European Journal of Pharmaceutics and Biopharmaceutics 77 (2011) 438–449

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Polyethylenimines for RNAi-mediated gene targeting *in vivo* and siRNA delivery to the lung

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ARTICLE INFO

Article history:
Available online 18 November 2010

Keywords:
Poly(ethylene imine)
PEI
Gene targeting
RNAi
siRNA
Lung

ABSTRACT

RNA interference (RNAi) is a promising strategy to inhibit the expression of pathologically relevant genes, which show aberrant (over-)expression, e.g. in tumors or other pathologies. The induction of RNAi relies on small interfering RNAs (siRNAs), which trigger the specific mRNA degradation. Their instability and poor delivery into target tissues including the lung, however, so far severely limits the therapeutic use of siRNAs and requires the development of nanoscale delivery systems. Polyethylenimines (PEIs) are synthetic polymers, which are able to form non-covalent complexes with siRNAs. These nanoscale complexes ('nanoplexes') allow the protection of siRNAs from nucleolytic degradation, their efficient cellular uptake through endocytosis and intracellular release through the 'proton sponge effect'. Chemical modifications of PEIs as well as the coupling of cell/tissue-specific ligands are promising approaches to increase the biocompatibility, specificity and efficacy of PEI-based nanoparticles.

This review article gives a comprehensive overview of pre-clinical *in vivo* studies on the PEI-mediated delivery of therapeutic siRNAs in various animal models. It discusses the chemical properties of PEIs and PEI modifications, and their influences on siRNA knockdown efficacy, on adverse effects of the polymer or the nanoplex and on siRNA biodistribution *in vivo*. Beyond systemic application, PEI-based complexation allows the local siRNA application to the lung. Biodistribution studies demonstrate cellular uptake of PEI-complexed, but not of naked siRNAs in the lung with little systemic availability of the siRNAs, indicating the usefulness of this approach for the targeting of genes, which are pathologically relevant in lung tumors or lung metastases.

Taken together, (i) PEI and PEI derivatives may represent an efficient delivery platform for siRNAs, (ii) siRNA-mediated induction of RNAi is a promising approach for the knockdown of pathologically relevant genes, and (iii) when sufficiently addressing biocompatibility issues, the locoregional delivery of PEI/siRNA complexes may become an attractive therapeutic strategy for the treatment of lung diseases with little systemic side effects.

1. Introduction

Since its discovery ~10 years ago, RNA Interference (RNAi) has been quickly recognized as an efficient strategy for the specific knockdown of any given target gene [1–3]. This is particularly attractive for the treatment of pathologies, which rely on the aberrant (over-)expression of functionally relevant genes. Even gene products, which are otherwise considered 'undruggable' by conventional means (e.g., low molecular weight inhibitors, therapeutic antibodies), can be specifically targeted and inhibited in their protein expression. Thus, among others, nucleic acid-based strategies may offer novel therapeutic approaches in various pathologies including viral diseases and cancer.

As an alternative to systemic routes of administration, the local delivery of therapeutic nucleic acids is possible, in the case of the lung as target organ through intranasal instillation or aerosol, and may offer an optimal approach to reach the airway and alveolar epithelium. Besides of maintaining high local concentrations, which is relevant if the lung is the target organ for pharmacological intervention, the pulmonary application may also be attractive for achieving systemic drug availability due to the large alveolar surface, the thinness of the epithelial barrier, the extensive vascularization, the low enzymatic activity in the alveolar space and the absence of a first pass metabolism. In the case of nucleic acids, those locoregional therapeutic strategies, however, still require a formulation in nanocarriers for efficient deposition and delivery into the lung tissue, and certain issues still need to be addressed.

Abbreviations: PEI, Polyethylenimine; siRNA, Small interfering RNA; RNAi, RNA interference.

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doi:10.1016/j.ejpb.2010.11.007

Those include effects like beating of the cilia/mucociliary clearance, interactions of the nanoparticles with the airway surface liquid, which covers the airway epithelial cells and possibly affects nanoparticle stability and nucleic acid uptake, the deposition of the nanoparticles in the correct lung areas, inefficient cellular uptake and intracellular release of the intact nucleic acids, and the uptake by alveolar macrophages.

In lung tumors as well as in other cancers, there is a strong need to develop specific, molecularly based therapies which, in contrast to classical treatment (surgery, chemotherapy, radiation), aim at exploring special biological of molecular features of the tumors (see e.g. [4,5]). Lung cancer is one of the most frequent tumors worldwide with regard to incidence rates and mortality. In Europe, 370,000 new cases are diagnosed every year, with ~70% being non-curable. Although the knowledge regarding the genetic and molecular basis of lung cancer has constantly increased, the median survival time of primarily non-curable tumors is still less than 12 months (see e.g. [6]). Furthermore, in many tumors outside the lung, e.g. colorectal carcinoma or melanoma, the formation of lung metastases is a major problem. Although the surgical removal of lung tumors is nowadays a standard therapy concept, only ~30% of all patients qualify for this treatment. The major problem is the insufficient treatment of this disease by local surgical intervention. Taken together, this emphasizes the need for the development of novel treatment concepts based on the inhibition of proteins, which are overexpressed in tumors and may be critically important for invasion and motility of tumor cells (e.g., matrix metalloproteases (MMPs), components of the extracellular matrix, or integrines), for tumor angiogenesis (e.g., vascular endothelial growth factor (VEGF) or fibroblast growth factors (FGFs) and their receptors) or for tumor cell proliferation (e.g., various growth factors like epithelial growth factor (EGF) or pleiotrophin (PTN), receptors of the HER family or protein kinase C). As stated earlier, this inhibition may be achieved through the selective knockdown of gene expression by RNAi.

2. RNA interference (RNAi) and siRNAs

RNAi is induced by 21–23 bp 'small interfering RNAs' (siRNAs), which are either delivered directly (see below) or processed from longer double-stranded RNA molecules (e.g. small hairpin RNAs, shRNAs) through the action of the enzyme complex 'Dicer'. Upon their incorporation into the RNA-induced silencing complex (RISC), they catalyse the sequence-specific cleavage of their target mRNA. To this end, the siRNA, which is single-stranded upon RISC activation, binds to its target mRNA by Watson–Crick base pairing, thus bringing RISC with its nuclease activity into close proximity to the mRNA. Since the subsequent cleavage leads to unprotected ends, the mRNA is rapidly degraded and thus unavailable for translation ([7–10]; see [11] for review).

RNAi is a naturally occurring mechanism and the cells provide all necessary components except for the siRNA and thus the therapeutic delivery of siRNAs is a necessary and sufficient condition for the induction of RNAi. Based on its mechanism of action, RNAi also allows the inhibition of otherwise 'undruggable' genes. This, however, relies on efficient delivery systems for either siRNAs or plasmids encoding for shRNAs, and this has been a major limitation for RNAi therapeutics [12].

3. Delivery of nucleic acids mediating RNAi *in vivo*

One approach for the delivery of viral or non-viral DNA-based expression is plasmids encoding shRNAs which, upon transcription, are processed to siRNAs [13–15]. For analytical purposes, i.e. for functional studies through permanent inhibition of a selected target gene and subsequent *in vivo* analyses, these plasmids can also be transfected ex vivo, creating stable knockdown cell lines (see e.g. [16] for Gα12/Gα13 knockdown in small lung cancer cells). In principle, the delivery of shRNA expression vectors can also be employed therapeutically *in vivo*. The transfer of DNA in gene therapy can be achieved through viral systems, which show high efficacy, but have major limitations with regard to immunogenicity, risk of insertional mutagenesis, large-scale production, loading capacity and often poor pharmacokinetics (see e.g. [12,17] and references therein, [18]). Thus, alternatively, non-viral vectors have been developed with the aim of condensing DNA for protection against degradation, for cellular uptake and intracellular release (see e.g. [19]). In addition to high bioactivity, the absence of cytotoxicity is of major importance.

In the case of the non-viral delivery of shRNA expression plasmids, these approaches may lead to a significant inhibition of the target mRNA (see e.g. [19]), but they may suffer from issues including non-specific toxic effects, low transfection efficacy and the unsolved safety concerns mentioned above. Thus, the direct delivery of siRNAs may be more promising, but critically relies on the protection of the instable siRNAs against degradation, efficient cellular internalization and intracellular release, as well as correct subcellular localization and release of the siRNA from its formulation. Furthermore, siRNA formulations need to be highly biocompatible and efficient and must exhibit favourable profiles regarding pharmacokinetics and toxicity. Comparable to DNA, various systems for the delivery of unmodified or chemically modified siRNA molecules *in vivo* have been described, which rely on the formation of nanoscale particles. These systems include the covalent conjugation to lipids, the covalent conjugation to peptides, aptamers or antibodies, the encapsulation in lipids, the complex formation with liposomes/lipoplexes or the complex formation with cationic polymers.

4. Polyethylenimines (PEIs) and PEI derivatives for nucleic acid delivery

Among polycationic polymers used for nucleic acid delivery, polyethylenimines (PEIs) take a prominent position based on their relatively high gene transfer efficacy, which was initially shown for DNA [20,21]. PEIs are water-soluble polymers with a high cationic charge density at physiological pH due to their protonable amino groups in every third position [22,23]. Since under physiological conditions, ~20% of the PEI nitrogens are protonated [23,24], this allows PEIs to form non-covalent complexes with nucleic acids and to change its degree of ionization over a broad pH range and dependent on chemical modifications [25]. PEI-based complexes ('polyplexes') are able to enter the cells via caveolae- or clathrin-dependent routes, with only the former leading to efficient transfection [26]. It was also shown that the mechanism of uptake is dependent on the PEI and the target cell [27]. Once internalized, the high gene transfer efficiency of PEI polyplexes is governed by their facilitated release from endosomes due to the so-called "proton sponge effect" [28,29] and, in the case of DNA, by the uptake into the nucleus [30,31].

Various PEIs and PEI derivatives have been used successfully as non-viral vectors for the *in vivo* gene transfer of therapeutic DNA molecules. This also includes delivery to the lung. The overexpression of the tumor suppressor gene p53 upon nebulization of PEI/DNA complexes led to a reduction of growth of metastatic lung cancer in a mouse model [32]. Using a reporter gene system, the same group reported optimal nebulization conditions and N/P ratios for maximum gene expression and found little systemic availability since no detectable expression was found in other organs [33]. This is in agreement with our data on siRNA delivery (see

below), indicating the absence of systemic delivery upon application of PEI complexes into the lung. Time course experiments of DNA expression in the lung, however, also indicated that peak levels were already reached after 24 h with subsequent reduction of expression levels, indicating a rather poor persistence of gene expression [33] as also shown by other groups [34]. Notably, this time course as well as the level of transgene expression seems to be dependent on the presence of CpG motifs. More specifically, a recent study demonstrated that, although equal DNA amounts were deposited in the lung after aerosol delivery, the luciferase expression 24 h after administration was 60-fold higher in the case of a PEI-complexed CpG-free luciferase plasmid when compared to a non-CpG-free PEI/pCMVLuc nanoparticle. However, the CpG-free luciferase plasmid was also taken up more extensively by cells in the bronchoalveolar lavage fluid and was cleared more rapidly from the lung, translating into less prolonged transgene expression [35]. Apart from the nature of the DNA, transfection efficacy as well as persistence of gene expression also seems to be strongly dependent on the mode of delivery. In general, intranasal administration, intratracheal instillation, aerosol delivery as well as intravenous injection have been employed for the delivery of PEI complexes and have been found to result in marked differences with regard to expression levels, persistence of gene expression and localization of transfected cells in the lung. Upon aerosolization of the complexes, longer persistence was observed [36] as well as a ~10-fold higher expression when compared to instillation [37]. Despite this fact, and although intratracheal instillation can hardly be envisaged to be a suitable administration route for human applications, it is still extensively employed in pre-clinical models for the assessment of vector efficacy and biocompatibility, as well as for functional studies. The mode of administration also determines which cells are transfected: While after nebulization of branched PEI/DNA complexes gene expression is mainly localized in the epithelial cells in the conducting airways including the peripheral airways [36,38], intravenous injection leads rapid crossing of the pulmonary endothelial barrier and to transfection mostly in the lung alveolar tissue [39–41].

Beyond the mode of administration, physicochemical properties of the PEI-based complexes, which may well be altered by the chemical nature of the PEI as well as by the conditions of complex preparation and delivery, play a pivotal role. The chemical structure (linear vs. branched) and the molecular weight of PEI, and in particular the N/P ratio during complex formation, determine the size and zeta potential of PEI-based complexes (see e.g. [21] for review). *In vitro*, complexes based on higher molecular weight PEIs display increased cytotoxicity, which is probably based on the formation of larger aggregates, while very low molecular weight PEIs show poor transfection efficacy [22,42]. In general, branched PEIs lead to stronger complexation and form smaller complexes, with the smaller size, dependent on the cell line, translating into reduced transfection efficacy *in vitro* [43,44]. Higher N/P ratios lead to smaller sizes and increased zeta potential of the PEI complexes, while the molecular weight of the PEI and of the nucleic acid does not play a major role [45,46]. Also important are buffer conditions during complexation, with complexes formed in low ionic strength conditions (water, 5% glucose) being smaller and physically rather stable when compared to PEI complexes prepared in 150 mM saline, which are larger and tending to aggregate [37,47]. PEI/siRNA complexes based on a low molecular weight branched PEI allowed lyophilization and reconstitution when formed in 5% glucose, but not in 150 mM NaCl [48].

While it has been extensively shown how complexation conditions alter complex properties and how this affects *in vitro* transfection activity, the effects of these parameters on the biological activity upon *in vivo* delivery are less clear and no direct correlations are observed. This indicates that for example PEI complexes may aggregate *in vivo* independently of their initial size after formation [44] and that in general *in vitro* data only poorly reflect the *in vivo* situation (see below). Important with regard to lung application, complexes formulated and nebulized under hypoosmotic conditions (i.e., distilled water) resulted in higher expression levels than complexes prepared in HEPES-buffered saline or isotonic 5% glucose. This effect, however, was also attributed to a temporary hypoosmotic shock induced by the water, which led to a temporary permeabilization of the epithelium thus enhancing complex uptake [37]. Likewise, upon intratracheal instillation in the mouse lung, complexes prepared in water led to higher expression levels than complexes prepared in 150 mM NaCl [34]. Conflicting results exist when comparing complexes based on linear vs. branched PEI, with results probably again being dependent on the details of complex formulation and on the mode of delivery [38,49–51]. Finally, aerosol application requires optimization of the nebulization process, with the addition of 5% CO_2, drying of the aerosol by silica gel or reducing the median mass aerodynamic diameter of the aerosol by interposition of an aerosol spacer leading to increased gene delivery [38].

If similar effects also exist for PEI-mediated siRNA delivery, and which of those are relevant for RNAi applications, remains to be elucidated.

As shown earlier, only certain PEIs are suited for *in vitro* transfection and *in vivo* applications (see e.g. [48,52,53]), and optimal transfection efficacies are achieved when the polymeric nanoparticles possess an overall positive charge, which allows them to bind to the negatively charged heparansulphate proteoglycans on the cell surface [54]. However, this may lead to major drawbacks regarding toxicity/biocompatibility, complex aggregation and undesired non-specific complex interactions with cellular and non-cellular components, particularly *in vivo* [54–56]. Adverse effects reported in the literature include liver necrosis and an age influence on lethality, activation of lung endothelium, adhesion of aggregated platelets and shock after systemic injection of higher doses [57]. Earlier studies showed that PEI complexes are able to stimulate the immune system and can cause various degrees of inflammation of the lungs [34,58,59]. A recent *in vitro* study focused on stress and toxicity pathways triggered by 25 kDa PEI-based vector systems to be used for pulmonary application. It was found that the intrinsic apoptotic pathway (mitochondrial signaling) was induced 24 h after treatment, probably caused by endosomal swelling and rupture upon endocytotic 25 kDa PEI uptake, followed by intracellular stress and mitochondrial alterations, and finally leading to apoptotic cell death at higher doses. PEG modifications (see below) reduced the 25 kDa PEI cytotoxicity but increased proinflammatory signaling, which may cause unwanted side effects related to respiratory and cardiovascular disorders [60]. However, despite moderate proinflammatory effects attributed to the PEG-PEI nanocarrier, no histological abnormalities were observed upon instillation, and a preliminary *in vivo* knockdown experiment suggested that PEG-PEI/siRNA complexes are promising nanomedicines for pulmonary siRNA delivery [61]. In another study, cytotoxic effects and inflammatory responses of two probably more biocompatible PEIs, the low molecular weight branched PEI F25-LMW (see below) and the linear jetPEI, which both have been used previously *in vivo* [53,62], were evaluated in three murine pulmonary target cell lines, the alveolar epithelial (LA4), the alveolar macrophage (MH-S) and the macrophage-monocyte-like (RAW 264.7) cell line. For both PEIs, cytotoxicity was detected most prominently in the alveolar epithelial cells, but only at higher doses. Cytokine responses in contrast were observed already at low PEI concentrations and could be divided into three groups, induced (i) by free PEI (IL-6, TNF-alpha, G-CSF), (ii) by PEI/siRNA complexes (CCL2, -5, CXCL1, -10), or (iii) unaffected by either treatment (IL-2, -4, -7, -9, and CCL3). It was

concluded that both PEIs represent powerful siRNA delivery tools with reduced cytotoxicity and minor proinflammatory potency even in the respiratory tissue, but that further *in vivo* investigations are warranted [63].

To improve the biocompatibility and/or to alter biological properties particularly *in vivo*, modifications to the PEI molecules have been introduced, which aim at the shielding of the PEI particle surface or the introduction of alterations in complex architecture and stability. Examples include the grafting of PEI with poly(ethylene glycol)/poly (ethylene oxide, PEO) [21,64–69], hyaluronic acid [70], chitosan [71,72], polyglycerol [73] or with various hydrophobic moieties (cholesteryl, cholanic acid, fatty acid residues, hydrophobic chains) [74–76]. Also, grafting of PEI with mono- or oligosaccharide units has been explored. Grafting with saccharides, which do not act as ligands for specific cell structures, led to PEIs with altered physicochemical properties, in particular surface charge [25,77], and allow the study of structure–function relationships for the transfection of cells without the involvement of specific recognition patterns. Recently, hyperbranched PEIs with various oligosaccharide architectures were described [78], and those (oligo)maltose-grafted PEIs mediate the uptake of free nucleotides and show altered properties *in vitro* and *in vivo* [25,78]. In contrast, the chemical coupling of galactose, mannose or lactose as ligands on PEI surfaces resulted in enhanced cell-specific uptake through selective binding [79–88]. Beyond that, other ligands to cellular surface structures, like peptides, proteins or antibodies have been extensively explored to increase the binding and internalization of the PEI complexes as well as their intracellular release from the endosomal compartment or nuclear import (see e.g. [55,89–96]). Non-covalent modifications of PEI complexes with liposomes have been described as well. These include the encapsulation of PEI complexes into various liposomes [97,98] or the formation of ternary complexes (lipopolyplexes) containing PEI and different liposomes [99–101]. Lipopolyplex formulations of PEI/DNA complexes combined with multivalent cationic lipids (DOCSPER, DOSPER) [101–103], or with a mixture of cationic or anionic and neutral lipids + cholesterol and PEG lipids [104] have been shown to be promising reagents for transfection or *in vivo* delivery of DNA.

Furthermore, the development of biodegradable PEI-based carriers may be beneficial to enhance bioactivity, increase biocompatibility and/or prevent accumulation especially upon multiple treatments. Examples of biodegradable copolymers, among several others, include poly(d,l-lactide-co-glycolide)-PEI (PLGA-PEI) [105,106], poly(propylene glycol) diglycidylether (PPGDGE)-PEI [107], polycaprolactone (PCL)-PEI [108], chitosan-PEI [109], PEI polyplexes bioreversibly crosslinked to PEG via a redox sensitive, biodegradable crosslinker [110] or PEIs reversibly crosslinked through reducible disulfide bonds [111].

5. PEIs for siRNA delivery *in vivo*

While PEIs were initially introduced as DNA transfection reagents, the PEI complexation of nucleic acids has more recently been extended towards the *in vivo* delivery of small RNA or DNA molecules including ribozymes [112,113], antisense oligonucleotides [113] and, most importantly, siRNAs (Table 1). This includes linear and branched PEIs as well as modified PEI derivatives. Studies aim at the analysis of biodistribution/pharmacokinetics upon PEI/siRNA application and at the therapeutic knockdown of pathologically relevant genes.

PEI/siRNA complexes based on a linear ~22 kDa PEI (jetPEI, *in vivo* jetPEI) were shown *in vivo* to exert low toxicity and little inflammatory responses, while exerting reporter gene knockdown in the lung or in brains of luciferase-positive newborn mice [114–116]. Bolcato-Bellemin et al. employed sticky siRNAs (ssiRNAs) and demonstrated that, when combined with linear PEI, the reversible concatemerization of siRNAs through annealing of the short sticky overhangs should be particularly suited for gene silencing in the lung [116]. One of the earliest studies on therapeutic PEI/siRNA delivery showed that the systemic application through intraperitoneal injection of the complexes comprising linear PEI and HER-2 specific siRNAs led to the knockdown of the tumor-relevant target gene in subcutaneous ovarian carcinoma xenografts with inhibition of tumor growth [53]. Other tumor-related studies relied on systemic or intratumoral administration of the PEI/siRNA complexes and demonstrated the inhibition of s.c. glioblastoma xenograft growth upon knockdown of the growth factor pleiotrophin (PTN) [46] or the inhibition of subcutaneous liver and lung xenograft growth upon knockdown of RecQL1 DNA helicase [117]. Likewise, the PEI/siRNA-mediated reduction of PTN expression led to decreased growth of orthotopic glioblastoma xenografts [46]. While this finding confirmed earlier results, which had shown that PTN is functionally relevant and rate-limiting in glioblastoma [118], a later study on the (ribozyme-mediated) simultaneous stable knockdown of PTN and its receptor, anaplastic lymphoma kinase (ALK), demonstrated enhanced anti-tumor effects upon this double targeting. In small cell lung carcinoma (SCLC), similar findings were reported for the parallel knockdown of the G proteins Gα12 and Gα13 [16]. This suggests that the combination of siRNAs targeting different genes may be even more efficient than single knockdown strategies. More recently, *in vivo* jetPEI was chosen by the RNAi company 'siRNAsense' as a delivery system for their synthetic siRNAs targeting tissue factor, to interfere with metastasis of melanoma, primarily by inhibiting circulating cancer cells' ability to attach to other cell membranes (Polyplus, Press Release).

Beyond tumor models, siRNAs complexed with the linear 'jetPEI'/'*in* vivo jetPEI' were used for the treatment of other pathologies as well. In a study on the post-exposure protection of guinea pigs against a lethal Ebola virus challenge, the i.p. injection of PEI/siRNA led to targeting of the polymerase (L) gene of the Zaire species of EBOV and to protective effects due to a significant reduction in plasma viremia levels [119]. Hassani et al. demonstrated that '*in* vivo jetPEI' efficiently delivered nucleic acids to cells, but, when used to introduce siRNAs into the mouse brain *in vivo*, did not lead to siRNA activity at least in the low dose ranges tested. This suggests an siRNA dose dependence, which is determined by the siRNA amount applied and the efficacy of PEI-mediated siRNA delivery [115]. Intraperitoneal application of PEI/siRNA complexes led to the downregulation of hypoxia inducible factor 1alpha and plasminogen activator inhibitor 1 and to a statistically significant reduction of post-operative abdominal adhesion formation [120]. In a physiological study, the knockdown of mouse anoctamin 1 (ANO1, TMEM16A) led to markedly reduced native Ca^{2+}-activated chloride currents and decreased saliva production, elucidating its role in various physiological processes [121]. Most recently, Kramer et al. employed PEI/siRNA complexes targeting FCgamma RIII in arthritic temporomandibular joints and demonstrated, upon local injection, reduced nociceptive and inflammatory responses [121].

With regard to the lung, the use of PEI/siRNA complexes has, beyond reporter gene studies (see above), also been explored for therapeutic applications. Ge et al. demonstrated that the administration of PEI-complexed siRNAs specific for the conserved regions of influenza virus genes allows the treatment and prevention of lethal influenza infections in the mouse. More specifically, upon administration of the complexes before or after initiation of the virus infection, reduced virus production in the lungs of infected mice was observed [122]. Concomitantly, the lung was identified as one preferential organ of PEI/siRNA complex delivery upon their intravenous injection [122], which was later on confirmed in

Table 1
Application of PEI/siRNA complexes to induce RNAi *in vivo*.

PEI/PEI derivative	Aim of study/disease/*in vivo* model	Target gene	Mode of administration	Reference
Branched PEI 25 kDa	SPECT and Real-time-γ camera imaging (biodistribution/ pharmacokinetics)	(Luciferase siRNA)	Intravenous	[127]
Branched PEI 25 kDa	Delayed progression of diabetes in accelerated diabetes animal model	Fas (CD95)	Intravenous	[126]
Branched PEI 25 kDa	Inhibition of s.c. prostate carcinoma xenograft growth	VEGF (PEGylated siRNA)	Intratumoral, intravenous	[129]
Branched PEI 25 kDa	Inhibition of s.c. pancreatic carcinoma xenograft growth	Mutant K-ras	Intratumoral	[128]
Branched PEI 25 kDa	Inhibition of melanoma lung metastases	Wilm's tumor gene 1 (WT1)	Local (lung) through aerosol	[151]
Branched PEI	Reduction of formalin-induced nociception	NMDA-R2B	Intrathecal	[125]
Branched PEI F25-LMW	Inhibition of s.c. prostate/pancreas xenograft growth	VEGF	Intraperitoneal	[62]
Branched PEI F25-LMW	Inhibition of s.c. pancreas xenograft growth	CUX1	Intratumoral	[131]
Linear PEI	Test for induction of inflammatory response	(Luciferase siRNA)	Intravenous	[114]
Linear PEI	Prevention of lethal influenza virus infections in the lung	Influenza virus genes	Intravenous	[122]
Linear PEI	Reduction of airway resistance in allergen-induced hyperresponsiveness	IL-13	Intravenous	[123]
Linear PEI	Inhibition of s.c. liver and lung xenograft growth	RecQL1 DNA helicase	Intratumoral	[117]
Linear PEI	Reporter gene targeting in the lung	Luciferase	Retroorbital	[116]
Linear PEI	Ca^{2+}-activated chloride currents/saliva production	ANO1	Retroorbital	[121]
Linear PEI	Inhibition of s.c. ovarian carcinoma xenograft growth	HER-2	Intraperitoneal	[53]
Linear PEI	Reduction of plasma viremia levels	ZEBOV L	Intraperitoneal	[119]
Linear PEI	Reduction of post-operative adhesion formation (uterine horns)	HIF-1alpha, PAI-1	Intraperitoneal	[120]
Linear PEI	Inhibition of s.c. and orthotopic glioblastoma xenograft growth	PTN	Intraperitoneal, intrathecal	[46]
Linear PEI	Reduced luciferase expression in Luc-positive newborn mouse brains	Luciferase	Intracerebral	[115]
Linear PEI	Reduction of nociceptive and inflammatory response in arthritic joints	FcgammaRIII (CD16)	Local injection	[155]
Deacylated linear PEI	Drop in influenza virus titer in the lung	Influenza nucleocapsid protein	Retroorbital	[124]
PEGylated branched PEIs	Biodistribution/pharmacokinetics	([32P]-labeled siRNA)	Intravenous	[68]
RGD-PEG-PEI nanoplexes	Inhibition of s.c. N2A neuroblastoma xenograft growth	VEGF R2	Intravenous	[90]
RGD-PEG-PEI nanoplexes	Biodistribution	(Luciferase siRNA)	Intravenous	[132]
Glycol chitosan/PEI	Reporter gene targeting in s.c. melanoma xenografts	RFP	Intravenous	[76]
Linear PEI-pullulan	Tissue uptake in liver and lung	(Fluorescein-labeled siRNA)	Intravenous	[156]
Hyaluronic acid (HA)-PEI	Inhibition of s.c. melanoma xenograft growth	VEGF	Intratumoral	[133]
(Oligo-)maltose-grafted PEIs	Biodistribution	([32P]-labeled siRNA)	Intravenous	[25]

biodistribution studies on a branched low molecular weight PEI ([62]; see below). This is mainly attributed to the fact that the lung is reached rather quickly after i.v. injection and that the physical properties of the complexes allow the efficient interaction with the pulmonary vasculature. Additionally, a certain degree of PEI complex aggregation under physiological conditions may be beneficial for efficient cell transfection in the lung. Indeed, anti-aggregant treatments (aspirin, EDTA, heparin or clopidogrel) decreased the PEI-mediated DNA transfection or siRNA delivery, supporting the hypothesis that platelets participate in the blocking of PEI complexes in the lung capillaries [57,68].

Intravenous injection was employed for targeting IL-13 as well. PEI-complexed, chemically modified siRNAs were administered to sensitized mice just before airway challenge with allergen, and the treatment significantly reduced airway resistance in the sensitized and challenged mice suggesting that PEI/siRNA-mediated IL-13 knockdown may prevent the induction of allergen-induced airway dysfunction [123]. Another study showed that systemic delivery of complexes based on fully deacetylated PEI resulted in a marked improvement of siRNA delivery to the lung as well as in reduced toxicity. This was demonstrated by the downregulation of luciferase as a model gene or of the influenza viral nucleocapsid protein gene with a subsequent 94% drop of virus titers in the lungs of influenza-infected animals [124].

Complexes based on branched PEIs were employed for *in vivo* siRNA delivery as well. The knockdown of the N-methyl-D-aspartate (NMDA) receptor subunit protein NR2B in a rat model was achieved through intrathecal injection of the complexes, leading to the modulation of pain. This study also demonstrated the receptor subtype specificity of the siRNA-mediated gene targeting and thus the advantages over less selective NMDA antagonists with regard to the absence of non-specific binding [125]. In an accelerated diabetes animal model, Fas (CD95) knockdown upon intravenous injection of PEI-complexed siRNAs led to delayed progression of diabetes [126]. By SPECT and real-time γ-camera imaging, the biodistribution and pharmacokinetics of siRNA complexes based on branched 25 kDa PEI were analysed upon intravenous injection [127], and intratumoral injection of PEI/siRNA complexes targeting mutant K-ras in subcutaneous pancreatic carcinoma xenografts led to anti-tumor effects [128]. Kim et al. employed modified (PEGylated) siRNAs for 25 kDa PEI complexation and observed an inhibition of subcutaneous prostate carcinoma xenograft growth upon intratumoral or intravenous injection [129].

In order to decrease toxicity and enhance bioactivity, Werth et al. described the purification of a low molecular weight polyethylenimine, PEI F25-LMW, from the commercially available 25 kDa PEI through size exclusion chromatography [48]. Complexes based on PEI F25-LMW can be stored lyophilized [48] or frozen [130], thus avoiding the need to prepare complexes freshly and allowing the preparation of standardized aliquots. Most recently, the intratumoral injection of PEI/siRNA complexes targeting CUX1 into subcutaneous pancreatic cancer xenografts demonstrated the functional relevance of CUX1 in pancreas carcinoma [131]. When used for the systemic delivery of siRNAs targeting the tumor-relevant growth factor VEGF, marked anti-tumor effects in s.c. prostate and pancreatic carcinoma xenografts in mice were observed upon

intraperitoneal, but not upon intravenous administration of the PEI F25-LMW/siRNA complexes. This effect was mirrored by the downregulation of VEGF expression, as demonstrated on mRNA and protein levels, and was comparable or additive to the parallel treatment with the therapeutic humanized anti-VEGF antibody bevacizumab (Avastin®) [62]. Biodistribution assays showed that the level of siRNA delivery into the tumor xenografts was indeed dependent on the mode of administration of the PEI/siRNA complexes and thus explained the differences in anti-tumor efficacy. Thus, the application strategy is critical for successful PEI/siRNA-mediated gene targeting, but also allows the preferred delivery of the complexes to certain organs. Regarding the lung, the administration through the tail vein, in contrast to intraperitoneal injection, resulted in high siRNA levels in this organ. These biodistribution patterns have also been analysed e.g. for various PEG-modified PEIs (see below).

6. PEI modifications for the alteration of *in vivo* siRNA biodistribution

Beyond changing their physicochemical properties, the PEGylation of PEIs has also been used for further ligand decoration to enhance the target cell specificity of siRNA effects. Self-assembling nanoparticles with the PEG chains additionally coupled to the RGD (Arg-Gly-Asp) peptide at their distal end were described by Schiffelers et al. (2004) as preferentially binding to the integrins on the tumor neovasculature. Consequently, the intravenous injection of these nanoparticles containing siRNAs directed against the VEGF receptor-2 (VEGF-R2) led to the selective uptake into subcutaneous tumor xenografts, and reduced tumor growth and angiogenesis was observed upon downregulation of the target protein within the tumor [90]. In a later study, the biodistribution upon intravenous injection was further analysed and compared with the cationic liposome formulation DOTAP/DOPE. Notably, the different carriers changed the intratumoral distribution of siRNA within the tumor [132]. Another tumor-homing nanosized carrier was generated from glycol chitosan (GC) polymer and PEI, modified with hydrophobic 5beta-cholanic acid. Due to their tumor-targeting ability, GC-PEI/siRNA nanoparticles led to significant inhibition of red fluorescent protein expression in tumor-bearing mice [76]. Likewise, hyaluronic acid (HA)-modified PEI was developed as a target-specific intracellular delivery system for siRNAs, based on the presence of HA receptors on tumor cells. Intratumoral injection of PEI-HA-based complexes delivering siRNAs directed against VEGF resulted in reduced growth of melanoma xenografts [133].

Independent of specific ligands, alterations in biodistribution have been observed already upon grafting of PEI e.g. with PEG or (oligo-)maltose. Those surface modifications often aim at the enhancement of the biocompatibility and reduction of toxicity of PEI-based complexes through covalent coupling with non-ionic, hydrophilic polymers (see [134] for review) or other chemical modifications (see e.g. [135]). Additionally, they may translate into increased efficiency for gene delivery *in vitro*, based on enhanced endocytosis or facilitated intracellular siRNA release, or altered pharmacokinetic properties *in vivo*. As mentioned above, the full deacetylation of PEI resulted in reduced toxicity and a marked improvement of siRNA delivery to the lung upon systemic delivery [124]. PEG-PEI copolymers condense nucleic acids due to the cationic charge of PEI, with the non-ionic PEG graft shielding the positive surface charge of complexes. This effect reduces non-specific interactions of the complex with biological structures [136], and DNA complexes based on some PEGylated PEIs have revealed prolonged blood circulation times when compared to non-PEGylated PEI, with this effect being dependent on the structure of PEG-PEI copolymer [137]. Furthermore, the systematic analysis of the influence of the PEG chain length and graft density on the physicochemical properties of siRNA-copolymer complexes *in vitro* and *in vivo* correlated the PEG-PEI structure to physicochemical and biological features of siRNA-copolymer complexes [61,66–68]. Again, the *in vivo* behaviour of PEI(-PEG)/siRNA complexes, which is also reflected by physiological properties including e.g. erythrocyte aggregation or induction of hemorrhage in the lung [68], cannot be fully deduced from physicochemical properties or from tissue culture data, indicating that the precise evaluation of delivery reagents requires *in vivo* experiments. More specifically, beyond their physicochemical and *in vitro* bioactivity characteristics, PEG-PEI/siRNAs complexes displayed marked differences *in vivo* with regard to biodistribution and efficacy of siRNA delivery to the lung and other organs, and optimal PEG-PEIs were identified based on siRNA biodistribution studies [68]. Biological effects like hemorrhage or erythrocyte aggregation, however, were also dependent on the complex amounts and N/P ratio used for complexation [68]. The same was true for the induction of immunostimulatory responses *in vitro*, as shown in a broad panel of pulmonary target cell lines (alveolar macrophage, macrophage-monocyte-like cells and alveolar epithelial cells). Despite this dose dependence, immunostimulatory or toxic effects were generally rather moderate under therapeutic conditions [63]. Nevertheless, gene changes *in vivo* have been observed by microarray analysis of tumors treated with branched or linear PEIs, which may contribute to siRNA off-target effects [138].

Comparable to PEG-PEIs, the grafting of PEIs with maltose, maltotriose or maltoheptaose has led to reduced cytotoxicity of those (oligo-)maltose PEIs, (OM-)PEIs, and their complexes, but also to poorer delivery of siRNAs and especially of DNA *in vitro*. However, upon their intravenous application in mice, marked differences in siRNA levels were observed for different (OM-)PEI/siRNA complexes, which were dependent on the pattern of (oligo-)maltose grafting. Notably, some modifications led to markedly reduced siRNA levels in the liver and increased uptake for example into skeletal muscle [25].

7. PEI-mediated siRNA delivery into the lung upon instillation

Various PEIs or PEI derivates have already been successfully employed as non-viral vectors with little immunogenicity for the *in vivo* gene transfer of therapeutic DNA molecules. Among others, this also included the intrapulmonary gene transfer [32,33,38,85,88,139–145]. The intrapulmonary overexpression of the tumor suppressor gene p53 upon nebulization of PEI/DNA complexes led to reduced growth of metastatic lung cancer [32]. Increased expression rates in alveolar epithelial cells were achieved through PEI modifications, e.g. through coupling of specific ligands [146] or particle adsorption of human insulin [147] or albumin [148].

Therapeutic RNAi effects in the lung were explored as well. The i.v. application of PEI-complexed siRNAs targeting influenza virus genes prevented a lethal virus infection in the lung [122]. Likewise, upon retroorbital application of siRNAs targeting an influenza nucleocapsid protein and complexed with deacetylated PEI, a reduction of the influenza virus titers in the lung was observed [124]. The knockdown of reporter genes upon retroorbital application was demonstrated for luciferase [115,116] and for EGFP [61]. The latter study also demonstrated the advantage of PEG-PEI/siRNA complexes for lung application over 25 kDa PEI/siRNA complexes. The same group described the nebulization of PEI/DNA complexes and identified ultrasound-based methods of nebulization as advantageous [141]. Comprehensive data regarding the optimized aerosol-based delivery of PEI/DNA complexes also exist from other studies [37,38]. Smaller PEI-complexed DNA

oligonucleotides were employed as antisense molecules for the knockdown of IL-4 to therapeutically interfere in asthma-associated inflammatory reactions [149].

Apart from nebulization strategies, the administration of PEI/siRNA complexes through instillation is an efficient strategy for pulmonary siRNA delivery. This is particularly true since more recent tissue distribution experiments demonstrated cellular uptake in the lung, but very little systemic availability, thus suggesting that knockdown effects will be confined to the lung upon pulmonary, PEI-mediated delivery. More specifically, a comprehensive, time-dependent analysis of the biodistribution of radiolabeled siRNAs complexed with the branched low molecular weight PEI F25-LMW [48,62,130] was performed upon instillation in mice (Lipka et al., submitted). Scintillation counting showed high tissue siRNA levels only in the lung, which remained almost constant over the whole observation period (8 h; see Fig. 1a). In contrast, in the case of naked siRNA levels were also high after 1 h, but markedly dropped thereafter, indicating poor cellular internalization of intact siRNAs (Fig. 1a, left panels). To further address this issue, a bronchoalveolar lavage was performed at 1 h, 3 h or 8 h after instillation and siRNA levels were analysed in the lavaged vs. non-lavaged lung as well as in the cells and in the supernatant of the lavage. The analysis of the lavaged lung confirmed the poor uptake of naked siRNA indicated by rapidly decreasing and after 8 h very low scintillation counts, as opposed to PEI/siRNA treatment where levels in the lavage supernatant remained constantly high. In sharp contrast, cellular uptake of siRNAs was only observed upon PEI complexation (Fig. 1b, right). While the lavaged cells may represent mainly macrophages, the constantly high siRNA levels in the lavaged lung observed upon PEI/siRNA treatment, but not in the case of naked siRNA, also indicate siRNA uptake into the lung tissue (Fig. 1b; see lavaged lung after 8 h).

To further address the question if scintillation signals really represent intact siRNA molecules rather than free [32P] label, RNA was isolated from the various tissues and analysed by gel electrophoresis and autoradiography of the blotted bands as described previously [53,62]. Bands representing full-length siRNAs showed a strong signal in the lung 1 h after PEI/siRNA instillation and a weak

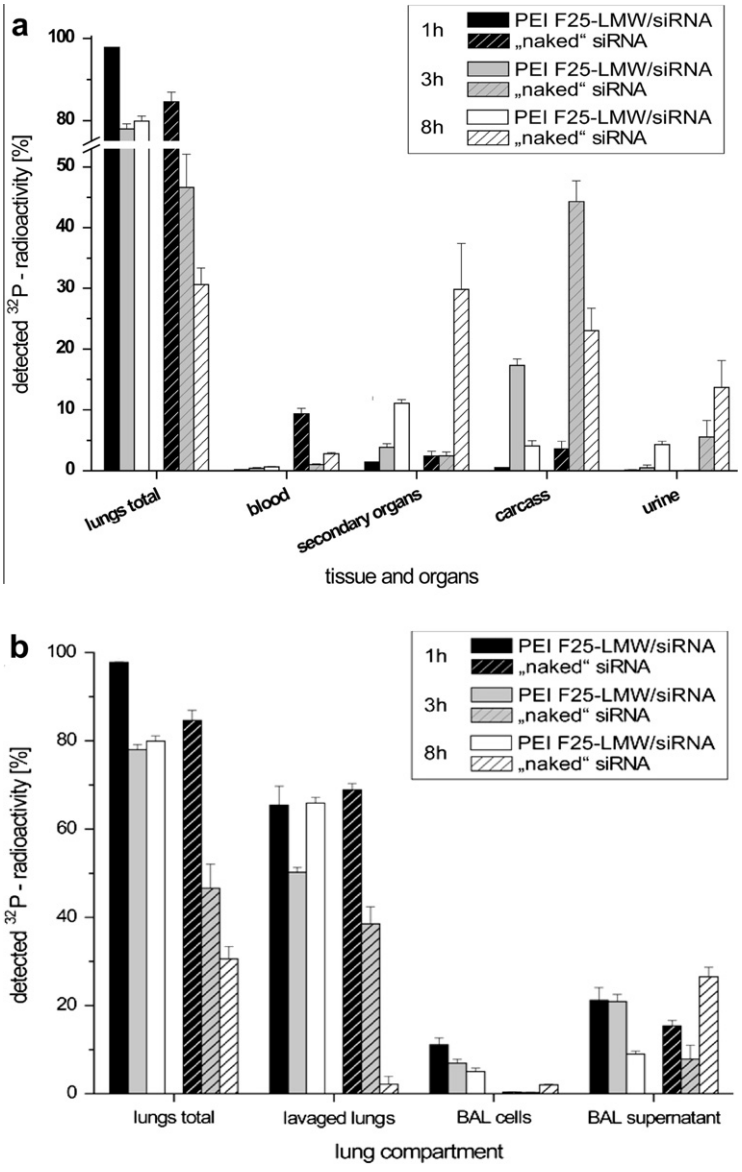

Fig. 1. (a) Biodistribution of [32P]-labeled siRNAs at different time points after instillation. PEI-complexed or naked siRNAs were directly applied into mouse lungs, and levels were determined by scintillation counting of various organs/samples as indicated in the figure. (b) Levels of [32P]-labeled siRNAs in the total vs. lavaged lung, and in the supernatant and the cells of the lavage.

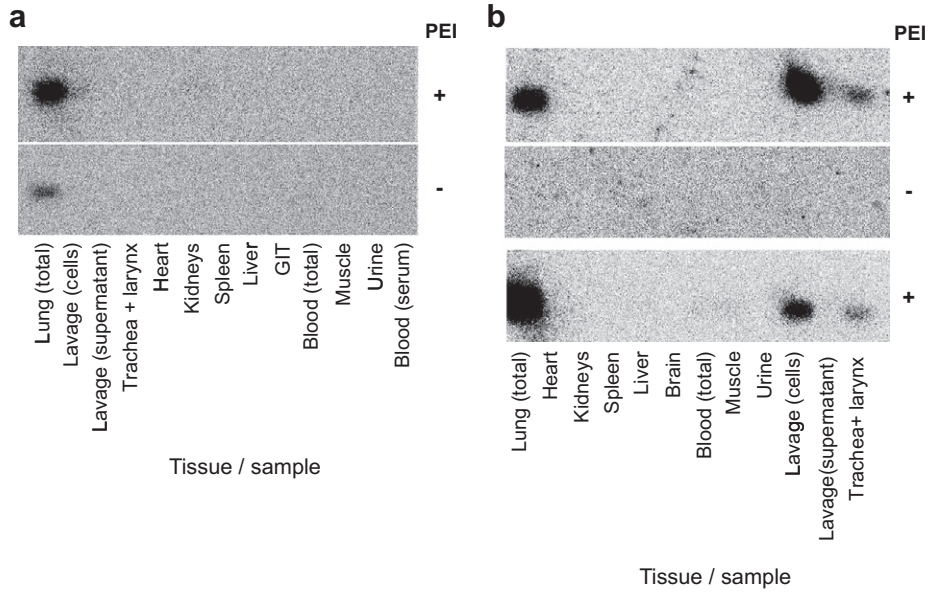

Fig. 2. (a) Levels of intact [32P]-labeled siRNAs at 1 h after instillation, as determined by gel electrophoresis and autoradiography in various organs/samples. Upper panel: PEI-complexed siRNA, lower panel: naked siRNA. (b) Biodistribution and cellular uptake of intact siRNA molecules at 3 h (upper panel) or 8 h (lower panel) after PEI/siRNA instillation, with peritoneal lavage prior to analysis. In contrast to the complexes, no bands are detected at 3 h after instillation of naked siRNAs (center panel).

band in the case of naked siRNA (Fig. 2a). A more detailed analysis at 3 h or 8 h after instillation confirmed the uptake of intact siRNA into cells of the bronchoalveolar lavage and the lavaged lung (Fig. 2b). Weak signals were also observed in the trachea/larynx, but not in any other tissue, again demonstrating the absence of systemic availability of the complexes. No signals at all were observed in the case of naked siRNAs, indicating that, without PEI formulation, no intact siRNAs were taken up by cells. Additionally, the comparison with Fig. 1a and b reveals that scintillation counting may also pick free nucleotides/free [32P] label after siRNA degradation and may thus rather over-estimate the cellular uptake of naked siRNA molecules. Since this may also be true for the microscopic analysis of the cellular internalization of fluorophor-labeled siRNAs, leading to results obscured by fluorescence signals from free nucleotides/free fluorophor, histological data should always be analysed with special care.

The PEI-mediated siRNA uptake and therapeutic efficacy in lung tumors has been demonstrated as well. Preliminary data from our group show that the knockdown of PTN through instillation of PEI/siRNA complexes in an experimental melanoma lung metastasis model leads to decreased metastasis burden (Günther et al., unpublished results). For more quantitative detection of metastases, we have recently established a quPCR-based method for the quantitation of genomic DNA from metastases vs. surrounding tissue [150], which will allow to precisely assess the effect of PEI/siRNA-based therapeutic interventions. Likewise, an aerosol-based delivery of PEI/siRNA complexes targeting the Wilms tumor gene 1 (WT1) resulted in a statistically significant reduction in the number and size of lung tumor foci in a melanoma lung metastasis mouse model. These effects were based on increased apoptosis in tumor cells and led to an enhanced survival of mice over control groups [151].

8. Conclusion and outlook

In the past years, RNAi has been shown to allow the efficient and specific knockdown of pathologically relevant genes, including the inhibition of otherwise 'undruggable' gene products. The therapeutic applicability and success of siRNAs, however, will largely depend on their efficient and safe *in vivo* delivery, while avoiding unwanted side effects. This is particularly true for the lung. In general, the studies and data reviewed in this paper establish (i) PEI and PEI derivates as efficient and biocompatible delivery platform for siRNAs, (ii) the siRNA-mediated induction of RNAi as a promising approach for the knockdown of pathologically relevant genes, and (iii) the locoregional delivery of PEI/siRNA complexes as an attractive therapeutic strategy for the treatment of lung diseases with little systemic side effects. Still, several issues will have to be addressed, which will be critical for the transfer of PEI-based systems into a safe drug formulation, especially with regard to the lung. While toxic and immunological reactions are rather low in the case of some PEIs, they are still present and need to be addressed. This applies to the polymer, to the nucleic acid as well as to the combination of both, i.e. the nanoparticle. The clearance of PEI from the lung tissue will have to be a matter of intense investigation. This is also true in the case of so-called biodegradable carriers, since, notably, the term 'biodegradable' often only refers to one or a few cleavage sites within a copolymer. On the other hand, the authors' own results showed that also upon repeated systemic application in mice no accumulation of PEI was observed, thus indicating an efficient elimination of PEI from the system. If this also applies to the lung, needs further investigation. The choice of the best mode of application will be dependent on the cell type to be hit in the lung, and, as pointed out above, with regard to efficacy and biocompatibility only certain PEIs will qualify for further development. Among those, linear jetPEI has already been successfully introduced into clinical studies for the delivery of BC-819, a plasmid, which is comprised of the H19 gene regulatory sequences that drive the expression of Diphtheria Toxin A (DTA) [152], and it is in the clinical trial stage for the treatment of ovarian cancer, pancreatic cancer and superficial bladder cancer. Likewise, PEI is explored in clinical studies for HIV immune therapy [153]. Also, a stable formulation is a central pre-requisite for any (PEI-based) nanocarrier to be considered a drug. While aggregation is a major issue here, some studies have demonstrated that certain PEI complexes, upon lyophilization or freezing, can be stored for a prolonged time while preserving their physical stability and biological

activity [48,130,154]. The addition of copolymers or ligands may enhance their biocompatibility, alter pharmacokinetic properties and increase their tissue specificity; however, any modification adds further complexity to the system. The direct application of the complexes through inhalation may be preferential in many ways, especially with regard to a direct delivery and the largely absence of systemic bioavailability, but will require that PEI complexes efficiently overcome the extracellular barriers in the lung. To sufficiently address these issues, further studies in appropriate animal models are warranted.

Statement

We hereby state that the own work described in this article was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

Acknowledgments

The own work described in this review was supported in part by grants from the Deutsche Krebshilfe to A.A. and from the Deutsche Forschungsgemeinschaft (DFG Forschergruppe 627 'Nanohale' to A.A. and to W.K.).

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