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# Stabilizing protein formulations during air-jet nebulization

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ABSTRACT

Whilst some proteins can be effectively administered to the lungs using a nebulizer, others, such as lactate dehydrogenase (LDH) are degraded during air-jet nebulization. In order to deliver LDH by nebulization a protective delivery system or carrier may therefore be appropriate. The aim of this study was to produce and characterize a formulation of LDH, which retains enzyme activity during nebulization. Chitosan, a biocompatible, biodegradable and bioadhesive polysaccharide polymer, was included in the formulations studied as a potential protective agent. Complexes of LDH with chitosan of different molecular weights and concentrations were assessed for size, zeta potential, aerosol droplet size and delivery from a jet nebulizer. The highest molecular weight chitosan had the greatest complex size and a net positive charge of +29.7 mV. Jet nebulization resulted in aerosol droplets with median size in the range 2.36–3.52 μm. Nebulization of LDH solution resulted in enzyme denaturation and reduced activity. The stability of LDH was greatly improved in formulations with chitosan; with greater than 50% total LDH available in a nebulizer delivered to the lower stage of a two-stage impinger, with up to 62% retained enzyme activity. The nonionic surfactant Tween 80 also improved the stability of LDH to nebulization and had an additive protective effect when included, with chitosan, in formulations. These findings suggest chitosan may be a useful excipient in the preparation of stable protein formulations for jet nebulization.



## 1. Introduction

The majority of therapeutic proteins and peptides are delivered via the parenteral route due to the deactivation and instability caused by gastrointestinal enzymes and first pass metabolism in the liver, leading to poor bioavailability following oral administration (Hussain et al., 2004; Amidi et al., 2008; Tan et al., 2010). Pulmonary delivery has been extensively studied as a non-invasive administration route for macromolecule delivery (Adjei and Gupta, 1994; Coldrons et al., 2003; Kumar et al., 2006; Malik et al., 2007). The large pulmonary surface area, high blood supply and relatively low enzymatic activity compared to the oral route make the lungs a particularly attractive delivery route for proteins and peptides (Takeuchi et al., 2001; Groneberg et al., 2003; Pilcer and Amighi, 2010). However, delivery of proteins via the pulmonary route may present difficulties, for instance, deposition in the oropharyngeal region, the mucus barrier layer and the mucociliary clearance process (Patton, 1996; Amidi et al., 2010). Moreover, a suitable delivery system may be required to ensure that the physicochemical stability of the biopharmaceutical is maintained and that it is delivered to the appropriate region of the respiratory tract (Hardy and Chadwick, 2000).

Whilst jet nebulizers have been used successfully to generate protein aerosols (Cipolla et al., 1994), some proteins are inactivated in a time-dependent manner (Niven and Brain, 1994). The loss of protein activity during jet nebulization has been attributed to high shearing forces within the nebulizer, deactivation at the surface of aerosolized droplets and the increase in protein concentration in the nebulizer reservoir due to preferential solvent loss during atomization (Phipps and Gonda, 1990; Niven et al., 1994; Fangmark and Carpin, 1996).

To overcome the stability issues associated with nebulizing some macromolecules, a number of formulation strategies have been employed; including liposomes (Gilbert and Knight, 1996; Kanaoka et al., 1999; Ten et al., 2002; Huang and Wang, 2006), lipid or polymeric complexes (Birchall et al., 2000; Kleemann et al., 2004; Lentz et al., 2005), surfactants (Niven et al., 1996; Flament et al., 1997) and nanocarriers (Kawashima et al., 1999; Liu et al., 2008; Patlolla et al., 2010).

In this paper, a jet nebulizer was used to convert protein formulations into aerosol droplets using a compressed gas. Lactate dehydrogenase (LDH) was chosen as a model hydrophilic protein due to its ready availability and the presence of well established quantitative in vitro assays for enzymatic activity. Chitosan was employed in our formulations as it was hypothesized that complexation of chitosan with LDH might enhance its stability. The inclusion of chitosan might also prolong the residence time at the targeted site in the airways and enhance absorption. This has been reported

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to enhance the bioavailability of intranasally administered human growth hormone (Cheng et al., 2005) and pulmonary delivered interferon alpha (Yamada et al., 2005). Chitosan is a biocompatible, biodegradable and bioadhesive polysaccharide cationic polymer that has been used in macromolecular delivery systems. Chitosan may prolong the contact time of formulations with mucosal tissues, without causing toxic effects or irreversible morphological changes to pulmonary cells (Alpar et al., 2005; Martinac et al., 2005; Douglas et al., 2006; Grenha et al., 2007).

The aim of this study was to produce and characterize formulations based on chitosan with or without non-ionic surfactant as a formulation strategy to enhance the stability and retain the activity of the protein, LDH during jet nebulization.

## 2. Materials and methods

### 2.1. Materials

QuantiPro BCA Assay Kit, lactate dehydrogenase (LDH, type II from rabbit muscle), β-nicotinamide adenine dinucleotide (NADH), sodium phosphate, sodium hydroxide, pyruvic acid sodium salt, Tween 80 (Polysorbate 80; polyoxyethylenesorbitan monooleate) and bovine serum albumin (BSA) were obtained from Sigma–Aldrich, UK. Pari LC Sprint air-jet nebulizers and Pari TurboBoy N compressor were purchased from Pari, GmbH, Germany. Visking dialysis membranes were purchased from Medicell International Ltd., UK. Chitosan (chitooligosaccharide (1 → 4) 2-amino-2-deoxy-β-D-glucose) of average molecular weight 1k, 3–5k and 10k was purchased from KITTO life Co., Ltd., Korea. Precision Plus Protein™ Standards were purchased from Bio-Rad Laboratories, Inc., UK.

### 2.2. Methods

#### 2.2.1. Preparation of LDH–chitosan complexes

LDH was dialyzed into phosphate buffer, pH 7.4, following the method of Harris and Angel (1989) and its concentration was determined by the BCA protein assay. Chitosan solutions of different molecular weights (1k, 3–5k and 10k) or different concentrations (0.05%, 0.1% and 0.2%, w/v) were prepared, and LDH solution (1.4 mg/ml) was added to the chitosan solutions dropwise using a peristaltic pump (Gilson, France) while the polymer solutions were stirred on a magnetically stirred plate.

#### 2.2.2. Size distribution and surface charge of the complexes

The size distribution of LDH–chitosan complexes was obtained as $Z_{Ave}$ hydrodynamic diameter and polydispersity index (PI) by photon correlation spectroscopy, using a Malvern 3000 spectrometer (Zetasizer Malvern Instruments Ltd., UK), which was also used to measure the zeta potential of formulations.

#### 2.2.3. Aerosol size analysis by laser diffraction

The size distribution of aerosol droplets was determined using a Malvern 2600C laser diffraction size analyser (Malvern Instruments Ltd., UK) with a 63 mm lens. Preparations (2.5 ml) were placed into a Pari LC Sprint jet nebulizer attached to the TurboBoy N compressor. The nebulizer was clamped 2.5 cm from the laser beam and aerosols traversed the beam 2.5 cm from the lens of the instrument. A vacuum was applied to draw aerosols through the beam. The volume median diameter (VMD) and Span (Span = 90% undersize − 10% undersize/VMD) were recorded at time intervals until aerosol output from the nebulizer ceased.

#### 2.2.4. Aerosol delivery of LDH containing formulations

The two-stage glass impinger (TSI, Copley Instruments, UK) comprises two stages that 'represent' the upper and lower respiratory tract. 5 ml of LDH solution (1.4 mg/5 ml) or LDH–chitosan/Tween 80 formulation (1.4 mg/5 ml) was placed in a Pari LC Sprint nebulizer and attached to a TurboBoy N compressor and directed towards the throat of the TSI. 7 ml and 30 ml of phosphate buffer (pH 7.4; 100 mM) were placed in the upper and the lower stages respectively to collect the nebulized aerosols. The aerosol was drawn through the apparatus at a rate of 60 L/min, with a cut-off aerodynamic diameter between stages of 6.4 μm. Following nebulization to "dryness", samples from each stage and the nebulizer were assayed for total mass of protein and the activity of deposited LDH. In all cases, the total mass balance using the TSI was within the European Pharmacopeial limit of 75–125% of average delivered dose.

#### 2.2.5. Determination of total protein concentration

Total protein concentration was measured using a QuantiPro BCA Assay Kit, which comprises Reagent A: 250 ml of a solution containing sodium carbonate, sodium tartate and sodium bicarbonate in 0.2 M NaOH; Reagent B: 250 ml of 4% (w/v) bicinchoninic acid solution and Reagent C: 12 ml of 4% (w/v) copper II sulphate. Bovine serum albumin (BSA) was used as the standard for this assay. Using 96 well microtitre plates, 150 μl from each standard and sample were placed in the plate, and 150 μl of the working reagent (Reagent A:Reagent B:Reagent C, 5:5:0.2) was added to the plate which was kept in an oven at 60 °C for 1 h. After 1 h, the plate was removed and the absorbance determined at 560 nm using an Opsys MR microplate reader (Dynax Technologies, UK). A calibration curve of absorbance against BSA standard concentrations was constructed and the gradient of the linear portion of the graph calculated. The concentration of the protein was then calculated using the BSA calibration curve.

#### 2.2.6. LDH activity assay

LDH activity was determined by continuous spectrophotometeric rate determination (Wroblewski and LaDue, 1955). Briefly, LDH was added to the 3 ml reaction solution (100 mM sodium phosphate buffer, pH 7.4, 0.13 mM β-NADH and 69 mM sodium pyruvate) and the decrease in the absorbance of β-NADH at 340 nm at 25 °C was immediately measured for 3 min at 15 s time intervals. LDH activity was estimated by the initial reaction rate. The recovered LDH activity was calculated as LDH activity of the tested formulation as a percentage of the initial LDH activity of the formulation.

#### 2.2.7. Statistical analysis

In addition to Student's *t*-tests, SPSS Version 17.0 (SPSS Inc.) software was used for non-parametrical statistical analysis. A Kruskal–Wallis test, followed by a post hoc Nemenyi's statistical test, was used to compare results between different groups. A value of $P < 0.05$ denotes a statistically significant difference for all statistical tests used. All experiments were undertaken in triplicate.

## 3. Results and discussion

### 3.1. Nebulization of LDH solution

When LDH solution (1.4 mg/5 ml) was nebulized, more than 60% of the enzyme was deposited in the lower stage of the TSI (Fig. 1), indicating the ability of the air-jet nebulizer to produce aerosol droplets having a small median size. However, the LDH activity was greatly reduced during nebulization, with 5.9 ± 0.7%, 4.8 ± 2.0%, and 2.3 ± 1.3% of the protein being active in the nebulizer reservoir and the upper and lower stages of the TSI respectively following nebulization.

The large loss of protein activity during nebulization has been described previously (Byron, 1990; Thanoo et al., 1992; Niven et al.,

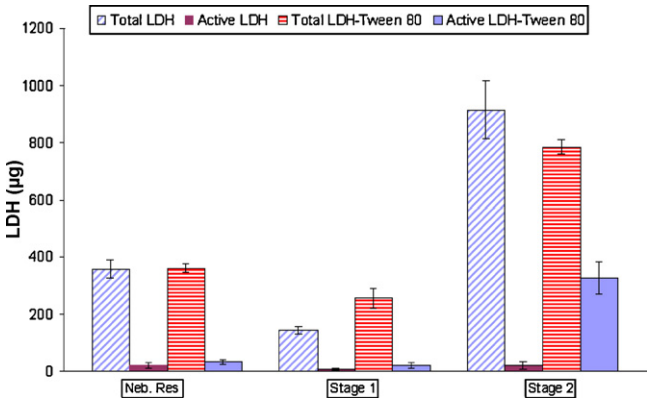


**Fig. 1.** Total and active LDH in the nebulizer reservoir and deposited in the TSI stages following nebulization of LDH solutions, with or with out 0.1% Tween 80. Each result is the mean (±SD) of 3 preparations.

1994; Khatri et al., 2001; Steckel et al., 2003). Loss of activity can be attributed to shear forces (Charm and Wong, 1970) which occur during nebulization (Niven et al., 1994). Denaturation may also occur at the air–water interface of nebulized droplets (Andrews, 1991), or as a result of increases in enzyme concentration due to enhanced solvent loss during nebulization (Niven et al., 1994).

### 3.2. Nebulization of formulations of LDH with Tween 80

The non-ionic surface active agent, Tween 80, was used above its critical micelle concentration (CMC) of 0.005% (w/v) in order to investigate whether alone, or in combination with chitosan, it would reduce enzyme instability and denaturation during nebulization. Tween 80 was employed as it has been studied previously as a stabilizer for nebulized proteins (Hillgren et al., 2002; Steckel et al., 2003; Niven et al., 1996) and is present in a commercially available, licensed nebulizer product (Pilcer and Amighi, 2010). Approximately 60% of the nebulized enzyme (784.7 ± 2.4 μg) was deposited in the lower stage of the TSI, and if compared to LDH solution, the surfactant significantly increased the active LDH delivered to the TSI stages, with 40.8% of deposited enzyme (320.2 ± 5.7 μg) remaining active ($P < 0.05$; Fig. 1). The improved retention of LDH activity can be attributed to the surface active properties of Tween 80, resulting from its amphiphilic character. Niven et al. (1994, 1996) showed previously that Tween 80 enhanced the stability of LDH and granulocyte-colony stimulating factor during nebulization. The protective effect was explained in terms of the surfactant being preferentially concentrated at the air–water interface during droplet production, excluding the protein, and thereby protecting the protein from denaturation at that interface. Above the CMC, Tween 80, unlike sodium dodecyl sulphate, has a weak interaction with the hydrophobic regions of the LDH molecule, which is not sufficiently strong to induce LDH denaturation (Hillgren et al., 2002). However, although Tween 80 has been incorporated in protein formulations to prevent denaturation, long term instability of such formulations is problematic (Wang et al., 2008).

### 3.3. LDH–chitosan complexes

As an alternative to the use of a surfactant, complexation of LDH with chitosan was studied as a formulation strategy to determine whether this would enhance enzyme stability

#### 3.3.1. Physical characterization of LDH–chitosan complexes

Table 1 shows the physical characteristics of the LDH–chitosan complexes with or without the inclusion of 0.1% Tween 80. The complexes prepared with the higher molecular weight chitosan had the greatest mean size, and all the formulations were polydispersed. The cationic polymer, chitosan, produced complexes having a net positive charge, which was proportional to the molecular weight of the polymer. There was a significant difference in the complexes' hydrodynamic size, and surface charge across groups ($P < 0.05$). Post hoc statistical testing revealed that 10k chitosan complexes were largest ($P < 0.05$), had highest zeta potential ($P < 0.05$) and on nebulization produced droplets of largest median size ($P < 0.05$).

Increasing the molecular weight of the chitosan complexed with LDH in the absence or presence of Tween 80 resulted in the production of complexes with higher hydrodynamic diameter and surface charge (Table 1). However, the positive zeta potential reduced after incorporating Tween 80 in the complexes, which is due to the association and interaction of the surfactant with the enzyme, as reported previously (Gang and Wang, 2007).

### 3.4. Nebulization of LDH–chitosan complexes

The droplets of the all formulations nebulized in this study had a median size less than 3.6 μm, and were polydispersed, as determined by laser diffraction. The volume median droplet size did not differ significantly ($P < 0.05$) from equivalent formulations from which LDH was omitted. Median size ranged from 2.20 ± 0.13 μm for the 0.1% 1k chitosan formulation up to 3.26 ± 0.40 μm for 0.1% 10k chitosan with 0.1% Tween 80 (full data set not shown). Formulations prepared with highest molecular weight chitosan produced the largest aerosol droplets (Table 1). This may result from these solutions having higher viscosity (McCallion et al.,

**Table 1**
Complex size, zeta potential and aerosol droplet size for complexes of LDH mixed with different molecular weight chitosan, with and without 0.1% Tween 80.

| Formulation | Hydrodynamic diameter (nm ± SD) (PI ± SD) | Zeta potential (mV ± SD) | Droplet VMD (μm ± SD) (Span ± SD) |
|---|---|---|---|
| 0.1% 1k chitosan | 237.8 ± 0.9 (0.304 ± 1.0) | +6.4 ± 5.7 | 2.36 ± 0.87(2.51 ± 0.45) |
| 0.1% 1k chitosan + Tween 80 | 274.6 ± 3.2 (0.454 ± 0.25) | +4.1 ± 2.3 | 2.59 ± 1.3(1.31 ± 0.14) |
| 0.1% 3–5k chitosan | 285.0 ± 2.8 (0.261 ± 0.3) | +18.2 ± 3.2 | 2.81 ± 2.7(1.68 ± 0.12) |
| 0.1% 3–5k chitosan + Tween 80 | 359.3 ± 4.0 (0.344 ± 0.16) | +13.7 ± 0.8 | 2.96 ± 0.44(2.11 ± 1.9) |
| 0.1% 10k chitosan | 291.9 ± 6.0 (0.523 ± 0.1) | +29.7 ± 2.9 | 3.01 ± 1.1(1.80 ± 2.1) |
| 0.1% 10k chitosan + Tween 80 | 386.5 ± 4.8 (0.652 ± 1.7) | +21.6 ± 1.8 | 3.52 ± 0.62(2.64 ± 0.05) |
| 0.1% Tween 80 | N/A | N/A | 2.40 ± 0.53(1.72 ± 0.32) |

Each result is the mean (±SD) of 3 preparations.

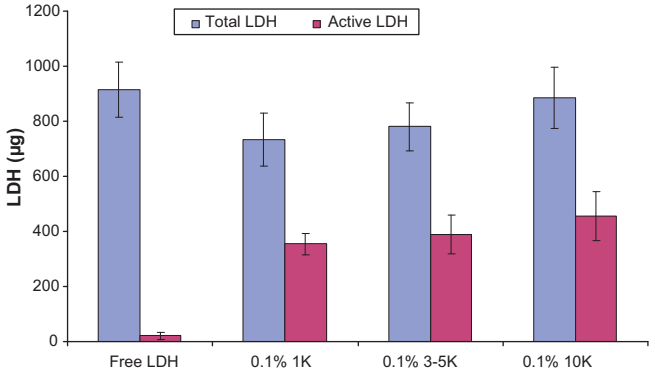


**Fig. 2.** Total and active LDH delivered to the lower stage of the TSI following nebulization of LDH solution and LDH with 0.1% chitosan 1k, 3–5k and 10k. Each result is the mean (±SD) of 3 preparations.

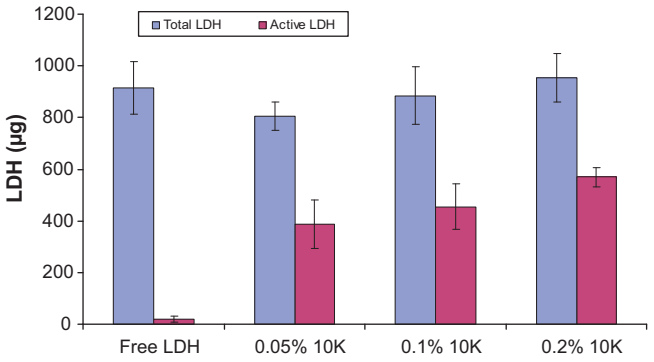


**Fig. 3.** Total and active LDH delivered to the lower stage of the TSI following nebulization of LDH solution, LDH with 0.05%, 0.1% and 0.2% 10k chitosan. Each result is the mean (±SD) of 3 preparations.

1996). The aerosol droplets generated by nebulizing LDH–chitosan complexes were of size appropriate for deep lung deposition. This was achieved due to the small size of the disperse phase (less than 390 nm) combined with a nebulizer producing aerosols of small median size (Bridges and Taylor, 2000; Dailey et al., 2003).

### 3.4.1. *Effect of molecular weight of chitosan on LDH stability during nebulization*

Compared to nebulized LDH solution, enzyme activity was retained to a greater extent when chitosan was included in the nebulized formulation, suggesting that the electrostatic interaction between the negatively charged enzyme and positively charged chitosan provides some protection during nebulization (Fig. 2). Between 47.9% and 59.8% of the protein delivered to the lower stage of the TSI remained active. The protective effect is due to the ionic interaction between chitosan and LDH. Such an effect has been shown previously for complexes of cationic polyethylenimine (PEI) and anionic DNA, which is degraded when nebulized as a solution (Rudolph et al., 2002).

There were statistically significant differences in the deposition of total and active enzyme in the TSI stages following air-jet nebulization of LDH solution and complexes prepared with chitosan of different molecular weights ($P < 0.05$). Increasing the molecular weight of chitosan complexed with LDH significantly increased total and active enzyme delivered to the second stage of the TSI ($P < 0.05$; Fig. 2). More LDH was delivered to stage 2 with 10k chitosan than with 1k ($P < 0.05$). Conversely, more LDH remained in the nebulizer ($P < 0.05$) for complexes prepared with 1k chitosan, compared to 10k chitosan. In the nebulizer reservoir, the LDH remaining active was significantly higher for complexes prepared with the highest molecular chitosan ($P < 0.05$).

### 3.4.2. *Effect of chitosan concentration*

When chitosan of average molecular weight 10k was used to form the LDH complexes, the total mass of LDH deposited in the lower stage increased with increasing concentration ($P < 0.05$) (Fig. 3). The total amount of LDH delivered to the lower stage of the TSI was significantly higher ($P < 0.05$) for complexes prepared with the highest concentration of chitosan (0.2%), while this concentration resulted in a decrease in the total amount of the enzyme in the nebulizer reservoir following nebulization ($P < 0.05$). The highest concentration of chitosan used in the LDH complex preparation significantly increased ($P < 0.05$) the amount ($570.0 \pm 2.8$ µg and proportion; 59.8%) of active enzyme delivered to the second stage of the TSI.

### 3.5. *Nebulization of LDH–chitosan complexes with Tween 80*

Inclusion of Tween 80 in formulations of LDH–chitosan (1k, 3–5k and 10k) complexes significantly ($P < 0.05$) enhanced the delivery of LDH in its active form to the lower stage of the TSI (Fig. 4). The greatest delivery ($P < 0.05$) of active LDH to the lower stage of TSI was achieved when Tween 80 was incorporated with the chitosan with higher molecular weights (3–5k and 10k). The addition of Tween 80 to the chitosan 1k formulation increased the recovery of active LDH in the lower stage of TSI by 3.7%. The proportion of active protein delivered to the second stage for formulations with 1, 3–5 and 10k chitosan was respectively 48.3%, 50.0% and 51.4% without Tween 80 and 52%, 57% and 62% when Tween 80 was included.

Although chitosan has some surface activity (Schulz et al., 1998; Geng et al., 2005; Pepic et al., 2008), it is likely that its main protective effect on LDH during nebulization results from the ionic interaction between the positively charged polymer and negatively charged enzyme. Tween 80, which will be present at the surface of aerosolized droplets, provided an additional protection to the nebulized protein, via a different mechanism.

The mean size of LDH–chitosan formulations, with or without Tween 80, was less than 400 nm in all cases, prior to nebulization (Table 1). Air-jet nebulization reduced the measured size of the complexes remaining in the nebulizer and deposited in both stages of the TSI ($P < 0.05$), with the smallest complexes deposited in the lower stage of the TSI (Table 2). The mean diameter of complexes deposited in the lower impinger stage was largest for formulations prepared with 10k chitosan ($P < 0.05$). The reduction in mean size for the formulations has previously been described for liposomes, and is likely to be the result of deaggregation, size reduction or

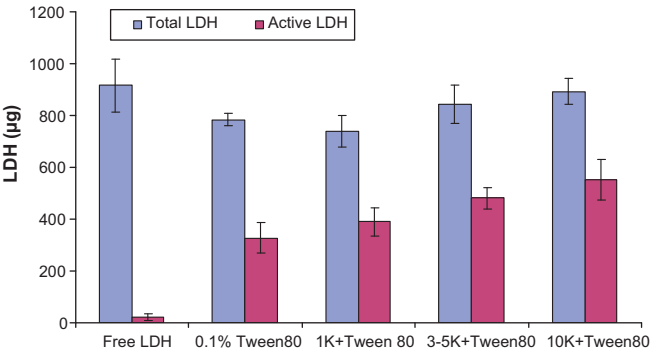


**Fig. 4.** Total and active LDH deposited in the lower stage of the TSI following nebulization of LDH solution, LDH with 0.1% Tween 80 and LDH with 0.1% chitosan 1k, 3–5k and 10k and 0.1% Tween 80. Each result is the mean (±SD) of 3 preparations.

**Table 2**
Size characteristics of LDH–chitosan complexes delivered to the TSI and remaining in the nebulizer.

| Formulation | Mean size in nebulizer reservoir (nm ± SD) (PI ± SD) | Mean size in Stage 1 (nm ± SD) (PI ± SD) | Mean size in Stage 2 (nm ± SD) (PI ± SD) |
|---|---|---|---|
| 0.1% 1k chitosan | 192.5 ± 2.8 (0.277 ± 0.4) | 141.0 ± 3.9 (0.390 ± 0.84) | 130.6 ± 5.0 (0.377 ± 1.4) |
| 0.1% 1k chitosan + Tween 80 | 184.4 ± 3.9 (0.362 ± 1.3) | 157.3 ± 2.6 (0.498 ± 0.9) | 142.5 ± 1.7 (0.422 ± 0.5) |
| 0.1% 3–5k chitosan | 203.6 ± 4.3 (0.456 ± 2.0) | 183.6 ± 0.7 (0.441 ± 1.5) | 144.9 ± 3.2 (0.239 ± 0.16) |
| 0.1% 3–5k chitosan + Tween 80 | 224.8 ± 1.1 (0.211 ± 0.04) | 170.6 ± 4.7 (0.292 ± 0.1) | 156.8 ± 2.7 (0.451 ± 2.0) |
| 0.1% 10k chitosan | 226.3 ± 1.9 (0.387 ± 0.5) | 180.0 ± 1.3 (0.219 ± 0.07) | 169.3 ± 4.2 (0.179 ± 0.03) |
| 0.1% 10k + Tween 80 | 238.2 ± 2.6 (0.544 ± 0.2) | 191.9 ± 3.4 (0.643 ± 0.31) | 178.0 ± 0.5 (0.384 ± 2.6) |

Each result is the mean (±SD) of 3 preparations.

fractionation of complexes during air-jet nebulization (Bridges and Taylor, 2000).

The aerosols generated from all the formulations investigated had median droplet sizes in the range 2.4–3.5 μm, with complexes in the size range 130–178 nm deposited in the lower stage of the TSI which represents the lower respiratory region. These findings indicate that the LDH formulations studied would be predicted to penetrate into and deposit in the peripheral airways (alveoli and bronchioles) of man (Taylor, 2007). Moreover, the net positive charge of the chitosan containing formulations has previously been shown to result in binding to anionic lipids, such as phosphatidylglycerols in pulmonary surfactant (Acosta et al., 2010) in the respiratory lung regions, and to mucus (Lai et al., 2009) within the conducting airways, potentially increasing residence time and enhancing bioavailability.

## 4. Conclusions

The formulation strategies used in this study may provide an effective means of increasing the stability of labile proteins and peptides to jet nebulization. Chitosan complexes of LDH were effectively delivered in aerosol droplets appropriate for deep lung delivery, and produced significantly enhanced enzyme activity compared to an aqueous LDH solution. The addition of Tween 80 also enhanced enzyme stability, but a different mechanism of stabilization, a surface effect at the aerosol–droplet interface, rather than an ionic interaction, is proposed. There was an additive effect with respect to LDH stability to nebulization when both Tween 80 and chitosan were included in formulations. Chitosan is biocompatible, considered safe for inhalation therapy and is known to enhance the residence times of materials in the airways, altering their pharmacokinetics. Chitosan would seem to have great potential as a stabilizer for labile proteins intended for delivery to the lungs using jet nebulizers.

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