Published in final edited form as:
Adv Drug Deliv Rev. 2014 August 30; 0: 112–128. doi:10.1016/j.addr.2014.05.018.

siRNA Delivery to the Lung: What's New?

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Abstract

RNA interference (RNAi) has been thought of as the general answer to many unmet medical needs. After the first success stories, it soon became obvious that short interfering RNA (siRNA) is not suitable for systemic administration due to its poor pharmacokinetics. Therefore local administration routes have been adopted for more successful *in vivo* RNAi. This paper reviews nucleic acid modifications, nanocarrier chemistry, animal models used in successful pulmonary siRNA delivery, as well as clinical translation approaches. We summarize what has been published recently and conclude with the potential problems that may still hamper the efficient clinical application of RNAi in the lung.

Keywords

siRNA; pulmonary delivery; nanocarrier; polymer; lung cancer; asthma

1. Introduction

At the end of the previous century, Fire and Mellow discovered the pathway of RNA interference (RNAi) [1], a sequence-specific post-transcriptional gene silencing (PTGS) mechanism that occurs intracellularly to degrade foreign double stranded RNA (dsRNA) and regulates endogenous RNA levels, and they received the Nobel Prize in Physiology for this discovery in 2006. RNAi can experimentally be triggered by either introducing so-called short hairpin RNA (shRNA) expressed by a plasmid, by introducing long double stranded RNA (dsRNA), or by directly delivering synthetic short interfering RNA (siRNA) (21-26 nucleotides) into target cells. In case of shRNA and long dsRNA delivery, the endogenous pathway of RNA processing into siRNAs by the enzyme complex Dicer is exploited [2],

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which is said to facilitate the incorporation of the siRNA into the RNA-induced silencing complex (RISC) [3]. In the activated RISC, the sense strand is removed and degraded by nucleases, whereas the antisense strand directs the RISC to the base-complementary sequence of the target mRNA [4]. Binding of mRNA to the antisense strand in the activated RISC eventually induces cleavage by the endonuclease Argonaute and post-transcriptional silencing of the target gene expression [5].

More than 15 years later, we are still struggling with translating this pioneering discovery into clinical applications. Efficient intracellular delivery of siRNA across biological barriers in general and delivery to target cells in particular is not at all straightforward since cells lack an efficient uptake mechanism for nucleic acids. Although siRNA can be spontaneously endocytosed by some cells to a very low extent via caveolae-mediated uptake [6], the major hurdle for advancing RNAi into clinical trials, however, is a lack of biocompatible siRNA carriers that overcome extracellular and intracellular barriers [7, 8] and that improve the currently inefficient targeting of siRNA to the desired organs and cell types [9].

In 2011, we described the ideal siRNA delivery system would be “biodegradable and biocompatible (neither cytotoxic nor immunogenic)” [10]. We expected that it would “protect the payload from degradation by nucleases during the delivery process, should maintain long circulation times after systemic administration and long retention times after local administration by avoiding recognition by macrophages, and should reversibly (or stimuli-responsively) bind siRNA to allow for efficient release of the siRNAs at the target site” [10]. In the meantime, a multitude of new formulations that compact siRNA into nano-sized particles suitable for intracellular uptake by endocytosis have been tested *in vitro*, and a smaller number of nano formulations have been assessed for *in vivo* delivery of siRNA.

To date, systemic administration of siRNA is still the most widely used administration route in preclinical settings although it is known that siRNA is rapidly degraded by nucleases and fast excreted via the kidneys upon intravenous (i.v.) injection [11]. Additionally, many siRNA delivery systems that incorporate their payload by electrostatic interaction are prone to instability problems and premature release of the nucleic acids [12, 13].

Local delivery of siRNA into the lung, however, allows for noninvasive access and avoids interactions with serum proteins that degrade siRNA after i.v. administration. However, serum is absent on the air-side of the lung, and nuclease activity is comparably low [10]. The lung also offers other advantages over systemic administration such as the large alveolar surface area with high vascularization and the thin air-blood-barrier which are beneficial factors for absorption [14]. Another superiority of local administration of drugs in general which also applies to siRNA is that they can be administered at a reduced dose compared to systemic delivery, which decreases side effects. In the case of pulmonary administration, the delivered therapeutic is immediately available in the lung [15]. Since pulmonary epithelial cells are potential target cells for gene knockdown in lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), asthma, pulmonary fibrosis and viral infections, their accessibility via pulmonary treatment is of great importance [8].

The pulmonary deposition of siRNA administered to the lung depends on the aerodynamic diameter of the particle in which the siRNA is formulated and on the patient's pulmonary function [16-18]. Particles with aerodynamic diameters larger than 6 μm are described to be deposited in the oro-pharynx due to impaction. Therefore, smaller droplet or particle sizes are required for efficient delivery of siRNA to the respiratory zone (bronchioles and alveoles) where the gas exchange occurs [19]. The fate of particles smaller than 1 μm in aerodynamic diameter needs to be differentially addressed. Text books in the past did not differentiate between particles smaller than 1000 nm and particles smaller than 100 nm and assumed that all particles below 1 μm are exhaled [20]. While it is true that particles between 100 nm and 1000 nm are mostly exhaled during normal breathing as their movement follows only Brownian motion, nanoparticles (<100nm) can successfully be deposited in the alveolar space due to diffusion deposition [16-18][21]. It is possible that these nanoparticles agglomerate and build up to aerodynamic diameters above 1 μm, and that their deposition is additionally affected by the pulmonary function in healthy and asthmatic subjects [22, 23]. Therefore, to date, optimal aerodynamic diameters for efficient deposition in the lung are assumed to range between 1 and 5 μm.

Despite of all advantages of the pulmonary route, the lung also imposes intrinsic anatomic, physiologic, immunologic, and metabolic hurdles to efficient siRNA delivery, such as cough and mucociliary clearance, the presence of the mucus and the clearance of particles by macrophages, which have been reviewed by us and others [24-27] and are shown in Figure 1.

What has been undertaken to better overcome these barriers in the lung? After eight years of screening advanced siRNA delivery systems, do we now understand the impact of nanocarrier chemistry, siRNA modifications, and administration route on cellular delivery and intracellular fate of siRNA in order to pave the way for clinical translation of RNAi? And what are the current therapeutic targets and indications for an RNAi-based therapy of pulmonary diseases? Although pulmonary delivery and absorption can as well be exploited for systemic effects, we will focus here on diseases of the lung.

2. Nucleic acid modifications

As described in the introduction, different types of nucleic acids can mediate RNAi. The most commonly used types are shRNAs expressed by plasmid DNA, Dicer substrate interfering RNAs (DsiRNA) which are slightly longer (25/27mers) dsRNAs, and chemically synthesized siRNAs. Since RNA is especially prone to degradation by nucleases [28], chemical modifications at the sugar, backbone or of the bases of the oligoribonucleotides are often introduced to increase the nuclease resistance. Modifications of siRNA such as the methylation of the hydroxyl group of the 2′ of the ribose are also described to decrease the off-target effects and activation of the immune system without reducing the siRNA's efficacy [29, 30]. Most of the clinical trials employ chemically modified siRNA, such as Alynam's ALN-RSV-01 siRNA which bears a cholesterol-coupled sense strand. Other modifications include phosphorothioate (PS) backbones or modified sugars such as locked nucleic acids (LNA).

2.1. RNA modifications for gene knockdown in the lung

A combination of PS backbones and LNA was described by Moschos et al. who investigated the intratracheal (i.t.) administration of 10 nmol free nucleic acids, which were either siRNA duplexes or PS LNA antisense oligonucleotides (ASO) [31]. More sophisticated RNA chemistry was used to design a packaging RNA (pRNA) dimer that carries both an siRNA sequence against signal transducer and activator of transcription 5b (STAT5b) and a CD4 aptamer for targeted delivery to spleen lymphocytes. pRNA is a component of the bacteriophage phi29-packaging motor which packages the double-stranded DNA of the Bacillus *subtilis bacteriophage* phi29 into a precursor capsid [32]. So far, this siRNA-aptamer construct has only been evaluated *ex vivo* in cells isolated from mice that were challenged in a model of allergic asthma. However, efficient gene knockdown on the mRNA level of hard-to-transfect primary lymphocytes is a promising result for a potentially new therapeutic approach [33]. Another new class of nucleic acids for RNAi are so-called “PnkRNAs” and “nkRNAs” which are synthesized on a solid phase resin as single-stranded RNAs (ssRNAs). PnkRNAs consist of an oligonucleotide that contains 2 proline derivatives and self-anneals to a structure of a central stem of the sense and antisense region, an unpaired part, and a loop. nkRNAs, on the other hand, self-anneals in absence of any proline derivatives into a central stem, a loop, and an unpaired site [34]. These novel RNAs have been used therapeutically in a model of acute lung injury [34] and in a lung cancer model, where mice were i.v. injected with luciferase expressing A549-luc-C8 cells on day zero. The tumor growth progression was followed by whole body luminescence imaging twice a week up to day 28. Mice were treated with 15 μg of the RNAi agent against human-ribophorin II (RPN2) by i.t. microsprayer administration on day 28. When the animals were sacrificed, the RPN2 mRNA levels were found to be significantly downregulated in the xenografts of mice that had received either RPN2 siRNA or PnkRNA but not in animals treated with control siRNA [35]. This novel class of RNAs therefore can be administered as free nucleic acid without the need of protection or formulation.

2.2. Therapeutic effects of free nucleic acid delivery

It is know that some cells can internalize small amounts of siRNA via caveolae-mediated uptake [6], and pulmonary surfactant may in fact act as an adjuvant in mediating cellular uptake [36]. As described above, Moschos et al. investigated the i.t. administration of 10 nmol free nucleic acids, which were either siRNA duplexes or phosphorothioate (PS) locked nucleic acid (LNA) antisense oligonucleotides (ASO) in luciferase expressing mice. They used *ex vivo* luminometry to determine the luciferase knockdown in various organs and IVIS imaging of Cy5-labeled oligos and confocal microscopy to evaluate the biodistribution of the different types of nucleic acids. With these methods, they observed rapid transcytosis to the systemic circulation and renal clearance for both types of oligos with the result of liver and kidney uptake of the PS LNA ASO, which caused gene knockdown in the latter organs. However, knockdown on the protein level in magnetic bead purified lung macrophages (CD11b+/F4/80+), epithelia (CD45-/CD326+), or other cell types was not achieved [31]. This report of a lack of therapeutic efficacy of free RNA is in line with the observations by Lomas-Neira et al. They showed that free siRNA against TNF-α delivered i.t. post hemorrhage did not efficiently reduce the symptoms of acute lung injury (ALI) in a septic

shock model, despite measurable TNF-α knockdown in cytokeratin 18+ epithelial cells. However, siRNA which was delivered i.v. did not only knock down the gene expression in CD31+ endothelial cells but also significantly reduced the expression of indices of ALI. It is thus possible that pulmonary endothelial cells play a greater role in mediating the TNF-α mediated lung injury than epithelial cells. However, the authors did not fully assess the uptake into any other cell types after i.t. or i.v. administration. Additionally, they did form liposomes for the i.v. administration but not for the i.t. route, which may not be a fair comparison, and they did not include scrambled siRNA controls in their experiments which may make interpretation of their results more difficult [37]. The same group had published a similar protocol a year before where they used 75 μg siRNA as free nucleic acids for the i.t. administration but 75 μg siRNA + 50 μg N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methysulfate (DOTAP) for the i.v. administration. In that paper, however, they found about 35% *in vivo* knockdown of FasL after i.t. but not after i.v. administration. Additionally, the inflammation markers TNF-α, MCP-1, IL-6, and IL-10 were all significantly decreased in i.t. treated animals compared to the group that received siRNA against GFP, which was used as negative control. The criticism here is that Robbins et al. had published in 2009 already that the use of GFP sequences in models of inflammation may be misleading [38]. Therefore, again the interpretation of the results may have some flaws. Nonetheless, there were two additional success stories in the recent literature where free nucleic acids were used. D'Alessandro-Gabazza et al. used i.t. delivery of 5 mg/kg siRNA against TGF-β1 or a scrambled control on days 3, 7, and 14 after induction of pulmonary fibrosis using bleomycin in mice with transgenic expression of human TGF-β1 in their lungs. They reported that siRNAs with sequences common to human and rodents significantly inhibited pulmonary fibrosis in mice and efficiently knocked down TGF-β1 expression in human cell lines [39]. And Hama et al. developed a needle-free jet injection for anti-C7orf24 siRNA which was injected to subcutaneous EBC-1 tumors in mice. The treatment significantly reduced the C7orf24 protein levels in the tumor tissue as assessed by immunohistochemistry (IHC), and additionally caused cytotoxicity in the tumors as shown by cell apoptosis/necrosis measured via flow cytometry [40].

3. Nanocarrier chemistry

While novel nucleic acids are being designed to circumvent the need of nanocarriers, unmodified synthetic siRNA is fast degraded and excreted upon *in vivo* administration. Additionally, despite the comparably easy accessibility of the lung, successful intracellular delivery requires the overcoming of many extracellular and intracellular barriers which we have discussed recently [10]. The main extracellular and cellular hurdles for pulmonary siRNA delivery are shown in Figure 1. Therefore, viral and non-viral carriers are urgently needed to efficiently deliver siRNA or other RNAi-mediating nucleic acids. And although viral vectors bear advantages in regards to cellular delivery of nucleic acids, their safety concerning toxicity, immunogenicity, tumorigenicity as well as uncontrolled virus replication [41] and their reproducible manufacturing are challenges for clinical applications. In preclinical settings, however, where new targets are identified, adeno viruses [42-44] and lenti viruses [45-48] have still found wide-spread applications in the last years.

Non-viral vectors offer the advantages of chemical modifications and tailoring to the needs of advanced siRNA delivery. Many cellular and intracellular barriers, that viruses overcome naturally, need to be considered, however, when engineering new nanomaterials. These materials are supposed to be biocompatible but also facilitate cellular internalization, escape from the endosome and the release of the siRNA to the cytoplasm [49, 50]. To understand how all these parameters can be met, a detailed characterization of existing and new nucleic acid nanocarriers concerning size, chemistry, surface charge, shape, biocompatibility, and their efficacy is necessary. If aerosols or dry powders for inhalation are formulated, additional requirements such as the compatibility with excipients, for example propellants or lyoprotectants [51-53], need to be factored in.

For the formulation with nanocarriers, unmodified as well as modified nucleic acids as described above can be used. The additional encapsulation with a nanocarrier can be advantageous for protecting the payload. Nevertheless, several reports in the last years described pulmonary administration of free nucleic acids for RNAi in the lung.

3.1. Lipids and liposomes

Despite of the successes with pulmonary delivery of free siRNA, most authors of the recent publications chose to formulate the nucleic acids into nanoparticles. Some made use of commercially available transfection reagents which are based on cationic lipids, such as Lipofectamine2000 (LF2000) [54-62], Lipofectamine RNAiMAX [63] or HiPerfect [64]. Many of the studies, in which these reagents are used, are functional genomics studies in which novel targets for the treatment of lung cancer [54, 55, 57-62, 64] or other diseases [56, 63] are identified. Others made lipoplexes with phospholipids and cholesterol of about 100 nm in size that showed higher uptake in lung cancer cells than LF2000 but decreased cell viability [65], with oxime ether lipids that are 150-220 nm in size and transfect in the presence of serum at higher levels than commonly used liposome formulations [66], or with ethylphosphocholine of about 200 nm in size that were administered intratracheally. Shim et al. administered 0.21 mg/kg myeloid cell leukemia sequence 1 (Mcl1)-specific siRNA using a microsprayer four times every other day starting 5 days after the i.v. injection of B16 or Lewis Lung Carcinoma (LLC) cells for the development of lung metastases. On day 15, the animals were sacrificed, and qPCR and Western Blots were performed to detect the Mcl1 knockdown. The best formulation achieved a reduction of lung metastases and significant gene knockdown, especially in B16 metastases, but also in LLC metastases [67]. Most lipoplexes, on the other hand were described to be administered by i.v. injection. One example is the DACC formulation by Silence Therapeutics which is made of their previous AtuFECT01 lipoplexes, cholesterol, and mPEG2000-DSPE. In their recent publication, they describe the knockdown of CD31, Tie-2, VE-cadherin or BMP-R-2 in the pulmonary endothelium after systemic administration. Therapeutic knockdown of CD31 led to reduced lung metastases and an increased life span in an LLC metastasis model similar to the one described above [68]. Similarly, “Staramin” lipoplexes also accumulated in pulmonary endothelial cells after i.v. injection. The lipopolyamine “Staramine” is a PEGylated, C18-modified triethanolamine. These lipoplexes were about 100 nm in size and showed target gene knockdown after i.v. administration of 40 μg siRNA per animal in the lungs of normal mice. The authors observed much lower knockdown in the liver, spleen, and kidneys due to

reduced clearance from the lung. However, one may ask if this lung accumulation and slow clearance could be a result of microemboli in the lung capillaries. Blood count and serum chemistry analyses of the treated animals proved minimal toxicity, reflected in an increase of granulocyte colony-stimulating factor upon treatment with mPEG550-modified Staramine nanocomplexes. A slight increase of the neutrophil count was observed, but no microscopic signs of acute inflammation in H&E stained tissue sections at the time of sacrifice were found. However, the described exclusive transfection in endothelial cells could explain the accumulation of the lipoplexes in the lung. To determine the biodistribution, the authors made use of a stem-loop qPCR protocol, which is a sophisticated, label-free method but is not able to detect degraded siRNA in contrast to label-based methods that may overestimate the presence of siRNA in tissues due to detecting the label only. While the GFP knockdown was only modest in the lung, CD31 was knocked down by 70%, which reflects the passive targeting of endothelial cells [69]. While the staramine lioplexes were PEGylated, Schlegel et al. decided to PEGylate half of their formulations and to decrease the surface charge of their 2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecyl carbamoyl methyl-acetamide (DMAPAP) lipoplexes by adding biodegradable anionic polymers of varying nature, namely alginic acid (AA), poly-L-glutamic, acid (PG), dextran sulfate (DS), polyacrylic acid (PAA), heparan sulfate (HS), sodium carboxymethyl cellulose (CMC) and hyaluronic acid (HA). They showed that PEGylated formulations did not accumulate in the lung, whereas about 10% of the lipids and varying amounts of the siRNA were extracted and recovered from the lung after retro-orbital injection. However, the authors did not show *in vivo* gene knockdown [70]. Other cationic liposomes that passively accumulated in the lung after i.v. injection were described by Bardita et al. [71, 72]. In their earlier paper, they introduced Intersectin-1s (ITSN-1s) as a general endocytic protein which is involved in regulating lung vascular permeability and endothelial cells (ECs) survival. They knocked down ITSN-1s expression in mice by repeated delivery of a specific siRNA targeting ITSN-1. Thus, they showed that 3 days post-siRNA treatment significant ECs apoptosis and lung injury occurred and that a week later, caspase-3 activity peaked [71] and confirmed in their later paper that ITSN-1s plays an important role in the function of lung endothelial cells and homeostasis [72]. Also, liposomes made of DOTAP, DOPE, and PEG via a hydration of a freeze-dried matrix (HFDM) method which were 190 nm in size were found to reach the lung, however, accumulated in both endothelial and epithelial cells. While one could argue that the uptake of the DiR loaded liposomes measured by flow cytometry could have been misinterpreted after dye leakage and uptake into the epithelium, the authors confirmed gene knockdown by PCR after magnetic bead isolation of CD146 (endothelial) and CD326 (epithelial) positive cells. Unfortunately, the authors only used two animals per group which is the only weak part of this publication [73]. Similarly to the controversial reports about the efficacy of delivering free nucleic acids to the lung, there are also reports about lipid particles that do not passively accumulate in the lung. One example was described by Shi et al. who used immunofluorescence (IF) staining and quantitative polymerase chain reaction (qPCR) to detect the biodistribution and kinetics of lipid nanoparticles (LNP) but only found marginal accumulation and no knockdown of Sjogren syndrome antigen B (Ssb) in the lung 0.5 or 24 h after injection [74]. Therefore, and in order to guide their lipoplexes to the target, Wang et al. made magnetic lipoplexes that they used for *in vitro* and *in vivo* liposomal magnetofection. They chose Type1 insulin-like growth

factor receptor (IGF-1R) as a target, which is frequently overexpressed in lung cancer and designed shRNAs targeting IGF-1R. To establish a lung tumor model, the authors subcutaneously (s.c.) injected 5×10^6 A549 cells in nude mice and placed a Nd–Fe–B magnet (400 mT) onto the subcutaneous tumor surface throughout the infusion of the magnetic lipoplexes for 1 min and an additional 14 min following the injection. Mice received 50 μg shRNA against IGF-1R, but no control shRNA was used in the experiments. Tumor accumulation was significantly higher after magnetofection compared to treatment with LF, and up to 72% gene knockdown was detected by Western Blot [75]. In a H292 s.c. xenograft model, passive liposome accumulation was exploited for the co-delivery of shRNA against survivin and to enhance cisplatin sensitivity. The authors reported tumor volume reduction by more than 80%, which was superior to the treatment with either shRNA or cisplatin, and inhibition of angiogenesis and tumor cell proliferation, as well as induction of apoptosis [76]. In the recent literature, only two examples of lipid based formulations were described for local delivery to the lung. One of the reasons why lipoplexes and liposomes are most commonly administered i.v. is that they often undergo structural changes that may lead to premature release of siRNA when aerosolized [77, 78]. One successful approach of intranasal delivery of liposomes was described by Clark et al. They developed novel siRNA sequences against the alpha subunit of the epithelial sodium channel (ENaC) and evaluated them *in vitro* in A549 cells by Western Blot, in human primary bronchial epithelial cells from nonsmokers by PCR, and in human primary nasal epithelial cells by transepithelial short-circuit current measurements. After intranasal administration of the liposomes, the authors found significant ENaC knockdown but no effects on GAPDH or 2′,5′-oligoadenylate synthetase 1 (OAS1). To ensure that the measured effects were RNAi-mediated, they also confirmed that there were no signs of TLR3, 8, and 9 activation [79]. Mainelis et al. went a step further and aerosolized their liposomes made of egg phosphatidylcholine:1,2-dipalmitoyl- *sn*-glycero-3-phosphatidylcholine:cholesterol for the delivery of DOX and ASO or siRNA against multidrug resistance-associated protein 1 (MRP1) and also evaluated the output of a small animal nose-only exposure chamber. They characterized the particles after aerosolization and described that the mean size of 130 nm of the liposomal particles did not change significantly during the continuous 60-min aerosolization. They also determined that 1.4% of the nebulized mass was available for inhalation at each port, and that despite the amount of drug delivered by inhalation being lower than the injected amount, the lung retention was higher. For the lung tumor model, the authors administered luciferase expressing A549-luc cells i.t. and detected the progression of the tumor growth by luminescence imaging. They did not only assess the retention of DOX and FITC labeled ASO by whole body fluorescence imaging with an IVIS system, but also the effect on tumor size after inhalation vs. injection 4 weeks after cancer cell instillation. Their protocol involved treatments on days 0, 3, 7, 11, 14, 17, 21, and 24. After this treatment regimen, the authors found that tumor volumes were reduced by 90% after inhalation of the ASO/DOX combination but only by 40% after i.v. injection of DOX. Unfortunately, the authors did not include an i.v. control with the ASO/DOX combination [80].

3.2. Polymers

Another large group of non-viral nanocarriers for RNAi are polymer-based delivery vectors. These nanocarriers are comparably cheap in their production, can easily be chemically modified to incorporate desirable characteristics such as biodegradability and cell- specific targeting effects [81-83] or to avoid the activation of the immune system or mutagenicity [84]. This class of vectors can be further subdivided into polycations that electrostatically self-assemble with negatively charged nucleic acids to form so-called polyplexes and polymeric solid nanoparticles which encapsulate their load. Although the toxicity generated from the charge of polycations is considered the main concern with the use of the latter, polyethylenimine (PEI) and chitosan as well as other positively charged biopolymers are still widely used for RNAi in the lung. Additionally, several new synthetic polyamines and block copolymers have been prepared and optimized and will be reviewed in this section.

3.2.1. Chitosan and other biopolymers—Chitosan is a natural polysaccharide that is generally accepted as biocompatible and biodegradable [85]. However, its poor water solubility and low transfection efficiency are challenges that need to be addressed in the optimization of advanced siRNA delivery systems. Several modifications have been introduced that will be discussed below. In a study by Okuda at al., the authors focused on the actual development of an inhalable chitosan/siRNA dry powder and used unmodified chitosan. The dry powder was prepared via a supercritical CO_2 technique and manual grinding. The biodistribution of Cy5.5 labeled siRNA was recorded after i.t. administration of either the dry powder, the nanoparticle suspension, or of free siRNA. A lung metastasis model was established by i.v. injection of colon26/Luc cells that metastasized in the lung within 9-14 days after inoculation. The silencing efficiency of the delivered siRNA was measured by luciferase knockdown in the lung metastases. The ground dry powder particles had the best knockdown efficiency although they seemed to be cleared from the lung faster than the nanoparticle suspension. The hydrodynamic diameters and zeta potentials of the particles were shown not to be affected by the steps involved in the powder formation [86]. Obviously, the supercritical antisolvent method chosen for the preparation of the dry powder, in which the solvents water, ethanol and CO_2 are mixed in a compressed column (35°C, 25 MPa), is suitable for precipitation of powders that can be reconstituted without loss of activity of the siRNA or significant effects on the physico-chemical parameters of the nanoparticles. This study needs to be highlighted as one of the few reports in the literature that describes the formulation of a dry powder containing siRNA which could potentially be administered to patients. To further optimize the properties of chitosan as siRNA carrier, Luo et al. synthesized guanidinylated chitosan (GCS). To target their polyplexes to the smooth muscle cells in the lung for potential applications in asthma or COPD, they also synthesized salbutamol-modified chitosan that was subsequently guanidinylated. To test the efficacy *in vivo*, the authors nebulized their formulations with an Aeroneb Pro device first and then administered the collected condensate i.t. with a PennCentury microsprayer to EGFP expressing mice. The animals were treated with 5 μg of siRNA each day for 3 consecutive days, and *in vivo* knockdown was quantified by confocal laser scanning microscopy (CLSM) of tissue sections and by Western Blot to confirm 40% gene knockdown with the targeted formulation [87]. Unfortunately, the authors did not include negative control siRNA in the *in vivo* study and did only check effects of negative control

siRNA *in vitro* with the guanidinylated chitosan but not with the salbutamol-modified polymer. These additional controls would be very helpful for the interpretation of the results, however. Another modified chitosan that mediated nucleic acid delivery to the lung was described by Park et al. who prepared O-carboxymethyl chitosan-graft-branched polyethylenimine (OCMPEI). Their copolymer was tested for *in vitro* gene silencing with siRNA and *in vivo* gene delivery with plasmid DNA. The polyplexes were injected i.v. and mediated excellent GFP expression in the lung [88]. Thus, it is possible that this delivery system may as well be successful for siRNA delivery to the lung after i.v. or even local administration.

A different biopolymer that is used for nucleic acid delivery and that has received attraction for pulmonary delivery is atelocollagen, a highly purified and pepsin-treated type I collagen obtained from calf dermis. Liu et al. formed polyplexes between the protein and siRNA by mixing and incubating for 16 hours before they were administered i.t. to Wistar or EGFP expressing rats. As a therapeutic gene, syntaxin4, one of the soluble membrane N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins was chosen as a novel target that regulates the function of mucosal-type mast cells. After it was shown that syntaxin4 knockdown with siRNA significantly decreases the release of granule contents from rat mucosal mast cells, rats were sensitized and challenged with ovalbumin (OVA) to establish symptoms of asthma. The rats were then treated i.t. with siRNA/atelocollagen complexes once a day for 5 days which decreased the levels of rat mast cell protease-II (RMCPII) in the bronchoalveolar lavage fluid (BALF), and asthmatic airway constriction was prevented. The authors therefore concluded that syntaxin4 knockdown stabilizes mucosal mast cells [89].

Similarly, cationic bovine serum albumin (CBSA) can be used as well to form polyelectrolyte complexes with siRNA. Han et al. injected their polyplexes of about 250 nm in size i.v. to the widely used B16-F10 melanoma/lung metastasis model and observed particles accumulation in the lung as determined by fluorescence imaging and RT-PCR due to aggregation in presence of serum. Free siRNA or LF2000 formulated siRNA, did not accumulate in the lung. Compared to free siRNA, the CBSA/siRNA polyplexes mediated significant knockdown of the target gene Bcl2 on the protein and mRNA levels, and the reduction of lung metastases was shown histologically [90]. Therapeutic effects of LF2000/siRNA formulations *in vivo* were not shown. It is not surprising, however, that the rather large polyplexes further aggregated and as a consequence accumulated in the lung. These results are in line with our investigations of polyplex stability [12] and aggregation [91] post i.v. injection. Although we found that the environment of the lung is more conducive for siRNA delivery with polyplexes, we were also concerned with the stability in the presence of mucus and surfactant [92]. In a similar approach, De Backer et al. tested dextran nanogels in the presence of pulmonary surfactants and showed that the cellular uptake of the dextran nanogel particles was decreased, but gene silencing was maintained in contrast to LF RNAiMAX [36]. This finding corroborates early findings by Rosenecker et al. who described better stability of polyplexes compared to lipoplexes in BALF [93].

Another biopolymer that is widely used for the delivery of nucleic acids is spermine. Spermines are naturally-occurring, small linear tetraamines with two primary amines and

two secondary amines and are considered safe. They endogenously aid in packaging cellular DNA into a compact state which is artificially exploited in nucleic acid delivery [94-98]. However, unmodified spermine does not efficiently compact siRNA, which may be due to the rigidity of the rather short molecules [99]. Therefore, we have recently investigated oligospermines for siRNA delivery to lung cancer cells and have showed that a minimum length of 4 spermines in a linear assembly is necessary for efficient intracellular siRNA delivery [100]. Others have prepared poly(amino ester) (PAE)-based spermine conjugates where the amines of spermines were reacted with the acrylate functionalities of glycerol propoxylate triacrylate (GPT) [101-103]. Jiang et al., for example, formed polyplexes with glycerol propoxylate triacrylate-spermine (GPTSPE) and shRNA that were around 160 nm in size and had a slightly positive zeta potential of +9 mV. They used a $Kras^{LA1}$ model to mimic human NSCLC and administered 8 mg of GPT–SPE and 0.8 mg of shRNA (scrambled or Akt1 shRNA) twice a week for 4 weeks as aerosol. The authors did not determine how much of the aerosolized dose actually reached the lung. However, they ran a proof of principle experiment in which they aerosolized a plasmid encoding GFP which successfully expressed the gene *in vivo* without microscopic signs of inflammation in H&E stained tissue sections. Additionally, the authors characterized the polyplexes before nebulization but did not determine if that process had effects on their physico-chemical characteristics. In the therapeutic model, delivery of shRNA against Akt1 with GPT-SPE mediated reduction of lung tumors and 60% Akt1 knockdown as measured by Western Blot. The authors did not observe significant changes in blood cell counts [101]. However, based on our experience with nanotoxicology, other levels of biocompatibility, such as the release of cytokines [92], the activation of the complement system [104], and genotoxicity [84] are factors that need to be evaluated before a nanocarrier can be considered safe. In a paper by Hong et al, the same group used GPT-SPE to deliver shRNA against the same target in the same mouse model. They used the same nose-only exposure chamber but this time delivered only 0.4 mg DNA (scramble or Akt1 shRNA) twice a week for 4 weeks. The authors confirmed that aerosol delivery of shRNA against Akt1 suppressed lung tumor growth and now focused further on the mechanism of action. They reported that apoptosis was induced in the neoplastic lung lesions through cell cycle arrest, as measured by a significant decrease of Cyclin B1 protein levels. They also saw a reduction in metalloproteinase-9 activity, a suppression of the expression levels of proliferating cell nuclear antigen (PCNA), vascular endothelial growth factors, CD31, Akt1 and Bcl-xL as well as an increase of Bax in Western Blots [102]. Another year later, the same group reported the use of their GTE-SPE polymer for siRNA delivery via nose-only inhalation. The authors chose sodium-dependent phosphate co-transporter 2b (NPT2b) as a target gene as it was shown to be overexpressed in $Kras^{LA1}$ mice. This time, they treated the animals with 0.5 mg of siRNA twice a week for four weeks and observed similar results as with the Akt1 knockdown. Aerosol-delivered siNPT2b decreased NPT2b expression levels as detected via Western blotting, densitometric analysis and qPCR, and significantly increased the levels of the pro-apoptotic proteins BAX and BAD. TUNEL positive cells confirmed the activation of apoptosis, and PCNA as well as VEGF levels were decreased as seen after knockdown of Akt1 [103].

3.2.2. Poly-methacrylates—One of the fully synthetic methacrylate based polymers that is now also used for pulmonary siRNA delivery is poly-dimethylaminoethyl methacrylate

(pDMAEMA). Boyer et al. synthesized biodegradable, well-defined star polymers based on pDMAEMA via reversible addition–fragmentation transfer polymerization (RAFT) with 4-cyanopentanoic acid dithiobenzoate as the RAFT agent and 2,2′-azobisisobutyronitrile (AIBN) as initiator. The linear RAFT polymers were then chain extended in the presence of the crosslinker N,N-bis(acryloyl)cystamine, AIBN, and DMAEMA. Polyplexes were formed by electrostatic interaction with siRNA, and sizes below 100 nm were measured by TEM and dynamic light scattering (DLS). A lung tumor xenograft was obtained by injecting GFP-expressing H460 NSCLC cells subcutaneously into the flank. The polyplexes were administered by intratumoral injection and mediated 50% *in vivo* GFP silencing on the mRNA level [105]. Other polymers synthesized by living polymerization were described by Yu et al. [106, 107]. They synthesized poly(2-(diisopropylamino)ethyl methacrylate) (PDMA-b-PDPA) di-block copolymers by atom transfer radical polymerization (ATRP) and loaded micelleplexes with siRNA and PTX using a solvent displacement technique. In their first paper, they showed that the co-delivery of siRNA against Bcl-2 was able to sensitize A549-Bcl-2 cells to PTX via down-regulation of the anti-apoptotic gene. The authors concluded that PDMA-b-PDPA micelleplexes are promising nanovectors for the combination delivery of siRNA and anti-cancer drugs to overcome chemoresistance in cancer [106]. However, for their *in vivo* studies, they synthesized a new pH-responsive diblock copolymer by ATPR, namely poly(methacryloyloxy ethyl phosphorylcholine)-block-poly(diisopropanolamine ethyl methacrylate) (PMPC-b-PDPA) that electrostatically interacted with siRNA to form polyplexes of 50 nm in size. Their anti-lung cancer efficacy was tested in an s.c. H2009 xenograft model in nude mice after the authors showed *in vitro* that delivery of siRNA against MDM2 with their polyplexes induced significant cell cycle arrest, apoptosis and growth inhibition in p53 mutant NSCLC H2009 cells through the up-regulation of p21 and the activation of caspase-3. Ten days after inoculating the cells, mice were injected i.v. every other day for 12 days with polyplexes containing 0.32 siRNA mg/kg. These repeated injections efficiently inhibited the growth of the H2009 xenografts through MDM2 knockdown as shown in IHC and also decreased luciferase expression in a xenograft model of luciferase expressing A549 cells [107].

3.2.3. Polyethylenimine and derivatives—The polycation that still enjoys most popularity for siRNA delivery to the lung is PEI. Since 2011, a total of ten publications have described nucleic acid delivery to the lung with unmodified PEI [108-110] or the development of copolymers that are expected to have better biocompatibility and targeting characteristics [88, 111-116]. PEI is often used in proof-of-principle studies or set-ups where a method is optimized. In 2013, we described a new method, for example, for detecting which cell types in the lung are transfected with siRNA polyplexes and show gene knockdown. We used a reporter gene model of Enhanced Green Fluorescent Protein (EGFP) expressing mice and developed a flow cytometry based technique to gate different cell types of the lung based on their antigen expression [108]. Although branched 25 kDa PEI did not achieve very strong knockdown in most of the cell types, the method can be used to screen various other nanocarriers. In a similar approach, linear PEI was used to identify a new target in a lung cancer model in combination with modified RNA. Kedinger et al. designed so-called “sticky siRNAs” (ssiRNAs) that polymerize via their complementary 3′ overhangs. Thus, complexation of the longer and less rigid RNA strand with PEI is facilitated, and the

evolving polyplexes are more stable than usually obtained siRNA/PEI complexes [12, 92]. The authors evaluated this new siRNA/PEI formulation in a model of lung metastases which were obtained after i.v. injection of B16-F10 cells. Polyplexes with 1 mg/kg ssiRNA at a charge ratio of N/P 8 (8 PEI nitrogens per siRNA phosphate) were injected i.v. on a daily basis for one week. The injection of siRNA against either survivin or cyclin B1 reduced the tumor growth of the primary melanoma tumor. But also the lung weight was reduced, reflecting the weight and progression of the lung metastases. Additionally, a significant reduction in melanocyte specific transcription factor was observed after systemic injection of either anti survivin or anti cyclin B1 siRNA. The authors describe that the sticky siRNAs can be delivered more efficiently to tumors and metastases [109]. The same group used a very similar approach of formulating ssiRNAs against survivin and cyclin B1 with linear PEI in a combination treatment with cisplatin. They were able to inhibit the target genes at the mRNA and protein levels, but also blocked the cell cycle and cell proliferation which had synergistic effects in combination with cisplatin. In this model, lung metastases were obtained after i.v. injection of luciferase expressing mammary tumor TSA-Luc cells. The treatment with RNA started 2 days after injection of the tumor cells and was repeated for a total of two injections per week. The cisplatin treatment started on day three after the tumor cell injection and was continued once a week until day 10 after tumor cell injection. The inhibition of lung metastasis progression was recorded by whole body luminescence imaging, and the number of metastases was counted in histological sections. The authors found significantly decreased luminescence after injection of luciferin in animals treated with ssiRNA against survivin, but an even more pronounced effect in the group that received ssiRNA and cisplatin. These results were reflected in the Kaplan–Meier-plots of animal survival [110]. Steele et al. did not modify the RNA but used an oligoethylenimine (OEI) and a PEGylated version of OEI instead of PEI for siRNA delivery *in vitro*. In an approach to optimize their formulations for pulmonary delivery, the effects of the presence of lung surfactants and/or non-ionic surfactants and of nebulizing the formulation were determined. The authors found that covalent PEGylation but also blending the polymer with PEG resulted in colloidal stability in isotonic saline buffers, and that formulations with the commercial non-ionic surfactant Pluronic™ P68 yielded the smallest particles of 140 nm which were stable in isotonic glucose solution. Formulations with the lung-derived surfactant Alveofact™ showed colloidal stability in glucose solution also and achieved transfection efficiencies comparable to the Pluronic formulations and better than those obtained with the plain OEI-HD/siRNA polyplexes. To address the shelf-live problem of polyplexes, the two components (polymer and siRNA) were flash frozen and lyophilized as separate droplets in the same reaction tube and rapidly formed polyplexes upon reconstitution. This formulation step did not have any adverse effects on the polyplex particle size or transfection efficiency, since similar hydrodynamic diameters compared to fresh polyplexes were obtained with particle sizes of 210 ± 30 nm and 200 ± 20 nm. Also the knockdown efficiency of up to 90% was maintained [117]. In 2009, we had reported that PEGylation of PEI has a beneficial impact on polyplex stability in the lung [92], which can be attributed to improved colloidal stability of the polyplexes, as described by Steele et al. Similarly, Hibbits et al. reported that PEGylated PEI/siRNA polyplexes were more efficient in facilitating cellular uptake and luciferase knockdown in Calu-3 cells than PEI/siRNA complexes, as measured in a high-content analysis (HCA) [112]. Shen et al. used a

combination of polymers similar to the ones reported by Steele et al. They covalently coupled PEI and Pluronic™ P68 to combine the nucleic acids complexing properties of the former and the micelle formation ability of the latter. Additionally, they added D-a-tocopheryl polyethylene glycol 1000 succinate (TPGS) to the formulation which is supposed to act as solubilizer and absorption enhancer. Micelles encapsulating paclitaxel (PTX) were formed by thin-film hydration, and shRNA against survivin (shSur) was complexed afterwards with the cationic portion of the micelles. The particle size was below 200 nm for this P85-PEI/TPGS/PTX/shSur (PTPNs) nanoparticle formulation. In a s.c. tumor model using A549T cells, PTPNs with 10 mg/kg PTX and 2 mg/kg shSur were injected every 4 days for 4 times and their therapeutic effect was compared to groups of animals that were treated with saline, only taxol, only micelles encapsulating shSur, taxol and micelles encapsulating shSur, or only micelles encapsulating PTX. Combining taxol with the shRNA micelles led to reduced tumor volumes compared to the single therapies. Interestingly, encapsulating PTX in micelles was as efficient in regards to tumor growth inhibition as the combination therapy. However, complexing shRNA with the PTX encapsulating micelles had an even stronger inhibitory effect. It was concluded that the combination therapy lowered the threshold taxol concentration for PTX-induced apoptosis, and that the encapsulation of PTX in micelles increased their tumor uptake [114]. These results can be easily understood in the context of improving the bioavailability of PTX by encapsulation. The control groups which are missing in this study, however, are groups of a scrambled shRNA sequence with and without PTX. In the paper by Choi et al., a conjugate of dexamethasone and polyethylenimine (DEXA-PEI) was synthesized to target the polyplexes toward the nucleus via interaction with the glucocorticoid receptor. This concept had been successfully exploited before [118, 119] but was now applied in a model of acute lung inflammation (ALI) induced by the instillation with baked SiO_2 nanoparticles. The authors complexed siRNA against macrophage migration inhibitory factor (MIF) with DEXA-PEI and obtained particles of about 355 nm in size, whereas PEI/siRNA complexes aggregated to over 600 nm. These particles were administered i.t. with either 2.5 μg or 10 μg siRNA per animal and decreased the extent of the pulmonary inflammation, of MIF mRNA and of Muc5ac expression, as measured by IHC, after treatment with conjugated and non-conjugated PEI complexes 72 h after polyplex administration. However, at the same siRNA concentration, the DEXAPEI/MIF siRNA complexes contributed more efficiently to the reparative lung response [111]. A different PEI conjugate used for siRNA to the lung was synthesized by Ganesh et al. who attached hyaluronic acid (HA) to PEI and mixed it with PEG functionalized lipids. HA is often used for targeting the CD44 receptor, which is overexpressed on several tumor cell surfaces. Their particles were below 100 nm and were injected i.v. with 0.5 mg/kg siRNA for 3 days after establishing a lung metastases model with i.v. injected B16 melanoma cells. The siRNA was directed against Polo-like-Kinase 1 (PLK1) and achieved about 55% target gene knockdown in the primary tumors but only 25% knockdown in the metastatic lesions in the lung [120]. Since the origin of lung metastases is an endothelial one, it is not clear if local delivery through the lung could have achieved an equivalent of more efficient targeting and gene knockdown in the lung lesions.

3.2.4. Poly-L-lactic-co-glycolic acid—In contrast to polyplexes, solid polymeric nanoparticles, on the other hand, encapsulate siRNA in a polymer matrix. Poly-L-lactic-co-

glycolic acid (PLGA) and other biodegradable polymers are used for siRNA encapsulation in such nanoparticles. However, in case of solid nanoparticles, siRNA is often precomplexed with a polycation to increase the encapsulation efficiency. Das et al., for example, described the pre-formulation of PEI/siRNA polyplexes in presence of 1% poly vinylalcohol (PVA) and the encapsulation of these polyplexes in PLGA using a solvent displacement technique. In this method, PLGA is dissolved in a water-miscible solvent, here acetone, and an aqueous stabilizer solution, here 1% pluronic F68, is added drop wise before the polyplexes are added drop wise also. The solvent is then evaporated by stirring the nanosuspension, and the PLGA nanoparticles precipitate in the aqueous environment. The particles obtained were found to be 145 nm in size with a polydispersity index (PDI) of 0.131, which is comparably low considering that polyplexes without further encapsulation tend to aggregate and thus exist as rather polydisperse suspensions [12]. To assess their siRNA particles *in vivo*, the authors force-fed balb/c mice with 25 mg benzo[a]pyrene (BaP) and 10 mg sodium arsenite (SA) per kg body weight twice a week for 4 weeks and induced lung cancer at week 16. Consequently, the nanoparticles or free siRNA against signal transducer and activator of transcription 3 (STAT3) were administered intraperitoneally (i.p.) at either 0.5 or 1 mg/kg body weight three times a week for 30 days. The authors chose STAT3 as a target as it is frequently constitutively activated in lung cancer and in turn upregulates bcl-xl, bcl2, cyclin D1, c-myc, and mcl-1, promoting cell proliferation and angiogenesis. The treatment of mice with anti-STAT3 siRNA nanoparticles, however, reduced the number of STAT3 positive cells in the lung as determined by IHC and the expression of caspase 9 as measured by immunoblot. The mRNA levels of Cyclin D1, VEGF, and IL6 were significantly downregulated *in vivo*, whereas caspase 3 was upregulated, which was interpreted as an activation of intrinsic apoptosis of the lung cancer cells. Das et al. concluded that their nanoparticles are a promising carrier for the delivery of siRNA targeting STAT3 which was reflected in the regression of tumor growth in balb/c mice [115]. The possible toxicity of PEI [104, 121] which may be shielded by encapsulation in PLGA was not differentially assessed, however. And it is possible that some of the apoptosis-mediating effects may stem from the carrier rather than from the siRNA. Su et al. on the other hand formed PEI-coated PLGA nanoparticles that were loaded with PTX (PLGA-PEI-TAX). The PTX loading was performed using a modified solvent displacement technique, and PEI was coated my electrostatic interaction. Subsequently, siRNA against STAT3 was added to electrostatically bind to the PEI surface. The nanoparticles were around 250 nm in size and were delivered to A549 cells and A549-derived PTX resistant cells *in vitro*. The combination therapy nanoparticles with siRNA induced stronger cellular apoptosis than the PTX loaded nanoparticles as measured in annexin-V assays. The effect of the combination therapy was additionally determined in microtubule assembly assays, and their toxicity was assessed in MTT assays. The uptake of PTX and siRNA was confirmed by CLSM, which revealed prolonged uptake of PTX and siRNA [116]. These findings are in line with the ones reported by Shen et al. [114] who showed that encapsulation of PTX increased its bioavailability and who reported a synergistic effect of survivin knockdown and co-encapsulation of PTX. However, it would be interesting to determine if the PEI-coated nanoparticles described by Su et al. can efficiently deliver siRNA *in vivo* since the nucleic acid load is only electrostatically complexed to the surface of the particles. This may eventually lead to premature release of the siRNA before reaching the target cells [12]. Although polymeric

nanoparticles have been widely used to deliver nucleic acids to the lung or to lung cancer and metastases, there are only a few reports that actually optimize their inhalation delivery. One example is a paper by Jensen et al. who designed a dry powder formulation and encapsulated siRNA into DOTAP-modified PLGA nanoparticles using a double emulsion solvent evaporation method. They kept the total concentration of DOTAP and PLGA in chloroform constant and varied the weight/weight (w/w) percentage of DOTAP. Their optimal formulation contained 25% (w/w) DOTAP and achieved 74% gene silencing *in vitro*. Subsequently, they spray-dried the particles with mannitol and measured an aerodynamic size of 3.69 ± 0.18 μm, which is within the optimal size range for deep lung deposition. The sugar alcohol excipient also acted as a stabilizer during spray drying and remained in its crystalline state, as determined by X-ray powder diffraction analysis, which could explain why it prevented the nanoparticles from aggregation and coalescence. The hydrodynamic diameters and cellular uptake were comparable before and after freeze-drying, which prompted the authors to conclude that spray-drying is an excellent technique for engineering dry powder formulations of siRNA nanoparticles [122]. However, although the cellular uptake of their particles was comparable before and after spray-drying, the authors did not determine gene silencing afterwards. It would be necessary to know if the siRNA remained intact during the formulation processes. Benfer et al. used the covalently modified PLGA poly[vinyl-3-(dialkylamino) alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(D,L-lactide-co-glycolide), abbreviated as DEAPA-PVA-g-PLGA, that contains positively charged side groups for the complexation of siRNA. It was shown that the cellular uptake of as DEAPA-PVA-g-PLGA/siRNA nanoparticles in H1299 NSCLC cells could be increased after formulation with surfactant and that the particles were predominantly taken up via clathrin-mediated endocytosis [123]. In 2011, we also showed that DEAPA-PVA-g-PLGA/siRNA nanoparticles are suitable for intratracheal administration and remain in the lung for prolonged periods of time compared to PEI-formulated or free siRNA [10]. In the meantime, we optimized a freeze-drying protocol for these nanoparticles to obtain dry inhalable powders. Although particles freeze-dried in the presence of 10% glucose as lyoprotectant were about 150 nm in size after reconstitution and smaller than particles freeze-dried in presence of 10% saccharose (about 250 nm), the larger particles showed more efficient and comparable cellular uptake as freshly prepared nanoparticles. However, most importantly, it was shown that the gene knockdown effect was preserved during the freeze-drying and after reconstitution of the nanoparticles (Figure 2). Our findings are in line with an investigation by Kasper et al. who showed that concentrations of 12% and higher of lyoprotectants such as trehalose, sucrose, lactosucrose, hydroxyproxypropyl-beta-cyclodextrin, dextrose and polyvinylpyrrolidone (PVP) can prevent the aggregation of PEI/DNA polyplexes which retain their transfection efficiency after reconstitution [53].

3.3. Peptides

While many polymers used for nucleic acid delivery are polyamines and thus structurally similar to proteins, we want to focus on short peptides, such as peptide transduction domains (PTDs) and cell-penetrating peptides (CPPs) in this section. These peptides are small positively charged molecules (10-30 amino acids and usually contain arginine and lysine which provide primary and secondary amines in their side chains that can be protonated for

electrostatic interaction with siRNA and enhanced the permeability across the cell membrane. One recent example of the application of such a short peptide was published by Oh et al. who used R3V6 peptides, which were composed of 3 arginines and 6 valines. The authors formed ternary complexes with siRNA against sphingosine-1-phosphate lyase (S1PLyase), a recombinant high mobility group box-1 box A peptide (HMGB1A), and R3V6 (siS1PLyase/HMGB1A/R3V6). These ternary complexes were tested *in vitro* and delivered siRNA into non-phagocytosing LA-4 lung epithelial cells more efficiently than polyethylenimine (PEI) and Lipofectamine. The treatment reduced levels of IL-6 and TNF-α more efficiently than HMGB1A only or the delivery of siS1PLyase/R3V6 complexes in LPS activated macrophages. I.t. administration of the ternary complexes reduced the S1PLyase level efficiently in an LPS-induced balb/c ALI model. The authors also described that the mixing sequence of the three components was important. Premixture of siRNA and HMGB1A was found to be advantageous to avoid aggregate formation and to form particles below 200 nm that mediate an anti-inflammatory effect. Interestingly, the synergistic effect of co-delivery was more pronounced regarding cytokine levels in the lung lavage fluid compared to the lung tissue samples [124]. Baoum et al. used a CPP, namely the HI-virus derived motif TAT and a longer ‘double’ TAT (dTAT) for the delivery of siRNA. When they formed their complexes, the authors mixed siRNA with the polycations and then additionally added $CaCl_2$ to decrease the hydrodynamic diameters of the particles and compact the siRNA. They observed very strong accumulation of the complexes in the lung after i.v. injection and high levels of gene knockdown in the lung and muscle tissue [125]. Based on these promising results, it would be very interesting to administer these particles locally to the lung and to determine their stability in presence of mucus and surfactant. The addition of calcium has been evaluated further in combination with phosphate and lipids, as described below.

3.4. Inorganic materials

Leaf Huang’s group at the University of North Carolina started research on hybrid Lipid/Calcium/Phosphate (LCP) nanoparticles for siRNA delivery a few years ago and published a multitude of papers applying this concept since. Some of the publications focus on delivery to lung cancer and metastases. As they successfully described anisamide (AA) modification of liposomes for targeting the sigma receptor in lung cancer cells in 2008 already [126], this motive remained a central component of their LCP nanoparticles. One of their recent papers by Li et al. describes the biodistribution of PEGylated LCP nanoparticles modified with AA that were loaded with Cy5.5 labeled siRNA. After optimizing the PEGylation degree of the surface from 16 to 23%, the liver uptake decreased, and the siRNA efficiently reached the primary H460 xenograft. After i.v. injection of 0.6 mg siRNA per kg body weight, about 50% luciferase knockdown was reported in the luciferase expressing primary tumor [127]. However, it needs to be noted that this accumulation in the xenografts does not necessarily reflect the accumulation and uptake into the lung or a lung tumor. Due to the small size of the particles (40 nm) the authors hypothesized that the tumor targeting can be explained by the enhanced permeability and retention (EPR) effect [128]. The actual intracellular uptake, however, was mediated by the AA targeting that causes preferential uptake in sigma receptor overexpressing cells. In another paper from the same group, Yang et al. reported the delivery of three different siRNA sequences with AA-targeted LCP nanoparticles. The three

siRNA sequences were chosen to target the oncogenes HDM2, c-myc and VEGF for simultaneous knockdown and significant inhibition of tumor cell growth in an A549 xenograft. The authors investigated the biodistribution of Texas Red labeled siRNA and molecular effects on tumor growth and angiogenesis. They showed reduced liver uptake but enhanced tumor accumulation in the non small cell lung cancer (NSCLC) xenograft and inhibition of tumor proliferation and angiogenesis upon repeated i.v. injection of 0.2 mg siRNA per kg body weight of the pooled siRNA formulated in the targeted LCP particles. They also achieved induction of tumor apoptosis, but no comparison with single siRNA sequences was included [129]. Thus, it is not clear if downregulation of a single target would have been enough for the same effects or if a combination of two or three sequences is necessary for the observed effects. In a later paper by Yang et al., a more relevant lung metastasis model was chosen for the evaluation of the co-delivery three siRNA sequences targeting MDM2, c-myc, and VEGF. The AA targeted, PEGylated LCP nanoparticles were described to encapsulate 91% of the siRNA into particles of 40 nm in size. The authors injected B16-F10 luciferase expressing cells to obtain luciferase expressing lung metastases in which 78% luciferase knockdown was achieved after a single i.v. injection of LCP nanoparticles. The co-delivery of the three therapeutic siRNA sequences encapsulated in the LCP particles reduced the abundance of lung metastases by 70–80% at a relatively low dose of siRNA (0.36 mg/kg) and significantly induced tumor apoptosis as detected by TUNEL assays. Additionally, the mean survival time of the animals treated with the LCP formulated siRNAs was prolonged by 27.8%, and liver enzymes remained unaffected. The authors concluded therefore that their nanoparticles are well tolerated. They included control siRNA and non-targeted nanoparticles in both the luciferase model and the therapeutic model to ensure that the observed effects were RNAi and sequence specific [130]. In 2013, Zhang et al. optimized the LCP particles for co-delivery of siRNA and a chemotherapeutic drug. The authors chose VEGF siRNA and gemcitabine monophosphate (GMP) for i.v. injection as one single nanoparticle formulation for effective NSCLC treatment. The LCP particles were injected i.v. three times daily with different doses of GMP (19.5 mg/kg or 13.2 mg/kg) and/or 0.2 mg/kg siRNA against VEGF. A day after the third injection, the mice were sacrificed, and the anti-tumor effect was measured in tumor cell apoptosis, reduction of tumor cell proliferation and decrease of tumor microvessel density (MVD). VEGF levels were determined on the protein and mRNA levels by Western Blot and RT-PCR for VEGF, and cleavage of PARP by caspases, which is considered to be a hallmark of apoptosis, was used to determine tumor apoptosis. The combination therapy was especially advantageous over the GMP monotherapy based on the read out of the molecular assays such as caspase activation and tumor vessel density. But a significant difference compared to the siRNA monotherapy was observed also regarding the tumor growth [131]. In a variation, Zhang et al. reported shortly later the co-encapsulation of GMP and siRNA specific to the “undruggable” c-myc oncogene in LCP nanoparticles for combination therapy in both subcutaneous and orthotopic models of NSCLC. The s.c. model was established by s.c. injection of 5×10^6 H460 cells into the right flank 11 days before the therapy started. For the orthotopic model, 5×10^6 A549 cells in Matrigel-PBS were injected to the dorsal side of the ribcage 4 weeks before the start of the therapy. The LCP nanoparticles were subsequently daily injected i.v. for 3 days. The combination therapy mediated not only effective induction of tumor cell apoptosis but also dramatic inhibition of tumor growth with

very little *in vivo* toxicity [132]. After the Huang group has efficiently shown the accumulation in xenografts, lung metastases, and in an orthotopic model of NSCLC after systemic administration, it would be interesting to see if these LCP nanoparticles are aerosolizable, and if they are efficient or could be administered at even further reduced doses if locally applied.

Another group of inorganic nanoparticles that gains interest for siRNA delivery are gold nanoparticles. In an *in vitro* study by Huschka et al., the authors used silica cores and gold nanoshells designed in a way so that the surface plasmon resonance occurs at 800 nm, which was the laser wavelength used for excitation of the particles. The surface of the shells was then modified with a Cysteine (C)-Tyrosine (Y)-Serine (S)-poly-L-Lysine(K)50 (PLL) peptide which was capable of electrostatically complexing single stranded DNA (ssDNA) or siRNA. After transfection of GFP/RFP expressing H1299 cells, the authors illuminated half of the transfected wells for 2 minutes with a near infrared (NIR) laser and showed that siRNA or oligonucleotide cargos can be released on demand upon illumination mediating about 50% gene knockdown [133]. So far, this particular delivery system has not yet been evaluated *in vivo*. But if the biocompatibility of the particles can be ensured, this approach may be a promising one. While Huschka et al. had used the thiol group of the cysteine modified PLL to form the Au-thiol bond with the gold shells, Conde et al. described the covalent attachment of thiolated siRNA to gold nanoparticles. These particles were additionally PEGylated and modified with the Arginine-Glycine-Aspartate (RGD) targeting peptide. They were about 14 nm in size and carried siRNA against mouse c-myc. Lung tumors in mice were grown for 4 weeks after instillation of LA-4 mouse tumor cells to the lung. Tissue sections of lungs treated with gold nanoparticle-coupled siRNA were stained with antibodies against c-myc and caspase 3, and the tumor burden was additionally measured in a tumor model with luciferase expressing cells. In both models, the delivery of siRNA against c-myc reduced the tumor cell proliferation and thus the tumor size. However, no scrambled siRNA sequence was used in any of the experiments. Additionally, the authors observed a strong proinflammatory effect of the intratracheal administration of the gold nanoparticles which may, in part, have contributed to the anti-tumor effect [134].

A third group of inorganic materials that becomes more and more important for drug and gene delivery is that of mesoporous silica nanoparticles (MSN), which can be used as a core for gold spheres but can also be loaded with nucleic acids as such. However, to help the loading process, it is beneficial to impregnate the particles with a polycation that electrostatically absorbs the cargo. Lin et al, for example, used DMAEMA for the coating of the pores and crosslinked it with N,N'-(dithiodi-2,1-ethanediyl)bis(acrylamide) (BAC) to form a bioreducible polymer that complexed siRNA against Plk1. After i.v. injection of particles containing 50 μg Cy5-siRNA, the biodistribution was investigated with the Kodak Imager in a s.c. HeLa xenograft model. For therapeutic effects, 2 mg siPlk1 per kg body weight were injected i.v. every three days starting 2 weeks after the tumor inoculation. The tumor uptake with the optimized formulation was as efficient as with LF2000, and delivery of siPlk1 yielded up to 60% gene knockdown as well as reduced tumor burdens and strong lung and liver uptake [135]. The authors explained that the success of the *in vivo* knockdown can be attributed to the intracellular siRNA release due to the reduction of the crosslinked

pDMAEM polymer. However, since they didn't include a control with non-biodegradable polymer, this hypothesis was not tested. Taratula et al. modified MSNs with 3-mercaptopropyl 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. The modified pores of the MSNs were loaded with either doxorubicin or cisplatin to achieve a combination therapy of chemotherapeutics with two types of siRNA. The siRNA sequences were chosen to target MRP1 and BCL2 mRNA for suppression of pump and nonpump cellular chemoresistance. An orthotopic model of lung cancer was established as described before [136] by i.t. installation of luciferase expressing A549 cells. The tumor progression was followed as described before [80], and the particles were administered by nose-only inhalation or by i.v. injection. The accumulation of the nanoparticles in the mouse lungs was detected by whole body fluorescence imaging and revealed that inhalation administration prevented the escape of the MSNs into the systemic circulation and thus limited their accumulation in other organs [137].

A summary of non-viral vectors used in pulmonary siRNA delivery *in vivo* is presented in Table 1.

4. Administration route

As described in the previous sections, several routes can be exploited to successfully deliver siRNA to the lung. While inhalation is considered the most clinically relevant administration route, it is also the least straightforward one when it comes to formulation development. This is reflected in the few studies that actually administer siRNA by inhalation [80, 87, 101-103, 137, 138]. Several *in vitro* studies have described the optimization of dry powders [122] or aerosolizable suspensions [117] but did not administer them *in vivo*. The challenges of developing inhalable formulations are related to the processes involved during the engineering of the formulation. In case of dry powders, usually freeze-drying or spray-drying steps are needed which can cause aggregation of the particles and problems regarding the preserving of the biological and physico-chemical stability of the siRNA and particles. While dry powder inhalers (DPIs) are one type of currently available inhalation devices, metered dose inhalers (MDI) and nebulizers are the other clinically relevant types. The problems of aerosolizing a nanoparticle suspension, however, are that the formulation is exposed to severe shear forces. Additionally, preferential nebulization of the solute and adhesion of positively charged polyplexes to plastic parts of the device can strongly limit the dose of siRNA emitted through the mouthpiece, as we described earlier [10]. However, there are recent reports in the literature where the authors successfully mediated RNAi after nebulizing their suspensions in a nose-only exposure chamber as shown in Figure 3a [80, 101-103, 137, 138]. While most studies focused on the therapeutic outcome of the nucleic acid inhalation, Mainelis et al. characterized their liposomal particles before and after aersolization and showed that their hydrodynamic diameters did not change significantly due to the nebulization [80]. Luo et al. in fact nebulized their guanidinylated chitosan polyplexes with an Aeroneb Pro but collected the condensate and then administered their formulation via intratracheal aerosolization with a PennCentury Microsprayer™ [87]. Other examples of studies in which the authors administered nanoparticle suspensions by intratracheal aerosolization are the publications by Shim et al. who delivered Mcl1-specific siRNA to

lung metastases [67] and by Fujita et al. who developed novel PnkRNA constructs against RPN2 [35]. As discussed earlier [10], intratracheal aerosolization can potentially increase the biological activity of siRNA-containing nanoparticles due to a more even deposition of siRNA throughout the entire lung in alveolar and bronchiolar regions, whereas intratracheal bolus administration usually leads to a patchy distribution pattern [139]. In the past, the Micro-Mist Nebulizer™[140] and the AeroProbe™ nebulizing catheter [141] have been used also, but in the recent years, the PennCentury Microsprayer™ seems to have gained the market leadership. Since the intratracheal administration of a bolus does not require the formulation of a dry powder or an aerosolizable suspension, it is a very popular route which, however, is slightly more invasive than the others (Figure 3b). Therefore, it is not a clinically relevant administration route but routinely used in animal studies. Amongst the studies discussed here, 15 ones used the i.t. route with [35, 67, 87] or without aerosolization [31, 37, 39, 42, 86, 89, 108, 119, 124, 134, 142, 143]. Another local and less invasive route for easily accessible administration of siRNA is the application of a nasal suspension in the nasal cavity. This approach is very simple but a certain amount of the administered dose usually gets lost in the nasal cavity or by swallowing [144, 145]. The disadvantage of the intranasal (i.n.) route is the less quantitative delivery of siRNA to the lung. Nonetheless, since we last reviewed the current literature [10], two more studies described siRNA delivery to the lung after i.n. administration. While Clark et al. delivered liposomes containing siRNA against ENaC and found significant ENaC knockdown [79], Barik et al. successfully standardized a procedure for using the nose as a specific route for siRNA delivery into the lung of laboratory animals [146].

Although the aim of this review is to discuss pulmonary delivery of siRNA, we did not want to disregard a variety of recent reports that target the lung, lung cancer or metastases via the systemic route [68-76, 88, 90, 107, 110, 113, 114, 125, 127, 129-132, 135, 147, 148]. Although i.v. injection does not provide the advantages of local delivery discussed in the introduction, many reports were able to reach the lung, lung xenografts or metastases from the endothelium. While accumulation of nanoparticles in the lung after systemic administration can be a sign of a “first-pass” effect of aggregated particles that are retained in the lung capillaries [149], lung metastases are expected to have an endothelial origin and therefore may be better accessible from the blood than from the air side. Therefore, the administration route may have to be carefully chosen based on the therapeutic application.

5. Disease models

Lung diseases are currently amongst the top ten causes of death worldwide according to the WHO [150]. Besides the more lethal lung diseases such as lung cancer, tuberculosis, COPD, cystic fibrosis, and lower respiratory tract infections, other diseases such as uncomplicated virus infections and asthma can also strongly affect the quality of life of patients. Therefore, intensive research is currently underway to address the shortcomings of current therapies. Reviewing the recent literature on RNAi in the lung (Figure 4), lung cancer (61%) and metastases (8%) are the main targets of the research conducted during the last years. While Das et al. force-fed balb/c mice with BaP and SA to provoke the development of lung cancer [115], others have used a $Kras^{LA1}$ model to mimic human NSCLC [101-103]. Besides these two models that seem more translationally relevant, most publications describe the use of

xenograft models where for example A549 NSCLC cells [35, 48, 75, 114, 120, 129], H460 cells [105, 127, 131, 132], H2009 cells [107], EBC-1 cells [40], H358 cells [48] or H292 cells [76] are injected s.c. Alternatives are the injection of A549 cells in matrigel into the dorsal side of ribcage for an orthotopic model [131, 132] or the intratracheal administration of A549 [80, 137, 138] or LA-4 mouse tumor cells [134] to the lung, which mimics the clinical reality of lung cancer better than xenograft models. A lot of *ex vivo* work is performed on tissue samples from patients [57-60, 151]. However, these experiments do not take into account administration barriers, biodistribution, side effects or metabolism in organs other than the lung. Lung metastasis models are usually obtained by intravenous injection of cancer cells that accumulate in the lung capillaries and form metastatic lesions. The most widely used model is that of injected B16-F10 melanoma cells, which was also used in several papers discussed here [67, 90, 113, 130, 147]. Other cells used in lung metastasis models are Lewis Lung Carcinoma (LLC) cells [67], TSA mammary tumor [110], and colon26 cells [86]. Whereas many of the reports describing lung tumor models evaluated new therapy strategies such as co-deliver of siRNA and chemotherapeutics [43, 76, 80, 114, 120, 131, 137, 138] or co-delivery of siRNA sequences targeting several different genes [129, 132], a lot of the lung metastasis studies were interested in finding out if the nanoparticles reach the lung lesions after i.v. or i.t. administration [67, 86, 113].

Since the establishment of tumor and metastasis models requires very sophisticated knowledge and handling, many studies performed simpler proof-of-principle experiments in which they knocked down the expression of the reporter genes (Enhanced) Green Fluorescent Protein ((e)GFP) [63, 69, 88, 108, 123] or Luciferase [31, 117]. These modes are especially helpful for *in vitro* studies in which the performance of a carrier is determined or optimized [117, 123], or for *in vivo* experiments that are interested in determining the biodistribution [31, 69, 88, 108, 148] or optimizing the formulation [148].

ALI is another disease that is strongly researched in animal models as it can be rather easily induced by infection with lipopolysaccharide (LPS) after hemorrhage [37, 42, 119, 124, 143]. In regards to siRNA delivery, five papers are discussed here that are mostly interested in identifying novel targets that can be therapeutically downregulated to reduce the secretion of pro-inflammatory cytokines. Peptides [124], polymers [119], viruses [42], even free nucleic acids [37, 143] have been used to achieve this effect. The results obtained with free siRNA that was administered locally were controversial between different papers. But virally or non-virally delivered nucleic acids were reported to be promising treatment options.

Considering the limited therapies for patients with Cystic Fibrosis (CF), it is surprising that in the past years, only 3% of the published research on RNAi in the lung addressed this genetic disease [63, 79]. It is even more interesting that one of the two papers discussed here is the result of industrial research [79]. Apparently, CF is more attractive for industry than lung cancer or other diseases of the lung. In contrast to the many animal models available for lung cancer, rodent models for CF are rather limited and allow for example the assessment of the cystic fibrosis transmembrane conductance regulator (CFTR) functions. A recent book chapter reviewed animal models in rodents and other species that are applied in CF research and highlighted pig and ferret models as more translationally relevant [152].

These animal models have not yet been introduced to RNAi research which may be explained by the larger doses of siRNA needed for the treatment of animals larger than mice and rats. Lung Fibrosis, on the other hand, can be easily induced by the treatment with bleomycin [56], and lung inflammation and remodeling can be achieved, for example, by knocking down Intersectin-1s (ITSN-1s) [64]. In line with lung inflammation and remodeling research are several models for allergic asthma which can be induced by sensitizing animals with ovalbumin (OVA) [153], house dust mite extracts, or pollen [154]. Interestingly, only one *in vivo* [89] and one *ex vivo* [33] paper were published recently on RNAi in asthma. Both examples used the OVA model to sensitize rats [89] or mice [33], although other models are considered more clinically relevant. While Liu et al. passively targeted mast cells to reduce the expression of rat mast cell protease-II (RMCPII) [89], Qiu et al. targeted T cells with a CD4 aptamer after isolating lyophocytes from sensitized mice [33]. They took on a very challenging task since primary T cells, the target cells in their paper, are difficult to transfect cells [155]. Exploiting CD4 as a gate for delivery of bacteriophage phi29 particles was successful *ex vivo* but could cause delivery to all CD4 positive cells *in vivo* and could thus mediate general immune suppression. To address this problem, we have described the differential expression of CD71 (transferrin receptor) on activated but not naïve T cells and thus targeted delivery of siRNA to activated T cells [156, 157].

Although pulmonary delivery of siRNA has been widely successful for treating viral infections and other respiratory disorders in the past [10], there has not been much progress in the last years [146]. It is not clear if the reason is that influenza, SARS and other viral infections did not make it to the news in the same extent as in the early 2000s or if some of the results published earlier are not reproducible when control sequences other than the less immunostimulatory GFP sequences used in the past [38] are incorporated in the study design. Despite the lack of reports in the literature, Respiratory Syncytial Virus (RSV) is a very important target in Alynlam's clinical trials, as discussed below.

6. Clinical translation

With the decoding of the human genome, the sequences of many genes so far believed to be "undruggable" with conventional therapeutics [158] have become commonly accessible. And the discovery of RNAi has been expected to facilitate the therapeutic knockdown of such genes. siRNA-based therapeutics are supposed to be broadly applicable and highly versatile and have the advantage that they can potentially target any given gene based on the knowledge of its sequence and secondary mRNA structure [159]. The reality of more than a decade of research on therapeutic siRNA delivery has taught us that many barriers, hurdles, and challenges need to be overcome for successful therapeutic RNAi, as described in the introduction. However, despite all odds, several siRNA-based therapeutics are currently being evaluated in clinical trials. While all of the trials on polymeric and lipid nanoparticle formulations reviewed by Khatri et al. make use of i.v. administration, the non-formulated, "naked" siRNA preparations are mostly administered locally [65]. In the context of siRNA being easily degraded by ubiquitous nucleases and being rapidly excreted upon systemic injection [11], it is understandable that they need to be formulated for more beneficial pharmacokinetics post i.v. administration. Local administration routes, such as inhalation,

intranasal administration, intraocular, intravitreal, and intradermal routes are thus preferred if “naked” siRNA is delivered. siRNA can be modified as described above, and as in the case of ALN-RSV01, Alnylam’s cholesterol-modified siRNA for RSV treatment which is currently in Phase IIb. After positive evaluation of safety studies in healthy volunteers with doses up to 150 mg daily for 5 days [160], the efficacy of ALN-RSV01 was tested in a randomized, double-blind, placebo-controlled trial in 88 adults and demonstrated effective anti-viral activity after experimental infection with RSV. However, these results may not be reflective of treatment outcomes in naturally infected patients [161]. In an additional study, safety and efficacy were therefore tested in 24 lung transplant patients with natural RSV infections. Another inhaled siRNA-based therapeutic that passed the phase I safety trial and entered a phase II efficacy trial is Excellair™ (ZaBeCor, Bala Cynwyd, PA, USA), an inhaled siRNA-based treatment of asthma. The target gene in this trial was Syk kinase which is a tyrosine kinase that plays a central role in cytokine receptor signalling. The downregulation of Syk kinase was reported to ameliorate lung inflammation not only in asthma but also acute lung injury models [162]. Although the results from the Excellair™ trial looked very promising, the current status of the trial is unknown. Currently, the clinicaltrials.gov database does not list any additional trials on siRNA delivery to the nose or lung, but a recent review on patent applications that aim to treat pulmonary disorders lists 12 publications that mainly focus on lung cancer, influenza, and asthma [65]. Since the review, additional patents have been granted, including patent US8574623 which describes liposomes carrying retinoid derivatives for targeting extracellular matrix producing cells for fibrosis therapy [163]. It can be expected, therefore, that new therapies may be translated into the clinics soon.

7. Conclusion

In the last decade, hundreds of groups all over the globe have tried to translate RNAi into therapeutics. The lab success stories especially regarding local administration have been plenty, and efficient intracellular delivery and therapeutic effects in lung cells and models of lung diseases have been described in over a hundred publications. Then why do we still struggle with the clinical translation? Are we using the wrong models? In section 5, we reviewed the animal models that have been described lately and highlighted that most of them focused on malignant diseases but mainly used xenograft models. It is understandable that with a large proportion of the funding supporting cancer research that also siRNA delivery has an emphasis on cancer models. However, reviewers nowadays demand more relevant models than xenografts. Although we can “cure” cancer in various s.c. models, we have not even seen a single clinical trial for pulmonary siRNA delivery to lung cancer or metastases. The animal models used for asthma research are not the most relevant models either. Although sensitization of mice or rats with ovalbumin is easy and straightforward, more relevant allergens such as house dust mite extracts would be better choices. To correctly interpret our results, the inclusion of all necessary controls, especially scrambled siRNA sequences is foremost important. But aside from the problems our animal models and experimental designs pose, do we know enough about the biocompatibility of our nanocarriers to responsibly justify clinical trials? Although most of the studies reviewed here did address the question of biocompatibility, it is not clear if the tests that were run are

conclusive enough to rule out complications. Often times, the authors assessed histology of tissues where only microscopic signs of toxicity problems can be observed. Other publications described the results of proliferation assays, which, however, do not address other levels of toxicity. Real biocompatibility studies are rather sparse and could be the reason why despite of good lab results, the authors do not dare to push the investigations further. Related to the biocompatibility issue, we need to ask ourselves if our delivery systems and targets suitable? Delivery systems that are not biocompatible or not stable *in vivo* are certainly not worth translating in to the clinics. For a clinical trial, the biocompatibility after chronic administration would have to be investigated in studies that are accepted by the FDA, EMA, and other agencies around the world. Nonetheless, in academic research, we still often make use of such nanocarriers, justifying our studies as proof-of-principle studies. But once the principle is proven, we subsequently need a delivery system that is really suitable for application in humans. One factor that cannot be underestimated is certainly the expenses and administration associated with clinical trials. They explain why most clinical trials are initiated by industry and not academia and the gap of interests. While in academia we often focus on publishable results that are not always clinically relevant, industry must translate their results into products. With the clinical trials currently going on and several patent applications in the pipeline, we are optimistic that further progress in pulmonary siRNA delivery will soon address the problems we have identified so that inhalable siRNA is not only a vision of the future.

Acknowledgments

The authors are thankful to Markus Benfer for lyophilizing the DEAPA-PVA-g-PLGA formulations described in this paper. OMM acknowledges the Wayne State Start-Up Fund, BOOST and FRAP Awards funding the *in vivo* studies of siRNA delivery as asthma therapy. IR acknowledges NIH grant 1U01NS083457 and DVA grant 1I01BX001514.

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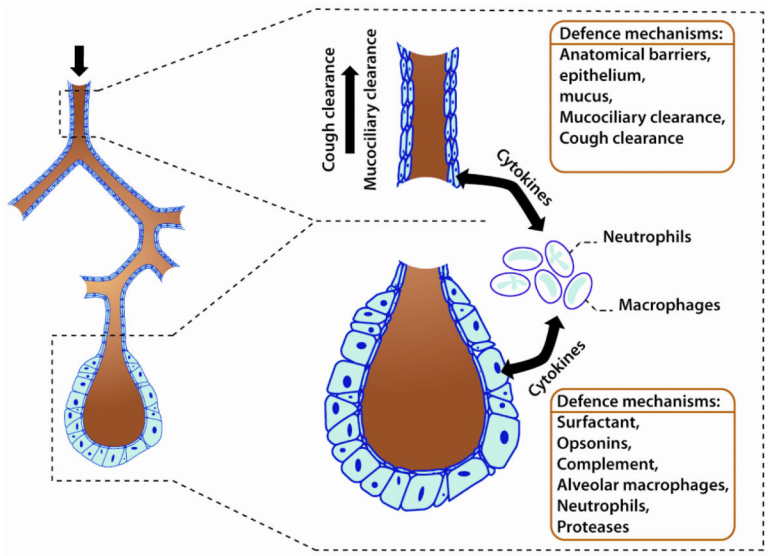

Figure 1.

Lung-intrinsic barriers to efficient pulmonary siRNA delivery. Reprinted with permission from Merkel and Kissel [10]. Copyright 2012 American Chemical Society.

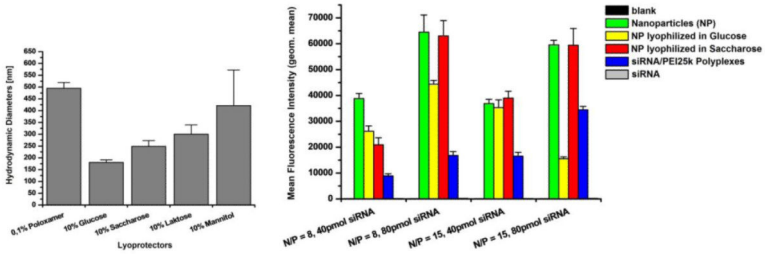

Figure 2.

a) Hydrodynamic diameters of DEAPA-PVA-g-PLGA nanoparticles after lyophilisation and reconstitution in water determined by DLS. b) Comparison of cellular uptake of lyophilized and freshly prepared nanoparticles containing AlexaFluor647 siRNA in H1299 NSCLC cells measured by flow cytometry.

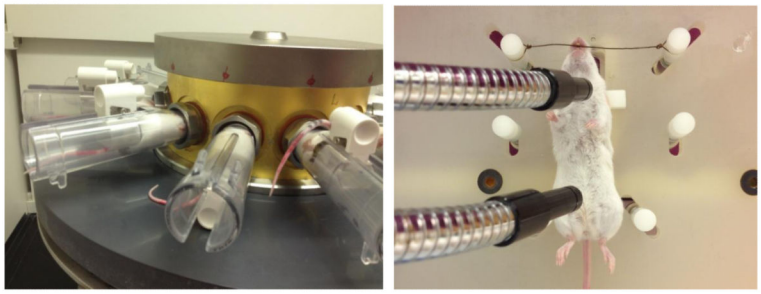

Figure 3a.

For inhalation exposure of mice in a nose-only exposure chamber (Battelle, Columbus, OH), the animals do not need to be anaesthetized. After placing the animals in individual tubes and restraining them, rodents can be exposed for hours. 3b: For intratracheal administration, the trachea of the experimental animal needs to be fully relaxed and the animal anaesthetized. After fixing the animal in a supine position on an intubation platform (PennCentury, Wyndmoor, PA), a gooseneck light can be placed on the lower part of the thorax, the tongue of the animal can be gently pullout out with tweezers, and the throat can be cleared so that the trachea can be recognized as a light spot. Magnification can be used if necessary, and the animal can be gently intubated with a microsprayer or a catheter-based trachea tube.

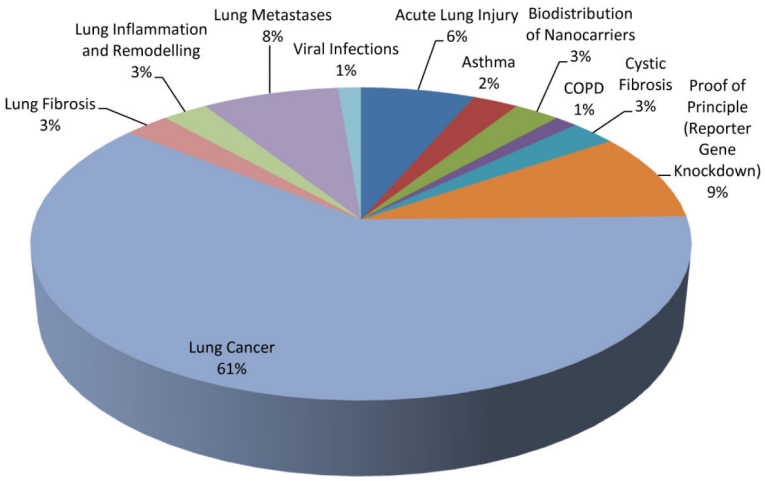

Figure 4.

Disease models described in 80 recent *in vitro* and *in vivo* studies on pulmonary RNAi published between 2011 and 2014.

Table 1

Overview of 47 recent *in vivo* studies of non-viral siRNA delivery to the lung grouped by the delivery system.

Type of siRNA delivery	siRNA/ Targeted gene	Route of administration	Animal model	Delivery system/ Polymer used	Ref.
Naked nucleic acids	Luciferase	Intratracheal	Luciferase expressing mice	Naked PS LNA ASO	[31]
	GAPDH, TGF-β1	Intratracheal	Acute lung injury	Naked PnkRNAs and nkRNAs	[34]
	RPN2	Intratracheal, microsprayer	Lung cancer model	Naked PnkRNAs and nkRNAs	[35]
	TNF-α	Intratracheal	Acute lung injury	Naked siRNA	[38]
	TGF-β1	Intratracheal	Pulmonary fibrosis	Naked siRNA	[39]
	C7orf24	Needle-free jet injection	Lung cancer model	Naked siRNA	[40]
	PAI-1	Intratracheal	Bleomycin-treated Male Wistar rats	Naked siRNA	[56]
Lipid	Mcl1	Intratracheal, microsprayer	Lung metastasis model	Ethylphosphocholine-based lipoplexes	[67]
	CD31, Tie-2, VE-cadherin, BMP-R-2	Intravenous	Lung metastasis model	AtuFECT01 lipoplexes	[68]
	GFP, CD31	Intravenous	Biodistribution	Staramin lipoplexes	[69]
	None	Intravenous	Biodistribution	DMAPAP lipoplexes	[70]
	ITSN-1	Intravenous	Lung endothelial cell function	cationic liposomes	[71, 72]
	Lamin	Intravenous	Biodistribution	PEGylated DOTAP, DOPE liposomes	[73]
	Ssb	Intravenous	Biodistribution	Lipid nanoparticles	[74]
	IGF-1R	Intravenous	Lung cancer model	Magnetic lipoplexes	[75]
	Survivin	Intravenous	Lung cancer model	Liposomes	[76]
	ENaC	Intranasal	Cystic fibrosis	Liposomes	[79]
	MRP1	Inhalation	Lung cancer model	Liposomes	[80]
Polymer	Luciferase	Dry powder Inhalation	Lung metastasis model	Chitosan	[86]
	EGFP	Intratracheal, microsprayer	Asthma, COPD	Salbutamol-modified chitosan	[87]
	GFP	Intravenous	Biodistribution	O-carboxymethyl chitosan-graft-branched polyethylenimine	[88]
	Syntaxin4	Intratracheal	Asthma	Atelocollagen	[89]
	Bcl2	Intravenous	Lung metastasis model	Cationic bovine serum albumin	[90]
	Aktl	Inhalation	$Kras^{LA1}$ model	Glycerol propoxylate triacrylate-spermine	[101] [102]

Type of siRNA delivery	siRNA/ Targeted gene	Route of administration	Animal model	Delivery system/ Polymer used	Ref.
	NPT2b	Inhalation	$Kras^{LA1}$ model	Glycerol propoxylate triacrylate-spermine	[103]
	GFP	Intratumoral	Lung cancer model	Star polymers based on pDMAEMA	[105]
	MDM2	Intravenous	Lung cancer model	PMPC-b-PDPA	[107]
	EGFP	Intratracheal	Biodistribution	PEI	[108]
	Survivin, cyclin B1	Intravenous	Lung metastasis model	PEI	[109, 110]
	Survivin	Intravenous	Lung cancer model	P85-PEI	[114]
	MIF	Intratracheal	Acute lung injury	Dexamethasone-coupled PEI	[111]
	PLK1	Intravenous	Lung metastasis model	HA-PEI	[120]
	STAT3	Intraperitoneal	Lung cancer model	PEI and PLGA	[115]
	EGFP	Intratracheal	Biodistribution	DEAPA-PVA-g-PLGA	[10]
Peptide	S1PLyase	Intratracheal	Acute lung injury	R3V6 and HMGB1A	[124]
	GAPDH	Intravenous	Biodistribution	TAT and ‘double’ TAT	[125]
Inorganic materials	Luciferase	Intravenous	Lung cancer model	LCP	[127]
	HDM2, c-myc, VEGF	Intravenous	Lung cancer model	LCP	[129]
	HDM2, c-myc, VEGF	Intravenous	Lung metastasis model	LCP	[130]
	VEGF	Intravenous	Lung cancer model	LCP	[131]
	C-myc	Intravenous	Lung cancer model	LCP	[132]
	C-myc	Intratracheal	Lung cancer model	RDG-modified, PEGylated gold nanoparticles	[134]
	Plk1	Intravenous	HeLa xenograft	DEMAEMA-coated MSN	[135]
	MRP1 and BCL2	Inhalation	Lung cancer model	LHRH-modified MSN	[137]