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Toxicity of Polymeric-Based Non-Viral Vector Systems for Pulmonary siRNA Application

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1. Introduction

Nanomedicine has the potential of clinical benefit by combination of engineering technologies and materials (Schatzlein, 2006). Development of nanometre scaled therapeutics which provides new and improved properties by specifically targeting the site of action and causing low level of side effects would be a big challenge to treat patients with severe and live-threatening diseases like cancer. Gene therapy provides a new way to treat patients and a lot of effort is made to improve the clinical benefit. But current gene therapy is still experimental and has not proven success in the clinics. Nevertheless there is a need for new approaches to treat „undruggable" disease sites and there are some clinical trials ongoing which using RNA inference (RNAi) as therapeutic mechanism (Table 1).

2. Gene silencing by siRNAs

2.1 RNA interference

RNA interference (RNAi), the Nobel Prize winning mechanism for gene silencing (Fire *et al.*, 1998), raises nowadays increasing attention of many researchers as a new way to treat life-threatening diseases like cancer (Akhtar, 2006) or other genetic disorders like cystic fibrosis (Griesenbach and Alton, 2009) or viral infection as respiratory syncytial virus (RSV) (Ge *et al.*, 2004) and as an in vitro research tool to investigate mechanisms which are involved in those diseases. Small interfering RNA (siRNA) duplexes of 19-23 base pairs could trigger sequence specific gene silencing in mammalian cells (Caplen *et al.*, 2001; Elbashir *et al.*, 2001; Hannon and Rossi, 2004; Meister *et al.*, 2004; Mello and Conte, 2004). The siRNAs are double stranded molecules, consisting of a guide strand that is perfectly complementary to a target mRNA and a passenger strand. Core components of this siRNA-mediated post-transcriptional silencing include the RNAse III enzyme Dicer and its co-factor transactivating response RNA-binding protein (TRBP) along with the Argonaute family of proteins, in particular Argonaute 2 (Ago 2) (Meister *et al.*, 2004), which is the catalytic engine of the RNA induced silencing complex (RISC). Dicer converts dsRNA into 21-25 nucleotide duplexes with 3' 2nt overhangs. The siRNA is incorporated into one or more of the Argonaute proteins in RISC for sequence specific target degradation or translational inhibition (Tuschl *et al.*, 1999). In general, perfect or near perfect base pairing between the siRNA guide strand and the target mRNA is required for Ago2 cleavage to occur. In

Company	siRNA	Target	Disease/ Disorder	Status	Administration / Formulation	Remarks
Acuity Pharmaceuticals (Opko Health)	Bevasiranib (Cand5)	VEGF	AMD, DME	Phase II	intravitreal injection, free siRNA	-
Alnylam Pharmaceuticals	ALN-RSV-01 ALN-RSV-02	RSV Pediatric RSV	RSV Pediatric RSV	Phase IIb	aerosolized siRNA, free siRNA	-
	ALN-VSP02	KSP and VEGF	Liver cancer	Phase I	i.v., free siRNA	-
Silence Therapeutics	Atu027	PKN3	Advanced solid cancer	Phase I	i.v., free siRNA	-
Sirna Therapeutics (Calando Pharmaceuticals)	CALAA-01	RRM2	Solid tumor cancer	Phase I	i.v., Cyclodextrin-adamantan-PEG-transferrin nanocomplex.	-
Sirna Therapeutics (TransDerm Inc.)	TD101	PC keratin K6a	Pachyonychia congenita	Phase Ib	Injection into a callus on the bottom of one foot, free siRNA	-
Sirna Therapeutics	AGN211745 (Sirna-027)	VEGFR1	AMD, CNV & AMD	Phase II	intravitreal injection, free siRNA	-
Quarks Pharmaceuticals	I5NP (QPI-1002)	p53	Delayed graft function, Kidney transplantation	Phase I Phase II	i.v., free siRNA	-
	QPI-1007	Caspase 2	Chronic optic nerve atrophy Non-Arteritic Anterior Ischemic Optic Neuropathy	Phase I	intravitreal injection, free siRNA	-
Tekmira Pharmaceuticals Corporation	PRO-040201	APOB	Hyperchol-esterol-emia	Phase I	i.v., liposomal formulation	study has been terminated due to potential for immune stimulation to interfere with further dose escalation

Silenseed Ltd	siG12D LODER (Local Drug EluteR)	KRAS G12D	Pancreatic cancer	Phase I	miniature biodegradable polymeric matrix, placed in the tumor using an endoscopic ultrasound biopsy needle	-

source: http://clinicaltrials.ifpma.org/no_cache/en/search-trials-ongoing/all/index.htm,

Table 1. Summary of ongoing clinical trials for siRNA delivery,
abbreviations used: AMD: age related macular degeneration; APOB: apolipoprotein B; CNV: choroidale neovascularization; DME: diabetic macular edema; i.v.: intravenous; KSP: kinesin spindle protein; PC: pachyonychia congenital; PKN3: protein kinase N3; RRM2: ribonucleotide reductase M2 polypeptide; RSV: respiratory syntical virus; VEGF: vascular endothelial growth factor

laboratory work and in clinical trials siRNAs are most often chemically synthesized, bypassing the Dicer cleavage step for entry into RISC and avoiding any immune responses and toxicity which is described for long double stranded RNAs (dsRNAs) (Behlke, 2008).
RNAi has widely been used in drug development and several phase I and II clinical trials (Table 1) are ongoing. However, for therapeutic applications still some concerns and challenges need to be overcome, e.g. off-target effects, innate immune response and most importantly specific delivery into the cytoplasm of target cells.

3. Small interfering RNAs (siRNA)

siRNAs are very attractive for therapy because they are easily designed and synthesized, and their versatility allows simultaneous use of multiple siRNAs or change of sequences to accommodate virus mutations. The negative charge of siRNA and their size of around 14 kDa make it difficult to cross the cell membrane without any carrier. There are various delivery strategies under investigation, which includes nanoparticular systems consisting of polymers and/or lipids of different compositions and with or without any conjugation like antibodies or ligands for achieving the most specific way to the target side of action. Davis et al. showed 2008 first evidence for RNAi mechanism of action in human with their self-assembling, cyclodextrin polymer-based nanoparticle system (CALAA-01) targeting the riboucleotide reductase subunit 2 (RRM2) which could be used for therapy of different types of cancers (Heidel *et al.*, 2007; Davis, 2009; Davis *et al.*, 2010). At the same time Zimmermann, MacLachlan and colleagues reported successful siRNA delivery using a different approach for delivery (Zimmermann *et al.*, 2006). They introduced so-called stable nucleic acid lipid particles (SNALP) generated by ethanol dilution technique and showed for the first time in non-human primate a successful targeting of ApoB in the liver (Soutschek *et al.*, 2004; Morrissey *et al.*, 2005; Zimmermann *et al.*, 2006). Ge and co-workers (Ge *et al.*, 2004) used PEI 25 kDa to complex and protect siRNA specific to influenza virus genes and they showed successful reduction of influenza virus infection in mice. Alton et al. gave first evidence for successful gene therapy by using a lipid-based system to delivery CFTR DNA in cystic fibrosis patients (Alton *et al.*, 1999). Thus, gene therapy approaches still need improvements

regarding specific targeting and successful delivery of the nucleic acid but clinical trials are ongoing and preclinical testing are conducted for different kind of diseases (Table 1).

4. Non-viral vector systems for siRNA delivery

RNA interference (RNAi) based therapeutics represent a fundamentally new way to treat human disease by addressing targets that are otherwise "undruggable" with existing medicines (Novina and Sharp, 2004; de Fougerolles *et al.*, 2007). The goal of RNAi-based therapy represents the activation of selective mRNA cleavage for efficient gene silencing. There are two possibilities to harness the endogenous pathway: either i) by using viral vector to express short hairpin RNA (shRNA) that resembles miRNA precursors, or (ii) by introducing siRNAs that mimic Dicer cleavage product into the cytoplasm. Synthetic siRNAs utilize the naturally occurring RNAi pathway in a manner that is consistent and predictable, thus making them particularly attractive as therapeutics. Since they enter RNAi pathway later, siRNAs are less likely to interfere with gene regulation by endogenous miRNAs (Jackson *et al.*, 2003; Grimm *et al.*, 2006). The most important characteristics for effective design and selection of siRNAs are potency, specificity, and nuclease stability. Two types of off-target effects need to be avoided or minimized: i) silencing of genes sharing partial homology to the siRNA and ii) immune stimulation induced by recognition of certain siRNAs by the innate immune system. The activation of the innate immune systems by siRNA could be induced by recognition of dsRNAs by the serine/threonine protein kinase receptor (PKR) (Schlee *et al.*, 2006). This pathway is normally triggered by dsRNAs that are more than 30 nucleotides long, but at higher concentrations also siRNAs may be able to activate this pathway resulting in global translational blockade and cell death. The potential to activate toll-like receptors (TLRs) in the endosomal compartment is more likely to occur after siRNA delivery due to recognition of specific nucleotide sequence motifs (e.g. GU) by TLRs. TLR activation could trigger the production of type I interferons and pro-inflammatory cytokines, and induce nuclear factor kappa B (NF-kB) activation (Hornung *et al.*, 2005; Judge *et al.*, 2005). For example, the presence of 2'-O-methyl modifications within the siRNA duplex could abrogate the binding to TLR7 in endosomes and abolish immunostimulatory response. In addition, these modifications also reduce sequence-dependent off-target silencing and may be particularly beneficial in enhancing siRNA target specificity (Judge *et al.*, 2006; Robbins *et al.*, 2008; Robbins *et al.*, 2009).

Due to increasing mortality and morbidity caused by several lung diseases, RNAi strategies have attracted particular attention and the lung as target organ provides an attractive tool because of the accessibility via non-invasive routes, e.g. nasal or pulmonary applications. The clinical success of siRNA-mediated interventions critically depends upon the safety and efficacy of the delivery methods and agents. Naked siRNAs are degraded in human plasma with a half-life of minutes (Layzer *et al.*, 2004; Choung *et al.*, 2006). Thus, the search for optimized nanocarriers to deliver siRNA is still under intensive investigation. The negative charge and chemical degradability of siRNA under physiologically relevant conditions make its delivery a major challenge (Gary *et al.*, 2007). Depending on their origin, two types of positively charged carriers could be distinguished: i) lipid–based and ii) polymeric-based carrier systems. Both systems provided several advantages to deliver siRNA. Liposome formation agents like Lipofectamine 2000 (Dalby *et al.*, 2004; Santel *et al.*, 2006) and cardiolipin analogues (Chien *et al.*, 2005; Pal *et al.*, 2005) have been successfully used for the delivery of siRNA. Negatively charged nucleic acids and positively charged lipids spontaneously form

nanoparticles, known as lipoplexes, of 50-200 nm in diameter (Sitterberg *et al.,* 2010). Interaction with serum components represents one of the major hurdles that influence the performance when used systemically (Zuhorn *et al.,* 2007). Recently, lipid-mediated delivery of siRNA against apolipoprotein B (ApoB) has been used to target ApoB mRNA to the (Soutschek *et al.,* 2004; Zimmermann *et al.,* 2006). The in vivo use of cationic lipids especially by i.v. administration presents significant problems as these reagents can be quite toxic. Despite problems with i.v. use, cationic lipids are employed for i.p. injection (Verma *et al.,* 2003; Flynn *et al.,* 2004; Miyawaki-Shimizu *et al.,* 2006), for CNS injection (Hassani *et al.,* 2005; Luo *et al.,* 2005) or in topical epithelial surface application (Maeda *et al.,* 2005; Palliser *et al.,* 2006) and intratracheal (Griesenbach *et al.,* 2006). Toxicity varies with the precise chemical composition of the lipids employed dose, and the delivering route. Variations in chemical composition can have a large impact on the functional properties of cationic lipid mixtures (Spagnou et al., 2004), and lipoplex/liposomal preparations have been devised with decreased toxicity that are more compatible with i.v. administration. Liposomes can be modified with ligands such as folate or small peptides, which assist with delivery and help target specific cell types or tissues (Meyerhoff, 1999; Dubey *et al.,* 2004). Through the use of neutral polyethylene glycol-substituted surfaces and other approaches, liposomes can be stabilized and made more "stealthy" showing reduced clearance and improved pharmacokinetics (Oupicky *et al.,* 2002; Moghimi and Szebeni, 2003). These kinds of lipid nanoparticles have been successfully used to deliver antisense oligonucleotides and siRNAs in vivo (Braasch *et al.,* 2003; Chien *et al.,* 2005).

Similar to the lipid-based non viral vector systems, the positive charges of polycations allow an efficient interaction with siRNAs to form so-called polyplexes, which can bind onto cell plasma membrane and be endocytosed. In contrast to the lipid-based systems that rely on the fusogenic property of the liposomes to mediate endosomal escape, polymeric carriers such as poly(ethylene imine) (PEI) use the so-called "proton-sponge" effect to enhance endosomal release of endocytosed polyplexes (Boussif *et al.,* 1995; Behr, 1997; Akinc *et al.,* 2005; Demeneix and Behr, 2005; Nel *et al.,* 2009). According to this mechanism, the deprotonated amines with different pK_a values confer a buffer effect over a wide range of pH. This buffering may protect the siRNA from degradation in the endosomal compartment during maturation of the early endosomes to late endosomes and their subsequent fusion with the lysosomes. The buffering property also allows the polycation to escape from the endosome. At lower pH the buffering capacity causes an influx of chloride ions and water into the endosomes, which burst due to osmotic pressure and facilitating intracellular release of PEI - siRNA polyplexes. PEI has been used for many years to facilitate nucleic acid delivery (Boussif *et al.,* 1995; Demeneix and Behr, 2005). However, due to toxicity and variable performance it has not found generalized acceptance as a delivery tool for either antisense oligonucleotides or siRNAs. Nevertheless, PEI can be used as a prototype for formulation of more complex particles with improved properties (Kim and Kim, 2009).

5. PEI-based non-viral vector systems

Polyethylene imine (PEI) is a simple repetition of the 43 Da CH_2-CH_2-NH ethylene imine motifs. It can be synthesized from ethylene imine (aziridine) via ring opening polymerization or by hydrolysis of poly(2-ethyl-2-oxazolium), leading to branched or linear polymeric backbones, respectively (Godbey *et al.,* 1999). PEI represents one of the most comprehensive investigated cationic polymer for gene delivery in vitro and in vivo (Godbey

et al., 1999; Fischer *et al.*, 2002; Brus *et al.*, 2004; Neu *et al.*, 2005; Gary *et al.*, 2007). PEI 25 kDa serves as gold standard for in vitro transfection experiments (Godbey *et al.*, 2000). The mechanism of cell entry and action for gene delivery is intensively analyzed. To enhance the endosomal release of endocytosed polyplexes PEI uses the so-called "proton-sponge" effect (Boussif *et al.*, 1995; Behr, 1997) Due to the high buffer capacity of PEI amino groups in PEI molecules will be protonated at lower pHs like in the endosomal-lysosomal environment, additional chloride influx into the vesicles increases the osmolarity and the vesicles begin to swell and under the increased osmotic pressure the vesicle will be disrupted and the nucleic acid protected from PEI will be released into the cytoplasm (Godbey *et al.*, 1999; Akinc *et al.*, 2005; Nel *et al.*, 2009). PEI has been used for many years to facilitate nucleic acid delivery (Demeneix and Behr, 2005). However, due to toxicity and variable performance a lot of research is undertaken to reduce the toxicity of PEI and maintain or improve the efficacy and specificity by modification PEI backbone and/or conjugation of hydrophilic molecules like polyethylene glycol (PEG) (Petersen *et al.*, 2002a; Petersen *et al.*, 2002b), disulfide linkages (Breunig *et al.*, 2008), or for specific targeting molecules like transferrin, galactose, TAT-peptide, RGD-motifs (Ogris *et al.*, 1999; Kunath *et al.*, 2003a; Kunath *et al.*, 2003b; Kleemann *et al.*, 2005). Other approaches are reduction of the molecular weight of PEI 25 kDa or purification of PEI 25 kDa via gel filtration (Boeckle *et al.*, 2004; Urban-Klein *et al.*, 2005; Werth, 2006; Fahrmeir *et al.*, 2007) or using instead of the branched PEI 25 kDa the linear form PEI22kDa (Breunig *et al.*, 2005). Thomas and colleagues showed that full deacylation of linear PEI dramatically improves the efficacy but on cost of increased cytotoxicity due to increased numbers of protonatable nitrogens in the PEI molecule (Thomas *et al.*, 2005).

6. Modifications of PEI

Modifications of PEI with the hydrophilic poly(ethylene glycol) (PEG) reduces dramatically the cytotoxicity of PEI 25 kDa but in part on cost of efficacy and increased immunomodulatory and proinflammatory effects (Kichler *et al.*, 2002; Petersen *et al.*, 2002b; Mao *et al.*, 2005; Glodde *et al.*, 2006; Beyerle *et al.*, 2010a; Beyerle *et al.*, 2010b). PEG provides polyplexes with improved solubility, lower surface charge, diminished aggregation, lower cytotoxicity, and possibly improved "stealth effect" in the bloodstream.

Glodde et al. synthesized a series of PEG-PEI copolymers and found that the molecular weight of PEG was found to be the major determinant of polyplex size, via its influence on particle aggregation and polyplex stability (Glodde *et al.*, 2006). Transfection efficiency was correlated to polyplex stability and low molecular weight PEI 2 kDa grafted with PEG showed higher activity than their counterparts with high molecular weight PEI 25 kDa (Williams *et al.*, 2006). In contrast, Petersen and Mao showed good transfection efficiencies for PEI 25 kDa - PEG copolymers with high molecular weight PEG and low numbers of grafting on PEI backbone compare to low molecular weight PEG with high grafting numbers on PEI 25 kDa (Mao *et al.*, 2005; Merkel *et al.*, 2009; Beyerle *et al.*, 2011a).

Grayson and colleagues investigated the siRNA transfection efficacy of different PEI polymers (branched 800 Da, branched 25 kDa and linear 22 kDa) in HeLa derivative cell line (Grayson *et al.*, 2006). They showed that the siRNA delivery and activity was mainly dependent on the biophysical and structural characteristics of the polyplexes and only

25 kDa PEI was able to effective deliver siRNA. The authors explained the high activity of PEI25kDa/siRNA with good stability of polyplexes, small size, and positively surface charge, but nevertheless the cytotoxicity was highest for PEI 25 kDa.

Succinylated PEI polymers for complexation of siRNA were introduced by Wagner and colleagues which showed 10-fold lower toxicity and higher knockdown efficacy compare to pure PEI polyplexes (Zintchenko *et al.*, 2008).

7. Toxicity of PEI-based non-viral vector systems

Synthetic polymers and nanomaterials display selective phenotypic effects in cells and in the body that affect signal transduction mechanisms involved in inflammation, differentiation, proliferation, and apoptosis. When physically mixed or covalently conjugated with cytotoxic agents, bacterial DNA or antigens, polymers can drastically alter specific genetically controlled responses to these agents (Kabanov, 2006). These effects, in part, result from cooperative interactions of polymers and nanomaterials with plasma cell membranes and trafficking of polymers and nanomaterials to intracellular organelles. Cells and whole organism responses to these materials can be phenotype or genotype dependent. In selected cases, polymer agents can bypass limitations to biological responses imposed by the genotype, for example, phenotypic correction of immune response by polyelectrolytes. Overall, these effects are relatively benign as they do not result in cytotoxicity or major toxicities in the body. Collectively, however, these studies support the need for thoroughly assessing pharmacogenomic effects of polymer materials to maximize clinical outcomes and understand the pharmacological and toxicological effects of polymer formulations of biological agents, i.e. polymer genomics. In addition, it is well described in the literature that cationic nanoparticles disrupt lipid bilayers (Hong *et al.*, 2006; Leroueil *et al.*, 2008), induce oxidative stress inside the cell as a result of cell-type interplay and cause in some cases acute lung inflammation when administered intratracheally (Tan and Huang, 2002; Beyerle *et al.*, 2010b; Beyerle *et al.*, 2011a and Beyerle *et al.*, 2011c). Intensive efforts will have to focus on the issue of cytotoxicity to obtain more insight in the exact mechanisms behind, which are multidimensional and largely depend on the application route as well as the formulation that is delivered. Therefore, tissue specific toxicity profiles are still needed and represent a great implement in improving non-viral delivery systems.

8. General toxicity

Hornung et al. described that any rupture or leakage of the endosomal or lysosomal membrane will release cathepsin B, which leads to an inflammasome activation associated with IL-1 production and apoptosis (Hornung *et al.*, 2008). Beyerle et al. found that application PEI/siRNA complexes caused release of proinflammatory cytokines like IL-6, G-CSF, TNF-a, IP-10 in murine lung cell lines (Beyerle *et al.*, 2010a; Beyerle *et al.*, 2010b; Beyerle *et al.*, 2011a and Beyerle *et al.*, 2011c). Cytokine release upon PEI/nucleic acid polyplex treatment has been also described by Gautam and Kawakami et al. (Gautam *et al.*, 2001; Kawakami *et al.*, 2006). Cubillos-Ruis and co-workers investigated linear PEI/siRNA complexes for antitumor immunity and identified linear PEI as TLR 5 agonist of mouse and human. They found that linear PEI/siRNA complexes induced a pattern of inflammatory cytokines which are triggered in vivo by flagellin in a TLR5 dependent manner (Cubillos-

Ruiz *et al.*, 2009). Thus, for in vivo use a lot of effort should be made to avoid the high proinflammatory effects caused by the rupture or leakage of the endosome caused by PEI. Godbey classified PEI-mediated toxicity in an immediate toxicity, associated with free PEI and a delayed form, connected with cellular processing of PEI/DNA polyplexes (Godbey *et al.*, 2001). To form stable and protective PEI nucleic acid polyplexes an excess of PEI polymer is needed, 60-80% PEI remains in a free form after nucleic acid escape and is mainly attributed to PEI toxicity. The high positively charged PEI molecule is able to disrupt cell membranes, disruption of the endosome is on one hand favourable with respect to the intended cytoplasmatic delivery, but on the other hand disruption of other cell membranes (e.g., lysosomal membranes, mitochondrial membrane, plasma membrane) is not favourable as it will cause stress responses or even apoptotic or necrotic cell death. In this context it has been shown that PEI causes apoptosis in an unspecific manner in all kinds of cells (Beyerle *et al.*, 2010a; Merkel *et al.*, 2011) which should be avoided with regard to human use. Therefore, a purification approach of the PEI polymer before and after complexation with nucleic acid is one possibility to reduce PEI-related toxicity (Boeckle *et al.*, 2004; Werth, 2006; Fahrmeir *et al.*, 2007).

9. Lung toxicity

Especially, when regarding the lung as target organ the activation of the inflammosome should be avoided. Lung targeting could in general be achieved by systemic delivery or pulmonary delivery. Pulmonary delivery enhances siRNA retention in the lungs, lowers the dose of siRNA required for efficient delivery, and therefore implicates reduced systemic toxic effects, and due to lower nuclease activity in the lung siRNA stability is increased. RNAi can be used to treat or prevent diseases affecting the lungs, such as lung cancer (Li and Huang, 2006; Tong, 2006; Jere *et al.*, 2008; Ren *et al.*, 2009; Zamora-Avila *et al.*, 2009), various types of respiratory infectious diseases (Ge *et al.*, 2004; Fulton *et al.*, 2009; DeVincenzo *et al.*, 2010), airway inflammatory diseases (Lee and Chiang, 2008; Seguin and Ferrari, 2009), and cystic fibrosis (Pison *et al.*, 2006).

Beyerle and co-workers investigated the effects of PEGylation on cytotoxicity and cell-compatibility of different PEG-PEI copolymers in murine lung cell lines and found a clear structure-function relationship (Fig. 1).

The higher the degree of PEGylation on PEI25kDa with low molecular weight PEG, the stronger was the reduction of cytotoxicity and oxidative stress, but the proinflammatory potential of PEI remained high (Beyerle *et al.*, 2010b). The same group evaluated the pulmonary toxicity of PEI/siRNA complexes and found at day three after intratracheal delivery still high numbers of neutrophils and high levels of proinflammatory cytokines in the airspace of polyplex treated mice (Beyerle *et al.*, 2011a and Beyerle *et al.*, 2011c). The higher inflammatory potential but lower toxicity of PEI modifications is still an issue to be overcome when targeting pulmonary diseases. There is an urgent need to balance the efficacy and toxicity of such nucleic acid carriers.

10. Toxicogenomics of PEI-based non-viral vector systems

Toxicogenomic and genotoxic information of non-viral vector systems is rare, but of great concern when nowadays focusing personalized medicine. Gene delivery systems should be
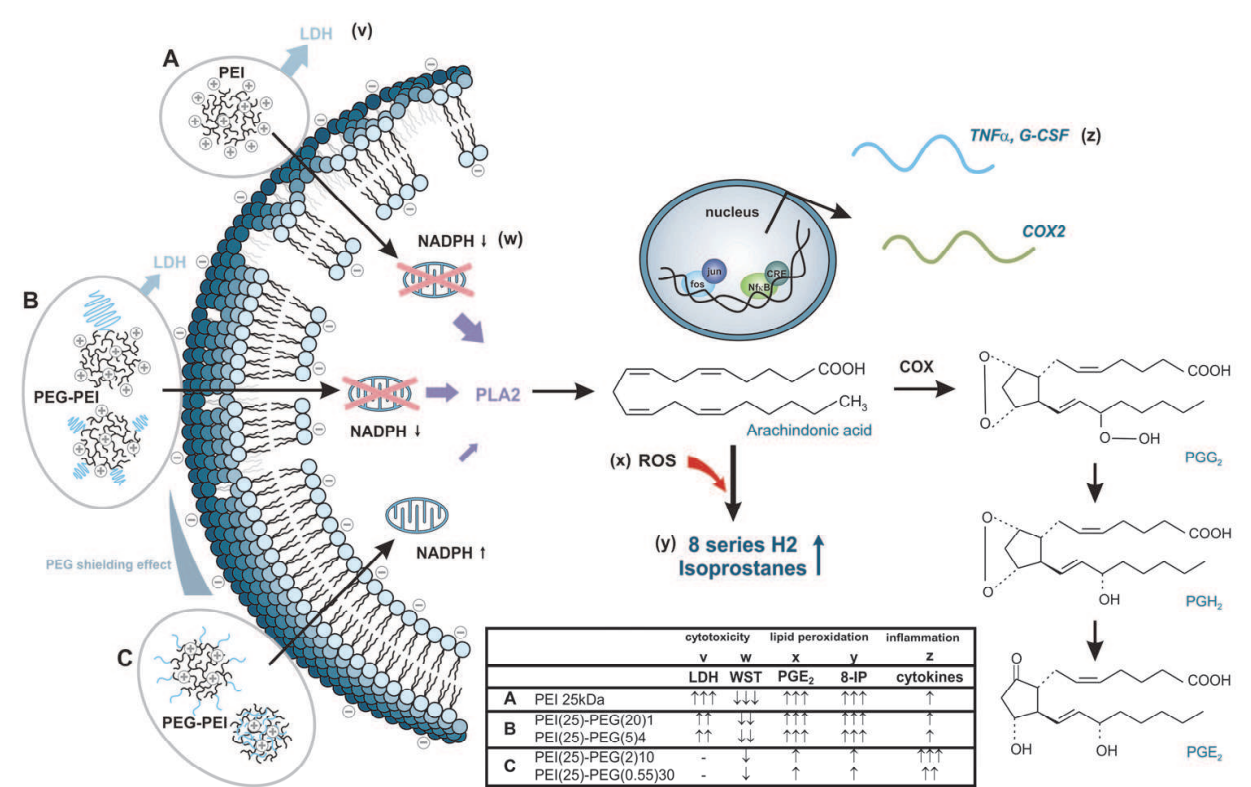
Fig. 1. Structure-function-relationships of PEG-PEI copolymers
Overview of the structure-function relationships of PEG modified PEI copolymers (B-C) in comparisonto PEI 25kDa (A) with regard to cytotoxic (v,w), oxidative stress (x,y) and proinflammatory responses (z). Arrows represent the up- or downregulation of the investigated molecules.

able to pass through biological membranes/barriers and transfer the desired information to target sites with minimal impact on the integrity of the target cell or tissue (Forrest and Pack, 2002; Omidi *et al.*, 2008). Viral vectors possess high efficacy accompanied by stimulation of the immune systems which is a limitation of these systems to deliver nucleic acids and human use. Therefore, non-viral vector systems should overcome these adverse side effects and represent safer and more efficient alternatives with improved bioavailability and reduced cellular toxicity in the clinics (Akhtar *et al.*, 2000; Somia and Verma, 2000; Panyam and Labhasetwar, 2003). It has been shown that cationic polymers and lipid-based transfection reagents could elicit cellular gene expression changes and complexation with siRNA increased these changes (Omidi *et al.*, 2003; Omidi *et al.*, 2005; Fedorov *et al.*, 2006; Hollins *et al.*, 2007; Tagami *et al.*, 2007; Tagami *et al.*, 2008). Beyerle et al. analyzed the expression changes of genes related to cytotoxicity, inflammation and oxidative stress in a pathway focused qRT-PCR array system upon treatment with different PEI-PEG copolymers in murine lung epithelial cells (LA-4 cell line) and could show that PEGylated PEI copolymers altered the gene expression profile on cost of upregulation of genes involved in inflammatory and oxidative stress processes while PEI 25 kDa mainly induced genes related to cytotoxicity and apoptosis (Beyerle *et al.*, 2010a). In addition, the potential of PEI and PEI-PEG copolymers to induce DNA damage and therefore their genotoxic potential was investigated in a lung epithelial cell line derived from the MutaMouse, but no indication for

genotoxicity of PEI 25 kDa and PEI-PEG copolymers was observed (Beyerle *et al.*, 2011b). These investigations showed that PEI uptake causes cellular oxidative stress which affects the cytoplasmatic compartment with subsequent gene expression responses, but PEI not necessarily penetrate the nuclear membrane and cause DNA damage.

11. Conclusion

In conclusion, for development of safe and efficient non-viral vector systems a lot of investigations are needed before enter clinical trials. In our book chapter we mainly focused on PEI-related polymers for siRNA delivery to the lungs and gave an overview of the ongoing research in this field with a great focus on toxicity. To improve the toxicity profile of such carriers for pulmonary application one of the biggest challenge is to overcome the inflammatory response besides reduction of the overall cytotoxicity. Future studies should implement basic toxicity testing like evaluation of cytotoxicity (cell viability, LDH release, erythrocytes aggregation, apoptosis), inflammation (cytokine release, gene regulation, in vivo analysis of relevant tissues and cells or liquids), oxidative stress (lipid mediators, GSH levels) before extensively improving the efficacy of such carriers.

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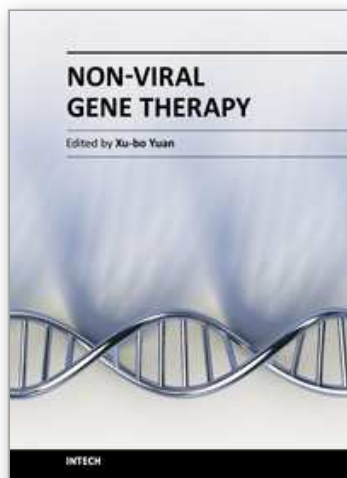
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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

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