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The processing of nanoparticles containing protein for suspension in hydrofluoroalkane propellants

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ABSTRACT

Nanoparticles delivered from pressurized metered dose inhalers (pMDIs) potentially offer a means of efficiently delivering proteins to the lung. Nanoparticles containing the model protein lysozyme have been produced using microemulsion and nanoprecipitation methods. Freeze-drying water in oil emulsions, with chloroform as the organic solvent, followed by washing of excess surfactant (lecithin) led to the production of lactose nanoparticles having approximately 300 nm mean size. Substitution of lactose with lysozyme led to a significant increase in the mean size of nanoparticles (645–750 nm). This may have been due to the surface activity of lysozyme which altered the emulsification properties. The retained biological activity of lysozyme increased with increased lactose concentration in the formulation, and approximately 99% biological activity was retained when 20% (w/w) lactose was used.

Ethanol used in the formulation in place of chloroform changed the production process from emulsification to nanoprecipitation. A monodisperse system (mean size approximately 275 nm, polydispersity index approximately 0.1) of spherical nanoparticles containing 80% (w/w) bioactive lysozyme (retained activity 99%) was generated. The nanoparticles washed with ethanol containing DPPC, oleic acid or Span 85 (2%, w/v) could readily be dispersed in HFA 134a without further processing, and a stable suspension was formed. Lysozyme remained stable (retained biological activity 98%) even after the nanoparticles were suspended in HFA 134a. This indicates the potential of nanoparticles for delivery of proteins from HFA-based pressurized metered dose inhaler formulations.

1. Introduction

Nanoparticles may be produced by milling or high pressure homogenization of large crystalline particles (Merisko-Liversidge et al., 2003; Kesisoglou et al., 2007), by controlled precipitation (Rabinow, 2004) or a combination of microprecipitation and homogenization (Keck and Muller, 2006). Previously, inhalation therapy consisting of drug-containing nanoparticles has been recommended as an alternative to conventional micronised particles or large porous microparticles, since nanoparticles can be deposited in the lung and escape both phagocytic and mucociliary clearance mechanisms (Tsapis et al., 2002).

The aerodynamic diameter, which determines the aerosolization properties of particles, is directly proportional to the geometric diameter of the particles. This suggests that nanoparticles could form a successful aerosol delivery system for macromolecules, as they possess an excellent geometric size that could positively influence the aerosolization properties of an aerosol delivery system. Ridder (2004) manufactured a pressurized metered dose inhaler (pMDI) formulation containing beclometasone dipropionate (BDP) nanoparticles which demonstrated an improved fine particle fraction of ≥50% compared to 37% obtained from a commercially available chlorofluorocarbon (CFC)-based BDP microparticle pMDI formulation (Becotide™).

Freeze-drying is frequently used when developing drugs with stability issues for human use. The process can also be used to form nanoparticles containing macromolecules and stabilising excipients such as surfactants, buffers, sugars, etc. However, prior to freeze-drying, techniques such as emulsification, crystallization, salting out, and precipitation may be applied to control the particle size of the freeze-dried product. Not only do these methods allow control of particle size, but the morphology, internal structure, drug loading, drug encapsulation efficiency, and release kinetics can also be modulated. The understanding of the relationship between physicochemical parameters and the effects on nanoparticle properties would allow nanoparticles to be designed with the desired physical and chemical properties for delivery to specific cells or organs in the body.

Emulsions are heterogenous systems that contain one immiscible liquid dispersed as droplets in another liquid. These systems are usually thermodynamically unstable and in most instances they are kinetically stabilised by addition of surfactant. A variety of

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synthetic and natural surfactants have previously been used to stabilise emulsions (Benita, 1998). However, it is critical to identify surfactants that are biocompatible with drug delivery via inhalation. Surfactants such as lecithin, oleic acid and Span 85 (sorbitan trioleate) are used commercially in pMDIs. Block copolymers of polyoxyethylene polyoxypropylene (poloxamer), polyoxyethylene castor oil derivatives (Cremophors®), polyoxyethylene sorbitans (Tweens®), and to a lesser extent, acetylated monoglycerides are already included in the various pharmacopoeias for parenteral administration and therefore can be considered for emulsion formulation design (Benita, 1998). However, further work is required to determine their safety upon inhalation.

Fessi et al. (1989) first described the nanoprecipitation (or solvent displacement) method for the manufacture of nanoparticles. This technique is mostly suitable for forming nanoparticles containing a hydrophobic drug and a polymer, usually PLGA, cellulose derivatives or poly ε-lactones. The polymer and drug are dissolved in a water miscible solvent (e.g. acetone or ethyl acetate) and introduced into a non-solvent of drug plus polymer (usually water containing surfactant). Rapid desolvation of the polymer and drug when the polymer–drug solution is added to the non-solvent, results in nanoprecipitate formation (Doelker et al., 2005). The nanoprecipitation method can be modified to produce nanoparticles containing hydrophilic drugs or biopharmaceuticals. Govender et al. (1999) efficiently entrapped procaine hydrochloride when the aqueous pH was set at a value that reduced drug ionization and lowered its aqueous solubility. Barichello et al. (1999) demonstrated the use of a biodegradable polymer (PLGA) to produce nanoparticles with surface-bound peptides by a solvent diffusion technique and consequently, improved their bioavailability.

The small size of bulk dry nanoparticles leads to high interparticle cohesive forces that negatively influence their aggregation behaviour, making it a challenging task to formulate nanoparticles as an inhaled dry powder delivery system. However, there is a promising option of formulating drug-containing nanoparticles as a pMDI formulation, where the dispersant (propellants such as hydrofluoroalkane, HFA 134a) can provide energy for deaggregating the nanoparticles (Dickinson et al., 2001).

The objective of this work was to explore the feasibility of producing pMDI formulations containing a bioactive model protein such as lysozyme. Emulsification and precipitation techniques coupled with freeze-drying process have been used to produce nanoparticles containing lysozyme. Factors that affect the emulsification and nanoprecipitation methods have been investigated with the aim of optimizing formulation and processing parameters in order to produce nanoparticles with desired characteristics (i.e. size and retained biological activity of lysozyme). The ability to disperse the nanoparticles in HFA 134a and retention of lysozyme's biological activity in the subsequent HFA 134a suspension have been evaluated.

2. Materials and methods

2.1. Materials

Dichloromethane, chloroform and absolute ethanol were obtained from VWR International Ltd. (Poole, Dorset, UK) while HFA 134a was purchased from Solvay Flour and Derivate GmbH (Hannover, Germany). Water was distilled via an Option 4 water purification system (Elga Ltd., High Wycombe, Buckinghamshire, UK). The pH meter used in the preparation of the buffer solutions (model pH 211, Hanna Instruments Ltd., Leighton Buzzard, Bedfordshire, UK) was calibrated in accordance to the manufacturer's instructions using pH 4 (phthalate) and pH 7 (phosphate) buffers obtained from Fisher Scientific Ltd. Salts (potassium hydroxide, and potassium phosphate), lactose, oleic acid, Span 85, lysozyme and *Micrococcus lysodeikticus* cells were obtained from Sigma–Aldrich Company Ltd. (Gillingham, Dorset, UK). Lecithin (Lipoid E80) was kindly donated by Lipoid GmbH (Ludwigshafen, Germany) and dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids, USA.

2.2. Methods

2.2.1. Production of lactose nanoparticles by an emulsification method

The work of Cook et al. (2005) was modified and followed to produce lactose nanoparticles. Briefly, 100 mg of lactose was weighed into a 7 mL vial and 1 mL of 50 mM phosphate buffer (pH 7) was added to form the aqueous phase of the emulsion. Lecithin was weighed in separate vials and 7 mL of chloroform added to form the oily phase. The concentration of lecithin in the oily phase was varied from 2% to 10% (w/v) (i.e. 140, 280, 420, 560 and 700 mg respectively) to investigate the optimal concentration for production of nanoparticles. The aqueous phase was then added into the oil phase drop-wise while homogenizing at low speed (10,000 rpm) using a ultra Turrax T25 homogeniser (IKA-Werke GmbH, Staufen, Germany) and later the emulsion was homogenized for 5 min at high speed (24,000 rpm). This was followed by immediate snap freezing using liquid nitrogen to immobilise the emulsion. The frozen emulsion was freeze-dried using a Heto-Holten A/S Drywinner 110 freeze dryer (Gydevang, Denmark) for a minimum of 12 h at −110 °C to −115 °C under vacuum to remove water and solvent from the frozen microscopic aqueous droplets. This resulted in dry matter containing nanoparticles covered with surfactant which was suspended in absolute ethanol in which lysozyme and lactose were insoluble, while lecithin was freely soluble, thus preserving the structure of nanoparticles. The nanoparticles in the raw suspension were separated from free surfactant by centrifugation using 3K30 Refrigerated centrifuge (Sigma Laborzentrifuges GmbH, Osterode am Harz, Germany). The sedimentation conditions were set as 17,000 rpm (equivalent to approximately 39,000 × g) at 25 °C. Oakridge Teflon® centrifuge tubes (50 mL, Nalge-Nunc Inc., Rochester, NY, USA) were selected for centrifugation due to their excellent solvent compatibility and ease of nanoparticle collection from the non-stick surface. The solvent plus surfactant were decanted and the sediments comprising nanoparticles were collected. The process was repeated twice to ensure maximum purification of the nanoparticles, and their size determined (Cook et al., 2005).

2.2.2. Production of lysozyme-containing nanoparticles by an emulsification method

200 mg of lecithin was dissolved in 5 mL of chloroform to form the oil phase (equivalent to 4% (w/v) lecithin in chloroform). Lysozyme and lactose were dissolved in 1 mL of phosphate buffer at a ratio of 100:0, 95:5, 90:10, 85:15, and 80:20 as the aqueous phase. In all cases the total weight of lactose and lysozyme was 100 mg. The aqueous phase was then added into the oil phase drop-wise while homogenizing at low speed (10,000 rpm) then for 5 min at high speed (24,000 rpm). This was followed by immediate snap freezing using liquid nitrogen to immobilise the emulsion. The frozen emulsion was freeze-dried to remove water and the lysozyme-containing nanoparticles were purified and recovered as above, and their size determined.

2.2.3. Assay of retained biological activity of lysozyme

Depending on the lysozyme concentration in samples, appropriate weights (equivalent to 4 mg lysozyme) were weighed in triplicate into vials. Control lysozyme (4 mg) was weighed in triplicate into respective vials. In each sample and control-containing

vial, 4 mL of a phosphate buffer solution (pH 7) was added and shaken well to form a clear solution. Then, 80 μL of each solution was measured into a further vial and diluted with 9920 μL of buffer solution. The UV/vis spectrophotometer (Model UV-1601, Shimadzu Corporation, Japan) was adjusted to 450 nm and 25 °C. A blank rate was determined by pipetting 2.5 mL of substrate suspension into 1 cm quartz cuvettes followed by incubation for 5 min in order to achieve temperature equilibration. Then 0.1 mL of buffer was added to the test cuvette and the solutions were mixed immediately by inversion. The decrease in $A_{450\,nm}$ was recorded for 5 min. The same procedure was followed for each sample and control solution except that addition of 0.1 mL buffer solution was substituted with the addition of either sample or control solutions. The change in absorbance units of samples, controls and blank were used to calculate the percent-retained activity of each sample.

2.2.4. The effect of solvent on nanoparticle characteristics

To investigate the effect of dichloromethane, chloroform was replaced by dichloromethane as an oily phase solvent. The lysozyme: lactose ratio was maintained at 80:20 while all other processing and formulation parameters were as described previously. The nanoparticles obtained were characterized and compared to those obtained using chloroform as the oily phase solvent.

2.2.5. Production of lysozyme-containing nanoparticles by nanoprecipitation

Lysozyme (80 mg) and lactose (20 mg) were weighed into a glass vial to form a composition of 80% (w/w) lysozyme. 1 mL of 50 mM phosphate buffer (pH 7) was added into the vial and the powder dissolved to form the aqueous phase. Egg lecithin (200 mg) was weighed into a separate vial and dissolved in 7 mL ethanol to form the oil phase. The aqueous phase was then added into the oil phase drop-wise while homogenizing at low speed (10,000 rpm) and later the nano-emulsion was homogenized for 5 min at high speed (24,000 rpm). The nano-emulsion was snap-frozen using liquid nitrogen to immobilise the nanoprecipitates and then lyophilised overnight to remove ethanol and water. The lysozyme-containing nanoparticles were purified and recovered, size analysed and the retained biological activity of lysozyme after processing was determined as outlined above.

2.2.6. Particle size analysis of nanoparticles

The nanoparticles were sized using photon correlation spectroscopy (Malvern Zetasizer 3000, Malvern Instruments, Malvern, UK). The instrument measures hydrodynamic diameter, expressed as *Z*-average diameter and a polydispersity index (PI), determined by cumulant analysis as described in the International Standard on DLS. The PI gives the information regarding the width of the size distribution and the values range between 0 and 1. Nanoparticles were suspended in chloroform (filtered through a 0.1 μm nylon membrane filter, Whatman®, UK) and bath sonicated for 5 min. The sample concentration was maintained at 5 mg of nanoparticles/mL of chloroform, which was enough to provide the required analytical count rate in the PCS (>50 kilocounts per second). The suspension was transferred into a non-frosted quartz cuvette and placed in the sample holder of the PCS. Each sample was measured in triplicate.

2.2.7. Scanning electron microscopy of nanoparticles

Lysozyme-containing nanoparticles, suspended in dichloromethane, were placed on the sample holder and dichloromethane allowed to evaporate. The samples were sputter-coated with gold using an Emitech K550 (Ashford, UK) and then visualized with a Philips XL20 (Eindhoven, Holland) scanning electron microscope.

2.2.8. Dispersion characteristics of nanoparticles in HFA 134a

In order to determine the dispersion properties of lysozyme-containing nanoparticles in HFA 134a propellant, four sets of nanoparticles were prepared and purified as previously described. The four sets of nanoparticles were separately dispersed in 40 mL of ethanol, 2% (w/v) oleic acid in ethanol, 2% (w/v) Span 85 in ethanol and 2% (w/v) DPPC in ethanol. The nanoparticles were separated from the medium by centrifugation. 100 mg lyophilisate (nanoparticles) of each set was placed into a plastic coated glass bottle (Wheaton, USA). A continuous spray valve (Valois, France) was immediately crimped using a manual bottle crimper 3000 (Aero-Tech Laboratory Equipment Company, USA) onto the vial and propellant filled through the valve using a pressure burette (Aero-Tech Laboratory Equipment Company, Maryland, USA). The pMDI suspension was then sonicated (XB6 Grant Instruments Ltd., UK) for approximately 1 min and the suspension stability was visually examined for any obvious separation, sedimentation, coalescence or flocculation of the suspended nanoparticles with time. The ability to re-disperse the nanoparticles in HFA 134a was visually evaluated by manually shaking the formulation to mimic patient use. This was repeated three times.

2.2.9. Stability of lysozyme in HFA 134a

Proteins are thought to be physically unstable when stored within a hydrophobic environment (Williams and Liu, 1999). In order to determine the retention of lysozyme activity after the nanoparticles were suspended in HFA 134a, three batches of lysozyme-containing nanoparticles were prepared and the activity of lysozyme was determined before the nanoparticles were suspended in HFA 134a. Each batch of nanoparticles was suspended in HFA 134a to form pMDI formulations that were left to stand overnight, at ambient temperature, before further analysis.

The pMDI formulations were placed in a beaker containing dry ice to chill the propellant. Once cold, the vials were removed from the beaker and their valves were removed manually to allow HFA 134a to escape slowly, leaving the nanoparticles at the base of the vials. The activity of lysozyme present in the nanoparticles was determined using the method described above.

3. Results

3.1. Effect of lecithin concentration on the size of lactose nanoparticles

Cook et al. (2005) showed that energy in the form of shear applied to the emulsion (homogenization speed), drug concentration in the aqueous phase and surfactant concentration determined the size of emulsion droplets and consequently had a direct influence on the size of nanoparticles produced by this method. However, in this work the type of surfactant and model drug used were different and hence, the concentration of the surfactant had to be re-optimized in order to produce nanoparticles of the desired size. Initially, this was done using a placebo (lactose) prior to the incorporation of the model protein drug.

The results (Fig. 1) indicate that high lecithin concentration (≥4%, w/w) in the organic phase of the emulsion resulted in production of nanoparticles of mean hydrodynamic diameter <500 nm compared to low (2%, w/w) lecithin concentration with a mean size >700 nm. Values for PI were in the range 0.1–0.4. It is important to note that high concentration of lecithin in the organic phase led to a high mass of surfactant covering the nanoparticles. This was detrimental to the purification and recovery process of nanoparticles as it led to increased viscosity of the suspension and therefore hindered the sedimentation of nanoparticles during ultracentrifugation. Thus, minimal lecithin was required to ensure nanoparticles

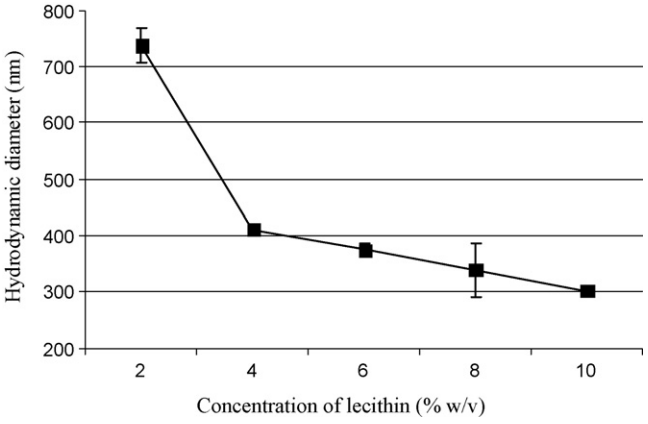

Fig. 1. Effect of lecithin concentration on the size of lactose nanoparticles. Each point is the mean (±S.D.) of five preparations.

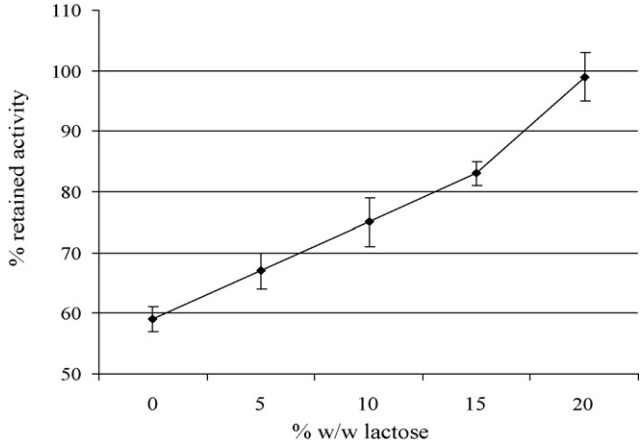

Fig. 2. Effect of lactose concentration on the retained biological activity of lysozyme. Each point is the mean (±S.D.) of three preparations.

were manufactured with the desired size. Therefore, a preparation containing 4% (w/v) lecithin in chloroform was selected for further investigation since the nanoparticles prepared using concentrations above 4% (w/v) lecithin were difficult to separate via a centrifugation process. Additionally, a lecithin concentration of 4% (w/v) produced smaller nanoparticles compared to 2% (w/v) as shown in Fig. 1.

3.2. Effect of lysozyme concentration on the size of nanoparticles

Proteins are known to be surface-active due to clusters of hydrophobic peptides into specific regions of the molecule. This may have an effect on emulsion characteristics and consequently influence the size properties of the nanoparticles. Therefore, the effect of the model protein (lysozyme) on the nanoparticle size was investigated.

No clear trend was found between nanoparticle size, PI and lactose concentration (Table 1). However, a high concentration of lysozyme (i.e. 95%, w/w) resulted in the largest nanoparticle size and highest PI compared to lower lysozyme concentrations.

3.3. Effect of lysozyme concentration on retained biological activity

The retention of biological activity was found to be a function of the lactose concentration used in the formulation (Fig. 2). The results indicate that lactose had a role in the preservation of the biological activity of lysozyme. When lysozyme nanoparticles were prepared in the absence of lactose, a minimal activity of around 59% (relative to unprocessed lysozyme raw material) was retained after processing. In contrast, addition of as little as 5% (w/w) lactose led to an increase (t-test, $p < 0.05$) of retained biological activity of lysozyme after processing (Fig. 2) and further increases were noted as the lactose concentration was increased to 20% (w/w). This was regarded as the optimal lactose concentration in the formulation and was kept constant in further investigations.

3.4. Effect of solvent on nanoparticles properties

Solvents have different physical and chemical properties that can influence a particular manufacturing process. For instance, the polar strength of a particular solvent can solubilise excipients in a biphasic system to form a solution, e.g. ethanol is used to form a co-solvent system to solubilise oleic acid/lecithin/sorbitan trioleate in HFA. On the other hand, addition of an anti-solvent in a solution can lead to precipitation of solutes. In this work, the effect of ethanol and halogenated solvents (i.e. dichloromethane and chloroform) in the production of lysozyme-containing nanoparticles has been investigated. Preliminary studies indicated that water in oil emulsions were formed with the halogenated solvents. However, inclusion of ethanol required a change in the production technique from emulsification to nanoprecipitation due to the miscibility of water and ethanol. Thus, emulsification and nanoprecipitation techniques were investigated in the production of lysozyme-containing nanoparticles by changing the type of solvent used to form the oily phase.

The retained biological activity of lysozyme (Table 2) of the formulations produced from emulsification and nanoprecipitation methods indicated similar results (t-test, $p < 0.05$). However, the size of nanoparticles produced by the emulsion method using both chloroform and dichloromethane was significantly larger (t-test, $p < 0.001$) compared to nanoparticles produced by the nanoprecipitation method employing ethanol (Table 2). Nanoparticles produced using dichloromethane had a higher PI compared to nanoparticles produced using chloroform (i.e. 1.000 ± 0.000 and 0.445 ± 0.176 respectively). This indicates that nanoparticles produced using chloroform had a narrow particle size distribution compared to those produced using dichloromethane. The nanoparticles produced by the nanoprecipitation method had a small PI compared to the nanoparticles produced by the emulsion method (Table 2). A typical size distribution curve of the nanoparticles produced by nanoprecipitation is shown in Fig. 3. An SEM of the nanoparticles produced by nanoprecipitation showed that the nanoparticles were spherical (Fig. 4). Therefore, lysozyme-

Table 1
Effect of lysozyme concentration on nanoparticle size distribution. Each result is the mean (± S.D.) of five preparations.

Lysozyme (%)	Lactose (%)	Hydrodynamic diameter (nm)	Polydispersity index
95	5	757.8 ± 13.5	0.280 ± 0.038
90	10	645.6 ± 0.9	0.101 ± 0.013
85	15	666.0 ± 2.3	0.157 ± 0.015
80	20	688.0 ± 18	0.155 ± 0.019

Table 2
Effect of solvent on size distribution and retained activity of lysozyme-containing nanoparticles. Each result is the mean (±S.D.) of three preparations.

Solvent	Hydrodynamic diameter (nm)	Polydispersity index	Retained activity (%)
Chloroform	693.0 ± 13.6	0.445 ± 0.176	99 ± 4
Dichloromethane	677.0 ± 12.3	1.000 ± 0.000	95 ± 6
Ethanol	275.4 ± 4.5	0.146 ± 0.022	99 ± 8

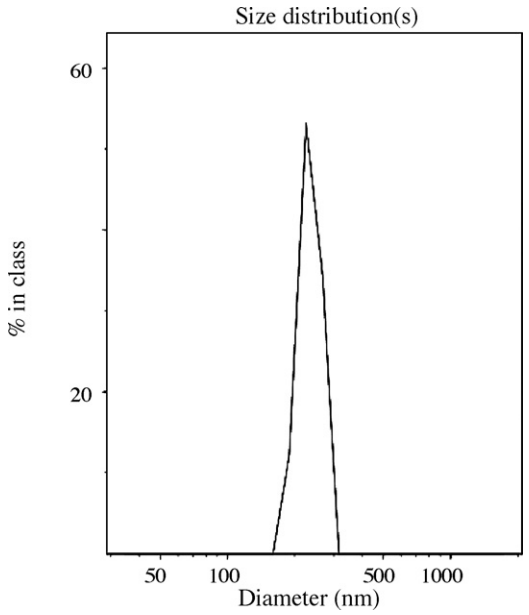

Fig. 3. Typical size distribution curve of lysozyme-containing nanoparticles produced by the nanoprecipitation method.

containing nanoparticles produced using the nanoprecipitation method were considered as an optimal formulation and further investigations were undertaken on nanoparticles produced by this method.

3.5. Dispersion of nanoparticles in HFA 134a

The addition of surfactant in the final rinsing solution led to suspension stability for at least 1 min (Table 3). The nanoparticles rinsed with ethanol led to an unstable suspension in HFA 134a, while nanoparticles rinsed with ethanol containing DPPC, oleic acid or Span 85 formed more stable suspensions in HFA 134a.

It was observed that agglomeration followed by creaming occurred immediately after shaking when absolute ethanol was used to rinse the nanoparticles, indicating that ethanol alone was insufficient to stabilise the suspension. A homogeneous dispersion was formed immediately after shaking and it was stable for at least a minute when the nanoparticles were rinsed with ethanol containing DPPC, oleic acid or Span 85. The creamed formulations readily re-dispersed upon shaking which is important for dose reproducibility.

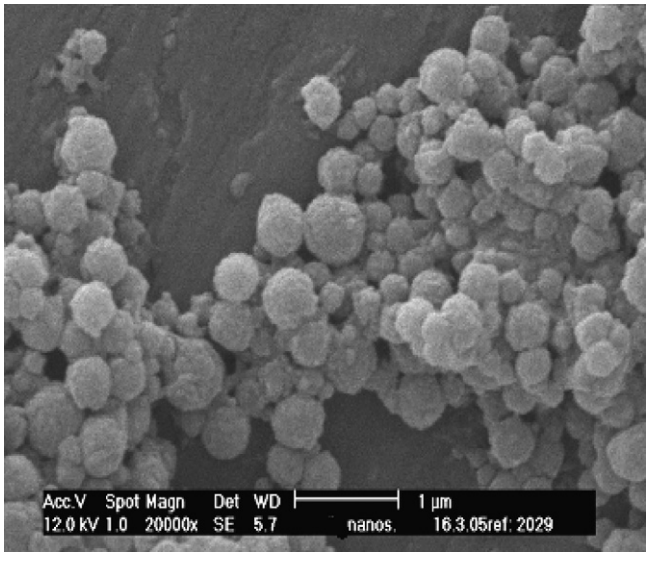

Fig. 4. SEM of lysozyme-containing nanoparticles produced by nanoprecipitation using ethanol as solvent.

Table 3
Effect of surfactant on suspension stability of nanoparticles in HFA 134a.

Final rinsing solution	Suspension stability at 1 min
Ethanol	Unstable
2% (w/v) DPPC in ethanol	Stable
2% (w/v) oleic acid in ethanol	Stable
2% (w/v) Span 85 in ethanol	Stable

3.6. Stability of lysozyme in HFA 134a

There was no significant difference (t-test, $p < 0.05$) between the retained biological activities of lysozyme before ($99 \pm 3\%$) and after ($98 \pm 4\%$) the nanoparticles (from the nanoprecipitation method) were suspended in HFA 134a ($n = 3 \pm$ S.D.). The results are in agreement with previous studies where stability of proteins in HFA pMDIs has been demonstrated.

4. Discussion

Degradation of a particular macromolecule is a function of the formulation, manufacturing process, packaging and storage conditions. In this work, these parameters have been investigated and optimized in order to develop a stable model protein therapeutic.

During homogenization, emulsifier (lecithin) adsorbs rapidly to the surfaces of the freshly formed droplets and reduces the interfacial tension sufficiently to facilitate droplet disruption and provide a protective coating that prevents droplets from aggregating with their neighbours (McClements, 2004). The emulsion droplet size has a direct influence on nanoparticle size and is governed by the concentration of lecithin and by the energy input from the homogenizer. With low lecithin concentrations, the droplet size is primarily determined by the maximum surface area that can be covered by the available lecithin rather than by the energy input of the homogenizer. This explains why there was a decrease in lactose nanoparticle size when lecithin concentration was increased. Excess lecithin led to more emulsifier than required to completely cover the droplet surface area created by the homogenizer, such that the droplet size was independent of lecithin concentration, and depends primarily on the energy input by the homogenizer. However, since the energy input of the homogenizer was constant, further reduction of nanoparticle size could not be achieved.

Addition of protein (lysozyme) to the emulsion changed the emulsion characteristics. Proteins are known to be surface-active and this may have altered the droplet size of the emulsion and/or emulsion stability leading to increased nanoparticles' size compared to lactose nanoparticles, as observed in the current results. Ionic surfactants may bind to protein molecules through a combination of electrostatic and hydrophobic interactions, while non-ionic surfactants may bind to a protein through hydrophobic interactions. Once a surfactant is bound to a protein molecule, it alters the molecular characteristics leading to changes in its ability to adsorb to interfaces and to stabilise the emulsion, subsequently altering emulsion droplet size (Kelley and McClements, 2003).

Protein adsorption at the water–oil interface is influenced by surface hydrophobicity and charge. Once proteins are adsorbed, they unfold and rearrange their secondary and tertiary structures, i.e. undergo surface denaturation, to expose hydrophobic residues to the hydrophobic phase, and in consequence lose their biological activity (Wilde et al., 2004). In addition, the extent of conformational changes has been reported to be larger for more non-polar oils, which may be because the hydrophobic driving force for protein unfolding is greater (McClements, 2004). The results for nanoparticles prepared using ethanol and halogenated solvents, which are characterized by different polarities, indicated similar results of retained biological activity for lysozyme. This suggests that surface denaturation induced by adsorption of lysozyme to the water–oil interface did not occur. This may be due to the presence of lecithin in the emulsion that is more surface-active than lysozyme, leading to the exclusion of some or all of the lysozyme from the water–oil interface, thereby protecting lysozyme from surface denaturation (Gunning et al., 2004).

Previously, it has been reported that a protein in a suspension can maintain its secondary and tertiary structures compared to protein in solution (Stevenson, 2000). This suggests that the nanoprecipitation method whereby a nano-suspension is formed favours the stability of protein during processing compared to the emulsification method where the protein is in solution while processing. The excipients (lactose and lecithin), as well as buffer, protect lysozyme from degradation during processing and therefore the activity of lysozyme is retained despite the manufacturing process, as indicated by the current results. However, it is worth noting that nanoprecipitation yielded nanoparticles with a smaller size compared to the emulsification method. This is in agreement with the work of Doelker et al. (2005) who have found that a nanoprecipitation method can sometimes be a good alternative to the classical and widely used emulsification method in the fabrication of protein-containing nanoparticles.

The formulation and the production process of the nanoparticles in this study ensured a low concentration of lactose in the formulation compared to the minimum sugar–enzyme mass ratios previously reported (Liao et al., 2002). The minimum mass ratio for glycerol, sucrose and trehalose to achieve a close maximum to stabilisation of catalase has been reported to be approximately 0.6, 2, and 2 respectively (Liao et al., 2002). The authors also found that the minimum mass ratio required for the stabilisation of lysozyme appeared to be twice those for the stabilisation of catalase. In the current formulation, a minimum lactose–lysozyme mass ratio of 0.25 has been found to stabilise lysozyme. The presence of a high amount of lecithin may have protected lysozyme from degradation during processing. This is an added advantage as high concentration of sugars in protein formulations could lead to degradation of the protein during storage due to sugar crystallization. The results indicate that around 99%-retained activity of lysozyme relative to the unprocessed raw material could be achieved when 20% (w/w) lactose was added to the formulation.

For pMDI suspension formulations, the drug should be insoluble in the suspending medium to reduce the potential for size changes during storage, as well as retaining the particle structure. In this work, surfactant in a co-solvent system containing ethanol was required to stabilise nanoparticles in HFA 134a. The presence of surfactant at the nanoparticle:HFA interface permitted production of suspensions which were physically stable for at least 1 min and following creaming on storage could be readily re-dispersed. Liao et al. (2005) have reported the retention of biological activity of lysozyme and catalase in pMDI formulations stored for up to 6 months at room temperature. In that study, the proteins were stabilised using sugar (sucrose or trehalose) and/or 80% hydrolyzed polyvinyl alcohol. Similarly, studies of lysozyme structures using FT-Raman spectroscopy have shown lysozyme largely exhibits structural stability in both HFA 134a and HFA 227 (Quinn et al., 1999). These positive literature reports and the present studies indicate that there is potential to develop pMDI formulations containing stable macromolecules for aerosol drug delivery.

5. Conclusion

In conclusion, nanoprecipitation and emulsification methods coupled with freeze-drying can be used to produce protein-containing nanoparticles of appropriate size for peripheral lung deposition. The nanoparticles have a narrow size distribution and the entrapped protein (lysozyme) is characterized by high retention of biological activity after manufacturing. Nanoprecipitation can be a more suitable alternative technology to the traditionally used emulsification method for the fabrication of protein-containing nanoparticles as it leads to the production of spherical nanoparticles containing bioactive protein. The nanoparticles can readily be dispersed in HFA 134a with the aid of ethanol as co-solvent although additional surfactant was required to ensure stability of the suspension. Additionally, the biological activity of entrapped lysozyme is retained after the nanoparticles are dispersed in HFA 134a. This suggests that there is a potential to produce pMDI formulations containing therapeutic proteins/peptides.

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