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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.

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 multicomponent 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 ($CaCl_2$), magnesium chloride ($MgCl_2$), 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 $CaCl_2$, 2 mM $MgCl_2$ 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 w_1/o emulsion which was subsequently added dropwise into 30 mL of an external aqueous phase (w_2) 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 $CaCl_2$, 2 mM $MgCl_2$, 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.