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Nanoscale particles for lung delivery of siRNA

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Small interfering RNAs (siRNAs) are potent molecules capable of blocking gene expression after entering cell cytoplasm. Despite their strong efficacy, they need to be carried by nanoscale delivery systems that can protect them against degradation in biological fluids, increase their cellular uptake and favor their subcellular distribution. Several studies have highlighted the potential of local pulmonary delivery of siRNAs for the treatment of lung diseases. For this purpose, nanoscale delivery systems were addressed to target passively or actively the target cell. This review discusses the possibilities of approaching lung delivery of nanoscale particles carrying siRNAs.

Key words: Small interfering RNA – Nanoparticles – Lipoplexes – Polyplexes – Lung.

Since its discovery, the mechanism of RNA interference via small interfering RNA (siRNA) has developed rapidly into a powerful tool in molecular biology for studying the downregulation of gene expression. Due to their high efficiency and selectivity, siRNAs became rapidly interesting for medical applications like the treatment of severe diseases. There are a number of potential applications for targeted local delivery of siRNA to the lungs including the treatment of inflammatory, immune and infectious diseases, cystic fibrosis (CF), and cancer [1]. However, siRNAs face the same obstacles for a successful application as other nucleotide-based therapeutics like plasmid DNA or antisense oligonucleotides i.e., a poor cellular uptake and stability in serum and the lack of selectivity for the target tissue [1-4]. Therefore, carrier features of nanoparticulate delivery systems have been applied to address these limitations. Encapsulation and specific targeting of lung cancer cells thus offer the possibility of improving the treatment of lung diseases. This review focuses on the local delivery of siRNA to the lungs, describing the different barriers and the state-of-the-art ways they can be circumvented.

I. MECHANISM OF ACTION OF siRNA

siRNAs were discovered by showing that the introduction of long double-stranded RNA (dsRNA) into a variety of hosts could induce post-transcriptional silencing of all homologous host genes and/or transgenes [5-8]. Within the intracellular compartment, the long dsRNA molecules are metabolized to small 21-23 nucleotide interfering RNAs by the action of an endogenous ribonuclease: dsRNA-specific RNase III enzyme Dicer [6, 9-12]. The siRNA molecules then assemble into a multiprotein complex, termed RNA-induced silencing complex (RISC) (Figure 1). Functional RISC contains four different subunits, including helicase, exonuclease, endonuclease, and homology searching domains. When siRNA binds to RISC, the duplex siRNA is unwound by helicase, resulting in two single strands (Figure 1) [13], allowing the antisense strand to bind to the targeted RNA molecule (Figure 1) [7, 14]. The endonucleases hydrolyze the target mRNA homologous at the site where the antisense strand is bound. RNA interference has an antisense mechanism of action as, ultimately, a single strand RNA molecule binds to the target RNA molecule by the Watson-Crick base pairing rules. Following binding, a cleavage enzyme present in RISC called argonaute 2, degrades the target RNA [15]. This mechanism makes feasible the use of small double stranded siRNA in therapeutics instead of AS-ODNs. When siRNA-mediated silencing occurs, the products are cleaved, released and degraded, allowing RISC complex to interact with other molecules from the mRNA pool [16]. It was also shown that small RNAs (called miRNAs for microRNAs) cause gene silencing in humans as well as in Caenorhabditis elegans, Drosophila melanogaster, and plants [17, 18]. miRNAs are processed from extended RNA hairpins, whereas siRNAs are produced from a range of RNA precursors, such as viral, transposon RNAs and transgenes [19]. The mechanisms of siRNAs and miRNAs for RNA interference have some similarities, for example, the synthesis of both of them is related to the activity of Dicer, although there are significant differences between them, such as siRNAs cleaving mRNA, whereas miRNAs in some cases translationally repressed mRNA [20, 21].

II. BARRIERS TO siRNA DELIVERY BY INHALATION

Direct instillation of siRNA into the lung offers several important benefits. As for any drug [22], the desired effect can be achieved with a total dose considerably lower than that required for systemic administration, resulting in a lower risk of toxicity and reduced adverse effects [23-24]. Also, instillation of siRNA (for instance as aerosol by intratracheal administration) offers direct access to lung epithelial...
...cells, important cell types in a variety of pulmonary disorders and virus infections [22] and to the malignant cells on the respiratory tract, that cannot be achieved via systemic administration [25]. Finally, in the case of cancer treatment, the specificity of the siRNA would also result in much lower side effects when compared to conventional chemotherapy.

In this review, we will focus on local delivery to the airways of chemotherapy. The specificity of the siRNA would also result in much lower side effects when compared to conventional chemotherapy. One other barrier is the size of the inhaled material [37]. For lung delivery, the geometric diameter, particle shape and density are taken into account resulting in the so called aerodynamic diameter (AD). The AD of the particles affects the magnitude of forces acting on them. While inertial and gravitational effects increase with increasing particle size, diffusion produces larger displacement as particle size decreases (Figure 2). Large particles (> 5 μm AD) usually impact on airway wall at bifurcations. They are usually deposited higher up in the airway such as the back of the throat or pharynx [38]. When the AD of particles ranges from 1 to 5 μm, they are subject to sedimentation by gravitational force that occurs in smaller airways and respiratory bronchioles (Figure 2). The optimal particle size for efficient deposition at the lower respiratory tract is considered to be between 1 and 3 μm [39, 40] (Figure 2). The movement of smaller particles (< 1 μm aerodynamic diameter) is controlled by Brownian motion. And as the particle size further decreases, deposition in the lung increases again due to the increasing mobility through diffusion [37]. However, only nanoparticles that are less than 100 nm appear to settle effectively to the alveolar region with a fractional deposition of around 50% [41, 42]. When the diameter gets larger in the nanoscale range, a high proportion, up to 80 %, can be exhaled [43, 44].

III. LUNG DELIVERY OF siRNA BY NANO SCALE DELIVERY SYSTEMS

In the effort to enhance gene silencing, various non-viral nanoscale delivery systems have been developed for siRNA delivery, and a large part, summarized in Table II, are based on liposomal or polymeric delivery systems [3, 4, 45, 46]. Most systems developed for this purpose have a size of one to several hundreds of nanometers, and should thus
**Table II - In vitro and in vivo intranasal or intratracheal delivery of siRNA by nanoscale lipidic or polymeric carriers.**

| Target | Delivery technology | Model | Biological action (in vivo or in vitro) | Ref. |
|--------|---------------------|-------|---------------------------------------|------|
| **In vitro experiments** | | | | |
| Human telomerase reverse transcriptase | Hyaluronic acid-bearing DOTAP/DOPE liposomes | A549 (human lung cancer cells) | Transfection of siRNA cells was markedly improved with HA-modified liposomes targeting the CD44 receptor. Telomerase activity was successfully inhibited after a treatment with lipoplexes prepared with antiTERT siRNA | [64] |
| BCL-2 | Antagonist G-bearing DOTAP/HSPE/CHOL/DSPE-PEG | Human variant small cell lung cancer (SCLC) cell | Increased internalization in SCLC | [69] |
| Cyclin B1 | Linear polyethyleneimine (PEI) | H460 (human lung cancer cell s) | Higher apoptotic effect | [105] |
| Enhanced green fluorescence protein (EGFP) | Poly(b-amino ester) (PAEs), composed of low molecular weight PEI and PEG | A549 | 79 % silencing was obtained by PAE, higher than the standard | [106] |
| Enhanced green fluorescence protein (EGFP) and Protein kinase B (Akt1) | Chitosan-graft-PEI copolymer composed of chitosan and low molecular weight PEI | A549 | Silencing of EGFP expression was approximately 2.5 folds more than that of PEI25K and efficient knockdown of Akt1 mRNA | [107] |
| Enhanced green fluorescence protein (EGFP) | Chitosan/siRNA nanoparticles | H1299 (human lung cancer cells) | Up to 80 % silencing was obtained | [115] |
| Enhanced green fluorescent protein (EGFP) and TNF-α (tumour necrosis factor) | Freeze-dried chitosan/siRNA nanoparticles | H1299 expressing EGFP and TNF-α expressing RAW murine macrophage cell line | EGFP knockdown efficiency increased with the siRNA concentration and depended on the presence of sucrose as lyoprotectant. Chitosan particles yielded more specific TNF-α knockdown than particles formed with lipid. | [116] |
| Luciferase | pHPMA-MPPPM (poly[(2-hydroxypropyl) methacryla- mide-1-methyl-2-piperidine methanol)] TMC (O-methyl-free N,N,N- trimethylated chitosan) | H1299 | Luciferase expression was silenced by 30–40% increasing up to 70–80% in the presence of an endosomolytic peptide or a photochemical internalizing agent | [122] |
| Luciferase | PVA/PLGA nanoparticles | H1299 | Luciferase knockdown of 80-90 % with minor to no cytotoxicity | [118] |
| **In vivo experiments** | | | | |
| BCL-2 | Cationic DOTAP lipid | Mice Intratracheal | Higher peak concentrations and longer retention of siRNA in the lungs when compared with systemic administration | [75] |
| β galactosidase | Cationic lipid Genzyme (GL) | Mice Intratracheal | Reduced βgal mRNA levels in the airway epithelium but no protein expression | [76] |
| SPARC (matricellular protein) | Cationic lipid Dharma-FECT | Mice Intratracheal | SPARC siRNA reduced gene and protein expression of collagen type 1 in fibroblasts. Lung fibrosis induced by bleomycin was reduced with SPARC siRNA and was accompanied by an inhibition of connective tissue growth factor expression in these same tissues | [77] |
| Highly conserved regions of the nucleoprotein or acidic polymerase in Influenza viruses | Cationic lipid oligofectamine (Invitrogen) | Mice Intravenous followed by intranasal | Delivery of the siRNAs reduced lung virus titers in infected mice. Protection was specific and not mediated by an antiviral IFN response | [78] |
| Respiratory syncytial virus (RSV)-P protein and parainfluenza virus (PIV) type 3 (HPIV3) P protein | TransIT-TKO (Mirus BioCorp) | Mice Intranasal | Specific reduction of pulmonary RSV and PIV titer, siRNAs free of transfection reagent also inhibited pulmonary viral titers | [79] |
| Severe acute respiratory syndrome (SARS) coronavirus | Infasurf® (calfactant) | Mice Intranasal | The siRNAs provided relief from SCV infection-induced fever, diminished SCV viral levels and reduced acute diffuse alveoli damage | [80] |
| GAPDH protein | Infasurf (calfactant) | Mice Intranasal | Reduction of lung concentration of GAPDH protein 50 % at 24 h and 67 % at 7 days | [81] |
| Protein kinase B (Akt1) | Poly(ester amine) | Mice | Silencing of Akt1 in the lungs without affecting Akt2 and Akt3 or affecting the protein expression of Akt1 in other organs | [109] |
but termed “sub-micron particles” rather than “nanoparticles” according to the latest European Commission recommendation of 18 October 2011 on the definition of nanomaterials.

1. Lipid-based delivery systems

Several commercially available cationic lipids, including Lipofectamine2000, DOTAP (N-[1,2-dioleoylpropyl]-N,N,N-trimethylammonium methosulfate) and DOTMA (N-[1,2-dioleoylpropyl]-N,N,N-trimethylammonium chloride), have been selected and applied to the delivery of nucleic acids from all types: plasmid, antisense oligonucleotides and siRNA [47]. At the same time, researchers are still designing and synthesizing new lipids [48, 49] mostly to reduce the adverse effects described for the first cationic lipids [50, 51]. Cationic lipids added in excess to nucleic acids form positively charged lipoplexes which strongly interact with the negatively charged cell membrane facilitating their endocytosis and further cytoplasmic delivery [52]. Dioleylphosphatidylethanolamine (DOPE), a lipid helper, is generally associated with cationic lipids since it participates on the formation of the complexes with nucleic acids [53] and facilitates endosomal membrane disruption by forming hexagonal phases at the acidic pH of the endosomes [54]. Strategies involving the covalent attachment of a targeting ligand at the extremity of poly(ethylene glycol) (PEG) chains grafted onto liposomes have also been explored. Ligands specifically promote intracellular accumulation of the nucleic acid-containing lipid particle into the target cells and further lead to improved gene silencing [55]. Regarding lung delivery, cationic liposomes carrying siRNA have the ability to be aerosolized [56] and they have shown, in vitro, to deliver intracellularly siRNA in the human lung cancer cell lines A549 and H661 [57], human bronchial epithelial cells [58] or type II rat alveolar epithelial cells [59].

Nevertheless, liposome-based carriers still face some challenges for optimal siRNA delivery. These particles may be inadequate as they are associated after systemic administration with an intrinsic problem of non-specific interactions with blood components [51]. Some improvements were tested, such as the inclusion of anionic charges in PEG, which gives a greater colloidal stability in presence of serum [60]. However, when covering lipoplexes with PEG to reduce aggregation, a decrease of transfection efficiency is observed in vitro [61, 62]. Therefore it is important to find other ways to achieve targeting of lung cells. We have designed hyaluronic acid (HA)-coated lipoplexes for the targeted delivery to cancer cells that overexpress the HA binding receptor CD44 [63, 64]. This approach is based on the properties of HA, that, besides being an endogenous polymer, fulfills important mechanical or structural functions [65], interacting with its principal cell surface receptor CD44. The latter plays an important role on cell-cell/cell-matrix interaction, cell adhesion and migration and signal transduction from the extracellular to the intracellular compartment [66-68]. siRNA transfection was improved with HA-modified liposomes, in CD44 positive A549 cells but not in Calu-3 cells that do not display the receptor. Santos et al. [69] have designed lipoplexes bearing as targeting ligand, antagonist G, a substance P analogue that competitively inhibits basal and neuropeptide-stimulated growth of Ctgf expression in these same tissues. The lung distribution of intratracheally injected fluorescent siRNA showed that intense fluorescence was distributed within epithelial cells of bronchi and bronchioles, and only weak fluorescence was detected in the parenchyma [77]. It is unclear so far why such a difference of lung distribution was observed in both studies. Physicochemical properties of complexes such as size, charge or other surface properties might play an important role in their distribution.

Complexes comprised of Oligofectamine and a siRNA specific for highly conserved regions of the nuclease protein or acidic polymers were found to inhibit influenza A virus replication in vivo after intranasal administration to mice [78]. The same inhibition was obtained on respiratory syncytial virus (RSV) and parainfluenza virus (PIV) using siRNAs against viral genes, instilled intranasally in mouse, associated or not with the transfection reagents TransIT-TKO [79]. Finally, suspensions of siRNAs, with or without the transfection reagent Lipofectamine were administered intranasally in an in vivo murine model of Equine Herpes Virus Type 1 (EHV-1) infection using an EHV-1-specific siRNA. Although the administration of free siRNA induces protection against clinical signs like weight loss, no significant difference could be observed between the effectiveness of siRNA complexed with the transfection reagent Lipofectamine or with buffer (PBS). This agrees with some reports where the activity of intranasally administered free siRNA in various animal models has been clearly demonstrated and in some cases superior to that of lipoplexes [80, 81]. Finally, a more recent study has demonstrated the inflammatory properties of lipoplexes carrying siRNA [82].

et al. [70] developed a promising novel non-glycerol based cationic lipid, DSGLA (N,N-dicetyl-N-methyl-N-[N2-guanidino-L-lysiny]l aminoethyl ammonium chloride) that was formulated in lipid-poly-cation-DNA (LPD) na-
2. Polymeric nanoparticles

Among the various siRNA delivery systems considered for pulmonary application are the nanoparticles [4, 83-85]. These nanoscale carriers present several advantages for the treatment of respiratory diseases, such as prolonged drug release, cell specific targeted drug delivery or modified biological distribution of the therapeutic agent, both at the cellular and organ level [86]. Nanoparticles composed of polymers show assurance in fulfilling the stringent requirements placed on these delivery systems, such as targeting of specific sites or cell populations in the lung, release of the drug in a predetermined manner, and degradation within an acceptable period of time [87]. Moreover, studies using inhaled nanoparticles dispersed in aqueous droplets suggest that the mucus clearance can be overcome by nanoparticles, and the drug efficiently transported to the respiratory epithelium [88].

2.1. Association of siRNA to cationic polymer systems by complexation

Several polymer nanoparticle systems have been developed to enhance intracellular delivery of nucleic acids [3, 4, 89]. As for cationic lipids, at physiological pH, siRNA can electrostatically interact with cationic charges from polymers to form polyplexes simply by mixing the components in optimal conditions. The positive charges can either come from soluble cationic polymers or cationic nanoparticles. As the complex generally possesses a global positive surface charge, they get easily attached to the negatively charged cell surface and subsequently undergo endocytosis [90].

One of the main polymers used is polyethyleneimine (PEI), a branched polymer with high cationic potential. The complexes formed between nucleic acids and PEI are usually stable, dispersible, non-covalent and have a net positive charge, which allows interaction with negatively charged cell membranes, followed by internalization by endocytosis [4]. The delivery of the nucleic acid to the cytoplasm occurs by the “proton sponge effect” [91], in which the protonation of the numerous amino groups in the polymer causes osmotic rupture of the endosome and a cytosolic release of the nucleic acid [92-95]. Additionally, after complexation, siRNA is efficiently protected from degradation both in vitro and in vivo in the presence of serum nucleases [99]. Consequently, PEI has been employed in several studies for efficient siRNA delivery in vitro and in vivo [4, 100]. Lin et al. [105] tested linear PEIs forming cationic complex with siRNA to target Cyclin B1, an indispensable protein for cell mitosis. After treatment with siRNA alone, lung cancer cells population in the G2/M phase of the cell cycle increased to around 5% (compared with control) whereas cells treated with PEI-siRNA complex had a significant increase to around 13%, indicating better arrest of the cell mitosis in the G2/M phase of cell cycles by treatment with the complex. Bolcato-Bellemine et al. [110] used linear PEI for the formation of complexes, and modified the siRNA structure adding short complementary A5-T8 overhangs to make the siRNA bind to each other, forming a large “gene-like” structure. With these new sticky overhangs, the PEI-siRNA complex showed increased stability and improved RNase protection and gene silencing in lung cancer cells. While linear PEI is capable of providing a high efficiency of systemic gene delivery to the lungs, branched PEI has been associated with higher levels of acute toxicity [101]. Indeed, higher molecular weight PEIs are related to increased cytotoxicity, due to aggregation of the polymer on the outer cell membrane, which thereby induces necrosis [102]. Also, PEI toxicity might be caused by the rapid adsorption of plasma proteins onto the positively charged polymers, which can lead to aggregation. Regarding siRNA, Beyerle et al. [103] showed that the toxicity is also dependant on cell type, and Thomas et al. [104] found that linear fully deacetylated PEI of 25 kDa and 87 kDa were the most effective for lung delivery. Cytotoxicity and transfection efficiency of PEI are directly proportional to its molecular weight [96]. Efforts to reduce the toxicity or improve stability of the polyplexes have been made by synthesis of PEI with graft copolymers such as linear PEG [97, 98]. A modified PEI system, poly (β-aminoo ester) (PAE) composed of low molecular weight PEI and PEG was complexed with siRNA [106]. The complex was shown to transfect A549 lung cancer cells with a siRNA targeting the enhanced green fluorescence protein (EGFP) achieving 79 % silencing, higher than the standard 25 kDa PEI complex. In a subsequent study, the authors combined the advantages of chitosan (another cationic polymer: for more details see below) and of a modified PEI system. They synthesized a chitosan-graft-PEI (CHI-g-PEI) copolymer, composed of chitosan and low molecular weight PEI [107]. The CHI-g-PEI carrier complexed with siRNA silenced EGFP expression approximately 2.5 times more than that of 25kDa PEI in A549 cells. The carrier was also used to study the silencing of Akt1 protein expression. Such a protein has a vital role in lung cancer, since its overexpression is related to the mechanisms of cancer cell survival, proliferation, and metastasis [108, 109]. Efficient knockdown of Akt1 mRNA as well as the decrease in Akt1 protein expression was observed using the new carrier. Also the addition of peptides such as HIV-TAT to cationic PEG-PEI copolymers has been studied to enhance transfection efficiency in bronchial cells in vitro [111]. Recently, noninvasive aerosolized siRNA delivery systems were developed for lung cancer treatment. Addegradable PEI-PEG copolymer was synthesized by reaction of low-molecular weight PEI with PEG diacylate [112]. An aerosol of poly(ester amine)/Akt1-targeting siRNA complex was delivered into mice with urethane-induced lung cancer through a nose-only inhalation system. Following aerosol delivery twice weekly for 4 weeks, Akt1 siRNA delivered in complexes with poly(ester amine) showed down-regulation of Akt related signals and inhibited the progression of tumors in the lung cancer model of K-rasLAI mice [109].

Beside PEI, chitosan is a natural polysaccharide both mucocoadhesive and mucopermeable, two qualities that are advantageous for targeted delivery of siRNA to the respiratory tract [113, 114]. Additionally, it is well tolerated, biodegradable, and forms cationic complexes with nucleic acids. Chitosan has been effective for siRNA delivery and gene silencing with low toxicity [22, 113]. In an interesting study, the high degree of deacetylation and high chitosan molecular weight were identified by Liu et al. [115] as important parameters for efficient siRNA-mediated knockdown, thus highlighting the importance of polymer chemical properties for the optimization of gene silencing using chitosan/siRNA nanoparticles. Andersen et al. [116, 117] delivered chitosan/siRNA nanoparticles targeting the enhanced green fluorescent protein (EGFP). They found that EGFP knockdown efficiency increased with siRNA concentration and that it could be achieved using freeze-dried nanoparticles, lyophilized directly within the cell culture dishes or onto surfaces, and used later by simply adding the cell suspension directly. At low siRNA concentration (< 25 nM) the highest knockdown was obtained at relatively high sucrose concentrations used as cryoprotectant (10 % knockdown at 10 % sucrose) whereas, at high siRNA concentration (50 nM), 5 % sucrose was sufficient to reach maximum knock down efficiency (70 %). Compared with lipoplexes using (TransIT-TKO), freeze-dried TransIT-TKO/siRNA were more effective than chitosan/siRNA at all concentrations tested, although chitosan particles yielded more specific TNF-α knockdown than particles formed with lipid, which also exhibited significantly lower viability of approximately 60 %, compared to 90-100 % of the chitosan/siRNA nanoparticles.

Other cationic polymers have been used. Nguyen et al. [118] designed a branched biodegradable polyester consisting of tertiary-amine-modified PLA backbones grafted to PLGA. This polymer was previously found to show high transfection efficiency [119], superior biocompatibility compared to PEI [120], rapid degradation rates [120] and low acute toxicity and inflammatory response after pulmonary application [121]. Nguyen et al. [118] found that small siRNA dosages (of 5 and 10 pmol) were necessary to achieve the aimed
luciferase knockdown of 80-90 % in H1299 human lung carcinoma cell with minor to no cytotoxicity. Also, at a polymer ratio of 10:1 the nebulized nanoparticles displayed comparable knockdown efficiency to the non-nebulized samples. Varkouhi et al. [122] evaluated the gene-silencing activity of two enzymatically-degradable designed polymers: trimethylated chitosan (TMC) and pHPMA-MPPM (poly(2-hydroxypropyl) methacrylamide 1-methyl-2-piperidinone methanol). Both compounds showed a rather important silencing effect (higher than current surfactants) with less toxicity, reaching 30 to 40 %. These values increased up to 70-80 % in the presence of an endosomolytic peptide or a photochemical internalizing agent.

2.2. Encapsulation of siRNA within polymer nanocapsules

Another established approach for lung delivery of siRNA involves poly(lactic-co-glycolic acid) (PLGA) nanoparticles. PLGA is approved for human use by the Food and Drug Administration (FDA), and several PLGA-based formulations have received worldwide marketing approval [123]. This synthetic biodegradable polymer was shown to be useful for drug delivery formulations with good biocompatibility, cellular uptake and controlled release characteristics [124-126], but also a high stability during nebulization [127]. According to Campolongo and Luo [128], PLGA nanoparticles have attracted much attention since they are assumed to meet the criteria required for successful siRNA delivery: i) they are sufficiently small for efficient tissue penetration and cellular uptake, ii) the siRNA can be entrapped into the PLGA matrix, which offers physical protection against RNase activity as well as a favorable colloidal stability of the system, and iii) the PLGA particles generally possess an excellent controllable and alterable degradation profile, allowing drug release to span from days to years depending on the molecular weight, the composition of the block copolymer and the structure of the nanoparticles [129]. Even though, regarding nucleotide encapsulation, formulation of PLGA nanoparticles also presents some challenges. One of them is that it is difficult to load smaller nucleic acid molecules like siRNA into PLGA nanoparticles and obtain a high loading and encapsulation efficiency with the use of classical preparation methods. As other low molecular weight compounds, siRNA easily leaks from the inner water phase into the outer water phase during preparation due to its relatively low molecular weight, its hydrophilic character and the electrostatic repulsion forces present between the phosphate groups in the siRNA backbone and the anionic acid groups in the PLGA polymer [118]. Also, the degradation of PLGA leads to the formation of acidic products, lactic and glycolic acid, which can cause DNA degradation and damage [130].

The optimal parameters for preparation of siRNA-loaded PLGA nanoparticles by the double emulsion solvent evaporation method were studied by Cun et al. [129]. The formulation and preparation process parameters were rationally optimized by an understanding of the effect of the parameters: i) the volume ratio between the inner water phase and the oil phase, ii) the PLGA concentration, iii) the sonication time for the primary emulsification, iv) the siRNA load and v) the amount of acetylated bovine serum albumin (Ac-BSA) added to the inner water phase to stabilize the primary emulsion. PLGA concentration and the volume ratio were the only significant main effects. Surprisingly, siRNA load was found not to contribute significantly to the encapsulation efficiency. The suggested explanations for the individual effects were that the increased PLGA concentration resulted in a further stabilization of the primary emulsion and limited the diffusion of siRNA through and out of the organic phase due to the increased viscosity of the organic phase, and that a higher inner water phase volume reduced the concentration gradient between the inner and the outer water phase.

2.3. Mineral nanoparticles

Calcium phosphate (CaP) nanoparticles have also been studied to improve nucleotide transfection [131-136]. CaP is a well used non-viral vector for in vitro transfection due to the fact that it is rapidly dissolved in the cytoplasm’s acidic pH, and that it presents little cell toxicity [134, 135, 137]. Recently, Li et al. [138] combined the advantages of a core/shell nanoparticle structure and CaP nanoparticle to achieve a prolonged circulation time and an elevated endosome release mechanism. The authors studied nanoparticles with a CaP core and PEG-phospholipid coating to target luciferase in human lung cancer H460 cells, and an improvement in the gene-silencing effect was observed. CaP dissolved at a low pH, causing nanoparticle de-assembly, the dissolved calcium and phosphate ions increased the osmotic pressure and caused endosome swelling to release the encapsulated siRNA.

2.4. Viral-like synthetic vectors

Lastly, virus-like synthetic vectors as influenza virus envelopes [139] or virosomes have been applied to deliver siRNA in vitro. The use of virosomes has some advantages for antiviral siRNA delivery in the lung, such as the fact that they are expected to be taken up by the cells infected with the virus [140] and their relatively high efficiency. For certain types of viral vectors, a relatively long duration of gene expression can also be achieved [101]. However, progress in virology research is hampered by the difficulty in manufacturing viral envelopes [140], inefficiency, impossibility of repeated administration and severe complications associated with their immunogenicity and oncogenic potential [101, 114, 141-143]. The well-documented case of the death of Jesse Gelsinger due to complications associated with an adeno viral vector highlights this problem [144].

IV. MEANS TO DELIVER NANOSCALE CARRIERS AS DRY POWDERS

The study of the nanoparticle delivery into the respiratory system is also important, whether they are administered as aerosol droplets or dry powders. As mentioned earlier in this review, the study of the aerodynamic diameter of the particles containing nanoparticles is crucial to an optimized delivery to the lower respiratory tract. Also, the characteristics of the liquid media or solid excipients that surround the nanoparticles will influence their release kinetics after deposition in the airways, and thus the time it will take until the nanoparticles themselves are in contact with epithelial cells or macrophages.

Our group have designed multistage or “trojan” delivery system that combines the advantages of microspheres (i.e., long term release) and polyplexes (i.e., enhanced nucleic acid intracellular penetration) [145-148]. Indeed, solid nanoparticulate complexes made of PEI and nucleic acids were encapsulated into a polymer matrix [146, 148]. ODN complexation with PEI improved the encapsulation efficiency probably by an electrostatic interaction between the cationic ODN/PEI complex and the anionic PLGA [145, 146, 148]. Release rate was shown to be dependent on the porosity of the microparticles: the higher the porosity, the faster the release. Low porous particles were shown to deliver constantly the nanoparticles with the consequence of improving the intracellular penetration once reaching the target. Microparticles consisting of aggregates of PLGA nanoparticles surrounding a hollow core were developed using non-biodegradable [39] and biodegradable PLGA polymers [149] because of their high aerodynamic properties, these particles seem very promising for lung delivery of nanoparticles.

Although lung delivery of siRNA represents an important field for therapeutic applications, the need of nanoscale carriers is essential to allow their delivery. A variety of systems have shown efficiency in vitro, as the systems based on cationic lipids. Other cationic polymers were able to complex siRNA, and biodegradable polymers were able to encapsulate siRNA, and they can be further surface-modified and functionalized to enhance targeting properties. Nevertheless, in vivo
delivery also requires the control of aerosol formulation of the nano-
particles. And despite some successful experiments reported here, the
studies carried out in vivo do not all show a real benefit of lung inhaled
nanoparticulate systems. Further research is needed in order to better
understand the distribution of such particles after lung administration.

ABBREVIATIONS

AA: anisamide. AD: aerodynamic diameter. AS-ODN: antisense oligo-
deoxy nucleotides. CaP: calcium phosphate. CF: cystic fibrosis. CQf:
connective growth factor. DEAPA: diethylaminopropylamine. DOPE:
dioleylphosphatidylethanolamine. DOTAP: N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methylsulfate. DOTMA: N-[1-(2,3-dioleyloxy) propyl]-N,N-trimethylammonium chloride. DSGLA: N,N-distearyl-N-
methyl-N-[2-(N2-guanidino-L-lysyl)] aminoethyl ammonium chloride.
dsRNA: double-stranded RNA. EGFP: enhanced green fluorescence protein. EHV-1: equine herpes virus type 1. ERK1/2: extracellular signal-regulated kinases. HA: hyaluronic acid. PEG: poly(ethylene glycol). PEI: polyethylenimine. PIV: parainfluenza virus. PLGA: poly(lactic-co-glycolic acid). RISC: RNA induced silencing complex. RSV: respiratory syncy-
tial virus. SCLC: small cell lung cancer. siRNA: small interfering RNA.
SPARC: secreted protein, acidic and rich in cysteine.

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