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# Pulmonary delivery of Nanocomposite Microparticles (NCMPs) incorporating miR-146a for treatment of COPD



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## Abstract

The treatment and management of COPD by inhalation to the lungs has emerged as an attractive alternative route to oral dosing due to higher concentrations of the drug being administered to site of action. In this study, Nanocomposite Microparticles (NCMPs) of microRNA (miR-146a) containing PGA-co-PDL nanoparticles (NPs) for dry powder inhalation were formulated using L-leucine and mannitol. The spray-drying (Buchi B290) process was optimised and used to incorporate NPs into NCMPs using mix of L-leucine and mannitol excipients in different ratios (F1; 100:0% w/w, F2; 75:25% w/w, F3; 50:50% w/w, F4; 25:75% w/w, F5; 0:100% w/w) to investigate yield %, moisture content, aerosolisation performance and miR-146a biological activity. The optimum condition was performed at feed rate 0.5 ml/min, aspirator rate 28 m<sup>3</sup>/h, atomizing air flow rate 480 L/h, and inlet drying temperature 70 °C which produced highest yield percentage and closest recovered NPs size to original prior spray-drying. The optimum formulation (F4) had a high yield (86.0 ± 15.01%), recovered NPs size after spray-drying 409.7 ± 10.05 nm (initial NPs size 244.8 ± 4.40 nm) and low moisture content (2.02 ± 0.03%). The aerosolisation performance showed high Fine Particle Fraction (FPF) 51.33 ± 2.9%, Emitted Dose (ED) of 81.81 ± 3.0%, and the mass median aerodynamic diameter (MMAD) was ≤ 5 μm suggesting a deposition in the respirable region of the lungs. The biological activity of miR-146a was preserved after spray-drying process and

miR-146a loaded NCMPs produced target genes *IRAK1* and *TRAF6* silencing. These results indicate the optimal process parameters for the preparation of NCMPs of miR-146a-containing PGA-co-PDL NPs suitable for inhalation in the treatment and management of COPD.

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**Keywords** :Nanoparticles; Nanocomposite Microparticles (NCMPs); MicroRNA (miRNA); Chronic obstruction pulmonary disease (COPD); Spray drying; Dry powder inhalation

## 1 Introduction

Chronic obstruction pulmonary disease (COPD) is a heterogeneous inflammatory disease characterised by airflow limitation, narrowing of the small airways and destruction of alveoli walls, which is considered a hallmark of emphysema (Cosio et al., 2009). An additional feature is chronic bronchitis which is associated with mucus and inflammation of the airways (Cosio et al., 2009). The treatment of COPD by inhalation to the lungs has emerged as an attractive alternative route to oral dosing due to higher concentrations of the drug being administered to site of action, which avoids degradation, by the strong acids and enzymes associated with the gastrointestinal tract.

MicroRNA (miRNA)-based therapeutics have emerged as a tractable approach for clinical intervention in respiratory diseases (Ebrahimi and Sadroddiny, 2015; Fujita et al., 2013; Kishore et al., 2014). The ability of miR-146a to downregulate the interleukin 1 receptor (IL-1R) and Toll-like receptor (TLR) signalling components IL-1 receptor-associated kinase (IRAK1) and tumour necrosis factor (TNF) receptor-associated factor (TRAF6) supports negative feedback regulation of IL-1 $\beta$ , IL-6 and IL-8 (Taganov et al., 2006). However, the delivery of miRNA to site of action considers one of the main concerns of developments of miRNA therapeutics. The physicochemical properties, such as hydrophilicity and negative charge, make it difficult for these molecules to cross biological barriers (Yin et al., 2014).

During the last few years, there has been a growing interest in nanotechnology for pulmonary drug delivery. The nanoparticles (NPs) have the ability to interact with intracellular and extracellular components of cells loaded with therapeutic agents for systemic and local delivery to treat diseases (Borm et al., 2006; Labiris and Dolovich, 2003; Merchant et al., 2016; Osman et al., 2018; Papay et al., 2017; Petkar et al., 2018; Rodrigues et al., 2018; Saleem et al., 2017). Therefore, using NPs is an interesting delivery strategy for small nucleic acids to treat respiratory diseases. However, NPs in the dry powder form do not deposit efficiently in the deep lungs, due to their low inertia and size (Kunda et al., 2013; Sung et al., 2007). Furthermore, the ideal particle size for optimal particle deposition in the deep lung ranges between 1 and 5  $\mu\text{m}$  in diameter (Sakagami, 2006). Therefore, NPs can be incorporated into dry powder microparticles (NCMPs) of aerodynamic particle size 1–5  $\mu\text{m}$  through spray-drying (Alfagih et al., 2015; Kunda et al., 2015a).

Spray-drying is a one step process that converts liquid emulsion solution or suspension to dry powder, and is controlled by different parameters to provide desirable physical characteristics of powders and aerosolisation properties, including the morphology, moisture content, particle size and density (Sakagami, 2006; Saleem et al., 2017; Ungaro et al., 2012). Moreover, excipients such as sugars and amino acids can be added to the spray dried liquid formulation to enhance aerosolisation properties powders (Bosquillon et al., 2001; Tawfeek et al.,

2013). The selection of appropriate excipients for inhalation leads to optimal and functional dry powder formulation and can help preserve NPs and nucleic acid integrity. If NPs aggregate and become very large i.e 500 nm, this can impact on diffusing through lung lining fluid and uptake by cells hence reducing miRNA uptake into cells (Alfagih et al., 2015).

L- Leucine is one of the amino acids that has been used as a dispersing agent that improves aerosolisation properties for dry powders, reduces contact cohesion between the particles and prevents aggregation (Tawfeek et al., 2011). Sugar excipients such as mannitol are commonly used in spray-drying with good safety characteristics, are less hygroscopic than lactose and they have European approval for dry powder inhalation (Burness and Keating, 2012; Jensen et al., 2010; Pilcer and Amighi, 2010).

Similarly, these excipients can protect, and stabilize the NPs and encapsulated materials during spray-drying process including operational high temperature and mechanical stress (Alfagih et al., 2015; Kunda et al., 2015a,b; Rodrigues et al., 2018; Tawfeek et al., 2011). In addition, excipients added to the spray-dried formulations induce the production of a desirable aerodynamic particle size 1–5 µm and release of the active ingredients after they disperse in the lung lining fluid (Jensen et al., 2010; Saluja et al., 2010). Taking into account the advantage of the promising biological effects of inhaled siRNA in the lung, there have been limited work exploring dry powder inhalation of miRNA via spray-drying. In this study, L-leucine and mannitol were mixed together as dispersion enhancer and protective excipients. To our knowledge this is the first time that miRNA was formulated into inhalable dry powder NCMPs using a mix of amino acid and sugar excipient. We optimised the production of NCMPs of miR-146a-containing PGA-co-PDL NPs for dry powder inhalation in terms of size, morphology, aerosol performance, moisture content and miRNA functionality.

## 2 Materials and methods

### 2.1 Reagents

Novozyme 435 (a lipase from *Candida antarctica* immobilized on a microporous acrylic resin) was purchased from Biocatalytics, USA and DOTAP was purchased from Avanti Polar lipids, Alabaster, AL, USA. Solvents were purchased from Fischer chemicals (Fischer Scientific, UK). Poly (vinyl alcohol) PVA, Mw of 13–23 kDa 87–89% hydrolysed, RPMI-1640 medium with L-glutamine, L-leucine, D-Mannitol and RNase-free diethyl pyrocarbonate (DEPC) water were purchased from Sigma Aldrich, UK. Human adenocarcinomic alveolar basal epithelial cell line, A549, was purchased from ATCC. A synthetic miR-146a mimic with a FAM-label on the sense 5' FAM-CCGGGCAAUUCAGUUUCUACA-dTdT-3', was purchased from Eurogentec, UK with the sequence: sense 5' FAM-CCGGGCAAUUCAGUUUCUACA-dTdT-3', antisense 5' dTdT-GGCCCCGUUAAGUCAAGAUGU-3'.

### 2.2 Nanoparticle preparation and miRNA adsorption

PGA-co-PDL NPs were prepared using an oil in water (o/w) single emulsion method, as previously described (Kunda et al., 2014) with modification. DOTAP 15% w/w was added to the organic phase to prepare cationic NPs. The miR-146a mimic was added to 1 ml solution of RNase free water containing NPs (to obtain a final NP:miRNA weight ratio of 250:1) and mixed using a HulaMixer Sample Mixer (Life Technologies, UK) at

20rpm and 25 °C at 2h. After adsorption, RNase free water was added to a total volume of 4ml prior to separation of free miR-146a from the adsorbed miR-146a by ultracentrifugation at 35000 ×g, for 40 min at 4 °C using an Optima L-80 Ultracentrifuge (Beckman, UK).

### 2.3 Particle size and miRNA adsorption characterisation

The mean particle size and polydispersity index (PDI) of the NPs were analysed by dynamic laser scattering using a Zetasizer Nano ZS, Malvern Instruments Ltd, UK. The NPs (10mg) were diluted with 4ml distilled water and 1 ml of the diluted sample was placed into a measuring cuvette. The concentration of adsorbed miR-146a was determined indirectly from the difference in miR-146a concentration before and after loading by UV absorbance at 260nm using a NanoDrop 2000C (Thermo Fisher Scientific, and USA).

### 2.4 Preparation of Nanocomposite Microparticles

The Nanocomposite Microparticles (NCMPs) were prepared by spray-drying the NPs suspension from different aqueous solutions containing L-leucine and mannitol in various ratios (F1; 100:0% w/w, F2; 75:25% w/w, F3; 50:50% w/w, F4; 25:75% w/w, F5; 0:100% w/w), with a NPs to L-leucine and mannitol (NCMPs) ratio of 1:1.5 w/w ([Alfagih et al., 2015](#)) using a Büchi B- 290 mini spray-dryer (Büchi Labortechnik, Flawil, Switzerland) containing a standard two-fluid nozzle (0.7mm diameter) applying the following spray-drying conditions; feed rate 0.5ml/min, aspirator rate 28m<sup>3</sup>/h, atomizing air flow rate 480 L/h, inlet drying temperature 70 °C (corresponding outlet temperature of approximately 47 °C). Dry particles were separated from the airstream using a high-performance cyclone (Büchi Labortechnik), collected and stored in desiccator at room temperature until further use.

### 2.5 Characterisation of Nanocomposite Microparticles

#### 2.5.1 Yield, Morphology, particle size and moisture content

The dry powder yield was determined as the percentage mass of expected powder (n=3) according to following equation (Eq. (1)):

$$\text{Yield\%} = \frac{\text{weight of powder collected after spray drying}}{\text{weight of total dry mass used for the preparation}} \times 100$$

(1)

The spray-dried powder was examined using a scanning electron microscope (SEM), Quanta 450, FEI, Oregon, USA). The spray-dried samples were mounted on an aluminium stub with adhesive, coated with gold (40–60nm) and then observed at high vacuum. The NPs recovery from NCMPs (2 mg) was determined by re-dispersing in 4ml water and vortexed for 20s at 100rpm to release the recovered NPs, 1 ml of the diluted sample was loaded into a measuring cuvette, and the measurements were recorded at 25 °C. The mean particle size and polydispersity index (PDI) of the NPs were analysed by dynamic laser scattering using a Zetasizer Nano ZS, Malvern Instruments Ltd, UK. The water content of the NCMPs powder was determined by thermogravimetric analysis (TGA), using a Linseis STA PT 1750 Model Thermo Analyzer system, Germany.

NCMPs powder (10mg) was heated between 25 and 650 °C at constant rate 10 °C/min in nitrogen gas. The weight loss (%) due to water evaporation, was recorded between 25 and 120 °C.

### 2.5.2 Powder density and aerodynamic diameter

The Tapped density of the NCMPs was determined by adding approximately 0.2 g of powder to a 5 ml measuring cylinder (Alfagih et al., 2015). The initial bulk volume ( $V_0$ ) was recorded and then again following mechanical tapping ten times ( $V_{10}$ ), then five hundred times ( $V_{500}$ ), then after one thousand and two hundred fifty ( $V_{1250}$ ) taps until no reduction in the particle volume was noticed. The theoretical aerodynamic diameter ( $d_{ae}$ ) was determined based on the data obtained from geometric particle size ( $d$ ) and tapped density ( $p$ ) according to Eq. (2).  $p_1$  is the unit density (1 g/cm<sup>3</sup>)

$$d_{ae} = d \sqrt{\frac{p}{p_1}} \quad (2)$$

### 2.5.3 In vitro aerosolisation studies

The aerosol performance of the NCMPs was evaluated using a next generation impactor (NGI). The spray-dried powder (10mg) was manually loaded into four hydroxypropyl methylcellulose HPMC size 3 capsules (Qualicaps, Japan). The capsules were pierced using a 2-pin Cyclohaler® (Teva pharma) and aerosolised into the NGI that was connected to a pump (Copley Scientific, UK). The airflow was measured and adjusted prior to experiment using a flow meter (Copley Scientific, UK). The flow rate was operated at 60 L/min for 4 s. The plates were coated with polyethylene glycerol (PEG-200) to decrease powder bounce (Edwards et al., 1998). The amount of particles deposited in each stage of the impactor was evaluated gravimetrically by measuring the difference in mass before and after powder deposition (Meenach et al., 2013a; Meenach et al., 2013b). The fine particle dose (FPD), fine particle fraction (FPF), respirable fraction (RF), and emitted dose (ED) were calculated according to Eqs. (3)–(6);

$$\text{Fine particle dose (FPD, mg)} = \text{mass of the drug deposited} \leq 4.46 \mu\text{m} \quad (3)$$

$$\text{Fine particle fraction (FPF\%)} = \frac{\text{Fine particle dose}}{\text{initial particle mass loaded into capsules}} \times 100 \quad (4)$$

$$\text{Respirable fraction (RF\%)} = \frac{\text{Mass of powder} < 4.46 \mu\text{m (stages 2 through 7)}}{\text{Total particle mass on all stages}} \times 100 \quad (5)$$

$$\text{Emitteddose(ED\%)} = \frac{\text{Initial mass in capsules} - \text{final mass remaining in capsules}}{\text{initial mass in capsules}} \times 100$$

(6)

The FPD is defined as the mass of the drug deposited in the NGI with  $d_{ae} \leq 4.46 \mu\text{m}$ , FPF % is the fraction of emitted dose deposited in the NGI with  $d_{ae} < 4.46 \mu\text{m}$ , RF % is the mass of powder  $\leq 4.46 \mu\text{m}$  and ED is the amount of powder exiting the inhaler. The mass mean aerodynamic diameter (MMAD,  $\mu\text{m}$ ) and geometric standard deviation (GSD,  $\mu\text{m}$ ) were calculated from log probability analysis (<http://www.mmadcalculator.com/>).

## 2.6 Semi-quantitative reverse transcriptase PCR

A549 cells ( $3.8 \times 10^5$  cells per well) were seeded on a 6 well plate. After 24 h growth, the cells were incubated in serum free medium with miR-146a-NCMPs for 1 h. The miR-146a- NCMPs mixture was then replaced with complete medium and the cells incubated for 24 h. Total RNA was extracted using the RNeasy Mini kit according to the manufacturer's instructions. Reverse transcription for cDNA generation was performed on 200 ng RNA using miScript reagents (Qiagen, Manchester, UK). Levels of *IRAK1* transcripts, were assessed using RT<sup>2</sup> qPCR Primer Assays in 20  $\mu\text{l}$  reactions composed of 10  $\mu\text{l}$  SYBR Green PCR master mix, 2  $\mu\text{l}$  primers, 2  $\mu\text{l}$  diluted cDNA and water to 20  $\mu\text{l}$ . The reactions were amplified for three-step method. Expression was normalised to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001), SYBR Green PCR reagents and PCR primers were purchased from Qiagen (Manchester, UK).

## 2.7 Statistical analysis

All statistical analysis were performed using Minitab® 16 Statistical Software. One-way analysis of variance (ANOVA) with the Tukey's comparison was employed for comparing the formulations with each other. Statistically significant differences were assumed when  $p < 0.05$ . All values were expressed as the mean  $\pm$  standard deviation.

# 3 Results and discussion

## 3.1 Spray-drying optimization, moisture content, recovered particle size and yield


The spray-drying optimisation process was utilised to incorporate NPs into NCMPs using different ratios of L-leucine and mannitol excipients. Spray-drying parameters used showed, that outlet temperature  $47^\circ\text{C}$  was below melting point of PGA-co-PDL NPs (Tawfeek et al., 2011, 2017). Monitoring and maintaining a low outlet temperature can reduce agglomeration, and reduced the risk of small nucleic acid denaturation (Mohajel et al., 2012).

Water content for formulations (F1–F5) were within the range of moisture content ( $2.02 \pm 0.03\%$ – $5.1 \pm 0.37\%$ ) of spray-dried particles intended for lung deposition as reported by others (Chew et al., 2005a; Ståhl et al., 2002), (Table 1). However, F4 (the optimum ratio of L-leucine to mannitol F4; 25:75% w/w) had the lowest moisture content, due to a higher percentage of mannitol (Jensen et al., 2012), there was a time to the particles



to be in spray drying chambers hence particles dry and reduced cohesion between particles resulting in increased powder respirability (Fig. 1) (Chew and Chan, 2002). Mannitol has hygroscopic properties enabling the formation of hydrogen bonds with water molecules causing water replacement, which stabilises the formulation (Chow et al., 2017; Clegg et al., 1982; Li et al., 2016; Sarmiento et al., 2006; Schüle et al., 2008). A study by Rohani *et al.* demonstrated that mannitol used in a combination with amino acid (L-alanine), found that the formulation containing mannitol and amino acid had a moisture content range between 4 and 6% (Rohani et al., 2014), which was similar to our results  $2.02 \pm 0.03$ – $5.1 \pm 0.37\%$  (Table 1).

Table 1

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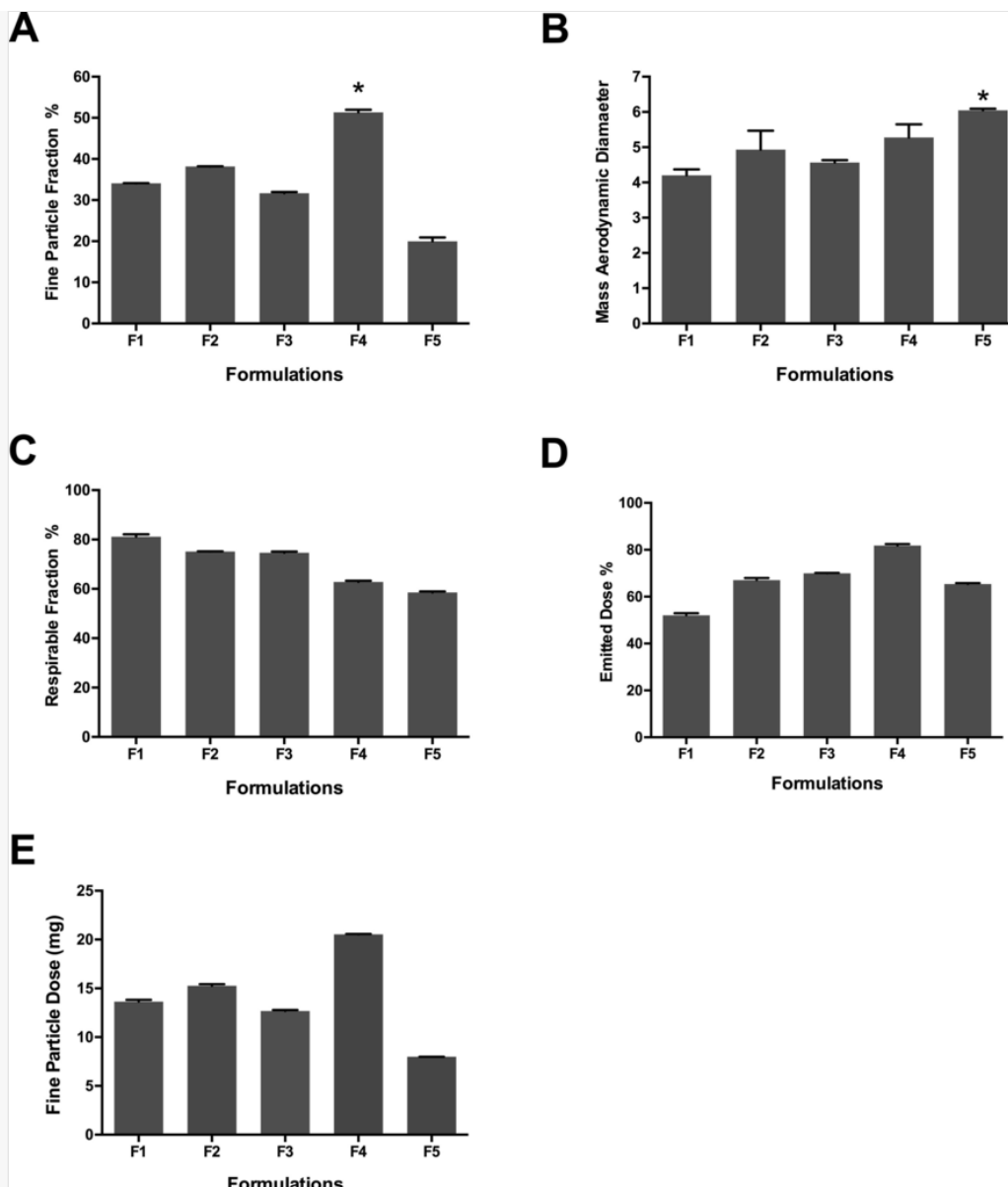
Physical properties of spray-dried nanocomposite microparticles. Mean  $\pm$  SD (n=3).

Formulations	Yield %	NPs recovery size (nm)	Tapped density (g/cm <sup>3</sup> )	Water content (%)
F1	56.0 $\pm$ 11.30	958.3 $\pm$ 21	(–)	5.10 $\pm$ 0.37
F2	79.2 $\pm$ 10.10	1810.66 $\pm$ 18	0.17 $\pm$ 0.01	4.91 $\pm$ 0.20
F3	95.6 $\pm$ 2.80	3252.06 $\pm$ 26	0.13 $\pm$ 0.20	3.80 $\pm$ 0.90
<b>F4</b>	<b>86.0 <math>\pm</math> 15.01</b>	<b>409.7 <math>\pm</math> 10.05</b>	<b>0.14 <math>\pm</math> 0.01</b>	<b>2.02 <math>\pm</math> 0.03</b>
F5	84.0 $\pm$ 2.20	2174.6 $\pm$ 13	0.20 $\pm$ 0.05	3.77 $\pm$ 0.11

Note: The size of PGA-co-PDL miR-146a NPs prior to spray-drying was  $244.80 \pm 4.40$  nm.

Fig. 1





(A) The fine particle fraction (%) spray dried powder (B) mass mean aerodynamic diameter ( $\mu\text{m}$ ) (C) Respirable fraction (%) (D) Emitted dose (%) (E) Fine Particle Dose ( $\mu\text{g}$ ). Data represent mean  $\pm$  SD ( $n=3$ ), (\* $p<0.05$ , ANOVA/Tukey's).

It was found that operational parameters such as feed rate, aspirator rate, atomised air flow, spray-drying inlet and outlet temperature had a significant impact on recovered particle size and yield of dry powders (Alfagih et al., 2015). A similar observation was noted in spray-drying condition inlet  $70^\circ\text{C}$  and outlet  $47^\circ\text{C}$  temperature. It was found that the inlet temperature had affected (increased/decreased) the recovered NPs particle size and yield. This was due to a faster rate of drying resulting in decreased particle aggregation (Mohajel et al., 2012) and residual moisture resulting in less particles sticking to the spray-drying chamber walls (Billon et al., 2000). However, high inlet temperature may affect the small nucleic acids stability (Mohajel et al., 2012).

When mannitol concentration was up to 50%, the yield varies from  $56.0 \pm 11.30\%$  (F1) to  $95.6 \pm 2.80\%$  (F3), whereas yield decreased to  $86.0 \pm 15.01$  (F4) with 75% to 100% mannitol. Despite this variation in yield percentage, the NCMPs reported here have high yield values, which indicate the significant potential of using a mixture of mannitol and L-leucine. The values are much higher than the yield values reported in previous

studies from 40 to 50% (Alfagih et al., 2015; Kunda et al., 2015a; Tawfeek et al., 2011). Formulation F4 produced the highest yield % of NCMPs  $86.0 \pm 15.01\%$  (Table 1) (Jensen et al., 2010). Moreover, the increase of mannitol concentration lead to an increase in particle size from  $244.80 \pm 4.40$  nm before spray-drying to  $409.7 \pm 10.05$  nm (Table 1) after spray-drying. F1-F3 showed large particle size recovery after spray drying ranges from  $958.3 \pm 21$  to  $3252.06 \pm 26$  nm, this large size particle was due to the mannitol crystallinity profile in the formulations and presence the different contents of mannitol and leucine in the formulations can affect the particle size (Pourshahab et al., 2011; Sou et al., 2011). As mentioned in the literature, low amount of mannitol or mannitol alone cause particle aggregation, therefore increase in particle size, whereas formulation containing more quantity of mannitol could prevent crystallisation of mannitol after spray drying (Lee et al., 2011; Sou et al., 2011). However, F4 when re-dispersed, the NPs size returned to particle size before spray drying. Moreover, this particle size increase after spray-drying may be a result of particle cohesiveness due to Van der Waals force, capillary force, combination of leucine and mannitol and particles aggregation under operating conditions of spray-drying (Beck-Broichsitter et al., 2012; Chew and Chan, 1999; Chow et al., 2017; Sosnik and Seremeta, 2015). The high yield of F4 was associated with reduced loss of dry powder during the collection process which relies on centrifugal forces for collection of final dry powder due effective separation capacity of cyclone (Sosnik and Seremeta, 2015). Furthermore, the high yield was also associated with higher percentage of mannitol which has hygroscopic properties and stabilise the formulation as mentioned above due to the formation of hydrogen bonds with water molecules causing water replacement (Schüle et al., 2008). Hence, F4 was selected as the optimum formulation according to the high yield and lower moisture content and taken forward for target gene and protein expression, based on results of tapped density and aerosolisation data (Section 3.1 and 3.2).

### 3.2 *In vitro* aerosolisation studies

The *in vitro* aerosol dispersion properties of NCMPs were determined using NGI. The mass median aerodynamic diameters (MMAD) ranged from  $4.20 \pm 0.15$  to  $6.03 \pm 1.08$   $\mu\text{m}$ . The NCMPs' formulations (F1-F4) showed that MMAD was less than  $\leq 5$   $\mu\text{m}$  with F5 significantly greater at  $6.03 \pm 1.08$   $\mu\text{m}$  ( $p < 0.05$ , ANOVA/Tukey's) (Fig. 1B). The corresponding GSD values between formulations were approximately similar in size from  $1.75 \pm 0.31$ – $2.15 \pm 0.53$   $\mu\text{m}$ . The difference suggests the spray-dried excipients influence MMAD, with F1–F4 possessing less mannitol and more L-leucine than F5. A study by Jensen *et al.* showed that spray-dried NPs with high concentration mannitol resulted in larger MMAD size compared with spray-dried NPs with lower mannitol concentration (Jensen et al., 2010). Another study by Chow *et al.* showed high amount of L-leucine with low amounts of mannitol led to the reduction of the MMAD size (Chow et al., 2017). Despite the NCMPs' formulations (F2-F4) containing different L-leucine and mannitol amounts, which affected and decreased MMAD values from  $5.28 \pm 0.71$   $\mu\text{m}$  (F4) to  $4.93 \pm 0.49$   $\mu\text{m}$  (F2) (Fig. 1B). Similar results were obtained by Chow *et al.* when various amounts of L-Leucine were used with mannitol in inhaled powder formulation, and they found the high amount of L-leucine in the formulation with low amount of mannitol led to reduction of the MMAD values (Chow et al., 2017). The amount of mannitol in F5 and absence of L-leucine may form aggregated powders which contributes to higher MMAD and a decrease in FPF (Fig. 1A) (Chow et al., 2017; Kaialy and Nokhodchi, 2013). This may be related to the surface activity of

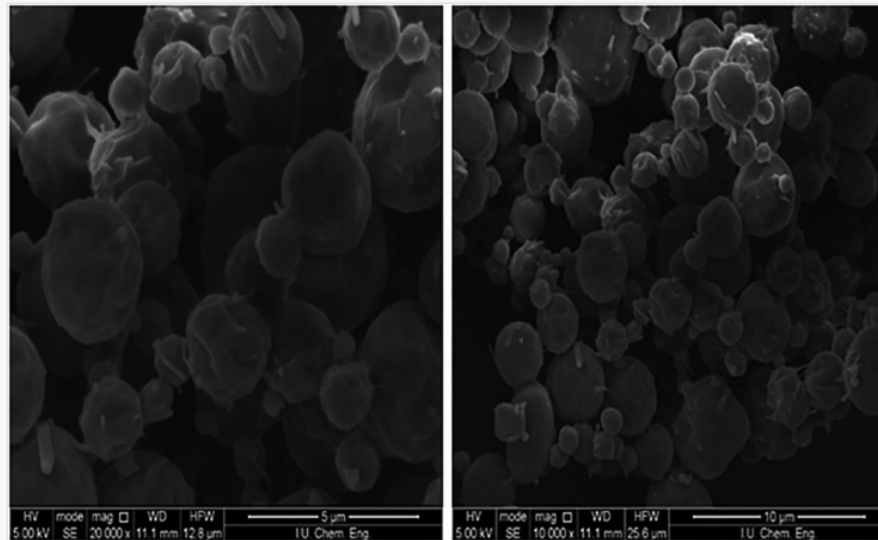
hydroxyl group of mannitol, which interacts with certain amine groups and effect the particle's surface (Bharate et al., 2016).

FPF % showed that F4 produced significantly higher FPF % ( $51.33 \pm 2.90\%$ ) compared to the other formulations ( $p < 0.05$ , ANOVA/Tukey's) (Lucas et al., 1999; Sou et al., 2013; Tawfeek et al., 2011). The ED values were all over 50% with highest value for F4  $81.81 \pm 3.0\%$  (Fig. 1D). Adding L-leucine to spray-dried powders reduces the forces of attraction due to less contact points between particles forming rough particle surface and lower spray-dried powder aggregation, which causes greater dispersibility (Alfagih et al., 2015; Kunda et al., 2015a; Tawfeek et al., 2011). Furthermore, the L-leucine amount in the spray-dried powder had an effect on aerosolisation performance. A study by Sou et al. showed that L-leucine enhanced aerosolisation performance of mannitol formulations (Sou et al., 2011). The incorporation of L-leucine into F4 increased the FPF % and FPD. F4 had 25% L-leucine, which resulted in more than double FPF %,  $51.33 \pm 2.9\%$  and FPD,  $20.53 \pm 2.90$  mg respectively compared to F5, 0% leucine with FPF %,  $19.96 \pm 1.2\%$  and FPD  $7.98 \pm 1.20$  mg (Fig. 1A and E). Similar results were obtained by Chow et al. which found that the absence of L-leucine and high amount of mannitol in the formulation decreased FPF % (Chow et al., 2017). This difference was due to less powder stickiness and cohesiveness, as indicated in a study conducted by Gervelas et al. in which leucine was added to spray-dried powder (Gervelas et al., 2007).

The FPF, FPD, ED and MMAD values obtained from F4 would suggest very good aerosolisation properties and a deep lung deposition profile which is in agreement with other studies that used PGA-co-PDL NPs-NCMPs (Kunda et al., 2015b). Hence, NCMPs when inhaled, L-leucine and mannitol will dissolve the lung lining fluid, subsequently releasing miR-146a-NPs to be taken up by lung cells and cause the required biological expression (Alfagih et al., 2015; Kunda et al., 2015b).

### 3.3 Morphology, powder density and aerodynamic diameter of formulation F4

The tapped density of PGA-co-PDL NCMPs F4 was  $0.14 \pm 0.01 \text{ g cm}^{-3}$  (Table 1) and theoretical dynamic diameter ( $d_{ae}$ ) was  $0.63 \pm 0.01 \mu\text{m}$ . The formulated NCMPs (F4) were analysed for morphology using SEM (Fig. 2) and micrographs showed that NCMPs possessed a spherical shape and corrugated surface, which suggested this shape was due to the reduction in the cohesion between particles (Chew and Chan, 2001; Chew et al., 2005b). This occurred due to water evaporation that happened during the spray-drying process causing high vapour pressure. In addition, the presence of L-leucine in spray-dried particles, which has low density was capable of forming a shell that encapsulate the particles (Alfagih et al., 2015; Lucas et al., 1999; Sou et al., 2013). These corrugated surface particles would have a larger surface area, leading to an increase in the particles' capability to disperse in lung fluid thus releasing miR-146a-NPs. The aerosolisation properties with mannitol has been shown to be better than lactose, because of the corrugated spray-dried particles produced have less contact points and van der Waal forces, thus it is easier to disperse (Bharate et al., 2016; Vehring, 2008). Furthermore, mannitol was also shown to be the best in formulation, a study by You et al. who investigated the use of spray-drying method by mixing mannitol and other sugars (trehalose, lactose and dextran) with amino acids (L-leucine, glycine and threonine), they found mannitol produce these corrugated particles with good aerosolisation behaviour (You et al., 2007).

**Fig. 2**

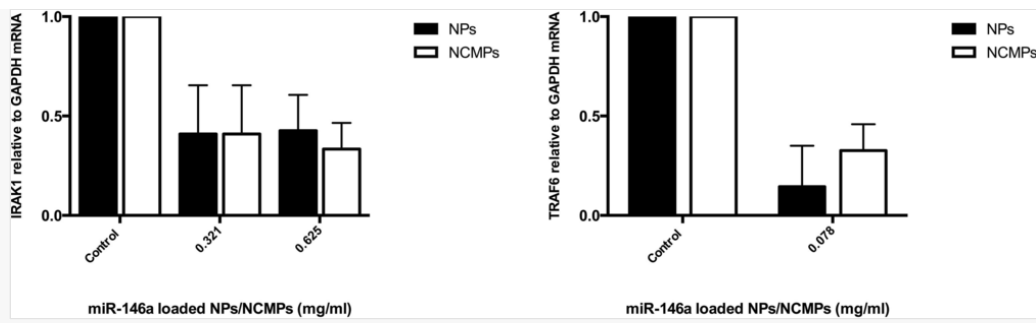
SEM images of F4 NCMPs, the scale bare represent 5  $\mu\text{m}$  and 10  $\mu\text{m}$ .

The morphology of F4 particles that contains combination of L-leucine and mannitol appear to behave differently. As the corrugated particles formed, dries, causing detention and does not interfere with coating. It is therefore speculated that the presence of mannitol altered the core structure of the spherical drying particles. This combination of L-leucine and mannitol may also be advantageous in terms of aerosolisation efficacy, hence the FPF % is high. These results are consistent with a study by Sou *et al.* in which they used combination of mannitol and leucine, they found this combination produced corrugated particles with high aerosolisation (Sou *et al.*, 2011).

### 3.4 Effect of miR-146a-NCMPs on target gene and protein expression

To confirm miR-146a-NCMPs biological functionality after spray-drying, the expression of target genes *IRAK1* and *TRAF6* was assessed in A549 cells. Analysis of transcript levels showed that miR-146a expression against targeted genes *IRAK1* and *TRAF6* (Fig. 3). The expression of *IRAK1* and *TRAF6* was normalised to *GAPDH* expression. The NCMPs results were comparable with the miR-146a-NPs. As shown in Fig. 3 that miR-146a activity was maintained after spray-drying, indicating the ability of miR-146a-NCMPs. There were different apparent sensitivities of *IRAK1* and *TRAF6* to the miR-146a-NCMPs, where *TRAF6* was significantly reduced with a relatively low dose due to miR-146a-binding sites in their 3'UTR. This suggests that the dry powder particles combining amino acid and sugar did not affect the miR-146a silencing activity despite aggregation of recovered NPs and protected biological activity of miR-146a.

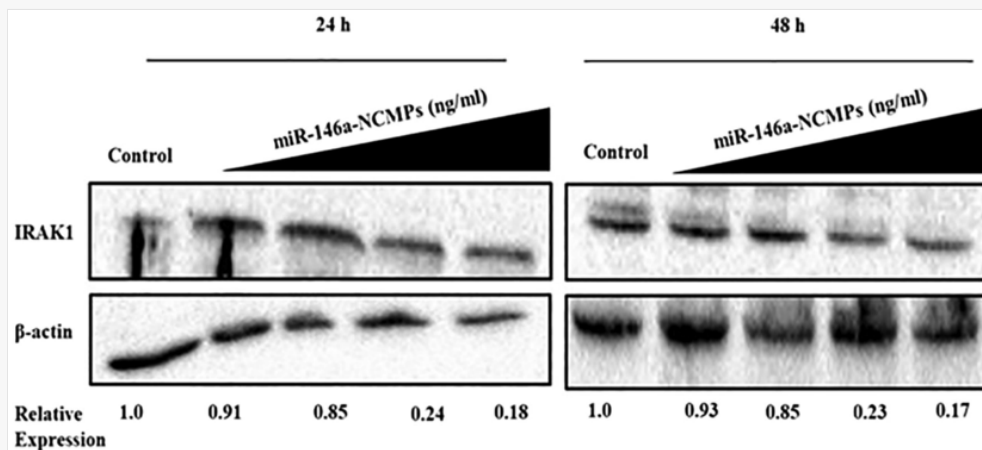
**Fig. 3**



MiR-146a-NCMPs reduce miR-146a target expression. Levels of IRAK1 and TRAF6 were assessed by sqRT-PCR in A549 cells that had been exposed to miR-146a-NPs or miR-146a-NCMPs for 1 h washed then incubated for 24 h. The doses yielding the most consistent downregulation of the target genes are shown. Mean  $\pm$  SD (n=3).

Immunoblotting (Fig. 4) supports that miR-146a activity was maintained after spray-drying, and reduced *IRAK1* protein levels in A549 cells after 24 h and 48 h treatment. The  $\beta$ -actin was used as control. As shown in (Fig. 4), the protein level decreased in a dose-dependent manner compared to untreated cells. This suggested that *IRAK1* protein levels were reduced in response to miR-146a-NCMPs. Despite nucleic acid being exposed to high temperatures of 47 °C during the drying process, and the use of the delivery formulation and excipients, the biological activity of miR-146a appears to have been preserved.

Fig. 4



Western Blot expression of miR-146a reduced IRAK1 protein levels in A549 cells for 24 h and 48 h. Dark triangle represents lowest (left) to highest (right) miR-146a-NCMPs concentrations. Numbers under each band represent the densitometric readings relative to control samples that normalized each band to its corresponding  $\beta$ -actin control.

It appears that NCMPs delivered miR-146a-NPs to site of action, and produced the required gene knockdown by inhibiting *IRAK1* and *TRAF6* genes, which is in line with other studies that indicated miR-146a role in inhibiting expression of *IRAK1* and *TRAF6* genes (Taganov et al., 2006). The local pulmonary delivery of miR-146a has played an important role by targeting multiple genes after inhalation with the dose spread over various parts in lung (Bhardwaj et al., 2009; He et al., 2005; Rossi, 2009). In fact, various studies have investigated siRNA and DNA delivery intended for inhalation (Jensen et al., 2010; Liang et al., 2015, 2014),

but limited research has been performed on miRNA pulmonary delivery. The miRNA-based therapy has showed interesting progress for treating different diseases, the MRX34 using miR-34 hepatocellular carcinoma and lung cancer have entered phase I clinical trials (Beg et al., 2017). In addition, miRagen using miR-29 to treat pulmonary fibrosis, through intravenous injection *in vivo* has reached the pre-clinical studies (Montgomery et al., 2014). Although NPs size was larger than before spray-drying, activity of miR-146a was still retained and gene knockdown reduced. The final payload of miR-146a would range between 30 and 35 µg for *in vivo* and clinical studies. Therefore, the current study provides the feasibility of using miR-146a-NCMPs for therapeutic purposes as pulmonary drug delivery that manages COPD rather than other medications.

## 4 Conclusion

The selected PGA-co-PDL NPs were incorporated into L-leucine and mannitol as a carrier to improve the powder's aerosolisation properties. Five different formulations were prepared with various excipients ratios. The NPs' size recovered after spray-drying ( $409.7 \pm 10.05$  nm) and geometric particle size is suitable for targeting the respiratory bronchiole. Although NPs size was larger than before spray-drying, activity of miR-146a was still retained and gene knockdown reduced. Moreover, the optimum formulation had a high yield ( $86.0 \pm 15.01\%$ ), and low moisture content ( $2.02 \pm 0.03\%$ ) which is essential for powder aerosolization and formulation stability. The aerosolisation performance showed high FPF  $51.33 \pm 2.9\%$ . The biological activity of miR-146a was preserved after spray-drying process and miR-146a loaded NCMPs produced gene silencing. The results indicated that the method has been optimised for the spray-drying of NCMPs with preserved miR-146a activity demonstrating potential gene therapy administered via dry powder inhalation treatment of COPD.

## Declaration of Competing Interest

None.

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## Graphical abstract

