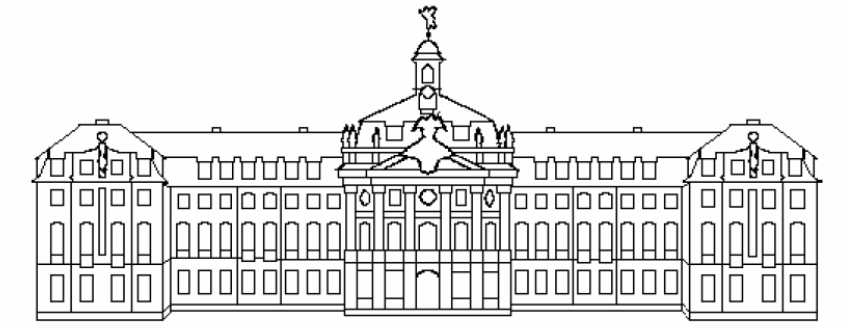

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Gene delivery to murine pulmonary epithelium by nonviral gene transfer agents

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LIST OF ABBREVIATIONS

AM - alveolar macrophage

AT I - alveolar epithelial cells type 1

AT II – alveolar epithelial cells type 2

brPEI - branched polyethylenimine

BW – body weight

DEP - diesel exhaust particles

DLPC - beclomethasone dipropionate (Bec)-dilauroylphosphatidylcholine

DOPE - 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine

DOTAP - 1,2-Dioleoyloxy-3-trimethylammoniumpropane chloride

DOTMA - N-[1-(2,3-Dioleyloxy)propyl]-N,N,N-trimethylammoniumchlorid

GTA - gene transfer agent

INF 7 – fusogenic hemagglutinin-derived peptide (23 aminoacids)

MAC- macrophage

MPO - myeloperoxidase

PAMAM - polyamidoamine

pDNA - plasmid DNA

PEEP – positive end-expiratory pressure

PEG – polyethylenglycol

PEI - polyethylenimine

pKa - acid-base ionization/dissociation constant

PLL – poly-L-lysine

PMN - polymorphonuclear cell

RT-PCR – real time polymerase chain reaction

SCID – severe combined immunodeficiency disorder

SPLL – super branched poly-L-lysine

1 INTRODUCTION

1.1 Gene therapy

Gene therapy is a treatment method based on nucleic acid delivery into the cell aiming to correct a variety of disorders including hereditary diseases. Unlike standard therapeutic methods dealing mainly with symptoms and consequences of gene-based disease, this approach is characterised by it's directivity towards the correction of the reason of the disease, e.g. the gene failure or deficiency (179).

To date several strategies for compensation of gene failure are developed. Absence of a functional gene caused by mutation may be compensated by introducing the wild type allele into cell. Gene vectors based on bacterial plasmids provide the delivery of functional gene into the cell, where it is transcribed thus restoring the missing protein. Besides, functional gene may be cloned into a viral vector (146). Gene delivery with viral vectors can provide not only DNA delivery into the nucleus but also insertion of a therapeutic gene copy into the host chromosome via random integration. This provides long term transgene expression also in subsequent cell population Besides, lots of efforts are devoted to development of viral vectors for site-specific integration (215). A separate branch in gene therapy research is represented by antisense therapy, where nucleic acid sequences (either RNA or gene constructs bearing corresponding DNA) are introduced into the cell to bind to certain mRNA molecules thus silencing them and preventing the expression of the protein (152). Apart from in vivo gene delivery methods described above, ex vivo gene transfer may be used for correction of a wide range of hereditary diseases. The main principle of this approach includes transformation of the cells outside the host with the following reimplantaion to the patient. Both high transformation efficiency and selection and multiplication of sucsessfully transformed cells may be achieved via ex vivo gene transfection (102). A certain number of gene delivery approaches developed to date have proved their efficiency on animal studies, while several have been accepted for clinical trials.

1.2 Clinical progress in gene therapy

Successful treatment of a patient with hereditary immunodeficiency (SCID), performed in 1990 by W.F. Anderson and his colleagues in U.S. National Institute of Health is considered to be the first milestone in gene therapy (32). Ex vivo gene transfer was performed using retroviral vectors, which still remain one of the basic trends in gene delivery.

Nowadays almost half of all gene therapy clinical trial test viral vectors, mainly adenoviruses and retroviruses. These vectors are tested intensively for different kinds of

cancers as well as for monogenic diseases like SCID and showed relative success in the USA, Britain, Italy and Japan (82). A clinical study for treatment of retinal disease using adeno associated virus (AAV) carrying RPE65 gene was started in 2007 in London, showing certain positive results and no side effects for the patient (125). Besides, viruses are applied successfully to treat pulmonary cancers. Fischer et al. showed that aerosol delivery of viral particles bearing reporter gene can provide high transgene expression with minimal associated inflammatory response (62). Adenoviral delivery of IL-12 gene via instillation could inhibit osteosarcoma metastases growth in the lung (95). Several combinations of inhalation therapies appear to be mostly promising.

Clinical trials with nonviral gene delivery systems involve mainly lipid-based GTAs (lipofection). Gene carriers of this group underwent first phase of clinical trial in USA, devoted to targeted delivery of HLA-B7/Beta 2-Microglobulin gene into the tumors for treatment of malignant melanoma. Besides, a clinical study for treatment of chronic renal insufficiency is carried through phase I (167). Phase-I immunotherapy study of IL-2-expressing allogeneic tumor cells as a vaccine in patients with progressive renal-cell carcinoma takes place in Germany (205).

Great attention is paid to delivery of naked plasmid DNA via physical methods like gene gun or electroporation. Hydrodynamic delivery combined with artificial ischemia was shown to deliver efficiently gene construct containing dystrophin gene into the muscles of Duchene muscular dystrophy patients (16).

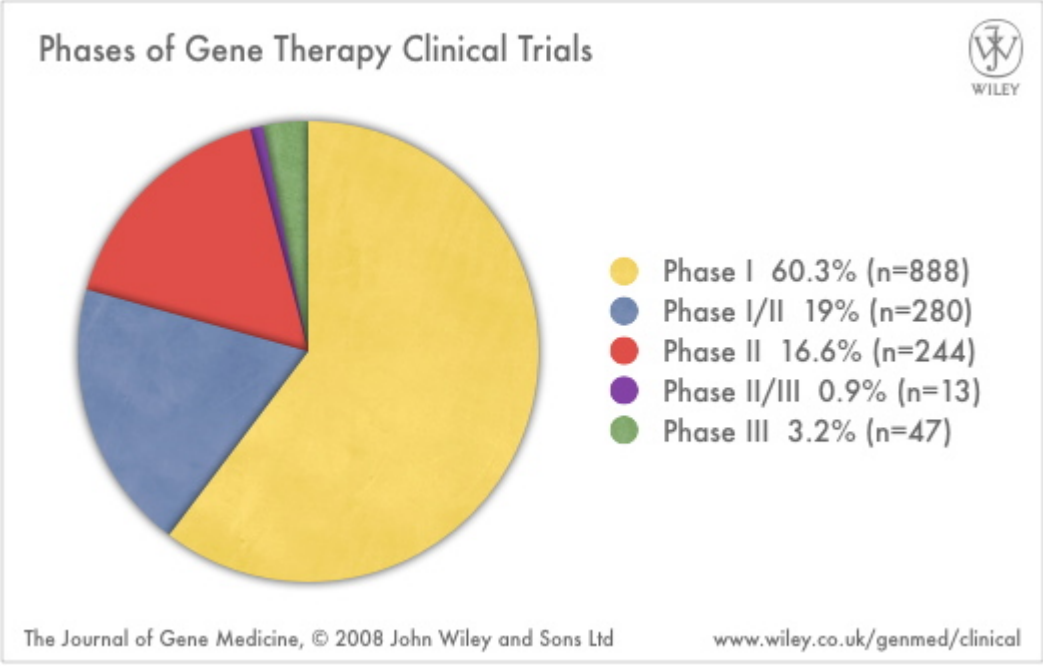

Figure 1. Phases of gene therapy clinical trials in 2008 worldwide

Antisense therapy approach is intensively investigated for treatment of cancers (including lung cancer, colorectal carcinoma, pancreatic carcinoma, etc.) malignant glioma and melanoma, diabetes, ALS, Duchene-Becker muscular dystrophy, as well as for diseases with inflammatory component, such as asthma and arthritis. Also short interfering RNAs (siRNAs) appear to be very promising. In 2003 this concept was proved to be perspective for treatment of Huntington's disease (109).

Most potential therapies have not yet produced significant clinical results, though one antisense drug, fomivirsen (marketed as Vitravene), has been approved by the US Food and Drug Administration (FDA) as a treatment for cytomegalovirus retinitis (189).

The majority of clinical studies are devoted to different cancers and cardiovascular diseases, while less attention is devoted to infectious and monogenic illnesses (Fig. 1). In total, almost 1500 gene therapy clinic trials now take place worldwide.

1.4 Gene delivery to the lung

Depending on the disease, gene delivery has to be performed to specific organs or tissues of the patient. Gene transfer to the lung is required for treatment of a wide variety of respiratory diseases. Not only hereditary disorders like cystic fibrosis or surfactant protein B deficiency may be treated via gene delivery, but also illnesses with genetic component like asthma and emphysema, as well as other somatic respiratory disorders, e.g. acute lung disease or SARS (acute lung respiratory syndrome)(4, 159). Besides, gene vaccination against certain infections may be performed via respiratory route (2).

Approximately twenty different cell types may be found in adult mammalian lung. The nature of an illness defines the target cell types for gene delivery. For treatment of SP-B deficiency gene delivery to the alveolar type II cells is required, which produce and excrete surfactant proteins. Transfection of airway epithelial cells is needed for treatment of cystic fibrosis patients to normalize the water-salt balance within the lung. Several populations of stem cells are to be found within the lung, e.g. BASC cells, located on broncho-alveolar junctions, or NEBs-the stem cells of sub mucosal glands (99). Depending on the cell type, involved into a certain disease, transfection of different stem cell populations of the lung would be favorable. Successful gene transfer to these cells would provide gene correction of all subsequent cell pollutions and could provide long-term therapeutic effect (98).

Efficiency of a certain gene transfer method is defined by the target cell type. Effective transfection of endothelial cells may be achieved through intravenous delivery,

while for lung epithelial cell types topical administration was proved to be most effective (227).

Administration route influences not only the toxic effects and immune response but also the clearance pattern of administered gene vectors and thus is extremely important for gene transfer efficiency. Intravenous administration encounters many physiological and mechanical barriers (107), which may be bypassed by direct administration into the lungs. Aerosol delivery, as well as intranasal instillation is also applied successfully to transport gene constructs into the lung (168). Gene transfer via aerosol was proved to be highly efficient in delivering certain therapeutic substances as well as gene constructs into the pulmonary area (70, 99, 219). Intratracheal instillation is a widely known method of pulmonary administration of therapeutic agents. This route was also shown suitable for delivery of gene vectors in high amounts, which provided higher gene transfer efficiency than aerosol but caused severe inflammation (34, 50). Still, despite its efficiency instillation is reported to cause swelling and cell damage within the lung along with neutrophils infiltration and strong immune response.

1.5 Thresholds to successful gene delivery into the lung

To deliver vectors into the lung cells successfully gene transfer agents have to overcome certain limitations. Apart from penetration into the cell and escape from the endosomes, which comprise a certain bottleneck for every GTA, pulmonary gene vectors have to withstand shear forces arising during aerosol gene delivery. In case of instillation uneven distribution of the liquid within the lung is a serious hindrance to successful gene delivery. Besides, DNA-containing particles have to escape lung resident phagocytes – macrophages and neutrophils. To enter the target cells gene constructs have to bypass the mucus layer covering the conducting airways. Afterwards, delivery of the particles into the target cell type gene has to be carried out.

Shear forces arising during aerosolization depend on the nebulization device and method, as well as on breathing depth and frequency. Besides, physical characteristics of lung functioning, like lung size and architecture, number of branching orders and the diameter of the airways also influence the efficiency of DNA delivery (140). Mechanical influence of the shear forces may destroy or damage the particles, thus decreasing the gene transfer efficiency. Dames et al. showed that application of magnetic field during aerosol transfection with paramagnetic particles allows obtaining equal distribution of GTAs within the desired lung region and provides high levels of transgene expression (33).

The **mucus layer** represents a serious barrier for GTAs. Its thickness varies between different regions of the lung and comprises in healthy individuals 10-30μm in trachea and 2-5μm in bronchi (216). Respiratory mucus contains 90-95% of water and approximately 5% of protein component, consisting of negatively charged mucins and other proteins, e.g. albumin, proteases, antiproteases (30, 120). Experiments involving particles of different origin and diameter showed that pulmonary mucus forms within the lung a three dimensional network with a mesh size of approximately 150 nm (177). Mucus layer not only prevents further movement of GTAs mechanically, but also changes the surface characters of the particles via binding of non-cross-linked macromolecules (174). The **surfactant layer** in alveolar region may as well retain incoming particles. Beating of alveolar type II cells microvilli cause constant movement of the surfactant layer towards esophagus providing its renewal, so that entrapped particles are removed form the lung (6). Certain increase in gene delivery efficiency may be obtained using mucolytics and mucus liquidising agents (18, 36). Besides, PEGilation of gene delivery vehicles decreases their binding to the mucus layer (115).

Alveolar fluid covers the alveolar region and may prevent gene transfer particles from penetration into the cell. Thickness of this layer of liquid may vary from 0.2 to 0.9 μm (76). It was shown that lipids and proteins of BALF interact with the incoming complexes. Negatively charged lipids may cause disintegration of lipoplexes thus making plasmid DNA accessible to nucleases (53). Nevertheless, synthetic gene delivery dendrimers PEI and PAMAM were proved to be resistant to pulmonary surfactant components (170). Intensive interaction with BALF proteins was showed for lipoplexes, leading to significant increase in their diameter, as well as to certain decrease in surface charge and transfection efficiency (169).

Alveolar macrophages (AM) represent a population of actively phagocyting lung resident cells. Incoming GTAs may be opsonised with surfactant proteins and immunoglobulins and than taken up macrophages. Nevertheless, AM were proved to be a highly specialized cell group which can actively take up both opsonised and naive particles (72). It was shown that the rapidity of particle clearance depends not only on their number and distribution within the lung, but also on their size. In particular, size threshold between micro- (> 100 nm) and nano- (<100 nm) particles plays an important role defining the dynamics and intensity of particle clearance (139).

1.6 Advances in gene delivery to the lung

Many efficient methods of pulmonary gene delivery exist today, involving different carriers and administration strategies.

Among existing non-invasive DNA transfer strategies, aerosol delivery is considered to be mostly safe; a lot of efforts are applied to raise the efficiency of this procedure. Already in 1994, nebulisation of plasmid DNA compelxed with lipoplexs like DC-cholesterol or DOTMA/DOPE was shown to result in prolonged transgene expression in mice (3, 23). Recently, a promising agent was developed, bis-guanidinum-tren-cholesterol (BGTC), able to transfect the lung efficiently via both instillation and aerosol application (41). The toxicity arising because of aerosol administration can be reduced by modifying the applied device. Deshpande et al. demonstrated that pDNA administered using AERx delivery system can withstand shear forces and provide transgene expression with minimal toxicity (42). Polymer **Polyethylenimine** (PEI), which will be described in detail below, can bind DNA efficiently and shows low toxicity when inhaled. Considerable advances were achieved with this polymer in the field of anti-cancer pulmonary gene therapy. Injections of its complexes with pDNA coding for p53 gene reduced tumor growth significantly. Also aerosolization of such complexes was beneficial for treatment of lung tumors (40). Aerosol delivery of DLPC liposomes with DNA coding for topoisomerase 1 inhibitor, prevents metastases in the lung and growth of subcutaneous tumors. This approach had recently entered the phase I clinical trial (108).

1.7 Gene constructs for gene delivery

Gene construct (or gene vector) is a molecule of DNA or RNA containing a selection marker or therapeutic gene. A gene of interest is connected to a 5'-regulatory area (promotor), which is supposed to control the intensity of transgene expression within eukaryotic cells. Besides, gene vectors usually contain regulatory elements and selection regions, which allow multiplication of plasmids in bacterial cells. In the last decade gene transfer systems were optimized via introduction of viral promoters as regulatory elements for therapeutic genes. Promotors from cytomegalovirus or Simian virus were shown to increase significantly the transgene expression (160).

For gene delivery with viral vectors 5'-regulatory area and the gene of interest are inserted into the viral genome. Retroviral and AAV-based vectors bear the integration elements within their genomes, which can provide specific or random integration into the eukaryotic chromosome (185).

For non-viral gene delivery non-integrating (transitive) vectors are used, based on bacterial plasmids. These vectors do not possess structures required for integration into the host genome, so that this genetic material remains exogenous within the nucleus. In nonviral vectors a gene of interest is cloned downstream of a suitable promoter. Besides, the construct may contain additional regulatory elements. Different reporter genes, e.g. fire-fly luciferase of beta-galactosidase are used in experimental work instead of therapeutic genes to analyze the efficiency of gene delivery and distribution of transgene product. It was shown that incorporation of introns into the coding DNA sequence may increase the transfection efficiency (89). Usefulness of polyadenilation signals (polyA) insertion was proved to enhance transgene expression by numerous studies (223).

1.8 Non-viral gene delivery systems

The main aim of a gene delivery system is to provide, firstly, efficient transportation of gene vector into the target cells and, secondly, penetration of exogenous DNA into the nucleus and expression of transgene. Wide variety of gene delivery strategies was exists nowadays. Gene transfer agents differ in their efficiency and safety, each possessing its unique combination of advantageous and negative features.

1.8.1. Delivery of naked DNA

Administration of naked DNA is considered to be the oldest gene delivery approach. Fazio et al. showed in 1995 that intraperitoneal and intravenous injections of plasmid DNA result in transcription and synthesis of transgene product (58). First significant result after intramuscular injection of naked plasmid DNA bearing beta-galactosidase gene was achieved by Wolf et al. They showed that pDNA may remain within the muscle several months after administration, being transcribed and providing synthesis of the reporter product (217). In 1996 a method of gene vaccination was developed, suitable to obtain anti-viral immune response after administration of pDNA coding for viral capsid protein.

Delivery of naked plasmid DNA would be favorable not only for therapeutic purposes like correction of gene deficiency, but also for development of systemic immune response required for gene vaccination. Lowest possible immune response should be provoked in the first case, while for the latter an efficient balance between safety and efficiency is required (202). Successful gene vaccination was shown via intradermal delivery on mice (203).

The main disadvantage of naked DNA delivery in therapeutic purposes is the low transfect ion efficiency, which in 1997 enhanced the search for agents which could protect

DNA form intracellular degradation (71). To improve the efficiency of naked pDNA delivery many invasive (e.g., gene gun) and noninvasive methods (liquid jet injectors, electroporation, sonoporation, magnetofection) were developed.

1.8.2 Mechanical gene delivery strategies

Gene gun is a promising method for both gene delivery and DNA vaccination. A load of gold colloid particles is involved, which is covered with naked or complexed DNA and may be activated with compressed helium (92). This method provides effective DNA delivery into epidermal and intradermal layers of the skin. Gold particles under pressure penetrate into the cells, so that DNA is delivered directly into the cytoplasm. Although slight tissue necrosis and damage are observed after this procedure, gene gun method is considered to be less invasive than subcutaneous injections. Besides, non-severe tissue damage may act as maturation signal for dendritic cells, thus increasing the effectiveness of DNA immunization (81). Gene gun was applied successfully for gene delivery into skin and muscles of rodents, dogs and cattle (104). Successful immunization of cows was reported, resulting in high antibody titters and INF-gamma secretion by peripheral lymphocytes. One of the main disadvantages of this method is that only limited amounts of DNA can be precipitated on gold particles. Besides, sensitive and vitally important muscles like heart and diaphragm may not be transfected with gene gun because of inflicting tissue damage.

Liquid Jet Injection implicates fine high-pressure stream, which is created by forcing the liquid though a syringe orifice by gas under high pressure. Depending on the device, pressure and orifice diameter, DNA may be delivered subcutaneously, intramuscularly or intradermally. Reports about liquid jet efficiency for DNA vaccination are contradictory; still, this method provides antibody responses compared to those after needle injections (204). This method is regarded to be perspective for transfection of vast tissue areas, for example, skin and skeletal muscles. Application of liquid jet injectors allows penetration into the deep dermal layer without causing severe tissue necrosis (31). In a clinical study devoted to DNA vaccination a group of volunteers preferred liquid jet injection to syringe, as it was less harmful and caused milder adverse reactions (51).

As the cell membrane is negatively charged, penetration of naked DNA or complexes with negative zeta-potentials into the cell is complicated and may be overcame via modification of complexes (e.g., with ligands to cell surface receptors) or using physical methods. Electroporation is among the most intensively investigated techniques for DNA

delivery for both in vitro and in vivo applications. Utilization of electric pulses with controlled duration, polarity and frequency permits penetration of plasmid DNA into the cells and, seemingly, forwards it towards the nucleus (86). Different configurations of electrodes have been designed for application on tissues with certain structure and location. This method was proved to achieve high cellular infiltration being perspective for both gene delivery and DNA vaccination. Babiuk et al. showed that electric impulses applied on swine muscle after plasmid injection enhance the expression of transgene protein (7). High efficiency of electroporation was shown on sheep model, where increased levels of transgene GFP protein were observed after using a syringe electrode device (180). Safety issues proved the method to be less invasive and causing only short time irritation. Still, configuration of electrodes and the pattern of electric impulses have to bee optimized for every application.

Although physical gene delivery strategies can provide transportation of plasmid DNA into the target tissue and certain transgene expression, their efficiency on the background of invasiveness and safety appears to be rather low. An alternative, highly effective trend in gene delivery is represented by viral gene delivery systems, which can efficiently modify the genetic status of the target cell.

1.8.3 Viral gene delivery vectors

Natural and highly effective mechanisms of penetration into the cells comprise the main privilege of viruses for gene delivery. Besides, certain viruses can provide integration of their genetic material into the host genome, meaning long term expression of transgene in subsequent cell populations. The most important characters of viruses used for gene delivery are the capability to transduce both proliferating cell lines and non-proliferating cells in culture, as well as targeted delivery into certain cell types. High immunogenicity is considered to be the main disadvantage of viral vectors. Absence of with site-specific integration capabilities in viral vectors reduces their applicability in vivo because of high risk of insertional mutagenesis.

The majority of viral gene delivery vectors for gene delivery in to the lung developed to date were created on the base of retro-, adeno- and adeno-assosiated viruses (200).

The genetic material of retroviruses is represented in form of RNA molecule, which DNA copy is supposed to be introduced into the host genome via reverse transcription. These vehicles represent the greatest success of gene delivery achieved to date, namely correction of X-linked severe combined immunodeficiency (X-SCID) (28). The successful integration of

retroviral genetic material into host chromosome provides stable transgene expression in all descendants of the cell. Nevertheless, random integration may lead to insertional mutagenesis and provoke cancer. This risk represents the main hindrance to utilization of these vehicles for therapeutic purposes. Attempts are being made to control the integration site. Introduction of certain sequences such as beta-globin control region into the retroviral genome is reported to direct the site of integration towards specific chromosomal sites (19).

Lentiviruses (for example, HIV virus) belong to a family of retroviruses. They can successfully transfect both proliferating and non-proliferating cells. Vectors based on these viruses may provide persistent (up to 6 month) transgene expression in slowly renewing cell populations (hepatocytes, myofibers). High rates of immune system recovery were observed in more than twenty patients, treated in France and Great Britain. Unfortunately, the main risk of insertional mutagenesis developed in four children provoking leukemia. Nevertheless, several clinical trials are being carries out in USA, Britain, Italy and Japan. Latest achievements in controlling of DNA integration sites should increase the safety of these effective gene delivery vehicles (176).

Application of zinc finger nucleases (ZFNs) is considered to be very promising. Viral genome encoding for combination of custom-designed zinc fingers with endonuclease offers an opportunity to induce site specific double strand breaks in genomic DNA, thus enhancing local homologous recombination by several orders of magnitude (78). Also ZFN-encoded plasmid-based approach was proved to have certain advantages.

Adenoviral vectors represent their genetic material in form of double-stranded DNA. Entering the host cell, they introduce their DNA into the nucleus without incorporation into the host genome (Fig. 2). As a result, viral genetic information may become damaged and destroyed with time, causing the necessity for repeated administrating of the vector. Although there vehicles do not represent insertional mutagenesis risk, their immunogenicity may cause complications during repeated administrations (45).. Besides, low efficiency of penetration into proliferating cells reduce their applicability for gene therapy treatment (188). Nevertheless, despite the disadvantages described above these vehicles are reported to be a powerful gene delivery tool. Certain progress was observed in clinical trial devoted to cancer treatment (239,163).

Adeno-associated viruses (AAV) belong to a parvovirus family and are well known for their ability to transfect non-proliferation cells efficiently. Their genome is represented as single-stranded DNA molecule. Although viruses of this group can introduce their genetic material into the specific site on chromosome 19. Nevertheless, preparation of viral vectors requires removal of all viral genes including Rep gene which is mandatory for this ability and thus leads to loss of this function.

A class of recombinant viruses has been developed, which genomes fuse at the ends via the ITR (inverted terminal repeats) recombination to form circular, episomal forms which are predicted to be the primary cause of the long term gene expression (163). The main disadvantages of these vehicles are the small amount of DNA they can transport and production technology, rather complicated and demanding. Because of their ability to transfect non-dividing cells these vectors are supposed to be especially favorable for gene delivery into the muscle and brain (into the neurons). Several clinical trials are being carried out in the moment, mainly in USA (21).

Aiming to unite positive qualities of different viruses, artificial gene delivery systems based on viruses are created. Artificial viral vectors are supposed to be able to enter a wider

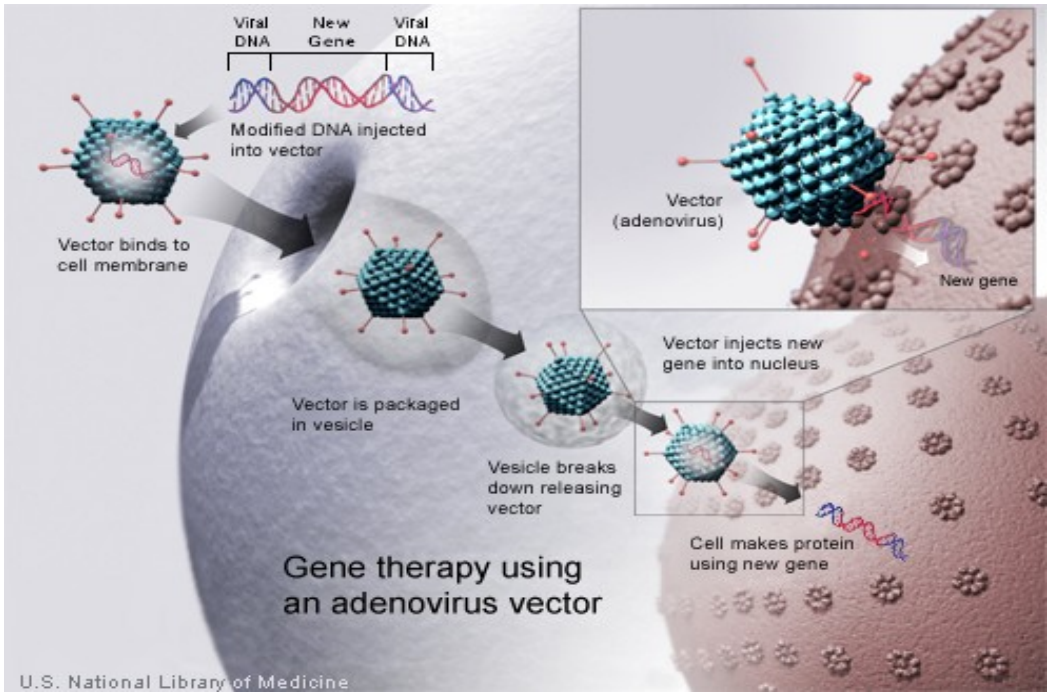

Figure 2. Gene delivery using an adenoviral vector. The figure represents packing of DNA material into the viral aprticle, penetration of the virus into the cell and transmission of DNA into the nucleus.

(or a restricted) range of cells compared to their natural predecessors. To achieve that, surface proteins are modified with those from other viruses or chimeric ones, so called “pseudotyping”. Among such “pseudotyped viruses” a lentivirus “Simian immunodeficiency virus” is mostly well known, which is able to infect a wide range of cell types. The restriction of possible target cell types may be achieved by modification of viral envelopes with antibodies fragments. This so called „magic bullet“concept has already showed it’s effectiveness in several studies (138).

Despite certain disadvantages as immunogenicity and risk of insertional mutagenesis, viral vectors remain one the most perspective groups of gene delivery vehicles. To date the majority of clinical trials are devoted to testing of different viral vectors. Still, in case of a wide variety of genetic diseases for which suitable therapies are already developed safety issues dominate irrevocably over the efficiency and effect continuance matters. Consequently, alternative gene therapy approaches including mechanical gene delivery methods and a plethora of nonviral gene transfer vehicles are being constantly developed.

1.8.4 Non-viral gene delivery systems

Development of effective gene delivery methods into the cell is one of the major trends in modern gene therapy research. Rapid progress in development of nonviral gene delivery vehicles observed within the last decades lead to the establishment of many effective carriers which possess certain advantages compared to mechanic or viral DNA delivery systems. Being able to provide targeted gene delivery and high levels of transgene expression, nonviral vectors are less immunogenic and possess lower toxicity (178), allowing their repeated administration (68, 122). Non-viral gene delivery was proved to have therapeutic effect on mouse model (69, 99, 219). Also several clinical trials devoted to non-viral gene delivery are undertaken at the moment (82, 168).

An effective nonviral gene delivery vehicle is supposed to possess certain characters, like the ability to bind DNA and protect it form enzymatic degradation; it should provide effective targeted delivery and intracellular penetration, as well as escape from the endosomes and effective transport of genetic construct into the nucleus (56, 152).

1.8.5 Polypeptide-based vehicles

Natural and synthetic polypeptide-based vehicles comprised the first generation of gene delivery systems among numerous polymers used for gene transfer (200). Although many proteins of natural origin like histones (179) or histatin5 (130) have shown high gene

transfer efficiency, synthetic amionacid –based vectors possess significant advantages over them. Structure and chemical composition of synthetic polypeptides may be modified easily, thus allowing to coordinate the features of different aminoacids (54). Vehicles based on lysine (poly-L-lysines, PLLs) were among the first synthetic GTAs and represent nowadays the most thoroughly investigated group of gene transfer agents (210). These polymers are biodegradable and possess low immunogenicity and toxicity which makes them perspective vehicles for in vivo delivery of nucleic acids. Free ε-amino groups of lysine within the polymer become protonated at physiological pH thus binding DNA via electrostatic interactions. PLLs may form with DNA, particles of different size and surface characteristics, depending on the polymer structure, in particular on the number and availability of e-amino-groups for interactions with DNA (112, 124). Polylysines not only can bind DNA under physiological conditions, but also release it within the cell thus opening it for transcription (12, 126, 127). Unfortunately, pKa values of lysine are too low to provide endosomal escape of gene constructs. That is considered to be one of the main reasons of relatively low transfection efficiency of PLLs compared to other synthetic cationic polymers like PEI (26, 197). That is why poly-L-lysines are often modified with imidazole-containing amino acids or endosomolytic agents, which can provide disruption of lysosomes and thus secure DNA form enzymatic degradation (211). Polyhistidines may also form particles with DNA under acidic pH, which enter the cells successfully, and release DNA form the endosomes (26, 119).Gluconylated poly-histidine was shown not only to deliver plasmid DNA into the cells but also to release it from the endosomes successfully (151). Nevertheless, efficiency of gene delivery using polyhystidines is low compared to that of polylysines mainly because of the unfavorable size of the conglomerates that these polymers form with plasmid DNA (9). Besides, electrostatic repulsion occurring at physiological pH complicates their application for in vivo gene transfer (83, 184). Covering with PEG is another type of modification described for PLL complexes with plasmid DNA. PEGilation prolongs the half-life of complexes within the blood stream and significantly reduces their toxicity (105). Modification of PLL/DNA complexes with ligands to cell surface receptors may provide cell-type-specific gene transfer (149). Estimation of gene delivery abilities of many PLLs showed that combination of these polymers with specific agents like chloroquine or JTS-1 peptide, as well as development of chimeric structures with other cationic polymers like PEI may significantly increase their gene transfer efficiency, providing high levels of transgene expression both in vitro and in vivo (143, 212). Successful clinical trials prove PLLs to be perspective candidates for future therapeutic applications (105).

1.8.6 Peptide-based dendrimers

Dendrimers (“branching molecules”) are used in many areas of pharmacology and may be easily modified in their structure and surface properties. Because of their structural similarity to many globular proteins they were often called “artificial proteins” (11).

Polypeptide-based dendrimers as well as linear aminoacid-based polymers are biodegradable gene transfection vehicles with low toxicity which have proved their efficiency on cell cultures and animal models (183). Besides, secondary modification with fatty acids residues (60), ligands for receptor-meditated gene delivery (175) or PEGilation (210, 222) enable the establishment of tailor-made gene delivery systems, highly specific for certain purposes (192). Tertiary structure of a globular protein is sensitive for denaturizing effects and unpredictable in location of hydrophobic and hydrophilic surface areas; while polypeptide based dendrimers possess stable flexible structure, inner space and dense homogenous surface (54). Such gene delivery agents as polylysine-based dendrimers became recently one of the main trends in development of biodegradable gene therapy vehicles (146, 181). Poly-L-lysine dendrimers were shown to deliver DNA efficiently into the cells providing long term transgene expression (142). Because of their ability to form spherical monodisperse structures these polymers possess unique capacities. Branching orders and thus the size may be controlled by stage synthesis. Because of their flexible structure polylysine dendrimers may bind DNA into dense structure which may be released within the cell, providing DNA with the access to transcription complexes. These vehicles may be easily modified with short polysaccarides, cytoplasm translocation signals, ligands for cell surface receptors and endosomolithic agents. Besides, polylysines can be easily combined with other polymers. For example, it was shown that co-polymers of polylysine dendrimers with polyethylenglicol bind DNA forming complexes of 15-100 nm and deliver it successfully (116). PEGilation of complexes of branching polylysines with plasmid DNA prolonged their circulation in bloodstream (116, 212).

1.8.7 Modification of peptides with histidine and arginine

Aiming to provide escape of plasmid DNA from the lysosomes the aminoacid composition of the polypeptide vehicle may be modified with endosomolytic agents of different origin (97). Modification of branching polylysines with histidine was proved to enhance DNA escape form endosomes, resulting in significant improvement of gene delivery.

It was shown that introducing histidine into polylysine core leads to higher transgene expression than that after cotransfection of standard polylysine dendrimer with endosome disrupting agent cloroquine (132). Presence of histidines within a polypeptide may provide so called “proton sponge” effect, which effectively disrupts the endosomes thus releasing plasmid DNA (201, 209). Under physiological pH histidine remains uncharged and thus does not participate in DNA binding (13, 35). As far as pH within the primary lysosomes drops, histidines become protonated and develop the proton sponge effect, resulting in overflow of ions into the endosome which leads to it’s disruption (147, 214). Thus presence of histidines within the vehicle may enhance the efficiency of gene delivery by protecting plasmid DNA form enzymatic degradation. Proton sponge effect is widely known not only for synthetic but for many natural proteins (152, 209, 215). For example, viruses possess many effective membrane active proteins capable of endosome disruption (214, 215). Also certain synthetic polymers like polyethylene amine and polyamidoamine exhibit proton sponge effect (79, 195).The toxicity of polylysine-based branching vehicles may be reduced by amphiphilic modification (20, 119) or by creating copolymers with PEG (61, 149, 197). Promising results obtained during in vivo studies prove this group of GTAs to be perspective for targeted delivery of therapeutic genes (5, 192).

Introduction of arginine residues into the polylysin-based vehicles is widely reported to improve the gene delivery capacities of the complexes. Effective targeted intracellular delivery of DNA complexes with polypeptides depends on many factors like the size and structure of the complexes, its surface charge, and presence of ligands to surface receptors or specific aminoacids (142). Many natural peptides, for example TAT-peptide of HIV-1 virus possess arginin-rich moieties, which enable them to enter the cells effectively via both endocytosis and endocytosis-independent pathways (66). The data about the mechanism of arginine-rich moieties penetration into the cell are rather contradictive. While early works reported it to be independent from endocytosis pathways (64, 65), more recent studies showed a significant influence of endocytosis blockaders on penetration of arginine-rich peptides into the cell (17, 133). Nevertheless, many arginine-rich peptides were proved to enter the cells successfully thus improving gene transfer. It was shown that presence of arginine within linear or branching polypeptides enhances the efficiency of complexes penetration into the cell (67).

1.8.8 A series of SPLL branching polypeptides

Based on our previous results obtained with a branching poly-L-lysine of five branching orders D2, (83, 206), a series of novel vehicles was synthesized, where additional aminoacids were introduced into the basic branching polylysine core.

Two dendrimers were modified with arginine residues which were supposed not only to enhance DNA binding but also to improve penetration of the complex into the cell. Other two vehicles were modified with histidin to provide endosome disruption and thus to prevent enzymatic degradation of DNA.

We have shown that both modifications have improved the DNA-binding and gene delivery capacities of the dendrimers and the transcfection efficiency was higher than that observed for basic D2 dendrimer. Arginin-modified polylysines demonstrated higher levels of transgene expression in vitro, showing that penetration into the cell plays a crucial role in gene delivery with modified branching polylysines. To date this group of polymers was tested only on cell cultures, meaning that extensive animals experiments are required to estimate their potential for gene delivery. Future extensive study of these polymers is planned.

To date, a wide variety of synthetic gene delivery vectors exist, which can efficiently deliver plasmid DNA to different organs and tissues of animals. A well known representative of this group is polyethylenimine (PEI), a highly efficient nonviral gene delivery vector.

1.8.9 Polyethylenimine

Polyethylenimine (PEI) is a synthetic cationic polymer which is widely reported to provide effective delivery of DNA and long term transgene expression (34, 69, 107). Gene transfer capacities were first described for a group of PEIs by Boussif in 1995, who had shown their efficiency in vitro and in vivo (14). Different variations involving CH2-CH_2-NH ethylenimine core molecule result in a variety of linear and branching polymers with unique characteristic features. The molecules most oftenly used for gene transfer purposes are 22kDa linear PEI and branched PEI of 25kDa (Fig. 3). Recently, gene delivery capacities were demonstrated for both branching and linear PEIs (70, 126). Linear PEI possesses mostly secondary amines, while primary, secondary and tertiary amines are known for branching polyethylenimines. It is the combination of amino groups of different orders that enables the branched PEI with its buffering capacity observed over a wide pH range (191). The osmotic swelling within the endosome combined with polymer swelling leads to endosome disruption (186). Because of it's ability to form small stable particles with plasmid DNA and to provide the proton sponge effect PEI may be applied as a single multifunctional gene delivery vehicle (48). Nevertheless, compared to many natural polymers used for gene delivery like histones or chitosan, PEI is not biodegradable and thus more toxic (164). Toxicity of PEI originates mostly form the high concentration of positive charges, and diminishes applicability of this polymer for in vivo gene transfer (75). Not only the amount of applied polymer, but also the

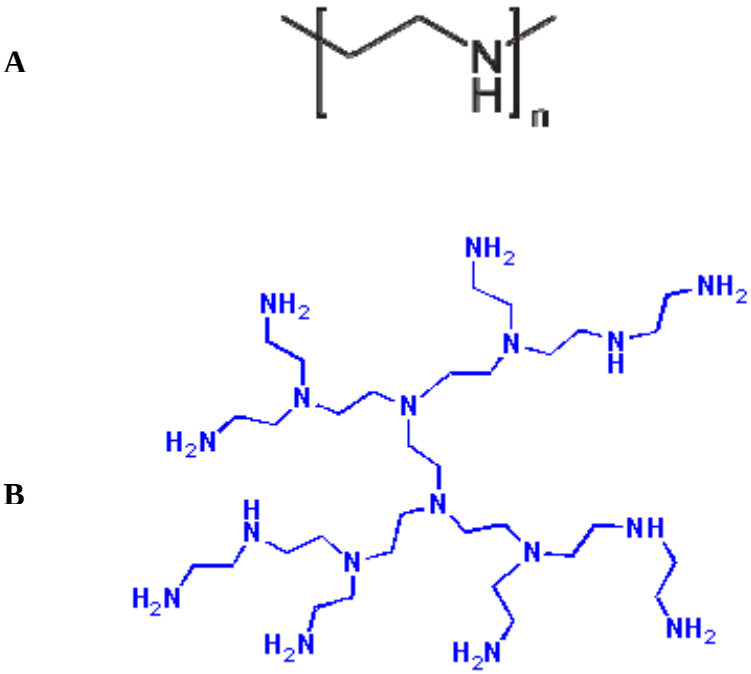

Figure 3. Structure of linear (A) and branching (B) polyethilenimine (PEI).

surface characteristics of it's particles with plasmid DNA, such as particle size, density and surface charge, as well as the solvent influence the development of toxic effects (173, 195). Administration procedure is also important for preventing toxicity.

While severe inflammatory reaction was shown for intravenous application (157), aerosol delivery was proved to be relatively safe for mice (70, 157). Apart from application route, toxicity of PEI may be reduced by different modifications like PEGilation (68). Being able to transfect a variety of cell types, PEI is used intensively to deliver gene constructs into the lung to alveolar and bronchial cells (107, 171), where it provides higher gene expression than liposomes (39). The recent success of PEI-pDNA gene delivery in bladder cancer clinical trial (145) proved that this vehicle is a perspective polymer for gene delivery.

For in vivo application of a certain gene delivery technique safety issues are mostly important. Many methods have been developed to analyze the safety of certain particles. Investigation of lung mechanics is nowadays considered to be one of the most reliable methods for estimation of the impact on the lung.

1.9 Estimation of lung mechanics

Measurement of lung mechanics represents a sensitive tool to observe the reaction of the lung on certain influences. As far as mice are nowdays considered to be the predominant laboratory species, development of suitable instruments for estimation of murine lung functioning parameters became an important milestone in lung physiology research (73). For adequate assessment of mouse lung functioning sensitive methods are needed, which allow quantitive evaluation of lung parameters. Measurement of pulmonary function in mice is challenging due to the small size of their airways (91). Several invasive and non-invasive techniques are developed to date, each possessing its advantages and limitations (46). Although non-invasive methods are quick, easy to handle and do not need anaesthesia or tracheal administration, their low sensitivity and tendency to form artefacts make them unsuitable for measuring fine alterations within the lung. On the other hand, invasive methods are rather demanding and time-consuming. Besides, anaesthesia and surgical preparation of the animals are required. Nevertheless, sensitivity and specificity of these methods, as well as the possibility to carry out long-term measurements in intubated mice make them a valid tool in estimation of lung functioning alterations (123, 162). Pulmonary mechanics is traditionally determined with such parameters as pulmonary resistance (R_L), describing the sum of airway and tissue resistance, and dynamic compliance (C_{dyn}). Already in 1988 the feasibility of these parameters was demonstrated for mechanically ventilated mice (128). Basing on these, other

functional parameters may be calculated such as tissue damping (GTiss), tissue elastance (HTiss) or hysteresivity (Eta). For example, tidal volume of the lung is usually not measured directly by transpulmoanry pressure but calculated according to the differentiation of the volume signal (91). Determination of R_L and C_{dyn} not only provides the classical determination of airway responsiveness, but also enables a more detailed insight into pulmonary mechanics. R_L reflects both narrowing of the conducting airways and parenchymal viscosity. In contrast, C_{dyn} is considered to primarily reflect the elasticity of the lung parenchyma, but is also influenced by surface tension, smooth muscle contraction and peripheral airway inhomogeneity (73).

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1.10 Inflammation in the lung

Gene delivery into the lung is reported to cause high toxicity either because of the administration route or because of the plasmid DNA used in the experiment. Especially the presence of CpG motifs in plasmid DNA is widely reported to influence the transfection dramatically provoking immune response via its adjuvant-like functioning (225). Yew et al. investigated the contribution of plasmid DNA to the toxic effects observed after transfection. They demonstrated that pulmonary administration of DNA with unmethylated CpG motifs induces highly elevated levels of the cytokines TNF-alpha, IFN-gamma, IL-6, and IL-12 in the bronchoalveolar lavage fluids (222). Resident alveolar macrophages are proved to be the major cell type reacting on the presence of unmethylated CpG-containing (=bacterial-like) DNA (103, 140). Internalization of complexes with CpG-containing DNA leads not only to their activation and cytokine excretion but also to active recruitment of fresh monocytes form the bloodstream (87).It was shown that even one CpG motif present within a plasmid may

induce lung immune response (90). Besides, presence of unmethylated CpG-motifs in the plasmid DNA is known to activate B-cells and natural killer cells (193, 222). Co-delivery of inflammation suppressors allows reducing the immune response caused by the vectors (122). Nevertheless, repeated application of strong anti-inflammatory substances may cause risk for the patient. That is why the reduction of immunostimulatory effects of both plasmid DNA and the vector is a crucial point for successful gene delivery (90, 122, 196).

Aiming to decrease the negative effects of gene delivery procedure, we have analysed the effect of plasmid DNA structure on safety and efficiency of gene transfer. We have shown that after instillation of standard DNA complexes with PEI significant increase in number of macrophages and neutrophils was observed within the lung. Activated macrophages also increased in number. Aerosol administration of the same complexes resulted in a much less pronounced and transient alteration in number an character of phagocyting cells. Besides, we have delivered via aerosol PEI particles formed with two CpG-motif-free plasmids, one of coding for luciferase and another representing only the plasmid backbone. Aerosol distribution of CpG-motif free DNA did not cause any alterations in number and type of lung resident cells.

1.11 Clearance of particles form the lung

Analysis of particles clearance from the pulmonary area clarifies not only their distribution within the lung but also the duration of their persistence indifferent areas of this organ.

Clearance of particles from the lung is a complicated process reflecting their metabolism within this organ. Intensity of particle clearance depends on their size, structure and toxic effects, as well as on the administration route (69, 188). Being constantly exposed to every kind of endogenous material like dust, bacteria, etc. lung has developed powerful mechanisms to eliminate incoming materials (15). The lipidic and protein components of the layer are constantly renewed, while beating of alveolar type II cells chilia forms a stream of surfactant in the airways towards oesophagus, the speed of which in human lung comprises 3,6 mm/min (221). Resident alveolar macrophages represent a population of actively wandering cells which bind effectively both opsonised and non-opsonised particles (15, 44). Incoming macroparticles are usually quickly opsonised and afterwards consumed by alveolar phagocytes (140).

Plasmid clearance from the lung tissue is one of the mostly important issues regarding effective gene transfection of lung epithelial cells. While titanium dioxide particles may penetrate through the alveolar wall into the blood stream, many gene transfer complexes are reported to be trapped into the surfactant or to become opsonised and cleared up by phagocytes (39, 153, 171). Also pDNA clearance from the lung cells has to be taken into account. In case of non integrating gene constructs half life of plasmid DNA within the cell defines the duration and intensity of transgene expression. After efficient penetration into the cell and than into the nucleus, subsequent damaging and degradation of plasmid DNA is a serious bottleneck for prolonged gene expression (56, 152). Degradation of plasmid DNA within the cell depends on its size and structure, as well as from presence of specific sequences (164, 173).

Analysis of particle clearance form lung resident cells is important for understanding of the pulmonary response to the gene transfer procedure. Lung resident cells represent the first row of lung defence against foreign materials and react intensively on very kind of incoming particles. Alterations in number of resident macrophages and neutrophils, as well as macrophage activation level represent the primary inflammatory response of the lung and thus may be used to characterise the safety of a certain administration procedure. Alveolar macropahge (AM) is able to take up particles as big in diameter as the cell itself and either destroy it or bring it to the oesophagus (196). While alveolar macrophages can clear up significant amount of macroparticles from the lung, for example coal dust, they react differently being challenged with nanoparticles (144, 148). PEI/DNA complexes which were used in our study for aerosol or intranasal delivery comprise around 100 nm in diameter and thus may be referred to the class of nanoparticles. Rosenecker et al. showed that within the lung PEI/DNA particles are rapidly coated with surfactant proteins, thus enhancing their uptake by resident PMNs (169) . Nanoparticles may block phagocytosis of resident macrophages and also the influx of fresh monocytes into the lung. Renwick et al. demonstrated that ultrafine particles cause not only epithelial damage and macrophage recruitment into the pulmonary area, but also alter the sensitivity of polymorphonuclear cells to chemoattractants (44, 165). Interestingly, significant differences in pulmonary response were found between different rodents both in clearance pattern and involvement of different resident cells (10). It was shown that AMs bind nanoparticles intensively to their surface, while duration of the internalization process depends on the size and number of the particles (154). Being exposed to nanoparticles, macrophages quickly reach the overload condition (90, 196). It is assumed that the ratio of mass to the surface area which is much higher in case of

nanoparticles than for bigger ones is the main disturbing factor for lung macrophages (24, 150). Thus AMs take up nanoparticles inefficiently, changing the clearance pattern from the lung.

Efficient DNA delivery and prolonged transgene expression was the main topis of investigation of the current thesis. Clearance of plasmid DNA from lung tissue, BALF liquid and lung resident cells was analyzed. Comparison of intranasal instillation and aerosol delivery of PEI/DNA particles showed significant differences in patterns of DNA release from PEI and its clearance. Clearance of CpG-free plasmid DNA delivered via aerosol was proved to depend on the structure of the plasmid.

Apart form safety matters which define the applicability of a certain procedure for therapeutical applications, the efficiency of transfection comprise the main issue of interest for gene delivery.

1.12 Efficiency of gene transfer

The efficiency of gene delivery depends on many factors, which include not only the structure of the complexes and plasmid DNA properties, but also physical parameters of particles and the administration route (56, 200). PEI is applied in gene delivery since 1985 and developed into the golden standard of gene deliver within the last decades. From many commercially available PEI forms both for linear (178) and branched PEI (186, 191) high gene transfer efficiency was demonstrated. In particular branched PEI was proved to provide high transgene expression in the lung (107, 168). It was shown that PEI stabilizes DNA within the complexes during nebulisation (171). Within the lung PEI/DNA particles interact with the surfactant layer and bind to cell surface proteoglycans (106). From all lung cell types the bronchial epithelial cells obtain the majority of administered particles (69, 69, 173, 173).

This work shows that delivery of CpG-free plasmid DNA provided much higher transgene expression that administration of standard plasmid. Nevertheless, in both cases luciferase expression decreased dramatically seen days after transfection.

Thus, we have shown that aerosol delivery of CpG-motif-free plasmid DNA complexes with branched PEI results in high levels of luciferase expression and causes no significant alteration in lung functioning or inflammation.

1.12 Aims of the research study

The main aims of investigating a group of branched modified polylysines were:

- to find out how different modifications of the basic peptide influence it's DNA – binding and protective capacities, as well physical characteristics of it's complexes with plasmid DNA
- to analyze the levels of transgene expression which these polymers could provide under different conditions.
- to elicit mostly promising representatives of the group, which could provide high levels of reporter gene expression on cell cultures.

Our final aim was to reveal the mostly promising candidates for further experiments on *in vivo* gene delivery on animals.

Comparing different strategies of pulmonary gene delivery, we aimed:

- to analyse the response of the lung to different gene delivery procedures depending on structure of plasmid DNA and administration route
- to determine the administration strategy with the lowest negative impact on the lung.
- to educe the influence of CpG-motifs within the plasmid on it's immunogenity and applicability for gene transfer.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Branching polypeptides SPLLs

A series of branching polypeptides based on lysine with different Lysine:Arginine or Lysine:Histidine ratios was kindly provided by Prof.Dr.Vlasov (Laboratory for Physiologically Active Polymers, High Molecular Compounds Institute, St. Petersburg, Russia). The following polymers were taken into the study:

Polymer	Weight ratio of lysin to an additional aminoacid	Molecular weight
SP-KR 1:1	Lys:Arg 1:1	690 kDa
SP-KR 10:1	Lys:Arg 10 :1	450 kDa
SP-KH 1:1	Lys:His 1:1	425 kDa
SP-KH 10:1	Lys:His 10:1	525 kDa

Table 1. A series of modified poly-L-lysines with different w:w ratios of lysine to additional aminoacids (arginine or histidine)

2.1.2 Branched PEI

Branched PEI (average MW = 25 kDa) was obtained from Aldrich (Deisenhofen, Germany), dialyzed in water (12-14 kDa MW cutoff), and adjusted to pH=7. Double distilled endotoxin free water for injection was purchased from Delta Pharma (Boehringer Ingelheim, Germany).

2.1.3 Plasmid DNA

pCMV-Luc containing the firefly luciferase cDNA driven by the CMV promoter was generously provided by Dr. E. Wagner (Department of Pharmacy, University of Munich, Germany). pCpGLuc was constructed by Manfred Ogris (Department of Pharmacy, Ludwig Maximilians University, Munich, Germany). Briefly, CpG-free plasmids pCpG-mcs and pMOD-LucSh were obtained from Invivogen (Toulouse, France). Luciferase cDNA was excised from pMOD-LucSh by BgllI-NheI digestion and cloned into respective sites of pCpG-mcs to generate pCpGLuc. pCpG-mcs was used as backbone plasmid. Both plasmids were propagated in *E.coli* and provided in a highly purified form (LPS content ≤ 0.1 E.U./μg

DNA) by PlasmidFactory (PlasmidFactory GmbH, Bielefeld, Germany). The amount of supercoiled pDNA was ≥ 98% ccc (covalently closed circular, ccc grade).

2.1.4 Cell cultures

For our experiments we used the following cells: epithelial cell line of a human alveolar carcinoma A549, human bronchial epithelial cell line 16 HBE, human hepatocellular liver carcinoma cell line HepG2 and human cervical carcinoma cell line Hela.

The cell lines were received from German Cell Culture Collection (DSMZ, Braunschweig, Germany). All the cell lines were cultivated in 100ml tissue culture flasks (Sarstedt, Hamilton, USA) at 37°C in an incubator with 100% air humidity and with 5% CO2 supply. The cells lines A549 and 16HBE were supplied with Dulbecco's modified Eagle's medium (D-MEM) (Invitrogen, Karlsruhe) with 10 % of fetal calf serum (FCS) (PAA, Pasching, Austria). Cell lines HepG2 and HeLa were cultivated in standard Eagle's minimal essential medium (MEM) with 10 % of foetal calf serum. After reaching 80% confluence the cellular medium was removed from the tissue culture flasks, the cell monolayer was rinsed with prewarmed PBS (Gibco, Karlsruhe, Germany) and detatched from the surface by incubation with 5 ml of Trypsin-EDTA (Invitrogen, Karlsruhe, Germany) for 10 minutes. The detatched cells were resuspended carefully, counted on haemocytometer and diluted with culture medium up to desired ratio. For gene delivery experiments the cells of not more than 20 passages were used.

2.1.5 Animals

Female Balb/c mice of 10-12 weeks old were obtained from Elevage Janvier, Le Genest St. Isle, France. After arrival the animals spent minimum two weeks in quarantine. Afterwards approximately 5 animals he animals were housed in Individually ventilated cages (IVC) and fed ad libitum.

2.2 Methods

2.2.1 DNA-binding assay for SPLLs

All peptides of the group were first of all tested for their DNA binding capacity. The predefined charge ratios, corresponding to negative charges of DNA molecules and positive charges of the lysine (and arginin) residues of the polypeptides were transformed into the weight-to-weight ratios according to their molecular weight and purity coefficients.

Basing on previous experiments with lysine-based dendrimers of the similar structure, a certain range of charge ratios was chosen for DNA binding experiments. For every charge ratio 5 µg of plasmid DNA were used for every experiment in a final volume of 100µl. DNA dissolved in 50µl was added drop wise into the 50µl of polymer solution and mixed gently by pipeting. Distilled water or 0,15 mM NaCl were used as solvents. After 20 minutes of incubation 30 µl of complexes suspension were mixed with 10 µl of standard loading buffer and electrophoresed in a 0,8% (w/v) agarose gel for 40 minutes at 100V. TBE (90mM Tirs-borate and 2mM EDTA, pH 8) buffer was used as electrophoresis buffer. The gel was stained with ethidium bromide (0,5 µg/ml) for 30 minutes and illuminated with UV illuminator for DNA location. One microgram of plasmid DNA was used as negative control in these experiments. Presence of smears or plasmid bands corresponding to a certain well meant that DNA was not fully bound by the polypeptide, while absence of DNA traces on the gel symbolised complete DNA binding.

2.2.2 "DNAse-protection" assay

The charge ratios of DNA to polypeptide, providing complete binding of plasmid, were used for the DNAse protection assay. The aim of this assay was to find out the charge ratios which provided stable and dense complexes, where plasmid would be well protected from intracellular enzymes.

Plasmid DNA was compelexed by a tested vehicle as described above. From a 100 µl of complexes solution, prepared for every charge ratio 10 µl of complexes were loaded on a gel to control the compactization efficiency, while 90 µl were mixed with bovine deoxyribonuclease 1 (DNase 1) (from Sigma Aldrich, Munich, Germany) according to manufacturers instructions (10 Units per 1µg DNA). After mixing the complexes were incubated for 3 hours at 37°C. The action of DNAseI was inhibited by 10 min incubation of a probe in a water bath by 70°C. After incubation DNA was extracted from the complexes according to the standard phenol-chloroform DNA-extraction procedure. Briefly, an equal amount of 0,5M Tris-HCl (pH=8) was added to a precooled solution, mixed gently by swirling and centrifuged 15 minutes at 5000g. The viscous phase was separated by centrifugation (5 minutes, 5000 rpm) and phenol extraction was repeated another two times. Afterwards two volumes of ethanol and 0,2 volume of 0,12 M ammonium acetate were added to a probe and mixed thoroughly. The DNA containing precipitate was washed twice with ethanol and dried. After drying DNA samples were resuspended in 20 µl of distilled water and loaded on a gel. In these series of experiments samples of uncompelxed plasmid DNA were

used as positive control. DNA degraded by DNAseI was used as negative control. A presence of characteristic bands on a gel, corresponding to the intact plasmid DNA, meant that DNA was well protected by the polypeptide and was not available to the DNAse during the incubation. The charge ratios, which provided not only compactization but also protection of DNA molecules, were used for further studies including gene delivery experiments *in vitro*.

2.2.3 "TOTO-displacement" assays

To analyse not only the dynamics of complexes formation but also the density of pDNA/SPLL complexes a "TOTO-excision" assay was performed. A florescent dye TOTO-1 iodide was purchased form Molecular Probed (Leiden, Netherlands). The dye was complexed with plasmid DNA acoording to manufacturer's instructions. An amount of TOTO-iodide corresponding to 1 molecule per 20 DNA base pairs was mixed with DNA (0,0025µg/µl in distilled water) and incubated for 10 minutes at 45°C. The TOTO-1 dye belongs to a family of intercalating dyes, which are symmetric dimers of cyanine dyes with exceptional sensitivity for nucleic acids. TOTO-1 posesses a very low fluorescence when not complexed to DNA. After binding to DNA it increases more that 1000-fold. In regard of double stranded DNA the dye acts as intercalator (25) and thus becomes replaced with molecules of polymer during DNA compactization. During the formation of DNA/SPLL complexes the TOTOdye became replaced by the moleculas of polymers. Thus the light excitation of DNA/SPLL complexes was at least 1000 fold lower than that of free plasmid DNA. Comparing the fluorescence intensity of plasmid DNA with that of the complexes formed at different conditions one could judge about the density of complexes as well as about the dynamics of DNA bunding.

To prove that the binding capacities of DNA did not change after compelxation with TOTO-1 a series of experiments devoted to electrophoretic mobility of TOTO-DNA/SPLL complexes was performed as described above. The DNA with integrated TOTO dye showed the same pattern of complexes formation as the standard plasmid. For the "TOTO-excision assay" a series of probed was prepared containing TOTO-labelled-pDNA compelxed with polymers at certain charge ratios. The complexes of 1µg pDNA in a final volume of 100 µl were prepared as described above. Distilled water and 0,15 mM NaCl were taken as solevents. TOTO-labelled DNA without polymer was used as positive control. After 20 of incubation the complexes were transported into the wells of 96well measuring plate and measured for light emission on Walloc-Victor 2 device (1420 multilabel counter, Perkin-Elmer Waltham, USA). The light emission was measured for 10 seconds, afterwards the values obtained form the control well were extracted. Every probe was taken in duplicate, prepared separately. The

experiments were repeated twice. Positive control probes, containing uncompelxed TOTO1-labelled DNA, were used to establish a 100% value. Every probe was measured in triplicate at 5 min, 15 min and 30 min time points.According to the positive control value light emission intensity of other probes was calculated as the per cent form positive control. The decrease of fluorescence was observed corresponding to different polymers, charge ratios and compactization conditions.

2.2.4 Analysis of Size and Zeta-potentials of complexes

The size of complexes of plasmid DNA with polymers was measured with the help of photon correlation spectroskopie (PCS). Zeta-potentials of the complexes were measured electrophretically. Both types of measurements were carried on a Zetasizer 3000HS (Zetasizer, Brookhaven Instrument Corporation) and analysed with ZetaPALS Particle Software (Version 3.42). For determination of the particle size 600µl of suspension of complexes were pipetted in a plastic cuvette (Greiner, Frickenhausen, Germany) and measured 10 minutes by dynamic light scattering. The principle of the measurement was based on Brown's law of particles movements, describing the smallest particles as the fastest. The scattered light was detected with the help of photomultiplier. The size of the particles was measured as a function of light intensity changing speed. Each variation of complexes was measured in triplicate. The compelxes were prepared freshly for every measurement.

Zeta-potentials of the complexes dissolved in distilled water were measured according to their electrophoretical mobility. The complexes in liquid move between positive and negative electrodes according to their surface charge. Measuring of electrophretical movement speed of particles is used for calculation of the "netto surface charge" (zeta-potential), which describes the summarized surface charge of a particle. For the measurement of zeta-potentials 1,6 ml of complexes solution were placed in a plastic cuvette and a palladium electrode was immersed into the liquid. Ten measurements for 30 sec long were performed for every probe. The viscosity of the distilled water or PBS solution (pH=7,4) comprised 0,0089P (Poise) and 0,14P, respectively. All measurements were performed at room temperature. A Henry's correction factor (Fka), reflecting the ratio between particle radius and width of the diffusion layer was set as 1.5. Zeta-potentials were calculated according to Debye-Hückel equation. Each variation of complexes was measured in duplicate. The complexes were prepared fresh for every measurement.

2.2.5 Transfection of the cells in culture using SPLL polymers

In our experiments we used branched PEI as positive control. This polymer has proved it's efficiency as a vehicle able to provide stable gene expression in vitro with a relatively low toxicity. Transfection with naked plasmid DNA was used as negative control. It is widely reported that addition of free plasmid DNA into the cell culture medium results in a certain value of transgene expression, which is significantly lower than that provided by effective gene delivery agents.

Twenty four hours prior to transfection the cells cultivated as described above (2.1.4) were seeded on cell culture 96 well plates (TPP, Peske) in amount of approximately 15 000 cells pro well and supplied with standard cell culture medium. The condition of cells in every well was controlled visually 1 hour before transfection. Ten minutes before transfection cell culture medium was removed and replaced with 150 µl serum free MEM. For transfection experiments 5 µg pCMV-Luc plasmid per well were complexed with a polypeptide corresponding to desired charge ratio. The experiments were preformed in triplicate. A single batch of SPLL/pDNA complexes was produced for every triplicate under sterilel conditions in final volume of 100 µl of PBS. Five mg of pDNA, dilutedin 25 µl PBS, were added drop wise to a 25µl of a polypeptide solution and mixed gently by pipeting. The solution was incubated 15 minutes at room temperature for formation of the complexes. The solution of complexes was added to the wells and mixed gently by rocking the cell culture plate. The pCMVLuc plasmid, compelxed with branched PEI was used as positive control in these experiments. Five microgram of DNA were diluted in 25 µl of distilled water and afterwards added drop wise to 25µl of brPEI solution in distilled water. The N/P ratio of 10 was used in all cases. After 15 minutes of incubation the complexes were added to the cell culture. As negative control free plasmid DNA was used. Half a microgram of pDNA was added to the serum-free culture medium into the wells and mixed with the culture medium by gentle rocking of the plate. After incubation for 4 hours in an incubator with standard conditions the medium was removed and replaced with MEM with 10% FCS and antibiotics (penicillin 10U/ml, sttreptomycin 10U/ml, gentamycin 50µg/ml). Afterwards the cells were incubated for another 24 or 48 hours. After incubation the cell medium was removed, cells were rinsed with PBS and incubated with a 100 µl of a lysis buffer (250 mM Tris, 0,1% Triton, pH=7,8) pro well 10 minutes at 37°C. The liquid was resuspended and 50µl were taken for luciferase measurment. Aliqutes from every well were placed on a measurment 96well plate and measured in a Wallac–Victor2 device (1420 multilabel counter, Perkin-Elmer Waltham, USA). During the measurement 50µl of luciferin solution (D-luciferin 100mg, coenzymeA 159,1mg, DTT 3894

mg, ATP 221mg, (MgCO3)4Mg(OH)2x5H2O 394mg, MgSo4x7H2O 498mg, Tricine 2715mg, EDTA 28,2mg, distilled water 757ml)) was added to every well individually and mixed by shaking. Light exitation was measured for 10 seconds once before and once after the addition of the luciferin. Ten µl of the cell lysate were used for protein concentration assay by Bradford performed in a Wallac-Victor device according to a standatd protocol. An aliquote from every well was mixed with 190µl of Bradford solution (Bio-Rad, München, Germany). Addtiotionally a correlation curve was established for every measurement using duplicates of BSA protein standard (Sigma Aldrich, Schnelldorf, Germany) serial dilutions. After data analysis the efficiency of the transfection was expressed as relative light units per 10 seconds per mg of protein (RLU/10sec/mg prot). Every set of transfections was performed in duplicate.

2.2.6 Aerosol and intranasal delivery of PEI/pCMVLuc, PEI/pCpG-free-mcs and PEI/pCpG-free-Luc comlexes

For this series of experiments the following plasmids were used: pCMVLuc, pCpG-Luc and pCpG-mcs.

2.2.6.1 Aerosol application of brPEI-pCMV-Luc complexes

Branched polyethileneimine (brPEI) with molecular weight of 25 kDa was purchased from Sigma Aldrich (Deisenhofen, Germany). The pH=7,4 was standardised with HCl. The complexes for aerosol transfection were produced as previously described (172). Two miligrams of plasmid DNA were diluted in 4 ml distilled water up to final concentration of 0.5 mg/ml. The N/P ratio of 10 was used for all gene delivery experiments. Branched PEI was also diluted in 4 ml of distilled water till the final concentration of 0.66 mg/ml. The prepared solutions were mixed carefully by addition of the DNA solution into the PEI solution and pipeting. The complexes were incubating for 20 minutes. Afterwards a control measurement of complexes size was performed by dynamic light scattering (Zetasizer, Brookhaven Instrument corporation, ZetaPals Particle Software Ver. 3.42). The size of complexes comprised approximately 150 nm.

Time point of the mesurment	brPEI-pCMV-Luc aerosol	brPEI-pCMV-Luc instillation	brPEI-pCpG-Luc aerosol	brPEI-pCpG-mcs aerosol
1h	n = 5	n = 5	n = 5	n = 5
24h	n = 5 +1	n = 5 +1	n = 5 +1	n = 5 +1
72h	n = 5	----------	n = 5	n = 5
7 days	n = 5 +1	n = 5 +1	n = 5 +1	n = 5 +1
14 days	----------	----------	n = 5	n = 5

Table2. The number of mice used for different time points in this series of experiments. A lung functioning parameters of all mice were measured, as well as transgene expression efficiency, cellular and histological markers for inflammation were analysed. At 24h and 7 days time points an additional animal in every group was used for histological analysis of the lungs.

Female BALB-C mice of 10-12 weeks old (Elevage Janvier, Le Genest St. Isle, France) were used for aerosol delivery experiments. Groups of mice, comprising approximately 20 animals, were placed into an inhalation chamber (9,8 x 13,2 x 21,5 Plexiglas box). The box was connected to a spacer (a Plexiglas pipe of 30 cm long and 6, 5 cm in diameter). Within the spacer 150 g of silica gel were deposited and distributed evenly on the lower part. The spacer was connected to a nebulizer (PARY BOY Jet Nebulizer, PARI GmBH, Germany). Silica gel placed into the spacer provided the drying of the aerosol droplets up to approximately 0,4μm in diameter. The solution of complexes was aerosolized with the help of synthetic air , containing 5% of CO2 under pressure of 3-6ml/min. That was performed to stimulate animals for deeper breath and thus to provide higher gene expression Two groups of mice containing 22 or 27 animals were inhaling 8 ml of aqueous aerosol containing PEI/pDNA complexes. Afterwards animals were analysed at certain time points (Table 2). Another group of 22 mice inhaled distilled water. These animals were used as control group. Animals were analysed 1h, 24hrs, and 72hrs and 7days after aerosol administration of PEI-pCMVLuc comlexes, while and additional time point (14 days after administration) was introduced in experiments with CpG-free pDNA. No alteration in animal condition or behaviour was observed.

2.2.6.2 Intranasal instillation of bePEI/DNA complexes

For intranasal instillation 50 μg of plasmid DNA pro mouse were delivered. The N/P ratio of 10 was used in these experiments. For the formation of complexes plasmid DNA was dissolved in 25 μl of distilled water to final concentration of 1 mg/ml and mixed with an equal

volume of PEI with the concentration of 2.6 mg/ml. To avoid precipitation of highly concentrated DNA the pH of the solution was decreased to pH=6, which provided higher concentrations of NR2H2 cations. After mixing the solution was incubated for 20 minutes by room temperature. Before the application the size of the complexes was controlled by dynamic light scattering as described above and comprised approximately 90nm.

Before instillation of brPEI/pCMVLuc compelxes (experimental group) or distilled water (contorl group) 17 mice were intraperitoneally inejcted with a mixture of Medetomidine (11, 5µg/kg), Midazolam (115µg/kg) and Fentanyl (1,15 µg/kg). Fifty µl of liquid (either suspension of complexes or water) were applied dropwise on the nostrils of anesthezised animals. Breathing frequency as ell as general conditions of animals were controlled visually during the procedure. After the whole volume applied mice were injected inraperitoneally with an antidote solution, containing Atipamezol (50 µg/kg), Flumazenil (10 µg/kg) and Naloxon(24µg/kg). Within 25 minutes all the animals woke up from the narcosis. Animals were analysed 1h, 24hrs and 7days after intranasal administration of PEI-pCMVLuc comlexes.

2.2.7 Measurment of lung parameters on mice

Measurement of pulmonary function of mice was performed in collaboration with Dr. Andreas Flemmer and Dr. Kerstin Hajek. At certain time points (table 2) mice were sacrificed by intraperetoneal injection of 50 mg/kg pentobarbital, weighed and subsequently intubated through a trachestomy. Lung functioning was measured with piston-ventilator (SAV-Flexivent, SCIREQ Inc, Montreal, Canada) as previously described (63). Prior to the measurements The flexiVent® software allowed continuous monitoring of tidal volumes and airway pressure. During tidal ventilation the respirator was set to a volume controlled, pressure limited ventilation mode (Vt= 10 µl/g; Pmax=30 cmH2O, PEEP 4 cmH2O) at 2 Hz and 100% oxygen. Measurements were taken in 5-minute intervals for 25 minutes at indicated times after whole body aerosol application of PEI-pDNA complexes or instillation. We also determined lung function parameters of mice at indicated time points after aerosol application of distilled water or intranasal instillation of 50 µl PEI-pCMVLuc (50 µg) complexes and distilled water, respectively. Additionally, lung function of 5 untreated mice was measured. In all animals lung mechanics reached a plateau after 15 to 20 minutes after the initiation of mechanical ventilation. Thus, data at 25 minutes were taken for comparison between groups.

Pressure transducers were calibrated by two point calibration and ventilator tubing and cannula were accounted for by open and closed calibration of the system for all perturbations,

prior to each experiment. Dynamic mechanics of the respiratory system, compliance (Crs) and resistance (Rrs), and lung input impedance were measured following a recruitment manoeuvre (two inflations to 15 μL/g over 1 second) to provide a standard volume history. For oscillatory measurement ventilation was halted at PEEP-level. At each time point after treatment a group of 5 mice was examined. To determine impedance of the respiratory system (Zrs) by forced oscillation technique (FOT), a forcing signal, consisting of an 8 second pseudorandom oscillatory signal, was applied with an amplitude of 3 ml/kg. The forcing signal contained frequencies between 0.5 to 19.6 Hz (110). Data were collected at 256 Hz and analysed within 4s windows with 66% overlap. Lung impedance data were displayed as resistance (real part, Rrs) and reactance (imaginary part, Xrs) of the respiratory system within the frequency domain.

Lung impedance data (Z_{rs}) were partitioned, applying the constant phase model of the lung, suggested by Hantos et al. (84).

In this model Z_{rs} consist of an airway resistance (R_{aw}), airway inertia (I_{aw}), tissue elastance (H_L), and tissue damping (G_L) according to the equation:

$$\mathbf{Z_{rs} = R_{aw} + j\omega\, I_{aw} + (G_L - jH_L) / \omega\alpha}$$

with ω being the angular frequency and α the frequency dependence of Z_{rs} ($\alpha = (2/\pi) \tan^{-1} (1/\eta)$). In this model lung hysteresivity (eta) = G_L / H_L, is a measure for lung tissue composition taking into account both tissue damping and tissue elastance (47,226).

For each measurement the fitting of the constant phase model is automatically tested. Fitting quality is displayed as coherence of determination (COD), Data were rejected when COD was below 0.85.

2.2.8 Preparation of Animals after Aerosol and Intranasal Applications

At certain time points after the application of PEI/DNA complexes or distilled water firstly the lung functioning parameters were measured. After the measurements were completed the peritoneum was opened. Perfusion was performed, for which the Vena Cava was cut and 3-5 ml of Koch's NaCl solution was injected with an 20-Gauge needle into the right ventricle. This procedure was performed to diminish the amount of blood in lung capillaries and vessels. Blood component haemoglobin may disturb the luciferase measurement (29). Besides, the presence of lung blood cells and monocytes in broncho-alveolar lavage would disturb the estimation of type and number of lung resident cells. Before

perfusion the ribcage was removed carefully to avoid the piercing of the lung by sharp rib edges. The trachea was released from surrounding connective tissues. Afterwards the 20-Gauge catheter (BD Venflon Pro, Beckton Dickinson, Franklin Lakes, NJ USA) was gently installed into the trachea approximately 1 cm deep. Afterwards the metallic needle was removed and the flexible plastic catheter was fixed on the trachea with the help of 2-0 polyester surgical thread. Broncheo-alveolar lavage was performed, for which 1 ml of PBS was instilled slowly into the trachea with an insulin syringe (BD MicroFinePlus, Beckton Dickinson, Franklin Lakes, NJ USA) and after 1 minute slowly removed. This procedure was repeated 10-12 times till the final volume of the obtained fluid had reached 10 ml. Afterwards the catheter was removed from the trachea. The lung-heart unit was dissected carefully. The heart was removed, the trachea and big bronchi were teased carefully form the lung lobes. Immediately after preparation lungs were frozen in liquid nitrogen and stored at -80 degrees. For further procedures the weight of the whole lung was measured. Afterwards the frozen lung was placed in a mortar cooled with liquid nitrogen and homogenized with a cooled pestle. An aliquot of approximately 30 mg was taken off further DNA isolation. The rest of the homogenized lung was used for measurement of luciferase expression.

2.2.9 Estimation of luciferase expression levels in probes from mice

For the measurement of the luciferase expression in the lung the homogenate of murine lungs was mixed with Lysis buffer and incubated on ice for 15 minutes. The lysis buffer was prepared as a ten fold solution and contained 15, 1 g Tris of pH=7,8; 0,5 g Triton X-100 and, diluted with distilled water to the final volume of 50 ml. A tablet of Complete Protease Inhibitor Mix (Roche Molecular Biochemicals, Basel, Switzerland) was added per 50 ml of buffer to avoid the degradation of luciferase by cellular enzymes during preparation of the probes. After incubation with the lysis buffer the probes were centrifugated at 10.000 g-force by 4°C for 10 minutes. Two aliquots 100 µl fo the supernatant were taken for the duplicate measurement placed in luminometer measurement tubes and placed into a Lumat LB9507device (Berthold, Bad Wildbach, Germany). To every probe 100 µl of luciferin substrate mix (60 mM DTT, 10 mM Mg2SO4, 10mM ATP, 30µM Luciferin in 25 mM Glycyl-Glycin buffer, pH=7,8) was added during the measurement. After the substrate mix was added to the probe the photon emission (relative light units, RLU) was measured for 10 seconds. The background value (photon emission of the probe before addition of the substrate mix) was automatically subtracted from the final value. An average mean between two measurements was calculated for every probe.

2.2.10 Quantification of cell type composition of BALF cells

The liquid obtained from broncheo-alveolar lavage was centrifugated for 10 minutes at 1000rpm to spin the cells down. The supernatant was removed and stored at -80°C t for further analysis. The cells were resuspended in 600 µl of prewarmed PBS and counted on haemocytomoter according to a two-field scheme. An aliquot of 200 µl was taken for DNA isolation. Four hundred µl were used for production of cytospin slides. A hundred µl of cell suspension were applied on a cytofunnel (ThermoScientific, Helsinki, Finland) and placed into a Shandon Cytospin 2 centrifuge for centrifugation (10 min, 1000 rpm). Four slides were produced for every mouse. After centrifugation the slides were dried and stored at 4°C.

For detection of cell type ratio the slides were stained with May-Grünwald (Sigma Aldrich Schnelldorf, Germany) and counterstained with ten fold diluted Giemsa stain (Sigma Aldrich Schnelldorf, Germany) according to a standard procedure described elsewhere (128). The following cell types were taken into consideration: macrophages, lymphocytes and neutrophils. For estimation of cell type ratio 500 recognizable cells were counted and percent ratios were calculated individually for every experimental animal. Microscopic observations were carried out on a Dialux light microscope (Leica, Solms, Germany) with x40 and x100 objectives.

2.2.11 Estimation of macrophage activation level

Cytospin slides prepared as described above were fixed with 3,7% Paraformaldehyde in 96% ethanol. After fixation the slides were stained with a benzidine-hydrochloride containing stain according to the method of Kaplow (94). Afterward the slides were counterstained with Giemsa and dried. After staining dark blue dots, representing the myeloperoxidase activity are to be seen on the surface of activated macrophages and allow to distinguish them from not activated. Five hundred macrophages in different filed of view were counted for every mouse and a percent level of activated macrophages was calculated.

2.2.12 Histological examination of the lungs

At certain time points the mice were sacrificed and dissected as described above. The perfusion was performed using 5 ml of Koch's solution with heparin (25000 I.E per 1000 ml). Afterwards the catheter was installed and the lungs were filled with 2 ml of 4% paraformaldehyde (PFA) solution. The trachea was tied tightly with the surgical thread and the whole unit of trachea, bronchi and lungs was dissected and placed into a tube with 4% PFA. The lungs were incubated overnight and then embedded in paraffin. The histological sections as well as their staining and analysis were performed by Dr. Charel , Institute for Pathology. The sections of 6 nm were fixed on glass slides by snap-freezing technique and than a standard haematoxylin-eosin staining was performed as described elsewhere (76). The microscopic analysis was performed according to a double blind system. Special attention was given to the signs of inflammation or swelling, influx of inflammatory cells, alterations in tissue structure and condition. Microscopic analysis was performed on an Axioplan 2 microskope with 20x and 40x objectives, supplied with Programm AxioVision software (Zeiss, Jena, Germany).

2.2.13 Isolation of DNA from the probes of mice

Approximately 30 mg of lung homogenate obtained as described in above were used for DNA isolation wit the help of DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). With the kit both genomic and plasmid DNA was extracted. The Tessie homogenate was mixed with Proteinase K and the digestion buffer and incubated overnight at 55°C. The following DNA isolation procedure was carried out according to the manufacturer's instructions. In case of cells of BALF a 200 µl aliquot of cell suspension was mixed with the 20µl of ten fold digestion buffer and Proteinase K solution. The following extraction was performed the same way as for lung tissue. For DNA isolation from the BALF supernatant a portion (either 1300µl or 1000 µl) was concentrated on Micropore YM3 Spin Columns (Millipore GmbH, Schwalbach, Germany) up to approximately 300 µl. The Proteinase K incubation step was omitted. The following isolation was carried out according to manufacturer's instructions. In case of experiments devoted to aerosol application and instillation of pCMV.Luc plasmid the samples of isolated DNA were digested for 3h with restriction endonuclease XbaI (Fermentas GmbH, St.Leon-Rot, Germany) at 37°C according to manufactirer's recommendations. After digestion the probes were purified from the

residual nucleotides with the help of QIAquick Nucleotide Removal Kit (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions.

2.2.14 Quantitative Real-Time PCR

The quantitative Real-Time PCR analysis was carried out on iCycler IQ Real-Time PCR Detection System (Bio-Rad GmbH, München, Germany). The exact calculation of DNA molecules between amplification cycles was performed through continuous estimation of SYBR Green I emission (asymmetrical cyanine dye [2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium]+°).

Primers were designed for the luciferse-coding region of the pCMVLuc plasmid. The sequenses of the primers were the following: the forward primer 5'-TCCATCTTCCAGGGATACG-3' 5'and the reverse primer -ATCCAGATCCACAACCTTGG-3', corresponding to the 1872-1891 nucleotides of the plasmid.

The real time polymerase chain reaction (RT-PCR) was carried out in triplicates, for which 80ng of extracted DNA were mixed with iQ Sybr Green Supermix (Bio-Rad) in a final volume of 50µl. For the creation of a standatd curve certain amounts of plasmid pCMVLuc were digested with DNAse 1 as described above (2.2.2). The concentrations of 1ng, 0,1 ng, 0,001 ng and 0,0001 ng/µl were used, from which 2.5 µl were taken for a real time PCR reaction. The PCR condiitons were the following: 95°C for 4min, 40 cycles including 95°C for 15 sec and 60°C for 1 min. The melting curve analysis was preformed immediately after the amplification. For that the temperature starting from 55°C was increased in 80 10sec-long steps for 0.5°C per step. The obtained data were analysed Potical System Software Version 3.1 (Bio-Rad).

For the real-time detection of CpG-motif free plasmids a set of primers was used, corresponding to a zeomycin-resistance area, which was present on both plasmids. Thus, for analysis of CpG-Luc and CpG-mcs plasmids the following primers were synthesised by MetaBion (Martinsread, Germany): the forward primer :
5'-GCCAAGTTGAYYAGTGCTGTC-3' and the reverse primer
5'-CCTCAGTCCTGCTCCTCTGCC-3'.

The real-time PCR was carried out in duplicated and repeated at least twice. For the reaction 2 µl of DNA-contatinig extraction buffer was taken and mixed with Sybr-green SuperMix (BioRad) in a final volume of 20 µl. The correlation curve was developed

individually for every plasmid. For establishment of the curve certain amounts of the plasmid DNA were taken, corresponding to a final concentration of 2ng, 0,2 ng, 0,02 ng, 0,002ng, 0,0002 ng and 0,00002ng. The experiment was carried out three times in duplicate. The RT PCR conditions were the following: 95°C for 3min, than 40 cycles including 95°C for 1 min, 67°C for 20sec and 72°C for 2min. The melting curve analysis was performed as described above.

2.2.15 Statistical methods

Results are reported as means±standard deviation. The statistical analyses between different groups of animals or cell cultures was carried out using Mann-Whitney-U-test. Probability ($p \leq 0.05$ was considered significant.

3 RESULTS

A series of branching polypeptides SPLL

A series of branching polylysines, modified with histidine and arginine at different proportions, was analyzed for their gene delivery capacities. This series represented modifications of a primary peptide D5 which was investigated by Dr. Vlasov et. al (62, 63). Two peptides of the group (SPKR1:1 and SPKR10:1) were modified with arginine residues in weight-to weight ratio of 1:1 and 10:1, respectively. The arginine residues were introduced into the branching polypeptide not only to enhance DNA binding, but also to provide effective penetration of the complexes into the cell which is widely reported for arginine-containing polymers (23,31). Two another polypeptides – SPKH1:1 and SPKH 10:1 – represent the modifications of a basic polycation with histidine in a weight-to-weight proportion of 1:1 and 10:1, respectively. Histidine is widely used in development of polyaminoacid gene carriers because of it's capacity to become protonated under low pH (12, 19). The function of histidine residues within the vehicle was do increase the buffer capacity of the polymer and thus to provide an effective endosomal escape of plasmid DNA.

First of all the ability of a vehicle to bind and to protect plasmid DNA was investigated using gel-retardation assays and DNase protection assay. The structure of complexes of SPLLs with pCMVLuc plasmid was investigated using TOTO-displacement test. Besides, the size and zeta-potentials of the complexes were measured. The gene delivery capacities of the series of synthetic polymers were analyzed in a series of experiments in vitro, using different cell types and transfection conditions.

3.1 Estimation of DNA-binding capacity of SPLL polypeptides

For investigation of DNA binding properties of the polypeptides plasmid DNA pCMVLuc was complexed by the polymers at different charge ratios. The charge ratios of plasmid DNA to polypeptide from 1:0,1 up to 1:2 were used. Distilled water and 0,15 mM NaCl (physiological solution) were utilized as solvents. In these experiment naked (uncomplexed) DNA was used as negative control. Free plasmid DNA is presented on a gel as a double strand, where the upper band contains open circular DNA and the lower is composed of super coiled plasmid. Typical double strands under the certain well represented the unbound DNA, thus meaning that under chosen conditions nearly all plasmid DNA remained uncomplexed. A smear under the well symbolized DNA degradation or partial

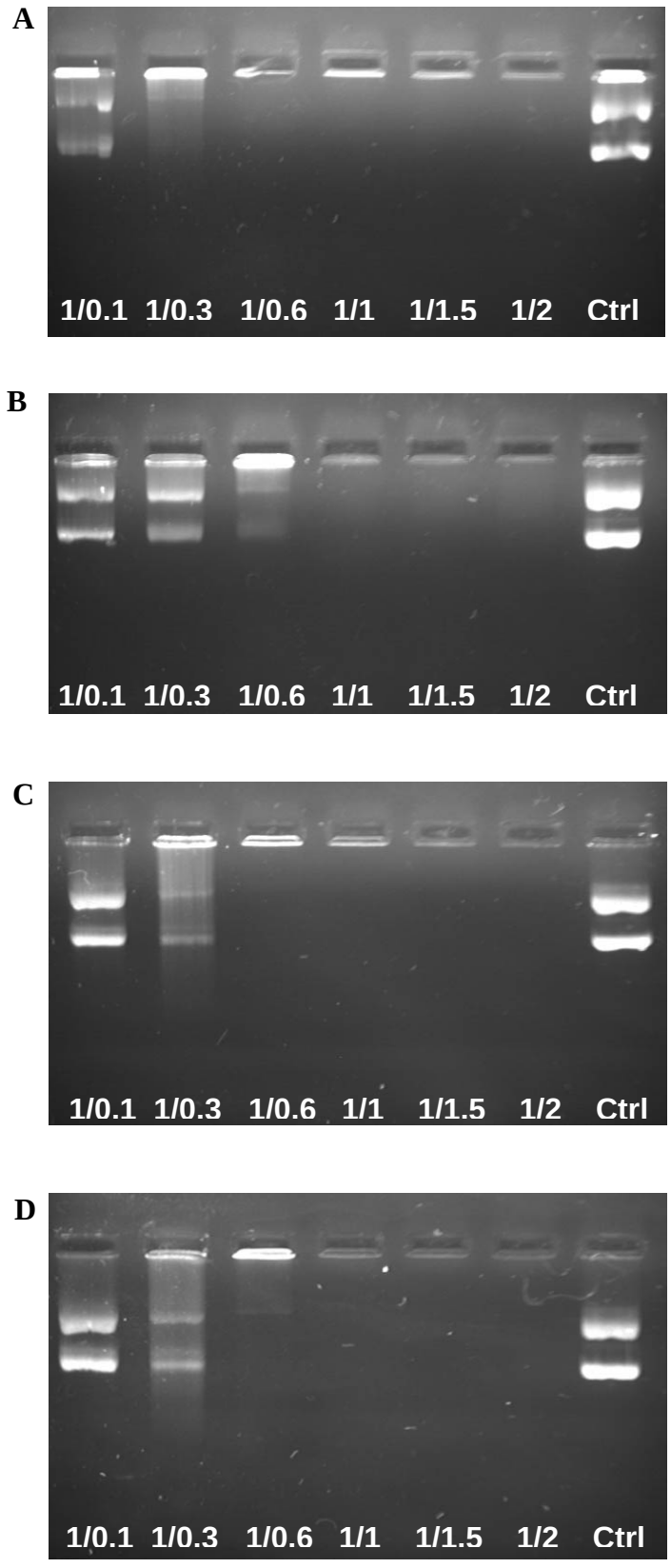


Figure 4. Estimation of DNA-binding capacities of branching polypeptides SPLL. The following polymers: SPKR10:1 (A), SPKR1:1 (B), SPKH10:1 (C), SPKH 1:1 (D) were mixed with plasmid DNA under marked DNA/polypeptide charge ratios. Naked DNA (Ctrl) was used as control.

DNA binding. Absence on any DNA traces on a gel, sometimes combined with a pronounced light signal within the well corresponded to complete binding of DNA. These charge ratios were proven to provide complete binding of the plasmid.

For all four polypeptides taken into the study similar results were shown for compactization in distilled water and in NaCl solution.

It was shown that the lowest charge ratio needed to bind DNA completely with SPKR10:1 polypeptide was 1:0,6 (Fig. 4). Under lower charge ratios DNA was either bound only partially (1:0,3) or remained mostly unbound (1:0,1). DNA compactization experiments with SPKR1:1 peptide showed, that using charge ratios from 1:0,1 to 1:0,6 a significant part of DNA was seen unbound on a gel. From the charge ratio of 1:1 all DNA remained in the well of a gel meaning it's complete binding. Experiments with SPKH10:1 showed that already at the charge ratio of 1:0,6 complete binding of plasmid DNA was observed, while complete DNA binding only at the charge ratio 1:1 was observed for SPKH1:1 polypeptide.

3.2 DNAse protection assays

DNase protection test was performed to investigate how efficiently the polypeptides can protect plasmid DNA from intracellular enzymes. Completely destroyed plasmid was present on a gel as a smear. For production of positive control DNA was complexed with polypeptides and extracted afterwards without DNAse digestion. These samples are present as clear double bands on a gel. Presence of a smear on a gel corresponding to an experimental well symbolized complete or partial DNA degradation, meaning that complexes formed at these charge ratios could not protect plasmid DNA form enzymatic degradation. Clear plasmid DNA double bands representing intact plasmid DNA meant that the corresponding DNA: polypeptide charge ratio could provide DNA protection within the complex and resist the action of intracellular enzymes. DNase protection assays showed that complexes formed under similar charge ratios in distilled water and saline differed significantly in their ability to provide DNA protection. Though SPKR 10:1 polypeptide could form stable complexes with pDNA with the charge ratio of 1:0,6 and higher, only starting from charge ratio 1:2 the complexes made in distilled water could provide DNA protection (Fig. 5). One may see that the smear symbolizing the digested plasmid reduces with the increasing DNA/polymer ratio. At the same time the complexes formed in saline showed higher protective ability (Fig.6). Starting from the charge ratio 1:1 clear pDNA double bands were observed on a gel. A polypeptide with a higher arginine ratio (SPKR1:1) could protect plasmid DNA starting from

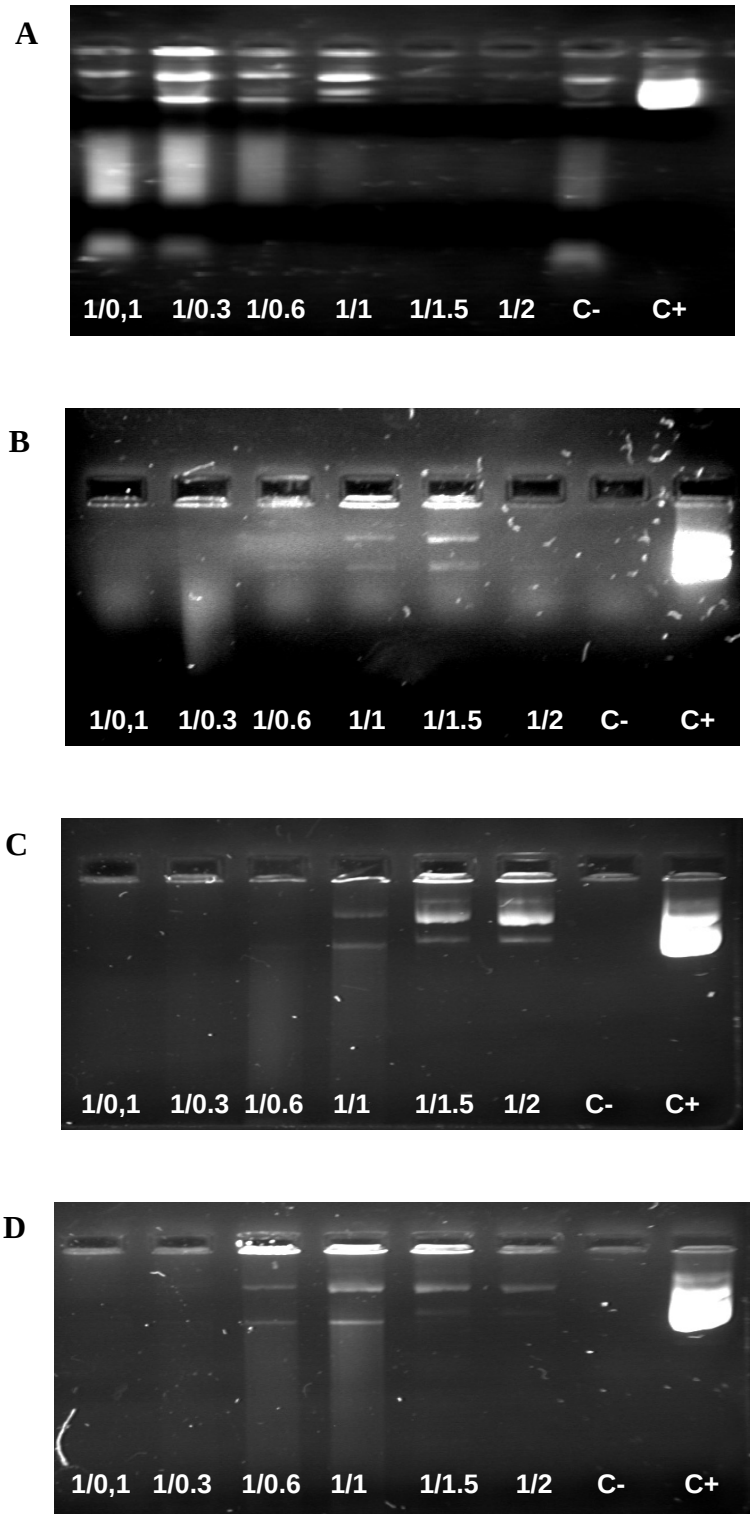


Figure 5. DNAse protection assays with SPLL polypeptides. The following polymers: SPKR10:1 (A), SPKR1:1 (B), SPKH10:1 (C), SPKH 1:1 (D) were mixed with plasmid DNA under marked DNA/polypeptide charge ratios. Distilled water was used as solvent. Digested uncomplexed DNA (C-) and naive DNA probe (C+) were used as controls.

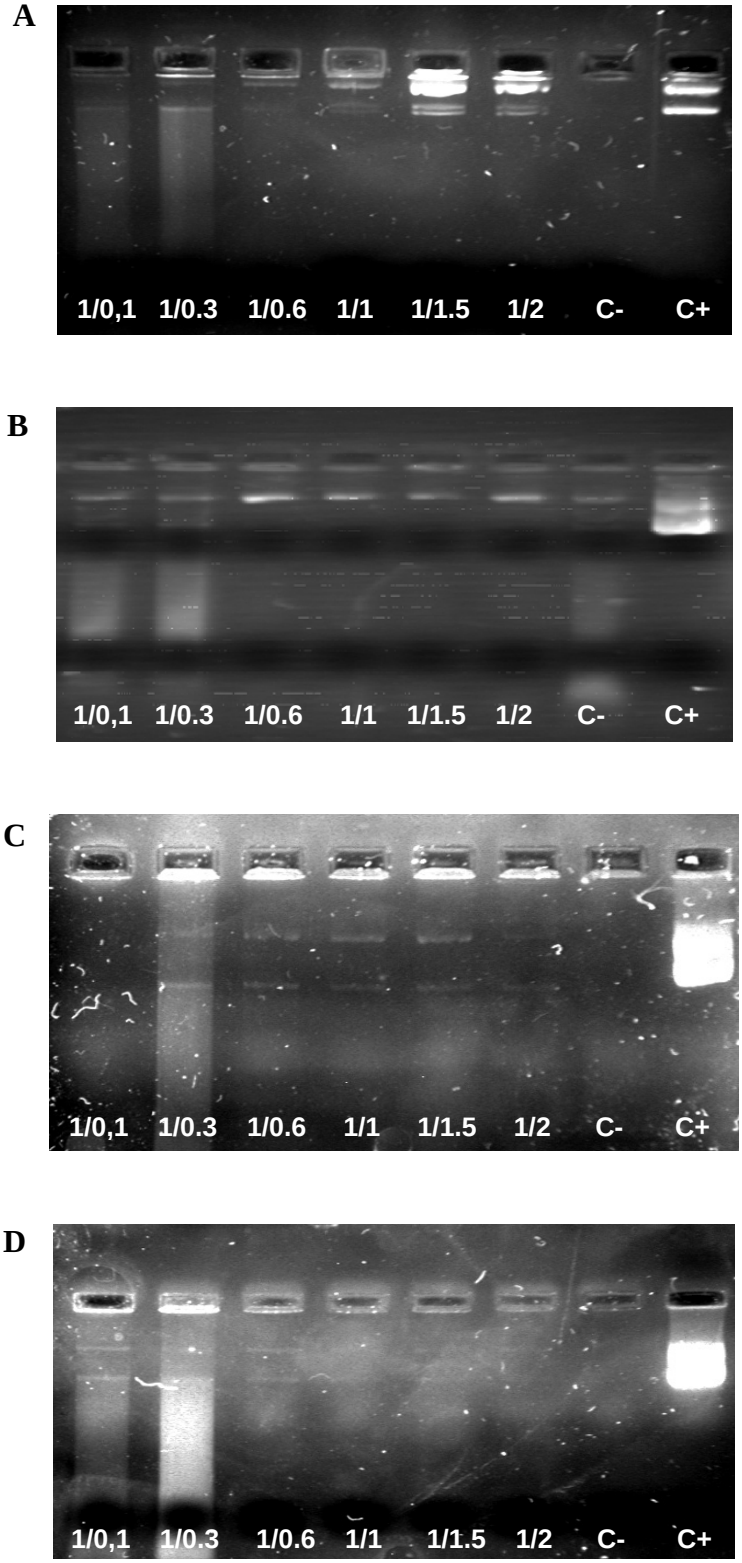


Figure 6. DNAse protection assays with SPLL polypeptides. The following polymers: SPKR10:1 (A), SPKR1:1 (B), SPKH10:1 (C), SPKH 1:1 (D) were mixed with plasmid DNA under marked DNA/polypeptide charge ratios. NaCl was used as solvent. Digested uncomplexed DNA (C-) and naive DNA probe (C+) were used as contorls.used as solvent.

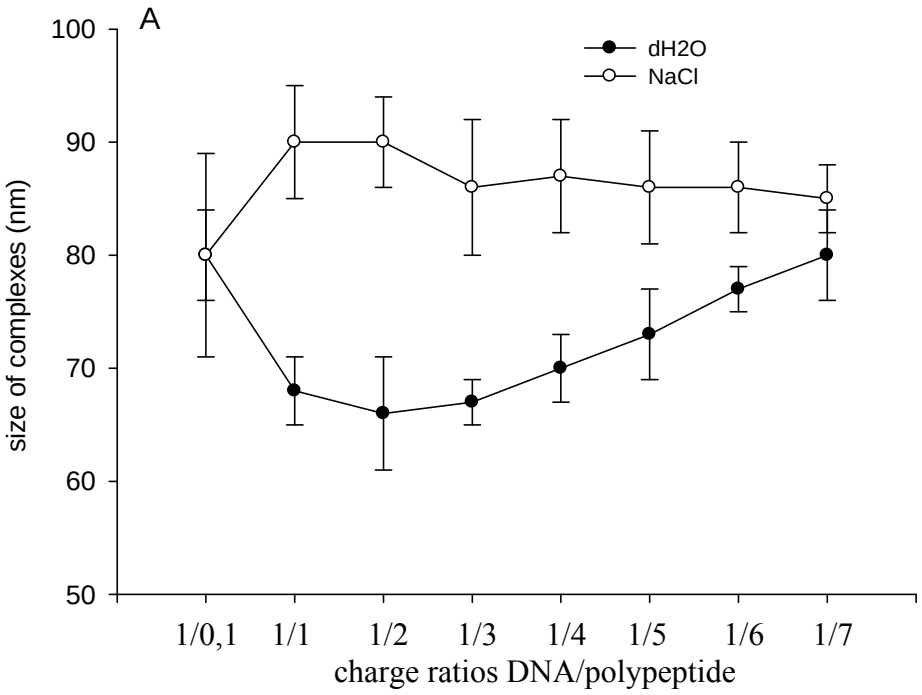
the charge ratio 1:1 for the complexes made in water (Fig.5) and from 1:0,6 for the complexes made in NaCl solution (Fig.6).

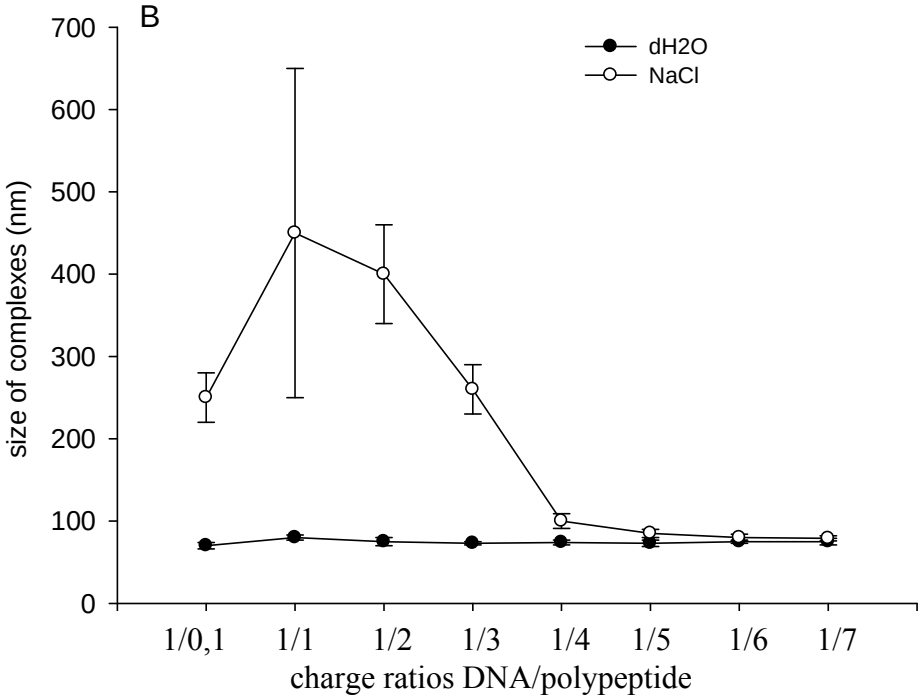
The differences between complexes formed in distilled water and those made in 0,15mM NaCl were even more pronounced in case of polypeptides modified with histidine (Fig. 6). For SPKH10:1 polymer it was shown that pDNA was protected starting from the charge ratio 1:2 in complexes made in water, while in case of complexes formed in saline clear pDNA bands were seen in a gel starting from the charge ratio 1/0,6. For SPKH1:1 polymer from all complexes formed in dH2O only highest charge ratios (1:1,5 and 1:2) could protect plasmid DNA form degradation by DNase 1, while for the complexes formed in NaCl already from the charge ratio 1:0,6 pronounced DNA double bands were observed meaning DNA was well protected form DNaseI.

3.4 Size measurement of SPLL complexes with DNA

The size of complexes formed by SPLL polymers with plasmid DNA was measured for different charge ratios and binding conditions. The ratios were used form 1:0,1 up to 1:7. The measurements were performed in duplicate.

We have shown that in case of SPKR10:1 polymer the complexes preformed in saline did not differ significantly form those formed in distilled water. The aggregation of plasmid DNA with the polymer observed at the charge ratio of 1:1 reached almost 300 nm in size for both compactization conditions (Fig. 7). The smallest complexes of approximately 70nm in size were observed for 1:2 charge ratio. With the increase of the charge ratio the formation of more compact complexes was observed, reaching the size of 250 nm at the charge ratio of 1:7. The differences between compactization conditions were insignificant in this case. For SPKR1:1 polylysine a vivid increase in the size of complexes was observed when the distilled water was substituted with NaCl solution. The complexes at the charge ration 1:2 in water had a size of around 95 nm, while those produced in saline comprised approximately 400nm. The differences between two solvents decreased with the following charge ratios and were completely diminished at 1:4.


A
dH2O
NaCl
size of complexes (nm)
100
90
80
70
60
50
1/0,1
1/1
1/2
1/3
1/4
1/5
1/6
1/7
charge ratios DNA/polypeptide


B
dH2O
NaCl
size of complexes (nm)
700
600
500
400
300
200
100
0
1/0,1
1/1
1/2
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1/5
1/6
1/7
charge ratios DNA/polypeptide

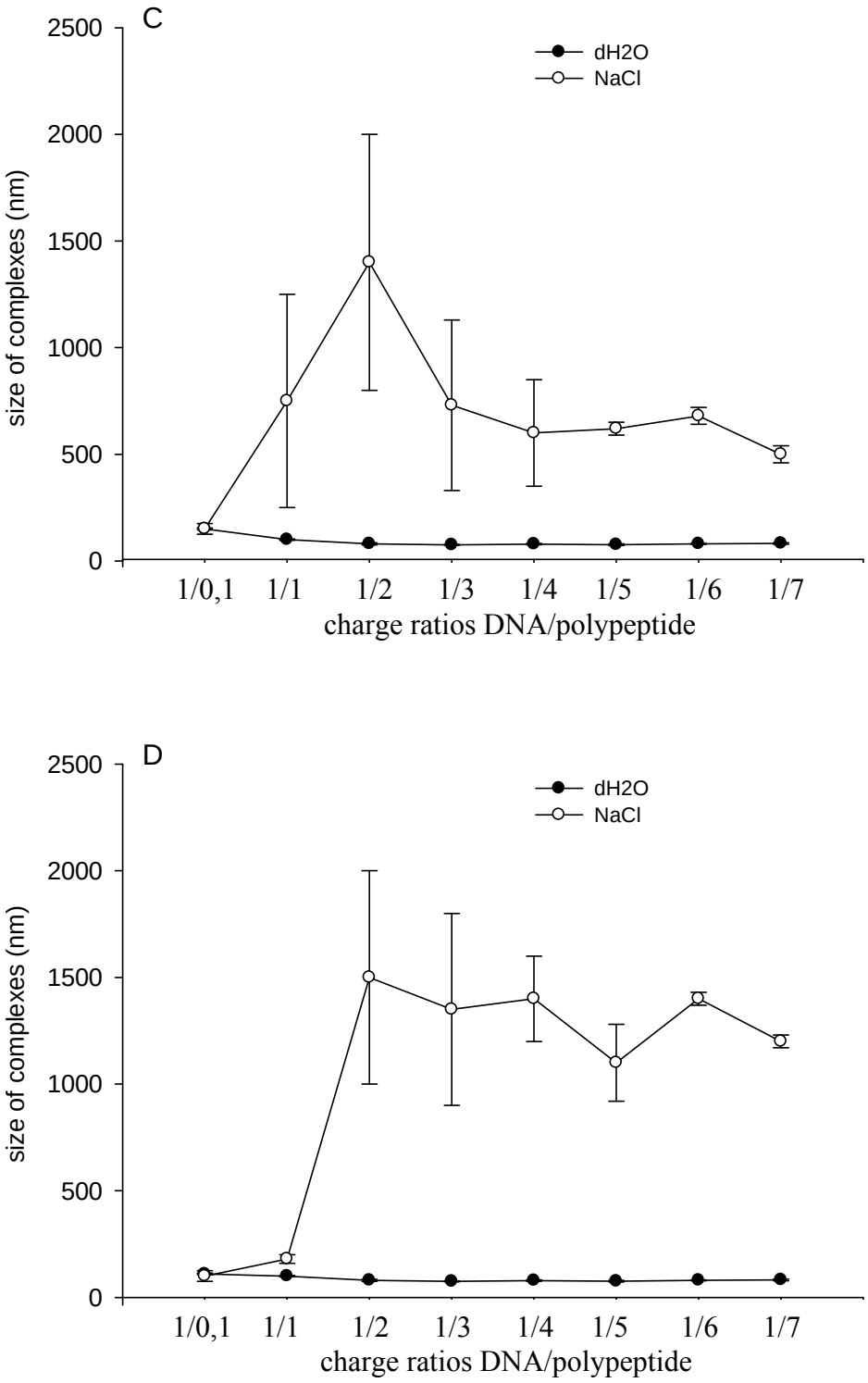


Figure 7. Sizes of SPLL compexes with DNA formed in distilled water (filled dots) or NaCl solution (blank dots). The following polymers: SPKR10:1 (A), SPKR1:1 (B), SPKH10:1 (C), SPKH 1:1 (D) were mixed with plasmid DNA under certain charge ratios and incubated 20min before measurments. Experiments were repeated in triplicate. Data are presented as average mean with standard deviation.

The size of complexes with all other charge ratios both in water and saline differed insignificantly from one another and comprised approximately 80nm. In case of histidine-enriched polymers the observed differences in size of complexes between water and saline were much more pronounced. For SPKH10:1 polymer charge ratio had no significant effect on the size of complexes formed in distilled water. The size of complexes stabilized at 78nm at 1:1 charge ratio and did not alter with the increase of polymer concentration.

At the same time complexes formed in saline showed greater variation in size and tendency for aggregation.

The complexes observed with SPKH10:1 polymer for charge ratio 1:1 and 1:2 comprised 650nm and 1300 nm respectively. High deviations were observed between several measurements. Increase of polypeptide concentration lead not only to a slight decrease in size of complexes but also to stabilization within the group between separate measurements. At charge ratio of 1:7 the size of complexes reached 500nm. Similar tendency was observed for SPKH1:1 polymer. While the size of complexes formed in distilled water was stable between the charge ratios from 1:1 and 1:7 and comprised approximately 70 nm, the size of complexes performed in saline increased dramatically for charge ratios 1:2 – 1:4 and comprised approximately 1400 nm with high deviations between measurements. Escalations of polymer concentration lead to slight decrease in size of complexes. Differences within the group also decreased. At the charge ratio 1:7 the size of complexes comprised 1170nm.

3.5 Zeta-potentials of SPLL complexes with plasmid DNA

Zeta potentials of the complexes were measured in order to understand better their structure and surface charge of the particles depending on the DNA/polypeptide charge ratio. The preceding analysis showed the complexes performed in distilled water to be better applicable for gene delivery than those performed in saline only the first one were taken for these demanding measurements. Only those charge ratios were studied which could provide complete DNA binding. The zeta-potential analysis was performed in duplicate for every charge ratio. It was shown that the complexes of SPKR10:1 peptide with plasmid DNA under all taken charge ratios were strongly positive with a tendency to increase the positive charge with the incensement of polypeptide concentration. The surface charge of complexes at 1:1 charge ratio comprised 18mV, slightly increasing with the next charge ratio3 from 1:2 to 1:6 to approximately 20 mV were shown (Fig.8A). For the charge ratio 1:7 a surface charge of 32 mV was measured.

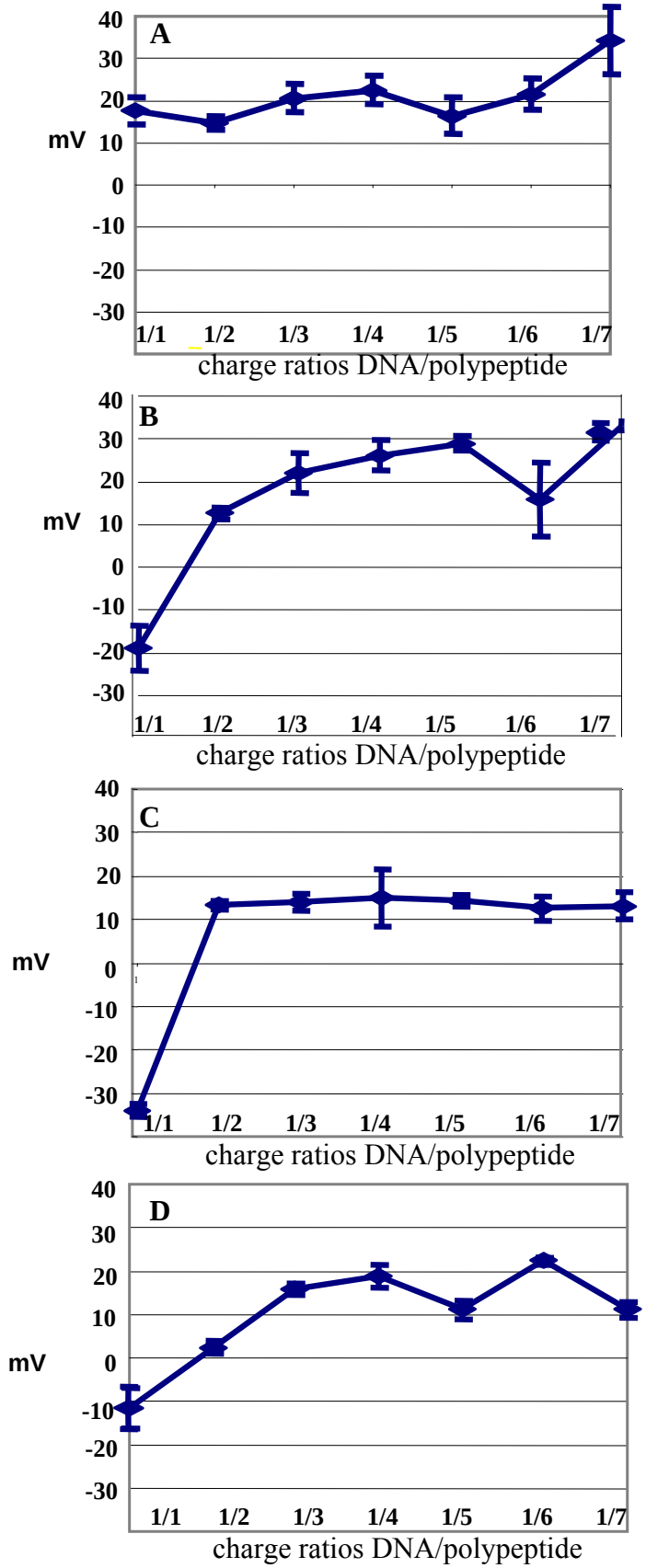


Figure 8. Zeta-potentials of SPLL compexes with DNA. The following polymers: SPKR10:1 (A), SPKR1:1 (B), SPKH10:1 (C), SPKH 1:1 (D) were mixed with plasmid DNA under certain charge ratios and incubated 20min before measurments. Experiments were repeated in duplicate. Data are presented as average mean with standard deviation.

In case of a more arginine-enriched polymer SPKR1:1 complexes produced at the charge ratio 1:0,1 had a negative charge which comprised -20 mV. Following the increase of polypeptide in complexes a tendency for rising was showed for the surface charges. The complexes at 1:1 charge ratio were already positive (12 mV) and for the 1:4 charge ratio a zeta-potential of approximately 30mV was shown (Fig. 8B). In case of polymers modified with histidine similar tendency was observed. The polymer SHKH10:1 bound to pDNA at the charge ratio of 1:0,1 showed negative zeta-potential of -40mV, while all higher charge ratios used in the test showed relatively similar surface charges. For all other charge ratio tested (from 1:2 to 1:7) surface charges of approximately 13mV were observed with insignificant differences between different ratios. Complexes of plasmid DNA with SPKH1:1 polymer showed a slightly negative surface charge of -10mV at the 1:1 charge ratio, while the complexes wt 1:1 were almost neutral. Further increase in polymer concentration led to establishment of strongly positive zeta-potentials, which reached approximately 20mV for charge ratios 1:4 and 1:6. For charge ratios 1:5 and 1:7 zeta-potential of around 10mV were shown.

3.6 Analysis of the dynamics of complexes formation

Aiming to analyze the dynamics of complexes formation and to observe the changes in complexes density TOTO1-excision assays were performed for each of four polymers. DNA labeled with TOTO-1 fluorescent dye was complexed with polymers at different charge ratios and residual fluorescence was measured. As for all preceding g experiments, complexes were formed in distilled water and in saline. To study the dynamics of DNA binding the charge ratios were taken which provided only partial DNA binding as well as those which were shown to bind DNA completely and to protect it from degradation.

Remarkably similar schemes of increasing of complexes density were shown for all four polymers when complexes were formed in in 0,15mM NaCl. Fluorescence intensity measured with SPKR10:1 at 1:0,1 charge ratio was close to 100% showing slight binding of a polymer to DNA. At the next charge ratio 1:1 DNA was proved to be bound completely as the fluorescent of TOTO-labeled molecules went down to the level of approximately 40%. All following charge ratios tested, namely from 1:2 to 1:7 showed similar levels of residual fluorescence. In case of higher arginine-enriched polymer SPKR1:1 complexes at the charge ratio of 1:1 were characterized with DNA fluorescence of approximately 80% from control (Fig. 9). All higher charge ratios showed significant reduction in TOTO-DNA fluorescence. In case of histidine-modified polymers SPKH10:1 and SPKH1:1 no incubation time effect

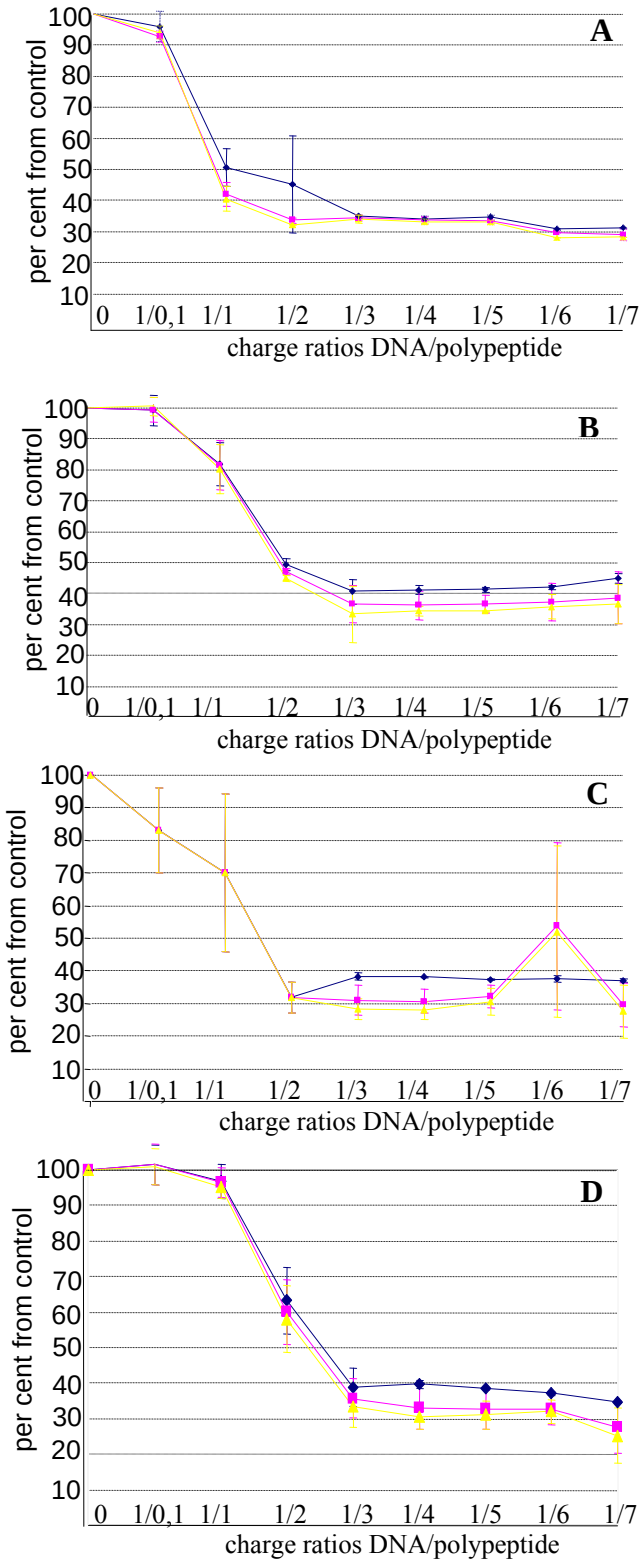


Figure 9. Analysis of complexes formation dynamics using TOTO-quenching tests. The following polymers: SPKR10:1 (A), SPKR1:1 (B), SPKH10:1 (C), SPKH 1:1 (D) were mixed with TOTO-labelled plasmid DNA under certain charge ratios. Distilled water was used as solvent. Light emission was measured 5 min(blue), 15min (purple) and 30min (yellow) after DNA was mixed with the polymer. Experiments wre repeated in triplicate. Data are presented as average mean with standard deviation.

was observed for any of the charge ratios applied. Both polymers showed high DNA fluorescence at the smallest charge ratio of 1/0,1 symbolizing the presence of only immature conglomerates of DNA with a polymer in solution. At the charge ratio of 1:1 TOTO-DNA fluorescence was eliminated completely and no fluctuations were observed between other charge ratios applied.

Different dynamics was shown for DNA/polypeptide complexes formed in distilled water. For the SPKR10:1 polypeptide a graduate decrease in fluorescence was shown with charge ratios 1:0,1 1:1 and 1:2, insignificantly changing with time (Fig. 9). Fluctuations observed after 5 minutes of incubation were shown to diminish with time, reaching minimal standard deviation at 30 min time point. At 1:2 charge ratio DNA fluorescence was reduced up to approximately 30%. The following increase of polymer concentration did not influence the fluorescence levels significantly. Even at the charge ratio 1:7, which corresponded to saturation with the polymer fluorescence remained on the same 30% level. Similar dynamics was shown for SPKR1:1 polymer. Charge ratio escalation caused decrease in fluorescence, which reduced to approximately 40% of control level in a range from unbound DNA to the charge ratio of 1:3. Further increase of the charge ratio did not cause significant alterations in fluorescence. Different dynamics of complexes formation was observed for histidine-containing polymers. In case of SPKH10:1 a decrease in fluorescence was shown reaching the lowest level of 30% at the charge ratio 1:2. Further charge ratio increase showed a slight effect of an incubation time on the complexes density. Fluorescence level after 5 minutes of incubation, which comprised approximately 40% for 1:3, 1:4, 1:5, 1:6 and 1:7 charge ratios, showed a tendency to decrease with time and reached the level of 30% to the 30min time point. In case of charge ratio 1:5 an increase of fluorescence was observed with high deviation. For SHKP1:1 polymer a rapid decrease in DNA fluorescence was observed between charge ratios 1:0,1 and 1:3, followed by an insignificant decrease tendency observed for other charge ratios (form 1:4 to 1:7).

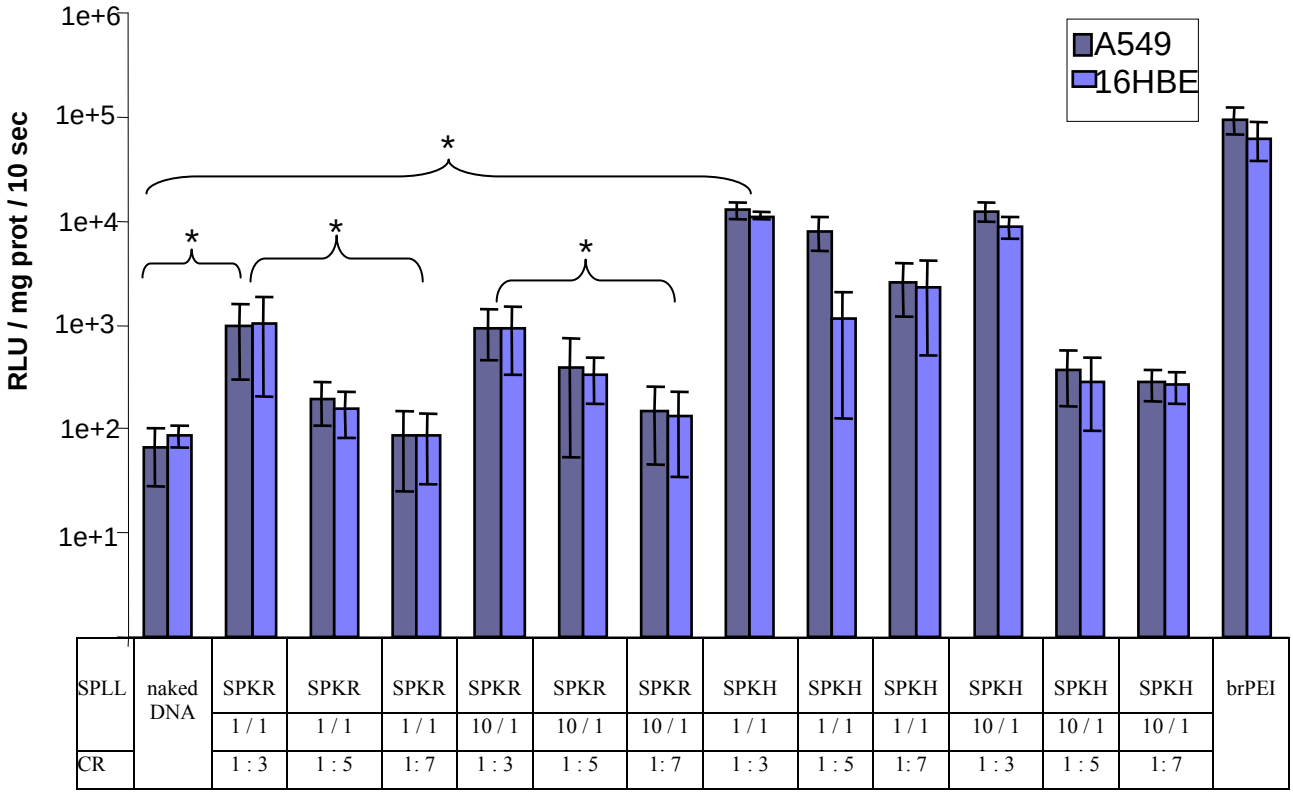
3.7 Transfection of A549and 16HBE cells with a series of branching polypeptides

The most important characteristic feature of the polymers taken into the study was their ability to deliver plasmid DNA into the cells in culture thus providing the expression of reporter protein. The gene delivery capacities of four branching modified polylysines were tested on different cell cultures. Taking into consideration the results obtained with the primary branching polymer D5 (62), two incubation tome point were chosen for transfection experiments: 24hours and 48 hours. The data obtained from the polymers were compared with

the negative control values (naked DNA delivery) as well as with the positive control (complexes of pDNA with branched PEI). Based on our own data about capacities of SPLL polypeptides, preceding studies of other polymers of this group (63) and published data (7, 25, 42) three charge ratios were chosen for gene delivery experiments: 1:3, 1:5 and 1:7.

Complexes produced at every of the named charge ratios could provide not only complete DNA binding but also protection of the plasmid from enzymatic degradation. According to the results of performed analysis the complexes formed in distilled water appeared to be more favorable candidates for gene delivery experiments (3.1-3.6).

It was shown that after 24 hours of incubation SPRK1:1 polymer could provide significantly higher expression of the luciferase gene at the charge ratio 1:3 that the delivery of uncomplexed DNA (Fig. 10). Other two charge ratios did not showed significant difference from the negative control. Also for SPKR10:1 polymer charge ratio 1:3 was shown to be the most effective, while charge ratios 1:7 was proved to possess significantly lower efficiency and was comparable to that of naked pDNA.

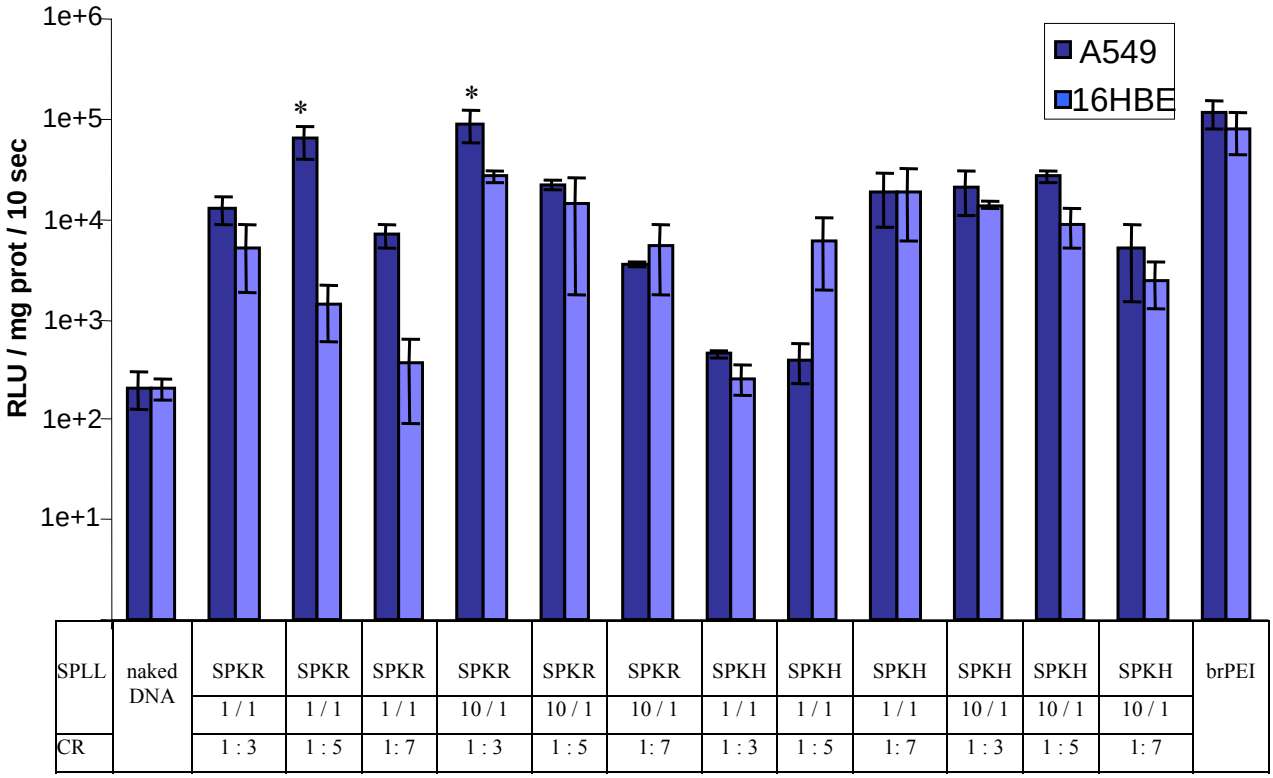


SPLL	naked DNA	SPKR	SPKR	SPKR	SPKR	SPKR	SPKR	SPKH	SPKH	SPKH	SPKH	SPKH	SPKH	brPEI
		1 / 1	1 / 1	1 / 1	10 / 1	10 / 1	10 / 1	1 / 1	1 / 1	1 / 1	10 / 1	10 / 1	10 / 1	
CR		1 : 3	1 : 5	1: 7	1 : 3	1 : 5	1: 7	1 : 3	1 : 5	1: 7	1 : 3	1 : 5	1: 7	

Figure 10. Transfection of A549 (dark) and 16HBE (light) cell lines with a series of branching polymers. Polypeptides were mixed with plasmid DNA under certain charge ratios (CR) and transferred to the cells. After 24 hours incubation cells were analysed for transgene (luciferase) expression. The data are scaled as relative light units (RLU) per mg of protein per 10 sec. Experiments were performed in triplicate. Data are presented as average mean with standard deviation. Significant differences ($p<0.05$) between values are marked with an asterisk.

Generally higher transfection efficiency was obtained with histidine-modified polymers. For 16HBE cells 1:3 charge ratio of DNA with SPKH1:1 was mostly effective, while for A549 cells comparable results were obtained with 1:3 and 1:5 charge ratios. For SPKH10:1 polymer 1:3 charge ratio was significantly more effective than 1:5 and 1:7. The highest values of luciferase expression were obtained with charge ratio 1:3 for SPKH10:1 polymer and 1:3 and 1:5 for SPKH1:1 polypeptide. Still the levels of luciferase expression obtained with all four SPLL polymers were significantly lower than the efficiency of brPEI polymer which was used as positive control.

For incubation time of 48 hours a significant increase in transgene expression was observed for both arginine-modified polymers. Nevertheless, the tendency of luciferase expression to decrease with the increment of charge ratio was observed for both arginine-modified polypeptides on 16HBE cells. The highest levels of luciferase expression were observed for SPKR1:1 with 1:5 charge ratio and for SPKR 10:1 with 1:3 and were as high as after transfection with branched PEI, used as positive control (Fig.11). For both histidine-modified polymers and increase in transgene expression was shown was shown for certain



SPLL	naked DNA	SPKR	SPKR	SPKR	SPKR	SPKR	SPKR	SPKH	SPKH	SPKH	SPKH	SPKH	SPKH	brPEI
		1 / 1	1 / 1	1 / 1	10 / 1	10 / 1	10 / 1	1 / 1	1 / 1	1 / 1	10 / 1	10 / 1	10 / 1	
CR		1 : 3	1 : 5	1: 7	1 : 3	1 : 5	1: 7	1 : 3	1 : 5	1: 7	1 : 3	1 : 5	1: 7	

Figure 11. Transfection of A549 (blue) and 16HBE (purple) cell lines with a series of branching polymers. Polypeptides were mixed with plasmid DNA under certain charge ratios (CR) and delivered to the cells. After **48 hours** incubation cells were analysed for transgene (luciferase) expression. Experiments were performed in triplicate. The data are scaled as relative light units (RLU) per mg of protein per 10 sec with standard deviation. Results statistically undistinguishable from positive control are denoted with an asterisk.

charge ratios compared with the 24 hours time point, while the effect of charge ratio on transfection efficiency remained the same for SPKH1:1 polypeptide .

For arginine-modified peptides significant differences between two cell cultures were shown. Transfection efficiency obtained woth SPKR1:1 polymer on 16HBE cells was at all used charge ratios significantly lower than that on A549 cell line, while with SPKR10:1 polymer only 1:3 charge ratio showed difference between two cell lines.

The highest transgene expression values were obtained for SHKR1:1 polymer with 1:5 and for SHKR10:1 with 1:3 charge ratios. These values were not only significantly higher than those obtained with histidine-modified polymers but also did not differ prom positive control (branched PEI).

3.8 Optimization of pulmonary gene delivery in mice

3.8.1 Preparation of PEI -pDNA polyplexes

A certain limitation for application of PEI/pDNA complexes for gene delivery is the amount of liquid which can be administered to an animal either by instillation or aerosol application. The necessity to use highly concentrated DNA requires special conditions which would allow concentrating plasmid DNA in a small volume avoiding precipitation. Stability of PEI/pDNA complexes was investigated under different pH levels. We have found that the threshold concentration of pDNA was 1 mg/ml while the highest possible pH level was 6. Precipitation of pDNA was observed at higher pH or higher DNA concentrations. We have shown that DNA concentration influences the size of complexes.

	Size (nm)		
[pCMVLuc] mg/ml	pH=5	pH=6	pH=7.4
0.25	90±1	97±2	98±1
0.5	121±1	127±2	105±1
0.75	172±1	148±2	161±2
1.0	197±3	196±2	precipitation

Table 3. Size of pCMVLuc plasmid with branched PEI formed under different pH and DNA concentration.

Complexes formed at DNA concentration of 0,25mg/ml had a diameter of around 100nm, while those produced at 0,75 mg/ml and 1,0 mg/ml had a diameter of 150nm and 200nm respectively (Table 3). The size of complexes was stable at least 50 min in all tested variations.

The particles for aerosol delivery of CpG-free plasmid DNA were made according to the same strategy as for aerosol delivery of pCMVLuc/brPEI complexes. The NP ratio was 10 and complexes were performed in distilled water. Final DNA concentration of 0,5 mg/ml was reached by mixing of 4ml brPEI solution with the same volume of DNA distilled water. The size of complexes was measured before every application and comprised approximately 100nm. The differences between CpGLuc- and CpGmcs-plasmids containing complexes were insignificant.

Complexes for intranasal instillation experiments were performed in final volume of 100µl of distilled water per animal as described above (2.2.6.2). The size of complexes was controlled prior to application.

3.8.2 Lung function measurements in mice after intranasal instillation and aerosol application of PEI-pDNA gene vectors comprising a first generation plasmid pCMVLuc

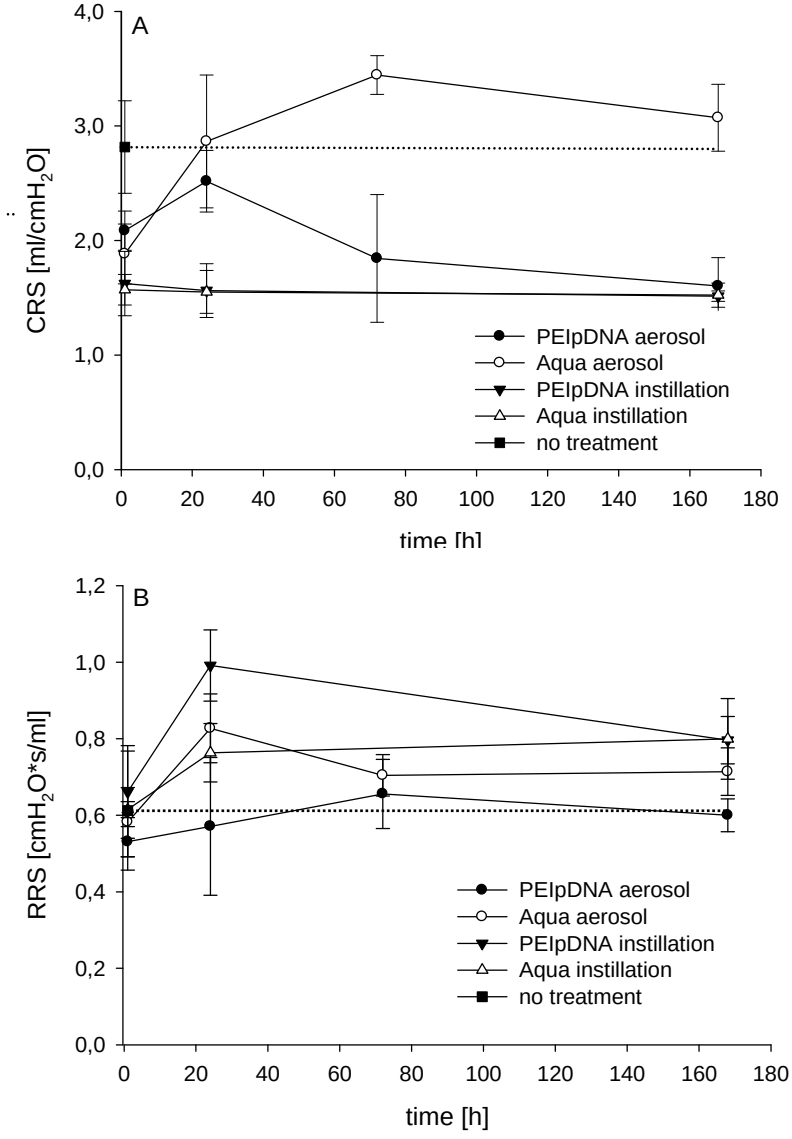
Dynamic lung mechanics were measured 1 hrs, 24 hrs, 3 days, and 7 days, both after nasal instillation and aerosol application of PEI-pDNA complexes. Mice treated with vehicle only, i.e. distilled water, and untreated mice were used as controls.

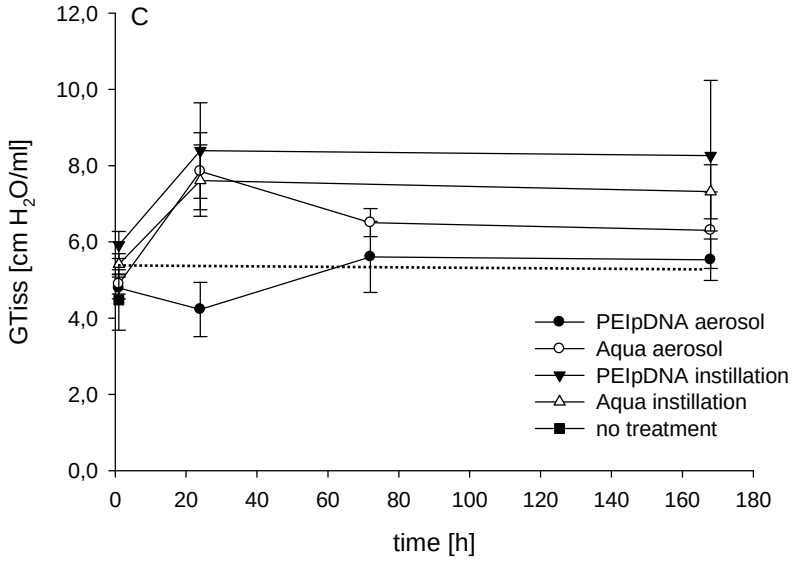
Compliance of the respiratory system (Crs/ bodyweight) was significantly decreased by 60%, one hour after instillation of PEI-pDNA complexes compared to untreated mice (1.6 ± 0.2 ml/cmH2O/kg BW vs. 2.8 ± 0.4 ml/cmH2O/kg BW, $p<0.001$) and stayed low for the remaining observation period (Fig. 12A). At one hour similar values were observed for mice treated with instilled vehicle only. In contrast to instillation, one hour after PEI-pDNA aerosol treatment, Crs was reduced only by 25% ($p=0.01$), recovered to values of untreated controls 24 hrs after application, but then declined to low values comparable to instillation. As observed for PEI-pDNA complexes, aerosol application of distilled water resulted in a significant decrease of Crs one hour after application but returned to normal values of untreated controls at later time-points. In untreated animals the level of lung compliance remain stable on 2.8 ± 0.4 ml/cmH2O/kg BW.

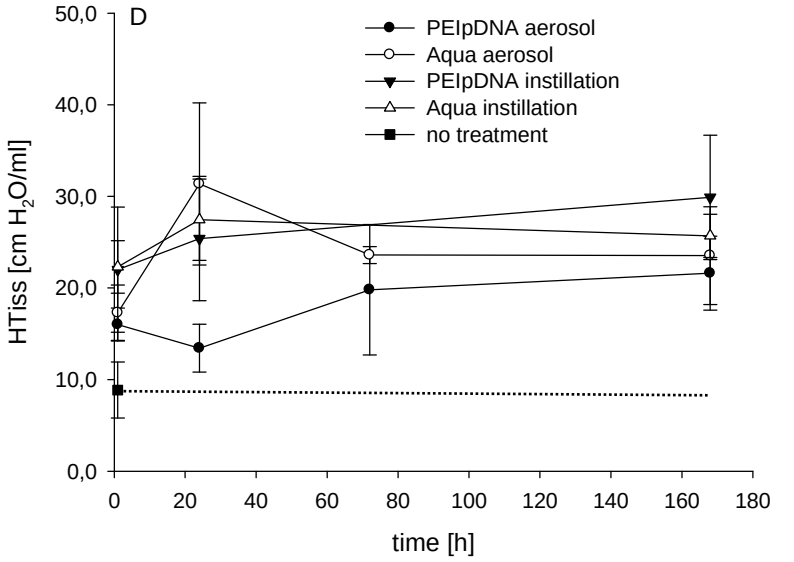
Resistance of respiratory system (Rrs) transiently increased after intranasal instillation of PEI-pDNA complexes or distilled water but returned to values which were not significantly different from untreated controls after seven days (Fig. 12B). Comparable Rrs values were

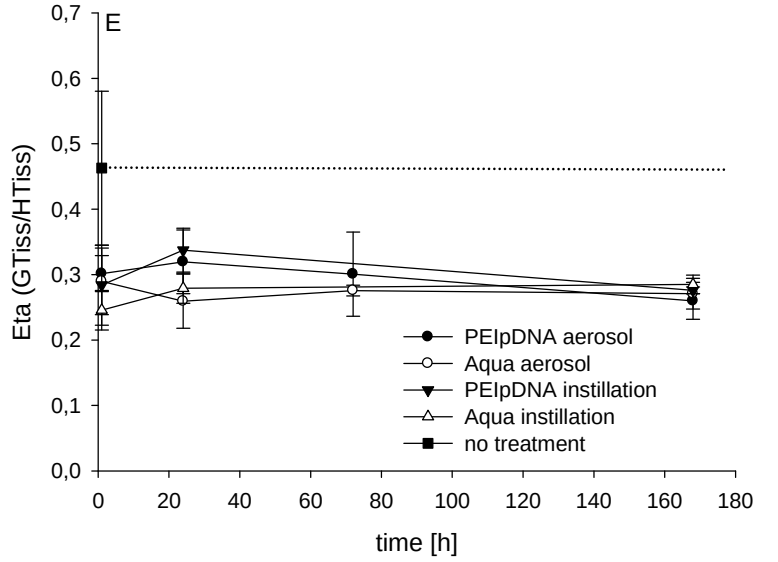
observed for mice treated with nebulized water. Aerosol application of PEI-pDNA did not alter Rrs compared to untreated controls.

Results from lung impedance measurements are outlined in figures 12C-12F. Tissue damping (GTiss), reflecting resistive forces of lung tissue, was not significantly different from control values after PEI-pDNA aerosol application, whereas after aerosol application of distilled water and intranasal instillation of PEI-pDNA complexes or vehicle only, GTiss significantly increased ($p<0.005$) compared to untreated controls 24 hrs after treatment (Fig 12C). After 7 days values returned to control after aerosol application of distilled water but not after intranasal instillation of PEI-pDNA complexes and distilled water. Tissue elastance (HTiss) was significantly affected by all treatments. However, the effect of inhaled PEI-pDNA was markedly delayed and only reached significance 7 days after treatment (Fig. 12D). The hysteresivity (Eta), which is the ratio of GTiss to HTiss, did not show any significant changes in treatment groups compared to untreated lungs (Fig 12E). Newtonian resistance (Rn), reflecting central airway resistance, was not significantly affected by any treatment (Fig 12F).




C
GTiss [cm H2O/ml]
time [h]
PEIpDNA aerosol
Aqua aerosol
PEIpDNA instillation
Aqua instillation
no treatment


D
HTiss [cm H2O/ml]
time [h]
PEIpDNA aerosol
Aqua aerosol
PEIpDNA instillation
Aqua instillation
no treatment


E
Eta (GTiss/HTiss)
time [h]
PEIpDNA aerosol
Aqua aerosol
PEIpDNA instillation
Aqua instillation
no treatment

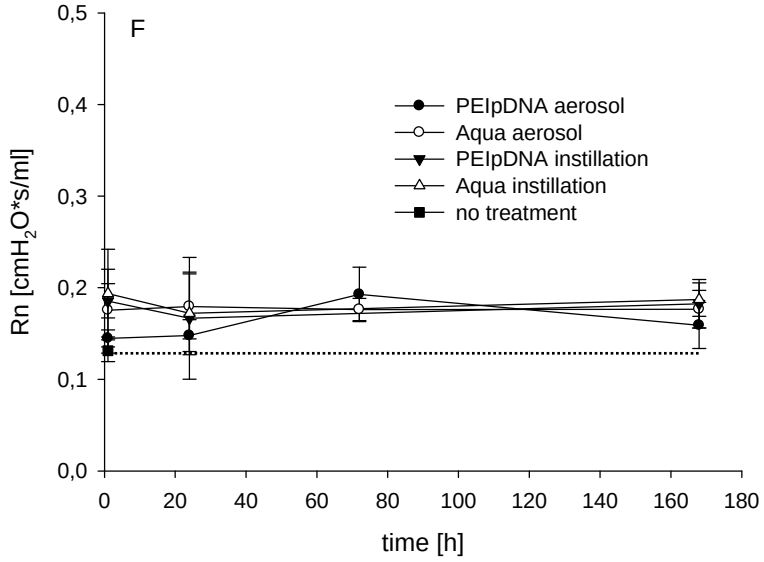
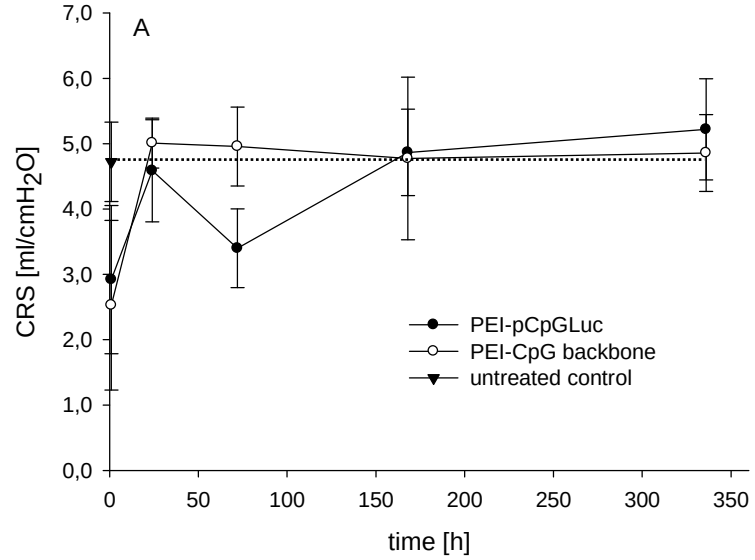
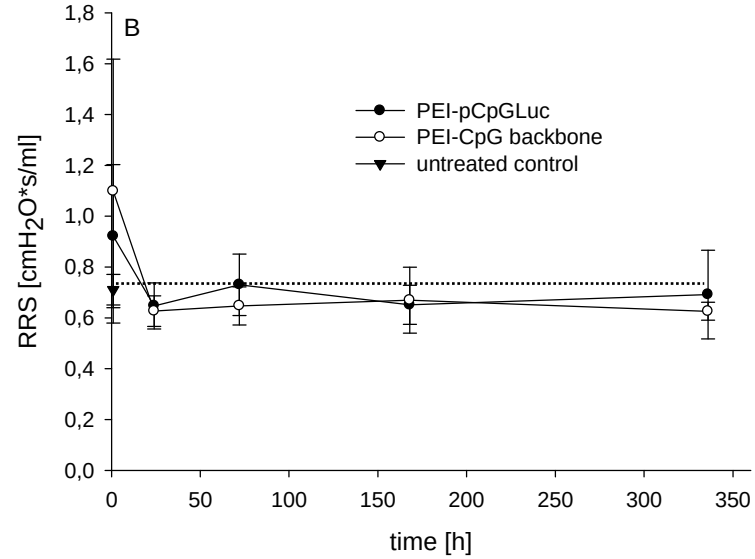


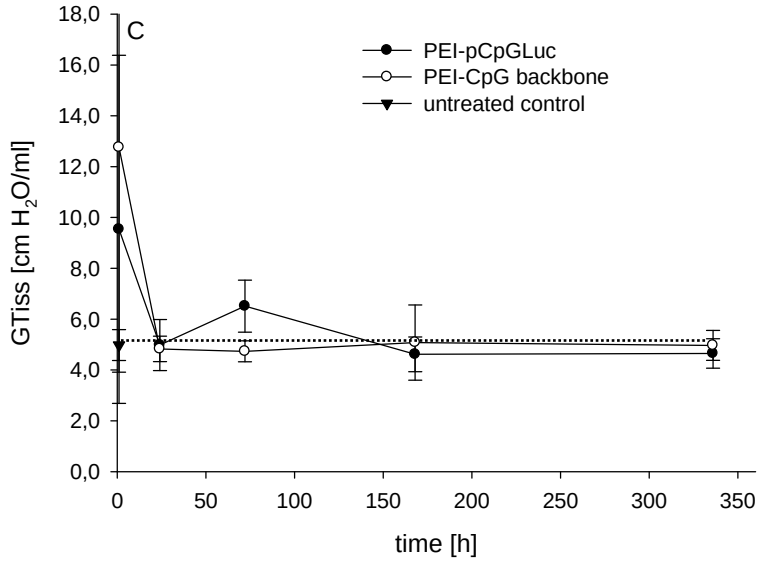
Figure 12. Lung function of mice measured at indicated time points after nebulization and nasal instillation of PEI-pCMVLuc particle and distilled water. (A) Compliance of mice lungs (ΔV/Δp) is given as [ml/cm H2O]. (B) The respiratory system resistance is shown [cm H2O*s/ml]. Tissue damping (GTiss, C), elasticity of collateral lung (HTiss, D), hysteresivity (Eta, E), and Rn (F) were detected. Results are reported as means ± standard deviation (n=5).

3.8.3 Lung function measurements in mice after aerosol application of PEI-pDNA gene vectors comprising CpG-free plasmid DNA

In order to investigate if the decline in lung function after aerosol treatment was a result of an acute unmethylated CG dinucleotide (CpG)-mediated inflammatory response or due to immune reactions against PEI-pCMVLuc compelxes or the luciferase transgene, respectively, analogue experiments were performed with a CpG-free luciferase reporter plasmid and its backbone. For each of the analyzed lung function parameters, values were not significantly different from untreated controls at time-points later than 24 hours (Fig. 13). Lung function abnormalities were only observed one hour after application, which may have resulted from transient airway epithelial swelling caused by distilled water inhalation (129). Thus, presence of CpG motifs in plasmid DNA had a dramatic influence on functioning of murine lung.


A
CRS [ml/cmH2O]
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
0
50
100
150
200
250
300
350
time [h]
PEI-pCpGLuc
PEI-CpG backbone
untreated control


B
RRS [cmH2O*s/ml]
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
50
100
150
200
250
300
350
time [h]
PEI-pCpGLuc
PEI-CpG backbone
untreated control


C
GTiss [cm H2O/ml]
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
0
50
100
150
200
250
300
350
time [h]
PEI-pCpGLuc
PEI-CpG backbone
untreated control

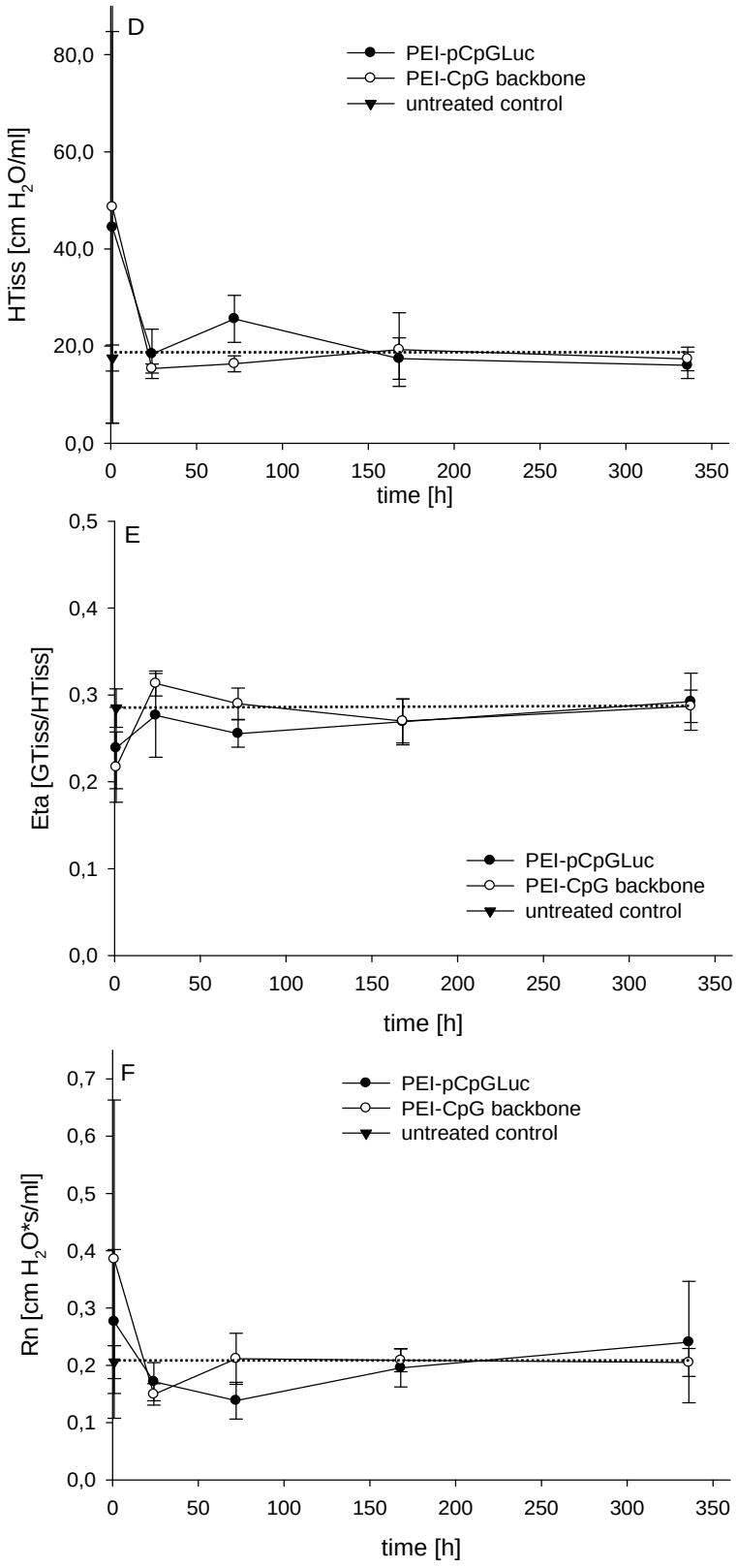


Figure 13. Lung function of mice after nebulization of PEI-pCpG-Luc and PEI-pCpG-mcs particles and distilled water. At indicated time-points after aerosol application pulmonary function of each mouse was measured. Compliance of mice lungs (ΔV/Δp) is given as [ml/cm H2O] (A). The respiratory system resistance is shown in figure B [cm H2O*s/ml]. Tissue damping (GTiss, C), elasticity of collateral lung (HTiss, D) and hysteresivity (Eta, E), and Rn (F) were detected. Results are reported as means ± standard deviation (n=5).

3.8.4 Histological examination of lung tissue after intranasal instillation and aerosol delivery of PEI-pDNA complexes.

Mice which received PEI-pDNA complexes by intranasal instillation showed a moderate inflammatory response which was associated with local immune cell infiltration. Twenty-four hours after treatment, restricted central parenchymal foci with strong capillary thrombus formation were observed (Fig. 14C-D). Seven days after treatment, newly formed foci of haemorrhage including fibrinthrombus were observed (Fig. 14E-F).

After aerosol delivery of PEI-pDNA complexes to the mice lung, a similar inflammatory response was observed which was less pronounced compared to intranasal instillation. Lungs showed subpleural focal haemorrhage associated with congestions and small capillary and pre-capillary fibrin thrombus after 24 hrs (Fig. 14G-H). Small subplural haemorrhage and congestions were still observed seven days after treatment (Fig 14I-J).

Mice which received distilled water or PEI-pDNA complexes with CpG-free plasmid DNA by aerosol application did not show differences to untreated controls (Fig 14K-L).

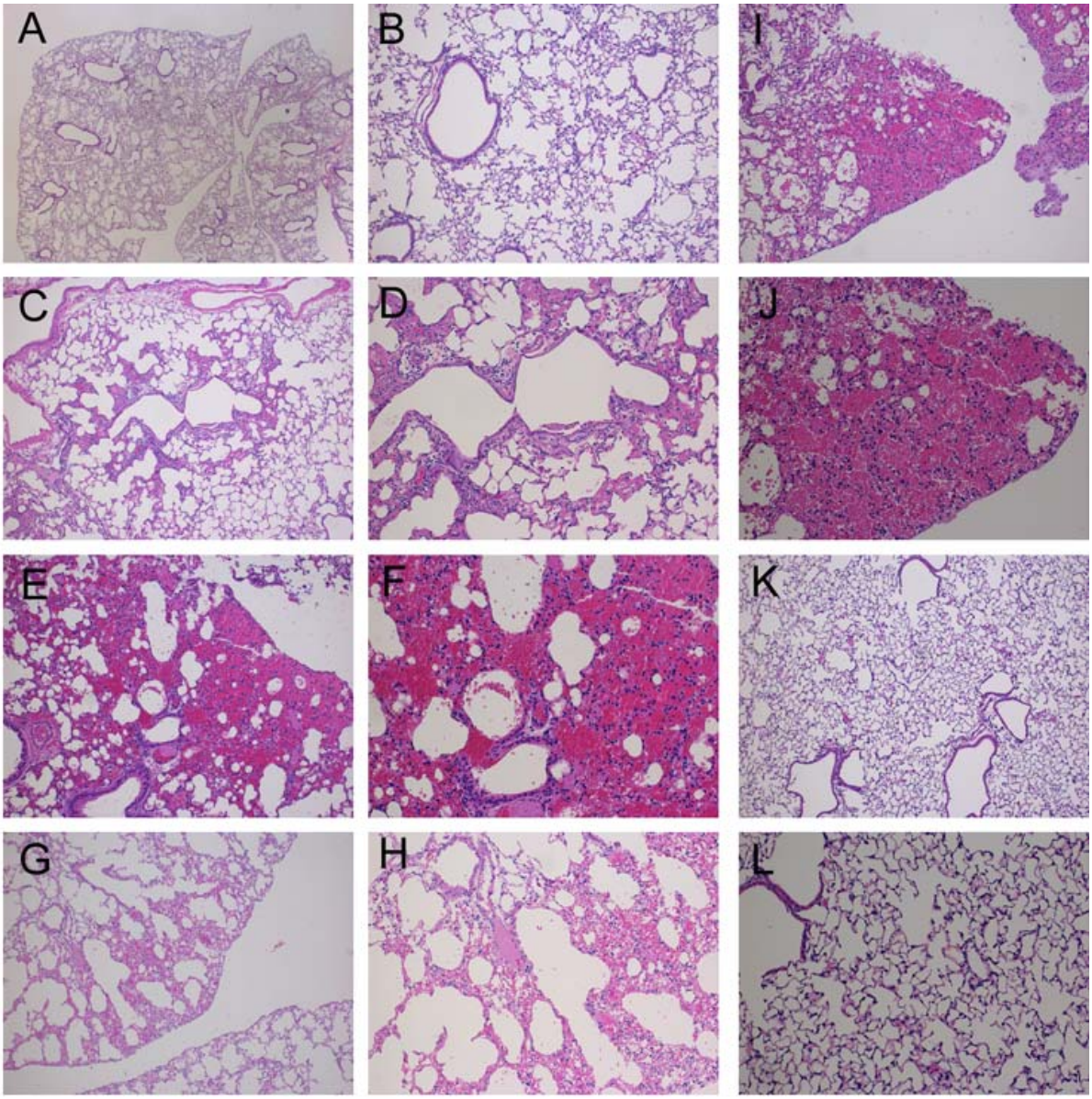


Figure 14. Histological examination of lung tissue after aerosol application and instillation of PEI-pDNA particles and distilled water. Histological examinations were performed using haematoxylin-eosin stain. A-B. Lung tissue of control mouse 24 hrs after distilled water aerosol treatment (10- and 20-fold magnification). C-D. Lung tissue from mouse 24 hrs after PEI-pCMVLuc instillation (10-and 20-fold magnification, respectively). E-F. Lung tissue from mouse 7 days after PEI-pCMVLuc instillation (10- and 20-fold magnification). G-H. Lung tissue from mouse 24 hrs after PEI-pCMVLuc aerosol treatment (10- and 20-fold magnification). I-J. Lung tissue from mouse 7 days after PEI-pCMVLuc aerosol treatment (10- and 20-fold magnification). K-L. Lung tissue from mice 24 hrs after PEI-pCpG-Luc aerosol treatment (10- and 20-fold magnification).

3.9 Pulmonary pharmacokinetics of PEI-pDNA complexes after aerosol application and intranasal instillation

At certain time points lung tissue as well as BALF cells and liquid were analyzed for presence of plasmid DNA with the help of real time polymerase chain reaction (RT PCR). analysis. The obtained data were used for calculation of such clearance parameters as maximal concentration, area under the curve AUC, half-clearance time T ½. We have found similar amounts of plasmid DNA in the lungs 1 hour after administration, which comprised 257ng and 293ng after aerosol application and instillation respectively (Table 4). After 24 hours of gene delivery the amount of DNA after aerosol application decreased to 180ng and remained on practically the same level up to 72hours, while that after instillation decreased dramatically after 24 hours up to approximately 30ng.

Significant differences ($p>0.001$) in DNA concentrations between instillation and aerosol delivery were found in BAL liquid already 1 hour after application. Whereas 740pg of plasmid DNA was present in BALF liquid after instillation, only around 1,3pg were found after aerosol delivery. The same tendency was observed for resident cells of lung (BALF cells).

Delivery route	Compartment	AUC$^{\#}$	C_{max}§	Clearance$^{\$}$	K_e*	$T_{1/2}$
Aerosol	Lung tissue	72,997	257,759	2.92	0.0092	75.4
	BALF cells	0.12	188	1,551	0.015	46.4
	BALF	12.0	1.3	0.11	0.016	44.2
Instillation	Lung tissue	28,861	293,519	10.17	0.0448	15.4
	BALF cells	4.55	9,772	2,149	0.0098	70.6
	BALF	5,212	740	0.14	0.020	34.0

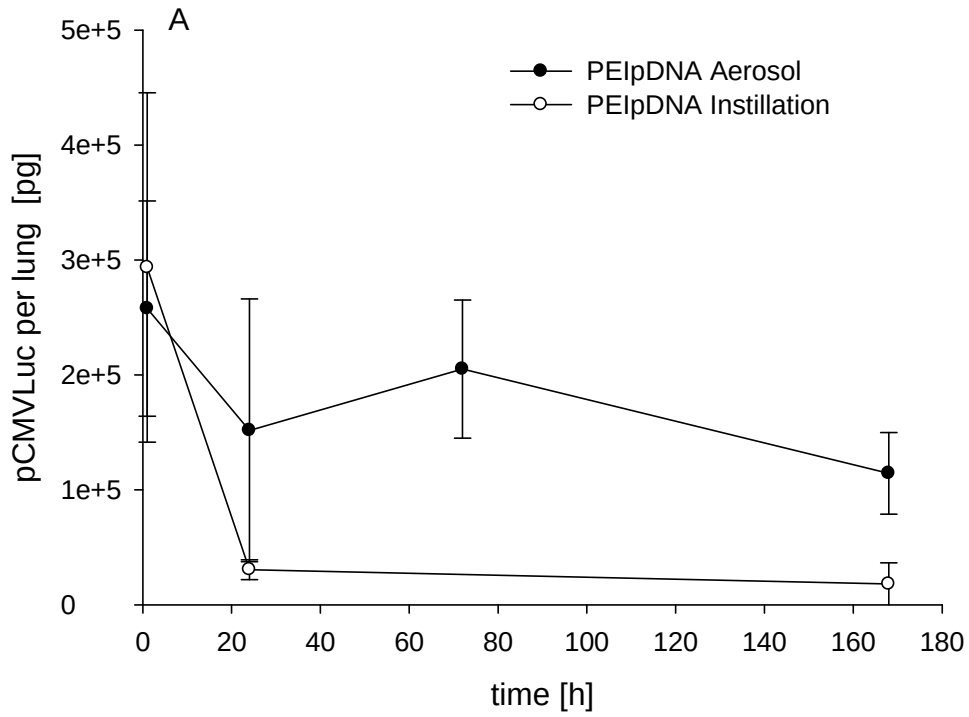
Table 4. Comparison of pulmonary pharmacokinetic parameters for PEI-pCMVLuc after aerosol application and intranasal instillation. $^{\#}$**AUC** for 7-day period was calculated by trapezoidal rule and is presented as pg pCMVLuc per mg lung tissue [pg pCMVLuc*h/mg lung tissue], pg pCMVLuc per BALF cell [pg pCMVLuc*h/single cell], or pg pCMVLuc per ml supernatant [pg pCMVLuc*h/ml supernatant]. §**C_{max}** was measured after 1 hour and is presented as pg pCMVLuc per lung tissue, per total BALF cells, or total supernatant (7 ml). $^{\$}$**CL** is presented as mg lung tissue cleared per hour, BALF cell cleared per hour, or ml supernatant cleared per hour and was calculated C_{max}(dose)/AUC. ***K_e** is CL/mg total lung tissue, CL/amount of total BALF cells, or CL/7 ml supernatant of BALF [1/h]. **$T_{1/2}$** is ln 2/K_e and is presented in hours.

While almost 10ng of pCMVLuc were found in BALF cells one hour after instillation, only 180pg were present in BALF cells after aerosol application at the same time point. The amount of plasmid DNA after instillation remained on the level of around 10ng for the next 24 hours and than decreased drastically to the seven days time point.

3.10 Comparing pDNA clearance after instillation and aerosol delivery of pCMVLuc plasmid

For comparison of DNA clearance patterns samples of 5 animals from each groups were tested for plasmid content. RT PCR experiments were repeated at least in triplicates with three probes pro set .

Patterns of plasmid DNA clearance from different lung compartments showed significant differences between instillation and aerosol application. Clearance coefficient from lung tissue after instillation was 10,17, which is 3,5-fold higher than after aerosol application. Significant differences were observed in clearance patterns between all lung compartments.



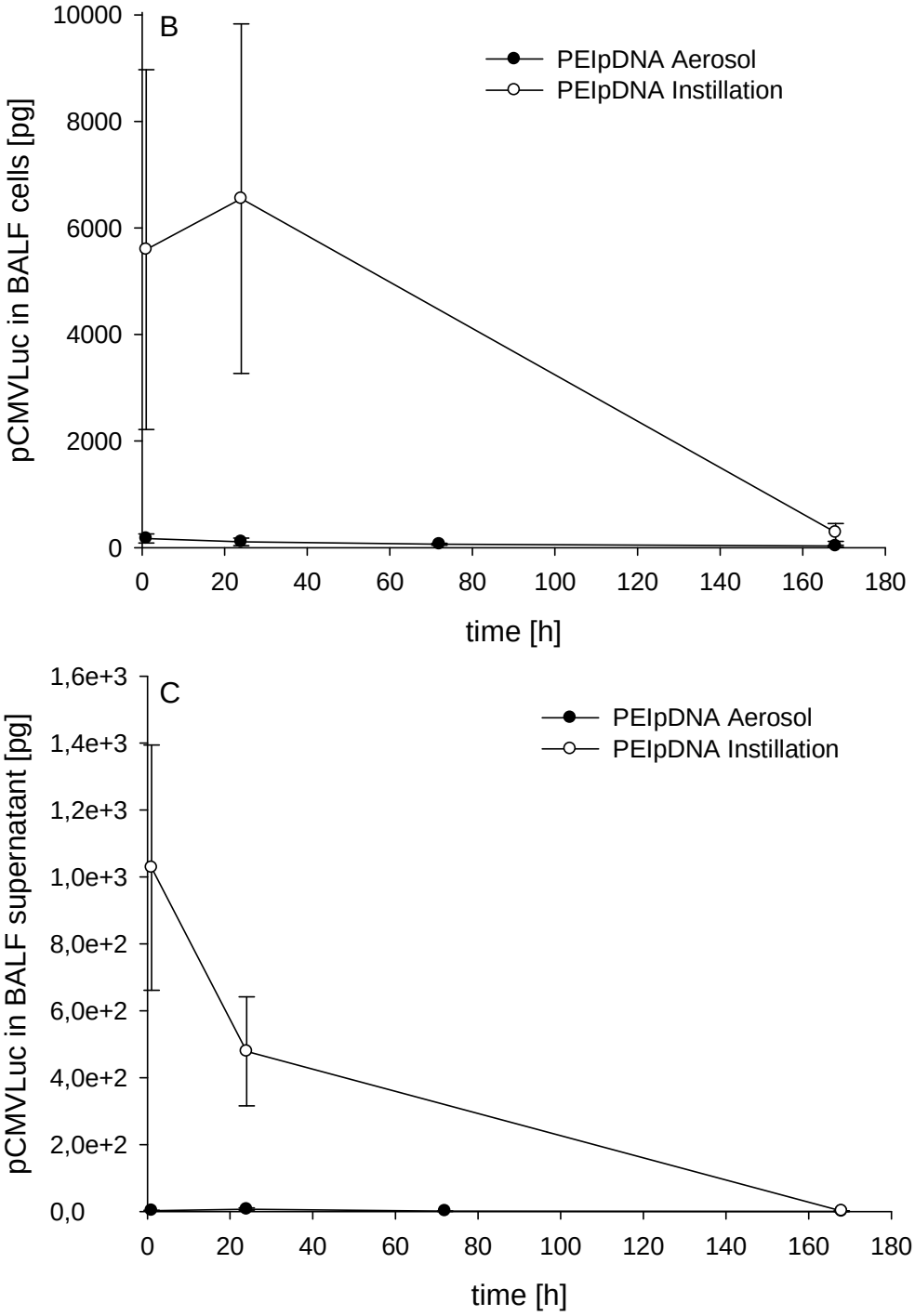


Figure 15. Plasmid DNA clearance from the lungs after nebulization and intranasal instillation of PEI-pDNA complexes. PEI-pDNA complexes were either nebulized or instilled to the lungs of mice. At indicated time points pDNA was analyzed in mouse lung tissue (A), BALF cells (B), and BALF fluid (C) by real time PCR. Results are reported as means ± standard deviation (n=5).

The resulting half clearance time form the lung tissue calculated for aerosol application and instillation comprised 75,4 and 15,4 hours, respectively (Fig. 15). Thus elimination from the lung after instillation was 4,9-fold higher after instillation than after aerosol distribution. Seven days after PEI-pDNA aerosol application still 47% of initially deposited pDNA could be detected in lung tissue resulting in a pDNA half-life (T1/2) of 75.4 hrs of inhaled pDNA in lung tissue In contrast, 90% of pCMVLuc was cleared from murine lung tissue within 24 hrs after intranasal instillation.

Clearance patterns form BALF and BALF cells also differed greatly between two delivery methods. Only small amounts of plasmid DNA were found in BALF and BALF cells after aerosol application, while maximal concentrations after instillation were 540-fold and 52-fold higher respectively. Half clearance time form the BALF cells after instillation was approximately twice higher than after aerosol, indicating that the pharmacokinetic profile of PEI-pDNA complexes in the lungs was dependent on the route of application.

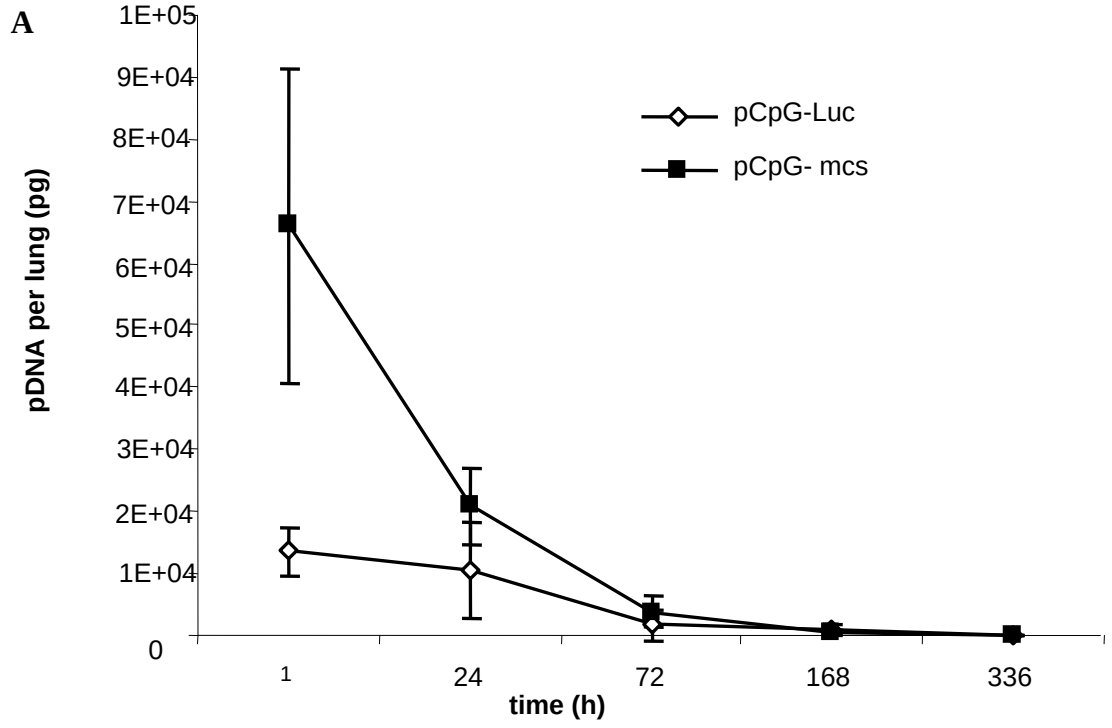
Plasmid DNA	Compartment	AUC[#]	C_{max}[§]	Clearance[$]	K_e[*]	$T_{1/2}$
pCpG-free-Luc	Lung tissue	3824,9	13499	3,53	0,01587752	43,7
	BALF cells	11,8	124206	10482,05	0,03253273	21,3
	BALF	133294,5	113423	0,85	0,0850917	8,1
pCpG-free-mcs	Lung tissue	8285,8	66101	7,98	0,03513819	19,7
	BALF cells	28,2	168959	5983,81	0,01332697	52,0
	BALF	70744,0	60646	0,86	0,08572587	8,1

Table 5. Comparison of pulmonary pharmacokinetic parameters after aerosol application of pCpG-free-Luc and pCpG-free-backbone plasmids. [#]**AUC** for 7-day period was calculated by trapezoidal rule and is presented as pg pCMVLuc per mg lung tissue [pg pCMVLuc*h/mg lung tissue], pg pCMVLuc per BALF cell [pg pCMVLuc*h/single cell], or pg pCMVLuc per ml supernatant [pg pCMVLuc*h/ml supernatant]. [§]$\mathbf{C_{max}}$ was measured after 1 hour and is presented as pg pCMVLuc per lung tissue, per total BALF cells, or total supernatant (7 ml). [$]**CL** is presented as mg lung tissue cleared per hour, BALF cell cleared per hour, or ml supernatant cleared per hour and was calculated C_{max}(dose)/AUC. [*]$\mathbf{K_e}$ is CL/mg total lung tissue, CL/amount of total BALF cells, or CL/7 ml supernatant of BALF [1/h]. $\mathbf{T_{1/2}}$ is ln 2/K_e and is presented in hours.

3.11 Pulmonary pharmacokinetics of PEI-pDNA complexes after aerosol application of CpG-free-plasmid containing complexes

In a series of experiments devoted to aerosol delivery of CpG-free plasmids, analysis of plasmid DNA concentrations was performed and the clearance coefficients were calculated.

One hour after aerosol application of pCpG-Luc- and pCpG-mcs–containing complexes 13ng and 66ng of plasmid DNA, respectively, were found per whole lung tissue. Twenty four hours after transfection the amount of pCpG-Luc had slightly decreased to proximately 11ng and after a week only residual amount of 1,5ng were found in the lung tissue (Fig. 16). Much more rapid clearance was observed for pCpG-mcs plasmid: after 24hours the amount of pDNA decreased 3-fold and reached the level of approximately 3,5 ng per lung already after 72 hours of incubation. In resident BAL cells relatively similar amounts of DNA were found: 125ng of pCpG-Luc and 168ng for pCpG-mcs. Clearance of pDNA from BALF cells and BALF liquid also showed great similarity between two plasmids. Around 30ng of pCpG-Luc were found 24 hours after administration and only residual amount of 3,7ng per all cells of BALF was observed after 72 hours. Almost 100ng of pCpG-mcs were found in resident lung cells after 24 hours of incubation, which decreased to 35ng after 72hours. In BALF liquid 113ng and 60ng of plasmid DNA were found for pCpG-Luc and pCpG-mcs respectively after 1 hour of incubation. A dramatic decrease in DNA concentration was observed in both cases in the next 24 hours and comprised 180-fold for pCpG-Luc and 273.fold for pCpG-mcs. Plasmid DNA concentrations after 7 days if incubation did not differ form the levels of negative control.



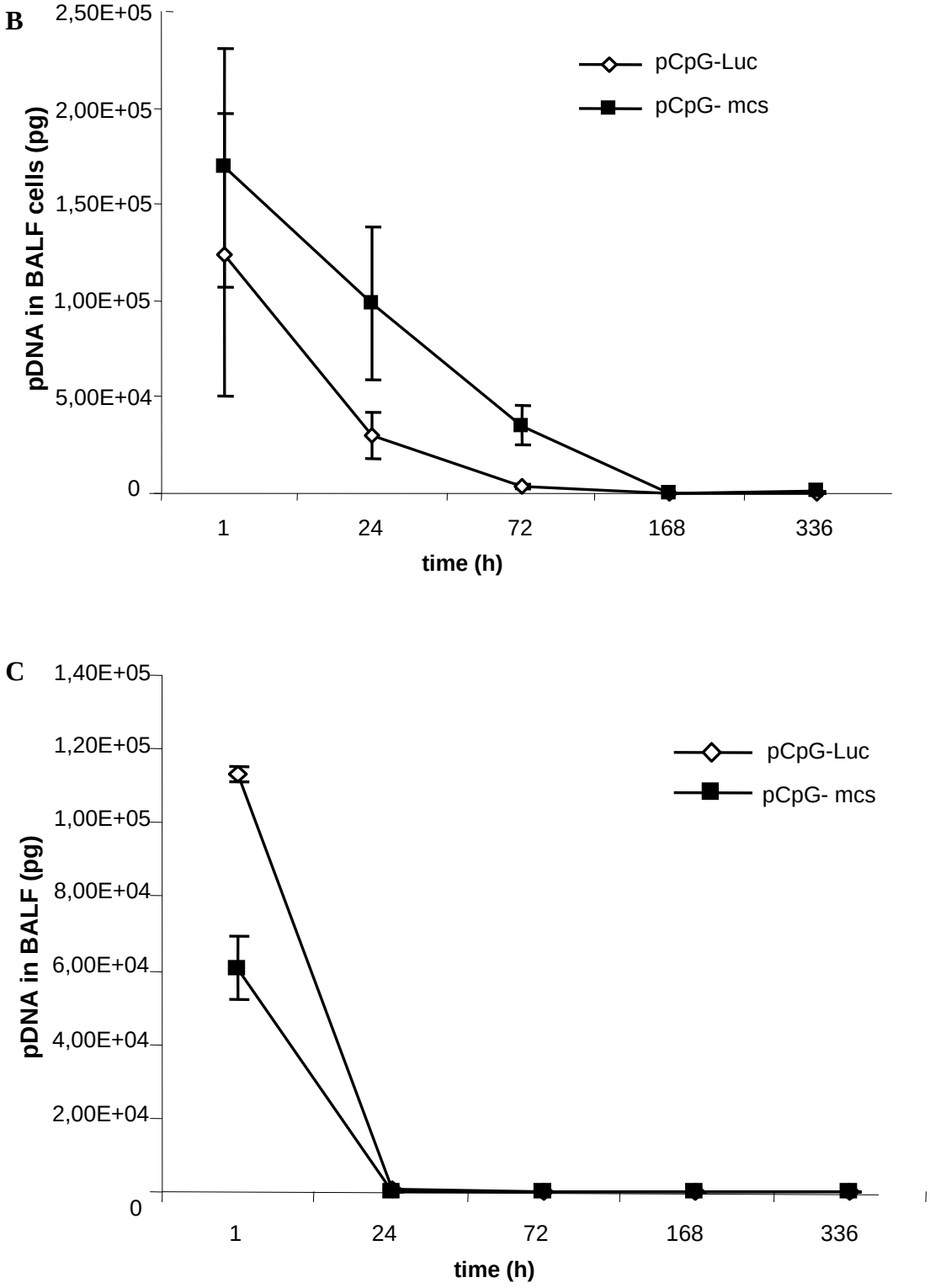


Figure 16. Plasmid DNA clearance from the lungs after aerosol administration of PEI complexes with pCpG-free-Luc and pCpG-free-mcs plasmids.
At indicated time points pDNA was analyzed in mouse lung tissue (A), BALF cells (B), and BALF fluid (C) by real time PCR.

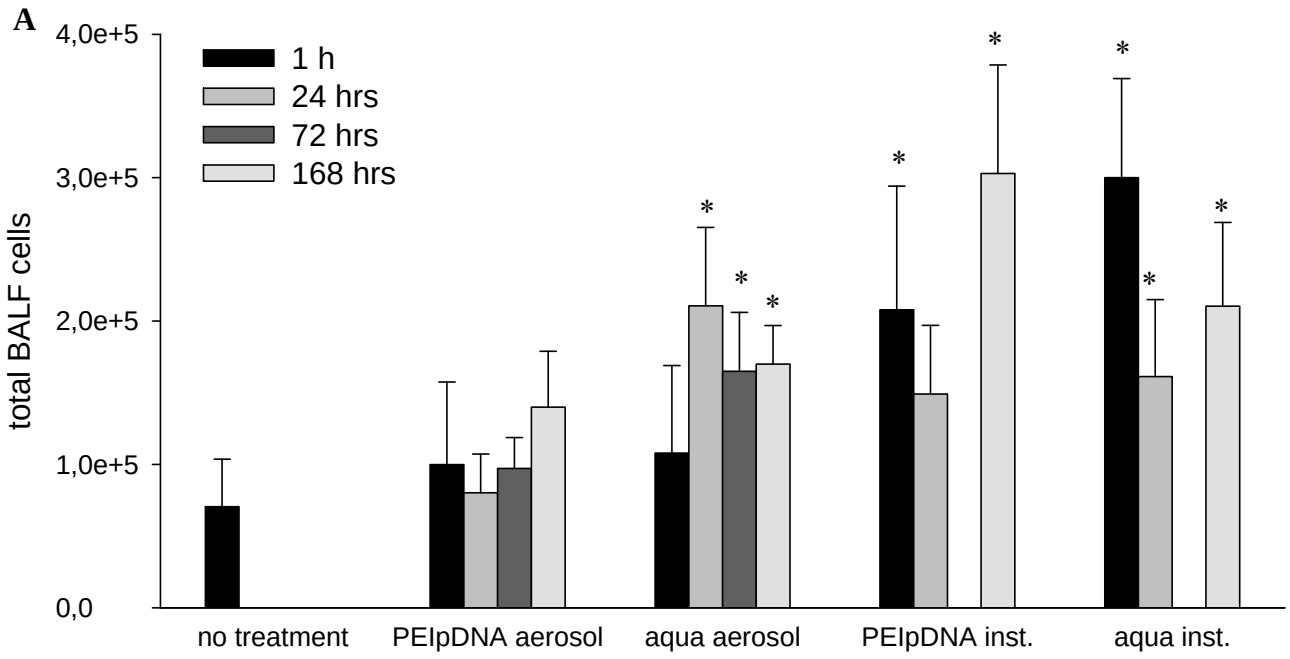
3.12 Comparing pDNA clearance of pCpG-Luc and pCpG-mcs plasmids after aerosol delivery of complexes

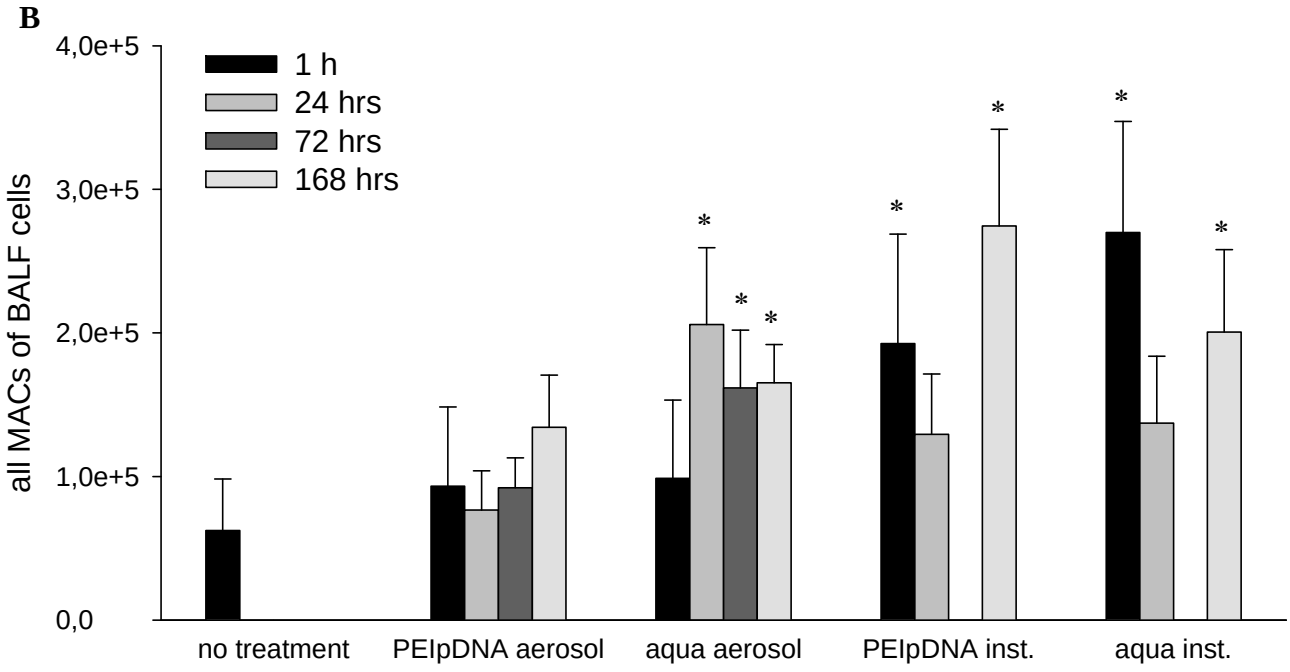
Pharmacokinetics coefficients calculated for pCpG-Luc and pCpG-mcs plasmids in the lung tissue showed significant differences in clearance patterns between too plasmids. The clearance coefficients for pCpG-Luc and pCpG-mcs plasmids comprised 43,7 and 19,7 respectively, resulting in approximately two fold difference in clearance half time (Table 5). Approximately 50% of pCpG-mcs were cleared from the lung tissue within 24 hours after application, while only slight decrease in pCpG-Luc concentration in lung tissue was observed for this time point. Even more pronounced differences in clearance patter were observed for BALF cells. Though maximal concentrations of plasmid DNA observed 1 hour after application were relatively close, for CpG-mcs two-fold higher clearance was observed than for pCpG-Luc. The calculated half clearance times for pCpG-Luc and pCpG-mcs comprised 21,3 and 52 respectively. For the pDNA clearance from BALF liquid no significant differences were observed between two plasmids.

3.13 Analysis of the number of BALF cells and activated AMs after aerosol and intranasal PEI-pCMVLuc application

Analysis of cellular composition of BALF as well as estimation of activated macrophages ratio were performed on every time point (3.8 – 3.12).

The analysis of cell types ratio showed that macrophages represented the major cell population and comprised around 90% of the resident cells. The majority of the rest cell population consisted of lymphocytes, neutrophiles represented ~0.3 % of BALF cells. The total numbers of BALF cells after aerosol application of PEI-pDNA particles was not significantly different from untreated controls. After intranasal instillation a significant 4-fold increase in cell number was observed compared to untreated controls (Fig. 17). Treatment of mice with vehicle only, i.e. distilled water, resulted in a significant increase in number of BALF cells after intranasal application. Whereas distilled water instillation resulted in an immediate 4-fold increase of BALF cells number compared with untreated controls which decreased to 3-fold after 24 hours and remained constant until day 7, aerosol application of distilled water did not lead to any significant changes one hour after treatment. Similar results were found for the numbers of AMs in BALF.


A
4,0e+5
3,0e+5
2,0e+5
1,0e+5
0,0
total BALF cells
1 h
24 hrs
72 hrs
168 hrs
no treatment
PEIpDNA aerosol
aqua aerosol
PEIpDNA inst.
aqua inst.


B
4,0e+5
3,0e+5
2,0e+5
1,0e+5
0,0
all MACs of BALF cells
1 h
24 hrs
72 hrs
168 hrs
no treatment
PEIpDNA aerosol
aqua aerosol
PEIpDNA inst.
aqua inst.

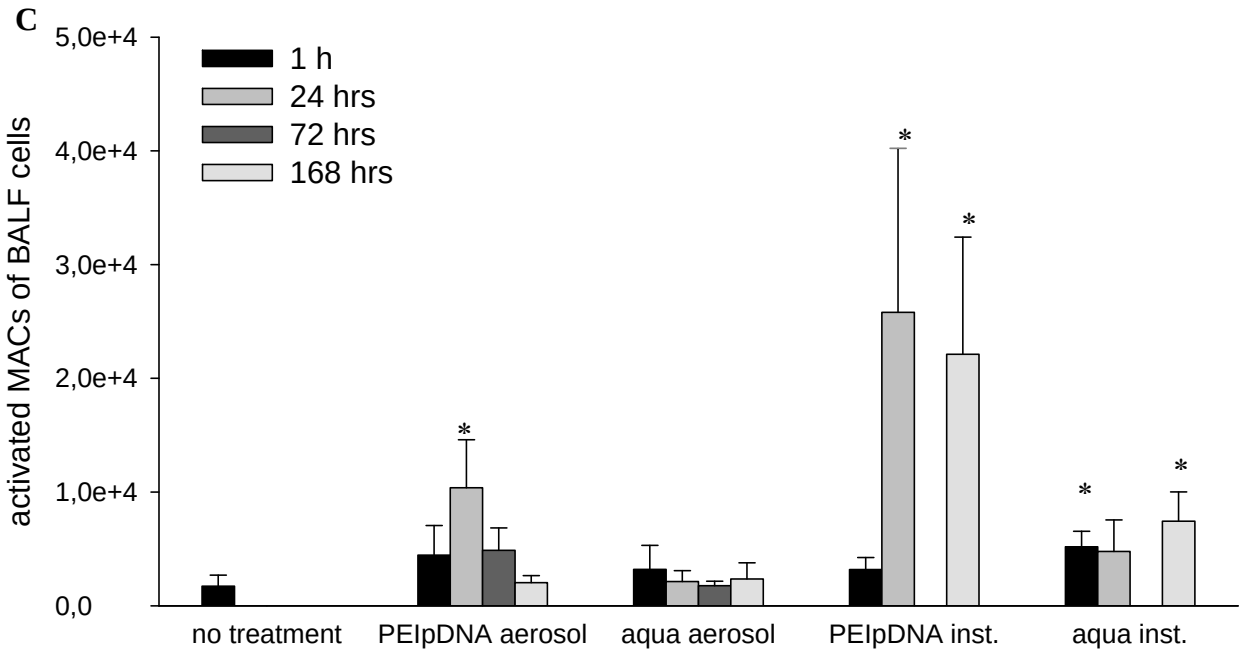


Figure 17. Number of BALF cells after nebulization and intranasal instillation of PEI-pDNA complexes. Total numbers of BALF cells (A) and alveolar macrophages (B) were counted at indicated time points. The number of activated alveolar macrophages was examined by benzidine staining (C). Results are reported as means±standard deviation of the mean (n=5). Statistically significant differences from cell numbers of untreated control mice to bars of treated mice are denoted with an asterisk.

To estimate AM activation, we performed benzidine-staining for myeloperoxidase activity according to method of Kaplow. Number of activated MACs increased 6-fold compared to untreated control 24 hours after aerosol application of PEI-DNA complexes and reached normal level to the seven days time point. After intranasal instillation of PEI/DNA complexes number of activated MACs increased 15-fold already 24 hours after administration and did not decrease significantly through the whole observation period. Aerosol application of distilled water did not alter the number of activated MACs, while intranasal delivery of 100 µl of dh2O induced 3-fold increase in number of activated MACs.

3.14 Analysis of BALF cells number and macrophage activation level after aerosol application of CpG-free plasmids

After aerosol application of PEI complexes with pCpG-Luc and pCpG-mcs plasmids estimation of cell number in BALF as well as alveolar macrophage activation analysis was performed (Table 6).

We have observed slight decrease in number of alveolar macrophages directly after aerosol application of PEI complexes with both plasmids, which restored to normal values within 72 hours. A significant increase in number of activated macrophages was observed 72 hours after PEI-pCPG- free-Luc complexes application, which returned to the normal values on 7 days time point. Also nebulization of PEI-pCpG-free-backbone complexes caused escalation in number of activated phagocytes 72 hours after administration ($p<0.005$). Nevertheless, the levels of macrophage activation observed on 7days time point did not differ from control level ($p<0.005$).

Treatment	Delivery	Time-point (hrs)	Total number of cells	AMs	Lymphocytes	Neutrophils	Activated MACs
Blank control		-	526,250± 198,893	519,303± 2,423	6,104± 2,512	830± 470	4,847± 1,412
PEI-pCPG-free-Luc	aerosol	1	176,500± 23,658	171,911± 2,058	4,024± 1,844	550± 473	3,574± 938
		24	314,250± 72,783	304,696± 3,594	10,918± 8,854	975± 562	4,818± 1,308
		72	510,750± 143,977	493,767± 19,396	14,301± 17,834	1,782± 2,265	10,087± 1,972
		168	246,500± 65,110	240,781± 1,285	5,225± 1,332	481± 348	2,958± 1,207
		336	363,300± 161,278	356,756± 18,930	5,082± 1,358	998± 649	3,775± 1,192
PEI-pCPG-free-mcs backbone	aerosol	1	200,000± 74,582	194,720± 1,110	4,240± 1,252	1,012± 536	3,000± 1,243
		24	505,500± 47,480	492,357± 9,293	10,918± 8,854	2,166± 1,318	10,514± 4,081
		72	559,750± 322,479	549,674± 3,450	8,508± 3,504	1,539± 1,276	13,769± 6,986
		168	246,500± 65,110	536,716± 3151	8,752± 2,046	1,502± 1,247	7,220± 1,467
		336	432,750± 180,983	424,095± 2,448	7,789± 2,870	848± 865	5,366± 1,664

Table 6. Numbers of different cell types in complete BALF after aerosol application of CpG-motif-free plasmids. On the certain time points mice were sacrificed and broncheo alveolar lavage was performed. Cell types and numbers were estimated using myelopwroxidase and May-Grünwald staining.

3.15 Time-course of luciferase gene expression after nebulization and intranasal instillation of PEI-pCVMLuc particles

One hour after application very low levels of luciferase gene expression were observed in the lungs after aerosol but not after intranasal delivery. Whereas highest luciferase expression levels after aerosol delivery were found after 72 hrs, which remained at comparable levels until day seven, highest transgene expression after intranasal instillation as observed after 24 hrs (Fig. 18). After intranasal delivery the expression values were highly variable. One hour after administration no transgene expression was observed. Whereas two out of five treated mice expressed luciferase at high levels, three mice did not express any luciferase at 7days time point..We could not detect any gene expression in BALF cells either (data not shown).

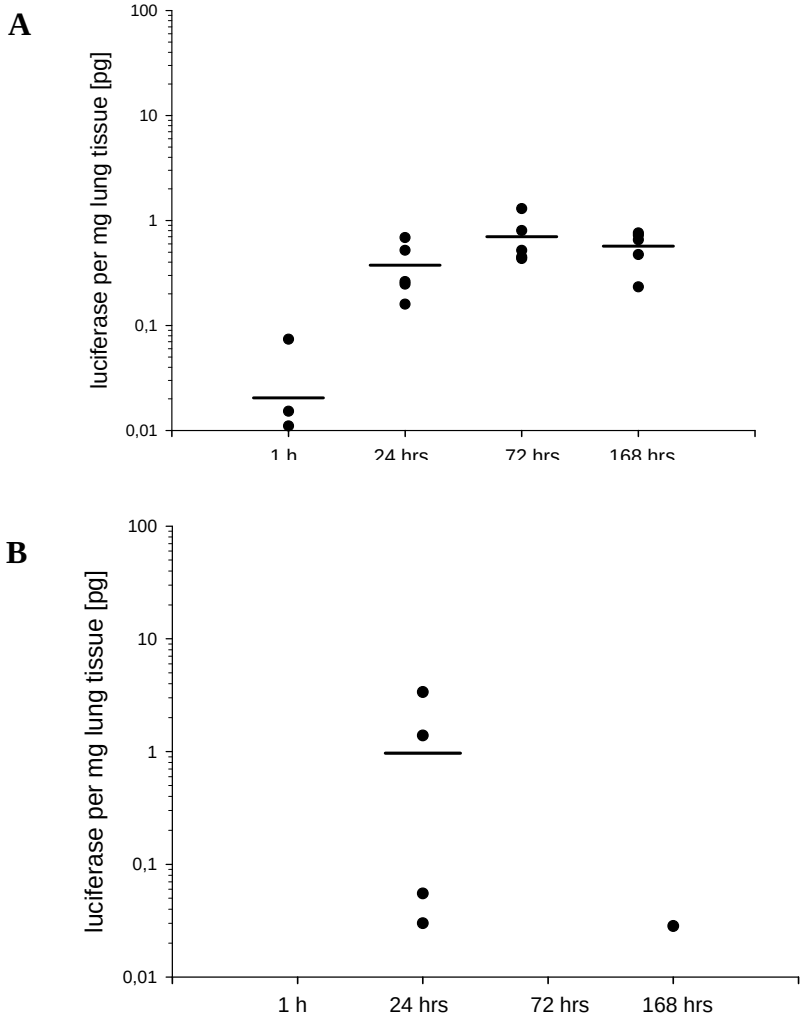


Figure 18. Time-course of luciferase gene expression after aerosol delivery and intranasal instillation of PEI-pDNA complexes. PEI-pDNA complexes were either nebulized (A) or instilled (B) to the lungs of mice. At indicated time points luciferase gene expression was examined in the lung tissue. Results are reported as absolute values (dots) with an average mean of the group (lines)(n=5).

3.16 Time-course of luciferase gene expression after aerosol application of PEI/pCpG-free-Luc complexes

The expression of luciferase reporter gene observed after transfection with CpG-motif free plasmid was significantly higher than that after delivery of standard plasmid DNA (Fig. 19). Already one hour after administration the level of luciferase reached approximately 8 pg luciferase per mg tissue, while 24 hours after nebulization around 10 pg were observed. However, on the third day of incubation the luciferase expression declined significantly. Further decrease was observed on 7days time points, where only 0,1-1 pg luciferase per mg tissue were registered. On 14 days time point the transgene expression level did not differ significantly form untreated control. No transgene expression was found in resident lung cells.

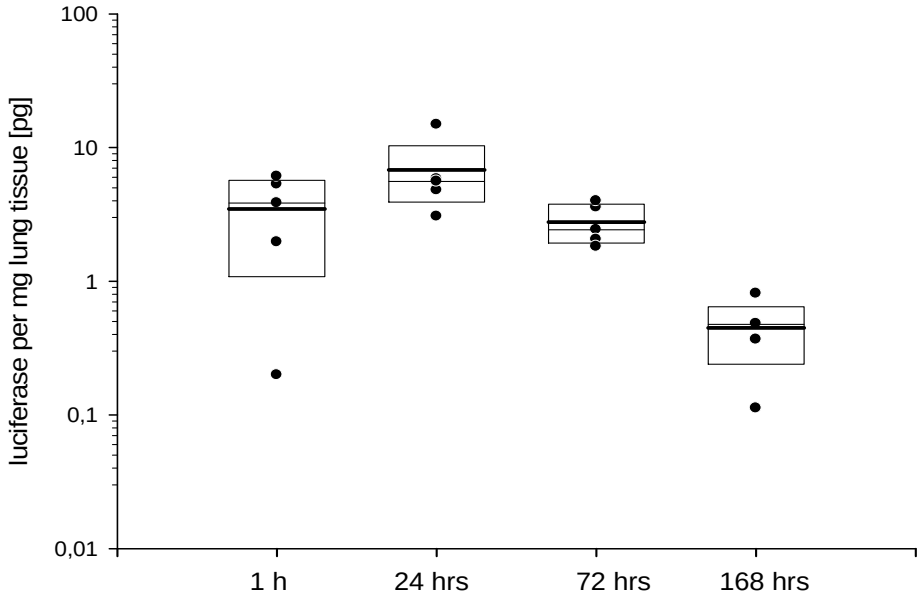


Figure 19. Time-course of luciferase gene expression after aerosol delivery of PEI complexes with pCpG-free-Luc plasmid.
PEI-pDNA complexes were either nebulized to the lungs of mice. At indicated luciferase gene expression was examined in the lung tissue. Every dot represents a single animal, a bold line represents the average mean.

4 DISCUSSION

4.1 A series of modified branching polylysines

Development of cationic polymers capable of DNA binding, protection and delivery into the living cell comprises nowadays one of the major trends in establishment of novel synthetic gene delivery systems. An ideal vehicle is supposed to: i) bind DNA molecules larger than 1kB, ii) transfect both proliferating and non-proliferating cells, iii) to provide tissue– or organ-specific gene delivery, iv) high transfection efficiency *in vivo*, and v) possess low toxicity and immunogenicity (56).

Compared to other gene delivery systems, for example viral, synthetic polymers possess several significant advantages. They can be modified easily and thus represent a class of flexible systems which can be adjusted for particular needs. Besides, with synthetic polymers the size of genetic construction is not restricted as it is in case of many viral vectors (152).

Ability of many natural proteins and peptides such as histones, nucleosome proteins, etc. to form particles with DNA molecules induced the investigation of their possible gene delivery capacities (200). In 1987 targeted delivery of plasmid DNA with polylysine and oligolisine was reported for the first time by Wu and Wu (218). Since than plenty of polymers have been used for gene delivery, like poly-L-lysine, poly-arginine, spermidin, spermin, polyamidamine dendrimers, protamin, polyethilenimine, poly-αγ-butanic acid and many others (117, 192). Depending on structure and aminoacid composition they are capable not only of binding DNA, but also of it's protection, and can enhance lysosome disruption thus providing DNA release form lysosomes, as well as to deliver DNA to the nucleus (38). At the same time, chemical composition, charge and length of a peptide may become the reason for cytotoxicity, immunogenicity of DNA/peptide complexes, as well as may cause the insolubility of peptide in water (47, 226). The structure may define not only the transfection efficiency of a peptide but also type of modifications it can bear (85). Nowadays many different modifications are applied which enhance gene transfer ability of the polypeptides (5, 158, 210). Although cationic peptide-based gene delivery vehicles can influence the permeability of cellular membrane, cell cycle, cause local toxicity and activate certain phospholipases (137), their main advantages – biodegradability and efficiency - make them to one of the most promising classes among gene delivery systems.

4.2 Development of SP-LL dendrimers series

A wide variety of linear (1, 65) and branching (93, 183) polypeptides, based on three basic aminoacids –lysine, histidine and arginine - have been developed and tested to date for their gene delivery capabilities. Polylysines are widely used in pharmacy to deliver therapeutic molecules (126). Epsilon-amino-group of lysine becomes positively charged at physiological pH, thus enabling the molecule to bind not only to polyanions like DNA, but also to ligands for cell surface receptors (121, 197). Many published data (146, 147, 155, 156) as well as our own previous results (83, 206) showed that a branching polylysine with five branching orders can provide efficient gene transfer. It was also shown, that the main problem of this vehicle was poor escape of complexes from endosomes and agglomeration of complexes. Basing on these data, a series of novel carriers was synthesized, where a branching polylysine with five branching orders was modified with histidine and arginine residues.

All four polymers were synthesized using “one-step” synthesis method developed by Dr. Vlasov (83, 206), providing equal distribution of hisitdine or arginine residues along the branching polylysine core, which was supposed to reveal fully the capacities of these aminoacids.

Introduction of arginine residues into the polylysine core aimed to increase the binding capacity of the polymer and to enhance its penetration into the cell; while modification of branching polylysine core with histidin residues was performed to enable the polymer to develop the “proton sponge” effect (147, 214, 220), thus providing escape of plasmid DNA form the endosomes (9, 147).

4.3 Analysis of DNA-binding capacity

A common method of gel retardation (26, 96, 127, 155) was utilized to estimate the ability of polymers to form complexes with plasmid DNA. We have observed significant differences in DNA- binding capacities between polypeptides of this group. At the same time, no influence of compactization conditions was observed in our experiments.

Both arginine-containing polymers showed high DNA-binding capacity which may be ascribed to positive charges of lysine and agrinine. The guanidine groups of arginine are strongly basic and thus positive in wide range of pH conditions. The pH levels of the solvents in our experiments comprised 7,0 for distilled water and 7,4 for physiological solution. As far as both alfa- and epsilon -amino groups of lysine and guanidine groups of arginine were

cationic (positively charged) under these conditions, we can assume that both aminoacids took part in DNA binding. That could explain the similarity observed between two applied pH values. Many factors can influence the efficiency of DNA binding by a polymer. It was shown that addition of tryptophan as well as of hydrophobic groups improves DNA binding, and in case of alpha-helix peptides the structure of the molecule is the main factor provodeing the possibility for effective binding (37). The differences observed between two polymers may be explained by their different dynamics of binding to DNA, depending on their chemical composition. The mechanism of polymer interactions with DNA depends greatly on the polymer structure. Indeed, the ratio of arginine residues within a polylysine core is reported to influence the DNA-binding behavior of the polymer (67, 133). On the electrophoresis image (Fig 4) one may see conglomerates of complexes forming at 1/0,6 charge ratio, while part of DNA remains uncompelxed. An excess of polypeptide molecules may have lead to formation of huge spongy conglomerates at this charge ratio, which are reported for many branching polypeptides as a stage preceding the formation of compact particles (127, 156).

In case of histidin-enriched polypeptides no differences in DNA-binding capacities were observed within the group. Presence of histidin residues within the polypeptide core is widely reported to have a dramatic influence on polymer's capacity to form complexes with DNA (79, 155). Yet, nearly all these observation were made at low pH (around 5), when histidin becomes protonated exhibiting negative charges and undergoes dramatic conformational change (194). Histidin-rich polylysine has two kinds of cationic groups on it's surface, namely, alfa-amino group of the lysine and the imidasole nitrogen of histidin, whose original pKa values comprise 9.33 and 6.04 respectively (35). This explains why at acidic pH polyhistidines possess either very low (147) or no (220) DNA- binding capacity, while at pH 7,4 histidin residues within a lysine-histidin polymer are mostly neutral and DNA is bound predominantly by positive charges of lysine. Consequently, under conditions we used for both polymers DNA was bound by lysine residues, which for both polymers were similar in number according to the similar charge ratio. The fact that complete binding of DNA was observed at a similar charge ratio for both polymers means that in both cases the structure of molecules provided the effective interactions of negatively charged DNA with positive charges of lysine, which were not significantly influenced by presence of varying amounts of histidin. Indeed, even low amounts of lysine within a polycation were noticed to provide effective compactization of plasmid DNA (79). The differences in structure of the complexes formed with these two polymers were revealed with the following tests.

DNA-binding capacity was proved for all four vehicles and no significant influence of solvent was observed on this stage. Presence of Na^+ and Cl^- ions in solvent is reported to influence the structure of complexes formed by polypeptides with DNA and thus has a dramatic impact on their transfection efficiency. In our following experiments we observed significant influence of the solvent on complexes size, structure and dynamics of their formation.

4.4 DNA-protecting capacities of the dendrimers

DNAse protection assay is a common method to estimate the ability of a polymer to protect plasmid DNA form enzymatic degradation. In particular, this method tests the structure of a complex formed under certain condition as it's permeability for DNA-disrupting agents. In the present study, have shown that complexes, formed at the same charge ratio but in different solvents exhibit different protective capacities.

Many authors report that presence of NaCl in solvent influences the dynamics of particles formation and thus the size and structure of DNA/polymer complexes (9, 166). Polymers composed of lysine and histidines were shown to form conglomerates in saline (13). It is widely reported that during complexes formation the dynamics of polymer attachment to the DNA defines both density and structure of the complex, thus making plasmid DNA more or less accessible for enzymatic degradation (79, 93, 127). Indeed, for SPKR 10:1 we have shown that presence of NaCl decreased the amount of digested DNA in case of smaller charge ratios, meaning improvement of protective capacities of the polymer. Similar dynamics was observed for the polymer with higher arginine ratio SPKR1:1. The described tendency was even more pronounced in case of histidine-enriched polymers. Many authors utilized stable (26, 147) or dynamic (119) NaCl concentrations in solvent to obtain compact and small complexes with histidin-rich polymers. It is widely reported that polyhistidines may change their interactions with DNA dramatically in physiological solution compared to distilled water (9, 156, 184). In our case, both histidine-enriched polymers exhibited a significant shift in DNA-protecting capacities towards smaller charge ratios in NaCl solution compared to distilled water. Further analysis of complexes formation dynamics revealed the reasons of these changes in polymers behavior.

4.5 Fluorescence assay

Different fluorescence assays are widely used to observe the dynamics of complexes formation. These methods are based on intercalating dyes like ethidium bromide (147),

propidium iodide (156), while we have utilized TOTO-1, another representative of the group of cyanine dyes. Gradual replacement of the intercalating dye with molecules of DNA-condensing polymer reflects the speed and the dynamics of complexes formation.

For both arginine-rich and histidine-rich polymers remarkably similar dynamics of complexes density incensement was observed. At small charge ratios used in distilled water, which were previously proved not to be able to provide complete DNA binding, no differences were found between measurements on three different time points. These results indicate that at small charge ratios polymer binds roomily to DNA without further compactization. These observations correspond to the major theory of complexes formation, which describes polymers to attach freely to DNA without interfering with one another during the first stage of DNA compactization (12, 13, 198). For our series of polymers only slight time-dependent shift in fluorescence efficiency was observed at small charge ratios for complexes formed in NaCl solution. Indeed, polylysins binding to DNA were proved to form toroids into which additional polycations incorporate during the second compactization phase (194). To these structures formed by histidin-enriched polylysines small size was demonstrated along with strongly positive charge (155, 166). With further increase of the charge ratio DNA fluorescence declined dramatically up to approximately 30% of initial intensity. It corresponds to the data of Yamagate et al., who showed a reduction of initial fluorescence up to 40% for histidin-enriched polylysine already at the charge ratio of 1:2, which remained on that level up to charge ratio 1:16 (220). For those charge ratios which were shown to provide complete DNA binding, time-dependent decrease in DNA fluorescence was observed. These changes may correspond to redistribution of polymer molecules within the preformed conglomerates. Such behavior was demonstrated for many other polymers (96, 156). This process may lead to formation of more compact particles and represents the second phase of DNA/polypeptide complexes formation (175, 184). No time dynamics was observed for complexes formed in NaCl solvent, where DNA fluorescence was completely quenched at 1/1 charge ratio within the first 5 minutes of incubation. These observations correspond well to the published data where rapid formation of big conglomerates of DNA and polypeptide in NaCl solution was reported (9, 155). For histidin-rich polymers the reduction of initial fluorescence up to 20%, at acidic pH and to around 60% at pH 7, 4 (147, 155) was demonstrated by several authors, which corresponds fully to our results.

4.6 Measurement of Size and Zeta-potential of the complexes

Aiming to select the vehicles mostly suitable for gene delivery, we have measured surface charges and size of the complexes formed by different polymers with plasmid DNA under certain charge ratios and compactisation conditions.

Heterogeneity of DNA complexes with polylysines is supposed to be one of the main reasons for their relatively low transfection efficiency. The efficiency of gene delivery depends greatly on the size of DNA/polymer particles. Several methods have been described to date to control the size of polymer/DNA complexes and thus to improve transfection efficiency (141, 142). Successful gene delivery with complexes of more than 300 nm in diameter was shown presumably for *in vitro* transfection on cell cultures, where precipitating conglomerates may be effectively taken up by the cells (131, 146, 179). It is assumed that the size of approximately 100nm is mostly favorable for gene transfer involving unspecific and receptor-mediated endocytosis (178). Such complexes are able not only to withstand better intravenous administration (96), but also to provide efficient targeted gene delivery when bound to certain ligands (79). It was shown that DNA condensation with polylysines may lead to a significant reduction of a plasmid of several thousands base pairs in a toroid or rod of around 100nm in diameter (13). Branching polymers tend to form bigger complexes with plasmid DNA than linear polypeptides (192, 220). The size and structure of particles formed by branching polypeptides with plasmid DNA are known to depend on molecular mass, number of branching orders and chemical composition of the polymer, as well as on compactization conditions (12, 13, 127, 175).

We have also observed a significant influence of polymer structure and DNA-binding conditions on the size and surface charge of the complexes. We have shown that arginine-rich polymers form relatively small particles the size of which was more influenced by charge ratio than by presence of NaCl in solvent. Under low charge ratios both polymers formed conglomerates with DNA which more varied in size when formed in saline and did not exceed 300nm. Basing on the results of fluorescence and DNAse protection assays, we can assume that these spongy structures represent the initial binding of the polymer to DNA molecules and may be characterized as relatively incoherent structures. Further increase in polymer concentration lead to formation of smaller and denser complexes, which were proved by our previous measurements to provide complete DNA binding and protection from DNAse. These results correspond to published data, which report the incensement of polymer local concentration to enhance formation of small and compact complexes with DNA (93, 127, 149).

For histidin-rich peptides significant influence of NaCl on the size of complexes was shown. Both polypeptides tended to form huge aggregates when compactization was performed in saline, while complexes performed in distilled water were characterized by relatively small sizes favorable for gene transfer. Our data correspond to the results of Pichon et al., who had observed histidin-rich polylysines forming strongly positive rods with DNA of approximately 130nm length (12). It was shown for histidin-containing polylysines, as well as for polyhistidines that presence of NaCl in solvent leads to aggregation of the complexes and thus to strong decrease in transfection efficiency (9, 93). Our measurements clearly showed that complexes of the hisitidin-rich polypeptides with DNA for gene delivery should be formed in distilled water.

Since the results of our previous measurements showed that complexes formed in distilled water should have been be more favorable for gene transfer, only these were taken for such a demanding analysis as zeta-potential measurement. We have shown that at charge ratios providing complete DNA binding all polymers formed positively charged complexes, with zeta-potential values varying between different charge ratios. The lowest positive charges were observed for SPKH10:1 polymer. These results correspond to the data of Okuda *et al.*, where polylysines with low hystidine number were shown to be slightly positive at neutral pH (147). The unique capacity of histidine to become protonated under acidic pH allows at the same time to obtain positively charged complexes with this polymer, which show high transfection efficiency (57, 178).

Interestingly, branching polylysines usually form more positive complexes with DNA than linear polylysines of similar molecular mass (220). According to numerous published data positively charged complexes have better chances to enter the cells because of the absence of charge repulsion with negatively charged surface membrane. Thus we have shown that complexes formed under conditions described above may enter the cell successfully.

4.7 Transfection efficiency of modified branching polylysines

Branching peptide-based polymers are widely reported for their ability to deliver plasmids efficiently into the cell (143), providing transport of DNA into the nucleus (141, 142) and expression of the transgene (146-147). Nevertheless, relatively low gene transfer efficiency, accompanied by certain toxicity and unclear delivery mechanisms comprise the bottlenecks for synthetic polypeptide-based gene vehicles (179).

We have tested a series of branching polylysines bearing different modifications for their gene transfer efficiency. Taking into consideration the results of preceding analysis we thoroughly selected the complexes which were used for gene transfer experiments. We used those charge ratios and compactization conditions which could provide complete DNA binding and protection, and formed small compact complexes.

We have shown that already after 24 hours of incubation certain charge ratios were able to provide significant levels of luciferase expression, which are higher than those after transfection with naked plasmid DNA. After two days of incubation, which is close to a doubling time of A549 and 16HBE cells in culture (93) , increase in transgene expression intensity was observed for several charge ratios. Based on this observation, we may suggest that in these cases plasmid DNA was entering the nucleus during cell division when nuclear membrane disappears. That means that the polymers could not provide an effective translocation of plasmid DNA into the nucleus. Transport of plasmid DNA into the nucleus is considered to be one of the most significant challenges of gene delivery, along with the targeted delivery and escape from the endosomes (17, 65). Therefore, an increase of transgene expression after 48 hours might have been mostly due to that the plasmids already delivered into the cells could finally contact the transcriptional apparatus. Our additional experiments devoted to transfection of two other cell lines, HeLa and HepG2, with the same complexes showed significantly lower gene transfer efficiency, which may be partly explained by characteristic features of these cells. Apart form metabolic characters, with which the differences in gene transfer efficiency between cell cultures are usually explained (184), the cells of these two cultures have higher doubling time than A549 and 16HBE cells, meaning that within 48 hours plasmid DNA had lower chances to be transcribed.

A tend to decline in transfection efficiency with the increase of the charge ratios was observed for all four polymers either on one or both cells cultures after 24 and 48 hours of incubation. Our measurements performed for the complexes with different charge ratios showed that within three of those chosen for gene delivery experiments DNA/polypeptide particles possessed relatively similar surface charge and size. With respect to these data we can assume, that formation of complexes with the excess of polypeptide leads to aggregation of molecules to very dense and compact structures, which prevent DNA from being released within the cell and thus make transfection inefficient. Indeed, release of plasmid DNA from the vehicle is considered to be one of the critical steps for successful gene transfer. Our earlier data also showed that polylysine-based dendrimers require certain modifications (e.g., with fatty acids residues) to transfect cells in culture sucsessfully (100). Many polylysines and

arginine-containing oligopeptides are reported to possess only low transfection efficiency despite their ability to form suitable complexes with DNA because of too strong plasmid compactization, which prevents the plasmid from being transcribed in the nucleus (9, 79, 127). Also complexes of DNA with polyhistidines formed with the excess of polymer are characterized as dense globular structures which can hardly release baceterial plasmids (57, 127).

High values of luciferase expression were observed for histidin-rich peptides SHKH10:1 and SHKH1:1. The fact that both peptides showed effective gene transfer may be due to the ability of histidines to become protonated under acidic pH and thus provide DNA escape from the endodomes via the "proton sponge" effect. Many published data describe endosomal escape as the major problem of polypetide-based synthetic gene carriers (79, 93, 96, 127). Along with well known synthetic (polyethileneimine and polyamidoamine (195)) and natural (histatin5 (130), Sea Urchin B18 (74) or LAH4 (208)) polymers, polylysines modified with histidin may show proton sponge effect, which helps plasmid DNA to escape lysosomes and thus enhances gene delivery (156, 201). Dendrimers may show buffering capacities at lower pH than the original pKa of the alfa-and epsilon-amino groups of lysine and the imidasole nitrogen of histidin. That occurs because nitrogen atoms on the surface of dendrimers are hard to protonate due to steric hindrance and electrostatic repulsion (155, 156). Nevertheless, the proton sponge effect shown by SHKH10:1 and SHKH1:1. peptides was high enough to provide the escape of at least part of plasmids from the endosomes. It is generally accepted, that for development of endocytosis-based gene delivery systems peptides exhibiting an acidic pH-dependent membrane destabilization are good candidates to favour DNA transport to cytozol (209). Still, the highest luciferase levels observed for these polymers were still lower that those observed for arginine containing polylysines. These results may mean that the escape form endodomes must have been not the crucial step in transfection.

Arginin-containing branching polypeptides have demonstrated the highest gene transfer abilities. One charge ratio for every of two vehicles had shown transgene expression as high as after delivery of PEI/DNA complexes, which were used as positive controls. Based on these observations we may speculate that modification of an initial branching polylsine with arginine had a more pronounced effect and improved dramatically it's gene delivery capacities. Arginines are reported to provide efficient penetration of complexes into the cells when they are integrated into the polypeptide or used in frame of peptides of natural origin (64, 66, 67). It is importaint to note, that branching polylysines themselves are able to develop

the proton sponge effect because of the reduction of pKa values of terminal amines towards 6, which can provide the escape of DNA from the endosomes (52, 220). Though the polylysines modified with arginine certainly lack the strong proton sponge effect which was exhibited by histidin-enriched polymers, part of the complexes definitely escaped enzymatic degradation. Arginine-enriched polymers probably were not able to provide efficient transportation of DNA into the nucleus. Nevertheless, higher levels of luciferase expression which exceeded those obtained with SPKH polymers shows that at least some of the previous hindrances were overcame by SPKR polymers more successfully. Additional studies should be performed to understand the action mechanisms of these vehicles.

The efficiency of both arginine-rich polymers was higher than that of initial branching polylysin D2, which was taken as a basis for creation of four modifications investigated in this study (206). Our observations showed that from all four modification of the initial polymer enrichment with arginine showed to increase transfection efficiency much better than integration of histidine residues. Our results demonstrate that arginine-enriched branching polylysine can efficiently deliver plasmid DNA into dividing cells in culture. Vectors of these family are reported to possess low toxicity and biodegradability (54. 46), thus appearing as a safer gene delivery vehicle. Many possible modifications are known which may improve the efficiency of a synthetic oligolysine. Modification of polylysine-based vehicles with PEG (113) or combination of PEGilation and glycopeptide cross-linking (114) is reported not only to reduce toxicity but also to provide their longer circulation in blood. An alternative way to enhance the gene delivery efficiency with polylysines is their modification with fatty acids residues. It was shown that polylysine complexes with fatty acids (lipophilic polylysin) can provide high transgene experession by protecting DNA from lysosomal degradation (141). Our previous results also demonstrated the usefullness of polylysines modification with fatty acids residues (207). Another possible way to increase transfection efficiency of an oligolysin is modification with ligands for cell surface receptors. For example, gene transfer into the liver cells was enhanced by galactozilation and mannosilation of polycationic peptide-based carriers (142, 222), while the stability of polyplexes may be increased by cross-linking via disulfide bonds (198). Amphiphilic peptides of INF family such as INF7 and of the E5 family such as E5CA have been shown to increase transfection efficiency of DNA/polylysin complexes (132). Many successful modifications of branching poylpeptides are reported which have improved their gene transfer abilities.

From all four polypepties investigated in this study SPKR10:1 and SPKR1:1 appear to be the most promising polymers. Further studies should be performed to select modifications which could improve gene delivery using these vehicles.

Nonviral gene delivery proved to be a promising trend within the last decades. Synthetic polypeptide -based vectors were investigated intensively and applied in many fields. Along with such advantages as biocompatibility and relatively low toxicity and immunogenicity, the transgene expression provided by these vehicles remains inefficient to establish a therapeutical effect. A number of other perspective polymers were investigated within the last years, which were demonstrated to provide high long-term expression of transgene *in vitro* and *in vivo*. One of these synthetic gene delivery agents is polyethylenimine (PEI), which has shown gene transfer abilities so significant that it is referred as a "golden standard" in nonviral gene delivery. Aiming to find a safe and effective way to deliver plasmid DNA into the lung, we have compared two methods of complexes administration – aerosol inhalation and intranasal instillation- for their safety and efficiency, as well for clearance parameters and deviations in lung functioning. Knowing that plasmid DNA may provoke toxic effects, we have tested the effect of CpG motifs present in plasmid DNA on safety of the administration procedure.

4.8 Lung function

In this study, we have demonstrated for the first time that intranasal instillation of a plasmid containing unmethylated CG dinucleotides (CpG) leads to deviations in pulmonary functioning in mice, in addition to the previously reported inflammatory lung response. The adverse effects that we have observed were markedly reduced at early time-points upon aerosol delivery. Nevertheless, comparable deviations were observed as late as one week after treatment. Only using CpG-free plasmid DNA was capable of preventing these adverse effects. These results therefore suggest that aerosol delivery together with CpG-free plasmid DNA is critical to minimize potential inflammatory response upon nonviral gene delivery to the lungs. Our findings demonstrate that instillation does not represent a suitable technique for gene vector application as evidenced by the inflammatory response and severe deterioration of pulmonary function. These observations confirm the previous findings from Davies et al. who recently reported a stronger inflammatory response to PEI gene vectors after instillation than aerosol application using histology and BALF cell counts (34). Furthermore, these results are in agreement with observations made from instillation of cationic lipid gene vectors into the sheep lungs which led to a dose-dependent toxicity with severe lesions,

whereas after aerosol delivery only mild inflammation with a few scattered areas of moderate inflammation were observed (50, 129). It may be suggested that instillation leads to inhomogeneous particle distribution with areas of high and low PEI-pDNA particle concentrations. The areas of high PEI-pDNA particle concentrations may result in stronger lung response than after aerosol delivery, where homogenous particle distribution has been observed (69, 173). Alternatively, the total PEI-pDNA complex burden which was higher after intranasal instillation than aerosol delivery may explain the different lung response together with the relatively large volume of distilled water used as solvent for PEI-pDNA particles. Indeed, our results demonstrate that immediately after instillation of distilled water, pulmonary function severely declined and did not return to normal even within one week. These results therefore suggest that distilled water instillation does not only induce transient airway epithelial swelling by a so-called hyposomotic shock, but leads to long-term side-effects in the lungs. However, associated swelling and permeabilization of lung tissue have been proposed to be critical for efficient gene transfer to the lungs (118, 173). Unlike with instillation, low amounts of nebulized distilled water have been previously reported to result in only transient airway swelling (134, 135). Our results confirm these observations as evidenced by returning the pulmonary function to within twenty-four hours after treatment.

Most of the previous studies addressed inflammatory response after nonviral gene transfer to the lungs by means of standard histology, BALF analysis, measurement of proinflammatory cytokines (69, 90), and more recently by gene expression profiling (164). Although together these methods are appropriate to characterize the pattern of the inflammatory response in the lungs as evidenced by infiltrating cells, changes of the lung architecture and genes involved in reaction to stress, they are not capable of assessing any effects on a relevant functional level. We demonstrate that forced oscillation technique can be successfully applied to display changes of pulmonary function after nonviral gene delivery to the lungs. Applying the constant phase model as suggested by Hantos *et al*. (84), our data indicate that the inflammatory effect of unmethylated CpG-motifs is located to the peripheral lung tissue (GTiss and HTiss) rather than to the central airways. An important finding of our study is that the FOT measurements could be well correlated with standard histology and BALF cell counts. Interestingly, neither of them correlated with the results from an inflammation antibody array of the lung tissue, which covered forty inflammatory markers (unpublished data of Dr. Petra Dames). None of the markers was significantly altered at any of the measured time-points. A possible explanation for this observation could be the choice of inappropriate measurement time-points which did not cover the relevant period of initial

tissue response. Indeed, it has been previously shown that the cytokines IL-1β and TNF-α are only increased in the lung tissue 5-12 hours after aerosol treatment (69). As a result, this observation suggests that any of the inflammatory response which is observed at later time-points is not associated with significant upregulation of the major inflammation pathways. It may therefore be suggested that the observed deterioration of pulmonary function and histology are the delayed result of an inflammatory response with an early onset. Consequently, our results suggest that potential inflammatory response should not only be examined at early time-points but should be additionally considered at late time-points. Furthermore, our results demonstrate that not only large amounts of instilled PEI-pDNA gene vectors induce an inflammatory response and deterioration of pulmonary function, but similar effects were observed for nanogram-amounts of aerosolized PEI-pDNA at later time-points. This observation may suggest a potentially high intrinsic toxicity of PEI-pDNA gene vectors. The potential toxicity could be caused by either the PEI-pDNA nanoparticles themselves or by one of their components, i.e. PEI or pDNA, and the transgene product. In particular, unmethylaed CpG motifs have been previously shown to largely contribute to the inflammatory response, although PEI itself has also been shown to induce lung inflammation (199). We therefore performed identical experiments but instead of a first generation CpG-rich plasmid, CpG-free plasmid DNA either encoding for luciferase, or only the backbone was aerosolized to the lungs of mice. None of the CpG-free pDNA caused impairment of pulmonary function or inflammation assessed by histology. We therefore conclude that neither the PEI-pDNA nanoparticles themselves nor PEI and the luciferase transgene are the reasons for the observed abnormalities of lung function, but that unmethylated CG sequences are the major reason for the observed impairment of lung function. These observations highlight the importance of using CpG-free pDNA for gene delivery to the lungs. Moreover, our resulta demonstrate that the cationic polymer PEI may be used for aerosol gene delivery without induction of alteration in lung functioning. We therefore suggest that PEI can be safely delivered to the lungs and could become a potential candidate for clinical trials in the future. This seems to be important in particular with respect to successful gene expression levels previously observed in a large sheep animal model (129).

4.9 Histilogical examination

The common method of histological analysis is utilized widely to estimate the influence of a certain procedure on the lung architecture and tissue condition.

We have observed a series of abnormalities in the lung histology, which were developing within the week after administration. Twenty four hours after instillation inflammatory response was observed together with cellular infiltration. That corresponds to our results of cell counting where significant increase in number of resident macrophages in the lung was observed at this time point. The overstress of the small blood vessels caused by intensive monocyte influx could have caused the strong capillary thrombus formation observed at 24 hours time point. Development of hemorrhage seven days after treatment may be explained as the remote effect of continuous presence of distilled water in alveolar region shortly after administration (136, 188). Nevertheless, analysis of lung function parameters on later time point after nebulization of water revealed no abnormalities, meaning that despite structural changes the function of the organ remained normal. Our analysis of pDNA content in lung resident macrophages showed residual amounts of plasmid as late as seven days after instillation. The surfactant layer within the lung renews constantly, and lung resident macrophages contribute to this process along with the mucocilliary movement (187, 190). We can assume that some residual volumes of liquid containing complexes were still present within the alveoli, causing the prolonged disturbance of the tissue (22). On the other hand, aerosols are widely reported to be a safer delivery route to the lung (140). This method avoids disposition of large volumes of water or solvent within the lung, thus omitting one of the main inflammation reasons (55). Besides, comparing the histological observations after aerosol delivery of standard plasmid DNA with that after CpG-free plasmid delivery one may see clearly that plasmid structure influenced the lung tissue response. As far as the size of the complexes and nebulization conditions were similar in both cases, we may assume that presence of CpG motif in plasmid DNA provokes the inflammatory response which was observed 24 hours after aerosol administration of PEI/pCMVLuc complexes. Causing strong immune response and macrophage activation (103), unmethylated CpG motifs of plasmid DNA stimulate macrophage activation and influx of fresh monocytes into the alveolar region. These effects were not observed after aerosol administration of CpG-free plasmids, proving that combination of a safe delivery rout with a safe plasmid DNA results in a harmless administration of the complexes within the lung (128, 129).

4.10 Clearance of pCMVLuc from the lung after aerosol delivery and intranasal instillation

Studying the plasmid DNA clearance form the lung we have compared three lung compartments, namely lung tissue, BALF cells and BALF liquid.

Aerosol application of PEI-pDNA particles resulted in slower and reduced pDNA clearance from the lung. This may have been the main reason for prolonged luciferase expression after aerosol delivery compared to intranasal instillation. At the same time aerosol application did not cause any significant increase in number of phagocytes in the lung contrary to intranasal instillation, where not only rapid migration but also higher rates of macrophage activation were observed.

The method of pDNA extraction used in this study was based on charge interactions and allowed the extraction of only those pDNA molecules which were released from PEI-pDNA complexes. According to our observations and also other published studies (187) around 50% of the initial dose of PEI-pDNA complexes can be found within the lungs of mice after intranasal instillation, which in our case comprised 25µg of DNA complexed with PEI. Interestingly, one hour after instillation only 290 ng of free (uncomplexed) DNA per mouse was detected by PCR. On the contrary, as it was shown in our previous study (173), standard aerosol delivery procedure deposited around 500 ng of complexed DNA to the lungs of an exposed animal. Nevertheless, the pDNA values after aerosol delivery were surprisingly close to those after intranasal administration and comprised around 213ng of free pDNA per mouse, despite the almost 100-fold difference in amount of delivered complexes between aerosol and intranasal delivery techniques. Comparing the amount of incoming complexes with maximal free pDNA levels, one may easily calculate the efficiency of pDNA release. While after instillation of complexes only about 1 % of pDNA was released, this was almost 50% after aerosol administration. Thus, administration route determines efficiency of pDNA release from the complexes. The amount of DNA released form the complexes defines the successful transgene transcription. The similarity in amounts of released pDNA after instillation and aerosol delivery - despite manifold difference in amount of delivered complexes – may explain the comparable luciferase expression levels 24 hours after aerosol and intranasal administration.

4.11 Clearance of pCMVLuc form BALF cells after aerosol delivery and intranasal instillation

We have found, that 3% of pDNA released from the complexes was absorbed by BALF cells during the first hour after instillation and only 0.1% after aerosol administration. The largest part of pDNA is supposed to have been associated with the cells and surfactant layer of the lung. Our observations suggest that both lung resident and tissue cells, as well as the extracellular components of the lung were engaged in clearance of PEI-pDNA particles.

Although the peculiarities of PEI-pDNA particles uptake by alveolar macrophages are still to be discovered, we extracted pDNA from lung resident cells (BALF cells) and tried to estimate the amount of corresponding PEI-pDNA complexes.

Although resident lung neutrophils also possess phagocyting ability (118), their small ratio and passiveness towards particles smaller than 1 μm reported by other authors (27) drove us to the conclusion, that clearance of the complexes in the lung was carried out mostly by lung resident macrophages. Based on our data on size and structure of our PEI/DNA complexes, we could calculate roughly the number of complexes corresponding to a certain amount of plasmid DNA. Real-Time PCR analysis showed that approximately 6000 and 250 molecules of free plasmid DNA were associated with each AM after intranasal instillation and aerosol delivery, respectively. The complexes of around 100 nm in diameter used for inhalation contained approximately 3.5 pDNA molecules per particle which corresponds to around 70 PEI-pDNA complexes per macrophage (27). The total volume of this batch of complexes comprises 0.036 μm3. We can only speculate that the complexes of 200 nm in diameter, which were used for intranasal instillation, could contain 7 molecules of pDNA. That would correspond to around 860 complexes of a total volume of 3.6 μm3 per phagocyting cell. The uptake capacity of a macrophage can under certain conditions exceed 10 μm in case of liposomes (154), which corresponds to a volume of ~500 μm^3. Taking into consideration that the complexes were taken up and destroyed by the cells not at once but gradually we can assume that the uptake capacity of AMs was not exceeded. However, in case of nanoparticles phagocytes are reported to reach the overload state far below their physical uptake limits (10). It is not only the volume but also the surface area of the particles which has a crucial influence on the efficiency of their uptake by phagocytes (182). As far as only uncomlexed DNA was in our case detectable by RT-PCR analysis, the calculated numbers of particles correspond to those destroyed by the phagocyting cells, thus releasing plasmid DNA. Efficiency of the gradual uptake of PEI/pDNA complexes by macrophages obviously depends on their number and distribution within the lung. Plasmids released form the complexes within a macrophage are supposed to be degraded. Uptake and degradation of complexes and of plasmid DNA is a dynamic process, influenced by many factors. The number of complexes per macrophage, calculated for the 1 hour time point, reflects not only the amount of destroyed but also the number of consumed particles during this hour, according to the speed of DNA degradation in macrophages. These calculations reflect actually the minimal amount of particles, DNA from which was recently released within a macrophage. Thus, our calculations describe also the intensity of complexes disruption within the phagocytes, which

can only partly correspond to the intensity of complexes uptake. The intensity of DNA degradation within phagocyting cells, as well the dependence of this process from the administration route of PEI/pDNA particles and physiological condition of the lung are to be investigated in future. Our on-line observations of FITC-labeled PEI/pDNA complexes uptake by alveolar macrophages showed, that only a small part of complexes is taken up and processed in cytoplasm, while the majority are attached to the surface of macrophages for a long time (our unpublished data). All that taken together shows that interaction of lung resident macrophages with PEI/pDNA compelexes are a complicated process, which can be only poorly described by categories of DNA clearance.

4.12 Clearance of CpG-free plasmids after aerosol administration

In our study we have compared the clearance patterns from the lung for two CpG-motif-free plasmids, which were compelxed with branched PEI and delivered via aerosol into the lungs of mice.

We have shown, that the half-clearance time (T1/2) observed for CpG-motif-free plasmids was not only significantly lower then the T1/2 of the standard pCMVLuc plasmid, but also differed significantly from one another. In particular, comparing clearance from the lung tissue, we have observed that pCpG-mcs plasmid was cleared twice faster than pCpG-Luc DNA and four times faster than pCMVLuc plasmid. It is also important to note, that, though the maximal concentrations of free plasmid DNA for pCMVLuc (aerosol delivery) and pCpG-free-Luc comprised 256pg and 13500pg, respectively, CpG-motif-free plasmid DNA was cleared from the lung twice faster than the first generation plasmid. Regarding all three series of experiments devoted to aerosol delivery, the size of complexes were proved to be relatively similar, as well as the administration procedure which delivered approximately similar amounts of complexes into the murine lungs.

In our previous study we have shown that standard aerosol delivery procedure deposited around 500ng of complexed DNA to the lungs of an exposed animal (173). Investigating the amount of free (released) DNA in lung tissue at 24 hour time point, we have found approximately 100ng and 15ng of free pCMVLuc and pCpG-free-Luc, respectively.

Comparing the amount of incoming complexes with maximal free pDNA levels, one may easily calculate the efficiency of pDNA release, which comprised approximately 20% and 5% of the initial dose for pCMVLuc and pCpG-free-Luc, respectively. Thus, our data show that structure of plasmid DNA determines the efficiency of pDNA release from the complexes. Interestingly, although at 24 hours time point the efficiency of pDNA release was

10-fold higher for first generation plasmid than for CpG-free one, the latter provided significantly higher levels of transgene expression. We might conclude, that the CpG-free plasmid was approximately 100-fold more efficient than pCMVLuc. Despite visual similarity of clearance patterns, pCpG-fre-Luc was cleared form the lung much faster than of pCMVLuc. Comparing transgene expression patterns with those of pDNA clearance, we may assume that gene silencing played an important role in case of pCMVLuc, while it was mostly pDNA degradation that was responsible for transiency of luciferase expression in case of CpG-depleted plasmid. Ummethylated CpG-motifs are widely reported to provoke gene silencing of plasmid DNA (229, 230, 231). Artificial methylation of pDNA prior to transfection reduces the immune response but also severely inhibits transgene expression and thus may not be a valuable solution (229). Reduction of CpG-content in plasmid DNA not only suppresses it's immuno-stimulatory effects (228 , 230), but also contributes to longevity of transgene expression. Depletion of CpG content is reported to help overcoming two major hindrances of gene transfer, namely acute toxicity and transient expression. Methylation of pDNA in the complex substantially decreases cytokine production, indicating that the mammalian immune system is recognizing the unmethylated pDNA and is likely triggering the cascade of response. Therefore, CpG-reduction should lessen the recognition of pDNA as foreign and reduce subsequent deleterious effects (231).

Comparing clearance of CpG-free-Luc and CpG-free-mcs plasmids we have observed that the backbone plasmid was cleared form the lung more rapidly the plasmid coding for luciferase. It is known that the clearance rates of plasmid DNA depend on their impact on the lung, namely, toxicity and provoked inflammation (87). However, our measurements of resident lung cells influx and activation of macrophages did not reveal any differences between these two plasmids. The half-clearance time of the backbone plasmid comprised only 19 hours compared to more than 40 in case of pCpG-free-Luc, which is surprising regarding it' smaller size and absence of encoded reporter gene. One would expect that CpG-free-Luc plasmid would be cleared faster because of its size and content. However, it is still little known about the clearance mechanisms of CpG-depleted DNA. This relatively new and very perspective trend in gene delivery had already achieved a lot in proving high levels of transgene expression, whereas mechanisms of CpG-free-pDNA clearance from the cells remain to be discovered(88). While plasmid DNA within the lung is metabolized according to it's structure, we could assume that also plasmid clearance depends on presence of CpG-motifs within it. Additional studies have to be performed to reveal the clearance mechanisms of CpG-rich and CpG-free plasmid DNA.

In case of both standard and CpG-free plasmids we have observed a two-phase clearance kinetics form the lung. For CpG-motif-free plasmids the first rapid phase took part within the first 72 hours after administration, while during the second phase presence of plasmid DNA within the lung decreased slowly till the time point of fourteen days. These results are close to the clearance pattern of pCVMLuc plasmid after aerosol administration, where relatively similar pDNA concentrations were observed within the first three days and than decreased significantly towards the last observation time point. Corresponding amounts of pCMVLuc observed within 72 hours after the application could be explained by its toxic effects which must have been the major hindrance to the clearance. Gradual decrease in amount of CpG-free plasmids observed during first three days after aerosol delivery proves this assumption. On the other hand, a break point between two clearance phases was observed already 24 hours after instillation, separating dramatic decrease in pDNA concentration within the first day form gradual decline within the following week. Our data demonstrate, that apart from the observed differences in clearance patterns between two plasmids, biophysical matters like amount of liquid and it's distribution within the lung determine the clearance pattern.

Analysis of DNA clearance from BALF resident cells revealed a different pattern. The elimination pCMVLuc and pCpG-free-Luc plasmids from the lung occurred with relatively similar speed so that the T1/2 comprised approximately two days, while pCpG-free-mcs was cleared much more rapidly with the T1/2 of 20 hours. Despite very similar half-clearance times observed for pCMVLuc and pCpG-free-mcs plasmids, other important clearance parameters like AUC and Cmax were 235-fold and 900-fold higher for CpG-free-Luc plasmid, respectively. These significant differences reveal that the CpG-motif free plasmid was cleared form the BALF cells much more effectively than the standard pCMVLuc. The dramatic influence of CpG-motif on lung resident cells, in particular on macrophages is widely reported (87, 103, 140). Murine macrophages were demonstrated to be activated with CpG-motifs in a Toll-like-1-receptop-mediated way. Yew et al. showed that addition of standard plasmid DNA after transfection with CpG-fre plasmid reduced transfection efficiency, revealing a deep influence of macrophage activation on basic biochemical processes within the lung (87, 140, 224). We can assume that presence of CpG motifs within plasmids plays an important role in pDNA clearance. We also can not exclude the influence of plasmid size on it's clearance, like we did it for the lung tissue. Macrophages are known to react differently on plasmid DNA of different size and structure which was shown by studying cytokine response (111). The fact that the pCpG-free-Luc DNA was cleared from

lung resident cells faster than the backbone-containing plasmid may be due to the presence of promoter and transgene sequence within it, which could have been recognized by phagocytic cells and thus influnec the clearance process.

Surprisingly similar T1/2 values were observed for both CpG-free plasmids in BALF liquid, which comprised approximately 8 hours and thus were five fold lower than that for pCVMLuc plasmid delivered by aerosol. Taking into consideration the influence of pCMV-Luc containing particles on lung mechanics and functioning we could assume that the lung swelling and changes in lung compliance and resistance could influence the metabolism of PEI/DNA complexes in the lung, thus preventing them form being rapidly cleared in case of pCMVLuc plasmid.

4.13 Analysis of BALF cells

Analyzing the resident lung cells for their number and type ratios we have found that both administration route and the CpG-content of plasmid DNA are important for provoking immune response.

In our study we have analyzed not only the total amount of phagocytic cells, but also activation of macrophages, for which myeloperoxidase revealing staining method by Kaplow was utilized (94). Myeloperoxidase (MPO) is a hem-containing enzyme, which is often used as an inflammation marker in the lungs (49, 77). Sugiyama et al. showed that granulocyte macrophage colony-stimulating factor regulates the ability of macropahges to express MPO (190). Stimulated phagocytes secrete this enzyme at inflammatory sites, where it generates a powerful reactive oxygen species, hypochlorous acid (HOCl), at physiological chloride concentrations (101, 213).

In our experiments both the amount of resident macrophages and the number of activated phagocyting cells increased significantly 24 hours after intranasal instillation of PEI/pCMVLuc complexes. These observations suggest that PEI-pDNA particles may induce cell infiltration and activation of macrophages when delivered intranasally. Histological observations also proved significant influx of neutrophils and monocytes to the alveolar region. Interestingly, instillation of distilled water also caused dramatic increase in number of activated macrophages, which was, however, less pronounced than in case of brPEI/pDNA particles instillation. Administration of high volumes of liquid into the lung is reported to provoke active infiltration of monocytes which clear the liquid rapidly, thus reducing the swelling and thu enhance lung functioning restoration (134). Therefore, we suggest that apart from the influence of PEI-pDNA particles a hypotonic character of the intranasal instilled

solution may contribute to cell infiltration and activation of phagocytosis. However, hypoosmotic solvents are necessary for PEI-pDNA complex formulation to guarantee high expression levels in lungs (118, 173). Regional distribution of PEI-pDNA complexes may be different after either instillation or aerosol delivery. This may lead to inhomogeneous particle distribution with areas of high and low PEI-pDNA particle concentrations, in particular after intranasal instillation. The areas of high PEI-pDNA particle concentrations may result in stronger lung response than after aerosol delivery, where homogenous particle distribution has been observed (148). Indeed, similar observations have been previously made for titanium dioxide particles (144). Histopathological techniques may be useful for studying this issue further. ii) Alternatively, the total PEI-pDNA complex burden which was higher after intranasal instillation than aerosol delivery may explain the different lung response. iii) The size of the PEI-pDNA particles was slightly different (90nm vs. 200 nm), which may result in different lung response. Complexes with the diameter of less than 100nm could be ascribed to the class of nanoparticles, which are widely reported to block the phagocytosis of lung resident macrophages and thus to increase the immune response (150). This mechanism could explain intensive influx of monocytes into the lung and activation of macrophages after instillation of complexes.

Comparing the behavior of lung resident cells after aerosol application of standard or CpG- free plasmids we have found that presence of CpG motifs plays an important role in inducing of immune response. Twenty four hours after aerosol delivery of PEI/pCMVLuc complexes a slight increase in amount of resident macrophages was observed which returned to it's normal level towards the next time point, while after aerosol administration of CpG-free plasmids no alterations in type or number of lung resident cells were observed. Both our and many published data prove aerosol delivery to be less immunostimulatory than instillation (134, 144, 148). Besides, considering equal characteristics of the particles and equal administration conditions, one may assume that it were the CpG motifs which defined the immune response in he lung. Indeed, CpG motifs of plasmid DNA are known to be highly immunogenic (111, 134), so that presence of even one motif may induce inflammatory response (90). Thus, our data show that exclusion of CpG motifs form plasmid DNA may provide a gene transfection method with a very low immunogenicity.

4.14 Luciferase expression in the lung after administration of pCVMLuc and pCpG-free-Luc plasmids

In our experiments we have observed three different patterns of luciferase expression. Twenty four hours after intranasal instillation the transgene expression levels were highly variable, while no transgene expression was observed at other time points. After aerosol administration of both pCMVLuc and pCpG-free-Luc plasmids a peak of luciferase concentration was observed 24 hours after administration followed by decrease in transgene levels observed for the next time points. Our observations demosntrate that administration route plays an important role for transfection efficiency. Intranasal instillation is repotted to be an efficient method of gene delivery (70). Nevertheless, in our case either the presence of high amounts of distilled water in lungs or the toxic effects of CpG motifs of the plasmid DNA prevented the lung cells from transgene expression. In all three cases the luciferase expression intensity corresponded to the clearance pattern of plasmid DNA, showing vividly that decrease in number of free pDNA moleucles within the lung cells leads to decrease in luciferase expression. Despite similar transgene expression patterns, aerosol administration of CpG-free-Luc plasmid resulted in at least 100 fold higher luciferase expression than it was observed for pCMVLuc. Other authors also demonstrated that plasmid DNA without CpG motifs can provide high levels of transgene expression combined with low toxicity (129). Unfortunately, even for CpG-free-Luc plasmid a decline in gene expression was observed, caused most probably by degradation of plasmid DNA within the lung cells.

Basing on our data, we can assume that it is mostly the structure of plasmid DNA that defines the levels of transgene expression in the lung cells, while the clearance pattern depends greatly on the route of administration.

In all our experiments we have not observed any transgene expression in lung resident macrophages. These cells, representing the primary defense mechanism of the lung, are extremely difficult to transfect because of their specialization to terminate the incoming bacteria and viruses (153). Vehicles for gene delivery into macrophages are studied intensively for gene vaccination (80). CpG-motifs are reported not only to provoke the immune response and macrophage activation but also to enhance the transfection of these cells. Nevertheless, our complexes of branched PEI with two different plasmids proved to be inefficient in transecting this particular cell type. Indeed, cationic liposomes were shown to be more effective for that than cationic polymers (43).

5 SUMMARY

Gene delivery is supposed to be a promissing strategy for treatment of a variety of inherited and somatic deseases. Correction of genetic defect within the cell should restore or improve the functioning of the cell thus eliminating the cause of an illness.

Development of efficient gene delivery vehicles is one of the basic objectives of contemporary gene therapy research. Among many existing synthetic vehicles branching polylysines are renouned for their DNA transfer capacities, as well as for biodegradability and low toxicity. We have investigated a group of branching polypeptides bearing different modifications of the basic polylysine core. The polymers were analysed for their ability to bind and protect DNA and to deliver it into the cells *in vitro* providing transgene expression. We have found that although all four modifications have improved the qualities of the primary polymer, introducing arginins had increased significantly gene transfer capacities of the polypeptides.

Safe and efficient adminstration route is as indispensable requirement for gene therapy treatement. Aiming to optimize the procedure for pulmonary gene delivery we have compared two administration strategies and two types of plasmid DNA structure. We have found that aerosol delivery is more efficient than intranasal instillation, being at the same time less harmful and immunogenic than the latter. We have shown that plasmid DNA without CpG-motifs did not alter normal lung functioning and was not immunogenic. Besides, particles containing CpG-free DNA delivered via aerosol were proved to provide higher levels of transgene expression than CpG-motif containing plasmid.

6 CONTRIBUTION TO THE WORK

Analysis of four polylysin-based dendrimers

Synthesis of a series of branching lysin-based dendrimers as well as the analysis of protein purity and aminoacid composition was performed in the laboratory of Dr. Vlasov in the institute of high molecular compounds, Laboratory of biologically active polymers, St.Petersburg, Russia. Analysis of DNA-binding, protective capacities of the polymers, as well as size- and zeta-potential measurements were performed by E. Leisna. Investigation of gene transfer abilities of the dendrimers was also performed by E. Lesina.

Intranasal instillation and aerosol administration of pCMVLuc/brPEI compelxes

Transfection of mice via aerosol nebulization or intranasal instillation, as well as the following animal preparation and transgene expression analysis were carried out in collaboration with Dr. Petra Dames. Preparation of animals for lung functioning analysis was performed by E. Ledina, while the measurement of lung functioning parameters was carried out by Dr. Andreas Flemmer and Dr. Kerstin Hajek. Broncheo-alveolar lavage and quantification of the cells, as well as the preparation of the samples for microscopy, histological staining and sample analysis were made by E. Lesina. Real-Time PCR was performed by Dr. Petra Dames. Histological analysis of the lung samples was made by Iris Bittmann.

Aerosol delivery of CpG-motif-free DNA complexed with brPEI

Aerosol administration was performed in collaboration with Dr. Dames. Analysis of lung functioning parameters was carried out in collaboration with Dr. Flemmer and Dr. Hajek. Histological analysis of the lung samples was made by Dr. Charel, Institute of Pathology, Berlin, Germany. The analysis of mice for luciferase expression and inflammatory parameters, animal preparation, broncheo-alveolar lavage and quantification and analysis of lung resident cells, as well as Real-Time PCR and clearance analysis were carried out by E. Lesina.

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Published aspects of this work

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Eugenia Lesina, Petra Dames, Andreas Flemmer, Kerstin Hajek, Thomas Kirchner, Iris Bittmann, and Carsten Rudolph **CpG-free plasmid DNA prevents deterioration of pulmonary function in mice** (submitted).

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