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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 6×10^{-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.