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*
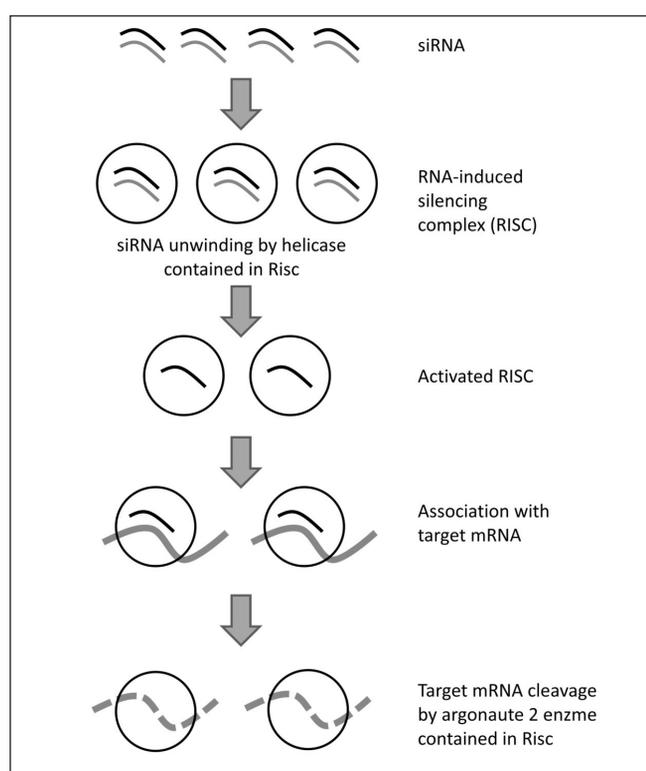
Figure 1 - Mechanism of action of intracellular siRNA.

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 translationaly 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

Table I - Tissue and cellular barriers for lung delivery of siRNA.

	Barriers to siRNA delivery to the lung	Consequences	SiRNA delivery strategies
Tissue level	Aerodynamic properties	Modulate the biodistribution in the different regions of the lung	**Aerosol formulation** Liquid droplets (nebulization) Dry powders (spray-drying)
	Muco-ciliary clearance	Limits the period of time the carrier has to cross the mucus barrier and reach the epithelial cells	
	Stability in the bronchoalveolar fluids	Degradation of the carrier or the siRNA	
Cellular level	Intracellular penetration	Insufficient amount of siRNA internalized	**Nanocarrier formulation** Lipid-based Polymer-based Virus-like vectors
	Intracellular stability	Quick endo-lyso-somal degradation of siRNA, reduction of siRNA amount in the cytoplasm	
	Subcellular distribution	SiRNA must be delivered to the cytoplasm in order to be active	

Figure 2 - Particle deposition along the pulmonary tract as a function of the aerodynamic diameter.

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 siRNA which are large molecular weight molecules, both ionized and hydrophilic [4]. Therefore, despite a large interest, they present some hurdles for their *in vivo* administration (*Table I*). Indeed, even by lung administration, if the aerosolized solution reaches sites close to the target, the *in vivo* applicability of siRNA will be limited by the apparent siRNA instability in biological fluids and their inadequate cellular uptake (*Table I*). In biological fluids, the siRNA half-life is very short and varies from seconds to minutes [26]. This is predominately due to their rapid degradation by endogenous RNases and their rapid clearance [26]. To reach their cellular target, siRNAs need to cross the cellular membrane, but because of their negative charge and size [2], the incubation of unmodified free siRNA with mammalian cells does not result in effective knockdown of the target gene [27]. This the reason why the most challenging innovation that is required for effective siRNA-mediated therapy in lung diseases is the targeting of siRNA to specific lung cell populations, using adequate delivery systems that will allow their delivery into the relevant intracellular compartment and further guarantee its protection against degradation. These systems should combine adequate intracellular delivery and permit lung delivery either by aerosol or dry powders.

The first obstacle to such a delivery is the resistance by respiratory mucus and alveolar fluids such as lung surfactant (*Table I*). Indeed, in the large airways, a continuously renewed mucus layer constitutes a barrier to assure the defence against inhaled materials [28, 29]. Mucus is composed mainly of water and mucins, long flexible highly glycosylated proteins that constitute around 1-5 % of the total weight of the whole mucus [30, 31]. Mucus traps the inhaled materials which are then effectively removed from the respiratory tract toward the upper end of the tracheal tube via the muco-ciliary clearance process
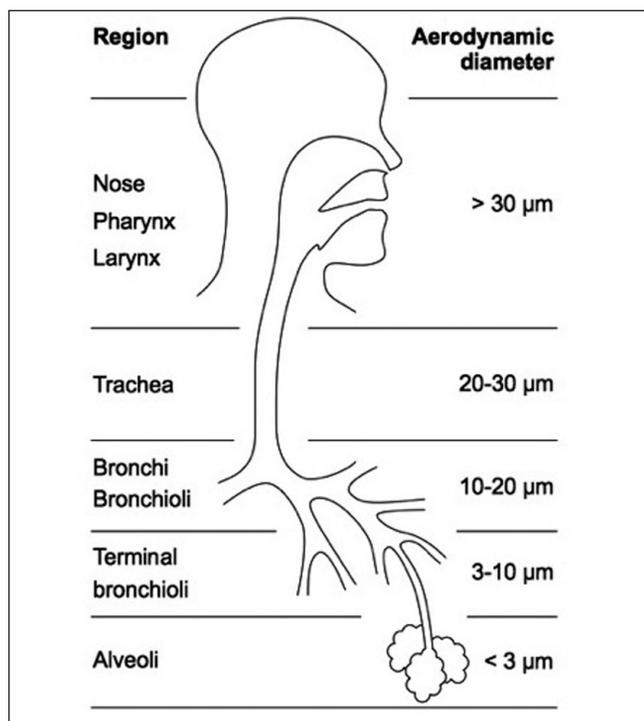
[31]. This complex mechanism, which guarantees lung integrity in physiological conditions, can undergo important changes associated with pathological diseases or in the case of inhalation of various toxic compounds. Chronic disorders of the respiratory tract, such as asthma and cystic fibrosis as well as exposure to cigarette smoke, pollutants and urban particular matter, are associated with impaired barrier function and changes in the composition of the mucus layer [32-36].

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 NANOSCALE 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 DO-TAP/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 antihTERT siRNA	[64]
BCL-2	Antagonist G-bearing DOTAP/ HSPC/CHOL/ DSPE-PEG	Human variant small cell lung cancer (SCLC) cell	Increased internalization in SCLC No enhanced down regulation of Bcl-2	[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)s (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 Akt1mRNA	[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-MPPM (poly((2-hydroxypropyl) methacryla-mide 1-methyl-2-piperidine methanol)) TMC (O-methyl-free N,N,N-trimethylated chitosan)	H1299	Luficerase expression was silenced by 30–40% increasing up to 70–80% in the presence of an endosomolytic peptide or a photochemical inter-nalizing 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-induced fibrosis 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 in-tranasal	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 parain-fluenza virus (PIV) type 3 (HPIV3) P protein	TransIT-TKO (Mirus Bio Corp)	Mice Intranasal	Specific reduction of pulmonary RSV and PIV titer. SiRNAs free of transfection reagent also inhibited pulmonary viral titers	[79]
Severe acute respiratory syn-drome (SARS) coronavirus	Infasurf® (calfactant) Extract of natural surfactant from calf lungs which includes phospholipids, neutral lipids, and hydrophobic surfactant-associated proteins B and C	Mice Intranasal	The siRNAs provided relief from SCV infection–in-duced fever, diminished SCV viral levels and reduced acute diffuse alveoli damage	[80]
GAPDH protein	Infasurf (calfactant) Extract of natural surfactant from calf lungs which includes phospholipids, neutral lipids, and hydrophobic surfactant-associated	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]

be 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-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) and DOTMA (N-[1-(2,3-dioleyloxy) propyl]-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 different small-cell lung cancer (SCLC) [70]. The targeted vesicles showed increased internalization in SCLC, as well as in other tumor cells and HMEC-1 microvascular endothelial cells, but the improved cellular association did not correlate with enhanced downregulation of the targeted Bcl-2. This absence of Bcl-2 downregulation was due to an inadequate release of the nucleic acids from the endocytic pathway, because of the presence of 10 mol % of PEG incorporated in the SLP formulation and/or to the absence of membrane destabilizing/fusogenic lipids.

To improve siRNA delivery, Chen *et al*. [71] developed a promising novel non-glycerol based cationic lipid, DSGLA (N,N-distearyl-N-methyl-N-2[N'-(N2-guanidino-L-lysinyl)] aminoethyl ammonium chloride) that was formulated in lipid-polycation-DNA (LPD) na-

noparticles containing DOTAP and targeted with PEG tethered with anisamide (AA). This new lipid contained both guanidinium and lysine residues as a cationic head group, that were chosen due to the fact that guanidinium groups are capable of forming hydrogen bonds with nucleic acid bases, thus further enhancing the capacity to deliver plasmid siRNA [71]. The lysine groups were selected for decreasing the cytotoxicity observed with the commercial lipid DOTAP contained in the LPD nanoparticles previously described by the authors [72, 73]. The presence of 10 μM DSGLA in AA-bearing LPD was found to decrease extracellular signal-regulated kinases (ERK1/2) activation in H460 cells. The lipids without guanidine group did not cause the inhibition of ERK1/2 activation, and on the contrary, due to the antiapoptopic effect of DOTAP, an increased ERK1/2 activation was observed.

Among all lipid systems developed for treating lung cancer or other pulmonary diseases only a few have been administered in animal models by local administration, either intranasally or intratracheally. Garbuzenko *et al*. [74, 75] compared intratracheal v/s intravenous delivery of DOTAP-siRNA lipoplexes in mice. Intratracheal administration led to higher siRNA peak concentrations and a much longer retention of liposome-siRNA in the lungs when compared with systemic administration. In another experiment, GL67, a cationic lipid from Genzyme, mediated the uptake of siRNAs into airway epithelium *in vivo* in mice. siRNAs were complexed to GL67 and the resulting suspension was placed as a single bolus into the nasal cavity and rapidly sniffed into the lungs [76]. Following *in vivo* lung transfection, siRNAs were visible only in alveolar macrophages. SiRNAs targeted to β-galactosidase reduced βgal mRNA levels in the airway epithelium of K18-lacZ mice by 30 % as shown by RT-PCR. However, this was insufficient to reduce protein expression [76]. In another experiment, C57BL/6 mice were induced for skin and lung fibrosis by bleomycin and followed by SPARC siRNA treatment through subcutaneous injection and intratracheal instillation, respectively using DharmaFECT 1 as transfection reagent. SPARC (secreted protein, acidic and rich in cysteine), is a component of the extracellular matrix which has been reported as a bio-marker for fibrosis in interstitial pulmonary fibrosis. After treatment, it was shown that lung fibrosis induced by bleomycin was markedly reduced by treatment with SPARC siRNA. The anti-fibrotic effect of SPARC siRNA *in vivo* was accompanied by an inhibition of connective growth factor (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 nucleoprotein or acidic polymerase 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 siRNA's, 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].

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* in the presence of RNase 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-Bellemin *et al*. [110] used linear PEI for the formation of complexes, and modified the siRNA structure adding short complementary A5-8/T5-8 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 deacylated 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-amino 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 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. A degradable PEI-PEG copolymer was synthesized by reaction of low-molecular weight PEI with PEG diacrylate [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-rasLA1 mice [109].

Beside PEI, chitosan is a natural polysaccharide both mucoadhesive 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 (60 % 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-piperidine 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 virosome 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 adenoviral 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 delivery 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 nanoparticles. 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 oligodeoxynucleotides. CaP: calcium phosphate. CF: cystic fibrosis. Ctgf: 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,N-trimethylammonium chloride. DSGLA: N,N-distearyl-N-methyl-N-2[N'-(N2-guanidino-L-lysinyl)] 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 syncytial virus. SCLC: small cell lung cancer. siRNA: small interfering RNA. SPARC: secreted protein, acidic and rich in cysteine.

REFERENCES

1. Devi G.R. - siRNA-based approaches in cancer therapy. - Cancer Gene Ther., **13**, 819-829, 2006.
2. Aagaard L., Rossi J.J. - RNAi therapeutics: principles, prospects and challenges. - Adv. Drug Deliv Rev., **59**, 75-86, 2007.
3. Fattal E., Bochot A. - State of the art and perspectives for the delivery of antisense oligonucleotides and siRNA by polymeric nanocarriers. - Int. J. Pharm., **364**, 237-248, 2008.
4. Fattal E., Barratt G. - Nanotechnologies and controlled release systems for the delivery of antisense oligonucleotides and small interfering RNA. - Br. J. Pharm., **157**, 179-194, 2009.
5. Fire A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C. - Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. - Nature, **391**, 806-811, 1998.
6. Elbashir S.M., Harborth J., Lendeckel W., Yalcin A., Weber K., Tuschl T. - Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. - Nature, **411**, 494-498, 2001.
7. Vaucheret H., Fagard M. - Transcriptional gene silencing in plants: targets, inducers and regulators. - Trends Genet., **17**, 29-35, 2001.
8. Kennerdell J.R., Yamaguchi S., Carthew R.W. - RNAi is activated during Drosophila oocyte maturation in a manner dependent on aubergine and spindle-E. - Genes Dev., **16**, 1884-1889, 2002.
9. Timmons L., Fire A. - Specific interference by ingested dsRNA. - Nature, **395**, 854, 1998.
10. Ketting R.F., Fischer S.E., Bernstein E., Sijen T., Hannon G.J., Plasterk R.H. - Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. - Genes Dev., **15**, 2654-2659, 2001.
11. Hannon G.J., Rossi J.J. - Unlocking the potential of the human genome with RNA interference. - Nature, **431**, 371-378, 2004.
12. Meister G., Tuschl T. - Mechanisms of gene silencing by double-stranded RNA. - Nature, **431**, 343-349, 2004.
13. Nykanen A., Haley B., Zamore P.D. - ATP requirements and small interfering RNA structure in the RNA interference pathway. - Cell, **107**, 309-321, 2001.
14. Martinez J., Patkaniowska A., Urlaub H., Lührmann R., Tuschl T. - Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. - Cell, **110**, 565-574, 2002.
15. Zamore P.D., Tuschl T., Sharp P.A., Bartel D.P. - RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. - Cell, **101**, 25-33, 2000.
16. Leung R.K., Whittaker P.A. - RNA interference: from gene silencing to gene-specific therapeutics. - Pharmacol. Ther., **107**, 222-239, 2005.
17. Lee R.C., Feinbaum R.L., Ambros V. - The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. - Cell, **75**, 843-854, 1993.
18. Hutvagner G., McLachlan J., Pasquinelli A.E., Balint E., Tuschl T., Zamore P.D. - A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. - Science, **293**, 834-838, 2001.
19. Jana S., Chakraborty C., Nandi S., Deb J.K. - RNA interference: potential therapeutic targets. - Appl. Microbiol. Biotechnol., **65**, 649-657, 2004.
20. Sontheimer E.J., Carthew R.W. - Silence from within: endogenous siRNAs and miRNAs. - Cell, **122**, 9-12, 2005.
21. Chu C., Rana T.M. - Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. - PLoS Biol., **4**, e210, 2006.
22. de Fougerolles A., Novobrantseva T. - siRNA and the lung: research tool or therapeutic drug? - Curr. Opin. Pharmacol., **8**, 280-285, 2008.
23. Koshkina N.V., Agoulnik I.Y., Melton S.L., Densmore C.L., Knight V. - Biodistribution and pharmacokinetics of aerosol and intravenously administered DNA-polyethyleneimine complexes: optimization of pulmonary delivery and retention. - Molecular Therapy, **8**, 249-254, 2003.
24. Sham J.O.H., Zhang Y., Finlay W.H., Roa W.H., Lobenberg R. - Formulation and characterization of spray-dried powders containing nanoparticles for aerosol delivery to the lung. - Int. J. Pharm., **269**, 457-467, 2004.
25. Zou Y., Tornos C., Qiu X., Lia M., Perez-Soler R. - p53 aerosol formulation with low toxicity and high efficiency for early lung cancer treatment. - Clinical Cancer Res., **13**, 4900-4908, 2007.
26. Soutschek J., Akinc A., Bramlage B., Charisse K., Constien R., Donoghue M., Elbashir S., Geick A., Hadwiger P., Harborth J., John M., Kesavan V., Lavine G., Pandey R.K., Racie T., Rajeev K.G., Rohl I., Toudjarska I., Wang G., Wuschko S., Bumcrot D., Koteliansky V., Limmer S., Manoharan M., Vornlocher H.P. - Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. - Nature, **432**, 173-178, 2004.
27. Dykxhoorn D.M., Lieberman J. - Knocking down disease with siRNAs. - Cell, **126**, 231-235, 2006.
28. Knowles M.R., Hohneker K.W., Zhou Z., Olsen J.C., Noah T.L., Hu P.C., Leigh M.W., Engelhardt J.F., Edwards L.J., Jones K.R., Grossman M., Wilson J.M., Johnson L.G., Boucher R.C. - A controlled study of adenoviral-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. - N. Engl. J. Med., **333**, 823-831, 1995.
29. Cone R.A. - Barrier properties of mucus. - Adv. Drug Del. Rev., **61**, 75-85, 2009.
30. Quraishi M.S., Jones N.S., Mason J. - The rheology of nasal mucus: a review. - Clin. Otolaryngol. Allied Sci., **23**, 403-413, 1998.
31. Samet J.M., Cheng P.W. - The role of airway mucus in pulmonary toxicology. - Environ. Health Perspect., **102**, Suppl. 2, 89-103, 1994.
32. Evans C.M., Koo J.S. - Airway mucus: the good, the bad, the sticky. - Pharmacol. Ther., **121**, 332-348, 2009.
33. Jany B., Gallup M., Tsuda T., Basbaum C. - Mucin gene expression in rat airways following infection and irritation. - Biochem. Biophys. Res. Commun., **181**, 1-8, 1991.
34. Ling S.H., van Eeden S.F. - Particulate matter air pollution exposure: role in the development and exacerbation of chronic obstructive pulmonary disease. - Int. J. Chronic Obstructive Pulmonary Disease, **4**, 233-243, 2009.
35. Ahn M.H., Kang C.M., Park C.S., Park S.J., Rhim T., Yoon P.O., Chang H.S., Kim S.H., Kyono H., Kim K.C. - Titanium dioxide particle-induced goblet cell hyperplasia: association with mast cells and IL-13. - Respiratory Research, **6**, 34, 2005.
36. Haswell L.E., Hewitt K., Thorne D., Richter A., Gaca M.D. - Cigarette smoke total particulate matter increases mucous secreting cell numbers *in vitro*: a potential model of goblet cell hyperplasia. - Toxicol *In vitro*, **24**, 981-987, 2010.
37. Yang W., Peters J.I., Williams R.O., 3rd - Inhaled nanoparticles, a current review. - Int. J. Pharm., **356**, 239-247, 2008.

38. Suarez S., Hickey A.J. - Drug properties affecting aerosol behavior. - Respir. Care, **45**, 652-666, 2000.

39. Tsapis N., Bennett D., Jackson B., Weitz D.A., Edwards D.A. - Trojan particles: large porous carriers of nanoparticles for drug delivery. - Proc. Natl. Acad. Sci. USA, **99**, 12001-12005, 2002.

40. Sakagami M. - *In vivo, in vitro* and ex vivo models to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. - Adv. Drug Deliv. Rev., **58**, 1030-1060, 2006.

41. Patton J.S., Byron P.R. - Inhaling medicines: delivering drugs to the body through the lungs. - Nat. Rev. Drug Discov., **6**, 67-74, 2007.

42. Byron P.R. - Prediction of drug residence times in regions of the human respiratory-tract following aerosol inhalation. - J. Pharm. Sci., **75**, 433-438, 1986.

43. Heyder J., Rudolf G. - Mathematical-models of particle deposition in the human respiratory-tract. - J. Aerosol. Sci., **15**, 697-707, 1984.

44. Heyder J., Gebhart J., Rudolf G., Schiller C.F., Stahlhofen W. - Deposition of particles in the human respiratory-tract in the size range 0.005-16-Mu-M. - J. Aerosol. Sci., **17**, 811-825, 1986.

45. Gilmore I.R., Fox S.P., Hollins A.J., Akhtar S. - Delivery strategies for siRNA-mediated gene silencing. - Curr. Drug Deliv., **3**, 147-145, 2006.

46. Li W., Szoka F.C., Jr. - Lipid-based nanoparticles for nucleic acid delivery. - Pharm. Res., **24**, 438-449, 2007.

47. Fattal E., Delattre J., Dubernet C., Couvreur P. - Liposomes for the delivery of nucleotides and oligonucleotides. - STP Pharma Sci., **9**, 383-390, 1999.

48. Han S.E., Kang H., Shim G.Y., Suh M.S., Kim S.J., Kim J.S., Oh Y.K. - Novel cationic cholesterol derivative-based liposomes for serum-enhanced delivery of siRNA. - Int. J. Pharm., 2007.

49. De Rosa G., De Stefano D., Laguardia V., Arpicco S., Simeon V., Carnuccio R., Fattal E. - Novel cationic liposome formulation for the delivery of an oligonucleotide decoy to NF-kappaB into activated macrophages. - Eur. J. Pharm. Biopharm., **70**, 7-18, 2008.

50. Plank C., Mechtler K., Szoka F.C., Jr., Wagner E. - Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. - Hum. Gene Ther., **7**, 1437-1446, 1996.

51. Lambert G., Fattal E., Brehier A., Feger J., Couvreur P. - Effect of polyisobutylcyanoacrylate nanoparticles and lipofectin loaded with oligonucleotides on cell viability and PKC alpha neosynthesis in HepG2 cells. - Biochimie, **80**, 969-976, 1998.

52. Lavigne C., Thierry A.R. - Specific subcellular localization of siRNAs delivered by lipoplex in MCF-7 breast cancer cells. - Biochimie, **89**, 1245-1251, 2007.

53. De Oliveira M.C., Fattal E., Couvreur P., Lesieur P., Bourgaux C., Ollivon M., Dubernet C. - pH-sensitive liposomes as a carrier for oligonucleotides: a physico-chemical study of the interaction between DOPE and a 15-mer oligonucleotide in quasi-anhydrous samples. - Biochim. Biophys. Acta, **1372**, 301-310, 1998.

54. Fattal E., Couvreur P., Dubernet C. - "Smart" delivery of antisense oligonucleotides by anionic pH-sensitive liposomes. - Adv. Drug Deliv. Rev., **56**, 931-946, 2004.

55. Cardoso A., Trabulo S., Moreira J.N., Duzgunes N., de Lima M.C. - Targeted lipoplexes for siRNA delivery. - Methods Enzymol., **465**, 267-287, 2009.

56. Gautam A., Waldrep J.C., Densmore C.L. - Aerosol gene therapy. - Mol. Biotechnol., **23**, 51-60, 2003.

57. Toulany M., Minjgee M., Kehlbach R., Chen J., Baumann M., Rodemann H.P. - ErbB2 expression through heterodimerization with erbB1 is necessary for ionizing radiation- but not EGF-induced activation of Akt survival pathway. - Radiother. Oncol., **97**, 338-345, 2010.

58. Han S.W., Roman J. - Fibronectin induces cell proliferation and inhibits apoptosis in human bronchial epithelial cells: pro-oncogenic effects mediated by PI3-kinase and NF-kappa B. - Oncogene, **25**, 4341-4349, 2006.

59. Ma X., Xu D., Ai Y., Ming G., Zhao S. - Fas inhibition attenuates lipopolysaccharide-induced apoptosis and cytokine release of rat type II alveolar epithelial cells. - Mol. Biol. Rep., **37**, 3051-3056, 2010.

60. Nicolazzi C., Mignet N., de la Figuera N., Cadet M., Ibad R.T., Seguin J., Scherman D., Bessodes M. - Anionic polyethyleneglycol lipids added to cationic lipoplexes increase their plasmatic circulation time. - J. Control. Release, **88**, 429-443, 2003.

61. Bally M.B., Harvie P., Wong F.M., Kong S., Wasan E.K., Reimer D.L. - Biological barriers to cellular delivery of lipid-based DNA carriers. - Adv. Drug Deliv. Rev., **38**, 291-315, 1999.

62. Shi F., Wasungu L., Nomden A., Stuart M.C., Polushkin E., Engberts J.B., Hoekstra D. - Interference of poly(ethylene glycol)-lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions. - Biochem J., **366**, 333-341, 2002.

63. Surace C., Arpicco S., Dufay-Wojcicki A., Marsaud V., Bouclier C., Clay D., Cattel L., Renoir J.M., Fattal E. - Lipoplexes targeting the CD44 hyaluronic acid receptor for efficient transfection of breast cancer cells. - Molecular Pharmaceutics, **6**, 1062-1073, 2009.

64. Taetz S., Bochot A., Surace C., Arpicco S., Renoir J.M., Schaefer U.F., Marsaud V., Kerdine-Roemer S., Lehr C.M., Fattal E. - Hyaluronic acid-modified DOTAP/DOPE liposomes for the targeted delivery of anti-telomerase siRNA to CD44-expressing lung cancer cells. - Oligonucleotides, **19**, 103-115, 2009.

65. Bastow E.R., Byers S., Golub S.B., Clarkin C.E., Pitsillides A.A., Fosang A.J. - Hyaluronan synthesis and degradation in cartilage and bone. - Cell. Mol. Life Sci., **65**, 395-413, 2008.

66. Turley E.A. - Extracellular matrix remodeling: multiple paradigms in vascular disease. - Circ. Res., **88**, 2-4, 2001.

67. Jothy S. - CD44 and its partners in metastasis. - Clin. Exp. Metastasis, **20**, 195-201, 2003.

68. Ponta H., Sherman L., Herrlich P.A. - CD44: from adhesion molecules to signalling regulators. - Nat. Rev. Mol. Cell Biol., **4**, 33-45, 2003.

69. Santos A.O., da Silva L.C., Bimbo L.M., de Lima M.C., Simoes S., Moreira J.N. - Design of peptide-targeted liposomes containing nucleic acids. - Biochim. Biophys. Acta, **1798**, 433-441, 2010.

70. Clive S., Webb D.J., MacLellan A., Young A., Byrne B., Robson L., Smyth J.F., Jodrell D.I. - Forearm blood flow and local responses to peptide vasodilators: a novel pharmacodynamic measure in the phase I trial of antagonist G, a neuropeptide growth factor antagonist. - Clin. Cancer Res., **7**, 3071-3078, 2001.

71. Chen Y., Sen J., Bathula S.R., Yang Q., Fittipaldi R., Huang L. - Novel cationic lipid that delivers siRNA and enhances therapeutic effect in lung cancer cells. - Mol. Pharm., **6**, 696-705, 2009.

72. Li S.D., Huang L. - Surface-modified LPD nanoparticles for tumor targeting. - Ann NY Acad. Sci., **1082**, 1-8, 2006.

73. Li S.D., Chen Y.C., Hackett M.J., Huang L. - Tumor-targeted delivery of siRNA by self-assembled nanoparticles. - Mol. Ther., **16**, 163-169, 2008.

74. Garbuzenko O.B., Saad M., Pozharov V.P., Reuhl K.R., Mainelis G., Minko T. - Inhibition of lung tumor growth by complex pulmonary delivery of drugs with oligonucleotides as suppressors of cellular resistance. - Proc. Natl. Acad. Sci. USA, **107**, 10737-10742, 2010.

75. Garbuzenko O.B., Saad M., Betigeri S., Zhang M., Vetcher A.A., Soldatenkov V.A., Reimer D.C., Pozharov V.P., Minko T. - Intratracheal versus intravenous liposomal delivery of siRNA, antisense oligonucleotides and anticancer drug. - Pharm. Res., **26**, 382-394, 2009.

76. Griesenbach U., Kitson C., Escudero Garcia S., Farley R., Singh C., Somerton L., Painter H., Smith R.L., Gill D.R., Hyde S.C., Chow Y.H., Hu J., Gray M., Edbrooke M., Ogilvie V., MacGregor G., Scheule R.K., Cheng S.H., Caplen N.J., Alton E.W. - Inefficient cationic lipid-mediated siRNA and antisense oligonucleotide transfer to airway epithelial cells *in vivo*. - Respir. Res., **7**, 26, 2006.

77. Wang J.C., Lai S., Guo X., Zhang X., de Crombrugghe B., Sonnylal S., Arnett F.C., Zhou X. - Attenuation of fibrosis *in vitro* and *in vivo* with SPARC siRNA. - Arthritis Res. Ther., **12**, R60, 2010.

78. Tompkins S.M., Lo C.Y., Tumpey T.M., Epstein S.L. - Protection against lethal influenza virus challenge by RNA interference *in vivo*. - Proc. Natl. Acad. Sci. USA, **101**, 8682-8686, 2004.

79. Bitko V., Musiyenko A., Shulyayeva O., Barik S. - Inhibition of

respiratory viruses by nasally administered siRNA. - Nat. Med., **11**, 50-55, 2005.

80. Li B.J., Tang Q., Cheng D., Qin C., Xie F.Y., Wei Q., Xu J., Liu Y., Zheng B.J., Woodle M.C., Zhong N., Lu P.Y. - Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. - Nat. Med., **11**, 944-951, 2005.

81. Massaro D., Massaro G.D., Clerch L.B. - Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice. - Am. J. Physiol. Lung Cell Mol. Physiol., **287**, L1066-1070, 2004.

82. Gutbier B., Kube S.M., Reppe K., Santel A., Lange C., Kaufmann J., Suttorp N., Witzenrath M. - RNAi-mediated suppression of constitutive pulmonary gene expression by small interfering RNA in mice. - Pulm. Pharmacol. Ther., **23**, 334-344, 2010.

83. Toub N., Malvy C., Fattal E., Couvreur P. - Innovative nanotechnologies for the delivery of oligonucleotides and siRNA. - Biomed. Pharmacother., **60**, 607-620, 2006.

84. Fattal E., Bochot A. - Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA. - Adv. Drug Deliv. Rev., **58**, 1203-1223, 2006.

85. Reischl D., Zimmer A. - Drug delivery of siRNA therapeutics: potentials and limits of nanosystems. - Nanomedicine, **5**, 8-20, 2009.

86. Beck-Broichsitter M., Gauss J., Packhaeuser C.B., Lahnstein K., Schmehl T., Seeger W., Kissel T., Gessler T. - Pulmonary drug delivery with aerosolizable nanoparticles in an ex vivo lung model. - Int. J. Pharm., **367**, 169-178, 2009.

87. Rytting E., Nguyen J., Wang X., Kissel T. - Biodegradable polymeric nanocarriers for pulmonary drug delivery. - Expert Opin. Drug Deliv., **5**, 629-639, 2008.

88. Schurch S., Gehr P., Im Hof V., Geiser M., Green F. - Surfactant displaces particles toward the epithelium in airways and alveoli. - Respir. Physiol., **80**, 17-32, 1990.

89. Fattal E., Vauthier C., Aynie I., Nakada Y., Lambert G., Malvy C., Couvreur P. - Biodegradable polyalkylcyanoacrylate nanoparticles for the delivery of oligonucleotides. - J. Control. Release, **53**, 137-143, 1998.

90. Gomes dos Santos A.L., Bochot A., Tsapis N., Artzner F., Bejjani R.A., Thillaye-Goldenberg B., de Kozak Y., Fattal E., Behar-Cohen F. - Oligonucleotide-polyethylenimine complexes targeting retinal cells: structural analysis and application to anti-TGFbeta-2 therapy. - Pharm. Res., **23**, 770-781, 2006.

91. Behr J.P. - The proton sponge: a trick to enter cells the viruses did not exploit. - Chimia, **51**, 34-36, 1997.

92. Boussif O., Zanta M.A., Behr J.P. - Optimized galenics improve *in vitro* gene transfer with cationic molecules up to 1000-fold. - Gene Ther., **3**, 1074-1080, 1996.

93. Zuber G., Dauty E., Nothisen M., Belguise P., Behr J.P. - Towards synthetic viruses. - Adv. Drug Deliv. Rev., **52**, 245-253, 2001.

94. Akinc A., Thomas M., Klibanov A.M., Langer R. - Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. - J. Gene Med., **7**, 657-663, 2005.

95. Werth S., Urban-Klein B., Dai L., Hobel S., Grzelinski M., Bakowsky U., Czubayko F., Aigner A. - A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes. - J. Control. Release, **112**, 257-270, 2006.

96. Godbey W.T., Mikos A.G. - Recent progress in gene delivery using non-viral transfer complexes. - J. Control. Release, **72**, 115-125, 2001.

97. Vinogradov S.V., Bronich T.K., Kabanov A.V. - Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. - Bioconjug. Chem., **9**, 805-812, 1998.

98. Petersen H., Fechner P., Fischer D., Kissel T. - Synthesis, characterization, and biocompatibility of polyethylenimine-graft-poly(ethylene glycol) block copolymers. - Macromolecules, **35**, 6867-6874, 2002.

99. Urban-Klein B., Werth S., Abuharbeid S., Czubayko F., Aigner A. - RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA *in vivo*. - Gene Ther., **12**, 461-466, 2005.

100. Malek A., Merkel O., Fink L., Czubayko F., Kissel T., Aigner A. - *In vivo* pharmacokinetics, tissue distribution and underlying mechanisms of various PEI(-PEG)/siRNA complexes. - Toxicol. Appl. Pharmacol., **236**, 97-108, 2009.

101. Kuruba R., Wilson A., Gao X., Li S. - Targeted delivery of nucleic-acid-based therapeutics to the pulmonary circulation. - AAPS J., **11**, 23-30, 2009.

102. Fischer D., Bieber T., Li Y., Elsasser H.P., Kissel T. - A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. - Pharm. Res., **16**, 1273-1279, 1999.

103. Beyerle A., Hobel S., Czubayko F., Schulz H., Kissel T., Aigner A., Stoeger T. - *In vitro* cytotoxic and immunomodulatory profiling of low molecular weight polyethylenimines for pulmonary application. - Toxicol. *In vitro*, **23**, 500-508, 2009.

104. Thomas M., Lu J.J., Ge Q., Zhang C., Chen J., Klibanov A.M. - Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. - Proc. Natl. Acad. Sci. USA, **102**, 5679-5684, 2005.

105. Lin S.Y., Lin F.S., Chen M.K., Tsai L.R., Jao Y.C., Lin H.Y., Wang C.L., Hwu Y.K., Yang C.S. - One-pot synthesis of linear-like and photoluminescent polyethylenimines for intracellular imaging and siRNA delivery. - Chem. Commun. (Camb)., **46**, 5554-5556, 2010.

106. Jere D., Xu C.X., Arote R., Yun C.H., Cho M.H., Cho C.S. - Poly(beta-amino ester) as a carrier for si/shRNA delivery in lung cancer cells. - Biomaterials, **29**, 2535-2547, 2008.

107. Jere D., Jiang H.L., Kim Y.K., Arote R., Choi Y.J., Yun C.H., Cho M.H., Cho C.S. - Chitosan-graft-polyethylenimine for Akt1 siRNA delivery to lung cancer cells. - Int. J. Pharm., **378**, 194-200, 2009.

108. Brognard J., Clark A.S., Ni Y., Dennis P.A. - Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. - Cancer Res., **61**, 3986-3997, 2001.

109. Xu C.X., Jere D., Jin H., Chang S.H., Chung Y.S., Shin J.Y., Kim J.E., Park S.J., Lee Y.H., Chae C.H., Lee K.H., Beck G.R., Jr., Cho C.S., Cho M.H. - Poly(ester amine)-mediated, aerosol-delivered Akt1 small interfering RNA suppresses lung tumorigenesis. - Am. J. Respir. Crit. Care Med., **178**, 60-73, 2008.

110. Bolcato-Bellemin A.L., Bonnet M.E., Creusat G., Erbacher P., Behr J.P. - Sticky overhangs enhance siRNA-mediated gene silencing. - Proc. Natl. Acad. Sci. USA, **104**, 16050-16055, 2007.

111. Kleemann E., Neu M., Jekel N., Fink L., Schmehl T., Gessler T., Seeger W., Kissel T. - Nano-carriers for DNA delivery to the lung based upon a TAT-derived peptide covalently coupled to PEG-PEI. - J. Control. Release, **109**, 299-316, 2005.

112. Park M.R., Han K.O., Han I.K., Cho M.H., Nah J.W., Choi Y.J., Cho C.S. - Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers. - J. Control. Release, **105**, 367-380, 2005.

113. Howard K.A., Rahbek U.L., Liu X., Damgaard C.K., Glud S.Z., Andersen M.O., Hovgaard M.B., Schmitz A., Nyengaard J.R., Besenbacher F., Kjems J. - RNA interference *in vitro* and *in vivo* using a novel chitosan/siRNA nanoparticle system. - Mol. Ther., **14**, 476-484, 2006.

114. Durcan N., Murphy C., Cryan S.A. - Inhalable siRNA: potential as a therapeutic agent in the lungs. - Mol. Pharm., **5**, 559-566, 2008.

115. Liu X., Howard K.A., Dong M., Andersen M.O., Rahbek U.L., Johnsen M.G., Hansen O.C., Besenbacher F., Kjems J. - The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing. - Biomaterials, **28**, 1280-1288, 2007.

116. Andersen M.O., Howard K.A., Paludan S.R., Besenbacher F., Kjems J. - Delivery of siRNA from lyophilized polymeric surfaces. - Biomaterials, **29**, 506-512, 2008.

117. Andersen M.O., Howard K.A., Kjems J. - RNAi using a chitosan/siRNA nanoparticle system: *in vitro* and *in vivo* applications. - Methods Mol. Biol., **555**, 77-86, 2009.

118. Nguyen J., Steele T.W., Merkel O., Reul R., Kissel T. - Fast degrading polyesters as siRNA nano-carriers for pulmonary gene therapy. - J. Control. Release, **132**, 243-251, 2008.

119. Oster C.G., Wittmar M., Unger F., Barbu-Tudoran L., Schaper

A.K., Kissel T. - Design of amine-modified graft polyesters for effective gene delivery using DNA-loaded nanoparticles. - Pharm. Res., **21**, 927-931, 2004.

120. Unger F., Wittmar M., Kissel T. - Branched polyesters based on poly[vinyl-3-(dialkylamino)alkylcarbamate-co-vinyl acetate-co-vinyl alcohol]-graft-poly(d,l-lactide-co-glycolide): effects of polymer structure on cytotoxicity. - Biomaterials, **28**, 1610-1619, 2007.

121. Dailey L.A., Jekel N., Fink L., Gessler T., Schmehl T., Wittmar M., Kissel T., Seeger W. - Investigation of the proinflammatory potential of biodegradable nanoparticle drug delivery systems in the lung. - Toxicol. Appl. Pharmacol., **215**, 100-108, 2006.

122. Varkouhi A.K., Lammers T., Schiffelers R.M., Van Steenbergen M.J., Hennink W.E., Storm G. - Gene silencing activity of siRNA polyplexes based on biodegradable polymers. - Eur. J. Pharm. Biopharm., 2011.

123. Jain R.A. - The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. - Biomaterials, **21**, 2475-2490, 2000.

124. Cartiera M.S., Johnson K.M., Rajendran V., Caplan M.J., Saltzman W.M. - The uptake and intracellular fate of PLGA nanoparticles in epithelial cells. - Biomaterials, **30**, 2790-2798, 2009.

125. Nie H., Ho M.L., Wang C.K., Wang C.H., Fu Y.C. - BMP-2 plasmid loaded PLGA/HAp composite scaffolds for treatment of bone defects in nude mice. - Biomaterials, **30**, 892-901, 2009.

126. Mura S., Hillaireau H., Nicolas J., Le Droumaguet B., Gueutin C., Zanna S., Tsapis N., Fattal E. - Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells. - International Journal of Nanomedicine, (in press), 2011.

127. Manca M.L., Mourtas S., Dracopoulos V., Fadda A.M., Antimisiaris S.G. - PLGA, chitosan or chitosan-coated PLGA microparticles for alveolar delivery? A comparative study of particle stability during nebulization. - Colloids Surf. B Biointerfaces, **62**, 220-231, 2008.

128. Campolongo M.J., Luo D. - Drug delivery: old polymer learns new tracts. - Nat. Mater., **8**, 447-448, 2009.

129. Cun D., Jensen D.K., Maltesen M.J., Bunker M., Whiteside P., Scurr D., Foged C., Nielsen H.M. - High loading efficiency and sustained release of siRNA encapsulated in PLGA nanoparticles: quality by design optimization and characterization. - Eur. J. Pharm. Biopharm., **77**, 26-35, 2011.

130. Walter E., Moelling K., Pavlovic J., Merkle H.P. - Microencapsulation of DNA using poly(DL-lactide-co-glycolide): stability issues and release characteristics. - J. Control. Release, **61**, 361-374, 1999.

131. Roy I., Mitra S., Maitra A., Mozumdar S. - Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. - Int. J. Pharm., **250**, 25-33, 2003.

132. Welzel T., Radtke I., Meyer-Zaika W., Heumann R., Epple M. - Transfection of cells with custom-made calcium phosphate nanoparticles coated with DNA. - J. Mater. Chem., **14**, 2213-2217, 2004.

133. Maitra A. - Calcium phosphate nanoparticles: second-generation nonviral vectors in gene therapy. - Expert Review of Molecular Diagnostics, **5**, 893-905, 2005.

134. Sokolova V., Kovtun A., Prymak O., Meyer-Zaika W., Kubareva E.A., Romanova E.A., Oretskaya T.S., Heumann R., Epple M. - Functionalisation of calcium phosphate nanoparticles by oligonucleotides and their application for gene silencing. - J. Mater. Chem., **17**, 721-727, 2006.

135. Sokolova V.V., Radtke I., Heumann R., Epple M. - Effective transfection of cells with multi-shell calcium phosphate-DNA nanoparticles. - Biomaterials, **27**, 3147-3153, 2006.

136. Olton D., Li J., Wilson M.E., Rogers T., Close J., Huang L., Kumta P.N., Sfeir C. - Nanostructured calcium phosphates (NanoCaPs) for non-viral gene delivery: Influence of the synthesis parameters on transfection efficiency. - Biomaterials, **28**, 1267-1279, 2007.

137. Bisht S., Bhakta G., Mitra S., Maitra A. - pDNA loaded calcium phosphate nanoparticles: highly efficient non-viral vector for gene delivery. - Int. J. Pharm., **288**, 157-168, 2005.

138. Li J., Chen Y.C., Tseng Y.C., Mozumdar S., Huang L. - Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery. - J. Control. Release, **142**, 416-421, 2010.

139. de Jonge J., Holtrop M., Wilschut J., Huckriede A. - Reconstituted influenza virus envelopes as an efficient carrier system for cellular delivery of small-interfering RNAs. - Gene Therapy, **13**, 400-411, 2005.

140. de Fougerolles A., Novobrantseva T. - siRNA and the lung: research tool or therapeutic drug? - Current Opinion in Pharmacology, **8**, 280-285, 2008.

141. Lehrman S. - Virus treatment questioned after gene therapy death. - Nature, **401**, 517-518, 1999.

142. Cavazzana-Calvo M., Fischer A. - Gene therapy for severe combined immunodeficiency: are we there yet? - J. Clin. Invest., **117**, 1456, 2007.

143. Di Gioia S., Conese M. - Polyethylenimine-mediated gene delivery to the lung and therapeutic applications. - Drug Design, Development and Therapy, **2**, 163, 2008.

144. Zallen D. - US gene therapy in crisis. - Trends in Genetics, **16**, 272-275, 2000.

145. De Rosa G., Quaglia F., La Rotonda M.I., Appel M., Alphandary H., Fattal E. - Poly(lactide-co-glycolide) microspheres for the controlled release of oligonucleotide/polyethylenimine complexes. - J. Pharm. Sci., **91**, 790-799, 2002.

146. De Rosa G., Bochot A., Quaglia F., Besnard M., Fattal E. - A new delivery system for antisense therapy: PLGA microspheres encapsulating oligonucleotide/polyethyleneimine solid complexes. - Int. J. Pharm., **254**, 89-93, 2003.

147. De Rosa G., Quaglia F., Bochot A., Ungaro F., Fattal E. - Long-term release and improved intracellular penetration of oligonucleotide-polyethylenimine complexes entrapped in biodegradable microspheres. - Biomacromolecules, **4**, 529-536, 2003.

148. Gomes dos Santos A.L., Bochot A., Doyle A., Tsapis N., Siepmann J., Siepmann F., Schmaler J., Besnard M., Behar-Cohen F., Fattal E. - Sustained release of nanosized complexes of polyethylenimine and anti-TGF-beta 2 oligonucleotide improves the outcome of glaucoma surgery. - J. Control. Release, **112**, 369-381, 2006.

149. Gomez-Gaete C., Fattal E., Silva L., Besnard M., Tsapis N. - Dexamethasone acetate encapsulation into Trojan particles. - J. Control. Release, **128**, 41-49, 2008.

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