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Graphical Abstract

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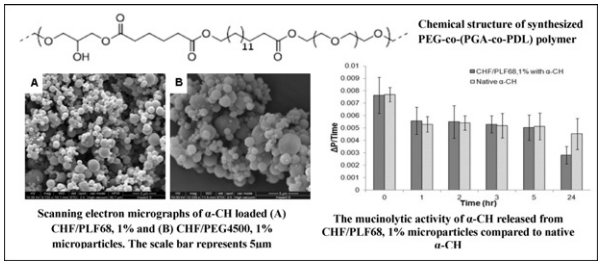

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Dry powder inhalation of macromolecules using novel PEG-co-polyester microparticle carriers

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ABSTRACT

This study investigated optimizing the formulation parameters for encapsulation of a model mucinolytic enzyme, α-chymotrypsin (α-CH), within a novel polymer; poly(ethylene glycol)-co-poly(glycerol adipate-co-ω-pentadecalactone), PEG-co-(PGA-co-PDL) which were then applied to the formulation of DNase I. α-CH or DNase I loaded microparticles were prepared *via* spray drying from double emulsion ($w_1/o/w_2$) utilizing chloroform (CHF) as the organic solvent, L-leucine as a dispersibility enhancer and an internal aqueous phase (w_1) containing PEG4500 or Pluronic® F-68 (PLF68). α-CH released from microparticles was investigated for bioactivity using the azocasein assay and the mucinolytic activity was assessed utilizing the degradation of mucin suspension assay. The chemical structure of PEG-co-(PGA-co-PDL) was characterized by ^{1}H NMR and FT-IR with both analyses confirming PEG incorporated into the polymer backbone, and any unreacted units removed. Optimum formulation α-CH-CHF/PLF68, 1% produced the highest bioactivity, enzyme encapsulation (20.08 ± 3.91%), loading (22.31 ± 4.34 μg/mg), FPF (fine particle fraction) (37.63 ± 0.97%); FPD (fine particle dose) (179.88 ± 9.43 μg), MMAD (mass median aerodynamic diameter) (2.95 ± 1.61 μm), and the mucinolytic activity was equal to the native non-encapsulated enzyme up to 5 h. DNase I-CHF/PLF68, 1% resulted in enzyme encapsulation (17.44 ± 3.11%), loading (19.31 ± 3.27 μg/mg) and activity (81.9 ± 2.7%). The results indicate PEG-co-(PGA-co-PDL) can be considered as a potential biodegradable polymer carrier for dry powder inhalation of macromolecules for treatment of local pulmonary diseases.

1. Introduction

Recent advances in inhalation therapy have provoked considerable interest in the development of novel formulations intended for pulmonary delivery of macromolecules (Tamber et al., 2005). This is primarily due to the lung having a favourable environment for delivery of macromolecules, including enzymes, compared to the low pH and high protease levels associated with the gastrointestinal tract (Fu et al., 2002). Furthermore, inhalation therapy of macromolecules has focused on dry powder inhalers (DPIs) due to their many advantages compared to pressurized metered dose inhalers (pMDIs) (Seville et al., 2002). Current research towards formulating macromolecules for pulmonary delivery has centred mainly upon biodegradable polymers as carrier based controlled release formulations (Kumar Malik et al., 2007), to increase the quantity of macromolecule reaching the site of action and prolong its residence time *in situ*, improve *in vivo* stability, and allow co-localized deposition with other therapeutic agents or helper excipients (i.e. absorption enhancers) (Cryan, 2005).

An alternative class of biodegradable polyester, poly(glycerol adipate-co-ω-pentadecalactone), PGA-co-PDL, have shown promise as sustained release carriers for large and small molecular weight compounds (Gaskell et al., 2008; Kallinteri et al., 2005), and the degree of hydrophobicity can be altered by varying the backbone chemistry or attaching chemical moieties to the free hydroxyl groups (Kallinteri et al., 2005). Recently, we have formulated PGA-co-PDL as a microparticle carrier for pulmonary delivery (Tawfeek et al., 2011) with small aerodynamic diameters (≤3.50 μm) (MMAD, diameter at which 50% of the particles by mass are larger and 50% are smaller than aerodynamic diameter (d_{ae} ≤ 4.6 μm)) to direct particles to the lung periphery for treatment of diseases. For example, in cystic fibrosis, the microparticle

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formulations delivered *via* DPIs should possess aerodynamic diameters be within 1–5 μm micron range (Westerman et al., 2007).

Many enzymes are not stable in phosphate buffered saline (PBS) pH 7.4 at 37°C, where chemical degradation (cleavage, oxidation, reduction etc.) and physical changes (conformational, aggregation and adsorption) on surfaces have been reported (Jiang et al., 2002). Moreover, enzyme aggregation may lead to incomplete release from microparticles (Jiang et al., 2002). For example, octreotide showed a slower non-complete release in acetate buffer with increasing ionic saline strength and PBS buffer (Bodmer et al., 1992), while another luteinizing hormone-releasing hormone (LHRH) analogue, orntide, showed nearly complete release in acetate buffer, but not in phosphate buffer (Kostanski et al., 2000). Furthermore, exposure of enzymes to organic solvents, sonication and mechanical shear forces during particle preparation results in the disruption of the three dimensional structure required to maintain their activity leading to complete inhibition of biological activity (Perez-Rodriguez et al., 2003).

Different strategies have been adopted to minimize such problems of protein and peptide inactivation, such as, increasing protein concentration during emulsification (Cleland and Jones, 1996; Perez and Griebenow, 2001), selection of solvent used in microparticle preparation (Castellanos and Griebenow, 2003) and incorporating amphipathic excipients (i.e. rat serum albumin), which competes with therapeutic protein for the interface between organic and aqueous phase (Srinivasan et al., 2005). Moreover, surfactants such as Tween 20, 80 (Cleland and Jones, 1996) and Pluronic® F68 (Blanco and Alonso, 1998) represent another group of amphipathic excipients, which can be applied to these systems. Another approach to prevent interface induced protein denaturation and aggregation is the addition of polyol or sugar excipients in the aqueous phase during preparation (Cleland and Jones, 1996; Perez and Griebenow, 2001). Poly(ethylene glycol) (PEG) has been studied as an emulsifier to decrease aggregate formation of α-CH loaded poly (lactic-co-glycolic acid) (PLGA) microspheres prepared by a soild-in-oil-in-water (s/o/w) technique (Castellanos et al., 2005).

The aim of the current investigation was to optimize the formulation and delivery of α-CH as a model enzyme being highly sensitive to unfolding and formulation conditions, *via* DPIs to the lungs using novel biodegradable carrier PEG-co-(PGA-co-PDL). PEG was incorporated into the polymer backbone to render the particles less susceptible to phagocytosis (Gref et al., 1994) and decrease polymer hydrophobicity making the particles more suitable for lung delivery (Fu et al., 2002). In addition, PEG4500 and Pluronic® F68 (PLF68) were incorporated in the internal aqueous phase (w_1) of the double emulsion in an attempt to prevent emulsification-induced denaturation and aggregation of α-CH. Different formulations were prepared by spray drying directly from a double emulsion ($w_1/o/w_2$), with L-leucine incorporated to enhance the dispersibility of the prepared dry particles within the respirable size range (Tawfeek et al., 2011) and investigated for their encapsulation efficiency, enzyme loading, particle size, zeta-potential, *in vitro* release, bioactivity, morphology, aerosolization performance, toxicity and mucinolytic activity. The optimum formulation parameters were then used to encapsulate deoxyribonuclease I (DNase I) within PEG-co-(PGA-co-PDL) microparticles.

2. Materials and methods

2.1. Materials

Novozyme 435 (a lipase from *Candida antarctica* immobilized on a microporous acrylic resin) was purchased from Biocatalytics, USA. Glycerol, ω-pentadecalactone (PDL), PEG (MWs 400, 1500 and 4500), α-CH (from bovine pancreas, type II), mucin (from porcine stomach, type II), poly(vinyl alcohol) (PVA, MW 9-10K, 80%), L-leucine, azocasein, trichloroacetic acid (TCA), RPMI-1640 medium with L-glutamine and sodium hydrogen carbonate ($NaHCO_3$), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ammonium persulphate (APS), TEMED, gelatin, isobutanol, bromophenol blue, trisma base, glycine, sodium dodecyl sulfate (SDS), colloidal coomassie blue, tris hydrochloride, sodium chloride, calcium chloride, methyl green, bovine serum albumin, thimerosal, Tween 20, and deoxyribonucleic acid (DNA) from salmon testes were all obtained from Sigma–Aldrich, UK. DNase I was obtained from Roche. Chloroform (CHF), dichloromethane (DCM), sodium hydroxide and N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES) were purchased from BDH laboratory supplies, UK. Tetrahydrofuran (THF), 75 cm^2/tissue culture flask with vented cap (IWAKI brand), 24-well tissue culture plates, 96-well flat bottom plates, antibiotic/antimycotic solution (100×) was purchased from Fisher Scientific, UK. Phosphate buffered saline tablets (PBS), pH 7.4, were obtained from Oxoid, UK. Divinyl adipate (DVA) was obtained from Fluorochem, UK and PLF68 was a gift from BASF Corp., USA. Foetal calf serum heat inactivated was purchased from Biosera, UK. Proto Gel, proto gel resolving buffer, proto gel stacking buffer was purchased from National Diagnostics, USA. Mini-protean tetra electrophoresis system was purchased from BioRad Laboratories, UK. Human bronchial epithelial (16HBE14o-) cells were produced by Dr Dieter Gruenert from the California Pacific Medical Center, University of California, San Francisco, USA.

2.2. Polymer synthesis

PEG-co-(PGA-co-PDL) was synthesized by a combination of enzyme catalyzed polycondensation and ring opening co-polymerization reactions for producing PGA-co-PDL using methods modified from that as described by Thompson et al. (2006) and He et al. (2003). Briefly, PEG (0.0005 mol, 1500 Da), and the monomers, glycerol (0.05 mol), divinyl adipate (DVA) (0.05 mol), and ω-pentadecalactone (PDL) (0.05 mol), were added to the reaction medium (THF) prior to addition of novozyme 435 (1 g) and the reaction allowed to proceed for 24 h. Upon completion, 300 ml of DCM was added and the enzyme was removed by Buchner filtration. The solvent was removed by rotary evaporation and the molten polymer re-dissolved in the minimum amount of DCM. Methanol (100 ml) was added and the mixture agitated to precipitate the polymer leaving the unreacted components in solution. The solid polymer was obtained by filtration and air dried before storing over molecular sieves. The synthesized co-polymer was characterized by gel permeation chromatography, GPC (Viscotek TDA Model 300 using OmniSEC3 operating software), calibrated with polystyrene standards (polystyrene standards kit, Supelco, USA). FT-IR spectra were obtained using a Perkin Elmer Spectrum BX spectrometer fitted with a PIKE technologies miracle sampling accessory and using Spectrum v5.0.1 for data processing. ^{1}H NMR spectroscopy was performed using a Bruker AVANCE 300, inverse probe with B-ACS 60 and autosampler with gradient chemming (Thompson et al., 2006).

2.3. Effect of release media on the bioactivity of α-CH

Native enzyme (α-CH) (10 mg) was added to different freshly prepared release media (PBS pH 7.4, distilled water pH 7.5, aqueous 1% w/v PLF68 and aqueous 1% w/v PEG 400, pH 7.15) and incubated for 24 h at 37 ± 0.5 °C in an orbital shaker (IKA KS 130) at 250 rpm.

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The concentration and the bioactive fraction of the enzyme were determined by the following tests:-

(A) The total enzyme content in the samples was determined using spectrophotometry at 282 nm (UV–vis spectrophotometer Lambda 40, Perkin Elmer).
(B) Enzyme bioactivity (expressed as bioactive fraction) was determined by measuring the proteolytic activity of α-CH using a chromogenic based technique, the azocasein assay, as described by Gaskell et al. (2008) and Jesse and Rudolph (1947). The bioactive fraction was calculated as the ratio of enzyme concentration recorded from the azocasein test to the enzyme concentration obtained from UV spectrophotometeric analysis at 0 h (the bioactivity of enzyme just after preparation) and 24 h (the bioactivity of enzyme after release from microparticles).
(C) SDS-PAGE zymography was employed to identify the proteolytic activity of α-CH separated in polyacrylamide gel using gelatin as the substrate under non reducing conditions (Kleiner and Stetlerstevenson, 1994). SDS-PAGE zymography gels were prepared as previously reported (Kleiner and Stetlerstevenson, 1994). Samples were prepared by mixing 30 μl of either non-encapsulated native enzyme (control) or enzyme released from microparticles before and after 24 h incubation in aqueous 1% w/v PLF68 with 30 μl sample buffer (10 ml glycerol, 1 g SDS, 4 ml Tris–HCl 1 M pH 6.8, 0.1% w/v bromophenol blue, 50 ml deionised water). Samples were loaded onto the gels placed within mini-protean tetra electrophoresis system and electrophoresis was conducted by applying a constant current of 26 mA per gel for approximately 60 min, followed by incubation at room temperature (25 °C) in 50 mM Tris–HCl buffer, pH 7.4 (121.14 g Tris base, 69.1 ml conc. HCl and 824 ml distilled water) with 2.5% Triton X-100, to remove SDS, followed by washing with distilled water. The gels were then incubated at 37 °C overnight in enzyme buffer (30 mM Tris–HCl pH 7.4, 200 mM NaCl, 10 mM $CaCl_2.2H_2O$) followed by colloidal coomassie blue staining for 90 min and then de-staining in distilled water for 2 h. Evidence of α-CH activity was demonstrated by the absence of staining in areas where the gelatin substrate had been degraded.

2.4. Microparticle preparation

Microparticles were prepared by spray drying directly from a double emulsion ($w_1/o/w_2$) as reported by Tawfeek et al. (2011) with modifications. 50 mg α-CH was dissolved in 1.5 ml distilled water (w_1) containing different concentrations (% w/v) of PLF68 (1 and 3.4) or PEG4500 (1, 3.4 and 6.7). The enzyme solutions were homogenized (IKA yellowline DI 25 basic at 8000 rpm for 1 min) in 15 ml DCM or CHF containing 450 mg polymer to form the first w_1/o emulsion. This was then added to a second aqueous phase (w_2) (150 ml distilled water containing 1% w/v PVA as an emulsifier and L-leucine (1.5% w/w of polymer weight) as a dispersing agent), under moderate stirring conditions (silverson L5RT mixer, 2000 rpm at room temperature, 25 °C) to form the $w_1/o/w_2$ double emulsion. The formulations will be represented within the text as: DCM alone, CHF alone, [CHF/PLF68, 1%], [CHF/PLF68, 3.4%], [CHF/PEG4500, 1%], [CHF/PEG4500, 3.4%], [CHF/PEG4500, 6.7%]. Microparticles incorporating DNase I were prepared using CHF/PLF68, 1%.

2.5. Microparticle characterization

2.5.1. Yield, encapsulation efficiency and enzyme loading

Spray dried PEG-co-(PGA-co-PDL) microparticles yields were quantified as the percentage mass obtained compared with the anticipated total powder yields. All analyses in the manuscript were conducted in triplicate unless otherwise specified. The enzyme loading (EL) and percentage encapsulation efficiency (EE) were calculated using Eqs. (1) and (2):

$$\mathrm{EL} = \frac{\text{weight of α-CH in microparticles}}{\text{microparticles sample weight}} \quad (1)$$

$$\mathrm{EL}\ (\%) = \frac{\text{actual weight of α-CH in sample}}{\text{theoretical weight of α-CH}} \times 100 \quad (2)$$

Briefly, 10 mg of microparticles were weighed and solubilized in a mixture of DCM/water (2:1) to dissolve the polymer and extract the enzyme. The two phases were separated by centrifugation (5 min at 16,200 × g, accuspin micro 17) and the aqueous layer analyzed for enzyme content spectrophotometrically at 282 nm. The same procedure was applied for formulations containing DNase I.

2.5.2. Particle size, zeta potential, powder density and theoretical aerodynamic diameter

Spray dried microparticles were sized using a Zetaplus, Brookhaven Instruments, UK. 100 μl of microparticles suspension was diluted to 4 ml using double distilled water and the measurements recorded at 25 °C. The zeta potential was determined using the same instrument with 50 μl of the suspension added to 2 ml of distilled water and the measurement was performed using a gold-plated zeta dip probe at 25 °C. The theoretical primary aerodynamic diameter (d_{ae}) was calculated using data acquired from geometric particle size (d) and tapped density (p) according to Eq. (3) (Tawfeek et al., 2011).

$$d_{ae} = d\sqrt{\frac{p}{p_1}} \qquad p_1 = 1 \ \ \mathrm{g\ \ cm^{-3}} \quad (3)$$

2.5.3. Degree of crystallinity and particle morphology

The degree of crystallinity for the polymer and spray dried microparticle formulations was determined using differential scanning calorimetry (DSC, Perkin Elmer Pyris 1) and the thermal data was determined from the second heating cycle (Thompson et al., 2007). Briefly, 3–5 mg of sample was placed into a hermetically sealed and crimped pan. Samples were purged with nitrogen at 20 ml/min and heated at a rate of 20 °C/min. Formulations were visualized by scanning electron microscopy (SEM) (FEI – Inspect S Low VAC SEM). Particles were mounted on aluminium stubs (pin stubs, 13 mm) using an adhesive conductive carbon tab and air dried. Samples were coated with gold (EmiTech K 550X Gold Sputter Coater, 10–15 nm) prior to examination at an accelerating voltage of 25 mA for 3 min.

2.6. In vitro release testing

Briefly, 10 mg of spray dried microparticles was added to a 1.5 ml microtube containing 1 ml pre-warmed (37 ± 0.5 °C) aqueous 1% w/v PLF68 release medium and incubated at 37 °C in an orbital shaker (IKA KS 130) at 250 rpm over 24 h. The supernatant was collected after centrifugation (5 min at 16,200 × g, accuspin micro 17) at designated time-points and stored at 2–4 °C until further analysis. The bioactivity of released α-CH from the different formulations was determined at 0 h and after 24 h using the azocasein assay described above. The enzyme activity of DNase I was determined at 0 h and 24 h using a colorimetric assay performed at room temperature (Sinicropi et al., 1994). Release samples and unprocessed DNase I (100 μl) were added to 96-well plate followed by 100 μl of DNA-methyl green substrate, and incubated at room temperature for 90 min. The reaction was quenched with 50 μl of EDTA–hydrogen peroxide solution (50 mM) and the absorption measured at 620 nm. This was immediately followed by incubation for a further 90 min before measuring the absorption at 620 nm. The

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difference in absorption was used to calculate the activity of DNase I compared to unprocessed DNase I.

2.7. *In vitro* aerosolisation investigations

Aerodynamic particle size distribution was determined using next generation impactor (NGI) (Copley Scientific, Nottingham, UK). Microparticle samples (20 ± 0.4 mg) were manually loaded into hydroxypropyl methylcellulose capsules (HPMC size 3) and placed in a HandiHaler®. A flow rate of 60 L/min for 4 s was applied using a pump (Copley Scientific, Nottingham, UK) to deposit the particles on the impactor plates which were coated with 1% w/w glycerol/methanol solution. Following inhalation all parts of NGI were washed with DCM/water (2:1), and analyzed as above. The fine particle fraction (%FPF) (defined as the mass of drug deposited (d_{ae} < 4.6 μm), was expressed as a percentage of the emitted dose. The mass median aerodynamic diameter (MMAD) was calculated by plotting cumulative percentage of mass less than stated aerodynamic diameter (probability scale) versus aerodynamic diameter (log scale). The fine particle dose (FPD) was expressed as the mass of drug deposited in the NGI (d_{ae} < 4.6 μm).

2.8. Cell viability study

The effect of microparticle formulations produced using CHF alone, optimum formulations [CHF/PL68, 1%] and [CHF/PEG4500, 1%] (0–5 mg/ml) (n = 6) on the viability of human bronchial epithelial (16HBE14o-) cells (passage no. 26) were evaluated over 24 h using the MTT assay as previously reported (Tawfeek et al., 2011). DMSO (10%) was used as a positive control. The relative cell viability (% of control cells) was calculated using Eq. (5):

$$\text{Viability } (\%) = \frac{A - S}{CM - S} \times 100 \quad (5)$$

where A is the absorbance of the test substance concentrations, S is the absorbance obtained for isopropanol and CM is the absorbance obtained for untreated cells incubated with medium (control).

2.9. Mucinolytic activity

The mucinolytic activity of α-CH was determined using mucin as a substrate and monitoring its degradation spectrophotometrically (400 nm). There are several factors that affect the rate of mucin degradation, such as substrate concentration, enzyme concentration, temperature and pH, which were investigated to obtain the optimum conditions for the assay (Gaskell et al., 2008). Briefly, mucin solution (1.45 ml, 3 mg/ml, 20 mM sodium phosphate buffer, pH 7.4) was added into a semi-micro, 2 ml cuvette and pre-incubated under controlled temperature conditions at 37 ± 0.5 °C for 20 min to eliminate any temperature variations. 50 μl native enzyme and 50 μl of α-CH released from optimum microparticle formulation [CHF/PLF68, 1%] and blank microparticles, which had been incubated in aqueous 1% w/v PLF68 release medium for different time intervals (0–24 h), were added to pre-incubated mucin solution and mixed by pipette (n = 6). The turbidity of mucin upon addition of enzyme was compared with PBS buffer (pH 7.4) as a negative control. Samples were analyzed immediately at 400 nm using a UV–vis spectrophotometer (Lambda 40, Perkin Elmer) and after 30 min incubation at 37 ± 0.5 °C. The change in absorbance reading taken at the two time points was considered as the enzyme's mucinolytic activity.

2.10. Statistical analysis

Statistical analysis comparing α-CH and DNase I was performed using the paired Student's t-test with two-tailed comparison. All subsequent formulations of [CHF/PEG] and [CHF/PLF68] were compared with control, CHF alone, by a one-way analysis of variance (ANOVA) with Dunnett multiple comparison test. CHF alone, [CHF/PEG] and [CHF/PLF68] formulations and release media were then compared with each other by means of a one-way ANOVA with the Tukey's comparison test. Differences of $p < 0.05$ are considered significantly different.

3. Results and discussion

3.1. Characterization of PEG-co-(PGA-co-PDL)

The chemical structure of PEG-co-(PGA-co-PDL) (Fig. 1A) was characterized by ^{1}H NMR and FT-IR. Analysis of the polymer by GPC confirmed a unimodal mass distribution corresponding to a polymer molecular weight of 19.9 kDa with no peaks relating to free PEG (1500 Da), indicating this was removed by the methanol during the washing stage of polymer synthesis. In conjunction with this data, NMR and FT-IR analysis (Fig. 1B and C) confirmed that the monomeric units were polymerized with PEG incorporated into the polymer backbone, and any unreacted units were removed. The integrals of the peaks for the CH_2 groups of PDL (1.3 ppm, 21H), DVA (2.39 ppm, 4H), glycerol (4.18 ppm, 4H) and PEG (3.67 ppm, 1.5H), were used to calculate the actual PEG and monomer ratio in the polymer using a ^{1}H NMR integration method (Kolhe et al., 2004). The actual ratio of PEG-co-(PGA-co-PDL), 0.01:1:1:0.75, was calculated as the ratio of theoretical to actual protons. Additionally, the percentage of PEG in the polymer was calculated as 3.89%, based on the molecular weight ratio of PEG to polymer.

3.2. Stabilization of α-CH in aqueous release medium

Degradation of enzyme resulting in loss of bioactivity can occur either during preparation or in conventional release media (PBS buffer pH 7.4 and distilled water) at 37 °C. Hence, *in vitro* release experiments under physiologically relevant conditions are difficult due to autolysis and protein fragmentation (Perez-Rodriguez et al., 2003). PBS (pH 7.4) and distilled water (pH 7.5) resulted in a low bioactive fraction, 0.21 ± 0.06 and 0.12 ± 0.04 respectively, whilst the incorporation of additives, 1% w/v PEG 400 (pH 7.15) and 1% w/v PLF68 (pH 7.15), to distilled water retained higher enzyme activity, with a bioactive fraction of 0.78 ± 0.09 and 1.02 ± 0.07 respectively after 24 h. Our data indicated the type and/or components of the medium used during *in vitro* release were influential, with aqueous 1% w/v PLF68 solution, being the optimum to preserve the native enzyme bioactivity during incubation at 37 °C over 24 h, compared to the other media (p < 0.05, ANOVA/Tukey's). Consequently, aqueous 1% w/v PLF68 solution was used in all subsequent studies to ensure that any loss in bioactivity was not associated with the composition of the release media. The enhanced bioactivity was most likely due to the stabilizing effects of PLF68 resulting in prevention of enzyme aggregation and adsorption to hydrophobic surfaces (Blanco and Alonso, 1998). Similar observations were reported (Wolf et al., 2003), indicating the preservation of L-asparaginase activity (100 ± 1.7%) after incubation in aqueous 1% w/v PLF68 medium for 24 h.

3.3. Microparticle characterization

DSC analysis of PEG-co-(PGA-co-PDL) alone produced a crystalline endothermic peak at 60.66 °C with an onset of melting at 55.01 °C (Fig. 2, Trace A). In contrast, spray dried microparticles CHF alone, [CHF/PLF68, 1%] and [CHF/PEG4500, 1%] exhibited a significant reduction in the area under the melting endotherm peak, lowered melting point (56.97 °C, 57.30 °C and 56.95 °C respectively)

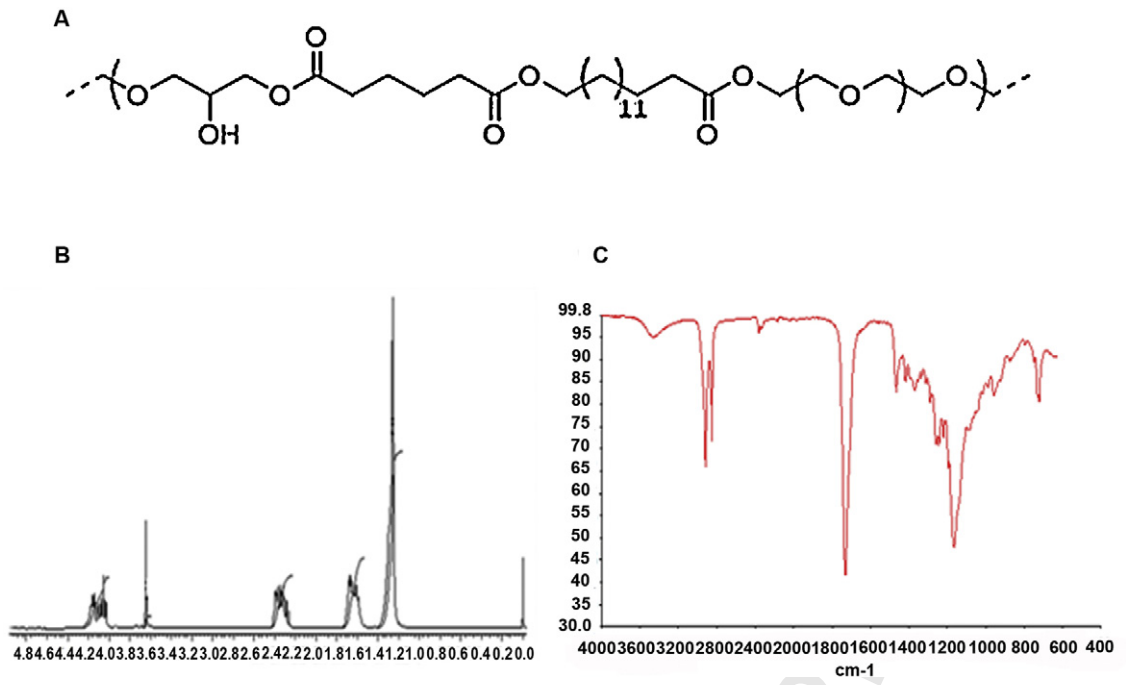

Fig. 1. (A) Chemical structure of PEG-co-(PGA-co-PDL) polymer, (B) ^{1}H NMR spectrum of PEG-co-(PGA-co-PDL), and (C) FT-IR spectrum of PEG-co- (PGA-co-PDL).

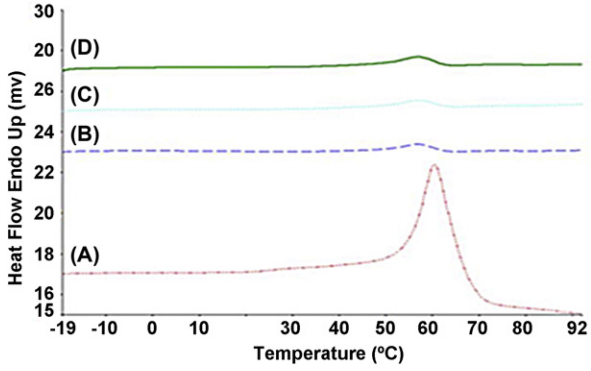

Fig. 2. DSC thermograms of (A) PEG-co-(PGA-co-PDL) polymer, (B) Spray dried CHF alone microparticles, (C) spray dried CHF/PLF68, 1% microparticles and (D) spray dried CHF/PEG4500, 1% microparticles.

and earlier onset of melting (50.84 °C, 50.52 °C and 49.54 °C respectively) (Fig. 2, Traces B–D) indicating a largely amorphous nature, which is characteristic of spray dried products due to the rapid drying of droplets (Corrigan, 1995). Consequently, spray drying parameters were set to preserve the outlet temperature in the range of 44–47 °C due to the low melting of PEG-co-(PGA-co-PDL).

Spray drying the emulsion formulations with L-leucine produced dry powders with yields between 66.6 ± 7.1 and 88.8 ± 5.4% (Table 1). Microparticles produced using DCM alone resulted in significantly lower enzyme loading, 13.35 ± 0.45 μg/mg particles, compared to microparticles produced using CHF alone, 22.85 ± 2.56 μg/mg ($p < 0.05$, *t*-test/two-tailed) (Table 1). The improvement was related to the low water miscibility of CHF (0.8 g/100 ml) compared to DCM (1.32 g/100 ml). This corresponded to reduced enzyme adsorption at the CHF/water interface compared to DCM/water interface (Castellanos et al., 2001), resulting in reduced leaching of α-CH to the external aqueous phase (w_2) during secondary emulsification (o/w_2 stage). Furthermore, the reduced partitioning to the external aqueous phase (w_2) significantly enhanced the encapsulation compared with microparticles produced using DCM alone ($p < 0.05$, *t*-test/two-tailed). Consequently, CHF was chosen as the organic solvent for preparing the remaining formulations. However, only addition of PEG4500 (1.0 or 3.4% w/v) to the internal phase resulted in an increase in enzyme loading compared to CHF alone ($p < 0.05$, ANOVA/Dunnett), whereas the addition of PEG4500 (6.7% w/v) resulted in a significant reduction to 16.57 ± 0.78 μg/mg particles ($p < 0.05$, ANOVA/Dunnett). A similar trend was observed with encapsulation efficiency, which ranged from 12.01 ± 0.40 to 25.32 ± 0.64% (Table 1). This has been associated with PLF68 and PEG4500 decreasing the surface tension of α-CH solution, leading to reduced protein aggregation and adsorption to hydrophobic surfaces (Bilati et al., 2005; Blanco and Alonso, 1998). The spray dried yield of DNase I encapsulated microparticles was similar to the formulations of α-CH with [CHF/PLF68, 1%]. However, the enzyme loading and encapsulation efficiency were lower ($p > 0.05$, *t*-test/two-tailed) than the corresponding α-CH formulations.

All microparticle formulations had a geometrical particle size suitable for pulmonary delivery, with microparticles produced using DCM alone (0.95 ± 0.12 μm) generated significantly smaller particles compared to using CHF alone (1.43 ± 0.27 μm) ($p < 0.05$, *t*-test/two-tailed) (Table 1). This was associated with CHF having a higher boiling point compared to DCM, resulting in a slower rate of removal (due to a higher solvency) and greater packing density of the polymer chain during spray drying (Bain et al., 1999). Furthermore, addition of PLF68 or PEG4500 to the internal aqueous phase significantly increased the particle size, 1.95 ± 0.14 to 2.66 ± 0.76 μm, compared to CHF alone (1.43 ± 0.27 μm) ($p < 0.05$, ANOVA/Dunnett) (Table 1). The theoretical aerodynamic diameter (d_{ae}) for all formulations was between 0.38 ± 0.07 and 1.08 ± 0.37 μm. Moreover, microparticles formed using DCM alone had an anionic surface charge with a zeta potential of −19.67 ± 1.20 mV, which was reduced to −14.99 ± 1.46 mV upon changing to CHF, with no significant change upon incorporation of PLF68 or PEG4500 (Table 1) ($p > 0.05$, ANOVA/Dunnett). The negative surface charge demonstrated the anionic nature of the produced microparticles, which may be associated with incomplete removal of the PVA emulsifier incorporated in the external aqueous phase (w_2) of the double emulsion.

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Table 1
The physical characteristics of spray dried microparticles (data represent mean ± SD, $n = 3$).

Formulation	Yield (%)	EL (μg/mg particles)	EE (%)	Particle size (μm)	Zeta potential (mV)	d_{ae} (μm)
DCM alone	80.5 ± 5.4	13.35 ± 0.45*	12.01 ± 0.40*	0.95 ± 0.12*	−19.67 ± 1.20	0.38 ± 0.07
CHF alone	66.6 ± 7.1	22.85 ± 2.56	20.57 ± 2.30	1.43 ± 0.27#	−14.99 ± 1.46	0.56 ± 0.18
CHF/PLF68, 1%	67.4 ± 4.9	22.31 ± 4.34	20.08 ± 3.91	2.05 ± 0.66	−16.60 ± 0.93	0.87 ± 0.30
CHF/PLF68, 3.4%	68.4 ± 5.5	22.23 ± 1.65	20.01 ± 1.49	2.44 ± 0.13	−17.58 ± 1.98	0.97 ± 0.21
CHF/PEG4500, 1%	83.2 ± 2.6	27.12 ± 3.01	24.41 ± 2.70	2.66 ± 0.76	−14.56 ± 0.50	1.08 ± 0.37
CHF/PEG4500, 3.4%	88.8 ± 5.4	28.13 ± 0.71	25.32 ± 0.64	1.95 ± 0.14	−17.76 ± 1.77	0.78 ± 0.10
CHF/PEG4500, 6.7%	72.8 ± 3.2	16.57 ± 0.78**	14.92 ± 0.70**	2.29 ± 0.35	−15.56 ± 0.93	0.92 ± 0.17
DNase I CHF/PLF68, 1%	69.1 ± 3.5	19.31 ± 3.27	17.44 ± 3.11	2.21 ± 0.93	−15.50 ± 0.71	1.16 ± 0.23

* DCM alone *vs* CHF alone ($p < 0.05$, *t*-test/two-tailed).
** CHF/PEG4500, 6.7% *vs* CHF/PLF68, 3.4%, CHF/PEG4500, 1% and CHF/PEG4500, 3.4% ($p < 0.05$, ANOVA/Tukey's).
\# CHF alone *vs* CHF/PLF68, 1%, CHF/PLF68, 3.4%, CHF/PEG4500, 1%, CHF/PEG4500, 3.4% and CHF/PEG4500, 6.7% ($p < 0.05$, ANOVA/Dunnett).

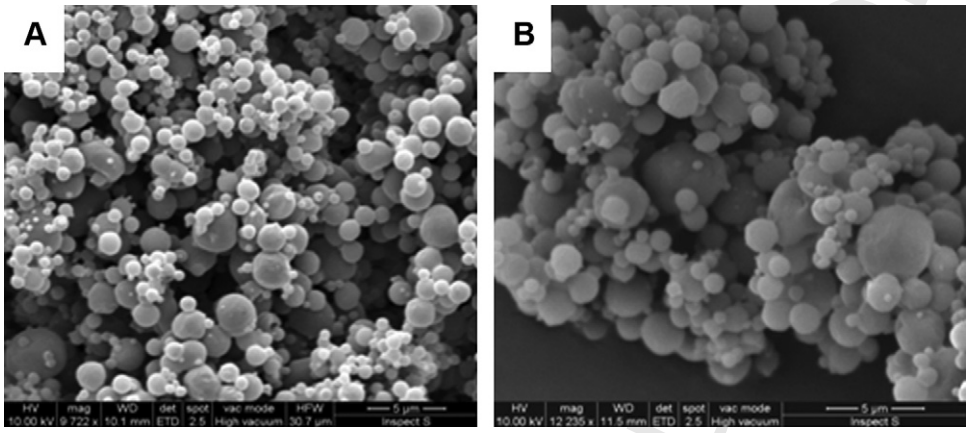

Fig. 3. Scanning electron micrographs of (A) α-CH loaded CHF/PLF68, 1% microparticles and (B) CHF/PEG4500, 1%. The scale bar represents 5 μm.

The optimum formulations in terms of bioactivity (see Section 3.4, bioactivity of released enzyme), [CHF/PLF68, 1%] and [CHF/PEG4500, 1%], were visualized using SEM (Fig. 3A and B, respectively). The spray dried microparticles appeared uniform spherical and regular in shape. The smooth surface possibly occurred due to rapid microparticle hardening and evaporation of CHF rather than solvent partitioning into water. Hence, there was limited possibility of water ingress during manufacturing or aggregation of particles which often occurs in emulsion solvent evaporation techniques. The optimum formulation incorporating PLF68, 1% was used to encapsulate DNase I, and had a similar geometrical particle size, zeta potential and aerodynamic diameter to α-CH microparticles formulated with [CHF/PLF68, 1%] ($p > 0.05$, *t*-test/two-tailed) (Table 1).

3.4. *In vitro* release and bioactivity of released α-CH

The *in vitro* release behaviour for all investigated formulations is represented in Fig. 4A and B up to 5 h as no further release of α-CH was noted beyond this time point. A significantly higher enzyme burst release was noted from microparticles prepared using DCM alone (95.33 ± 5.51%) compared to CHF alone (45.70 ± 8.25%) ($p < 0.05$, *t*-test/two-tailed). This was due to the low boiling point of DCM and faster rate of evaporation, resulting in the presence of higher amounts of enzyme on the surface of spray dried microparticles.

Incorporating PLF68 and PEG4500 to the internal phase (w_1) of double emulsion, [CHF/PLF68, 1%] (41.85 ± 7.34%) and [CHF/PLF68, 3.4%] (58.12 ± 13.29%), [CHF/PEG4500, 1%] (43.06 ± 6.87%), [CHF/PEG4500, 3.4%] (40.15 ± 6.56%), did not significantly affect the burst release of α-CH compared to microparticles produced using CHF alone ($p > 0.05$, ANOVA/Tukey's). However, a significantly higher burst release was observed with [CHF/PEG4500, 6.7%] and [CHF/PLF68, 3.4%] compared to the other formulations (Fig. 4A and B) ($p < 0.05$, ANOVA/Tukey's). This was due to the solubilizing effect of PEG4500 and PLF68 on the polymer matrix, influencing its plasticity and porosity (Jiang et al., 2002), resulting in more rapid entry of the release medium into the microparticles, eventually accelerating the release of the enzyme (Castellanos et al., 2005; Blanco and Alonso, 1998). It has been reported that complex formations between PLF68 and polyester polymers in organic solution may be responsible for changes in physicochemical properties of microparticles, such as encapsulation efficiency, particle size and release (Blanco and Alonso, 1998). Hence, the release pattern of α-CH from microparticles can be tailored by optimizing the additive concentration and selection of organic solvent. The release study indicated that both formulations [CHF/PLF68, 1%] and [CHF/PEG4500, 1%] were considered optimum, with reduced burst release. The formulation of DNase I with [CHF/PLF68, 1%] had a similar release profile to that composed of α-CH [CHF/PLF68, 1%], with no significant difference in the burst release (25.21 ± 13.29%) ($p > 0.05$, *t*-test/two-tailed) (Fig. 4C).

Table 2 represents the bioactive fraction of α-CH at 0 h and after 24 h release in 1% w/v PLF68 medium. The shear stresses involved in the preparation of microparticles during primary emulsification (w_1/o stage) and contact with DCM have been recognized as the major cause of α-CH aggregation and loss in activity (Castellanos et al., 2005). The results clearly indicated that microparticles prepared using CHF alone significantly improved the bioactive fraction compared to particles produced using DCM alone from 0.29 to 0.46 after 24 h release ($p < 0.05$, *t*-test/two-tailed). The improvement in bioactivity was again related to the low water miscibility (see Section 3.3, microparticle characterization); however, the low bioactive fractions for both formulations indicated a high percentage of enzyme degradation during preparation.

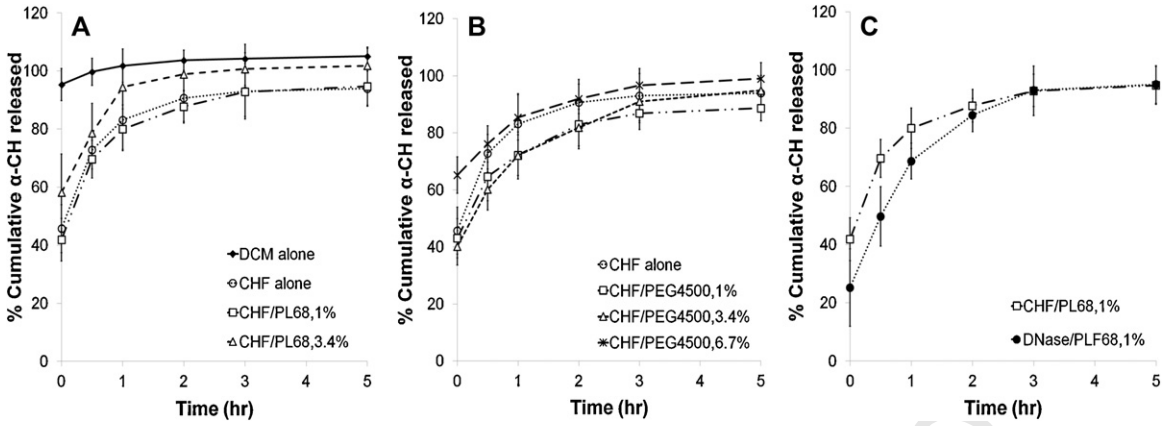

Fig. 4. *In vitro* release of α-CH from spray dried microparticles in aqueous 1% w/v PLF68 medium, (A) microparticles prepared using DCM alone, CHF alone and different concentrations of PLF68 in the internal phase (w_1) of double emulsion, (B) microparticles prepared using CHF alone and different concentrations PEG4500 in the internal phase (w_1) of double emulsion and (C) *in vitro* release of α-CH and DNase I from microparticles prepared using CHF/PLF68, 1% in the internal phase (w_1) of double emulsion (data represent mean ± SD, $n = 3$).

In this study the incorporation of PLF68 and PEG4500 in the internal aqueous phase (w_1) not only improved the bioactivity (Perez and Griebenow, 2001), but was also shown to be concentration dependent, with low concentrations of PLF68 (1% w/v) (1.08 ± 0.07 at 0 h and 0.9 ± 0.02 at 24 h) and PEG4500 (1% w/v) (0.84 ± 0.08 at 0 h and 0.83 ± 0.02 at 24 h) enhancing the bioactivity compared to the other formulations ($p < 0.05$, ANOVA/Tukey's) (Table 2). This occurred due to the properties of PLF68 and PEG4500 decreasing the surface tension of α-CH solution, leading to reduced protein aggregation and adsorption to hydrophobic surfaces (Bilati et al., 2005; Blanco and Alonso, 1998). In addition, PEG4500 has the potential to prevent the dehydration-induced protein structural perturbations during the emulsification process (Prestrelski et al., 1993). However, the reduced α-CH bioactivity with increased PLF68 (3.4% w/v) and PEG4500 (3.4 and 6.7% w/v) concentrations have been related to interactions with the enzyme resulting in its destabilization (Katakam et al., 1995). Furthermore, PLF68 (6.7% w/v) was not used due to separation of the w_1/o emulsion and visual aggregation of the enzyme. Due to [CHF/PLF68, 1%] and [CHF/PEG4500, 1%] maintaining the bioactive fraction of α-CH more efficiently than the other formulations, they were carried forward for aerosolization performance and cell toxicity studies.

3.5. *In vitro* aerosolisation performance

The use of L-leucine as a dispersibility enhancer has been investigated in our research group (Tawfeek et al., 2011) and by others (Chew et al., 2005; Najafabadi et al., 2004) to enhance the aerosolization performance. The deposition of α-CH from microparticles, [CHF/PLF68, 1%] and [CHF/PEG4500, 1%], in the capsule, inhaler and mouthpiece is represented in Fig. 5A, with no significant difference after aerosolization ($p > 0.05$, *t*-test/two-tailed). However, [CHF/PLF68, 1%)] microparticles had a significantly lower throat deposition compared to [CHF/PEG4500, 1%] ($p < 0.05$, *t*-test/two-tailed) (Fig. 5A). Both optimized microparticle formulations, [CHF/PLF68, 1%] and [CHF/PEG4500, 1%], illustrated high deposition on stages 2–4 (cut-off diameter 4.6–1.6 μm) of the NGI, indicating these formulations would be expected to deliver the majority of the emitted dose to the respirable regions of the lung periphery, which is the target site for CF patients (Westerman et al., 2007) (Fig. 5B) ($p > 0.05$, *t*-test/two-tailed).

Table 3 represents the FPF (%), FPD (μg) and MMAD (μm) of α-CH loaded [CHF/PLF68, 1%] and [CHF/PEG4500, 1%] spray dried microparticles, with significant difference noted only in the FPF (37.63 ± 0.97% and 33.69 ± 0.90%, respectively) ($p < 0.05$, *t*-test/two-tailed). The theoretical aerodynamic diameters (Table 1) calculated from tapped density indicate the suitability of PEG-co-(PGA-co-PDL) microparticles for targeting the deep lungs, generally ≤1 μm (Patton and Byron, 2007). However, the MMAD of the optimum formulations, [CHF/PLF68, 1%] and [CHF/PEG4500, 1%], was much larger at 2.95 and 2.75 μm respectively, indicating deposition in the lung periphery as noted above. This probably occurred due to formation of particle aggregates during aerosolization, which under the aerosolization parameters studied (flow rate 60 L/s, using the Handihaler) could not overcome van der Waals forces between particles.

3.6. Cell viability study, proteolytic and mucinolytic activity

Spray dried microparticles (0–5 mg/ml) CHF alone (96.99 ± 8.70 to 80.01 ± 4.01% cell viability), [CHF/PLF68, 1%] (94.56 ± 6.20 to 86.57 ± 4.39% cell viability) and [CHF/PEG4500, 1%] (96.65 ± 3.43 to 88.16 ± 3.55% cell viability) appeared to be well tolerated by normal

Table 2
The bioactive fraction of α-CH released from different formulations at 0 h and after 24 h release in aqueous 1% w/v Pluronic® F68 medium, determined using the azocasein assay (data represent mean ± SD, $n = 3$).

Formulation	Bioactive fraction at 0 h	Bioactive fraction after 24 h
DCM alone	0.29 ± 0.07	0.29 ± 0.06
CHF alone	0.46 ± 0.12*	0.46 ± 0.03*
CHF/PLF68, 1%	1.08 ± 0.07**	0.90 ± 0.02**
CHF/PLF68, 3.4%	0.63 ± 0.03	0.64 ± 0.05
CHF/PEG4500, 1%	0.84 ± 0.08**	0.83 ± 0.02**
CHF/PEG4500, 3.4%	0.75 ± 0.10	0.75 ± 0.03
CHF/PEG4500, 6.7%	0.63 ± 0.09	0.63 ± 0.07

* CHF alone *vs* DCM alone ($p < 0.05$, *t*-test/two-tailed).
** CHF/PLF68, 1% and CHF/PEG4500, 1% *vs* CHF alone ($p < 0.05$, ANOVA/Dunnett), CHF/PLF68, 1% and CHF/PEG4500, 1% *vs* CHF/PLF68, 3.4%, CHF/PEG4500, 3.4%, CHF/PEG4500, 6.7% ($p < 0.05$, ANOVA/Tukey's).

Table 3
The aerosolization characteristics of spray dried α-CH encapsulated microparticles (data represent mean ± SD, $n = 3$).

Formulation	FPF (%)	FPD (μg)	MMAD (μm)
CHF/PLF68, 1%	37.63 ± 0.97*	179.88 ± 9.43	2.95 ± 1.61
CHF/PEG4500, 1%	33.69 ± 0.90	175.05 ± 3.57	2.75 ± 0.23

* CHF/PLF68, 1% *vs* CHF/PEG4500, 1% ($p < 0.05$, *t*-test/two-tailed).

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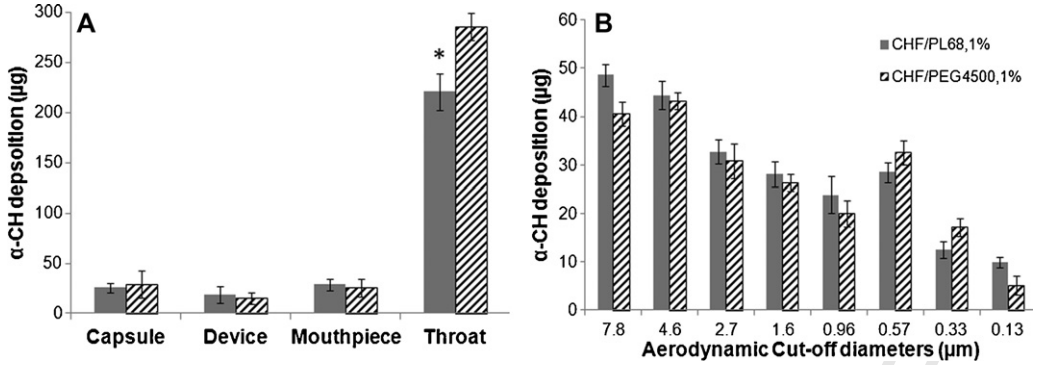

Fig. 5. Aerosol deposition of α-CH from CHF/PLF68, 1% and CHF/PEG4500, 1%, (A) comparing the deposition of α-CH in capsule, device, mouthpiece and throat, (B) the deposition of α-CH on different stages of NGI (data represents mean ± SD, $n = 3$). *CHF/PLF68, 1% *vs* CHF/PEG4500, 1% ($p < 0.05$, *t*-test/two-tailed).

lung bronchial epithelial cells (16HBE14o-) *in vitro*, even at high concentrations (5 mg/ml) following 24 h exposure, 80.01 ± 4.01, 86.57 ± 4.39 and 88.16 ± 3.55% cell viability were obtained respectively. Consequently the tolerance of normal bronchial epithelial cells to high concentrations of microparticles provides an indication to the feasibility of using PEG-co-(PGA-co-PDL) polymers as novel safe carriers for pulmonary delivery of α-CH and other macromolecules.

As there was no significant difference in cell toxicity and aerosol performance between [CHF/PLF68, 1%] and [CHF/PEG4500, 1%] microparticles, [CHF/PLF68, 1%] was chosen as the optimum formulation due to higher bioactive fraction, and released enzyme was subjected to further analysis at 0 h and after 24 h in 1% w/v PLF68 release medium using SDS-page gelatin-zymography (Fig. 6). It appeared that the bands obtained for α-CH released from [CHF/PLF68, 1%] microparticles were similar to the bands of the native enzyme at 0 h and after 24 h incubation, indicating incorporation of PLF68 (1% w/v), [CHF/PLF68, 1%], in the internal aqueous phase (w_1) of the double emulsion retained the proteolytic activity of α-CH after preparation, processing and release (Fig. 6). In addition, comparable mucinolytic activity was observed for α-CH released from [CHF/PLF68, 1%] microparticles and an equivalent concentration of native non-encapsulated enzyme incubated in aqueous 1% w/v PLF68 medium up to 5 h ($p > 0.05$, *t*-test/two-tailed), which was lower than the native non-encapsulated enzyme after 24 h ($p > 0.05$, *t*-test/two-tailed) (Fig. 7). Gaskell et al. have previously reported using mucin as a substrate for enzymes having mucinolytic activity, as a reproducible, reliable spectrophotometric assay (Gaskell et al., 2010). In this method light is transmitted and not absorbed as in standard photometric assays due to the turbidity of the mucin suspension. The results of this assay coincide with the release data obtained from [CHF/PLF68, 1%] microparticles with the burst release at time 0 h responsible for the initial higher mucinolytic activity followed by a gradual reduction over 24 h as the release concentration falls. Activity of DNase I was analyzed by monitoring DNA-methyl green degradation following release studies at 0 h and 24 h. The activity was found to be 90.1 ± 4.5% and 81.9 ± 2.7% respectively, compared to unprocessed DNase I.

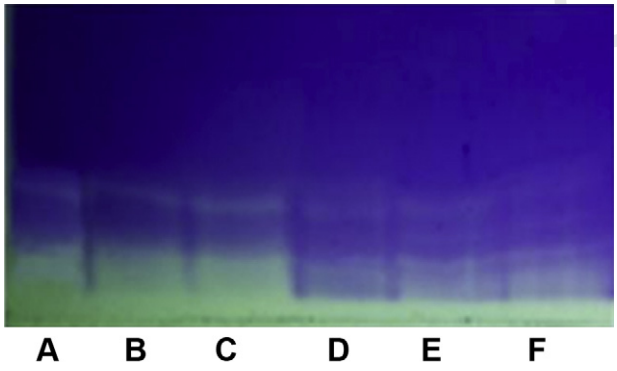

Fig. 6. SDS page-zymography of α-CH released from (A, B) CHF/PLF68, 1% at 0 h and after (D, E) 24 h compared to equal amounts of native, non-encapsulated enzyme, at (C) 0 h and after (F) 24 h incubation in aqueous 1% w/v PLF68 medium at 37 °C.

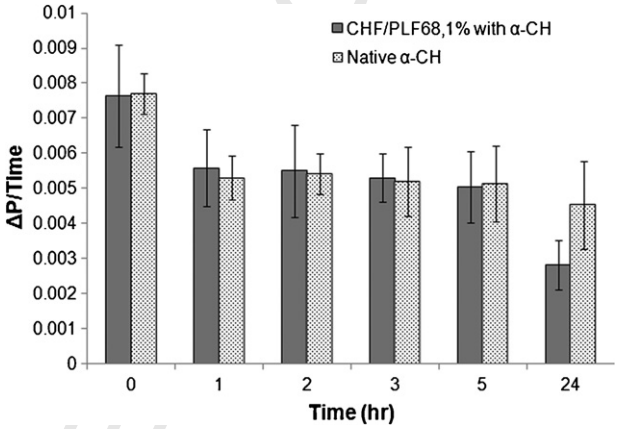

Fig. 7. The mucinolytic activity of native α-CH enzyme and encapsulated α-CH within optimum microparticle formulation CHF/PLF68, 1% obtained from the mucinolytic degradation spectroscopic assay of mucin (data represent mean ± SD, $n = 6$).

4. Conclusion

The findings from our investigations indicate that PEG-co-(PGA-co-PDL) can be used successfully to encapsulate α-CH, and possibly other macromolecules, in biocompatible microparticles utilizing a double emulsion/spray drying technique with a good yield, encapsulation efficiency and aerosolization performance. Moreover, incorporation of PLF68 (1% w/v) in the internal aqueous phase (w_1) of the double emulsion prior to emulsification/spray drying can maintain the bioactivity of α-CH and protect it from the harsh preparation conditions. The burst phase can be reduced to below 40% by incorporation of PLF68 or PEG4500 in the internal aqueous phase, and this is followed by gradual release over 5 h *in vitro*.

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