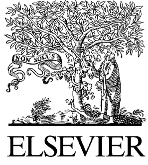

ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT

Journal of Controlled Release xx (2006) xxx–xxx

journal of controlled release

www.elsevier.com/locate/jconrel

1

Stabilisation of deoxyribonuclease in hydrofluoroalkane using miscible vinyl polymers

Stuart A. Jones *, Gary P. Martin, Marc B. Brown

Department of Pharmacy, Pharmaceutical Sciences Research Division, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London, SE1 9NN, UK

Received 16 March 2006; accepted 1 June 2006

UNCORRECTED PROOF

Abstract

A mix of biocompatible macromolecules (poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP)) has been shown previously to enhance the physical stability of non-aqueous pharmaceutical suspensions. The aim of this work was to assess the feasibility of employing such a combination to facilitate the formulation of deoxyribonuclease I (DNase I) in a metered dose inhaler (MDI) using hydrofluoroalkane (HFA) propellants. DNase I was combined with the selected excipients and formed into an inhalable microparticle by spray-drying. When spray-dried alone DNase I lost almost 40% of its original biological activity, but stabilising DNase I with trehalose and PVA (DTPVA) retained 85% biological activity and trehalose, PVA and PVP (DTPVAPVP) retained 100%. Suspending the DTPVAPVP microparticles within a HFA pMDI for 24 weeks led to no further reduction in the biological activity of DNase I and the formulation delivered almost 60% of the dose expelled to the second stage of a twin-stage impinger. The solubility of PVP in HFA propellants suggests that the enhanced physical stability observed with PVA and PVP may partially be as a result of steric stabilisation. However, the large zeta potential associated with the suspensions suggested that charge stabilisation may also influence the pMDI physical stability.
© 2006 Elsevier B.V. All rights reserved.

1. Introduction

Deoxyribonuclease I (DNase I) is a hydrophilic glycosylated protein with a molecular mass of approximately 33 kDa [1]. It is an endonuclease, which degrades DNA through the hydrolysis of the P–O3′ bond, yielding 5′-oligonucleotides [2]. The biological activity of DNase I is catalysed by divalent cations and it has previously been shown to exhibit optimal activity in the presence of Ca^{2+}, Mg^{2+} or Ca^{2+} and Mn^{2+} [3]. DNase I readily digests both single and double stranded DNA, but the enzyme reaction occurs at a rate of at least four orders of magnitude slower with a single stranded substrate [2].

DNase I has been employed clinically as a mucolytic since the 1960's because the breakdown of DNA within purulent sputum leads to a dramatic reduction in the viscosity of airway secretions. However, after a reported case of bronchospasm caused by an immunogenic response to the bovine form of the protein, the clinical applications of DNase I fell into disuse [4]. Subsequent development of recombinant DNA technology has allowed the production of a humanised form of DNase I (rhDNase I). rhDNase I lacks the immunogenicity of bovine DNase I and as a result rhDNase I is now licensed in numerous countries worldwide for the symptomatic relief of cystic fibrosis [5–7].

Whilst rhDNase I is highly effective in breaking down viscous mucus in the airways, its clinical use is limited by the fact that it is delivered using a nebuliser (Pulmozyme®). Although atomisation of a drug solution into droplets using a nebuliser avoids the often-problematic fabrication of a particulate-based drug delivery system, these devices are not favoured by patients due to their lack of portability and low delivery efficiency.

Pressurised metered dose inhalers (pMDIs) incorporate compounds within an air tight, light protective, low moisture environment and therefore provide excellent protection for labile therapeutics such as proteins [8]. However, hydrophilic proteins such as rhDNase I are typically poorly soluble within non-polar HFA propellants. Thus, to enable the delivery of such agents via pMDIs, the therapeutic protein must be formulated as

* Corresponding author. Tel.: +44 20 7848 4791; fax: +44 20 7848 4800.
E-mail address: stuart.jones@kcl.ac.uk (S.A. Jones).

0168-3659/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2006.06.003

ARTICLE IN PRESS

59 a particulate that can be suspended within the pMDI propellant. 60 In order to minimise chemical and physical instability within a 61 suspension pMDI a desirable compatibility between the 62 propellant and the particulate (containing the protein) must be 63 achieved within the formulation reservoir. In addition, upon 64 dose actuation, the pMDI must reproducibly release a homo- 65 geneous aliquot of the particulate suspension which upon 66 evaporation of the propellant must produce particles with 67 suitable aerodynamic characteristics to deposit within the 68 airways. Furthermore, it is essential that the protein's biological 69 activity is maintained during the formulation manufacturing 70 process and over the product shelf-life.

71 Despite the complexity of formulating proteins within 72 pMDIs, preliminary studies by Quinn and co-workers [9] 73 have found (using Fourier transform Raman spectroscopy) that 74 lysozyme undergoes no change in the presence of the pMDI 75 propellant HFA 134a. However, whilst the potential of pMDIs 76 to delivery proteins effectively has been reported previously, 77 few details have been published on the suspension stability, 78 biological activity, structural integrity and aerodynamic char- 79 acteristics of such formulations during long-term storage. In 80 addition, the mechanism by which these hydrophilic labile 81 macromolecules remain physically and chemically stable in 82 hydrophobic solvents is currently unknown.

83 As such, the objective of this study was to investigate the 84 feasibility of formulating DNase I within an HFA-based pMDI 85 for pulmonary delivery. In an attempt to try and achieve this 86 aim, a number of stabilising excipients, previously shown to 87 improve the physical stability of HFA suspensions, were 88 combined with the protein using spray-drying [10,11]. The 89 excipients were selected with a view to improving both the 90 chemical and physical stability of the protein during manu- 91 facture and storage within the pMDI.

92 2. Materials and methods

93 2.1. Spray-drying the DNase I formulations

94 Approximately 500 mg of DNase I (deoxyribonuclease I 95 isolated from the bovine pancreas, high purity, Rnase free, 96 14,200 U/mg, defined by Sigma Aldrich as Genotech® units, 97 supplied by Sigma Aldrich, UK) was added to 100 ml sodium 98 chloride solution (10 mM) containing a number of different 99 combinations of excipients (Table 1) which included PVA 80% 100 hydrolysed (M_w of 8000–10,000, Sigma Aldrich, UK), 101 trehalose dihydrate (Sigma Aldrich, UK) and PVP K15 102 (10,000 M_w Sigma Aldrich, UK). The excipients were dissolved 103 in solution by simple stirring using a magnetic hotplate (Stuart 104 Scientific, UK). When PVA was included within the solution it 105 required heating for 30 min at 90 °C to facilitate dissolution of 106 the polymer after which the solution was allowed to cool to 107 room temperature prior to addition of the protein. DNase I was 108 carefully added to the solution containing the excipients using 109 gentle rotation by hand to facilitate dissolution.

110 DNase I microparticles were produced from the solutions 111 using a 191 mini spray-dryer (Buchi, Germany). The protein/ 112 excipient(s) solutions were pumped through a spray atomisation 113 nozzle that combined the liquid with a 700 ml h^{-1} airflow 114 delivered to the drying chamber. The aspiration rate was set as 115 70%, the material feed rate was 3 ml min^{-1} and the inlet 116 temperature was set to 95 °C. The outlet temperature was found 117 to be in the range of 65–70 °C. The final spray-dried product 118 was collected on wax paper and stored in a desiccator 119 containing phosphorous pentoxide (Sigma Aldrich, UK) prior 120 to use. The yield was calculated as the solid mass collected at 121 the end of the spray-drying process as a percentage of the initial 122 solid weight of the excipients in the feed stock solution.

123 2.2. Microparticle characterisation

124 Approximately 2 mg of spray-dried powder was dispersed in 125 1 ml of 0.1% w/v lecithin (Sigma Aldrich, UK)–cyclohexane 126 (BDH, Germany) solution and sonicated in a water bath (Model 127 F5100b; Decon Laboratories, UK) for 30 s to disperse any 128 possible agglomerates before a small sample of the suspension 129 was added to a stirred sample cell again containing approxi- 130 mately 0.1% w/v lecithin–cyclohexane solution as the disper- 131 sion media. The particle size of the sample was measured using 132 a laser diffraction analyser (Malvern Instruments, UK) fitted 133 with a 63 mm focal length lens at an obscuration of 0.165–0.25. 134 Particle size distributions were expressed in terms of D(v,0.9), D 135 (v,0.5), and D(v,0.1) which were the respective diameters at 90, 136 50 and 10% cumulative volumes.

137 2.3. pMDI manufacture

138 The pMDIs were manufactured by adding the equivalent of 139 15.0 mg of the raw drug (calculated using the % of the DNase I 140 content of the microparticles) directly into a poly(ethylene 141 terephthalate) canister (donated by Astrazeneca, UK). A 25 μl 142 canister valve (donated by Astrazeneca, UK) was crimped in 143 place using a manual pMDI filler (Pamasol, Switzerland) and 144 20.0 g of HFA 134a (Dupont, Germany) or 17.5 g HFA 227 145 (Solvay, Germany) was pressure-filled into the can via the valve. 146 The formulation was then sonicated in an ultrasonication bath 147 (Decon Laboratories, UK) for 15 s to ensure particle separation 148 and stored, valve up, at room temperature. Several batches of 149 each formulation were manufactured and utilised in the study.

150 2.4. DNase I biological stability in HFA

151 The DNase I pMDIs were stored at room temperature 'valve 152 up' for 24 weeks. Immediately upon manufacture and after 2, 4,

t1.1 Table 1
t1.2 Compositions of the spray-dried microparticulate formulations containing DNase I

Formulation	Composition
DNase I SD	DNase I 500 mg
DT	DNase I 500 mg–trehalose 500 mg
DTPVA	DNase I 500 mg–trehalose 500 mg–PVA 80% hydrolysed 500 mg
DTPVAPVP	DNase I 500 mg–trehalose 500 mg–PVA 80% hydrolysed 500 mg–PVP K15 500 mg

UNCORRECTED PROOF

12 and 24 weeks, the ~~secondary structure,~~ deposition characteristics and biological activity of DNase I were determined using the methods detailed below.

2.5. DNase I quantification

The Pierce Protein Assay® was performed as per the manufacturer's instructions. BSA was used as the protein standard and a set of BSA solutions between 2 and 20 μg ml^{-1} was prepared by diluting the 2.0 mg ml^{-1} standard with deionised water (conductivity 0.5–1.0 μS). The working reagent was prepared by mixing 25 parts of Micro BCA reagent A (sodium carbonate, sodium bicarbonate, sodium tatrate in 0.2 M sodium hydroxide), 24 parts of reagent B (aqueous solution of BCA detection reagent) and 1 part of reagent C (4% cupric sulfate pentahydrate). An aliquot of 150 μl of each standard or test sample was transferred into separate wells of a 96-well microplate in duplicate and 150 μl of the working reagent was subsequently added to each well and the plate mixed on a plate shaker for 30 s. The plate was covered with its lid (to prevent significant evaporation) and incubated at 50°C for 90 min, after which it was cooled to room temperature and the UV absorbance in each well determined using a plate reader at a wavelength of 562 nm. The amount of enzyme in each test sample was determined as a nominal concentration against the BSA protein standard calibration curve.

2.6. Assay of DNase I biological activity

The biological activity of DNase I was monitored by assessing the ability of the enzyme to digest the substrate, DNA. The substrate was constituted in a 0.1 M, pH 5.0 acetate buffer, containing 5 mM Mg^{2+}. The buffer was prepared by dissolving 1.165 g of anhydrous sodium acetate (BDH, Germany), 0.355 g of acetic acid (Sigma Aldrich, UK) and 0.203 g of $MgCl_2 \cdot 6H_2O$ (Sigma Aldrich, UK) in 150 ml of deionised water (conductivity 0.5–1.0 μS). Approximately, 2 mg of fibrous DNA isolated from a calf thymus (Sigma Aldrich, UK) was dissolved in 52 ml of the acetate buffer by gently shaking overnight. The absorbance of this substrate solution at 260 nm was determined to be between 0.630 and 0.690. Prior to assessing the test samples, a DNase I standard, 2000 Kunitsz units mg^{-1} (Sigma Aldrich, UK), was used as a calibrant for the activity assay. This standard was constituted by dissolving the supplied powdered enzyme in 1.0 ml of 0.15 M NaCl solution. The solution was diluted further with 0.15 M NaCl to obtain five separate standard solutions within the concentration range of 20–80 units ml^{-1}. All dilutions were performed using 0.15 M NaCl solution. The wavelength of a lambda 5 UV spectrophotometer (Perkin-Elmer, UK) was adjusted to a 260 nm and 2.5 ml of substrate was placed into a cuvette (10 mm light path) and incubated in a thermostatic cell (25 °C) for 3–4 min to allow temperature equilibration. Then, 0.5 ml of diluted standard, or sample, was added and the solutions were immediately mixed by inversion. The increase in the UV absorbance per min (ΔA260) was recorded as a function of time for 10–12 min. An activity calibration curve was constructed by plotting the maximum ΔA260 vs. Kunitz units mg^{-1} of the five DNase I activity standards. The DNase I samples were diluted to attain a ΔA260 within the calibration range and activity measured as before. The Pierce Protein Assay® was then used to quantify the protein, to obtain the activity per mg. This was compared to the activity of the lyophilised raw DNase I to produce the % relative activity.

2.7. Impaction analysis

The twin-stage impinger (TSI) was employed to determine the deposition characteristics of the microparticles after release from the pMDIs. The TSI apparatus was set up and run using a flow rate of 60 l min^{-1} in accordance with the method set out in the British Pharmacopeia [12]. The pMDIs were primed prior to use by discharging approximately 10 shots into a fume cupboard. A total of 20 actuations were sprayed into the apparatus from each inhaler. The pump was allowed to run for 5 s after each discharge and then switched off for 5 s whilst the inhaler was shaken by hand. After the completion of each run the impinger was dismantled and the drug assayed according to the amounts deposited on the device, stage 1 and stage 2. The device was washed with 50 ml of deionised water (conductivity 0.5–1.0 μS) and both stage 1 and 2 with 100 ml of deionised water. The Pierce Protein Assay® (described above) was used to quantify the DNase I within the washing solutions.

The quantities of DNase I delivered by the pMDI formulations on each stage of the impinger were determined as a percentage of the total quantity of the protein recovered from the device and the impinger. For example, in order to calculate the stage 2% deposition, Eq. (1) was used:

$$\%\text{stage2} = \left(\frac{Q_a}{Q_a + Q_b + Q_c}\right)100 \qquad (1)$$

where %stage2 is the percentage of the DNase I on stage 2 of the impinger, Q_a is quantity of the DNase I on stage 2 of the impinger, Q_b is the quantity of the DNase I on stage 1 of the impinger, Q_c is the quantity of the DNase I on the device. The fine particle fraction (FPF) was defined as the % of the DNase I on stage 2 of the impinger, i.e. the % of particles <6.4 μm.

2.8. Zeta potential

A Zeta Sizer Nano NS® (Malvern instruments, UK) was used to measure the zeta potential. Samples were prepared by suspending approximately 2 mg of the powder in a surrogate solvent dichloromethane (DCM) (selected on the basis of a previous study [13], BDH, Germany) and sonicating the mixture for 1 min in a 5300b ultrasonication bath (Decon Laboratories, UK). The zeta potential of the samples was measured immediately after suspension within the solvent using a DTS1070 non-aqueous dip cell (Malvern Instruments Ltd, UK). A total of 20 runs were performed for each sample using a cell voltage of 20 V. Three different suspensions were made up and measured for each of the batches of microparticles. The conversion of the measured electrophoretic

UNCORRECTED PROOF

ARTICLE IN PRESS

mobility (U_e) into zeta potential (z) was carried out using Henry's equation (Eq. (2)):

$$U_e = \frac{2\varepsilon z f(\mathrm{Ka})}{3\eta} \quad (2)$$

where ε is the dielectric constant, η is the sample viscosity and f(Ka) is Henry's function. The parameter K, termed the Debye length is a measure of the thickness of the electrical double layer. In aqueous solutions this f(Ka) is approximated to be 1.5, known as the Smoluchowski approximation. However, for small particles in non-aqueous solvents this approximation becomes 1.0 (referred to as the Huckel approximation) and this was used in this study.

3. Results

3.1. Microparticle characterisation

The particle size measurements of the spray-dried microparticles immediately after preparation indicated that all of the batches were of a suitable size for drug delivery to the airways, i.e. the median diameters of all the particles were less than 5 μm (Table 2). The smallest median particle diameter (Dv, 0.5–1.94 ±0.14 μm) was produced by spray-drying the protein with PVA, PVP and trehalose (DTPVAPVP). Compared to spray-drying DNase I alone, the incorporation of additional stabilising excipients increased the yield of the manufacturing method from 15.40% to ca. 40%. All of the DNase I microparticles exhibited <5% variance in their protein content implying that they incorporated a very uniform distribution of the protein. DT PVA contained the highest DNase I content at 50.17±2.95% which was significantly more ($p<0.05$, ANOVA) than the DTPVAPVP microparticles which had the lowest DNase I content at 43.84% (Table 2).

3.2. DNase I biological stability in HFA

The biological activity of the spray-dried DNase I microparticles was compared to the original material immediately after manufacture and at four time points after suspension within a HFA propellant, with a view to separating the effects of the manufacturing process and storage within the HFA propellants on the DNase I. The DNase I SD lost almost 40% of its original activity as a consequence of the spray-drying process however, suspension within HFA did little to degrade the protein any further (Fig. 1A). The addition of trehalose to DNase I within the spray-dried microparticulates (Fig. 1B) significantly improved ($p<0.05$ Mann–Whitney test) the biological activity of the protein during the 26 week stability study, but the DT microparticles did still loose approximately 20% of their original enzymatic activity after spray-drying. Storage of DNase I stabilised with trehalose in HFA 134 propellant over 24 weeks did not induce any further reduction of biological activity (Fig. 1B). Using PVA and trehalose to stabilise DNase I (DTPVA Fig. 1C) significantly improved ($p<0.05$, ANOVA) the retention of the enzymes biological function during spray-drying (85.34±2.18%) compared to DNase I stabilised with trehalose alone (78.33±3.20%). When the DTPVA microparticles were suspended within both HFA 134a and HFA 227 some of the activity that appeared to have been lost as a result of spray-drying was recovered (Fig. 1C and E). DTPVA recovered less of its original activity when suspended in HFA 134a compared to when suspended in HFA 227 and retained a significantly higher biological activity over the 24 week period ($p<0.05$ Mann–Whitney test). The DNase I microparticles containing trehalose, PVA and PVP (DTPVAPVP) were the only spray-dried formulation to retain 100% ~~of its~~ activity after the manufacturing process (Fig. 1D). The activity of the enzyme within the DTPVAPVP microparticles immediately after manufacture was significantly higher ($p<0.05$, ANOVA) than any of the other DNase I microparticles produced using spray-drying.

3.3. Impaction analysis

The pMDI containing DNase I SD microparticles emitted the highest FPF compared to any of the other DNase I HFA formulations, depositing ca. 60% of its metered dose onto stage 2 of the TSI (Table 3). The pMDI containing the DT microparticles produced a significantly lower ($p<0.05$, ANOVA) FPF compared to the protein formulated alone through out the 24 week stability study. In a similar manner to DNase I SD there was no significant difference ($p>0.05$, ANOVA) in FPF emitted from the DT pMDI throughout the time frame of the stability study (Table 3). Using trehalose and PVA to suspend DNase I (DTPVA) within HFA 134a resulted in a similar FPF to the DT formulation for the first 2 sample time points in the stability study (ca. 42%). However, the FPF for the DTPVA HFA 134a formulation dropped to ca. 30% at the 24 week time point which was significantly lower ($p>0.05$, ANOVA) than that any of the other DNase I pMDIs. In addition to the low FPF, the deposition variability of the DTPVA 134a formulation increased dramatically during 24 weeks of storage at room temperature (Table 3). The suspension of DTPVA within HFA 227 produced an FPF of approximately 45% which was not significantly higher ($p>0.05$, ANOVA) ~~compared to~~ that from the DT or DTPVA 134a formulations. In addition, neither the FPF ($p<0.05$, ANOVA) nor the deposition variability changed dramatically over the 24 week time frame (Table 3). The combination of PVP, PVA and trehalose (DTPVAPVP) proved to be the best set of excipients with which to physically stabilise the DNase I within a HFA pMDI (Table 3).

Table 2
Particle size, manufacture yield and DNase I content of the spray-dried microparticles (mean±SD, yield $n=1$, rest $n=3$)

Sample	Yield (%)	Dv, 0.1 (μm)	Dv, 0.5 (%)	Dv, 0.9 (μm)	DNase I (%)
DNase I SD	15.40	1.00±0.02	2.25±0.15	4.52±0.52	–
DT	40.00	1.34±0.03	2.87±0.17	5.65±0.59	48.36±4.76
DTPVA	34.75	1.24±0.01	3.06±0.12	6.57±0.43	50.17±2.95
DTPVAPVP	39.60	1.09±0.12	1.94±0.14	3.35±0.30	43.84±2.95

UNCORRECTED PROOF

ARTICLE IN PRESS

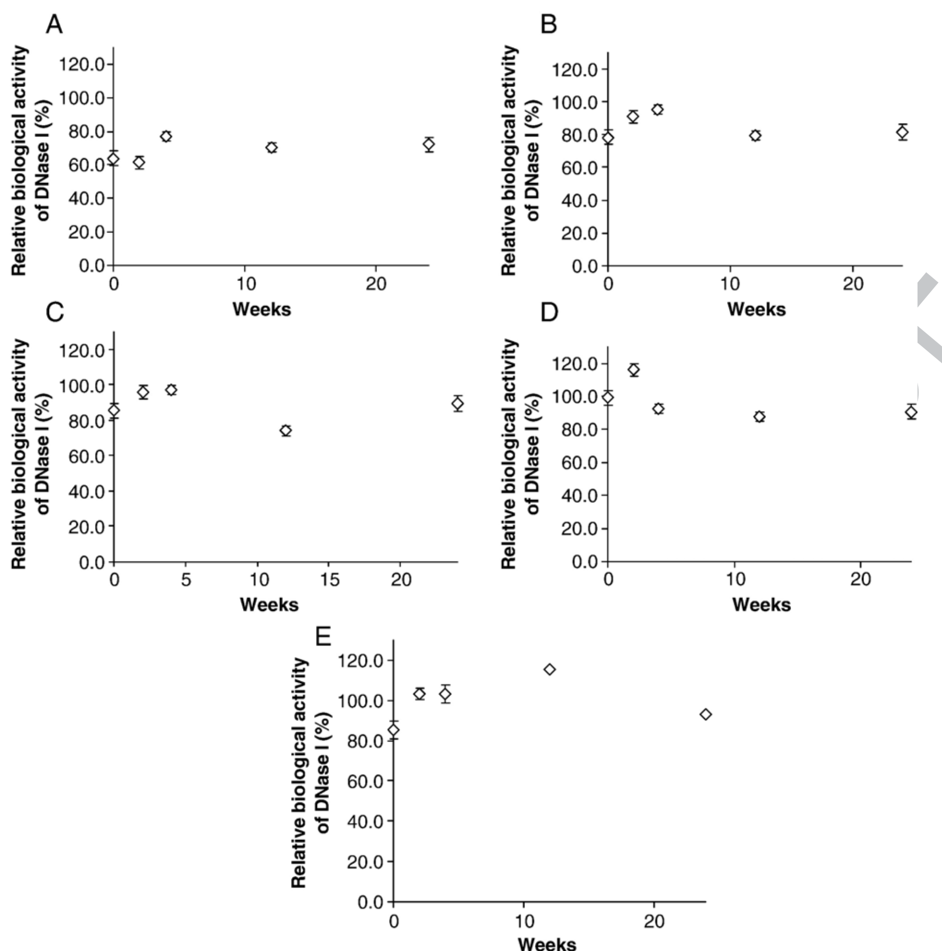

Fig. 1. Biological activity of DNase I microparticles alone (A) or formulated with trehalose, DT (B), trehalose and PVA, DTPVA 134a (C) trehalose and PVA and PVP DTPVAPVP (D), when stored at room temperature for 24 weeks within an HFA 134a metered dose inhalers. In addition (E) displays the biological activity of DNase I formulated with trehalose and PVA, DTPVA 227, when stored at room temperature for 24 weeks within a HFA 227 metered dose inhaler (n=3, mean±standard deviation).

3.4. Mechanism of excipient mediated stabilisation

Although the zeta potential distribution was found to be consistently broad when it was measured for the DNase I microparticles suspended in DCM, the measurements were highly reproducible (Fig. 2). The phase plot was clearly defined, with mobility in both directions (inferred by the positive and negative gradient in Fig. 2) when using a fast reversing micro-electrophoresis method. Spray-dried DNase I alone suspended in DCM produced a zeta potential of 66±4 mV, DT 67±3 mV, DTPVA 61±18 mV, DTPVAPVP 70±9 mV. There was no significant difference between the measured zeta potentials of the three batches of the excipient stabilised DNase I microparticles compared to DNase I SD (p>0.05, ANOVA).

Table 3
The fine particle fraction of the pMDI formulations obtained from the five DNase I HFA pMDIs after 2, 4, 12 and 24 weeks of storage at room temperature (mean±SD, n=3; the recovery was >75% in all cases)

Formulation	FPF week 2 (%)	FPF week 4 (%)	FPF week 12 (%)	FPF week 24 (%)
DNase I SD	59.6±4.5	61.8±3.5	69.2±6.5	52.4±9.5
DT	44.6±6.7	42.2±5.1	37.5±5.2	42.8±0.4
DTPVA 134a	42.0±0.6	42.0±0.6	23.0±5.6	31.6±12.4
DTPVA 227	45.1±7.2	50.6±5.2	47.2±5.2	50.1±0.7
DTPVAPVP	51.0±0.9	48.3±5.6	56.9±1.7	57.8±1.7

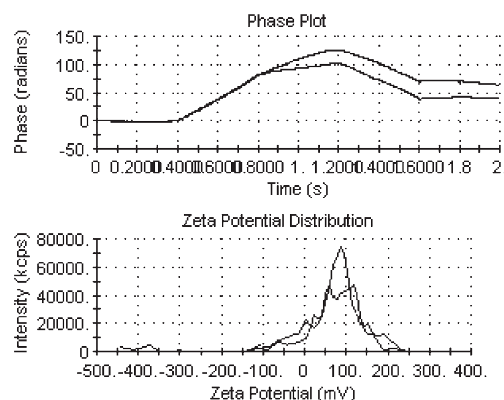

Fig. 2. Zeta potential distribution and phase plot for the suspension of DNase I SD microparticles within dichloromethane, n=3.

UNCORRECTED

4. Discussion

Although DNase I was initially combined with different ratios of stabilising excipients this ratio was not maintained during spray-drying. As is often the case when generating microparticles from solution feed stocks the excess excipients form very small <1 μm size particles and lost waste whilst the excipients associated with the protein form the microparticles >1 μm that can be collected in the cyclone of the spray-drying apparatus. As a result the final microparticles in each case contained approximately 50% of DNase I.

Microparticulate fabrication methods involving spray-drying can render peptides and proteins susceptible to inactivation. Trehalose is commonly used as a protectant during protein particle engineering techniques and was used in this study to protect DNase I against the stresses encountered during spray-drying [14,15]. It is thought that sugars stabilise proteins during rapid dehydration through either vitrification or water substitution mechanisms. Water substitution, a thermodynamic process, involves the formation of hydrogen bonds between the sugar and protein, which is believed to be responsible for the inhibition of the unfolding of the proteins. Vitrification, a kinetic process, depends upon the immobilisation of protein molecules during glass formation [14,16]. Although the exact mechanism of trehalose stabilisation was not identified in this work the sugar was able to conserve 90% of the proteins biological activity compared to 60% when the DNase I was spray-dried alone.

IN addition to heat induced dehydration, the process of spray-drying exposes macromolecules to high shear and rapidly forming air–water interfaces. Whilst trehalose is known to protect DNase I against the removal of water, there is little evidence that it is effective in protecting therapeutic agents against the surface effects caused by atomisation. In the current study, even when trehalose was employed as a stabiliser the DNase I lost >ca. 10% of its biological activity. The exposure of the protein molecules to the high interfacial tension that occurs during spray-drying may account for this loss in activity. Surface active, high molecular weight compounds, such as PVA and bovine serum albumin (BSA), have previously been shown to act as sacrificial molecules in order to protect labile peptides and proteins agents against denaturation caused by high interfacial tension [17]. However, in this study, the addition of PVA to trehalose had little effect on the loss of biological activity of the protein during particulate manufacture. Adsorption of a compound at the air–liquid interface of liquid droplets, such as those formed during the process of spray-drying, is dependent upon both diffusion and convection. Whilst the PVA used in this study is smaller than DNase I, and therefore should diffuse to the droplet surface more rapidly, this is based on the assumption that the two molecules do not interact. In addition, both compounds are macromolecules which adopt only a discrete number of conformations and it is difficult to predict if certain functionalities within the molecules will adsorb to the interface preferentially. As the addition of PVA to the microparticles had little effect on the 10% loss in the DNase I activity when formulated with trehalose, it appears that whilst PVA might adsorb to the surface of the droplets as they dry, this does not occur exclusively, otherwise it would be expected to protect the protein from denaturation. Therefore, DNase I is still potentially being denatured by adsorption at the air–liquid interface when trehalose and PVA are used as excipients during the spray-drying process.

In contrast to DNase I formulated with trehalose and PVA, the addition of both PVA and PVP did enhance the protection of the protein during spray-drying. PVA has been shown to interact with PVP and should such an interaction between the polymers occur within the aqueous environment of the droplet, any preferential adsorption of the polymers to the air–liquid interface simply driven by diffusion may be inhibited as the movement of the PVA/PVP composite would be slowed [18]. However, in the event of PVA and PVP interacting during the spray-drying of DNase I, the surface migration of the composite may be a consequence of the greater hydrophobicity of the associated polymers which would increase the driving force acting on the polymers to orientate at the air interface. Whilst the protein is also known to possess significant hydrophobic regions these will be shielded within a structure exhibiting a high order of conformation. In contrast, the two polymers, even if associated in some way, are likely to exhibit a less constrained conformation, thus preventing the protein from distributing at the surface of the spray-dried droplets by reducing the interfacial tension.

The DNase I microparticles were suspended in a volatile HFA solvent after spray-drying. In-situ analysis of the structural stability of heat sensitive proteins within solvents exhibiting a high vapour pressure such as hydrofluoroalkanes at present using commercially available equipment is impossible. Preliminary work by Quinn et al. [9,19] and other workers have demonstrated that model proteins such as lysosyme did not undergo structural modifications upon suspension within HFA propellants [20]. However, since this initial discovery, there has been little subsequent work to formulate therapeutic proteins within pMDIs. DNase I is a much more labile protein compared to lysosyme; it is physically unstable upon storage within aqueous solution at room temperature and it is susceptible to glycation in the dry-state. Regardless of its greater susceptibility to denaturation, the high stage 2 deposition of the DNase I when formulated without stabilising excipients in the TSI implied that the microparticle suspension was physically stable in the propellant. Furthermore, the unchanging biological integrity of the protein upon storage within the propellant suggested that the protein was structurally stable [21]. It is however difficult to draw definitive conclusions on the compatibility of DNase I alone with the hydrofluroalkane solvents as the protein microparticles formed without stabilising excipients had been significantly denatured during spray-drying. If the denaturation of the protein was as a result of a change in secondary structure then hydrophobic moieties within the protein's structure may have been externalised changing the microparticle surface and this could enhance the protein–HFA compatibility. Clearly further work is required to investigate the influence of protein denaturation upon HFA compatibility in more detail.

UNCORRECTED PROOF

ARTICLE IN PRESS

In low dielectric solvents the barrier to charging is up to forty times larger compared to a polar suspension vehicle and therefore, the charge effects within non-polar systems should be insignificant. According to Henry's equation if the Debye length is assumed constant (Huckel approximation) then in a low dielectric constant media a low electrophoretic mobility would indeed result in a low zeta potential. However, the zeta potential measurements of the spray-dried microparticles were all in excess of −60 mV. DNase I microparticles exhibited a similar charge when suspended within DCM irrespective of the included stabilising excipients. This however, again may not be a true reflection of what is occurring in the non-polar suspensions as the DNase I alone was shown to be denatured and therefore may present a more hydrophobic surface.

The ionisation of the folded protein in the buffer solution prior to spray-drying should have been negative according to theoretical calculations based on its amino acid sequence. However, the DNase I when spray-dried alone produced a +ve zeta potential. The surface charge will be dependent upon the conformation the protein has taken when it dried into the microparticle droplet. The charging of particles in non-polar solvents such as DCM is not necessarily due to the location of amino- and carboxy-terminus of the peptide as according to the dielectric constant of the non-polar solvent it would contain very few ionisable species. When the DNase I was spray-dried with stabilising excipients the protein should have taken a different conformation (as it is biologically active), but the zeta potential is very similar irrespective of the type of excipient included, this suggests therefore that it may not be the amino acids on the surface of the microparticle that are inducing the surface charge, but other ionic processes such as charging induced by absorption of impurities or proton exchange as described by Farr et al. (1994) [21].

Whilst care has to be taken when extrapolating the effects observed in a 'surrogate' non-polar system (in this case DCM) to those that can occur in HFA propellants it is evident that charge is present when the DNase I particles are suspended in non-polar systems and this effect may contribute to the excellent physical suspension stability of the suspension. Even in inert non-polar systems counterions are present, possibly in part due to trace impurities. Yu et al. [22] showed the effect of water on zeta potential using a series of homologous solvents. The simple addition of 0.5% water to a non-polar system provided a one order of magnitude rise in zeta potential. These workers also linked a decrease in the magnitude of zeta potential within suspension systems to a decrease in dielectric constant. In contrast, Kosmulski [23] concluded that the role of water in non-polar systems is "overrated" whilst the role of trace impurities such as amines is often overlooked. Regardless of the source and nature of the counterions, the results obtained in the present study appeared to support the observations that charge can have an important role in the physical stabilisation of pharmaceutically relevant non-polar suspensions. However, further work is required to investigate the origin of this charge and to determine whether it can be controlled to promote physical stability in non-polar systems.

Ridder et al. [24] showed that HFA 227 (C_3HF_7) demonstrates stronger interactions with common surfactants such as Brij and Tween compared to HFA 134 ($C_2H_2F_4$) due to a greater capacity to form hydrogen bonds. Despite both PVA and PVP being water soluble polymers both molecules contain hydrophobic moieties. Solvation effects as a result of the presence of PVP, which has been shown to be sparingly soluble in the HFA, are thought to promote limited chain extension and steric stabilisation [10,25,26]. Steric effects usually result from the adsorption of surfactants or polymers on the surface of the particles and subsequent chain extension however, the very limited solubility of PVA in HFA would not be expected to result in this polymer extending into the HFA. More fundamental studies using model systems are required to characterise the mechanism of the protein microparticle stabilisation and to isolate the factors that might enhance the physical stability of the microparticles. However, the results from this study suggest that both the charge generated on the particle surface, perhaps as a result of proton exchange with HFA 227 as demonstrated previously with small molecular weight surfactants and the steric stabilisation via the use of appropriate polymer excipients could be of relevance to the case of DNase I [21].

5. Conclusion

The pMDI system developed in this work could provide a suitable alternative to the nebuliser solution currently used to administer DNase I. Incorporating this protein into an HFA system not only stabilised the macromolecule at room temperature, but in addition, produced a formulation that is both portable and easy to use. Protection gained from the non-polar vehicle probably due to the low availability of water, air and light, which are the most common degradations for pharmaceutical compounds, resulted in two of the formulations being both physically and biologically stable over a 6 month stability study.

This study builds on previous work to show that the combination of PVA and PVP can be used to aid the physical stability of suspension based pMDIs [10,27,28]. Although the technical challenges of measuring the zeta potential directly within HFA systems have not yet been solved, this work provides some evidence that both steric hindrance (due to the incorporation of PVP) and electrostatic stabilisation could be functioning to aid the stabilisation of microparticles within non-polar systems. The high zeta potential of the particles in DCM which has an identical dielectric constant to HFA 134a indicates that whilst the electrostatic forces did not seem to be the sole influence on the physical stability of DNase I, DT, PVA and PVP microparticles, charge may play an important role in pMDI stabilisation.

Acknowledgements

The authors wish to thank MedPharm and King's College London for the financial support of this project. In addition, special thanks goes to Malvern Instruments UK and in

UNCORRECTED PROOF

particular Michael Kaszuba for the use of the Nano Series® Zeta Sizer.

References

[1] I. Gonda, Inhalation therapy with recombinant human deoxyribonuclease I, Adv. Drug Deliv. Rev. 19 (1) (1996) 37–46.

[2] M. Laskowski, in: P.D. Boyer (Ed.), The Enzymes, Academic Press, New York, 1971, pp. 289–311.

[3] C. Oefner, D. Suck, Crystallographic refinement and structure of Dnase-I at 2A resolution, J. Mol. Biol. 192 (3) (1986) 605–632.

[4] P. Raskin, Bronchospasm after inhalation of pancreatic dornase, Am. Rev. Respir. Dis. 98 (1968) 597–598.

[5] M. Berge, E. Wiel, H.A.W.M. Tiddens, P.J.F.M. Merkus, W.C.J. Hop, J.C. de Jongste, DNase in stable cystic fibrosis infants: a pilot study, J. Cyst. Fibros. 2 (4) (2003) 183–188.

[6] P.L. Shah, S.F. Scott, R.A. Knight, C. Marriott, C. Ranasinha, M.E. Hodson, In vivo effects of recombinant human DNase I on sputum in patients with cystic fibrosis, Thorax 51 (2) (1996) 119–125.

[7] C. Ranasinha, B. Assoufi, S. Shak, D. Christiansen, H. Fuchs, D. Empey, D. Geddes, M. Hodson, Efficacy and safety of short-term administration of aerosolized recombinant human Dnase-I in adults with stable stage cystic-fibrosis, Lancet 342 (8865) (1993) 199–202.

[8] C. Vervaet, P.R. Byron, Drug–surfactant–propellant interactions in HFA formulations, Int. J. Pharm. 186 (1) (1999) 13–30.

[9] E.A. Quinn, R.T. Forbes, A.C. Williams, M.J. Oliver, L. McKenzie, T.S. Purewal, Protein conformational stability in the hydrofluoroalkane propellants tetrafluoroethane and heptafluoropropane analysed by Fourier transform Raman spectroscopy, Int. J. Pharm. 186 (1) (1999) 31–41.

[10] S.A. Jones, G.P. Martin, M.B. Brown, High-pressure aerosol suspensions —a novel laser diffraction particle sizing system for hydrofluroalkane metered dose inhalers, Int. J. Pharm. 302 (2005) 154–165.

[11] S.A. Jones, G.P. Martin, M.B. Brown, Metered Dose Inhaler Preparations of Therapeutic Drugs, PCT/GB2004/005172, 2004.

[12] British Pharmacopoeia, Stationary Office, London, UK, 2004.

[13] S.A. Jones, G.P. Martin, M.B. Brown. Manipulation of beclomethasone–hydrofluoroalkane interactions using biocompatible macromolecules, J. Pharm. Sci. (in press).

[14] T. Arakawa, S.J. Prestrelski, W.C. Kenney, F.C. Carpenter, Factors affecting short-term and long-term stabilities of proteins, Adv. Drug Deliv. Rev. 10 (1) (1993) 1–28.

[15] Y.H. Liao, M.B. Brown, T. Nazir, A. Quader, G.P. Martin, Effects of sucrose and trehalose on the preservation of the native structure of spray-dried lysozyme, Pharm. Res. 19 (12) (2002) 1847–1853.

[16] J.F. Carpenter, K. Izutsu, T.W. Randolph, in: L. Rey, J.C. May (Eds.), Freeze-Drying/Lyophilization of Pharmaceutical and Biological Products, New York, 1999, pp. 123–160.

[17] W. Wang, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm. 203 (1–2) (2000) 1–60.

[18] S.A. Jones, G.P. Martin, M.B. Brown, Biocompatible polymer blends: the effects of physical processing on the molecular interaction of polyvinyl alcohol and polyvinylpyrrolidone, J. Appl. Polym. Sci. 98 (2005) 2290–2299.

[19] E.A. Quinn, The stability of proteins in hydrofluoroalkane propellants, PhD thesis, University of Bradford, 2000.

[20] Y.H. Liao, M.B. Brown, S.A. Jones, T. Nazir, G.P. Martin, The effects of polyvinyl alcohol on the in vitro stability and delivery of spray-dried protein particles from surfactant-free HFA 134a-based pressurised metered dose inhalers, Int. J. Pharm. 304 (1–2) (2005) 29–39.

[21] S.J. Farr, L. McKenzie, J.G. Clarke, Drug–surfactant interactions in apolar systems: relevance to the optimised formulations of suspension MDIs, Respir. Drug Deliv. IV (1994) 221–230.

[22] J.C. Yu, Z.T. Jiang, H.Y. Liu, J. Yu, Influence of solvation interactions on the zeta potential of titania powders, J. Colloid Interface Sci. 262 (1) (2003) 97–100.

[23] M. Kosmulski, Zeta potentials in nonaqueous media: how to measure and control them, Colloids Surf., A Physicochem. Eng. Asp. 159 (2–3) (1999) 277–281.

[24] K.B. Ridder, C.J. vies-Cutting, I.W. Kellaway, Surfactant solubility and aggregate orientation in hydrofluoroalkanes, Int. J. Pharm. 295 (1–2) (2005) 57–65.

[25] B.A.D. Costello, I.T. Kim, P.F. Luckham, T.F. Tadros, Experimental investigations of the interaction forces in concentrated dispersions, Colloids Surf., A Physicochem. Eng. Asp. 77 (1) (1993) 55–63.

[26] D.H. Napper, Flocculation studies of non-aqueous sterically stabilized dispersions of polymer, Trans. Faraday Soc. 64 (1968) 1701.

[27] S.A. Jones, G.P. Martin, M.B. Brown, Development of a rapid pre-formulation screen for HFA suspension metered dose inhalers, Drug Deliv. Lung IX (2004) 529–532.

[28] S.A. Jones, Y.H. Liao, G.P. Martin, M.B. Brown, Metered dose inhaler preparations, WO 2004/043442, 2003.

UNCORRECTED PROOF