7, supplement). The relation between the particle size and the applied pressure was presented in Fig. 1. It is obvious that all applied pressures failed to disperse L1 and for L2, 0.3 bar was needed to start VMD decrease. The VMD measured at the highest pressure (1 bar) was 38.05 μm which was higher than that measured by the wet method (6 μm), see Table 1. Formula L4 showed better dispersion properties at lower pressures than formula L3. It is to be noted that at 1 bar, an optimum concentration of leucine amounting to 30% ensured efficient dispersion of the particles. This pressure was sufficient to aerosolize the powder as individual particles. This enhanced dispersibility might be attributed to leucine relatively strong hydrophobic alkyl chains altering the surface morphology and surface

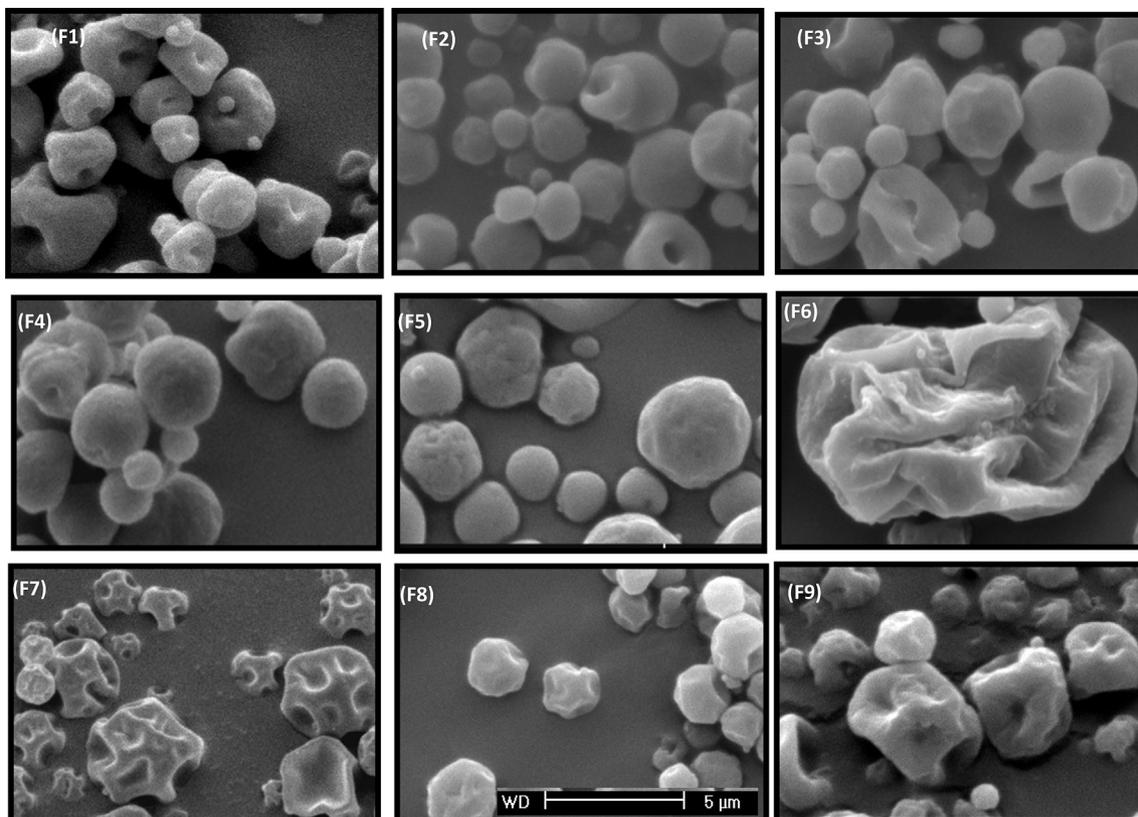


Fig. 2. SEM of DNase I loaded PLGA microparticles prepared by emulsion spray drying (ESD).

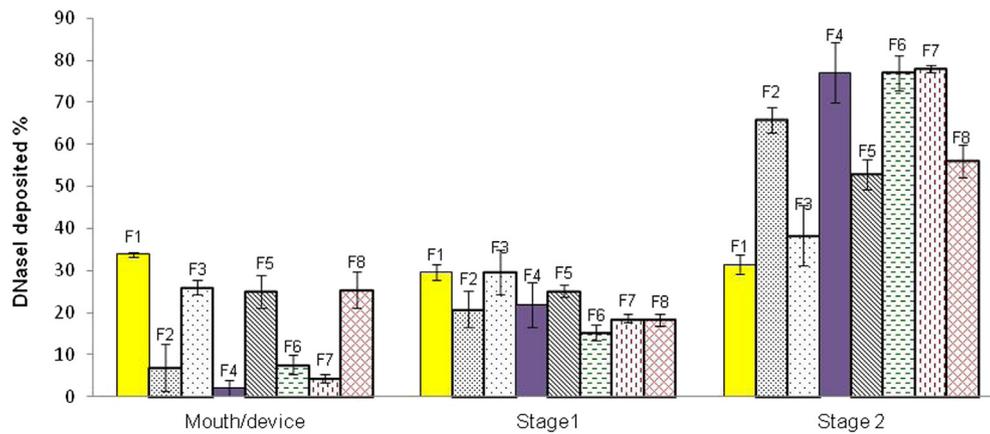


Fig. 3. *In vitro* deposition of DNase I loaded spray dried microparticles determined using a twin stage impinger. An Aerolizer® (DPI) was used to deliver the dose into the various parts of the TSI operated at a flow rate of 60 L/min for 2 × 5 seconds.

tension of fine particles produced during SD. This is beside its capacity to migrate to the droplet surface, precipitating on the surface of the particles, increasing its rugosity, reducing cohesiveness and preventing sintering between adjacent particles [9,35,37,38]. A concentration of 30% of leucine was then selected and used in subsequent work. Table 1 shows that after leucine addition, the yield ranged from 69 to 74% and the water content did not exceed 4%.

3.3. Particle size, zeta potential (ζ) and EE%

Laser diffraction data, shown in Table 1, reveal that all formulae had VMD less than 8 μm , indicating suitability for pulmonary delivery. A low span, indicative of narrow size distribution could be depicted. Although ζ measurement does not adequately reflect the charge in the dry state yet it allows for the examination of MP diffusion after administration especially in thick viscous sputum full of negatively charged macromolecules (DNA and F-actin) stabilized by multivalent cations, histones and peptides [16]. Particles of F1 showed a zeta potential of -7.4 due to the free carboxyl and hydroxyl groups of both PLGA and PVA. DNase I, carrying $-ve$ charge at this pH, could also have contributed to this negativity. Leucine alone or combined with lactose, OVA or DEX did not cause significant changes, as shown in F2, F3, F6 and F7, while the addition of PVP slightly increased particles negativity as seen in F5. A highly negative surface charge was seen with F8 and F9, surface

modified with PGA and PAA respectively. On the other hand, F4 containing CS-HCl exhibited positive ζ due to chitosan cationic nature. This cationic nature confers to chitosan its mucoadhesive properties and inhibition of the ciliary clearance constituting the base of its use in sustained release formulations targeting respiratory tract [9]. While the addition of leucine caused a non-significant increase in EE (from $63 \pm 3.8\%$ in F1 to $71 \pm 4.8\%$ in F2), the surface modifiers caused a significant increase. The highest EE increase was seen with PVP and can be explained by its probable interaction with PVA forming a composite with greater hydrophobicity and higher driving force for surface orientation than PVA alone led to the formation of a protective layer around the MP in F5, preventing possible protein diffusion outside them [12]. On the other hand, the water soluble OVA, with a Mw close to that of the enzyme, probably competed for encapsulation in MP.

3.4. Microparticles surface morphology

SEM of MP are shown in Fig. 2. F6, containing OVA, shows an irregularly wrinkled raisin-like surface similar to that obtained in previous work upon spray drying bovine serum albumin [39]. When present in sufficient amount, molecules with surface active properties, like OVA can form a complete surface layer inhibiting the passage of the water vapour that forms as result of high temperature and the surface layer expands like a balloon. After complete water evaporation, the surfactant layer collapses resulting in

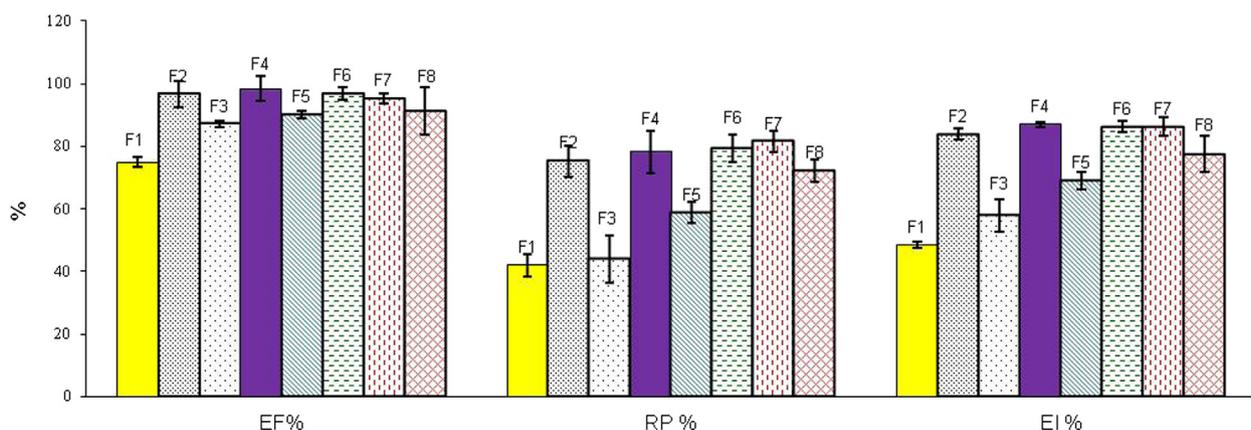


Fig. 4. Calculated inhalation indices of DNase I loaded spray dried microparticles determined using a twin stage impinger. The calculated inhalation indices are: $EF = (m_{\text{full}} - m_{\text{empty}}) / m_{\text{powder}} \times 100$, $RP\% = (St2/EF) \times 100$ and $EI\% = (EF \times St2)^{1/2}$. EF denotes the fraction of particles emitted from the inhalation system, RP denotes the respirable particle percentage of emitted particles and EI% is the effective inhalation index.

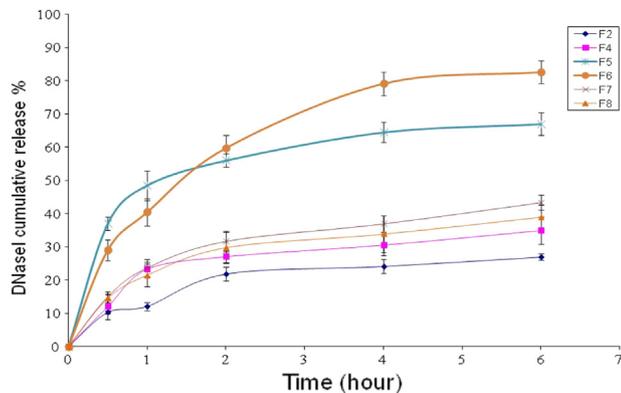


Fig. 5. *In vitro* release of DNase I from spray dried microparticles.

the wrinkled structure [40,41]. On the other hand, MP with smooth surfaces were obtained with F3 and F5 containing lactose and PVP respectively. When added to the external phase, these water soluble excipients uniformly spread on the particles surfaces giving them a smooth appearance. CS-HCl modified particles of F4 shows a cohesive powder with spherical particles having slightly undulating rough surfaces. Its relatively higher remaining water content (4%), together with the bipolar nature resulting from the presence of cationic CS together with negatively charged PLGA and PVA confers to this powder high aggregation tendency. Rough dented surface were obtained with F8 and F9 surface modified with PGA and PAA respectively, while irregular particles with dimpled surface could be depicted in formula F7 prepared with DEX.

3.5. *In vitro* deposition using the twin stage glass impinger

Fig. 3 shows the emitted fraction and its distribution after delivery of SDP with an *Aerolizer*[®] attached to TSI and Fig. 4 shows the inhalation indices calculated using Eqs. (2)–(4). In comparison with

F1, the presence of leucine in F2, raised the emitted fraction from 75 to more than 96% with a high stage 2 deposition corresponding to RP of 68%. The emitted fraction in the other SD formulae varied from 87.2% in F3 to 98.4% in F4 depending on the included surface modifier. Lactose and PVP surface modified particles, F3 and F5, showed high device and mouth/throat deposition (26.5 and 25.1 respectively). On the other hand, CS-HCl and dextran surface modified, F4 and F7, showed very little deposition in the mouth and throat regions. These formulae, upon inhalation, could deposit deep in the lungs avoiding the local side effects resulting from the enzyme deposition in the throat [3]. Formulae F4, F6 and F7 exhibited the best aerosolization properties as evidenced by their high RP% ranging from 77 to 81.6%. The use of CS-HCl, OVA and DEX to modify the surface of these particles, enhanced the effect of leucine in improving the SDP aerosolization. An explanation of this improved aerosolization properties could be withdrawn from the SEM of the MP. The asperities present on the surface of F6 particles could decrease the true surface area of contact between the particles reducing the cohesion between the individual particles. Similar observation was reported by Chew and Chan [42] who concluded that solid, non-porous corrugated particles can enhance aerosol performance over the smooth spherical particles of otherwise similar physical properties. Surface roughness or irregularity of CS and DEX modified SDP of F4 and F7 respectively could justify their enhanced aerosolization properties.

3.6. *In vitro* DNase I release from CR-MP

Fig. 5 shows the release profile of DNase I from MP formulae prepared with PLGA, 7 KDa, with estimated degradation time (2–3 weeks). About 24% of the enzyme was released during the first 6 h from the non-surface modified formula F2. Inclusion of the hydrophilic surface modifiers raised this amount to variable degrees. This released amount corresponded to surface located DNase I non-encapsulated within the PLGA particles during the emulsification step. It could be used to provide the immediate mucolytic effect.

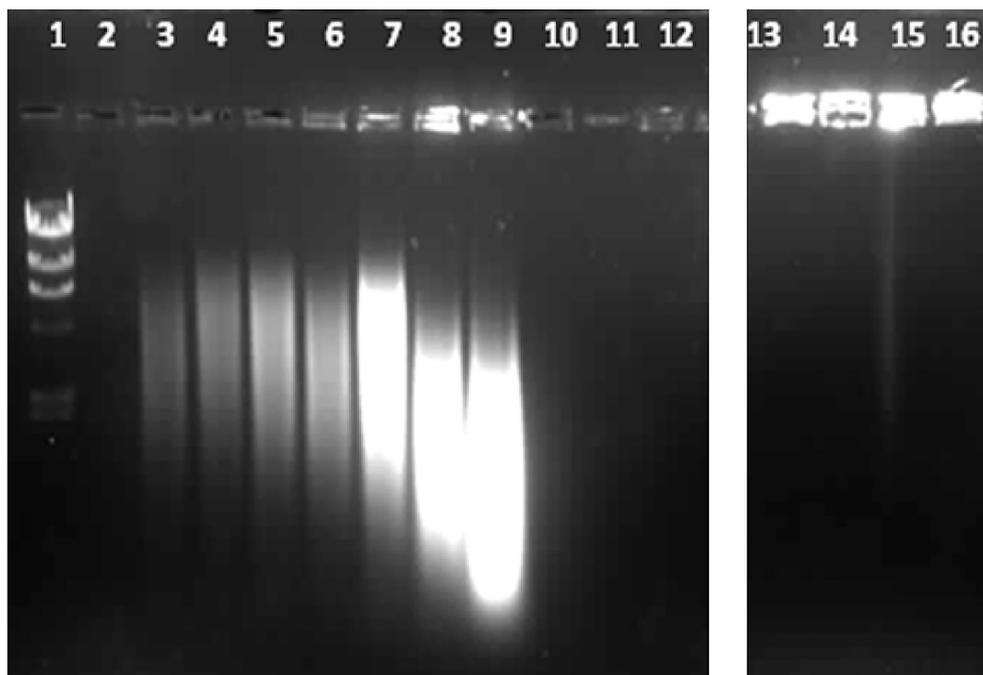


Fig. 6. Agarose gel electrophoresis showing artificial mucus sample containing DNA before and after treatment with DNase I (loaded and blank) MP. Lane1: λ DNA marker, 2: mucus treated with DNase I reference (10 $\mu\text{g}/\text{mL}$), 3–6: non treated control mucus sample at 0, 15, 30 and 60 min, 7–9: mucus treated with F2 blank for 15, 30 and 60 min Lanes 10–12: mucus treated with F2 for 15, 30 and 60 min 13,14: mucus treated with F4 for 30 and 60 min 15,16: mucus treated with blank of F4 for 30 and 60 min.

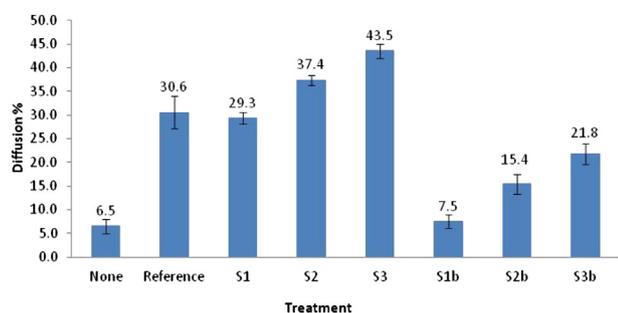


Fig. 7. *In vitro* diffusion of fluorescent nanospheres in sputum collected from cystic fibrosis patients.

However, the high amount released in case of F5 and F6 does not support the use of both OVA and PVP as surface modifiers for these controlled release particles. The release was followed for 15 days, after which, 77, 81.5, 85 and 84.8% were released from formulae F2, F4, F7 and F8 respectively while 95 and 98% were released from F5 and F6 respectively (Fig. S8, supplement).

3.7. Biological activity

To obtain a quick overview about the comparative activity of the encapsulated DNase I, agarose gel electrophoresis was done (Fig. S9, supplement). All formulae showed an enzyme concentration dependent decrease in DNA fluorescent signal except F4 where the wells remained illuminated showing DNA retention and protection. Electrostatic attraction between the positively charged CS-HCl and the negative phosphate backbone on the DNA molecules caused their protection against the enzyme and their retardation in the wells. A similar observation was seen with blank of this formula.

For enzyme activity quantification, a RED assay was conducted and examples of gel plates are shown in Fig. S10 (supplement). More than 80% of the enzyme activity was retained with all formulae as shown in Table 1. Formula F5, containing PVP in the external phase, showed the highest remaining activity (85%) and CS-HCl modified particles showed an increased activity with $83 \pm 4.5\%$ remaining activity.

Trying to simulate *in vivo* conditions and to test MP ability to dissolve and penetrate viscous CF mucus, a sample of artificial highly viscous mucus was prepared with mucin and salmon sperm DNA in which F2, F4 and F5 were tested. Non-surface modified particles of F2 dissolved in mucus and released their DNase I which degraded DNA as evidenced by the absence of DNA fluorescent signal which can still be clearly seen with blank F2 (Fig. 6). Visual examination revealed also an increased pourability and decreased viscosity of prepared mucus sample treated with F2. Similar results were obtained with F5 surface modified with PVP although the test did not allow to differentiate between both formulae (not shown).

On the other hand, with F4, surface-modified with CS-HCl, particles aggregation with decrease in mucus viscosity was seen. However, DNA fluorescent signal was evident in the gel (Fig. 6). The observed decrease in viscosity noted might be due to the precipitation of negatively charged DNA on the positively charged particles surface of F4 (as evidenced by particle aggregation) rather than degradation by DNase I. This was in contrast to the result of the RED. In this method, DNA immobilization inside the gel matrix led to a faster enzyme diffusion because of the positively charged CS. Although, the artificial mucus sample denoted that it was possible to condense DNA with CS, yet the release of the enzyme from the particles could not be achieved in such medium. A matter, which suggests the unsuitability of CS for use in preparation of DNase I controlled release particles.

The previous methods focused on evaluating activity of the enzyme and included excipients on DNA, one of the components of CF sputum. In fact, viscosity of CF patients' sputum is usually complicated due to the presence of bundles of actin, histones and other macromolecules. Therefore, diffusion of fluorescent nanospheres through sputum samples collected from CF patients, was taken as indication of the decrease in viscosity of the sample and hence activity of the enzyme and excipients. However, due to difference between the release profiles of the various formulae with consequent differences in the amount of enzyme present in the medium after certain time, we decided to carry out this experiment on IR-MP of similar composition to CR-MP with no PLGA guaranteeing thus same amount of enzyme and excipients at specified time. Formulae S1 (no activity enhancer), S2 (containing DEX) and S3 (containing PGA) were selected and their effects on the diffusion of nanospheres in sputum collected from CF patients were compared to reference DNase I solution in a concentration of $2.9 \mu\text{g}/\text{mL}$. It is obvious from Fig. 7 that reference DNase I, S1, S2 and S3 caused significant increases in nanospheres diffusion ($P \leq 0.001$) compared to negative control. Relative to the reference, S1 showed non-significantly different diffusion of nanospheres while the diffusion was significantly higher in case of S2 and S3. Increase in nanospheres diffusion was also noticed with the blanks of formulae S2 and S3 but the increase was higher in case of S3 blank which was only slightly lower than the reference. This observation potentially supported the role of PGA in the dispersion of DNA and F-actin containing bundles in CF sputum [18]. Similar results are to be expected with the other polyanion PAA. Although the effect of dextran was lower than that of PGA, yet we expect another potential advantage which is the decrease in bacterial adhesion as reported in previous studies [17–19].

3.8. Cytotoxicity evaluation

Non-significantly different cell viabilities ranging from 93 to 115.7% of the negative control were obtained with F5, F6 and F8 at $P \geq 0.05$ as shown in Table 2. Moreover, the obtained values were

Table 2

A549 cell viability measured by MTT cytotoxicity assay after exposure to PLGA microparticles prepared by spray drying.

Particle conc (mg/mL)	% Cell viability						
	F5	F6	F8	Blank F5	Blank F6	Blank F8	PEI
0	100	100	100	100	100	100	100
0.03125	98.1(3.6)	93.4(5.7)	97.4(15.3)	94.6(2.5)	96.7(11.1)	96.7(9)	15(1.1)
0.065	111.8(11.5)	94.2(8)	99.6(14)	103.1(0.9)	95.5(7.8)	95.5(7.8)	14.9(0.2)
0.125	115.7(12.8)	95.6(11.1)	106(1.1)	94.8(12)	96.5(1.9)	96.5(1.9)	17.9(3.2)
0.25	114.6(14.1)	98.7(7.6)	93(5.5)	100.4(2)	93(15)	94(13.5)	6.2(0.5)
0.5	110.9(9.1)	95.3(8.2)	104.7(6.3)	103.2(4.8)	99.3(9.7)	99.3(8.6)	6.2(0.8)
1	103.3(13.5)	103.5(9.3)	110.7(2.3)	102.4(3.5)	103.2(6)	103.2(5.9)	5.5(1.2)
2	96.1(14.5)	103.4(3)	99.2(2.3)	102.1(2.7)	104.4(13.2)	103.5(5.5)	4(1.9)

Values are presented as mean (SD).

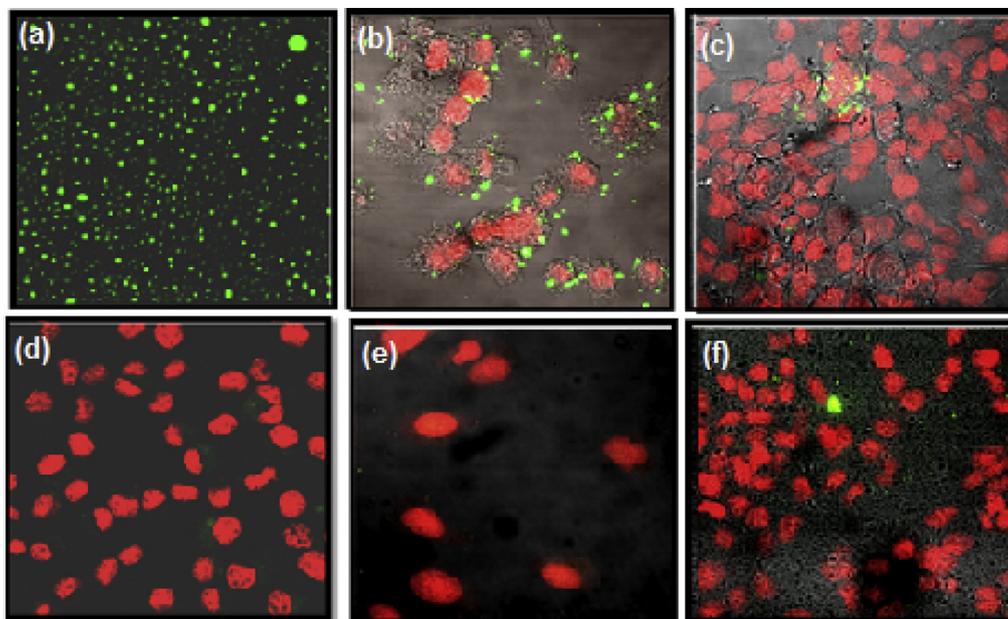


Fig. 8. Confocal microscopic images of (a) FITC labelled DNase I loaded PLGA-MP prepared by ESD (formula F2 without DPPC), murine macrophages (J774.1A) after exposure for 30 min to (b) F2 (without DPPC), (c) F2 (with DPPC), (d) F7 surface modified with dextran (e) F8 surface modified with PGA and (f) F9 surface modified with PAA.

significantly different from those obtained with PEI at the same concentrations ($P \geq 0.001$). Furthermore, the cell viabilities did not vary among all the tested SDP suggesting that the used excipients did not affect lung epithelial cells confirming their safety.

3.9. Phagocytic uptake

For a controlled release aerosol to be effective, it must be retained in the lung for a period, so that the therapeutic drug can be released. Confocal Laser scanning microscopy was used for the evaluation of the particle uptake by macrophages. Fig. 8b shows that a significant number of DNase I loaded PLGA-MP of formula F2 prepared without DPPC and labelled with DEX-FITC (Fig. 8a) showed evidence within the cytoplasm after exposure to a concentration of 2 mg/mL of particles for 30 min. It should be stressed that the images were taken at Z (depth) of 0.76 μm so that the nucleus was centred and only the internalized particles appear. The results indicated thus that the particles were internalized within 30 min. Using DPPC in the formulation caused a significant reduction in the number of phagocytosed particles as shown in Fig. 8c–e when compared to Fig. 8b. The use of DEX, PGA or PAA in the formulae did not cause any detectable effect. By virtue of its surface properties, DPPC migrates to the surface and can camouflage the macrophages, being an endogenous lung surfactant [21,22]. The included surface and activity enhancers did not interfere with DPPC effect.

4. Conclusions

The choice of the concentration of the dispersibility enhancer was based on PLGA amount since it was the most important parameter affecting SDP properties. Leucine, in a concentration of 30% w/w, caused a 40% increase in the yield, 9% in EE% and more than 20% in the fraction emitted from inhalation device. Surface modifiers greatly increased EE% with the superiority of PVP. The chosen excipients were able to conserve 80% of the enzyme activity after emulsification and spray drying. The results showed also that the use of CS-HCl in DNase I formulations was not recommended.

Dextran and PGA surface modified particles showed enhanced mucolytic activity on CF sputum. The formulations prepared with these DNase I adjuvants exhibited superior physico-chemical and aerosolisation properties than those containing conventional hydrophilic surface modifiers (lactose, PVP). The obtained results elect these formulations for further preclinical testing.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pupt.2013.07.010>.

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