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'Smart' non-viral delivery systems for targeted delivery of RNAi to the lungs.

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Citation

Smart non-viral delivery systems for targeted delivery of RNAi to the lungs

Summary

The emergence of RNA interference (RNAi) offers a potentially exciting new therapeutic paradigm for respiratory diseases. However effective delivery remains a key requirement for their translation into the clinic and has been a major factor in the limited clinical success seen to-date. Inhalation offers tissue–specific targeting of the RNAi to treat respiratory diseases and a diminished risk of off-target effects. In order to deliver RNAi directly to the respiratory tract via inhalation “smart” non-viral carriers are required to protect the RNAi during delivery/aerosolisation and enhance cell-specific uptake to target cells. Herein, we review the state of the art in therapeutic aerosol bioengineering (TAB) and specifically non-viral siRNA delivery platforms for delivery via inhalation. This includes developments in inhaler device engineering and particle engineering including manufacturing methods and excipients used in TAB that underpin the development of “smart” cell-type specific delivery systems to target siRNA to respiratory epithelial cells and/or alveolar macrophages.

Key terms: RNAi, siRNA, respiratory, inhalation, therapeutic aerosol bioengineering, polymers, lipids, particle engineering, bioresponsive, respiratory

Defined key terms:

Therapeutic aerosol bioengineering (TAB): encompasses both particle and device engineering to produce an inhaled product that is more advanced than conventional aerosols.

Small interfering RNAs (siRNA) that trigger sequence specific degradation of mRNA via the Argonaute 2 protein

Respiratory disease: Respiratory diseases are among the leading causes of morbidity and mortality worldwide according to the WHO
Introduction

RNA interference in respiratory disease

Respiratory diseases are a major world health problem in the 21st century and include pulmonary fibrosis, lung cancer, inflammatory lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), alpha-1-antitrypsin deficiency and cystic fibrosis (CF) along with bacterial and viral respiratory infections. Very limited treatment options are available for diseases like CF and lung cancer and new treatment modalities are urgently needed. For others, such as respiratory infections, current therapies have limitations and need to be improved. The highly bifurcating structure of the lung produces tortuous passages for airflow causing inhaled drug particulates to deposit on the airway walls before even reaching the target site of the alveolar region of the lungs. Furthermore, invasion of foreign material in the lungs is prevented by an overlying mucus layer, a tight packing of epithelial cells, mucociliary clearance and cough as well as recognition and subsequent destruction by alveolar macrophages and polymorphic neutrophils [1].

RNA interference (RNAi) could prove to be a powerful therapeutic tool for a wide range of diseases boasting superior specificity and potency over other forms of therapy. Endogenous RNAi, in particular short-interfering RNA (siRNA – the focus of this review) functions by silencing the expression of target genes at the post-transcription stage, and by understanding this pathway translational science has been able to utilize this mechanism in the design of therapeutics (reviewed in [2-4]). It shows promise in treating previously untreatable or difficult to treat diseases and has the potential to be used prophylactically and therapeutically. Clinical trials are ongoing for conditions including cancer, neovascular age-related macular degeneration (AMD) [5] and respiratory viral infections. Concerns over off-target effects, immunogenicity and toxicity remain due to the adverse effects witnessed during previous gene therapy trials [6].

Small, hydrophobic drug molecules are readily absorbed in the lung owing to its large surface area and high blood perfusion, while large hydrophilic macromolecules such as nucleic acids, are poorly absorbed in the lungs a factor that can be harnessed to enhance local exposure [7]. The synthetic
siRNA molecule is a 21-23 nucleotide dsRNA duplex about 7nm in length and 13kDa in weight, making it approximately 50 times larger in molecular mass than typical small drug molecules [8]. Additionally, the 40 phosphate groups in its backbone give the siRNA molecule a strong anionic charge leading to poor cellular uptake. In addition to challenges related to the molecule itself, the lung presents several biological barriers to siRNA [9]. Due to its chemical nature it is necessary to encapsulate siRNA in a carrier vector for protection against nucleases. Therefore, in order to fully realize the commercial and clinical potential of these molecules “smart” delivery systems that protect and target the RNA molecules to their intracellular site of action are critical. Due to the toxicity and immunogenicity issues associated with viral vectors there has been an increased requirement for non-viral, biocompatible siRNA delivery systems.

**Respiratory delivery of RNAi**

Success of a therapeutic is greatly dependent on the physiological and anatomical properties of the target tissue, as well as the disease type and severity, and the characteristics of the therapeutic agent itself and its carrier if any. Respiratory delivery of siRNA offers the benefits of targeted delivery to the disease site but also presents many challenges as outlined above such as the complex anatomy of the organ, the presence of mucous and other alveolar fluids and rapid clearance mechanisms [10]. Additionally, many chronic obstructive diseases such as CF and asthma are associated with mucus hypersecretion and severe inflammation that can impact on the delivery of therapeutic agents [10]. Delivery efficiency of any therapeutic, including RNAi, is dependent on a number of key factors including the nature of the drug molecule, the excipients/carrier and inhaler device combination. The drug must reach the desired site in the airways and once there be protected from rapid clearance and degradation. Due to the specificity and potency of siRNA low doses can be used to treat respiratory disease via inhalation, offering the benefit of direct delivery to the target organ. The area of therapeutic aerosol bioengineering (TAB) of “smart” RNAi particles aims to (1) enhance their delivery via inhalation (2) ensure stability during aerosolisation and in vivo and (3) improve targeting and cellular uptake into target cell types.
Delivering siRNA to the target site within the lungs i.e. the lung alveolar region with high efficiency and specificity is a major challenge. Whilst ‘naked’ siRNA has shown efficacy in certain physiological settings, it can often have a very poor pharmacokinetic and ultimately pharmacodynamic profile. This is primarily due to the rapid excretion and degradation of siRNA as well as its low level of uptake into the cell. For instance, it has been found that naked siRNA has a half-life of less than one hour in human plasma and circulating siRNA is rapidly excreted by the kidneys due to its small size. Furthermore, naked siRNA is unable to cross the lipid membrane of a cell due to its size and negative charge leading to insufficient tissue bioavailability. There has also been evidence of a certain level of cytotoxicity associated with ‘naked siRNA’ brought on by interferon activation via toll-like receptor 7 (TRL-7) following systemic administration with non-specific cellular uptake further compounding the issue [11-15].

To date no siRNA-based therapeutics have been approved as commercial products with all studies only reaching the clinical trial stage. However, some success has been reported by Alnylam Pharmaceuticals® with an anti-RSV N gene siRNA, ALN-RSV01, reaching the Phase II clinical stage. Alnylam® reports ALN-RSV01 to be safe and well tolerated with significant antiviral activity. A Phase IIb clinical trial has recently been completed in adult lung transplant patients where despite failure in achievement of the primary endpoint, they did observe a 50% reduction in bronchiolitis obliterans syndrome (BOS) incidence at day 180 in comparison to placebo [201]. Reports from animal studies have shown more success for instance, Bitko et al. (2005) showed very promising results using siRNA targeted at P proteins of both RSV and parainfluenza virus-3 (PIV-3) in infected mice [16]. This study demonstrated that intranasally administered siRNA alone or complexed with TransIT-TKO prior to RSV or PIV challenge inhibited viral proliferation, reduced infection and prevented disease. In addition, when administered after viral inoculation the siRNA had a curative effect [16].

**Therapeutic aerosol bioengineering (TAB) of smart delivery systems for siRNA**

The main goal of therapeutic aerosol bioengineering (TAB) is to overcome some of these pharmaceutical and biopharmaceutical barriers and produce an efficiently inspired therapeutic aerosol
either through the design of better inhalation devices or modification of the aerosol particle itself [17].

TAB encompasses both particle and device engineering to produce an inhaled product that is suitable for the therapeutic agent and the patient population it is being targeted for. Key to this is an understanding of the drug properties and a brief overview of some of the recent efforts in this area are given below.

**Device engineering**

A number of new devices for inhalation of dry powders and liquid formulations have been developed. With many of these devices, the focus of research has primarily been on three main areas [18]:

(A) Improving the fine particle fraction (FPF) in the generated aerosol: FPF is the fraction of administered drug dose that is of a size suitable for deposition in the deep lungs. In conventional dry powder inhalers (DPIs) for example, particle deagglomeration was mainly achieved by drawing the powder through a mesh or grid located within the inhaler. FPF in newer devices has been improved through the use of battery-driven impellers, pressurized air or cyclone chambers and tortuous passages within the device. These designs subject the particles to various centrifugal or impaction forces causing them to disaggregate and disperse better.

(B) Increasing drug penetration into the lungs and minimizing oropharyngeal deposition: A significant problem with conventional metered dose inhalers (MDI) is the need for the patient to coordinate actuation of the inhaler with inhalation. Failure to do this is one of the main reasons for the poor delivery efficiency seen with MDIs. High velocity of the aerosol also leads to significant oropharyngeal deposition. In the newer generation of inhalers, these problems have been overcome either by using breath operated systems in which actuation of the inhaler occurs only when the patient inhales or by reducing the velocity at which the aerosol is released from the device e.g. soft mist inhalers [18].

(C) Improving patient compliance: Efforts are made to produce devices that are simple and inexpensive to use, easy to carry, provide feedback to the patient about the success of the inhalation manoeuvre.
Particle Engineering

Although device engineering has been able to resolve many of the delivery issues that plagued the first generation of inhalers [19] the performance of a delivery system depends on both the device and the formulation. Superior delivery efficiency can also be achieved or enhanced by developing optimized particulate formulations that can be used with simple, user-friendly inhalers. This strategy has been referred to as “particle engineering (PE)”. The goal of PE is to incorporate desirable attributes into an inhaled formulation such as narrow particle size distribution, improved powder dispersibility, enhanced drug stability and bioavailability, sustained drug release and site-specific drug targeting, whilst taking into consideration the design limitations of the inhaler and dosing requirements of the drug. For RNAi medicines PE offers the potential to enhance cellular delivery and target specific cell types within the airways.

Manufacturing techniques

The choice of manufacturing method is closely aligned with the size of particle to be produced. So-called “nanoparticles” or particles <1micron size would generally be regarded as below the respirable size in their dry state but can be delivered to the airways using nebulizer technology that delivers them in a liquid droplet of respirable size. Dry particles of inhalable size have been prepared using a range of techniques. Air-jet milling is a traditional and well-established method which involves acceleration of drug particles in a spiral fashion inside a milling chamber using compressed air or nitrogen, reducing particle size by inter-particulate collision. However, this method has a number of drawbacks including lack of control over the size and morphology of the particles, drug degradation during comminution and poor powder dispersivity due to highly cohesive, electrostatic forces between the particles [19]. To overcome these issues, other particle formation technologies have been explored primarily spray drying, spray freeze drying and supercritical fluid technologies [20]. Spray drying involves atomisation of a drug solution into a current of gas (air or nitrogen) which has been heated to a temperature sufficient to evaporate the solvent. The solvent in the sprayed droplets is thus removed and the dry drug/excipient particles are collected in a chamber (Figure 1) [21]. In spray-freeze drying, a solution containing the drug of interest is sprayed into a vessel containing a cryogenic
liquid such as nitrogen. Droplets generated are quickly frozen as they make contact with the liquid nitrogen. Lyophilization of the frozen droplets results in particles suitable for inhalation. Supercritical fluids (SFs) are gases and liquids at temperature and pressure above their critical points (Tc, critical temperature; Pc, critical pressure). At this critical temperature and pressure, they exist as a single phase with several advantageous properties of both liquids and gases. CO₂ is the most widely used SF for pharmaceutical applications because of its low Tc (31.2°C) and Pc (7.4MPa). One of the many methods employed is RESS (Rapid expansion of supercritical solution) where the drug is dissolved in the SF which is then forced through a heated orifice. As it comes out of the orifice, the SF expands rapidly reducing the solubility of the drug leading to precipitation and formation of inhalable drug particles [20].

**Excipients used in TAB**

Many of the particle engineering approaches cited above involve combining the drug with one or more pharmaceutical excipients (e.g. polymers, lipids, amino acids, and sugars) to serve different purposes in the final product. For example, excipients might be used as diluents or bulking agents, as powder dispersibility enhancers, drug stabilizers, matrices that control drug release rate, for drug targeting or as bioavailability enhancers. Conventional inhaler formulations mainly involved dissolving or suspending the drug in a propellant (for an MDI) or blending the drug particles with a larger inert carrier (for a DPI). While this strategy was adequate for low molecular weight drug molecules intended for local action, more sophisticated formulation approaches are required for the newer peptide, protein and nucleic-acid based drugs and those intended for systemic delivery. Two of the most exhaustively studied classes of excipients for this purpose have been lipids and polymers [1]. They have been used primarily to create inhalable particulate carrier systems in which each particle acts as a micro-reservoir for the drug. The rationale being that this not only protects the drug from in vivo degradation but also allows it to be targeted to the site of action at a controlled rate [1]. The application of lipids and polymers for pulmonary drug delivery is briefly introduced below.
Phospholipids that are endogenous to the lungs such as dipalmitoyl phosphatidyl choline (DPPC) are used to prepare liposomes. These spherical vesicles in which the amphipathic lipids are arranged in spherical bilayer(s) surrounding an aqueous compartment can be manufactured in the micron or nano size range and can be used to encapsulate drug molecules (discussed in more detail below). There are currently 18 active clinical candidates in the RNAi therapeutics pipeline being delivered via a number of routes of administration and several of these clinical trials are using lipid-based carriers e.g. Stabilized Nucleic Acid Lipid Particles SNALP platform [22-24]. A number of in vivo pre-clinical studies have harnessed cationic liposomes for siRNA local delivery of siRNA to the lungs [43, 128, 135].

In addition to the use of lipids, microspheres made of biodegradable polymers have also been studied as pulmonary drug carriers. In comparison with liposomes, microspheres show greater physicochemical stability in vivo and also allow higher drug loading [25, 26]. Both naturally occurring polymers as well as synthetic polymers (e.g. polyesters) have been studied in the preparation of microspheres. Of the natural polymers, both proteins (e.g. collagen, gelatin and albumin) and polysaccharides (e.g. starch, dextran, chitosan, alginic acid) have been investigated extensively. Natural polymers remain attractive because of their abundance in nature, their biocompatibility, and the ability to be readily modified by simple chemistry [1]. Among the synthetic polymers, polyesters, poly (alkyl cyanoacrylates) and poly (ether anhydrides) have been investigated as pulmonary drug carriers. Inhaled microparticles made of polyesters such as poly (lactic acid) (PLA) and poly (lactic-co-glycolic acid) (PLGA) have been reported. For example, PLGA microparticles containing drugs such as rifampicin and isoniazid have been studied for treatment of tuberculosis [26]. Using proprietary spray drying techniques, PLGA has also been used to prepare large, porous particles with low mass density. Due to their greater aerosolisation efficiency and ability to avoid alveolar macrophage uptake, such particles have been shown to enhance bioavailability and also extend the duration of action of biomolecules such as insulin in the lungs [27]. Yet another class of polymers that have been studied for lung delivery are the poly (ether anhydrides) (PEAs). While polyesters such as PLGA degrade in the body via hydrolytic cleavage of the ester bonds in their backbone, degradation of PEAs occurs via
hydrolysis of their anhydride bonds. Like the cyanoacrylates, PEAs also display significantly faster 
degradation rates as compared to PLGA. These polymers are typically composed of the monomers 
sebacic acid (SA) and poly (ethylene glycol) (PEG). Large, low density particles of poly(SA-co-PEG) 
with aerodynamic diameters between 1.9-3.7μm were prepared. By varying the amount of PEG in the 
polymer backbone, particles with enhanced aerosolization efficiency were produced. The presence of 
PEG also decreased phagocytosis in the deep lung possibly due to steric effects [28].

Bioresponsive materials

In the majority of drug delivery systems currently in use, drug release occurs by simple diffusion of the 
drug or hydrolytic degradation of the biodegradable carrier. With many of these carriers, such as 
PLGA, the degradation rate can be tailored to some degree by altering factors such as the co-polymer 
composition, degree of crystallinity, or initial molecular weight. To achieve greater spatial and 
temporal control of drug release, there has been increasing interest in recent years in a new 
generation of synthetic biopolymers that can respond to environmental changes, such as 
temperature, pH, and presence of specific enzymes or molecules such as glucose [29, 30]. Many of 
these new polymers can form hydrogels, which are three dimensional networks of hydrophilic polymer 
chains that do not dissolve but can swell in water. Upon small changes in the environment, these 
‘smart’ hydrogels can undergo reversible changes in their structural and physical properties. Polymers 
that are cleaved by the enzymatic activity of serum proteins or cellular secretions can also be used as 
carriers in drug delivery. Using such polymers, site-specific drug delivery can be achieved by targeting 
the carrier to a site where the enzyme is produced. Accumulation of the carrier at the delivery site is 
avoided since enzymatic cleavage is faster than the normal hydrolytic mechanism. Such carriers 
would therefore behave as both an enzyme sensor and as an enzyme dependent delivery system 
[30]. While a number of such systems have been studied for oral or parenteral delivery, little or no 
work has been done on bioresponsive delivery systems for inhalation. Using the raised elastase 
levels present at sites of lung inflammation as a proof-of-concept model, we endeavored to develop 
prototypes of inhalable elastase sensitive microparticles (ESMs) (Figure 2) [31]. A novel particulate 
delivery platform capable of bioresponsive drug delivery in the presence of elastase was successfully
developed. These consisted of an interpenetrating network (IPN) of a polysaccharide, alginate and the elastase substrate, elastin [31].

Non-viral “smart” delivery systems for targeting airway epithelial cells.

One of the key cell types involved in the pathogenesis of respiratory disease are the airway epithelial cells. “Smart”, non-viral delivery systems are being extensively explored for enhancing siRNA delivery to the lung epithelium. As previously stated, delivery to the lung epithelium presents its own set of challenges, most importantly, the presence of the mucociliary escalator which runs from the nostrils to the terminal bronchioles (reviewed in [32-34]). This acts to clear any inhaled particles such as dust, pathogens or any delivered medicines from the upper airways before there is a potential for infection (or therapeutic effect) in the body. Should any delivered particle succeed in reaching the alveolar region of the lungs the alveolar fluid itself consists of surfactants, mucins and macrophages [35-37] which also pose a formidable barrier to effective delivery. To overcome these limitations, efforts have been made to build on TAB technologies to enhance aerosol delivery and assist the entry of siRNA into the target cell. These delivery systems can range from small peptides of just 10 amino acids to high molecular weight synthetic polymers and lipids and are derived from a wide variety of sources. A summary of these carriers that have been assessed in vitro and/or in vivo is provided below:

Liposomes for targeting airway epithelial cells

Liposomes are spherical, self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers. These range in size from 100 to 5,000 nm and can consist of a single lipid bilayer (unilamellar) or several concentric bilayers (multilamellar) [38]. While the use of lipid based systems for nucleic acid delivery has been investigated since the 1980s [39, 40], it is only in recent years that the use of highly specialized liposomes for siRNA delivery to the lungs has been brought to the preclinical and clinical stages.

One of the earliest “smart” approaches developed was in response to issues associated with cationic liposomes such as 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) delivery to airways. Use of
these lipids had previously resulted in adverse effects such as inflammation and toxicity [41]. To address these issues, shielding moieties such as PEG were grafted to cationic lipids in order to develop “stealth” liposomes. This has been found to increase half-life and stability in a number of systems including intratracheal administration to the lungs [42, 43]. Specifically, in the case of Garbuzenko et al. it was found that the use of neutral PEGylated liposomes for siRNA delivery resulted in increased uptake in mouse lungs compared to traditional cationic liposomes. However, PEGylation of liposomes has also been found to interfere with cellular uptake and result in decreased endosomal release [44, 45]. To address these issues, alternative approaches have been developed, examples of this include grafting cell penetrating peptides (CPPs) such as octa arginine and TAT directly to the neutral lipids such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in order to increase uptake. This has been found to result in increased uptake in bronchial epithelium cells in in vitro studies when compared with both neutral and traditional cationic liposomes [46]. Finally, liposomes have now been developed to incorporate targeting moieties for specific targets on the lung epithelial surface. In a recent study by Allon et al. a 7-amino acid peptide targeting endothelin receptors was conjugated to the neutral lipids 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Use of this ligand is especially promising since on binding, the endothelin-receptor complex internalizes immediately [47, 48]. This resulted in significantly increased gene transfer to lung epithelial cells both in vitro and in vivo [49].

Peptides for targeting airway epithelial cells

Synthesized peptides have also been investigated as gene delivery agents for siRNA in their own right. Numerous strategies for delivering siRNA using CPPs have been examined, primarily focusing on either covalent bonding of the CPP to the siRNA [50, 51] or nanocomplex formation via electrostatic interaction [52]. Some of the most common of these peptides to be used for siRNA delivery to the lungs are the HIV-derived TAT trans-activator proteins. These have been synthesised in a variety of molecular weights stemming from the original 86 amino acid long domain [53-56]. In recent studies it was found that the use of TAT peptides for siRNA delivery to the lungs was capable of eliciting high levels of gene knockdown. For example, a 30-45% knockdown in p38 MAP kinase
expression was obtained when siRNA conjugated to cholesterol and TAT was delivered intratracheally to BALB/c mice however, this elicited an inflammatory response 6hrs post-delivery [55]. In addition, in a more recent study, using TAT-siRNA nanocomplexes and calcium as a condenser, Baoum et al. were able to achieve in vitro levels of knockdown as high as 93% in human epithelial lung cells with little to no evidence of toxicity [56]. Even taking into account the expected drop-off in knockdown efficiency going from in vitro to in vivo, this remains a very promising delivery strategy. Another set of CPPs that have been investigated as siRNA delivery vectors are the polyarginine peptides, specifically, octa-arginine (R8) and nona-arginine (R9). These peptides are chemically derived and, in a similar fashion to TAT, have been investigated through covalent bonding to siRNA and through the formation of electrostatic nanocomplexes for siRNA delivery. From preliminary studies it was found that poly-arginine-siRNA nanocomplexes were capable of inducing gene knockdown levels similar or better than those observed with TAT [52, 57].

Although these are just two selected peptides, there are numerous CPPs currently in development such as antennapedia, transportan and poly-lysine [58, 59], most of which have the potential to successfully deliver siRNA to the lung epithelium. It should be noted however; that there have been recent studies that indicate CPP mediated siRNA internalization of cells does not result in endosomal escape using either covalently bound or electrostatically formed nanocomplexes [60, 61]. Efforts are now focusing on addressing this issue by combining to CPPs with the muco-inert qualities of PEG and endosomal escape peptides to form the next generation CPP siRNA delivery vectors [59]. Finally, new lung specific peptide sequences are constantly being developed using techniques such as phage display technology [62].

**Polymers for targeting airway epithelial cells**

**Polyethylenimine (PEI)**

One of the most commonly used polymers in siRNA delivery is polyethylenimine (PEI). These synthetic polymers are highly cationic and have been found to possess a strong ability to compact and concentrate any nucleic acid material. These properties have resulted in PEI demonstrating
siRNA mediated gene knockdown in lung epithelium both in vitro and in vivo [63, 64]. PEI is also capable of exerting a buffering effect following endocytosis which protects against lysosomal degradation. This is due to the fact that PEI consists of an amino nitrogen at every third atom which can be further protonated making it an effective “proton sponge” at virtually any pH [65]. PEI can also be synthesized in either a branched or linear conformation ranging from 25kDa up to 800kDa for branched formations allowing greater flexibility in the amount of siRNA that can be encapsulated [66, 67]. The ability of PEI to form branched units means it can be conjugated with other ligands e.g. transferrin or PEG [68, 69]. Unfortunately, PEI has been known to exert a cytotoxic effect in proportion to its increasing molecular weight [70]. In addition uptake of PEI-nucleic acid complexes into cells is known for some time to take place non-specifically via adsorptive endocytosis [71]. To overcome the poor specificity and cytotoxicity of PEI, much research has been undertaken in linking PEI with other polymers. A large proportion of research has involved conjugation of PEI to PEG with the possibility of further conjugation to other polymers. This has resulted in a significant improvement in the activity of siRNA-PEI complexes. Toxicity and aggregation levels of PEI-PEG complexes were found to be reduced when tested in vitro and in vivo [72, 73]. Interestingly, it was found that the behavior of PEI-PEG was dependent on both the level of PEG grafting to PEI and the molecular weight of the PEG chain [74]. In the case of PEI-PEG siRNA nanoparticle delivery to the lungs, it would appear that the higher the degree of PEGylation and the shorter the PEG chain length, the less cytotoxic effects were observed [75]. Unfortunately, while PEGylation of PEI results in decreased toxicity, it has also been shown to lead to a dose-dependent increase in pro-inflammatory and pro-immunomodulatory mediators [63, 75]. However, from subsequent preliminary studies it would appear that there was no evidence that PEI and PEI-PEG polymers induce oxidative DNA damage or mutation in cultured murine lung cells at therapeutic doses [76]. Finally, PEGylation of PEI was also found to protect cells in the lung from lipid peroxidation via the non-enzymatic pathway (8-IP) and cyclooxygenase mediated pathway (PGE2) [75]. In addition to improving the toxicological profile of PEI, PEGylation has also been found to increase the transfection efficiency for siRNA in the lung. In our recent work we found that through a high level of PEGylation of PEI it was possible to obtain significantly higher levels of in vitro gene knockdown compared to PEI using polarized, fully differentiated airway
epithelial cell monolayers (Figure 3c) [77]. This is in accordance with previous studies which had previously noted the difficulties involved in transfecting mucous secreting cell lines with unmodified PEI [78]. This ability of PEI-PEG to increase internalization across mucous layers also builds upon pre-existing research describing the formation of “muco-inert” PEGylated particles (Figure 3a&b) [79, 80]. In addition to PEGylation, PEI specificity is also being improved through the coupling of targeting peptides to the polymer e.g. the endothelial-specific integrin binding domain RGD [81].

**Chitosan**

Chitosan is a linear derivative of the naturally occurring animal protein chitin comprised of a beta (1–4) linked 2-amino-2-deoxy-b-Dglucose (GlcN; D-unit) and the N-acetylated analogue (GlcNAc; A-unit) [82]. It has been proposed as a biocompatible alternative to synthetic, cationic polymers for non-viral gene delivery. This is due to chitosan possessing attractive qualities such as low toxicity, low immunogenicity, excellent biodegradability and a high level of biocompatibility [83, 84]. In addition, chitosan has a high positive charge that can easily form polyelectrolyte complexes with negatively charged nucleotides, resulting in efficient encapsulation. Different varieties of chitosan are also available; these range from low molecular weight (LMWC) to high molecular weights (HMWC). Of the two, LMWC has been found to exhibit more favorable characteristics than the conventional HMWC equivalents. These include greater water solubility, lower toxicity, and a narrower molecular weight distribution. Chitosan/ siRNA formulations prepared with LMWC (~10kDa) exhibited almost no knockdown of endogenous GFP when tested in H1299 human lung carcinoma cells. This was compared to those prepared with HMWC (64.8–170kDa) which had a greater level of gene silencing (between 45% and 65%) [85]. The highest gene silencing efficiency (80%) was achieved using chitosan/siRNA nanoparticles of Mw 114 and 170kDa [85]. Recently, efforts have been made to address the disadvantages associated with unmodified chitosan. These drawbacks are especially pronounced with increasing chitosan molecular weights. Similar to PEI, research has been undertaken to conjugate chitosan to PEG to improve its pharmacological attributes. This has been achieved at the amino terminus of chitosan and most recently at the hydroxy-terminus [86, 87]. Conjugation of chitosan to both PEG and a poly-arginine CPP has been found to increase gene-
knockdown with improved cell viability compared to that of unmodified chitosan [88]. A novel approach recently demonstrated conjugation of the β2-adrenoreceptor agonist salbutamol to a guanidinylated chitosan combining the increased rate of siRNA uptake associated with the addition of guanidinium to chitosan with a high rate of cell specificity. On examination, it was found that siRNA systems formed resulted in significant decreases in gene expression both in respiratory cell lines and also when delivered in vivo to mice using vibrating mesh nebulizers [89]. Further possible improvements include the use of conjugated ligands such as transferrin, folate, mannose and galactose. These ligands have previously been demonstrated to improve chitosan-mediated transfection and would be a reasonable starting point for future work [90-92].

**Cyclodextrins**

Cyclodextrins (CDs) are a series of natural cyclic oligosaccharides composed of 6, 7, or 8 D(+)-glucose units linked by α-1,4-linkages, named α-, β-, or γ-CD, respectively. The geometry of CDs gives a hydrophobic inner cavity having a depth of ca. 7.0 Å, and an internal diameter of ca. 4.5, 7.0, and 8.5 Å for α-, β-, and γ-CD, respectively [93]. This hydrophobic cavity gives CDs the ability to encapsulate hydrophobic moieties within their cavities whilst retaining their solubility in an aqueous environment. In addition to their favorable solubility characteristics, CDs also show resistance to degradation by human enzymes. The toxicities of CDs are dependent on their route of administration and in some cases are well established. For example, the dose that causes 50% death (LD50) values of α-, β-, and γ-CD administered intravenously into mice are approximately 1.0 g per kg, 0.79 g per kg and more than 4.0 g per kg, respectively [94]. In spite of the potential for cytotoxicity, research is ongoing in the development of modified CD-siRNA nanoparticles. Recent advances in the formation of modified cationic and PEGylated CDs for siRNA delivery has yielded promising results in epithelial cell lines [95].

Finally, it is interesting to note that preliminary research has begun to investigate the potential in synergizing all of the above described polymers in a PEGylated supramolecular structure. On examination, PEGylated Chitosan-graft-(PEI-β-cyclodextrin) was found to induce up to 84% siRNA
knockdown in cell lines with a much improved cell viability compared to PEI alone [96]. This flexibility in formulation of siRNA nanoparticles demonstrates the abundance and potential for the development of a safe and efficient non-viral means of delivering siRNA to the lungs. This synergy and drive for new “smart” delivery systems is especially relevant today considering the cost of developing and scaling up production for clinical trials and releasing a drug now runs at approximately $1.8 billion over a period of 13.9 years [97, 98]. Many of the polymeric gene delivery systems harnessed for siRNA delivery in vivo to-date (Table 1) are readily available in large quantities including PEI, PEG and chitosan. For the modified polymeric carriers described herein e.g. PEI-PEG, scale up is feasible since, in the most part, the underlying chemistry is either relatively simple, uses mild/ aqueous reaction conditions, results in minimal side products and can be achieved to a high degree of purity [86,95,99-100]. The control and regulation of inhaled excipients is particularly rigorous and the current list of excipients approved for inhalation is very limited and does not include the common non-viral transfection polymers outlined here. The regulatory agencies such as the Food and Drug Administraion (FDA) and the European Medicines Evaluation Agency (EMEA) favour the use of established excipients and those generally regarded as safe (GRAS) but there is a growing appreciation for the need to expand the range of excipients that can be used for inhalation to enable effective delivery of new drugs including siRNA. There is a good deal of guidance to determine if a new excipient is safe for use in human pharmaceuticals including International Conference of Harmonisation (ICH) guidelines. Comprehensive toxicological evaluation of “smart” delivery systems will be critical and there is surprisingly little literature and information available in the field to-date on many commonly used polymeric systems.

**Non-viral “smart” delivery systems for targeting alveolar macrophages (AMs)**

Another major cell-type of interest in the lungs for siRNA targeting is the alveolar macrophage. Mononuclear phagocytes (MPs) such as monocytes, macrophages and dendritic cells play a central role in innate immunity. The major role of these cells is phagocytosis thereby engulfing and destroying apoptotic cells, pathogens and other targets generally by opsonin receptor-dependent mechanisms via complement- and Fc-receptors or opsonin receptor –independent mechanisms via lectin-
receptors, SRs, stearylamine (SA) receptors or CD14. Targeting siRNA to the alveolar macrophages that reside in the alveolar region of the airways would enable immune-modulatory siRNA therapies to be developed including anti-inflammatory agents, anti-cancer and those targeting *Mycobacterium tuberculosis*, which resides in the AM (101, 133).

**Liposomes for targeting AMs**

Liposomes are the most extensively explored delivery system for macrophage-targeted therapies and offer many benefits such as biocompatibility, low immunogenicity, cell targeting and drug protection. However, some liposomal drug carriers can be associated with poor scale-up and shelf-life, high cost and in some cases toxicity (reviewed in [102]). Liposomes targeting other cell types are generally modified to evade the phagocytic system for instance PEGylated “stealth liposomes” as mentioned previously [103]. There is now a greater understanding of the mechanisms of binding and uptake by macrophages that can be utilized for their targeting. Targeting of liposomes to AMs can be facilitated by (1) controlling the liposome physicochemical properties including size and surface charge or (2) the incorporation of ligands such as proteins, peptides, antibodies, polysaccharides, glycolipids, glycoproteins and lectins specific for AMs (Figure 4). Macrophages are notoriously difficult to transfect and although their location and phagocytic nature are advantageous in terms of alveolar macrophage targeting and pulmonary delivery, strategies that enhance cellular uptake are important.

A number of physicochemical parameters can be controlled to enhance AM targeting. Uptake of small liposomes (< 100nm) by phagocytic cells has been reported [104] however many other studies have shown liposome uptake to be improved with increased size [106, 107]. Surface charge may also promote macrophage targeting for instance phosphatidylinerine (PS) and phosphatidylglycerol (PG) both anionic lipids are preferentially recognized by macrophages [104]. Uptake studies comparing PG (neutral) and PS composed liposomes have long shown enhanced macrophage internalization of the anionic liposome formulations [108]. Apoptosis results in PS being exposed on the outer surface of cells and the initiation of phagocytosis. It is thought that macrophage Scavenger Receptors (SRs) recognize PS with SR classes A, B and D most likely to be involved. A 3.4-fold increase in rifampicin
lung retention in rats was determined by Vyas et al. using negatively charged DCP, PG and cholesterol composed liposomes in comparison to free drug following aerosol administration [109]. Furthermore, work by our group to deliver recombinant Secretory Leukocyte Protease Inhibitor (rSLPI) encapsulated in DOPS:Chol (7:3 molar ratio) liposomes has shown enhanced monocyte/macrophage uptake of liposomes both in vitro and in vivo [110].

In addition to controlling the physicochemical properties of liposomes, ligands such as lectins, antibodies and peptides can be incorporated into liposome formulations for improved macrophage targeting and uptake via lectin receptors, Fc receptors and integrins respectively. Using a ligand targeting strategy for liposome drug delivery has many advantages including increasing target specificity and avoiding the need for cationic lipids to trigger intracellular delivery. Ligands such as antibodies for example anti-CC531 antibodies [111], polysaccharides such as O-stearyl amylopectin (O-SAP), glycolipids such as Man3-DPPE and mannosylated cholesterol (Mann-C4-Chol) [112-114], peptides such as Arg-Gly-Asp (RGD) [115] and glycoproteins can be incorporated into liposome formulations for macrophage targeting. Hasida and colleagues have evaluated mannosylated liposomes targeting macrophages and DCs for the delivery of anti-inflammatory agents such as dexamethasone palmitate (dex) [116] and NFκB decoy and anti-cancer agents including CpG oligonucleotides and DNA [117]. Intratracheally administered Man-C4-Chol liposomes were shown to be preferentially taken up by alveolar macrophages mediated via macrophage mannose receptors. The degree of this mannosylation significantly improved liposome internalization by macrophages. Additionally the delivery efficiency using bubble liposomes and ultrasound in combination with mannosylated liposomes to deliver plasmid DNA to mouse peritoneal macrophages was assessed [118]. Initial studies by us have explored their application for siRNA delivery and found that they can significantly enhance uptake into alveolar macrophages (Figure 5).

**Microparticles for targeting AMs**

Microparticle carrier systems made from polymers such as PLA and PLGA can be generated within the size range of 1 µm to 250 µm, and size is an important factor in effective targeting of macrophage
cell populations where desired [119]. Previous work carried out by us demonstrated that microparticles of 2.1 µm in size were optimal for cell uptake in primary alveolar macrophages and differentiated THP-1 cells, and can be further enhanced through coating with gelatin [120]. For efficient pulmonary delivery the optimum mass median aerodynamic diameter (MMAD) of particles ranges between 1 µm to 5 µm that can be achieved using a range of manufacturing processes for PLGA microparticles [121]. Whilst size can be optimized through variations in manufacturing, encapsulation efficiency for siRNA in PLGA microparticles is poor. To overcome this, cationic agents such as PEI [122] and the cationic lipid N-[1-2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP) (figure 6) have been incorporated into formulations with siRNA. Whilst particles with optimal MMAD and size for cell internalization can be engineered, the aerodynamic inhalation capacity of the particles must also be considered. A recent study by our group demonstrated through cascade impaction analysis that incorporation of mannitol and then flash-freezing the microparticles prior to lyophilisation resulted in a significant improvement in aerosol performance and respirable dose [101]. Therefore, siRNA loaded PLGA microparticles can be bioengineered for both optimal lung delivery and macrophage cell internalization.

**Assessment of “smart” siRNA Therapies In Vivo**

Pulmonary drug delivery in humans generally involves the use of inhalers or nebulizers. Prior to clinical testing, drug formulations must be assessed in suitable in vivo models to examine efficacy, toxicity and immunogenicity and siRNA therapies are no different. The most common routes for pulmonary drug administration particularly in rodents are intratracheal, intranasal and inhalation [123]. A recent study by Gutbier et al. compared intratracheal, intranasal and intravenous administration of siRNA both naked and complexed with cationic liposomes in C57BL/6 mice [124]. Intratracheal instillation of fluorescently tagged naked siRNA showed efficient distribution throughout the lung while a higher dose was necessary to reach a similar level of siRNA delivery via the intranasal route with non-uniform distribution resulting. Although desired knockdown of E-cadherin was observed, siRNA rapidly entered the systemic circulation and was detected in the kidneys. Pharmacokinetic analysis performed by sandwich-hybridization ELISA determined the distribution of siRNA in the kidneys and
demonstrates the importance of not only manufacturing for effective respiratory delivery, but also the need for tailoring in vivo distribution of siRNA using particle engineering approaches. Interestingly siRNA cationic lipid (AtuFECT01) lipoplexes caused an inflammatory response characterized by a leukocyte influx in the lungs following intratracheal administration. However, following intravenous administration lipoplexes mediated significant targeted gene knockdown in lungs devoid of pulmonary inflammation.

In rodents intratracheal administration can offer many benefits over other routes of pulmonary administration due to their smaller anatomy such as increased dose reaching the smaller airways. It can give a more reproducible and higher deliverable dose than inhalation and improved pulmonary distribution compared to intranasal administration. Inhalation leads to variable respired doses between animals which will depend on the animal model being used and may lead to exposure, for example of the eyes, to aerosolized drug formulations. Also stable and functional siRNA formulations for inhalation are complex to develop. Intratracheal administration is invasive as it requires the animal to be anesthetized and formulations to be instilled via a tube surgically inserted between the tracheal rings or the endotracheal insertion of a tube through the mouth (without surgery and therefore less invasive). Intratracheal administration works best for proof of concept work though due to its invasive nature it is not routinely used in humans.

**Future Perspective**

The rapid development in the fields of molecular medicine and biomaterials over the last 20 years, now provide us with the key tools required to produce responsive and targeted medicines to treat patients. While controlled and targeted delivery systems are very well established for certain routes of administrations e.g. oral, parenteral, the concept of controlled or “smart” delivery systems in respiratory delivery is in its infancy. Biomaterials-based delivery systems have the potential when combined with the appropriate device to address key delivery and pharmacokinetic issues in inhaled products. Market reports predict a global pulmonary drug delivery technologies market of up to $40Billion by 2016 with a significant portion of this growth supported by technological advances in
biomaterials-based delivery systems which has lagged behind device development to-date. The strategies above relate to the current state of the art in developing siRNA for inhalation using non-viral vectors. The concept of TAB encompasses both device and particle engineering to produce advanced inhaled therapies and in the future will be a key part of translating these molecular therapies into clinically and commercially viable products. Using advanced materials the pharmacokinetics and ultimately pharmacodynamics of RNA-based therapeutics can be controlled in vivo. The focus in the short term will be to expand the range of materials that are approved for inhalation to encompass these more advanced materials. To-date the list of approved excipients for inhalation is small and inadequate for the delivery of macromolecules to or via the lungs. Investment in acute and chronic toxicology studies will be required to support their application in inhaled medicines. In the longer term the prospects of controlled and targeted delivery of siRNA harnessing bioresponsive materials capable of delivering the molecular therapy specifically to the disease site within the lungs e.g. cancer, inflammation and then intracellularly targeting a specific cell type in the airways e.g. epithelial cells and/or macrophages is an achievable goal. By combining these materials with cutting edge manufacturing processes inhalable medicines can be produced that enable sophisticated medicines to be delivered via a well-established clinical route of administration.

Executive Summary

**RNA interference in respiratory disorders**
- Inhalation therapy offers a non-invasive route for formulated-siRNA delivery for the treatment of respiratory diseases.
- A myriad of anatomical, physiological and cellular barriers must be overcome in the design of biocompatible, efficiently targeting and inhalable siRNA therapeutics.
- To-date no siRNA-based therapeutics have been approved as commercial products but many are in the clinical trial stage of development

**TAB of “smart” delivery systems for RNAi**
• Therapeutic aerosol bioengineering (TAB) encompasses both particle and device engineering to produce an inhaled product that is suitable for the therapeutic agent and the patient population it being targeted.

• Various approaches for therapeutic aerosol bioengineering of siRNA have been trialed to improve drug deposition and targeting.

• Device engineering of both dry powder and liquid spray devices has focused on increasing the FPF, minimizing oropharyngeal deposition and improving patient compliance.

• Particle engineering aims to incorporate desirable properties such as tailored size, shape, porosity, and drug release and targeting characteristics into aerosol particles, often composed of a mixture of drug and excipient.

• Excipients used in TAB have helped to improve the efficacy of pulmonary drug therapy however problems with non-specific targeting and inadequate cellular uptake remain a scientific challenge and issues around cytotoxicity and irritancy remain a regulatory hurdle that must be overcome to reach the clinic.

Future perspective

• A significant portion of the growth in the inhaled drug products market over the next 5-10 years will be supported by technological advances in biomaterials-based delivery systems which has lagged behind device development to-date

• A growing number of RNAi technologies being developed in biomedical discovery companies and academic laboratories including siRNA, shRNA and miRNA modulators will drive the need for cell-type specific targeting in the airways to underpin biomedical research and its translation into the clinic.

• An increasing number of advanced excipients will be approved for delivery to the lungs to overcome poor delivery and targeting but the speed of this approval is heavily dependent on the development of better in vitro and in vivo respiratory models and the funding for toxicology studies to fulfill regulatory requirements.
• With advancements in device and particle engineering the tools to manufacture “smart” inhalable RNAi therapeutics are now available to facilitate the full clinical and commercial potential of these therapies being realized.

Acknowledgements
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References


   **Comprehensive review of polymeric non-viral gene vectors**


*Commercial view of the area


** Review of the key technical challenges in pulmonary delivery of siRNA


Websites:


Phase IIb clinical trial of an inhaled siRNA therapy

Reference annotations:

* = of interest

** = of considerable interest
Table Legends

Table 1: Non-viral siRNA delivery to the lungs *harnessed for in vivo studies to-date*

Figure Legends:

Figure 1: Scanning electron micrographs of spray-dried microparticles prepared using:
Alginate (A); Chitosan (B); Gelatin (C) and suitable for inhalation (modified with permission from [14])

Figure 2: A) Scanning electron micrographs of protein-loaded bioresponsive microparticles
B) Elastase-mediated release of proteins (BSA) from bioresponsive microparticles in the
presence/absence of elastase or trypsin (n=3±SD) (modified with permission from [24])

Figure 3: Transport of poly(sebacic acid)-co-poly(ethylene glycol) (PSA-PEG) and
polystyrene (PS) nanoparticles in human cystic fibrosis sputum (CFS). (A) Transport mode of
particles in CFS. (B) The fraction of particles predicted to penetrate a 10 nm thick CFS layer
over time using Fick’s second law and diffusion coefficients obtained from tracking
experiments (redrawn from [76]) (C) PEGylation of PEI results in an increase in endogenous gene
knockdown in fully-differentiated, mucous-producing Calu-3 cells (modified from [75]).

Figure 4: Examples of particle targeting strategies to alveolar macrophages

Figure 5: Targeted liposome uptake by mouse macrophage RAW 264.7 cells. RAW 264.7
cells were incubated with 0 or 200μM rhodamine tagged anionic (DOPS), neutral (DOPC) or
mannosylated liposomes for 2 hours at 37°C, fixed and stained with hoescht (nucleus; blue)
and phalloidin-FITC (F-Actin; green). Image acquisition and analysis was carried out using
an INCELL1000 Cell Analyser system. (A) shows RAW264.7 cells treated with 200μM
anionic liposomes and (B) shows liposomes counted per cell. Statistical significance was
determined * p < 0.05, ** p < 0.01, *** p < 0.001 vs. DOPC treated counterparts. Data
represented as means ± SD (n = 6).
Figure 6: (A) Scanning electron micrograph of siRNA loaded PLGA microparticles (B) Primary human macrophages were isolated and allowed to adhere to 96 well plates. Cells were transfected with fluorescently tagged siRNA encapsulated in PLGA microparticles (siRNAMP) and after 24 hour incubation cells were fixed and counterstained with phalloidin- TRITC (F-actin, red) and Hoecsh (nucleus, blue). Images were acquired at 10x using an INCELL 1000.

Table 1: Non-viral siRNA delivery to the lungs *harnessed for in vivo studies to-date*

<table>
<thead>
<tr>
<th>siRNA Target</th>
<th>Delivery Carrier</th>
<th>Animal Model</th>
<th>Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-catenin</td>
<td>Naked siRNA</td>
<td>Pulmonary Fibrosis induced in C57BL/6N mice</td>
<td>Significant reduction in β-catenin expression and induced pulmonary fibrosis</td>
<td>[125]</td>
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<td>XCL1</td>
<td>Naked siRNA</td>
<td>C57BL/6 mice</td>
<td>Transient 50% knockdown of XCL1 gene giving long-term benefit</td>
<td>[126]</td>
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<td>GFP</td>
<td>Naked siRNA</td>
<td>C57BL/6-TgN(ACTbEGFP)1OsB</td>
<td>GFP, Fas and Caspase-8 each reduced in expression. Fas siRNA treatment</td>
<td>[127]</td>
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<tr>
<td>Gene</td>
<td>Treatment</td>
<td>Model</td>
<td>Result</td>
<td></td>
</tr>
<tr>
<td>------</td>
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<tr>
<td>Caspase-8</td>
<td>Naked siRNA</td>
<td>C57BL/6-TgN and C3H/HeN mice</td>
<td>showed greatest physiological benefit</td>
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<tr>
<td>GFP</td>
<td>Naked siRNA</td>
<td>C3H/HeN mice</td>
<td>Lung GFP, KC and MIP-2 expression reduced in a hemorrhage-induced sepsis mouse model</td>
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<tr>
<td>KC</td>
<td>Naked siRNA</td>
<td>C3H/HeN mice</td>
<td>Reduced GRP78 expression and decreased bronchial hyper-responsiveness</td>
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<td>MIP-2</td>
<td>BALB/C mouse OVA induced asthma model</td>
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<tr>
<td>GRP78</td>
<td>Naked siRNA</td>
<td>BALB/C mouse OVA induced asthma model</td>
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<tr>
<td>Liposomes</td>
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<tr>
<td>Cy3-tagged VE-Cadherin</td>
<td>naked, stabilized siRNA (AtuRNAi)</td>
<td>C57BL/6 mice</td>
<td>21% reduction in E-Cadherin, however no significant reduction in VE-Cadherin</td>
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<tr>
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<td>Cationic liposomes</td>
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<tr>
<td>siGLO red</td>
<td>Cationic liposomes</td>
<td>Athymic nu/nu mice</td>
<td>Higher siRNA concentration and retention in the lungs compared to systemic delivery</td>
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<td>siGLO Green</td>
<td>DharmaFect (lipid based transfection reagent)</td>
<td>Pulmonary Fibrosis induced in C57BL/6 mice</td>
<td>Fluorescent siRNA detected in epithelial cells of bronchi and bronchioles</td>
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<tr>
<td></td>
<td>Pulmonary Fibrosis induced in C57BL/6 mice</td>
<td>Collagen content of the lung reduced by up to 68%</td>
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<td>Peptides</td>
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<tr>
<td>p38 MAP kinase</td>
<td>Naked siRNA Cholesterol Peptides</td>
<td>BALB/c mice</td>
<td>30-45% knockdown of p38-MAP kinase localized within macrophage and epithelial cells</td>
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<tr>
<td>Polymers</td>
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<tr>
<td>eGFP</td>
<td>PEI</td>
<td>C57BL/6J-Tg(Bos/GFP)CaBa-Bii011Dcm mice</td>
<td>Significant knockdown of up to 75% however non-specific knockdown of up to 58% was observed</td>
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<tr>
<td>siGL3</td>
<td>PEI-PEG</td>
<td>C57BL/6J-Tg(Bos/GFP)CaBa-Bii011Dcm mice</td>
<td>60% knockdown in GFP expression</td>
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<td>antisense 2'-O-Methyl RNA (OMR)</td>
<td>chitosan-coated poly(lactide-co-glycolide)</td>
<td>Male WISTAR rats</td>
<td>Significant increase in OMR uptake in nanoparticles compared to naked siRNA</td>
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<td>siEGFP</td>
<td>PEI-PEG</td>
<td>C57BL/6-Tg(CAG-EGFP)1Os/J mice</td>
<td>42% knockdown in GFP expression</td>
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<tr>
<td>eGFP</td>
<td>Chitosan</td>
<td>B6;129P2- RAGE tm1.1 mice</td>
<td>82% knockdown in eGFP expression</td>
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<td>siRNA-GFP duplex</td>
<td>Salbutamol-guanidinylated chitosan</td>
<td>EGFP-transgenic mice (C57BL/6-Tg) (ACTb-EGFP)</td>
<td>Significant (~40%) decreases in GFP expression</td>
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<tr>
<td>eGFP</td>
<td>PEI</td>
<td>C57BL/6 Tg(CAG-EGFP)1Os/J mice</td>
<td>42% decrease in eGFP expression</td>
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Figure 1:
Figure 2:

A) ........................................ B)
Figure 4:
Figure 5
Figure 6: