Aerosol Delivery of siRNA to the Lungs.  
Part 2: Nanocarrier-based Delivery Systems†

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Abstract

In this article, applications of engineered nanoparticles containing siRNA for inhalation delivery are reviewed and discussed. Diseases with identified protein malfunctions may be mitigated through the use of well-designed siRNA therapeutics. The inhalation route of administration provides local delivery of siRNA therapeutics to the lungs for various pulmonary diseases. A siRNA delivery system can be used to overcome the barriers of pulmonary delivery, such as anatomical barriers, mucociliary clearance, cough clearance, and alveolar macrophage clearance. Apart from naked siRNA aerosol delivery, previously studied siRNA carrier systems include those of lipidic, polymeric, peptide, or inorganic origin. These delivery systems can achieve pulmonary delivery through the generation of an aerosol via an inhaler or nebulizer. The preparation methodologies for these siRNA nanocarrier systems will be discussed herein. The use of inhalable nanocarrier siRNA delivery systems have barriers to their effective delivery, but overcoming these constraints while formulating a safe and effective delivery system will offer unique advances to the field of inhaled medicine.

Keywords: siRNA, nanocarrier, nanoparticle, aerosolization, pulmonary, lung

1. Introduction

siRNA has potential therapeutic applications in treating ‘undruggable’ diseases via post-transcriptional downregulation of target gene expression. The mechanisms of RNAi have been comprehensively reviewed (Fellmann C. and Lowe S.W., 2014; Fire A. et al., 1998; Hannon G.J., 2002; Kim D.H. and Rossi J.J., 2007). The siRNA possess a specific sequence that is complementary with its target mRNA and induces site-specific cleavage and subsequent inhibition of intracellular protein synthesis. The synthesis of siRNAs is relatively simple compared to other therapeutic classes because it does not require a cellular expression system, complex protein purification, or refolding schemes (Amarzguioui M. et al., 2005). A major advantage of siRNA over small molecule drugs or protein therapeutics is that the sequences can be rapidly designed for highly specific inhibition of the target of interest.

Pulmonary diseases such as asthma, lung cancer, cystic fibrosis, pulmonary hypertension, and chronic obstructive pulmonary disorder (COPD) have potential siRNA therapeutic targets (Amarzguioui M. et al., 2005; Burnett J.C. and Rossi J.J., 2012; Kanasty R. et al., 2013). However, due to the high negative charge density and the relatively large size of the siRNA molecules, naked siRNA molecules are not able to enter cells efficiently (Reischl D. and Zimmer A., 2009). Thus, delivery systems for siRNA need to be developed to successfully protect and deliver these agents. Nevertheless, siRNA delivery systems are likely to have instability issues that cause premature release of the nucleic acids, especially with systems that incorporate their cargo through electrostatic interactions. Pulmonary delivery of siRNA faces major challenges that involve decreased correlation between in vitro and in vivo experiments, difficulty in translation from animal models to humans, and administration routes used in animal studies that are non-applicable for human use (Lam J.K.-W. et al., 2012). Another challenge in siRNA therapy is the possibility of off-target effects induction.

This second part of a two part review article focuses on the pulmonary route of administration, siRNA loaded non-viral particulates for pulmonary or nasally inhaled delivery systems, and preparation techniques for siRNA
loaded nanoparticles (Youngren-Ortiz S.R. et al., 2016). While the previous part covered the rationale for the use of various siRNA delivery systems, this part will focus on the preparation of siRNA loaded nanocarrier systems including examples of their pulmonary delivery.

2. Non-viral delivery of siRNA to the lung

siRNA is a highly negative charged, hydrophilic, and large-sized (approximately 13.3 kDa) macromolecule that cannot cross biological membranes to reach their target sites. Viral vectors have previously demonstrated cell uptake and siRNA efficacy, however, major limitations to human therapeutic delivery exist, such as uncontrolled viral replication, immunogenicity, tumorigenicity, and toxicity (Thomas C.E. et al., 2003). Due to these concerns, non-viral delivery systems have been developed and successfully used to deliver siRNA. An ideal siRNA delivery system should (1) condense siRNA into nanosized particles, (2) protect siRNA from enzymatic degradation, (3) facilitate cellular uptake, (4) promote endosomal escape to release siRNA to the cytoplasm where the RNA-induced silencing complex (RISC) is located, (5) have negligible effects on gene silencing activity or specificity and (6) have negligible toxicity (Lam J.K.-W. et al., 2012; Merkel O.M. and Kissel T., 2011). Non-viral delivery systems include naked siRNA, and delivery vectors such as lipids, polymers, peptides, and inorganic materials, as shown in Fig. 1.

2.1 siRNA delivery

2.1.1 Introduction

Unformulated or naked siRNA involves the delivery of siRNA without the use of a delivery vehicle or carrier. Advantages of this strategy include the ease of preparation and the facility of delivery by inhalation, intratracheal, or intranasal routes. A major disadvantage of this strategy is that the delivered siRNA is susceptible to poor cell targeting and uptake, and to degradation within the airways. As discussed in part 1 of the review article, the major barriers for the delivery of siRNA to the lung include the presence of mucus, alveolar fluid, alveolar macrophages, and mucociliary clearance. Unfavorable physicochemical properties of siRNAs (negative charge, large molecular weight) and instability in plasma (half-life 10 min) also poses major delivery challenges (Ren Y. et al., 2010). Furthermore, after being transported intracellularly into the lysosomes via endocytosis, siRNA gets degraded in the lysosomes which diminishes the activity of siRNA therapeutics (Tseng Y.-C. et al., 2009). Since unmodified siRNA is prone to enzymatic degradation, new methods such as chemical modification of siRNA have been developed to increase stability. The chemical modification also improves specificity and potency, and reduces the immune response and off-target effects (Watts J.K. et al., 2008).

2.1.2 RNA modifications & preparation methods

The local delivery of siRNA is particularly well-suited for lung disease and infection therapy (De Fougerolles A. and Novobrantseva T., 2008). The direct instillation of siRNA into the lungs through intranasal or intratracheal routes results in direct contact with lung epithelial cells. Non-modified siRNA’s can also induce nonspecific activation of immune system through the Toll-like receptor 7 pathway (Whitehead K.A. et al., 2009a). However, chemical modifications can be introduced into the RNA duplex structure which can enhance biological stability without adversely affecting gene-silencing activity and prevent nonspecific immune activation. Some of the modifications include incorporation of 2’-O-methyl modifications into the sugar structure of selected nucleotides within both the sense and antisense strands. The therapeutic efficacy of delivered siRNA can also be improved using conjugation of small molecules or peptides to the sense strand of siRNA. Several other modifications have been reported to eliminate the off-target effects such as phosphorothioate or boranophosphate introduction (Gandhi N.S. et al., 2014).

In a recent study, Antagomir-122 was synthesized from a hydroxyproline-linked cholesterol solid support and 2’-O-methyl phosphoramidites. On administration a marked decrease in endogenous mir-122 levels in the liver was observed (Krützfeldt J. et al., 2005). This and such other newer strategies have shown promise in protecting the siRNA against degradation by endonucleases thus allowing them to reach their site of action.

There are 3 main methods used for the production of siRNA in vitro which includes 1) chemical synthesis, 2) in vitro transcription of small RNA’s and 3) in vitro transcription of long RNA’s produced by the digestion with Dicer enzyme (Aalto A.P. et al., 2007). The in vitro chemical synthesis of siRNAs involves in vitro transcription and digestion of long dsRNAs by an RNase III family enzyme (e.g. Dicer, RNase III). These production methods

![Fig. 1 Schematic of different non-viral siRNA delivery vectors.](image-url)
require the design of siRNA sequences before siRNA preparation. Recently, Aalto et al. utilized an in vitro system using the combination of T7 RNA polymerase and RNA-dependent RNA polymerase (RdRP) of bacteriophage f6 to generate siRNA molecules. They further used an in vivo RNA replication system to produce siRNA. This system was based on carrier state bacterial cells containing the f6 polymerase complex which can result in unlimited amounts of siRNA up to 4.0 kb in size. Unmodified or modified siRNA can be prepared with simple reconstitution within normal saline or 5% dextrose solution for inhalation delivery. Aerosolization of the siRNA solution can be conducted using a nebulizer. A new class of naked siRNA has been developed by Hamasaki et al., termed ribophorin II (PnkRNA™) and nkRNA ® as shown in Fig. 2 (Hamasaki T. et al., 2012). This novel class of RNAi agents was synthesized as single-stranded RNA on a solid phase that, following synthesis, self-anneal as shown by Hamasaki et al. Nucleotides shown in red indicate sense strand of the target (GAPDH), nucleotides shown in violet are the antisense strand, and nucleotides shown in blue are the loop cassettes. P indicates a proline derivative. Reprinted through open access agreement under the Creative Commons Attribution (CC BY) license from Ref. (Hamasaki T. et al., 2012).

2.1.3 Examples of siRNA delivery

Luciferase expressing mice were treated by intratracheal administration of 10 nmol siRNA duplex or 10 nmol phosphorothioate locked antisense oligonucleotide free nucleic acids (Moschos S.A. et al., 2011). Ex vivo luminometry elucidated organ associated luciferase knockdown. IVIS imaging of oligonucleotides labeled with Cy5 combined with confocal microscopy was used to determine their biodistribution (Moschos S.A. et al., 2011). Upon administration, the oligonucleotides underwent fast systemic distribution by transcytosis and renal clearance. The kidney and liver uptake of the phosphorothioate locked nucleic acid antisense oligonucleotides caused gene knockdown in these organs. However, protein expression was not downregulated in purified lung tissue cells (Moschos S.A. et al., 2011). The reduced therapeutic efficacy of free RNA supports observations by another group, who concluded that intratracheally delivered free TNF-α siRNA post-hemorrhage was inefficient in reducing the symptoms in a septic shock model of acute lung injury (Lomas-Neira J. et al., 2012). After bleomycin triggered pulmonary fibrosis was induced in mice with transgenic expression of human TGF-β1, 5 mg/kg TGF-β1 siRNA or a scrambled control was delivered intratracheally. This study found that siRNAs sequences shared by human and rodents successfully knocked down TGF-β1 expression in human derived cell lines and significantly inhibited pulmonary fibrosis in vivo (D’alessandro-Gabazza C.N. et al., 2012).

2.2 Lipid-based delivery systems

2.2.1 Introduction

Lipid-based delivery systems are commonly used to deliver siRNA in vitro or in vivo (Tseng Y.-C. et al., 2009). Most often, cationic lipids or liposomes are used to form complexes, termed lipoplexes with anionic siRNA through spontaneous electrostatic interaction. Commercial siRNA transfection agents are commonly lipid-based systems, including Oligofectamine™, TransIT-TKO, Lipofectamine® RNAiMAX, and DharmaFECT (Bitko V. et al., 2005; Heidel J.D. et al., 2007; Tompkins S.M. et al., 2004; Wang J.-C. et al., 2010). A major challenge of using lipid-based siRNA delivery systems is their toxicity and non-specific activation of inflammatory cytokines and interferon responses (Wu S.Y. and Mcmillan N.A., 2009). Since aerosolization is a high shear stress process, the stability of the liposomes should be monitored since this process may cause physical and chemical changes that can lead to early siRNA release and degradation of the siRNA (Gaspar M.M. et al., 2008). Lipid based delivery systems for siRNA include liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC).

SLN are prepared by replacing the oil of the fat emulsion by a solid lipid or a blend of solid lipids, which makes the lipid matrix of the SLN solid at room and body temperature (Cavalli R. et al., 1997; Kalaraiya M. et al., 2005; Schwarz C. et al., 1994; Siekmann B. and Westesen K.,...
1994). SLN are composed of 0.1–30 % w/w lipid dispersed in an aqueous solution of 0.5–5 % w/w surfactant as a stabilizing agent (Pardeike J. et al., 2009; Weber S. et al., 2014). SLN provide physical stability, chemical stability, controlled release, and low cytotoxicity if appropriate excipients are utilized (Wissing S. et al., 2004). SLN can be produced without the use of organic solvents and can be scaled up. Disadvantages of SLN are low loading capacity and premature release during storage (Mehnert W. and Maeder K., 2012). These events occur because the low ordered lipid modification of the particle matrix after production transforms to the highly ordered β-modification during storage. The β-modification is characterized by perfect crystal lattice with few imperfections and therefore, little room is left for siRNA storage. In order to overcome these issues, a second generation of lipid nanoparticles, the NLC were developed. NLC have a solid lipid matrix at room and body temperature and consist of a blend of solid lipid and oil, preferably at a ratio of 70:30 up to a ratio of 99.9:0.1 (Mehnert W. and Maeder K., 2012; Patel A.R. et al., 2013; Patlolla R.R. et al., 2010). Through mixing different kinds of lipids, a less ordered matrix with more room for active compounds is achieved (Mehnert W. and Maeder K., 2012).

2.2.2 Preparation methods
2.2.2.1 Cationic lipoplexes

Cationic lipids or liposomes can spontaneously form complexes with negatively charged siRNA through electrostatic interactions to form lipoplexes (Fig. 3). Complexation of cationic lipids and siRNA occurs through the negatively charged siRNA interaction with the positively charged lipids. This interaction causes neutralization of the cationic lipids, causing them to aggregate and encapsulate the siRNA (Bochicchio S. et al., 2014). It is necessary to optimize the lipid composition, lipids to siRNA ratio and the lipoplexes preparation methods, in order to improve stability and delivery efficiency while reducing possible adverse effects. Lipoplexes often have good transfection efficiency due to their efficient interaction with the negatively charged cell membranes as shown in Fig. 3. However, lipoplexes generally display poor stability and reproducibility when compared to other lipid based siRNA delivery systems, such as liposomes or SLN (De Fougerolles A.R., 2008). Another disadvantage is that cationic lipids are typically more toxic than neutral lipids (Dokka S. et al., 2000). To shield the positive charge of the cationic lipids, hydrophilic polymers such as PEG have been used to reduce inflammatory response. Lipoplexes are not stable in liquid suspension for long-term storage, as shown by several studies in which the lipoplexes aggregate (Anchordoquy T.J. et al., 1997; Felgner P.L. et al., 1995; Gao X. and Huang L., 1996; Hofland H. et al., 1996; Hong K. et al., 1997). Lyophilization is another method for intervening this issue that has been shown to inhibit aggregation for long-term storage of nucleic acid formulations (Allison S.D. and Anchordoquy T.J., 2001; Allison S.D. et al., 2000; Anchordoquy T.J. et al., 1997). Lyophilization allows for storage at room temperature, and therefore is preferred over freezing due to cost reduction in transportation and storage, and improved stability of biomolecules resulting from the removal of non-freezable water associated with most biomolecules (Umrania Y. et al., 1995). Lyophilization can cause liposome fusion and phase separation during the drying and rehydration steps (Crowe J.H. and Crowe L.M., 1988). To overcome these problems, cryoprotectants, such as carbohydrates, are used. Cryoprotectants limit mechanical damage and rupture of the lipid bilayer, caused by ice crystals, during the freeze-drying and the rehydration process by maintaining the membrane in a flexible state (Allison S.D. and Anchordoquy T.J., 2000).

2.2.2.2 Liposomes

The basic steps of loaded liposome production include (1) lipid hydration, (2) regulation of liposome size, and (3) removal of non-encapsulated drug (Samad A. et al., 2007).

Lipid hydration can be achieved through mechanical methods, organic solvent methods, or detergent removal.
Methods (Bochicchio S. et al., 2014; Youngren S. et al., 2013). Mechanical methods for lipid hydration are based on using a rotating evaporator to form a thin-layer of phospholipids onto the wall of the rotating vessel. The layer is hydrated with an aqueous buffer solution that contains the siRNA to load while the vessel rotates. The process forms multimellar vesicles, which can further be sonicated, extruded, or homogenized to form small unilamellar vesicles. Glass beads may be used to optimize the process by allowing the creation of thinner lipid films by increasing surface area. These thinner lipid films allow for higher hydration efficiency and siRNA entrapment.

Lipid hydration by organic solvent methods requires the melting of lipids within an organic solvent followed by exposure to an aqueous phase that is then separated by evaporation. If the starting point is an organic solvent immiscible with the aqueous phase, then it is possible to use the reverse-phase evaporation vesicles technique, where the intermediate state is represented by emulsions. The lipid solution in an organic solvent with the aqueous buffer is sonicated to obtain a water-in-oil (W/O) emulsion. Controlled evaporation of the organic solvent leads to the formation of unilamellar vesicles. When most of the organic solvent is removed, a gel is formed, and it has to be agitated to transform into a viscous fluid that contains liposomes. The gel collapse coincides with the conversion of the W/O emulsion in the liposome form. If the starting point is an organic solvent miscible with water, then it is possible to continue with a precipitation stage. An aqueous buffer is added to the miscible organic solvent to dilute it, leading to the precipitation of lipids which then aggregate to form liposomes. The speed of the dilution passage can be adjusted to obtain desirable liposomal particle size, with faster dilution passages being associated with smaller particle size of formed vesicles.

Detergent removal methods for lipid hydration form micellar structures that engulf more lipids as the detergent is removed to form unilamellar vesicles. This method is less efficient for low molecular weight compounds than other lipid hydration methods (Tarkunde S.V. et al., 2014). Since this method has not been studied for large hydrophilic compounds like siRNA molecules, it is assumed that liposomal formulation of siRNA using this method is not suitable (Bochicchio S. et al., 2014).

Size optimization and removal of free siRNA should be performed following the lipid hydration step. Size of liposomes should be adjusted based on the siRNA that needs to be incorporated. This may be achieved through (1) extrusion at low or medium pressure through dimensionally defined pores, (2) fractionation of a heterogeneous population by centrifugation or size-exclusion chromatography, and (3) homogenization to obtain smaller sized liposomes (Bochicchio S. et al., 2014). Examples of extrusion devices are the Avanti Mini-Extruder and Nano DeBEE high pressure homogenizer shown in Fig. 4. Other mechanical dispersion methods include sonication, freeze-thawed liposomes, lipid-film hydration by shaking or freeze drying, or dried reconstituted vesicles (Çağdaş M. et al., 2014). The non-encapsulated siRNA can be removed by dialysis, ultrafiltration via ultracentrifugation, gel chromatography, and ionic-exchange resins (Bochicchio S. et al., 2014).

Methods of encapsulating siRNA in liposomes include simple mixes of siRNA with pre-assembled liposomes, pre-condensation of siRNAs before liposome encapsulation, direct hydration of a lipid thin-film layer with a siRNA solution, and the ethanol dilution method (Bochicchio S. et al., 2014). A pre-assembled liposome can be complexed with siRNA with a simple mixture method. This method is not suitable for PEGylated liposomes, since siRNA is unable to penetrate the PEGylated lipid bilayer efficiently (Gutbier B. et al., 2010). In this case, the siRNA would bind to the liposomal surface, which causes premature release of the siRNA from the liposome in vivo. To achieve a siRNA PEGylated liposome loaded using the simple mixture method, the siRNA and the non-PEGylated liposomes can be incubated to form the siRNA lipidosome complex and subsequent incubation with PEG-lipids at elevated temperatures. The PEG-lipids would be inserted within the lipid bilayer, which can be verified by measuring zeta potential (Whitehead K.A. et al., 2009b). An alternative is to covalently bind PEG chains to the siRNA lipidosome complexes after simple mixture loading of the siRNA. An example of this surface modification is the binding of PEG to the amine functional groups of the cationic cholesterol polyamine (CDAN) component of a liposomes using a pH-sensitive oxime linkage that decomposes at pH < 5.5 (Carmona S. et al., 2009). This technique, known as the “ABCD paradigm”, overcomes drawbacks of other PEGylation methods, in that there is adequate siRNA encapsulation, PEGylation elicits a stealth behavior, and the PEG chain release of the liposomes within the endosomal compartments leads to a pH triggered endosomal escape (Kostarelos K. and Miller A.D., 2005).

Another technique involves pre-condensation of siRNA with protamine, a natural cationic polypeptide, along with high molecular weight polyanions, such as hyaluronic acid, at a ratio allowing for negatively charged nanoparticles (Li S.D. and Huang L., 2009). The high molecular weight polyanion is used to provide resistance to reticuloendothelial clearance instead of PEG (Kim S.S. et al., 2010). These complexes are then incubated with cationic liposomes to form the siRNA encapsulated liposomes. Alternatively, siRNAs can be pre-condensed with calcium phosphate nanoparticles followed by the cationic liposomal coating and PEG-lipids (Li J. et al., 2010).

The direct hydration of a thin-film lipid layer with...
siRNA solution allows for encapsulation of siRNA within the aqueous core of a liposome. Addition of a concentrated solution of siRNA to a dry thin lipid film layer allows for the incorporation of the siRNAs into the internal cationic space of a lipidic bilayer (Buyens K. et al., 2009). Since siRNA is present during the formation of the liposomes, approximately 50% of the siRNA is encapsulated as it is uniformly distributed across cationic lipids, including the internal and external lipid bilayers. An alternative approach involves the formation of a monophasic solution of siRNAs and DOTAP in water/methanol/chloroform mixture that leads to a perfect complex between the negative charge of siRNA and the positive charge of the cationic lipids (Podesta J.E. and Kostarelos K., 2009). Once excess water and chloroform are added, a phase separation occurs. siRNA/DOTAP micelles stay in the organic phase and the aqueous/methanol phase can be discarded. The neutral PEGylated lipids are added along with water and then the organic solvent can be removed by evaporation. The liposomes can then be extruded for further size control (Bochicchio S. et al., 2014).

The ethanol dilution method for directly hydrating the lipid layer is an alternative method of direct hydration. Cationic PEGylated liposomes are mixed with siRNA in the presence of a critical concentration of ethanol (approximately 40%) in the aqueous buffer (Semple S.C. et al., 2001). At this ethanol concentration, liposomes are destabilized and their membrane structural integrity is compromised, allowing siRNA to uniformly penetrate the liposomes and associate with the positive charges on the lipids. Ethanol concentration is pivotal since if it is at an inadequate level, the siRNAs would not be able to efficiently penetrate the lipid bilayer. If the ethanol concentration is too high, liposomal aggregation would occur. The ethanol is removed by dialysis or tangential filtration following siRNA loading, forming the PEGylated cationic liposomes containing approximately 50% of the originally added siRNA. Stabilized Nucleic Acid Lipid Particles, or SNALPs, is a widely recognized example of liposomal complexes prepared using the ethanol dilution method (Morrissey D.V. et al., 2005). A cationic lipid is protonated at low pH of 4–5 within the ethanol of the complex formation buffer, but is neutral when the acidic buffer is exchanged with a physiological buffer at pH 7.4. The advantages of cationic lipids are that siRNAs bound to the external surface of the liposome are detached when neutralized. The released siRNAs can be washed away from the complexes, together with ethanol, during the

![Avanti Mini-Extruder](A), Avanti Mini-Extruder internal schematic (B), and Nano DeBEE high-pressure homogenizer (C).

Fig. 4 Avanti Mini-Extruder (A), Avanti Mini-Extruder internal schematic (B), and Nano DeBEE high-pressure homogenizer (C).
other exchange procedure. The final complexes can have a final siRNA complexation and encapsulation efficiency of around 90%. Other complexes of siRNA/PEGylated liposomes are prepared similarly to SNALPs only that they contain other cationic lipids and termed as Lipidoid Nanoparticles (Akinc A. et al., 2008; Love K.T. et al., 2010). This process is carried out with a combination of siRNAs in aqueous buffer with an equal volume of lipids (cationic, neutral, and PEGylated lipids), dissolved in butanol, the mixture is lyophilized and the lyophilized matrix is rehydrated (Akinc A. et al., 2008; Wu S.Y. and Mcmillan N.A., 2009).

2.2.2.3 Solid lipid nanoparticles and nanostructured lipid carriers

SLN formulations are composed of solid lipids, emulsifiers, and water (Mehnert W. and Maeder K., 2012). A schematic of SLN preparation detailing the types of components and feed solutions used is shown in Fig. 5. NLC formulations are composed of solid lipids, oil, surfactant, and water (Mehnert W. and Maeder K., 2012). Lipids are used in a broad sense to include triglycerides, partial glycerides, fatty acids, steroids, and waxes. All classes of emulsifiers have been used to stabilize the lipid dispersion. Particle agglomeration may be efficiently prevented through the combination of emulsifiers selected in respect to charge and molecular weight. The use of physiologic lipids within the lipid matrix decreases the possibility of acute or chronic toxicity. The choice of emulsifier heavily depends on the administration route.

Initially, high shear homogenization and ultrasound are dispersing techniques used for the production of solid lipid nanodispersions. Despite both methods being common due to their ease of use, dispersion quality is poor due to the presence of microparticles and metal contamination.

High pressure homogenization is a reliable and powerful technique for the preparation of SLN. High pressure homogenization has been used for the production of nanoemulsions for parenteral nutrition; therefore these homogenizers are available from several manufacturers with varying sizes. The scalability of this technique is highly efficient. High pressure homogenizers force liquid with 100–2000 bar pressure through a narrow orifice with size of approximately a few micrometers. Due to the narrowed orifice, the fluid accelerates on a very short distance to a high velocity of over 1000 km/h. High shear stress and cavitation forces disrupt the particles down to the nanoparticle size range. Typically, the lipid contents are between 5–10% and even higher lipid concentrations up to 40% have been successfully homogenized to lipid nanodispersions. A preparation step that incorporates drug into the bulk lipid by dissolving or dispersing the drug in the lipid melt followed by hot or cold homogenization techniques are used to prepare SLN.

Hot homogenization, or the homogenization of an emulsion, is performed at temperatures above the melting point of the lipid. The pre-emulsion of siRNA loaded lipid melt and the aqueous emulsifier phase is obtained using a high shear mixing device, such as an Ultra-Turrax homogenizer. The quality of the pre-emulsion affects the quality of the final product to a high degree since it is desirable to obtain droplets within the size range of a few micrometers. The higher temperature leads to a lower particle size because of the decreased viscosity of the inner phase. However, high temperatures may increase the degradation of the siRNA or the SLN. The homogenization step may be repeated several times to achieve the desired particle size, keeping in mind that the high pressure homogenization equipment increases the temperature of the sample approximately 10°C for 500 bar. Increasing the homogenization pressure and number of cycles over the recommended 500–1500 bar 3–5 cycles, respectively causes a particle size increase of the lipid nanocarriers due to particle coalescence as a result of high kinetic energy of the particles. The primary product of hot homogenization is a nanoemulsion due to the liquid state of the lipid. Cooling the nanoemulsion sample to a lower temperature forms the solid particles. Lipid crystallization is slowed due to the small particle size and the incorporation of emulsifiers, therefore the sample may remain as a supercooled melt for several months.

Cold homogenization is a high pressure milling of a suspension of solid lipids. Effective temperature control and regulation is needed in order to ensure the unmolten state of the lipid due to the increase in temperature during homogenization. Cold homogenization has been developed to overcome temperature induced drug degradation, drug distribution into the aqueous phase during homoge-
tion, and the complexity of the crystallization step of the nanoemulsion leading to several modification or supercooled melts associated with hot pressure homogenization. The first preparatory step that includes the dispersing of the drug in the melt of bulk lipid is followed by a rapid cooling step within liquid nitrogen or dry ice. In this step, the high rate of cooling causes the formation of a homogenous distribution of the drug within the lipid matrix. The solid, siRNA containing lipid is then milled to microparticles. Ball or mortar milling typically results in particle sizes of 50–100 micrometers. Low temperatures increase the fragility of the lipid and causes particle commination, or the reduction in particle size. The solid lipid microparticles are dispersed in a chilled emulsifier solution. Then the pre-suspension is subjected to high pressure homogenization at or below room temperature. Compared to hot homogenization, generally larger particle sizes and broader size distribution are obtained in cold homogenized samples. The method of cold homogenization minimizes the thermal exposure of the sample, but it does not fully avoid it due to the melting of the lipid/siRNA mixture during the initial step.

SLN can be prepared by solvent emulsification or evaporation techniques by precipitation in o/w emulsions. Lipophilic material is dissolved in a water-immiscible organic solvent that is emulsified within an aqueous phase. Once the solvent evaporates, precipitation of the lipid in the aqueous phase forms a nanoparticle dispersion.

SLN can be prepared by the dilution of microemulsions. Since there are varying opinions on the structure and dynamics of a microemulsion, a detailed review on the subject has been published (Moulik S. and Paul B., 1998). A microemulsion can be envisioned as a two-phase system composed of an inner and outer phase, such as o/w microemulsions. They are prepared by stirring an optically clear mixture at 65–70°C composed of a low melting fatty acid, an emulsifier, co-emulsifiers, and water. The hot microemulsion is dispersed in cold water (2–3°C) under stirring. Typical volume ratios of the hot microemulsion to cold water are between 1:25 to 1:50. The dilution process is driven by the composition of the microemulsion. The droplet structure is already contained in the microemulsion, therefore no energy is required to achieve nanoparticle sized particles. The temperature gradient, pH, and the composition of the microemulsion determine the product quality. High-temperature gradients facilitate rapid lipid crystallization and prevent aggregation. The dilution step constrains the lipid to a considerably lower concentration level than high pressure homogenization-based formulations.

Lyophilization increases the chemical and physical stability of SLN over an extended period of time. Transformation of the SLN into a solid form prevents crystal growth by Ostwald ripening and avoids hydrolysis reactions. Lyophilization is also possible for SLN formation of dry powders for inhalation or for reconstitution for nebulizer delivery. The solid state of the lyophilized SLN will have increased chemical and physical stability compared to the aqueous lipid dispersion. Two additional transformations between the formulations are necessary and may cause stability problems. The first transformation from aqueous dispersion to powder involves the freezing of the sample and the evaporation of water under vacuum. Freezing of the sample may cause stability problems due to the freezing out effect which results in changes in the osmolarity and the pH. The second transformation, resolubilization, involves low water and high particle content and high osmotic pressure that could facilitate particle aggregation.

The lipid content of the SLN dispersion should not exceed 5% to prevent an increase in particle size because direct content of lipid particles is decreased in more diluted samples. The protective effect of the surfactant can be compromised during lyophilization. Diluted SLN dispersions will also have higher sublimation velocities and a higher specific area. The addition of cryoprotectants will be necessary to decrease SLN aggregation and to obtain a better redispersion of the dry product. Typical cryoprotective agents are sorbitol, lactose, mannose, trehalose, glucose, and polyvinylpyrrolidone. They decrease the osmotic activity of water and crystallization and favor a glassy state of the frozen sample. Cryoprotectants are place holders that prevent contact between discrete lipid nanoparticles and they interact with polar head groups of the surfactants and serve as a pseudo hydration shell.

As an alternative to lyophilization, spray drying can be used to transform an aqueous SLN dispersion into a dry product (Chougule M. et al., 2007, 2008; Patel G. et al., 2009). Spray drying can cause particle aggregation due to high temperatures, shear forces, and partial melting of the particles, therefore it is recommended to use lipids with melting points > 70°C. The incorporation of carbohydrates and low lipid content improve the preservation of the colloidal particle size during spray drying. The melting of the lipid can be minimized by using ethanol-water mixtures as a dispersion medium instead of pure water due to the lower inlet temperatures.

2.2.3 Examples of lipid-based delivery systems

Functional genomic studies to study novel targets for lung cancer or other diseases have commonly utilized commercially available cationic lipid transfection reagents, including Lipofectamine RNAiMAX (Ramachandran S. et al., 2013), Lipofectamine2000 (Cao W. et al., 2012; Chen G. et al., 2012; Fourtounis J. et al., 2012; Huang B. et al., 2013; Liu Y. et al., 2013; Matsubara H. et al., 2012; Nishimura Y. et al., 2014; Rahman M.A. et al., 2013; Wang Y. et al., 2013; Zhang Y.-P. et al., 2012) and HiPerfect (Fourtounis J. et al., 2012). Other groups have made
liposomes composed of phospholipids and cholesterol with particle size of approximately 100 nm that have shown higher cellular internalization in lung cancer cells than with Lipofectamine2000, but had higher cytotoxicity. Oxime ether lipid nanoparticles with particle size of 150–220 nm transfected cells more efficiently with serum present than conventional liposomal formulations (Biswa S. et al., 2011).

A myeloid cell leukemia 1 (Mcl1)-specific siRNA administered in a lung metastases model at 0.21 mg/kg was delivered by a microsprayer intratracheally four times on alternating days starting at 5 days post intravenous (i.v.) injection of either B16 or Lewis Lung Carcinoma cells (Shim G. et al., 2013). In this study cationic nanoliposomes with particle size of 200 nm were prepared using the thin-film hydration technique and were composed of the various lipid ratio of EDOPC, DOTAP, DOTMA, DC-Cholesterol, DOPE, and Cholesterol (Shim G. et al., 2013). The thin-films were hydrated with 1 ml of 20 mmol/l HEPES (pH 7.4), and the resulting nanoliposomes were extruded through 0.2 μm polycarbonate membrane filters three times using an extruder (Shim G. et al., 2013).

An intranasally delivered liposome formulation containing a novel siRNA sequence against the epithelial sodium channel (ENaC) alpha subunit was developed and evaluated by Clark et al. (Clark K.L. et al., 2013). Liposomes administered intranasally caused significant ENaC downregulation without TLR3, 8, or 9 activation, thereby supporting that the effects were induced by RNAi (Clark K.L. et al., 2013).

An aerosolized liposomal formulation was developed by Mainelis et al., who prepared liposomes composed of egg phosphatidylcholine: 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine:cholesterol and antisense oligonucleotides or siRNA targeted against multidrug resistance-associated protein 1 (MRP1). This group used these formulations to evaluate the delivery of a nose-only small animal exposure chamber (Mainelis G. et al., 2013). Mainelis et al. characterized the aerosolized particles and found that the change of mean particle size was insignificant during the 1 hour of continuous aerosolization. It was shown that, of the nebulized formulation, 1.4 % at each port was available for inhalation and the lung retention was higher than in the injected samples (Mainelis G. et al., 2013). The lung tumor model was prepared by intratracheal administration of luciferase expressing A549-luc cells followed by detection of tumor growth by luminescence imaging. After inhalation treatment with antisense oligonucleotides and doxorubicin combination, the tumor volumes were 90 % reduced, compared to 40 % after doxorubicin i.v. injection (Mainelis G. et al., 2013).

2.3 Polymer-based delivery systems

2.3.1 Introduction

Polymers for use in nanoparticle drug delivery systems should be biocompatible in terms of non-toxicity, non-antigenicity, biodegradability, and biocompatibility. The natural polymers that have commonly been used for preparation of polymeric nanoparticles are albumin, chitosan, dextran, gelatin, lectins, and sodium alginate (Tekade R.K. and Chougule M.B., 2013; Tekade R.K. et al., 2014; Youngren S.R. et al., 2013). There are many synthetic polymers available, while some are still under development. The well-known synthetic polymers include Poly (ε-caprolactone) (PECL), polylactide (PLA), polystyrene, polyglycolide (PGA), Poly(lactide co-glycosides (PLGA), poly anhydrides, polyetherester, polycyanoacrylates, polycaprolactone, polyglutamic acid, polymaleic acid, poly(N-vinyl pyrrolidone), poly(methyl methacrylate), poly(vinyl alcohol), poly(acrylic acid), polyacrylamide, polyethylene glycol, and polyethylene acrylacid. Polymeric nanoparticles include nanocapsules, which contain a reservoir core surrounded by a solid material shell, or nanoparticles, which are solid matrix particles (Vauthier C. and Bouchemal K., 2009). Polymeric siRNA carriers are capable of swelling due to hydration and release siRNA by diffusion. Another physicochemical mechanism of release is by enzymatic degradation of the polymer at the delivery site, thus releasing the siRNA from the core. The siRNA may also dissociate causing de-adsorption or release. An advantage of the use of polymers as a siRNA delivery systems is the possibility of chemical modifications, which include the synthesis of block polymers and comb polymers or attachment of targeting moieties (Glasgow M.D. and Chougule M.B., 2015). Disadvantages of polymer delivery systems stem from the use of organic solvents in production processes that leave residues, polymer cytotoxicity, and obstruct scalability of the production process. Another disadvantage of peptide based polymer inhaled delivery systems is their ability to increase the elastic and viscous moduli of lung fluid and mucus, decreasing mucociliary clearance, which can complicate many diseases states (Innes A.L. et al., 2009; List S.J. et al., 1978; Marriott C. et al., 1982).

2.3.2 Preparation methods

The preparation methods of polymeric nanoparticles from a polymeric dispersion include emulsification/solvent diffusion, solvent evaporation, nanoprecipitation, salting out, dialysis, and supercritical fluid (SCF) technology methods. The preparation methods of polymeric nanoparticles from polymerization of monomers are emulsion (including mini- and micro-), interfacial polymerization, and controlled/living radical polymerization (C/LRP). Ionic gelation and coacervation are methods for
2.3.2.1. Emulsion and diffusion techniques

Emulsions are typically formed by two immiscible phases and a surface active agent and some form of dispersive force. Polymer precipitation from emulsion droplets can be achieved by removing the polymer solvent through solvent evaporation, fast diffusion after dilution, or salting out (Vauthier C. and Bouchemal K., 2009). These methods lead to formation of nanospheres when performed on simple oil-in-water emulsions. Oil containing nanocapsules can be obtained by adding oil in the polymer solution composing the emulsion droplets.

Emulsification and solvent diffusion is a modified version of the solvent evaporation method (Niwa T. et al., 1994). The encapsulating polymer is dissolved in a partially water soluble solvent such as propylene carbonate and saturated with water to ensure the initial thermodynamic equilibrium of both liquids. In order to produce the precipitation of the polymer and the consequent formation of nanoparticles, it is necessary to promote the diffusion of the solvent of the dispersed phase by dilution with an excess of water when the organic solvent is partly miscible with water or with another organic solvent in the opposite case. The polymer-water saturated solvent phase is emulsified in an aqueous solution containing stabilizer, leading to solvent diffusion to the external phase. Resultant nanospheres or nanocapsules will be formed dependent on the oil-to-polymer ratio. The solvent is removed by evaporation or filtration.

This technique allows for high encapsulation efficiencies (> 70%), high batch-to-batch reproducibility, eliminates the need for homogenization, ease of scale-up, and narrow size distribution. A disadvantage of this method is that it requires high volumes of water that has to be removed from the suspension and the leakage of water-soluble entrapped therapeutic agent drugs into the external saturated-aqueous phase during emulsification causes a reduction in entrapment efficiency (Reis C.P. et al., 2006). This method has moderate efficiency in the entrapping hydrophilic molecules, therefore it may have limited applications for siRNA delivery systems.

Emulsion polymerization can be used to prepare nanoparticles by monomer polymerization. It is a fast and easily scalable nanoparticle preparation method. The continuous organic phase methodology involves the monomer dispersion into an emulsion, inverse microemulsion, or into a non-solvent of the monomer (Nagavarma B. et al., 2012; Reis C.P. et al., 2006). Surfactants or protective soluble polymers were used in the initial stages of polymerization to prevent aggregation. Since this method utilizes toxic organic solvents, surfactants, monomers, and initiators that can be leached from the formed nanoparticles, this method has been used sparingly in recent years (Nagavarma B. et al., 2012).

An alternate approach was developed because of the conventional method disadvantages of having non-biodegradable polymer and difficult procedure. Poly(methylmethacrylate) (PMMA), poly (ethylcyanoacrylate) (PECA), and poly (butylcyanoacrylate) nanoparticles were produced by a surfactant dispersion within an organic phase consisting of cyclohexane, n-pentane, and toluene (Nagavarma B. et al., 2012). The monomer is dissolved in the aqueous continuous phase and surfactants or emulsifiers are not required. Initiation occurs when collision happens between the monomer molecules and the initiator molecule, which could be an ion or a free radical, within the continuous phase (Nagavarma B. et al., 2012). Alternatively, the monomer molecule itself can be converted into an initiating radical by high-energy radiation, such as g-radiation, ultraviolet, or strong visible light. Polymer chains form, according to a mechanisms of anionic polymerization, when collision occurs between the initiated monomer ions or monomer radicals and other monomer molecules. Solid particle formation and phase separation may occur before or after the polymerization reactions (Kreuter J., 1982; Vauthier C. et al., 2003).

The mini-emulsion polymerization method has been developed using a wide range of polymer materials (Arias J. et al., 2001; Ham H.T. et al., 2006; Ziegler A. et al., 2009). A typical mini-emulsion polymerization formulation contains water, monomer mixture, co-stabilizer, surfactant, and initiator. The key difference between emulsion polymerization and mini-emulsion polymerization is the use of a low molecular mass compound as the co-stabilizer and also the use of a high-shear device. Mini-emulsions are critically stabilized, require a high-shear to reach a steady state, and have an interfacial tension much greater than zero (Rao J.P. and Geckeler K.E., 2011).

Micro-emulsion polymerization is used to prepare nanosized polymeric particles that involves a kinetically different process than other emulsion methods (Mishra S. and Chatterjee A., 2011). Although these processes both produce high molar mass colloidal polymeric particles, the particle size achievable for micro-emulsion based techniques is much smaller than for other emulsion based techniques. For micro-emulsion polymerization, typically a water soluble initiator, is placed into the aqueous phase of a thermodynamically stable micro-emulsion containing swollen micelles (Nagavarma B. et al., 2012). These spontaneously formed thermodynamically stable micelles are where polymerization commences. This process relies on large quantities of surfactant systems, which have an interfacial tension at the oil/water interface close to zero. The particles formed are completely coated with surfactant because of this high concentration of surfactant.
Polymer chains are first formed only in some droplets, as the initiation cannot be attained in all droplets simultaneously. Then, the osmotic and elastic influence of the chains destabilize the fragile micro-emulsions and typically lead to an increase in the particle size, the formation of empty micelles, and secondary nucleation. Very small lattices of particle size of 5–50 nm are formed with the presence of empty micelles. Critical factors of the kinetics of micro-emulsion polymerization and the resultant polymeric nanoparticle properties are the concentration of initiator, surfactant, monomer, and reaction temperature (Fessi H. et al., 1989; Nagavarma B. et al., 2012).

2.3.2.2 Solvent evaporation

The first step of the solvent evaporation technique involves the preparation of a polymer solution prepared in volatile solvents and emulsions. Ethyl acetate is the solvent of choice because it has a better toxicological profile when compared to the other widely used solvents, dichloromethane and chloroform (Rao J.P. and Geckeler K.E., 2011). The primary emulsion is transformed into a colloidal nanoparticle suspension following the evaporation of the solvent from the polymer, which is facilitated through the diffusion of the emulsion’s continuous phase (Nagavarma B. et al., 2012). The conventional methods involve the formation of primary single emulsions, such as oil-in-water (o/w) or the formation of double-emulsions, such as (water-in-oil)-in-water (w/o)/w. Nanocarriers formed through this method utilize high-speed homogenization or ultra-sonication with subsequent solvent evaporation through continuous magnetic stirring or under vacuum. The resultant semi-solid particles may undergo ultracentrifugation and be washed with distilled water to collect and purify the nanocarriers. The resultant nanocarriers may then be freeze-dried with a suitable cryoprotectant and stabilizing agent. Particle sizes of these nanocarriers are dependent on the type and concentration of stabilizer, homogenizer speed, and polymer concentration. Reduction of particle size can be achieved through high-speed homogenization and ultra-sonication.

2.3.2.3 Nanoprecipitation

Nanoprecipitation, or the solvent displacement method, involves the precipitation of preformed polymer from an organic solution and the diffusion of the organic solvent in the aqueous medium with or without a surfactant (Barichello J.M. et al., 1999; Fessi H. et al., 1989; Galindo-Rodriguez S. et al., 2004; Ganachaud F. and Katz J.L., 2005). The polymer is dissolved in a water miscible solvent of intermediate polarity, leading to the precipitation of spherical nanocarriers. This phase is injected into a stirred aqueous solution containing a stabilizer as a surfactant. Polymer deposition on the interface between the water and the organic solvent, caused by fast diffusion of the solvent, leads to instant formation of stable nanocarriers in suspension (Quintanar-Guerrero D. et al., 1998). In order to facilitate the suspension formation, the first step is to perform phase separation with a completely miscible solvent that is also a non-solvent for the polymer (Vauthier C. et al., 2003). This nanoprecipitation technique allows the preparation of nanocapsules when a small volume of nontoxic oil is incorporated in the organic phase. Considering the oil-based central cavities of the nanocapsules, high loading efficiencies of hydrophobic drugs are often reported. This technique spontaneously produces emulsification because of the high diffusion rates in result of limiting water-miscible solvents (Nagavarma B. et al., 2012). Spontaneous emulsification is not observed if the coalescence rate of the formed droplets is sufficiently high due to certain instability when mixed in water of some water-miscible solvents. Acetone/dichloromethane is used to dissolve drugs and increase their entrapment. However, dichloromethane increases particle size and has toxicological issues. This method is appropriate for lipophilic drugs; therefore its usefulness for siRNA nanocarriers is limited by low entrapment efficiency.

2.3.2.4 Salting out

The salting out effect causes the separation of a water miscible solvent from an aqueous solvent (Reis C.P. et al., 2006). This procedure is considered a modified emulsification/solvent diffusion method. Drug and polymer are first dissolved in a solvent, like acetone, which is then emulsified with the salting-out agent into an aqueous gel. Salting-out agents include electrolytes, such as magnesium chloride or calcium chloride, or non-electrolytes, such as sucrose with a colloidal stabilizer such as polyvinylpyrrolidone or hydroxyethylcellulose (Nagavarma B. et al., 2012). Induction of nanoparticle formation occurs when the oil/water emulsion is diluted with enough water or aqueous solution to enhance the diffusion of acetone into the aqueous phase (Reis C.P. et al., 2006). Salting out agent selection is highly influential in the drug encapsulation efficiency (Nagavarma B. et al., 2012). Formed polymeric nanoparticles are purified by cross-flow filtration to remove the solvent and salting out agent (Nagavarma B. et al., 2012).

An advantage of the salting out method is that it minimizes stress to protein encapsulates (Jung T. et al., 2000; Lambert G. et al., 2001; Nagavarma B. et al., 2012). The salting out procedure is useful for heat sensitive substances since it does not require increased temperature. The multiple washing steps pose a disadvantage when considering entrapment efficiency. The greatest disadvantage of this method is that it is used exclusively for lipophilic drugs, therefore its application to siRNA is low.
2.3.2 Dialysis

Nanocarriers with small particle size and narrow particle size distribution can be prepared by dialysis (Fessi H. et al., 1989; Kostag M. et al., 2010; Rao J.P. and Geckeler K.E., 2011). Polymer dissolved in an organic solvent is placed within a dialysis tube with desired molecular weight cut off (Nagavarma B. et al., 2012). Dialysis is then performed against a non-solvent miscible with the former miscible solvent. The use of the dialysis membrane or semi-permeable membranes allows the passive transport of solvents to slow down the mixing of the polymer solution with a non-solvent (Nagavarma B. et al., 2012). The displacement of the solvent inside the membrane is followed by the progressive aggregation of the polymer due to a loss of solubility and the formation of homogeneous suspensions of nanoparticles. The mechanism of nanocarrier formation by dialysis method is based on a mechanism similar to that of nanoprecipitation. The solvent used in the preparation of the polymer solution affects the morphology and particle size distribution of the nanoparticles.

2.3.2.6 Supercritical fluid technology (SCF)

Supercritical fluid technology has allowed for the use of more environmentally friendly solvents for the preparation of polymeric nanocarriers with high purity (Kawashima Y., 2001; York P., 1999). Supercritical fluid and dense gas technology are expected to offer an effective technique of nanoparticle preparation because it avoids most of the drawbacks of traditional techniques, such as presence of organic solvent (Nagavarma B. et al., 2012).

The production of nanoparticles using supercritical fluids has two principles. The first is the rapid expansion of supercritical solution (RESS). The second principle is the rapid expansion of supercritical solution into liquid solvent (RESOLV). In traditional RESS, the solution is dissolved in a supercritical fluid, followed by the rapid expansion of the formed solution across a capillary nozzle or an orifice into ambient air. The resultant high degree of super saturation and rapid pressure reduction in the expansion results in homogenous nucleation and formation of dispersed particles (Nagavarma B. et al., 2012). Mechanistic studies of different model solutes for the RESS process indicate that within the expansion jet, both micrometer-sized and nanometer particles are present (Weber M. and Thies M.C., 2002). A few studies were carried out on the production of nanoparticles using RESS. Poly (perfluoropolyetherdiamide) droplets produced from the rapid expansion of CO2 solutions. The RESS experimental apparatus consists of three major units: a high pressure stainless steel mixing cell, a syringe pump, and a pre-expansion unit. A solution of polymer in CO2 is prepared at ambient temperature. The solution is heated isobarically to the pre-expansion temperature while pumped through pre-expansion unit before the solution reaches the nozzle. At this point, the supercritical solution is now allowed to expand through the nozzle, at ambient pressure. The concentration and degree of saturation of the polymer have a considerable effect on the particle size and morphology of the particles for RESS (Blasig A. et al., 2002; Chernyak Y. et al., 2001; Lim K.T. et al., 2005; Sane A. and Thies M.C., 2007).

A RESS modification, known as RESOLV, involves the expansion of the supercritical solution into a liquid solvent instead of ambient air (Rao J.P. and Geckeler K.E., 2011; Sun Y.P. et al., 2005). The RESS technique results in microscaled sized particles rather than nanoparticles. In order to overcome this drawback, RESOLV was developed. In RESOLV, the liquid solvent suppresses the particle growth in the expansion jet, thus making it possible to obtain primarily nanosized particles (Meziani M.J. et al., 2004; Nagavarma B. et al., 2012; Sun Y.P. et al., 2005).

2.3.2.7 Interfacial polymerization

Interfacial polymerization is one of the most established methods for the preparation of polymeric nanoparticles (Gaudin F. and Sintes-Zydowicz N., 2008; Landfester K. et al., 2010). In interfacial polymerization, there is step-wise polymerization of two reactive monomers or agents, which are dissolved respectively in two phases (continuous and dispersed), and the reaction takes place at the interface of the two liquids (Karode S. et al., 1998). Hollow polymeric nanoparticles were synthesized with the use of interfacial cross-linking reactions as polyaddition, polycondensation, or radial polymerization (Crespy D. et al., 2007; Danicher L. et al., 2000; Sarkar D. et al., 2005; Scott C. et al., 2005). Polymerization of monomers at the oil/water interface of an oil-in-water micro-emulsion produces oil-containing nanocapsules (Fallouh N.a.K. et al., 1986). The organic solvent, which is completely miscible with water, served as a monomer vehicle and the interfacial polymerization of the monomer was believed to occur at the surface of the oil droplets that formed during emulsification (Aboubakar M. et al., 1999; Gallardo M. et al., 1993; Vauthier C. and Bouchemal K., 2009). The use of acetone or acetonitrile, which are aprotic solvents, was recommended for nanocapsule preparation (Nagavarma B. et al., 2012). Alternatively, protic solvents, such as ethanol, m-butanol and isopropanol, were found to also induce the formation of nanospheres (Nagavarma B. et al., 2012; Puglisi G. et al., 1995). Nanocapsules containing aqueous water can be formed by the interfacial polymerization of monomers in water-in-oil interface and precipitated out forming the nanocapsule shell (Gasco M. and Trotta M., 1986; Watnasirichaikul S. et al., 2000). Therefore, siRNA containing nanocapsules can be formed using the interfacial polymerization of monomers in water-in-oil microemulsions.
The primary restrictions of radical polymerization are due to unavoidable radical to radical termination reactions and cause a lack of control over the molar mass, the end functionalities, and the overall macromolecular structure (Nagavarma B. et al., 2012). The C/LRP processes are based on previous polymerization techniques and opens new prospects in polymeric nanoparticle preparation (Matyjaszewski K. and Xia J., 2001; Nagavarma B. et al., 2012; Zetterlund P.B. et al., 2008; Zetterlund P.B. et al., 2007). The growing public concern over environmental impacts and the growth of hydrophilic polymer applications in medicine have sparked interest in C/LRP process. These factors have given rise to green chemistry techniques and created a demand for environmentally and chemically benign solvents such as water and supercritical carbon dioxide (Nagavarma B. et al., 2012). Industrial radical polymerization in aqueous dispersed systems and specifically in emulsion polymerization is widely used. The primary goal was to control the characteristics of the polymer to modulate their function, molar mass, molar mass distribution, and structure. Implementation of C/LRP in aqueous dispersed systems, results in the formation of polymeric nanoparticles with precise particle size and size distribution control (Braunecker W.A. and Matyjaszewski K., 2001; Nagavarma B. et al., 2012). Among the available controlled/living radical polymerization methods successfully and extensively studied methods include nitroxide-mediated polymerization (NMP), atom transfer radical polymerization (ATRP), and reversible addition and fragmentation transfer chain polymerization (RAFT) (Dire C. et al., 2008; Min K. et al., 2009; Nicolas J. et al., 2007; Rieger J. et al., 2010; Siegwart D.J. et al., 2009; Zhou X. et al., 2007). The size of the resultant polymeric nanoparticles depends on the type of control agent, control agent concentration, monomer, initiator, and the emulsion type.

2.3.2.9 Hydrophilic polymer ionic gelation/coacervation

Polymeric nanoparticles are prepared by using biodegradable hydrophilic polymers such as chitosan or gelatin as shown in Fig. 6. In this method, the positive charged amino groups of the polymer interacts with the negatively charged triphosphate to form coacervates with a size in the nanometer range (Calvo P. et al., 1997a; Calvo P. et al., 1997b; Dustgania A. et al., 2008). Coacervates are formed as a result of electrostatic interaction between two aqueous phases, whereas ionic gelation involves the material undergoing transition from liquid to gel due to ionic interaction conditions at room temperature (Nagavarma B. et al., 2012).

2.3.3 Examples of polymer-based delivery systems

Chitosan is a generally regarded as safe (GRAS) excipient and a natural polysaccharide (Keefe D., 2011; Mao S. et al., 2010). However, its applications in siRNA delivery systems have been hindered due to poor water solubility and low transfection efficiency (Merkel O.M. et al., 2014). An inhalable chitosan/siRNA dry powder composed of unmodified chitosan was prepared using the supercritical CO$_2$ technique followed by manual grinding in an investigation by Okuda et al. (Okuda T. et al., 2013). Water, ethanol and CO$_2$ solvents were combined in a compressed column (35 °C, 25 MPa). This supercritical CO$_2$ technique embodies tolerable conditions for the precipitation of powders capable of reconstitution without siRNA or nanoparticle degradation. The hydrodynamic particle sizes and zeta potentials of the developed chitosan particles were shown to be maintained throughout and after the powder formation process (Okuda T. et al., 2013). Biodistribution of intratracheally administered Cy5.5 labeled siRNA formulations, including the nanoparticle suspension, the dry powder, and the free/naked siRNA were monitored. The efficiency of the delivered siRNA was measured in a lung metastases model of colon26/Luc cells by luciferase downregulation. The dry powder particles had highest knockdown efficiency, however they had higher lung clearance than the nanoparticle suspension (Okuda T. et al., 2013). This study is one of the few reports that describe the formulation of an inhalable dry powder containing siRNA.

Another dry powder formulation prepared using a double emulsion solvent evaporation method was designed by Jensen et al., where siRNA was entrapped within DOTAP-modified PLGA nanoparticles (Jensen D.K. et al., 2012; Jensen D.M. et al., 2010). The total concentration of DOTAP and PLGA in chloroform was kept constant and the optimal formulation contained 25 % (w/w) DOTAP by varying the weight/weight (w/w) percentage of DOTAP. The particles were spray dried with mannitol and an aerodynamic size of 3.69 ± 0.18 μm obtained, which is within the optimal size range for deep lung deposition, and did not exhibit aggregation or coalescence.

![Diagram showing the preparation of polymeric nanoparticles using the coacervation (desolvation) technique.](image)
(Jensen D.K. et al., 2012; Jensen D.M. et al., 2010). During spray drying, the sugar alcohol remained in its crystalline state, as determined by X-ray powder diffraction analysis, and therefore functioned as a stabilizer. The hydrodynamic diameters and cellular uptake remained unchanged before and after freeze-drying according to statistical analysis, which supported the conclusion that spray-drying is a powerful technique for engineering siRNA nanoparticle dry powder formulations (Jensen D.K. et al., 2012).

PLGA poly[vinyl-3-(dialkylamino) alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,Lactide-co-glycolide), (DEAPA-PVA-g-PLGA), that contained covalently modified positively charged side groups for siRNA complexation was prepared by Benfer et al. (Benfer M. and Kissel T., 2012). The H1299 NSCLC cellular uptake of DEAPA-PVA-g-PLGA/siRNA nanoparticles increased after addition of surfactant and was internalized mostly by clathrin-mediated endocytosis (Benfer M. and Kissel T., 2012). Merkel et al. also showed that DEAPA-PVA-g-PLGA/siRNA nanoparticles remain in the lung for extended periods of time when compared to gold-standard PEI-formulated or free siRNA. Thus, making the formulation applicable for intratracheal administration (Zheng M. et al., 2012). To prepare the inhalable dry DEAPA-PVA-g-PLGA/siRNA nanoparticle powder, the freeze-drying protocol was optimized. Cryoprotectants, such as dextrose, hydroxypropyl-beta-cyclodextrin, lactosucrose, polyvinylpyrrolidone (PVP), sucrose, and trehalose can be used to stabilize the nanoparticles. Although particles freeze-dried in the cryoprotectant 10% glucose were about 150 nm in particle size after reconstitution, the particles freeze-dried in the cryoprotectant 10% saccharose were much larger with a particle size of 250 nm. However, the freeze-dried larger particles showed more similar and efficient cellular uptake when compared to freshly prepared nanoparticles (Zheng M. et al., 2012). It was demonstrated that gene downregulation efficacy was preserved through the freeze-drying process as assessed after reconstitution of the nanoparticles (Zheng M. et al., 2012). These findings are similar to those of a study by Kasper et al. who showed that cryoprotectant concentrations of 12% and higher can prevent PEI/DNA polyplex aggregation which retains their transfection efficiency after redispersion (Kasper J.C. et al., 2011).

Luo et al. synthesized salbutamol-modified guanidylated chitosan and subsequently formed polyplexes targeted to lung smooth muscle cells for potential applications in asthma or COPD (Luo Y. et al., 2012). The formulations were nebulized with an Aeroneb® Pro nebulizer (Aerogen, Galway, Ireland) and then the collected condensate was administered intratracheally with a PennCentury microsprayer to transgenic mice expressing enhanced green fluorescent protein (EGFP) (Luo Y. et al., 2012). The animals were treated daily with 5 µg of siRNA 3 days, and in vivo knockdown was quantified by confocal laser scanning microscopy (CLSM) of tissue sections and by Western blot. The targeted formulation achieved a 40% gene downregulation (Luo Y. et al., 2012).

Atelocollagen, a highly purified and pepsin-treated type I collagen obtained from calf dermis also raised interest for its use in pulmonary delivery of siRNA (Merkel O.M. et al., 2014). Protein and siRNA polyplexes were prepared by mixing during a 16h incubation and were administrated intratracheally to Wistar or EGFP expressing rats (Liu S. et al., 2012). The therapeutic gene target was syntaxin4, which regulates the function of mucosal-type mast cells and is one of the soluble membrane N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Following siRNA treatment, syntaxin4 knockdown significantly decreased granule contents release from rat mucosal mast cells. Rats were sensitized and challenged with ovalbumin (OVA) following the allergic mouse model protocols to elicit symptoms of asthma. The rats were then intratracheally instilled with atelocollagen siRNA complexes once a day for 5 days. These treatments decreased the rat mast cell protease-II (RMCPII) levels in the bronchoalveolar lavage fluid (BALF) and prevented asthmatic airway constriction. Therefore, the authors concluded that syntaxin 4 knockdown prevents degranulation and stabilizes mucosal mast cells (Liu S. et al., 2012).

Spermine, a naturally occurring polymer consisting of small linear tetra-amines with two primary and two secondary amines, is popular for the delivery of nucleic acids (Merkel O.M. et al., 2014). Endogenous spermines play a key role in the compact packaging of cellular DNA, this property is exploited artificially in nucleic acid delivery (Eliyahu H. et al., 2006; Jiang H.L. et al., 2011). Due to the short molecules and the resultant rigidity of spermine, unmodified spermine does not efficiently compact siRNA (Merkel O.M. et al., 2014; Pavan G.M. et al., 2010). Jiang et al. prepared glycerol propoxylate triacrylate-spermine (GPT-SPE) and shRNA polyplexes that had a particle size of 160 nm and had a zeta potential of +9 mV. Human NSCLC was mimicked using a KrasLA1 model and administered 8 µg of GPT-SPE and 0.8 µg of shRNA (scrambled or Akt1 shRNA) twice a week for 4 weeks as aerosol (Jiang H.L. et al., 2011). The authors did not discuss whether the preparation and delivery processes had an effect on the polyplex physico-chemical characteristics. In a later study, GPT-SPE delivered shRNA against the same target. The same nose-only exposure chamber was used but administered half of the previous dose (0.4 µg) DNA as an aerosol (scrambled or Akt1 shRNA) twice a week over a timeframe of 4 weeks (Hong S.-H. et al., 2012). Aerosol delivery of Akt1 shRNA suppressed lung tumor growth...
and the authors elucidated the mechanism of action (Hong S.-H. et al., 2012). The same group conducted another later study, which used the GTE-SPE polymer for sodium-dependent phosphate co-transporter 2b (NPT2b) siRNA delivery via nose-only inhalation (Hong S.-H. et al., 2013). The animals were treated for four weeks with 0.5 mg of siRNA twice a week. Aerosolized NPT2b siRNA downregulated NPT2b expression levels as detected via Western blotting, densitometric analysis and qPCR, and significantly increased the pro-apoptotic protein levels. The activation of apoptosis was confirmed in TUNEL positive cells, and PCNA and VEGF levels were decreased after knockdown of Akt1 (Hong S.-H. et al., 2013).

Choi et al. synthesized a conjugate of dexamethasone and PEI (DEXA-PEI) polyplex to target the cellular nucleus via interaction with the glucocorticoid receptor (Choi M. et al., 2013). This concept was previously explored, but the novelty of this approach was induction of nuclear translocation via interaction with the glucocorticoid receptor and PEI (DEXA-PEI) polyplex to target the cellular nucleus of baked SiO$_2$ nanoparticles (Choi M. et al., 2013; Mi Bae Y. et al., 2007). siRNA against macrophage migration inhibitory factor (MIF) was complexed with DEXA-PEI and particles obtained had a particle size of 355 nm, whereas PEI/siRNA complexes aggregated to a particle size of >600 nm. These particles were administered intratracheally with either 2.5 µg or 10 µg siRNA per animal and were shown to decrease pulmonary inflammation and the MIF mRNA and Muc5ac expression after treatment with conjugated and nonconjugated PEI complexes 72 h post-administration of the polyplexes (Choi M. et al., 2013).

2.4 Peptide-based delivery vectors

2.4.1 Introduction

Protein transduction domains (PTDs) are short amino acid chains that interact with the plasma membrane that allows for cellular uptake that can be used for siRNA delivery. Obtaining controlled siRNA cellular delivery through the use of peptides as carriers can overcome siRNA delivery constraints, which include poor bioavailability and clinical efficacy. Chemical conjugation of cationic peptide cell penetrating peptides (CPP) to siRNA has facilitated enhanced siRNA tissue delivery and cellular internalization. The TAT protein from HIV-1 which was discovered to be responsible for the cellular uptake of the virus was of interest of drug delivery scientists. Since that time, a variety of CPPs have been developed to facilitate the cellular uptake transport of therapeutic molecules including siRNA (Lam J.K.-W. et al., 2012). Covalent peptide to siRNA disulfide bond formation attachment, or non-covalent electrostatic interactions between the siRNA and peptide to form complexes can be used to prepare siRNA peptide conjugates (Lam J.K.-W. et al., 2012). Their mechanism of action is also expected to vary due to the sequence diversity of different CPPs. These peptides function to improve cellular delivery by efficient transport across the cellular membrane or promote endosomal escape (Endoh T. and Ohtsuki T., 2009). The activities of different CPPs have been reviewed elsewhere (Deshayes S. et al., 2005; Meade B.R. and Dowdy S.F., 2007).

2.4.2 Preparation methods

Double stranded siRNA, with or without modification, can form stable or cleavable peptide conjugates using a range of different conjugation chemistries (Jeong J.H. et al., 2008). Designing conjugation schemes include selection of linkage chemistry, spacer molecule, and orientation of the siRNA and peptide components (Zatsepin T. et al., 2005). There are two main strategies for synthesizing siRNA peptide conjugates: the total stepwise solid-phase synthesis and the solution-phase or solid-phase fragment coupling of peptides with oligonucleotides that have been prepared individually on solid supports (Corey D.R., 2004). Stable linkages may be prepared from either method, but fragment coupling of peptides is necessary for cleavable disulfide linkages.

The most common conjugation chemistries have involved fragment coupling in aqueous solution. Peptide and siRNA fragments are prepared on their own solid supports by conventional automated solid-phase synthesis procedures. Each fragment requires a masked functional group to be incorporated during the assembly, which can be released chemoselectively during a simple deprotonation step. A specific chemical reaction is then initiated in aqueous medium between the functional group on the oligonucleotide and that on the peptide to produce the desired conjugate. An advantage of the fragment-coupling method is that both components can be purified, for example by HPLC, before conjugation so that the conjugation product can be easily identifiable. This method’s disadvantage is that the aqueous conditions of conjugation sometimes need to be modified to maintain the solubility of both components and the conjugate product. A reaction of a cysteine-substituted siRNA with a thioester-substituted peptide produces a stable amide linkage through the mechanism of native ligation. Another method involves the reaction of an aldehyde-containing siRNA with a cysteine-containing peptide, forming a thiazolidine linkage, or with an aminoxy peptide forming an oxime linkage, or with a hydrazinopeptide forming a hydrazine. An aldehyde oligonucleotide is unstable and must be generated by periodate treatment of a cis-diol substituted siRNA just before conjugation (Hermsen G.T., 2013).

The most straightforward method of fragment conjugation involves the synthesis of the siRNA component with an alkyl thiol linker on either of the 5’ or 3’-end
forming a 3'-thiopropyl or a 5'-thiohexyl linker (Turner J.B. et al., 2006). Following activation with pyridylsulfide, the siRNA can be coupled to a peptide. These thiol-functionalized siRNA can be reacted with a cysteine-containing peptide to form a cleavable disulfide linkage or with a bromoacetyl-substituted peptide to form a stable thioether linkage. A maleimide derivative of the peptide can also be prepared for the reaction with a thiol-substituted siRNA via a Michael-type addition reaction (Hermanson G.T., 2013).

2.4.3 Examples of Peptide-based Deliver Vectors
The PTDs and cell penetrating peptides are small (10–30 amino acid), positively charged molecules that usually contain arginine and lysine. Arginine and lysine amino acids provide primary and secondary amine functional groups in their side chains that can be protonated for electrostatic interaction with siRNA and cellular membranes to enhance the cellular permeability. Positively charged molecules that usually contain arginine and lysine amino acids provide primary and secondary amine functional groups in their side chains that can be protonated for electrostatic interaction with siRNA and cellular membranes to enhance the cellular permeability and form micellar aggregates and formed particles with sizes below 200 nm that elicited an anti-inflammatory effect (Oh B. and Lee M., 2014). These ternary complexes were tested in vitro and delivered siRNA into non-phagocytosing LA-4 lung epithelial cells more efficiently than PEI and Lipofermamine, inferring that their uptake mechanisms were not based on phagocytosis (Merkel O.M. et al., 2014; Oh B. and Lee M., 2014). Intratracheal administration of the ternary complexes reduced the SIPLyase level efficiently in an LPS-induced BALB/c ALI model. The cell penetrating peptides, TAT and double TAT (dTAT) was used for siRNA delivery by Baoum et al. (Baoum A. et al., 2012). To form the complexes, siRNA was mixed with the polycations and then CaCl$_2$ was added to the mixture in order to compact the siRNA and to decrease the hydrodynamic diameters of the particles (Baoum A. et al., 2012). Following i.v. injection of the complexes, high gene knockdown was observed in the lung (Baoum A. et al., 2012). These results suggest that formulating these siRNA complexes for pulmonary delivery by inhalation, would allow for high local concentration while keeping high accumulation in the lungs.

2.5 Inorganic-based delivery systems

2.5.1 Introduction
Various promising inorganic delivery systems have been investigated for the delivery of siRNA devised for diagnostic and therapeutic purposes. They include carbon nanotubes and metals such as iron oxide, quantum dots, gold and silica (Chen S. et al., 2014). Mesoporous silica based nanoparticles (MSN) have been widely investigated as carriers for siRNA based targeted drug delivery systems (Chen A.M. et al., 2009; Radu D.R. et al., 2004). The MSN are chemically stable, safe and biodegradable which makes it a promising gene delivery carrier. (Borm P. et al., 2006; Finnie K.S. et al., 2008). MSN also possess several advantages over other inorganic carriers, such as capability to encapsulate higher amounts of drugs due to large pore volumes and improved stability due to their inorganic oxide framework (Slowing, Ii et al., 2007).

2.5.2 Examples of inorganic-based delivery systems
Taratula et al. prepared MSNs with a 3-mercaptopropyl modification for labeling with Cy5.5 and with pyridylthiol for conjugation of a lutein hormone releasing hormone (LHRH) peptide via a PEG spacer and for attaching thiol modified siRNA (Merkel O.M. et al., 2014; Taratula O. et al., 2011b). The MSNs pores were loaded with either doxorubicin or cisplatin with two types of siRNA for a chemotherapeutic combination therapy, as shown in Fig. 7. The MRPI and BCL2 siRNA were selected based on their target mRNA's role in suppression of pump and non-pump cellular chemoresistance. An orthotopic model of lung cancer was established by intratracheal installation of luciferase expressing A549 cells (Garbuzenko O.B. et al., 2010). Nanoparticle accumulation in the mouse lungs was detected by whole body fluorescence imaging and revealed that inhalation administration prevented the MSN systemic circulation delivery and thus limited their accumulation elsewhere in the body (Taratula O. et al., 2011b).

The same group further utilized the modified MSN's to...
These results helped propel Excellair™ while placebo patients reported no such improvement. Improvement of breathing or reduced rescue inhaler use, factors. Thus, the specific Syk inhibition by Excellair™ the activation of several proinflammatory transcription downstream signaling cascades that ultimately leads to the signaling from the B-cell receptor and regulates the transcription factors regulated by Syk. Syk is involved in Cynwyd, Pennsylvania, USA) is an inhaled siRNA for the effects. Moreover, 75% of the patients treated reported (Watts J.K. and Corey D.R., 2010). The drug was well tol-
erated in all asthma patients, with no serious adverse side
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results of MSN was retained in lung compared to 5% when i.v. injected (Taratula O. et al., 2011a). Also, after i.v. administration MSN-based DDS was found to be accumulated mainly in liver (73%), kid-
neys (15%) and spleen (7%) while inhalation delivery resulted in significant reduction in accumulation to only 17%, 9% and 1% in liver, kidneys and spleen respectively.

3. Ongoing clinical trials on aerosolized siRNA based medicines

Excellair™ from ZaBeCor Pharmaceuticals (Bala Cynwyd, Pennsylvania, USA) is an inhaled siRNA for the treatment of asthma. This siRNA functions as a spleen tyrosine kinase (Syk) inhibitor thereby inhibiting the transcription factors regulated by Syk. Syk is involved in the signaling from the B-cell receptor and regulates the downstream signaling cascades that ultimately leads to the activation of several proinflammatory transcription factors. Thus, the specific Syk inhibition by Excellair™ is designed to reduce the inflammation associated with asthma. In a phase I study, patients with asthma received the inhaled siRNA therapeutic for 21 consecutive days (Watts J.K. and Corey D.R., 2010). The drug was well tolerated in all asthma patients, with no serious adverse side effects. Moreover, 75% of the patients treated reported improvement of breathing or reduced rescue inhaler use, while placebo patients reported no such improvement. These results helped propel Excellair™ into a phase II clinical trials in 2009, however these results are not available (De Backer L. et al., 2015).

Alnylam Pharmaceuticals (Cambridge, Massachusetts, USA) developed a nebulizer and nasal spray delivered siRNA therapeutic. The ALN-RSV01 siRNA was designed for the treatment of respiratory syncytial virus (RSV) targeting the nucleocapsid protein is indicated for prophylactic treatment against RSV infections in healthy patients (NCT00496821) and for treating RSV infection in lung transplant patients (NCT00658086 and NCT01065935). The ALN-RSV01 siRNA is composed of a double-stranded RNA duplex with 19 base pairs of complementary and 2-nt dT overhangs at both 3’ ends (Alvarez R. et al., 2009, Devincenzo J. et al., 2008).

RSV is the leading cause of hospitalization of infants, infecting approximately 70% of infants under a year old. A RSV vaccine is not available for pediatric patients due to the potential teratogenicity and reduced effectiveness (Burnett J.C. et al., 2011). RSV may also produce severe respiratory diseases like pneumonia in immunocompromised adolescents and adults, and in the elderly (Devincenzo J. et al., 2010). RSV replicates in the outermost layer of the airway epithelium, including regions lining the nasal passages, trachea, and the bronchioles (Devincenzo J.P., 2009). The anti-viral efficacy of ALN-RSV01 was demonstrated by the reduced infection rate in healthy patients and by reducing the daily symptoms in lung transplanta-
tion patients (Devincenzo J. et al., 2010; Zamora M.R. et al., 2011). This example clearly shows the advancement that siRNA brings to clinical care by providing safe and efficacious treatment options.

4. Conclusions and future directions

The therapeutic potential of siRNA in the treatment of lung diseases has yet to be fully explored. The use of inhalable nanoparticulate siRNA delivery systems have barriers to their effective delivery, such as degradation by RNase, mucociliary clearance, cough clearance, and alveolar macrophage clearance, but overcoming these constraints will offer unique advances to the field. Important parameters to consider in aerosolized siRNA formulation design and development are the particle, aerosol, physicochemical, stability, targeted site, and lung physiological properties.

siRNA carrier systems for aerosol delivery include those of naked siRNA formulation, lipidic, polymeric, peptide, or inorganic origin. Lipid-based delivery systems, such as liposomes or solid lipid nanoparticles have received attention for inhaled pulmonary delivery by using endogenously present phospholipids and surfactant-like lipids. Polymer-based siRNA delivery systems can be composed of biocompatible polymers which may be chemically modified to improve cellular delivery, internalization, and intracellular release. Peptide-based siRNA delivery vectors improve the poor bioavailability and clinical efficacy associated with naked siRNA delivery. Inorganic-based delivery systems allows for the unique delivery of siRNA for diagnostic and therapeutic purposes at higher payloads compared to other delivery sys-
tems. Safety of these formulations must be proven through various laboratory and clinical studies. In vitro techniques for characterizing aerosolized pulmonary siRNA delivery systems include dissolution tests, inertial cascade impaction, delivered dose uniformity assays, laser diffraction, and laser Doppler velocimetry. Ex vivo techniques such as the isolated perfused lung model can be used to identify inhaled particulate distribution. In vivo techniques like scintigraphy and pharmacokinetic/
pharmacodynamics analysis can give even more insight to the distribution, safety, and efficacy of a pulmonary inhaled siRNA nanocarrier. The vigorous work researchers have completed regarding the local pulmonary inhaled delivery of siRNA in various models of lung disease. Two inhalable therapeutic siRNA products have entered clinical trials. More clinically relevant preclinical studies utilizing biocompatible and safe siRNA delivery vehicles will provide inhalable aerosolized siRNA delivery vehicles for future clinical studies. Overall, recent work focused on inhalation delivery of siRNA for the treatment of pulmonary disorders has demonstrated the feasibility of this approach. The rapid progress in this area of research would facilitate the translation to the clinic.

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References

Aalto A.P., Sarin L.P., Van Dijk A.A., Saarma M., Poranen M.M., Arumae U. and Bamford D.H., Large-scale production of dsRNA and siRNA pools for RNA interference utilizing bacteriophage phi6 RNA-dependent RNA polymerase, RNA, 13 (2007) 422–429.

Aboubakar M., Puisieux F., Couvreur P., Deyme M. and Vauthier C., Study of the mechanism of insulin encapsulation in poly (isobutylcyanoacrylate) nanocapsules obtained by interfacial polymerization, Journal of biomedical materials research, 47 (1999) 568–576.

Akine A., Zumbuehl A., Goldberg M., Leshchiner E.S., Busini V., Hossain N., Bacallado S.A., Nguyen D.N., Fuller J. and Alvarez R., A combinatorial library of lipid-like materials for delivery of RNAi therapeutics, Nature biotechnology, 26 (2008) 561–569.

Allison S.D. and Anchordoquy T.J., Mechanisms of protection of cationic lipid DNA complexes during lyophilization, Journal of pharmaceutical sciences, 89 (2000) 682–691.

Allison S.D. and Anchordoquy T.J., Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: Insight from other structural indicators, Journal of pharmaceutical sciences, 90 (2001) 1445–1455.

Allison S.D., De Molina M. and Anchordoquy T.J., Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: The particle isolation hypothesis, Biochimica et Biophysica Acta (BBA)-Biomembranes, 1468 (2000) 127–138.

Alvarez R., Elbashir S., Borland T., Toudjarska I., Hadwiger P., John M., Roehl L., Morskaya S.S., Martinello R., Kahn J., Van Ranst M., Tripp R.A., Devincenzo J.P., Pandey R., Maier M., Nechev L., Manoharan M., Koteliandi V. and Meyers R., RNA interference-mediated silencing of the respiratory syncytial virus nucleocapsid defines a potent antiviral strategy, Antimicrob Agents Chemother, 53 (2009) 3952–3962.

Amarzguioui M., Rossi J.J. and Kim D., Approaches for chemically synthesized siRNA and vector-mediated RNAi, FEBS Letters, 579 (2005) 5974–5981.

Anchordoquy T.J., Carpenter J.F. and Kroll D.J., Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration, Archives of biochemistry and biophysics, 348 (1997) 199–206.

Arias J., Gallardo V., Gomez-Lopera S., Plaza R. and Delgado A., Synthesis and characterization of poly (ethyl-2-cyanoacrylate) nanoparticles with a magnetic core, Journal of Controlled Release, 77 (2001) 309–321.

Baoum A., Ovcharenko D. and Berkland C., Calcium condensed cell penetrating peptide complexes offer highly efficient, low toxicity gene silencing, Int J Pharm, 427 (2012) 134–142.

Barichello J.M., Morishita M., Takayama K. and Nagai T., Encapsulation of hydrophilic and lipophilic drugs in PLGA nanoparticles by the nanoprecipitation method, Drug development and industrial pharmacy, 25 (1999) 471–476.

Benfer M. and Kisse1 T., Cellular uptake mechanism and knockdown activity of siRNA-loaded biodegradable DEAPA-PVA-g-PLGA nanoparticles, European Journal of Pharmacaceutics and Biopharmaceutics, 80 (2012) 247–256.

Biswas S., Knipp R.J., Gordon L.E., Nandula S.R., Gorr S.U., Clark G.J. and Nantz M.H., Hydrophobic oxime ethers: A versatile class of pDNA and siRNA transfection lipids, ChemMedChem, 6 (2011) 2063–2069.

Bitko V., Musiyenko A., Shulyayeva O. and Barik S., Inhibition of respiratory viruses by nasally administered siRNA, Nature medicine, 11 (2005) 50–55.

Blasig A., Shi C., Enick R.M. and Thies M.C., Effect of concentration and degree of saturation on RESS of a CO2-soluble fluoropolymer, Industrial & engineering chemistry research, 41 (2002) 4976–4983.
Bochicchio S., Dalmoro A., Barba A.A., Grassi G. and Lamberti W., Liposomes as siRNA delivery vectors, Current drug metabolism, 15 (2014) 882–892.

Borm P., Klaessig F.C., Landry T.D., Moudgil B., Pauluhn J., Thomas K., Trottier R. and Wood S., Research strategies for safety evaluation of nanomaterials, part V: Role of dissolution in biological fate and effects of nanoparticle scales, Toxicol Sci, 90 (2006) 23–32.

Braunecker W.A. and Matyjaszewski K., Controlled/living radical polymerization: Features, developments, and perspectives, Progress in Polymer Science, 32 (2007) 93–146.

Burnett J.C. and Rossi J.J., RNA-based therapeutics: Current progress and future prospects, Chemistry & biology, 19 (2012) 60–71.

Burnett J.C., Rossi J.J. and Tiemann K., Current progress of siRNA/shRNA therapeutics in clinical trials, Biotechnology Journal, 6 (2011) 1130–1146.

Buyens K., Demeester J., De Smedt S.S. and Sanders N.N., Elucidating the encapsulation of short interfering RNA in pegylated cationic liposomes, Langmuir, 25 (2009) 4886–4891.

Çağdaş M., Sezer A.D. and Bucak S., Liposomes as potential drug carrier systems for drug delivery, 2014.

Calvo P., Remuñan-López C., Vila-Jato J.L. and Alonso M.J., Chitosan and chitosan/ethylen oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines, Pharmaceutical Research, 14 (1997a) 1431–1436.

Calvo P., Remunan Lopez C., Vila Jato J. and Alonso M., Novel hydrophilic chitosan polyethylene oxide nanoparticles as protein carriers, Journal of Applied Polymer Science, 63 (1997b) 125–132.

Cao W., De Oliveira Ribeiro R., Liu D., Saintigny P., Xia R., Xue Y., Lin R., Mao L. and Ren H., EZH2 promotes malignant behaviors via cell cycle dysregulation and its mRNA level associates with prognosis of patients with non-small cell lung cancer, PloS one, 7 (2012) e52984.

Caplen N.J., Alton E.W., Middleton P.G., Dorin J.R., Stevenson B.J., Gao X., Durham S.R., Jeffery P.K., Hodson M.E. and Coutelle C., Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis, Nature medicine, 1 (1995) 39–46.

Carmona S., Jorgensen M., Kolli S., Crowther C., Salazar F.H., Marion P.L., Fujino M., Natori Y., Thanou M. and Arbuthnot P., Controlling HBV replication in vivo by intravenous administration of triggered PEGylated siRNA-nanoparticles, Molecular pharmacology, 6 (2009) 706–717.

Cavalli R., Caputo O., Carlotti M.E., Trotta M., Scarneccchia C. and Gasco M.R., Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles, International journal of pharmacaceutics, 148 (1997) 47–54.

Chen A.M., Zhang M., Wei D., Stueber D., Taratula O., Minko T. and He H., Co-delivery of doxorubicin and Bcl-2 siRNA by mesoporous silica nanoparticles enhances the efficacy of chemotherapy in multidrug-resistant cancer cells, Small, 5 (2009) 2673–2677.

Chen G., Kronenberger P., Peugel E., Umelo I.A. and De Grève J., Targeting the epidermal growth factor receptor in non-small cell lung cancer cells: The effect of combining RNA interference with tyrosine kinase inhibitors or cetuximab, BMC medicine, 10 (2012) 28.

Chen S., Ni B., Huang H., Chen X. and Ma H., siRNA-loaded pegylated porous silicon nanoparticles for lung cancer therapy, Journal of Nanoparticle Research, 16 (2014) 1–8.

Chernyak Y., Henon F., Harris R.B., Gould R.D., Franklin R.K., Edwards J.R., Desimone J.M. and Carbonell R.G., Formation of perfluoropolyether coatings by the rapid expansion of supercritical solutions (RESS) process. Part I: Experimental results, Industrial & engineering chemistry research, 40 (2001) 6118–6126.

Choi M., Lee M. and Rhim T., Dexamethasone-conjugated polyethylenimine/mif siRNA complex regulation of particulate matter-induced airway inflammation, Biomaterials, 34 (2013) 7453–7461.

Chougule M., Padhi B. and Misra A., Nano-liposomal dry powder inhaler of tacrolimus: Preparation, characterization, and pulmonary pharmacokinetics, Int J Nanomedicine, 2 (2007) 675–688.

Chougule M., Padhi B. and Misra A., Development of spray dried liposomal dry powder inhaler of dapsone, AAPS PharmSciTech, 9 (2008) 47–53.

Clark K.L., Hughes S.A., Bulsara P., Coates J., Moores K., Parry J., Carr M., Mayer R.J., Wilson P. and Gruenloh C., Pharmacological characterization of a novel ENaCα siRNA (GSK2225745) with potential for the treatment of cystic fibrosis, Molecular Therapy—Nucleic Acids, 2 (2013) e65.

Corey D.R., Synthesis of oligonucleotide-peptide and oligonucleotide-protein conjugates, (Eds), Bioconjugation protocols, Springer, 2004, pp. 197–206.

Crespy D., Stark M., Hoffmann-Richter C., Ziemer U. and Landfeuster K., Polymeric nanoreactors for hydrophilic reagents synthesized by interfacial polyecondensation on miniemulsion droplets, Macromolecules, 40 (2007) 3122–3135.

Crowe J.H. and Crowe L.M., Factors affecting the stability of dry liposomes, Biochimica et Biophysica Acta (BBA)-Biomembranes, 939 (1988) 327–334.

D’alessandro-Gabazza C.N., Kobayashi T., Boveda-Ruiz D., Takagi T., Toda M., Gil-Bernabe P., Miyake Y., Yasukawa A., Matsuda Y. and Suzuki N., Development and preclinical efficacy of novel transforming growth factor-β1 short interfering RNAs for pulmonary fibrosis, American journal of respiratory and molecular biology, 46 (2012) 397–406.

Danicher L., Frère Y. and Le Calvé A., Synthesis by interfacial polyecondensation of polyamide capsules with various sizes. Characteristics and properties, Macromolecular Symposia, Wiley Online Library, 2000.

De Backer L., Cerrada A., Pérez-Gil J., De Smedt S.C. and Raemdonck K., Bio-inspired materials in drug delivery: Exploring the role of pulmonary surfactant in siRNA inhalation therapy, Journal of Controlled Release, (2015).

De Fougerolles A. and Novobrantseva T., siRNA and the lung: Research tool or therapeutic drug?, Curr Opin Pharmacol, 8 (2008) 280–285.

De Fougerolles A.R., Delivery vehicles for small interfering RNA in vivo, Human gene therapy, 19 (2008) 125–132.

Deshayes S., Morris M., Divita G. and Heitz F., Cell-penetrating
Devincenzo J., Cehelsky J.E., Alvarez R., Elbashir S., Harborth J., Toudjarska I., Nechev L., Murugaiah V., Van Vliet A. and Vaishnav A.K., Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV), Antiviral research, 77 (2008) 225–231.

Devincenzo J., Lambkin-Williams R., Wilkinson T., Cehelsky J., Nochur S., Walsh E., Meyers R., Gollob J. and Vaishnaw A., A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus, Proceedings of the National Academy of Sciences, 107 (2010) 8800–8805.

Devincenzo JP., Harnessing RNA interference to develop neonatal therapies: From Nobel prize winning discovery to proof of concept clinical trials, Early Hum Dev, 85 (2009) S31–35.

Dure C., Magnet S., Couvreur L. and Charleux B., Nitroxide-mediated controlled/living free-radical surfactant-free emulsion polymerization of methyl methacrylate using a poly (methacrylic acid)-based macroalkoxymine initiator, Macromolecules, 42 (2008) 95–103.

Dokka S., Toledo D., Shi X., Castranova V. and Rojasakul Y., Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes, Pharmaceutical Research, 17 (2000) 521–525.

Dustgania A., Vasheghani Farahani E. and Imani M., Preparation of chitosan nanoparticles loaded with dexamethasone sodium phosphate, Iranian Journal of Pharmaceutical Sciences, 4 (2008) 111–114.

Eliyahu H., Siani S., Azzam T., Domb A.J. and Barenholz Y., Relationships between chemical composition, physical properties and transfection efficiency of polysaccharide–spermine conjugates, Biomaterials, 27 (2006) 1646–1655.

Endoh T. and Ohtsuki T., Cellular siRNA delivery using cell-penetrating peptides modified for endosomal escape, Advanced drug delivery reviews, 61 (2009) 704–709.

Fallouh N.a.K., Roblot-Treupel L., Fessi H., Devissaguet J.P. and Puisieux F., Development of a new process for the manufacture of polyisobutylcyanoacrylate nanocapsules, International Journal of Pharmaceutics, 28 (1986) 125–132.

Felgner P.L., Tsai Y.J., Sukhu L., Wheeler C.J., Manthorpe M., Marshall J. and Cheng S.H., Improved cationic lipid formulations for in vivo gene therapy, Annals of the New York Academy of Sciences, 772 (1995) 126–139.

Fallmann C. and Lowe S.W., Stable RNA interference rules for silencing, Nature cell biology, 16 (2014) 10–18.

Fessi H., Puisieux F., Devissaguet J.P., Ammoury N. and Benita S., Nanocapsule formation by interfacial polymer deposition following solvent displacement, International journal of pharmaceutics, 55 (1989) R1–R4.

Finnie K.S., Waller D.J., Perret F.L., Krause-Heuer A.M., Lin H.Q., Hanna J.V. and Barbé C.J., Biodegradability of sol–gel silica microparticles for drug delivery, Journal of Sol-Gel Science and Technology, 49 (2008) 12–18.

Fire A., Xu S.Q., Montgomery M.K., Kostas S.A., Driver S.E. and Mello C.C., Potent and specific genetic interference by double-stranded RNA in caenorhabditis elegans, Nature, 391 (1998) 806–811.

Fourtounis J., Wang I.-M., Mathieu M.-C., Claveau D., Loo T., Jackson A.L., Peters M.A., Herien A.G., Boie Y. and Crackower M.A., Gene expression profiling following Nrf2 and KEAP1 siRNA knockdown in human lung fibroblasts identifies CCL11/Eotaxin-1 as a novel Nrf2 regulated gene, Respir Res, 13 (2012) 92.

Galindo-Rodriguez S., Allemann E., Fessi H. and Doelker E., Physicochemical parameters associated with nanoparticle formation in the salting-out, emulsification-diffusion, and nanoprecipitation methods, Pharmaceutical research, 21 (2004) 1428–1439.

Gallardo M., Couarraze G., Denizot B., Treupel L., Couvreur P. and Puisieux F., Study of the mechanisms of formation of nanoparticles and nanocapsules of polyisobutyl-2-cyanoacrylate, International journal of pharmaceutics, 100 (1993) 55–64.

Ganachaud F. and Katz J.L., Nanoparticles and nanocapsules created using the ouzo effect: Spontaneous emulsification as an alternative to ultrasonic and high shear devices, ChemPhysChem, 6 (2005) 209–216.

Gandhi N.S., Tekade R.K. and Chougule M.B., Nanocarrier mediated delivery of siRNA/miRNA in combination with chemotherapeutic agents for cancer therapy: Current progress and advances, J Control Release, 194 (2014) 238–256.

Gao X. and Huang L., Potentiation of cationic liposome-mediated gene delivery by polycations, Biochemistry, 35 (1996) 1027–1036.

Garbuzenko O.B., Saad M., Pozharov V.P., Reuhl K.R., Mainelis G. and Minko T., Inhibition of lung tumor growth by complex pulmonary delivery of drugs with oligonucleotides as suppressors of cellular resistance, Proceedings of the National Academy of Sciences of the United States of America, 107 (2010) 10737–10742.

Gasco M. and Trotta M., Nanoparticles from microemulsions, International journal of pharmaceutics, 29 (1986) 267–268.

Gaspar M.M., Bakowsky U. and Ehhardt C., Inhaled liposomes—current strategies and future challenges, Journal of Biomedical Nanotechnology, 4 (2008) 245–257.

Gaudin F. and Sintes-Zydowicz N., Core–shell biocompatible polyurethane nanocapsules obtained by interfacial step polymerisation in miniemulsion, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 331 (2008) 133–142.

Glasgow M.D. and Chougule M.B., Recent developments in active tumor targeted multifunctional nanoparticles for combination chemotherapy in cancer treatment and imaging, Journal of Biomedical Nanotechnology, 11 (2015) 1859–1898.

Guthier B., Kube S.M., Reppe K., Santel A., Lange C., Kaufmann J., Suttrop N. and Witzentrah M., RNAi-mediated suppression of constitutive pulmonary gene expression by small interfering RNA in mice, Pulmonary pharmacology & therapeutics, 23 (2010) 334–344.

Ham H.T., Choi Y.S., Chee M.G. and Chung I.J., Singlewall carbon nanotubes covered with polystyrene nanoparticles by
carrier in vitro and in vivo, Eur J Pharm Biopharm, 77 (2011) 36–42.
Jung T., Kamm W., Breitenbach A., Kaiserling E., Xiao J. and Kissel T., Biodegradable nanoparticles for oral delivery of peptides: Is there a role for polymers to affect mucosal uptake?, European Journal of Pharmaceutics and Biopharmaceutics, 50 (2000) 147–160.
Kalariya M., Padhi B.K., Chougule M. and Misra A., Cobetosol propionate solid lipid nanoparticles cream for effective treatment of eczema: Formulation and clinical implications, Indian J Exp Biol, 43 (2005) 233–240.
Kanasty R., Dorkin J.R., Vegas A. and Anderson D., Delivery materials for siRNA therapeutics, Nature materials, 12 (2013) 967–977.
Karlo D., Kulkarni S., Suresh A. and Mashelkar R., New insights into kinetics and thermodynamics of interfacial polymerization, Chemical engineering science, 53 (1998) 2649–2663.
Kasper J.C., Schaffert D., Ogris M., Wagner E. and Friess W., Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability—a step closer from promising technology to application, Journal of Controlled Release, 151 (2011) 246–255.
Kawashima Y., Nanoparticulate systems for improved drug delivery, Advanced drug delivery reviews, 47 (2001) 1–2.
Keefe D., Agency response letter GRAS notice no. GRN 000397, 2011, FDA (U.S. Food and Drug Administration) <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm287638.htm> accessed 29.02.2016.
Kim D.H. and Rossi J.J., Strategies for silencing human disease using RNA interference, Nature Reviews Genetics, 8 (2007) 173–184.
Kim S.S., Peer D., Kumar P., Subramanya S., Wu H., Asthana D., Habiro K., Yang Y.G., Manjunath N., Shimaoka M. and Shankar P., RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevents HIV infection in BLT mice, Mol Ther, 18 (2010) 370–376.
Kostag M., Köhler S., Liebert T. and Heinze T., Pure cellulose nanoparticles; a structural paradigm for viable synthetic polymerization, Journal of Polymer Science: Polymer Letters Edition, 20 (1982) 543–545.
Kreuter J., On the mechanism of termination in heterogeneous polymerization, Journal of Polymer Science: Polymer Letters Edition, 20 (1982) 543–545.
Krutzwald J., Rajewsky N., Braich R., Rajeev K.G., Tusche T., Manoharan M. and Stoffel M., Silencing of microRNAs in vivo with ‘antagomirs’, nature, 438 (2005) 685–689.
Lam J.K.-W., Liang W. and Chan H.-K., Pulmonary delivery of therapeutic siRNA, Advanced drug delivery reviews, 64 (2012) 1–15.
Lambert G., Fattal E. and Couvreur P., Nanoparticulate systems for the delivery of antisense oligonucleotides, Advanced drug delivery reviews, 47 (2001) 99–112.
Landfester K., Musyanovych A. and Mailänder V., From polymeric particles to multifunctional nanocapsules for
biomedical applications using the miniemulsion process, Journal of Polymer Science Part A: Polymer Chemistry, 48 (2010) 493–515.

Li J., Chen Y.-C., Tseng Y.-C., Mozumdar S. and Huang L., Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery, Journal of Controlled Release, 142 (2010) 416–421.

Li S.D. and Huang L., Nanoparticles evading the reticuloendothelial system: Role of the supported bilayer, Biochim Biophys Acta, 1788 (2009) 2259–2266.

Lim K.T., Subban G.H., Hwang H.S., Kim J.T., Ju C.S. and Johnston K.P., Novel semiconducting polymer particles by supercritical fluid process, Macromolecular rapid communications, 26 (2005) 1779–1783.

List S.J., Findlay B.P., Forstner G. and Forstner J., Enhancement of the viscosity of mucin by serum albumin, Biochem. J, 175 (1978) 565–571.

Liu S., Nugroho A.E., Shudou M. and Maeyama K., Regulation of mucosal mast cell activation by short interfering RNAs targeting syntaxin4, Immunology and cell biology, 90 (2012) 337–345.

Liu Y., Dong Q.-Z., Wang S., Fang C.-Q., Miao Y., Wang L., Li M.-Z. and Wang E.-H., Abnormal expression of pyogopus 2 correlates with a malignant phenotype in human lung cancer, BMC cancer, 13 (2013) 346.

Lobovkina T., Jacobson G.B., Gonzalez-Gonzalez E., Hickerson R.P., Leake D., Kaspar R.L., Contag C.H. and Zare R.N., In vivo sustained release of siRNA from solid lipid nanoparticles, ACS Nano, 5 (2011) 9977–9983.

Lomas-Neira J., Perl M., Venet F., Chung C.-S. and Ayala A., The role and source of TNF-α in hemorrhage induced priming for septic lung injury, Shock (Augusta, Ga.), 37 (2012) 611.

Love K.T., Mahon K.P., Levins C.G., Whitehead K.A., Querbes W., Dorkin J.R., Qin J., Cantley W., Qin L.L. and Racie T., Lipid-like materials for low-dose, in vivo gene silencing, Proceedings of the National Academy of Sciences, 107 (2010) 1864–1869.

Luo Y., Zhai X., Ma C., Sun P., Fu Z., Liu W. and Xu J., An inhahal bear σ-loadreceptor ligand-directed guanidinylated chitosan carrier for targeted delivery of siRNA to lung, J Control Release, 162 (2012) 28–36.

Mainelis G., Seshadri S., Garbuzea Nut, Han T., Wang Z. and Minko T., Characterization and application of a nox-only exposure chamber for inhalation delivery of liposomal drugs and nucleic acids to mice, Journal of aerosol medicine and pulmonary drug delivery, 26 (2013) 345–354.

Mao S., Sun W. and Kissel T., Chitosan-based formulations for delivery of DNA and siRNA, Advanced drug delivery reviews, 62 (2010) 12–27.

Marriott C., Beeson M.F. and Brown D.T., Biopolymer induced changes in mucus viscoelasticity, (Eds), Mucus in health and disease—ii, Springer, 1982, pp. 89–92.

Matsubara H., Sakakibara K., Kunimitsu T., Matsuoka H., Kato K., Oyachi N., Dobashi Y. and Matsumoto M., Non-small cell lung carcinoma therapy using mTOR-siRNA, International journal of clinical and experimental pathology, 5 (2012) 119.

Matyjaszewski K. and Xia J., Atom transfer radical polymerization, Chemical reviews, 101 (2001) 2921–2990.

Meade B.R. and Dowdy S.F., Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides, Advanced drug delivery reviews, 59 (2007) 134–140.

Mehnert W. and Maeder K., Solid lipid nanoparticles production, characterization and applications, Advanced Drug Delivery Reviews, 64 (2012) 83–101.

Merkel O.M. and Kissel T., Nonviral pulmonary delivery of siRNA, Accounts of chemical research, 45 (2011) 961–970.

Merkel O.M., Rubinstein I. and Kissel T., siRNA delivery to the lung: What’s new?, Adv Drug Deliv Rev, 75 (2014) 112–128.

Meziani M.J., Pathak P., Hurezeanu R., Thies M.C., Enick R.M. and Sun Y.P., Supercritical fluid processing technique for nanoscale polymer particles, Angewandte Chemie International Edition, 43 (2004) 704–707.

Mi Bae Y., Choi H., Lee S., Ho Kang S., Tae Kim Y., Nam K., Sang Park J., Lee M. and Sig Choi J., Dexamethasone-conjugated low molecular weight polyethyleneimine as a nucleus-targeting lipopolymer gene carrier, Bioconjugate Chemistry, 18 (2007) 2029–2036.

Min K., Gao H., Yoon J.A., Wu W., Kowalewski T. and Matyjaszewski K., One-pot synthesis of hairy nanoparticles by emulsion ATRP, Macromolecules, 42 (2009) 1597–1603.

Mishra S. and Chatterjee A., Novel synthesis of polymer and copolymer nanoparticles by atomized microemulsion technique and its characterization, Polymers for Advanced Technologies, 22 (2011) 1593–1601.

Morrisssey D.V., Lockridge J.A., Shaw L., Blanchard K., Jensen K., Breen W., Hartsough K., Machemer L., Radka S. and Jadhav V., Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs, Nature biotechnology, 23 (2005) 1002–1007.

Moschos S.A., Frick M., Taylor B., Turnpenny P., Graves H., Spink K.G., Brady K., Lamb D., Collins D. and Rockel T.D., Uptake, efficacy, and systemic distribution of naked, inhaled short interfering RNA (siRNA) and locked nucleic acid (LNA) antisense, Molecular Therapy, (2011).

Moulik S. and Paul B., Structure, dynamics and transport properties of microemulsions, Advances in Colloid and Interface science, 78 (1998) 99–195.

Nagavarma B., Yadav H.K., Ayaz A., Vasudha L. and Shivakumar H., Different techniques for preparation of polymeric nanoparticles—a review, Asian J Pharm. Clin. Res, 5 (2012) 16–23.

Nicolas J., Ruzette A.-V., Farcet C., Gérard P., Magnet S. and Charleux B., Nanostructured latex particles synthesized by nitroxide-mediated controlled/living free-radical polymerization in emulsion, Polymer, 48 (2007) 7029–7040.

Nishimura Y., Takiguchi S., Ito S. and Itoh K., Evidence that depletion of the sorting nexin 1 by siRNA promotes HGF-activated MET endocytosis and MET phosphorylation in a gefitinib-resistant human lung cancer cell line, International journal of oncology, 44 (2014) 412–426.

Niwa T., Takeuchi H., Hino T., Kunou N. and Kawashima Y., In vitro drug release behavior of D, L-lactide/glycolide copolymer (PLGA) nanospheres with nafarelin acetate prepared...
Oh B. and Lee M., Combined delivery of HMGB-1 box a peptide and siPLyase siRNA in animal models of acute lung injury, J Control Release, 175 (2014) 25–35.

Okuda T., Kito D., Oiwa A., Fukushima M., Hira D. and Okamoto H., Gene silencing in a mouse lung metastasis model by an inhalable dry small interfering RNA powder prepared using the supercritical carbon dioxide technique, Biological and Pharmaceutical Bulletin, 36 (2013) 1183–1191.

Pardeike J., Hommoss A. and Mueller R.H., Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products, International Journal of Pharmaceutics, 366 (2009) 170–184.

Patlolla R.R., Chougule M.B., Townley I., Patlolla R., Wang G. and Singh M., Efficacy of aerosolized celecoxib encapsulated nanostructured lipid carrier in non-small cell lung cancer in combination with docetaxel, Pharmaceutical research, 30 (2013) 1435–1446.

Patel G., Chougule M., Singh M. and Misra A., Nanoliposomal dry powder formulations, Methods in enzymology, 464 (2009) 167–191.

Patlolla R.R., Chougule M., Patel A.R., Jackson T., Tata P.N.V. and Singh M., Formulation, characterization and pulmonary deposition of nebulized celecoxib encapsulated nanostructured lipid carriers, Journal of Controlled Release, 144 (2010) 233–241.

Pavan G.M., Mintzer M.A., Simanek E.E., Merkel O.M., Kissel T. and Danani A., Computational insights into the interactions between DNA and siRNA with “rigid” and “flexible” triazine dendrimers, Biomacromolecules, 11 (2010) 721–730.

Podesta J.E. and Kostarelos K., Chapter seventeen-engineering cationic liposome: siRNA complexes for in vitro and in vivo delivery, Methods in enzymology, 464 (2009) 343–354.

Puglisi G., Fresco M., Giammona G. and Ventura C., Influence of the preparation conditions on poly (ethyleneoxide) nanocapsule formation, International Journal of Pharmaceutics, 125 (1995) 283–287.

Quintanar-Guerrero D., Allemann E., Fessi H. and Doelker E., Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers, Drug Development and Industrial Pharmacy, 24 (1998) 1113–1128.

Radu D.R., Lai C.Y., Jef tinjia K., Rowe E.W., Jef tinjia S. and Lin V.S., A polyamidoamine dendrimer-capped mesoporous silica nanosphere-based gene transfection reagent, J Am Chem Soc, 126 (2004) 13216–13217.

Rahman M.A., Amin A.R., Wang D., Koenig L., Nannapaneni S., Chen Z., Wang Z., Sica G., Deng X. and Shin D.M., RRM2 regulates Bcl-2 in head and neck and lung cancers: A potential target for cancer therapy, Clinical Cancer Research, 19 (2013) 3416–3428.

Ramachandran S., Krishnamurthy S., Jacob A.M., Wohlford-Lenane C., Behlike M.A., Davidson B.L. and Mccray P.B., Efficient delivery of RNA interference oligonucleotides to polarized airway epithelia in vitro, American Journal of Physiology-Lung Cellular and Molecular Physiology, 305 (2013) L23–L32.

Rao J.P. and Geckeler K.E., Polymer nanoparticles: Preparation techniques and size-control parameters, Progress in Polymer Science, 36 (2011) 887–913.

Reis C.P., Neufeld R.J., Ribeiro A.J. and Veiga F., Nanocapsulation. i. Methods for preparation of drug-loaded polymeric nanoparticles, Nanomedicine: Nanotechnology, Biology and Medicine, 2 (2006) 8–21.

Reischl D. and Zimmer A., Drug delivery of siRNA therapeutic: Potentials and limits of nanosystems, Nanomedicine: Nanotechnology, Biology and Medicine, 5 (2009) 8–20.

Ren Y., Kang C.S., Yuan X.B., Zhou X., Xu P., Han L., Wang G.X., Jia Z., Zhong Y., Yu S., Sheng J. and Pu P.Y., Codelivery of as-miR-21 and 5-FU by poly(aminodiamine) dendrimer attenuates human glioma cell growth in vitro, J Biomater Sci Polym Ed, 21 (2010) 303–314.

Rieger J., Zhang W., Staffelbach F.O. and Charleux B., Surfactant-free RAFT emulsion polymerization using poly (n, n-dimethylacrylamide) trithiocarbonate macromolecular chain transfer agents, Macromolecules, 43 (2010) 6302–6310.

Samad A., Sultana Y. and Aqil M., Liposomal drug delivery systems: An update review, Current drug delivery, 4 (2007) 297–305.

Sane A. and Thies M.C., Effect of material properties and processing conditions on RESS of poly (l-lactide), The Journal of supercritical fluids, 40 (2007) 134–143.

Sarkar D., El-Khoury J., Lopina S.T. and Hu J., An effective method for preparing polymer nanocapsules with hydrophobic acrylic shell and hydrophilic interior by inverse emulsion radical polymerization, Macromolecules, 38 (2005) 8603–8605.

Schwarz C., Mehnert W., Lucks J. and Muller R., Solid lipid nanoparticles (SLN) for controlled drug delivery I. Production, characterization and sterilization, Journal of Controlled Release, 30 (1994) 83–96.

Scott C., Wu D., Ho C.-C. and Co C.C., Liquid-core capsules via interface polymerization: A free-radical analogy of the nylon rope trick, Journal of the American Chemical Society, 127 (2005) 4160–4161.

Semple S.C., Klumk S.K., Harasym T.O., Dos Santos N., Ansell S.M., Wong K.F., Maurer N., Stark H., Cullis P.R. and Hope M.J., Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: Formation of novel small multilamellar vesicle structures, Biochimica et Biophysica Acta (BBA)-Biomembranes, 1510 (2001) 152–166.

Shim G., Choi H.-W., Lee S., Choi J., Yu Y.H., Park D.-E., Choi Y., Kim C.-W. and Oh Y.-K., Enhanced intrapulmonary delivery of anticancer siRNA for lung cancer therapy using cationic ethylphosphocholine-based nanolipoplexes, Molecular Therapy, 21 (2013) 816–824.

Siegrist D.J., Srinivasan A., Bencherif S.A., Karunanidhi A., Oh J.K., Vaidya S., Jin R., Hollinger J.O. and Matyjaszewski K., Cellular uptake of functional nanogels prepared by inverse miniemulsion ATRP with encapsulated proteins, carboxy-
drugs, and gold nanoparticles, Biomacromolecules, 10 (2009) 2300–2309.

Siekmann B. and Westesen K., P234 solid lipid nanoparticles stabilized by tyloxapol, European Journal of Pharmaceutical Sciences, 2 (1994) 177.

Slowing, I., Trewyn B.G. and Lin V.S., Mesoporous silica nanoparticles for intracellular delivery of membrane-impermeable proteins, J Am Chem Soc, 129 (2007) 8845–8849.

Sun Y.P., Meziani M.J., Pathak P. and Qu L., Polymeric nanoparticles from rapid expansion of supercritical fluid solution, Chemistry-A European Journal, 11 (2005) 1366–1373.

Taratula O., Garbuzenko O.B., Chen A.M. and Minko T., Innovative strategy for treatment of lung cancer: Targeted nanotechnology-based inhalation co-delivery of anticancer drugs and siRNA, J Drug Target, 19 (2011a) 900–914.

Taratula O., Garbuzenko O.B., Chen A.M. and Minko T., Innovative strategy for treatment of lung cancer: Targeted nanotechnology-based inhalation co-delivery of anticancer drugs and siRNA, Journal of Drug Targeting, 19 (2011b) 900–914.

Tarkunde S.V., Gujar K., Harwalkar M. and Gambhire M., Liposome—a novel drug delivery, Int. J. Pure App. Biosci, 2 (2014) 92–102.

Tekade R.K. and Chougule M.B., Formulation development and evaluation of hybrid nanocarrier for cancer therapy: Taguchi orthogonal array based design, BioMed Research International, 2013 (2013) 18.

Tekade R.K., Youngren-Ortiz S.R., Yang H., Haware R. and Chougule M.B., Designing hybrid onconase nanocarriers for mesothelioma therapy: A taguchi orthogonal array and multivariate component driven analysis, Molecular pharmaceutics, 11 (2014) 3671–3683.

Thomas C.E., Ehrhardt A. and Kay M.A., Progress and problems with the use of viral vectors for gene therapy, Nature Reviews Genetics, 4 (2003) 346–358.

Tompkins S.M., Lo C.-Y., Tumpey T.M. and Epstein S.L., Protection against lethal influenza virus challenge by RNA interference in vivo, Proceedings of the National Academy of Sciences of the United States of America, 101 (2004) 8682–8686.

Tseng Y.-C., Mozumdar S. and Huang L., Lipid-based systemic delivery of siRNA, Advanced drug delivery reviews, 61 (2009) 721–731.

Turner J.J., Williams D., Owen D. and Gait M.J., Disulfide conjugation of peptides to oligonucleotides and their analogs, Current protocols in nucleic acid chemistry, (2006) 4.28. 21–24.28. 21.

Umrana Y., Nikjoo H. and Goodfellow J., A knowledge-based model of DNA hydration, International journal of radiation biology, 67 (1995) 145–152.

Vauthier C. and Bouchemak K., Methods for the preparation and manufacture of polymeric nanoparticles, Pharmaceutical research, 26 (2009) 1025–1058.

Vauthier C., Dubernet C., Fattal E., Pinto-Alphandary H. and Couvreur P., Poly (alkylcyanoacrylates) as biodegradable materials for biomedical applications, Advanced Drug Delivery Reviews, 55 (2003) 519–548.

Wang J.-C., Lai S., Guo X., Zhang X., De Crombrugghe B., Sonnynal S., Arnett F.C. and Zhou X., Attenuation of fibrosis in vitro and in vivo with SPARC siRNA, Arthritis research & therapy, 12 (2010) R60.

Wang Y., Chen L., Huang G., He D., He J., Xu W., Zou C., Zong F., Li Y. and Chen B., Klotho sensitizes human lung cancer cell line to cisplatin via PI3k/Akt pathway, PloS one, 8 (2013) e57391.

Watts M.R., Susanne R. Youngren-Ortiz et al. / KONA Powder and Particle Journal No. 34 (2017) 44–69

Whitehead K.A., Langer R. and Anderson D.G., Knocking down barriers: Advances in siRNA delivery, Nat Rev Drug Discov, 8 (2009a) 129–138.

Whitehead K.A., Langer R. and Anderson D.G., Knocking down barriers: Advances in siRNA delivery, Nature Reviews Drug Discovery, 8 (2009b) 129–138.

Wissing S., Kayser O. and Müller R., Solid lipid nanoparticles for parenteral drug delivery, Advanced drug delivery reviews, 56 (2004) 1257–1272.

Wu S.Y. and Mcmillan N.A., Lipidic systems for in vivo siRNA delivery, The AAPS journal, 11 (2009) 639–652.

York P., Strategies for particle design using supercritical fluid technologies, Pharmaceutical science & technology today, 2 (1999) 430–440.

Youngren-Ortiz S.R., Gandhi N.S., España-Serrano L. and Chougule M.B., Aerosol delivery of siRNA to the lungs. Part 1: Rationale for gene delivery systems, KONA Powder and Particle Journal, 33 (2016) 63–85.

Youngren S., Mulik R., Jun B., Hoffmann P., Morris K. and Chougule M., Freeze-dried targeted mannosylated selenium-loaded nanoliposomes: Development and evaluation, AAPS PharmSciTech, 10.1208/s12249-013-9988-3 (2013) 1–13.

Youngren S.R., Tekade R.K., Gustilo B., Hoffmann P.R. and Chougule M.B., STAT6 siRNA matrix-loaded gelatin nanocarriers: Formulation, characterization, and ex vivo proof of concept using adenocarcinoma cells, Biomed Res Int, 2013 (2013) 858946.

Zamora M.R., Budev M., Rolfe M., Gottlieb J., Hunnar M., Devincenzo J., Vaishnaw A., Cehelsky J., Albert G., Nochur S., Gollob J.A. and Granville A.R., RNA interference therapy in lung transplant patients infected with respi-
Zatsepin T., Turner J., Oretskaya T. and Gait M., Conjugates of oligonucleotides and analogues with cell penetrating peptides as gene silencing agents, Current pharmaceutical design, 11 (2005) 3639–3654.

Zetterlund P.B., Kagawa Y. and Okubo M., Controlled/living radical polymerization in dispersed systems, Chemical reviews, 108 (2008) 3747–3794.

Zetterlund P.B., Nakamura T. and Okubo M., Mechanistic investigation of particle size effects in TEMPO-mediated radical polymerization of styrene in aqueous miniemulsion, Macromolecules, 40 (2007) 8663–8672.

Zhang Y.-P., Li W.-B., Wang W.-L., Liu J., Song S.-X., Bai L.-L., Hu Y.-Y., Yuan Y.-D. and Zhang M., siRNA against plasminogen activator inhibitor-1 ameliorates bleomycin-induced lung fibrosis in rats, Acta Pharmacologica Sinica, 33 (2012) 897–908.

Zheng M., Librizzi D., Kilic A., Liu Y., Renz H., Merkel O.M. and Kissel T., Enhancing in vivo circulation and siRNA delivery with biodegradable polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol) copolymers, Biomaterials, 33 (2012) 6551–6558.

Zhou X., Ni P. and Yu Z., Comparison of RAFT polymerization of methyl methacrylate in conventional emulsion and miniemulsion systems, Polymer, 48 (2007) 6262–6271.

Ziegler A., Landfester K. and Musyanovych A., Synthesis of phosphonate-functionalized polystyrene and poly (methyl methacrylate) particles and their kinetic behavior in miniemulsion polymerization, Colloid and polymer science, 287 (2009) 1261–1271.

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