Chitosan nanoparticles for siRNA delivery to the lungs using pressurised metered dose inhalers and nebulisers

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Plagiarism Statement

This thesis describes research conducted in the School of Pharmacy, University of London between 2007 and 2010 under the supervision of Prof. Kevin Taylor and Dr. Satyanarayana Somavarapu. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication. The thesis has been approved by AstraZeneca (UK) for publication; authorisation to publish number: 11/2029.

Signature: ____________________________________________ Date: _______________________

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To my wonderful family...
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Abstract

Purpose: To produce and characterise high molecular weight chitosan (HMC; glutamate derivative) and low molecular weight chitosan (LMC) nanoparticles associated with different grades of PEG (PEG600, PEG1000 and PEG5000) and chitosan-grafted PEG (LMC-g-PEG5000) nanoparticles as carriers for pulmonary delivery of siRNA using pMDIs and nebulisers.

Materials and Methods: Nanoparticles encapsulating siRNA were prepared using an ionic crosslinking technique at a chitosan to siRNA weight/weight ratio of 10:1, 30:1 and 50:1. They were characterised for their functional groups, physicochemical properties and siRNA encapsulation efficiency using FTIR, Malvern Zetasizer and PicoGreen reagent respectively. Formulation physical stability was studied in propellants (HFA-227 and HFA-134a) using visual and infrared (Turbiscan) techniques. Gel electrophoresis was used to assess the association of siRNA with nanoparticles, including after aerosolisation. The aerosolisation properties of the formulations were tested using cascade impaction techniques and laser diffraction. Cell viability, cellular uptake and gene silencing were performed using A-549/H-292 cells.

Results: Nanoparticles were positively charged with mean diameters less than 250 nm, with and without PEG, at all chitosan to siRNA ratios. FTIR confirmed PEG association and PEG grafting to chitosan. Nanoparticles were non-aggregated at the pH of the airways (6.5) and showed high siRNA loading efficiency (>89% PEG associated and 57% PEG grafted nanoparticles). PEG1000 associated nanoparticles displayed acceptable stability (~10 min) as dispersions with both grades of chitosan in HFA-227, whereas other formulations either rapidly creamed or sedimented. Complete binding of siRNA was observed when the w/w ratio approached 50:1 for PEG associated particles. Nebulisation studies showed ~50-70% fine particle fraction (FPF) for all formulations. Following actuation from pMDIs, the FPF for HMC-PEG1000 nanoparticles, was 34 (± 1.4) % whereas for LMC-PEG1000 nanoparticles, the FPF was 38 (±2.7) % (without siRNA) and 25% (with siRNA). The volume median diameter (VMD) of LMC-PEG1000 particles; post-actuation was 1.43 (±0.08) μm (without siRNA) and 4.41 μm (with siRNA). Cell viability was high (>80%) at the highest chitosan concentration, with PEG1000 associated nanoparticles. LMC-g-PEG5000 nanoparticles were taken up within A-549 cells and showed gene silencing activity (58.20 ±18.6 %), however gene silencing with other formulations could not be demonstrated due to poor RNA recovery.

Conclusion: The results suggest that PEG-1000 associated chitosan nanoparticles have potential for delivery of siRNA to the lungs using a pMDI, whereas LMC-g-PEG5000 was potentially suitable as a siRNA carrier for use with nebulisers.
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## Abbreviations and Acronyms

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<th>Definition</th>
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<tbody>
<tr>
<td>AMD</td>
<td>Age related macular degeneration</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary strand of DNA</td>
</tr>
<tr>
<td>CFC</td>
<td>Chlorofluorocarbon</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CoV</td>
<td>Coronavirus</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell penetrating peptide</td>
</tr>
<tr>
<td>d</td>
<td>Physical diameter of particle</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>Dae</td>
<td>Aerodynamic diameter</td>
</tr>
<tr>
<td>DAD</td>
<td>Diffused alveolar damage</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DFP</td>
<td>2H,3H-decafluoropentane</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-dioleoyl-3-trimethylammonium-propane</td>
</tr>
<tr>
<td>DOTMA</td>
<td>N-[1-{2,3-dioleyloxy}propyl]-N,N,N-trimethylammonium chloride</td>
</tr>
<tr>
<td>DPI</td>
<td>Dry powder inhaler</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded ribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GSD</td>
<td>Geometric standard deviation</td>
</tr>
<tr>
<td>HFA</td>
<td>Hydrofluoroalkane</td>
</tr>
<tr>
<td>HFA-134a</td>
<td>1,1,1,2-tetrafluoroethane</td>
</tr>
<tr>
<td>HFA-227</td>
<td>1,1,1,2,3,3,3-heptafluoropropane</td>
</tr>
<tr>
<td>HMC</td>
<td>High molecular weight chitosan</td>
</tr>
</tbody>
</table>
PLGA  Poly(lactic-co-glycolic acid)
PLL  Poly-L-Lysine
PLR  Poly(L-arginine hydrochloride)
pMDI  Pressurised metered dose inhaler
PPI  Polypropylenimine
PTD  Peptide transduction domains
PVA  Polyvinyl alcohol
PVP  Polyvinylpyrrolidone
Q  Quencher moiety
RISC  Ribosomal induced silencing complex
RNAi  Ribonucleic acid interference
RSV  Respiratory syncytial virus
+RT mix  Positive reverse transcriptase mix
-RT mix  Negative reverse transcriptase mix
RT-PCR  Reverse transcriptase polymerase chain reaction
SARS  Severe acute respiratory syndrome
SEM  Scanning electron microscopy
siRNA  Short interfering ribonucleic acid
SLN  Solid lipid nanoparticles
TAE  Tris-acetate ethylenediaminetetraacetic acid
TAT  Transactivator of transcription
TE  Tris-hydrochloride ethylenediaminetetraacetic acid
TEM  Transmission electron microscopy
TPP  Sodium tripolyphosphate
TSI  Twin stage impinger
VEGF  Vascular endothelial growth factor
VMD  Volume median diameter
\( \rho_0 \)  Unit density
\( \rho \)  Particle density
1. Introduction

1.1. General introduction

Biopharmaceuticals are active substances used for diagnostic purpose (Gary 2002). These include recombinant proteins, monoclonal antibodies and nucleic acid-based molecules (siRNA). The first biopharmaceutical product (Humulin®), a recombinant human insulin, was introduced in 1982 and developed by Genentech and Eli lilly (Johnson 1983). More than 160 biopharmaceuticals have gained medical approval and the majority of them are protein based (Gary 2005). Nucleic acid therapy shows immense potential for the treatment of many genetic and acquired diseases. It is highly selective to a particular disease and minimises unwanted side effects. Conventional drugs act on proteins, whereas nucleic acid therapeutic agents act at the level of gene expression producing those proteins, and the effect is long lasting (Woodle and Lu 2005). Hence nucleic acid therapy shows promise in the treatment of diseases which cannot be cured by conventional drugs. Nucleic acids such as antisense oligodeoxynucleotides (ODN), ribozymes (ribonucleic acid enzyme) and the more recently introduced double-stranded, short interfering ribonucleic acid (siRNA) are employed in gene silencing techniques that can lead to specific knock down (silencing) of the targeted cellular proteins and functions (Akhtar et al. 2000; Leung and Whittaker 2005). The major limitations for the use of siRNA are rapid degradation and poor cellular uptake (Behlke 2006). Vectors (carriers) have been employed to overcome such barriers. The use of both viral and non-viral vectors has been extensively investigated. In the past few decades, non-viral vectors have received particular attention because of their safety and cost-effective delivery compared to viral vectors (Kong et al. 2007).

Gene silencing has attracted attention as an approach to treat pulmonary infections (Durcan et al. 2008). The lung is an attractive route of drug delivery since it is non-invasive, has a large surface area, it is easily accessible, avoids first pass metabolism, and has enhanced permeability (Bisgaard et al. 2002; Birchall 2007). The therapeutic agent can be delivered through the nose or by mouth, and can be formulated in different forms such as solutions, suspensions or powders. It can also be delivered using different devices, such as nebulisers, pressurised metered dose inhalers (pMDIs) and dry powder inhalers (DPIs). The research work presented here was performed to explore the use of non-viral vectors (chitosan with
polyethylene glycol) for the pulmonary delivery of nucleic acids (siRNA) using pMDIs and nebulisers.

1.2. Anatomy of the lungs

The human respiratory tract is a complex organ system for gaseous exchange, where oxygen is inhaled in exchange for carbon dioxide from blood. A diagrammatic representation of the human respiratory tract is shown in Figure 1-1.

![Diagram of the human respiratory tract](image)

Figure 1-1. Anatomy of the lungs (a) Upper respiratory tract and (b) Lower respiratory tract (Martini 2001).
Chapter 1. Introduction

It comprises the upper respiratory tract (a), known as the conducting region, which consists of the trachea, main bronchi, lobar bronchi, segmental bronchi, small bronchi and terminal bronchioles. The lower respiratory tract (b), consists of respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli (Washington et al. 2001). The airways reduce in length and diameter from upper airways to bronchioles. For example, the diameter of the trachea is about 1.8 cm and the alveolar diameter is about 0.04 cm (Hickey and Thompson 1992). The pulmonary organ is highly vascularised and composed of epithelial cells with a surface area as large as 140 m². It is estimated that the whole system consists of 23 generations considering the trachea as generation-0 and alveolar sac as generation-23 (Taylor and Kellaway 2001).

1.3. Drug deposition in the lung

Factors such as lung morphology and airstream velocity affect drug deposition. The size of particles is also an important factor in determining the site of deposition as shown in Figure 1-2.

![Figure 1-2. Average predicted total and regional lung deposition based on particle size in human respiratory tract following oral breathing of unit density spheres at a breathing cycle of 15 s period and flow rate of 300 cm³ s⁻¹ (Bisgaard et al. 2002).](image)

In pulmonary delivery, the size of particles is often expressed as aerodynamic diameter which is a major factor in determining the performance of particles as an aerosol. It incorporates particle physical diameter, shape and density. It is by definition, the diameter of a sphere with unit density (p=1) as described in equation 1.1 (De Boer et al. 2002; Carvalho et al. 2011). For non-spherical particles correction for shape factor can be applied.
\[ D_{ae} = \left( \frac{\rho}{\rho_0} \right)^{1/2} d \]  
\textit{Equation 1.1}

where; \( D_{ae} \) = Aerodynamic diameter, \( \rho_0 \) = unit density, \( \rho \) = Particle density, \( d \) = physical diameter of particle

The importance of aerodynamic diameter instead of geometric diameter has been previously demonstrated, as light-weight large porous particles \((\rho = 0.1 \text{ g/cm}^3; d = 8.5\mu\text{m})\) were delivered deep into the lungs with a respirable fraction of 50 ±10% (Edwards et al. 1997). Small non-porous particles \((\rho = 0.8 \text{ g/cm}^3; d = 3.5\mu\text{m})\) were less efficient and exhibited a respirable fraction of 20.5 ±3.5%.

Particles of size >6 \( \mu \text{m} \) are considered to be too large for deep lung deposition and deposit at the upper airways whereas particles between 1 and 5 \( \mu \text{m} \) are likely to deposit in the lower airways (McDonald and Martin 2000). As the size further decreases (1 to 0.1 \( \mu \text{m} \)), these particles are mainly exhaled. However, studies have shown that particles of size less than 0.1 \( \mu \text{m} \) are highly deposited in the alveolar regions (Hinds 1999; Bisgaard et al. 2002).

When an aerosolised drug is inhaled, three principal mechanisms of drug deposition occur; inertial impaction, gravitational sedimentation and diffusion. Large drug particles are deposited by impaction and sedimentation whereas the smaller drug particles reach the lower respiratory tract (where airflow is minimal or absent) by diffusion (Brownian motion) (Taylor and Kellaway 2001). Drug deposition in the lungs following inhalation can be assessed in two ways: pharmacokinetic methods and imaging techniques such as gamma scintigraphy (2D) and single photon emission computed tomography (3D) (Cryan et al. 2007). The pharmacokinetic method determines total lung dose based on plasma concentrations and urinary recovery, whereas lung imaging quantifies deposition in the lungs and also provides a visual image of the different zones of deposition.

1.4. Modes of pulmonary delivery

Pulmonary delivery has been used for many years to treat local diseases such as asthma, chronic obstructive pulmonary disease (COPD), lung cancer, severe acute respiratory syndrome (SARS), respiratory syncytial virus (RSV). This route may also be used for systemic administration. Essentially, there are three types of devices available for pulmonary drug delivery: dry powder inhalers (DPIs) which are breath actuated devices, nebulisers which
deliver drug during tidal breathing and pressurised metered dose inhalers (pMDIs) which are usually non-breath activated devices (Claudio 2001).

1.4.1. Dry powder inhalers

DPIs are devices through which a dry powder formulation of an active drug is delivered into the lungs utilising the patient’s inhalation for local or systemic effect (Islam and Gladki 2008). This technology was introduced in 1967 (Bell et al. 1971). Most dry powders for inhalation consist of micronised drug blended with carrier particles, which enhance flow, reduce aggregation and aid in dispersion (Figure 1-3). After the formulation has been blended, it is filled into capsules, multi-dose blisterst or reservoirs for use with the inhaler device. Breath assisted inspiration, force drug particles to separate from the carrier particles and entrains particles with aerodynamic size less than 5 μm into the patient’s airways (Hersey 1975). DPI carrier particles generally lactose (blended with drug) enhances the powder flow of the formulations and increase the powder bulk for capsule filling. Recently other carrier materials have been studied such as, antistatic agents and lubricants (magnesium stearate), amino acids (leucine), surfactants, phospholipids, derivatised carbohydrates and sugars (glucose and mannitol) (Chougule et al. 2007).

Figure 1-3. Principle of DPI design (Chrstyn 2003).
Chapter 1. Introduction

Formation of weak conglomerate between drug, ternary component (a lubricant) and carrier particle was essential as it provides a discontinuous covering as opposed to a "coating" which is an important and advantageous feature in inhalation (Patton et al. 1999). Successful delivery of drugs into the deep lungs depends on particle size, powder flow properties, formulation, drug-carrier adhesion, respiratory flow rate and design of DPI devices (Hickey and Concessio 1997). Unlike pMDIs, DPIs avoid problems inherent in the use of propellant gases which were temporarily ozone depleting chlorofluorocarbons. Secondly, the need for coordination of inhalation and actuation as observed with pMDIs (Islam and Gladki 2008). Nevertheless, the downside of using a DPI is that the formulation may absorb moisture which could result in structural changes and formulation instability (Hersey 1975). Devices with higher internal resistance need a higher inspiratory effort by patients to achieve the desired air flow. This could be difficult for patients with severe asthma and for children and infants. Therefore, a reduction in moisture ingress and improvement in powder flow properties are necessary to achieve the desired therapeutic effect from DPI formulations.

1.4.2. Nebulisers

Nebulisers are most commonly used to produce aerosols for inhalation. These aerosols are produced from aqueous solutions or suspensions of drugs using ultrasound, a compressed gas or mesh technology. The process of converting liquid into small droplets is called atomisation. Nebulisers have an advantage over pMDIs or DPIs in patients who may find it difficult to master the operation of these latter devices. They are applicable for drugs which cannot be formulated within pMDIs or DPIs due to stability reasons. Other advantages include the delivery of drug during normal tidal breathing and the administration of relatively large volumes of drug solution (McCallion et al. 1996b). The three types of medical nebulisers currently available commercially are ultrasonic, vibrating mesh or plate and jet nebulisers (Elhissi et al. 2006).

a. Ultrasonic nebulisers

The mechanism of aerosol generation in ultrasonic nebulisers is based on the energy created from a piezoelectric crystal, generally a man-made ceramic material, vibrating at a high frequency between 1-3 MHz. The intense high-frequency vibrations are transmitted to the liquid medication above the crystal to generate aerosols (Taylor and McCallion 1997). The higher the frequency the smaller the droplet size produced. Ultrasonic nebulisers are more variable and less efficient in delivering drugs in suspension form (McCallion et al. 1996b).
Further, heat generated in the process can degrade heat labile medicaments such as biopharmaceuticals (Khatri et al. 2001).

b. Mesh nebulisers
The most recent nebuliser technology utilises a vibrating mesh or plate with multiple apertures to generate a fine-particle, low-velocity mist (Berger 2009). The vibrating mesh nebuliser offers efficient delivery of aerosols and minimal residual drug volume in the device (Elhissi et al. 2006). They are also useful in the nebulisation of suspensions, proteins and peptides. Since the energy for nebulisation is applied to the vibrational element in the aerosol generator rather than to the drug solution or suspension, the increase in temperature of the solution during operation is minimised (Kapitza et al. 2004).

c. Jet nebulisers
The operation of jet nebulisers requires compressed air which acts as a driving force for the atomisation of a liquid drug formulation. This high velocity gas is passed through a narrow venturi nozzle (0.3 – 0.7 mm in diameter). A region of negative pressure is created over the liquid solution. The drug solution is entrained into the gas stream by the Bernoulli effect, up a feed tube and breaks into droplets due to surface tension forces (Figure 1-4). The baffle placed above the venturi helps to produce smaller aerosols which are taken up by the patient through inspiration. The remaining large non-respirable droplets impact on baffles or the walls of the nebuliser chamber and are recycled into the reservoir fluid (McCallion et al. 1996b; Hess 2000).

![Diagram of jet nebuliser](image-url)

Figure 1-4. Operation of an open-vent (Pari Turboboy) jet nebuliser (O’Callaghan and Barry 1997).
Open-vent jet nebulisers avoid the wastage of aerosols during continuous operation as they have inhalation and exhalation valves. During inspiration, a valve situated on top of the nebuliser cap opens and air is drawn in. Upon exhalation this inspiratory valve closes decreasing the pressure and flow of air inside the chamber. At the same time, the expiratory valve on the mouthpiece opens to expel the exhaled air (McCallion et al. 1996b). These modifications to conventional nebulisers help reduce the wastage of drug aerosols during exhalation.

The two types of liquid-gas mixture during atomisation are described as internal mixing and external mixing (Figure 1-5). Internal mixing is where the liquid mixes with gas before leaving the exit port and external mixing is where the liquid and gas mix after leaving the venturi. Both have similar effects over aerosol formation (Hess 2000).

Nebuliser design, formulation composition and patient-related factors are important parameters governing aerosol size and fluid output. It has been observed that an increase in gas velocity decreases droplet size (Hess 2000). Increasing the viscosity of the formulation may be beneficial as the aerosol size is reduced up to a critical solution viscosity value, but it takes longer to aerosolise high viscosity liquids to dryness, hence more is retained in the nebuliser reservoir (McCallion et al. 1996b).

The suitability of nebulisers for delivering liposomes has been tested previously. It was reported that both air-jet nebulisers resulted in loss of entrapped drug during nebulisation and damage to liposome structures (Taylor et al. 1990). Changing the composition of liposomes by adding cholesterol or DPPC made liposomes more resistant to disruptive forces (Bridges and...
Taylor 1998). Latex spheres have been atomised using both jet and ultrasonic nebulisers. Relatively large particles could be aerosolised using nebulisers but smaller particles were more efficiently delivered. Ultrasonic nebulisers were less efficient in nebulising latex spheres than air-jet nebulisers (Mc Callion et al. 1996a). Jet nebulisers have demonstrated a fine particle fraction of 40 to 52% when used to nebulise nanomicelle formulations composed of conjugated chitosan-stearic acid encapsulating amphotericin B (Gilani et al. 2011).

The downside of nebulisation is that during atomisation, the temperature of the formulation decreases which may cause poorly soluble drugs to precipitate and results in changes to fluid physicochemical properties potentially leading to variable aerosol size. Nebulisers have been reported to show erratic drug output due to a change in concentration of solute in the solution during nebulisation (Phipps and Gonda 1990). The aerosols generated by nebulisers vary significantly in size from each other, due to the nebuliser design, droplet aggregation, solvent evaporation and condensation (O'Callaghan and Barry 1997). At the end of nebulisation of drug solution or suspension, a small volume of residual fluid remains trapped inside the nebuliser and is not made available for inhalation. This is known as 'dead volume' or 'residual volume'. It is minimised by tapping the walls of the nebuliser periodically during therapy and using a reservoir with a conical shape (Niven and Brain 1994; Dalby et al. 2006).

d. Nebulisers for delivery of biopharmaceuticals

Since it is easy to formulate and deliver new entity by nebulisers hence researchers have investigated the applicability of nebulisers for the delivery of gene formulations (Gautam et al. 2003). ALN-RSV, produced by Alnylam Pharmaceuticals Inc. (USA), is an cholesterol-attached siRNA (Merkel and Kissel 2011) formulated in saline targeting the RSV N gene transcript which has reached phase 2b clinical studies. Extensive evaluation has demonstrated structural and functional integrity of the gene before and after nebulisation (Alnylam Pharmaceuticals Inc. 2012). Some studies have reported the degradation of proteins (lactate dehydrogenase; LDH) during air-jet nebulisation. The stability of LDH was greatly improved in formulations with chitosan suggesting that this may be a useful excipient in the preparation of stable protein formulations for jet nebulisation (Albasarah et al. 2010b).

Formulations have also been developed to improve the aerosolisation efficiency and delivery of biopharmaceuticals through nebulisers. Published studies of PEI a cationic polymer/carrier when complexed with DNA and delivered using nebulisers have shown high lung deposition, transfection and pulmonary gene expression (Gautam et al. 2000). Amine-modified-PVA-
PLGA/siRNA nanoparticles as therapeutic carriers for pulmonary gene delivery using nebulisers were assessed. Studies showed 80-90% *in-vitro* knockdown of luciferase reporter gene in the human lung epithelial cell line, H1299 luc after nebulisation (Nguyen et al. 2008).

### 1.4.3. Pressurised metered dose inhalers

pMDIs are a pressurised dosage form, designed to deliver therapeutic drugs to the human respiratory tract. This pharmaceutical preparation was introduced in 1955 (Thiel 1996) but the idea of delivering active compounds using pressurised propellant 12 (chlorofluorocarbon) was exploited in 1943 for delivering an insecticide by the US Department of Agriculture (Shoyele and Slowey 2006). pMDIs consist of: the active substance formulated with propellant and excipients, a canister, a metering valve crimped onto the canister, an actuator that connects the metering valve to an atomisation or actuator nozzle, and a mouth piece (Smyth 2003) (Figure 1-6).

![Components of a pMDI](Fromer et al. 2010)

pMDIs offer advantages of portability, protection of the therapeutic drug from oxidative and light degradation, dose reproducibility, exclusion of microbiological contamination, disposability, low cost and a high number of doses (upto 200) per device (Jones et al. 2006). On the down side, pMDIs deliver small doses than nebulisers and upon actuation most of the drug deposits in the oropharyngeal region (Berger 2009). In order to get an efficient spray for lung delivery, device characteristics and formulation factors play an important role.
Chapter 1. Introduction

a. Device characteristics

The key components of the pMDI device that play a significant role in aerosol formation are canister, actuator, valve and actuator nozzle. Canisters are containers, internally coated with aluminum to avoid corrosion, chemical degradation and possible interaction with the formulation (Crowder et al. 2001). They should be robust and strong enough to withstand the high pressure generated by propellants. These canisters are crimped with a valve to prevent propellant escape, oxidative degradation and moisture ingestion. The valve has two components; metering chamber which has a volume ranging from 25 μL to 100 μL and a valve stem which fits directly on top of a plastic actuator with nozzle diameter ranging from 0.14 mm to 0.6 mm (Lewis et al. 2004).

The main basic principle for the function of a pMDI is shown in Figure 1-7. The channel between the canister and the metering chamber is open before actuation. As the canister is pressed, this channel closes, and the channel connecting the metering chamber and atmosphere opens. The formulation is expelled into the valve stem and then flows through the actuator nozzle into the expansion chamber from where it is directed into the desired site of action along with the propellant. Upon the release of pressure from the top of the canister the inverted position of the valve helps it to refill under gravity.

![Figure 1-7. Schematic representation of aerosols generated through actuator nozzle of pMDI (Newman 2005).](image)

b. Formulation factors

The formulation content of a pMDI is a blend of drug substance, excipients and propellant. The drug is either solubilised or dispersed in a propellant medium which is liquified under pressure.
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(i) Propellants

Chlorofluorocarbons (CFCs) were the first propellants to be used in pMDIs. They are being phased out due to their reported ozone-depleting properties (Molina and Rowland 1974). After the Montreal protocol, more environmentally friendly, biocompatible, chemically inert, non-toxic and non-ozone depleting hydrofluoroalkanes (HFAs) were developed as replacements for CFCs. HFA and CFC have similar density and vapour pressure, but several of their physicochemical properties are significantly different. The two HFA propellants that are currently used in pMDIs are HFA-134a (1,1,1,2-tetrafluoroethane) and HFA-227 (1,1,1,2,3,3,3-heptafluoropropane). HFA-134a and 227 both have high vapour pressure and low boiling points. Vapour pressure determines the speed and rate of propellant evaporation. 2H,3H-decafluoropentane (DFP), is a good model for HFA-134a as it correlates with its solubility properties and is liquid at room temperature, permitting its use in early formulation studies. The physicochemical properties of these propellants are shown in Table 1-1.

Table 1-1. Comparison of the physicochemical property of HFA-134a, HFA-227 and DFP (Smyth 2003).

<table>
<thead>
<tr>
<th>Property</th>
<th>HFA-134a</th>
<th>HFA-227</th>
<th>DFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiling point (°C)</td>
<td>-26</td>
<td>-16</td>
<td>53.6</td>
</tr>
<tr>
<td>Vapour pressure (kPa)</td>
<td>572</td>
<td>390</td>
<td>30</td>
</tr>
<tr>
<td>Dielectric constant</td>
<td>9.5</td>
<td>4.1</td>
<td>15.05</td>
</tr>
<tr>
<td>Dipole moment</td>
<td>2.1</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>Induced polarization</td>
<td>6.1</td>
<td>6.1</td>
<td>-</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
<td>1.23</td>
<td>1.42</td>
<td>1.58</td>
</tr>
<tr>
<td>Viscosity (mPa.s)</td>
<td>0.21</td>
<td>0.27</td>
<td>0.537</td>
</tr>
</tbody>
</table>

(ii) Surfactants

pMDIs are either solutions/suspensions depending on the solubility of the active drug in propellant. Suspension-based systems are more frequently encountered since the majority of drugs are insoluble in the propellant, and a drug’s chemical degradation is reduced compared to a solution (Smyth 2003). Instability and aggregation of suspensions within pMDIs may require the addition of stabilising excipients such as surfactants. These are incorporated in pMDIs as they stabilise the dispersion and provide valve lubrication. Surfactants tend to associate at the air–liquid interface which may lead to an increase in mass median aerodynamic diameter (MMAD) due to decreased evaporation rates from aerosol droplets.
(Dalby and Byron 1988). Some of the stabilisers that have been used in HFA-based pMDI formulations are perfluorinated carboxylic acids or esters and polyethoxylated surfactants, e.g. PEG (Ashayer et al. 2004). Suspensions correspond to more than 50% of the commercially available pMDI (Rogueda 2005). Well documented literature about surfactants and its properties has been detailed in (Vervaet and Byron 1999; Ridder et al. 2005).

(iii) Co-solvents
One of the differences between HFA and CFC lies in the inability of HFA to solubilise conventional surfactants, previously licensed for use in pMDI formulations. Co-solvents such as ethanol improve the solubility of surfactants, aiding dispersion. Commercially the FP of beclometasone dipropionate (corticosteroid) was improved by the inclusion of a limited amount of ethanol to HFA-134a propellants (Vervaet and Byron 1999). Increasing ethanol concentrations to 10% w/w has a significant detrimental impact on MMAD and droplet size (Smyth et al. 2004). The introduction of co-solvents also brings problems such as chemical instability of the drug substance, extraction of elastometric components from valves and the undesirable taste of alcohol (Dellamary et al. 2000). They may also change the solubility profile of drugs in propellant, altering the preparation of solution formulations.

c. pMDIs for delivery of biopharmaceuticals
Not much work has been published in the field of gene delivery to the lungs using pMDIs. Structural stability and bio-structural integrity of protein (lysozyme) in hydrofluoroalkanes (HFA-134a or HFA-227) was studied. The protein maintained its robustness in both types of propellants (Quinn et al. 1999). This is not applicable to all types of amino acid bases but is useful as a point of investigation into biopharmaceuticals as therapeutic agents for pulmonary diseases. A study was conducted on a protein (Bovine serum albumin) formulation within a pMDI system using excipients such as surfactants, a dispersing aid or co-solvent and a propellant (WilliamsIII and Liu 1999). The aqueous solution of a protein and surfactant was lyophilised and suspended in HFA-134a with ethanol as a dispersing aid. Surfactants such as Span 80 and Span 85, were not soluble in HFA-134a to any appreciable extent, regardless of the presence of ethanol. On the other hand Brij 98 and Tween 80 were soluble in HFA-134a. The formulation containing Tween 80 as a surfactant produced the highest respirable fraction. Ethanol acted as a wetting agent, and increasing the ethanol level above 7.89% w/w resulted in a decrease in vapour pressure of the propellant, reducing the evaporation rate of the propellant and lowering the respirable dose. Using differential scanning calorimetry it was found that Brij 98 and Tween 80 both increased the stability of bovine serum albumin in the
lyophilised solid complex, with Brij 98 being a slightly better stabiliser. In a study pEGFP-N1 (green fluorescent protein reporter plasmid), incorporated in aqueous phase of micro-emulsion, snap frozen and lyophilised along with sucrose (lyoprotectant) were dispersed in HFA-134a (along with ethanol). The study demonstrated maintained biological activity of the pEGFP-N1 post particle processing, propellant dispersion and post-actuation from a pMDI canister. A significant increase in the percentage of cells expressing fluorescence (p<0.001) was also observed following aerosolisation (pMDI) of the washed pEGFP-N1 particulates into DOTAP-containing media (24.75) compared with control cells (0.96) (Bains et al. 2010).

1.5. Pulmonary delivery of nanoparticles

Nanoparticles are engineered carriers with one or more components ranging in size from 10 to 1000 nm (Soppimath et al. 2001; Sung et al. 2007). Nanoparticles have been considered particularly attractive for pulmonary delivery of drugs because of their small size and since it has been established that epithelial cells rapidly internalise nanoparticles (direct penetration or endocytosis) compared to microparticles. Further, these nanoparticles interact with components of living cells (ribosomes and receptors) which are also constructed at nano-level (Labhasetwar 2005).

A variety of pharmaceutical materials have been used to fabricate a range of nanocarriers, including polymers, liposomes, solid-lipid, sub-micron emulsions and dendrimers (Mansour et al. 2009). Nanoparticulates are prepared either by precipitating particles out of solution (bottom-up), or milled from larger particles (top-down). Technology to engineer these nanoparticles include wet milling, spray drying, electrospraying, high pressure homogenisation, recrystallisation via solvent displacement, etc. (Bailey and Berkland 2009). The type of pharmaceutical material chosen to manufacture nanoparticles and the steps taken to prepare those nanoparticles are very important in deciding the fate of nanoparticles and associated therapeutic agents upon delivery.

The potency of a poorly soluble drug to provide therapeutic effectiveness may be limited. This can be improved by reducing the particle size of the drug to the nanometer level. With a decrease in particle size (<1 μm), the dissolution rate of a drug increases as the surface to volume ratio increases (Müller et al. 2001; Merisko-Liversidge and Liversidge 2008). Poorly soluble drugs, which cannot be dispersed in a media, such as non-polar (pMDI) or polar (nebulisers) for pulmonary drug delivery, can be dispersed using a suitable nanoparticle with
adequate dispersion qualities in that medium (Müller et al. 2001; Mansour et al. 2009). For example, salbutamol sulphate and bovine serum albumin do not disperse in HFA-227 (non-polar), whereas when encapsulated within oligo(lactide)-g-chitosan (medium molecular weight) nanoparticles demonstrated excellent colloidal stability in HFA-227 (Wu et al. 2008b).

The use of nanoparticles for pulmonary delivery applications is receiving increasing attention for targeting both systemic and local disease using pMDIs, DPIs and nebulisers. Many studies have explored the delivery of nanoparticles from pMDIs (Dickinson et al. 2001; Nyambura et al. 2009b; Bains et al. 2010; Selvam et al. 2011). Nanoparticles have been dispersed in CFC (Baran et al. 2008) and HFA propellants (Wu and Da Rocha 2011) using Tween, dipalmitoylphosphatidylcholine (DPPC), span 85, cineole and citral as dispersants (Nyambura et al. 2009a; Tam et al. 2010; Tan et al. 2011). These studies have shown an FPF upto 60% (Nyambura et al. 2009a; Bharatwaj et al. 2010). More detail on pMDI technology is described in Section 1.4.3.

DPI, have been investigated for the delivery of nanoparticles as aerosols. Reported FPF values have ranged from 30% to 60% (Sham et al. 2004; Corrigan et al. 2006; Pilcer et al. 2009) with one study demonstrating up to 90% FPF (Ungaro et al. 2009). Other modifications associated with nanoparticle technology are the innovation of porous nanoparticle-aggregate particles (PNAPs) for DPIs. This involved formulating nanoparticles within large hollow porous micron scale particles. Upon inhaling PNAPs, the lung fluid and humidity dissolves the micrometer sized structures, resulting in the release of nanoparticles (Tsapis et al. 2002). More detail on DPI technology is described in Section 1.4.1.

There is a comprehensive literature available where nanoparticles provide new formulation options for the delivery of “active” using various types of nebulisers for their delivery as aerosols. These include; ultrasonic nebulisers (Dailey et al. 2003), vibrating mesh nebulisers (Beck-Broichsitter et al. 2009) and jet nebulisers (Liu et al. 2008a). Generally, it has been demonstrated that nebulisation of nanoparticles result in a FPF around 60% (Yang et al. 2008; Nielsen et al. 2010). More on nebulisation technology has been outlined in Section 1.4.2.

1.6. RNA interference (RNAi)

In 2006, Professor Andrew Z. Fire and Professor Craig C. Mello, were awarded a Nobel Prize for the discovery of a process named RNA interference (RNAi). This technique has evolved as a novel therapeutic pathway by which deleterious genes can be “degraded” by delivering RNA
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that binds to a complementary mRNA molecule and stops the expression of genes in cells. It was first discovered in plants and was later demonstrated in roundworm *caenorhabditis elegans* (Fire et al. 1998). RNAi pathways are guided by double stranded RNA (dsRNA) and small RNAs such as short interfering RNA (siRNA) and micro-RNA.

1.6.1. Mechanism of RNAi

Figure 1-8 shows the molecular mechanism of RNAi. Double stranded RNA (dsRNA; typically 500-1000 base pairs) delivered intracellularly is cleaved into 21-23 base pair siRNA fragments by the enzyme Dicer (ribonuclease III). A multi-protein complex called RISC (RNA Induced Silencing Complex) assembles with the siRNA, retaining the guide strand (antisense strand) and discarding the passenger strand (sense strand) (Zhang et al. 2007). The RISC is guided to the corresponding mRNA by siRNA within the cytoplasm and it selectively binds the complementary deleterious mRNA. The mRNA is then degraded by the endonuclease region of the RISC complex, thus making it unavailable for translation into protein, leaving the disengaged RISC complex to further survey the mRNA pool (Leung and Whittaker 2005).

Double stranded RNA is known to induce an interferon response rendering it unsuitable for RNAi therapy (Gil and Esteban 2000). The use of siRNA instead of dsRNA can bypass the earlier steps of gene silencing and it can be delivered directly for RISC complexation (Lee and Ambros 2001).
1.6.2. siRNA

siRNA is an anionic, hydrophilic 21-23 base pair nucleotide (Figure 1-9). It is naturally generated from dsRNA, but it is possible to produce synthetic siRNA for sequence specific cleavage of mRNA and treatment of incurable diseases (De Fougerolles et al. 2007). siRNA is highly potent as only a few molecules are required to produce effective gene silencing (Bumcrot et al. 2006). Since siRNA circumvents the dicer protein mechanism, it reduces activation of the innate immune response (Grimm et al. 2006).
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The design and selection of siRNA for therapeutic application should be such that the siRNA is potent, specific and stable (De Fougerolles et al. 2007). The potency can be demonstrated if nanomolar quantities of siRNA are sufficient to silence any gene. Web-algorithms have been developed to predict the effectiveness of siRNA (Pei and Tuschl 2006). Such applications are not always perfect, hence experimental testing is undertaken, whereby synthetic siRNA candidates are screened. The specificity of siRNA is based on selective silencing of complementary mRNA (Figure 1-9). This specificity is achieved through the seed region where two to eight nucleotides from the 5' end of siRNA antisense strand show complementarity to the target mRNA strand. Large quantities of siRNA can induce an immune response. This can be avoided by the use of delivery carriers that avoid immune stimulation. Naked siRNA is degraded in human plasma with a half life of minutes. Greater than 50% of synthetic 2'-OH siRNA is degraded within 1 min of exposure to plasma and virtually all is degraded after four hours (Layzer et al. 2004). The stability of siRNA can be enhanced through chemical modifications, by introducing a phosphorothioate (P=S) backbone linkage at the 3'-end which protects against exonuclease degradation and a 2'-sugar modification (2'-fluoro) provides endonuclease resistance (Figure 1-9) (Choung et al. 2006). siRNA grafted to cholesterol at the 5'-end of the sense strand targeting apolipoprotein B (ApoB) in mice silenced ApoB mRNA by 55% in the liver and 70% in the jejunum upon systemic administration (Soutschek et al. 2004). Results demonstrated that the unconjugated ApoB siRNA was rapidly cleared and inactive.

Figure 1-9. Schematic illustration of siRNA structure with sense/antisense strand, two base pair overhangs, cleavage site and seed region. Optimised siRNA with chemical modifications such as P=S backbone and 2'-base sugar modification (De Fougerolles et al. 2007).
1.6.3. siRNA in clinical trials

In just a decade RNAi has advanced to pre-clinical and clinical studies (Table 1-2). siRNA therapeutics have been developed using mostly novel formulations, often employing carriers. Alnylam Pharmaceuticals have successfully performed the first pulmonary studies in the treatment of RSV. ALN-RSV01, a siRNA could target viral nucleocapsid gene without any toxicity and has successfully entered clinical trials (De Fougerolles et al. 2007) and is currently under phase 2b (Alnylam Pharmaceuticals Inc. 2012). Treatment of age-related macular degeneration (AMD); a cause of blindness due to excessive blood vessel growth and rupture within the cornea has been treated by targeting vascular endothelial growth factor (VEGF) using siRNA (Bevasiranib) manufactured by Opko Health, who have initiated phase 3 clinical trials (Oh and Park 2009). Allergan Pharmaceuticals demonstrated minimal side effects and improved vision of adult patients after the treatment of AMD using siRNA (AGN211745). This nucleic acid has entered phase 2 clinical trials (Whitehead et al. 2009). Silence Therapeutics had also developed a siRNA for the treatment of AMD but they have refocused their phase 2 clinical trials to treating diabetic macular oedema, a condition caused by leaky vasculature within the eye (Whitehead et al. 2009). Calando Pharmaceuticals have recently begun phase 1 clinical trials for the treatment of solid tumours using CALAA-01 (siRNA) in a cyclodextrin based polymeric nanoparticle that targets the M2 subunit of ribonucleotide reductase (Oh and Park 2009). The use of nucleic acids for the treatment of diseases and a major change in pharmacopoeia will soon then be a reality.

Table 1-2. siRNA therapeutics in clinical trials.

<table>
<thead>
<tr>
<th>Company</th>
<th>Disease</th>
<th>Mode of administration</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alnylam</td>
<td>Respiratory syncytial virus</td>
<td>Local/direct</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Allergan</td>
<td>Age-related macular degeneration</td>
<td>Topical</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Silence/Quark/Pfizer</td>
<td>Diabetic macular oedema</td>
<td>Topical</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Calando</td>
<td>Tumor</td>
<td>Systemic</td>
<td>Phase 1</td>
</tr>
<tr>
<td>Quark/Pfizer</td>
<td>Acute renal failure</td>
<td>Systemic</td>
<td>Phase 1</td>
</tr>
<tr>
<td>Opko Health</td>
<td>Age-related macular degeneration</td>
<td>Topical</td>
<td>Phase 3</td>
</tr>
</tbody>
</table>
1.6.4. Delivery of siRNA to the respiratory tract

Many studies have been performed to explore the potential of RNAi therapeutics for the treatment of respiratory disorders by the delivery of naked or formulated siRNA in-vivo. Respiratory pathogens, such as respiratory syncytial virus (RSV) have been targeted with siRNA using a RNA interference (RNAi) approach. The siRNA complexed with a transfection agent, Transit-TKO (a proprietary polycation), when instilled intranasally in a mouse model reduced viral infection (RSV) three-fold, with no side effects (Bitko et al. 2005). siRNA has been studied as a therapeutic agent for severe acute respiratory syndrome corona viral infection (SCV) in-vitro for efficacy and safety in a non-human primate model. When formulated in 5% dextrose solution, and instilled intranasally, siRNAs provided relief from infection-induced fever, reduced viral levels and reduced acute diffuse alveoli damage. No sign of siRNA-induced toxicity was observed (Li et al. 2005). Safe and effective treatment of lung cancer using siRNA has been reported previously; Akt1, a siRNA bound to a nanosized poly(ester amine) polymer complex was delivered into K-ras\textsuperscript{IA1} and a urethane-induced lung cancer model in mice through a nose-only inhalation system with the aid of nebuliser. The nucleic acid suppressed lung tumor progression significantly (Xu et al. 2008). These studies suggest that inhaled siRNA in small amounts, in a properly designed formulation might offer a fast, potent and easily administrable cure against respiratory diseases in humans.

1.6.5. Limitations of siRNA delivery

Despite the advantages offered by siRNA there are barriers to overcome. The barriers to using siRNA as a therapeutic agent include: enzymatic degradation of siRNA once administered, poor cellular uptake both in-vitro and in-vivo and rapid excretion from the kidney depending on the size of siRNA (Bumcrot et al. 2006). Triggering of a nonspecific immune response upon administration is another issue. One of the other challenges is the inhibition of an mRNA, the expression of which should not be targeted since the mRNA shares a partial nucleotide sequence to siRNA. This can be avoided by the careful design and selection of potent siRNA (Oh and Park 2009). It is important that the delivered siRNA is prevented from being distributed too widely, so that it can be concentrated at the pathological tissue. Regardless of the administration route, it is very important to maintain siRNA stability and biological activity during manufacturing and delivery. In order to overcome these hurdles and for the successful delivery of the nucleic acids, a variety of carriers have been investigated.
1.7. siRNA delivery using carrier systems

The carrier should be able to target the tissue of interest and facilitate uptake. It should be designed to protect the siRNA from degradation. These carriers which have been used for several years for delivery of therapeutic nucleic acids are either viral or non-viral:

1.7.1. Viral vectors

Various viral vectors such as adeno-associated virus, retrovirus and lentivirus have been used mainly for the delivery of short hairpin RNA (shRNA). The shRNA structure is cleaved by the cellular machinery into siRNA, which is then bound to the RISC and result in gene silencing as discussed in section 1.6.1. The major advantage associated with viral vectors is the high transfection efficiency, though there is a possibility of generating an immunogenic and toxic effect upon administration into the lungs. In one of the early gene therapy trials in 1999 adenoviral vector caused the death of Jesse Gelsinger due to multiple-organ-system failure (Teichler Zallen 2000). Scientists claimed that the adenovirus triggered an overwhelming inflammatory reaction resulting in an immune-system revolt. Hence the use of viral vectors in gene silencing is still under investigation (Reischl and Zimmer 2009). Modified viral vectors are being engineered with the aim to overcome toxicity and immunogenic response. Progress has been made in the use of adeno-associated virus vectors for clinical trials (phase 2) for the treatment of cystic fibrosis (Grieger and Samulski 2005). The attractive feature of this virus is its ability to infect both non-dividing and dividing cells with persistent expression and lack of pathogenicity (Daya and Berns 2008).

1.7.2. Non-viral vectors

Non-viral vectors are more attractive than the viral vectors for the following reasons (Kong et al. 2007); (1) With non-viral vectors chemical and physical interactions are utilised for the synthesis and assembly of transfection material, whereas viral vectors need to be assembled by cells in a microbiological area, therefore being restricted to a proteinaceous composition. (2) The non-viral vectors can be produced by assembly of few, defined components and can be adjusted depending on the size of the nucleic acid to be transported, (3) Synthetic materials are less expensive compared to viral vectors. (4) Safety testing of transfection reagents (non-viral vectors) is less laborious than testing of recombinant materials. (5) These transfection reagents cause low immunogenecity when administered, have high gene encapsulation
capability, are reproducible, and are considered safe (Wagner 1999). Some of the limitations associated with non-viral nucleic acid delivery are the low transfection efficiency and precipitation problems. Generally non-viral vectors fall into five broad categories and are described in detail below:

a. **Cationic liposomes / lipid based systems**

Cationic lipids consist of hydrophobic groups (either one or two fatty acids or alkyl moieties of 12-18 carbons in length) in addition to an amine group. The amine group increases the transfection efficiency as these sites interact electrostatically with DNA and condense the large anionic molecules into small carrier-lipoplexes (Wheeler et al. 1996). Some commercially available lipids include; Lipofectamine 2000, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). Plasmid-DNA has been observed to degrade during nebulisation due to the shear forces of the jet nebuliser (Birchall et al. 2000). The author and his co-workers demonstrated enhanced physical stability and biological activity of pDNA when complexed with the cationic lipid (DOTAP) and peptide (protamine). Lipofectamine 2000 has been shown to be an efficient gene carrier. Its cationic nature provides high transfection efficiency and high levels of transgene expression *in-vitro*. Studies performed with lipid particles such as lipofectamine 2000 as a carrier for siRNA have shown cellular toxicity *in-vitro* and *in-vivo* (Spagnou et al. 2004; Lv et al. 2006). Unlike cationic polymers which simply form a complex with negatively charged siRNA upon mixing, liposomes also require several processing steps. Upon pulmonary administration, lung surfactants may inhibit cationic liposome-mediated gene delivery to respiratory epithelial cells (Duncan et al. 1997).

b. **Cell penetrating peptides**

Cationic cell penetrating peptides (CPPs) have been developed to enhance the cellular uptake of siRNA. They are also known as peptide transduction domains (PTDs). They can effectively condense siRNA because of the presence of charged amino acids such as histidine, lysine and arginine (Meade and Dowdy 2007). These CPPs can be categorised into; amphipathic helical peptides (containing positively charged lysine) and arginine-rich peptides; TAT (transactivator of transcription) (Nam et al. 2011) and penetratin (Hällbrink et al. 2001). Heterologous proteins including RNase A have been delivered into the cytoplasm of different cell types *in-vitro* using TAT peptides (Fawell et al. 1994). siRNA delivery mediated by CPP can be achieved by non-covalent complexation of CPP to siRNA through electrostatic interactions (Sandgren et
al. 2002). It is also achieved by covalent attachment of CPP to siRNA duplexes through disulfide bond formation (Gupta et al. 2005). There are concerns that the conjugation is not sufficiently effective, since it leads to neutralisation of the positively charged peptide and negatively charged nucleic acid, reducing the penetrating efficacy of CPPs (Meade and Dowdy 2007). Intratracheal administration of siRNA conjugated with cholesterol and cell penetrating peptides such as TAT(48-60) and penetratin as carriers, caused an innate immune response and showed no knockdown of p38 MAP kinase mRNA in mouse lung. These studies vary in results from in-vitro studies which were performed in the mouse fibroblast L929 cell line where limited knockdown was achieved (Moschos et al. 2007).

c. Modification of carriers – polyethyleneglycol (PEG)-based polycations

These include the presence of ligands, formation of nanoparticles and PEG-modified polycations. Amongst these particular attention is given to PEG (polymer of ethylene oxide) for the reasons outlined below. PEG is widely used in the pharmaceutical industry, including as an excipient in FDA-approved nasal spray formulations (Traini et al. 2006). PEG chain length plays an important role in the stability and nuclease resistance of PEGylated carriers. PEG grafted to PLL (Poly-L-Lysine) and complexed with DNA was coated with a positively charged fusogenic peptide (KALA) to form nanocarriers of size <200 nm. PEG suppressed aggregation of these complexes (KALA/polymer/DNA complexes) due to steric stabilisation whereas PLL without PEG induced severe aggregation (Lee et al. 2002). A positively charged carrier is essential for targeting and facilitating uptake into negatively charged cells, and to form effective complexes with polyanionic nucleic acids. In-vivo the negatively charged serum proteins bind to carriers and make them ineffective. The presence of PEG on the surface of the polymer helps by providing serum stability and modulates particle size by preventing aggregation (Auguste et al. 2008). A study has demonstrated stable dispersions of polar drugs (salbutamol sulphate) in HFAs by entrapping HFA philic PEG groups using a modified emulsification–diffusion method (Wu et al. 2008a). PEG acted as stabilising agent, thus preventing flocculation of the otherwise unstable colloidal polar drug particles. Protection against RNase digestion was highest for poly(ethylenimine)/PEG based formulations; PEI(25k)-g-PEG(5k) and PEI(25k)-g-PEG(20k), while siRNA/PEI provided insufficient protection (Mao et al. 2006).

Lactosylated PEG micelles have also been used to deliver siRNA, where they are conjugated by an acid labile β-thiopropionate linkage, which is cleaved under the acidic condition of endosomes. This causes a build-up of free PEG chains and thereby increases osmotic pressure,
resulting in disruption of the endosome and release of siRNA (Oishi et al. 2005). This last, but very important step in the delivery process ensures that siRNA will be available in the cytosol to initiate RNAi.

Alveolar regions of the lung contain macrophages which engulf and degrade pathogens and particles deposited in the deep lung. Particles of size less than 260 nm probably escape these macrophages (Groneberg et al. 2003) hence in order to overcome macrophage clearance and for alveolar lung delivery, it is desirable to formulate nanoparticles of size <260 nm. Another additional step is the surface modifications with polymers such as PEG (Klibanov et al. 1990). Covalent grafting, entrapment, or adsorption of PEG chains onto nanoparticles gives a hydrophilic surface which reduces macrophage recognition and complement activation, thereby prolonging nanoparticle circulation-time. Microparticles of PEG-g-chitosan formulated with Pluronic F108 efficiently evaded macrophage activity due to the stealth characteristics offered by PEG5000 (El-Sherbiny et al. 2010).

d. **Lipopolyplexes (combination of lipid and polymer)**

Lipopolyplexes are non-viral carriers formed by combining liposomes and polycations. DNA when condensed with a polycation such as polyethylenimine (Guo and Lee 2000), forms polyplexes. These can be further entrapped within anionic liposomes to form lipopolyplexes. This method of packaging DNA is reported to result in a less toxic and more efficient *in-vitro* gene transfer particle which is resistant to nuclease degradation when compared with cationic liposomes alone. Lipid/polycation condensed pDNA chitosan particles were developed and it was observed that *in-vitro* deposition of chitosan-modified powders, and also that the level of reporter gene expression was enhanced due to the presence of chitosan (Li and Birchall 2006).

e. **Cationic polymers**

Cationic polymers are positively charged polymers which vary in their chemical structure and molecular interaction. Some of the commonly used cationic polymers include: polyethylenimine (PEI), poly (L-Lysine) (PLL), chitosan, poly (vinyl) pyridine, polyamidoamine dendrimer (PAMAM), polypropyleneimine (PPI), polyallylamine (PAA), cationic dextran and poly (lactide-co-glycolide) (PLGA). Depending on their chemical structure, cationic polymers occur as linear (PLL) or highly branched chains (Dendrimers). Some cationic polymers have the positive charge on the backbone (PEI) while others have it on side groups (PLL) (De Smedt et al. 2000). The positive nature of the polymer helps to condense large negatively charged nucleic
acids into small structures and mask the negative charge. Moreover they can be formulated as nano or micro particles based on the type of cationic polymer chosen and method of preparation. The size and charge of cationic polymers vary and help optimise the carrier for nucleic acid delivery to the target site (Merdan et al. 2002; Zhang et al. 2007). Cell entry mainly occurs via adhesion of the positively charged carrier systems to the negatively charged cell membrane followed by endocytosis (De Smedt et al. 2000). Among these cationic polymers, polyethylenimine (PEI) and chitosan have been widely investigated as nucleic acid carriers (Gautam et al. 2000; Mao et al. 2010).

(i) Polyethylenimine (PEI)

PEI is considered as the gold standard in many in-vitro and in-vivo applications for the delivery of nucleic acids (Lungwitz et al. 2005). It is an effective gene carrier due to its high charge density and endosomal disruption function, which help in achieving high transfection efficiency (Boussif et al. 1995). However, the major drawback of the unmodified polymer is that it is non-degradable and toxic, due to the high positive surface charge which limits its applicability both in-vitro and in-vivo (De Smedt et al. 2000).

(ii) Chitosan

Chitosan is a derivative of chitin (extracted from the exoskeleton of insects), deacetylated to a degree between 40 and 98% (Illum 1998) with d-glucosamine and N-acetylglucosamine units as end products (Figure 1-10).

![Structure of chitosan](Figure 1-10. Structure of chitosan.)

This natural amino polysaccharide has distinctive biological properties such as non-toxicity and antimicrobial activity (Mourya and Inamdar 2008) and has been widely used in various pharmaceutical applications such as contact lenses, drug delivery, wound healing and sustained release (Paul and Sharma 2000). Chitosan is also useful for gene delivery, since it is...
by nature biodegradable and biocompatible. It is obtained in various molecular weights, degree of deacetylation and represents a class of cationic carriers that are potentially safe, efficient and cost-effective (Nordtveit et al. 1996).

Chitosan with a high degree of deacetylation (90%) develops a high positive charge which allows more efficient interaction with negatively charged mammalian cells (Lee et al. 2005) and negatively charged siRNA through electrostatic interaction. This property allows several siRNA molecules to be complexed with chitosan, rather than one molecule per chitosan molecule (Katas and Alpar 2006). It has also been observed that chitosan enhances the immunity of the diphtheria vaccine by providing longer residence times and bioavailability in the nasal cavity and by transiently opening the tight junction between the epithelial cells (Illum 2003).

Chitosan is available in various molecular weights but is generally classified as low (<100 kDa) and high molecular weight (>100 kDa). Low molecular weight chitosan (LMC) used in formulation preparation in this thesis is water soluble with a molecular weight >10 kDa. The use of high molecular weight chitosan (HMC) in drug delivery has been limited, due to its poor solubility in physiological media. Chitosan derivatives such as chitosan glutamate and chitosan hydrochloride have been developed to overcome this drawback. Of these, chitosan glutamate (HMC; 150k - 200 kDa) has been shown to be a suitable vector for siRNA delivery (Katas and Alpar 2006) and is used in this thesis. In a study, chitosan of various molecular weight (8.9, 11.9, 64.8, 114.2, 170, 173 kDa) and degree of deacetylation (95, 77, 78, 84, 84, 54%) were complexed with siRNA and were compared for their in-vitro gene silencing ability (Liu et al. 2007). The highest gene silencing efficiency (80%) was achieved using chitosan/siRNA nanoparticles at nitrogen:phosphate (150:1) using higher molecular weight (114 and 170 kDa) and degree of deacetylation (84%). This was suggested to be due to the long chain length of high molecular weight chitosan that provide an efficient balance between appropriate protection and release, resulting in high in-vitro gene silencing efficiencies. Chitosan derivative such as N-sulfonato-N,O-carboxymethylchitosan (SNOCC) have proved their potential in the permeation enhancement of macromolecules (low molecular weight heparin; Reviparin) across epithelial cell lines (intestinal) (Thanou et al. 2007). Modified chitosan has been explored as vectors for gene delivery and shown enhanced transfection efficiency. Trimethyl chitosan (TMC93) complexed with pGL3 luc plasmid DNA were able to transfect MCF-7 cells with 50-fold greater efficiency than PEI (Kean et al. 2005).
Chapter 1. Introduction

Chitosan forms a semi-rigid film or hydrogel and shows swelling properties because of its porous network in aqueous medium (Kumbar et al. 2002). This property affects the drug release rate and particle size, hence a chitosan network needs to be crosslinked. Other reasons for crosslinking are to improve the mechanical strength of the polymer and to attain free diffusion of water/biopharmaceutical compounds. Increasing the amount of crosslinker decreases the ability of chitosan to form hydrogen bonds with water molecules and leads to the formation of a 3D network (Berger et al. 2004). Ionic gelation of chitosan with sodium tripolyphosphate (TPP), used as a cross linking agent, results in the formation of nanoparticles.

Chitosan has been used in various in-vitro and in-vivo studies as a non-viral delivery system for both pDNA and siRNA (Alameh et al. 2010). Nucleic acid–chitosan nanoparticles show high transfection levels because of the high binding property of cationic chitosan, which condenses the nucleic acid into nanosized particles. It also shows high endosomolytic activity because of the overall positive charge of the carrier and is able to mediate gene silencing activity (Erbacher et al. 1998). The low immunogenicity provides a good alternative to viral or lipid mediated transfection. Chitosan pre-condensed with DNA further complexed with an emulsion; composed of 3β [N-(N’,N’-dimethylaminoethane) carbamoyl] cholesterol, dioleoylphosphatidyl ethanolamine, castor oil and Tween 80, enhanced cell viability and transfection efficiency, resulting in prolonged mRNA expression in liver and lung until day 6 (Lee et al. 2005). Chitosan/polyguluronate encapsulating siRNA nanoparticles were stable in neutral pH and protected siRNA from degradation in the presence of serum when compared to naked siRNA (Lee et al. 2009). The author also demonstrated negligible gene silencing (using plasmid DNA encoded luciferase) of ~10% with naked siRNA in absence of carrier over HEK293FT cells whereas, in presence of carrier the gene silencing efficiency raised to 60%.

The use of chitosan as a carrier has been explored in the field of inhalation. It has been demonstrated that salbutamol sulphate/chitosan when co-spray dried, resulted in particles with a high FPF compared to micronised salbutamol sulphate when delivered as a powder from a capsule-based Rotahaler DPI device (Corrigan et al. 2006). Aerosolisation of chitosan/siRNA nanoparticles to mouse lung administered using an intratracheal catherter led to alveoli and bronchiolar depositions. A reduction of 62% in fluorescence was observed after aerosolisation of siRNA to silence enhanced green fluorescent protein (EGFP) expressing H1299 cells (Nielsen et al. 2010). It has been previously tested that chitosan/DNA particles adhered to bronchiole epithelia and facilitated gene expression in this region after intratracheal administration (Koping-Hoggard et al. 2001). This was further confirmed, where
chitosan (114 kDa)/siRNA complexes showed knockdown of endogenous enhanced green fluorescent protein (EGFP) in both H1299 human lung carcinoma cells and murine peritoneal macrophages (77.9% and 89.3% reduction in EGFP fluorescence, respectively) *in-vitro*. Nasal administration of these formulations showed effective *in-vivo* RNAi in bronchiole epithelial cells of transgenic EGFP mice with 43% reduction compared to untreated control (Howard et al. 2006). Pulmonary administration of chitosan–plasmid DNA (pCMV–Luc) powders (nitrogen/phosphate ratio = 5) to mice led to increased luciferase activity in mouse lung compared to pCMV–Luc powders. The use of chitosan also suppressed the degradation of pCMV–Luc (Okamoto et al. 2003). The preparation with pulmonary delivery of chitosan:siRNA nanoparticles are described in more detail in Chapter 4. Given the numerous examples of *in-vivo* siRNA delivery (Behlke 2006; Howard et al. 2006; De Fougerolles et al. 2007; Andersen et al. 2009), the use of chitosan as a safe delivery systems seems to be promising.
1.8. Scope of the thesis

In the case of respiratory treatment of diseases, very few studies have been performed to investigate siRNA delivery from nebulisers using chitosan as carriers, and none using pMDI devices. The aim of this work was to formulate siRNA associated nanoparticles suitable for delivery using nebulisers and pMDI.

The first objective was to engineer and characterise chitosan nanoparticles as siRNA carriers stable at lung pH (6.5) and good dispersion properties within propellant HFAs. Chitosan particles are susceptible to aggregation in non-polar media (Williams III et al. 1998). Hence the physical stability of nanoparticles suspension was investigated using different grades of hydrophilic stabiliser (PEG) in propellant, to enhance dispersion stability.

The developed formulations were investigated for their suitability as carriers for siRNA. For this purpose, siRNA associated nanoparticle formulations were prepared and their physicochemical properties and encapsulation efficiency was determined. Additionally, the binding affinity of siRNA towards formulations with different compositions was analysed to investigate whether association was strong or weak.

Engineers of siRNA loaded nanoparticles for pulmonary delivery are faced with the challenge of producing particles with the optimal properties for deep lung deposition without degradation of siRNA molecules. Therefore, in this study (siRNA loaded) nanoparticles were investigated for their aerodynamic behaviour, particle size and morphology to assess their suitability for aerosolisation using nebulisers and pMDIs.

The final study investigated the toxic effects of increasing amounts of chitosan formulations towards lung epithelial cells. The nanoparticle formulations were also tested for cellular uptake characteristics. siRNA loaded formulations were screened on the basis of their gene silencing ability in-vitro. More investigation is further needed to verify the in-vitro gene silencing experiment.
CHAPTER 2

PREPARATION AND CHARACTERISATION OF CHITOSAN NANOPARTICLES
Chapter 2. Preparation and characterisation of chitosan nanoparticles

2. Preparation and characterisation of chitosan nanoparticles

2.1. Introduction

The use of nanoparticles is a particularly attractive approach for pulmonary delivery, as their size not only permits access to the peripheral airways (Bailey and Berkland 2009), but also ensures they escape phagocytic and potentially mucociliary clearance mechanisms (Tsapis et al. 2002).

Incorporating drugs into or onto nanoparticles potentially provides protection against intracellular and extracellular barriers, degradation and may overcome formulation challenges, such as delivery of poorly aqueous soluble and unstable drugs without compromising the native conformation of these molecules (Mayer 2005; Vauthier and Labarre 2008). The small size of dry nanoparticles leads to high inter-particle cohesive forces that impact on their aggregation behaviour, which is particularly problematic for DPI formulation. Advantages of pMDIs as inhalation devices include, their convenient use and sealed environment, providing protection from air, light, moisture and microbial degradation. A number of groups have studied pMDI nanoparticle delivery, though adequate dispersion of such small particles in liquefied aerosol propellants is a major formulation challenge (Williams III et al. 1998; Bailey and Berkland 2009; Nyambura et al. 2009b). Oleic acid, Span 85, dipalmitoylphosphatidylcholine and volatile oils have been used within HFA propellants, permitting the successful dispersion of protein nanoparticles (Nyambura et al. 2009a) which maintained protein integrity and were successfully delivered in an aerosol having appropriate aerodynamic characteristics for therapeutic activity.

Chitosan has attracted considerable interest as a polymer for preparing nanoparticles because of its biodegradable, biocompatible, non-toxic and mucoadhesive properties (Felt et al. 1998; Illum 1998). The successful application of chitosan for \textit{in-vitro} and \textit{in-vivo} gene delivery has demonstrated its potential for pharmaceutical and biomedical applications (Chae et al. 2005). The cationic polymer has also been formulated as nanoparticles through ionotropic gelation with TPP anions designed to improve the delivery of therapeutically active molecules across mucosal surfaces (Fernandez-Urrusuno et al. 1999). Ionic crosslinking to form nanoparticles offers advantages over covalent crosslinking since, preparation is easier and the conditions are
Chapter 2. Preparation and characterisation of chitosan nanoparticles

mild, which may prevent change in drug morphology during encapsulation (Liu et al. 2008b). TPP is non toxic and has multivalent anions (Liu et al. 2008b). Other crosslinking agents such as glutaraldehyde, glyoxal and ethylene glycol diglycidyl ether have been explored as good crosslinkers but are not preferred due to their physiological toxicity (Bhumkar and Pokharkar 2006). The potential for pulmonary delivery has been recognised and chitosan nanoparticles encapsulated in mannitol microspheres have been demonstrated to be biocompatible with Calu-3 and A-549 human respiratory epithelial cell lines for up to 48 h (Grenha et al. 2007). Crosslinked chitosan microparticulates containing the bronchodilator, salbutamol sulfate prepared by spray drying, were able to achieve controlled release of the drug (Corrigan et al. 2006) and could be formulated into DPIs, with good aerosolisation properties. Chitosan has not been extensively studied as a carrier for delivery from pMDI systems; hence in this study the formulation and characterisation of chitosan nanoparticles for delivery using pMDIs are explored, as has their potential for nebuliser delivery as an aqueous dispersion.

Previous studies (Williams III et al. 1998) suggested that TPP crosslinked chitosan microspheres were not suitable for HFA-134a pMDI systems because their density differs greatly from that of the propellant. The dispersibility of such particles within a non-polar medium, such as HFA-227 which has higher density than HFA-134a, can be improved by controlling the steric repulsive forces between particles (Pugh et al. 1983), for instance by inclusion of hydrophilic polymers, such as polyethylene glycol (PEG) and poly(vinylpyrrolidone) (PVP) (Paul et al. 2005; Traini et al. 2006). PEG acts as a polymeric surfactant and helps to reduce the cohesive interactive forces between drug particles that are suspended in a fluorinated solvent (2H, 3H-perfluoropentane) used as a model for HFA propellants (Traini et al. 2006). PEG was chosen in the formulation of chitosan nanoparticles in this study, since it has appreciable solubility in HFAs (Vervaet and Byron 1999) and is approved by the FDA as an excipient for use in pMDIs (Pilcer and Amighi 2010). In one study (Wu et al. 2008a), inclusion of PEG300 within particles of salbutamol sulfate ensured PEG molecules trapped at the particles surface, prevented cohesive interactions between particles dispersed in 2H, 3H-perfluoropentane.

The objective of the experiments described in this chapter was to prepare and characterise chitosan nanoparticles and to study the applicability of PEG of different molecular weights as stabilisers for these particles in dispersions in HFA-227 and 134a. A study was performed to see the effect of pH on nanoparticle size and surface charge representative of the pH environment of the lung. For clinical applications of this drug delivery system, formulations
should have acceptable suspension stability within a pressurised propellant, this was analysed both visually and using the Turbiscan, an optical analyser.

2.2. Materials

High molecular weight water soluble chitosan glutamate (HMC); Protasan UP G 113, molecular weight (m.w) 150 k-200 kDa, degree of deacetylation 75-90% was purchased from Novamatrix (Norway). Low molecular weight, water soluble chitosan (LMC) with m.w >10 kDa and degree of deacetylation 97.0% was purchased from Kittolife Co. Ltd (Korea). Deuterium oxide (D₂O), methylene chloride (CH₂Cl₂), acetic acid, methanol, acetone (HPLC grade), dimethyl sulfoxide (DMSO), Dichloromethane (DCM), PEG 1000 and TPP 85% were purchased from Sigma-Aldrich (Germany). PEG 5000 monomethyl ether and methoxy PEG 5000 succinate N-hydroxy succinimide (mPEG-NHS) was purchased from Fluka (UK). PEG 600 and HPLC grade water were purchased from Fischer Scientific (UK). HFA-134a (>99.9%) and HFA-227 (>99.9%) were obtained from Solvay Fluor (UK). Model propellant 2H, 3H Decafluoropentane (DFP) was supplied by Apollo Scientific (UK). Dialysis tubing with molecular weight cut-off (MWCO) of 3.5 kDa and 12 k to 14 kDa was purchased from Medicell Intl. Ltd., (UK). Before use, the dialysis tubing was submerged in water for 15 min.

2.3. Methods

2.3.1. Synthesis of LMC-g-PEG5000 copolymer

Preparation of LMC grafted PEG (LMC-g-PEG5000) followed an established procedure (Jeong et al. 2008). LMC (100 mg) was weighed and mixed with 0.2 ml of deionised water. To this 9.8 ml of DMSO was added to dissolve LMC. mPEG-NHS (100 mg) was weighed and dissolved in 2 ml DMSO. Both samples were mixed together under constant stirring and were kept overnight under nitrogen. The resulting solution was dialysed using a dialysis membrane (MWCO 3.5 kDa) with constant replacement of water every 3 h, four times, and later 7 h for the remainder of the 48 h. The dialysed sample was freeze-dried (Section 2.3.5) using a freeze-dryer (Virtis advantage high vacuum, UK) and stored in a desiccator. Freeze-dried PEG conjugated LMC was washed three times with DCM to remove excess mPEG-NHS. DCM dissolved free mPEG (methoxyPEG) and the LMC-g-PEG5000 was dissolved in water, which was then separated out as a layer from DCM in a separating funnel and freeze-dried to obtain pure LMC-g-PEG5000 copolymer. Synthesis and structure of LMC-g-PEG5000 are shown schematically in Figure 2-1.
Chapter 2. Preparation and characterisation of chitosan nanoparticles

Introduction of PEG to chitosan was performed by reacting the N-Hydroxysuccinimide group of activated methoxyPEG and the amine group of chitosan resulting in an amide bond.

![Chemical structure](image)

Figure 2-1. Chemical process for grafting LMC to PEG5000; A) LMC, B) mPEG-NHS, and C) LMC-g-PEG5000.

2.3.2. Analytical confirmation of formulation structural association

a. Fourier transform infrared spectroscopy

The chemical structure of PEG physically associated with chitosan and PEG grafted chitosan were analysed using a Fourier transform infrared (FTIR) spectrophotometer; Spectrum-100 (Perkin Elmer, UK). Freeze-dried samples were placed on a small round disc and a background check was performed prior to sample analysis to nullify the signal to noise ratio.

b. Nuclear magnetic resonance spectroscopy

A 3 mg sample was dissolved in 0.6 ml deuterated water (D₂O) and loaded into a sample tube. This was tested in nuclear magnetic resonance spectrometer (¹H NMR) using an Ultra shield instrument (Bruker, UK) at 400 MHz.
2.3.3. Preparation of crosslinked nanoparticles

a. High and low molecular weight chitosan nanoparticles
HMC nanoparticles were produced using the ionic gelation method (Calvo et al. 1997a; Li et al. 2011; Trapani et al. 2009) whereby, 4 ml TPP solution (0.5 mg/ml) was added dropwise to a 10 ml HMC (chitosan derivative; Chitosan glutamate) solution (1 mg/ml) in the HMC:TPP ratio of 5:1 (w/w) using a peristaltic pump (Gilson, France) with constant stirring. TPP is an anion which reacts with positively charged amine groups of chitosan to form nanoparticles. These nanoparticles were formed immediately upon interaction due to ionic gelation.

LMC nanoparticles were produced using the same method with 2 ml TPP solution (0.5 mg/ml) and 10 ml of chitosan solution (1 mg/ml). Forming a LMC:TPP ratio of 10:1 (w/w)

b. PEG associated high and low molecular weight chitosan nanoparticles
Three molecular weights of PEG; 600, 1000 and 5000 were used in nanoparticle formulation and the same protocol as above was followed by adding 15 mg/ml of PEG to the TPP solution prior to addition to the chitosan solution. This leads to a 1:6 (w/w) HMC:PEG ratio and 1:3 (w/w) LMC:PEG ratio.

c. Low molecular weight chitosan grafted PEG nanoparticles
2 ml TPP solution (0.5 mg/ml) was added dropwise to 10 ml LMC-g-PEG5000 solution (1 mg/ml) in the w/w ratio of 1:10 using a peristaltic pump (Gilson, France) with constant stirring.

2.3.4. Dialysis of nanoparticles
HMC nanoparticles were dialysed against deionised distilled water to remove excess PEG using a dialysis membrane (MWCO 12 k - 14 kDa). Dialysis was performed over a period of 48 h under constant stirring, with water being replaced every hour for the first 3 h period and every 7 h for the remainder of the 48 h. The dialysed solution without free PEG was freeze-dried using a freeze-dryer (Virtis advantage high vacuum, UK) as described in Section 2.3.5.

2.3.5. Freeze-drying of nanoparticles
Freeze-drying is a process employed for drying heat sensitive materials. It is also used to improve the stability of materials which are not stable in the presence of moisture. Freeze-drying was performed using a Virtis freeze-drier (Advantage-EL, UK) by preparing the
nanoparticle suspension in glass vials (14 ml) covered with parafilm (with holes). The method of freeze-drying was carried out in stages:

a) Freezing- The samples were placed in the freeze-drying chamber and the chamber was closed using a quick-seal valve. The shelf temperature was lowered to -40°C and a visual check was made to ensure that the samples appeared frozen.

b) Vacuum- The air pressure above the sample was lowered for 10 to 20 s and then stopped.

c) Primary and secondary drying- The condenser was switched on so that the condenser coil reached a temperature of -60°C. This reduced the moisture in the sample and was followed by heat transfer, removal of vapour and secondary drying by turning on the vacuum which was set at 200 mT. The step was performed before setting the shelf temperature at -20°C and later to +20°C for fast drying. The whole process depends on the type of material used in the sample. The freeze-drying process required 32 h to complete.

After freeze-drying the samples were kept in a moisture free vacuum chamber (desiccator) at ambient temperature.

2.3.6. Measurement of nanoparticle size

The particle size was measured using dynamic light scattering, also called photon correlation spectroscopy with a ZetasizerNano ZS (Malvern Instruments, UK). This consists of a laser which focuses on the sample (loaded in a cuvette). Due to Brownian motion of particles the laser light is scattered towards a detector which is placed at 90 degrees to the laser. Light scattering is dependent on the size of the particles which are present in the illuminated area and is a representation of the bulk sample (Malvern Instruments 2011a).

The samples were not diluted for particle size analysis. The instrument parameters such as refractive index (1.333) and viscosity (0.8872 cP) were set according to the medium (water) in which the nanoparticles were dispersed (25°C). Each sample was tested in triplicate.

2.3.7. Measurement of surface charge of nanoparticles

The surface charge of nanoparticles (with and without PEG) dispersed in deionized water was measured using the laser doppler micro-electrophoresis technique with a ZetasizerNano ZS (Malvern Instruments, UK). The technique for measuring the surface charge is based on
electrophoresis; where the movement of the charged particles is related to the medium in which they are suspended and the electric field applied to the system (Malvern Instruments 2011b). The velocity of charged particles was measured using an inbuilt laser interferometric technique called M3-PALS (Phase Analysis Light Scattering). Each sample was tested in triplicate.

2.3.8. Transmission electron microscopy of nanoparticles

The morphology of nanoparticles was determined using transmission electron microscopy (TEM) prior to freeze-drying. One drop of the sample was placed on a coated copper grid (3.5 mm) support and excess sample was removed by touching the meniscus with filter paper. The sample was then covered with 1% w/v uranyl acetate solution and allowed to air dry. The prepared sample was placed in a Philips FEI CM120 BioTwin TEM (Philips, Netherlands) for visualisation. TEM images were captured using a digital camera (Advanced Microscopy Techniques, UK) using the camera software AMTV542.

2.3.9. Scanning electron microscopy of nanoparticles

A small amount of each freeze-dried formulation was placed on a scanning electron microscopy (SEM) stub and these samples were sputter-coated with gold (Emitech K550; Quorum, UK). Primary electrons were targeted over the sample and these were deflected to a secondary electron detector. This deflection is the image visualised on screen as examined using SEM (FEI XL30 TMP; Philips, Netherlands).

2.3.10. Effect of pH on nanoparticle properties

The effect of pH on the size and surface charge of nanoparticles was studied using a ZetasizerNano ZS (Malvern Instruments, UK) equipped with an auto-titration unit; MPT-2 (Malvern Instruments, UK). The aqueous dispersion of nanoparticles (12 ml) was titrated with 0.1M sodium hydroxide solution (NaOH) with constant stirring over a range of pH (5.5 to 8). The titrated dispersion was transferred to a measuring capillary cell by a spinning disc and changes in the properties of the nanoparticles were measured as a function of pH. Measurements were conducted in quadruplicate.
2.3.11. Dispersion of nanoparticles within model propellant

7 mg of freeze-dried, un-dialysed HMC nanoparticle formulation was added to a glass vial and 0.5 g of model propellant; 2H,3H-decafluoropentane (DFP) was added using a pipette. The mixture was vortexed (SA7, Stuart Vortex Mixers, UK) for 30 s and sonicated (50-60 hertz, Ultrawave, UK) in a bath sonicator for 5 min at 25°C. This step was repeated twice, at each stage a greater weight of propellant was added to give a total weight of the contents of the vial of 8.4 g and a total sonication time of 15 min. The glass vial was capped with parafilm and a plastic cap to prevent propellant from evaporation. This stepwise approach, referred to as a slurry technique, was adopted to ensure homogeneous dispersion of nanoparticles within propellant. To an identical batch of formulation the same procedure was repeated but with 20 min sonication at each stage when adding the propellant resulting a total sonication time period of 60 min.

A comparison of dialysed and un-dialysed nanoparticle formulations was undertaken to determine the effect of excess PEG on nanoparticle dispersion. Dialysed and un-dialysed suspensions in DFP were prepared using a total sonication time of 15 min.

2.3.12. Dispersion of nanoparticles within commercial propellants

A uniform dispersion of nanoparticles in commercial propellants; HFA-227 and HFA-134a was prepared. Un-dialysed formulation (HMC nanoparticles, 7 mg; LMC nanoparticles, 4.8 mg) was added to a clear, transparent pre-weighed polyethylene terephthalate (PET) vial (supplied by AstraZeneca, UK). Vials were crimped with a continuous valve (Valois, France) using a pneumatic crimper (P2002/20, Pamasol, Switzerland) as shown in Figure 2-2. Propellant (0.5 g) was manually added to the vial using cold-filled propellant cans which were supplied by AstraZeneca, UK. The mixture was vortexed for 30 s (Vortexer VWR Mini, USA) and sonicated (U300H, Ultrawave, UK) for 5 min at 20°C. The process was repeated twice, and at each step additional propellant was added until the desired weight of 8.4 g was achieved. These PET vials were used for studies of nanoparticle dispersion in HFA propellants.
2.3.13. Visual analysis of nanoparticle suspension stability

Nanoparticles suspended in propellant within the transparent vials were vigorously hand-shaken for 5 s and then analysed for their physical dispersion characteristics such as sedimentation, creaming, flocculation and coalescence. This was performed by placing the vial in a light box; the AstraZeneca in-house built light box has a black and white background with an attached light. The dispersion appearance against contrasting colours allows for easy visualisation. The stability and dispersion behaviour were monitored by naked eye observation after manual shaking ceased, until sedimentation or creaming was apparent. Photographic images were taken at various time points for all propellant-suspended formulations.

Images were taken after 5 min following hand shaking for nanoparticles dispersed in model propellant and were compared to determine the optimum sonication time to attain a homogeneous dispersion. For comparing dialysed and un-dialysed nanoparticles dispersed in model propellant, images were taken at 1 min time points whereas they were taken at 10 s and 1 min time points for dispersions in HFA-134a and HFA-227.
2.3.14. Sample preparation for Turbiscan analysis

Once the visual analysis was performed, the PET vial was placed over a hollow valve which was conical at one end and attached to a glass tube at the other end (Figure 2-3). The vial was manually pushed down through a lever and the contents of each vial were transferred, via the valve, into a pressure sealed glass tube which was maintained at a temperature of ~-20°C to avoid propellant loss. The glass tube was dismantled from the apparatus (supplied by AstraZeneca) and was equilibrated to room temperature before performing optical analysis using the Turbiscan (Section 2.3.15).

![Figure 2-3. Propellant transfer apparatus under cold conditions for samples studied using the Turbiscan.](image)

2.3.15. Turbiscan analysis of nanoparticle suspension stability

In order to develop a nanoparticle colloidal suspension within a non-aqueous media for pMDI delivery it is important to determine their suspension and storage stability. The nanoparticle dispersion in HFA were investigated using an optical analyser; the Turbiscan® MA2000 (Formulaction, France). Figure 2-4 shows the principle of operation, which has been described previously (Snabre and Arhaliass 1998; Mengual et al. 1999; Bru et al. 2004).
The Turbiscan glass tube was well shaken to disperse the suspended formulation before placing it within the optical analyser where the entire length of the glass tube was scanned 4 times for 1 min with near infrared light ($\lambda = 850$ nm). The near infrared beam was either backscattered or transmitted from the glass tube depending on the nature of the suspension, and was received by two synchronous optical sensors placed at an angle of 45° and 180° to the incident beam. The principle of suspension stability measurement is based on particle migration (sedimentation or creaming) and/or particle size variation (coalescence or flocculation) expressed as a percentage of the total height of the suspension in the tube. A formulation is deemed to be unstable if the variation in its scan intensity on a time scan graph is >10% whereas if it is <2% it’s considered to be stable (Celia et al. 2009). Turbiscan measurements are dependent on particle size, concentration and the refractive index of the medium. If there is creaming or sedimentation within the glass tube, then the backscattering or transmission profile changes accordingly and a scan is produced. For the analysis of nanoparticle suspension stability, backscattering profile was analysed.

The recorded profile (Figure 2-5) is a qualitative or semi-quantitative indication of the distribution of nanoparticles within HFA propellant along the height of the glass tube, to a maximum height of 60 mm. The profile is a macroscopic finger print of sample behaviour within a non-polar medium at pre-determined time intervals (1 min) for a total period of 3 min. For a creaming formulation the backscattering profile increases after every scanned measurement at the upper section of the glass tube (~40 to 45 mm tube height) whereas if the suspension showed sedimentation the backscattering profile at the lower section is higher. Scans obtained below 2.5 mm and above the meniscus of the system (~40 mm) in the glass tube were not taken into account (Marianecci et al. 2010). Superimposable scans are indicative...
of homogeneous systems, whereas non superimposable scans are indicative of flocculation, coalescence or aggregation. The ability of the Turbiscan to detect instability earlier than the naked eye is useful for screening suspension formulations in propellants within pressurised HFA systems, especially where the formulations are turbid. Calibrated polystyrene latex beads have been used as a non-absorbing reflectance standard material for the measurement of bead colloidal stability within aqueous media using the optical analyser Turbiscan MA 2000 (Mengual et al. 1999). The mean diameter of polystyrene latex bead suspensions (3.157 μm) was in good accordance with the manufacturer’s data (3.189 μm).

Figure 2-5. Schematic representation of a Turbiscan profile, as the sample creams within a pressure sealed glass tube.

2.3.16. Statistical analysis

The data are presented as a mean ± standard deviation. The physicochemical properties of chitosan nanoparticle formulations (with and without PEG) were compared statistically using a non-parametric Kruskal-Wallis test followed by a Nemenyis post hoc test. A Mann-Whitney non-parametric test was performed to compare nanoparticle size and surface charge. The results were considered significantly different based upon 95% probability values (p<0.05).
### 2.3.17. Summary of experiments conducted

Table 2-1. Summary of experiments conducted in chapter 2.

<table>
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<tr>
<th>Experiments</th>
<th>Results Section</th>
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<th>LMC</th>
<th>LMC-PEG</th>
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<td>*Scanning electron microscopy</td>
<td>2.4.6</td>
<td></td>
<td></td>
<td></td>
<td>PEG5000</td>
<td>✓</td>
</tr>
<tr>
<td>*Transmission electron microscopy</td>
<td>2.4.7</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>*Transmission electron microscopy</td>
<td>2.4.8</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>*Effect of pH on size and charge</td>
<td>2.4.9</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Dispersion within DFP</td>
<td>2.4.10</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- *Effect of sonication on nanoparticle dispersion</td>
<td>2.4.10</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Effect of dialysis on nanoparticle dispersion</td>
<td>2.4.10</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Dispersion within HFA (134a and 227)</td>
<td>2.4.11</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>- Visual analysis</td>
<td>2.4.11</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>*Dispersion within HFA (134a and 227)</td>
<td>2.4.12</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>- Turbiscan</td>
<td>2.4.12</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

✓ = Experiment performed

* = Un-dialysed
Chapter 2. Preparation and characterisation of chitosan nanoparticles

2.4. Results and discussion

2.4.1. Characterisation of LMC-g-PEG5000 copolymer

a. Fourier transform infrared spectroscopy

Figure 2-6 shows the FTIR spectra of LMC, mPEG-NHS and PEG-g-LMC5000. Chitosan has a characteristic IR spectrum with a peak at 3286 cm\(^{-1}\) which corresponds to the stretching vibration of hydroxyl groups via hydrogen bonds (Figure 2-6A). Another peak originating at 2887 cm\(^{-1}\) is characteristic of the C-H stretch region of the spectrum and is assigned to the symmetric modes of CH\(_2\). The amide bonds of chitosan at 1615 cm\(^{-1}\) (Amide 1) and 1515 cm\(^{-1}\) (Amide 2) were also clearly visible. The peak at 1150 cm\(^{-1}\) is assigned to the C-O-C group within the chitosan structure.

![FTIR spectra of A) LMC, B) mPEG-NHS and C) LMC-g-PEG5000 copolymer.](image)

For mPEG-NHS (Figure 2-6B), characteristic peaks occurred at 2882 cm\(^{-1}\), 1466 cm\(^{-1}\), 1096 cm\(^{-1}\), 1059 cm\(^{-1}\), 946 cm\(^{-1}\) and 841 cm\(^{-1}\). These were also present in the LMC-g-PEG5000 spectrum (Figure 2-6C) indicating the presence of PEG with chitosan polymer. The conjugation caused the peak intensity at 2878 cm\(^{-1}\) to be more sharp and strong due to the presence of PEG. The
decrease in intensity and shift in amide 1 and amide 2 bands after conjugation has been reported previously (Peng et al. 2010), and confirmed the conjugation of PEG to chitosan.

The FTIR spectra of LMC, mPEG-NHS and LMC-g-PEG5000 have been previously described and correspond to the findings discussed in this work (Hu et al. 2005; Ganji and Abdekhodaie 2008; Jeong et al. 2008; Zhang et al. 2008a; Kong et al. 2010).

b. Nuclear magnetic resonance (NMR) spectroscopy

NMR was used to confirm the conjugation of PEG to chitosan. The N-hydroxysuccinimide group of activated mPEG and the amine group of chitosan react together to form a grafted copolymer.

Figure 2-7 shows the integrated NMR scan of individual polymers and co-polymers. Peaks specific to chitosan (LMC) were seen between 1.8 and 5.0 ppm (Figure 2-7A). The carboxylic acid peak and NHS peak specific to mPEG-NHS polymer were observed between 2.5 and 3.0 ppm (peak number 8 to 11) (Figure 2-7B). mPEG peaks were present between 3.5 and 3.7 ppm. LMC-g-PEG5000 can be assigned peaks at 4.6 ppm (H1); 2.9 to 3.0 ppm (H2); 3.5 to 4.0 ppm (H3 to H6); 3.7 ppm (MPEG) (Figure 2-7C).

The degree of substitution (DS) of mPEG to chitosan was calculated from integration of the peak ratio between H2 (2.9 ppm; 60.71) of chitosan, acetyl group (1.9 ppm; 3.04) of chitosan and the methyl group (3.6 to 3.7 ppm; 2.98) of mPEG as shown in Equation 2.1. The degree of PEG substitution was found to be 1.6%. mPEG2000 was previously successfully grafted to LMC (10 kDa) using the same conjugation method resulting in degree of substitution of 4.6 mol% using \(^1\)H NMR (Jeong et al. 2008).

\[
DS = \frac{\text{Proton integration ratio of methyl group of mPEG/3}}{\text{Proton integration ratio of H1 of chitosan} + \left(\frac{\text{Proton integration ratio of acetyl group of chitosan/3}}{3}\right) \times 100}
\]

*Equation 2.1 (Jeong et al. 2008)*

where; DS = Degree of substitution

NMR spectra of LMC-g-PEG5000 copolymer correlates well with a previous report (Kong et al. 2010).
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Figure 2-7. $^1$H spectra of A) LMC, B) mPEG-NHS and C) LMC-g-PEG5000 copolymer in D$_2$O.
2.4.2. Fourier transform infrared spectroscopy to characterise HMC nanoparticles

FTIR analysis of dialysed and un-dialysed formulations was performed to analyse the interaction (adsorption or entrapment) of different grades of PEG with chitosan nanoparticles. In Figure 2-8, the FTIR spectrum of chitosan nanoparticle (un-dialysed) exhibited characteristic peaks at 2877 cm$^{-1}$ (C-H), 1549.17 cm$^{-1}$ (N-H bending), 1397.59 cm$^{-1}$ (CH$_2$ scissoring), 1071.91 cm$^{-1}$ (C-O stretching) (Ganji and Abdekhodaie 2008). No change was observed in FTIR spectra of chitosan nanoparticles at peak intensity 2877 cm$^{-1}$ before dialysis and 2872.33 cm$^{-1}$ after dialysis. This peak of dialysed sample (2872.33 cm$^{-1}$) when compared with FT-IR spectrum of chitosan alone (2875.56 cm$^{-1}$) suggested chitosan nanoparticles stayed within the dialysis bag, since the large molecular weight of chitosan (150 k to 200 kDa) did not allow it to escape through the dialysis membrane (MWCO 12 k to 14 kDa).

Figure 2-8. FTIR spectra of dialysed and un-dialysed formulations; A) TPP before dialysis, B) HMC before dialysis, C) HMC-TPP before dialysis and D) HMC-TPP after dialysis.
For chitosan-PEG600 before removal of excess PEG, a sharp peak was obtained at 2867.31 cm\(^{-1}\) which could be attributed to the C-H stretching vibration of PEG molecules (Figure 2-9B). A similar peak was observed for PEG600 (2865.36 cm\(^{-1}\); Figure 2-9A). On comparing chitosan-PEG600 nanoparticles before and after dialysis, an absence of a peak at 2867.31 cm\(^{-1}\) suggested the complete removal of PEG 600 from the formulation. A smaller peak obtained at 2871.18 cm\(^{-1}\) (Figure 2-9C) was probably due to the chitosan peak as seen at 2872.33 cm\(^{-1}\) (Figure 2-8D). This suggests the method employed to form nanoparticles did not result in association of PEG600 with the particles. Peaks associated with PEG appeared at \(~840 \text{ cm}^{-1}\), \(~950 \text{ cm}^{-1}\) and \(~2880 \text{ cm}^{-1}\) (Hu et al. 2005; Jeong et al. 2008).

Figure 2-9. FTIR spectra of dialysed and un-dialysed formulations; A) PEG600 before dialysis, B) HMC-PEG600 before dialysis and C) HMC-PEG600 after dialysis.
In the case of Chitosan-PEG1000 (Figure 2-10) and PEG5000 (Figure 2-11) nanoparticles, FTIR showed the presence of C-H stretch at 2884 cm$^{-1}$ and 2882 cm$^{-1}$ respectively which were present before and after dialysis. The peak intensity decreased after dialysis suggesting removal of free PEG from the formulation, whereas PEG associated within the nanoparticles remained.

Figure 2-10. FTIR spectra of dialysed and un-dialysed formulations; A) PEG1000 before dialysis, B) HMC-PEG1000 before dialysis and C) HMC-PEG1000 after dialysis.
Theoretically since all grades of PEG studied had molecular weights less than 12 kDa, non-associated PEG should be expected to diffuse out of the dialysis bag. Such diffusion would not be likely for chitosan since it had a molecular weight greater than 150 kDa. These results confirmed the successful association of high molecular weight PEG such as PEG1000 and PEG5000 with chitosan nanoparticles, whereas PEG600, because of its low molecular weight chain length, leads to weak entrapment and hence does not remain associated with chitosan nanoparticles.
PEG-associated chitosan nanoparticles were prepared by incorporating PEG in chitosan solution and forming nanoparticles through slow addition of TPP to the chitosan solution (Wu et al. 2005). They suggested that a chitosan-PEG semi-interpenetrating network is formed through intermolecular hydrogen bonding between the electro-positive amino hydrogen of chitosan and electro-negative oxygen atom of PEG. This network is weak with respect to chitosan nanoparticles without PEG. This explanation can be one of the reasons for PEG 1000 and PEG 5000 remains within the nanoparticles during ionic gelation but does not explain the escape of PEG600 during dialysis.

2.4.3. Nanoparticle size and surface charge

The physicochemical properties of nanoparticles directly influence nanoparticle behaviour in-vitro and in-vivo (Sun et al. 2008a).

a. HMC nanoparticles

The ionic interaction between the positively charged amine groups of chitosan and the negatively charged phosphate groups of TPP helped to generate nanoparticles. The size and surface charge of chitosan nanoparticles with, and without PEG are shown in Table 2-2.

The mean surface charge for all formulations was positive, ranging from +24 to +28 mV as a result of the amine groups in chitosan. The cationic nature of these nanoparticles make them useful for production of complexes with negatively charged nucleic acids for biopharmaceutical applications (Mao et al. 2010) and for promoting high transfection efficiency within negatively charged cells, as a result of electrostatic interactions (Sato et al. 2001). There was no significant difference (p>0.05) in surface charge between the four formulations.

The mean hydrodynamic diameter of the nanoparticles ranged from 170 to 210 nm. Sizes in this range are essential if such particles are intended for the delivery of biopharmaceuticals such as genes and siRNA, since nanoparticles have a significantly higher intracellular uptake than particles in the micrometer size range (Bivas-Benita et al. 2004). A significant difference (p<0.05) in nanoparticle size was observed. Post hoc Nemenyi test showed that HMC-PEG5000 nanoparticles were significantly larger (p<0.05) than HMC particles without PEG. This indicates that PEG (molecular weights 600-5000) had no influence on nanoparticle surface charge, but that the high molecular chain of PEG5000 increased particle size, presumably due to the interlinkage of the polymer with the particle. For all formulations, the polydispersity
index (PDI) was less than 0.3 which is an indication of a narrow particle size distribution in all instances.

Table 2-2. Hydrodynamic diameter and surface charge of HMC nanoparticles (with and without PEG) (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic diameter (nm ± S.D)</th>
<th>Polydispersity index (±S.D)</th>
<th>Surface charge (mV ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMC</td>
<td>170 ± 6</td>
<td>0.26 ± 0.04</td>
<td>+26 ± 3</td>
</tr>
<tr>
<td>HMC-PEG600</td>
<td>175 ± 5</td>
<td>0.27 ± 0.00</td>
<td>+24 ± 1</td>
</tr>
<tr>
<td>HMC-PEG1000</td>
<td>194 ± 7</td>
<td>0.25 ± 0.00</td>
<td>+28 ± 4</td>
</tr>
<tr>
<td>HMC-PEG5000</td>
<td>210 ± 9</td>
<td>0.26 ± 0.02</td>
<td>+25 ± 3</td>
</tr>
</tbody>
</table>

Chitosan nanoparticles have previously been produced by incorporating PEG in chitosan solution during ionic interaction with TPP (Wu et al. 2005). Results for that study showed an increase in nanoparticle size with an increase in the amount of PEG included. Reduced surface charge was also observed with these preparations. They also suggested that the interaction between chitosan and PEG was through intermolecular hydrogen bonding between the electro-positive amino hydrogen of chitosan and the electro-negative oxygen atom of PEG, forming a chitosan/PEG semi-interpenetrating network; also confirmed by (Kim et al. 1995). This was not the case with PEG600. The concentration of the polymer, molecular weight of chitosan and type of chitosan all contribute to polymer-polymer interaction causing variation in nanoparticle size (Wu et al. 2005; Katas and Alpar 2006)

b. LMC nanoparticles

LMC nanoparticles (with and without PEG) were prepared with hydrodynamic diameter ranging from 123 to 165 nm (Table 2-3). The LMC nanoparticles were significantly (p<0.05) different in hydrodynamic diameter from LMC-PEG5000 nanoparticles indicating the influence of high molecular weight PEG on nanoparticle size. The size distribution was unimodal with PDI <0.2. There was no trend observed between the PDI values for LMC nanoparticles; with and without PEG. The hydrodynamic diameter of LMC nanoparticles was smaller than the hydrodynamic diameter of HMC nanoparticles as also presented in previous studies (Janes and Alonso 2003; Csaba et al. 2009). This is because of the high molecular weight of HMC per anionic crosslinking with respect to low molecular weight of LMC.
All formulations showed a positive mean surface charge ranging from +37 to +39 mV. The surface charge was similar within formulations and showed no significant (p<0.05) difference amongst them.

Table 2-3. Hydrodynamic diameter and surface charge of LMC nanoparticles (with and without PEG) (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic diameter (nm ± S.D)</th>
<th>Polydispersity index (±S.D)</th>
<th>Surface charge (mV ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC</td>
<td>123 ± 8</td>
<td>0.19 ± 0.01</td>
<td>+37 ± 2</td>
</tr>
<tr>
<td>LMC-PEG600</td>
<td>134 ± 8</td>
<td>0.15 ± 0.06</td>
<td>+38 ± 1</td>
</tr>
<tr>
<td>LMC-PEG1000</td>
<td>152 ± 4</td>
<td>0.16 ± 0.03</td>
<td>+39 ± 1</td>
</tr>
<tr>
<td>LMC-PEG5000</td>
<td>165 ± 4</td>
<td>0.20 ± 0.00</td>
<td>+38 ± 4</td>
</tr>
</tbody>
</table>

**c. LMC-g-PEG5000**

The hydrodynamic diameter of LMC-g-PEG nanoparticles was 179 (±7.4) nm and PDI was less than 0.2. As observed from these results, LMC-g-PEG5000 nanoparticles were larger than LMC and LMC-PEG5000 nanoparticles (Table 2-3). The increase in particle size is attributed to the long polymer chain of grafted PEG (5000). Particle surface charge was found to be +31 (±1.4) mV. The small number of amino functional groups due to PEG attachment reduced the surface charge of nanoparticles after ionotropic gelation with TPP with respect to LMC and LMC-PEG5000 nanoparticles (+37 ±2 mV and +38 ±4 mV respectively). It could also be possibly due to charge shielding effect of PEG in this particular formulation. This indicates successful grafting of PEG to chitosan polymer. Insulin-loaded PEG-g-chitosan nanoparticles with similar hydrodynamic diameter (150-300 nm) and surface charge +16 to +30 mV prepared by the ionotropic gelation using TPP as the crosslinking agent have been previously produced (Zhang et al. 2008b). The group prepared nanoparticles of PEG-g-chitosan with TPP in the ratio of 4.2:1 (w/w).

**2.4.4. Size and surface charge of dialysed nanoparticles**

In order to study the association of different grades of PEG with nanoparticle formulation, dialysis of the particles was performed, and the nanoparticles subsequently characterised.
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a. Hydrodynamic diameter

In general, among the formulations studied, Figure 2-12 indicates that chitosan nanoparticles without PEG showed smallest particle size. Inclusion of PEG within chitosan nanoparticle formulation caused a gradual increase in their mean size. After dialysis all formulations showed a reduction in particle size.

![Figure 2-12. Hydrodynamic diameter of HMC nanopartides (with and without PEG) before and after dialysis (mean ± S.D., n=3).](image)

The nanoparticle diameter for all HMC formulations was 170 to 210 nm (before dialysis) and 157 to 184 nm (after dialysis) (Figure 2-12). In the case of dialysed particles PEG-600 nanoparticles were significantly (p<0.05) smaller than PEG5000 nanoparticles. Presumably these results could be due to entrapment of PEG5000 molecules within nanoparticles which cause an increase in nanoparticle size. Mann Whitney test between corresponding un-dialysed and dialysed formulations indicated that dialysed forms of chitosan-PEG600 and chitosan-PEG5000 nanoparticle showed a statistically significant (p<0.05) reduction in nanoparticle size following dialysis indicating removal of excess PEG from the formulation.

b. Surface charge

The surface charge (Figure 2-13) for all HMC formulations was positive and in the range of +24 to +28 mV (before dialysis) and +19 to +29 mV (after dialysis). Un-dialysed formulations showed no difference in surface charge (p<0.05). This could be because PEG does not have any charge of its own (Zhang et al. 2008b). Statistical studies showed a significant difference (p<0.05) in surface charge between dialysed chitosan-PEG600 and chitosan-PEG5000...
nanoparticle formulations. Significant difference (p<0.05) was also seen between chitosan-PEG600 un-dialysed and dialysed nanoparticles. This is un-attributable at this stage and needs further investigation.

Figure 2-13. Surface charge of HMC nanoparticles (with and without PEG) before and after dialysis (mean ± S.D., n=3).

2.4.5. Transmission electron microscopy of HMC nanoparticles

Figure 2-14. TEM of HMC nanoparticles.
TEM was used to examine the morphology of nanoparticles. Crosslinked chitosan showed dark spots of nanometer size which were likely to be nanoparticles (Figure 2-14).

TEM of PEG-associated crosslinked chitosan (Figure 2-15) showed nanoparticles were very small, spherical and well dispersed structures, with a size less than 50 nm (Figure 2-16). The surface morphology of TPP crosslinked chitosan nanoparticles was not clearly shown by TEM whereas the inclusion of PEG within the formulation improved dispersion properties and brought segregation of particles within the system as consistent discrete structures were obtained. The size recorded using the zetasizer was 210 nm (Table 2-2). This may be due to collective small sized nanoparticles to form a size of around 200 nm, or changes in the sample morphology when exposed to the high vacuum of electron microscope.

2.4.6. Transmission electron microscopy of LMC-g-PEG5000 nanoparticles

LMC-g-PEG5000 nanoparticles were visualised as dark structures in close contact with each other (Figure 2-17). These particles did not appear spherical in morphology. They were of inconsistent shape and had a size of around 20-50 nm (Figure 2-18). These particles may aggregate hence the measured size increases to 100-200 nm. LMC-g-PEG nanoparticles formed in another study were spherical with the individual particles larger in size (~100 nm) (Zhang et al. 2008b). Zetasizer results showed nanoparticle size of 179 nm (2.4.3.c) which may be due to nanoparticles coming in close proximity to each other resulting in an increase in over all size.
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2.4.7. Scanning electron microscopy of HMC nanoparticles

HMC nanoparticles following freeze-drying were spherical, of approximately uniform size and had a smooth surface. They were small with narrow size distribution (Figure 2-19).

PEG1000 (Figure 2-20) and PEG5000 (Figure 2-21) associated nanoparticles showed similar morphology but with a size ~800 to 1 μm. An increase in size was also confirmed through sizing.
experiments using the zetasizer where HMC nanoparticle (without PEG) size was significantly different ($p<0.05$) from HMC-PEG5000 nanoparticle size, as described in Table 2-2.

The PEG600 associated nanoparticle appeared to have similar particle size but with unassociated PEG around spheres (Figure 2-22). The particle size distribution is small. Taking the TEM and SEM images into account, these chitosan nanoparticles with and without PEG showed suitable size and shape for pulmonary delivery as a therapeutic carrier.
2.4.8. Scanning electron microscopy of LMC nanoparticles

LMC nanoparticles were spherical in appearance with uniform size distribution and seemed to be aggregated (Figure 2-23).

pDNA loaded crosslinked chitosan irrespective of molecular weight formed well defined spherical structures (Csaba et al. 2009), observed by TEM. The authors suggested that compact and uniform nanoparticles were formed in comparison to undefined chitosan-pDNA polyplexes because of the controlled gelation process between TPP and chitosan.
2.4.9. Effect of pH on nanoparticle properties

a. HMC nanoparticles

In order to explore the potential of chitosan nanoparticles for lung delivery it is important to consider their behaviour at lung pH; 6.5 (Mohri et al. 2010). The change in surface charge of nanoparticles over the pH range 5.5 to 8.0 is shown in Figure 2-24.

The measured surface charge for all four formulations was very similar at any single measured pH. The high positive surface charge density on crosslinked chitosan at lower pH is due to the free surface amine groups of chitosan. As the pH of the nanoparticle suspension was increased, a greater proportion of amine groups were deprotonated resulting in a decrease in the measured positive surface charge for the particles.

The influence of pH on nanoparticle size is shown in Figure 2-25. At a pH range of 5.5 to 6.5 the mean nanoparticle size was constant. The positive charge of chitosan in acidic medium results in repulsion between nanoparticles (Kiang et al. 2004). However, as the pH was increased the mean measured particle size increased, which suggested the occurrence of aggregation. Such increases were more marked for formulations without PEG, compared to those with PEG, suggesting that the association of PEG with chitosan nanoparticles provides steric hindrance, preventing nanoparticles from aggregating. At pH 7.5 and greater, the measured particle size was greatly increased due to decreased surface charge (Figure 2-24) leading to aggregation of
all formulations, and dispersions became turbid in appearance. This agrees with a study, where chitosan and enoxaparin complexes showed physicochemical stability at a pH range of 3 to 6.5, but aggregated at higher pH (Sun et al. 2008b).

From these studies it was observed that the physical stability of chitosan nanoparticles is pH dependent, but considerable aggregation did not occur at lung pH: 6.5 (Mohri et al. 2010). Further, these findings suggest that PEG-associated chitosan nanoparticles are more resistant to aggregation as a result of changing pH than formulations without PEG.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2-26.png}
\caption{Hydrodynamic diameter of HMC nanoparticle (with and without PEG) at range of pH (mean ± S.D., n=4).}
\end{figure}

\textit{b. LMC nanoparticles}

In acidic pH of around 5.5 the nanoparticle formulations showed maximum surface charge of \(^{\sim}+45\) mV (Figure 2-26). The surface charge gradually and constantly reduced as the pH of the solution was increased. The nanoparticles were still positively charged even at a pH as high as 7.5. Positive charge is essential for a condensed and compact association with negatively charged biopharmaceutical compounds. LMC (10 kDa)/DNA particles at pH6.5 showed higher transfection efficiency in HEK293 cell lines in comparison to pH7.1 since the nanoparticle surface charge is positive and this helps in adherence to negatively charged cells (Lavertu et al. 2006). The decrease in surface charge may also cause dissociation of nucleic acid from the nanoparticles (Ishii et al. 2001).
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The results of pH on the hydrodynamic diameter of LMC nanoparticles at different pH values are shown in Figure 2-27. Nanoparticles over a range of pH from 5.5 to 7 did not show much change in hydrodynamic diameter. The particle size remained constant and less than 250 nm. The size increased to >1 μm when pH of the medium was raised to 7.5. With increase in pH particle aggregation occurred, leading to a turbid dispersion. This study is a clear indication that the particles are mainly stabilised due to electrostatic repulsion. As the pH increased the steric stability due to positive surface charge decreased further resulting in particles being aggregated. The results of variation in surface charge and hydrodynamic diameter correlated well with each other over the pH range 5.5 to 8.
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c. LMC-g-PEG5000 nanoparticles

As shown in Figure 2-28, LMC-g-PEG5000 nanoparticles were positively charged (+32 mV) at pH 5.5. As the pH increased the surface charge continuously reduced indicating the effect of pH on the cationic amine groups of chitosan.

![Figure 2-28. Surface charge of LMC-g-PEG5000 nanoparticles at a range of pH (mean ± S.D., n=4).](image)

Figure 2-29 shows that chemical modification of chitosan with PEG helped to maintain the physical stability of nanoparticles. Nanoparticle size was between 106 to 233 nm between pH 5.5 and 7. Adding more base (sodium hydroxide) to the nanoparticle suspension caused the particles to aggregate drastically to a value greater than could be accurately determined by PCS. The stability of nanoparticles up to pH 7 is essential when incorporated in physiological conditions of lung.

![Figure 2-29. Hydrodynamic diameter of LMC-g-PEG5000 nanoparticles at a range of pH (mean ± S.D., n=4).](image)
2.4.10. HMC nanoparticle suspension stability within a model propellant

Preliminary nanoparticle dispersion studies were performed within model propellant; 2H, 3H decafluoropentane (DFP; which is liquid at room temperature) before testing formulation with conventional propellants HFA-227 or HFA-134a. The study was performed in two stages:

a. Effect of sonication on nanoparticle dispersion

Initial studies (Table 2-4), conducted using a 15 min sonication period showed that the dispersion of freeze-dried chitosan nanoparticles DFP resulted in flocculation (Figure 2-30). Nanoparticles containing PEG600 displayed better dispersion stability as it slowed the onset of instability but they were still loosely flocculated. The cohesive force between particles was high in both these formulations resulting in physically poor dispersion. PEG1000 nanoparticles produced a homogeneous dispersion after shaking and preparations were visibly stable for around 10 min (Table 2-4). The stability was due to PEG 1000 providing steric stabilisation within the system. This is an indication of a well dispersed pMDI system which easily re-suspends after sedimentation. The stabilising effect of PEG1000 and PVP within the model propellant 2H, 3H-perfluoropentane, reducing particle-particle and particle-canister wall surface interactions has been previously described (Ashayer et al. 2004). PEG5000 associated nanoparticles formed a milky dispersion, without aggregates. It was observed that this formulation creamed within 1 min. This might be due to low PEG5000 density to propellant.

Similar results with slightly greater flocculation were also observed in formulations prepared using 60 min sonication time (Figure 2-30). This confirms that a total sonication period of 15 min provides better results of formulation dispersion. Another disadvantage of a long period of sonication is the potential instability of biopharmaceutical compounds which may in future be associated with the particles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Suspension in DFP</th>
<th>Appearance</th>
<th>Time to visible phase change</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMC</td>
<td>Flocculation</td>
<td>≤30 s</td>
<td></td>
</tr>
<tr>
<td>HMC-PEG600</td>
<td>Flocculation</td>
<td>~1 min</td>
<td></td>
</tr>
<tr>
<td>HMC-PEG1000</td>
<td>Homogeneous</td>
<td>≥10 min</td>
<td></td>
</tr>
<tr>
<td>HMC-PEG5000</td>
<td>Creaming</td>
<td>≥1 min</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-4. Dispersion properties of HMC nanoparticles (with and without PEG) in DFP. Prepared using a total sonication period of 15 min (n=3) at room temperature.
Chapter 2. Preparation and characterisation of chitosan nanoparticles

Figure 2-30. Images of un-dialysed formulation dispersed within DFP prepared over a total sonication period of 15 min and 60 min. The images were taken 5 min following hand shaking: A) HMC, B) HMC-PEG600, C) HMC-PEG1000 and D) HMC-PEG5000 nanoparticles.

b. Effect of dialysis on nanoparticle dispersion

Dialysed chitosan and chitosan-PEG600 nanoparticle formulations showed flocculated dispersion which creamed within one min (Figure 2-31). Instability also occurred in un-dialysed preparations with flocculation. Previously, the inability of PEG600 to stabilise silica particles dispersed in a fluorinated solvent (2H,3H-perfluoropentane) has been reported (Paul et al. 2005). PEG 1000 associated nanoparticles showed stability (~10 min) and PEG 5000 associated nanoparticles resulted in creaming (<1 min) within the system. It was observed that both dialysed and un-dialysed PEG1000 and PEG5000 associated formulations were visually similar in their suspension behaviour. Based on these results, later work was performed using undialysed preparations.

Figure 2-31. Images of un-dialysed and dialysed formulation dispersed within DFP. The images were taken 1 min following hand shaking: A) HMC, B) HMC-PEG600, C) HMC-PEG1000 and D) HMC-PEG5000 nanoparticles.
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2.4.11. Visual analysis of nanoparticle suspension stability within HFA propellants

Chitosan nanoparticle formulations with and without PEG showed either sedimentation or creaming when dispersed within HFA-134a or HFA-227 over a period of 10 s and 1 min once the manual shaking was stopped.

a. HMC nanoparticles

The onsets of change in the nanoparticle phase from the dispersed media are summarised in Table 2-5. HMC nanoparticles without PEG aggregated readily and phase separation occurred with clearly visible aggregates settling rapidly (Figure 2-32A). The inclusion of PEG600 within the nanoparticles did not result in any apparent change in their dispersion properties (Figure 2-32B). Both formulations were physically unstable, resulting in aggregation in less than one minute. It has been previously reported that the use of PEG400 within HFA suspension formulations decreased the forces of adhesion between particles (Reid et al. 2008). Previously, PEG300 has been explored as a surfactant with appreciable solubility within non-polar media such as HFA-134a and HFA-227 (Blondino and Byron 1998).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Suspension in HFA-134a</th>
<th>Suspension in HFA-227</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dispersion Phase change Rate</td>
<td>Dispersion Phase change Rate</td>
</tr>
<tr>
<td>HMC</td>
<td>Sedimentation &lt;1 min</td>
<td>Sedimentation &lt;1 min</td>
</tr>
<tr>
<td>HMC-PEG600</td>
<td>Sedimentation ~3 min</td>
<td>Sedimentation &lt;1 min</td>
</tr>
<tr>
<td>HMC-PEG1000</td>
<td>Homogeneous ~10 min</td>
<td>Homogeneous ~10 min</td>
</tr>
<tr>
<td>HMC-PEG5000</td>
<td>Sedimentation ~5 min</td>
<td>Creaming &lt;1 min</td>
</tr>
</tbody>
</table>

HMC-PEG1000 nanoparticles produced a homogeneous translucent dispersion in HFA-227, which exhibited no phase separation for up to 10 min (Figure 2-32C). In addition to exhibiting the slowest rate of sedimentation, these nanoparticles readily re-dispersed upon shaking. This property is essential for acceptable dose reproducibility from a pMDI. A good stabilisation excipient for pMDI formulation should be well solvated and prevent particle-particle interaction (Thiel 1996). A previous study demonstrated that PEG1000 is able to solvate well within HFA-227 (Peguin and Da Rocha 2008).
Chapter 2. Preparation and characterisation of chitosan nanoparticles

Figure 2-32. Images of formulations dispersed within HFA-227 propellant at 10 s and 1 min time points following hand shaking: A) HMC, B) HMC-PEG600, C) HMC-PEG1000 and D) HMC-PEG5000 nanoparticles.

Figure 2-33. Images of formulations dispersed within HFA-134a propellant at 10 s and 1 min time points following hand shaking: A) HMC, B) HMC-PEG600, C) HMC-PEG1000 and D) HMC-PEG5000 nanoparticles.
A commercially available pharmaceutical product such as Proventil® has suspension stability less than 30 s in pressurised HFA system (Dellamary et al. 2000). The preparation is readily redispersible just like our preparation but stays suspended for a short period of time indicating the suitability of HMC-PEG1000 for HFA. HMC-PEG5000 nanoparticles produced an opaque, milky dispersion in HFA-227, which separated into two phases (Figure 2-32D), with particles creaming to the surface of the liquid propellant, presumably as the density of these particles is less than the density of the propellant. Although this formulation creamed in less than 1 min, it was re-dispersible with gentle shaking.

Similar results (to HFA-227) were observed with non-PEG and HMC-PEG1000 formulations when dispersed in HFA-134a (Figure 2-33A and Figure 2-33C). It was observed that HMC-PEG600/HFA-134a suspension (Figure 2-33B) was stable for ~3 min whereas HMC-PEG5000/HFA-134a (Figure 2-33D) showed turbidity with onset of particles settling at the bottom at ~5 min.

These findings, undertaken using pressurised apparatus to investigate properties in the clinically relevant propellants HFA-227 and HFA-134a, indicate that PEG1000 incorporated into chitosan nanoparticles, is an effective formulation strategy to produce viable nanoparticle dispersion for delivery from a pMDI.

The variation in particle behaviour in a propellant depends on the type of propellant used and the composition of the formulation. Previous studies have reported that DFP proved to be a good model for HFA-134a only (Ridder et al. 2005). It was further suggested in their work that, generally non-ionic surfactants show higher solubility in HFA-227 than compared to HFA-134a and DFP and hence they got similar results of surfactant solubility in DFP and HFA-134a. Visually screening formulation dispersion in HFA-134 or 227 in our study was not particularly helpful to postulate DFP as a good model for any of these propellants.
Chapter 2. Preparation and characterisation of chitosan nanoparticles

b. LMC nanoparticles

The onset of separation of nanoparticles from the dispersed media is summarised in Table 2-6.

Table 2-6. Dispersion properties of LMC nanoparticles (with and without PEG) in pressurised propellants (HFA-134a and HFA-227) at room temperature.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Suspension in HFA-134a</th>
<th>Suspension in HFA-227</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dispersion</td>
<td>Phase change Rate</td>
</tr>
<tr>
<td>LMC</td>
<td>Sedimentation</td>
<td>~6 min</td>
</tr>
<tr>
<td>LMC-PEG600</td>
<td>Sedimentation</td>
<td>~4 min</td>
</tr>
<tr>
<td>LMC-PEG1000</td>
<td>Sedimentation</td>
<td>~3 min</td>
</tr>
<tr>
<td>LMC-PEG5000</td>
<td>Sedimentation</td>
<td>~9 min</td>
</tr>
</tbody>
</table>

LMC nanoparticles when dispersed within HFA-134a (Figure 2-34A) or HFA-227 (Figure 2-35A) showed an onset of sedimentation at ~6 mins. The sample was translucent but was still clearer than other formulations. The system showed slight particle sedimentation at the bottom of the vial indicating instability but at a slow and steady rate. Similar results were also observed with LMC-PEG600 nanoparticles with no increase in particle stability when dispersed in both HFA-134a (Figure 2-34B) and HFA-227 (Figure 2-35B).

The particles sedimented in ~4 mins but the sample was more opaque and white in appearance. This could be due to the presence of PEG600 within the formulation changing its optical properties. LMC-PEG1000 nanoparticle formulations were unstable in HFA-134a with ~3 mins after which the sample sedimented (Figure 2-34C). The same formulation showed its highest stability period of >10 mins in HFA-227 propellant (Figure 2-35C). It seems the sample is less dense than LMC-PEG600 nanoparticle formulation dispersions. This high stability property of LMC-PEG1000 formulation within HFA-227 is the same as with HMC-PEG1000 nanoparticles. Change in nanoparticle behaviour with HFA-134a could be due to the differing properties of propellant HFA-134a and HFA-227. LMC-PEG5000 nanoparticle formulations also showed similar differences in nanoparticle behaviour with both hydrofluoroalkanes. LMC-PEG5000 nanoparticle formulation dispersed in HFA-134a formed a highly opaque suspension. The sedimentation of the particles was not visible until ~9 mins (Figure 2-34D) whereas in HFA-227 the particles creamed in <1 min (Figure 2-35D).
Figure 2-34. Images of formulations dispersed within HFA-134a propellant at 10 s and 1 min time points following hand shaking: A) LMC, B) LMC-PEG600, C) LMC-PEG1000 and D) LMC-PEG5000 nanoparticles.

Figure 2-35. Images of formulations dispersed within HFA-227 propellant at 10 s and 1 min time points following hand shaking: A) LMC, B) LMC-PEG600, C) LMC-PEG1000 and D) LMC-PEG5000 nanoparticles.
Chapter 2. Preparation and characterisation of chitosan nanoparticles

All formulations re-dispersed homogenously immediately upon slight shaking implying that the engineered formulations do get solvated and have suitable properties for dispersion within non-polar media. LMC-PEG1000 nanoparticle formulation showed greatest stability and suitability for further tests of aerosolisation. Comparing LMC nanoparticles with HMC nanoparticles it was observed that the onset of sedimentation or creaming and the bulk of nanoparticles phasing out of the media were very different. Relatively, LMC supported nanoparticles were better dispersed than HMC supported nanoparticles.

Similar to chitosan nanoparticles, salbutamol sulfate spheres dispersed in HFA-134a and HFA-227 formed unstable dispersions (Wu et al. 2008a). Upon addition of PEG300, the formulations showed stability for a longer period. According to the authors, the science behind PEG stability is its solvation within semifluorinated systems. Similar studies were performed to stabilise terbutaline hemisulfate (THS) modified using PEG300 within HFA-134a and HFA-227. The formulations showed similar good dispersion properties in HFAs (Wu et al. 2008a).

c. LMC-g-PEG5000 nanoparticles

Physical stability of LMC-g-PEG5000 nanoparticles within HFA-134a and HFA-227 was assessed (Figure 2-36). Upon manual shaking the particles aggregated rapidly followed by sedimentation in less than 1 min when dispersed within HFA-134a. Similar observations were made when particles were suspended within HFA-227 but the process was slower. This is not useful as the patient cannot ensure uniform dose after each actuation. Chitosan (medium m.w) grafted PEG5000 particles encapsulating PLGA loaded curcumin nanoparticles suspended within HFA-227 propellant showed an onset of flocculation after 1 min of shaking (Selvam et al. 2011).

This performance downgraded from LMC nanoparticle HFA suspensions where the particles were atleast stable for ~6 min in both HFA-134a and HFA-227 (Table 2-6). This dissimilarity in dispersion properties of LMC nanoparticles form LMC-g-PEG5000 nanoparticles might be due to the change in LMC structural orientation after being grafted by PEG. It was observed that PEG did not improve the dispersion properties of the nanoparticles. This could be due to fusion or aggregation of nanoparticles in the high pressure system within the canister. It can also be due to the molecular weight of PEG (5000) since PEG5000 associated LMC nanoparticles were also not stable in HFAs causing a lot of turbidity within the system (Table 2-6). This requires further investigation.
2.4.12. Turbiscan analysis of nanoparticle suspension stability

a. HMC nanoparticles

The acquired scans for the percentage of backscattered light obtained for each formulation suspended in HFA-227 as a function of time and sample height is shown in Figure 2-37. Turbiscan results obtained with HMC nanoparticle suspension showed a sharp increase in backscattering (>10%) between the first scan (time = 0) and all the consecutive scans with greatest increase at the lower regions of the tube (Figure 2-37a). This is due to particle size variation and particle migration to the bottom of the glass tube which causes a variation in light transmission through the glass tube and an increase in backscattering. Similar results were also observed for these formulations suspended within HFA-134a (Figure 2-38a) where the consecutive scans after time point 0 s were elevated because of formulation sedimentation in less than 1 min. The nanoparticle suspension signal was modified in the presence of PEG600, indicated by a step-wise increase in backscattering signal level between each successive scan (Figure 2-37b). This signifies a delay in the separation between two different phases compared to the HMC/HFA formulation. The variation in the percentage of
backscattering between the first and the final scan was greater than 10%, indicating that PEG600 does not prevent aggregation, but only delays the onset of aggregation. PEG600 associated formulation when suspended within HFA-134a showed greater stability, and improvement in dispersion properties (Figure 2-38b) when compared to chitosan-PEG600 suspended within HFA-227.

The scan for PEG1000-chitosan dispersions in HFA-227 showed less than 2% variation in backscattering for the entire scan at all time points (Figure 2-37c), indicating high stability for this formulation, i.e. no settling or creaming behaviour within the HFA. There was no apparent change in particulate size and volume fraction for the dispersion, even after 3 min post shaking, leaving the product homogeneous. It is likely that the nanoparticle bound PEG1000 covers the surface of the nanoparticles suspended within HFA and reduces the interfacial energy between particulates and propellant in order to prevent suspension instability. Slightly different results were obtained for chitosan-PEG1000 nanoparticle formulation dispersed
within HFA-134a (Figure 2-38c). The first scan at time 0 s did not superimpose with all other scans indicating slight sedimentation in particles which was not visible to the naked eye as shown in Figure 2-33c. This indicates the sensitivity of Turbiscan compared to visual analysis.

The dispersion of chitosan-PEG5000 nanoparticles in HFA-227 was opaque and milky in appearance. The backscattering signal increased to 25% between 40 and 45 mm, reflecting the creaming behaviour of the sample (Figure 2-37d), and suggesting that chitosan-PEG5000 nanoparticles had lower density than HFA-227 causing them to cream. PEG5000 associated formulations dispersed within HFA-134a showed similar physical appearance as HFA-227 dispersed formulations but the sample showed sedimentation which was visually apparent at ~5 min (Figure 2-38d). The variation in interaction at molecular level and dispersion behaviour of PEG5000 associated formulation within HFA-134a and HFA-227 is due to differences in propellant physicochemical properties.

![Graphs](image)

**Figure 2-38.** Backscattering profiles of HMC nanoparticle formulations dispersed within pressure sealed HFA-134a as a function of time; a) HMC, b) HMC-PEG600, c) HMC-PEG1000 and d) HMC-PEG5000 nanoparticles.

These results demonstrate that chitosan nanoparticles that incorporate PEG1000 were successful in stabilising the particle dispersion at all time points. These results are in line with the data obtained by visual analysis of dispersions, as described in Table 2-5. Taking these
results into consideration, particles suspended within HFA-227 were used for other characterisation studies.

b. **LMC nanoparticles**

The Turbiscan studies showed that LMC nanoparticles within HFA-134a (Figure 2-39a) or HFA-227 (Figure 2-40a) propellants had partial stability over a longer period than HMC. This may be due to HMC not solvating well within the non-polar media. Slightly raised scans from the initial scan are an indication of particles settling to the bottom of the glass tube, at a slow rate but most likely are due to high density in comparison to the propellant.

![Figure 2-39. Backscattering profiles of LMC nanoparticle formulations dispersed within pressure sealed HFA-134a as a function of time; (a) LMC (b) LMC-PEG600 (c) LMC-PEG1000 and (d) LMC-PEG5000 nanoparticles.](image)

PEG600 was included to the engineered nanoparticle formulation (Figure 2-39b and Figure 2-40b). The results obtained are very similar to the LMC nanoparticle dispersion preparations. Instability of LMC-PEG600 nanoparticles was slightly higher in HFA-134a propellant (based on % backscattering) in comparison to HFA-227, indicating better suspension properties of the formulation in HFA-227. LMC nanoparticle/HFA suspension stability was further investigated by including PEG1000 within the formulation. Results showed prolonged dispersion stability of the formulation in HFA-227 maybe because of the optimum chain length (Figure 2-40c).
Chapter 2. Preparation and characterisation of chitosan nanoparticles

scan is uniform through the time period the suspension was analysed. Surprisingly the PEG1000 formulation when suspended within HFA-134a showed immediate instability with particles settling to the bottom of the glass tube (Figure 2-39c). PEG5000 associated LMC nanoparticles formed a highly opaque suspension when dispersed within HFA-134a (Figure 2-39d). The formulation was stable for a long period with slight sedimentation of particles. On the other hand the same formulation when dispersed in HFA-227 showed immediate creaming (Figure 2-40d). Differences were observed in scans due to particle migration rate with slight increase in backscattering at the upper part of the system indicating creaming of the particles within the system. It seems the particle density is in between the density of two different HFAs leading to sedimentation in HFA-134a and creaming in HFA-227.

For all formulations instability was reversible upon slight shaking. In the present study the same concentration of polymer was used for all formulations. The results revealed LMC-PEG1000 nanoparticle formulation comparatively as the most stable in HFA-227.
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c. LMC-g-PEG5000 nanoparticles

Figure 2-41 is a backscatter profile of LMC-g-PEG5000 nanoparticle formulations dispersed within HFA-134a and shows an inconsistent and irregular profile (along the sample height) at $t = 0$ due to aggregation. These particles quickly aggregate and sediment at the bottom of the tube. The other scans showed slight higher backscattering but were all of the same intensity to each other at all time periods. This aggregation prevents the use of LMC-g-PEG5000 nanoparticles within pressurised HFA-134a systems.

Upon suspension of LMC-g-PEG5000 nanoparticles within HFA-227 the particles showed aggregation with slow rate of sedimentation (Figure 2-42).

Figure 2-41. Backscattering profiles of LMC-g-PEG5000 nanoparticle formulations dispersed within pressure sealed HFA-134a as a function of time.

Figure 2-42. Backscattering profiles of LMC-g-PEG5000 nanoparticle formulations dispersed within pressure sealed HFA-227 as a function of time.
The scans at ~2.5 mm showed increase in backscattering intensity due to sedimentation of aggregated particles. These scans remained elevated throughout sample height since the particles were still suspended even though they were large aggregated lumps. This could be due to the nearly equivalent density of particles to HFA-227 propellant.

The suspension is not homogeneous and could not be used as carriers for biopharmaceutical delivery using pMDI. PEG is known for steric stabilisation property; interestingly it does not provide stability to particles both in terms of particle migration and flocculation upon suspension within propellants. This could be due to the effect of PEG grafting to LMC which might change its properties and result into a molecular structure not suited for steric stabilisation. The stability findings by Turbiscan measurements were also supported by visual appearances recorded over a period of time after manual shaking (Figure 2-36). The profiles were similar and the nanoparticle dispersion within propellant was highly unstable.

2.5. Conclusion

The study has successfully demonstrated the preparation of non-PEG, PEG associated and PEG grafted chitosan nanoparticles. These nanoparticles of high and low molecular weight chitosan were sized <220 nm and had a positive surface charge. All these preparations stayed physically stable at lung pH; 6.5 which indicates their potential for lung delivery. FTIR analysis of dialysed and un-dialysed HMC showed that PEG1000 and PEG5000 were successfully retained within the nanoparticles following crosslinking.

It was observed that the composition of formulation including molecular weight of polymer, type of polymer, concentration and type of propellant used in a pMDI suspension are interdependent and play a major role in achieving optimal dispersion of nanoparticles in HFAs. Nanoparticles prepared from HMC (without PEG) showed aggregation (~1 min) when suspended within HFA-227 or HFA-134a pressurised systems, whereas LMC nanoparticles (without PEG) behaved differently with slightly longer stability (~6 min). These nanoparticles in presence of PEG1000 showed the most effective steric stabilisation and improved dispersion in HFA-227 (~10 min). Upon grafting PEG with LMC the preparations were unstable (aggregation) when suspended within HFA-227 or HFA-134a propellants. Turbiscan scanning and visual appearance assessment correlated well in determining the formulation’s physical stability. We demonstrated that co-formulating PEG with chitosan helped in easy dispersion in HFA propellants without the need for co-solvents. Based on the findings, HFA-227 was considered
Chapter 2. Preparation and characterisation of chitosan nanoparticles

as a more suitable propellant for carrying out future pMDI experiments with nanoparticle formulations in comparison to HFA-134a. This could be due to the large molecule of HFA-227 than HFA-134a allowing better solvation of these meandering chains of PEG molecule, and hence, these dispersion forces are expected to be higher.

The objective has been achieved whereby small particle size, positively charge chitosan nanoparticles were produced which were dispersible within propellant in the presence of PEG1000 showing potential application in delivery of drugs and biopharmaceuticals (negatively charged nucleic acids) to the lungs.
CHAPTER 3

PREPARATION AND CHARACTERISATION OF
siRNA LOADED NANOPARTICLES
3. Preparation and characterisation of siRNA loaded nanoparticles

3.1. Introduction

siRNA is gaining increasing popularity for its potential as a therapeutic tool for the treatment of lung diseases (Durcan et al. 2008), refer Chapter 1. It is based on RNAi mechanism which is a naturally occurring gene regulatory technique with specificity of inhibition, potency and reduced toxicity. In order to achieve successful gene silencing, careful siRNA delivery to the target lung cells is paramount. This is difficult due to two main barriers. The first is the complex branched anatomy of the lung and the second is the airway cell membrane (Durcan et al. 2008). Associating siRNA with cationic polymers can aid in the protection of negatively charged siRNA and enhanced interaction with the surface of negatively charged cells (Grenha et al. 2007). Chitosan is a cationic polymer which is mucoadhesive and has permeation enhancing properties (Chopra et al. 2006), which is potentially advantageous for targeting siRNA delivery to the respiratory tract. It has also been shown to be effective in gene silencing (Howard et al. 2006). More information on chitosan has been detailed in Chapter 1.

These formulations were designed with consideration of the charge ratio between the number of primary amine (nitrogen) groups in chitosan and the number of phosphate groups in siRNA also known as N:P ratio. For experiments performed in this thesis, varying weight/weight ratios of siRNA to chitosan were assessed. The cationic nature of chitosan and anionic nature of siRNA results in formation of nanocomplexes by ionic interaction. This process in presence of a crosslinking agent TPP gives rise to nanoparticles. The use of this simple and cost effective technique (ionic gelation) in forming hydrophilic biodegradable carriers has been suggested to be extremely mild, not requiring high temperatures or chemical synthesis (Calvo et al. 1997). In recent years there has been considerable interest in formulating chitosan as nanoparticles for siRNA delivery (Katas and Alpar 2006). It has been demonstrated that chitosan nanoparticles showed better uptake with epithelial cells (A-549) compared to chitosan molecules as carriers (Huang et al. 2004). Drug formulations containing chitosan base and derivative (chitosan hydrochloride) showed non-crosslinked chitosans swelled in contact with water and crosslinking chitosan with crosslinking agents formaldehyde and glutaraldehyde maintained the integrity of the particles (He et al. 1999). Crosslinking chitosan has been shown to result in formulations with controlled release properties (Ganza-González et al. 1999).
The objective of this study was to explore the size and surface charge of siRNA loaded nanoparticle systems and nanocomplexes. Different molecular weight chitosan particulate systems were investigated for siRNA loading efficiency. The study also assessed the interaction of chitosan (in the presence and absence of a crosslinking agent) with siRNA at different weight/weight ratios using gel electrophoresis.

3.2. Materials

Synthetic PTENV10-23-hmr (phosphatase and tensin homolog) with a molecular weight 7.4118 kDa and a duplex sequence (Sense: 5'-UAAGUUCUAGCUGUGGGUUA-3', antisense: 3'-AUUCAAGAUCGACACCACCCAAU-5' (specific) was donated by Integrated DNA technologies via AstraZeneca (UK). Non-specific negative control siRNA (Stealth RNAi control) was purchased from Invitrogen, UK. Tris acetate- ethylenediaminetetraacetic acid (TAE) buffer (10x), ethidium bromide, agarose, acetic acid, methanol, acetone (HPLC grade), PEG 1000 and TPP 85% were supplied by Sigma Aldrich (UK). Gel loading dye (Blue 6x) was purchased from Biolabs (UK). High molecular weight water soluble chitosan glutamate (HMC); Protasan UP G 113, m.w 150 k-200 kDa, degree of deacetylation 75-90% was purchased from Novamatrix (Norway). Low molecular weight, water soluble chitosan (LMC) with m.w >10 kDa and degree of deacetylation 97.0% was purchased from Kittolife Co. Ltd (Korea). PEG 5000 monomethyl ether was obtained from Fluka (UK). PEG 600 and HPLC grade water were purchased from Fischer Scientific (UK). Model propellant 2H, 3H Decafluoropentane (DFP) was supplied by Apollo Scientific (UK). PicoGreen reagent (Quan-iT™) was bought from Molecular Probes (USA)

3.3. Methods

3.3.1. Preparation of siRNA nanocarriers

Three approaches were followed to form siRNA nanocarriers using non-specific siRNA (Stealth RNAi control) and specific siRNA (PTEN; tumor suppressor gene). These are schematically presented in Figure 3-1.
a. siRNA adsorption on to nanoparticles

Crosslinked HMC nanoparticles loaded with negative control Stealth siRNA were prepared by ionic gelation as described in Section 2.3.3.

20 µl TPP solution (0.5 mg/ml) was added to 50 µl chitosan solution (1 mg/ml), vortexed (SA7, Stuart Vortex Mixers, UK) for 1 min then allowed to stand for 30 min, for complete polymeric interaction and nanoparticle formation to occur. siRNA was diluted to 5 fold in RNAse-free water and 30 µl of this solution was added to the prepared nanoparticles for anionic interaction of siRNA with the surface of polycationic chitosan nanoparticles, resulting in the formation of siRNA adsorbed nanoparticles.
Chapters. Preparation and characterisation of siRNA loaded nanoparticles

Three grades of PEG (600, 1000 and 5000) were used at a concentration of 15 mg/ml within the TPP solution prior to nanoparticle preparation, to form PEG-chitosan formulations. The experiment was performed under a sterile laminar flow hood (Walker, UK). Following siRNA nanoparticle preparation, samples were stored for 1 h at room temperature before analysis using gel electrophoresis.

b. siRNA encapsulation within nanoparticles

Chitosan or chitosan-PEG nanoparticles were formed through ionic gelation as described in Section 2.3.3.

(i) HMC nanoparticles loaded with negative control Stealth siRNA: A preliminary investigation

siRNA (20 μM) was diluted to 5 fold in RNAse-free water and 30 μl of this solution was added to 20 μl TPP solution, vortexed (SA7, Stuart Vortex Mixers, UK) for 1 min then allowed to stand for 5 min. This solution was added to 50 μl chitosan solution (1 mg/ml) to form siRNA encapsulated nanoparticles. PEG associated formulations were prepared in a similar manner, where 15 mg/ml PEG (600, 1000 and 5000) was added to the TPP solution prior to nanoparticle preparation. Following siRNA nanoparticle preparation, samples were stored for 1 h at room temperature prior to analysis with gel electrophoresis.

(ii) HMC nanoparticles loaded with PTEN siRNA

285.7 μl of TPP solution (0.5 mg/ml) was mixed with siRNA (15.38 μg/μl) prior to nanoparticle formation. This was added dropwise to 714.2 μl chitosan solution (1 mg/ml) with constant stirring to form siRNA encapsulated nanoparticles. The total volume of the formulated sample was made up to 1014.12 μl with nuclease-free water. siRNA solution was added in volumes of 4.6 μl, 1.5 μl and 0.92 μl to form a chitosan to siRNA w/w ratio of 10:1, 30:1 and 50:1. These represent N:P ratios of 20:1, 60:1 and 100:1. Three grades of PEG (600, 1000 and 5000) were used to prepare PEG associated HMC formulations by adding PEG (15 mg/ml) to siRNA-TPP solution. All nanoparticle formulations were stored for 1 h at room temperature prior to use in future experiments.

(iii) LMC and LMC-g-PEG5000 nanoparticles loaded with PTEN siRNA

166.6 μl TPP solution (0.5 mg/ml) was mixed with siRNA (15.38 μg/μl) prior to nanoparticle formation. This was added dropwise to 833.3 μl of LMC solution (1mg/ml) dropwise with
constant stirring to form siRNA encapsulated LMC nanoparticles. The total volume of the nanoparticles was made up to 1013.3 µl with nuclease-free water. siRNA solution was added in volumes of 5.4 µl, 1.8 µl and 1.08 µl to form a chitosan to siRNA w/w ratio of 10:1, 30:1 and 50:1. These represent N:P ratios of 20:1, 60:1 and 100:1. Three grades of PEG (600, 1000 and 5000) were used to prepare PEG associated LMC formulations by adding PEG (15 mg/ml) to siRNA-TPP solution.

Similarly, LMC-g-PEG5000 nanoparticles were produced where 166.6 µl TPP solution (0.5 mg/ml) was mixed with siRNA (15.38 µg/µl) prior to nanoparticle formation. The solution was added dropwise to 833.3 µl LMC-g-PEG5000 solution (1mg/ml). siRNA was added to produce a chitosan to siRNA ratio of 10:1, 30:1 and 50:1 w/w. All nanoparticle formulations were stored for 1 h prior to use in future experiments.

c. **siRNA nanocomplexes**

siRNA complexes were prepared in a similar manner to nanoparticle preparation but without the inclusion of TPP (crosslinker).

(i) **HMC nanocomplexes**

15.38 µg/µl of siRNA and siRNA premixed with PEG1000 and 5000 (285.7 µl; 15 mg/ml), were added dropwise to 714.2 µl of HMC solution (1 mg/ml) with constant stirring to produce HMC nanocomplexes with and without PEG. The solution was made up to the desired volume (1014.5 µl). siRNA was added to produce a chitosan to siRNA ratio of 10:1, 30:1 and 50:1 w/w. The sample was left undisturbed for 1 h to allow complete siRNA interaction with chitosan.

(ii) **LMC and LMC-g-PEG5000 nanocomplexes**

15.38 µg/µl of siRNA and siRNA premixed with PEG1000 and 5000 (166.6 µl; 15 mg/ml), was added dropwise to 833.3 µl of LMC solution (1 mg/ml) with constant stirring to produce LMC nanocomplexes with and without PEG. The solution was made up to the desired volume (1013.3 µl). siRNA was added to produce a chitosan to siRNA ratio of 10:1, 30:1 and 50:1 w/w. Similarly, LMC-g-PEG5000 nanocomplexes were produced where siRNA (15.38 µg/µl) was added dropwise to 833.3 µl LMC-g-PEG solution (1mg/ml) at w/w ratio of 10:1, 30:1, 50:1 and 80:1. The nanocomplexes were left undisturbed for 1 h for complete siRNA interaction with chitosan.
3.3.2. Size and surface charge of nanoparticles and nanocomplexes

The particle size was measured by dynamic light scattering using a ZetasizerNano ZS (Malvern Instruments, UK) as described in Section 2.3.6.

The surface charge of non-PEG and PEG containing nanoparticles dispersed in deionized water was measured by laser doppler micro-electrophoresis technique also using the ZetasizerNano ZS (Malvern Instruments, UK) as described in Section 2.3.7.

3.3.3. Loading efficiency

The loading efficiency of siRNA onto or within nanoparticle/nanocomplexes was determined using the PicoGreen reagent (Quant-it™, Molecular Probes, USA) according to a standard protocol. 900 µl of siRNA loaded nanoparticles in the chitosan:siRNA w/w ratio of 10:1, 30:1 and 50:1 (w/w) were centrifuged at 17,000 xg for 15 min at 10 °C (refrigerated centrifuge Sigma 1-15PK, Osterode am Harz, Germany). Centrifugation causes siRNA loaded nanoparticles to settle at the bottom of the tube, with free siRNA in the supernatant. For each measurement, 500 µl of supernatant was mixed with 500 µl of TE (Tris-hydrochloride ethylenediaminetetraacetic acid) buffer supplied with the kit and 1 ml of PicoGreen reagent (1/200 dilution with TE buffer) to quantify free siRNA, unbound to nanoparticles. After an incubation period of 10 min, the sample was transferred to a quartz microcurvette and the amount of siRNA associated with nanoparticles was assayed using a luminescence spectrometer (LS55, Perkin Elmer, USA) with excitation and emission wavelengths of 480 nm and 520 nm respectively. siRNA reacts with PicoGreen to emit fluorescence. The detected fluorescence corresponds to siRNA in the supernatant whereas the nanoparticle associated siRNA settled as pellet and hence is unable to react with the dye. Blanks containing no siRNA were also prepared. A calibration curve ranging from 0 to 1000 ng/ml siRNA was prepared and equation 3.1 used to determine the encapsulation efficiency. Each sample was assayed in triplicates.

Encapsulation efficiency = \[
\frac{[\text{Total siRNA} - \text{siRNA unbound}]}{\text{Total siRNA}} \times 100
\]

Equation 3.1
3.3.4. Gel electrophoresis of HMC nanoparticles with negative control Stealth siRNA

In order to assess the association of siRNA with nanoparticles, gel electrophoresis was performed. The studies were conducted using 4% agarose gel formed with 1x TAE buffer at pH 8. Separately, agarose (4 g) was weighed and dissolved in 100 ml TAE in a conical flask by heating in a microwave oven. As soon as the solution was warm, ethidium bromide (4 μl) was added to the agarose solution (100 ml) and was swirled to dissolve. This was then transferred to the gel tray and a comb (size: 1.5 mm thick) was inserted to form a well for sample loading. When the solution cooled, the combs were removed. The gel was then completely submerged in TAE solution. Prior to siRNA loading in gel, the samples were mixed with a loading dye in a ratio of 1:6 in order to help weigh down the nucleic acid, so that it can sink into the bottom of the wells and not float in the buffer solution.

siRNA was diluted to 5 fold, then 3.3 fold using RNAse-free water. 10 μl of this solution was loaded into one of the wells as a control. Nanoparticle formulations were associated with siRNA by adsorption or encapsulation as described in Section 3.3.1. Electrophoresis was carried out at 65 V for 30 min. The movement of stained siRNA was detected when exposed to ultraviolet light using a UV transilluminator based image analyser; G-Box (Syngene, UK). The presence of a cathode electrode at one end of the tray caused the unbound negatively charged siRNA to move out of the well. The migration of siRNA from the sample and the standard (control) were then compared.

3.3.5. Gel electrophoresis of nanoparticles and nanocomplexes with PTEN siRNA

The stability of siRNA to various conditions encountered during the manufacture of siRNA associated nanoparticles and nanocomplexes were examined by monitoring degradation with gel electrophoresis. The gels were prepared with 4% (w/v) agarose in TAE buffer. More detail on preparation of gel has been described in Section 3.3.4. siRNA (0.2 μg) was loaded onto each well encapsulated with different ratios of chitosan (with and without PEG) as detailed in Section 3.3.1. The gel was run at 60 V for 1 h. siRNA movement was determined using ethidium bromide which was dissolved within the gel. Gel images were produced using a UV transilluminator image analyser (Syngene G-Box, UK).
3.3.6. Gel electrophoresis to study siRNA stability within model propellant

30 μl siRNA was dispersed within 985 μl model propellant (DFP). The sample was then placed in an oven at 30°C until the propellant evaporated. This sample was re-dispersed with water and made up to 1000 μl. This was then loaded onto 4% agarose gel to investigate the stability of siRNA following in model propellant. An equal amount of siRNA was loaded as control. Gels were run and imaged as described in Section 3.3.4.

3.3.7. Summary of experiments conducted

Table 3.1. Summary of experiments conducted in chapter 3.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Results Section</th>
<th>HMC</th>
<th>HMC-PEG</th>
<th>LMC</th>
<th>LMC-PEG</th>
<th>LMC-g-PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PTEN siRNA encapsulated nanoparticles</td>
<td>3.4.1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>- PTEN siRNA associated nanocomplexes</td>
<td>3.4.3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Surface charge</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- PTEN siRNA encapsulated nanoparticles</td>
<td>3.4.2</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>- PTEN siRNA associated nanocomplexes</td>
<td>3.4.4</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Loading efficiency of nanoparticles (PTEN siRNA)</td>
<td>3.4.5</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Gel electrophoresis of Stealth siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Adsorbed onto nanoparticles</td>
<td>3.4.6</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- Encapsulated in nanoparticles</td>
<td>3.4.6</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel electrophoresis of PTEN siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Encapsulated in nanoparticles</td>
<td>3.4.7</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>- Associated with nanocomplexes</td>
<td>3.4.8</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

✓ = Experiment performed
Chapter 3. Preparation and characterisation of siRNA loaded nanoparticles

3.4. Results and discussion

3.4.1. Particle size analysis of nanoparticles with encapsulated siRNA

a. HMC nanoparticles

The HMC nanoparticles produced had a mean size less than 250 nm for all formulations with siRNA in three different chitosan to siRNA ratios; 10:1 30:1 and 50:1 w/w (Figure 3-2). These particles were of narrow size distribution with a PDI less than 0.3. No significant difference was observed between chitosan:siRNA ratios for all the formulations tested (p>0.05) except HMC:siRNA and HMC-PEG600:siRNA where nanoparticles of ratio 10:1 had significantly larger mean hydrodynamic diameter (p<0.05) than 30:1 and 50:1. At 10:1, siRNA is present in much greater amounts than at 50:1 resulting in siRNA linkage and hence bridging inbetween particles. Secondly, high amount of siRNA also reduced positive surface charge resulting in particle aggregation.

It was also observed that at 10:1; HMC:siRNA and HMC-PEG600:siRNA had significantly larger mean hydrodynamic diameter than HMC-PEG1000:siRNA and HMC-PEG5000:siRNA formulations (p<0.05).

b. LMC nanoparticles

Inclusion of siRNA within LMC nanoparticle formulations in various ratios did not produce any difference (p>0.05) in mean nanoparticle size (Figure 3-3). The hydrodynamic diameter was in the range of 135 to 164 nm in all cases. The PDI (<0.3) indicated a relatively narrow size...
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distribution of the nanoparticles. The largest size was observed with all formulations with the chitosan:siRNA ratio of 10:1, although the difference was not significant (p>0.05). A similar study demonstrated that crosslinked nanoparticles with a chitosan (20 kDa) to siRNA weight ratio of 10:1 showed a larger particle size than other higher ratios (Rojanarata et al. 2008). Comparing hydrodynamic diameter of HMC nanoparticle with LMC nanoparticles it was observed that particle size decreases with the decrease in chitosan molecular weight (Wu et al. 2005).

![Graph showing mean hydrodynamic diameter of nanoparticles](image)

Figure 3-3. Hydrodynamic diameter of LMC:siRNA nanoparticles (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).

**c. LMC-g-PEG5000 nanoparticles**

The size of LMC-g-PEG5000 nanoparticles encapsulating siRNA is summarised in Table 3-2.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>LMC-g-PEG5000:siRNA (w/w)</th>
<th>10:1 Hydrodynamic diameter (nm±S.D) PDI±S.D</th>
<th>30:1 Hydrodynamic diameter (nm±S.D) PDI±S.D</th>
<th>50:1 Hydrodynamic diameter (nm±S.D) PDI±S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC-g-PEG5000</td>
<td></td>
<td>198 ± 4 0.26 ± 0.01</td>
<td>193 ± 3 0.23 ± 0.01</td>
<td>165 ± 7 0.22 ± 0.03</td>
</tr>
</tbody>
</table>

The mean hydrodynamic diameter ranged from 165 to 198 nm for all nanoparticles prepared in chitosan to siRNA (w/w) ratios of 10:1, 30:1 and 50:1. Particle size reduced with increased ratios, with 10:1 and 50:1 being significantly different (p<0.05). The PDI measured was...
approximately 0.2 in all cases. We hypothesize that decrease in siRNA amount results in more positive surface charge resulting in particle stability within media and further reducing the possibility of aggregation within the system.

### 3.4.2. Surface charge of nanoparticles with encapsulated siRNA

**a. HMC nanoparticles**

The mean surface charge for all formulations at all three chitosan:silRNA ratios was between +14 to +26 mV (Figure 3-4). This compares to a report, where the surface charge of crosslinked chitosan:silRNA nanoparticles was determined as approximately +30 mV (Katas and Alpar 2006). As the chitosan:silRNA ratio decreased the nanoparticle positive surface charge decreased indicating the influence of negatively charged phosphate ions in silRNA reducing polymer surface charge. The effect was not significant except for chitosan-PEG5000:silRNA nanoparticles, where the charge of 10:1 nanoparticles was significantly lower (p<0.05) than 50:1. These results were in agreement with a previous study (Rojanarata et al. 2008). The surface charge of silRNA loaded nanoparticles was lower than un-loaded nanoparticle which ranged from +24 to +28 mV (Table 2-2). The surface charge of all silRNA encapsulated nanoparticle formulations in the ratio of 10:1 (w/w) except HMC-PEG1000, was significantly different (p<0.05) to corresponding un-loaded nanoparticles. This was due to the influence of negatively charged silRNA over positively charged chitosan.

![Figure 3-4. Surface charge of HMC:silRNA nanoparticles (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).](image-url)
Chapter 3. Preparation and characterisation of siRNA loaded nanoparticles

b. **LMC nanoparticles**

The surface charge of siRNA loaded nanoparticles ranged from +35 to +41 mV (Figure 3-5). These values were not significantly different ($p<0.05$) when compared to unloaded nanoparticles ranging from +37 to +39 mV (Table 2-3). When comparing formulations for their siRNA loading it was found that in general as the siRNA loading decreased, a higher surface charge was determined. Significant differences ($p<0.05$) in surface charge were found for LMC-PEG600:siRNA and LMC-PEG5000:siRNA at ratios of 10:1 and 50:1.

![Figure 3-5. Surface charge of LMC:siRNA nanoparticles (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).](image)

c. **LMC-g-PEG nanoparticles**

The surface charge of LMC-g-PEG nanoparticle formulation associated with siRNA is shown in Table 3-3. The surface charge ranged from +28 to +31 mV for all nanoparticles prepared with chitosan to siRNA w/w ratio of 10:1, 30:1 and 50:1. This increase in surface charge was directly related to an increase in chitosan:siRNA ratio. Thus indicating an increase in surface charge with a decrease in siRNA amounts used. No statistical difference ($p>0.05$) was observed in surface charge at different w/w ratios. Surface charge of non-siRNA loaded LMC-g-PEG5000 nanoparticles was +31 mV (Section 2.4.3.c) which was similar to LMC-g-PEG5000:siRNA at w/w ratio of 50:1.
Table 3-3. Surface charge of PEG grafted LMC:siRNA nanoparticles in w/w ratio 10:1, 30:1 and 50:1 (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>10:1 Zeta potential (mV±S.D.)</th>
<th>30:1 Zeta potential (mV±S.D.)</th>
<th>50:1 Zeta potential (mV±S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC-g-PEG5000</td>
<td>+28 ± 3</td>
<td>+29 ± 1</td>
<td>+31 ± 4</td>
</tr>
</tbody>
</table>

3.4.3. Hydrodynamic diameter of siRNA associated nanocomplexes

a. HMC nanocomplexes

The mean particle size for HMC:siRNA complexes prepared using the complexation technique, without the use of TPP, was less than 285 nm (Figure 3-6). In a study chitosan glutamate complexes of size less than 300 nm with siRNA were produced (Katas and Alpar 2006). Similar to these results, a study showed decrease in the size of chitosan glutamate:siRNA complexes with increasing weight ratio from 16:1 to 32:1 (w/w) (Techaarpornkul et al. 2010). It was observed that in presence of the higher amount of siRNA, the complexes were larger (significantly different; p<0.05), with greatest mean hydrodynamic diameter at a ratio of 10:1 w/w. The PDI was also higher ranging between 0.4 and 0.5 indicating a more polydispersed nanoparticle population. Comparing hydrodynamic diameters of siRNA nanoparticles (Figure 3-2) with siRNA complexes, it was observed that the PDI was of broader size distribution with siRNA nanocomplexes. Secondly, the hydrodynamic diameter of nanoparticles at different chitosan to siRNA ratios (10:1, 30:1 and 50:1 w/w) for the same formulation was of similar values in comparison to corresponding nanocomplex formulations.

Figure 3-6. Hydrodynamic diameter of HMC:siRNA nanocomplexes (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).
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b. LMC nanocomplexes

The mean hydrodynamic diameter for siRNA complexes unlike for siRNA loaded nanoparticles showed a large variation ranging from 78 to 218 nm (Figure 3-7). No significant difference ($p>0.05$) was observed in the hydrodynamic diameter of LMC and LMC-PEG5000 nanocomplexes at different w/w ratios. LMC-PEG1000:siRNA at a ratio of 10:1 (w/w) was significantly larger ($p<0.05$) compared to 50:1 w/w. The PDI ranged between 0.2 to 0.4.

In a study, chitosan (50 - 150 kDa) siRNA complexes (12:1 w/w) were prepared with a hydrodynamic diameter of 117.5 nm ($\pm 4.9$) which is in good agreement with the LMC:siRNA nanocomplexes produced here (Noh et al. 2010).

![Figure 3-7. Hydrodynamic diameter of LMC:siRNA nanocomplexes (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).](image)

Figure 3-7. Hydrodynamic diameter of LMC:siRNA nanocomplexes (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).

c. LMC-g-PEG5000 nanocomplexes

LMC-g-PEG5000 nanocomplexes showed a particle size in the range of 177 to 219 nm Table 3-4. LMC to siRNA in w/w ratio of 10:1 and 50:1 showed significant difference ($p<0.05$) in size from each other. The PDI measured was approximately 0.3 in all cases. LMC-g-PEG5000 nanocomplexes were not significantly different ($p>0.05$) from LMC-g-PEG5000 nanoparticles at same chitosan to siRNA ratios.
Table 3-4. Hydrodynamic diameter of PEG grafted LMC:siRNA nanocomplexes in w/w ratio 10:1, 30:1 and 50:1 (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>10:1 Hydrodynamic diameter (nm±S.D)</th>
<th>30:1 Hydrodynamic diameter (nm±S.D)</th>
<th>50:1 Hydrodynamic diameter (nm±S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC-g-PEG5000</td>
<td>219 ± 8</td>
<td>202 ± 5</td>
<td>177 ± 6</td>
</tr>
<tr>
<td></td>
<td>0.24 ± 0.02</td>
<td>0.35 ± 0.15</td>
<td>0.25 ± 0.07</td>
</tr>
</tbody>
</table>

3.4.4. Surface charge of siRNA associated nanocomplexes

a. HMC nanocomplexes

The surface charge of complexes was higher than that of corresponding nanoparticles, and increased to approximately +59 mV as the chitosan to siRNA w/w ratio increased (Figure 3-8). Similar results were also observed with chitosan (114 kDa):siRNA complexes with increasing N:P ratios (6:1 to 71:1) where the surface charge increased from +18.8 to +26.8 mV reflecting high positive charge due to chitosan at higher N:P ratios (Howard et al. 2006).

![Figure 3-8. Surface charge of HMC:siRNA nanocomplexes (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).](image-url)
Chapter 3. Preparation and characterisation of siRNA loaded nanoparticles

b. LMC nanocomplexes

110:1
130:1
150:1

LMC:siRNA
LMC-PEG1000:siRNA
Chitosan:siRNA (w/w)
LMC-PEG5000:siRNA

Figure 3-9. Surface charge of LMC:siRNA nanocomplexes (with and without PEG) in ratio 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).

The surface charge of formulations was in the range of +33 to +53 mV (Figure 3-9). These formulations showed a narrow charge distribution with significant differences (p<0.05) in surface charge in between chitosan to siRNA w/w ratio of 30:1 and 50:1 for LMC-PEG1000:siRNA and 10:1 and 50:1 for LMC-PEG5000:siRNA nanocomplexes. The absence of polyanion; TPP in these nanocomplexes caused an increase in their surface charge from comparable nanoparticles.

c. LMC-g-PEG5000 nanocomplexes

The results of LMC-g-PEG nanocomplexes showed a slight increase in surface charge from +32 to +35 mV as the amount of siRNA decreased (p>0.05) (Table 3-5). Similar results were observed where siRNA was loaded to PEGylated chitosan (50 k - 150 kDa) conjugated poly-l-arginine (PLR) and resulted in a surface charge of +23.9 mV (±3.6) (Noh et al. 2010).

Table 3-5. Surface charge of PEG grafted LMC:siRNA nanocomplexes in w/w ratio 10:1, 30:1 and 50:1 (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>LMC-g-PEG5000:siRNA (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:1</td>
</tr>
<tr>
<td></td>
<td>Zeta potential (mV±S.D.)</td>
</tr>
<tr>
<td>LMC-g-PEG5000</td>
<td>+32 ± 3</td>
</tr>
</tbody>
</table>
3.4.5. Encapsulation efficiency of nanoparticles

A calibration curve of siRNA concentration against siRNA intensity using the PicoGreen assay was plotted (Figure 3-10). The relationship was linear from 0 to 1000 ng/ml, and a calibration curve equation was derived ($y=0.4381x - 1.313$) with $R^2 > 0.999$.

![Calibration curve of naked siRNA using the PicoGreen assay.](image)

Figure 3-10. Calibration curve of naked siRNA using the PicoGreen assay.

a. HMC nanoparticles

An encapsulation efficiency of greater than 90% was achieved for almost all nanoparticle formulations (Figure 3-11).

![siRNA loading efficiency of HMC:siRNA nanoparticles (with and without PEG) at ratio of 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).](image)

Figure 3-11. siRNA loading efficiency of HMC:siRNA nanoparticles (with and without PEG) at ratio of 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).

This compares well with findings by another group where HMC:pDNA 20% w/w ratio resulted in a 100% encapsulation efficiency (Csaba et al. 2009). The high loading efficiency may be due...
to the favourable interaction between cationic amine of HMC and the anionic phosphate group of siRNA. Previous studies have also established HMC to be suitable for siRNA delivery since it can bring in the chain entanglement effect for effective siRNA binding (Rojanarata et al. 2008).

HMC nanoparticles without PEG showed highest encapsulation efficiency, although Kruskal-Wallis statistics showed that the values were not significantly different ($p>0.05$) from other formulations at 10:1 w/w ratio. As the ratio increased to 30:1 and 50:1 (i.e the amount of siRNA reduced), there was a small decrease in loading efficiency among all four formulations with a significant difference ($p<0.05$) in PEG1000 associated HMC nanoparticles.

The loading efficiency of a biopharmaceutical compound in chitosan nanoparticles is dependent on several parameters such as molecular weight of chitosan, the nature of the biopharmaceutical compound, stirring speed, formulation composition, concentration and volume ratio of TPP and type of chitosan used (Kang et al. 2006). In conclusion, the siRNA load does not contribute significantly to the difference in loading efficiency within all four formulations at a ratio of 10:1, although greater variation was seen at 30:1 and 50:1.

b. LMC nanoparticles

An encapsulation efficiency of $>90\%$ was achieved for all LMC:siRNA nanoparticles (Figure 3-12). When comparing siRNA loaded nanoparticle formulation in w/w ratios of 10:1, 30:1 and 50:1, no significant difference ($p>0.05$) in loading efficiency was observed. All four formulations (with and without PEG) when compared together showed no significant difference ($p>0.05$) except LMC and LMC-PEG1000 nanoparticle formulation which showed difference in loading efficiency at a ratio of 30:1 w/w. Dehouss and co-workers, prepared chitosan (50 k – 150 kDa) nanoparticles using TPP as an ionic crosslinker in w/w ratio of 6:1. These chitosan particles encapsulating siRNA at an N:P ratio of 30:1, also showed an encapsulation efficiency of $>95\%$ (Dehouss et al. 2010). PLGA as a carrier is not efficient in complexation with siRNA resulting into a complexation efficiency of 4.3% since both siRNA and PLGA are negatively charged (Yuan et al. 2010). Modifying PLGA by addition of chitosan resulted in a loading efficiency up to 77.7%. 

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Figure 3-12. siRNA loading efficiency of LMC:siRNA nanoparticles (with and without PEG) at ratio of 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3).

c. LMC-g-PEG5000 nanoparticles

The mean encapsulation efficiency achieved for LMC-g-PEG5000 nanoparticles was 57% (Figure 3-13). However, the encapsulation efficiency was comparable for all chitosan:siRNA ratios. This indicates partial encapsulation of siRNA within LMC-g-PEG nanoparticles. The lower loading efficiency (%) of LMC-g-PEG nanoparticles in comparison to LMC nanoparticles might be due to PEG resisting encapsulation as a result of a steric mechanism (Ohya et al. 2000). Insulin loaded PEG (750 Da) grafted chitosan (6 kDa) nanoparticles were reported to have a loading efficiency of 23.4 (±1.9) % (Zhang et al. 2008b).

Figure 3-13. siRNA loading efficiency of LMC-g-PEG5000:siRNA nanoparticles at ratio of 10:1, 30:1 and 50:1 w/w (mean ± S.D., n=3)
3.4.6. Evaluation of the negative control Stealth siRNA association with nanoparticles

Gel electrophoresis was performed to assess the association (adsorption and encapsulation) of siRNA with nanoparticles (Figure 3-14). Lanes 1 and 7 show that naked siRNA migrated to the positive electrode under the applied electric field. A clear bright uniform band was visualised. The band is due to ethidium bromide which is an intercalating agent used as a fluorescent dye for nucleic acids. Upon siRNA interaction with chitosan the positively charged nanoparticles retarded the movement of negatively charged siRNA towards the positive electrode. All siRNA was retained within the well for all HMC and HMC-PEG nanoparticles investigated, confirming the successful and complete association of siRNA with non-PEG and PEG containing chitosan nanoparticles. It is seen that siRNA, when either adsorbed to or encapsulated within the nanoparticles, showed no difference in association. Lane 6 confirms that TPP did not show any interaction with siRNA, since siRNA migrated the same distance as the siRNA control. Lane 2 shows spots of siRNA when adsorbed onto nanoparticles. This could be due to dissociation of double stranded siRNA which was unable to show any band in the presence of ethidium bromide. This was not observed in siRNA encapsulation, and was also not evident in the presence of PEG.

The potential advantages of nucleic acid encapsulation within chitosan nanoparticles compared to adsorption are; a) protection from degradation; b) overcoming internal and external barriers by modifying the surface of the particles without affecting the loading efficiency; and c) controlled release (Csaba et al. 2009). The nanoparticle encapsulated siRNA
band is similar to siRNA control, being continuous, clear and bright. This implies that siRNA is structurally intact.

### 3.4.7. Evaluation of PTEN siRNA encapsulated nanoparticles

This experiment was performed to determine whether this carrier system could be used for a specific sequence siRNA (PTEN) for later gene silencing work.

#### a. HMC nanoparticles

Lanes 1 show that naked siRNA migrated to the positive electrode under the applied electric field (Figure 3-15). All formulations at a chitosan to siRNA ratio of 10:1 w/w (lane 2, 5, 8 and 11) showed migration of siRNA from the wells to the same extent as siRNA control (Lane 1) suggesting the presence of free siRNA within the formulations. siRNA gave a very slight, trailing band and complete siRNA binding as the w/w ratio varied from 30:1 to 50:1 suggesting effective affinity of siRNA for the nanoparticles. This difference in siRNA association at high and low ratios is due to the presence of large amount of siRNA in formulations with chitosan to siRNA ratio of 10:1, with less for a ratio of 50:1.

![Figure 3-15. Gel electrophoresis of siRNA and siRNA encapsulated nanoparticles in HMC to siRNA weight ratio of 10:1, 30:1 and 50:1. Lane 1 and 14: Naked siRNA (Control); Lane 2,3 and 4: HMC:siRNA (10:1), (30:1) and (50:1); Lane 5, 6 and 7: HMC-PEG600:siRNA (10:1), (30:1) and (50:1); Lane 8, 9 and 10: HMC-PEG1000:siRNA (10:1), (30:1) and (50:1); Lane 11, 12 and 13: HMC-PEG5000:siRNA (10:1), (30:1) and (50:1).](image)

Nanoparticle formulations of same chitosan to siRNA ratio showed similar siRNA association and hence similar siRNA migration behaviour or retardation. The bright band of siRNA is an indication that the siRNA nanoparticle preparation was successful, maintaining the integrity of siRNA during the stage of encapsulation.
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b. LMC nanoparticles

Lane 1 showed siRNA control, as negatively charged free siRNA migrated towards a positive electrode (Figure 3-16). At a chitosan:siRNA weight ratio of 10:1, siRNA is present in high amount with respect to formulation and is unable to be retained completely encapsulated within the nanoparticles in the well, and hence showed movement to the same level as the control siRNA (lane 1).

At a ratio of 30:1, siRNA stayed almost completely within the well with a slight ‘smear’ away from the well. At the highest ratio of 50:1 complete siRNA binding was observed, indicating higher potential of nanoparticles to retain siRNA within the well. All formulations at these polymer:siRNA ratio (with or without PEG) showed a similar extent of interaction. This suggests that as the amount of siRNA is reduced the nanoparticle binding affinity improves. PEG does not have any effect on the extent of nanoparticle siRNA interaction.

Poor retention of siRNA within crosslinked chitosan (20 kDa):siRNA nanoparticles at a weight ratio of 80:1 has been described in the literature (Rojanarata et al. 2008). This suggests that siRNA:chitosan affinity depends not only on weight ratio of polymer: siRNA used but also on the salt form, degree of deacetylation and molecular weight of chitosan used.

Figure 3-16. Gel electrophoresis of siRNA and siRNA encapsulated nanoparticles in LMC to siRNA weight ratio of 10:1, 30:1 and 50:1. Lane 1: Naked siRNA (Control); Lane 2,3 and 4: LMC:siRNA (10:1), (30:1) and (50:1); Lane 5, 6 and 7: LMC-PEG600:siRNA (10:1), (30:1) and (50:1); Lane 8, 9 and 10: LMC-PEG1000:siRNA (10:1), (30:1) and (50:1); Lane 11, 12 and 13: LMC-PEG5000:siRNA (10:1), (30:1) and (50:1).
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c. LMC-g-PEG5000 nanoparticles

Naked siRNA in free form was loaded in lane 1 and showed movement towards the positive electrode without any retardation (Figure 3-17).

[Figure 3-17: Gel electrophoresis of siRNA and siRNA encapsulated nanoparticles in PEG grafted LMC to siRNA weight ratio of 10:1, 30:1, 50:1 and 80:1. Lane 1: Naked siRNA (Control); Lane 2, 3, 4 and 5: LMC-g-PEG5000:siRNA nanoparticles (10:1, 30:1, 50:1 and 80:1).]

Lanes 2, 3, 4 and 5 show PEGylated LMC nanoparticle with encapsulated siRNA, in chitosan to siRNA weight ratios of 10:1, 30:1, 50:1 and 80:1. In order to investigate complete binding of siRNA to LMC-g-PEG5000 nanoparticles a ratio of 80:1 was also tested. The bands in lane 2 moved to the same extent as the naked siRNA in lane 1 indicating no association of LMC-g-PEG5000 nanoparticles with siRNA (10:1 w/w). As the chitosan to siRNA ratio increased, siRNA showed less migration towards the positive electrode. At a ratio of 80:1 (w/w) a very small amount of siRNA movement was observed. The streak of siRNA movement in lane 3, 4 and 5 is an indication that prolonged release of siRNA was obtained with the use of this polymeric complex. In a study good binding affinity of siGLO (fluorescently labelled control siRNA) towards PEGylated (5000)-LMC nanoparticles was demonstrated in the chitosan:siRNA ratio of 200:1 (w/w) (Malhotra et al. 2011).

The results shown in Section 3.4.5.c showed some loading of siRNA in PEGylated chitosan nanoparticles. The positive (amine) and negative (phosphate) interaction between chitosan in LMC-g-PEG5000 formulation and siRNA respectively was not however strong enough for siRNA to be retained within the well. One of the reasons could be the steric hindrance of the association of PEG with the chitosan which limits interaction with siRNA in comparison to LMC without PEG. This suggests that LMC-g-PEG5000 nanoparticles are successful in encapsulating siRNA and may be appropriate to release siRNA upon administration to the target site.
In comparison to LMC nanoparticles, LMC-g-PEG5000 nanoparticles behave very differently in terms of the interaction with siRNA, as seen through loading efficiency and gel electrophoresis experiments. This indicates a change in the physicochemical characteristics of the polymer upon PEG conjugation.

3.4.8. Gel electrophoresis of PTEN siRNA nanocomplexes

a. HMC nanocomplexes

The complexation of siRNA with HMC was examined using agarose gel electrophoresis (Figure 3-18). siRNA nanocomplexes showed similar siRNA association as for HMC nanoparticles (Figure 3-15). Chitosan glutamate (20 kDa)/siRNA complexes were previously characterised using 2% agarose gel (Techaarpornkul et al. 2010). A smear band at a w/w ratio of 8:1 was observed, whereas a completely retarded bright band remained in the well at w/w ratio of 32:1. The inclusion of PEG did not alter the siRNA-chitosan interaction as similar band movements were observed with both PEG (different molecular weights) and non-PEG nanocomplexes. In another study it was observed that the interaction between siRNA and chitosan by simple mixing was weak (Rojanarata et al. 2008). For a carrier, it is important to provide siRNA stability and protection for effective delivery, but it might be possible that the carrier restricts the release of siRNA and results in low gene knockdown. Hence a balance needs to be established between protection and release of siRNA which will be studied in future gene silencing experiments.

Figure 3-18. Gel electrophoresis of siRNA and siRNA nanocomplexes in HMC to siRNA weight ratio of 10:1, 30:1 and 50:1. Lane 1: Naked siRNA (Control); Lane 2, 3 and 4: HMC:siRNA (10:1), (30:1) and (50:1); Lane 5, 6 and 7: HMC-PEG1000:siRNA (10:1), (30:1) and (50:1); Lane 8, 9 and 10: HMC-PEG5000:siRNA (10:1), (30:1) and (50:1).
Chapter 3. Preparation and characterisation of siRNA loaded nanoparticles

b. LMC nanocomplexes

In contrast to LMC nanoparticles; LMC:siRNA complexes showed slight movement of siRNA at higher chitosan:siRNA ratios (50:1) w/w (Figure 3-19). From these results it is clear that siRNA addition during ionic gelation and formulation of nanoparticles is better shielded and results in good interaction both in and around the nanoparticle when compared to siRNA complexes.

![Figure 3-19. Gel electrophoresis of siRNA and siRNA nanocomplexes in LMC to siRNA weight ratio of 10:1, 30:1 and 50:1. Lane 1: Naked siRNA (Control); Lane 2, 3 and 4: LMC:siRNA (10:1), (30:1) and (50:1); Lane 5, 6 and 7: LMC-PEG1000:siRNA (10:1), (30:1) and (50:1); Lane 8, 9 and 10: LMC-PEG5000:siRNA (10:1), (30:1) and (50:1).](image)

In a study it was showed that siRNA was quickly displaced from chitosan (10 kDa)/siRNA complexes at N:P ratios of 50:1 upon addition of poly-l-aspartic acid via the polyanion displacement method (Liu et al. 2007). This highlights the weak interaction of chitosan:siRNA complexes in comparison to chitosan:siRNA nanoparticles. High molecular weight and low molecular weight chitosan/siRNA complexes (with and without PEG) showed a slight difference in their association with siRNA. These results were similar to the observations made by (Techaarpornkul et al. 2010) where HMC (glutamate) bound siRNA slightly better than LMC.

c. LMC-g-PEG5000 nanocomplexes

Naked siRNA in free form in lane 1 moved towards positive electrode without any retardation (Figure 3-20). Lanes 2, 3, 4 and 5 show LMC-g-PEG5000 complexed with siRNA in the ratio of 10:1, 30:1, 50:1 and 80:1 respectively. The gel showed bands in lane 2 moving to the same extent as the naked siRNA in lane 1, indicating no association of siRNA with the LMC-g-PEG5000. As the weight ratios increased, siRNA showed slight retardation of movement. At a weight ratio of 80:1 a very small amount of siRNA movement was observed. siRNA interaction with LMC-g-PEG5000 nanoparticles showed similar results (Figure 3-17).
3.4.9. Evaluation of siRNA stability in model propellant

In a separate experiment, gel electrophoresis was conducted to determine the stability of siRNA following exposure to the model propellant, DFP (Figure 3-21). The siRNA band (Lane 2) moved out of the well, in a similar manner to the siRNA control (Lane 1). They both were of similar band strength indicating similar siRNA stability before and after dispersion within the propellant. The use of DFP propellant instead of HFA was preferred due to low vapour pressure of DFP which allows it to slowly evaporate at room temperature allowing time for siRNA incubation with propellant. This demonstrates siRNA stability and potential for lung delivery following dispersion in a non-polar media. It has been previously confirmed that propellant does not affect the biological activity of biopharmaceuticals (plasmid DNA) (Conti et al. 2011).
3.5. Conclusion

The electrostatic interaction between polymer nitrogen and siRNA phosphate help in the formation of compact siRNA associated nanocomplexes or nanoparticles (using crosslinking agent). These formulations were of mean size less than 300 nm. siRNA loaded systems showed decrease in surface charge as the siRNA amount increased. HMC nanoparticles were bigger in size but less positively charged when compared to LMC nanoparticles. Nanocomplexes showed similar size and charge as nanoparticles of the same composition but with larger differences in chitosan:siRNA w/w ratios of the same formulation. Loading efficiency was observed to be >80% for HMC and LMC nanoparticles but with LMC-g-PEG the loading efficiency was much lower. The successful electrostatic interaction of siRNA with positively charged chitosan and chitosan-PEG nanoparticles was confirmed through gel electrophoresis. siRNA when adsorbed or encapsulated showed no difference in association with chitosan or chitosan-PEG nanoparticle as determined by gel electrophoresis. The migration behaviour or retardation of siRNA encapsulated in nanoparticle formulations (with and without PEG) showed the same association at same chitosan:siRNA ratios. From here on chitosan nanoparticles will be prepared instead of chitosan nanocomplexes for future studies, where the siRNA will be encapsulated instead of adsorbed or associated. Due to different chain length and derivatives of HMC (glutamate) and LMC, slight differences in siRNA interactions were observed with both types of chitosan. LMC-g-PEG5000 was unable to effectively and completely overcome the strong forces of attraction on siRNA by the positive electrode in gel electrophoresis. siRNA was observed to be stable in non-polar media hence indicating the potential for delivery by a pMDI.
CHAPTER 4

AEROSOLISATION OF NANOPARTICLES
4. Aerosolisation of nanoparticles

4.1. Introduction

Challenges in aerosol delivery of biopharmaceuticals include forming stable dispersions in HFA propellants or aqueous media for uniform dosage using pMDIs or nebulisers respectively and protecting labile biopharmaceuticals such as DNA, proteins and siRNA from degradation during aerosolisation (Gautam et al. 2003). Nanocarriers can be employed to protect and stabilise such biopharmaceuticals during preparation, storage, delivery and cellular uptake (Woodle and Lu 2005; Lavertu et al. 2006; Nyambura et al. 2009b). Various types of nanocarriers and their properties have been outlined in Chapter 1.

Previously published studies have investigated nanocarriers for biopharmaceutical delivery using nebulisers. Analysis of the stability and functionality of siRNA after jet nebulisation of siRNA complexed with non-viral vectors; PEI and oligofectamine showed that complexed siRNA, unlike naked siRNA was not damaged by the nebulisation process, and was still able to down regulate green fluorescent protein expression (Huth et al. 2006). In another study, chitosan (126 kDa)-DNA-FAP-B (fibronectin attachment protein of mycobacterium bovis) nanoparticles proved good candidates for gene delivery to mice lung epithelial cells (Mohammadi et al. 2011). Jet nebulisation did not produce changes to the physicochemical properties of the nanoparticles and high gene expression was achieved.

Biological activity of biopharmaceuticals delivered from pMDIs has also been investigated. The biological activity (>98%) of Lysozyme was retained in nanoparticles even after the nanoparticles were dispersed in HFA-134a based pMDI (Nyambura et al. 2009b). Likewise, freeze-dried microemulsions complexed with pDNA and aerosolised from pMDI maintained pDNA biological functionality (Bains et al. 2010). Recent studies have demonstrated the use of chitosan as gene carriers using pMDI technology (Conti et al. 2011). Chitosan (31 kDa)-DNA nanoparticles prepared as dispersions in HFA-227 propellant showed good physical stability and excellent aerosol performance (FPF of up to 63%) (Conti et al. 2011). In-vitro studies showed that the chitosan-DNA nanoparticles were able to transfect A-549 cells, even after several weeks of storage in the HFA-227, suggesting that the DNA maintained its biological functionality. These results suggest that formulating carriers with HFA propellants is an appropriate approach to deliver genes to the lungs.
The present study has reported the use of chitosan nanoparticles as a potential carrier for siRNA to the lungs. Since chitosan is hydrophillic and it is to be suspended in a hydrophobic environment (HFA); there is a need of an HFAphilic moiety to stabilise the nanoparticle dispersion in HFA. PEG is a polymer with appreciable solubility in HFA due to the presence of ether groups in its chemical structure (Selvam et al. 2006). It has previously been explored for dispersion stability in HFAs (Traini et al. 2006). Hence this work also explored the effect of different grades of PEG in improving dispersion and aerosolisation of nanoparticles for delivery from nebulisers and pMDIs. In addition to patient’s pulmonary morphology, inspiratory flow rate and breathing pattern, other factors that govern deep lung deposition are aerosol characteristics such as particle size, size distribution, shape, charge and density (Pilcer and Amighi 2010). The characterisation of particle deposition according to their aerodynamic size was performed in this study using the NGI (De Boer et al. 2002). This is an in-vitro instrument used to assess the likely performance of an inhalation product in-vivo.

To quantify the amount of chitosan delivered to different regions of the NGI, chitosan was conjugated with a fluorescent marker (FITC). Previous studies have reported the preparation of stable FITC conjugated chitosan (hydrochloride; 180 kDa) nanoparticles (Huang et al. 2002). They confirmed the successful use of FITC to track the extent and mechanism of uptake of labelled chitosan nanoparticles in pulmonary epithelium cells (A-549). Successful grafting of FITC to chitosan glutamate (30 kDa) has been established and similar physicochemical properties of FITC-labelled and unlabelled chitosan glutamate nanoparticles were confirmed (Colonna et al. 2008). It has also been reported that FITC conjugated chitosan (800 kDa) nanoparticles were stable against photobleaching (Zhao and Wu 2006). The physical adsorption of a marker to a nanoparticle was not employed as it may dissociate from the nanoparticle (Suh et al. 1998).

### 4.2. Materials

Synthetic PTENV10-23-hmr with a molecular weight 7.4118 kDa and a duplex sequence (Sense: 5'-UAAGUUCUAGCUGUGGGUUA-3', antisense: 3'-AUUCAAGAUCGACACCACCAU-5' (specific) was donated by Integrated DNA technologies through AstraZeneca (UK). Fluorescein 5-isothiocyanate (FITC) with m.w 398.38 and ≥90% purity, glycerol (≥99% purity), tris acetate-ethylenediaminetetraacetic acid (TAE) buffer (10x), ethidium bromide, acetic acid, DMSO, methanol, agarose, acetone (HPLC grade), PEG 1000 and TPP 85% was bought from Sigma Aldrich (UK). High molecular weight chitosan (HMC); Protasan UP G 113, m.w 150 k - 200 kDa,
degree of deacetylation 75-90% was purchased from Novamatrix (Norway). Low molecular weight, water soluble chitosan (LMC) with m.w >10 kDa and degree of deacetylation 97.0% was bought from Kittolife Co. Ltd (Korea). Gel loading dye (Blue 6x) was purchased from Biolabs (UK). PEG 5000 monomethyl ether and brij 35 was obtained from Fluka (UK). PEG 600 and HPLC grade water were purchased from Fischer Scientific (UK). HFA-227 (≥99.9%) was obtained from Solvay Fluor (UK).

4.3. Methods

4.3.1. Preparation of FITC-labelled nanoparticles

Different techniques were employed in order to conjugate FITC to HMC and LMC. This is because the physicochemical interaction of chitosan with any compound is based on its composition, molecular weight and degree of deacetylation. Two different types of chitosan polymer were used to prepare nanoparticles. Glutamate substituted HMC with a molecular weight of 150 k to 200 kDa (degree of deacetylation; 75 to 90%) and LMC (unmodified) with molecular weight >10 kDa (degree of deacetylation; 97%).

The percentage of FITC, which is the amount of FITC within FITC-labelled chitosan, was determined by measuring the fluorescence intensity of FITC-labelled chitosan (pre-weighed) solution against standard solutions of FITC. Labelling efficiency (percent) was calculated as the percentage of FITC by weight of FITC-chitosan. All steps associated with FITC were carried out in the dark and under ambient temperature.

a. High molecular weight chitosan

Chitosan was stably labelled with FITC by conjugating the primary amine group of the chitosan with the isothiocyanate group of FITC (Huang et al. 2002; Colonna et al. 2008). 1% (w/v) of chitosan (16 ml) was dissolved in 0.1 M acetic acid, then an equal volume of methanol (16 ml) was added. To this, 2.5 ml of FITC in methanol (2 mg/ml) was added dropwise with constant stirring for 3 h. The solution was precipitated by the addition of 0.5 M sodium hydroxide solution (pH 10.0), and then centrifuged at 25,000 xg; using a Heraeus biofuge stratos centrifuge (Thermo Scientific, UK) for 10 min. The FITC-labelled chitosan precipitate was washed with methanol:water (70:30) mixture and centrifuged until no fluorescence was detected in the supernatant at excitation and emission maxima of 492 and 518 nm respectively, using a fluorescence spectrometer (LS 55, Perkin-Elmer, UK).
The precipitate was freeze-dried (Virtis Advantage, SP Scientific, USA) using the parameters detailed in Section 2.3.5, to obtain a dry powder. Chitosan conjugated FITC was used to prepare fluorescent nanoparticles as described in Section 2.3.3, and these particles were used in subsequent *in-vitro* aerosolisation studies.

**b. Low molecular weight chitosan**

(i) FITC conjugated to preformed nanoparticles

FITC-labelling was performed using an established method (Zhao and Wu 2006). 5 mg of freeze-dried crosslinked chitosan nanoparticles (Section 2.3.3) were dispersed in 50 ml DMSO followed by addition of 5 ml 0.1M sodium hydroxide. To this dispersion 10 mg/ml of FITC dissolved in DMSO was added under constant stirring for 10 h in the dark to label the nanoparticles. The labelled nanoparticles were centrifuged at 15,000 xg; (Heraeus biofuge stratos centrifuge; Thermo Scientific, UK) for 15 min and washed with DMSO until no fluorescence was detected in the supernatant. The fluorescent nanoparticles were examined on a fluorescence spectrometer (LS 55, Perkin-Elmer, UK) with an excitation wavelength of 492 nm and an emission wavelength of 518 nm. Finally, FITC-labelled nanoparticles were freeze-dried using the parameters described in Section 2.3.5, and stored in a desiccator at room temperature.

(ii) FITC conjugated to LMC and subsequent formation of nanoparticles

In order to quantify chitosan, a synthetic procedure was followed to covalently conjugate free amine groups of chitosan to the isothiocyanate group of FITC using a method modified from previously published studies (Chae et al. 2005; Zeng et al. 2008). 500 mg of chitosan was dissolved in DMSO:water in the ratio 90:1. Separately, 20 mg of FITC (1 mg/ml) was dissolved in DMSO and added slowly to the chitosan solution. The sample was left in the dark overnight (10 h) for the chemical reaction to complete at room temperature. The FITC-bound chitosan was precipitated in excess acetone (8 ml) and separated from free FITC and DMSO using a millipore filter paper. The precipitate collected was then freeze-dried (Virtis Advantage, SP Scientific, USA) as detailed in Section 2.3.5 to yield FITC-labelled chitosan. The FITC percentage was measured at maximum excitation 492 nm and λ maximum emission 518 nm using a fluorescence spectrometer (LS 55, Perkin-Elmer, UK). Labelled chitosan was used to prepare fluorescent crosslinked nanoparticles using the protocol described in Section 2.3.3.
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c. LMC-g-PEG5000 polymer

In order to produce FITC conjugated chitosan nanoparticles, a modified chemical synthesis procedure was followed (Chae et al. 2005; Zeng et al. 2008). 500 mg of LMC-g-PEG5000 was dissolved in DMSO/water (90:10). Separately, FITC (1 mg/ml) was dissolved in DMSO and added slowly to the LMC-g-PEG5000 solution. The sample was left in the dark overnight (10 h) for the chemical reaction to complete. Free FITC was removed using acetone (washed 8 times) and the precipitate was collected by filtration. The sample was then freeze-dried (Virtis Advantage, SP Scientific, USA; Section 2.3.5) to yield FITC-labelled LMC-g-PEG5000. FITC percentage was measured at $\lambda_{\text{max}}$ excitation 492 nm and $\lambda_{\text{max}}$ emission 518 nm using a fluorescence spectrometer (LS 55, Perkin-Elmer, UK). Nanoparticles of labelled polymer were prepared using the protocol described in Section 2.3.3.

4.3.2. Preparation of pMDI nanoparticle formulations

FITC-labelled nanoparticles (HMC; 7 mg, LMC; 4.8 mg) were transferred to aluminium canisters (donated by AstraZeneca, UK). These were crimped with metering valves (Valois, France) having a delivery volume of 50 $\mu$L per actuation using automatic pressure filling equipment (Pamasol P2011, Switzerland), followed by propellant (HFA-227) transfer (1 g) to the canister. The mixture was vortexed (Vortexer VWR Mini, USA) for 30 s and bath sonicated (Ultrawave U300H, UK) for 5 min at 20 °C. The process was repeated twice, and at each step additional propellant was added until the desired weight of 8.4 g was achieved. All pMDI canisters were vigorously shaken for 10 s; stored inverted for one month at ambient temperature and later, after insertion into a plastic actuator (supplied by AstraZeneca, UK) investigated for exactuator aerosol particle size distribution and FPF.

siRNA (20.27 pg/ml) encapsulated FITC-labelled nanoparticles in a chitosan to siRNA ratio of 10:1 (w/w) (Section 3.3.1.b.iii) were freeze-dried and loaded into pMDI canisters and dispersed within HFA-227 utilising the protocol detailed above.

4.3.3. SEM of nanoparticles following actuation of pMDIs

SEM was performed using the method detailed in Section 2.3.9. The morphology and integrity of nanoparticles, post-aerosolisation, was determined by actuating pMDI formulations of nanoparticles from a distance of approximately 15 cm onto a glass slide, which was cut into pieces, mounted on an SEM stub and visualised by SEM following sputter coating with gold.
4.3.4. Aerosol-particle size distribution

In order to determine the aerosol-particle size distribution, a laser diffraction particle size analyser Sympatec (GmbH System-Partikel-Technik, Clausthal-Zellerfeld, Germany) was employed (Figure 4-1).

![Figure 4-1. Aerosol dispersion of pMDIs for particle size analysis with the device, mounted in the measuring zone of the laser diffraction sensor HELOS (Sympatec).](image)

The pMDI was connected using a rubber gasket; a specially designed mouthpiece to prevent any loss of aerosol, into the central adapter (measuring zone). This central unit was connected to a vacuum pipe with a solenoid valve on the opposite end. A constant air pressure (~53 mBar) was maintained through a vacuum control unit which automatically regulates the inspiratory flow rate (60 L/min). The actuated aerosol crossed a laser beam at a 90 degree angle and the diffracted light was collected by an optical lens (R2; 0.45–87.5 μm size range). The vacuum pump was switched on at least 15 min prior to the first run and the Sympatec unit was set to differential pressure method (nebuliser / pMDI mode). The flow rate was attained (solenoid valve open) within the central adapter and a reference measurement was performed with an empty inhaler connected to the central adapter prior to the actual measurement, to calibrate the instrument. This was also essential for the instrument to achieve target values for the pressure. The flow rate was set to create a controlled suction on the device which mimics a human inhalation. An obscuration of 5% was achieved.

In operation, the pMDI canister was shaken for 10 s before placing within the rubber gasket, and actuation into the central adapter. The aerosol cloud, generated under a constant flow of air passes through the measuring zone and an aerosol size distribution was determined by
laser diffraction. The solenoid valve closes after the inhalation time (10 s) has elapsed. This was 
sufficient to draw the aerosol from the pMDI through the laser beam. A mean of four sets of 
data generated for four different devices of the same formulation were obtained and 
analysed. The first ten doses of each new pMDI were fired to waste. A time interval of 60 s was 
allowed between each actuated dose to prevent excessive cooling of the pMDI canister.

The particle size distribution was obtained in terms of volume median diameter (VMD, μm) 
which is a statistical measure of the cumulative particle diameter at which 50% of the particle 
volume is contributed by particles larger than the VMD and 50% by particles smaller than the 
VMD. Span is a representation of the width of the distribution of particle sizes within the 
sample. Span has no units and is calculated as the difference in particle diameters at 10% and 
90% of the cumulative volume, divided by the VMD (Elhissi et al. 2006). The technique does 
not take in to account the aerodynamic diameter of particles unlike impactor methodologies. 
WINDOX 5 software was used to set the parameters of the instrument and to process the data 
for the evaluation of aerosol particle size distribution.

4.3.5. Analysis of nebulised formulations using the TSI

Formulations were nebulised using a jet nebuliser into a twin stage impinger (TSI; Copley 
Scientific Ltd., Nottingham, UK) (Figure 4-2). The method followed was officially specified 
standard procedure (European Pharmacopoeia 2008).

Figure 4-2. Twin stage impinger (Mendes et al. 2007).
Nanoparticles suspended within 5 ml aqueous medium (water) were placed in the nebuliser reservoir (Pari LC Sprint nebuliser, Pari GmbH, Germany). Formulations comprised either HMC at a concentration of 0.7142 mg/ml or LMC/LMC-g-PEG5000 at a concentration of 0.8333 mg/ml. The nebuliser reservoir was attached to the TSI via a moulded rubber mouthpiece and aerosols were generated using a Turbo boy N compressor (Pari GmbH, Germany) (Zaru et al. 2007). The aerosolised nanoparticles passed into the TSI which contained deionised water in the upper stage (stage 1, 7 ml) and lower stage (stage 2, 30 ml). The flow rate was set at 60 L/min using a digital flowmeter (DFM 2000, Copley Scientific Ltd., UK) achieved using a vacuum pump (HCP 5, Copley Scientific Ltd., UK). The TSI was covered with aluminum foil to prevent the degradation of FITC by light. The pump was allowed to run for 10 s before starting the nebuliser and after stopping the nebuliser. In total the whole nebulisation process took 10 min; allowing the sample in the nebuliser reservoir to reach “dryness”. Towards the end of nebulisation the nebuliser was tapped to maximise fluid output. The aerosolised formulation was collected in stage 1 and 2 of TSI which was dismantled and the stages washed separately with deionised water (in the dark) and made up to the desired volume in a volumetric flask. In addition the TSI moulded rubber mouthpiece and the nebuliser reservoir (containing residual fluid) were also washed and collected in one flask. These samples were then analysed for their fluorescence (λmax excitation = 492 nm, λmax emission = 518 nm) using a fluorescence spectrometer (LS 55, Perkin-Elmer, UK) in a 10 mm quartz cuvette. Based on the fluorescence in each stage, the concentration of FITC was calculated by reference to a standard curve prepared from serial dilutions of a stock solution of FITC in water. This concentration value was used to back calculate the FITC original concentrations before subsequent dilutions were made. Finally, FITC concentration was converted to chitosan concentration using previously calculated FITC percentage. Each experiment was performed in triplicate.

The amount of chitosan added initially to the nebuliser reservoir was considered the nominal dose or original dose. The recovered dose (RD) is defined as the sum of the total amount of chitosan determined in the nebuliser reservoir, moulded rubber mouth piece, stage 1 and stage 2. Mass balance is the percentage of the recovered dose with respect to the nominal dose. In all cases a mass balance was within 75 to 125% for all formulations (European Pharmacopoeia 2008). The cut off diameter for stage 2 (lower stage) is 6.4 μm and the amount of chitosan detected in this stage is considered the fine particle dose (FPD). Fine particle fraction (FPF) is the percentage of chitosan present in the “respirable stage” (stage 2) of the impinger over the recovered dose (Li and Seville 2010).
4.3.6. Analysis of nebulised siRNA nanoparticles using the TSI

PTEN siRNA (81.9 µg/ml) encapsulated within LMC nanoparticles (FITC-LMC 833.3 µg/ml) in chitosan to siRNA ratio 10:1 w/w were nebulised (5 ml) into the TSI using the methodology described in Section 4.3.5. The liquid was collected from stage 2 and centrifuged at 17,000 xg for 30 min (Sigma 1-15PK, Osterode am Harz, Germany). The pellet was re-suspended in 4 ml of DNAse and RNAse free water and assessed for siRNA stability on 4% agarose gel dispersed within 1xTAE buffer (Section 3.3.5). The sample was run at 60 V for 45 min and visualised using a UV transilluminator image analyser (Syngene G-Box, UK).

4.3.7. Analysis of pMDI nanoparticle aerosols using the TSI

LMC-PEG1000 nanoparticles dispersed in HFA-227 within pMDIs (as described in Section 4.3.2) were vigorously shaken for 10 s before each actuation. Five shots were made to waste initially to ensure the consistency of emitted dose. Figure 4-3 shows a TSI-pMDI set up. A total of 20 actuations were made at a flow rate of 60 L/min with a 10 s run time and a 60 s interval between each actuation (Section 4.3.5). The aerosolised formulation was collected and washed with water from actuator, mouthpiece, stage 1 and stage 2. These samples were then tested for their fluorescence as described in Section 4.3.5.

![Figure 4-3. TSI attached to a pMDI.](image-url)
4.3.8. Analysis of pMDI: siRNA nanoparticle aerosols using the TSI and gel electrophoresis

siRNA encapsulated within LMC-PEG1000 nanoparticles dispersed in HFA-227 pMDIs (as described in Section 4.3.2) were tested at a flow rate of 60 L/min. Prior to this, five shots were made to waste. A total of 20 actuations were made into the TSI with 60 s interval between each actuation (Section 4.3.5). siRNA loaded LMC nanoparticles were collected from stage 2 of the TSI, and were recovered by centrifugation at 17,000 xg for 30 min (refrigerated centrifuge Sigma 1-15PK, Osterode am Harz, Germany). The supernatant was removed and the pellet was re-suspended in 0.5 ml DNAse and RNAse free water. The sample was examined using a 4% agarose gel dispersed within 1xTAE buffer (Section 3.3.5) at 60 V for 45 min and visualised using a UV transilluminator image analyser (Syngene G-Box, UK).

4.3.9. Analysis of pMDI: nanoparticle aerosols using the NGI

The aerosol performance of FITC-labelled nanoparticles and siRNA loaded nanoparticle pMDI formulations was determined using a NGI (Copley Scientific, UK; Figure 4-4), operated in accordance with the specifications for pMDIs described in the official guide (European Pharmacopoeia 2008).

Figure 4-4. pMDI attached to a NGI.

The collection plates of the NGI were uniformly coated with a coating solution. Coating of NGI plates is a standard procedure implemented to avoid particle bounce upon impaction on the NGI plates (Mitchell 2003). The coating material was prepared according to an AstraZeneca internal protocol. 3 g Brij 35 was weighed and dissolved in 20 ml water. 5 g of this solution was
mixed with 25 g glycerol to obtain the final coating solution. After spreading the coating material (sufficient to form a thin layer) on the NGI plates they were dried in a fume cupboard (Extract Technology, UK). Stage 8 comprised a micro-orifice filter, used to collect very fine particles. The NGI was operated at an air flow rate of 30 L/min for preset 10 s intervals following pMDI actuation, to allow particle deposition on the plates. The pMDI was vigorously shaken for 10 s and thereafter five shots were fired to waste. The pMDI was then actuated into the NGI, with 60 s intervals between each actuation. Twenty actuations were cumulatively collected on the coated plates for each pMDI canister. The collection plates, micro-orifice filter, throat and actuator were rinsed with water and washings collected and made up to volume. The amount of FITC-labelled chitosan nanoparticles collected from each stage was determined using a fluorescence spectrophotometer as described in Section 4.3.5. The parameters determined using NGI were recovered dose (RD) which is the total emitted dose (actuator to stage 8); fine particle dose (FPD)- the amount of drug deposited in stage 3 to stage 8 (Carvalho et al. 2011; Selvam et al. 2011). Fine particle fraction (FPF), which is the percentage of active deposited on stages 3 to 8 (cutoff diameter range: <6.4 μm) with respect to the recovered dose. MMAD and GSD were calculated through a web based application (Holt 2011). MMAD is the cut off particle size in which 50% of the mass of the aerosol is smaller and the other 50% is larger than the referred parameter (Jaafar-Maalej et al. 2009). The GSD of a lognormal distribution is determined by dividing the mass median particle diameter by the particle size at the 15.78 % probability or by dividing the particle size at the 84.13 % probability by the mass median particle diameter (Finlay et al. 1997).

4.3.10. Mean size and size distribution of nanoparticles post-aerosolisation

The nanoparticles collected in each stage of NGI and TSI were tested for size (hydrodynamic diameter and PDI) using the ZetasizerNano ZS (Malvern Instruments, UK) as described in Section 2.3.6. Each sample was tested in triplicate.

4.3.11. Statistical analysis

A non parametric Kruskal-Wallis test and Nemenyi post hoc test were used to perform the statistical analysis. All analysis was performed using the SPSS statistical program (version 19) and differences were considered to be significant at a level of $p<0.05$. 

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### 4.3.12. Summary of experiments conducted

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<td>✓</td>
</tr>
<tr>
<td>SEM of fluorescent nanoparticles</td>
<td>4.4.3</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Nebulisation of fluorescent nanoparticles into the TSI</td>
<td>4.4.4</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Nanoparticle size following nebulisation into the TSI</td>
<td>4.4.5</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Gel electrophoresis of siRNA following nebulisation</td>
<td>4.4.6</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Nebulisation of siRNA encapsulated nanoparticles into the TSI</td>
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<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>SEM of pMDI actuated particles</td>
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<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>pMDI actuated aerosol-particle size distribution</td>
<td>4.4.9</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>- Un-loaded nanoparticles</td>
<td>4.4.10</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>- siRNA encapsulated nanoparticles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>pMDI actuated nanoparticle aerosolisation performance using TSI</td>
<td>4.4.11</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Nanoparticle size following pMDI actuation into the TSI</td>
<td>4.4.12</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Gel electrophoresis of siRNA encapsulated within nanoparticle delivered using pMDI</td>
<td>4.4.13</td>
<td></td>
<td></td>
<td></td>
<td>PEG1000</td>
<td>✓</td>
</tr>
<tr>
<td>pMDI actuated nanoparticle aerosolisation performance using NGI</td>
<td>4.4.14</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Nanoparticle size following pMDI actuation into the NGI</td>
<td>4.4.15</td>
<td></td>
<td></td>
<td>PEG1000</td>
<td>PEG1000</td>
<td>✓</td>
</tr>
<tr>
<td>pMDI actuated siRNA encapsulated nanoparticles into the NGI</td>
<td>4.4.16</td>
<td></td>
<td></td>
<td>PEG1000</td>
<td>PEG1000</td>
<td>✓</td>
</tr>
</tbody>
</table>

✓ = Experiment performed
4.4. Results and discussion

4.4.1. Quantification of chitosan

Quantification of chitosan at low concentrations was important for aerosolisation studies; as aerosolised chitosan would deposit in small amounts on the eight stages of the NGI or the two stages of the TSI. Particles were collected, washed and diluted with water.

a. Percentage of FITC conjugated to HMC

Standard solutions of FITC, 12.5 to 200 ng/ml were produced. A calibration curve \( y = 1.2745x + 5.5744 \) was generated using fluorescence spectrometry with an \( R^2 \) value of 0.9987 (Figure 4-5). The FITC percentage was found to be 2.8% (w/w) and the labelling efficiency calculated to be 90%. The weight fraction of FITC per unit weight of chitosan (180 kDa) used by (Huang et al. 2002) and chitosan (300 kDa) used by (Colonna et al. 2008) was 2.7% (w/w). Another study demonstrated a percentage of 3.1% (w/w) for FITC when conjugated to chitosan (500 kDa) (Tallury et al. 2009).

\[
y = 1.2745x + 5.5744
\]

\( R^2 = 0.9987 \)

Figure 4-5. FITC calibration curve using fluorescence spectrometer to calculate percentage of FITC to HMC.

b. Percentage of FITC conjugated to LMC and LMC-g-PEG5000

Standard solutions of FITC, 12.5 to 200 ng/ml were produced. A calibration curve \( y = 1.292x + 11.131 \) was generated using fluorescence spectrometry with a \( R^2 \) value of 0.995 (Figure 4-6). The FITC percentage was found to be 3.51% (w/w) with a labelling efficiency of 91%. For LMC-g-PEG5000, the percentage was found to be 2.88% (w/w) with a labelling efficiency of 94%.
300 n

\[ y = 1.292x + 11.131 \]

\[ R^2 = 0.995 \]

Figure 4-6. FITC calibration curve using fluorescence spectrometer to calculate percentage of FITC to LMC and LMC-g-PEG5000.

These results vary slightly from results presented by (Chae et al. 2005) and (Zeng et al. 2008) where the percentage of FITC attached to the amine group of LMC was found to be 0.91% (w/w) and 1.07% (w/w) for chitosan of molecular weight 13 kDa and 32.7 kDa respectively.

4.4.2. Size and surface charge of FITC-labelled nanoparticles

a. HMC nanoparticles

Labelling of HMC nanoparticles had no significant effect (p>0.05) on their size or surface charge and hence both labelled (Table 4-2) and unlabelled (Table 2-2) nanoparticles would be predicted to behave in a similar manner when incorporated into pMDI or nebuliser formulations.

Table 4-2. Hydrodynamic diameter and surface charge of FITC-labelled HMC nanoparticles (with and without PEG) (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic diameter (nm ± S.D)</th>
<th>Polydispersity index (±S.D)</th>
<th>Surface charge (mV ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMC</td>
<td>166 ± 3</td>
<td>0.19 ± 0.06</td>
<td>+30 ± 2</td>
</tr>
<tr>
<td>HMC-PEG600</td>
<td>183 ± 8</td>
<td>0.21 ± 0.06</td>
<td>+25 ± 4</td>
</tr>
<tr>
<td>HMC-PEG1000</td>
<td>203 ± 9</td>
<td>0.14 ± 0.02</td>
<td>+24 ± 6</td>
</tr>
<tr>
<td>HMC-PEG5000</td>
<td>221 ± 11</td>
<td>0.22 ± 0.01</td>
<td>+27 ± 6</td>
</tr>
</tbody>
</table>

For all formulations, the PDI was less than 0.3 which is an indication of a narrow particle size distribution in all instances. There was no significant difference (p>0.05) in surface charge
between the four FITC-labelled formulations. A significant difference \((p<0.05)\) in nanoparticle size was observed, with post hoc Nemenyis test indicating that chitosan-PEG5000 nanoparticles were significantly larger than chitosan particles without PEG. This indicates that PEG (molecular weights 600-5000) had no influence on nanoparticle surface charge, but that the highest molecular weight PEG (5000) increased particle size, presumably due to the presence of the polymer on the particle surface.

b. LMC nanoparticles

(i) FITC-labelled pre-formed nanoparticles

The size and surface charge of the pre-formed nanoparticles labelled with FITC were measured (Table 4-3). Results suggested that aggregation had occurred and consequently the measured particle size was outside the range for accurate determination by PCS. The four formulations exhibited similar surface charges ranging from \(-0.08\) to \(+0.03\) mV indicating the overall charge to be approximately neutral. This is not suitable for siRNA association since it is necessary to have a positive polymer charge for electrostatic interaction with negative phosphate ions of siRNA (Katas and Alpar 2006). The results indicate that the method of FITC-labelling employed for these nanoparticles was not appropriate for further studies.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic diameter (nm ± S.D)</th>
<th>Polydispersity index (±S.D)</th>
<th>Surface charge (mV ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC</td>
<td>11200 ± 954</td>
<td>0.46 ± 0.09</td>
<td>-0.02 ± 0.03</td>
</tr>
<tr>
<td>LMC-PEG600</td>
<td>19400 ± 2261</td>
<td>0.79 ± 0.36</td>
<td>+0.017 ± 0.01</td>
</tr>
<tr>
<td>LMC-PEG1000</td>
<td>20100 ± 9163</td>
<td>0.90 ± 0.18</td>
<td>-0.08 ± 0.06</td>
</tr>
<tr>
<td>LMC-PEG5000</td>
<td>19800 ± 4158</td>
<td>0.36 ± 0.16</td>
<td>+0.03 ± 0.04</td>
</tr>
</tbody>
</table>

(ii) FITC-labelled LMC formed into fluorescent nanoparticles

FITC-labelled chitosan was used to prepare fluorescent nanoparticles which were investigated with regard to their size and surface charge (Table 4-4). Formulations (with and without PEG) were not significantly different \((p>0.05)\) from each other in terms of size or surface charge. Corresponding labelled formulations were also not significantly different \((p>0.05)\) from unlabelled formulations (Table 2-3).
Table 4.4. Hydrodynamic diameter and surface charge of FITC-labelled LMC formed into fluorescent nanoparticles (with and without PEG) (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic diameter (nm ± S.D)</th>
<th>Polydispersity index (±S.D)</th>
<th>Surface charge (mV ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC</td>
<td>141 ± 11</td>
<td>0.20 ± 0.03</td>
<td>+39 ± 2</td>
</tr>
<tr>
<td>LMC-PEG600</td>
<td>154 ± 14</td>
<td>0.22 ± 0.00</td>
<td>+39 ± 1</td>
</tr>
<tr>
<td>LMC-PEG1000</td>
<td>150 ± 8</td>
<td>0.18 ± 0.04</td>
<td>+39 ± 2</td>
</tr>
<tr>
<td>LMC-PEG5000</td>
<td>163 ± 6</td>
<td>0.21 ± 0.00</td>
<td>+37 ± 2</td>
</tr>
</tbody>
</table>

c. LMC-g-PEG5000 nanoparticles

There was no significant difference (p>0.05) in the size or surface charge of nanoparticles after FITC-labelling to LMC-g-PEG5000 (Section 2.4.3.c). The hydrodynamic diameter was 186 (±6.6) nm and surface charge +29 (±5) mV.

4.4.3. SEM of fluorescent LMC nanoparticles

a. FITC-labelled pre-formed LMC nanoparticles

FITC-labelled nanoparticles showed a collapsed and broken particle structure (Figure 4-7).

![SEM of freeze-dried FITC-labelled pre-formed LMC nanoparticles.](image-url)
The dried sample looked aggregated. SEM suggests that the FITC conjugation to formed nanoparticles was not a successful process and the particles were not suitable for further studies. This image should have been similar to SEM image of non labelled LMC nanoparticles as shown in previous studies (Figure 2-23).

\[\text{b. FITC-labelled LMC formed into fluorescent nanoparticles}\]

SEM of fluorescent particles where FITC was conjugated to LMC and then formed into nanoparticles showed spherical nanosized particles (Figure 4-8) as previously observed for unlabelled nanoparticles of the same composition (Figure 2-23). The size distribution (<500 nm) as observed using SEM was similar to hydrodynamic measurements determined using ZetasizerNano ZS (Malvern Instruments, UK). These fluorescent LMC nanoparticles were considered for future experimental studies.

\[\text{Figure 4-8. SEM of freeze-dried FITC-labelled LMC formed into nanoparticles.}\]

\[\text{4.4.4. Nebulisation of FITC-labelled chitosan nanoparticles into the TSI}\]

\[\text{a. HMC nanoparticles}\]

Figure 4-9 shows chitosan deposition profiles in the TSI following aerosolisation of each formulation using the Pari LC jet nebuliser. All formulations showed a high nanoparticle deposition in stage 2 of the TSI and lowest deposition in stage 1. Aerosol collected in stage 1 are considered to have a MMAD >6.4 μm. Aerosols reaching stage 2 of the TSI have MMAD
<6.4 μm and are predicted to reach the peripheral part of the lung (Hallworth and Andrews 1976). This suggests that the formulation is suitable as a carrier for drug deposition to the lower respiratory tract. The four formulations showed no difference (p>0.05) in chitosan deposition in stage 1 and nebuliser reservoir. Deposition of chitosan in stage 2 for all formulations was in the order of chitosan-PEG600 > chitosan-PEG5000 > chitosan-PEG1000 > chitosan nanoparticles. In stage 2, PEG600 nanoparticles showed highest (p<0.05) chitosan deposition.

Figure 4-9. Chitosan deposited on each stage of the TSI and nebuliser following nebulisation of FITC-labelled HMC nanoparticles.

Figure 4-10 shows the FPF of all formulations nebulised into the TSI. HMC-PEG1000 showed highest FPF of around 76 (±2) % compared to HMC 66 (±2) %, HMC-PEG600 73 (±4) % and HMC-PEG5000 68 (±4) %. These results show good dispersion properties of the formulation and effective delivery using the nebuliser.

Figure 4-10. FPF of nebulised formulations of HMC nanoparticles (with and without PEG) determined by TSI.
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The recovered dose or mass balance for HMC, HMC-PEG600, HMC-PEG1000 and HMC-
PEG5000 nanoparticles was 87 (±5) %, 90 (±2.7) %, 89 (±3) % and 89 (±10) % respectively. This
was within the European Pharmacopoeia recommendation of 75 to 125%. The chitosan
remaining in the nebuliser is the “residual or dead” -volume of the nebuliser, i.e. that
proportion of the fluid that remains in the nebuliser at the end of atomisation. All jet
nebulisers have a residual volume often upto 1 ml (McCallion et al. 1996b).

The advantage of using chitosan in nebulised particle delivery has been shown in a published
work where PLGA microparticles were poorly nebulised, whereas upon modification with
chitosan (150 kDa) the FPF was greatly improved to 51.3% due to the small particle diameter
produced (Yamamoto et al. 2005). The author suggests chitosan modified PLGA particles could
be aerosolised like a solution because the diameters of the particles were smaller than the
diameters of the droplets generated by the nebuliser. The nanoparticles generated and tested
for nebulisation in our study are also very small and are likely to be more efficiently delivered
from a jet nebuliser than larger particles (McCallion et al. 1996a).

b. LMC nanoparticles

A high amount of chitosan was deposited on stage 2, than stage 1 for nebulised LMC
nanoparticles with and without PEG, indicating suitable aerosolisation properties, for lung
delivery using nebulisers (Figure 4-11).

![Chitosan deposited on each stage of the TSI and nebuliser following nebulisation of FITC-labelled LMC
nanoparticles.](image)

All four formulations collectively in the nebuliser or stage 1 or stage 2 showed a very similar
trend in particle deposition using the nebuliser.
The FPF of LMC, LMC-PEG600, LMC-PEG1000 and LMC-PEG5000 nanoparticles was found to be 54 (±11) %, 57 (±1) %, 61 (±4) % and 61 (±3) % respectively (Figure 4-12). LMC formulations (with and without PEG) showed no significant difference (p>0.05) in particle aerosolisation properties. A previous study of chitosan (400 kDa) - stearic acid conjugate nanomicelles delivered from a nebuliser into a TSI also produced a similar FPF of 40% to 52% (Gilani et al. 2011). LMC nanoparticle formulations containing PEG showed narrow standard deviations for FPF whereas nanoparticles without PEG showed broader standard deviation values, perhaps suggesting improved dispersion properties and more uniform delivery of nanoparticles in the presence of PEG. LMC recovered from the nebuliser and all parts of TSI achieved a mass balance of 91 (±7) %, 99 (±2) %, 94 (±4) % and 92 (±7) % for LMC, LMC-PEG600, LMC-PEG1000 and LMC-PEG5000 nanoparticle formulations respectively.

c. LMC-g-PEG5000 nanoparticles

Following nebulisation of PEG grafted nanoparticles the amount of LMC deposited in the nebuliser reservoir and stages 1 and 2 of the TSI was 1.339 (±0.09) mg, 0.464 (±0.03) mg and 1.768 (±0.24) mg respectively. The FPF was 49 (±5) %. This was not significantly different (p>0.05) to LMC nanoparticles without PEG (54 ±11 %) but was significantly different (p<0.05) to LMC-PEG5000 nanoparticles (61 ±3 %), such that grafting of PEG resulted in poorer aerosolisation of nanoparticles than simple association. The recovered dose was 92 (±7) % which was within acceptable limits.
4.4.5. Nanoparticle size following nebulisation

a. HMC nanoparticles

Prior to nebulisation, the mean nanoparticle size was in the range 170 to 210 nm (Figure 4-13). Following nebulisation, the mean particle size collected from nebuliser reservoir was between 377 and 584 nm. This increase in particle size suggests association of nanoparticles during nebulisation. Aggregation is likely to occur in droplets deposited produced by the nebuliser as solvent evaporates from droplets and in the nebuliser due to excessive solvent loss, which results in the decrease in the temperature of fluid within the nebuliser reservoir and an increase in the concentration of solutes or dispersed particles (McCallion et al. 1996b; Elhissi et al. 2007).

Unlike our results, lactate dehydrogenase (LDH) loaded chitosan (10 kDa) nanocomplexes showed larger hydrodynamic diameter pre-nebulisation (292 ±6 nm) than those collected in nebuliser reservoir (226 ±2 nm) (Albasarah et al. 2010b). Another study demonstrated generation of aerosols by jet nebulisers resulted in reduction in the measured vesicle size of liposomes (Bridges and Taylor 1998) following aerosolisation. This was reasoned to be due to the damage of liposome and complex structures during nebulisation resulting in the production of smaller structures (Bridges and Taylor 1998; Elhissi et al. 2007; Albasarah et al. 2010b). Our study did not show a reduction in nanoparticle size, suggesting the nanoparticles generated in this study are robust and are not damaged by the shear forces during nebulisation. Similar to our studies, polyvinyl alcohol-grafted PLGA particles during air-jet nebulisation have reported the generation of cluster aggregations (Dailey et al. 2003) resulting in an increased measured size. Small sized suspended particles readily aerosolised as droplets (McCallion et al. 1996b). Aerosolised nanoparticles reaching stages 1 and 2 of TSI, were of mean particle size in the range 266 to 420 nm. These are larger than before nebulisation but smaller than particles collected from nebuliser reservoir. Similar results were found in another study, where lactate dehydrogenase loaded chitosan nanocarrier size decreased as the particles travelled from nebuliser reservoir (226 ±2 nm) to stage 2 (169 ±4 nm) of the TSI (Albasarah et al. 2010b). Formulations experiencing aggregation problems during nebulisation have also been observed with other types of nebulisers. Nebulisation of siRNA encapsulated within nanoparticles consisting of PVA grafted to PLGA, also known as P(68)-10 was carried out using an vibrating-mesh nebuliser (Aeroneb) (Nguyen et al. 2008). The nanoparticles had a 37% increase in the hydrodynamic diameter, following nebulisation. In this case, this increase was suggested to be due to ultrasound induced aggregation of the nanoparticles.
No significant difference (p>0.05) was observed between corresponding formulations deposited on stage 1 and stage 2. The cut-off diameter for stage 2 is <6.4 μm which means particles of size higher than this value should be retained in either nebuliser reservoir or stage 1 (Hallworth and Westmoreland 1987). Nanoparticles of size <6.4 μm were collected in stage 1. This indicates that during aerosolisation, deposition was dependent on the droplet size of the aerosols produced by the nebuliser, rather than size of the individual nanoparticles. The PDI of the nanoparticles prior to nebulisation was very low (range; 0.19 to 0.22) (Figure 4-14). Nanoparticles, after nebulisation, showed higher PDI (>0.4) for all formulations collected from nebuliser reservoir, stages 1 and 2, indicating a broad distribution of nanoparticle size after aerosolisation process, suggesting the occurrence of nanoparticle aggregation.
b. LMC nanoparticles

The hydrodynamic diameter of LMC nanoparticles with and without PEG was in the range 141 to 163 nm, pre-nebulisation (Figure 4-15). Following nebulisation, the particles were in the range 209 to 403 nm when collected from nebuliser reservoir, stages 1 and 2. This increase in size following nebulisation was also observed with HMC nanoparticle formulations and has been discussed (Section 4.4.5.a). Out of the four formulation particles collected from stage 2 of the TSI, PEG5000 associated nanoparticles showed highest particle size, and LMC without PEG had the smallest particle size (p<0.05).

![Figure 4-15. Hydrodynamic diameter of LMC nanoparticles post-nebulisation on each stage of the TSI and in the nebuliser reservoir (mean ± S.D., n=4).](image)

The PDI (Figure 4-16) suggests aggregation and possibly multi-modality, since it was >0.3 for nanoparticles collected from nebuliser reservoir, stages 1 and 2 of TSI following nebulisation.

![Figure 4-16. PDI of LMC nanoparticles deposited at each stage of TSI and in the nebuliser reservoir following nebulisation (mean ± S.D., n=4).](image)
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c. LMC-g-PEG5000 nanoparticles

LMC-g-PEG5000 nanoparticles were of size 186 (±7) nm following preparation (Figure 4-17), with PDI less than 0.2 (Figure 4-18).

Particles showed higher particle size in nebuliser reservoir (689 nm), stage 1 (475 nm) and in stage 2 (503 nm) following nebulisation. These results are similar to results obtained with HMC and LMC nanoparticles (Sections 4.4.5.a and 4.4.5.b). The size is still small enough to reach deep down in the lung but is larger than its original size. The PDI of this nanoparticle formulation in our study was very high (0.6) at stage 2, indicating highly aggregated and non-uniform size distribution (Figure 4-18).
4.4.6. Gel electrophoresis of siRNA following nebulisation

In order to investigate the potential for delivery of siRNA (PTEN) to lung using a nebuliser, the formulations were aerosolised using a Pari nebuliser into the TSI. Materials collected in stage 2 and studied using agarose gel. Stage 2 is that part of TSI which represents lower regions of the lung (<6.4 μm) and is the area of interest, hence other parts such as nebuliser reservoir and stage 1 were not studied on gel electrophoresis. siRNA loaded HMC formulations were not tested for aerosolisation due to the limited amount of siRNA available and the associated expense.

a. LMC nanoparticles

Naked siRNA could not be detected in lane 1 indicating either siRNA degradation upon aerosolisation or inability to form aerosols using nebulisation (Figure 4-19). The bright bands in Lane 1 were the side numbering on the gel plate present by default. Pre-nebulised, LMC:siRNA (Lane 2) and LMC-PEG1000:siRNA (Lane 4) nanoparticles chitosan : siRNA (10:1 w/w) showed a bright clear band with slight retention in the well, but mostly moving away from the well. This was due to the higher ratio of negatively charged siRNA to positively charged chitosan molecules. These results are the same as Figure 3-16. Following nebulisation of siRNA loaded LMC (Lane 3) and LMC-PEG1000 (Lane 5) nanoparticles, the siRNA behaved in a parallel manner to the corresponding formulation prior to nebulisation. The band intensity was not exactly similar since volume of siRNA recovered from TSI apparatus either got diluted or too
concentrated. This is one of the experimental limitations. Other formulations were not tested for siRNA/carrier aerosolisation and stability due to limited amount of expensive siRNA available.

Biopharmaceuticals are unstable to nebulisation in naked form (Kanaoka et al. 1999; Albasarah et al. 2010b). They undergo stress including shearing during the generation of aerosol droplets and effects at the liquid/air interface. Stability can be improved by association with carrier particles. Previously it was demonstrated that complexed siRNA, unlike naked siRNA, was not harmed during the nebulisation process when used with non-viral vectors such as PEI and Oligofectamine (Huth et al. 2006). Pulmonary gene silencing in transgenic EGFP mice using nebulised chitosan/siRNA nanoparticles resulted in nanoparticle deposition throughout the entire lung in both alveoli and bronchiolar regions. Transgenic EGFP mice dosed with the aerosolised nanoparticle formulation showed 68% EGFP gene silencing (Nielsen et al. 2010) indicative of stable delivery of the siRNA to the lungs. Multi-lamellar lipid coated particles comprising lipid:peptide:DNA (LPD) along with protamine (polycationic peptide), when nebulised also showed unaltered physical stability (particle size distribution) and biological activity of the complexes (Birchall et al. 2000). In our study, the advantage of using nanoparticles as a carrier for the delivery of intact siRNA using a jet nebuliser has been demonstrated. This confirmed the successful association of siRNA with the chitosan nanoparticles, and further indicated nanoparticle potential protective ability for the nucleic acid against the physical stresses encountered during nebulisation.

b. LMC-g-PEG5000 nanoparticles

Following nebulisation, siRNA did not show any band in lane 3 (Figure 4-20). Hence the need of a carrier to ensure integrity of siRNA is maintained during nebulisation. Pre-nebulised siRNA bound LMC-g-PEG5000 nanoparticles when loaded on to a gel showed siRNA movement away from well (Lane 1) indicating no siRNA retention by the polymer. These results are the same as shown in Figure 3-17. Nebulised siRNA encapsulated within nanoparticles showed a faint siRNA band on gel but at the same distance from well as that of naked siRNA (Lane 2). The siRNA band in lane 2 is not very clear, which may be due to the dilution of the loaded formulation in the TSI. This confirms successful siRNA aerosolisation by a nebuliser and delivery to stage 2 using a new class of co-polymer nanoparticle formulation, indicating its potential for in-vivo studies. The bright bands on the side of lane 3 are the numbering on the gel by default and are not a part of the results.
4.4.7. Deposition of nebulised nanoparticles encapsulating siRNA into the TSI

The in-vitro aerosol performance of nebulised siRNA encapsulated within LMC nanoparticles (chitosan to siRNA ratio of 10:1 w/w) showed a high FPF (57.3 ±1.9%) in the TSI with a mass balance of 92.9 (±1.8)% (Figure 4-21).
Comparing the FPF of unloaded nanoparticles (53.5 ±11.4%) with siRNA loaded chitosan nanoparticles (57.3 ±1.9%), showed no significant difference ($p>0.05$). This signifies that the presence of siRNA within the system did not have any effect on aerosolisation behaviour.

### 4.4.8. SEM of nanoparticles delivered from pMDIs

#### a. HMC nanoparticles

Figure 4-22 shows that HMC-PEG1000 nanoparticles collected post-actuation from a pMDI canister, via the metering valve, were spherical with similar morphology to the freeze-dried HMC-PEG1000 nanoparticles prior to pMDI manufacture (Figure 2-20). Other formulations apparently either aggregated or creamed in HFA propellants and were not visualised by SEM. The size of particles should be less than 2 μm for deep lung deposition and the size obtained using the HMC-PEG1000 formulation was apparently sub-micron and hence within this range (Stahlhofen et al. 1980).

![SEM of HMC-PEG1000 nanoparticles](image)

**Figure 4-22.** SEM of HMC-PEG1000 nanoparticles; following actuation from a pMDI.

#### b. LMC nanoparticles

It was observed that following actuation LMC nanoparticles were deposited as discrete entities with nearly spherical structure (Figure 4-23). These occur in close proximity giving an appearance similar to the same particles before loading into pMDI canisters (Figure 2-23).
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LMC-PEG600 nanoparticles actuated from the pMDI resulted in slightly deformed shaped samples (Figure 4-24). The sample is relatively distorted with high surface roughness and aggregated form. No discrete particles could be observed. This aggregated morphology may be an indication of unsuitability of LMC-PEG600 nanoparticles as biopharmaceutical carriers using pMDIs.

Actuation of LMC-PEG1000 nanoparticles resulted into extremely small sized particles (Figure 4-25). The particles are present in close proximity to each other, likely to be due to the evaporation of propellant and presence of excess PEG.
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Figure 4-25. SEM of LMC-PEG1000 nanoparticles; following actuation from a pMDI.

Post-actuation of LMC-PEG5000 nanoparticles resulted into structures with a network-like appearance (Figure 4-26). The long molecular chain length of PEG5000 may have caused bridging between the individual particles.

Figure 4-26. SEM of LMC-PEG5000 nanoparticles; following actuation from a pMDI.

4.4.9. Particle size distribution of pMDI aerosols generated from nanoparticle formulations

a. HMC nanoparticles

The size distribution determined by laser diffraction, of the aerosol clouds generated by the pMDIs is shown in Figure 4-27. HMC nanoparticles without PEG showed a wide size distribution (Span; 78.52 ±1.6), with some multi-modality and a VMD of 54.24 (±4.7) µm. This suggests that the nanoparticles were highly aggregated within HFA-227 propellant, and largely
remained aggregated following actuation and subsequent rapid evaporation of the propellant. HMC-PEG600 formulations showed a bi-modal distribution, having a VMD of 23.16 (±3.0) μm and span of 57.71 ±2.3. The inability of PEG600 to stabilise silica particles has previously been reported (Paul et al. 2005), which may be related to the relatively short length of the polymer chains, or the physical state of PEG600, which is liquid at room temperature, whilst PEG 1000 and 5000 are solids. There was a small sub-population of particles having a mode less than 3 μm. Similar observations were made for HMC-PEG5000 particles, which had a multimodal size distribution (Span; 67.65 ±5.0), with a VMD of 28.43 (±6.5) μm.

These data suggest that aerosolisation of these three formulations using a pMDI is not appropriate for lung delivery, as their median size greatly exceed the size (1-5 μm) required for peripheral lung deposition. By contrast, the HMC-PEG1000 particle formulation produced a more uniform size distribution (Span; 2.29 ±0.3), with a much smaller VMD of 1.53 (±0.1) μm, which is appropriate for alveolar delivery (Stahlhofen et al. 1980). The improvement in dispersibility of HMC nanoparticles, when incorporating PEG1000 observed in previous sections (2.4.11.a and 2.4.12.a), is thus reflected in an improved performance in delivery from a pMDI. There is a fundamental difference in between cascade impactor and laser diffraction techniques. The cascade impactor measures the aerodynamic size distribution based on mass and density of drug particles whereas the laser diffraction measures on the basis of light scattering by droplets. Proventil®, a commercially available HFA-134a based suspension formulation containing albuterol sulphate (active), ethanol and oleic acid (suspending aid)
when tested for particle size distribution using laser diffraction resulted in a VMD of 2.5 μm (Haynes et al. 2004). This was higher than VMD produced using HMC-PEG1000/HFA-227 nanoparticle formulation system. These results confirm previous findings which suggest that PEG act as a suitable polymeric stabiliser for HFA systems when used in an appropriate concentration and molecular weight (Ashayer et al. 2004; Paul et al. 2005; Traini et al. 2006).

b. **LMC nanoparticles**

Figure 4-28 shows the particle size distribution of aerosolised pMDI formulations of LMC nanoparticles (with and without PEG). Aerosols of LMC nanoparticles without PEG showed a bimodal distribution. The majority of nanoparticles formed a population size less than 5 μm with a VMD of 4.09 (±0.5) μm and span of 19.51 ±3.5. The inclusion of PEG600 in the chitosan nanoparticle formulations altered the aerosolisation characteristics by increasing the VMD up to 15.49 (±2.1) μm and span to 53.10 ±23.1. The distribution was broad with the majority of particles greater than 5 μm. The VMD (9.01 ±7.8 μm) and span (48.60 ±5.3) obtained for LMC-PEG5000 formulations was also too large to make the formulation eligible as a pulmonary carrier for active agents. With inclusion of PEG1000, nanoparticles showed a decrease in VMD to 1.43 (±0.1) μm and span to 1.91 ±0.2. Thus the presence of PEG1000 provided good dispersion in propellant and deaggregation during atomisation, generating a suitable size for pulmonary delivery.

![Figure 4-28. Mean size distribution data for aerosols generated from pMDI systems containing LMC nanoparticles (with and without PEG) (n=4).](image-url)
Chapter 4. Aerosolisation of nanoparticles

c. **LMC-g-PEG5000 nanoparticles**

Figure 4-29 shows the size distribution of aerosols of LMC-g-PEG5000 nanoparticles with a VMD of 36.98 (±18.0) μm and span of 73.61 ±3.8. This behaviour could be attributed to poor dispersion properties of high m.w PEG (5000) as observed in previously detailed studies (Sections 2.4.11.c and 2.4.12.c). PEG 5000 has been shown not to be a good stabiliser in a recently published study. Chitosan (medium m.w) grafted PEG5000 particles encapsulating PLGA loaded curcumin nanoparticles suspended within HFA-227 propellant showed an onset of flocculation after 1 min of shaking (Selvam et al. 2011). Total creaming occurred at a concentration of 0.2 mg/ml at >10 min and this reduced to 5 min when tested at a concentration of 2 mg/ml (Selvam et al. 2011). It might be the case that our nanoparticle formulation was at too high a concentration (HMC; 7 mg, LMC; 4.8 mg) for an efficient dispersion within HFA-227. The author further suggested that the solvating capacity of HFA decreases with increasing concentration. This formulation might have improved dispersion in HFA with the inclusion of ethanol as a co-solvent. This has not been investigated but could be a point of interest for future studies.

![Figure 4-29. Mean size distribution data for aerosols generated from pMDI systems containing LMC-g-PEG5000 nanoparticles (n=4).](image-url)
4.4.10. Particle size distribution of pMDI aerosols generated from siRNA encapsulated nanoparticle formulations

a. LMC nanoparticles

siRNA encapsulated LMC-PEG1000 nanoparticles were aerosolised using the pMDI. The size distribution of the aerosols produced is shown in Figure 4-30. The VMD and span was found to be 4.42 μm and 40.0 respectively, indicating an increase in particle size (p<0.05) compared to unloaded nanoparticle (VMD; 1.43 ±0.1 μm, Span; 1.91 ±0.2) (Figure 4-28). At less than 5 μm range, this should be acceptable for pulmonary delivery; with potentially different regional distribution in lung compared to the nanoparticles without siRNA. The hydrodynamic diameter of blank LMC-PEG1000 nanoparticles (Table 2-3) in aqueous media was raised from 152 (±4) nm to 161 (±4) nm upon siRNA inclusion (Figure 3-3) at a LMC to siRNA ratio of 10:1 w/w. The presence of large amount of siRNA is likely to have bridged between nanoparticles resulting in a broader aerosol size distribution upon actuation in comparison to unloaded nanoparticles. Biopharmaceuticals (protein) are known to be surface-active and this could also have been the reason for a change in droplet size upon actuation (Nyambura et al. 2009b). The dispersion and aerosolisation behaviour of siRNA encapsulated in LMC-PEG1000 nanoparticles suggests they may be suitable for delivery using a pMDI.

Figure 4-30. Mean size distribution data for aerosols generated from pMDI systems containing LMC-PEG1000:siRNA (10:1 w/w) nanoparticle (n=1).
b. LMC-g-PEG5000 nanoparticles

The VMD and span of LMC-g-PEG5000 nanoparticles encapsulating siRNA (10:1 w/w) was 38.02 µm and 70.90 respectively (Figure 4-31), which was almost the same as those without 36.98 ±18.0 µm (VMD) and 73.61 ±3.8 (span) (Figure 4-29). The study suggested that LMC-g-PEG5000 particles were not likely to be suitable for delivery of siRNA using a pMDI approach.

![Figure 4-31. Mean size distribution data for aerosols generated from pMDI systems containing LMC-g-PEG5000:siRNA (10:1 w/w) nanoparticles (n=1).](image)

4.4.11. Nanoparticle aerosolisation from a pMDI into the TSI

Results from the TSI indicate that actuation of pMDI formulations led to appreciable FPFs for some of the formulations (Figure 4-32). LMC and LMC-PEG1000 showed FPFs of 16.0 (±1.1) % and 45.2 (±1.2) % respectively. On the other hand HFA suspended formulations containing PEG600 and PEG5000 showed smaller FPFs, of 5.1 (±0.8) % and 11.6 (±3.3) % respectively. The mean delivered dose as percent of label claim from all four formulations suspended in pMDI was >75%. These results with LMC-PEG1000 nanoparticle formulation are as expected. The dispersion stability of LMC-PEG1000 nanoparticle formulation in HFA-227 has been observed to be the best amongst all tested formulations (Table 2-6). Moreover Sympatec results indicated LMC-PEG1000 nanoparticle pMDI formulations showed lowest VMD (Figure 4-28) suggesting their potential as delivery vehicles for nanoparticle therapeutics. The FPF of the commercial inhaler pMDI Proventil® (HFA-134a) has been determined to be 47.5% (Dellamary et al. 2000) which is comparable to the formulation LMC-PEG1000 nanoparticles suspended in
Chapter 4. Aerosolisation of nanoparticles

HFA-227. Aerosolisation studies with curcumin loaded PLGA nanoparticles encapsulated within chitosan (medium m.w) grafted PEG, when dispersed in HFA227 had a FPF of 65% (Selvam et al. 2011) which was considered highly suitable for pulmonary delivery. Previous studies on ethoxylated surfactant solubility within HFAs have indicated that PEG solubility decreases with an increase in molecular weight, being almost insoluble for molecular weights greater than 1000. This has been reasoned to be due to a change in orientation of PEG from a “zig-zag” to a “twisted” configuration, reducing the solvation capacity of the propellant (Ridder et al. 2005; Rogueda 2005; Wu et al. 2008a).

![Fine particle fraction (%)](image)

Figure 4-32. FPFs of pMDI formulations of LMC and LMC-PEG nanoparticles determined by TSI (mean ± S.D., n=3).

### 4.4.12. Nanoparticle size following pMDI actuation into the TSI

Aerosolisation of LMC nanoparticles (with and without PEG) into a TSI using pMDI resulted in a measured nanoparticle size less than 1 μm (Table 4-5). Particles collected from actuator and mouthpiece had a hydrodynamic diameter larger than those collected in stage 2 (p<0.05). Pre-actuation particle size was in the range of 123 to 165 nm (Table 2-3) whereas particles upon post-actuation (stage 2) showed particle size in the range of 344 to 513 nm with wide particle size distribution (PDI; 0.4 to 0.7). It has been reported that an increase in particle size of TPP crosslinked chitosan (50 kDa) was observed after exposure to propellant (P134a) (Williams III et al. 1998). This was due to the presence of water in P134a which hydrated chitosan microspheres causing aggregation during storage of the pMDI formulations this might be a possible mechanism here, though the water content of HFA-227 used in this study is unknown.

Upon actuation, HFA evaporates to leave tight clusters of nanoparticles (Figure 4-25). Based on post-aerosolisation nanoparticle physicochemical and stability properties, particles are likely to travel to the desired site, interact with mucosal surface and enter the cells (De Campos et al.
2003). Good dispersion, high FPF and the small size of LMC-PEG1000 nanoparticles suggest these may be suitable for pulmonary delivery of active molecules.

Table 4-5. Hydrodynamic diameter and PDI of LMC nanoparticles post-actuation from pMDI on each stage of the TS1 (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic diameter in actuator and mouthpiece (nm ± S.D.)</th>
<th>Hydrodynamic diameter in stage 1 (upper stage) (nm ± S.D.)</th>
<th>Hydrodynamic diameter in stage 2 (lower stage) (nm ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC</td>
<td>721 ± 134 (0.68 ± 0.09)</td>
<td>568 ± 227 (0.63 ± 0.09)</td>
<td>496 ± 48 (0.67 ± 0.07)</td>
</tr>
<tr>
<td>LMC-PEG600</td>
<td>711 ± 117 (0.43 ± 0.04)</td>
<td>327 ± 51 (0.39 ± 0.03)</td>
<td>344 ± 42 (0.46 ± 0.07)</td>
</tr>
<tr>
<td>LMC-PEG1000</td>
<td>699 ± 162 (0.55 ± 0.02)</td>
<td>418 ± 59 (0.55 ± 0.22)</td>
<td>440 ± 30 (0.42 ± 0.06)</td>
</tr>
<tr>
<td>LMC-PEG5000</td>
<td>820 ± 129 (0.36 ± 0.03)</td>
<td>435 ± 64 (0.41 ± 0.04)</td>
<td>513 ± 13 (0.48 ± 0.08)</td>
</tr>
</tbody>
</table>

4.4.13. Assessment of nanoparticle encapsulated siRNA delivery from a pMDI

a. LMC nanoparticles

Gel electrophoresis showed delivery of stable siRNA to stage 2 of the TS1 when encapsulated within LMC-PEG1000 nanoparticles (Figure 4-33).

Figure 4-33. Gel electrophoresis of siRNA; Lane 1: Pre-actuation naked siRNA (Control) and Lane 2: Post-actuation siRNA in LMC-PEG1000 nanoparticles; chitosan to siRNA ratio of 10:1 (w/w).
Encapsulated siRNA showed retardation within the well with partial movement away from the well (Lane 2) due to high association of siRNA with chitosan (1:10 w/w). This movement away from well is of the same distance as that of naked siRNA (Lane 1; control). siRNA bands observed in lane 1 and lane 2 were bright and uniform without any spots of degradation. The particle is similar to that of the non-aerosolised LMC-PEG1000:siRNA (10:1 w/w) as shown in Figure 3-16 (Lane 8). These HFA dispersed siRNA encapsulated nanoparticle formulations were stored for a month before performing gel electrophoresis and it is clear from these studies that the siRNA-carrier particles were robust and that siRNA did not lose its integrity during delivery from pMDI. The study was performed using a chitosan to siRNA (w/w) ratio of 10:1 since that formulation is loaded with maximum amount of siRNA, which was essential in this preliminary study to detect siRNA deposited in stage 2 of the TSI. This formulation may require further investigation and optimisation but from results obtained, the LMC-PFG1000 nanoparticle formulation is acceptable for further in-vivo studies. To our knowledge this is the first successful delivery of siRNA from a pMDI though; successful delivery of macromolecules by pMDI has been previously demonstrated in various studies (Brown and Pickrell 1995; Quinn et al. 1999; Nyambura et al. 2009b; Bains et al. 2010).

**b. LMC-g-PEG5000 nanoparticles**

Naked siRNA (lane 1) showed a bright siRNA band away from the well, towards the positive electrode (Figure 4-34).

![Figure 4-34](image)

**Figure 4-34.** Gel electrophoresis of siRNA; Lane 1: Naked siRNA (Control); Lane 2: Post-actuation siRNA in LMC-g-PEG5000 nanoparticles in weight ratios of 10:1.

LMC-g-PEG5000 nanoparticles encapsulating siRNA when actuated using a pMDI (lane 2) did not show a band for siRNA. LMC-g-PEG5000 nanoparticles formed aggregates and sedimented when dispersed in the non-polar system, assessed visually (Figure 2-36) and using laser
diffraction (Figure 2-41 and Figure 2-42). The poor aerosolisation of this system was also demonstrated by studies performed using the Sympatec, where a VMD of 38.02 μm upon pMDI actuation was achieved (Figure 4-29). Hence siRNA in LMC-g-PEG5000 nanoparticles could not be effectively aerosolised, and they were not delivered in stage 2 of the TSI.

4.4.14. Nanoparticle deposition in the NGI following aerosolisation from a pMDI

a. HMC nanoparticles

The measured FPF (<6.4 μm) was 5.7 (±0.9) %, 11.8 (±2.7) % and 17.0 (±2.1) % for HMC nanoparticles, HMC-PEG600 and HMC-PEG5000 nanoparticles respectively (Figure 4-35). The relatively poor performance of these formulations as aerosols may be attributed to aggregation of particles within the liquefied HFA and/or an inability to disperse during actuation and propellant evaporation following actuation of the device. HMC-PEG1000 nanoparticles showed highest (p<0.05) FPF of 34.0 (±1.4) %, indicating this was the most suitable crosslinked chitosan nanoparticle formulation for lung delivery, delivered using a pMDI. These results correlate well with the aerosol sizing study described earlier in Figure 4-27, where VMD of HMC-PEG1000 nanoparticle formulation dispersed within HFA-227 was lower than all other formulations. The dose of nanoparticles per actuation (50 μl) was 59 μg.

The MMAD for the aerosols generated was 4.92 (±0.29) μm with a GSD of 3.35 (±0.92). Generally a MMAD of 1 to 5 μm is suitable for lung delivery (Zeng et al. 1995). These systems are claimed to be polydisperse since the GSD is greater than 1.22. If the GSD was less than
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1.22 the system have been considered to be monodisperse system (Finlay et al. 1997). A mass balance >80% was achieved. Chitosan deposition in all stages of the NGI and accessories (actuator, mouthpiece and throat) was 85.8 (±2.6) %, 77.9 (±3.1) %, 90.1 (±2.5) % and 78.7 (±2.1) % for HMC, HMC-PEG600, HMC-PEG1000 and HMC-PEG5000 nanoparticle formulations respectively. Mass balance between 75 to 125% of the anticipated label claim, is considered to be in accordance with the criteria of the pharmacopoeia (European Pharmacopoeia 2008). In comparison with the other formulations, the HMC-PEG1000 formulation in HFA demonstrated high and consistent aerosolisation performance and good dose recovery (mass balance). The mass deposition of HMC at each stage of the NGI is presented in Figure 4-36, for the HMC-PEG1000 formulation. Overall maximum deposition was seen in the induction port (actuator, mouthpiece and throat) indicating that these particles will remain in the device or are likely to deposit in the oropharynx region in-vivo (Particles > 11.7 μm). The remaining particles may be predicted to reach the lungs, with an FPF of 34% indicative of the potentially therapeutically useful aerosol. This may be considered adequate to exhibit a therapeutic effect, as commercially available pMDI products have been reported to deliver about 30% of the total emitted dose to the lungs (Hirst et al. 2002; Rau 2005).

![Deposition pattern of chitosan for HMC-PEG1000 nanoparticles delivered from a pMDI into the NGI (mean ± S.D., n=3).](image)

Previous reports of nanoparticle delivery from HFA-134a based pMDI systems reported a FPF of approximately 45% for insulin loaded nanoparticles, employing a volatile oil (cineole) as a
dispersant (Nyambura et al. 2009a) and lysozyme nanoparticles with oleic acid, Span 85 or dipalmitoylphosphatidylcholine as dispersants (Nyambura et al. 2009b). Lecithin-based nanoparticles (microemulsion of water/propan-2-ol/iso-octane) were also successfully dispersed in HFA-227 and achieved a high FPF (>58%) (Dickinson et al. 2001). Successful pMDI formulation depends on the properties of the dispersed material, dispersant and propellant. This study has demonstrated the potential of a novel HMC-PEG1000 formulation, capable of delivery from a pMDI, as a nanocarrier for pulmonary drug delivery.

b. LMC-PEG1000 nanoparticles

After testing LMC nanoparticles (with and without PEG) for their aerosolisation characteristics it was observed that LMC-PEG1000 showed the best results, with aerosols having the lowest VMD of 1.43 (±0.08) μm (Figure 4-28) and highest FPF of 45.2 (±1.2) % (Figure 4-32) in the TSI. Hence, the LMC-PEG1000 nanoparticle formulation was selected and evaluated for performance using the NGI. LMC-PEG1000 nanoparticles when delivered from a pMDI gave a FPF of 38 (±2.7) %. The MMAD and GSD values of nanoparticle aerosols were 3.5 (±0.48) μm and 3.6 (±0.42) respectively. The dose of nanoparticles per actuation (50 μl) was 41 μg. Chitosan deposition within the NGI is shown in Figure 4-37.

![Figure 4-37. Deposition pattern of chitosan for LMC-PEG1000 nanoparticles delivered from a pMDI into the NGI (mean ± S.D., n=3).]
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The highest amount of chitosan was deposited in the actuator, mouthpiece and throat, with least deposition in the filter stage. Aerosolised particles with aerodynamic diameter <6.4μm showed maximum chitosan deposition in stage 3, 4 and 5 indicating high potential for LMC-PEG1000 formulations as carriers of biopharmaceuticals for pulmonary delivery.

4.4.15. Nanoparticle size following pMDI actuation into the NGI

a. HMC nanoparticles

The mean particle size for HMC-PEG1000 nanoparticles was >6 μm; in the pre-NGI stage i.e actuator, mouthpiece and throat (Figure 4-38). The measured size was less than 2 μm and was similar in the lower stages (stage 1 to stage 7) of the NGI with similar size distribution. This particle size is useful to deliver siRNA to the peripheral areas of the lung. This increase in measured particle size compared to the same nanoparticles prior to formulation in the pMDI and aerosolisation (Table 2-2) may be due to clusters of nanoparticles forming during aerosolisation resulting in aggregates upon propellant evaporation.

![Figure 4-38. Hydrodynamic diameter and PDI of HMC-PEG1000 nanoparticles deposited at each stage of the NGI following delivery from a pMDI (mean ± S.D., n=3).](image)

This cluster formation was also observed using SEM, for HMC-PEG1000 nanoparticles collected from a pMDI canister (Figure 4-22). The particles were still small enough to penetrate deep into the impactor resulting in reduced deposition in the induction port and leading to a FPF of
34.0 (±1.4) % (Section 4.4.14.a). It may be postulated that in the physiological environment of the lung, aggregates of nanoparticles may deaggregate to the primary particles. The PDI of nanoparticle post-aerosolisation (0.8) was higher in all stages in comparison to pre-aerosolisation (0.3) indicating some aggregation during the whole process. Particles collected on the filter showed the smallest particle size (<400 nm) with a narrow size distribution (PDI 0.4). These data indicates acceptable aerosol delivery of HMC-PEG1000 nanoparticles in-vitro showing potential for pulmonary delivery but further investigation to look at dispersion of aggregates should be conducted in the future.

b. LMC nanoparticles

The deposition profile of LMC-PEG1000 nanoparticles delivered from a pMDI into the NGI is shown in Figure 4-39. Hydrodynamic diameter was largest; 978 nm in the actuator, mouthpiece and throat. The distribution was fairly uniform <1 µm on all stages, showing deposition depended on droplet size. These aerosol droplets contained small particles, which may have aggregated upon aerosolisation as they were smaller in size before aerosolisation (Table 2-3).
Chapter 4. Aerosolisation of nanoparticles

The formation of aggregated particles or clusters of LMC-PEG1000 nanoparticles upon actuation using pMDI was also suggested previously from the SEM studies (Figure 4-25). The PDI of the nanoparticles collected on different stages of NGI was 0.3 to 1 (Figure 4-39). The particles were small enough to penetrate deep into the impactor resulting in reduced deposition in the induction port and leading to a FPF of 38 \( \pm 2.7 \) % (Section 4.4.14.b).

4.4.16. Aerosolisation of LMC-PEG1000 nanoparticles with siRNA using a pMDI into the NGI

LMC-PEG1000 nanoparticles with siRNA were evaluated for their deposition in the NGI following delivery from a pMDI (Figure 4-40). It is clear that, with the inclusion of siRNA the aerodynamic properties changed. FPF reduced from 38 \( \pm 2.7 \) % (Section 4.4.14.b) to 25% with the inclusion of siRNA. The reduction in FPF might be due to bridging of excess siRNA between particles, causing them to group together and form larger particles. The test was performed once \( (n=1) \) with 20 actuations. siRNA encapsulated HMC nanoparticles (with and without PEG) were not tested following delivery from a pMDI due to the limited amounts of the expensive siRNA available.

Figure 4-40. Deposition pattern of chitosan for LMC-PEG1000:siRNA (10:1 w/w) nanoparticles delivered from a pMDI into the NGI \( (n=1) \).
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siRNA based nanoparticle preparation seem to be suitable for further studies. The mass balance achieved was >80%. The MMAD and GSD values for siRNA loaded nanoparticles were 4.54 μm and 7.45 respectively. Since the dose uniformity through canister could not be measured therefore the siRNA deposition in different stages of NGI is not known. siRNA deposited at each stage is presumably bound to chitosan and therefore the release pattern of siRNA from its carrier needs to be investigated.

4.5. Conclusion

A method of preparing inhalable siRNA encapsulated nanoparticles, to yield aerosol particles possessing optimal aerodynamic sizes for deep lung administration has been developed. FITC-labelled and unlabelled nanoparticles had similar physicochemical characteristics and were thus considered to be comparable in terms of likely aerosol performance. SEM studies also showed similar morphology of labelled and unlabelled nanoparticles. Chitosan formulations (with and without PEG) show good nebulisation characteristics since the FPF was >50%. Naked siRNA could not be aerosolised without degradation confirming the need of a delivery carrier. LMC-siRNA encapsulated nanoparticles showed a FPF (54 ±11 %) not significantly different than nanoparticles without siRNA (57 ±2 %). These siRNA nanoparticles formed sufficiently stable carriers for nebulisation of the nucleic acid and would be suitable for future studies using nebulisation.

Formulations tested using pMDIs showed that not all PEGs were suitable as an aerosolisation enhancer in non-polar pressurised systems. PEG1000 modified particle properties, resulting in a mean geometric particle size of <5 μm. Both HMC and LMC associated PEG1000 formulations showed a narrow and uniform post-actuation size distribution. LMC-PEG1000 nanoparticles encapsulating siRNA displayed suitably high FPF and siRNA stability following delivery from a pMDI. The structural integrity of siRNA was maintained after nanoparticle encapsulation and following aerosolisation using a pMDI suggesting, it may be suitable for future studies. LMC grafted PEG formulations were not suitable for delivery using pMDI. Further work would be needed to optimise the formulations developed here in terms of its long term stability in polar and non-polar media and to understand the science behind the aggregation of nanoparticles upon nebulisation or delivery from a pMDI. This study has though demonstrated the applicability of crosslinked chitosan-PEG particle based technology for carriers of biopharmaceuticals, suitable for aerosolisation using pMDIs and nebulisers.
CHAPTER 5

NANOPARTICLE CELL CULTURE STUDIES FOR CELLULAR VIABILITY, UPTAKE AND GENE SILENCING
Chapter 5: Nanoparticle cell culture studies for cellular viability, uptake and gene silencing

5. Nanoparticle cell culture studies for cellular viability, uptake and gene silencing

5.1. Introduction

siRNA-mediated knockdown of protein expression at the messenger RNA (mRNA) level, also known as RNA interference (RNAi) offers a new therapeutic strategy to overcome diseases (Flynn et al. 2004). This technique can be used to target any gene being expressed in a cell (Chapter 1). Preferably, a target gene is one involved in, or associated with, the progression of cellular activities important to diseases. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor-suppressing gene that inhibits the proliferation and promotes the apoptosis of tumor cells (Stahl et al. 2003). PTEN gene is found in almost all tissues in the body. PTEN gene is beneficial since it is infrequently deleted or mutated (Zou et al. 2011). The effectiveness of PTEN siRNA is determined by its ability to migrate through the body and reach the target site at therapeutically relevant levels. It is used as a model in this study and has no clinical relevance.

Due to the strong anionic charge of the RNA phosphodiester backbone, naked siRNA does not freely cross the cell membrane. Therefore, delivery systems are required to facilitate its access to intracellular sites (Kim and Kim 2009). Cationic polymers and lipid-based siRNA vectors have been shown to enter cells and mediate specific RNAi in-vitro (Takahashi et al. 2005) and in-vivo (Sørensen et al. 2003). Effective in-vivo RNAi has been achieved in bronchiole epithelial cells of transgenic EGFP mice after nasal administration of chitosan/siRNA nanoparticles, indicating the potential application of chitosan nanoparticles in RNA mediated therapy of systemic and local diseases (Howard et al. 2006). Chitosan has also been studied as an absorption enhancer without causing any damage to the nasal mucosa (Illum 2003). Studies have shown the successful transport (paracellular) of polar drugs using chitosan across epithelial cell line by opening the tight junctions of the cells (Thanou et al. 2001). Hence chitosan was chosen for nanoparticle formulation of siRNA.

The composition of a polymeric carrier plays a vital role in the delivery of a nucleic acid. In order to improve the pharmacological effect of the carrier, certain surface characteristics can be modified. It has been previously established that PEG prolongs circulation time within the biological system, improves stability of the carrier and reduces cytotoxicity (Niidome et al.
Chapter 5. Nanoparticle cell culture studies for cellular viability, uptake and gene silencing

2006; Veronese and Mero 2008; Sheng et al. 2009). Hence PEG has been used in the modification of chitosan nanoparticles in these studies to form chitosan-PEG nanoparticles. A detailed description of formulation and physicochemical characterisation significant to in-vitro cell culture use has been detailed in Chapters 2 and 3. We have also demonstrated aerosolisation characteristics of siRNA loaded and unloaded chitosan-PEG nanoparticles in Chapter 4.

In order to formulate siRNA associated nanoparticles, both nanocarrier and the nucleic acid should be able to meet the following criteria: (1) Degradation of the carrier into non toxic by products once administered in to the lungs, (2) Fast uptake by the cells and (3) high specific gene knockdown. Following pulmonary administration, the first line of contact of such systems is the epithelial cells, lining the respiratory tract. This work investigates the novel chitosan-siRNA nanoparticle formulations developed in preceding chapters for cell viability, which was analysed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and WST-1 reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) on A-549 and H-292 epithelial cells respectively. Nanoparticles were also fluorescently labelled to track them for cellular (A-549) uptake studies using confocal microscopy. The effectiveness of siRNA delivery into cells is generally assessed by evaluating gene silencing (RNAi). Quantitative assessment of gene silencing was performed by real time polymerase chain reaction (RT-PCR) analysis of targeted genes (Bustin 2000). The in-vitro gene silencing experiment is a suitable surrogate for in-vivo gene silencing of siRNA and has been widely used to evaluate various polymeric carriers (Hagan et al. 2009). Interference has been reported during RNA purification prior to analysis when chitosan loaded siRNA was tested (Alameh et al. 2010), therefore in this chapter, we have attempted different optimisation techniques to explore the possibilities of minimising the interference and enabling gene silencing evaluation using RT-PCR.

5.2. Materials

Synthetic PTENV10-23-hmr with a molecular weight 7.4118 kDa and a duplex sequence (Sense: 5'-UAAGUUCUAGCUGUGGGGUA-3', antisense: 3'-AUUCAAGAUCGACACCACCCAAU-5' (specific) was donated by Integrated DNA technologies via AstraZeneca (UK). The human caucasian lung carcinoma cells (A-549; part number 32) were purchased from ECACC (UK). MTT, fetal bovine serum (FBS), penicillin streptomycin (Pen Strep), ultrapure DNase/RNase-Free distilled water, Dulbecco’s Modified Eagle’s Medium (DMEM), Quant-iT RiboGreen RNA reagent and kit were bought from Invitrogen (UK). RPMI medium 1640 (developed by Roswell
Chapter 5. Nanoparticle cell culture studies for cellular viability, uptake and gene silencing

Park Memorial Institute), phosphate buffered saline (PBS) and trypsin-EDTA was purchased from Gibco (USA). H-292 cells (Part number 22) were kindly donated by AstraZeneca (UK). PEG600 was bought from Fischer Scientific (UK). WST-1 reagent was supplied from Roche Diagnostics (Sweden) and kindly donated by AstraZeneca (UK). Agarose, DMSO, trypan blue, PEG 1000, TPP (85%), ethanol (≥95%), hydrochloric acid solution (2 M), paraformaldehyde and 96-well plates were purchased from Sigma-Aldrich (Germany). Nail varnish was purchased from a local shop (L’Oreal, France). High molecular weight water soluble chitosan glutamate (HMC); Protasan UP G 113, m.w 150 k-200 kDa, degree of deacetylation 75-90% was purchased from Novamatrix (Norway). Low molecular weight water soluble chitosan (LMC) with m.w >10 kDa and deacetylation degree 97.0% was purchased from Kittolife Co. Ltd (Korea). Polyethylene glycol 5000 monomethyl ether was obtained from Fluka (UK). Atufect transfection reagent was supplied by Silence Therapeutics (Germany).

5.3. Methods

5.3.1. Preparation of blank or siRNA encapsulated nanoparticles

Nanoparticles of grafted or physically associated PEG with chitosan were prepared by the ionotropic gelation method using TPP as a crosslinking agent (Section 2.3.3). A series of PEG grades (600, 1000 and 5000) were used in this study to achieve steric stabilisation of particles within the dispersions. siRNA was also encapsulated within these nanoparticles at different w/w ratios (10:1, 30:1 and 50:1) by dispersion within TPP or TPP-PEG solutions during ionic interaction. Chapters 2 and 3 gives more detail on the composition of nanoparticles.

5.3.2. In-vitro cell culture studies

a. Cell splitting and seeding

Flasks confluent with A-549 or H-292 cells containing DMEM or RPMI media respectively were placed inside a HEPA class 2 microbiological safety cabinet (Walker, UK). For cell splitting, media (25 ml) was removed and the cells were washed gently with 5 ml PBS (pH 7.4). PBS was aspirated and 5 ml trypsin was added to the flask which was incubated with 5% CO₂ at 37°C for 1 min in a cell culture incubator (Heracell, USA). The cells attached to the bottom of the flask were tapped and detached. In order to inactivate or neutralise trypsin, 5 ml media was added and mixed thoroughly.
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100 µl of cell-media was withdrawn and mixed with 100 µl of trypan blue. A drop from the stained solution was then placed on the cover slide of a haemocytometer (Section 5.3.6). This made the solution spread uniformly over the middle (5x5) square cross section of the haemocytometer and cells were counted using an optical microscope (Olympus CK40, Japan). Once the calculations were performed, known volume of cell-media suspension (~0.3 ml) were added to a new flask containing fresh media (25 ml) and incubated for cell growth in an incubator.

The remainder of the cell-media suspension was transferred to a falcon tube. Required dilution with media was performed to the cell-media suspension to attain 1x10^5 cells/ml. From this, 100 µl of media containing cells were withdrawn and added to each well of a 96-well plate to achieve a cell density of 1x10^4 cells/well. The prepared well plate was placed in the incubator for cells to grow.

b. Cell feeding

The cell culture flask was placed under a light microscope (Culture microscope CK40, Olympus, UK) to observe the growth and confluence of cells. This flask was placed inside the HEPA class 2 microbiological safety cabinet (Walker, UK) and the media (DMEM or RPMI) was replaced with fresh media. The flask was then placed inside the cell culture incubator (Heracell, USA) for cells to grow.

5.3.3. Influence of nanoparticles on cellular viability

a. HMC nanoparticles

A-549 cells (lung adenocarcinoma cells) were incubated (Heracell, USA) at 37°C and 5% CO₂ overnight in 100 µl DMEM media which consisted of 10% FBS and 0.5% antibiotics (Pen Strep), at a seed density of 1x10^4 cells per well. The media was removed and replenished with fresh media (100 µl) containing nanoparticles with chitosan concentration ranging from 0.05 to 0.42 mg/ml and incubated for 48 h. Post incubation, 20 µl MTT (5 mg/ml) dissolved in sterile PBS (pH 7.4) was added to each well and incubated for 4 h. Water soluble, yellow-coloured MTT is metabolised by cells to the water insoluble, purple-coloured formazan. Thereafter, the MTT solution was aspirated and 100 µl DMSO was added to each well to dissolve formazan crystals. The plate was incubated at 37°C for 5 min and DMSO was aspirated. The plates were read at 540 nm using a microplate reader (Wallac Victor2 1420 Multilabel Counter; Perkin Elmer, UK).
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The loss of cell viability was indicated by a reduced absorbance in formulation loaded wells compared to control (without formulation). Analysis was performed in triplicates.

b. LMC and LMC-g-PEG5000 nanoparticles

H-292 cells (lung muco-epithelial cells) were incubated (Heracell, USA) at 37°C and 5% CO₂ overnight in 100 μl RPMI media which consisted of 10% FBS, 1% Pen Strep and 1% glutamine at a seed density of 1x10⁴ cells per well. The media was removed and replenished with fresh media (100 μl) containing a range of nanoparticle formulation/siRNA (chitosan to siRNA w/w ratio of 30:1) for 24 h period in an incubator (37°C and 5% CO₂). The concentration of chitosan ranged from 2.6 to 83.3 μg/ml (n=3). The plate also contained non-treated cells, 40 nM of luciferase siRNA and PTEN siRNA delivered using atufect® (carrier) to ensure identical conditions and comparison studies with an established carrier system. After 24 h, the incubation media was aspirated off the cells. 10% WST-1 reagent along with media was added to each well and incubated for 15 min. WST-1 is reduced to form soluble, coloured formazan products by dehydrogenase present in intact mitochondria. Absorbance of the formazan product was measured at a wavelength of 540 nm using a microplate reader (SPECTRAmax M5; Molecular devices, USA).

5.3.4. Cellular uptake studies

Cover glasses (16 mm diameter, thickness number 1, FB58700, Fisher, UK) were cleaned in HCI (2 M), rinsed with deionised water and sterilised in ethanol (70 %) overnight, dried and coated with poly-lysine solution (100 μg/ml, P4832, Sigma-Aldrich, UK). A-549 cells in 0.5 ml indicator free medium (GIBCO 31053, Invitrogen, UK) were seeded over a sterilised and dry cover glass in a six-well plate (Nunc, Wiesbaden, Germany) at a density of 2x10⁵ cells/well. After incubation overnight leading to growth and attachment of cells to the cover glasses, a concentration of 916.6 μg/ml of FITC conjugated nanoparticles (20 μl) was added, and the cells incubated for 4 h. The media was then removed, the cells washed with PBS three times and fixed with fresh paraformaldehyde solution (2% in PBS) for 10 min at room temperature. After washing with PBS (5 x 2 ml), the cover glasses were placed face down on microscopy slides (631-0114, VWR international) using mounting medium (6 μL, Vectashield, Vector Laboratories, CA, USA). The edges of the cover glasses were sealed with nail varnish. Visualisation was performed with a confocal scanning laser microscopy (CSLM; 510 META confocal microscope, Zeiss, Germany). Within confocal microscopy a z-stack was performed to observe nanoparticle uptake at different cell depth.
5.3.5. Plate layout and operating protocol

The nanoparticle formulations were added to the 96-well plate with the layout as shown in Figure 5-1. The border wells surrounding the plate were loaded with media to avoid moisture ingress during incubation and temperature change from the rest of the wells. Non-treated cells (NTC) were used to act as the target RNA amount upon RNA isolation. Atufect® is a standard carrier with established results for the delivery of both luciferase (siRNA) and PTEN (siRNA). The control (c) was used to form a standard curve for RNA isolation calculations in gene silencing studies. The term “x” denotes media alone, whereas wells 1 to 12 in the middle denote chitosan:siRNA nanoparticles with and without PEG.

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Figure 5-1. Layout of prepared 96-well plates for in-vitro studies; NTC: Non treated cells, Luc: Luciferase siRNA, PTEN: siRNA, x: media alone, c: Control, 1 to 12 in the middle: chitosan:siRNA nanoparticles with and without PEG.

The protocol used to perform gene silencing studies required cell seeding in 96-well plates on day 1, formulation loading followed by 24 h incubation on day 2, cell viability assay and RNA isolation on day 3 and gene silencing on day 4.

5.3.6. Cell counting

Cell counting studies were performed to determine the number of cells present in the flask. A drop of cell solution stained with trypan blue was spread over a cross section of haemocytometer. The number of cells counted in the middle portion was attributed to that value times 10^5 cells/ml. This was diluted with media to attain 1x10^5 cells/ml as a final cell solution.
5.3.7. RNA isolation for gene silencing studies

H-292 cells were seeded (1x10^4 cells/well) in a 96-well plate with 100 μl RPMI media (containing 10% FBS, 1% Pen Strep) at 37°C and 5% CO_2 for 24 h prior to transfection. Following attachment, the medium was removed and the cells were transfected with chitosan/siRNA (PTEN) nanoparticles (30:1 or 50:1 w/w) and Atufect® transfection reagent with siRNA (non-specific siRNA; luciferase and specific siRNA; PTEN) at 40 nM. These samples were prepared in the respective growth medium (100 μl) containing FBS. The plates were then incubated for 24 h for complete siRNA transfection.

a. mRNA extraction

It is essential to treat transfected cell samples to eliminate contaminants such as DNA or other cellular matrix and extract RNA before subsequent use in the RT-PCR which is a technique for quantifying changes in gene expression across samples using total RNA extracted. This RNA isolation step was performed using spin column (silica) technology, following the protocol described in the RNeasy 96 handbook (Qiagen, UK). The medium was aspirated from the cells and 100 μl RLT buffer (pH 6.5) containing β-mercaptoethanol in the ratio of 100:1 (v/v) was added to each well to achieve cell lysis. RLT is a cell lysis buffer containing guanidine isothiocyanate. Complete media removal was essential to avoid RLT buffer dilutions which might cause inhibition of cell lysis or binding of RNA to the RNeasy membrane which could further reduce the yield. With RLT buffer, the plate was shaken back and forth for 10 s for complete spreading of the RLT buffer. To this plate, 100 μl of 70% ethanol was added with thorough mixing using a pipette (3 times). The RNeasy 96-well plate was placed on top of a square well block. The plate was marked with respect to 96-well plate (as described in Figure 5-1) and samples were transferred from the 96-well plate to the RNeasy 96-plate (kit) using a multichannel pipette without touching the sides of the well. Non-treated cells were all transferred into one well to prepare RNA standards and the plate was sealed using an air-pore tape sheet. The plate was centrifuged (Jouan centrifuge, UK) at ~5,600 xg for 4 min at room temperature. In order to remove DNA from the lysed cells, the wells were further exposed to a DNase1 incubation mix (80 μl). The plate was incubated at room temperature for 15 min and sealed with air-pore tape on top of the wells. At this stage, 0.8 ml of washing buffer RW1 was added to each well, sealed with air-pore tape and incubated at room temperature for 5 min before centrifugation (Jouan centrifuge, UK) at ~5,600 xg for 4 min. The period of 15 min and 5 min allowed DNA digestion to prevent DNA interference during RT-PCR. Another clean kit, square well block was placed beneath the RNeasy 96-plate, and in each well 0.8 ml of buffer
RPE was added. Buffer RPE is supplied as a concentrate. Before using RPE four volumes of ethanol (96–100%) was added to RPE to obtain a working solution. The RNeasy 96-plate was centrifuged (with an air-pore seal on top of the well) at \(~5,600 \times g\) (Jouan centrifuge, UK) for 4 min at room temperature. This step was repeated but using a 10 min centrifugation time to remove traces of cellular matrix apart from RNA and to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The RNeasy 96-plate kit was placed on top of a 1.2 ml microtube elution rack. This step was performed to elute RNA by adding 45 - 70 µl of RNase-free water to each well and incubating for 1 min at room temperature with an air pore seal on top of the plate. The plate was centrifuged as above. The step was repeated with 30 - 55 µl RNase-free water for complete elution. Pure RNA was isolated in microtubes at this stage, and these were sealed and stored at \(-20^\circ C\).

b. Pre-treated with chitosanase for mRNA extraction

Chitosan has been known to interfere with techniques used in nucleic acid (RNA) purification. It binds to nucleic acid and prevents it from binding to the silica column. This results in elution of the nucleic acid during centrifugation, and it not being available for quantification. RNA isolation in the presence of chitosan has also been found to be inefficient, using the following kits; Ambion, Roche, JENA and TRIzol (Tchemtchoua et al. 2009). One of the ways to improve RNA recovery was to treat the transfected cells with chitosanase, an enzyme that can digest chitosan and helps in the release of mRNA which was bound to chitosan (Alameh et al. 2010). Hence, cells incubated with chitosan:siRNA nanoparticles for a period of 24 h, were treated with chitosanase (Sigma Aldrich, UK) prior to RNA extraction. A final concentration of 6.12 mU of chitosanase per µg of chitosan, along with 100 µl RLT lysis buffer was used in each well. These plates were shaken on a bench for 20 s and incubated at 37°C and 5% CO₂ for 2 h. Following this the whole procedure for RNA isolation was the same as above.

c. Pre-treated at different pH for mRNA extraction

Another technique, involving a change in the media pH was implemented to achieve complete RNA purification and isolation. Since the cationic nature of chitosan helps binding with anionic nucleic acid, increasing the pH of the solution would be expected to result in a reduction of cationic charge on chitosan, and might result in RNA release from chitosan. Hence in this set of experiments formulation transfected cells (incubated for 24 h) were treated with 1.25 µl of 0.2M NaOH in 100 µl RLT lysis buffer (pH raised to 8.41). These plates were shaken on a bench for 20 s and incubated at 37°C and 5% CO₂ for 2 h. Following this the whole procedure for RNA isolation was the same as above.
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d. Pre-treated with reduced formulation and chitosanase for mRNA extraction

One of the other RNA recovery strategies was to reduce the formulation strength with respect to chitosanase. In this set of experiments; chitosan:siRNA nanoparticle strength was reduced to half with each well containing 20 nM of siRNA and incubated for 24 h. Before RNA purification at the cell lysis stage, these formulation transfected cells were treated with chitosanase. A final concentration of 6.12 mU of chitosanase per μg of chitosan along with 100 μl RLT lysis buffer was used in each well. These plates were shaken on a bench for 20 s and incubated at 37°C and 5% CO₂ for 2 h. Following this the whole procedure for RNA isolation was the same as above.

5.3.8. RNA quantification for gene silencing studies

Gene expression measurements were normalised against total RNA concentration. Normalisation of gene expression data was used to correct sample-to-sample variation (Bustin 2000). RNA was quantified using the RiboGreen assay (Invitrogen, UK) based on the manufacturer’s instructions (Jones et al. 1998). An aqueous solution of Quant-iT RiboGreen reagent was prepared by mixing 25 μl RiboGreen reagent with 9.975 ml of 20 times diluted RNase-free TE buffer (10 mM Tris–HCl and 1 mM EDTA at pH 8.0). 2 μl RNA samples were mixed with 50 μl aqueous solution of RiboGreen reagent and incubated for 5 min at room temperature, protected from light, in microplate wells. Fluoroscence measurements were acquired at an excitation wavelength of 500 nm and emission wavelength of 525 nm using a plate reader (Molecular Devices, UK) at room temperature. RNA prep standards were serially diluted and a calibration curve was plotted to quantify RNA within samples.

5.3.9. Real time reverse transcriptase polymerase chain reaction

Reverse transcriptase synthesizes a complementary strand of DNA (cDNA) using RNA as a template. This step precedes the polymerase chain reaction (PCR) which is a technique for cloning a particular piece of DNA. Quantitative RT-PCR was performed using a QuantiTect probe RT-PCR (Qiagen, UK) on a Stratagene real-time cycler (Quanta Biosciences, MX3000P, USA). The reactants (Figure 5-2) were assembled in a 96-well plate. Each well contained 18 μl of stock solution composed of 800 μl master mix (Taq polymerase), 6 μl forward primer, 6 μl reverse primer, 6 μl TaqMan probe and 622 μl RNase free water. To this, 2 μl of sample RNA was added. To these reactants 5 μl of positive reverse transcriptase mix (+RT mix) was added. Positive RT mix was composed of 180 μl RNase free water, 200 μl master mix and 20 μl RT. To
selected wells, 5 µl of negative reverse transcriptase mix (-RT mix) was added which comprised 50 µl RNase free water and 50 µl master mix. The total volume in a well was up to 25 µl. These reactants were centrifuged at 4000 xg at room temperature using a Jouan RC 10.22 TT centrifuge (ThermoFisher Scientific, UK) for 30 s.

Figure 5-2. Reactants used in the amplification of gene within RT-PCR (Biosystems 2011).

The plates were placed in the real-time cycler and the instrument was programmed with stages of high and low temperature for a set period of time. At the following parameters; 30 min at 50°C, mRNA is copied to cDNA by the reverse transcriptase technique using a reverse transcriptase enzyme. This removes the mRNA, allowing the second strand of DNA to be formed. cDNA is denatured at 90°C so that the two strands separate, and to activate heat stable Taq polymerase at this initial step. The sample was cooled to 60°C, a forward and reverse primer (synthetic sequence) anneals (binds) to the target strand of DNA (Figure 5-3).

Figure 5-3. Forward and reverse primers anneal to the gene (Biosystems 2011).
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The TaqMan probe is a sequence-specific oligonucleotide with a fluorophore (R) at either the 5’ or 3’ end of an oligo and a quencher moiety (Q) at the opposite end (Figure 5-4). The probe is designed to bind to the target DNA sequence. While the dye and quencher is intact, there is no fluorescence.

![Figure 5-4. TaqMan probe binds to the target RNA (Biosystems 2011).](image)

The temperature was raised to 72°C and at this point Taq polymerase which is stable at very high temperatures, extend the primer (forward and reverse) along the strand (Figure 5-5).

![Figure 5-5. Polymerase chain reaction (Biosystems 2011).](image)

This elongation causes the separation of the reporter from its quencher and allows the reporter to fluoresce upon using a light source in the real-time PCR instrument (Figure 5-6). At this point, four cDNA strands have been produced from the original two.
Each replication will result in the cleavage of a probe: as a result the fluorescent signal will increase proportionally to the amount of amplification product. This fluorescent signal is captured by the instrument after every cycle. The whole process was performed in around 40-45 cycles. The signal is further translated into a real-time PCR graph. The results were subdivided into three amplification stages; a) Exponential, b) Linear and c) Plateau (Figure 5-7).

In the exponential phase the reagents are in abundance and the PCR product doubles in every cycle. In the linear phase the reagents begin to exhaust and the PCR starts to slow down. In the plateau stage the reagents are depleted and the PCR reaction stops. The exponential phase was selected for analysing PCR amplification efficiency since it gives the most precise and accurate data.

Within the exponential phase, two values are calculated (Figure 5-8). The threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above
background noise. The fractional cycle number at which the fluorescence passes the fixed threshold is called the cycle threshold (Ct). All experiments were performed in triplicate. The PCR procedure can make virtually unlimited copies of a single DNA molecule even though it is initially present in a mixture containing many different DNA molecules.

![Graph of RT-PCR data showing threshold line and cycle threshold (Biosystems 2011).](image)

5.3.10. Summary of experiments conducted

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✔ = Experiment performed
5.4. Results and discussion

5.4.1. *In-vitro* assay of nanoparticle effect over cell viability

Chitosan toxicity has been extensively investigated by other research groups and reported in this section, but each published study has different assay conditions employed, in particular different degrees of deacetylation, molecular weight and salt form of chitosan. Although chitosan has not as yet achieved GRAS (Generally Recognised as Safe) status, it is considered a safe biomaterial and further studies are needed to demonstrate its safety for use (Casettari et al. 2011). This study also helps in evaluating the amount of formulation suitable for gene silencing without affecting the cell viability. Nanoparticles utilised for cell viability assay were not fluorescently labelled.

*a. HMC nanoparticles*

As shown in Figure 5-9, at 48 h post incubation chitosan concentrations up to 0.2 mg/ml for all formulations with and without PEG were not toxic to cells, presumably because the total polymer concentration was sufficiently low. The cell viability reduced greatly in comparison to other formulations beyond 0.2 mg/mL (chitosan concentration) with the lowest cell viability of around 40% attained at 0.42 mg/ml. This effect was not seen with PEG associated chitosan at this concentration as the cell viability stayed at 80%.

![Figure 5-9. A-549 cell viability post-exposure to nanoparticles with HMC (without siRNA) ranging from 0.05 to 0.42 mg/ml (with and without PEG) over a 48 h incubation period (mean ± S.D., n=3).](image-url)
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Excess chitosan in nanoparticles formed at high chitosan (114 kDa) to siRNA ratio (N:P/285:1) decreased cell viability and was related to the possible toxicity associated with HMC (Howard et al. 2006). Significant toxicity (p<0.05) was observed with nanoparticles having chitosan concentrations above 0.741 mg/ml when incubated with A-549 cells for a period of 4 h (Huang et al. 2004). The same group confirmed that chitosan solution did not show any significant difference (p>0.05) in cytotoxicity from chitosan nanoparticles at the same concentration.

Chitosan (150 kDa)/siRNA cytotoxicity with HEK293 cells was previously investigated using the MTT method for a period of 24 h and it was observed that at a chitosan concentration of 14 μg/ml, the cell viability was 100% (Dehousse et al. 2010). This could not be compared with our studies since the starting concentration in our study was 50 μg/ml showing a cell viability of ~80%. Cell viability using MTT assay following 24 h incubation with A-549 cells at a HMC (1900 kDa) concentration of 50 μg/mL was around 50% (Park et al. 2011). It was suggested that chitosan cytotoxicity over cells seems to depend not only on molecular size of chitosan but also on their chemical structure. This is the basis for our results not correlating with the studies of Park et al., (2011) as our studies involved a chitosan derivative (glutamate) with a relatively much lower molecular weight in comparison to the above stated study (1900 kDa).

In our study, the inclusion of PEG improved the cell viability. The differences in viability with different grades of PEG were not statistically significant (p>0.05). In general many studies reported that the introduction of PEG chain to the chitosan backbone reduces its cytotoxicity (Mao et al. 2005; Jiang et al. 2006). Due to limited amounts of siRNA available, the formulations were not loaded with siRNA for cell viability studies.

b. LMC nanoparticles

As shown in Figure 5-10, siRNA loaded nanoparticles (with and without PEG) at a chitosan nanoparticle concentration of 2.6 μg/mL did not show cellular toxicity (H-292 cells) over a period of 24 h exposure time, comparable with non-treated cells (no formulation loaded). At the highest chitosan nanoparticle strength (83 μg/mL) the cell viability reduced to ~85% for both non-PEG and PEG600 associated chitosan nanoparticles. LMC-PEG1000 and PEG5000 formulations showed a cell viability >90% and were not significantly different (p>0.05) to the cell viability results of formulations without PEG.
Figure 5-10. H-292 cell viability post-exposure to nanoparticles with LMC (encapsulated siRNA) ranging from 2.6 to 83.3 μg/ml (with and without PEG) over a 24 h incubation period. Luciferase (40 nM) was used as negative control and PTEN siRNA was also included (mean ± S.D., n=3).

Nanoparticles prepared at chitosan to siRNA w/w ratios of 30:1 were selected for cytotoxicity studies, because at this ratio particles showed almost complete binding to the siRNA, had a positive mean surface charge and nanosized structure (Section 2.4.3 and 3.4.7). Notably, both luciferase siRNA and PTEN siRNA showed 100% cell viability along with Atufect®, an agent commonly used to transfect cells. These observations collectively demonstrate that non-PEG and PEG containing LMC nanoparticles loaded with siRNA, at the LMC concentrations and siRNA ratios studied here do not affect cell viability and may be used for in-vivo applications.

Furthermore, the chitosan has shown molecular weight-dependent cytotoxic effects where water soluble chitosan (<10 kDa) at a low concentration (<1 mg/mL) showed negligible cytotoxic effect on the Caco-2 cells (incubated over 2 h) (Chae et al. 2005). The use of LMC in the development of safe gene carriers has also been long established (Lee et al. 2001). Complexes of 5 μg of pDNA and 10 μg of LMC (22 kDa) were formulated and added to 293T cells and incubated for 4 hours. The formulation showed negligible cytotoxicity (100% cell viability) for 293T cells. LMC were found not to be toxic (~100%) against CCRF-CEM human lymphoblastic leukaemia cells up to concentrations of 100 μg/ml (Richardson et al. 1999). These observations suggest that LMC are suitable as carriers for administration.

c. **LMC-g-PEG5000 nanoparticles**

LMC-g-PEG5000 nanoparticles loaded onto H-292 cells in increasing amounts of chitosan nanoparticle showed ~100% cell viability at concentration up to 5.2 μg/ml (Figure 5-11). An increase in chitosan nanoparticle concentration caused the cell viability to reduce constantly,
down to 71% (±1) measured at a concentration of 83.3 μg/ml. This reduction in cell viability in between the highest and the lowest chitosan nanoparticle concentration was statistically significant (p<0.05). Results with non-treated cells (no nanoparticle loading) Atufect®-luciferase and Atufect®-PTEN which are used as controls showed no cytotoxicity with 100% cell viability. Another chitosan derivative was studied and results suggest not so similar cell viability. Trimethylated chitosan oligomers (TMO) with degrees of quaternization of 40% (TMO-40) at a concentration of 1mg/ml have shown a cell viability of 100% over Caco-2 cell line (Thanou et al. 2002).

A study showed similar results where PEG (5 kDa) grafted trimethyl chitosan (~100 kDa) copolymers incubated for 24 h period, tested on a mouse connective tissue fibroblast cell line (L929) at a concentration 100 μg/ml showed a cell viability of ~80% (Mao et al. 2005). In another study it was reported that PEG (5 kDa)-g-chitosan (47 kDa)/DNA complexes exhibited a slightly lower acute toxicity to the liver than chitosan/DNA complexes (Jiang et al. 2006).

5.4.2. Cellular uptake studies

Confocal scanning laser microscope images demonstrated cellular internalisation of FITC-labelled LMC-g-PEG5000 nanoparticles (Figure 5-12c). High distribution of nanoparticles was observed not only in the cell cytoplasm but also in to the nucleus suggesting efficient cellular uptake of the formulation. The presence of FITC (labelled to chitosan) produced a strong fluorescence (green) detected using the confocal microscope. A control of A-549 cells without any nanoparticle loading and with FITC-labelled LMC polymer solution was also run and no
fluorescence was detected on confocal microscope. The study is an indication of intracellular uptake and is strong evidence of the movement of nanoparticles through cytoplasm to the nucleus. This property of novel LMC-g-PEG5000 nanoparticles is not a necessity for siRNA therapeutics since siRNA acts on mRNA which is present in the cytoplasm but could be highly advantageous for other biopharmaceuticals where the active needs to be transfected to the nucleus. Images were also taken at different depths of cell height (Figure 5-12 a - f). The green fluorescence caused due to FITC, increased gradually from image a to c and decreased from d to f. This confirms that the labelled nanoparticles are present deep within the cells and not just on the outer surface of the cell. The cellular uptake properties of chitosan nanoparticles can be attributed to the small particle size, positive charge (Section 2.4.3) and nanoparticle composition that facilitate interaction with cellular membrane.

FITC-labelled HMC and LMC nanoparticles with and without PEG did not show any cellular uptake. The reason to this observation is supported by the fact that chitosan has shown cell type dependency when transfecting across cell membranes (Mao et al. 2001). Similar studies performed demonstrated, HT-29 cell line was more difficult to transf ect with the specific chitosan (10 kDa)/siRNA formulation, at the cell uptake level (Alameh et al. 2010). In another study it was confirmed that the cellular uptake of the chitosan (80 kDa)-mediated siRNA was significantly enhanced in HT1080 (human fibrosarcoma) cells through the inclusion of poly(y-glutamic acid) (20 kDa) into complexes (Liao et al. 2010). They proposed that there might be a y-PGA-specific receptor-mediated pathway involved in the internalisation of these complexes. Likewise PEG may enhance the uptake of nanoparticles when grafted in comparison to associated PEG but the mechanism requires further investigation.
Figure 5-12. Confocal images of A-549 cells incubated for 4 h with FITC-labelled LMC-g-PEG5000 nanoparticles. Image a, b, c, d, e and f were taken at a cell depth of 8.3 μm, 11.1 μm, 13.9 μm, 16.7 μm, 19.5 μm, and 22.2 μm respectively.
5.4.3. mRNA quantification

It is essential to purify and isolate RNA from the cells before mRNA expression analysis. Complete purification and isolation of RNA, not only increases the mass of RNA obtained for downstream processing, but presents it in a more concentrated form. Table 5-2 shows total mRNA obtained from each well on a 96-well plate.

Table 5-2. Total mRNA quantification; post RNA isolation using various parameters (mean ± S.D., n=3).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Formulation</th>
<th>Total mRNA (ng/µl)</th>
<th>Non-optimised</th>
<th>Chitosanase pH change (pH8.4)</th>
<th>Reduced formulation with chitosanase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non-treated cells (Control)</td>
<td>20.0 (±1.9)</td>
<td>20.7 (±2.8)</td>
<td>19.4 (±2.2)</td>
<td>22.4 (±1.1)</td>
</tr>
<tr>
<td>2</td>
<td>Atufect®-Luciferase</td>
<td>22.0 (±2.4)</td>
<td>22.3 (±0.2)</td>
<td>22.7 (±2.3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Atufect®-PTEN</td>
<td>19.8 (±1.4)</td>
<td>20.5 (±3.3)</td>
<td>21.7 (±1.4)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HMC:siRNA (30:1)</td>
<td>3.0 (±0.6)</td>
<td>10.95 (±0.4)</td>
<td>7.20 (±2.5)</td>
<td>16.9 (±2.5)</td>
</tr>
<tr>
<td>5</td>
<td>HMC:siRNA (50:1)</td>
<td>2.6 (±0.6)</td>
<td>11.24 (±0.4)</td>
<td>10.7 (±1.7)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HMC-PEG1000:siRNA (30:1)</td>
<td>3.0 (±0.6)</td>
<td>12.14 (±0.9)</td>
<td>15.8 (±1.9)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HMC-PEG1000:siRNA (50:1)</td>
<td>2.1 (±0.2)</td>
<td>12.58 (±1.8)</td>
<td>2.6 (±1.4)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>LMC:siRNA (30:1)</td>
<td>7.4 (±1.5)</td>
<td>9.70 (±1.4)</td>
<td>9.91 (±0.9)</td>
<td>11.1 (±2.6)</td>
</tr>
<tr>
<td>9</td>
<td>LMC:siRNA (50:1)</td>
<td>3.9 (±0.5)</td>
<td>7.97 (±1.2)</td>
<td>13.3 (±0.9)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>LMC-PEG1000:siRNA (30:1)</td>
<td>9.0 (±1.5)</td>
<td>15.38 (±1.7)</td>
<td>10.5 (±0.5)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>LMC-PEG1000:siRNA (50:1)</td>
<td>5.5 (±1.0)</td>
<td>13.38 (±0.9)</td>
<td>11.2 (±1.0)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>LMC-g-PEG5000:siRNA (30:1)</td>
<td>8.0 (±0.5)</td>
<td>13.30 (±1.5)</td>
<td>22.4 (±1.4)</td>
<td></td>
</tr>
</tbody>
</table>

a. Non-optimised

Non-optimised, non-treated cells achieved a RNA level of 20.0 ng/µl (±1.9) which also acts as target level to be achieved by other cells incubated with chitosan:siRNA formulations (with and without PEG). Similar levels were obtained for Atufect®-Luciferase and Atufect®-PTEN. This showed that RNA could successfully attach to the silica column which was then further isolated...
and quantified using the RiboGreen technique. Cells incubated with HMC formulations (with and without PEG) showed a very low level of RNA recovery (around <4 ng/μl). Similarly, total RNA recovered from cells incubated with LMC formulations (with and without PEG) was low, though higher than for HMC (around <10 ng/μl). This could be due to the interference of chitosan during RNA isolation, causing RNA to elute through the silica filters. Since gene silencing is back calculated from the total RNA recovered from each well containing the formulation, HMC, LMC and LMC-g-PEG formulations cannot be considered for gene silencing testing by this procedure. Nucleic acids binding to chitosan when extracting RNA for subsequent quantitative PCR evaluation of silencing resulted in low RNA yield has been observed by other research groups working with chitosan (Hagan et al. 2009; Tchemtchoua et al. 2009; Alameh et al. 2010).

b. Chitosanase enzyme

Upon inclusion of chitosanase, it was observed that HMC, LMC and LMC-g-PEG formulations gave improved total RNA levels (Table 5-2). Total RNA levels quantified in non-treated cells were the same in the absence and in the presence of chitosanase. Total RNA recovered in chitosan formulations (with and without PEG) was still less than total RNA obtained from non-treated cells (20.7 ±2.8 ng/μl). This recovery level was too low to proceed with gene silencing studies. Experimental investigation of the effect of chitosanase on LMC performed by another group has led to conflicting results (Alameh et al. 2010). RNA level quantified from HepG2, HT-29 and caco-2 cells incubated with chitosan formulation (in absence of chitosanase) was 2.6, 1.7 and 3.9 ng/μl respectively whereas in the presence of chitosanase, the RNA levels increased to 78, 83 and 76 ng/μl (Alameh et al. 2010). The difference in results with the use of chitosanase could be due to the type of chitosan, molecular weight and degree of deacetylation of chitosan used within the system. The effect of chitosan on nucleic acid binding is so efficient that an approach to coat silica particles with chitosan was taken to improve total mRNA recovery in a microfluidic device (Hagan et al. 2009). A recovery efficiency of 71% (under aqueous conditions) with chitosan coated silica particles in comparison to standard silica phase under chaotropic conditions (53%) was observed. Another alternative was the use of cesium chloride cushion (during centrifugation). These have demonstrated to provide a yield of 95.11% in comparison to other kits such as Ambion (2.8%), Roche (4.91%), Jena (1.03%), TRizol (3.36%) (Tchemtchoua et al. 2009).
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c. pH controlled optimisation

In order to retrieve total mRNA from the cells, a pH controlled approach was implemented. Increasing the system pH from 6.5 to 8.4, promotes chitosan to lose cationic nature. This can help anionic RNA to be released from chitosan and attach to the column during RNA isolation and purification stage. Non-treated cells incubated with and without 0.2M NaOH showed similar RNA recovery (Table 5-2). This suggested that pH change does not interfere with RNA retention. The results showed an increase in RNA recovery for both HMC:siRNA (7.2 ±2.5 ng/µl) and LMC:siRNA (9.91 ±0.9 ng/µl) formulations in comparison to non optimised formulations. This increase in RNA recovery still did not compare favourably to non-transfected cells (19.4 ±2.2 ng/µl) indicating that chitosan continued to support RNA elution from the column during RNA isolation and purification. A similar approach was adopted by another group, proposing DNA showed strong binding affinity to positively charged chitosan but was released at a pH above the pKa of the amino group (6.3) (Hagan et al. 2009).

d. Reduced formulation with chitosanase enzyme

In order to enhance RNA extraction, another method was tested. This involved reduction in siRNA bound nanoparticle strength to half incubated with chitosanase enzyme (Table 5-2). All formulations showed low RNA recovery except, LMC-g-PEG5000:siRNA formulation (22.4 ±1.4 ng/µl) which showed equivalent RNA recovery to non-treated cells (22.4 ±1.1 ng/µl). Since, RNA recovery at low siRNA and chitosan amounts (formulation 12) show difference from other optimisation parameters, it seems that there exist a window within which the formulation (at appropriate ratio of chitosan to siRNA) show maximum RNA recovery.

5.4.4. Gene silencing

Taqman (RT-PCR) was further performed to determine the gene silencing ability of siRNA (PTEN) loaded chitosan formulations (Figure 5-13). Cells transfected with Atufect®-PTEN showed a gene silencing of 97 ±2.7%. No knockdown was detected with Atufect®-Luciferase (1.6 ±12.5%). This is due to the non-specific nature of luciferase. Results with LMC-g-PEG5000:siRNA in ratio of 30:1 w/w showed a gene silencing of 58.20 ±18.60%. The gene knockdown ability of this nanoparticle formulation as shown in this work supports its potential use for in-vivo pulmonary RNAi-based therapies. The gene silencing results of rest of the formulations could not be taken into consideration since the total RNA content was significantly (p<0.05) less than the total RNA content of the non-treated cells.
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Figure 5-13. Gene silencing data for siRNA (PTEN) loaded chitosan formulations tested using taqman RT-PCR. These results are based on total RNA content and only confirm the gene silencing of Atufect-Luciferase, Atufect-PTEN and LMC-g-PEG5000:siRNA (30:1 w/w).

PEGylation of polymeric carriers have been employed in the past for efficient delivery of siRNA to cells. PEGylated poly-l-arginine (15-70 kDa) grafted chitosan (50-150 kDa)/siRNA complexes produced a significant reduction (down to 10.4 ± 4.5%) in levels of the target protein (red fluorescent protein), in tumor tissues of the mice (Noh et al. 2010). It was confirmed that chitosan conjugates (PLR and PEG) provided an enhanced delivery of siRNA compared with chitosan alone. Similar to this observation, a previous study reported that the complex of siRNA with LMC (about 10 kDa) showed little knockdown of endogenous EGFP in H1299 human lung carcinoma cells (Liu et al. 2007). In another study chitosan (80 kDa)/luciferase-siRNA/poly(y-glutamic acid) (20 kDa) complexes showed greater gene silencing efficiency (50%) within 24 h after treatment onto HT1080 (human fibrosarcoma) cells (Liao et al. 2010). Chitosan along with its conjugates have also been tested as a carrier for RNAi therapeutics to brain cancer cells (Veiseh et al. 2010). Iron oxide nanoparticles coated with chitosan, PEG, and PEI was prepared. This was further functionalised with siRNA and a tumor-targeting peptide, chlorotoxin (CTX), to improve tumor specificity. The research group suggested that the surface chemistry of a nanovector regulates the internalisation of the nanoparticles. These magnetic nanoparticles were internalised into C6 rat glioma cells and reduced GFP mRNA levels to 0.23 relative to those in untreated cells, a 4.3 fold knockdown in gene expression. In another study it was demonstrated that chitosan/polyguluronate nanoparticles encapsulating siRNA were more efficient to deliver siRNA into HEK 293FT cells expressing luciferase (55% gene silencing),
Chapter 5. Nanoparticle cell culture studies for cellular viability, uptake and gene silencing compared with chitosan/siRNA nanoparticles (60% gene silencing) (Lee et al. 2009). In some studies the successful use of chitosan for gene silencing has also been demonstrated. Chitosan (114 kDa)/siRNA (N:P 57) nanoparticle mediated knockdown of EGFP (77.9%) was demonstrated in H1299 human lung carcinoma cells 48 h post-transfection (Howard et al. 2006).

It was concluded that in-vitro gene silencing of chitosan/siRNA nanoparticles are strongly dependent on chitosan molecular weight, degree of deacetylation, chitosan conjugates and chitosan:sirNA ratio. The application of chitosan/siRNA nanoparticle aerosols for improved pulmonary delivery and gene silencing in-vitro has been studied recently (Nielsen et al. 2010). EGFP knockdown in EGFP-stably-expressed H1299 cells were performed to evaluate nebulisation effects on nanoparticle-mediated gene silencing. Chitosan/siRNA (N:P 57) nanoparticles, in-vitro demonstrated a 68% reduction in fluorescence, which did not alter significantly after nebulisation (62%). PTEN gene is a universal anti-tumor gene and has proven to be a good therapeutic target for the development of gene therapy strategies. These encouraging results have proved the effective in-vitro gene silencing ability of PTEN siRNA when in particular delivered through chitosan-grafted PEG nanoparticles as gene carriers.

5.5. Conclusion

We have successfully demonstrated appropriate concentrations of PEG associated HMC (420 μg/ml) and LMC (83.3 μg/ml) formulations as non-toxic siRNA carriers with cell viability >80% for the delivery of siRNA. These formulations did not show cell uptake properties and could not be quantified for gene silencing due to chitosan interference in the process of mRNA extraction.

LMC-g-PEG5000 appears to be an ideal drug delivery system for siRNA as it was prepared in an aqueous environment without the use of detrimental forces and encapsulated siRNA using electrostatic interactions. Under the conditions employed in the cell viability assay, LMC-g-PEG5000 nanoparticles demonstrated >80% viability up to chitosan concentrations of 10.4 μg/ml. The formulation showed highly efficient cellular uptake. Experimental studies showed that mRNA is difficult to extract from chitosan/siRNA-transfected cells for subsequent quantification of gene expression. A relatively simple technique based on enzymatic degradation of chitosan was employed but still the recovery of total RNA was low for most of
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the formulations, except LMC-g-PEG5000:siRNA in ratio of 30:1 w/w which showed a gene silencing of 58.20 (±18.60)%.

In conclusion, the application of chitosan as a non-viral carrier for siRNA with no toxic implications and the formulation which showed gene silencing was verified. More investigation is required in the recovery of mRNA so that gene silencing of other formulations could be tested.
CHAPTER 6

GENERAL DISCUSSION
6. General discussion

6.1. Nanoparticle preparation

In the research work of this thesis, biodegradable crosslinked nanoparticles were produced and evaluated in-vitro as potential carriers of biopharmaceuticals, such as siRNA, for pulmonary delivery. The study was performed using crosslinked high (150 k - 200 kDa) and low (>10 kDa) molecular weight chitosan as nanoparticles. The molecular weight of chitosan may be considered as a critical parameter of chitosan transport across cell layers. Polar materials with molecular weights below 1 kDa generally pass through the epithelial cell membranes by the paracellular route, i.e through the tight junctions between the cells (Illum 2003). HMC cross membranes by the transcellular route (Chae et al. 2005). In this study chitosan and chitosan glutamate were used to prepare nanoparticles, since they do not require additional processing or solubilising acids such as acetic acid within neutral aqueous media (water). This is useful to avoid the toxicity and harmful effects of solubilising acids on the integrity and activity of biopharmaceuticals (Liu et al. 2008b).

Chitosan has been used both as a solution and as particles to deliver drug molecules to target sites. Generally it has been observed that chitosan powders or particle formulations are better absorption enhancers, compared to chitosan solutions (Illum 2003). Chitosan nanoparticles were prepared using inter and intra molecular linkages created between the polyanion TPP and the polycation (chitosan), resulting in ionic gelation. Care is essential during the processing of biopharmaceutical drugs as most of them are labile (Shoyele and Cawthorne 2006); hence ionic gelation, a cost effective, simple, single step, low energy method for nanoparticle preparation was utilised. Ionic gelation is beneficial compared to crosslinking with glutaraldehyde since the biopharmaceuticals could otherwise suffer from covalent cross linkages during nanoparticle preparation (Ohya et al. 1993). Alginates are an alternative crosslinking agent, though they swell when exposed to acidic medium (Calvo et al. 1997) which could be problematic given the slightly acidic nature of the lung (Mohri et al. 2010). One of the other advantages of the ionic gelation method is that the physicochemical properties of the nanoparticles can be modulated by changing the ratio of TPP to chitosan (Calvo et al. 1997). High amounts of TPP can lead to aggregation, since large numbers of anions neutralise the polymer. Experimental studies have shown that the nanoparticle formulations were
reproducible and stable at the following ratios; HMC:TPP ratio (5:1) w/w and LMC:TPP ratio (10:1) w/w. These optimised formulations were used in further investigations.

PEG was either grafted on to the chitosan molecules or was physically associated with the chitosan. Chemical modification of chitosan with PEG has been seen as a way of improving the biocompatibility of chitosan (Zhang et al. 2002). It has also been shown to facilitate the transport of a tetanus toxoid across the nasal epithelium (Tobio et al. 1998). Nanoparticle surface modification using non ionic polymers, such as PEG, has been shown to reduce irritation to lung epithelia and bronchial tissues (Suzuki et al. 2000). FTIR (Figure 2-6) and NMR (Figure 2-7) results confirmed that PEG5000 was successfully grafted to LMC in these studies. The successful grafting of PEG with chitosan has also been reported by other groups using the same synthetic and analytical techniques (Jeong et al. 2008). Different molecular weight PEG (600, 1000 and 5000) was physically associated with both HMC and LMC during ionic gelation to prepare crosslinked chitosan-PEG nanoparticles. HMC-PEG preparations were characterised for their physical interaction with PEG both before and after dialysis using FTIR (Figure 2-8). Results indicated an absence of PEG600 in the nanoparticles whereas PEG1000 and PEG5000 were both present after dialysis. This indicates that PEG1000 and PEG5000 were entrapped within chitosan nanoparticles and could not pass freely through the relatively large pores of the dialysis bag. Thus, the method employed to form nanoparticles did not result in association of PEG600 with the particles.

6.2. Nanoparticle physicochemical characterisation

The hydrodynamic diameter for all nanoparticle formulations was less than 230 nm (Section 2.4.3). A small particle size is essential since aerosolised particles must reach the deep lung and enter the cells to achieve the desired therapeutic outcome. Secondly, it has been reported that particles of diameter less than 260 nm are minimally taken up by alveolar macrophages (Shoyele and Cawthorne 2006). HMC nanoparticles showed a significantly larger particle size than LMC. This could be due to the long chains of the polymer forming bigger particles. The inclusion of high molecular weight PEG (5000) increased the particle size for both HMC and LMC. Similarly it was previously observed that the increase in molecular weight of PEO (poly ethylene oxide) and PEO-PPO (poly propylene oxide) led to an increase in the size of crosslinked chitosan nanoparticles (Calvo et al. 1997). The surface charge of nanoparticles was investigated, since this property is important for association with negatively charged siRNA and interaction with negatively charged lung cells (Section 2.4.3). The degree of deacetylation of
LMC (97%) is higher than HMC (75 to 90%), hence LMC nanoparticles were more positively charged. A positive surface charge should induce a repulsive particle-to-particle ionic interaction, which may enhance the physical stability of nanoparticle dispersions.

HMC-PEG5000 and LMC-g-PEG5000 nanoparticles, viewed using TEM were similar in appearance, being small and of varying shape (Figure 2-15, Figure 2-16, Figure 2-17 and Figure 2-18). It was not possible to observe HMC nanoparticles without PEG using TEM (Figure 2-14). All nanoparticle formulations were freeze-dried. The advantage of freeze-drying over spray-drying is that it is reproducible and easy to scale up. Spray-dried chitosan microspheres have been reported to swell quickly in water and release the encapsulated drug immediately (Desai and Park 2005). SEM studies showed that LMC nanoparticles and HMC nanoparticles (with and without PEG) were between 200 nm and 1 μm (Sections 2.4.7 and 2.4.8). The pH of the bio-relevant media could potentially change the physicochemical properties of the nanoparticles upon administration; hence the effect of pH on the surface charge and size of nanoparticle formulations was studied. It was found that the physical stability of non-PEG and PEG-chitosan nanoparticles was pH-dependent and particles were not aggregated from pH 5.5 to 7 (Section 2.4.9). This suggests that the delivery of chitosan nanoparticles (with and without PEG) to the lung where the pH is 6.5 (Mohri et al. 2010), should not bring any change in the size of nanoparticles bearing a positive surface charge.

6.3. Nanoparticle dispersion within propellant

Preliminary investigation of the physical stability of nanoparticles dispersed within model propellant (DFP) was performed visually, using the naked eye. For a thorough dispersion of the nanoparticles in the non-polar medium, sonication was performed. It was observed that a 15 min total duration for nanoparticle/propellant sonication was sufficient to achieve a good dispersion (Figure 2-30). Dialysed and un-dialysed nanoparticle formulations suspended in DFP differed little in the stability of their dispersions (Figure 2-31). Nanoparticle dispersibility was found to vary with molecular weight of PEG used in the formulation. PEG1000 associated nanoparticle dispersions showed good dispersion properties in DFP, being readily dispersible and with ≥10 min suspension stability after shaking (Table 2-4). Further, it seems that PEG is even less soluble in HFA at molecular weight >1000 since PEG5000 was turbid. Similar observations were made by another group where polyoxyethylene (POE) solubility decreased with increasing molecular weight, becoming insoluble at molecular weight >1000 (Ridder et al. 2005). The degree of steric stabilisation of particles suspended within a pMDI system is
dependent on the surface layer thickness (Traini et al. 2006) and it seems PEG1000 is more effective at reducing cohesion between nanoparticles due to a long unfurled chains, which was not improved by using PEG5000. Previously, PEG1000 and PVP K25 copolymers in the model propellant 2H, 3H perfluoropentane (HPFP) reduced the interactions between formoterol fumarate dihydrate particles (Ashayer et al. 2004). The results also indicated that charge (<30 mV) does not play an important role in stabilisation of nanoparticles within non-polar media. This study correlates with the findings of another study where it was established that carbon particles with charge <100 mV were not effective in stabilising suspension in a non-aqueous vehicle (Pugh et al. 1983). These observations also agree with an earlier study, which stated that high surface charge is essential for electrostatic stabilisation in an aqueous media (Kitahara 1974). Further investigations were performed to determine the dispersion properties of nanoparticle formulations within commercial HFA propellants.

Long term stability, dispersibility and ease of re-dispersion of pMDI suspension formulations are key parameters for the quality of an inhalation product. It was visually observed that LMC nanoparticles without PEG was better dispersed in propellant (HFA-134a and 227) in comparison to HMC nanoparticles. This could be due to the polymer composition and molecular weight which are probably the main factors affecting the destabilisation rate of a polymer (Wang et al. 2000). PEG1000 associated HMC and LMC nanoparticles suspended within HFA were stable for the longest period of time (~10 min) except for LMC-PEG1000 suspended within HFA-134a (Section 2.4.11). The polymeric chain length of PEG1000 seems optimal to provide steric stabilisation and to minimise particle-particle interaction between nanoparticles. It has been claimed that suspension homogeneity of the formulation should persist for at least 5-10 min, providing sufficient time for patients to inhale medication following shaking of the canister (Selvam et al. 2011). It is hypothesized that PEG1000 is able to interact with both particles and the medium in a way that the polymer is adsorbed to the particle substrate yet is freely soluble in the liquid propellant. The beneficial effect of PEG in pMDI suspension formulations has also been highlighted previously (Steckel and Wehle 2004). Hydrophilic PEG300 covered the surface of particulate budesonide and HP-β-CD (hydroxypropyl-β-cyclodextrin) which reduced interfacial energy between the propellant (HFA-227) and the particles resulting in dispersion stability of around 3 months. Our studies suggest PEG600, PEG5000 and LMC-g-PEG5000 were not able to achieve the same degree of physical stability resulting in either sedimentation or creaming of the nanoparticles. Another observation is the variation in the dispersion properties of some formulations when suspended
in either HFA-134a or HFA-227. This could may be because HFA-134a has lower density than HFA-227.

The crosslinked chitosan nanoparticles, with and without PEG, did not visibly adhere to the walls of polyethylene terephthalate (PET) vials, even after one month’s storage. The stabilising effect of different molecular weight PEG (200, 400 and 600) at different concentrations (0.05%, 0.1%, 0.25%, 0.5% v/w) for salbutamol sulphate in a model pMDI systems has been explored previously (Traini et al. 2006). It was suggested that a high concentration of PEG200 (above 0.25% v/w) was required to achieve the same degree of steric hindrance as provided by PEG600 at the lowest concentration. This might be due to an increase in particulate surface coverage and polymer protrusion in HFA, with increased molecular weight. Our study suggests chitosan-PEG1000 nanoparticles are adequately dispersed in HFA and should be readily delivered from pMDI.

Further dispersion studies were performed using a near infra red light scattering technique (Section 2.4.12). The Turbiscan results supported the observations made visually. The smaller the particles, the stronger the cohesive forces (French et al. 1996), resulting in aggregation. This aggregation behaviour was observed for most HMC and LMC nanoparticles when dispersed either in HFA-134a or HFA-227. No aggregates were visible in chitosan-PEG1000 HFA systems due to the reduction in inter-particle attractive forces. PEG, associated with nanoparticles was able to stabilise chitosan nanoparticle in HFAs without the need of additional surfactants or co-solvents such as ethanol. Generally PEGs show higher solubility in HFA-227 than to HFA-134a, which suggests stronger interactions between surfactant (PEG) and HFA-227 (Ridder et al. 2005; Peguin and Da Rocha 2008). Our results agree with this study since most of the formulations in HFA-227 were stable for longer than in HFA-134a. Hence HFA-227 was used as a propellant of choice for formulation studies using pMDIs. The data suggest that an optimal PEG size for dispersion stability in HFA is required. Other additional factors influencing dispersion stability are; the type of propellant, polymer concentration, molecular weight and type of polymeric stabilising excipients used to reduce interparticulate forces. PEG1000-chitosan nanoparticles showed particular promise for the administration of therapeutic such a siRNA to the airways.
6.4. Preparation and characterisation of siRNA loaded nanoparticles

Chitosan was evaluated as a carrier for siRNA in the form of crosslinked nanoparticles (siRNA entrapped and adsorbed) and as nanocomplexes (simple complexation without a crosslinking agent). These approaches were used since they employ mild preparation conditions and were relatively simple to prepare. Chitosan nanoparticles with encapsulated siRNA were of mean size less than 250 nm and had a positive surface charge in the range of +14 to +41 mV (Sections 3.4.1 and 3.4.2). When comparing unloaded nanoparticles with nanoparticles encapsulating siRNA at a chitosan to siRNA ratio of 10:1 w/w a significant increase (p<0.05) in the hydrodynamic diameter for non-PEG, PEG600 associated chitosan and LMC-g-PEG5000 formulations was observed. PEG1000 and PEG5000 associated formulations did not show a significant change (p>0.05) in hydrodynamic diameter and surface charge. Previously it has been suggested that the lecithin/lactose nanoparticles showed an increase in particle size upon inclusion of model protein (lysozyme) (Nyambura et al. 2009b). Nanocomplexes were of mean size less than 280 nm and had a positive surface charge in the range of +32 to +58 mV (Sections 3.4.3 and 3.4.4). It was observed that in presence of higher amount of siRNA in HMC with and without PEG, LMC-PEG1000 and LMC-g-PEG5000 nanocomplexes, the hydrodynamic diameter was larger (p<0.05). Simultaneously the surface charge reduced with higher amount of siRNA for almost all formulations.

The loading efficiency of siRNA into chitosan nanoparticles was determined using a PicoGreen assay. Recent studies have shown that electrostatic interaction of chitosan with siRNA yielded 83% to 94% loading efficiency (Liu 2010). Previously it has been reported that siRNA loading of chitosan nanoparticles prepared by ionic gelation reached 100%, protecting siRNA from nuclease degradation (Katas and Alpar 2006). Almost all formulations in this study except LMC-g-PEG5000 nanoparticle had ≥90% siRNA encapsulation efficiency (Section 3.4.5) indicating that PEG association with chitosan does not affect siRNA binding. The low loading efficiency of LMC-g-PEG5000 (57%) could be due to the grafting of the amine groups of chitosan to PEG which hindered siRNA binding to chitosan.

Agarose gel electrophoresis confirmed the ionic interaction of chitosan with siRNA making the nanoparticle suitable for siRNA adsorption and/or entrapment (Figure 3-14). Similar ionic interaction was demonstrated in another study, where negatively charged bovine serum albumin (PH 4.5 to 5) was encapsulated within positively charged chitosan nanoparticles (Calvo et al. 1997). The activity of the nanoparticle bound siRNA molecules remained similar to siRNA.
control indicating that siRNA was not degraded during nanoparticle preparation (Section 3.4.6). Compared to the control (naked siRNA), chitosan (HMC and LMC) binding retarded the mobility of siRNA on the gel. No difference in the interaction of siRNA with nanocarrier was observed for adsorbed or entrapped siRNA. It was previously demonstrated using gel electrophoresis that adsorption of siRNA results in weak binding between chitosan and siRNA (Katas and Alpar 2006). These authors also suggested that siRNA entrapment gave better gene silencing compared to particles prepared by simple complexation and adsorption of siRNA onto the preformed crosslinked chitosan nanoparticles.

The interaction of siRNA with crosslinked chitosan (nanoparticles) and non-crosslinked chitosan (nanocomplexes) is similar, with little difference in association observed by gel electrophoresis (Sections 3.4.7 and 3.4.8). Unlike LMC and HMC nanoparticles with and without PEG, LMC-g-PEG5000 polymer showed weak siRNA association at almost all weight by weight ratios (Figure 3-17). Nucleic acids generally have biological origin and can induce immune response once administered. Hence using nanoparticles for encapsulation of nucleic acids might help attenuate the activation of immune response (Skjerringe et al. 2009). The activity of siRNA following dispersion in the model propellant (DFP) (Figure 3-21) was similar to naked siRNA (not exposed to DFP) as assessed by gel electrophoresis, indicating that siRNA could potentially be delivered using a pMDI.

6.5. Aerosolisation of nanoparticles with and without siRNA

The aerodynamic characteristics of the nanoparticles were evaluated, following delivery, from a pMDI and an air-jet nebuliser. Covalent conjugation of the fluorescent probe (FITC) to chitosan is a rapid, simple and sensitive means to quantify the extent of nanoparticle deposition in-vitro upon aerosolisation. FITC-labelled chitosan nanoparticles showed negligible difference in hydrodynamic diameter, surface charge and morphology when compared to unlabelled chitosan nanoparticles (Sections 4.4.2 and 4.4.3).

6.5.1. Jet nebuliser

In order to identify the most suitable formulation for jet nebulisation, a TSI was employed (Section 4.4.4). Amongst the investigated formulations, the FPF (<6.4 μm) of non-PEG and PEG associated HMC nanoparticles ranged from 66% to 76% whereas for non-PEG and PEG associated LMC nanoparticles the FPF ranged from 54% to 61%. Corresponding formulations of
PEG600 associated with HMC and LMC nanoparticles, PEG1000 associated with HMC and LMC nanoparticles differed in their FPF significantly (p<0.05). LMC-g-PEG5000 nanoparticles showed a FPF of 49%. Nanoparticle deposition in TSI is dependent on nebuliser properties and not so much on nanoparticle properties as previously reported for liposomes (Bridges and Taylor 1998). Nebulised formulations containing different molecular weight of PEG and chitosan were analysed for their morphology following aerosolisation. Particles appeared structurally stable and were of measured diameter <1 μm. This measured size was larger than before nebulisation but such nanoparticle aggregates would still be suitable for lung delivery (Section 4.4.5). Some biopharmaceuticals undergo denaturation and degradation during nebulisation. This loss of activity during jet nebulisation has been attributed to the high shearing forces within the nebuliser (Thanoo et al. 1992; Niven and Brain 1994; Fängmark and Carpin 1996). Following nebulisation, gel electrophoresis results showed loss of siRNA integrity in absence of carrier whereas siRNA activity was similar before and after nebulisation in presence of a carrier signifying siRNA stability and successful aerosolisation using nanoparticles (Section 4.4.6). The FPF of nebulised siRNA encapsulated LMC nanoparticles determined using the TSI was 57% which was similar to unloaded LMC nanoparticles (54%) indicating no significant effect of siRNA loading on aerosolisation of the nanoparticles from jet nebulisers (Section 4.4.7). A FPF of 40% to 52% was achieved when chitosan-stearic acid conjugate nanomicelles encapsulating amphotericin B (Gilani et al. 2011) were aerosolised using a jet nebuliser.

6.5.2. pMDI

For the preparation of nanoparticles suspended within propellant, only one concentration of nanoparticles was investigated (HMC nanoparticles, 7 mg; LMC nanoparticles, 4.8 mg) in 8.4 g propellant. Further optimisation may be required for different concentrations of the nanoparticles. The SEMs of LMC-PEG1000 nanoparticles dispersed in HFA-227 and actuated from a pMDI showed smallest particles (<500 nm) which appeared slightly fused together (Section 4.4.8). Laser diffraction sizing technique was used at the pre-formulation stage of suspension pMDI development (Sections 4.4.9 and 4.4.10). HMC-PEG1000 and LMC-PEG1000 produced aerosols having VMD of 1.53 μm and 1.43 μm respectively. LMC-PEG1000 suspension pMDI formulations were prepared with siRNA because of their high dispersibility, stability and aerosolisation properties. The presence of a relatively high amount of siRNA (1.0279 μg/actuation) might be the reason for an increase in VMD from 1.43 μm to 4.42 μm.
Chapter 6. General discussion

(Figure 4-30). This is still acceptably suitable for pulmonary delivery though such particles may not penetrate to the alveoli.

Initial experiments to investigate the in-vitro aerosolisation performance of different chitosan-PEG nanoparticles in pMDI formulations were performed using the TSI (Section 4.4.11). PEG1000 associated LMC nanoparticle dispersions in HFA-227 had longer system stability (>10 min) compared to non-PEG nanoparticles and different molecular weight PEG associated LMC nanoparticles (Table 2-6) and hence the aerosol characteristics of the formulation were good with the highest FPF of 45.2 (±1.2) %. The FPF is similar to commercially available Ventolin HFA® pMDIs (45.9%), which contains micronised salbutamol sulfate in HFA-134a (Wu et al. 2008a). The author also dispersed micronised salbutamol sulfate in HFA-134a in the presence of PE 300 and a higher FPF of 65.3% was obtained. Our studies show that following aerosolisation the measured mean nanoparticle size was less than 500 nm (Section 4.4.12). LMC-PEG1000/siRNA collected from the TSI following delivery from a pMDI and studied by gel electrophoresis, showed that the integrity of siRNA was preserved within the canister and following actuation (Section 4.4.13). Based on these properties, siRNA loaded LMC-PEG1000/HFA systems seemed to be the most suitable of those investigated to deliver siRNA to the desired site in the airways.

The NGI has recently been introduced, and is used routinely within pharmaceutical industry for the assessment of aerodynamic particle size. In the NGI, the LMC-PEG1000 had a FPF (<6.4 μm) of 38% with a MMAD and GSD values of 3.5 μm and 3.6 respectively (Section 4.4.14). HMC-PEG1000 had a FPF of 34%, with MMAD of 4.92 μm and GSD of 3.35, indicating that these were suitable chitosan nanoparticle formulations for lung delivery using a pMDI. These systems are polydispersed since the GSD is greater than 1.22 (Finlay et al. 1997).

PCS was used in determining particle size at each stage of the NGI (Section 4.4.15). HMC-PEG1000/HFA-227 systems showed fine particles of size less than 2 μm whereas, LMC-PEG1000/HFA-227 systems showed particles of size less than 1 μm, in stage 3 (6.4 to 3.99 μm) to filter (<0.54 μm). These particles were small and did not fuse together to form particles bigger than 6.4 μm. Upon inclusion of siRNA, the FPF of LMC-PEG1000 nanoparticles was reduced from 38% (±2.7) to 25% (Section 4.4.16).

Overall, the nanoparticle physical stability, dose uniformity and relatively high aerosolisation efficiency makes chitosan-PEG1000 a suitable candidate as a platform formulation technology for metered dose inhalers in HFA propellants and nebulisation using nebulisers.
6.6. Nanoparticle cell culture studies for cellular viability, uptake and gene silencing

Cytotoxicity of novel engineered siRNA-nanoparticle formulations was studied towards the epithelial cell lines. HMC-PEG1000 and LMC-PEG1000 nanoparticle formulations were found to have an absence of overt toxicity to A-549 cells and H-292 cells respectively following exposure at chitosan concentration of upto 420 µg/ml (48 h) and upto 83.3 µg/ml (24 h) respectively (Section 5.4.1. a and b). Incorporation of PEG within HMC nanoparticles reduced the potential toxicity of HMC to the cells at higher HMC concentrations probably due to the shielding effect of PEG over HMC molecules. The presence of different grades of PEG in the nanoparticles did not significantly impact on cell viability when incubated with LMC nanoparticles in different LMC concentrations (Section 5.4.1.b). Both A-549 and H-292 respiratory cell lines have been previously used for in-vitro evaluation of safety, efficacy and uptake of particulate drug delivery systems. Chitosan hydrochloride was formed into nanoparticles using TPP as a crosslinking agent in a similar manner to chitosan glutamate in these studies. These nanoparticles were investigated for their safety as powders for lung delivery. The formulation showed 70% cell viability with A-549 cells at a concentration of 0.5 mg/ml for up to 48 h (Grenha et al. 2007) unlike chitosan glutamate nanoparticles which showed a cell viability of approximately 40%. The results obtained here are encouraging with respect to the development of crosslinked chitosan based nanoparticles for pulmonary administration of siRNA. Not only do some of the formulations (HMC/LMC-PEG1000 nanoparticles) possess suitable aerodynamic characteristics (Chapter 4) and the capacity to encapsulate siRNA (Chapter 3), they also have been shown to exhibit in-vitro biocompatibility (Chapter 5).

RNAi can effectively knockdown targeted transcripts (mRNA). This can be achieved by efficient intracellular delivery of preformed siRNA. The micrographs (Figure 5-12) show sections of A-549 cells after incubation with FITC-labelled LMC-g-PEG5000 nanoparticles. Efficient intracellular uptake of the formulation was observed in the cytosol as well as nucleus of the cells indicating good uptake of the nanoparticles. Quantitative assessment of gene silencing using these formulations as siRNA carriers was performed by quantitative real time PCR analysis (Section 5.4.3). Various methods of RNA extraction were investigated, such as; TRizol®, where a phase separation technique was followed by RNA precipitation. Another RNA extraction technique is the use of RNeasy®, utilising silica which binds and recovers RNA through washing and elution steps. A major drawback with these methods is that when extracting RNA for subsequent quantitative PCR evaluation of silencing, there was an apparent
poor recovery of total RNA, as chitosan interfered with the assay by binding to the nucleic acid during extraction. This effect of chitosan is due to its chemical structure and charge. Another reason for poor recovery is the presence of guanidinium thiocyanate buffer (approximate pH 5) used in the extraction kits (in RA1 lysis buffer) which caused chitosan to become more positively charged, and thus to bind strongly to negatively charged siRNA. Enhancement of RNA extraction was attempted using various techniques such as enzymatic treatment (chitosanase). Chitosanase treatment prior to RNA extraction greatly enhanced the yield and the integrity of extracted RNA from the LMC-g-PEG5000 nanoparticle formulation (Table 5-2). The RNA recovery with LMC-g-PEG5000 formulation was similar to non transfected cells (22.4 ng/μl) unlike other formulations. The gene silencing effect was found to be 58.20%. Another group has demonstrated 82% gene silencing (RNA recovery 78 ng/μl) of DPP-4 (dipeptidyl peptidase4) gene in HepG2 cells with LMC (10 kDa)/siRNA in the presence of chitosanase (Alameh et al. 2010). In the absence of chitosanases, the RNA recovery was 2.6 ng/μl.

The combined features of low cytotoxicity, efficient cellular uptake and high specific knockdown make PEG grafted LMC a promising siRNA carrier for pulmonary (by nebulisation) gene therapy and future in-vivo studies. We have also established PEG1000 associated HMC and LMC nanoparticle formulations as good suspension (HFA-227) pMDI systems without the need for additional excipients, such as suspending agents or co-solvents. Both formulations showed high affinity towards siRNA and high loading efficiency. LMC-PEG1000 nanoparticle formulations have proved to be aerosolisable carriers (pMDI or nebuliser) when loaded with siRNA. This indicates that other siRNA variants can also be successfully formulated using this approach. In conclusion, the current study suggests that the inhaled delivery of siRNA using biodegradable nanoparticles may be a viable approach for pulmonary siRNA therapy.

6.7. Thesis summary

- Chitosan (HMC and LMC) nanoparticles of <220 nm size were prepared with and without PEG bearing a positive surface charge.
- The nanoparticles stayed stable at physiological pH of the lung (pH 6.5).
- Polymer type, its concentration, molecular weight and type of propellant were the major determinants of the dispersion efficiency of these nanoparticles within propellant.
PEG1000 improved HMC and LMC nanoparticle dispersion in the propellant (HFA-227) and eliminated the need for co-solvents.

These nanoparticles encapsulating siRNA resulted in size <300 nm and a high loading efficiency was observed for HMC and LMC nanoparticles (>80%) compared to LMC-g-PEG5000 nanoparticles (~50%).

Gel electrophoresis confirmed the electrostatic interaction between negatively charged siRNA and positively charged chitosan.

No differences were observed between formulations with siRNA adsorbed or encapsulated within the nanoparticles. However, LMC-g-PEG5000 was unable to retain the siRNA during gel electrophoresis.

siRNA was successfully aerosolised using chitosan nanoparticles compared to naked siRNA. High nebulisation was achieved (fine particle fraction >50%) for all chitosan nanoparticles with or without PEG. LMC-PEG1000 siRNA encapsulated nanoparticles when delivered using a pMDI, exhibited good fine particle fraction (FPF) and siRNA stability, whereas LMC-g-PEG5000 nanoparticles were found to be unsuitable. However, aerosolisation may need to be further optimised to increase the FPF so that a greater proportion of the aerosol generated from the pMDI/nebuliser will reach the deep lung.

PEG associated HMC and LMC nanoparticles displayed a good cell viability (>80%), however gene silencing properties could not be evaluated due to an interference during mRNA extraction process. Therefore, there is a need for a more robust method, such as western blot, to assess silencing from these formulations.

LMC-g-PEG5000, however, showed a good cell viability (>80%), an efficient cellular uptake and a gene silencing of ~58%, however the mechanism of cellular uptake needs to be understood in more details.

6.8. Future work

In this thesis, in-vitro work highlighted the successful production of safe, stable, aerosolisable siRNA loaded crosslinked chitosan carriers for pulmonary delivery. In view of the findings some additional experimental work is suggested for a better understanding of the system in achieving therapeutic response in the airways following delivery.

The prime concern in formulating nucleic acids, such as siRNA for pulmonary delivery is to maintain their biological activity. The three dimensional structure may undergo changes during
formulation preparation, freeze-drying and delivery from pulmonary drug delivery devices (Cherng et al. 1997; Shoyele and Slowey 2006). siRNA physical stability was analysed using gel electrophoresis which is a semi quantitative technique. It is essential in the future to perform a complete quantification of the physical and chemical stability of therapeutic siRNA, during processing and post-aerosolisation, using techniques such as real-time polymerase chain reaction.

The dispersion stability and aerosolisation behaviour of siRNA loaded nanoparticles was analysed over short term storage, within polar and a non-polar pressurised system. Future work will involve long term stability studies and determination of product shelf-life which is desired by international conference on harmonisation (ICH) for a product to be eligible for clinical trials.

PEG associated with HMC and LMC interfered with RNA purification and isolation, hence an alternative RNA silencing protocol needs to be investigated to determine gene silencing ability of these siRNA loaded carriers.

This work introduced a versatile siRNA nanocarrier that was suitable for pulmonary delivery using both pMDI and nebulisers. It is also essential to determine the optimum respirable dose of siRNA for a particular disease, to be efficacious and provide absolute gene knockdown. In order to achieve this objective it is vital to further perform in-vivo studies in a clinical setting. This would clear whether the carriers are able to bypass the extracellular and intracellular barriers in animal models. The novel siRNA loaded formulation system could be aerosolised into a chamber with test animal inhaling the aerosolised formulation and providing a new stage of development for the siRNA therapy of diseases.
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