MicroRNA Nanocarriers for the Treatment of Chronic Obstructive Pulmonary Disease

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List of abbreviation

Anti-Rb IgG (H+L)	Anti-Rabbit Immunoglobulin G (Heavy + Light chains)
Antibody HR	Antibody Horseradish peroxidase
AGO	Argonaute
BCA	Bicinchoninic acid
β-actin	Beta actin
BSA	Bovine serum albumin
COPD	chronic obstruction pulmonary disease
CMV	Cytomegalovirus
COX-2	Cyclooxygenase-2
DCM	Dichloromethane
DDS	Drug Delivery System
DMAB	Didodecyldimethylammoniumbromide
DMSO	Dimethyl Sulfoxide
DOTMA	N-[(1-(2,3-dioleyloxy)propyl)]-N-N-Ntrimethylammonium chloride
DOTAP	Dioleoyltrimethylammoniumpropan
DNA	Deoxyribonucleic acid
pDNA	Plasmid Deoxyribonucleic acid
DEPC	RNase-free diethyl pyrocarbonate
DVA	Divinyl adipate

DPIs	Dry Powder Inhalers
DAPI	4′,6-diamidino-2-phenylindole, dihydrochloride
ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
ED	Emitted Dose
EMEM	Eagle's Minimum Essential Medium
FPF	Fine Particle Fraction
FAM	6-Carboxyfluorescein
FPD	Fine particle dose
FBS	Foetal Bovine Serum
F1–F5	Formulations 1 to 5
GPC	Gel Permeation Chromatography
GSD	Geometric standard
GFP	Green Fluorescence protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOLD	Global Initiative on Obstructive Lung Disease
^{1}H-NMR	Proton Nuclear Magnetic Resonance
IL	Interleukin
IRAK1	Interleukin-1 receptor-associated kinase 1
IRES	Internal Ribosome Entering Sequence
ICAM	Intercellular Adhesion Molecules

LPS	Lipopolysaccharides
MDIs	Metered Dose Inhalers
MMAD	Mass Median Aerodynamic Diameter
MTT	3-4,5-Dimethylthiazol-2-yl-2,5-Diphenyl Tetrazolium Bromide
Mw	Molecular Weight
MeOH	Methanol
miRNA	Micro Ribonucleic acid
mRNA	Messenger Ribonucleic acid
N/A	Not Applicable
NCMPs	Nanocomposite Microparticles
NGI	Next Generation Impactor
NPs	Nanoparticles
NR	Nile Red
ncRNAs	Noncoding Ribonucleic acids
o/w	Oil in water
PFA	Paraformaldehyde
PCR	Polymerase Chain Reaction
pDNA	Plasmid Deoxyribonucleic acid
PBS	Phosphate Buffered Saline
PDI	Poly Dispersity Index
PEG	polyethylene glycol
PEI	Polyethylene-imine

PGA-co-PDL	poly (glycerol adipate-co- ω-pentadecalactone)
PLGA	Poly Lactic-co-Glycolic-Acid
PVA	Poly (vinyl alcohol)
PDL	Pentadecalactone
PGSS	Precipitation from Gas Saturated solutions
qRT-PCR	Quantitative Real- Time Polymerase Chain Reaction
RNA	Ribonucleic acid
RT- qPCR	Real-time quantitative polymerase chain reaction
RF	Respirable fraction
RFP	Red fluorescent protein
RT	Room Temp
RISC	RNA-Induced Silencing Complex
RESS	Rapid Expansion of Supercritical Fluid Solution
RIPA	Radio Immuno Precipitation Assay
siRNA	Small Interfering RNA
SEM	Scanning electron microscope
SDS	Sodium dodecyl sulphate
TRAF6	TNF receptor-associated factor 6
TGA	Thermo Gravimetric Analysis
THF	Tetrahydrofuran
TNF-α	Tumor Necrosis Factor-alpha
TBE	Tris-Borate electrophoresis

3’ UTR	Untranslated region
WHO	World Health Organization
WB	Western blot
ZP	Zeta potential

Abstract

Chronic obstruction pulmonary disease (COPD) is a major cause of morbidity and mortality across the world. COPD is currently the fourth leading cause of death in the world and is predicted to become the third leading cause of chronic illness and death worldwide by 2030. There are several therapeutic strategies to reduce COPD symptoms and complications such as; bronchodilator medications, antibiotics, inhaled corticosteroids and rehabilitation. However, none of the available pharmacological or non-pharmacological treatments for COPD have been shown to delay or correct long-term defects in lung function. Small nucleic acids such as non-coding RNA (ncRNA) and interference microRNA (miRNA) have recently gained attention as a new class of therapeutics for various genetic diseases. Modulation of miRNA expression and function represents a promising strategy for therapeutic intervention in disorders such as inflammatory lung disease including COPD. In this study the aim was to design, formulate and characterise polymeric nanoparticles (NPs) containing miR-146a. This was followed by spray-dying using L-leucine and mannitol to prepare dry powder nanocomposite microparticles (NCMPs) for pulmonary delivery.

Anionic and cationic poly (glycerol adipate-co- ω-pentadecalactone), (PGA-co-PDL), NPs were produced using poly (vinyl alcohol) and dioleoyltrimethylammoniumpropane (DOTAP) respectively. The particle size of the anionic NPs was 266.10±20.80 nm and the incorporation of DOTAP resulted in NPs of 244.80±4.40 nm at 15 % DOTAP concentration. The zeta potential (ZP) of 15 % DOTAP NPs was +14.8±0.26 mV. Fluorescently labelled synthetic miRNA (miR-146a) was adsorbed onto the surface of the optimum 15 % DOTAP NPs. The cell viability studies indicated that over 65 % of A549 cells remained viable after 24 h exposure to cationic NPs at a concentration of 1.25 mg/ml.

The spray drying process was optimised to produce NCMPs with recovered NPs of 409.7±10.05 nm, yield of 86.05±15.01 % and low moisture content 2.02±0.03 %. The NCMPs produced had a spherical shape and corrugated surface. The *in vitro* aerosolisation analysis showed a mass mean aerodynamic diameters (MMAD) of less than 6 µm indicating the NCMPs would be deposited in the middle to deep lung region and a fine particle fraction (FPF) of 51.33±2.90 %. Internalisation of miR-146a loaded cationic NPs was observed in A549 cell lines using both fluorescence and confocal microscopy.

The miR146a delivered to A549 cells as miR-146a-NPs and miR146a-NCMPs had a dose dependent reduction on target gene repression; interleukin 1 receptor-associated kinase *(IRAK1)* expression to 40 % and TNF receptor-associated factor *(TRAF6)* expression to over 20 %. Moreover, the miR-146a biological activity was maintained after spray drying. These findings demonstrate the promise of miR-146a-NPs/NCMPs as a dry powder pulmonary for the treatment of COPD, protecting miR-146a from degradation and enzymatic activity in the lung airways.

1 General introduction

1.1 Pulmonary disease; COPD

Lung inflammation is a common symptom in various lung diseases, which can be either acute or chronic in nature. For example, pneumonia is an acute inflammatory disease, whereas chronic lung diseases include asthma, cystic fibrosis and chronic obstruction pulmonary disease (COPD). 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). In a healthy lung, when a person inhales and exhales, each air sac fills up and deflates with air and the airflow passes through the lung airway smoothly (*Fig* 1–1). Whereas, in a lung afflicted with COPD, there will be less air flow in and out of the airways due to; the air sacs and airways having lost their elastic quality, destruction of the walls between air sacs, the walls of airways become thick and inflamed, and there is a greater presence of mucus than usual which can prevent air entering and leaving the lung (Pauwels et al. 2001). One of the mechanisms that affect COPD pathogenesis is that alveolar epithelial cells start looking fibroblastic, due to epithelial-mesenchymal transition (Nishioka et al. 2015).

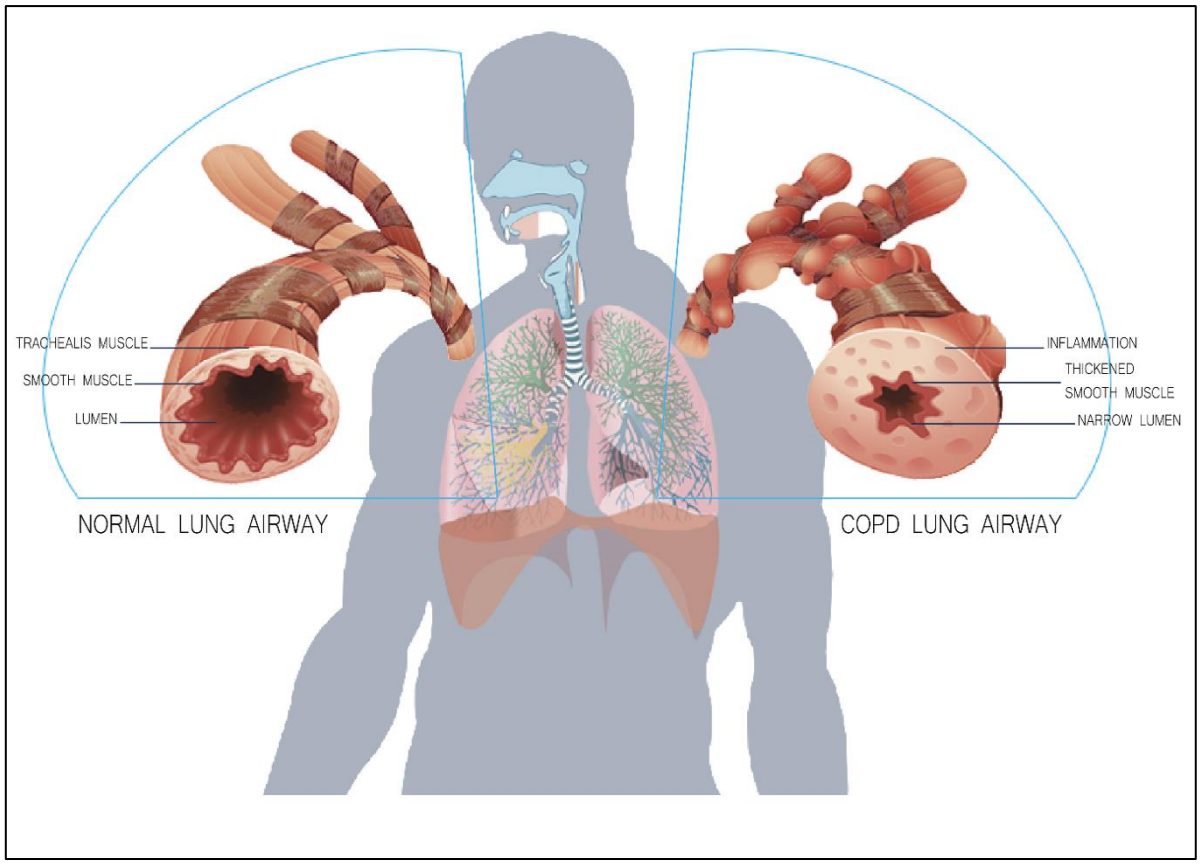

Figure 1 – 1. Illustrative diagram of a healthy normal and COPD lung airways. In a healthy lung, during inhalation and exhalation airflow passes through the lung airway smoothly, whereas, a lung afflicted with COPD, there will be less air flow in and out of the airways.

1.2 Statistics for COPD

COPD is a major cause of morbidity and high mortality rates throughout the world. COPD is currently the fourth leading cause of death in the world and is predicted to become the third leading cause of chronic illness and death worldwide by 2030 (World Health statistics 2008).

The World Health Organization (WHO) estimated that 251 million people were affected by COPD in 2016, and the Global Initiative on Obstructive Lung Disease (GOLD) indicated the disease is more prevalent among males than females (Global Strategy for the Diagnosis, Management and Prevention of COPD and GOLD 2016). A study among participants in developed countries found that a healthy male at 40 years of age has a 12.7 % chance of contracting COPD in the next forty years of his life, whereas for a healthy female the corresponding probability is 8.3 % (Halbert et al. 2006). Moreover, similar data in low middle income counties indicated almost a quarter of adults aged over 40 years were diagnosed with mild airflow obstruction, defined as a reduced post-bronchodilator ratio of forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC) (Halbert et al. 2006). Thus, the incidence of COPD occurs in both genders and in both developed and low middle income countries which indicates the marked global burden of this disease (Afonso et al. 2011).

1.3 COPD inflammatory causes

Primarily, COPD is contracted as a consequence of tobacco smoking, which progressively damages the lungs through nicotine and tar deposition, consequently leading to airflow constriction. Despite smoking being a major risk factor for COPD, only 20 % of smokers develop this pathology (Pauwels and Rabe 2004). Bronchial epithelial cells are the first anatomical barrier to noxious cigarette smoke particles and are involved in the initiation of airway remodelling through the production of proinflammatory mediators (Jeffery 2004).

The exposure to cigarette smoke releases pro-inflammatory mediators, cytokines, which leads to the release of proteases and high oxidant concentrations that damage lung tissue (Heijink et al. 2013). Other factors such as exposure to indoor and outdoor air pollutants, bacterial infections, occupational hazards and genetic abnormalities contribute towards emphysema in the form of alpha-1-antitrypsin (A1AT) deficiency, have also been implicated in the

pathogenies of COPD ((Mannino and Buist 2007). There is also a relationship between exposure to indoor biomass fuels and a number of respiratory diseases including COPD (Ezzati 2005, Orozco-Levi et al. 2006), particularly in low middle income countries that rely on biomass fuels as source of everyday domestic energy (Umoh and Peters 2014). The smoke released from burning biomass fuels releases toxic substances, and causes extremely high levels of air pollution that affects and irritate the respiratory system. Desalu *et al*. showed the increased risk of respiratory symptoms and chronic bronchitis in women using biomass fuels in Nigeria (Desalu et al. 2010). Various studies have revealed that biomass fuel smoke is one of the causes of obstructive airway diseases (van Gemert et al. 2011, Oluwole et al. 2013, Zhou et al. 2014).

1.4 Current treatment

There are several clinically appropriate therapeutic strategies to reduce COPD symptoms and frequency for example, bronchodilator medications which are used to reduce bronchial spasms, antibiotics to reduce respiratory infections, inhaled corticosteroids which are recommended for reducing inflammation, as well as influenza and pneumococcal vaccines to reduce serious illness and death. There is also rehabilitation available to reduce symptoms and enhance quality of life and oxygen can be administered to keep mucous membranes moist and provide adequate hydration. However, none of these pharmacological and non-pharmacological treatments for COPD have been shown to delay and correct the long-term defects in lung function (Global Strategy for the Diagnosis, Management and Prevention of COPD and GOLD 2016).

In parallel to inflammatory and pro-inflammatory mediators involved in COPD pathogenesis, micro RNA (miRNAs) have recently been attributed to the pathogenesis of COPD (Angulo et al. 2012). miRNAs are endogenous and noncoding functional mediators of RNA interference (RNAi). They are an essential group of 18-25 single strand nucleotides (Bartel 2004). Almost

2,000 human miRNAs that have been discovered, despite some of their biological functions being unknown (Li and Belmonte 2015). The first miRNA (*Lin*-4) for cellular development was discovered in 1993 in *Caenor-habditis elegans* (Lee et al. 1993). Later, Reinhart *et al*. discovered let-7 and its association with cellular development timing (Reinhart et al. 2000). The following year Hutvágner *et al.* performed a similar study which indicated the relationship between let-7 and development regulation timing in both humans and animals (Hutvágner et al. 2001). The role of these miRNAs is in the post-transcriptional regulation of gene expression by binding to targeting messenger RNA (mRNA), promoting translation repression and mediating a cleavage and degradation of the mRNA target, and consequently blocking the translation of mRNAs into proteins (Tomankova et al. 2010).

1.5 Biogenesis of miRNA

miRNA genes are generally found in intergenic areas, and they tend to be highly concentrated in the vicinity of the centromere of each chromosome (Meurant 2012). There are thousands of miRNAs that have been identified in various organisms and have been added to miRNA databases e.g miRBase (www.miRBase.org), which is a centralized repository for miRNAs and miRNA annotation. For example, the hsa-miR-146a entry into miRBase, has a chromosome where it is located and the loss of the function of miR-146 leads to an individual suffering from chromosome 5q deletion syndrome (*Fig.* 1–2).

Figure 1 – 2. Example miRNA entry in miRBase, hsa-miR-146 from human genome (http://www.mirbase.org/). The mature miRNAs derived from the 5' and 3' arms are in pink, corresponding to miR-145a-5p and miR-146a-3p. The miR-146 mimic used throughout this project corresponds to miR-146a-5p.

miRNA is transcribed in the nucleus by RNA polymerase II, to produce miRNA primary transcripts (pri-miRNAs). The pri-miRNA is cleaved by Drosha which partners with RNA-binding protein DGRC8 forming pre-miRNA which is then exported to the cytoplasm by Exportin-5. In the cytoplasm, the pre-miRNA is cleaved by RNAase type III Dicer to produce 21-23 nucleotides long, with two nucleotide 3' overhanging, called a miRNA/miRNA* duplex. This duplex is subsequently incorporated in a RNA-induced Silencing Complex (RISC), which contains Argonaute protein (*Fig* 1–3).

1.5.1 The RISC Complex

There are four Argonaute (AGO) proteins that represent a key component of RISC, which contains two domains; PIWI and PAZ. PIWI structurally resembles RNaseH, which is essential for target cleavage, and PAZ, recognizes the miRNA 3' end (Kuhn and Joshua-Tor 2013). Nevertheless, AGO protein is loaded with double stranded miRNA precursors that form the RISC loading complex, and forms a passenger strand (miR*) that is degraded. A guide strand (miR) then guides the miRNA:AGO protein complex to the target 3' untranslated region (UTR) of mRNA and promotes mRNA transitional inhibition or degradation depending on the complementarity between miRNA seed and 3'UTRs (Brodersen and Voinnet 2009, Rajewsky 2006, Elkayam et al. 2012, Schirle et al. 2014).

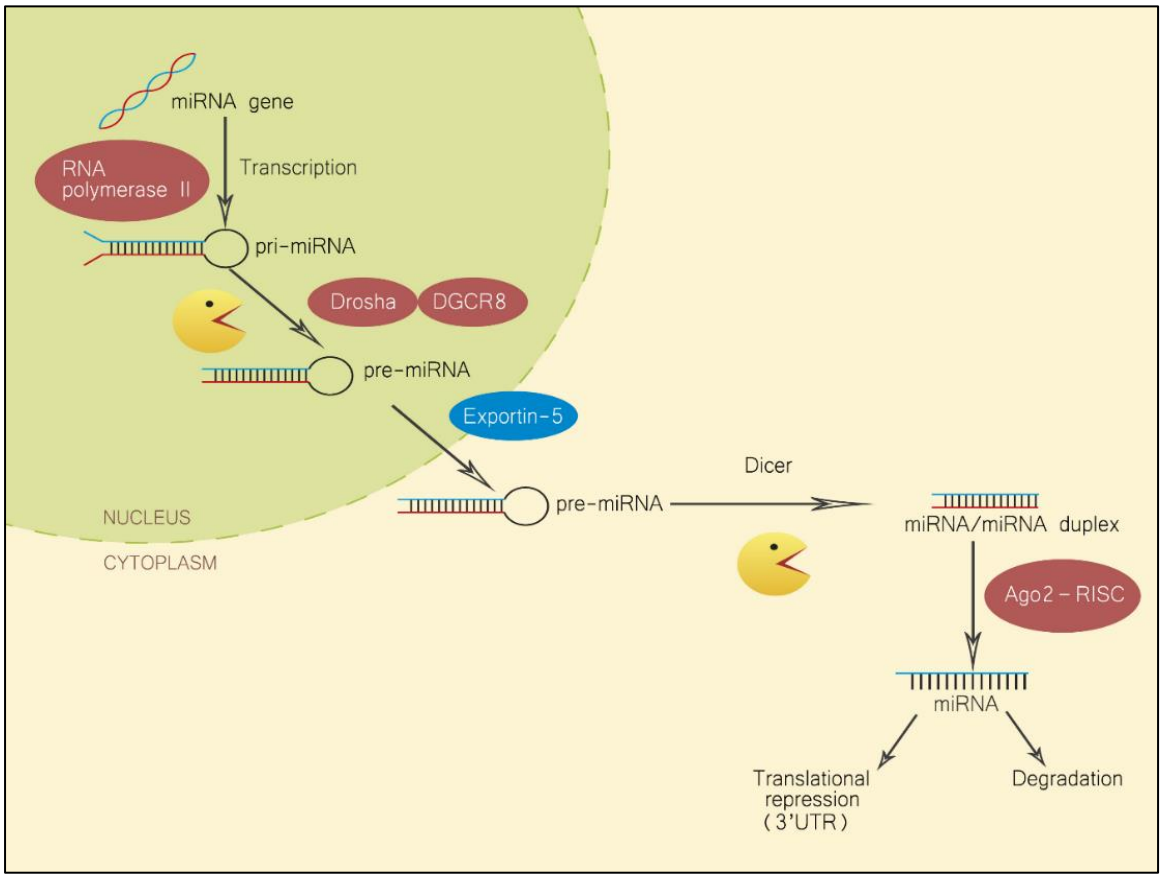

Figure 1 – 3. Schematic overview of miRNA biogenesis (adapted from (Winter et al. 2009). miRNA biogenesis starts with transcription of miRNA gene by RNA polymerase II to pri-miRNAs. The pri-miRNA cleaved by Drosha which partners with RNA-binding protein DGRC8 forming pre-miRNA. The pre-miRNA is exported to cytoplasm by Exportin-5, which cleaved by RNAase type III Dicer in cytoplasm to produce 21-23 nucleotides long, with two nucleotide 3' overhanging, called a miRNA/miRNA* duplex. This duplex incorporated in RISC which contain AGO proteins which forms a passenger strand (miR*) that is degraded and a guide strand (miR) that target 3' untranslated region (UTR) of mRNA and promotes mRNA transitional repression.

1.6 miRNA as a biomarker for COPD

miRNAs play an important role in the regulation of gene expression for various normal and pathological mechanisms. Hence miRNAs are good candidates to act as potential biomarker agents in diagnostic and therapeutic clinics.

Studies have detected miRNAs in various body fluids, tissue and cell types in both animal models and humans, and their involvement in lung development and progression, indicates their significant role in inflammatory responses (*Fig.* 1–4), (Akbas et al. 2012). These studies have shown the presence of miRNA in lung tissue and bodily fluids such as sputum, plasma and urine. The stability of miRNAs in sputum and plasma varies with the level of degradative enzymes in blood, which reflects the biological importance of miRNA. Chen *et al*. found that miRNAs levels in serum are stable and consistent among individuals (Chen et al. 2008). Akbas *et al.* showed that miR-7 was up-regulated whereas another four miRNAs (miR-20a, -28-3p,34c-5p, and 100) were down-regulated when comparing COPD patients with a control population (Akbas et al. 2012). Similarly a study by Perry *et al.* showed that the changes in expression of miR-146a could regulate the inflammatory response in human alveolar epithelial lung cells (Perry et al. 2008). Therefore, with the presence and stability of miRNA, it is possible to differentiate between healthy individuals with healthy lung tissue and those suffering from lung inflammation by using miRNA as a diagnostic and therapeutic biomarker.

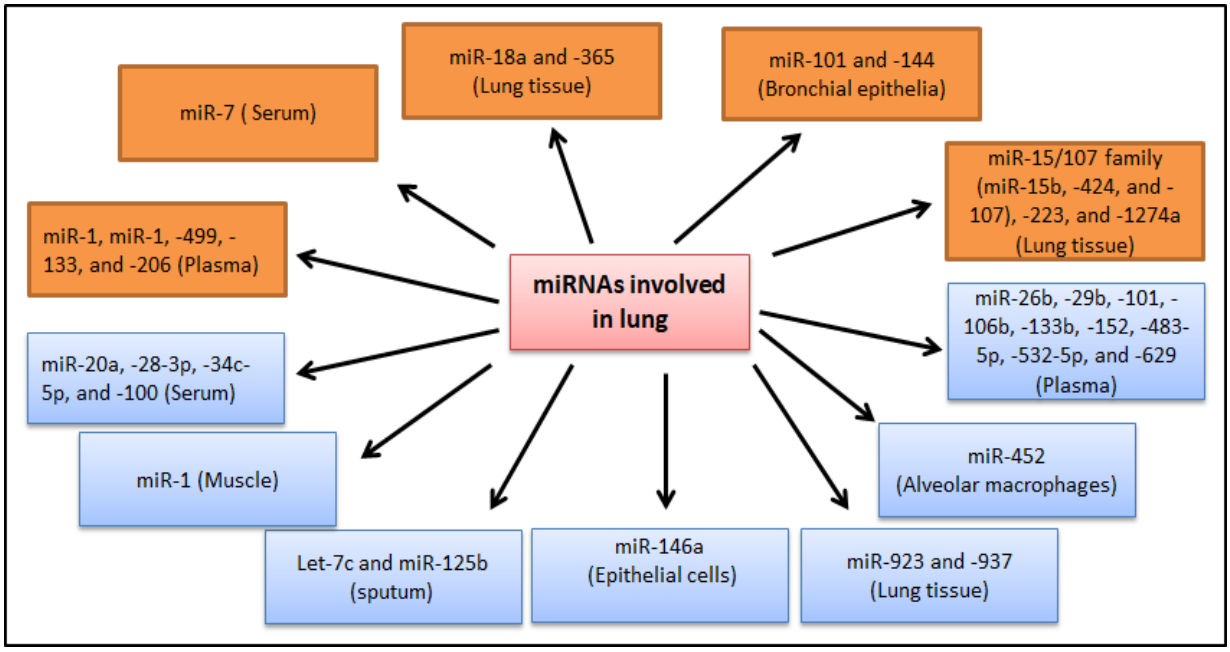

Figure 1–4. Illustration of the main miRNAs involved in COPD. Orange boxes show up-regulated miRNAs (cell, tissue and body fluids). Blue boxes indicate down-regulated miRNAs (cell, tissue and body fluids).

1.7 Role of miRNA in inflammatory response

In the relationship between miRNA and the inflammatory response in COPD, miR-146a is precisely linked to COPD pathogenesis. Sato *et al.* demonstrated that miR-146a and the down-regulation involved in pathogenesis increases the abnormal inflammatory cyclooxygenase (COX-2) half-life in COPD and enhances production of prostaglandin E2. These expressed proteins are considered a hallmark of chronic inflammation in COPD (Sato et al. 2010). These findings were evaluated through *in vitro* studies using a miR-146a mimic and Western Blot analysis to determine COX-2 protein expression in cytokine-treated COPD fibroblasts. A Quantitative Real- Time Polymerase Chain Reaction (qRT-PCR) assay was used to quantify miR-146a expression and the luciferase reporter assay demonstrated a binding of miR146a to COX-2 mRNA 3'UTR (Sato et al. 2010).

miR-146a was shown to be involved in the targeting of interleukin-1 and Toll-like receptor (TLR) signalling, of which NF-κB activation is a primary downstream effector, known as interleukin 1 receptor-associated kinase (IRAK1) and TNF receptor-associated factor (TRAF6). Taganov *et al.* reported miR-146a base-pairs with sequences in the 3' UTRs of IRAK1 and TRAF6, and that these UTRs inhibit expression of a linked reporter gene. miR-146a is capable of controlling TLR and cytokine signalling through negative feedback regulation, associating down regulation of IRAK1 and TRAF6 protein levels (Taganov et al. 2006, Nahid et al. 2009). Moreover, miR-146a plays a crucial role in the negative feedback regulation of interleukins, IRAK1 and TRAF6 (*Fig*. 1–5) (Bhaumik et al. 2009). In addition, there are various genes associated with inflammation of COPD airways including intercellular adhesion molecules (ICAM)-1 (Papi and Johnston 1999), tumour necrosis factor TNF-α (Leeper-Woodford and Detmer 1999), monocyte chemoattractant MCP-1 (Ueda et al. 1997), endothelin-1 (Quehenberger et al. 2000), and secretory leucocyte proteinase inhibitor (Sallenave et al. 1997).

There are also other miRNAs present, which are not directly related to the inflammatory response of COPD. Guo *et al.* used a murine model to evaluate miR-125b which caused a significant reduction in neutrophil counts and pro-inflammatory cytokines corresponding with lipopolysaccharide (LPS) induced pulmonary inflammation (Guo et al. 2014). The LPS was targeted through the myeloid differentiation factor 88 (My88), which showed the capability of miR-203 to regulate inflammation (Wei et al. 2013). In addition, other miRNAs involved in COPD inflammation include miR-150, miR-181a and miR-20a. These were down regulated in COPD compared to non-COPD models *in vitro* and *in vivo* (Manoharan et al. 2014, Xie et al. 2014). Manoharan *et al.* found that an increase of miR-150 levels correlated with reduced chemokine CXCL 1 expression in mice (Manoharan et al. 2014). A study by Xie *et al.* used qRT-PCR to measure expressed miR- 21 and miR-181a levels in enforced smoking rat models

to predict the occurrence of COPD. They demonstrated that levels of miR-21 were significantly higher, whereas levels of miR-181a were significantly lower, in COPD patients than in healthy control subjects. This suggests that levels of miR-21 and miR-181a can be useful for predicting the development of COPD in heavy smokers (Xie et al. 2014).

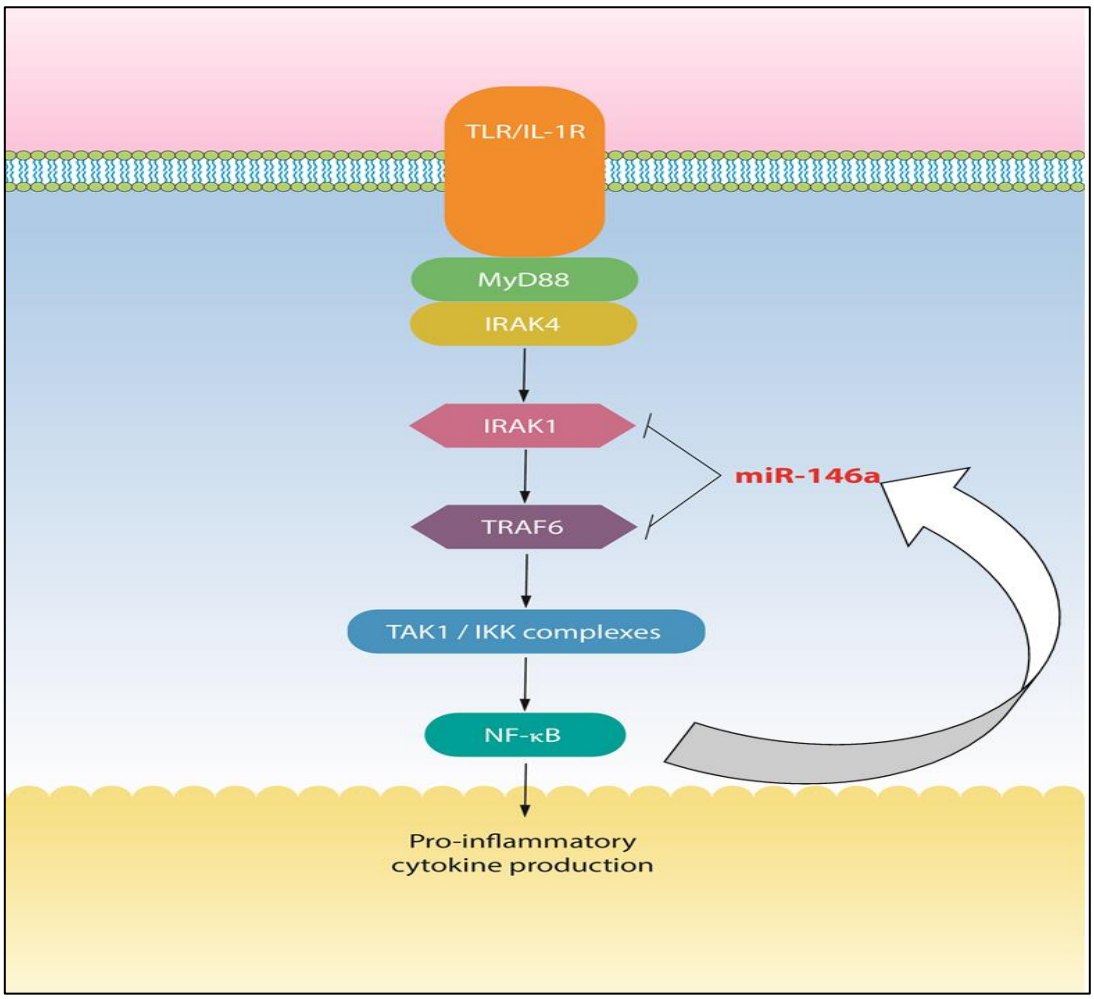

Figure 1–5. The role of miR-146a in TLR4 and IL-1R mediated signal transduction (adapted from (Oglesby et al. 2010, Bhoj and Chen 2009). The TLR4 and IL-1R signalling is started after binding with adaptor protein MyD88 which in turn recruits IRAK4 leads to activation of IRAK1 and TRAF6. This activates the protein kinases TAK1 and IKK complexes as a result allowing NF-κB starts transcriptional regulation and produce pro-inflammatory cytokines. miR-146a downregulates IRAK1 and TRAF6 protein levels and leads to a change in signalling pathway.

1.8 miRNA as a therapeutic agent

The ability of miRNAs to concurrently target multiple genes involved in inflammatory pathways is an advantage over siRNAs, which typically target a single gene transcript (Pasquinelli et al. 2005, Rossi 2009) and miRNAs can also be used therapeutically to target multiple cell types and multiple inflammatory processes (Conde et al. 2016). For this reason, the study of miRNAs may potentially generate new therapies for targeting and treating COPD rather than symptomatic relief.

miRNA replacement therapy can be administered using two distinct approaches. The first method, involves introducing double-stranded synthetic mimic miRNA known as anti-miRs. These mimics are complementary to the miRNAs of interest and following uptake by cells they bind to RISC in the cytoplasm (Bader et al. 2010) restoring the loss of miRNA function due to down expression. This method is more specific for restoring miRNA function, as it is associated with decreased off target effect and offers the ability of personalization according to the miRNA required, and has less side effects compared to miRNA antagonists (Bader et al. 2010). The significant therapeutic action of miRNA mimics enabled miR-34 to be used for the treatment of many cancers such as; colon, ovarian, cervical, non-small cell lung cancer, and hepatocellular carcinoma, and miR-34 has been tested in phase I clinical trials (Bouchie 2013b).

The second method is known as the miRNA-expression vector where oligonucleotide miRNA mimics are combined with a vector. The viral vectors have been employed to deliver let-7 to decrease lung tumour and breast cancer cells (Esquela-Kerscher et al. 2008, Yu et al. 2007). Moreover, Trang *et al.* demonstrated the use of a neutral lipid emulsion delivery system of both miRNA-34a and let-7 mimics in a xenograft model to reduce tumour growth (Trang et al. 2011). The use of these mimics has also been explored in lung cancer; Wu *et al.* therapeutically

delivered miR-29b in cationic lipoplexes for lung cancer in both *in vitro* using A549 cell lines and *in vivo* (Wu et al. 2013c). This suggests that miRNA mimics could also be successful in COPD therapeutics. Although COPD alveolar epithelial cells undergo a epithelial-mesenchymal transition, miRNA mimics can target this transition and restore their function (Nishioka et al. 2015).

miRNA replacement therapy is considered an attractive target for clinical therapeutic development and there are various miRNAs currently in pre-clinical and clinical trials. The success of these clinical trials is crucial for the development of strategies for miRNA therapies. Pre-clinical studies using miR-34 for hepatocellular carcinoma and lung cancer progressed, in 2013, to the clinical trial stage where MRX34 was the first miRNA replacement therapy in clinical trials (Bouchie 2013a). MRX34 development from the biopharmaceutical company *Mirna Therapeutics,* has completed phase I and the study is ongoing in terms safety, pharmacokinetics and pharmacodynamics (Beg et al. 2015).

1.9 Delivery of miRNA to the lung

When developing pulmonary delivery systems, it is important to consider the unique anatomical and physiological characteristics of the lung. The lung has a large alveolar surface area (80 sq. m) and offers a controlled low enzyme environment, ideal for the systemic absorption of macromolecules promoting efficient local and systemic delivery of drugs (Muralidharan et al. 2014). The lung is divided into two main parts; the central and peripheral regions. The former includes the trachea, bronchi and bronchioles, which act as defence mechanisms against airborne particles, and these particles are cleared by cilia within the mucus layer that lines the epithelia. The peripheral region, contains alveolar sacs whose primary function is to enable the lung to perform gas exchange during the breathing process (Ong et al. 2013).

When air is inhaled through the nose and mouth it passes through the larynx and trachea, which represent generation zero of the airways, proceeding until the sixteenth generations where the central bronchi and bronchioles end. At the beginning of the seventeenth generation of bronchioles, the alveoli start to appear which contain the alveoli ducts (20^{th} generation) and alveoli sacs end at the 23^{rd} generation of Weibel model (*Fig* 1–6) (Effros 2006). The Weibel airway model of human lung refers to upper and lower airways regions with respect to deposition of inhaled particles of different size (Weibel 1963).

The deposition of inhaled particles into the epithelial and alveolar regions or cells of the lung is influenced by various parameters such as particle size and shape, airway dimension, flow dynamic, breathing rate, respiratory volume and health of the individual. Particle deposition occurs through one of the following mechanisms; impaction, sedimentation, interception or diffusion (Heyder 2004) and is dependent on the aerodynamic diameter (d_a). The aerodynamic diameter is defined as the diameter of a sphere of a given geometric diameter, which is equivalent to the settling velocity of the particle in question and represented by the following equation:

$$d_a = d_g \sqrt{\frac{p}{X.po}}$$

d_a is the aerodynamic diameter, d_g is geometric diameter, p is the particle density, reference density (usually from water) and x is shape factor (Bailey and Berkland 2009, Carvalho et al. 2011b). The inhaled particles would target lung epithelial and alveolar cells.

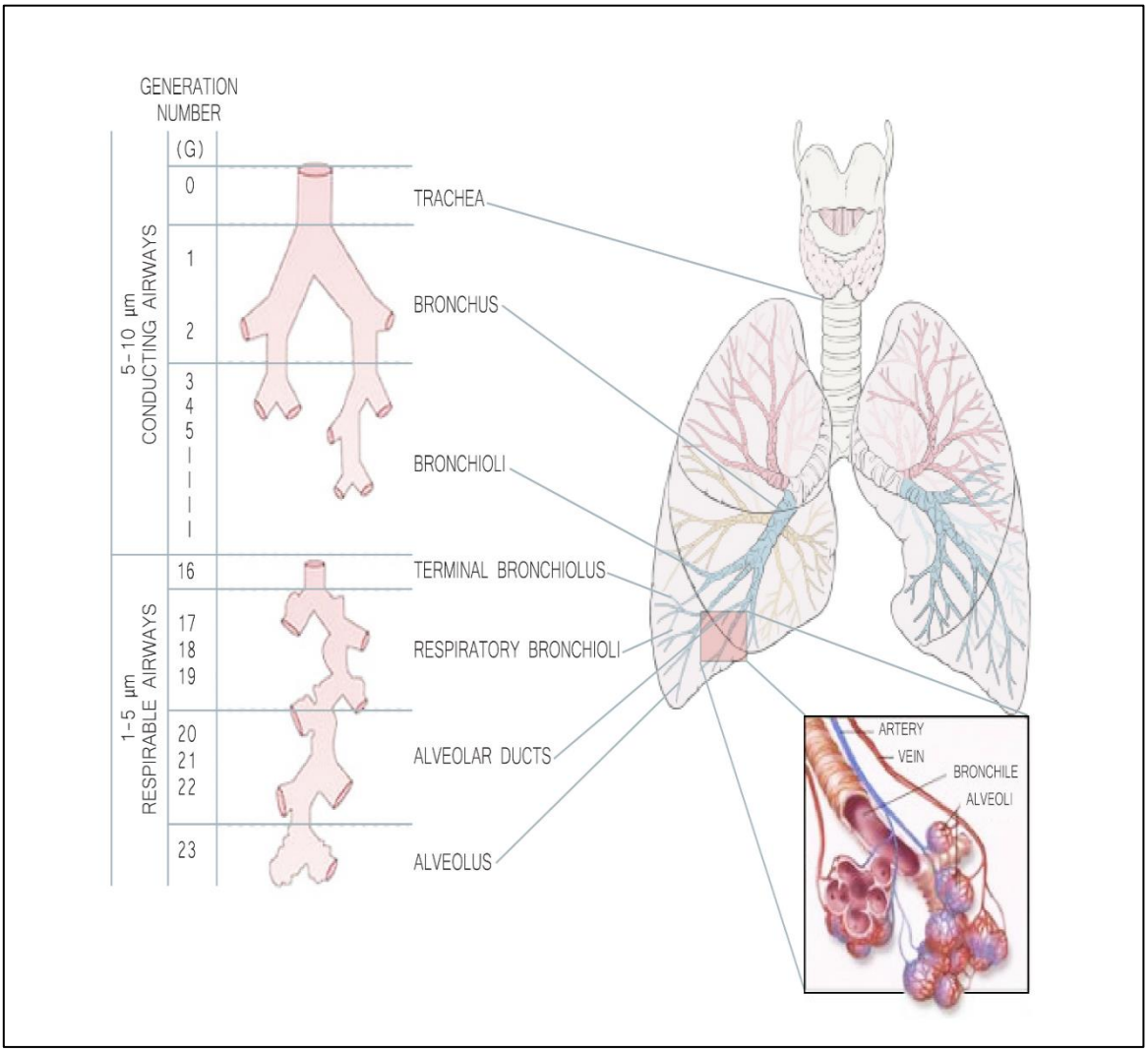

Figure 1–6. Diagram of human lung and deposition of inhaled particles dependent on particles size (adapted from (Kunda et al. 2013).

Numerous delivery routes for small nucleic acids have been reported, ranging from local injection (ophthalmic drops, intradermal injection and intranasal spray) into target tissue to systemic applications (intravenous injection), (Fujita et al. 2013). Let-7 miRNA has previously

been delivered locally to the mouse lungs by intra-tumour injection into lung cancer tumours *in vivo* in order to improve bio-distribution (Trang et al. 2010). However, this resulted in limitations and challenges such as peripheral tumour cells remaining present and relative knockdown of let-7 targets by immunohistochemistry (Trang et al. 2010). Nevertheless, each route of administration has different pharmacological effects. The pulmonary delivery of small nucleic acids e.g siRNA to treat lung disease, asthma and cystic fibrosis, showed significant advantages when compared with local injection delivery (Valle et al. 2007, Labiris and Dolovich 2003a). ZaBeCor Pharmaceuticals have developed an inhaled siRNA for local delivery via the lungs to treat asthma and phase I clinical trials showed 75 % of patients who received the treatment experienced less laboured breathing (Watts and Corey 2010, Xie and Merkel 2015). The pulmonary delivery of nucleic acids to the lung is non-invasive and offers various advantages over the other non-parenteral oral, buccal, transdermal and nasal routes (*Table* 1–1). Although much research has been conducted on siRNA there is a limited amount of studies that have investigated the inhalation of miRNA.

Table 1– 1. Advantages and disadvantages of different routes of delivery of small nucleic acids therapeutics

Route of administration	Nucleic acid	Advantages	Disadvantages	Reference
Pulmonary	siRNA	(a) Nucleic acid can be administered in a reduced dose thereby decreasing local and systemic side effects. (b) Rapid clinical response due to locally targeting lung cells. (c) Avoiding degradation in serum due to lower nuclease activity in the airways.	Limited lung deposition depending upon carrier, formulation and device.	a-(Patton and Byron 2007) (Labiris and Dolovich 2003a) b- (Agu et al. 2001) c- (Takei et al. 2004)

Dry powder inhalation	siRNA	(d) Higher pharmacokinetics, which in association with higher retention time can achieve the maximal therapeutic. (e) Bypass of the first hepatic metabolism	Stability of siRNA associated with high temperature conditions and final powder loss in spray drying.	(Jensen et al. 2010) and (Jensen et al. 2012)
Intratumoural Injection	Let-7 miRNA	Strong inhibition of tumour distribution	Limited gene knock down.	(Wiggins et al. 2010)
	miR-34a	Clinical trial data showed ability to block lung tumour growth.	None	(Bouchie 2013a)
Intravenous (systemically)	siRNA	Improved siRNA bio-distribution.	Accumulation in organs such as kidney and liver.	(Braasch et al. 2004) and (Santel et al. 2006)
	miR-200c	Improved radio-sensitivity.	None	(Cortez et al. 2014)

Intraocular	siRNA	Reduction in inflammatory cells of 50%.	Invasive, and requires specialists such as clinicians.	(Hérard et al. 2006)

1.10 Nanocarriers for miRNA delivery

The delivery of miRNA to a site of action is a major challenge especially in diseased lungs, for the development of miRNA therapeutic. The physicochemical properties, such as hydrophilicity and negative charge, make it difficult for these molecules to cross biological barriers (Yin et al. 2014). Viral vectors such as lentiviral (Liu et al. 2012, Lian et al. 2012) and adenoviral (Xia et al. 2012, Gu et al. 2013) vectors have been used as carriers for the delivery of RNAs but they are known to cause immunologic inflammatory responses (Wang et al. 2012b).

During the last few years, there has been a growing interest in nanotechnology for drug delivery. Components of living cells are commonly nano-sized, for example membrane transporters, ribosomes and receptors (Labhasetwar 2005) so nanoparticles (NPs), defined as small particles ranging from 1 to 1000 nm in diameter (Sung et al. 2007), can readily interact with intracellular and extracellular components of cells (Borm et al. 2006). NPs loaded with therapeutic agents can be utilised as a drug delivery system (DDS) for systemic and local delivery to treat diseases (Labiris and Dolovich 2003b). Inhalation is a favourable non-invasive route for lung targeting, providing high bioavailability (Cefalu 2004). Therefore, using NPs is an interesting delivery strategy for small nucleic acids to treat respiratory diseases.

Advantages of inhaled NPs

NPs have several properties which make them good candidates for nucleic acid delivery, for example; the small particle size and positively charged surface. These properties allow the NPs to interact with cells, enhance intracellular uptake and release of miRNA to achieve the required gene silencing (Anwer et al. 2000, Stuart et al. 2006). The lung has barriers such as lung lining fluid, epithelial cells and enzymes e.g endonucleases. Inhaled miRNA may be

degraded by these barriers. NPs, due to their size 200 nm, have the ability to diffuse through lining fluid. A study by Patton *et al*. showed the uptake of smaller particles 200 – 500 nm size could efficiently cross the lung barriers (Patton et al. 2004).

The surface of NPs can be modified with other molecules and functional groups and can be complexed with proteins, carbohydrate and antibodies (Davies et al. 2008). This can enhance NP miRNA delivery and cellular uptake compared to that of naked miRNA (Yuba et al. 2008). Yin *et al*. showed the attachment of polyethylene glycol (PEG) to NPs protected nucleic acids from degradation and provided greater serum stability (Yin et al. 2014). miRNA complexed with cationic NPs protected against degradation, improved circulation and used in treatment of pulmonary disease such as cystic fibrosis (Yin et al. 2014, Konstan et al. 2004). Similarly, N-[(1-(2,3-dioleyloxy)propyl)]-N-N-Ntrimethylammonium chloride (DOTMA), in combination with a lipid, formed a complex with plasmid DNA (Anwer et al. 2000).

As a DDS to treat severe lung disease, poly (D L-lactide-co-glycolide acid), (PLGA), NPs with surface coated DOTAP were loaded with siRNA and incorporated into inhalable carrier particles by spray drying. The siRNA loaded NPs had a particle size of 216.0±6.0 nm and surface charge of +33.1±2.2 mV. *In vitro* assays showed reduced gene expression and gene silencing of 73 % was achieved (Jensen et al. 2012) indicating that small nucleic acid-loaded NPs within a microcarrier can be used efficiently as a DDS in a pulmonary delivery platform to enable targeting of lung disease.

1.11 Polymer based Nanoparticles

In the last few years, several *in vitro* and i*n vivo* studies have described different NP DDS used in gene delivery including lipid, inorganic materials and polymers (*Table* 1–2) and some that have included delivery technologies using non-viral carriers for appropriate safe delivery of miRNAs for different diseases (Fortunato et al. 2014).

Table 1– 2: Examples of nanocarriers used for gene delivery

Type of NPs		**Oligonucleotide**	**Disease type**	**Particle size (nm)**	**Reference**
Polymer based NPs	Polyethylene-imine (PEI)	miR-145 and miR-33a	Colon cancer	N/A	(Ibrahim et al. 2011)
	Chitosan	siRNA	Lung cancer	40-600	(Howard et al. 2006a)
	poly(lactic-co-glycolic acid)- Polyethylene-imine (PLGA-PEI)	miR-26a	Liver cancer	60	(Liang et al. 2011)
	Dendrimers (Poly amidoamine)	miR-21	Glioblastoma	<100	(Ren et al. 2010)
Inorganic NPs	Gold	miR-205 and miR-20a	Prostate Cancer	13	(Hao et al. 2011)
	Quantum dots	miR-491	Breast cancer	10	(Yoon et al. 2010)
	Silicon oxide	DNA	Ovary	50	(Liu et al. 2009)
	Iron oxide	siRNA	Cancer	15	(Lee et al. 2009)
Lipid based NPs	Cationic lipids	miR-107	Head and neck cancer	150.1	(Piao et al. 2012)

1.12 Nanoparticle surface charge and adsorption

Treatment of various diseases face hurdles for the efficient delivery of drugs necessary to alleviate the associated symptoms and/or eradication such as cellular uptake, degradation and the ability to target specific cells (Pack et al. 2005). A great deal of interest has been shown in the viability of NPs for gene delivery and specific cell targeting (Green et al. 2007). However, uncoated NPs result in poor bio-compatibility and bio-distribution.

The physicochemical characteristics of NPs such as the electrostatic charge on their surface affects interactions between the particles and the cell's surface. The modification of NPs' surfaces was shown to have an effect on particle uptake and bio-distribution. Moradi *et al.* modified NPs surface by adsorption of ligand which affects the level of cell internalisation and enhanced the NPs cellular uptake (Moradi et al. 2012). Polycationic NPs with hydrophilic polymers containing amine groups, interacted with negatively charged phosphate groups of nucleic acids led to neutralisation and increase in bio-distribution (Hwang and Davis 2001) (Garnett 1999).

Coating NPs with lecithin and albumin can improve cellular uptake due to the electrostatic charge, where the positively charged particles bind to a negatively charged membrane and undergo endocytosis. Zeta Potential is considered as an important property in measuring the particle surface that helps in particle adsorption, cell interaction and delivery. The electrical potential and surrounding surface charge prevents aggregation of NPs (Somasundaran et al. 2004).

Nucleic acids can bind and adsorb onto the cationic NPs via electrostatic interactions. Studies revealed that additionally attaching molecules such as chitosan, polyethylene-imine (PEI) or cationic lipids such as dioleoyltrimethylammonium propane (DOTAP) promote siRNA transfection efficacy. These cationic additives can be added to the NPs' surface pre or post

formation. DOTAP has been successfully used for the modification of single-walled carbon nanotubes surfaces which lead to efficient small nucleic acid loading which improved cellular interactions (Li et al. 2014) and formed lipoplexes with negatively charged small nucleic acids when used for siRNA delivery *in vitro* (Taetz et al. 2009). The NPs surface modification lead to high efficiency accompanied with safe siRNA delivery (Yezhelyev et al. 2008). At LJMU we have been investigating adsorption of macromolecules onto polymer based NPs to maintain stability and structural activity. An example is PGA-co-PDL NPs adsorbed bovine serum albumin (BSA) onto the surface as proof of successful protein adsorption for subsequent development as a NP vaccine delivery system (Kunda et al. 2014b). The coating of NPs with macromolecules can control many features such as release rate, cytotoxicity and cellular uptake (Yogasundaram et al. 2012). In fact, the small nucleic acids adsorption on NPs provided an effective potential DDS as incorporating siRNA during the chemical synthesis of siRNA-conjugated NPs may affect small nucleic acid integrity. The siRNA must remain as a duplex to bind to RISC and cause gene silencing (Fire et al. 1998). However, other methods that added siRNA directly during synthesis lead to siRNA being affected by high temperature, and strong solvents resulting in the loss of function (Soutschek et al. 2004).

1.13 Dry powder microcarriers

Inhaled NPs in the dry powder form smaller than 1 µm (diameter) are not capable of reaching the alveolar part of the lung and are likely to be exhaled during inspiration. However, particles larger than 10 µm in diameter are most likely to deposit on the throat tissue and sediment in upper lung mucus (Heyder et al. 1986). Therefore, the ideal particle size for optimal particle deposition in the deep lung ranges from between 1 to 5 µm in diameter (Sakagami 2006). Due to these limitations, the NPs required for cellular uptake cannot be aerosolised effectively. Hence, NPs require formulation within an aerosolisable micron-sized carrier for efficient pulmonary delivery.

Spray drying can be used to formulate NPs and naked drugs into micro-sized dry powders providing a mechanism for delivery to the lung via dry powder inhalation (DPI) (Jensen et al. 2012). There are three different types of inhalation devices available for pulmonary delivery; nebulizers, pressured metered dose inhalers (MDI) and dry powder inhalers (DPIs). DPIs are the most common and offer advantages such as; overcoming issues with solubility, bioavailability, and stability, compared to other inhalation modes. Spray-drying parameters can be optimised to achieve microparticles with a desirable particle size, particle size distribution and aerosolisation properties suitable for pulmonary delivery (Shoyele and Cawthorne 2006).

1.13.1 Dry powder inhalers in gene delivery

Carrier particles containing polymeric NPs have been used to deliver pDNA to the lung. Incorporating NPs (mean diameter 100–250 nm) containing pDNA into mannitol microparticles was successfully achieved by Takashima *et al.* and subsequently delivered to the bronchial and alveoli segments of the lung using DPI (Takashima et al. 2007). Spray drying of small nucleic acids also showed potential for inhalation (Jensen et al. 2010). Nanocomposite microparticles (NCMPs) of PLGA nanospheres containing siRNA were formed by Ditte Marie *et al.* using a variety of excipients including trehalose, mannitol and lactose. The PLGA-siRNA NPs were spray dried producing dry powders of low moisture content and a desired inhalable aerodynamic particle size for inhalation. Importantly, the siRNA remained biologically active (Jensen et al. 2010).

1.14 Dry powder preparation techniques

There are several approaches to producing dry powders e.g freeze drying (Abdelwahed et al. 2006), spray drying (Pilcer and Amighi 2010), spray freeze drying (Shoyele and Cawthorne 2006) and super critical fluid techniques (Johnson 1997, Kaialy and Nokhodchi 2015, M Al-fagih et al. 2011). These methods can be optimised to provide desirable attributes such as

narrow particle size distribution, improved dispensability, enhanced drug stability, optimised bioavailability and controlled release (Kunda et al. 2013, Kunda et al. 2015b), The different techniques can be compared in terms of cost, yield, powder particle size and morphology (*Table* 1–3). To date, there are no studies reporting the use of these dry powder techniques with miRNA. However, some studies reported using these techniques on other nucleic acids such as siRNA (YT Chow and KW Lam 2015) suggesting formulated miRNA will be suitable for inhalation.

1.14.1 Spray drying

Spray-drying is a one-step drying technique in which dry powder can be produced by converting the liquid solution to dry powder through mixing the evaporated liquid with a drying hot gas medium. The spray-drying technique has four distinct stages; atomisation, spray air contact, drying and separation (Pilcer and Amighi 2010). The advantage of this technique compared with the milling process, is the ability to generate higher respirable particles with a spherical shape in terms of decreasing drug and carrier adhesive forces, and more particle size distribution homogeneity (Steckel and Brandes 2004). All these characteristics have an effect on particle deposition, particle adhesion and drug delivery in respiratory airways. Such properties can be achieved by controlling spray-drying parameters such as solvent composition, coating excipients, drying and gas feed rate, solute concentration, liquid viscosity, solution feed rate and humidity (Johnson 1997).

Another advantage with this technique is the propensity for laboratory-scale work and the higher drug loading ability (Peltonen et al. 2010). Spray drying using different carriers has been used widely for the preparation inhalable dry powders for delivering peptides, proteins, genes, and small nucleic acid delivery. Jensen *et al*. used mannitol as an excipient to prepare dry powders containing PLGA NPs loaded with siRNA to target lung disease (Jensen et al. 2012).

The study found that these dry powders released siRNA to lung tissue but didn't alter the gene silencing activity. This may be due to the thermal and mechanical stresses during the manufacturing process, which may affect the nucleic acid transfecting efficacy. However, DPI is still a promising technique for inhaled nucleic acid dependant on choosing the right excipient and vector as protective agents (Berghe et al. 2000, YT Chow and KW Lam 2015).

Table 1– 3: Different types of techniques to produce dry powder

Method	Cost (commercial)	Powder Yield	Powder particle size and morphology
Spray Drying	Cost-effective (expensive in early stages), commercially available (Peltonen et al. 2010)	Relatively low recovery approximately 50%, It may increase depending on type of spray dryer (> 70 %) formulation (Shoyele and Cawthorne 2006)	Small, within range of inhalation and spherical shape (Liang et al. 2014)
Spray Freeze drying	High, and time consuming (Shoyele and Cawthorne 2006).	High recovery (YT Chow and KW Lam 2015)	Controllable size, relies on the excipients used and spherical in shape (Mohri et al. 2010, Amorij et al. 2008)
Super Critical fluid drying	Commercially, less available equipment (YT Chow and KW Lam 2015, Gradon and Sosnowski 2014)	High, approximately 80 % (Okamoto et al. 2005)	Controllable, depends on the excipients (M Al-fagih et al. 2011)

1.15 Thesis Aim and objectives

Aim

To design, formulate and characterise miRNA-containing NPs formulated as an inhalable dry powder microcarrier (*Fig* 1–7) for the treatment of Chronic Obstructive Pulmonary Disease (COPD).

Objectives

The aim of the thesis was achieved by the following objectives to;

1. Formulate and develop polymeric NPs for miRNA delivery.
    a. Design and prepare optimized cationic PGA-co-PDL NPs using the cationic surfactant, DOTAP.
    b. Investigate the influence of particle size and charge on lung cell uptake and toxicity.
    c. Adsorb miRNA onto the optimum NP formulation.
    d. Investigate the cellular uptake and the biological functionality of the miRNA-NPs.
2. Formulate NCMPs for pulmonary delivery.
    a. Incorporate optimum NPs into NCMPs via spray drying with L-leucine and mannitol as microcarriers.
    b. Evaluate NCMPs containing miRNA-NPs for inhalation in terms of size, morphology, moisture content and aerosol performance.
3. Evaluate *in vitro*, miRNA-NP NCMPs.
    a. Investigate miRNA functionality in terms of cellular uptake, interactions and gene knockdown.

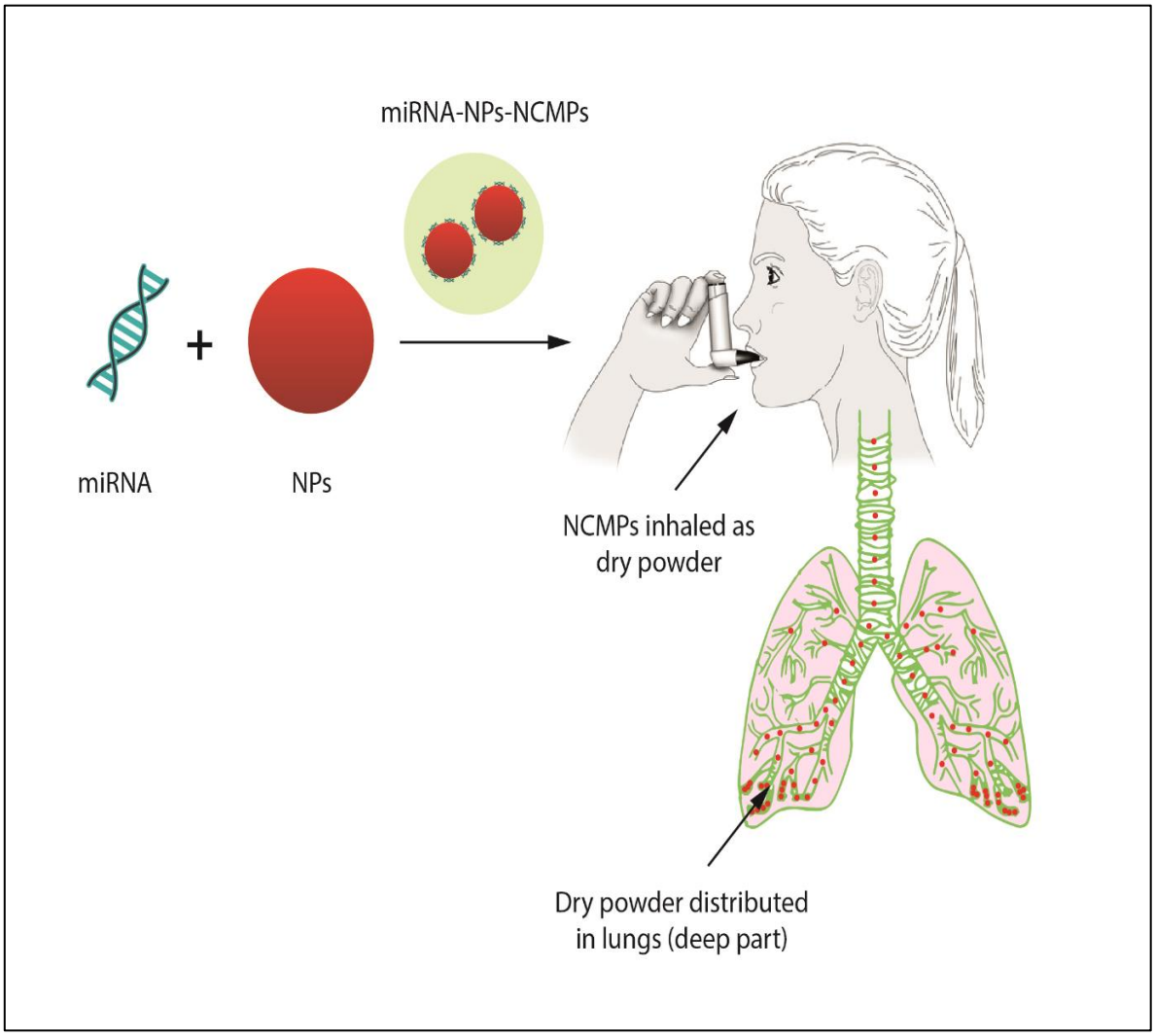

Figure 1 – 7. Illustration of pathway for formulation and pulmonary delivery of miRNA-NPs incorporated into NCMPs.

2. Materials and General Methods

Materials

Materials and Properties	Supplier
Poly (vinyl alcohol) PVA, Mw of 13- 23 kDa 87-89% hydrolysed	Clariant GmbH, Frankfurt am Main, Germany
Divinyl adipate (DVA)	Fluorochem, UK
Novozyme 435 (a lipase lipase from *Candida antartica* immobilized on a microporous acrylic resin)	Biocatalytics, USA
Dichloromethane (DCM), Tetrahydrofuran (THF) Methanol (MeOH), Chloroform, Dimethyl sulfoxide (DMSO)	Fischer chemicals (Fischer Scientific, UK)
8 well chambered slides Opti-MEM® I Reduced Serum Medium Anti-actin beta rabbit monoclonal antibody Anti-Rb IgG (H+L) cross adsorbed secondary antibody HRP conjugates	Thermo Fisher, scientific, UK

SuperSignal™ West Dura Extended Duration Substrate	
Bicinchoninic acid (BCA) protein assay kit	
Bovine serum albumin (BSA)	
L-glutamine	
6-well and 24-well tissue culture plates	
25 and 75 cm^2 tissue culture flasks	
Dioleoyltrimethylammoniumpropane (DOTAP)	Avanti Polar lipids, Alabaster, AL, USA
Glycerol	Sigma Aldrich (now Merck), UK.
ω-pentadecalactone (PDL)	
RPMI-1640 medium with L-glutamine and $NaHCO_3$,	
Thiazolyl blue tetrazolium bromide (MTT),	
Phosphate Buffered Saline PBS tablets, pH 7.4,	
Nile red and	
RNase-free diethyl pyrocarbonate (DEPC) water	
Paraformaldehyde (PFA)	

Triton X-100

10× TBE buffer (Tris-Borate electrophoresis

buffer: 108 mg/mL Tris base,

55 mg/mL Boric acid,

9.3 mg/mL EDTA)

9.4 Ethidium bromide (5 mg/mL) (EtBr)

LB broth (Lennox) powder

Anti-Mouse IgG (Fab specific) antibody

Anti-Actin-Beta antibody, Rabbit monoclonal

Penicillin-Streptomycin

Trypsin-EDTA

Sodium deoxycholate

L-leucine

D-Mannitol

Complete™ ULTRA Tablets, Mini,

EASYpack Protease Inhibitor Cocktail

Sodium orthovanadate

ß-mercaptoethanol

Triton X-100

sodium dodecyl sulphate (SDS) Tris PH 8.0 Unlabelled miR146a mimics	
Human adenocarcinomic alveolar basal epithelial cell line (A549). Calu-3 cell lines Eagle's Minimum Essential Medium (EMEM)	ATCC (Middlesex, UK)
A synthetic miR-146a mimic with 6-carboxyfluorescein (FAM) -label on the sense 5' FAM-CCGGGCAAUUCAGUUUCUACA-dTdT-3' with the sequence: sense 5' FAM-CCGGGCAAUUCAGUUUCUACA-dTdT-3', antisense 5' dTdT-GGCCCGUUAAGUCAAAGAUGU-3'	Eurogenetec, UK
Plasmid pMirTarget vector contains Firefly Luciferase and empty pMirTarget vector (Control) cDNA generations, PCR reagents and primers (IRAK, TRAF) 3'-UTR reporter	Origene
Luciferase assay kit (cell lysis reagents) SOC media	Promega's, UK

Plasmid maxi prep purification kit	Qiagen, UK
Interleukin-1 receptor-associated kinase 1 (IRAK1) RT² qPCR Primer	
TNF receptor-associated factor 6 (TRAF6) RT² qPCR Primer	
RT² qPCR Primer Assay for Human GAPDH	
miScript SYBR® green PCR KIT	
miScript II RT Kit	
RNeasy Plus Mini Kit	
AllPrep DNA/RNA/miRNA Universal Kit	
Lipofectamine 3000 and reagents	Invitrogen (Life technology, UK).
4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI)	Invitrogen, Ltd.,UK.
Precision Plus Protein™ Kaleidoscope™ Protein Standards	Bio Rad, UK
Mini-PROTEAN® TGX Stain-Free™ Precast Gels	
Nitrocellulose membranes	
Mouse monoclonal Anti-IRAK1 antibody	Abcam, UK
Rabbit monoclonal Anti-TRAF6 antibody	
10 X Tris/Glycine/SDS	Geneflow Ltd , UK

Foetal Bovine Serum (FBS)	Biosera, UK.
96-well tissue culture plates, black, flat bottom	Griener Bio-one, UK
213 - pIL8/d2EGFP and 269 - pDSRED mono promoters	A kind gift from Dr. Endre Kiss-Toth, Sheffield, UK.
Interleukin 8 (IL8) promoter and tumor necrosis factor-a (TNFa)	Peprotech, UK.

General Methods

2.1 Polymer synthesis and characterisation

Poly (glycerol adipate-co-ω-pentadecalactone), (PGA-co-PDL) was produced using an enzyme catalysed condensation and ring opening co-polymerisation reaction of equimolar quantities of the three monomers: divinyl adipate, glycerol, and ω-pentadecalactone *(Fig.* 2–1*)* as described by Thompson *et al.* (Thompson et al. 2006). Briefly, a dry 250 ml two-necked round bottom flask, equipped with a central stirrer attached to a teflon paddle and open condenser, was charged with equal molar amounts (125 mmol) of divinyl adipate (DVA), pentadecalactone (PDL), glycerol and 25 ml of Tetrahydrofuran (THF). The flask was immersed in a water bath at 50 °C left to stir (210 rpm) for 20 minutes to equilibrate the temperature. Novozyme 435 (1.25 g) was added, using the remaining THF (10 ml) to wash all the solid into the flask and the mixture stirred for 6 hours. Warm dichloromethane (DCM) (300 ml) was added to the flask followed by Buchner filtration to remove the solid enzyme. The solvent was then removed by rotary evaporation (60 °C) until around 20 ml of DCM remained. Methanol (100 ml) was added to precipitate the polymer and leave unreacted monomers and oligomers in solution which were

filtered using Buchner filtration. The precipitate was broken up and left to dry under vacuum for 24 h before grinding into a powder and storing in a desiccator at room temperature.

For further explanation to *Fig.* 2–1, the PGA-co-PDL was prepared from co-polymerisation of three monomers; PDL, DVA and glycerol with aid of a hydrolytic enzyme (Novozyme 435) to produce PGA-co-PDL as a linear polyester. The enzyme chosen for this polymerisation reaction has a regio-selectivity for primary hydroxyl (OH) groups (Kline et al. 1998).

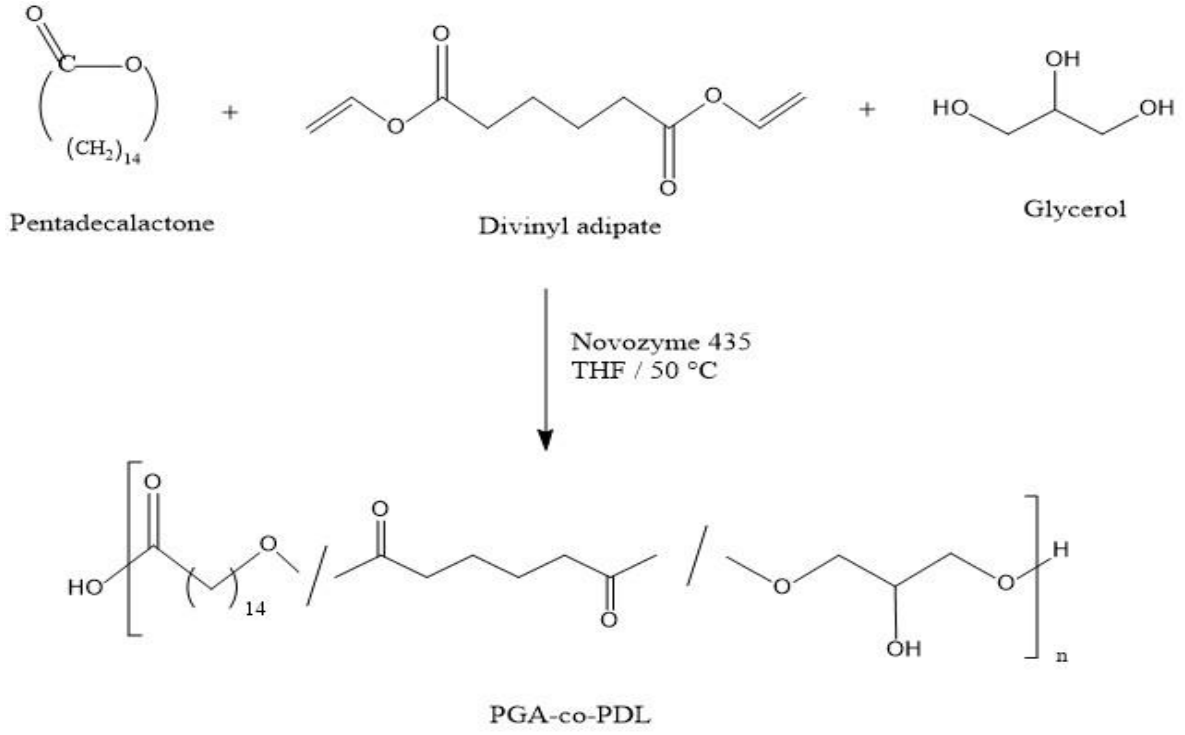

Figure 2– 1. The reaction scheme for the enzymatic synthesis of PGA-co-PDL (Thompson et al. 2006).

The PGA-co-PDL was analysed by Nuclear Magnetic Resonance (^{1}H NMR), using a Bruker Avence 300 MHz spectrometer, inverse probe with B-ACS 60 and auto sampler with gradient shimming. Gel permeation chromatography (GPC) using a Viscotek TDA Model 300

(OmniSEC3 software), the system was fitted with two PLgel 5 µm MIXED-D 300x7.5 mm columns (Varian, Polymer Laboratories, UK), stored in the detector oven at 40 °C, and a flow rate of 1 ml/min using chloroform as a solvent. The system was pre-calibrated with polystyrene standards.

2.2 Nanoparticle preparation and miR-146a adsorption

PGA-co-PDL nanoparticles (NPs) were prepared using an oil in water (o/w) single emulsion method as previously described (Kunda et al. 2014a). Briefly, 200 mg PGA-co-PDL and optimum 15 % dioleoyltrimethylammoniumpropan (DOTAP) were dissolved in 2 ml DCM, and upon adding 5 ml 10 % w/v poly (vinyl alcohol) (PVA) (1st aqueous solution) drop wise on ice using probe sonicated (20 µm amplitude) for about 2 minutes to obtain an emulsion. This was immediately added drop wise to 20 ml of a 2nd aqueous solution (0.75 % w/v PVA) under magnetic stirring at a speed of 500 RPM and stirred at room temperature for 3 h to facilitate the evaporation of DCM. The NPs suspensions were collected by ultracentrifugation at 35,000 x g, for 40 min at 4 °C using (Beckman L-80 Ultracentrifuge, UK) and washing twice with distilled water (4 ml) to remove excess surfactants.

For adsorption, miR-146a was adsorbed onto the NPs suspension obtained after centrifugation following removal of excess surfactant. 6-carboxyfluorescein (FAM) labelled miR-146a, and unlabelled miR146a-NPs (for IL-8 Promoter Reporter assay), (40 µg) was added to a 1 ml solution of RNase free water containing 10 mg of NPs (to obtain a final NP:miRNA weight ratio of 250:1) and mixed using a HulaMixerTM Sample Mixer (Life technologiesTM, UK) at 20 rpm and 25 °C for 2 h. RNase free water was then added to a total volume of 4 ml prior to separation of non-adsorbed miR-146a by a single ultracentrifugation at 35,000 x g, for 40 min at 4 °C using (Beckman L-80 Ultracentrifuge, UK) (*Fig* 2–2). The concentration of free miR-146a in the supernatant was analysed by UV prior further use.

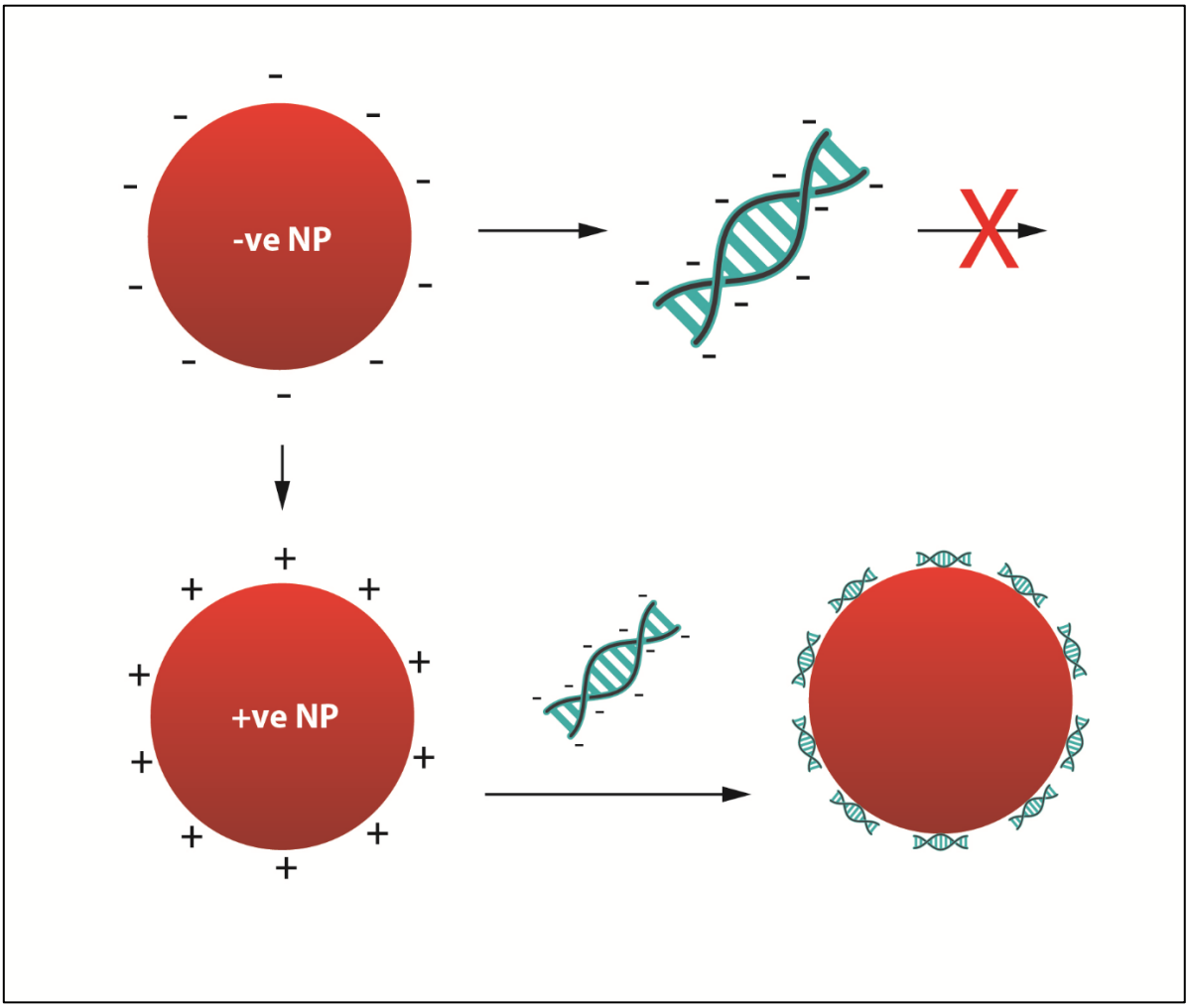

Figure 2– 2. Adsorption of negatively charged miRNA onto positively charged NPs.

2.3 Nanoparticle characterisation

2.3.1 Particle size and zeta potential

The mean particle size and polydispersity index (PDI) were analysed by dynamic laser scattering, and the surface charge of the particles was determined by measuring the zeta-potential (Zetasizer Nano ZS, Malvern Instruments Ltd, UK) in triplicate and for three different NPs batches. NPs (10 mgs) were diluted with 4 ml distilled water and 1 ml of the diluted sample was loaded into a measuring cuvette and the measurements were recorded at 25 °C.

2.3.2 miRNA adsorption

To determine the amount of miRNA adsorbed to the NPs, the concentration of adsorbed miR-146a determined indirectly from the difference in miR-146a concentration before and after loading by UV absorbance at 260 nm using a NanoDrop 2000C (Thermo Fisher Scientific, and USA). In addition, the concentration of 6-carboxyfluorescein (FAM) labelled miR-146a was determined by fluorescence using a plate reader (CLARIOstar®, BMG) at λEX: 495 nm; λEm; 520 nm. In both cases, a calibration curve was generated using known concentrations of miR146a. Excitation wavelength 496 nm green laser.

2.4 Real time quantitative polymerase chain reaction RT-qPCR

2.4.1 RNA extraction

miR-146a-NPs/NCMPs in serum free medium were incubated with A549 cells for 1 h. The miR146a-NPs/NCMPs mixture was then replaced with complete medium and the cells incubated for 24 h. The total RNA was extracted from the cells using the miRNA mini kit II according to the manufacturer's instructions (Qiagen, Manchester, UK). Briefly, cells were trypsinised by trypsin-EDTA, washed with PBS twice and collected by adding PBS and serum free medium and collecting the cells by centrifugation at 300 x g for 5 minutes. The cells were disrupted by adding buffer RLT plus containing β-mercaptoethanol (100:1). The homogenised

lysate was vortexed for 30 s and transferred to an AllPrep DNA spin column and centrifuged for 30 s at 8000 x g. The aqueous flow-through was mixed by pipetting with 350 µl of 70 % ethanol. a 700 µl of sample was transferred to an RNeasy spin column and centrifuged for 15 s at 8000 x g. The aqueous flow-through was discarded and 700 µl buffer RW1 was added to RNeasy spin column and centrifuged for 15 s at 8000 x g. The flow-through was discarded and 500 µl buffer RPE added to the RNeasy spin column, and centrifuged for 15 s at 8000 x g. The RNeasy spin column was washed again with 500 µl buffer RPE and centrifuged for 2 min at 8000 x g then re-centrifuged again for 1 min at 8000 x g to dry and eliminate any possible carryover of buffer RPE. The RNeasy spin column was placed in a new microcentrifuge tube and 40 µl RNase-free water added followed by centrifugation for 1 min at 8000 x g to elute the RNA. RNase-free water (40 µl) was added and then centrifuged for 1 min at 8000 x g. The RNA extract was tested for purity and concentration at 260 nm using a NanoDrop spectrophotometry (Thermo scientific).

2.4.2 cDNA generation

Qiagen miRScript reagents were used to convert mature mRNA to cDNA (*Fig*. 2–3). miScript HiFlex buffer was used with 1 µg RNA and incubated with a master mix of reverse transcriptase, dNTPs and both oligo-dT and random primers (*Table* 2–1). The reaction was heated to 37°C for 60 min followed by 5 min incubation at 95°C to inactivate the reverse transcriptase. cDNA was stored at -20°C for further experimental work.

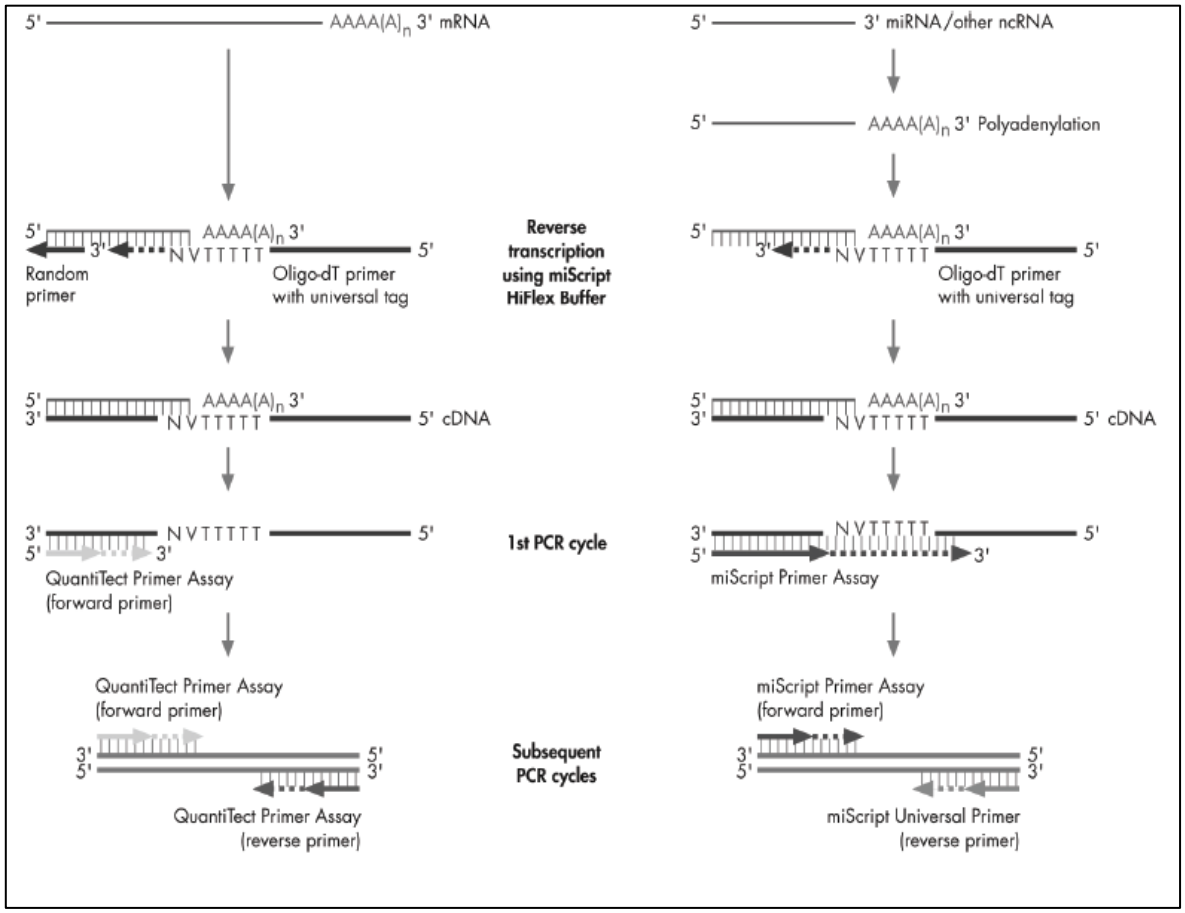

Figure 2– 3. Conversion RNA into cDNA in miScript HiFlex buffer. In a reverse transcription reaction with miScript HiFlex Buffer. mRNAs are converted into cDNA by reverse transcriptase using both oligo-dT and random priming. Detection of mRNA can be performed using real-time PCR (Qiagen miScript PCR handbook).

Table 2– 1. Reverse transcription reaction components

Component	Volume
5x miScript HiFlex Buffer	4 µl
10x miScript Nucleics Mix	2 µl
miScript Reverse Transcriptase Mix	2 µl

2.4.3 Polymerase chain reaction

cDNA reaction was diluted with 200 ul of RNAse-free water. Levels of *IRAK1* and *TRAF6* transcripts were assessed using RT^2 qPCR Primer Assays in 20 µl reactions composed of 10 µl SYBR Green PCR master mix, 2 µl primers, 2 µl diluted cDNA and water to 20 µl. The reactions were amplified for three-step method (*Table* 2–2). The expression of *IRAK1* and *TRAF6* was normalised to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression. Data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 2– 2. Cycling conditions for RT-PCR

Step	Time	Temperature
PCR initial activation step	15 min	95 °C
Denaturation	15 s	94°C
Annealing	30 s	55 °C
Extension	30 s	70 °C

2.5 Effect of miR-146a-NPs/NCMPs on protein expression

2.5.1 Western Blot

A549 cells were seeded at a density of 3.8×10^5 cells per well in a 6 well plate. After 24 h growth, the cells were incubated in serum free medium with 1 ml of pre-mixed freshly prepared miR146a-NPs and miR146a-NP-NCMPs at a range of concentration (0 – 0.625 mg/ml), (*Table* 2–3) for 1 h. The miR-146a-NPs/NCMPs mixture was then replaced with complete medium and the cells incubated for 24 h and 48 h respectively. The medium was discarded and the plates were rinsed twice with PBS, followed by adding lysis buffer (Radio Immuno Precipitation Assay, RIPA) on ice. RIPA buffer contains 150 mM sodium chloride, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate (SDS), 50 mM Tris PH 8.0, sodium orthovandate and protease inhibitor tablet (Roche), then left to agitate for 15 minutes at room temperature. A plastic cell scraper was used to scrape and collect the homogenate which was then centrifuged at 10,000 x g for 1 min at 4˚C and the supernatant collected. The protein content of the supernatant was determined using a bicinchoninic acid assay, BCA (Sigma, UK). Samples (4 parts) were diluted with an equal volume of 1 part Laemmli buffer (0.5 M Tris-HCL PH 6.8, glycerol, SDS, 0.25 % bromophenol blue, β-mercaptoethanol) and boiled for 5 min at 95 ˚C. For each lane protein standards and extracts (20 µg) were resolved on 12 % precast gel (Bio-Rad, UK) and transferred to nitrocellulose membranes (Bio-Rad, UK) in Tris-glycine buffer with 20 % methanol. After transfer, membranes were blocked for 1 h at room temperature in 5 % fat free milk in TBST (TBS and Tween-20). The membranes were then washed for 3 x 5 min in TBST before probing overnight at 4˚C with antibodies anti-IRAK1 (1:1000, Abcam), anti-TRAF6 (1:1000, Abcam), β-Actin (1:1000, ThermoFischer). Membranes were washed for a further 3 x 5 min in TBST then incubated with horseradish Peroxidase conjugated secondary antibodies (1:5000, sigma) for 1 h at room temperature. After a further 3 x 5 min washes in TBS, membranes were exposed in

a chemiluminescence Pierce ECL reagents (Thermo Fisher,UK) according to manufacturer's protocol. Densitometry was performed using ImgaeJ software, and protein of interest values were used to normalize against β-Actin values.

Table 2– 3. Shows miR-146a-NPs/NCMPs concentrations used

Lane	NPs/NCMPs concentration (mg)	miR-146a concentration (µg)
1	Control	Control
2	0.078	0.25
3	0.156	0.50
4	0.321	1.00
5	0.625	2.01

2.6 Statistical analysis

All statistical analysis was 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 $p<0.05$. Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, Inc., San Diego,CA). All values are expressed as their mean ± standard deviation (SD).

3. Polymeric Nanoparticles for the Delivery of miRNA to Treat Chronic Obstructive Pulmonary Disease (COPD)

3.1 Introduction

Small nucleic acids such as miRNA have recently gained significant attention as a new class of therapeutics for various genetic diseases. Modulation of miRNA expression and function represents a promising strategy for therapeutic intervention in disorders such as inflammatory lung disease, particularly COPD. There is a relationship between miRNA (miR-146a) and inflammatory response in COPD pathogenesis (Sato et al. 2010).

Pulmonary drug delivery is a non-invasive route that can be utilised to deliver molecules to lung cells. However, one of the main issues with delivering miRNAs to the inflammatory lung tissue is that upon delivery of the naked, negatively charged miRNAs to the site of action, the molecules cannot cross the anionic cell membranes, and are degraded by physiological enzymes (Guzman-Villanueva et al. 2012). Therefore, the challenge is to design a delivery system capable of protecting and transporting the miRNA through these biological barriers to reach the site of action (Yin et al. 2014).

Viral vectors have historically been used as carriers for small nucleic acid delivery but have the disadvantagee of causing an immunogenic inflammatory repsonse (Wang et al. 2012b). Consequently, more attention has been given to non-viral delivery vectors such as biodegradable polymeric NPs. These NPs have previosuly been considered for delivery small mucleic acids (Labiris and Dolovich 2003b).

Polymeric NPs can offer properties such as targeted delivery, sustained release, biodegradation, and low toxicity (Panyam and Labhasetwar 2003). NPs have an electrostatic charge on their surface that affects the interaction between the particles and cell membrane surfaces as the neutralization of the charge can lead to increased bio-distribution. Furthermore, the surface electrostatic charge can enhance particle uptake into cells when positively charged particles bind to the negatively charged cell membrane and undergo endocytosis (Somasundaran et al.

2004). Additionally, the electrical potential and surrounding surface charge prevent aggregation of NPs (Somasundaran et al. 2004) and can interact with negatively charged small neuclic acid leading to particle uptake and increasing bio-distribution (Hwang and Davis 2001, Hamdy et al. 2011). Compared to encapsulation of nucleic acids within NPs, which includes exposure to solvent, sonication and mechanical stress during processing, the adsorption of small nucleic acid s onto the NP surface can provide enhanced stability and activity, (Cun et al. 2010). The difficulty in loading of miRNA into NPs can be attributed to the hydrophobic nature of NPs and the absence of electrostatic interaction between miRNA and NPs.

The cationic lipid dioleoyltrimethylammoniumpropane (DOTAP) has previously been used as a transfection agent, forming lipolexes with negatively charged small-interfering RNA and increasing their cellular interaction (Li et al. 2014, Ozpolat et al. 2010). It has also be used to preapre cationic NPs that showed biocompitability with small nuclic acid and genes (Kumar et al. 2012, Díez et al. 2009b). Therefore, cationic NPs may have the potential to serve as small nucleic acid carriers.

PGA-co-PDL NPs has previously been investigated as carriers for both small molecules and macromolecules by encapsulation of the molecules within the particle (Kallinteri et al. 2005, Gaskell et al. 2008, Tawfeek et al. 2013) or adsorption to the surface (Kunda et al. 2014b, Tawfeek et al. 2011).

3.2 Aim

The aim of this study was to adsorb miRNA on to the surface of cationic PGA-co-PDL NPs, and evaluate toxicity, and cell uptake.

To obtain the aim of the study, the following objectives were considered;

1. Design and prepare optimized cationic PGA-co-PDL NPs using cationic surfactants (DOTAP).
2. The influence of particle size and charge on A549 and Calu-3 lung cells, toxicity and cell uptake.
3. Adsorption of miR-146a on to the surface of cationic PGA-co-PDL NPs.

3.3 Methods

3.3.1 Polymer synthesis and characterisation

The PGA-co-PDL was synthesised and characterised as described in section 2.1.

3.3.2 Nanoparticle preparation and miRNA adsorption

PGA-co-PDL NPs were prepared using an oil in water (o/w) single emulsion method as described in our previously published method, incorporating 0.4 mg Nile red dye in the inner organic phase for visualization experiments (Kunda et al. 2014b) and different concentrations of DOTAP to prepare cationic NPs (DOTAP is % w/w of polymer) (*Table* 3–1).

Different quantities of miR-146a (10, 20, 30 and 40 µg) were added to a 1 ml solution of RNase free water containing 10 mg of NPs (to obtain a final NP:miRNA weight ratio of 250:1 and mixed using a HulaMixerTM Sample Mixer (Life TechnologiesTM, UK) at 20 rpm and 25°C at different time points (0.5, 1, 2, 4 and 24 h). After adsorption, RNase-free water was added to a total volume of 4 ml prior to separation of free miRNA from the adsorbed miRNA by ultracentrifugation at 35,000 x g, for 40 mins at 4°C using (Beckman L-80 Ultracentrifuge, UK).

Table 3– 1. Different DOTAP to PGA-co-PDL concentration.

DOTAP Conc. (%)	PGA-co-PDL (mg/ml)	DOTAP (mg/ml)
0	200	0
5	190	10
10	180	20
15	170	30
20	160	40
25	150	50

3.3.3 Particle size, zeta potential and miRNA adsorption characterisation

The particle size, zeta potential and miRNA adsorption were characterised as detailed in section 2.3.

3.3.4 In vitro release

The miR146a-adsorbed PGA-co-PDL NPs (10 mg) were suspended in a tube containing 4 ml PBS (pH 7.4) and incubated at 37°C, rotating at 20 rpm on a HulaMixer™ Sample Mixer. At various time points, the samples were centrifuged at 35000 x g for 40 min and 1 ml of supernatant was collected for quantification, replaced with fresh aliquot (1 ml) of PBS and incubation resumed. The supernatant was analysed as described in 2.3.2. The amount of released miR146a was calculated as percentage of cumulative released miR146a to the total amount of adsorbed miR146a (Eq.1).

$$\% \text{ Cumulative miR146a released} = \frac{\text{Cumulative miR146a released}}{\text{miR146a loaded}} \times 100 \quad \text{(Eq.1)}$$

3.3.5 Cell Viability study

3.3.5.1 Cell culture and toxicity studies

Adenocarcinomic human alveolar basal epithelial cell lines (A549) were maintained in RPMI-1640 supplemented with 100 U/ml penicillin, 100 µg /ml streptomycin, 2 mM L-glutamine and 10% (v/v) fetal bovine serum (FBS). Epithelial cell lines (Calu-3) were grown in Eagle's Minimum Essential Medium (EMEM) containing balanced salt solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate and supplemented with 100 U/ml penicillin, 100 µg /ml streptomycin and 10% (v/v) fetal calf serum (not heat treated). The cell culture medium was changed every 2 days and the cells were checked under a microscope to determine the confluency and to confirm the absence of contamination. Both cells were grown in an atmosphere of 5 % CO_2 and 95 % O_2 at 37°C. The cells were trypsinised with trypsin-EDTA once 80-90 % confluency was reached, then, either passaged to a new flask or plated in 96 well plates for toxicity and transfection experiments.

3.3.5.2 MTT toxicity assay

The cytotoxicity of the NPs was determined *in vitro* with the MTT (3[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay, using A549 and calu-3 cells. MTT is a tetrazolium dye that upon reduction by mitochondrial enzymes changes colour from yellow to blue. The colour intensity is directly related to the viability of the cells. The cells were seeded in a 96-well plate in growth medium at density of 1.2×10^5 cells per well. After 24 h growth (80 % confluence), the cells were incubated with 100 µl of pre-mixed freshly prepared NPs at a range of concentration (0 - 2.5 mg/ml) using 10 % dimethyl sulfoxide (DMSO) as a positive control. The medium was then removed after 18 h and 40 µl of the MTT solution (5 mg/ml in PBS) added to each well and incubated for 2 h. The MTT medium was removed and the formazan precipitate dissolved using 100 µl DMSO. The absorbance of each well was

measured at 570 nm and the cell viability percentage calculated as the absorbance ratio between treated and blank cells (control). The assay was performed on three occasions with three replicates for each concentration.

3.3.6 Cell Imaging

3.3.6.1 Confocal and Fluorescence Microscopy

A549 cells were seeded on a 8-well chambered slide (Fisher Scientific, UK) at a seeding density of 5×10^5 cells per well and incubated overnight. The cell culture media was removed and replaced with 500 µl of FAM-labelled miR-146a-NPs and Nile Red NPs in serum free medium, RPMI-1640 for 1 h (37°C, 5 % CO_2). After this time, the media was removed from the cells to allow confocal imaging. In brief (*Table* 3–2*)*, cells were washed with 3 x PBS for 5 min each and fixed using 4% paraformaldehyde (PFA) or ice-cold (-20°C) methanol was used for 15 min. Methanol or PFA was then removed and 0.1% Triton-X 100 was added for 10-15 min at room temperature for permeabilization. In order to stain the nucleus, 1 µg/ml DAPI was added for 5 min, then removed and washed with PBS and mounting medium was added on coverslip. Confocal images were acquired using a Zeiss LSM 710 confocal microscope using an oil immersion objective 40x and 60x. Images of FAM labelled miR146a-NPs were captured using an excitation wavelength 496 nm (green laser), Nile Red NPs were captured at 543 nm (green, red and blue channels). Additionally, the slides were observed under an Olympus BX51 Fluorescent microscope. Images were collected in a 8-bit format. These procedures were performed in the dark.

Table 3– 2. Workflow for staining A549 cells for confocal microscope

Step	Treatment	Composition
1. Washing	3 x rinse, Room Temp (RT)	PBS
2. Fixation	15 min, RT	4% PFA or -20°C methanol
3. Washing	1 x Rinse, RT	PBS
4. Permeabilization	15 min, RT	0.1% Triton-X 100
5. Washing	1 x Rinse, RT	PBS
6. Nucleus stain	5 min, RT	1 µg/ml DAPI
7. Washing	1 x Rinse, RT	PBS

3.3.7 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 $p<0.05$. Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, Inc., San Diego,CA). All values are expressed as their mean ± standard deviation (SD).

3.4 Results

3.4.1 Polymer synthesis and characterisation

The PGA-co-PDL (a ratio of monomers, 1:1:1) obtained was a white powder with a molecular weight of 16 kDa, as determined by GPC. The structure of PGA-co-PDL was determined by

^{1}H-NMR as described previously (Thompson et al. 2006) (δH CDCl3, 300 MHz): 1.34 (s, 22H, H-g), 1.65 (m, 8H), 2.32 (m, 6H), 4.05 (q)-4.18 (m) (6H), 5.2 (s, H). The Infra-red spectrum showed a typical broad shallow –OH band at 3447.0 cm^{1}, –CH2 groups of DVA, PDL, at 2915.7 cm^{1}, –CH group of glycerol at 2848.4 cm^{1}, the carbonyl group of DVA and lactone monomers at 1730.7 cm^{1}, C–O group of lactone, glycerol at 1417.0 and 1164.8 cm^{1}.

3.4.2 Particle size and Zeta Potential

NPs with and without DOTAP were prepared using a single emulsion solvent evaporation method adding the DOTAP, where relevant, in the organic phase at various DOTAP to PGA-co-PDL concentration. Varying the concentration of DOTAP incorporated into the PGA-co-PDL NPs resulted in a change in particle size from 266.10±20.80 nm at 0 % (w/w) DOTAP to 197.90±1.70 nm at 20 % (w/w) DOTAP and, a change in surface charge from -18.9±0.9 mV to +16.7±0.1 mV as the DOTAP concentration increased (*Fig.* 3–1). The particle size and zeta potential of the DOTAP-NPs varied-only slightly above a concentration of 15 % (w/w) DOTAP. Therefore 15 % (w/w) DOTAP was subsequently used in all NPs formulations.

At this DOTAP concentration miR-146a loaded cationic NPs (miR-146a-NP), were the same size as unloaded NPs (244.8±4.4 nm and 242.4±0.3 nm, respectively). The charge after adsorption of miR-146a onto the NP surface ranged between +5.9 mV to +11.1 mV depending on the amount of miR-146a adsorbed, compared to +14.8±0.2 mV for unloaded NPs, showing the miR-146a loaded NPs retained a positive charged. The observed reduction in Zeta potential confirmed the miR-146a was adsorbed.

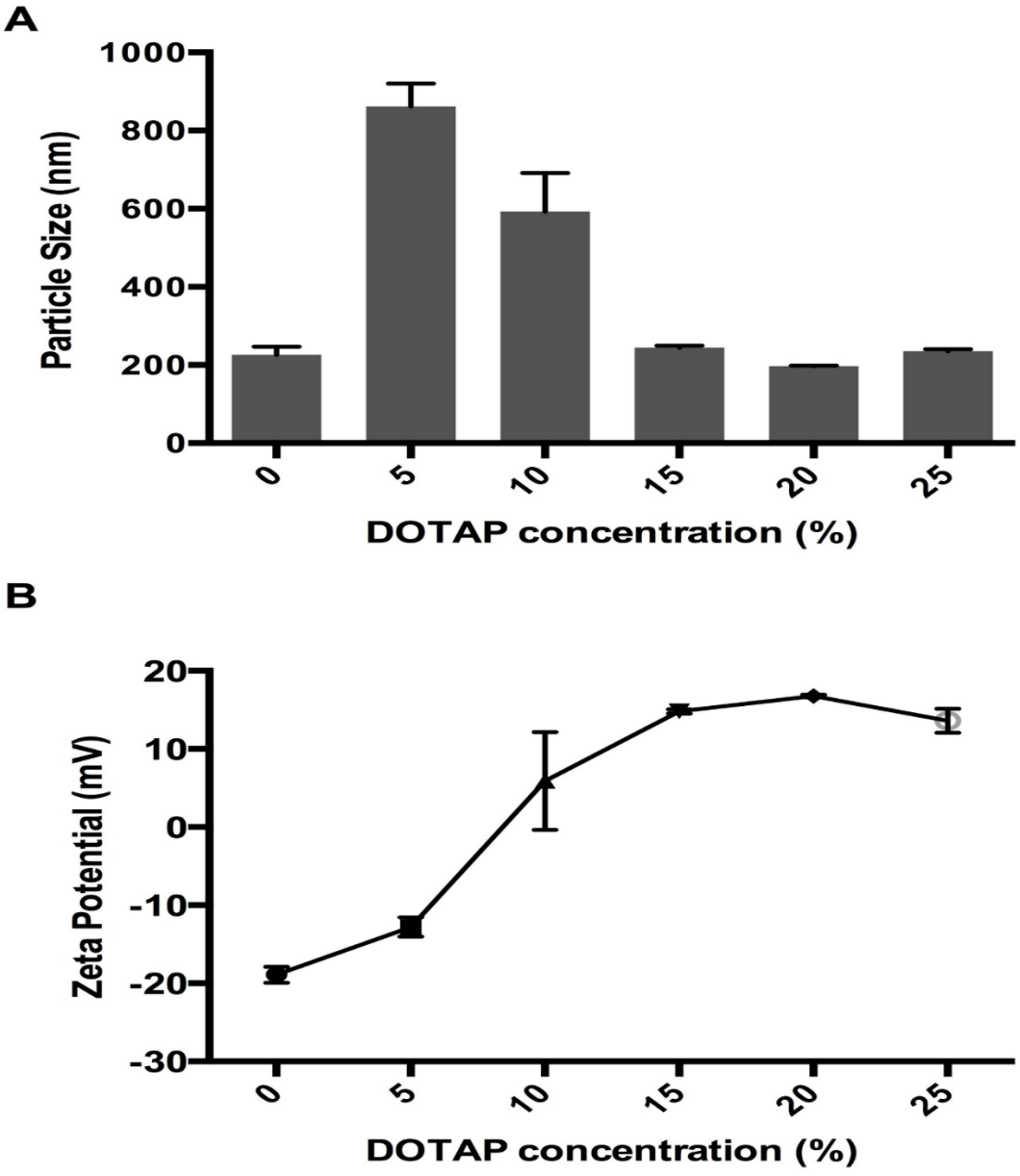

Figure 3 – 1. (A) The effect of the concentration of DOTAP on particle size of PGA-co-PDL NPs and (B) The effect of the concentration of DOTAP on the particle surface charge of PGA-co-PDL NPs. Data represented in A and B are Mean ± SD (n=3).

3.4.3 miR-146a adsorption

The 15 % (w/w) DOTAP NPs were used to study miR-146a adsorption by first using a fixed miR-146a concentration (40 µg/ml) at different time points 0.5, 1, 2, 4 and 24 h (*Fig*. 3–2A). After 0.5 h, 12.05 ± 1.3 µg of miR-146a (40 µg/ml) was adsorbed onto 10 mg of NPs. The maximum miR-146a adsorption was 36.25±0.35 µg miR-146a per 10 mg NPs after 24 h. Beyond 2 h there was no significant difference in miR-146a adsorption with a maximum of 32.25±2.0 µg miR-146a per 10 mg NPs (p <0.05, ANOVA/ Tukey's comparison). Furthermore, adsorption of miR-146a at different concentrations with a fixed time of 2 h was investigated. As shown in (*Fig.* 3–2B) over 50% of the miR-146a was adsorbed at concentrations of 20, 30 and 40 µg miR-146a. The positively charged NPs attract the negatively charged miR-146a by electrostatic interaction. Therefore, the results indicate that 15 % (w/w) DOTAP NPs can be effectively adsorbed with miR-146a.

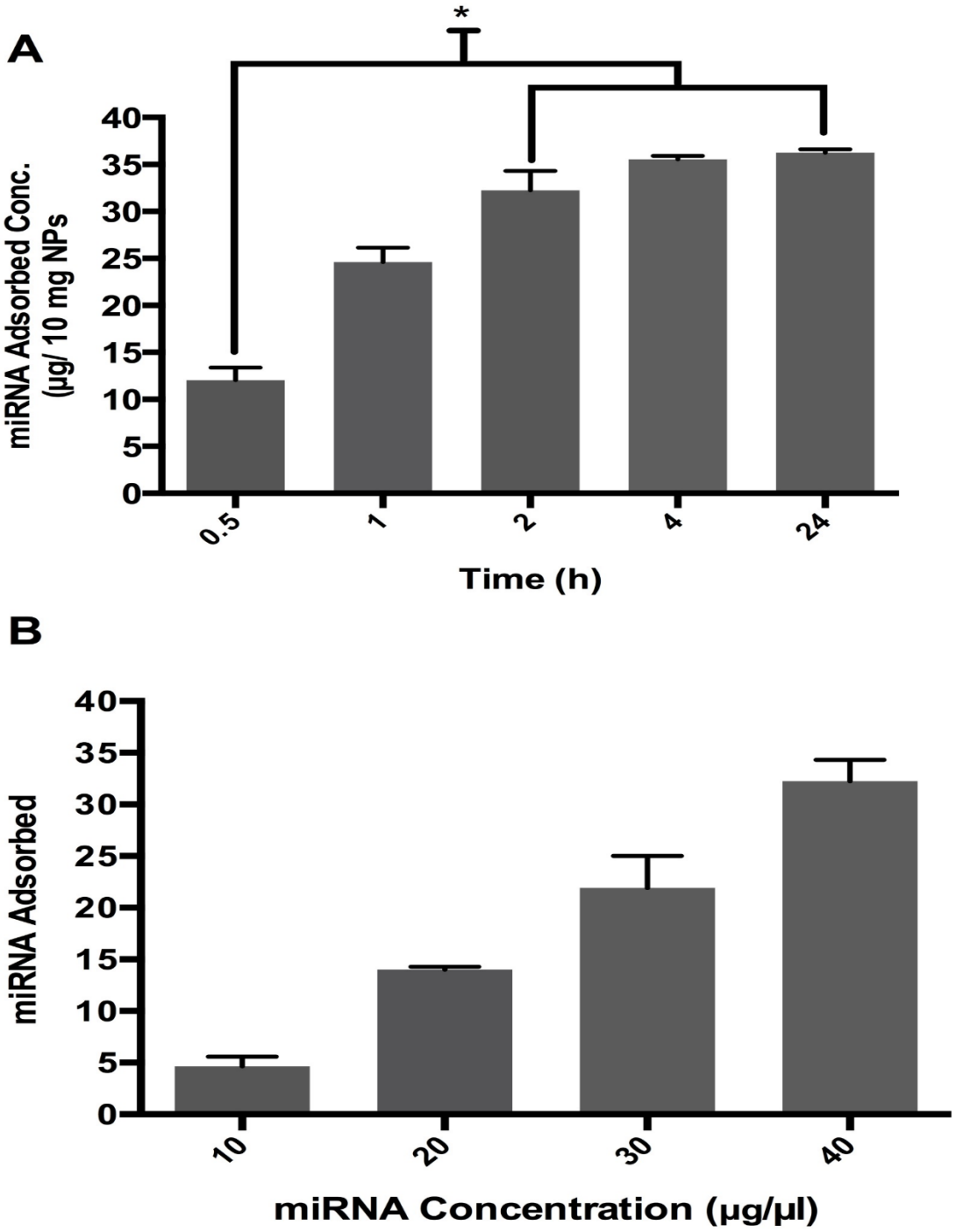

Figure 3 – 2. (A) Adsorption of miRNA (40 µg/ml) at different time points up to 24 h onto 15 % DOTAP NPs, * is p <0.05, ANOVA/ Tukey's comparison. (B) miRNA adsorption onto 15 % DOTAP NPs at various miRNA concentrations over 2 h, µg miRNA per 10 mg NPs. Data is presented as Mean ± SD (n=2).

Confirmation that the miRNA was associated with the NPs was achieved using Fluorescence microscopy. *Fig.* 3–3 indicates the fluorescently labeled NPs (Nile Red dye) with labelled FAM-miR-146a (green) were bound and adsorbed.

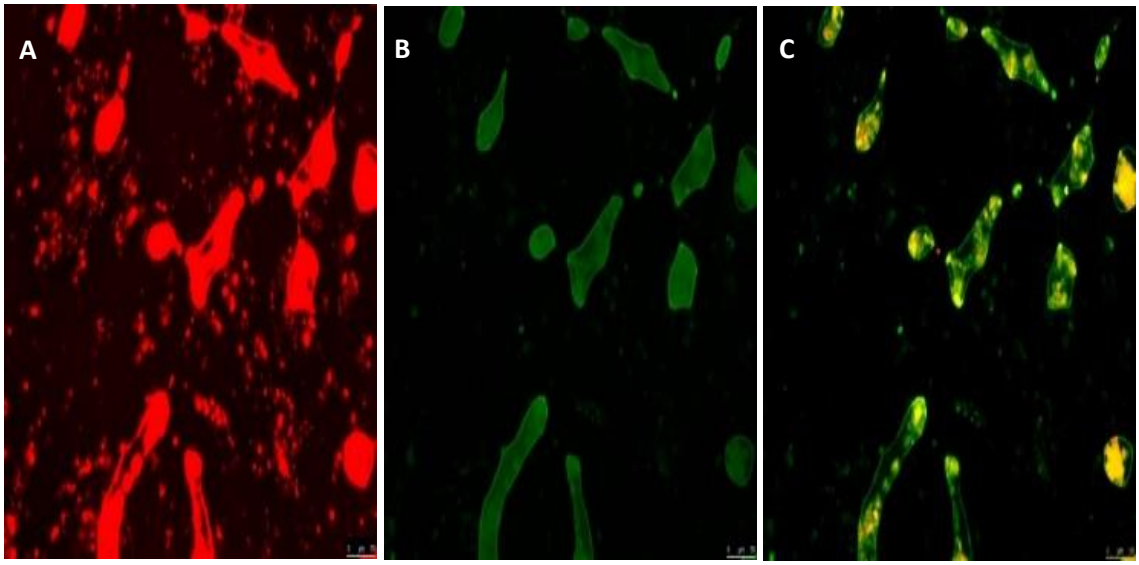

Figure 3 – 3. Image (A) shows red colour collection of NPs (Nile Red dye), (B) the labelled FAM-miRNA (Green) and (C) the merged image of both A and B (scale bar represents 50 µm).

3.4.4 *In vitro* cytotoxicity

To assess the toxicity profile of the PGA-co-PDL NPs with DOTAP, different DOTAP concentrations (0 – 25 %) were investigated using the MTT assay. *Fig.* 3–4A illustrates that decrease in cell viability and an increase in concentration and the 15 % DOTAP particles were

then further investigated for toxicity based on size (section 3.4.2) and compared with unloaded NPs. Blank cells (control) had 100 % cell viability, whereas cell viability at 1.25 mg/ml were around 90 % (unloaded NPs) that decreased to 65 % (15 % DOTAP NPs) (*Fig.* 3–4B), indicating the NPs appear to cause cell death with an increase in concentration after 18 h exposure (Fischer et al. 2003, Bose et al. 2015). 15 % DOTAP NPs at 1.25 mg/ml concentration showed calu-3 cells remained viable following 18 h exposure (*Fig.* 3–4C).

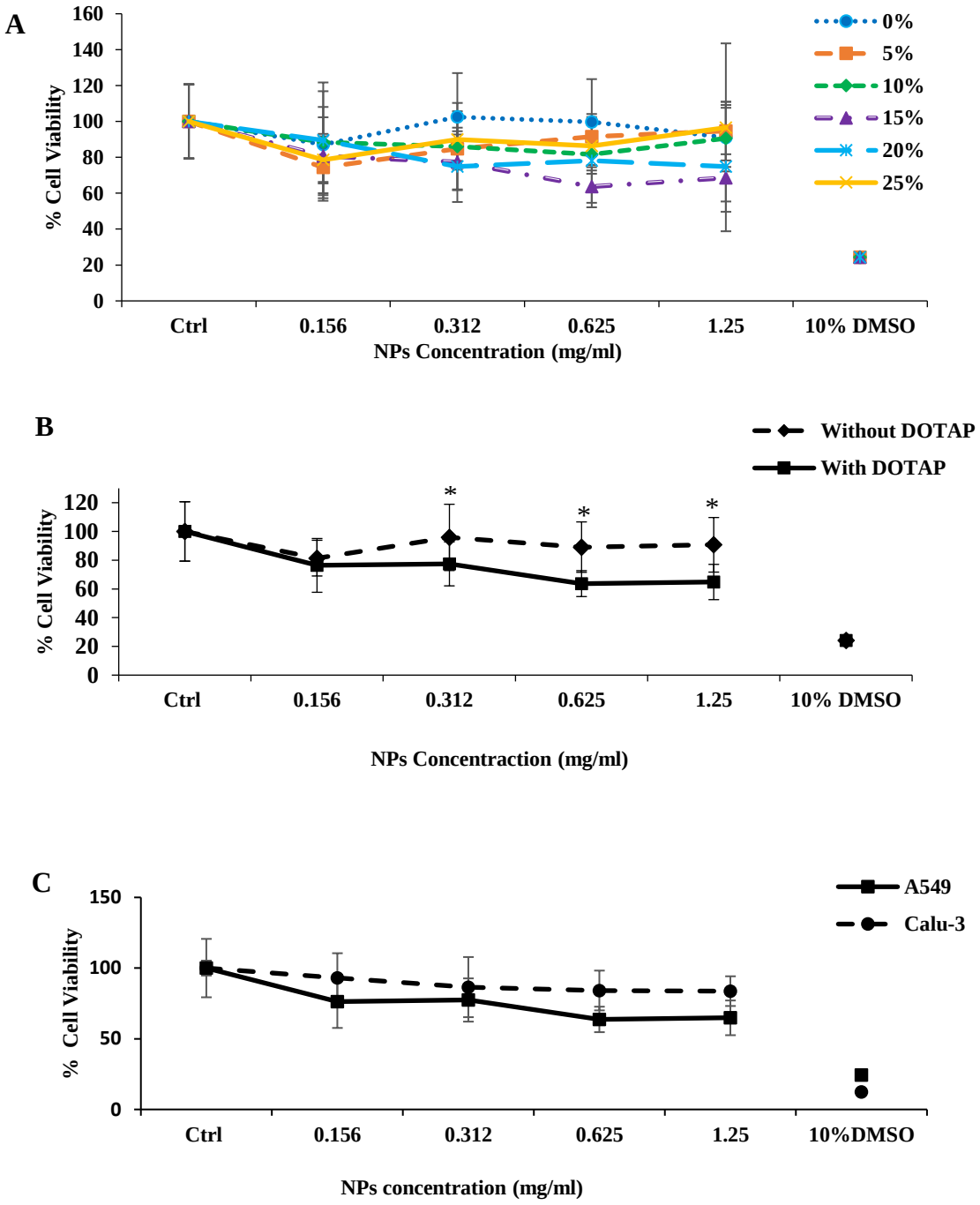

Figure 3– 4. (A) Cytotoxic effect of cationic PGA-co-PDL NPs (0 % - 25 % DOTAP) after 18 h incubation. (B) Cytotoxic effect of unloaded NPs (0 % DOTAP) and 15 % DOTAP NPs on A549 cells after 18 h incubation. (C) Cell viability of A549 and calu-3 cells with 15% DOTAP NPs after 18 h incubation. DMSO was used as positive control, the cell viability was measured using MTT assay. The experiments were repeated three times and data represented as mean ± SD (n=3), (*p<0.05, ANOVA/Tukey's).

3.4.5 *In vitro* release

The *in vitro* release profile of the miR-146a -loaded NPs (40 µg/ml) showed that the release could be divided into two stages (*Fig.* 3–5). The miR-146a was initially rapidly released in the first 4 h with a 51±1.5 % cumulative release. Followed by a second stage during which the miR-146a was constantly released from 4 h up to 24 h, to a cumulative release of 77±1.5 %.

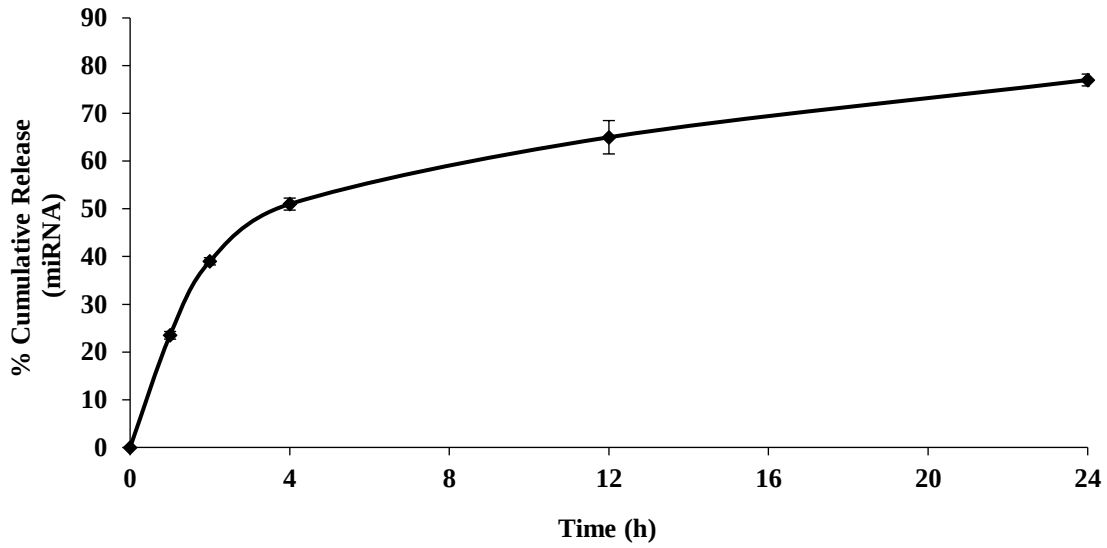

Figure 3 – 5. miRNA in vitro release from 15 % DOTAP NPs in phosphate buffer saline at pH 7.4. Data presented as Mean ± SD (n=3).

3.4.6 Cellular uptake

To visualise the cellular uptake of the NPs for delivery to A549 cells, NPs encapsulating Nile red dye were prepared and added to A549 cells. The NPs were observed to be distributed in the perinuclear region, in large populations of cells and single cells, indicating that the NPs were taken up by the cells (*Fig.* 3–6).

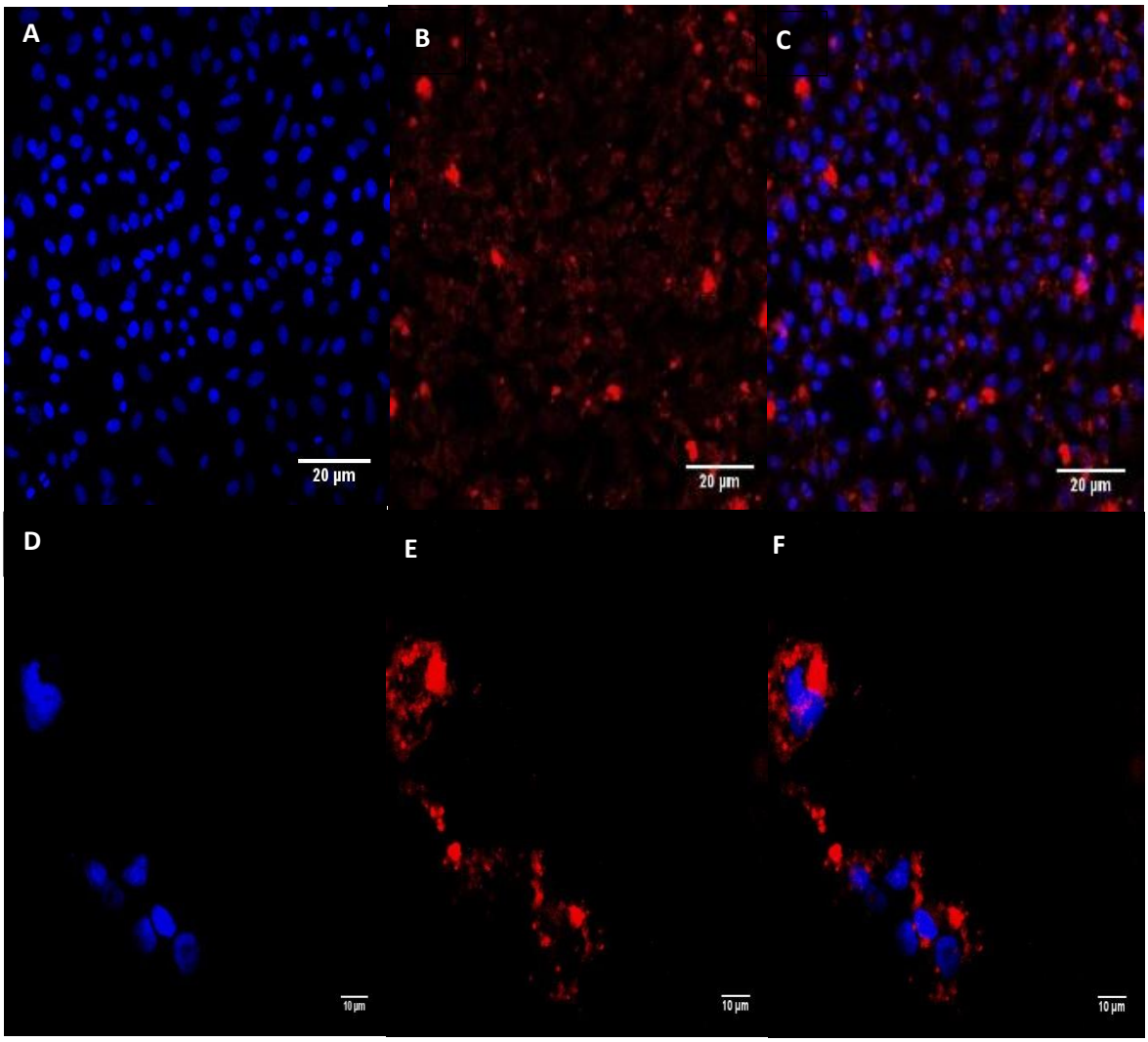

Figure 3 – 6. Fluorescence (A–C) and confocal (D–F) images of Nile Red NPs in A549 cells. (A&D) Nucleus is stained with DAPI, (B & E) NPs stained with Nile Red dye and (C&F) merged image after 1 h of incubation. The scale bars (A–C) represent 20 µm and (D–F) represent 10 µm.

Furthermore, to visualize the intracellular uptake of FAM-labelled miR-146a-NPs for delivery to A549 cells, FAM-labelled miR-146a-NPs were prepared and exposed to A549 cells. Large populations of cells were stained with DAPI, and particles were distributed differentially across a population of cells with a clear variation (*Fig*. 3–7 A, B and C). The same results were obtained with a single cell where the particles can be observed around perinuclear distribution (*Fig*. 3–7 D, E and F).

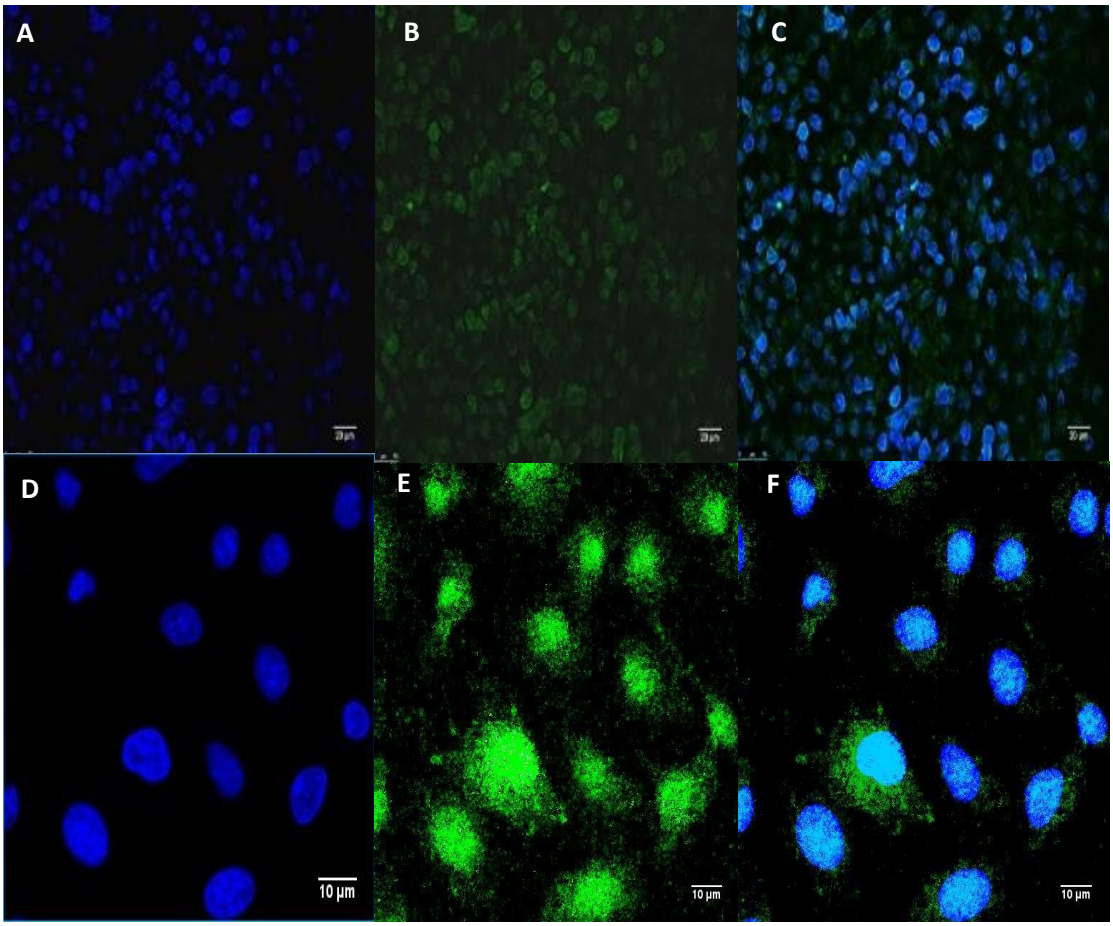

Figure 3– 7. Fluorescence (A–C) and confocal (D–F) images in A549 Cells. (A&D) Nucleus is stained with DAPI, (B&E) FAM-labelled miR-146a-NPs and (C&F) merged image after 1 h of incubation. The scale bars (A–C) represent 20 µm and (D–F) represent 10 µm.

3.5 Discussion

3.5.1 Optimization of cationic NPs

In this study, the effect of cationic lipid DOTAP on particle size and surface charge of NPs was investigated. The physicochemical properties of cationic NPs were consistent with those previously reported in the literature (Jensen et al. 2012). DOTAP has previously been used in other studies as a cationic material to modify polymeric NP properties such as particle size, charge and improve gene transfection (Kumar et al. 2004). DOTAP is thought to limit the enlargement of polymeric NPs due to its surfactant and condensation characteristics (Jensen et al. 2012). Another reason for the change in particle size is that the cationic material has the ability to decrease interfacial tension between the particle surface and the aqueous phase during formation (Song et al. 2006a). This is similar to the effect of PVA on particle size during preparation of NPs (Murakami et al. 1997), where the particle size decreased due to an adequate amount of surfactant covering the surface of PGA-co-PDL NPs (Kunda et al. 2014b).

The electrostatic interaction between the DOTAP cationic moiety, quaternary amine and the negatively charged PGA-co-PDL neutralises the PGA-co-PDL and the remaining amine groups cause the positive charge (Hagigit et al. 2008, Campbell et al. 2001, Mura et al. 2011). This positive charge on the particle surface can potentially interact with biomolecules. PGA-co-PDL NPs have the ability to combine with other cationic compounds leading to a change in particle size and charge. Kunda *et al.* used DMAB as a cationic surfactant to prepare positively charged PGA-co-PDL NPs and observed similar results to ours in which PGA-co-PDL NPs ability to become cationic (Kunda et al. 2014b).

The data presented in this study indicate that PGA-co-PDL mixed with DOTAP formed cationic NPs using 10 % to 25 % (w/w) DOTAP. It was noted that increasing the concentration of DOTAP decreased particle size and caused the zeta potential to become more positive (*Fig* 3–1).

The particle size and zeta potential of the DOTAP-NPs showed only slight differences above a concentration of 15 % (w/w) DOTAP. However, there was little change in particle properties from 15 % to 20 % (w/w) so given the increased cost and toxicity of using higher DOTAP concentrations, 15 % (w/w) DOTAP was chosen for all future work and was selected for the subsequent miRNA adsorption studies. The particle size of 5 % and 10 % (w/w) DOTAP was significantly higher compared with other concentrations (15 % - 25 % (w/w) DOTAP), suggesting that this larger particle size was due to particle aggregation and surface tension (Cui and Mumper 2001, Bose et al. 2015). This particle aggregation was due to the electrostatic interaction of negatively charged PGA-co-PDL and positively charged DOTAP, which caused electrostatic repulsion making the low DOTAP concentrations 5 % and 10 % (w/w) produce larger particle sizes. It appears that the particles with higher DOTAP concentrations (15 % - 25 % (w/w) stabilized PGA-co-PDL more efficiently than at lower concentrations 5 % and 10 % (w/w). Thus, the particle size was proportionally related to the DOTAP concentration (Bose et al. 2015).

This data agrees with previous research indicating that the use of DOTAP during particle preparation affects the particle size. Jensen *et al.* used DOTAP with PLGA for siRNA delivery, and reported that particle size was decreased from 260.8±14.1 nm to 207.7±0.1 nm after use of various DOTAP concentrations, included during preparation (Jensen et al. 2012).

Also, DOTAP reduced the size of spontaneously-forming PC-containing liposomes, allowing

the liposomes to become positively charged and enhance cellular interaction (Campbell et al. 2001). The particle surface charge is another important factor for miRNA adsorption and cellular uptake. The effect of DOTAP concentration on NPs surface charge was investigated (*Fig* 3–1). As DOTAP concentration increased, the surface charge increased changing to a positive charge compared with negatively charged NP formulated without DOTAP -18.9±0.90 mV.

This electrostatic surface charge affects the adsorption of miR-146a, and addition of negatively charged miR-146a lead to a slight charge reduction in cationic NPs. This could be attributed to the decreased surface area of cationic NPs and ionic interactions (Bose et al. 2015, Kumar et al. 2012). The charge of cationic NPs remained positive after miR-146a adsorption, and the amount of miR-146a adsorbed on cationic NPs (*Fig*. 3–2A) over different time points indicated that the surface of cationic NPs was saturated with miR-146a after 2 h, suggesting the maximum adsorption time, which results in good affinity to anionic cell surface. However, when the amounts of miR-146a are added to cationic NPs indicated (*Fig*. 3–2B) there was an equilibrium condition which did not reach the point of saturation and which suggests a positive charge of cationic NPs after miR-146a adsorption.

3.5.2 Toxicity studies and *in vitro* release

The MTT assay was used to determine the cytotoxicity of PGA-co-PDL NPs with 15 % (w/w) DOTAP concentration on A549 and calu-3 cells. It was previously reported that PGA-co-PDL NPs had a little effect on A549 cell viability (>75 % at 1.25 mg/ml) on lung bronchial epithelial cells with cell viability studies (Alfagih et al. 2015), whereas our unloaded NPs had 90 % at 1.25 mg/ml.

The concentration of optimum 15 % DOTAP NPs caused changes in cell viability, those without DOTAP had 96 % viability at 0.312 mg/ml, which decreased to 77 % with 15 % DOTAP NPs at 0.312 mg/ml. The toxicity measured here is of a high particle concentration in

a relatively small surface area in 96 well plate, whereas in the lungs, the NPs will be more dispersed, thus the high level of particle toxicity shown here is unlikely to be seen in lungs (Kunda et al. 2015a). These results support previous literature studies that have used A549 and calu-3 respiratory cell lines to evaluate the *in vitro* toxicological effect of particulate drug delivery systems (Foster et al. 2001, Seagrave and Nikula 2001). Grenha *et al.* used A549 and calu-3 cells to study particle toxicity, where chitosan NPs entrapped in mannitol reduced cell viability (~ 65 %) and were *in vitro* compatible with A549 and calu-3 cells (Grenha et al. 2007).

The cationic lipid DOTAP also influences the cytotoxicity, and Bose *et al.* found that different DOTAP concentrations, when added to lipid polymer hybrid nanospheres, affected cell viability of HEK293, HeLa, HaCaT, and HepG2 cells but did not cause severe cytotoxicity > 70 % cell viability (Bose et al. 2015). However, our 15 % DOTAP NPs at 0.156 and 0.312 mg/ml showed lower cell viability > 76 % in both A549 and calu-3 cell lines (*Fig.* 3–4C).

The *in vitro* release profile of miRNA showed release of miR-146a from 15 % DOTAP NPs over 24 h which was similar to other *in vitro* release studies reported by other researchers (Kumar et al. 2015, Sanna et al. 2012, Mohamed and van der Walle 2008). The miR-146a was adsorbed on 15 % DOTAP NPs via electrostatic interactions. The *in vitro* release curve suggests the weakly attached miR-146a was liberated rapidly over the first 4 h, while the remaining strongly attached miR-146a was slowly released between 4 h up to 24 h. This slow release rate of miR-146a is desirable in DDS to allow miR-146a cellular uptake (Huang and Brazel 2001, Jagani et al. 2013).

Polymeric NPs are biodegradable under physiological condition, and PGA-co-PDL degrades slower than PLGA when subjected to *in vitro* condition (Tawfeek et al. 2011). This particle degradation might affect the surface of particles over time, causing a change in miRNA release, similar to a study by Jagni *et al*. where chitosan coated PLGA NPs were used to adsorb siRNA,

which showed that particle degradation occurred over a period of time, affecting siRNA release and this particle degradation was influenced by particle surface charge and polymer hydrophobicity (Jagani et al. 2013).

However, the *in vitro* release of miR-146a from 15 % DOTAP NPs is affected by the presence of DOTAP in NPs, cationic DOTAP has amine group that provide opportunity of intermolecular hydrogen bonding with PVA and PGA-co-PDL, so DOTAP forms a network on the particle's surface which affects miR-146a release. Comparing our results with other studies that used adsorbed biomolecules such as siRNA, the *in vitro* release of siRNA from cationic PLGA was more than 60 % over 24 h (Jagani et al. 2013) whereas more than 70 % of our miR-146a was released from cationic NPs after 24 h. These biomolecules (siRNA and miRNA) and their potential *in vitro* release depends on the polymer hydrophobicity and the type of cationic material used, which is likely to result in a lower dose making it possible that this delivery system may also result in lower toxicity for the lung. The concept of using NPs as biomolecule or drug carrier for a specific targeting cells, the release of drug from particles influenced by surface modified particles (Lin et al. 2001). Kunda *et al*. conducted a similar *in vitro* release study using PGA-co-PDL NPs adsorbed BSA protein, and found that more than 90 % of BSA was released because of weaker hydrophobic interactions between BSA and NPs (Kunda et al. 2014a).

The *in vitro* release of miR-146a after 24 h was 77±1.5 %, which was the desired concentration for release and correlated with the change in gene silencing and protein levels in the Western Blot data, suggesting that miR-146a maintained its biological efficacy and was not affected by the degradation of NPs where protein level bands were intense at lower miR-146a-NPs concentration and the bands of intensity decreased when NPs concentration is increased. The difference in protein level bands show that miR-146a-NPs produce intense bands which indicates a slight decrease in protein level, whereas the less intense band caused a significant

decrease in protein levels, which will be discussed further in chapter 4, section 4.5.1. This suggests that miR-146a was successfully released from miR-146a-NPs within 24 h and was able to supress the gene expression. A similar study by Li *et al*. which used DOTAP as a cationic lipid to modify single-walled carbon nanotube as non-viral siRNA delivery system, found that cationic DOTAP electrostatically interact with negatively charged siRNA, protecting siRNA against degradation, siRNA was released from single-walled carbon nanotube-DOTAP and was able to fully realise its gene silencing potential in the cancer cells (Li et al. 2016a, Gibbings et al. 2009).

miRNAs that have undergone clinical application have shown the potential value of miRNA therapeutics, for example, miR-34 for treating lung and prostate cancer, reached phase I clinical trial (Wiggins et al. 2010, Liu et al. 2011), miR-208/499 for chronic heart failure (van Rooij et al. 2007) and miR-122 specifically for liver cancer, which has completed phase I and is currently in phase II (Jopling et al. 2005).

3.5.3 Cellular uptake

Fluorescence and confocal microscopies are capable of imaging different cellular and molecular components (Lichtman and Conchello 2005, Wang et al. 2012a) and allow for imaging fluorescently labelled cells and particles for greater depth of visualisation (Amos and White 2003). The rational for using two microscopes is that one microscope (fluorescence) can show cellular uptake as shown in *Fig*. 3–7 A, B and C, whilst the other (confocal) uses particular optical components to generate high-resolution 3-D images of material stained with fluorescent probes, to allow further uptake study in greater depth, to provide wider knowledge of co-localization and the interaction between NPs and cells as shown in *Fig*. 3–7 D, E and F. However, most of the research that has been conducted to study fluorescently labelled NPs and their interaction with cells using either microscopes (Alfagih et al. 2015, Kunda et al. 2014b) or both of these microscopes (Böse et al. 2014, Xu et al. 1996, Win and Feng 2005). Therefore,

applying these two valuable tools were used to determine the cellular uptake of NPs and nucleic acid loaded NPs.

Conventionally, miRNA are very limited in their ability to cross the cellular membrane, without use of polymer based carrier DDS (Yin et al. 2014). The cationic NPs, with adsorbed miR-146a, were distributed in perinuclear region in A549 lung fibroblasts. This distribution of FAM labelled miR-146a suggest that miR-146a could interact with RISC complex with AGO2 and target mRNA in cytoplasm and achieve a good level of gene silencing (Wu et al. 2013b, Kuhn and Joshua-Tor 2013) .

FAM-labelled miR-146a -NPs and NPs encapsulating Nile red dye were taken up by A549 and Calu-3 cells, which were used as model for alveolar and epithelial cells respectively. Nile Red NPs were used to show the ability of cationic NPs cellular uptake and to deliver miRNA to the cell. The uptake of polymer based NPs had previously been demonstrated in A549 (Kunda et al. 2015a), dendritic cells (Alfagih et al. 2015, Kumar et al. 2012), breast adenocarcinoma MCF7 cells (Abulateefeh et al. 2013), liver cancer cells, HepG2 and Hela cells (Díez et al. 2009a), lung carcinoma cells, H1299 (Jensen et al. 2012, Howard et al. 2006b) and Chinese hamster ovary cells (Katas and Alpar 2006). In the present study, NPs and FAM-labelled miR-146a –NPs ended up successfully inside perinuclear region in both A549 and calu-3.

The NPs were used in cellular uptake study were positively charged and in nano particle size (section 3.4.2). The surface charge and particle size, which are reflected by the zeta potential and dynamic laser scattering, influences NPs and helped in their cellular uptake (Stuart et al. 2006). The particles in nanoscale size influence the cell interaction, a study by Jiang *et al.* showed the cellular internalization of Herceptin-gold NPs was size-dependant (Jiang et al. 2008) and this size can also have implication on toxicity (Verma and Stellacci 2010, Nel et al. 2009, Garnett and Kallinteri 2006). Moreover, the NPs' surface charge contributes significantly to their interaction with cells, and the presence of cationic DOTAP facilitates the positively

particle surface interaction to negatively charged cellular membrane allowing NPs cellular uptake (Díez et al. 2009a). A study by Kedmi *et al*. showed the adjusted use of cationic DOTAP in the formulation improved NPs' cellular uptake (Kedmi et al. 2010, Mukherjee et al. 2005). Moreover, it is notable that uptake and distribution of FAM labelled miR-1464a-NPs in cells that the NPs quantity is sufficient, indicating an active intracellular NPs transport, and miR-146a reached perinuclear to initiate efficient gene silencing (Portis et al. 2010).

3.6 Conclusion

The cationic DOTAP was successfully used to produce cationic NPs with particle size 244.8±4.4 nm which was similar to unloaded NPs 242.4±0.3 nm. Moreover, cationic NPs offer positive surface charge for miR-146a adsorption. The miR-146a adsorption was 32.25±2.0 µg miR-146a per 10 mg NPs after 2 h (the optimum conditions were 15 % DOTAP and miR146a adsorbed after 2 h). The *in vitro* release of miR-146a after 24 h was 77±1.5 %, the NPs were taken up by the cells and delivered miR-146a into the cell.

4. Evaluation of the miR-146a-NPs effect on target gene and protein expression

4.1 Introduction

Based on the results of chapter 3, the optimum formulation had shown to successfully adsorb miR-146a on NPs, with low toxicity and uptake into cells. However, in this chapter we will investigate the functionality and activity of miR-146a from the optimum formulation.

miR-146a has been associated with inflammatory pathway in the targeting of interleukin-1 and Toll-like receptor (TLR) signalling, of which NF-κB activation is a primary downstream effector, known as interleukin 1 receptor-associated kinase (IRAK1) and TNF receptor-associated factor (TRAF6) (Taganov et al. 2006). Taganov *et al.* described miR-146a as capable of controlling TLR and cytokine signalling through negative feedback regulation, with associated down regulation of IRAK1 and TRAF6 protein levels (Taganov et al. 2006). In addition, Bhaumik *et al.* reported that miR-146a showed significantly downregulated IRAK1 and TRAF6 in the IL-1 and TLR signalling pathway (Bhaumik et al. 2008) using Western Blot to determine protein expression. Similar studies have been investigated with miRNA for inflammatory diseases through controlling signalling pathway. Sato *et al.* determined pathogenesis increase of the abnormal inflammatory cyclooxygenase (COX-2) half-life in chronic obstructive pulmonary disease (COPD) and enhanced production of prostaglandin E2, Quantitative Real- Time Polymerase Chain Reaction (qRT-PCR) assay was used to quantify miR-146a expression (Sato et al. 2010).

Post-transcriptional regulation of IRAK1 and TRAF6 by microRNA has been associated with the regulation of physiological responses as described by Perry *et al*. that showed the changes in expression of miR-146a could regulate the inflammatory response in human alveolar epithelial lung cells (Perry et al. 2008). The miRNAs could be delivered to the site of action using nanocarriers, as a study by Wu *et al.* showed therapeutically delivered miR-29b in cationic lipoplexes for lung cancer in both *in vitro* using A549 cell lines and *in vivo* (Wu et al. 2013c).

4.2 Aim

The aim of this study was to determine the effect of miR-146a loaded NPs on *IRAK1*, *TRAF6* and protein expression.

4.3 Methods

4.3.1 Transformation and Quantification

In order to assess direct interaction of miR-146a-NP with the 3' UTR of *IRAK1*, a firefly luciferase plasmid reporter was used (*Fig.* 4–1) (Origene, UK).The plasmids were replicated in DH5-α *Escherichia coli* strain grown on Luria-Bertani (LB) agar supplemented with kanamycin at (10 µg/ml). After overnight incubation on the agar, a single colony was transferred into LB broth and incubated overnight at 37 °C. The bacterial cells were harvested, the plasmid isolated and purified by Qiagen plasmid maxi kit (Qiagen, UK) according to the manufacturer's protocol. The Purified DNA was quantified by UV spectrophotometry at 260 nm and 280 nm (NanoDrop).

The purified reporter plasmid was also characterized by (1 %) agarose gel electrophoresis. The agarose gel was run at 60–80 V for 60 min, lane for DNA ladder 200–10,000 bp as standards was used (HyperLadder, Bioline), and the DNA bands were visualized under UV light and photographed on a UV transilluminator (Syngene®, UK).

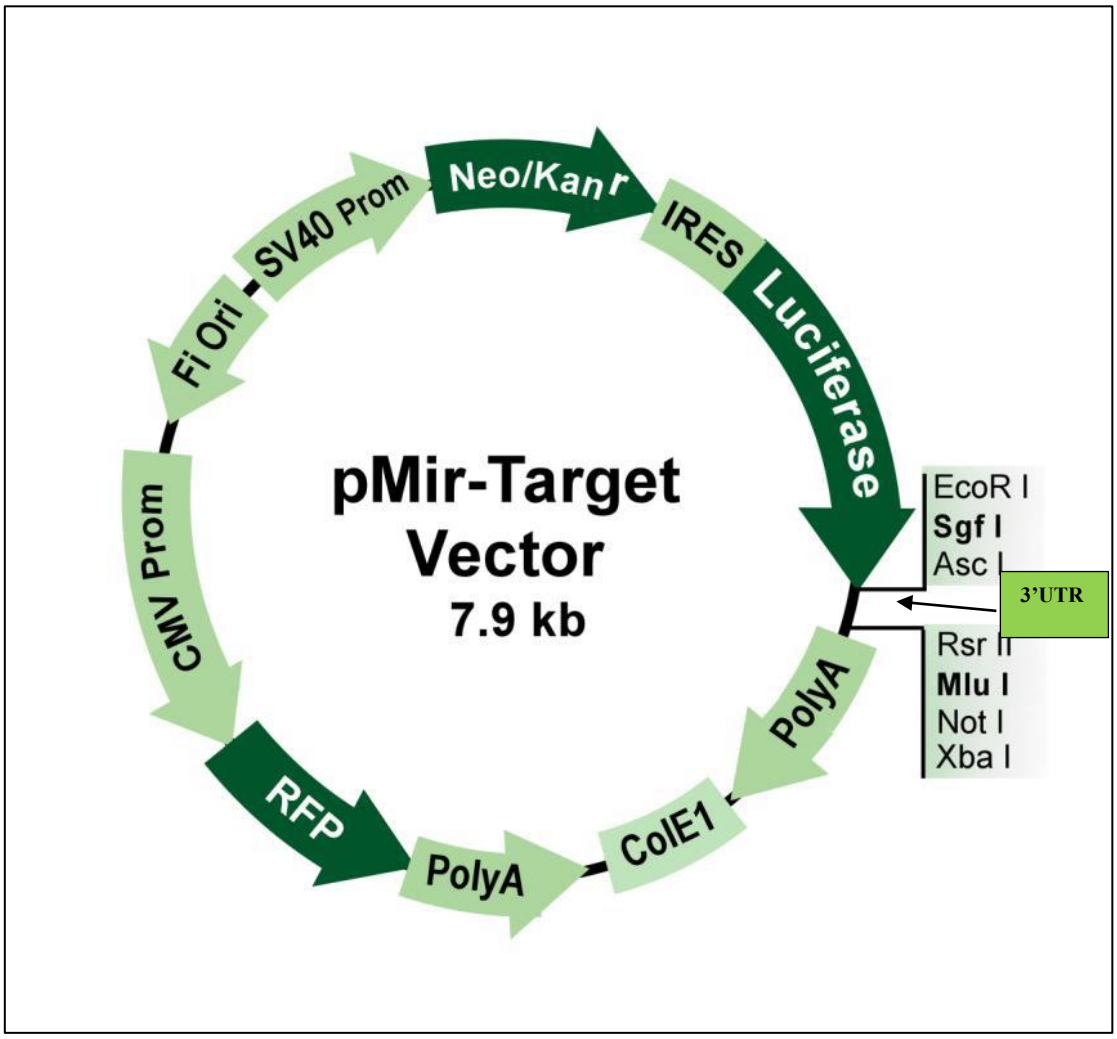

Figure 4– 1. pMirTarget vector map. Depiction of plasmid with firefly luciferase upstream of the 3' UTR miRNA targeting sequence. Expression is driven by a simian virus SV40 promotor and translated with an internal ribosome entering sequence (IRES). Vector contains red fluorescent protein (RFP), kanamycin resistance gene and cytomegalovirus (CMV) (Jin et al. 2013, Petersen et al. 2006).

4.3.2 Transfection and Luciferase assay

Serum free Opti-MEM medium (Thermo Fisher, scientific, UK) was used for transfection of A549 cells seeded in 96-well white plates at a density of 2×10^5. After 24 h incubation, the cells were transfected with 100 ng 3' UTR reporter plasmid (purified firefly luciferase plasmid reporter from section 4.3.1, *Fig.* 4–1). This vector has 3'UTR of *IRAK1* which has two conserved binding sites for miR-146a and this 3'UTR was downstream of a firefly luciferase gene. The plasmid was designed with an SV40 promoter to drive constitutive expression of a selection marker (neomycin/kanamycin) fused to the firefly luciferase gene, with an IRES for translation of the luciferase transcript into protein. The exogenous miR-146a binding to the 3'UTR should lead to a reduction in luciferase output. The vector has a region encoding RFP (driven by a CMV promoter) for validation of transfection by fluorescence microscopy. Plasmids encoding green fluorescent protein (pmaxGFP; Lonza) or encoding a red fluorescent protein endoplasmic reticulum marker (dsRed-ER; Clontech) were used as controls for transfection and fluorescence. Plasmids were combined with 0.15 or 0.3 µl Lipofectamine 3000 reagent (Life technology) in Opti-MEM medium per well and incubated 6 – 48 h (37°C, 5 % CO_2) before removal of the complexes and further incubation with complete culture medium 24 – 48 h.

Luciferase expression was performed using the luciferase assay system (Promega) according to the manufacturer's protocol. Briefly, after 48 h incubation the medium was removed and the cells washed once with PBS, lysed by adding 20 µl lysis buffer. Then, 100 µl luciferase assay reagent was added. Luminescence was detected on a CLARIOstar® plate reader emission wavelength was adjusted at 580–80 nm to measure firefly luciferase activity. The luminescence values were represented as relative light units (RLU), also used red fluorescent protein (RFP) values for transfection monitoring and normalization. Fluorescence microscopy was used to visualise GFP and RFP expression.

4.3.3 Semi-quantitative reverse transcriptase RT-qPCR

RT-qPCR was performed as described in section 2.4.

4.3.4 miR-146a-NPs effect on protein expression

Immuno western blotting is outlined as in section 2.5.

4.3.5 IL-8 Promoter Reporter assay

Cells were seeded on an 8 well chambered slide (Fisher Scientific, UK) at seeding density of 5 × 10^5 cells per well and incubated for 24 h prior to transfection. Transfections were performed using lipofectamine 3000 reagent following the manufacturer's protocol (Life technology, UK) in serum free DMEM and a total amount of 0.5 µg of DNA per well was used. The DNAs used were 213 - pIL8/d2EGFP and 269 - pDsRED mono promoters. The cells were transfected for 6 h before removal of the mixture and washed twice with PBS. A prepared mixture of unlabelled miR146a-NPs (100 µl) was added and incubated for 1 h. After this time, the media was removed from cells and washed twice with PBS. Then, cells were stimulated with 1 ng/ml Interleukin beta and tumour necrosis factor-a (TNFα) and incubated for further 6 h. Cells were washed with PBS and subsequently fixed using ice-cold (-20°C) methanol. Images were acquired using an Olympus BX51 Fluorescent microscope.

4.3.6 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 $p<0.05$. Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, Inc., San Diego,CA). All values are expressed as their mean ± standard deviation (SD).

4.4 Results

4.4.1 Effect of miR-146a-loaded NPs on target gene expression

To confirm miR-146a function after adsorption onto the NPs, the expression of target genes *IRAK1* and *TRAF6* was assessed in A549 cells. Analysis of transcript levels showed that miR-146a delivered via NPs (miR-146a mimic) led to dose dependent suppression of *IRAK1* (*Fig.* 4–2) and *TRAF6* (*Fig.* 4–3) compared with untreated cells. The expression of *IRAK1* and *TRAF6* was normalised to *GAPDH* expression. The average of all concentrations was pooled from three independent experiments (Appendix–1 and Appendix–2).

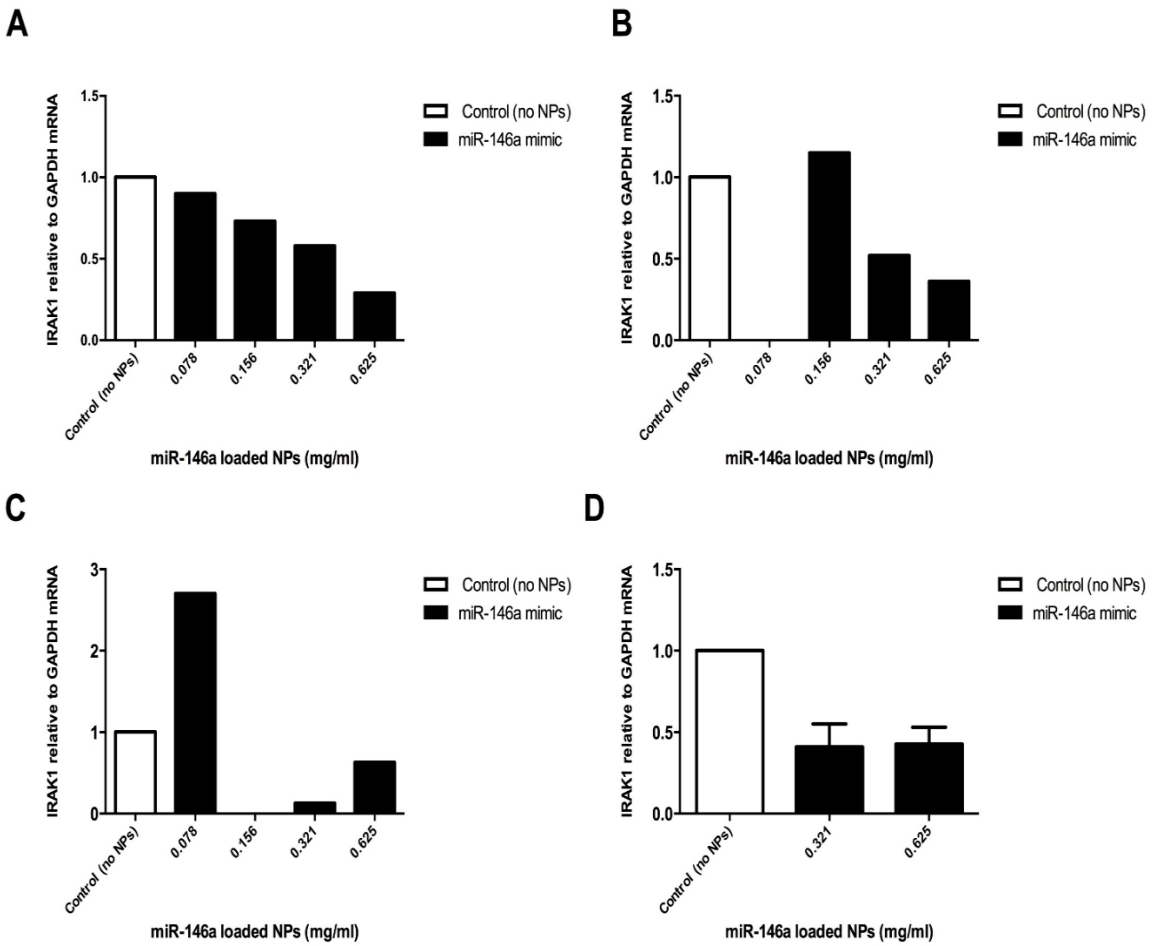

Figure 4– 2. Effect of miR-146a loaded NPs on IRAK1 expression. Data were pooled from three independent experiments (A, B & C). The highest two concentrations are shown at (D) were pooled from the three independent experiments. The expression of IRAK1 was normalised to GAPDH expression. Values in graph D are Mean ± SD (n=3).

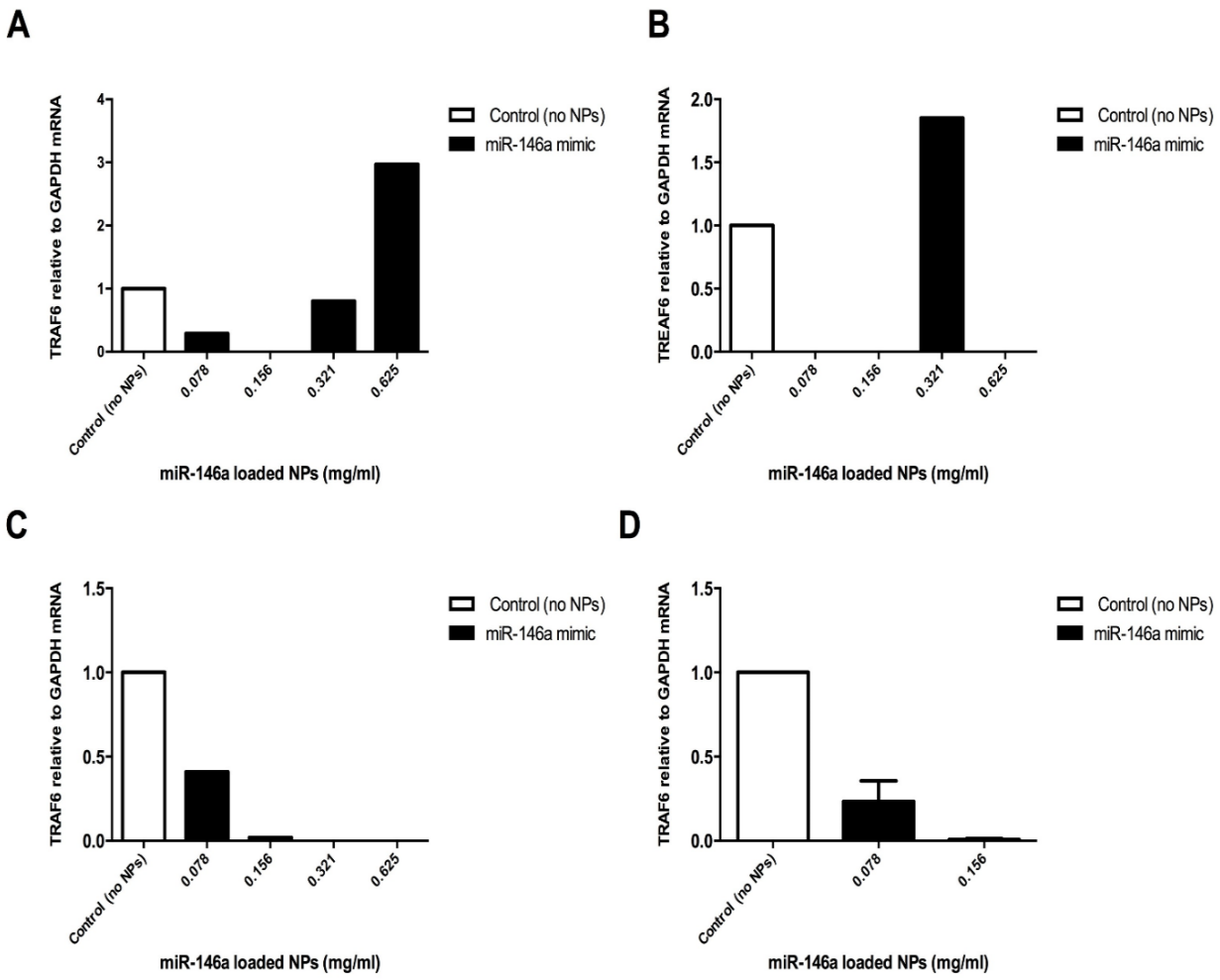

Figure 4– 3. Effect of miR-146a loaded NPs on TRAF6 expression. Three independent experiments (A, B & C). Lowest two concentrations (D) were pooled from the three independent experiments. The expression of TRAF6 was normalised to GAPDH expression. Values in graph D are Mean ± SD (n=3).

4.4.2 Effect of miR-146a-loaded NPs on protein expression

To confirm the downregulation of *IRAK1* and *TRAF6* occurred at the protein levels, miR-146a-loaded NPs were applied to A549 cells and lysates evaluated by immunoblotting. As shown in *Fig.* 4–4, the miR-146a-NPs reduced *IRAK1* protein levels in A549 cells after 24 h and 48 h treatment. The decrease in protein levels occurred in a dose dependent manner compared to untreated cells, suggesting that *IRAK1* protein levels reduced in response to miR-146a-NPs.

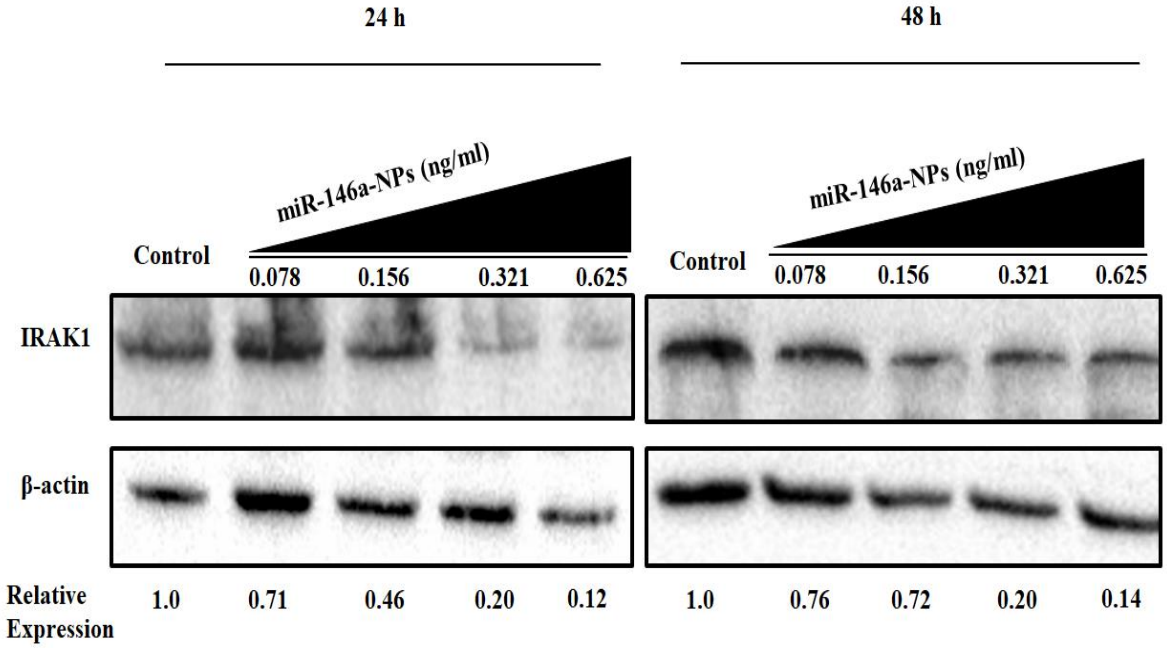

Figure 4– 4. Effect of miR-146a on IRAK1 protein levels in A549 cells. Dark triangle represents lowest (left) to highest (right) miR-146a-NPs concentrations. The numbers under each band represents the densitometric readings relative to control samples that are normalized to each band and to its corresponding β -actin control.

4.4.3 Transfection and Luciferase assay

To assess miR146a-NPs biological function, A549 cells were transfected with luciferase using lipofectamine 3000 as a transfection reagent with purified plasmid to identify the transfection toxicity and effectivity. However, when Lipofectamine 3000 was used, it did not cause any transfection with a longer incubation time of more than 6 h (*Fig.* 4–5). After 24 - 48 h of incubation, the A549 cells died despite using different cell densities of 10,000 and 12,000 cell per well which is in line with other studies indicating the toxic behaviour of Lipofectamine 3000 (Zhong et al. 2008). Therefore, the incubation period of 6 h is considered as the optimum incubation time for Lipofectamine 3000.

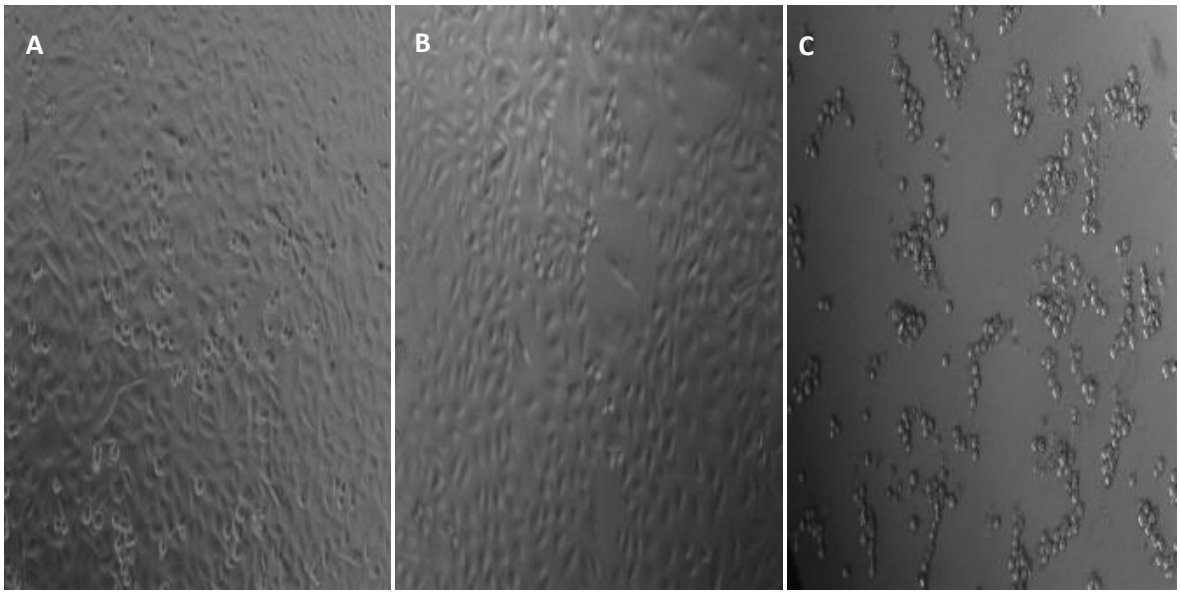

Figure 4– 5. Shows the effect of transfection reagent (Lipofectamine 3000) on A549 cells after incubation at different time points (A) un-transfected cells 24 h, (B) transfected and incubated for 6 h (C) after 24 h transfection.

Further analysis of transfection reagent toxicity, with Lipofectamine 3000 was carried out on A549 cells with different concentrations to transfect plasmid. The fluorescence images in *Fig.* 4–6A, B and C, showed a transfection expression occurred in GFP and ds-Red. However, there was no expression detected of RFP despite the same procedure followed with the GFP and ds-Red.

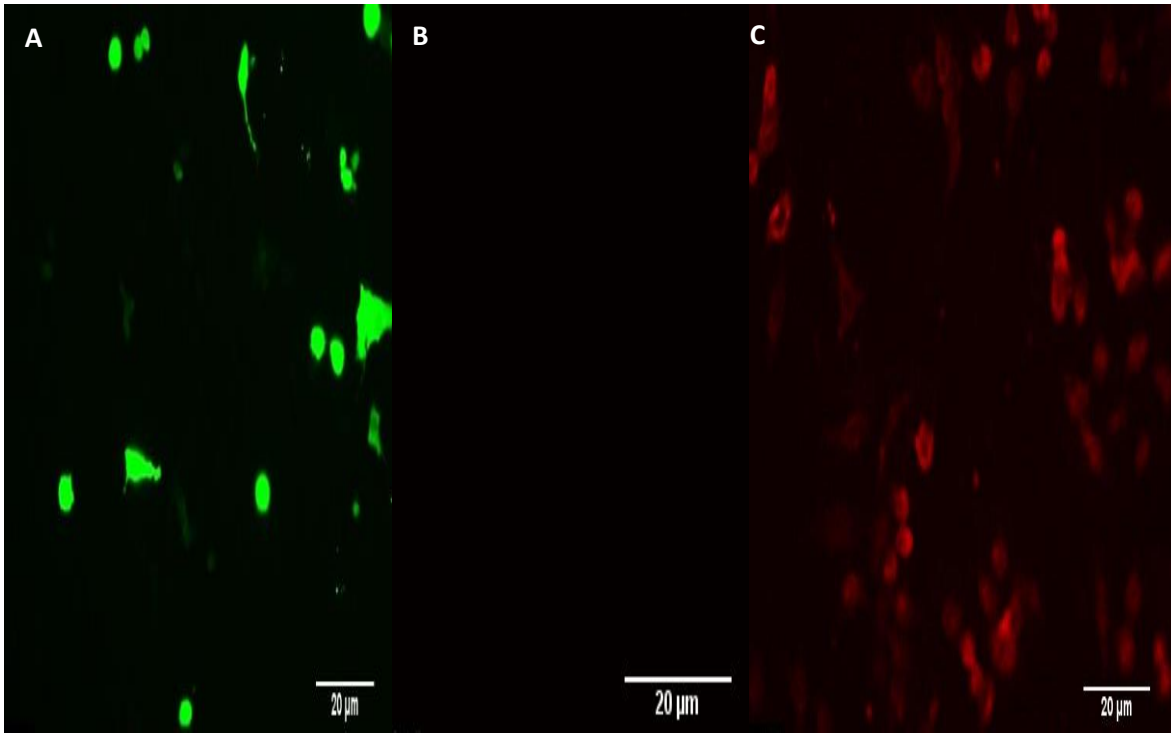

Figure 4– 6. Fluorescence images of fixed A549 cells. Expression (A) GFP, (B) RFP and (C) dsRed-ER. The scale bars represent 20 µm.

Gel electrophoresis was used to find the presence of plasmid. The results in *Fig*. 4–7 showed that the presence of plasmid and purity of plasmid also have been confirmed

spectrophotometrically by NanoDrop that determined A260/280 ratio and the concentration was within a ratio of 0.1 – 1.

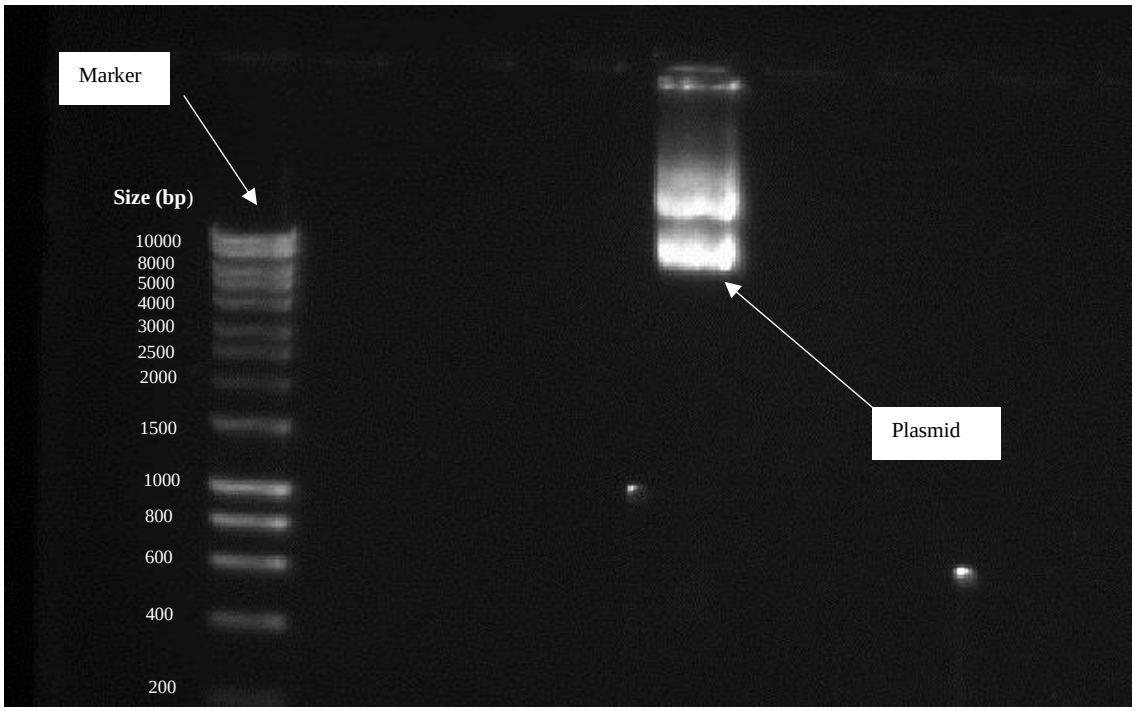

Figure 4– 7. Gel electrophoresis of plasmid after purification using Qiagen plasmid maxi kit. The left band shows the marker and the right band represents pure plasmid (7.9 bp).

4.4.4 Reporter assay

To determine miR-146a-NPs biological function, the IL-8 promoter reporter assay was used. The pIL-8 promoter- GFP reporter was transfected into A549 cells, which are noted to express the functional IL-1 receptors (Ding et al. 1998). The promoter's response after IL-1β stimulation produced an intense fluorescent signal *Fig.* 4–8A, while IL-8 promoter reporter output (GFP) was dampened by miR-146a-NPs *Fig.* 4–8B.

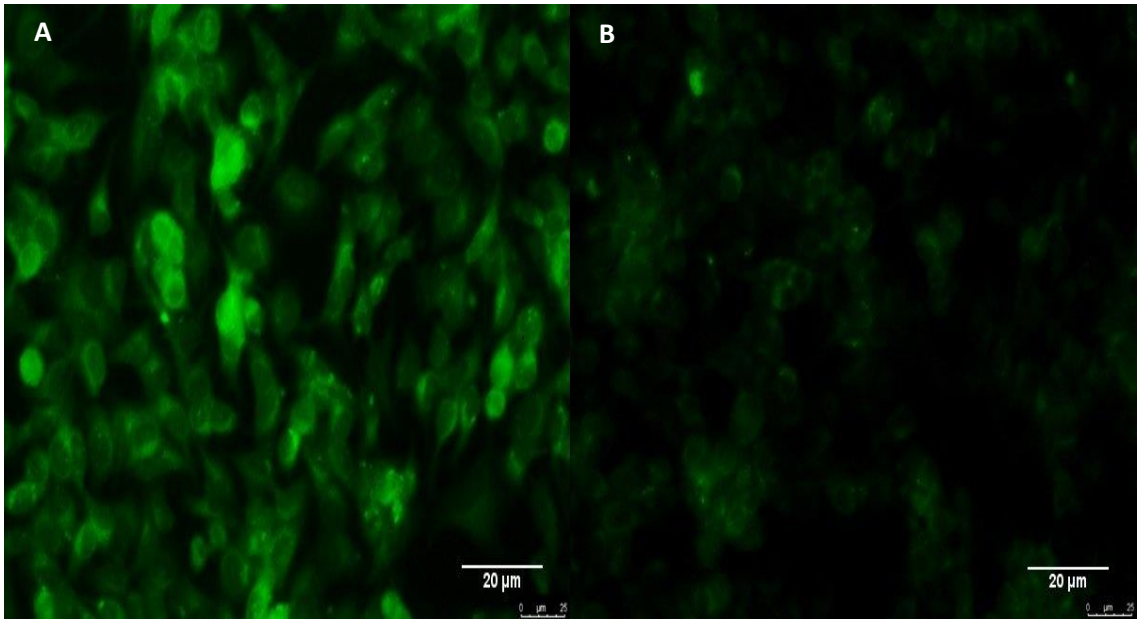

Figure 4– 8. Fluorescence images of (A) response of pIL8 reporter to cells stimulated with IL-1β, (B) Cells loaded with miR-146a-NPs prior to stimulation with IL-1β. The scale bar represent 20 µm.

4.5 Discussion

4.5.1 Functional evaluation of miR-146a loaded NPs

To confirm the functional activity of miR-146a loaded NPs, their impact was assessed on two leading miR-146a target genes, *IRAK1* and *TRAF6.* A study by Taganov *et al.* indicated that miR-146a inhibit the expression of *IRAK1* and *TRAF6* genes and the significant function of miR-146a as negative regulator of inflammation (Taganov et al. 2006). The responses varied across the three independent experiments performed in each case but reduced expression of the *IRAK1* (*Fig.* 4–2) or *TRAF6* transcripts was observed in response to most doses (*Fig.* 4-3). This suggest that NPs delivered miR-146a to site of action and produced the expected downregulation effect. However, the highest NPs concentrations (*Fig*. 4–2D), reduced target

gene *IRAK1* expression to 40 %. The highest NPs concentrations were used as an overall average, as they showed a very strong downregulation effect compared with the lowest NPs concentrations. In the case of *TRAF6* the lowest NPs concentration (*Fig.* 4–3D) caused the expression to be reduced to over 20 %. The lowest NPs concentrations were used as overall average, as they showed a very strong downregulation effect compared with the highest NPs concentrations. Thus, low doses of miR-146a-NPs appeared to be more effective at downregulating *TRAF6* than *IRAK1*.

TRAF6 has three conserved miR-146a binding sites in the 3'UTR of mRNA and *IRAK1* has two conserved sites in the 3'UTR. This suggests that miR-146a-NPs downregulated the *TRAF6* target gene more than *IRAK1*. There are computational algorithm programmes that provide a valuable resource in predicting the biological target of miRNAs in relation to gene regulatory networks. The TargetScan programme was used to predict conserved sites for miRNA with their target regions (Agarwal et al. 2015). Assessment of the impact of the miR-146a-NPs on *IRAK1* and *TRAF6* protein levels revealed understanding the effect of miR-146a-NPs (*Fig. 4–4*). However, protein band intensity decreased when the NPs' concentration is further increased. Thus, it is clear that miR-146a delivery using NPs dependent on miR-146a and NPs amount, which indicates the capability of miR-146a loaded NPs ability to control COPD disease.

To determine the difference in *IRAK1* expression, and whether the miR-146a-NPs influenced protein of interest in lung cell lines. As presented in the results (*Fig* 4–4), anti-IRAK1 antibody appear to bind specifically to the protein of interest. This is in agreement with the results of Chen *et al.* where the anti-IRAK antibody was suitable for WB (Chen et al. 2012).

There was no significance difference found between 24 h and 48 h after miR-146a-NPs treatment, suggesting that treatment with as little as miR-146a-NPs concentration were enough to exhibit the maximum targeting capacity of cellular environment (Ghosh et al. 2013, Song et

al. 2006b), affecting *IRAK1* protein levels the physiological pathway (Taganov et al. 2006). However, *TRAF6* protein expression was not tested, and whether the miR-146a-NPs influenced this protein in the lung cell lines we don't know, but we expect the same modulation as *IRAK1* protein expression in these cell lines, which was confirmed by the functional activity of miR-146a loaded NPs which inhibit the expression of *TRAF6* genes. These functional studies confirm that miR-146a-NPs delivered to site of action in the lung cell lines, and miR-146a have modulated both genes and protein levels (Ezzie et al. 2011). With these results, miR-146a-NPs appeared to be a promising therapeutic approach for treatment of disease by targeting genes that involved in unusual pathological way.

The NPs have played important role in terms of delivery DNA into dendritic cells and protected the DNA from degradation (Yuba et al. 2008). However, miR-146a molecules without delivery carrier are rapidly affected by enzymes activity in lung airways (Takei et al. 2004). Although intratumoural injection of Let-7 miRNA into tissue can reduce tumour distribution with some limited therapeutic potential, however, miRNA delivery using delivery carrier could significantly improve the gene knockdown (Wiggins et al. 2010).

4.5.2 Transfection and Luciferase assay

The expression occurred in GFP and dsRed-ER in the transfected cells (*Fig.* 4–6A and C). These two have small size plasmids of approximately 4.7 kb while RFP plasmid size has 7.9 kb and its expression was not detected (http://www.origene.com/MicroRNA/3-UTR-Clone/). The use of luciferase RFP reporter was designed to monitor the transfection and interaction with 3'UTR miRNA. Tanganov *et al.* used luciferase reporter to show the role of miR-146a and its target in 3' UTRs of *IRAK1* and *TRAF6* (Taganov et al. 2006). Our experimental design was to transfect the GFP, dsRed-ER and RFP into lung cell lines (*Fig.* 4–6A, B and C).

If any marked fluorescence in the reporter's expression were expected, the miR-146a-NPs would have been used to determine the expression. Kumar *et al*. used a fluorescence reporter to identify siRNA inhibition of target gene using enhanced GFP and RFP. There was a significant reduction in enhanced GFP but not in RFP expression (Kumar et al. 2003). Our data indicated there was no expression detected for the required RFP in A549 cell line. Consequently, then 3' UTR luciferase reporter will not be reliable and due to limited funding available for the continuation of this project Luciferase assay was not further tested on miR-146a-NPs.

4.5.3 Reporter assay

To determine the effect of miR-146a-NPs in the expression of target genes *IRAK1*, protein levels, pIL-8 promoter reporter assay was used. The pIL-8 promoter-GFP reporter was stimulated with proteins noted to be involved in IL-1β signalling (*Fig.* 4–8). The IL-1β has the ability to stimulate *IRAK1* phosphorylation in cytoplasm of A549 cells. The phosphorylated *IRAK1* is associated with *TRAF6*, and this association activates the ligase function of *TRAF6* leading to ubiquitin-mediated activation of NF-κB in Toll-like receptor (TLR) signalling (Bhoj and Chen 2009). Kiss-Toth *et al.* have described the protein functional expression from pIL-8 promoter that was induced by IL-1 and tumour necrosis factor TNF-α which underlined the physiological inflammatory response of the given promoter (Kiss-Toth et al. 2000). However, miR-146a was shown to be involved in targeting of the IL-1 and TLR signalling involving NF-κB, *IRAK1* and *TRAF6* (Taganov et al. 2006, Bhaumik et al. 2009). Perry *et al.* have determined the miR-146a action mediated through regulation of proteins involved in IL-1β signalling pathway and showed that the changes in expression of miR-146a can regulate the inflammatory response in human alveolar epithelial lung cells (Perry et al. 2008). When miR-146a-NPs were delivered to A549 cells prior to IL-1 stimulation, this led to a decrease in the fluorescence

intensity. As such, miR-146a-NPs' delivery to cytoplasmic target proteins and regulated biological inflammatory process is based on the functional GFP expression which is in line with other studies indicating the biological process (Kiss-Toth et al. 2000, Bhoj and Chen 2009, MacKenzie et al. 2001).

4.6 Conclusion

The miR-146a maintained its functional structure under gene silencing and protein level. The high miR-146a-NPs concentration reduced target gene *IRAK1* expression to 40 % and *TRAF6* the lowest NPs concentration reduced to over 20 %. We have showed that the optimum transfection reagent incubation period of A549 cell is 6 h. The GFP and dsRed expression occur in A549 cells. The miR-146a-NPs reduced IL-8 promoter reporter GFP via IL-1β signalling pathway suggestion that miR-146a-NPs can be used to target proteins, regulate the inflammatory process. These successful studies and results show the potential of cationic NPs for delivery of miR-146a in the treatment and management of COPD. These results can allow further research on these NPs to be nanocomposite microparticle and form potential spray dry powder and evaluate the cellular functionality.

5. Formulation of Dry Powder Nanocomposite Microparticles (NCMPs) for pulmonary delivery

5.1 Introduction

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 a site of action avoiding degradation by the strong acids and enzymes in the oral route. The nanoparticles (NPs) delivered to the lungs for pulmonary drug delivery essentially require an appropriate size that allows the small nucleic acid reach to the target site and correct the faulty genes. In fact, NPs in the dry powder form do not deposit efficiently in the alveolar part of the lungs, which results in the exhalation of the majority of the inhaled dose (Sung et al. 2007). However, the ideal particle size for optimal particle deposition in the deep lung ranges from between 1 to 5 µm in diameter (Sakagami 2006). Therefore, NPs can be incorporated into dry powder microparticles (NCMPs) of aerodynamic particle size 1-5 µm through spray drying (Alfagih et al. 2015). Spray drying is a one step process that converts liquid solution or suspension to dry powder, which are designed to diffuse in the lung lining fluid and release its contents (Ungaro et al. 2012). The spray drying process is controlled by different parameters to provide desirable particle size and aerosolisation properties (Sakagami 2006). It is important to take into consideration, when designing an experiment that aims to determine the influence of various parameters on the properties of the dosage formulation being studied and these parameters optimized in order to obtain desirable results (Yang and Zhu 2002). Liang *et al*. have prepared a powder formulation using pH responsive peptides for influenza treatment, and it was found that the integrity and biological activity of siRNA were well preserved after spray drying (Liang et al. 2015).

Moreover, excipients such as sugars and amino acids can be added to the formulation resulting in changes to physical characteristics of powders, including the morphology, moisture content, particle size and density (Bosquillon et al. 2001). The selection of appropriate excipients for inhalation leads to optimal and functional dry powder formulation, can help preserve NPs and

nucleic acid integrity. It is important to maintain NPs integrity that allows recovery of NPs size, can cross biological barriers e,g lung lining fluid and be up taken by cells. If particle aggregate and become larger can impact on diffusing through lung lining fluid and uptake by cells hence reducing miRNA uptake into cells.

L- Leucine is one of the amino acids that has been used as a cryoprotectant and dispersing agent that improves aerosolisation properties for dry powders, reduces contact cohesion between the particles and prevents aggregation (Alfagih et al. 2015, Tawfeek et al. 2011, Cruz et al. 2011, Lucas et al. 1999, Rabbani and Seville 2005, Kunda et al. 2015a). L-leucine is considered as a hydrophobic amino acid that accumulates at the air water interface (Vehring 2008). Sugar excipients such as mannitol are commonly used in spray drying with good safety characteristics, are less hygroscopic than lactose, have muco-adhesive properties and they have European approval for dry powder inhalation (Jensen et al. 2010, Pilcer and Amighi 2010, Burness and Keating 2012).

Furthermore, different forms and ratios of amino acids and sugars can be used alone or as a combination in the same formulation which produces positive results. You *et al*. performed a study using the spray drying method by mixing amino acids (L-leucine, glycine and threonine) and sugars (trehalose, lactose, dextran and mannitol) , which resulted in these excipients having affected the dry powder flow rate, aerosolisation behaviour, median aerodynamic diameters, moisture content and yield (You et al. 2007).

Similarly, these excipients can protect, and stabilize the NPs and encapsulated materials against the intense spray-drying process including operational high temperature and mechanical stress (Alfagih et al. 2015). 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 decompose 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 has been very limited work exploring miRNA spray drying. In this chapter, L-leucine and mannitol were mixed together as dispersion enhancer and protective excipients. L- Leucine was chosen due to cryoprotectant, dispersing properties and high aerosolisation properties (Alfagih et al. 2015, Tawfeek et al. 2011), whereas mannitol was chosen owing to its mucho-adhesive properties, the fact it is a protective excipient for small nucleic acids, and its good safety characteristics and European approval for dry powder inhalation (Jensen et al. 2010, Pilcer and Amighi 2010, Bhowmik et al. 2009). This is the first time that miRNA was formulated into inhalable dry powder using a mix of amino acid and sugar excipient.

5.2 Aim

The aim of this chapter was to optimise 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.

To obtain the aim of the study, the following objectives were considered;

1. Formulation of NCMPs via spray-drying technique and evaluating;
    a. The influence of L-leucine to mannitol ratio on the NPs recovery size and yield %.
    b. The influence of spray drying parameters on morphology, aerosol performance, and moisture content of NCMPs.
2. Determining the impact of biological activity of miR-146a loaded NCMPs.

5.3 Methods

5.3.1 Preparation and characterisation of NPs and miRNA adsorption

PGA-co-PDL NPs were prepared and miRNA adsorbed as described in section 2.2. Characterisation of PGA-co-PDL NPs and miRNA adsorption were performed as described in section 2.3. The optimum NPs formulation used for NCMPs.

5.3.2 Preparation of Nanocomposite Microparticles

The NCMPs preparation were conducted in Department of Pharmaceutical Technology, Faculty of Pharmacy with Associate Prof Dr Ayca Yildiz Pekoz during my Erasmus exchange at Istanbul University May-July 2016. The NCMPs were prepared by spray drying the NP 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 NP to micro carrier ratio of 1:1.5 w/w (Alfagih et al. 2015) using a Büchi B- 290 mini spray-dryer (Büchi Labortechnik, Flawil, Switzerland) using the conditions of experimental design (section 5.3.3). The optimised condition used for spray drying was performed at feed rate 0.5 ml/min, aspirator capacity 70 %, atomizing air flow rate 480 L/h, inlet drying temperature 70 ºC (corresponding outlet temperature of approximately 47 ˚C). Dry particles were separated from the airstream by centrifugal forces using a high-performance cyclone (Büchi Labortechnik) (*Fig* 5–1), collected and stored in desiccator at room temperature until further use.

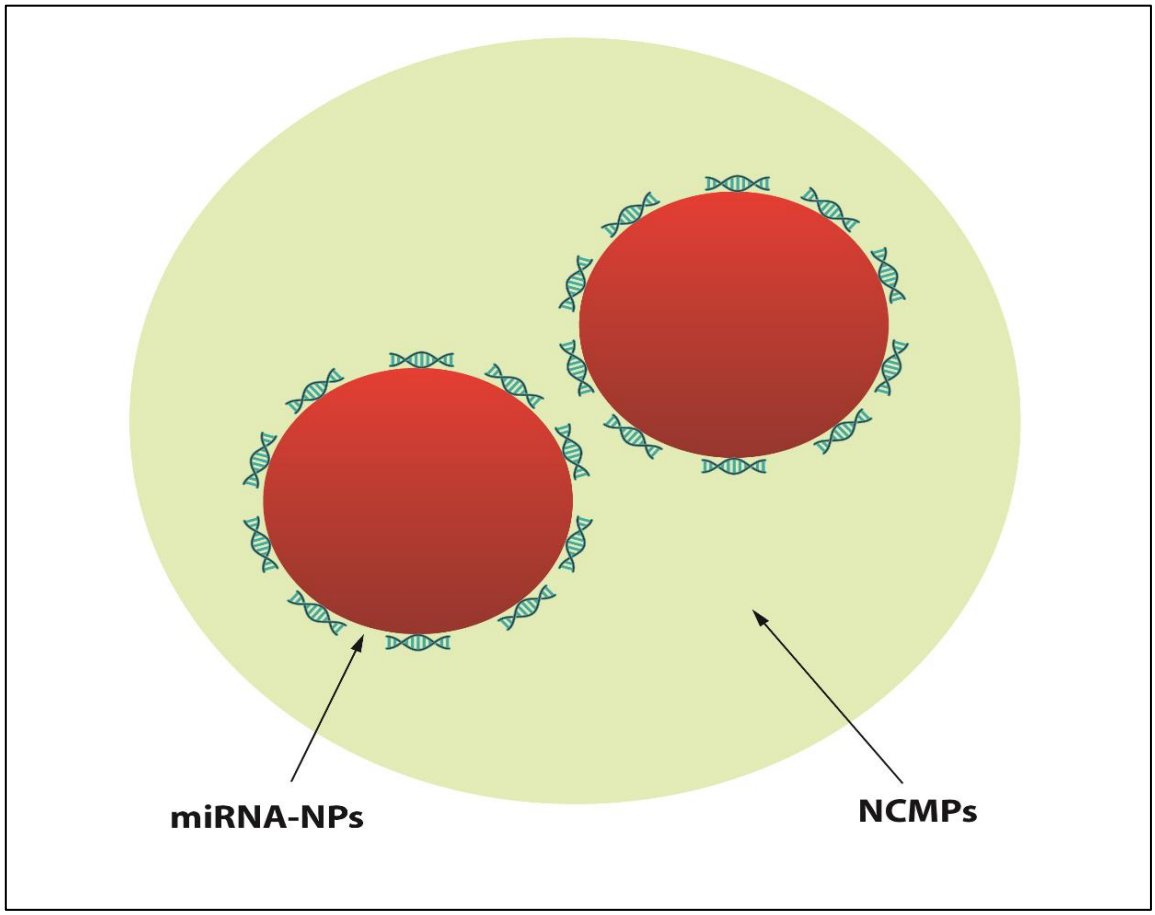

Figure 5–1 Diagram illustrates miRNA-NPs incorporated NCMPs.

5.3.3 Experimental design

The experimental design of the current formulation was optimized according to previous laboratory work and pre-formulation studies (Alfagih et al. 2015), combined with further parameters from the literature (Jensen et al. 2012, Edwards et al. 1998, Ståhl et al. 2002). It was composed of five variables and four levels (*Table* 5–1). The rational for using different parameters and levels is that some parameters were used for preparing protein loaded PGA-co-PDL NCMPs using L-leucine, but these parameters for example use high temperature, which

would damage miR-146a biological functionality. However, it was combined with further parameters derived from the literature that have used mannitol for siRNA and DNA, assuming that that will not affect miR-146a. The use of mannitol also preserves the biological activity and identifies the influence of spray drying parameters on recovered NPs size and yield %.

Table 5– 1. Nanocomposite microparticles process variables

Parameters	Unit	Level 1	Level 2	Level 3	Level 4
Feed Rate	ml/min	0.3	0.5	1	1
Atomized air flow	L/h	480	480	400	400
Aspirator capacity	%	70	70	100	100
Inlet temperature	°C	45	70	100	75
Approximate Outlet temp	°C	30	47	75	60

5.3.4 Characterisation of Nanocomposite Microparticles

The NCMPs characterisation were conducted in International Turkish pharmaceutical company- DEVA with assistance from Prof Dr Ayca Yildiz Pekoz during my Erasmus exchange at Istanbul University.

5.3.4.1 Yield

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

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

5.3.4.2 Morphology

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 (40–60 nm) and then observed at high vacuum.

5.3.4.3 Particle size

NCMPs (2 mg) were re-dispersed in 4 ml water to release the NP. The NP size was measured as described in section 2.3.1.

5.3.4.4 Moisture content

The water content of the NCMPs powder was determined by thermogravimetric analysis (TGA), using a Linseis STA PT 1750 Model Thermo Anaylzer system, Germany. NCMPs powder (10 mg) was heated between 25 °C to 650 °C at constant rate 10 °C/min in nitrogen gas. The weight loss (%) due to water evaporation, was recorded between 25 °C and 120 °C.

5.3.4.5 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. The initial bulk volume (Vo) was recorded and then again following mechanical tapping ten times (V10), then five hundred times (V500), then after one thousand and two hundred fifty (V1250) 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). $p1$ is the unit density (1 g/cm^3)

$$d_{ae} = d\sqrt{\frac{p}{p1}} \qquad \text{(Sou et al. 2011)}$$

5.3.4.6 Flowability and Carr's index

The particle flowability was analysed by Carr's index (compressibility index) and the Hausner ratio, using the values obtained for bulk density ($\mathbf{P}_{bulk}$) and tapped density ($\mathbf{P}_{tapped}$) (Louey et al. 2004) as follow;

$$\text{Compressibility Index} = \frac{\mathbf{P}\,\text{tapped} - \mathbf{P}\,\text{bulk}}{\mathbf{P}\,\text{tapped}} \times 100$$

$$\text{Hausner Ratio} = \frac{\mathbf{P}\,\text{tapped}}{\mathbf{P}\,\text{bulk}}$$

5.3.4.7 *In vitro* aerosolisation studies

The aerosol performance of the NCMPs was evaluated using a next generation impactor (NGI). The powder (10mg) was manually loaded into each of four hydroxypropyl methylcellulose HPMC size 3 (Qualicaps, Japan). The capsules were pierced by a needle pierce using a 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 every experiment by using a flow meter (Copley Scientific, UK). The flow rate was operated at 60 L/min for 4 seconds. 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 effective cut-off diameter for each impaction stage was calibrated by manufacturer and stated as shown in *Table* 5–3 (Hess 2005, Wu et al. 2013a). The fine particle dose (FPD) defined as the mass of the drug deposited in the NGI with $d_{ae} < 4.46$ μm, fine particle fraction (FPF %) is the fraction of emitted dose deposited in the NGI with $d_{ae} < 4.46$ μm, respirable fraction (RF) is the mass of particles < 4.46 μm and emitted dose (ED) is the amount of drug exiting the inhaler.

Table 5– 2. The effective cut-off diameter for NGI impactor at 60 L/min

Stage	cut-off diameter (µm)
1	8.06
2	4.46
3	2.82
4	1.66
5	0.94
6	0.55
7	0.34

The fine particle dose (FPD), fine particle fraction (FPF), respirable fraction (RF), and emitted dose (ED) were calculated as;

Fine particle dose (FPD, mg) = mass of particles < 4.46 µm (stages 2 through 7)

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

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

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

The mass mean aerodynamic diameter (MMAD, µm) and geometric standard (GSD, µm) were calculated from log probability analysis using the following website (http://www.mmadcalculator.com/).

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

The prepared optimum miR-146a-NPs in section 2.2 were spray dried as described in section 5.3.2 and biological functionality determined through semi-quantitative reverse transcriptase RT-qPCR and Western blot as described in section 2.4 and 2.5 respectively.

5.3.6 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 $p<0.05$. Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, Inc., San Diego,CA). All values are expressed as their mean ± standard deviation (SD).

5.4 Results

5.4.1 Spray drying optimization

Process parameters (T*able* 5–1) adopted from (Alfagih et al. 2015), for L-leucine NCMPs were applied alongside published parameters related to mannitol (Jensen et al. 2012), to identify the influence of spray drying parameters on NPs size and yield % (*Table* 5–3). The recovered NPs had sizes ranging from 175.93 nm to 3252.06 nm and a yield from 20.80 % to 96.00 % was obtained (*Table* 5–3). Based on the recovery of the NPs' size, yield (*Table* 5–5) response, and spray drying outlet temperature of 47 °C, *Level 2* parameters were chosen to be applied further.

Table 5– 3. Nanocomposite microparticles different levels Influence on particle size and yield.

		Excipient		Response	
	Formulations	**Leucine (%)**	**Mannitol (%)**	**Yield %**	**Recovery of NPs size (nm)**
Level 1	F1	100	0	20.80	251.06
	F2	75	25	Non	Non
	F3	50	50	84.80	289.80
	F4	25	75	48.00	175.93
	F5	0	100	76.00	319.13
Level 2	F1	100	0	64.00	774.26
	F2	75	25	86.40	1373.33
	F3	50	50	93.60	823.13
	F4	25	75	96.00	327.96
	F5	0	0	85.60	992.53
Level 3	F1	100	0	36.00	958.30
	F2	75	25	84.00	1810.66
	F3	50	50	91.20	3252.06
	F4	25	75	86.40	471.64
	F5	0	100	80.80	2174.60
Level 4	F1	100	0	36.00	669.10
	F2	75	25	84.00	600.90
	F3	50	50	91.20	747.10
	F4	25	75	86.40	297.10
	F5	0	100	80.80	682.40

5.4.2 Moisture content, powder flow and Carr's index

Water content for formulations (F1–F5) were within the range of moisture content (2.02±0.03 % – 5.1±0.37 %) of spray-dried particles intended for lung deposition (Ståhl et al. 2002, Chew et al. 2005a), (*Table* 5–4). However, F4 had the lowest moisture content, due to the presence of a higher percentage of mannitol (Jensen et al. 2012).

To measure flow properties of NCMPs, Carr's compressibility index was used and determined from tapped and bulk density (*Table* 5–4). Values of more than 25 % indicated poor followability (Learoyd et al. 2008).

Table 5– 4. Physical properties of spray-dried nanocomposite microparticles. Mean± SD (n=3).

Formulations	Tapped density (g/cm^3)	Water content (%)	Carr's Index	Hausner Ratio	Flow character
F1	(-)	5.1±0.37	N/A	N/A	N/A
F2	0.17±0.01	4.91±0.20	33.30	1.30	Poor
F3	0.13±0.20	3.8±0.90	0 (≤10)	1.00	Excellent
F4	**0.14±0.01**	**2.02±0.03**	**33.30**	**1.30**	**Poor**
F5	0.20±0.05	3.77±0.11	66.60	1.60	V. Very poor

5.4.3 *In vitro* aerosolisation studies

The *in vitro* aerosol dispersion properties of NCMPs were determined using NGI. The mass mean aerodynamic diameters (MMAD) ranged from 4.20±0.15 to 6.03±1.08 µm. The NCMPs' formulations (F1-F4) showed that MMAD was less than 6 µm except F5 which was significantly greater 6.03±1.08 µm (p<0.05, ANOVA/Tukey's) (*Fig.* 5–2B). The corresponding geometric standard deviation **(**GSD) values were approximately similar in size from 1.75± 0.31 – 2.15±0.53 µm.

FPF % showed that F4 produced significantly higher FPF % (51.33±2.90 %) compared to the other formulations (p<0.05, ANOVA/Tukey's). The highest FPF % means the best aerosolization characteristics in term of deposition. The presence of leucine weight ratio to mannitol ratio in F4 resulted in high FPF compared to F1, which has no mannitol. Moreover, the incorporation of leucine into formulations has increased FPF %. F4 has 25 % leucine which resulted in more than double values of FPF %, 51.33±2.9 % and FPD, 20.53±2.90 mg respectively compared to F5, 0 % leucine with FPF %, 19.96±1.2 % and FPD 7.98±1.2 mg (*Fig.* 5–2A and *Fig.* 5–2E). The ED values were all over 50 % with highest value for F4 81.81±3.0 % (*Fig.* 5–2D).

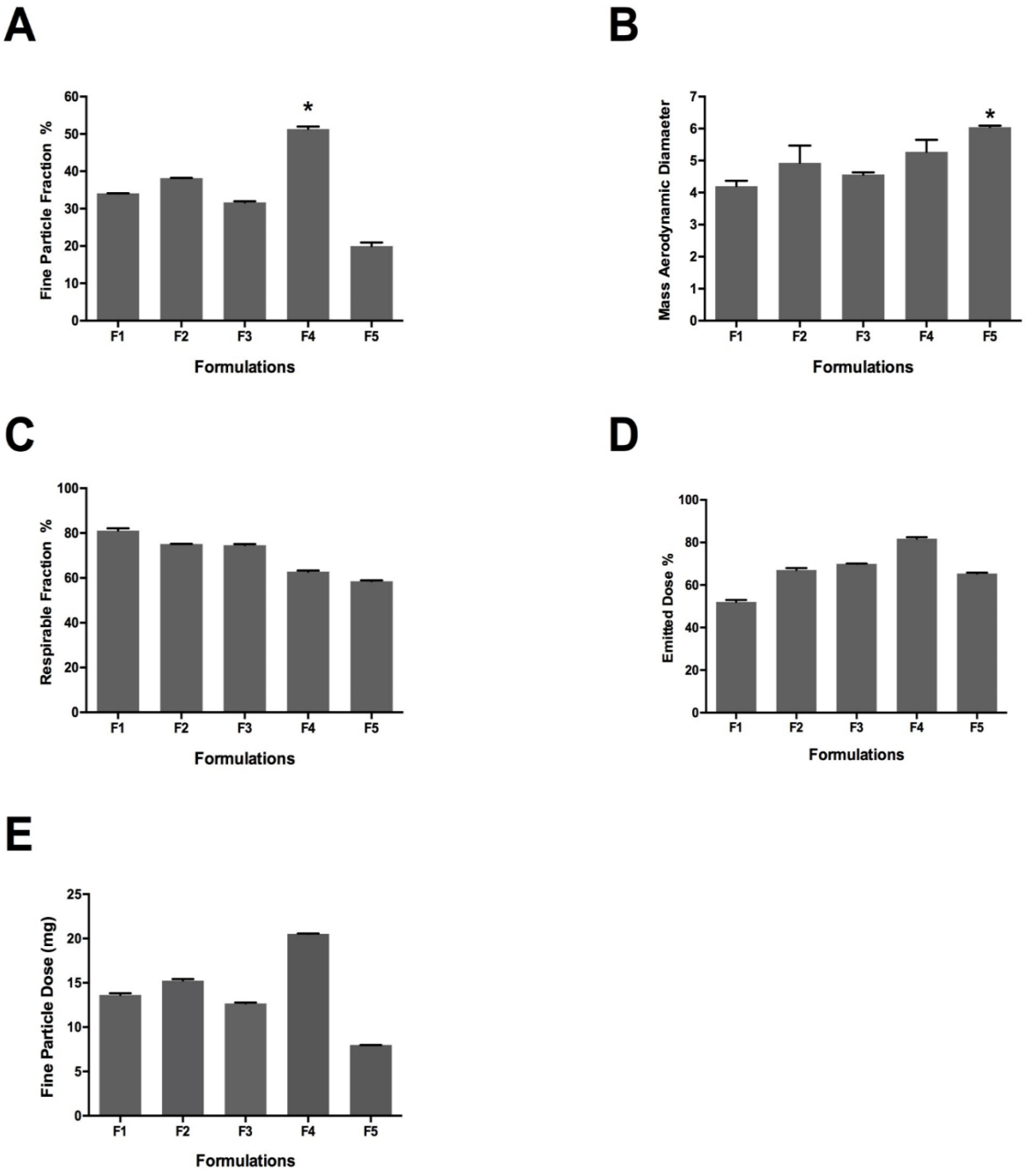

Figure 5– 2. (A) The percentage fine particle fraction spray dried powder (B) mass mean aerodynamic diameter (µm). (C) Respirable fraction percentage. (D) Emitted dose. (E) Fine Particle Dose. Data represent mean ±SD (n=3), (*p<0.05, ANOVA/Tukey's).

5.4.4 Recovered particle size and yield

From *Table* 5–3, *Level 2* is the best in terms of yield percentage (Level *2*, 96.00 % of dry powder compared with other level 3, 86.40 %) and recovered size of NPs (where level 3 has high NPs size after recovery), of which F4 is the optimum and taken forward, based on results of moisture content, powder flow (section 5.4.2), and aerosolisation data (section 5.4.3). The results of the recovery of F4 NPs' size and yield percentage were repeated to gain more replicates as shown in (*Table* 5–5). The particle size for all the levels and formulations were different, however, the smaller particle size recovered after spray drying was preferred and needs to be similar to prior spray drying (Alfagih et al. 2015).

Table 5– 5. Optimum Spay dried particles size and yield. Mean± SD (n=3).

	Particle size (nm)		
Formulation	**Before spray drying**	**After spray drying**	**Yield %**
F4	244.8±4.40	409.7±10.05	86.0±15.01

5.4.5 Morphology, Powder density and Aerodynamic diameter of formulation F4

The formulated NCMPs (F4) were analysed for shape and morphology using SEM (*Fig.* 5–3) and micrographs showed that NCMPs possessed a spherical shape and corrugated surface. The tapped density of PGA-co-PDL NCMPs varied between 0.132±0.03 to 0.20±0.01 g cm^{-3}, and F4 0.14±0.01 g cm^{-3} (*Table* 5–4). The Geometric particle size and tapped density was then used to calculate the theoretical dynamic diameter (d_{ae}). The optimum formulation (F4) d_{ae} was 0.63±0.01 µm.

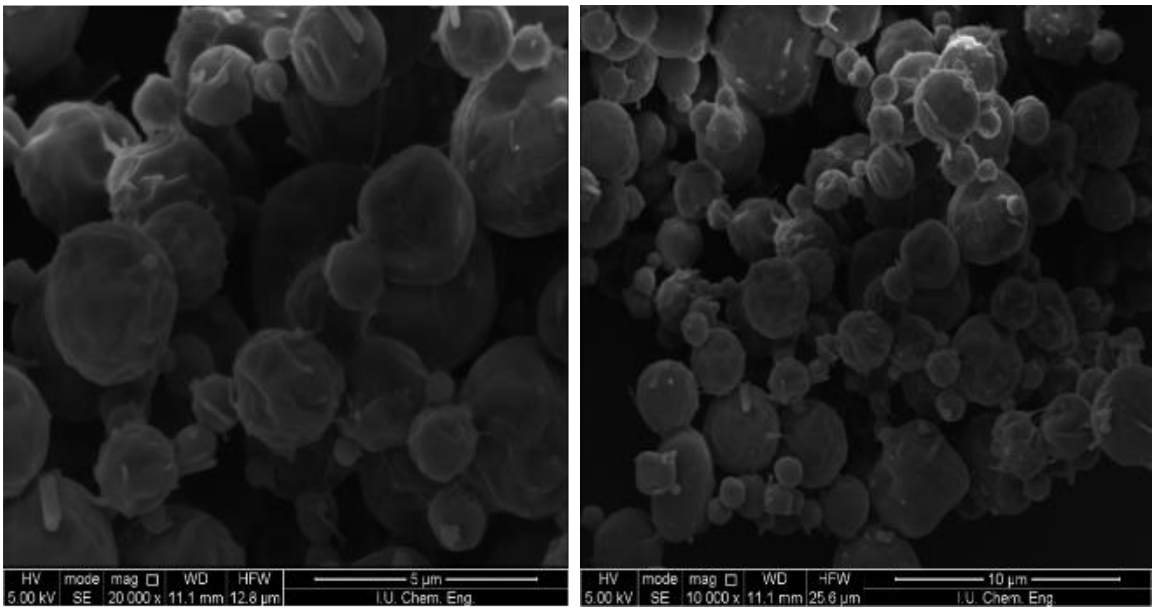

Figure 5–3. SEM images of F4 NCMPs, the scale bars represent 5 µm and 10 µm.

5.4.6 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.* 5–4). The expression of *IRAK1* and *TRAF6* was normalised to *GAPDH* expression. These results were comparable with the miR-146a-NPs data that was mentioned in section 4.4.1.

In Western Blot, miR-146a reduced *IRAK1* protein levels in A549 cells after 24 h and 48 h treatment. The β-actin was used as control. As shown in (*Fig.* 5–5), 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.

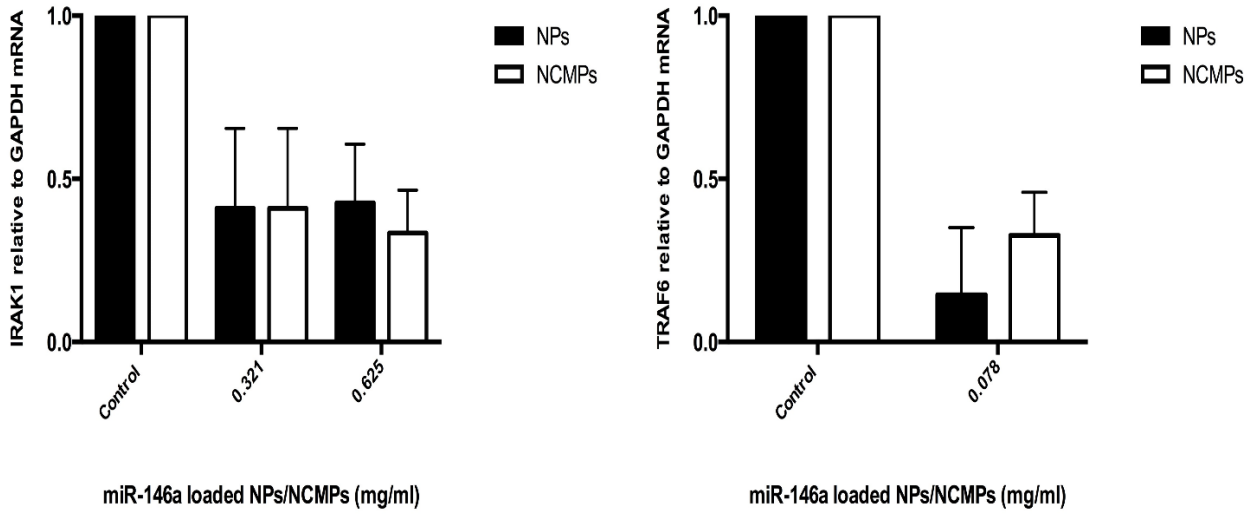

Figure 5– 4. MiR-146a-NCMPs reduce miR-146a target expression. Levels of IRAK1 and TRAF6 were assessed by sqRT-PCR in A549 cells that had been exposed to miR-146a-NPs or miR-146a-NCMPs for 1 h washed then incubated for 24 h. The doses yielding the most consistent downregulation of the target genes are shown. Mean ± SD (n=3).

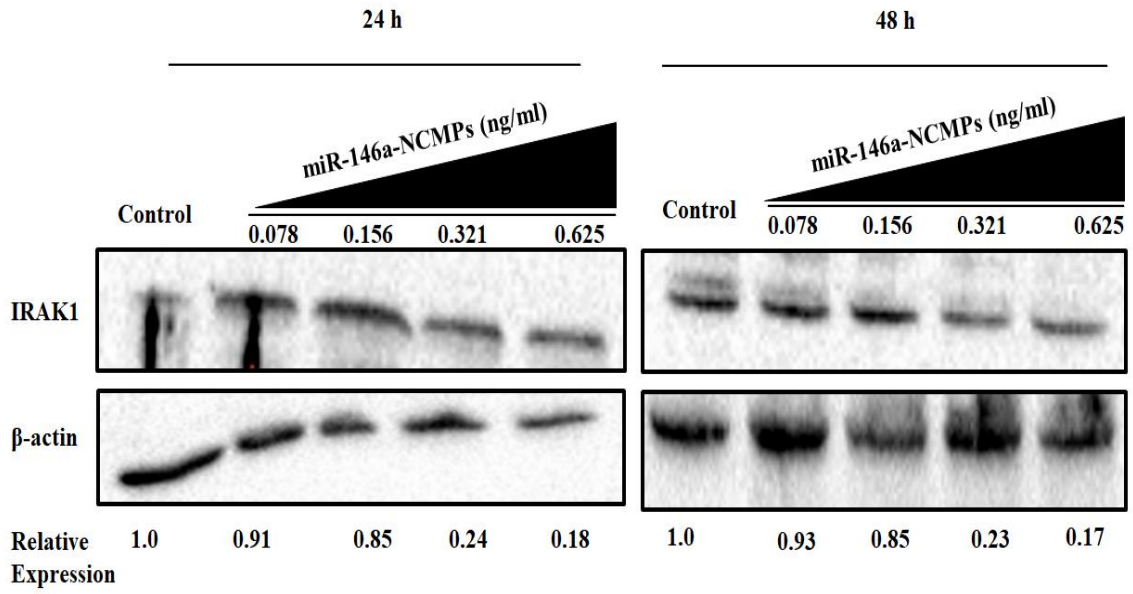

Figure 5– 5. Western Blot expression of miR-146a reduced IRAK1 protein levels in A549 cells for 24 h and 48 h. Dark triangle represents lowest (left) to highest (right) miR-146a-NCMPs concentrations. Numbers under each band represent the densitometric readings relative to control samples that normalized each band to its corresponding β -actin control.

5.5 Discussion

5.5.1 Optimization of spray drying process

The spray drying optimisation process was utilised to incorporate NPs into NCMPs using different ratios of L-leucine and mannitol excipients. From *Table 5–3*, it can be seen that all of levels (1–4) had variable yield % and NPs recovered particle size results. The different spray drying parameters (*Table* 5–1) were used to identify the best level that preserved the recovered particle sizes and produced the highest yield % during the spray drying process. Generally, levels 1, 3 and 4 produced acceptably high yields as a % and large particle size. However, *Level 2* showed the highest yield 96.00 % accompanied with the smallest particle size < 500 nm

(Table 5– 2), which would maintain miRNA biological functionality. Spray drying parameters used in *level 2* showed, that outlet temperature 47 °C *(Table* 5–1) was below melting point of PGA-co-PDL NPs (Tawfeek et al. 2011, Tawfeek et al. 2017). Monitoring and maintaining a low outlet temperature can reduce agglomeration, as shown by the study of Mohajel *et al.* using polymer based NPs as dry powder to deliver DNA, where it was found that using a low outlet temperature reduced the risk of DNA denaturation and decreased particle aggregation (Mohajel et al. 2012).

It was found that operational parameters such as feed rate, aspirator capacity, atomised air flow, spray drying inlet and outlet temperature have a significant impact on recovered particle size and yield % of dry powders (Rohani et al. 2014a). The highest yield % of level 2 was relying on operation parameters (*Table* 5–1), Alfagih *et al.* had optimised for the spray drying process of PGA-co-PDL NPs using BSA a model protein incorporated into NCMPs for pulmonary delivery. It was found that flow rate was the most significant factor that had an effect on yield (Alfagih et al. 2015). Technically, during the spray drying process atomized air was used which should penetrate the liquid stream. However, if there was insufficient atomized air due to low feed rate, the dry powder sticks on the drying chambers and cannot be collected adequately (Motlekar and Youan 2008).

A similar observation was noted in *level 2* inlet 70 °C and outlet 47 °C temperature (*Table 5–1*). It was found that the inlet temperature used in *level 2* had affected the recovered NPs particle size and yield. Comparing the inlet temperature in *level 1* and *level 2* (*Table 5–1*) showed an increase from 45 °C to 70 °C. This inlet temperature increase can lead to a reduction in the drying time and can inhibit particle aggregation (Mohajel et al. 2012). In addition, the high inlet temperature can lead to a decrease of residual moisture by enhancing water evaporation which cause less particles to stick in drying chamber walls (Billon et al. 2000). However, high inlet temperature may affect the small nucleic acids stability (Mohajel et al. 2012).

5.5.2 Water content and flowability

Table 5–4 showed the water contents for all the formulations, which were within the spray dried powder range. However, F4 had the lowest moisture content, due to a higher percentage of mannitol (Jensen et al. 2012). Adding mannitol as an excipient enabled the formulation to have less water content, and the powder with the least water content reduces the cohesion between particles and therefore increases the powder respirability (Chew and Chan 2002). Mannitol has hygroscopic properties enabling the formation of hydrogen bonds with water molecules causing water replacement which stabilises the formulation (Clegg et al. 1982, Schüle et al. 2008, Sarmento et al. 2006). The high water content may also have a negative impact on product stability (You et al. 2007), however by keeping the water content low it is likely to result in high storage stability. Adi *et al*. reported that the presence of mannitol in spray dried powder reduced water content, which prevented powder recrystallization, where the mannitol is thought to have decreased the amount of water bound in spray dried powder (Adi et al. 2010).

As a consequence, adding mannitol to the formulation was successful and kept moisture content low, and the optimum ratio of L-leucine to mannitol F4; 25:75, (*Table 5–3*) was also shown to have an effect on water content, which is in accordance with other studies (Li et al. 2016b, Chow et al. 2017). Additional research showed that mannitol used in a combination with amino acid (L-alanine), a study by Rohani *et al*. found that the formulation containing mannitol and amino acid had a moisture content range between 4 – 6 % (Rohani et al. 2014b) compared to our results 2.02±0.03 % – 5.1±0.37 % (*Table 5–4*). Mannitol is a commonly-used excipient in spray drying powders which has a good safety record, which produces NCMPs that are compatible, dissolve easily in lung fluid and has regulatory approval (Jensen et al. 2010, Pilcer and Amighi 2010, Burness and Keating 2012).

The NCMPs' flowability was determined by Carr's index, which showed F4 formulation has poor powder flow 33.30 % (more than 25 %). This property could be due to powder aggregation and the particles could not re-form again. Due to the limited mass of powder, larger volume cylinders could not be used, hence a 5 ml measuring cylinder was used instead. This is not ideal and may have resulted in poor flow as determined by Carr's index as this is associated with narrow cylinder during tapping that does not allow the powder particles to flow thus they become compact and restricted in movement. The F4's poor powder flow, could be due to powder adherence to walls of measuring cylinder compared to other formulation resulted due to strong van der Waal force between particles (Tawfeek et al. 2011). Similar results were reported by Alfagih *et al.* when PGA-co-PDL NPs-NCMPs were used and leucine as an excipient, where they found high Carr's index values and poor powder flowability (Alfagih et al. 2015). However, inhalation of the NCMPs with mannitol and leucine, will results in dissolution of the excipients in lung lining fluid, subsequently releasing NPs for uptake by lung cells. NCMPs composed of PLGA NPs and mannitol have been shown to decompose in the lung lining fluid and release NPs (Jensen et al. 2010).

5.5.3 *In vitro* aerosolisation studies

Incorporation of L-leucine into the formulations improved both FPF % and FPD. By adding leucine to the spray dried formulation, the aerosolisation performance was impacted (Tawfeek et al. 2011). F4 of optimum condition containing leucine had the highest FPF ratio 51.33±2.9 % (*Fig* 5–2A), which indicated it had the best aerosolisation properties and improved aerosolisation performance (Rabbani and Seville 2005, Li et al. 2005, Seville et al. 2007, Sou et al. 2013, Lucas et al. 1999). Thus, adding leucine to the spray dried formulation had a positive impact on the aerosolisation performance (Tawfeek et al. 2011).

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. This property also affects the shape of spray dried powders which will be discussed further in section 5.5.4. Furthermore, the L-leucine amount in the spray dried powder has an effect on aerosolisation performance, comparing FPF % of F4 and F5 as L-leucine ratio in F4 (25 % L-leucine) was higher than F5 (0 % L-leucine), difference was observed where, F4 had FPF %, 51.33±2.9 % and F5 had FPF %, 19.96±1.2 % (*Fig* 5–2A). This difference was due to less powder stickiness and cohesiveness, which is in line with another study conducted by Gervelas *et al.* when leucine was added to spray dried powder, and it was found that the higher leucine concentration in the formulation the better aerosolisation performance (Gervelas et al. 2007).

As seen in *Fig* 5–2B, the MMAD results showed that F1-F4 were less than 6 µm whereas, F5 was 6.03±1.08 µm. The difference here suggests excipient influences MMAD, F1-F4 had mannitol percentage of less than F5. A study by Jensen *et al.* showed that spray dried NPs with high concentration mannitol resulted in higher MMAD compared with spray dried NPs with lower mannitol (Jensen et al. 2010). The amount of mannitol in formulation and absent of L-leucine may form aggregated powders and a rougher surface which contributes to higher MMAD. Kaialy and Nokhodchi used mannitol as excipient in spray dried powder, and they found that the presence of a high mannitol concentration in the formulation increased MMAD, and that was reflected by powder cohesive force aggregation (Kaialy and Nokhodchi 2013). Another reason for the difference between F1-F4 and F5 MMAD, was due to F5 had no L-leucine and 100 % concentration of mannitol, and it was observed that there was a decrease in FPF % and an increase in MMAD > 6 µm. Similar results were obtained by Chow *et al.* when various concentrations of L-leucine were used with mannitol in inhaled powder formulation,

and they found that absence of L-leucine in powder reduced FPF % and increased MMAD (Chow et al. 2017).

The MMAD values were less than 6 µm, which means within the range of 1 – 5 µm, is necessary for it to be deposited in the middle to deep lung regions by sedimentation due to gravity (Carvalho et al. 2011b), whereas those particles with MMAD of more than 6 µm are considered to deposit in the upper conducting airways (Carvalho et al. 2011a). Although F4 MMAD is within 1 – 5 µm, the deposition profile from NGI data showed that 20 % of the formulation deposited in NGI stage is less than 5 µm. This can predict the amount of miR-146a that will be deposited in the regions of the lungs and have potential cell uptake, and can also produce change in targeted gene expression as shown in section 5.4.6.

The FPF, ED, and MMAD values obtained 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. 2013). 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. This mechanism was also proposed by Kunda *et al.* and Alfagih *et al.* (Kunda et al. 2014b, Alfagih et al. 2015).

5.5.4 Morphology, yield and recovered NPs particle size

The surface morphology of NCMPs was carried out using SEM, and the micrographs showed that NCMPs were corrugated, which suggested this shape was due to the reduction in the cohesion between particles (Chew et al. 2005b, Chew and Chan 2001). 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 space thereby filling and packing particle's structure (Sou et al. 2013, Alfagih et al. 2015, Lucas et al. 1999). These corrugated surface particles would have a larger

surface area, leading to an increase in the particles' capability to diffuse and disperse in lung fluid thus releasing miR-146a-NPs. The formulation was more controlled by choosing sugar excipient such as mannitol rather than lactose, which interacts with certain amine groups and affect the particle's morphology (Vehring 2008, Bharate et al. 2016).

Analysis of F4, produced a high yield 86.05±15.01 (*Table* 5–5) of dry powder accompanied with the desired small particle size recovery < 500 nm. The high yield was due to reduced loss of dry powder during the collection process which also affected on recovered particle size that relies on centrifugal forces for collection of final dry powder due effective separation capacity of cyclone. Another reason for producing a high yield is that the spray dryer used (section 5.3.2) in preparing spray dried powder can produce high yield, when the process and formulation parameters e,g flow rate, inlet temperature, atomized air flow, aspiratory capacity undergo appropriate optimisation (Motlekar and Youan 2008, Sosnik and Seremeta 2015).

Hence, F4 was chosen as optimum to use *level 2* parameters. There was an inverse correlation observed between increased mannitol concentration and yields. The increase of mannitol concentration lead to an increase in particle size from 244.8±4.4 nm before spray drying (Section 3.4.2) to 409.7±10.05 nm (*Table* 5–5) and 327.96 nm (*Table* 5–3) after spray drying. This particle size increase after spray drying may be a result of change in the conformation under operating conditions of spray drying, and similar results were also found by Sham *et al.* for spray dried powder prepared with sugar as excipient for gelatine NPs, and the size after spray drying which were in nano-sized (Sham et al. 2004).

The NCMPs' yield obtained a good production yield for all the formulations. However, spray-dried microparticles' yield was changeable and affected by the ratio of excipients. When the optimum parameters were applied with no mannitol, there was a lesser yield % (64 %). The

optimum parameters used for the production of the highest yield % of dry powder resulted in reasonable yield of NCMPs 86.0±15.01 % (*Table* 5–5) (Jensen et al. 2010).

F4 was chosen as the optimum formulation despite its poor flowability comparing to F3 that had showed better flow character, (*Table* 5–4) but the recovered NPs' size of F3 was almost 1 µm (823.13 nm), (*Table* 5–3) which was too large. Alfagih *et al.* have obtained results with poor flowability when added leucine to NCMPs but managed to achieve a significantly higher FPF % and the smallest recovered particle size (Alfagih et al. 2015). As mentioned above, F4 was selected as the optimum formulation according to the high yield, lower moisture content, good aerosol properties and the recovered NPs' size which was similar to the starting NPs' size (*Table* 5–5). It can be noted from *Tables* 5–2 and 5–4 the NCMPs reported here have high yield values, which indicate the significant potential of using a mixture of mannitol and L-leucine properly, with effective parameters. However, the yield values reported in previous studies were to be from 40 – 50 % (Alfagih et al. 2015, Tawfeek et al. 2011, Kunda et al. 2015a). Therefore, the prepared formulation would effectively deliver the small nucleic acid to the lung as inhaled dry powder.

5.5.5 Impact of miR-146a on target expression

The miR-146a-NCMPs lowered expression of t target genes *IRAK1* and *TRAF6*. As shown in *Fig. 5*–4 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 *TRAF*6 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 also protected biological activity of miR-146a.

Immunoblotting (*Fig.* 5–5) supports that miR-146a activity was maintained after spray drying, with protein level changes after 24 h and 48 h of miR-146a-NCMPs' treatment occurring in a dose dependent manner. 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.

It appears that NCMPs delivered miR146a 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 had played important role by targeting multiple genes after inhalation the dose that spread over various parts in lung (Rossi 2009, He et al. 2005, Bhardwaj et al. 2009). In fact, various studies have investigated siRNA and DNA delivery intended for inhalation (Jensen et al. 2010, Liang et al. 2015, Liang et al. 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. 2015). 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). 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.

5.6 Conclusion

The results indicated that the method has been optimised for the spray drying of NCMPs. 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 has been recovered after spray drying (409.7±10.05 nm) and geometric particle size is suitable for targeting the respiratory bronchiole. Moreover, the optimum formulation had a high yield (86.0±15.01 %), and low moisture content (2.02±0.03 %) which is essential for powder aerosolization and formulation stability. The aerosolization performance showed high FPF 51.33±2.9 %. The biological activity of miR-146a persevered after spray drying process and miR-146a loaded NCMPs caused gene silencing.

6.General Discussion

6.1 Overview

Chronic obstructive pulmonary disease COPD is heterogeneous inflammatory disease and is a major cause of morbidity and higher mortality rates throughout the world**.** COPD is currently the fourth leading cause of death in the world and is predicted to become the third leading cause of chronic illness and death worldwide by 2030 (World Health statistics 2008). The current therapeutic strategies including pharmacological and non-pharmacological treatments for COPD have not been shown to delay and correct the long-term defects in lung function (Global Strategy for the Diagnosis, Management and Prevention of COPD and GOLD 2016).

However, the expression of small non-coding RNA molecules known, as microRNAs (miRNAs) has been associated with neoplastic and inflammatory lung disease. Recent work suggests cytokine-dependent induction of miR-146a is impaired in COPD fibroblasts, leading to overexpression of cyclooxygenase (COX)-2 and enhanced production of PGE2 (Sato et al. 2010). miR-146a is capable of controlling the Toll-like receptor and cytokine signalling through negative feedback regulation, associated with down regulation of *IRAK1* and *TRAF6* protein levels (Taganov et al. 2006, Nahid et al. 2009).

The ideal route of the pulmonary delivery of nucleic acid is non-invasive and offers various advantages over the more conventional oral, buccal, transdermal and nasal routes. miRNA can be administered in reduced doses for lung cell targeting, avoiding degradation by serum, and bypass of the first hepatic metabolism (Patton and Byron 2007, Takei et al. 2004)

The promise of RNA interference mediated miRNA has provided a novel strategy to formulate non-viral intracellular carriers to knock down target messenger RNA (mRNA). Various studies have investigated the delivery of siRNA and DNA to target lung cells (Takashima et al. 2007, Jensen et al. 2010, Cherng et al. 1997, Andersen et al. 2008) but there is a very limited research has been investigated on miRNA delivery, which has the benefit of being able to regulate and

inhibit protein expression without interfering with other proteins. The aim of this project was to formulate and characterise miRNA containing NPs formulated as an inhalable dry powder microcarrier for the treatment Chronic Obstructive Pulmonary Disease (COPD).

6.2 Optimisation of cationic nanoparticles

The NPs size and surface charge play an important role in interaction with intracellular and extracellular components of cells. The interaction between the positive NPs charge and the negative cellular charge help in the particle uptake (Borm et al. 2006). Incorporation of DOTAP to NPs during NPs preparation methods was successful and produced cationic NPs. The use of DOTAP to produce cationic material have been previously explored, resulting in modified particle size and charge (Kumar et al. 2004, Jensen et al. 2012)

It was found that DOTAP concentrations affected particle size and charge, the increase of DOTAP concentration reduced the particle size and increased surface charge to a more positive value. Similar results were reported by Kunda *et al.* when DMAB was used as a cationic surfactant, to prepare positively charged PGA-co-PDL NPs. They found that polymeric NPs have the ability to combine with other cationic compounds (Kunda et al. 2014b). The particle size decreased when the concentration of DOTAP increased to 30 mg/ml DOTAP (15 % w/v). The decrease of particle size has been associated with the ability of cationic material to reduce the interfacial tension between particle surface and aqueous phase, as well as DOTAP properties to restrain the enlargement of polymeric NPs (Song et al. 2006a, Jensen et al. 2012).

When DOTAP incorporation to NPs applied for the preparation of cationic NPs, the adsorption of miR-146a occurred. The optimum adsorption condition and parameters on surface has ability to protect miR-146a over encapsulation, due to preventing from contact with solvent,

sonication and mechanical stress used during NPs preparation process (Cun et al. 2010, Soutschek et al. 2004).

The positive charge formed on the NPs allowed the negatively charged miR-146a for electrostatic interaction (Li et al. 2014). The adsorption of negatively charged miR-146a on the cationic NPs slightly decreased the surface charge. The cationic NPs charge remained positive after the miR-146a adsorption, the maximum level of miR146a adsorption was after 2 h, suggesting the saturation time, which help in effective ability to bind to anionic cell surface (Yuba et al. 2008).

6.3 Spray drying of miRNA-containing NPs into NCMPs

Spray drying has been shown to be a potential technique for small nucleic acids intended for inhalation with the use of excipients such as mannitol, trehalose, L-leucine and lactose alone or in combination (Jensen et al. 2010, Takashima et al. 2007, YT Chow and KW Lam 2015). The spray drying optimisation process was used to incorporate NPs into NCMPs using a mix of L-leucine and mannitol excipients. The optimum *level 2* produced highest yield percentage 96.00 % and smallest recovered particle size.

It was noted that the operational parameters such as feed rate, spray drying inlet, outlet, aspirator capacity, and atomised airflow have a significant effect on the yield and recovered particle size. Rohani *et al.* have optimised spray dry powder intended for pulmonary delivery, they found that the processing parameters affected particle size and achieved high yield (Rohani et al. 2014a). Others reported similar observations (Alfagih et al. 2015, Motlekar and Youan 2008, Mohajel et al. 2012, Billon et al. 2000).

The NPs were successfully spray dried using a mix of L-leucine and mannitol. The NCMPs showed a corrugated surface shape this occurred due to the reduction in cohesion between

particles, water evaporation that happened during spray drying process causing high vapour pressure. In addition, the presence of L-leucine in spray-dried particle, which has low density was capable of forming space filling thereby and packed particle structure (Chew et al. 2005b, Lucas et al. 1999, Sou et al. 2013, Alfagih et al. 2015).

Moreover, the NCMPs formulations produced high values of FPF % and FPD. This could be attributed to L-leucine excipient in the formulation, which improved aerosolisation performance (Kunda et al. 2014b, Tawfeek et al. 2011, Li et al. 2005). FPF, ED, MMAD values would suggest very good aerosolisation properties that should allow NCMPs formulation to deposit in the middle to deep lung regions.

Others reported similar results when L-leucine was used in the formulation (Kunda et al. 2013, Alfagih et al. 2015, Kunda et al. 2014b). The NCMPs formulation has low moisture content, which reflects the efficient spray drying conditions performed leading to helping in powder storage. This provides an indication of the use of mannitol as an excipient in the formulation that increases the powder respirability (Chew and Chan 2002). Therefore, the optimum spray dried parameters achieved high yield good recovery of NPs and maintained miRNA functionality.

6.4 The biological activity of miR-146a

The highest miR-146a loaded NPs concentration showed a reduction in target gene *IRAK1* expression to 40 %. While, target genes *TRAF6* were determined and the lowest NPs concentration caused reduction in expression to over 20 %. The miR-146a showed activity as a negative regulator of inflammation by inhibiting the expression of *IRAK1* and *TRAF6* genes (Taganov et al. 2006). Another a study by Zhao *et al.* showed miR-146a implicated as a negative feedback regulator of NF-κB and important component of immune cell gene regulation (Zhao et al. 2011).

In addition, the miR-146a-NPs affected *IRAK1* protein levels, and decreased the protein levels. This suggest that miR-146a released in the cell successfully targets IRAK1 mRNA, and leads to translational repression (Kuhn and Joshua-Tor 2013). These results show the promising NPs delivery carrier, protecting miR-146a from degradation and enzymatic activity in the lung airways (Lam et al. 2015, Yuba et al. 2008). Similar results were reported by Liang *et al.* they showed that polymer based NPs used as carrier to deliver miR-26a to HepG2 cells (Liang et al. 2011).

The miR146a biological activity maintained after spray drying. The miR-146a-NCMPs knockdown the targeted *IRAK1* and *TRAF6* genes, accompanied with protein level changes in a dose dependent manner. This could be attributed to the appropriate use of the delivery formulation and excipients (Jensen et al. 2010, Liang et al. 2015).

7. Future work

7.1 Future work

Further studies will evaluate the biological studies of miR-146a in terms of pro-inflammatory cytokine such as pIL-8 and TNF-α. ELISA assay indicate the measurement of pIL-8 promoter-GFP reporter protein level (MacKenzie et al. 2001). The optimum miR-146a-NPs incorporated NCMPs which would be helpful to examine the biochemical response, as well as the activity and stability of miR146a. To further expand the delivery potential of miR-146a-NCMPs for *in vivo* applications the promising *in vitro* studies need to move to animal models. The best experimental animal models for miR-146a-NCMPs administrated via inhalation for COPD studies are mice, guinea pigs and rats (Ghorani et al. 2017).

The stability of NCMPs will be determined using X-ray powder diffraction and differential scanning calorimeter (DSC) to evaluate changes in the crystallinity of the particles. Further studies should also examine the long-term stability of NCMPs at various temperature and humidity conditions.

Further immunoblotting studies will also evaluate the effect of miR-146a-NPs/NCMPs on protein expression of *TRAF6*. If these results could be acquired in addition to the obtained findings that showed the effect of miR-146a-NPs/NCMPs on target genes and protein expression of *IRAK1*, this will help provide full insight into miR-146a-NPs/NCMPs biological function activity. Further optimised studies to evaluate luciferase reporter, monitor the transfection and interaction with 3'UTR miRNA and determine the expression effect of miR-146a-NPs.

miR-146a regulate Toll-like receptor (TLR) biological signalling through interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6). However, there are likely to be other targets for miR-146a (Taganov et al. 2006). Transcriptome analyses will enable large-scale identification genes modulated by miR-146a-NCMPs.

The delivery of miRNAs offer an exciting field for NPs, inhalation and inflammatory diseases. There are various studies in literature linked to miRNAs role being used as biomarkers for diagnosis and regulation. This highlight the importance of miRNAs to be useful and reach the clinical trial stage. The significant therapeutic action of miRNA mimics enabled miR-34 to be used for the treatment of many cancers such as; colon, ovarian, cervical, non-small cell lung cancer, and hepatocellular carcinoma, and miR-34 has been tested in phase I clinical trials (Bouchie 2013b).

In order to align the present work to the pre-existing literature, the cationic DOTAP was successfully used to produce cationic NPs that offer positive surface charge for miR-146a adsorption. The selected PGA-co-PDL NPs were incorporated into L-leucine and mannitol as a carrier to improve the powder's aerosolisation properties. The biological activity of miR-146a persevered after spray drying process and miR-146a loaded NCMPs caused gene silencing. The use of polymeric NPs/ NCMPs in the delivery of miR-146a to lung cells offers important therapeutic potential to cellular dysregulations with less off-target effects. However, taking into account the advantage of the promising biological effects of inhaled siRNA in the lung there has been very limited work exploring miRNA spray drying. In the field of COPD and inhaled miRNA there is need of more future work to be done of using more miRNA spray drying due to their therapeutic potential as pulmonary drug delivery that manages COPD rather than other medications.

It is important to take into consideration, the practical considerations of patient compliance with repeated doses and the probability of using combination of miR-146a-NCMPs with other chemotherapy or immunotherapy. In the future, NPs containing miR-146a will gain importance in lung diseases and other human disease.

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ZHAO JL, RAO DS, BOLDIN MP, TAGANOV KD, O'CONNELL RM AND BALTIMORE D. 2011. NF-κB dysregulation in microRNA-146a–deficient mice drives the development of myeloid malignancies. Proceedings of the National Academy of Sciences 108: 9184-9189.

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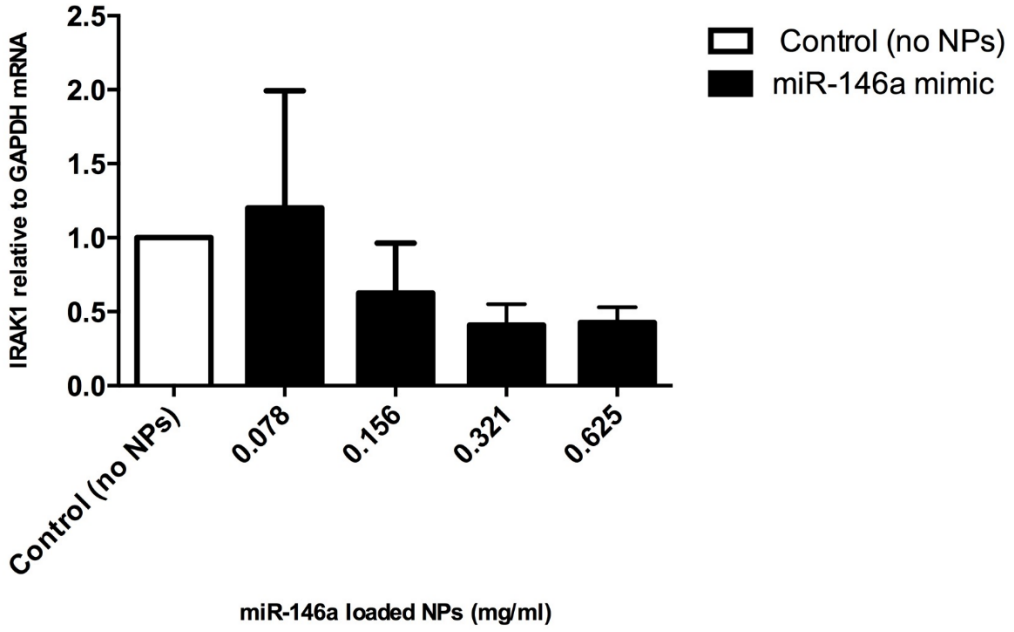
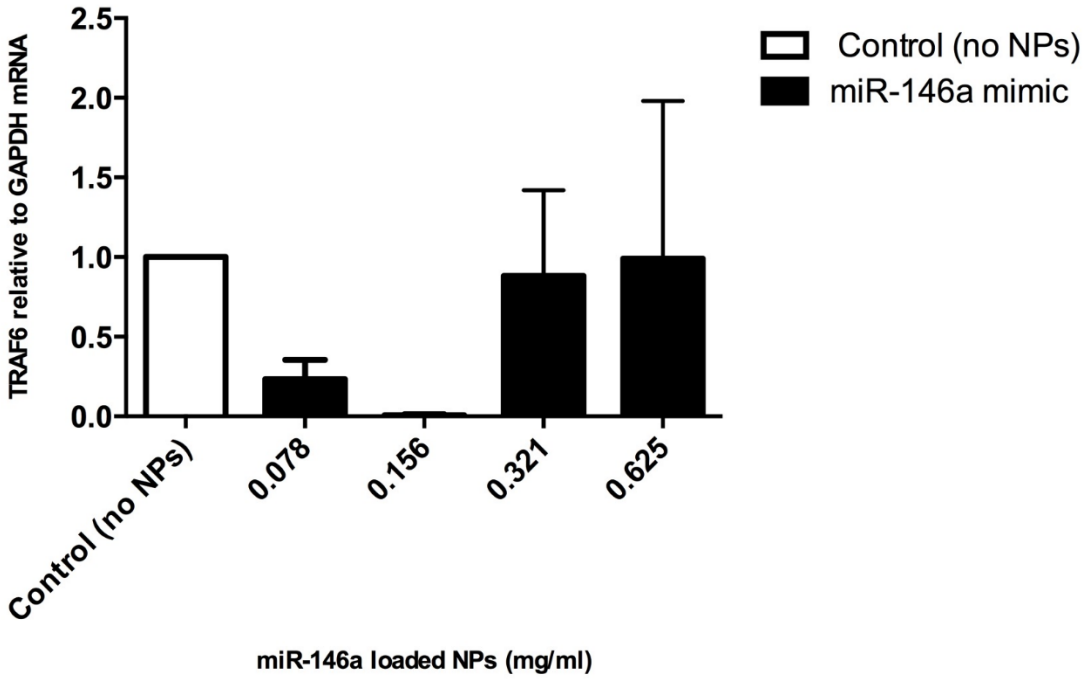
Appendices



Appendix–1. Effect of miR-146a loaded NPs on IRAK1 expression. Data of all concentrations were pooled from three independent experiments. The expression of IRAK1 was normalised to GAPDH expression. Values are Mean ± SD (n=3).



Appendix–2. Effect of miR-146a loaded NPs on TRAF6 expression. Data of all concentrations were pooled from three independent experiments. The expression of TRAF6 was normalised to GAPDH expression. Values are Mean ± SD (n=3).

List of publications

Conference Proceeding

1. A Mohamed, K Ross, I Saleem, G Hutcheon. Polymeric Nanoparticles For The Pulmonary Delivery Of miRNA To Treat Chronic Obstructive Pulmonary Disease (COPD). 42nd International annual meeting and exposition of the Controlled Release Society (CRS), Edinburgh, 2015.
2. A Mohamed, K Ross, I Saleem, G Hutcheon. Pulmonary Delivery of Therapeutic miR-146a Nanoparticles. National Biotechnology Conference of American Association of Pharmaceutical Scientists, Boston, USA, 2016
3. Valeria Carini, Ayca Pekoz, Gillian Hutcheon, Imran Saleem, Adel Mohamed. Dry powder intended for pulmonary delivery: Comparison between PGA-co-PDL and chitosan Nano Composite Microparticles. Journal of aerosol medicine and pulmonary drug delivery, 2017.

Awards and Honours

Associate Fellow of the Higher Education Academy (AFHEA), UK.

Posters Judge, the Faculty Research Seminar Day, (April and Jun) 2017, Liverpool John Moores University.

Biology teacher at the International Study Centre, Liverpool John Moores University.

Researcher at Liverpool John Moores University, 2017.

Chief examination invigilator at the International Study Centre, Liverpool John Moores University.

American association of Pharmaceutical Scientists (AAPS), 2015-2018: member.

Aerosol Society, 2014-2018: member.

British Society for Nanomedicine (BSNM), 2015-2018: member.

Controlled Release Society (CRS), 2015-2018: member.

United Kingdom Ireland Controlled Release Society (UKICRS), 2014-2018: member.

UIKICR graduate network committee rep, 2014-2018.

Postgraduate alternate student representative, School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University.

Conferences, courses and workshops

1. Oral presentation on Pulmonary Drug Delivery workshop, Istanbul, Turkey, September 2017.
2. Poster on Drug Delivery to the Lungs, Edinburgh, UK, December 2016.
3. Abstract accepted for poster on American Association of Pharmaceutical scientists, Colorado, Denver, USA, November 2016.
4. Abstract accepted for poster on European Federation for Pharmaceutical Sciences, Turkey November 2016.
5. ERASMUS ambassador on traineeship Placement-Turkey as part of study related to PhD, May-July 2016.
6. Poster on American Association of Pharmaceutical scientists (AAPS), Boston, MA, USA, May 2016.
7. Oral presentation "3 minute thesis, 3MT "on LJMU graduate school research conference April 2016.
8. Oral Presentation on Postgraduate research forum, LJMU, UK, October 2015.

9. Second workshop on Pulmonary Drug Delivery, Trinity College, Dublin, Ireland, September 2015.
10. Poster on British Society Nanomedicine young researchers BSNM, Liverpool, UK, August 2015.
11. Poster on 42nd annual international controlled release society CRS, Edinburgh, UK, July 2015.
12. Oral presentation on annual nano pharmaceutics symposium, Keele University, UK, July 2015.
13. Poster on United Kingdom Ireland Controlled Release Society Symposium UKICRS, Nottingham, UK, April 2015.
14. Poster on the Academic Practice and Technology (APT), AAPS student chapter Conference, University of Greenwich, UK, April 2014.

Awards and Grands

2015 Best second poster award. Faculty of Science, postgraduate research seminar and poster day. Liverpool John Moores University.

2016 Travel conference award. Graduate school. Liverpool John Moores University.

2016 The Sir Richard Stapley Educational Trust grant, the award made to help in university tuition fees.

2016 Early career scientists travel awards from Aerosol Society to attend overseas conference.

2016 Erasmus traineeship placement-Turkey as part of study related to PhD.