

## Pharmaceutical Nanotechnology

## Stability and aerosolization of pressurized metered dose inhalers containing thymopentin nanoparticles produced using a bottom-up process

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## ABSTRACT

The objective of this study was to investigate the stability and aerosolization of pressurized metered dose inhalers (pMDIs) containing thymopentin nanoparticles. Thymopentin nanoparticles, fabricated by a bottom-up process, were suspended in hydrofluoroalkane (HFA) 134a together with cineole and/or *n*-heptane to produce pMDI formulations. The stability study of the pMDIs obtained was carried out at ambient temperature for 6 months. The amount of thymopentin and the aerosolization properties of pMDIs were determined using high-performance liquid chromatography (HPLC) and a twin-stage impinger (TSI), respectively. Based on the results, thymopentin nanoparticles were readily suspended in HFA 134a with the aid of cineole and/or *n*-heptane to form physically stable pMDI formulations, and more than 98% of the labeled amount of thymopentin and over 50% of the fine particle fraction (FPF) of the pMDIs were achieved. During storage, it was found that for all pMDIs more than 97% of the labeled amount of thymopentin and FPF greater than 47% were achieved. Moreover, the size of thymopentin nanoparticles in propellant containing cineole and *n*-heptane showed little change. It is, therefore, concluded that the pMDIs comprising thymopentin nanoparticles developed in this study were stable and suitable for inhalation therapy for systemic action.

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## 1. Introduction

The application of nanoparticles is gaining momentum in pulmonary drug delivery in recent years (Zhang et al., 2011). Nanoparticles are considered to be an alternative to conventional micronized particles or large porous microparticles in inhalation therapy since they can be well deposited in the lung and escape both phagocytic and mucociliary clearance mechanisms (Tsapis et al., 2002). Proteins and peptides exhibit negligible solubility in hydrofluoroalkane (HFA) propellants used in the preparation of pMDI systems (Li and Seville, 2010). Therefore, preparation of suspensions of nanoparticles is necessary in order to deliver proteins and peptides using pMDIs.

Nanoparticles for pulmonary drug delivery are usually created by milling down larger particles (top-down process) or precipitating out of liquid phase (bottom-up process) (Rabinow, 2004).

However, there have been very few reports on the preparation of protein/peptide nanoparticles using the bottom-up processes. Recently, Nyambura et al. (2009a,b) reported the preparation of nanoparticles containing lysozyme or insulin using microemulsion or nanoprecipitation methods coupled with freeze-drying. The processes are relatively complex and have several disadvantages such as high-energy consumption, high-speed shearing and the application of toxic organic solvents with low freezing points. To overcome those disadvantages, a novel bottom-up process was developed by authors' research group where lysozyme nanoparticles were prepared and subsequently dispersed in HFA 134a (Tan et al., 2011). However, it is necessary to determine the potential of this method with a therapeutic peptide delivered via the pulmonary route. Moreover, the long-term stability and in vitro deposition efficiency of nanoparticles containing proteins and peptides in pMDI formulations should be investigated. For these purposes, thymopentin was chosen because it is a therapeutic peptide, sensitive to enzymatic degradation and suitable for pulmonary administration. Thymopentin, a synthetic pentapeptide, has been used clinically as a modulator for immunodeficient patients through intramuscular administration for one to six months, exhibiting a similar biological activity to thymopoietin, the native hormone

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isolated from thymus gland (Goldstein et al., 1979). Both an inhalable thymopentin formulation reported by Wang et al. (2009) and inhalable microparticles containing thymopentin-loaded solid lipid nanoparticles researched by Li et al. (2010) were used as dry powder inhalation (DPI). To date, there have been no reported investigations into thymopentin nanoparticles suspended in HFA propellants for delivery from pMDIs in order to improve the *in vitro* deposition efficiency of thymopentin and keep its stability in pMDI formulation.

The ability of nanoparticles to disperse and form a physically stable suspension in propellant used is one of the major properties related to dose reproducibility in a suspension-type pMDI (Byron, 1992). Unstable suspensions can result in uncontrolled emitted dose and size characteristics, which lead to poor inhalation therapy (Nyambura et al., 2009a). It has not reported that nanoparticles could be dispersed in pure HFA propellants to form self-stabilized nanosuspensions. The application of stabilizers is the most commonly used strategy in achieving a stable nanoparticle formulation (Wu et al., 2011). Alcohols such as ethanol have always been used as co-solvents to increase the solubility of surfactants to improve the physical stability of suspensions in pMDIs when HFAs are used as propellants (Williams et al., 1998). However, the presence of ethanol may affect the stability of protein and peptide and/or solubility of other stabilizers presented in protein pMDI formulations during storage (Nyambura et al., 2009a). Surfactants such as dipalmitoylphosphatidylcholine (DPPC), Span 85 and oleic acid have been used in suspension-type pMDIs to improve the physical stability of nanoparticles suspended in HFA propellants (Chokshi et al., 2009; Engstrom et al., 2009; Nyambura et al., 2009b; Tam et al., 2010; Tan et al., 2011; Wu et al., 2008a,b). Nevertheless, the surfactants used in HFA-based pMDI formulations containing nanoparticles are insufficient to stabilize the suspension systems during storage, which may be explained as the following. Firstly, the physico-chemical properties of HFA propellants are different from those of chlorofluorocarbons (Brindley, 1999; Vervaet and Byron, 1999). Secondly, the properties of nanoparticles such as surface charges and zeta potential in HFA propellants may differ from those of common particles. Although some techniques such as atomic force microscopy, inverse gas chromatography, goniometry and computer simulations have been employed to explain the solvation properties of HFA and surfactant interaction in HFAs (Verma et al., 2009; Wu and da Rocha, 2007; Wu et al., 2007), understanding of solvation of drug nanoparticles in the pMDI nanosuspension using HFA 134a as a low-dielectric medium is still in difficulty, which makes stabilizer selection even more challenging (Wu et al., 2011). There are a multitude of formulation factors to consider when developing a pMDI (Brambilla et al., 1999, 2011; Smyth, 2003). However, for nanosuspension-typed pMDIs, it is most important to choose suitable stabilizers to maintain the stability.

It was reported that some novel stabilizers were utilized in suspension-type pMDI formulations to stabilize nanoparticles (Dickinson et al., 2001; Nyambura et al., 2009a). Nyambura et al. (2009a) have studied essential oils such as cinnamaldehyde, cineole (eucalyptol) and citral (3,7-dimethyl-2,6-octadienal or lemonal) by dissolving them in HFA 134a and found both cineole and citral were beneficial in stabilizing the dispersion of insulin nanoparticles in HFA 134a of pMDI. Homogeneous dispersions were formed upon shaking, and remained visibly stable for approximately 2–3 min. Cinnamyl derivatives and citral are generally considered safe as they are widely used as flavoring materials in food products (Nyambura et al., 2009a). No adverse effects have been reported about them because they are rapidly absorbed, metabolized and excreted in man. Toxicological studies revealed little potential in causing significant carcinogenicity, genotoxicity and mutagenicity (Adams et al., 2004; Nyambura et al., 2009a). Additionally, Dickinson et al. (2001) reported that nanoparticles produced from

lecithin-based microemulsions could readily be dispersed in HFA propellants forming a homogeneous fine suspension by using 5% (w/w) *n*-hexane. Unfortunately, in those studies the storage stability or long-term stability of pMDIs containing nanoparticles was not reported.

The objective of this study was to investigate the stability and aerosolization of thymopentin nanoparticles from pMDIs. Thymopentin nanoparticles were produced by the bottom-up process reported previously by authors' research group (Tan et al., 2011). Subsequently, the nanoparticles were dispersed in HFA 134a with the aid of cineole and/or *n*-heptane to form suspension-type pMDI formulations. The aerosolization properties, physical stability and storage stability of the produced pMDI formulations comprising thymopentin nanoparticles were also evaluated in this study. In addition, a novel combination of cineole and *n*-heptane in HFA 134a propellants was investigated for their ability to improve the stability and aerosolization properties of the pMDI formulations.

## 2. Materials and methods

### 2.1. Materials

Tert-butyl alcohol (TBA), isoctane, isopropanol, sodium hydroxide, monopotassium phosphate, and phosphotungstic acid were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China) while HFA 134a (Pharmaceutical grade, purity 99.99%) was purchased from INEOS Flour Ltd. (Runcorn, Cheshire WA74JE, UK). HPLC grade methanol was purchased from Fisher Scientific Ltd. (NJ, USA). Thymopentin (purity 99.5%) was purchased from Chengdu Kaijie Biopharm Co., Ltd. (Chendu, China). Cineole and *n*-heptane were obtained from Aladdin Reagent Database Inc. (Shanghai, China). Lecithin (Lipoid E80) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Water was distilled via a water purification system (PureLAB Option, ELGA Lab Water Inc., UK).

### 2.2. Methods

#### 2.2.1. Preparation of thymopentin nanoparticles

Thymopentin nanoparticles were generated by the bottom-up process previously developed by authors' research group (Tan et al., 2011). Briefly, 25 mg of thymopentin and 15 mg of lactose were dissolved in 1.5 mL of purified water as the aqueous phase. The organic phase was prepared by dissolving lecithin (0, 100, 200, 300, 400, 500, 600, and 700 mg, respectively) in 3.0 mL of TBA at 26–30 °C. Various lecithin concentrations in the organic phase (0–23.3%, w/v) were used to investigate the optimal lecithin concentration for the production of nanoparticles. The aqueous phase was then added into the organic phase under vortexing or stirring at 26–30 °C, which resulted in a light yellow colored solution. Immediately, the solution was snap frozen by immersing it in liquid nitrogen and then lyophilized using a CHRIST ALPHA 1-4LSC freeze dryer (Osterode, Germany) for a minimum of 12 h at –55 °C under 0.25 mbar to remove water and TBA. The above resulted in lyophilizate containing nanoparticles covered with surfactant. Then the nanoparticles were suspended in isopropanol, in which lecithin was freely soluble while thymopentin and lactose were insoluble, and thus the structure of nanoparticles could be preserved. The nanoparticles in the raw suspension were separated from free surfactant by centrifugation using a TGL-16C desktop centrifuge (Feige Brand, Shanghai Anting Scientific Instruments Factory, Shanghai, China) at 25 °C. The supernatant was removed and the sediment portion containing nanoparticles was collected, which was subject to centrifugation again to ensure maximum purity (Cook et al., 2005).

In order to investigate the effect of water content in TBA/water co-solvent on the formation of thymopentin nanoparticles, 25 mg of thymopentin and 15 mg of lactose were dissolved in different amount of purified water (1.5, 2.0, 2.5, 3.0 and 3.5 mL, respectively). Lecithin (600 mg) was dissolved in 3.0 mL of TBA to form the organic phase at 26–30 °C. The rest of procedures of preparing thymopentin nanoparticles were the same as described above.

#### 2.2.2. Particle size analysis for thymopentin nanoparticles

Particle size distribution of thymopentin nanoparticles prepared was determined using Photon Correlation Spectroscopy (PCS) (Malvern Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK). Hydrodynamic diameter, expressed as Z-average diameter (Z-ave), was measured, and polydispersity index (PI) implying the width of particle size distribution was determined by cumulant analysis as described in the International Standard on PCS. At first, nanoparticles were uniformly dispersed in isoctane, which had been initially filtered through a 0.1 µm nylon membrane filter (Tengjin®, China), by sonication (Ningbo Xinzhi Biotechnology Co. Ltd., Zhejiang, China) in a water bath for 5 min. Then the concentration was adjusted to 5 mg of nanoparticles/mL of isoctane, which was necessary to provide required analytical count rate in the PCS (>50 kilocounts per second). Finally, the suspension was transferred into a non-frosted quartz cuvette, placed in the sample holder of PCS, and stood for 2 min to reach equilibrium prior to measurement. Each sample was measured in triplicate.

#### 2.2.3. Morphological study of thymopentin nanoparticles

Thymopentin nanoparticles, suspended in isoctane, were put on copper grids to allow the isoctane to evaporate. Then the nanoparticles were negatively stained with a phosphotungstic acid (PTA) solution (1%, w/v). Subsequently, the samples were dried under conditions of less than 45% relative humidity at ambient temperature before being examined by a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

#### 2.2.4. Preparation and visual observation of pMDI formulations containing thymopentin nanoparticles

Dispersing stabilizers are usually added in pMDI suspensions due to the physical instability of particles suspended in HFA propellants. In order to investigate the dispersing or suspending stability of thymopentin nanoparticles in HFA 134a propellant, seven groups of nanoparticles were prepared and purified following the procedure described previously. Then 40 mg of lyophilized nanoparticles from each group was moistened with 500 µL of cineole, cineole and *n*-heptane at different ratios (i.e. 9:1, 4:1, 3:2, 2:3 and 1:4) and *n*-heptane, respectively, (referred as formulations F1, F2, F3, F4, F5, F6 and F7) by ultrasonic treatment for 1 min. Then, the mixture was transferred into a plastic coated glass bottle (Shandong Jewim Pharmaceutical Co., Ltd., Shandong, China) after drying. Propellant HFA 134a (10 g) was added through a valve stem using an aerosol filling machine (Zhongshan Zhihua Aerosol Equipment Co., Ltd., Guangdong, China) after a 50 µL valve (Valois (Suzhou) Dispensing Systems Co., Ltd., China) was immediately fixed onto the bottle using a semi-automatic bottle crimer (Zhongshan Zhihua Aerosol Equipment Co., Ltd., Guangdong, China) at 22–26 °C and 40–50% relative humidity. The pMDIs obtained were then sonicated in a water bath for approximately 2 min and the suspension stability was visually checked for any obvious separation, flocculation, coalescence or sedimentation of the dispersed nanoparticles as time went on. The redispersibility of the nanoparticles in HFA 134a was visually evaluated upon shaking by hand. This operation was repeated twice.

#### 2.2.5. Determination of thymopentin in pMDI formulations

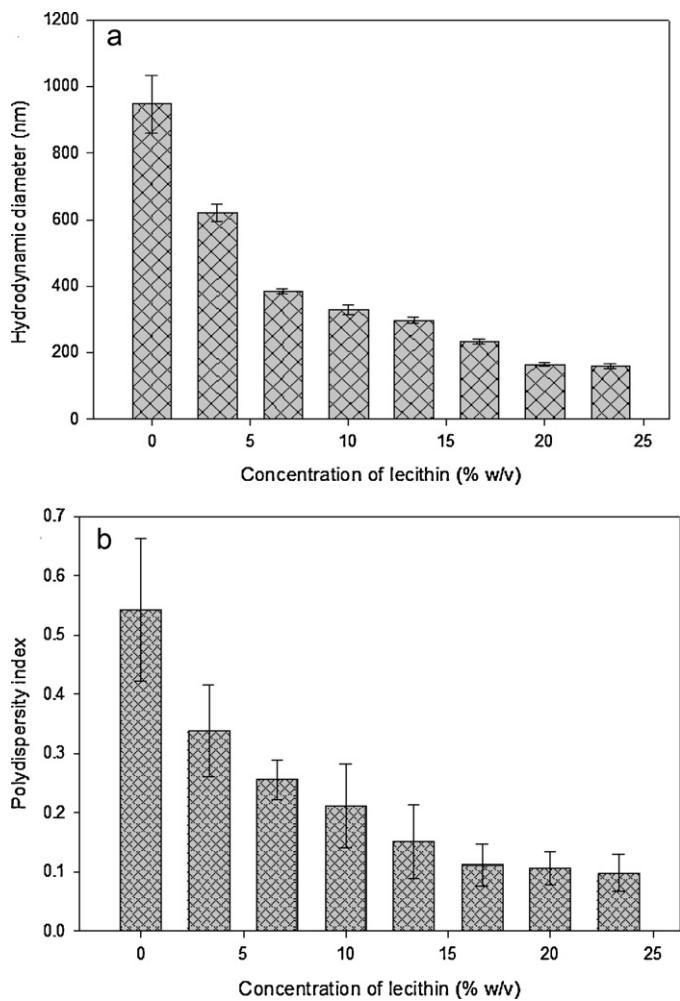
In order to determine thymopentin in the pMDI formulations, the pMDIs were immersed in liquid nitrogen to chill the propellants. Once cold, the bottles were removed from liquid nitrogen, and the valves were removed to allow HFA 134a to evaporate slowly, leaving the nanoparticles at the bottom of the bottles. The thymopentin nanoparticles in the bottles was dissolved in the solution of mobile phase and diluted to 50 mL with it. The amount of thymopentin was assayed by HPLC (Shimadzu, Japan) using a reverse-phase C<sub>18</sub> column (5 µm, 4.6 mm × 250 mm, Phenomenex, Inc., Torrance, USA) at ambient temperature with UV detection at 275 nm and methanol–PBS (pH 7.0) 85:15, v/v, at 1 mL/min as mobile phase. A calibration curve was constructed using thymopentin standard solutions of 100–500 µg/mL ( $R^2 = 0.9996$ ). Each sample was analyzed three times. The relative amount of thymopentin was defined as the percentage of the amount of thymopentin assayed to the labeled amount of thymopentin (i.e. 25 mg) in each pMDI formulation.

#### 2.2.6. Aerosolization properties of pMDI formulations

The aerosolization properties of the pMDI formulations were determined using the twin stage impinger (TSI, National Center for Pharmaceutical Engineering, Shanghai, China) in accordance with the specifications defined by British Pharmacopoeia. PBS (pH 7.0) was introduced into the upper and lower stages (7 and 30 mL, respectively), and the flow rate through the TSI was adjusted to 60 L/min using a glass rotameter (Model LZB-10, Changzhou Chengfeng Flow Meter Co., Ltd. Jiangsu, China). Actuators with 0.4 mm orifice diameter (Valois (Suzhou) Dispensing Systems Co., Ltd., China) were used. The pMDI formulations to be tested were primed prior to use by actuating five shots to waste. Ten actuations were sprayed into the TSI from each pMDI, and there was an approximate 10-s break between actuations to prevent the cooling effect of HFA 134a on the actuators. The pump was allowed to run for 5 s after each discharge and then switched off for 5 s while the inhaler was shaken by hand. After completing each run, the impinger was dismantled and the amount of thymopentin deposited in Stage 1 and Stage 2 of the TSI was determined. In addition, the pMDI actuator and the TSI throat were separately rinsed with PBS (pH 7.0), which were analyzed for the amount of thymopentin by the HPLC method described previously. Each aerosolization test was performed in triplicate. The recovered dose (RD) was defined as the total mass of thymopentin detected (i.e. actuator + throat + Stage 1 + Stage 2). The fine particle dose (FPD) was defined as the mass of thymopentin recovered from Stage 2 of the TSI with the effective cut-off diameter of 6.4 µm. The percentage of FPF<sub><6.4 µm</sub> was calculated as the ratio of FPD to RD multiplied by 100.

#### 2.2.7. Stability study of pMDI formulations during storage

Based on the results from Section 2.2.4 where formulations F1, F2 and F3 demonstrated a better suspension stability, they were used to investigate the stability of pMDIs during storage with valve-up for 2 days, 6 weeks, 12 weeks, 26 weeks and the results obtained at these time points were designated as week-1, week-6, week-12 and week-26, respectively. The samples of pMDI formulations containing thymopentin nanoparticles were placed in a case at ambient temperature while the particle size of nanoparticles in pMDI and the relative amount of thymopentin and FPF were determined by sampling at corresponding time points. Meanwhile, the suspension stability or the redispersibility of the nanoparticles in HFA 134a was visually evaluated following the method described in Section 2.2.4. For nanoparticle size analysis, HFA 134a propellant and stabilizers in pMDI containing thymopentin nanoparticles were firstly removed following the procedure in Section 2.2.5. Then, 5 mL of



**Fig. 1.** Effect of lecithin concentration on (a) the particle size and (b) polydispersity index (PI) of thymopentin nanoparticles. Data shown are mean  $\pm$  S.D.,  $n=3$ .

isooctane was added into the glass bottle of pMDI and the rest of procedures was identical to the one in Section 2.2.2.

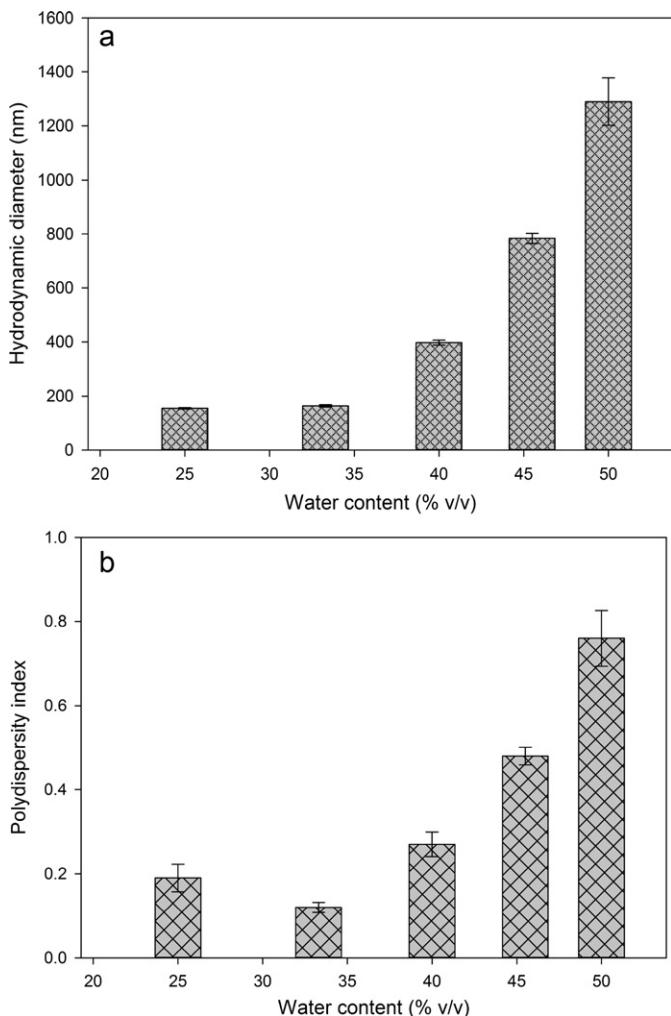
#### 2.2.8. Statistical analysis

All data were expressed as mean  $\pm$  S.D. and analyzed using Student's *t*-test or one-way ANOVA/Dunnett's test. In all cases, *p* values less than 0.05 or 0.01 were considered statistically significant or very statistically significant, respectively.

### 3. Results

#### 3.1. Effect of lecithin concentration on the particle size of thymopentin nanoparticles

During the preparation of thymopentin nanoparticles, the concentration of surfactant (lecithin) was optimized in order to produce nanoparticles in the desired size range. The average hydrodynamic diameter of the nanoparticles produced was in the range of 159–948 nm (Fig. 1a) with PI ranged 0.10–0.54 (Fig. 1b). As lecithin concentration in the organic phase increased, the average particle size and PI values were reduced until the lecithin concentration reached 20.0% (w/v). Further increase of lecithin concentration had little effect on the particle size and PI of thymopentin nanoparticles. When lecithin was absent, the average hydrodynamic diameter and PI were the highest. It is believed that higher concentration of lecithin leads to more

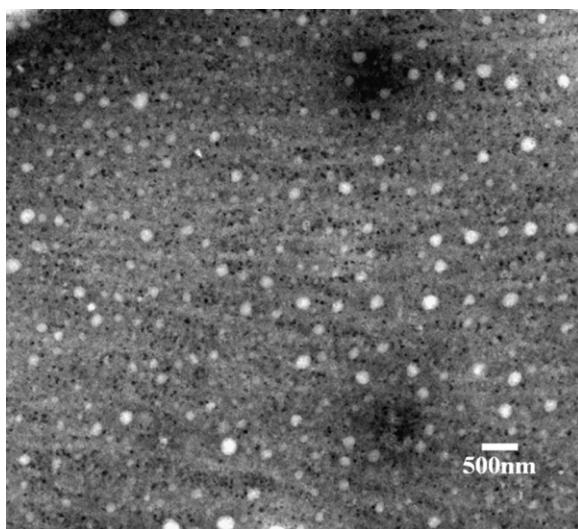


**Fig. 2.** Effect of water content in TBA/water co-solvent system on (a) the particle size and (b) PI of thymopentin nanoparticles. Data shown are mean  $\pm$  S.D.,  $n=3$ .

surfactant covering the surface of nanoparticles and thus reducing the average hydrodynamic diameter and PI. However, the presence of surfactant also increased the difficulty of product purification and recovery as the surfactant increased the viscosity of the suspension, hampering the sedimentation of produced nanoparticles during centrifugation. Thus, in choosing the proper concentration of lecithin, both the particles size of the nanoparticles and viscosity of the preparation need to be considered. Based on the results, preparation containing 20.0% (w/v) lecithin in the organic phase was selected for subsequent investigations.

#### 3.2. Effect of water content on the particle size of thymopentin nanoparticles

The water content in TBA/water co-solvent system was also optimized in order to produce nanoparticles in the desired size range. The results in Fig. 2a showed the average hydrodynamic diameter of nanoparticles increased from  $154.7 \pm 2.7$  nm to  $1289.4 \pm 87.9$  nm with water content increasing from 25.0% (v/v) to 50.0% (v/v). The mean size of nanoparticles slightly increased as water content in the range of 25.0–33.3% (v/v), but rapidly increased when water content was above 33.3% (v/v). PI values were in the range 0.12–0.76 with a minimum of 0.12 corresponding to 33.3% (v/v) of water content as shown in Fig. 2b. It was found that when water content was below 25.0%, thymopentin and lactose could not be fully dissolved in TBA/water co-solvent



**Fig. 3.** TEM micrograph of thymopentin nanoparticles (light dots represent the nanoparticles).

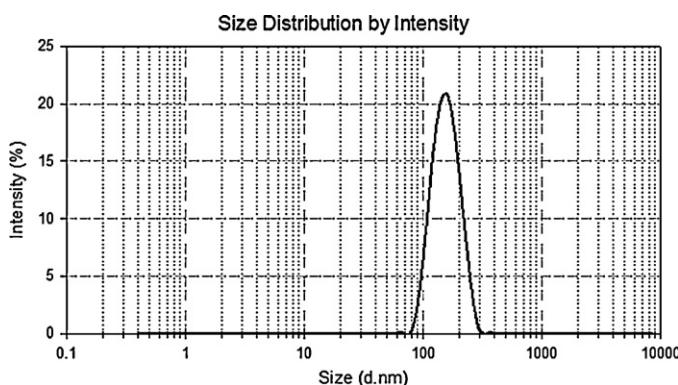
to form a single-phase solution (data not shown). Therefore, the thymopentin nanoparticle preparation using 33.3% (v/v) water was selected for subsequent investigations.

### 3.3. Morphology of thymopentin nanoparticles

TEM micrograph showed that thymopentin nanoparticles were in spherical shape with approximately 150 nm in mean size (Fig. 3). Typical size distribution curve of the nanoparticles measured via Malvern Zetasizer Nano ZS90 is shown in Fig. 4. The particle size and size distribution of thymopentin nanoparticles determined from TEM micrograph are in agreement with those from Malvern curve measured via PCS.

### 3.4. Physical stability of nanoparticles in HFA 134a

Good redispersibility of creamed suspensions upon shaking is important for dose reproducibility of suspension-type pMDIs. The results of dispersibility studies showed that the addition of cineole, *n*-heptane, or cineole and *n*-heptane mixed at different ratios resulted in suspensions of nanoparticles in HFA 134a stable for at least 3 min (Table 1), which would ensure inhaler users to acquire an accurate dose every time from pMDI. Homogeneous suspensions were formed immediately after shaking by hand and the suspensions were stable for 3 min when the nanoparticles were moistened with cineole alone. After 3 min, there was slight



**Fig. 4.** Typical particle size distribution curve of thymopentin nanoparticles ( $Z_{ave} = 152.4$  nm,  $PI = 0.11$ ).

**Table 1**

Effect of co-solvents on the physical stability of suspension containing thymopentin nanoparticles in HFA 134a determined visually (mean  $\pm$  S.D.,  $n = 3$ ). The volume of co-solvent in each formulation was 500  $\mu$ L, and the labeled amount of thymopentin in each pMDI formulation was 25 mg.

Formulation	Co-solvents	Time of suspension stability	Relative amount of thymopentin (%)
F1	Cineole alone	3 min	99.2 $\pm$ 1.80
F2	Cineole: <i>n</i> -heptane 9:1	2 weeks	99.6 $\pm$ 1.74
F3	Cineole: <i>n</i> -heptane 4:1	Over 2 weeks	98.9 $\pm$ 1.41
F4	Cineole: <i>n</i> -heptane 3:2	Over 2 weeks	99.6 $\pm$ 1.20
F5	Cineole: <i>n</i> -heptane 2:3	Over 2 weeks	98.4 $\pm$ 2.11
F6	Cineole: <i>n</i> -heptane 1:4	Over 2 weeks	98.8 $\pm$ 2.90
F7	<i>n</i> -Heptane alone	Over 2 weeks	99.1 $\pm$ 2.31

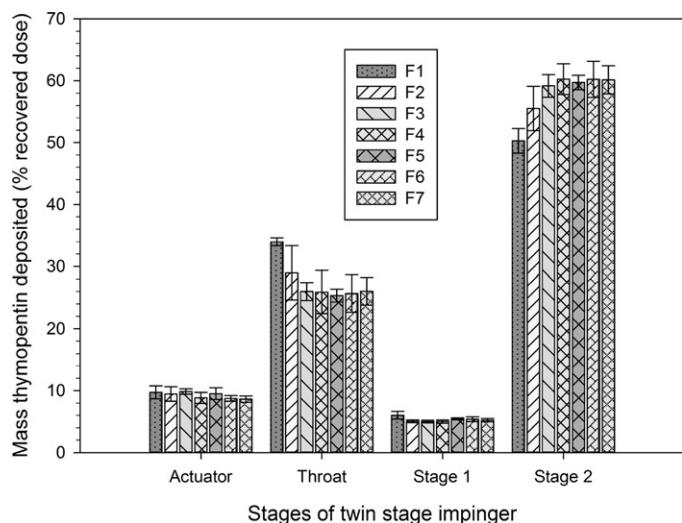
particle separation visible at the bottom of pMDI bottles, and this is in agreement with the results reported by Nyambura et al. (2009a). However, when the nanoparticles were moistened with *n*-heptane or *n*-heptane and cineole mixed at various ratios, milk-like pMDI suspensions were formed without obvious particle separation and the stability of pMDIs containing thymopentin nanoparticles was greatly prolonged to at least 2 weeks. The results demonstrated that the addition of *n*-heptane could greatly improve the physical stability of pMDI suspensions containing thymopentin nanoparticles. However, the dose of *n*-heptane used in the pMDI formulations should be limited due to its toxicity and irritating effect. Therefore, formulations F1 (as control), F2 and F3 were chosen for further investigation concerning the stability of suspension-type pMDIs during storage.

### 3.5. The relative amount of thymopentin in pMDI formulations

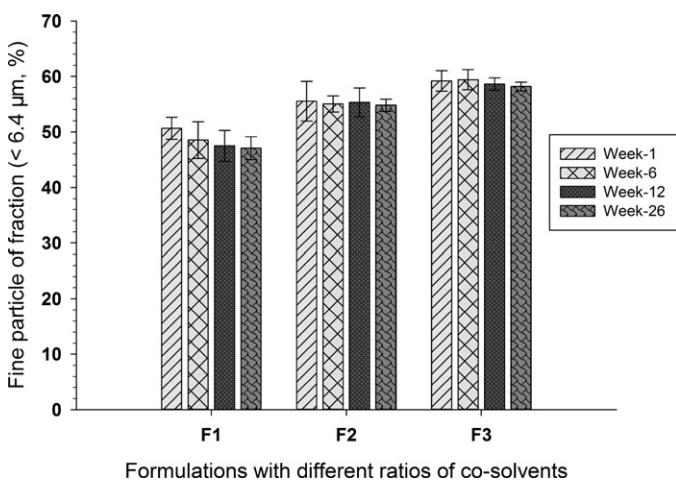
The relative amount of thymopentin in each of the pMDI formulations (Table 1) showed little difference (Student's *t*-test,  $p > 0.05$ ) between different formulations, F1–F7. The relative amount of thymopentin in each pMDI formulation was greater than 98% of the labeled amount.

### 3.6. Effect of cineole and/or *n*-heptane on aerosolization properties of pMDI formulations

Thymopentin nanoparticles were found to disperse well in pMDIs, and physical stability was maintained for at least 3 min upon shaking. Fig. 5 shows the deposition profiles following



**Fig. 5.** Effect of cineole and/or *n*-heptane on aerosolization properties of pMDI formulations (mean  $\pm$  S.D.,  $n = 3$ ).



**Fig. 6.** Fine particle fraction of pMDI formulations with different ratios of co-solvents during 26-week storage. F1, F2 and F3 represent the formulations using cineole, cineole:*n*-heptane 9:1 and cineole:*n*-heptane 4:1, respectively.

aerosolization of 10 actuations from each of the formulations into the TSI. Encouragingly, all formulations exhibited a high deposition in Stage 2 of the TSI (effective cut-off diameter 6.4  $\mu\text{m}$ ), with a minimal deposition on the pMDI actuator and Stage 1 of the TSI. All the formulations resulted in an FPF in excess of 50% of the total recovered dose, suggesting that all of these formulations would result in a high deposition in the lower respiratory tract. Fig. 5 also showed that pMDIs from formulations F2 to F7 resulted in statistically higher FPFs (55.5%, 59.2%, 60.3%, 59.7%, 60.2%, 60.1% vs. 50.6%) and lower depositions in the throat (28.9%, 26.0%, 25.9%, 25.3%, 25.7%, 26.0% vs. 34.0%) than formulation F1 (one-way ANOVA/Dunnett's test,  $p < 0.05$ ). As the concentration of *n*-heptane increased, the FPF increased. However, when the ratio of *n*-heptane to cineole increased above 2:3, there was little change in FPF observed. The results indicated that *n*-heptane added in cineol could significantly improve the aerosolization performance from pMDI formulations containing thymopentin nanoparticles.

### 3.7. Stability of pMDI formulations containing thymopentin nanoparticles during storage

Good redispersibility of pMDI formulations after shaking by hand was observed during the 26-week storage period. In details, it was observed that formulations F2 and F3 showed at least 2-week suspension stability after shaking by hand. However, formulation F1 showed approximately 2-min suspension stability under the same condition, which became shorter than the initial stability of 3 min. The results indicated the suspension stability of pMDI formulations during storage was retained by using the mixture of *n*-heptane and cineole but decreased by using cineole alone. The analytical results showed that for all pMDI samples during storage, the relative amount of thymopentin was above 97% (Table 2) and the FPF was above 47% (Fig. 6). The FPF values were well maintained during 26-week storage except for formulation F1 showing a slight decrease (Student's *t*-test,  $P > 0.05$ ). As to the relative amount of thymopentin of each formulation (Table 2), all of formulations had a slight decrease during storage without a significant difference (Student's *t*-test,  $P > 0.05$ ). These results indicated the aerosolization properties of pMDI formulations, expressed as the fine particle fraction, were well kept with time.

The particle size and size distribution of thymopentin nanoparticles in F2 and F3 samples during 26-week storage (Table 2) were almost not changed (Student's *t*-test,  $P > 0.05$ ). However, a great increase in particle size (Student's *t*-test,  $P < 0.01$ ) appeared at

0-week (when samples were just prepared) followed by a slow increase in particle size during the next weeks (Student's *t*-test,  $P < 0.01$ ) for F1 (as control). Nevertheless, the size distribution of thymopentin nanoparticles for F1 showed no significant change (Student's *t*-test,  $P > 0.05$ ).

## 4. Discussion

In this study, thymopentin nanoparticles were produced using the bottom-up process of freeze-drying a solution of thymopentin, lactose and lecithin in TBA/water co-solvent system and washing off excess lecithin in lyophilizate by centrifugalization. The results indicated that the concentration of lecithin and water content had significant effects on the size and PI of thymopentin nanoparticles produced. It was found that 20.0% (w/v) lecithin in the organic phase and 33.3% (v/v) water in TBA/water produced thymopentin nanoparticles of desired particle size, with a mean particle size of approximately 150 nm and PI of 0.1. The results demonstrated that the bottom-up process developed by this research group (Tan et al., 2011) was able to produce protein/peptide nanoparticles of desired size and size distribution. Although the optimal formulation for thymopentin nanoparticles is similar to that for lysozyme nanoparticles reported earlier (Tan et al., 2011), there are some differences such as the mean particle size of nanoparticles produced and the amount of lactose used in the formulations. These are likely due to the difference between lysozyme and thymopentin. Thymopentin is a small peptide and does not have secondary and tertiary structures. In addition, it is associated with little surface activity, which may be responsible for the smaller average diameter size observed.

Lyophilization or freezing-drying is widely used for the preparation of parenteral preparations and allows for the preservation and stabilization of thermolabile drugs and biologicals that have a limited shelf-life in solution (Telang and Suryanarayanan, 2005). Most lyophilization products are amorphous, and exhibit porous and network-like physical appearance under microscopy (Qian et al., 2008). However, in this study the bottom-up process of freeze-drying of thymopentin, lactose and lecithin in TBA/water followed by centrifugation to remove free lecithin produced thymopentin nanoparticles of approximately 150 nm in diameter.

Lecithin was added in TBA/water as a surfactant to protect peptides from degradation during lyophilization due to its low phase-change temperatures. Lecithin plays an important role as it coats on the surface of the internal phase during freeze-drying and provides a steric hindrance to reduce the possibility of coalescence of thymopentin nanoparticles formed. Snap-freezing using liquid nitrogen was one of the critical steps that could have caused molecular structure of thymopentin damage. When a solution of insulin was frozen, it suffered from the increased concentrations of both insulin and other additives from the formulation that could have led to the aggregation of insulin (Eckhardt et al., 1991). It was also reported that nucleation and crystallization of ice crystals formed during freezing may disrupt insulin molecules (Koseki et al., 1990). However, in this study excipients such as lactose and lecithin that play the roles of cryoprotectant and lyoprotectant were concentrated but not crystallized, and therefore, protected thymopentin from being damaged.

Stable pMDI suspensions were achieved with the aid of cineole and *n*-heptane in the present study. The mechanism by which cineole and *n*-heptane stabilized the pMDI suspension was not investigated. However, Nyambura et al. (2009a) hypothesized that cineole molecules have surface-active properties that enable them to stabilize a colloidal suspension, in which the non-polar end of these molecules orientates towards HFA 134a while their polar end orientates towards the nanoparticle. This is very likely in the case when thymopentin nanoparticles are made of hydrophilic

**Table 2**Stability of thymopentin nanoparticles in pMDI samples during 26-week storage (mean  $\pm$  S.D.,  $n=3$ ).

Formulation	Relative amount of thymopentin (%), Z-ave (nm) and PI			
	Week 0	Week 6	Week 12	Week 26
F1	99.2 $\pm$ 1.80 <sup>a</sup>	98.7 $\pm$ 1.62	98.1 $\pm$ 1.29	97.9 $\pm$ 2.20
	378.3 $\pm$ 15.1 <sup>b</sup>	435.3 $\pm$ 24.2	479.3 $\pm$ 43.14	488.3 $\pm$ 36.76 <sup>##</sup>
	0.29 $\pm$ 0.049 <sup>c</sup>	0.31 $\pm$ 0.050	0.29 $\pm$ 0.062	0.32 $\pm$ 0.057
F2	99.6 $\pm$ 1.74	98.5 $\pm$ 1.74	98.1 $\pm$ 2.05	97.7 $\pm$ 2.66
	156.4 $\pm$ 2.6 <sup>**</sup>	155.3 $\pm$ 1.9 <sup>**</sup>	159.3 $\pm$ 2.27 <sup>**</sup>	164.9 $\pm$ 3.86 <sup>**</sup>
	0.11 $\pm$ 0.010 <sup>***</sup>	0.12 $\pm$ 0.013 <sup>***</sup>	0.11 $\pm$ 0.023 <sup>***</sup>	0.11 $\pm$ 0.033 <sup>**</sup>
F3	98.9 $\pm$ 1.41	98.3 $\pm$ 1.01	98.0 $\pm$ 2.62	97.9 $\pm$ 1.89
	157.3 $\pm$ 1.3 <sup>***</sup>	162.7 $\pm$ 5.93 <sup>**</sup>	165.7 $\pm$ 5.68 <sup>**</sup>	168.3 $\pm$ 3.26 <sup>**</sup>
	0.10 $\pm$ 0.003 <sup>***</sup>	0.11 $\pm$ 0.042 <sup>**</sup>	0.12 $\pm$ 0.036 <sup>***</sup>	0.12 $\pm$ 0.041 <sup>**</sup>

Note: a, b and c are presented the data of relative amount of thymopentin, Z-ave and PI in 0-week for F1, respectively. The other data were displayed as the same order. \*\* $P < 0.05$ , \*\*\* $P < 0.01$  vs. F1 at the corresponding time point. ##  $P < 0.05$  vs. 0-week for F1.

materials while HFA 134a is hydrophobic. This is in agreement with the co-surfactant properties of aldehydes and ketones reported by Meziani et al. (2000, 1997). Furthermore, *n*-heptane was also used as a stabilizer to stabilize the pMDI suspensions containing thymopentin nanoparticles in the present study. *n*-Heptane may change the polarity of the medium of suspension system resulting in more stabilized nanoparticles in suspension-type pMDIs. Interestingly, even when a very small fraction of *n*-heptane was used in combination with cineole, the stability of thymopentin pMDI suspension was prolonged for at least 2 weeks and maintained during storage. However, although *n*-heptane is less toxic than *n*-hexane (Takeuchi et al., 1980, 1981), it is still an organic solvent with irritating properties and other problems. Therefore, only approximately 0.7% (w/w) *n*-heptane in HFA 134a propellants (i.e. F3) was used in the present study opposed to 5% (w/w) *n*-hexane as reported by Dickinson et al. (2001).

The results on the aerosolization of pMDIs from the initial 2 days to the end of 26-week storage showed that stabilizer dosage of 500  $\mu$ L per 40 mg of thymopentin nanoparticles was effective. It was found that the FPF increased from 50.6% (w/w) to 60.3% as the ratio of *n*-heptane to cineole increased from zero to 2:3. When the ratio of *n*-heptane to cineole exceeded 2:3, the FPF of formulations reached to a plateau of 60% (w/w) with little change as further increase in *n*-heptane. A typical pMDI formulation results in 10–20% peripheral lung deposition (Anderson, 2001). It should be noted that the pMDI formulations developed in this study showed approximately 50% (w/w) deposition in the area corresponding to the peripheral lung. The FPF of all the pMDI formulations was over 55% during the 26-week storage except the pMDI formulation using cineole alone as a stabilizer, which showed a decrease from 50 to 47% (w/w). Nevertheless, an FPF of 47% is still reasonable when compared to commercially available inhalation products. The present study demonstrated that a mixture of cineole and *n*-heptane was able to better stabilize the suspension-type pMDIs during storage.

It is necessary to use appropriate suspending agents to dispense nanoparticles into nanosuspensions. In theory, nanoparticles possess an excellent geometric size that could positively influence the aerodynamic diameter of a pulmonary drug delivery system so that pMDI formulation containing drug nanoparticles could improve aerosolization performance of drug (Nyambura et al., 2009b). However, aerosolization performance of the formulation will be compromised following separation, flocculation, coalescence or sedimentation after the nanoparticles are blended with the propellant HFA 134a to form suspension-type pMDIs. Therefore, nanoparticles should be dispersed uniformly in HFA 134a to form a nanosuspension pMDI to achieve an ideal drug deposition in the peripheral lung. In the present study, a milk-like suspension was formed, and it remained stable during storage when the

combination of cineole and *n*-heptane was used in pMDI formulations containing thymopentin nanoparticles. It was found that the thymopentin nanoparticles were well dispersed in HFA 134a forming a nanosuspension with the aid of cineole and/or *n*-heptane as stabilizers. The FPF of pMDI formulations using cineole alone was lower than that of the other two formulations in the initial 2 days and during the storage. It is likely that cineole alone as a stabilizer could not completely prevent thymopentin nanoparticles from aggregation in HFA 134a, and thus there may be a slow aggregation of the nanoparticles, which may lead to a decrease in the suspension stability and deposition efficiency during storage. Although the aerosolization performance, physical and chemical stability of pMDI formulations using cineole alone are satisfied to deliver thymopentin to deposit in the peripheral lung in the present study, it is still necessary to choose appropriate stabilizers to disperse the nanoparticles in HFA 134a to form nanosuspension and maintain its stability in order to achieve a higher deposition efficiency.

## 5. Conclusion

In this study, the bottom-up process of freeze-drying a solution of TBA/water co-solvent system was used to produce stable thymopentin nanoparticles with desired size and size distribution appropriate for peripheral lung deposition. The nanoparticles were of spherical shape and were dispersed in HFA 134a with the help of cineole and *n*-heptane forming stable pMDI suspensions. The aerosolization properties and storage stability of thymopentin nanoparticles of pMDIs indicated that the nanoparticles were largely deposited to areas corresponding to lower respiratory tract suggesting a good lung delivery, and the pMDI formulations maintained stability for 6 months. The pMDI formulations containing thymopentin nanoparticles using cineole alone or together with *n*-heptane as stabilizers were stable and suitable to deliver peptide therapeutics via inhalation for systemic action. However, more work is needed to find safer stabilizers to stabilize pMDI formulations containing protein nanoparticles for pulmonary drug delivery.

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