Dissertation

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Novel degradable pseudodendritic oligoamines for *in vitro* and *in vivo* gene delivery

vorgelegt von

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Professor Dr. Ernst Wagner betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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Für meine Mutter

(1950 – 2003)

Es ist nicht genug zu wissen,
man muss es auch anwenden.

Es ist nicht genug zu wollen,
man muss es auch tun.

Johann Wolfgang von Goethe

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1 Introduction

1.1 Gene therapy – a brief history

Gene therapy holds much promise to treat or to prevent diseases by delivering therapeutic nucleic acids into patients, although the field has experienced intense criticism and skepticism within recent years.[1,2]

The conceptually new approach to the treatment of human diseases using gene therapy arose during the 1960s and 1970s.[3,4] At this point in time, the first evidences for uptake and expression of exogenous DNA in mammalian cells have been found. Since then, the concepts and techniques of gene therapy rapidly progressed and the idea that human genetic disorders, infections and some degenerative diseases may be amenable to correction at the genetic level has cleared from theoretical ideas and became practically more feasible (see **Table 1**).

Year	Result	Reference
1960-70	Evidence of uptake and expression of exogenous DNA in mammalian cells	Szybalska, E.H. and Szybalski, W.: *Genetics of human cell lines IV. DNA-mediated heritable transformation of a biochemical trait*. PNAS (1962) Majumdar, A. and Bose, S.K.: *DNA mediated genetic transformation of a human cancerous cell line in cultured in vitro.* Ind. J. med. Res. (1968)
1980-81	Cline human study	Cline, M.J. *et al.*: *Gene transfer in intact animals.* Nature (1980) Mercola K.E., Stang, H.D., Browne, J. and Cline, M.J.: *Insertion of a new gene of viral origin into bone marrow cells of mice.* Science (1980) Kolata GB, Wade N.: *Human gene treatment stirs new debate.* Science (1980) Wade N.: *Gene therapy caught in more entanglements.* Science (1981)
1980	Public discussion "Gene therapy in human beings: When is it ethical to begin?"	Anderson WF, Fletcher JC.: Sounding boards. *Gene therapy in human beings: when is it ethical to begin?* N Engl J Med. (1980)
1989-90	First approved human gene therapy trial	Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, et al.: *Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction.* N Engl J Med. (1990)

Table 1: List of significant points in the early development of gene therapy[5]

However, after the severely, on both scientific and ethical ground, criticized first gene transfer study by Cline and colleagues in 1980[6-9], science, ethics and public policy aspects were

catalyzed and the question was no longer *if* human gene therapy can be developed, but rather *how.*[10] In 1989, Rosenberg and his colleagues performed the first approved human gene therapy clinical trial using retroviruses to introduce the gene encoding for resistance to neomycin into human tumor-infiltrating lymphocytes prior to re-infusion into patients with advanced melanoma.[11] This study demonstrated at first the feasibility of gene therapy using viral systems and set a basis for over 1300 clinical gene therapeutic trials that have been either completed, are still ongoing or are approved in 28 countries worldwide so far (2007).[2]

Nevertheless, there have been a few regrettable cases of severe adverse effects after gene therapeutic treatment (unexpected inflammatory reaction to adenovirus vectors causing death or leukemia-like clonal lymphocyte proliferation related to retroviral chromosomal integration), which had an inevitable effect on the progress of and enthusiasm for the clinical implementation of gene therapy. This has prompted scientists to undertake more detailed investigations into the behavior of vector systems and encouraged them for more careful testing of various approaches and technologies to ameliorate the field, resulting in greater steps toward safe and efficient gene therapeutic applications in the future.

The diseases targeted by gene therapy are various but the vast majority has addressed cancer, cardiovascular and inherited monogenetic diseases. While the different strategies applied in cancer gene therapy range from inserting tumor-suppressor genes, to immunotherapy and gene-directed enzyme prodrug therapy (GDEPT), cardiovascular gene therapy mainly addresses therapeutic angiogenesis by over expressing e.g. the fibroblast growth factor (FGF) or the vascular endothelial growth factor (VEGF). The aim in treating monogenic disorders is the permanent correction of the malfunctioning gene. Other indications addressed by gene therapy include human immunodeficiency virus (HIV) infections, tetanus and cytomegalovirus (CMV) infections as well as some neurological diseases, multiple sclerosis, glaucoma and age-related macular degeneration.[2]

Nucleic acids transferred in clinical trials are genes encoding for antigens, cytokines, tumor-suppressor or suicide genes, growth factors or different receptors while recently much interest has also sparked in RNA interference (RNAi) including especially the delivery of short interfering RNA (siRNA) for therapeutic application.[2]

1.2 Synthetic delivery systems for gene therapy

An ideal gene carrier should protect the nucleic acid against nucleases and should deliver the payload efficiently and exclusively to the target site. Since the delivery of naked DNA is only successful when injected directly into the target tissues or cells, several gene transferring vectors have been developed to improve transfection efficiency. Generally, these delivery devices can be divided into two main categories: viral vectors and synthetic transfer systems.

Viral vectors are the most commonly used gene transfer systems employed in clinical trials due to their high potency in gene transfer.[1,2] The limitations associated with viral vectors in terms of safety, immunogenicity, low transgene loading size and high costs have encouraged researchers to focus on different alternatives: synthetic gene delivery systems.

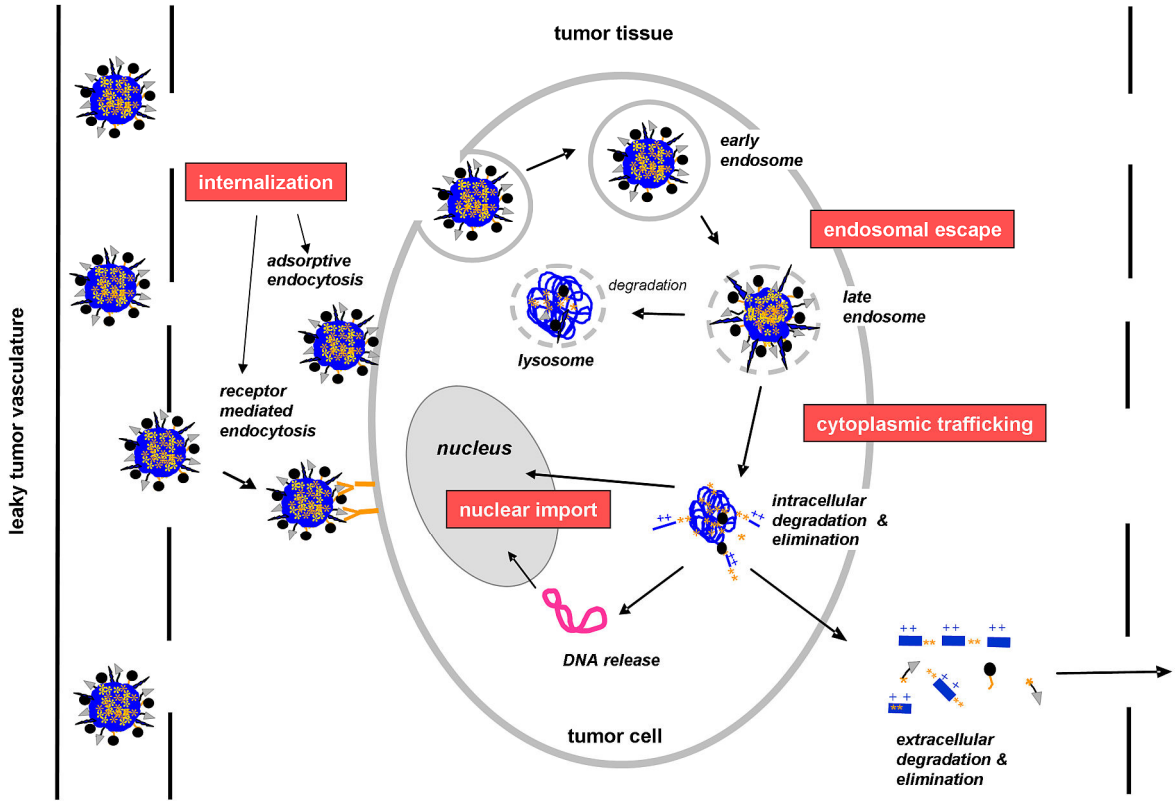

Fig. 1: Schematic representation of the different barriers that have to be overcome by a gene carrier system. After systemic application, polyplexes have to survive in the bloodstream without being degraded or captured by cellular immunogenic defense mechanisms (e.g. complement system). At the tumor site they have to extravasate into the tissue and bind – ideally – specifically to the target cell. After internalization, additional intracellular hurdles appear, such as endosomal escape, cytoplasmic trafficking and nuclear import.

The limitations of viral vectors, in particular their safety concerns, render synthetic vectors as alternatives progressively interesting. Moreover, they offer an enhanced biosafety profile since they can be generated protein-free or by only non-immunogenic or humanized proteins/peptides resulting in lower immunogenicity. A further benefit of synthetic transfer systems is that they can easily be synthesized in large quantities at rather low cost and offer a higher loading capacity for DNA. However, the major drawback of synthetic vectors is their limited efficacy compared to viral vectors after *in vivo* application, which is currently mainly compensated by the application of larger quantities of vector formulation.

Most synthetic vectors are generally based on formulations of chemically defined, positively charged polymers (polycations or cationic lipids), which interact electrostatically with the negatively charged nucleic acids. The resulting “polyplexes” or “lipoplexes” protect DNA from degradation and are positively charged themselves due to an excess of polymer used to form compact nano-sized complexes suitable for cell entry. Cell entry mainly occurs due to interactions with the negatively charged cell membranes and polyplexes are endocytosed afterwards (see **Fig. 1**).

It is quite obvious that no polymer is able to meet all of these requirements. Thus, it is often necessary to generate a so called “synthetic virus” system consisting of a cationic compacting part in combination with various functional domains in order to meet all demands.

1.3 Polymers used as synthetic vectors

Over the last decades a huge variety of polymers, mainly cationic polymers or cationic lipids have been investigated to generate synthetic gene carriers. The following section will focus on cationic polymeric compounds that were recently developed for systemic gene delivery. For cationic lipids as delivery vectors see the review by Li and Szoka (2007).[12]

To date, systemic administration of polycationic polymers often resulted in a toxic response, which is mostly linked to the positive surface charge of the vectors making them incompatible for large clinical applications. Therefore, the main objective in gene therapy for systemic administration is the development of stable and non-toxic gene vectors that can compact and deliver genetic materials into specific cell types exhibiting transfection efficiencies comparable to viral vectors under physiological conditions. The existing synthetic cationic systems can be divided into two groups: non-degradable and degradable polymers.

1.3.1 Non-degradable polymers – polyethylenimines (PEI)

PEI can be synthesized at various molecular weights, in a branched or linear manner or can undergo functionalization by group addition or substitution. PEI is the most commonly used polycation and has a privileged place in the line of synthetic gene delivery devices due to its superior transfection efficiency in a broad range of cell types compared to other systems. As PEI is able to form stable nano-scaled, positively charged polyplexes with DNA molecules, it is capable to enter cells after cell adhesion, followed by endocytosis and can promote its efficient escape from the endo-/lysosomal compartment by the so called "proton sponge effect".[13]

Studies showed that linear PEI (LPEI) with a molecular weight of 22 kDa is the most potent polycationic transfection agent and is thus defined as the "golden standard" for efficient *in vitro* gene delivery. Its excellent transfection/cytotoxicity–relation is much better compared to its branched PEI (BPEI) counterpart in a similar molecular weight range (25 kDa). However, the huge amount of positive surface charges[14], the non-degradability of the polymer and a variety of undesired, unspecific interactions with the biological environment[15,16] results in a fairly high toxicity of PEI polyplexes after systemic administration *in vivo* no matter whether a linear or a branched conjugate is used.[17,18]

1.3.2 Degradable polymers

Non-degradable polymers like LPEI or BPEI are "static" structures, which cannot be degraded, metabolized and eliminated by the body. In consequence they can accumulate in cells or organs (mainly in excretion organs like liver or kidney) leading to undesired and uncontrollable long-term toxicity in living systems. Consequently, there is the urgent need for biodegradable polymers. Their potential advantages should be their reduced toxicity (provided that degradation products result in non-toxic compounds) and the avoidance of accumulation. Furthermore, the polymer degradation pattern could also be used to improve the intracellular release of the nucleic acid payload in order to enhance the desired therapeutic effect.

The importance of using degradable polymers for gene delivery has been particularly recognized within the last years. Naturally occurring polymers like chitosans[19] or their derivatives[20-23] as well as peptidic polymers like poly(L-lysine) (PLL)[24-26] or the PLL analogue poly (α-[4-amino-butyl]-L-glycolic acid) (PAGA)[27-29] are biodegradable and therefore possess an advantage in terms of their toxicity profile for *in vivo* application exhibiting a better

biocompatibility compared to PEIs.[30] However, for example in case of PLL, although low molecular weight (LMW) compounds have been found to result in a strongly reduced toxicity compared to their high molecular weight (HMW) counterparts, they do not present a sufficient stability of the formed polyplexes under physiological conditions. Consequently, they are rapidly removed from the blood circulation e.g. by Kupffer cells in the liver.[26,31,32] Other non-toxic and easily excretable LMW polyamines like e.g. the naturally occurring spermine (Mw 202 Da) or spermidine (Mw 145 Da) as well as oligoethylenimines (e.g. OEI, 800 Da) exhibit the same effect of a low complex stability under physiological conditions and cannot be used as gene transfer agents.[33] Hence, the main goal in the design and development of degradable polymers is to obtain polymers that show high extracellular stability of the formed polyplexes while, importantly, these should be easily destabilized intracellularily in order to release their nucleic acid payload efficiently while a low acute *and* long-term toxicity should be maintained.

In **Fig. 2** one approach to reach this goal is shown. The reversible oligomerization of LMW oligocations by crosslinking them with biodegradable linkers was already successfully shown by several researchers.[31,34-37] Alternatively, degradable polymers could also be synthesized *de novo* by the addition of labile bonds within the repetitive monomeric units during synthesis.

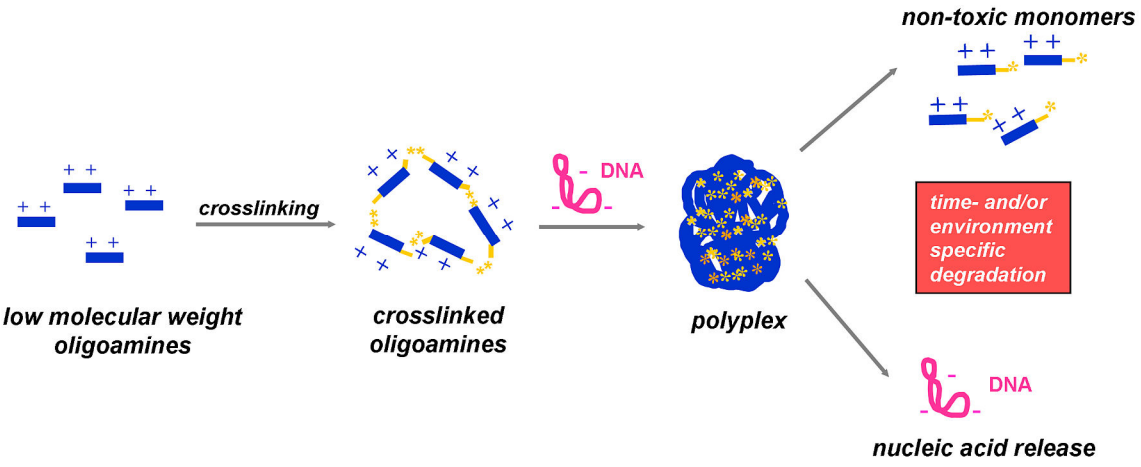

Fig. 2: Reversible oligomerization of low molecular weight oligoamines using degradable crosslinkers. Low molecular weight (LMW) oligoamines are crosslinked via reversible linkers to form oligomerized polyamines, which are capable to form stable polyplexes protecting DNA from degradation by nucleases. In a time- and/or environment-specific manner the labile bonds can be degraded in order to release the DNA resulting in non-toxic monomers that can easily be eliminated. (adopted from PhD thesis of Julia Kloeckner)

Reversible linkages may include degradable bonds that break down due to enzymatic or chemical degradation (see **Fig. 3**). Hence, most of the degradable polymers contain

hydrolytically sensitive bonds including e.g. esters, acetals or hyrdazones. The use of disulfide bonds which are cleaved in the reductive intracellular environment has also been investigated by several groups.

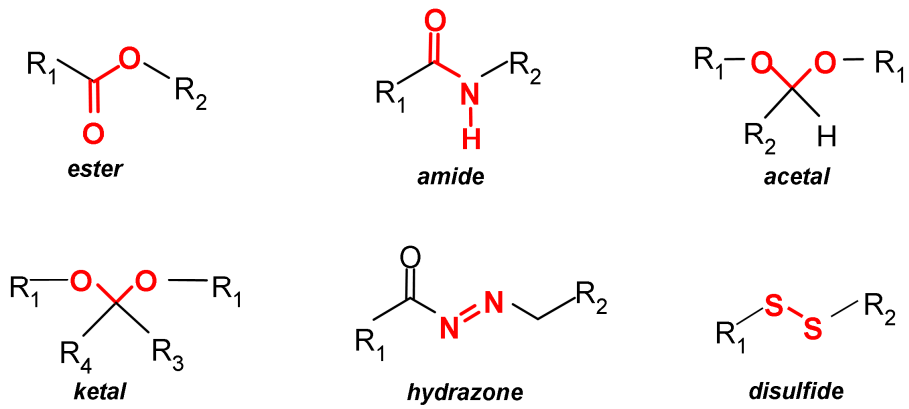

Fig. 3: Overview on different degradable bonds. Hydrolyzable bonds include ester, amide, acetal, ketal and hydrazone bridges, whereas disulfide bonds are degraded under reductive environment.

In order to deliver *and* release the DNA efficiently, the gene carrier should be constructed in a way that uses the properties of the target site, to get triggered at the "right" point in time (e.g. by the presence of enzymes, pH, redox potential, etc). Moreover, the polymers should be degraded within one or a few days in order to reduce the potential accumulation at undesired sites.

1.3.3 Dendrimers

A technical weakness many of the synthesis approaches described above share is the polydispersity, i.e. the heterogeneity in molecular weight, which is inherent to the procedure of the applied random polymer synthesis. The design of dendrimers is an elegant solution to obtain well-defined conjugates with narrow molecular weight distribution. Dendrimers (from the Greek *dendron*: "tree" and *meros*: "part") are spherical, highly branched structures that have a hierarchical, three-dimensional design. The core of the molecule provides a central point from which monomers ramify in well-ordered and symmetrical manner. The tree-like construction is created by repetition of the same sequence leading to a new dendrimer generation at the end of every reaction cycle.[38] Currently, the preferred dendrimers are polyamines, polyamides and polyesters. As an example, polyamidoamine (PAMAM) dendrimers have already successfully been applied for gene delivery *in vivo*.[39-41]

1.4 Optimization of polyplexes toward “synthetic viruses”

Even the most potent synthetic gene delivery devices can still not compete with viral vectors. Key issues for the optimization of polyplexes include an improved specificity for the target tissue, an accelerated intracellular and nuclear trafficking and in addition the already mentioned low toxicity of compounds. Furthermore, they should also provide good pharmacokinetic parameters. Hence, fundamental steps toward the ‘synthetic virus’ could only be achieved if the polyplexes were stable under physiological conditions, providing an increased specificity toward the target tissue and were able for an efficient overcome of most of the given cellular hurdles (see **Fig. 1**). Central steps to meet these goals are i) to imply methods to increase the colloidal stability of polyplexes under *in vivo* conditions and ii) to enhance their specificity toward the target site by introducing targeting ligands while a high biocompatibility of the gene delivering system should be maintained.

1.4.1 Stabilization of polyplexes for *in vivo* application

Despite their versatility and improved safety, synthetic gene delivery systems still lack high *in vivo* transfection efficiency. Therefore, developing a gene transfer system raises the challenge of finding solutions to efficiently deliver DNA to the target tissue (or target cell) but also being capable of effectively releasing the genetic payload for high gene expression. The low colloidal stability of polyplexes as well as possible unspecific interactions with blood components or proteins of the extracellular matrix are some of the reasons leading to hindrance of this property.[15,42] Hence, an emerging theme in recent studies is the use of crosslinked, stabilized polyplexes to achieve an optimized balance between the stability of particles in the extracellular environment (e.g. in the bloodstream) and the controlled release of DNA within the cytosol of the target site.[43,44] The idea of a transient, at best, bioreversibly triggered stabilization/destabilization strategy of polyplexes is quite favorable with regard to improving the pharmacokinetic properties of synthetic gene delivery devices.

1.4.2 Targeting polyplexes for site-specific gene delivery

Targeting – by definition – includes any strategy to improve the specificity of gene expression and/or the delivery of nucleic acids toward e.g. the tumor site, while highest transfection levels should be achieved. Multiple targeting methods have been investigated in the past including the targeted delivery of nucleic acids as well as transductional and transcriptional targeting strategies at the intracellular site of tumor cells (see **Fig. 4**). Transductional targeting comprises of all methods which improve the intracellular release and transport of transgenes toward the nucleus of the target cell. Triggered endosomal release, enhanced

cytosolic trafficking and the specific nuclear import of transgenes[45] are the major objectives of this targeting policy. As soon as the genes are delivered into the nucleus they can only be expressed if adequate promoter and/or enhancer elements are included in the gene expression cassette. Transcriptional targeting utilizes tumor-specific promoter/enhancer systems resulting in specifically high levels of transcription controlled transgene expression.

The cellular uptake of particle-based gene delivery systems usually occurs, as already mentioned, via charge-mediated interactions with the cell surface followed by endocytosis (see **Fig. 1**). Upon systemic administration, however, such non-specific interactions also take place with blood components and non-target tissues and hamper gene delivery. Specific delivery of polyplexes toward the tumor site can be mediated by physical (for review see Russ and Wagner, 2007)[46] or biological targeting technologies.

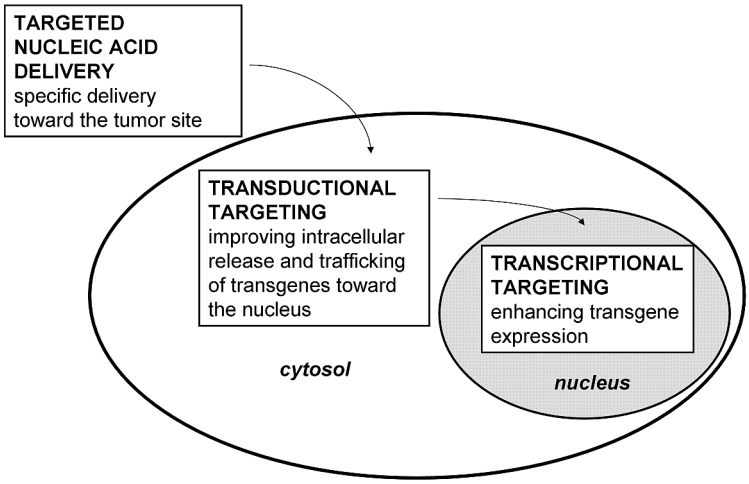

Fig. 4: Tumor targeting principles for therapeutic nucleic acids.

Biological targeting strategies toward the tumor site are achieved by taking advantage of the special tumor architecture and unique tumor properties. Basically, these strategies can be divided into two main groups: *passive* and *active* tumor targeting. The imperfect and leaky tumor vasculature, due to abnormal neovascularization required to serve fast-growing tumors, combined with an inadequate lymphatic drainage leads to passive targeting of polyplexes to the tumor site. This effect is termed “enhanced permeability and retention” (EPR effect)[47]. To exploit target cells specifically, gene delivering particles can be modified with numerous cell-targeting ligands such as peptides, proteins, vitamins and carbohydrates.[46,48] All these ligands recognize specific receptors on the cell membrane of target cells bind specifically and result in receptor-mediated endocytosis of polyplexes (see **Fig. 1**). One commonly used ligand is the serum glycoprotein transferrin (Tf) using the

transferrin receptor (TfR) for targeted delivery. TfR required for iron uptake into cells is over-expressed in tumor cells due to the higher demand of iron for their growth.[49] Tf as protein ligand combines both a shielding function of the vectors and a targeting moiety toward the tumor site.[50,51] It was found for example, that the incorporation of Tf or anti-Tf receptor single-chain antibody Fv fragments into polyplexes and lipoplexes resulted in enhanced gene transfer efficiencies *in vitro* as well as *in vivo*.[52-57] PEGylation of PEI/DNA polyplexes containing Tf as targeting ligand further improved the *in vivo* application. The epidermal growth factor (EGF) is another widely investigated ligand for tumor targeting[58-61] as well as folate or several other proteins and peptides that target surface markers of for example endothelial cells including integrins like αvβ3 and αvβ5 or the CD 13 receptor.[62]

1.5 Aim of the thesis

The major focus in the development of novel degradable polymers for synthetic gene delivery – both for *in vitro* and *in vivo* gene delivery – is to synthesize highly efficient and low-toxic compounds. Up to now, many non-toxic and (bio-) degradable polymers have been generated.

1.5.1 Design of novel pseudodendritic oligoamines for synthetic gene delivery

The aim of this thesis was to develop a novel family of hyperbranched polymers, namely pseudodendrimers, which exhibit a better defined chemical structure compared to the randomly synthesized hitherto existing polycations (see **Fig. 5**). These compounds consist of a low molecular weight polycation (branched oligoethylenimine 800 Da (OEI) or polypropylenimine dendrimer (PPI)) in the center, functionalized with an excess of degradable dioldiacrylates to form a pseudodendritic core. The pseudodendritic core had to be subsequently modified on its surface with different oligoamines. Although these conjugates do not have the typical symmetric dendritic structure based on AB_2 monomers, they follow the dendritic concept based on a branched structure, assembled by adding a generation of monomer to a core unit.[38]

Used as gene delivery devices these pseudodendrimers may have several advantages: (i) amines, presented in the periphery of the pseudodendrimers can interact well with DNA and form nano-scaled pseudodendrimer / DNA complexes; (ii) the high density of secondary and tertiary amines within the polymer may facilitate endosomal escape of

pseudodendrimer / DNA complexes (“proton sponge” effect) inside the cell;[13] (iii) the dendritic branches containing ester bonds allow degradation of pseudodendrimers under physiological conditions increasing their biocompatibility; (iv) pseudodendrimers can be easily modified on their surface to improve transfection efficiency or pharmacokinetic properties e.g. using crosslinkers for lateral stabilization of the polyplexes and/or targeting ligands.

Physicochemical and biological screening studies of several pseudodendrimers *in vitro* were planned to discover promising candidates for more detailed *in vivo* studies in tumor-bearing mice.

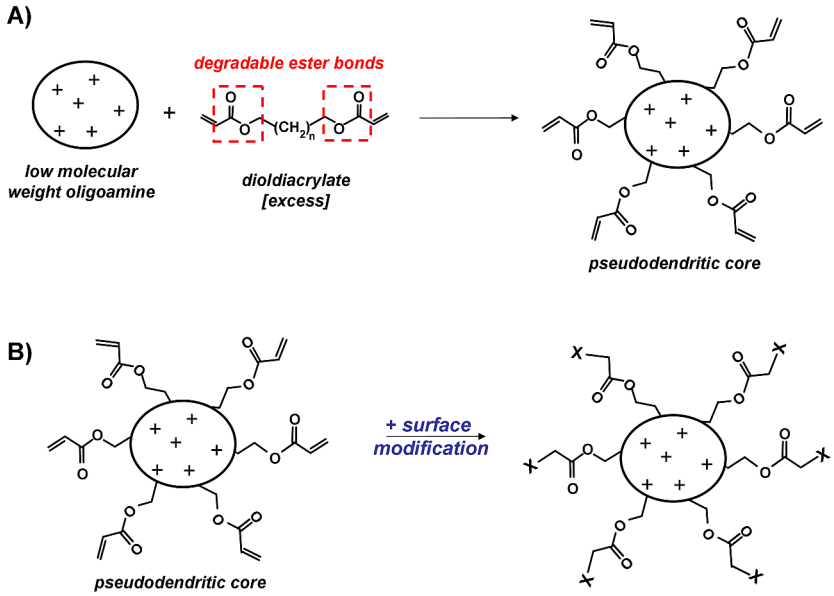

Fig. 5: Pseudodendritic concept. Schematic illustration of the two steps in pseudodendrimer synthesis. In the first step, the pseudodendritic core is formed by an excess of dioldiacrylate and a low molecular weight oligoamine. Subsequently, the pseudodendritic core is modified upon its surface with different oligoamines as surface modification units.

1.5.2 Establishment of a structure-activity relationship *in vitro*

To elucidate what is needed for efficient DNA gene delivery using pseudodendrimers a study on their structure-activity relationship was performed using the OEI core based pseudodendrimers. Both the influence of the pseudodendritic core characteristics and the influence of the surface modification unit on transfection efficiency versus their cytotoxicity had to be investigated and were set into relation. A further aim was to unravel the reason why some polymers result in high gene expression at low cytotoxicity whereas others are characterized by a lack of transfection activity. Further requirements to achieve high transfection efficiency were investigated using PPI core based pseudodendrimers.

1.5.3 Gene transfer and biocompatibility studies *in vivo*

The most promising degradable pseudodendrimers as found in *in vitro* studies had to be investigated for their *in vivo* transfection efficiency in a subcutaneous tumor model in mice. After studies regarding their biocompatibility, polyplexes were applied systemically. The transfection pattern in main organs and tumor tissue was examined finding predominant reporter gene expression in tumor tissue. Another aim was to improve the polyplex formulation of HD O particles to improve the reporter gene expression level. This had to be investigated by intratumoral application of polyplexes.

2 Materials and methods

2.1 Chemicals and reagents

Low molecular weight polyethylenimine 800 Da waterfree (oligoethylenimine, OEI), 1.6-hexandioldiacrylate (HD), 1.4-butandioldiacrylate (BD), 1.2-ethyleneglycoldiacrylate (ED), branched PEI 25 kDa (BPEI), spermidine (Sp), spermine (S), ethylenediamine (EDA), triethylenetetramine (TETA), pentaethyleneheximine (PEHI), DAB-Am-8, polypropylenimine-octaamine dendrimer, generation 2.0 (PPI G2) and DAB-AM-16, polypropylenimine-hexadecaamine dendrimer, generation 3.0 (PPI G3) were purchased from SIGMA-Aldrich (Steinheim, Germany). PPI G2 and PPI G3 are also available from SyMO-Chem (Eindhoven, The Netherlands). Ethanolamine (E) was obtained from Fluka (Buchs, Switzerland). Polyethylenimine 1200 Da (O1200) and polyethylenimine 1800 Da (O1800) were from Polysciences Inc. (Warrington, PA). Linear PEI 22 kDa (LPEI) was synthesized by acid-catalysed deprotection of poly(2-ethyl-2-oxazoline) (50 kDa, Aldrich) in analogous form as described[63] and is also available from Polyplus Transfections (Strasbourg, France). All reagents were used without further purification. LPEI and BPEI were adjusted to pH 7 using 1N HCl (SIGMA-Aldrich, Steinheim, Germany) prior to use. Dithiobis (succinimidylpropionate) (DSP) and disuccinimidyl suberate (DSS) were purchase from Thermo Fisher Scientific Inc. (Rockford, IL).

Deuterium oxide (D_2O) and dimethyl sulfoxide puriss. (DMSO) were purchased from SIGMA-Aldrich (Steinheim, Germany). Water was used as purified, deionized water.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was obtained from SIGMA-Aldrich (Steinheim, Germany). Bafilomycin A1 was purchased from Alexis Biochemicals (Lausen, Switzerland). Luciferase cell culture lysis buffer 5 x reagents and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany).

Cell culture media, antibiotics and fetal calf serum (FCS) were purchased from Life Technologies (Karlsruhe, Germany).

Plasmid DNA pCMVLuc for *in vitro* studies (Photinus pyralis luciferase under control of the CMV enhancer / promoter) described in Plank et al.[64] was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). pEGFPLuc as used for *in vivo* studies (a fusion

of enhanced green fluorescent protein (EGFP) and Photinus pyralis luciferase under control of the CMV enhancer / promoter) was obtained from Clontech Laboratories (Mountain View, CA) and was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). pEGFP-N1 (Clontech) was produced and purified by ELIM Biopharmaceuticals (San Francisco, CA).

2.2 Pseudodendrimer synthesis

Pseudodendrimers were synthesized in two steps. In the first step, the pseudodendritic core was formed by Michael addition of low molecular weight branched oligoamines and an excess of dioldiacrylate. In the second step, the surfaces of the pseudodendritic cores were modified using a variety of oligoamines.

2.2.1 Synthesis of OEI core based pseudodendrimers

For the synthesis of the various OEI core based pseudodendrimers, polyethylenimine 800 Da waterfree (oligoethylenimine, OEI) was coupled with a 20-fold molar excess of three different dioldiacrylates. For this, OEI and dioldiacrylate stock solutions were prepared in anhydrous DMSO at concentrations of 0.1 M and 2 M, respectively. For surface modification each oligoamine stock solution was prepared at a concentration of 0.6 M in anhydrous DMSO.

2.2.1.1 1.2-Ethyleneglycoldiacrylate (ED)-core conjugates

100 µL OEI stock solution (0.01 mmol) was mixed with 800 µL anhydrous DMSO and subsequently 100 µL ED stock solution (0.2 mmol) was added. The reaction occurred at 45 °C for 24 h under constant shaking (1000 rpm, Eppendorf Thermomixer). The resulting mixture was used for further synthesis without isolation of the product. The ED core was modified with different oligoamines including ethanolamine (E), spermidine (Sp), spermine (S) and OEI (O) by adding 0.3 mmol of each oligoamine stock solution (0.5 mL) to the reaction mixture. Reaction proceeded for 24 h at 22 °C under constant shaking (1000 rpm, Eppendorf Thermomixer).

2.2.1.2 1.4-Butandioldiacrylate (BD)-core conjugates

100 µL OEI stock solution (0.01 mmol) was mixed with 800 µL anhydrous DMSO and subsequently 100 µL BD stock solution (0.2 mmol) was added. The reaction occurred at 45 °C for 24 h under constant shaking (1000 rpm, Eppendorf Thermomixer). The resulting mixture was used for further synthesis without isolation of the product. The BD core was

modified with different oligoamines including ethanolamine (E), spermidine (Sp), spermine (S) and OEI (O) by adding 0.3 mmol of each oligoamine stock solution (0.5 mL) to the reaction mixture. Reaction proceeded for 24 h at 22 °C under constant shaking (1000 rpm, Eppendorf Thermomixer).

2.2.1.3 1.6-Hexandioldiacrylate (HD)-core conjugates

100 µL OEI stock solution (0.01 mmol) was mixed with 800 µL anhydrous DMSO and subsequently 100 µL HD stock solution (0.2 mmol) was added. The reaction occurred at 45 °C for 24 h under constant shaking (1000 rpm, Eppendorf Thermomixer). The resulting mixture was used for further synthesis without isolation of the product. The HD core was modified with different oligoamines including ethanolamine (E), ethylenediamine (EDA), spermidine (Sp), spermine (S) triethylenetetramine (TETA), pentaethyleneheximine (PEHI), OEI (O), oligoethylenimine 1200 Da (O1200) and oligoethylenimine 1800 Da (O1800) by adding 0.3 mmol of each oligoamine stock solution (0.5 mL) to the reaction mixture. Reaction proceeded for 24 h at 22 °C under constant shaking (1000 rpm, Eppendorf Thermomixer).

2.2.2 Synthesis of PPI core based pseudodendrimers

For the PPI core synthesis, polypropylenimine dendrimer generation 2 (G2), polypropylenimine dendrimer generation 3 (G3) and HD stock solutions were prepared in anhydrous DMSO at concentrations of 0.1 M, 0.045 M and 2 M, respectively. For surface modification units, OEI and G2 surface modification stock solutions were prepared at a concentration of 0.6 M in anhydrous DMSO.

2.2.2.1 PPI dendrimer generation 2 (G2) core conjugates

100 µL G2 stock solution (0.01 mmol) was mixed with 800 µL DMSO and subsequently 100 µL HD stock solution (0.2 mmol) was added. The reaction occurred at 45 °C for 24 h under constant shaking (1000 rpm, Eppendorf Thermomixer). The resulting mixture was used for further synthesis without isolation of the product. Grafting the acrylate terminated cores proceeded for each oligoamine by adding 0.5 mL (0.3 mmol) of either OEI or G2 surface modification stock solution to the reaction mixture. Reaction proceeded for 24 h at 22 °C under constant shaking (1000 rpm, Eppendorf Thermomixer).

2.2.2.2 PPI dendrimer generation 3 (G3) core conjugates

100 µL G3 stock solution (0.0045 mmol) was mixed with 800 µL DMSO and subsequently 100 µL HD stock solution (0.2 mmol) was added. The reaction occurred at 45 °C for 24 h under constant shaking (1000 rpm, Eppendorf Thermomixer). The resulting mixture was used for further synthesis without isolation of the product. Grafting the acrylate terminated cores proceeded for each oligoamine by adding 1.1 mL (0.66 mmol) of either OEI or G2 surface modification stock solution to the reaction mixture. Reaction occurred for 24 h at 22 °C under constant shaking (1000 rpm, Eppendorf Thermomixer).

2.2.3 Purification

After synthesis, the obtained products were diluted 1:10 in water and the pH was immediately adjusted to pH 7 using 1N HCl. Purification was done via dialysis using a Spectra/Por® membrane (Spectrum Medical Industries, Inc, CA) against 4L of water at 4 °C for 24 h. In general a 1 kDa MWCO dialysis membrane was used for purification, except for the OEI core based pseudodendrimers modified with O1200 and O1800 as well as for G3 PPI core derivatives where dialysis membranes with a MWCO of 3.5 kDa were used. Water was exchanged twice, i.e. after 2 h and 20 h of dialysis. The dialysates were freeze-dried. Yields of obtained products were listed as weight of product / weight of educts [w/w] percent.

2.3 Pseudodendrimer characterization

2.3.1 Structural analysis

^{1}H-NMR spectra were recorded in D_2O on an Eclipse +500 spectrometer from JEOL, Tokyo, Japan operating at 500 MHz.

ED E: ^{1}H NMR (D_2O): δ 4.2-4.5 (COO*CH_2*, ester linker, 4H, di-ester, 2H, mono-ester), δ 3.8-3.9 (NCH_2*CH_2*OH, ethanolamine, 2H), δ 3.7 (HO*CH_2*, mono-ester, 2H), δ 2.4-3.4 (82H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2OH of ethanolamine, N*CH_2**CH_2*COO linker)

molar ratio (diacrylate / OEI) = 8.3 : 1; molar ratio (surface amine / OEI) = 1.5

residual ester content after synthesis: 90%

yield: *33% (weight of product / weight of educts [w/w] percent)*

ED Sp: ^{1}H NMR (D_2O): δ 4.2-4.5 (COO*CH_2*, ester linker, 4H, di-ester, 2H, mono-ester), δ 3.8 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (88H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2*CH_2*N*CH_2*CH_2*CH_2*CH_2N of spermidine, N*CH_2**CH_2*COO linker), δ 2.1 (NCH_2*CH_2*$CH_2NCH_2CH_2CH_2CH_2N$, 2H, spermidine), δ 1.7-1.8 ($NCH_2CH_2CH_2NCH_2$*CH_2**CH_2*CH_2N, 4H, spermidine)

molar ratio (diacrylate / OEI) = 6.3 : 1; molar ratio (surface amine / OEI) = 1.4

residual ester content after synthesis: 88%

yield: *26% (weight of product / weight of educts [w/w] percent)*

ED S: ^{1}H NMR (D_2O): δ 4.3-4.4 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.8 (HO*CH_2*, mono-ester, 2H), δ 2.4-3.4 (92H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2*CH_2*N*CH_2*CH_2- of spermine, N*CH_2CH_2*COO linker), δ 2.1 (NCH_2*CH_2*$CH_2NCH_2CH_2$-, 4H, spermine), δ 1.7 ($NCH_2CH_2CH_2NCH_2$*CH_2*-, 4H, spermine)

molar ratio (diacrylate / OEI) = 6.3 : 1; molar ratio (surface amine / OEI) = 2

residual ester content after synthesis: 85%

yield: *30% (weight of product / weight of educts [w/w] percent)*

ED O: ^{1}H NMR (D_2O): δ 4.2-4.4 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.8 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.6 (80H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2OH of ethanolamine, N*CH_2CH_2*COO linker)

molar ratio (diacrylate / OEI) = 0.9 (* ratio of diacrylate to total OEI (core plus surface OEI)*

residual ester content after synthesis: 71%

yield: *30% (weight of product / weight of educts [w/w] percent)*

BD E: ^{1}H NMR (D_2O): δ 4.1-4.3 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.9 (NCH_2*CH_2*OH, ethanolamine, 2H), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (82H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2OH of ethanolamine, N*CH_2CH_2*COO linker), δ 1.8 ($COOCH_2$*CH_2,* 4H di-ester, 2H mono-ester),δ 1.6 (2H $HOCH_2$*CH_2* mono-ester)

molar ratio (diacrylate / OEI) = 8.6 : 1; molar ratio (surface amine / OEI) = 2.1

residual ester content after synthesis: 94%

yield: *32% (weight of product / weight of educts [w/w] percent)*

BD Sp: ^{1}H NMR (D_2O): δ 4.1-4.3 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.7 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (88H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2*CH_2*N*CH_2*CH_2CH_2*CH_2*N of spermidine, N*CH_2CH_2*COO linker), δ 2.1 (NCH_2*CH_2*$CH_2NCH_2CH_2CH_2CH_2$N, 2H, spermidine),δ 1.9 ($NCH_2CH_2CH_2NCH_2$*CH_2CH_2*CH_2N, 4H, spermidine), δ 1.7-1.8 ($COOCH_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.6 (2H $HOCH_2$*CH_2* mono-ester)

molar ratio (diacrylate / OEI) = 8.1 : 1; molar ratio (surface amine / OEI) = 1.8

residual ester content after synthesis: 88%

yield: *24% (weight of product / weight of educts [w/w] percent)*

BD S: ^{1}H NMR (D_2O): δ 4.1-4.3 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (92H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2*CH_2*N*CH_2*CH_2- of spermine, N*CH_2CH_2*COO linker), δ 2.1 (NCH_2*CH_2*$CH_2NCH_2CH_2$-, 4H, spermine), δ 2.0 ($NCH_2CH_2CH_2NCH_2$*CH_2*-, 4H, spermine), δ 1.7-1.8 ($COOCH_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.6 (2H $HOCH_2$*CH_2* mono-ester)

molar ratio (diacrylate/ OEI) = 9.6 : 1; molar ratio (surface amine / OEI) = 3.1

residual ester content after synthesis: 94%

yield: *38% (weight of product / weight of educts [w/w] percent)*

BD O: ^{1}H NMR (D_2O): δ 4.1-4.2 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.5 (80H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2OH of ethanolamine, N*CH_2CH_2*COO linker), δ 1.7-1.8 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.6 (2H HOCH$_2$*CH_2* mono-ester)

molar ratio (diacrylate / OEI) = 1.3 (* ratio of diacrylate to total OEI (core plus surface OEI)*

residual ester content after synthesis: 85%

***yield:** 25% (weight of product / weight of educts [w/w] percent)*

HD E: ^{1}H NMR (D_2O): δ 4.0-4.2 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.8-3.9 (NCH$_2$*CH_2*OH, ethanolamine, 2H), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.4-3.4 (82H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2OH of ethanolamine, N*CH_2CH_2*COO linker), δ 1.6-1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5-1.6 (2H HOCH$_2$*CH_2* mono-ester), δ 1.3-1.5 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate/ OEI) = 10 : 1; molar ratio (surface amine / OEI) = 3

residual ester content after synthesis: 97%

***yield:** 35% (weight of product / weight of educts [w/w] percent)*

HD EDA: ^{1}H NMR (D_2O): δ 4.1 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5-3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (84H N*CH_2*CH_2 of OEI and EDA ethylenes, N*CH_2CH_2*COO linker), δ 1.6-1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.3-1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 12 : 1; molar ratio (surface amine / OEI) = 8

residual ester content after synthesis: 99%

***yield:** 31% (weight of product / weight of educts [w/w] percent)*

HD Sp: ^{1}H NMR (D_2O): δ 4.1-4.2 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (88H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2*CH_2*N*CH_2*CH_2*CH_2*CH_2N of spermidine, N*CH_2CH_2*COO linker), δ 2.1 (NCH$_2$*CH_2*CH$_2$NCH$_2$CH$_2$CH$_2$CH$_2$N, 2H, spermidine), δ 1.9 (NCH$_2$CH$_2$CH$_2$NCH$_2$*CH_2CH_2*CH$_2$N, 4H, spermidine), δ 1.6-1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.3-1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 11.4 : 1; molar ratio (surface amine / OEI) = 4.1

residual ester content after synthesis: 98%

***yield:** 40% (weight of product / weight of educts [w/w] percent)*

HD S: ^{1}H NMR (D_2O): δ 4.1-4.2 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.5 (92H N*CH_2*CH_2 of OEI ethylenes, N*CH_2*CH_2*CH_2*N*CH_2*CH_2- of spermine, N*CH_2CH_2*COO linker), δ 2.1 (NCH$_2$*CH_2*CH$_2$NCH$_2$CH$_2$-, 4H, spermine), δ 1.8 (NCH$_2$CH$_2$CH$_2$NCH$_2$*CH_2*-, 4H, spermine), δ 1.6-1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.3-1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 12.5 : 1; molar ratio (surface amine / OEI) = 6.5

residual ester content after synthesis: 96%

***yield:** 46% (weight of product / weight of educts [w/w] percent)*

HD TETA: ^{1}H NMR (D_2O): δ 4.1 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5-3.6 (HO*CH_2*, mono-ester, 2H), δ 2.4-3.4 (92H N*CH_2*CH_2 of OEI and TETA ethylenes, N*CH_2CH_2*COO linker), δ 1.6-1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.3-1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 12 : 1; molar ratio (surface amine / OEI) = 7.2

residual ester content after synthesis: 97%

yield: *16% (weight of product / weight of educts [w/w] percent)*

HD PEHI: ^{1}H NMR (D_2O): δ 4.1 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (100H N*CH_2*CH_2 of OEI and PEHI ethylenes, N*CH_2CH_2*COO linker), δ 1.6-1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.3-1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 12 : 1; molar ratio (surface amine / OEI) = 6

residual ester content after synthesis: 98%

yield: *24% (weight of product / weight of educts [w/w] percent)*

HD O: ^{1}H NMR (D_2O): δ 4.1 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (80H N*CH_2*CH_2 of OEI 800 Da ethylenes, N*CH_2CH_2*COO linker), δ 1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 1.4 (* ratio of diacrylate to total OEI (core plus surface OEI)*

residual ester content after synthesis: 87%

yield: *40% (weight of product / weight of educts [w/w] percent)*

HD O1200: ^{1}H NMR (D_2O): δ 4.1 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.6 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.2 (188H N*CH_2*CH_2 of OEI 800 Da and OEI 1200 Da ethylenes, N*CH_2CH_2*COO linker), δ 1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.3-1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 1.7 (* ratio of diacrylate to total OEI (core plus surface OEI)residual ester content after synthesis: 88%*

yield: *62% (weight of product / weight of educts [w/w] percent)*

HD O1800: ^{1}H NMR (D_2O): δ 4.1 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (244H N*CH_2*CH_2 of OEI 800 Da and OEI 1800 Da ethylenes, N*CH_2CH_2*COO linker), δ 1.6-1.7 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester), δ 1.4 (-OCH$_2$CH$_2$C*H_2,* 4H, linker)

molar ratio (diacrylate / OEI) = 1.6 (* ratio of diacrylate to total OEI (core plus surface OEI)residual ester content after synthesis: 79%*

yield: *50% (weight of product / weight of educts [w/w] percent)*

G2-HD-OEI: ^{1}H NMR (D_2O): δ 4.0 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.4 (136H N*CH_2* of OEI ethylenes, N*CH_2*CH$_2$*CH_2* of PPI propylenes, N*CH_2CH_2*COO linker), δ 1.7-2.0 (28H of NCH$_2$*CH_2*CH$_2$ of PPI propylenes), δ 1.6 (COOCH$_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H HOCH$_2$*CH_2* mono-ester); δ 1.35 (4H –OCH$_2$CH$_2$*CH_2* linker).

molar ratio (diacrylate / PPI) = 8 : 1; molar ratio (surface amine / PPI) = 7

residual ester content after synthesis: 79%

yield: *32% (weight of product / weight of educts [w/w] percent)*

G2-HD-G2: ^{1}H NMR (D_2O): δ 4.0 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HO*CH_2*, mono-ester, 2H), δ 2.8-3.4 (64H N*CH_2*CH_2*CH_2* of PPI propylenes, N*CH_2CH_2*COO linker), δ 1.65-2.2 (28H of NCH_2*CH_2*CH_2 of PPI propylenes), δ 1.65 ($COOCH_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.5 (2H $HOCH_2$*CH_2* mono-ester); δ 1.35 (4H –OCH_2CH_2*CH_2* linker).

molar ratio (diacrylate / PPI) = 1.3 : 1 (* ratio of diacrylate to total PPI (core plus surface PPI)*

residual ester content after synthesis: 88%

yield: *62% (weight of product / weight of educts [w/w] percent)*

G3-HD-OEI: ^{1}H NMR (D_2O) : δ 4.0 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.3 (200H N*CH_2* of OEI ethylenes, N*CH_2*CH_2*CH_2* of PPI propylenes, N*CH_2CH_2*COO linker), δ 1.75-2.1 (60H of NCH_2*CH_2*CH_2 of PPI propylenes), δ 1.65 ($COOCH_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.55 (2H $HOCH_2$*CH_2* mono-ester); δ 1.4 (4H –OCH_2CH_2*CH_2* linker).

molar ratio (diacrylate / PPI) = 16 : 1; molar ratio (surface amine / PPI) = 11

residual ester content after synthesis: 83%

yield: *41% (weight of product / weight of educts [w/w] percent)*

G3-HD-G2: ^{1}H NMR (D_2O) : δ 4.0 (COO*CH_2,* ester linker, 4H, di-ester, 2H, mono-ester), δ 3.5 (HO*CH_2*, mono-ester, 2H), δ 2.5-3.3 (184H N*CH_2*CH_2*CH_2* of PPI propylenes, N*CH_2CH_2*COO linker), δ 1.75-1.9 (88H of NCH_2*CH_2*CH_2 of PPI propylenes), δ 1.65 ($COOCH_2$*CH_2,* 4H di-ester, 2H mono-ester), δ 1.55 (2H $HOCH_2$*CH_2* mono-ester); δ 1.4 (4H –OCH_2CH_2*CH_2* linker).

molar ratio (diacrylate / PPI) = 1.5 : 1 (* ratio of diacrylate to total PPI (core plus surface PPI)*

residual ester content after synthesis: 68%

yield: *23% (weight of product / weight of educts [w/w] percent)*

2.3.2 Molecular weight determination

Molecular weights were determined by gel permeation chromatography (GPC). GPC analysis was performed using an Agilent 1200 series HPLC system (Morges, Switzerland) equipped with a refractive index detector, a NOVEMA 10 µm precolumn and a NOVEMA 300 analytical column (10 µm, 8x300 mm) (PSS, Mainz, Germany). The mobile phase was maintained in formic acid and sodium chloride (0.1% (V/V) HCOOH, 0.1 M NaCl, pH 2.8) at a flow rate of 1 mL/min. 10 µL of methanol were added to 1 mL of sample (5 mg/mL) as internal standard. Results were evaluated using PSS WinGPC Unity software. Molecular weights were measured relative to Pullulan molecular weight standards that were used for preparing a standard calibration curve.

2.3.3 Degradation of pseudodendrimers

Hydrolysis of selected pseudodendrimers was investigated in an aqueous solution (10 mg/mL, 1 mL, D_2O) at pH 7 at 37 °C using ^1H NMR spectroscopy to determine the decrease of ester bonds (Eclipse +500 spectrometer, JEOL, Tokyo, Japan operating at 500 MHz). Remaining ester content was expressed as a percent relative to ester content determined after synthesis (t = 0d).

2.4 Physicochemical characterization of polymers and polyplexes

2.4.1 DNA binding ability

The ability of pseudodendrimers to bind DNA reduces DNA intercalation of ethidium bromide (EtBr). As only intercalated EtBr results in strong fluorescence (λ_{ex}= 510 nm, λ_{em}= 590 nm)[65], the DNA condensation can be expressed as relative fluorescence intensities to plain DNA, which results in 100% fluorescence intensity (% rel. F.= $F_{polyplex}$ / $F_{plain\ DNA}$). Fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian Deutschland GmbH, Darmstadt, Germany).

EtBr was diluted in either HEPES-buffered glucose (HBG, 5% [w/w] glucose, 20 mM HEPES, pH 7.1) or HEPES-buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.1) to a final concentration of 0.4 µg/mL. 2 mL of EtBr dilution were mixed with 20 µg of plasmid DNA and fluorescence intensity was measured after 3 minutes. Polycation was added stepwise every 20 seconds and % rel. F. was determined as described above.

2.4.2 Polyplex formation

Plasmid DNA was condensed with polymers at indicated conjugate / plasmid ratios (c/p-ratio) [weight/weight]. In general, HBG was used for polyplex formation unless otherwise indicated. In brief, polyplexes were prepared by adding the polymer solution at varying concentrations to DNA solution. Polyplexes were allowed to incubate for 20 min at room temperature prior to use.

2.4.3 Agarose gel retardation

Polyplexes were prepared as indicated in the corresponding experimental settings at a final DNA concentration of 20 µg/mL. 10 µL of complex mixed with loading buffer was loaded on a 0.8% agarose gel in TBE buffer containing ethidium bromide. The gel was run at 100 V for 1 h and then photographed under UV light.

2.4.4 Stability of complexes against sodium chloride

The stability of polyplexes to an increasing amount of sodium chloride was studied using a Cary Eclipse fluorescence spectrophotometer (Varian Deutschland GmbH, Darmstadt, Germany) at $\lambda_{ex} = \lambda_{em} = 600$ nm following a procedure as described by Oupický et al..[66] A significant reduction of scattering signal was attributed to dissociating complexes. Briefly, aliquots of 5 M NaCl were added to 2 mL of polyplex solution (10 µg/mL DNA; in HBG) and changes in the intensity of scattered light were followed.

2.4.5 Particle size measurement and zeta-potential determination

Particle size and zeta-potential measurements of polyplexes were performed in HBG or HBS using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 4 mW He-Ne laser at a wavelength of 633 nm at 25 °C. Scattered light was detected at a 173° backward scattering angle. The viscosity and refractive index of water at 25 °C was used for data analysis. Polyplexes of different c/p-ratios had a final DNA concentration of 10 µg/mL. Experiments were performed in a folded capillary cell (Malvern Instruments, Herrenberg, Germany). Dispersion Technology Software 5.0 (Malvern Instruments, Herrenberg, Germany) was used for data acquisition and analysis.

2.5 Biological properties of pseudodendrimers *in vitro*

2.5.1 Cell culture

All cultured cells were grown at 37 °C in 5% CO_2 humidified atmosphere. B16F10 murine melanoma cells (kindly provided by I. J. Fidler, Texas Medical Center, Houston, TX, USA) and murine neuroblastoma Neuro2a cells (ATCC CCI-131) were cultured in DMEM (1 g/l glucose) supplemented with 10% FCS. K562 (human chronic myeloid leukemia cells in blast crisis) were kindly provided by S. Kochanek, University of Ulm, Germany. Human prostate adenocarcinoma cells, PC3 (DSMZ No ACC 465), were purchased from the german collection of microorganisms and cell cultures (DSMZ). The latter cell lines were cultured in RPMI 1640 growth medium supplemented with 10% FCS and antibiotics.

2.5.2 Cytotoxicity of plain pseudodendrimers

Neuro2a cells were seeded at a density of $1x10^4$ cells in a 96-well plate in 200 µL medium (TPP, Transdingen, Switzerland). After 24 h, medium was replaced by 100 µL of fresh medium containing predetermined pseudodendrimer concentrations of 0.01 mg/mL to 1 mg/mL. Per well, 50 µL samples were collected after an incubation time of 4 h and lactate dehydrogenase (LDH) concentration was determined using a commercial kit (Promega,

Cytotox 96, Madison, WI, USA) following the manufacturer's protocol. The percent of LDH release was defined by the ratio of determined LDH concentration per well over total LDH content in untreated (intact) cells.

Remaining 50 µL of medium per well were used to analyze the ATP content utilizing a commercial kit (Promega, Cell Titer Glo Luminescent Viability Assay, Madison, WI, USA) for the determination of ATP following the manufacturer's protocol. The relative metabolic activity was defined by the ratio of measured ATP content over the ATP content of untreated cells.

2.5.3 Luciferase reporter gene expression studies

For screening transfection experiments (e.g. for OEI core based pseudodendrimers) the determination of reporter gene expression (firefly luciferase, pCMVLuc) was carried out in 96-well plates (TPP, Transdingen, Switzerland). LPEI and BPEI polyplexes at their optimized c/p-ratio of 0.8 (representing an N/P-ratio of 6) were prepared as references.[18]

In a standard experiment, 24 h prior to transfection B16F10 cells were seeded at a density of $5x10^3$ cells and Neuro2a cells at a density of $1x10^4$ cells in 200 µL medium per well. For studies involving PC 3 cell a cell density of $5x10^3$ cells was used. Transfections in K562 cells were performed in a 48-well plate (NUNC, flat bottom, Denmark) at a seeding density of $12.5x10^4$ cells per well. At the time point of transfection cells reached a confluency of 60% to 80%. Before cells were treated with complexes, medium was exchanged against 100 µL fresh medium containing 100 U/mL penicillin and 100 µg/mL streptomycin. 10 µL of complexes were added directly to the cells and after 4 h medium was exchanged against 100 µL fresh one. Transfection efficiency was evaluated 24 h after treatment. Cells were washed once with phosphate-buffered saline (PBS) and were subsequently lysed with 50 µL per well of 1:10 diluted cell culture lysis reagent (Promega, Mannheim, Germany). Luciferase activity was determined from 20 µL samples of the lysates using the Luciferase Assay system (Promega) at the luminometer (Lumat LB 9507, Berchtold, Germany). Two nanograms of recombinant luciferase (Promega, Mannheim, Germany) correspond to 10^7 light units. Values are given as relative light units (RLU) per seeded cells (mean +/- standard deviations of at least triplicates) or as relative light units (RLU) per mg protein.

2.5.4 Enhanced green fluorescent protein (EGFP) gene expression studies

EGFP expression studies were carried out in Neuro2a cells in a 24-well plate (NUNC, flat bottom, Denmark). 24 h prior to transfection $4x10^4$ cells were plated in 1 mL of medium reaching a confluency of 60 to 80% at the point of time of transfection. Transfection of HD O, LPEI and BPEI polyplexes at their optimized c/p-ratios (in HBS) was performed using 1 µg of pEGFP-N1. 24 h after transfection, cells were viewed on an Axiovert 200 inverted fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam camera using appropriate filters for EGFP excitation and emission. Digital image recording and image analysis were performed with the Axiovision 3.1 (Zeiss) software. For analysis of EGFP expression, cells were washed with PBS and harvested by trypsin treatment and analyzed using a CyanADP flow cytometer (DakoCytomation, Copenhagen, Denmark). Per sample, $3x10^4$ gated events were collected. Values shown are the average of four transfected wells. Mean fluorescence intensities (MFI) and the percent of transfected cells are indicated in the pictures.

2.5.5 Metabolic activity of transfected cells after polyplex treatment

The percent of metabolic activity of cells after complex treatment was evaluated 24 h after transfection using the methylthiazoletetrazolium/thiazolyl blue (MTT) assay. To each well 10 µL of a 5 mg/mL solution of MTT in sterile PBS buffer were added. Cells were incubated at 37 °C for 2 h, medium was completely removed and the samples were frozen at -80 °C for at least 1 h. 100 µL of DMSO was added and samples were incubated at 37 °C for 30 min under constant shaking. Optical absorbance was measured at 590 nm (reference wavelength 630 nm) using a microplate plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria). Metabolic activity was expressed relative to the MTT value for untreated cells taken as 100% metabolic activity of cells.

2.5.6 Determination of total protein content

For quantification of total protein concentration per well a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL) was used. 25 µL of cell lysate were transferred to a 96-well plate (NUNC, flat bottom, Denmark) and 5 µL of a 0.1 M iodacetamide (in water) was added for inactivation of DTT.[67] After 30 min of incubation at 37 °C, 200 µL of BCA working reagent were added following the manufacturers´ protocol. Optical absorbance was determined at 590 nm after 30 min of incubation at 37 °C. Bovine serum albumin (BSA) was used as standard.

2.5.7 Hemolytic activity of polymers

Freshly collected citrate buffered human blood was washed by four centrifugation cycles, each at 2200 rpm for 10 min at 4 °C with phosphate-buffered saline (PBS). Erythrocytes were resuspended in HBG / 10% FCS at a concentration of 4% (V/V). The suspension of red blood cells was always freshly prepared and used within 24 h. 75 µL of serial LPEI, BPEI and pseudodendrimer dilutions prepared in HBG / 10% FCS were mixed with 75 µL erythrocyte suspension in a 96-well plate (NUNC, V-bottom, Denmark). After 45 min at 37 °C under constant shaking, blood cells were removed by centrifugation (2200 rpm, 10 min, 4 °C) and 80 µL of the supernatant was transferred to a new 96-well plate. Hemoglobin absorption was determined at 405 nm using a microplate reader (Spectraflour Plus, Tecan Austria GmbH, Grödig, Austria). HBG/10% FCS and a 1% Triton-X100 solution in PBS were used as negative and positive controls, respectively.

Hemolysis is defined as percent ($OD_{polymer} - OD_{buffer} / OD_{Triton\text{-}X100} - OD_{buffer}$) *100.

2.5.8 Polymer-induced erythrocyte aggregation

The erythrocyte pellets were resuspended in 100 µL HBS for microscopic investigation and transferred to a 24-well plate (NUNC, flat bottom, Denmark) containing 1 mL HBS per well. Pictures were taken with a 20-fold magnification on an Axiovert 200 inverted fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam camera.

2.6 Stabilization and targeting of ester-degradable polyplexes

2.6.1 Formation of stabilized HD O / DNA polyplexes via DSP or DSS

HD O / DNA polyplexes were prepared at a c/p-ratio of 1 [w/w]. For comparative studies polyplexes of standard branched polyethylenimine BPEI (25 kDa) were prepared at a c/p-ratio of 0.8 (representing an N/P-ratio of 6). Complex formation was performed in HBG by adding the appropriate amount of polycation to DNA solution. Polyplexes were allowed to incubate for 15 min at room temperature. The lateral stabilization of HD O or BPEI polyplexes was achieved by adding different amounts of DSP or DSS (1 mM solution in DMSO) to the prepared polyplexes for crosslinking. Polyplexes were then allowed to incubate for further 30 minutes to form surface crosslinks. The indicated different ratios of crosslinker applied are reported as a molar ratio between crosslinking agent (DSP or DSS) and HD O or BPEI amine groups, respectively; assuming that HD O contains 54 amines per molecule and BPEI 580 amines per molecule.[68] The amount of amines for HD O was

calculated from ^{1}H NMR data assuming that only the primary amines of the surface OEIs are accessible for crosslinking reactions via DSP or DSS.

2.6.2 Formation of transferrin (Tf)-coated HD O / DNA polyplexes

After formation of laterally stabilized polyplexes via DSP using a crosslinker / amine ratio of 0.05 and incubation for 30 minutes, 3.5% (weight (Tf) / weight (HD O)) transferrin in its pure and unoxidized form (Tf_{unox}) or as oxidized transferrin (Tf_{ox}) (0.1 mg/mL in HBG) was added to form Tf–coated polyplexes. The transferrin solutions were supplemented with Fe citrate prior to use. Coated polyplexes were allowed to stand for additional 30 minutes prior to use.

2.7 *In vivo* studies in mice

2.7.1 *In vivo* gene transfer

Animal experiments were performed according to guidelines of the German law of protection of animal life and were approved by the local animal experiments ethical committee.

Male and female A/J mice were purchased from Harlan Winkelmann (Borchen, Germany) and housed in individually vented cages; food and water were provided *ad libitum.* For transfection studies 6-week-old mice were inoculated subcutaneously in the flank with $1x10^{6}$ Neuro2a cells. Experiments started when tumors reached diameter of 6 to 12 mm in size.

Gene transfer in tumor bearing mice was carried out using polyplexes containing 50 µg pEGFPLuc per 20 g body weight at a concentration of 200 µg/mL DNA (in HBG, pH 7.4). The polyplexes were injected into the tail vein and animals were sacrificed 24 h after application. Indicated tissues were resected and homogenized in 1:5 diluted cell culture lysis reagent (Promega, Mannheim, Germany) using an IKA-Ultra-Turrax and subsequently centrifuged at 4000 rpm at 4 °C for 20 minutes to separate insoluble cell components. Luciferase activity was determined at the luminometer (Lumat LB 9507, Berchtold, Germany) as described above using 20 µL of the supernatant. Two nanograms of recombinant luciferase (Promega, Mannheim, Germany) correspond to 10^{7} light units.

Intratumoral application of laterally stabilized HD O / DNA polyplexes was performed by using complexes containing 200 µg/mL pEGFPLuc. 100 µL of polyplex solution were slowly

injected into the tumor tissue. Luciferase gene expression was visualized using an IVIS® imaging system (Caliper Life Sciences GmbH, Rüsselsheim, Germany) at indicated points in time following the procedure: after intraperitoneal (i.p.) application of 100 µL luciferin sodium salt solution at a concentration of 60 mg/mL (in PBS, pH 7.4), mice were anaesthetized with isoflurane (2%) and luminescence due to luciferase reporter gene expression was imaged 13 min after i.p. application of luciferin. Photons/sec (total flux) signals were used for evaluation.

2.7.2 Blood sample analysis

To circumvent aggregation of blood samples, syringes were pre-drawn with 100 µL heparin. Immediately after sacrificing the animals, blood samples were collected by puncture of vena cava inferior. For the determination of liver enzyme levels in serum all samples were allowed to clot at 37 °C for 4 h and were then centrifuged at 1000 rpm for 20 min at 4 °C. The supernatants were collected for analysis and stored at -80 °C until they were further analyzed. Enzymes were quantified by the Institut für klinische Chemie at the Universitätsklinikum Großhadern. Alkaline and aspartate aminotransaminases (AST, ALT) as well as alkaline phosphatase (AP) were measured using a kinetic UV test from Olympus (Olympus Life and Material Science, Hamburg, Germany). Glutamate Dehydrogenase (GDPH) in plasma was analyzed using a kinetic UV test from Hitado (Hitado Diagnostic Systems, Möhnesee Delecke, Germany).

2.7.3 Histological examination

A small piece of liver tissue of each sacrificed animal was fixed for 24 h in formalin solution (4% paraformaldehyde in PBS). After washing the organs for 1 h in running water, they were embedded in paraffin. Sections of 5 µm thickness were cut and stained with hematoxilyn and eosin for histopathological examination.

2.8 Statistics

Where indicated, one-way analysis of variance (ANOVA) was conducted using Duncan´s test as post-hoc method. The statistic significance is indicated by p-values. As a statistical software package, WinSTAT® 2003 for Excel (R. Fitch Software) was used.

3 Results

3.1 Pseudodendritic oligoamines for gene delivery

3.1.1 Pseudodendritic concept – synthesis of OEI core based conjugates

Pseudodendrimers were synthesized in two steps (**Fig. 6** and **Fig. 7**). In the first step pseudodendritic cores were generated by Michael addition of oligoethylenimine 800 Da (OEI) with a 20-fold molar excess of dioldiacrylate. Due to the excess of dioldiacrylate, crosslinking between OEIs was prevented and a branched structure with reactive acrylate end groups was created. The different diacrylates of ethylenglycol, butane-1.4-diol and hexane-1.6-diol applied as shown in **Fig. 6** display increasing hydrophobicity (ED < BD < HD).

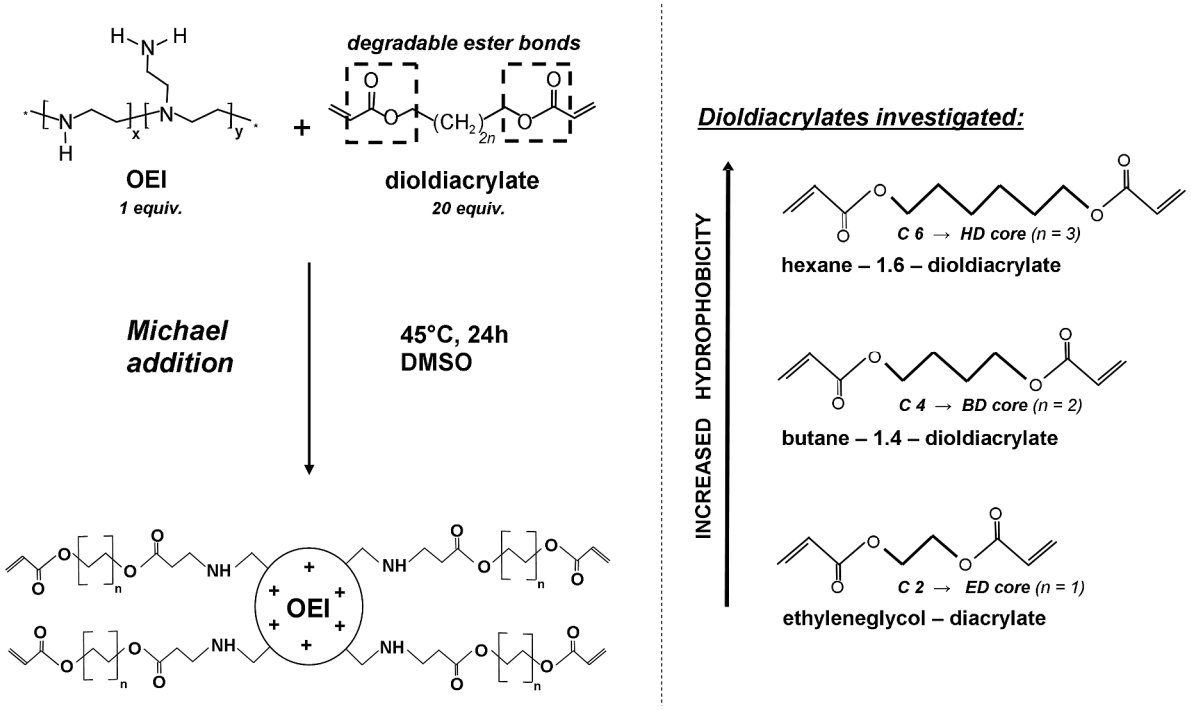

Fig. 6: Pseudodendritic core synthesis. Oligoethylenimine 800 Da (OEI) was coupled with a 20-fold molar excess of different dioldiacrylates in anhydrous DMSO. Reaction proceeded for 24 h at 45 °C. For graphical reasons, only four branches are shown.

In the following step, each OEI core (ED, BD and HD core) was further modified on its surface by adding an excess of either ethanolamine (E), spermidine (Sp), spermine (S) or OEI (O) as shown in **Fig. 7**. For further studies within the HD core conjugates also ethylenediamine (EDA), triethylenetetramine (TETA), pentaethyleneheximine (PEHI), oligoethylenimine 1200 Da (O1200) and oligoethylenimine 1800 Da (O1800) were used.

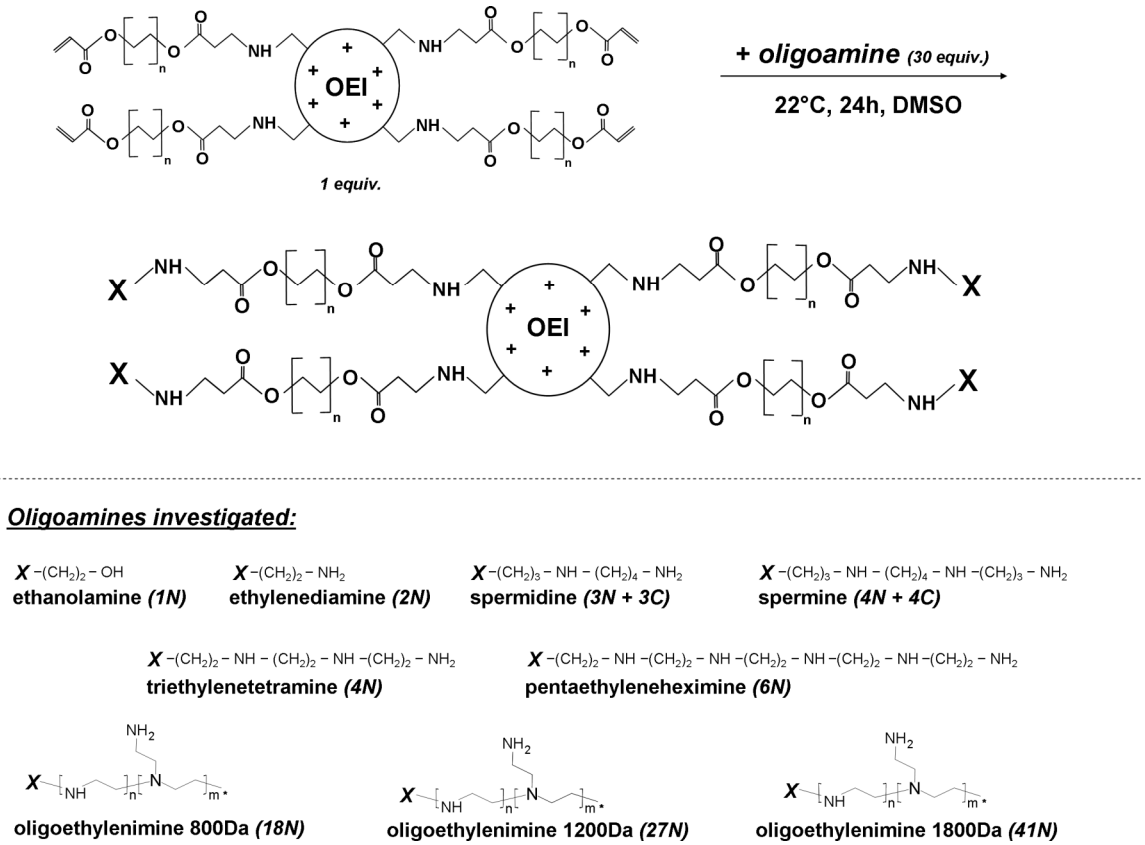

Fig. 7: Pseudodendritic surface modification. Various oligoamines were used as surface modification units. Surface modification of pseudodendrimers proceeded with a 30-fold molar excess of oligoamine to core OEI in anhydrous DMSO. Reaction proceeded for 24 h at 22 °C.

The different amines were selected to increase the number of nitrogens per surface modification unit step by step. Conditions for synthesis including reaction temperature, reaction time as well as concentrations of educts were optimized in preliminary studies (data not shown). Under the chosen reaction conditions, neither gelation nor precipitation occurred. After purification by dialysis and lyophilization, all products were well water-soluble. Yields are given in **Table 2**. The nomenclature of pseudodendrimers is derived from the core moiety followed by the surface amine used.

3.1.2 Structural characterization of pseudodendrimers

The structural composition of the resulting products was analyzed using ^{1}H NMR spectroscopy. The calculated molar diacrylate / OEI- and surface amine / OEI-ratios, the percentages of remaining esters and yields of pseudodendrimers after synthesis are listed in **Table 2**.

	diacrylate / OEI	surface amine / OEI	ester [%]	yield [%]
HD E	10	3	97	35
HD Sp	11.4	4.1	98	40
HD S	12.5	6.5	96	46
HD O	1.4 *	n.d.	87	40
BD E	8.6	2.1	94	32
BD Sp	8.1	1.8	88	24
BD S	9.6	3.1	94	38
BD O	1.3 *	n.d.	85	25
ED E	8.3	1.5	90	33
ED Sp	6.3	1.4	88	26
ED S	6.3	2	85	30
ED O	0.9 *	n.d.	71	30

Table 2: Composition of pseudodendrimers determined by ^{1}H NMR spectroscopy in D_2O. The diacrylate to OEI ratio was determined by comparing the integrals of signals at δ 4.0 and δ 2.5 – 3.3 that are attributed to the methylene protons of the ester group (COOCH_2) and the ethylene protons of oligoethylenimine (NHCH_2CH_2), respectively. The surface amine to OEI ratio was calculated by the relation of the integrals of signals at δ 2.5 – 3.3 that are attributed to the ethylene protons of oligoethylenimine (NHCH_2CH_2) and of the signals specifying the investigated oligoamine. * = ratio of diacrylate to total OEI (core plus surface OEI). N.d.: not determined. The percentage of residual ester content after synthesis was determined by comparing the integrals of signals at δ 4.0 and δ 3.5 that are attributed to the methylene protons of the ester group and hydroxyl group (HOCH_2), respectively. Yields are given as weight of product / weight of educts [w/w] percent.

In general approximately 10 diacrylates were coupled per OEI (diacrylate / OEI-ratio) resulting in a tree-like pseudodendritic core structure. The ratio of surface amine to OEI was lower compared to the ratio of diacrylate to OEI for all conjugates, indicating that more than one pseudodendritic branch reacted per surface modification unit (surface amine / OEI-ratio). In fact, it seemed that two to three amine functionalities have reacted per surface amine and pseudodendritic core. Interestingly, also ethanolamine that contains only one amine functionality per molecule had a lower surface amine / OEI-ratio indicating that intra-molecular side-reactions of the dioldiacrylate cannot be prevented during pseudodendritic core synthesis. The esters, which present the degradable bonds of the polymers, might aminolyze or hydrolyze during synthesis and purification. Therefore, the ester content of all conjugates was analyzed (**Table 2**); it was ranging around 90% of the theoretical value except for ED O, which contained only 71% of ester bonds after synthesis. As expected, all three different cores showed decreasing percentage of ester bonds as the number of

nitrogens per surface amine increased (HD core: 97% (E) to 87% (O), BD core: 94% (E) to 85% (O), ED core: 90% (E) to 71% (O)). This can be explained by aminolysis catalyzed by an increasing amount of unprotonated amino groups during pseudodendrimer synthesis.

The molecular weights of the pseudodendrimers were determined by gel permeation chromatography (GPC) measurements and are listed in **Table 3**.

	V_p [mL]	M_p	M_w	M_n	PDI
HD E	n.d.	n.d.	n.d.	n.d.	n.d.
HD Sp	11.5	1294	1410	1095	1.3
HD S	11.0	2264	2613	1729	1.5
HD O	10.8	3099	4138	2761	1.5
BD E	n.d.	n.d.	n.d.	n.d.	n.d.
BD Sp	11.3	1610	1989	1243	1.6
BD S	10.9	2850	5178	2163	2.4
BD O	10.8	3179	7905	3049	2.6
ED E	n.d.	n.d.	n.d.	n.d.	n.d.
ED Sp	11.0	2496	5289	1910	2.7
ED S	10.9	2763	6291	2221	2.9
ED O	10.5	4643	16207	5298	3.1
LPEI	8.3	60058	58499	26854	2.2
BPEI	8.8	35369	34823	9710	3.6
OEI	11.3	1628	1809	1581	1.1

Table 3: Determination of molecular weights of pseudodendrimers by GPC measurements. V_p: elution volume at peak maximum; M_p: molecular weight at peak maximum; M_w: weight average molecular weight; M_n: number average molecular weight; PDI: polydispersity index; n.d.: not detectable. Molecular weights were measured relative to Pullulan molecular weight standards, which were used for preparing a standard calibration curve.

In general, all pseudodendrimers had much lower molecular weights than the standard polyethylenimines LPEI and BPEI. With an increasing number of nitrogen per surface modification unit, all pseudodendrimers showed increasing molecular weights. For example, within HD core polymers molecular weights of 1410 Da for HD Sp, 2613 Da for HD S and 4138 Da for HD O were found. However, shorter hydrocarbon spacers in the coupled dioldiacrylates resulted in higher molecular weights.

For conjugate HD O degradation kinetic studies were carried out at physiological pH of 7 at 37 °C over a period of 7 days. The extent of degradation was determined using ^{1}H NMR spectroscopy. After 3 days, approximately 50% of the ester bonds were degraded and after

7 days almost no residual ester bonds were present, demonstrating that HD O is degradable under *in vitro* conditions (see **Fig. 8**).

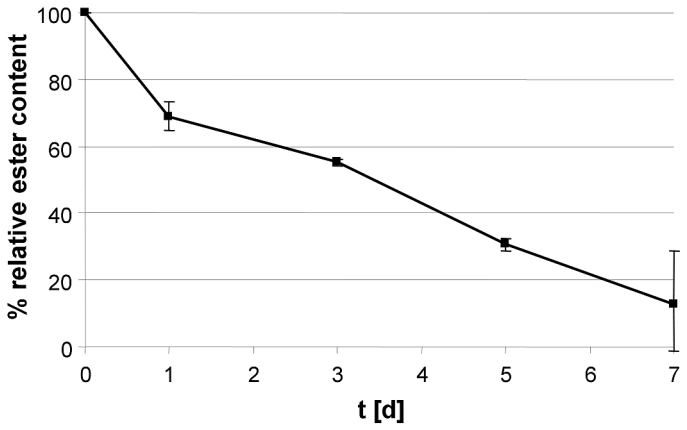

Fig. 8: Degradation kinetics of HD O. The decrease of remaining ester content was followed over time by ^{1}H NMR spectroscopy at 37 °C at physiological pH in aqueous medium (n = 2).

3.1.3 Physicochemical characterization of pseudodendrimers

3.1.3.1 DNA condensing ability of pseudodendrimers

For efficient gene delivery, the gene carrier must be able to condense DNA to form complexes suitable for cell entry. This formation was studied in an ethidium bromide (EtBr) exclusion assay measuring the reduction of relative fluorescence as a function of an increasing conjugate / plasmid weight / weight ratio (c/p-ratio) as shown in **Fig. 9**.

When investigating the influence of the surface modification on DNA binding ability (**Fig. 9A**), a similar DNA-binding pattern was found for ED, BD or HD cores: the surface modification with ethanolamine (E) containing one nitrogen showed the lowest DNA binding ability followed by spermidine (Sp) and spermine (S) modification, with three and four nitrogens per coupled oligoamine, respectively. OEI (O) surface modification (18 nitrogens per coupled oligocation) resulted in best DNA compacting properties indicating that an improved complex formation capability was found with an increasing number of nitrogens per coupled oligocation (**Fig. 9A**). This corresponded to the finding that an increasing number of nitrogen per coupled oligoamine resulted in stepwise increased zeta-potentials resulting in an enhanced DNA condensing ability (see below and **Table 4**).

Regarding the influence of the core characteristics on DNA binding, it was found that a larger hydrocarbon spacer in the coupled dioldiacrylate increased DNA compacting capabilities (**Fig. 9B**). Thus, HD core conjugates exhibited the best DNA compacting abilities, followed by BD and ED core conjugates, resulting in a stepwise decreased capacity for complex

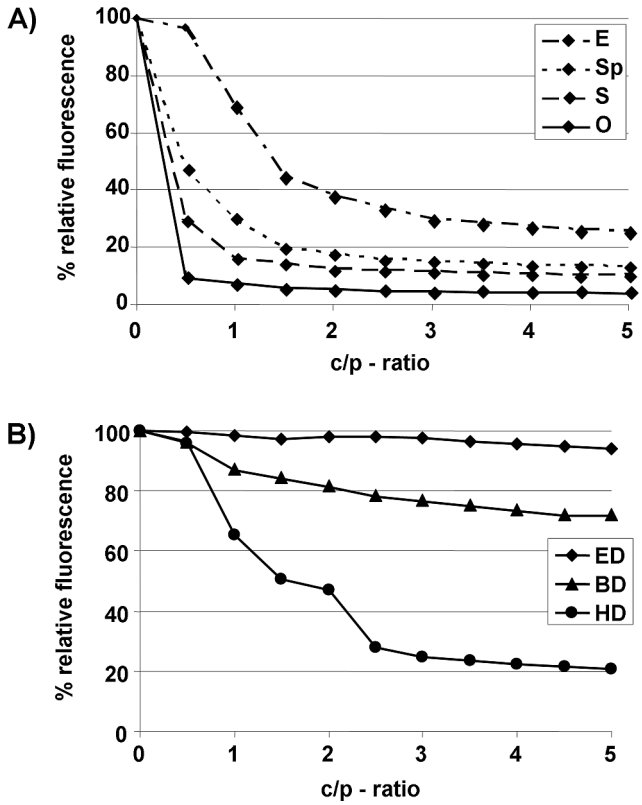

Fig. 9: DNA binding ability of pseudodendrimers demonstrated by ethidium bromide (EtBr) exclusion assay. A) Influence of the surface modification unit on DNA binding ability as demonstrated by a representative experiment with the HD core conjugates. B) Influence of the hydrocarbon spacer length in the pseudodendritic cores on DNA binding ability as demonstrated by a representative experiment with the ethanolamine (E) surface modification. All experiments were performed in HBG.

formation. Notably, this effect was representative for ethanolamine (E), spermidine (Sp) and spermine (S) surface modifications, whereas OEI (O) as coupled oligocation showed similar good DNA compacting abilities upon all pseudodendritic cores indicating that the influence of the surface modification unit was able to mask core characteristics.

3.1.3.2 Size and zeta-potential determination of polyplexes

Mean hydrodynamic diameters (DLS) and zeta-potentials of DNA polyplexes with pseudodendrimers at a c/p-ratio 2 are listed in **Table 4**. HD core conjugates exhibited polyplex sizes around 160 nm, except for the ethanolamine modified HD core (531 nm). Although loose DNA compaction cannot be excluded, the tendency of HD E to aggregate at low c/p-ratio is consistent with the fact that BD E results in small polyplex sizes although it

shows a lower DNA binding ability. BD core conjugates showed polyplex sizes around 200 nm while ED core conjugates resulted in decreasing polyplex sizes with an increasing number of nitrogens per surface modification unit (ED E: 894 nm; ED O: 181 nm) correlating with their increasing DNA condensing ability.

Pseudodendrimer	mean diameter [nm]	zeta-potential [mV]
HD E	531 *(± 62)*	2.7 *(± 1.5)*
HD Sp	144 *(± 17)*	12.8 *(± 3.7)*
HD S	167 *(± 38)*	19.1 *(± 3.6)*
HD O	162 *(± 22)*	29.0 *(± 2.0)*
BD E	247 *(±17)*	6.8 *(± 1.5)*
BD Sp	277 *(± 54)*	18.5 *(± 2.1)*
BD S	209 *(± 35)*	24.2 *(± 3.6)*
BD O	244 *(± 18)*	25.1 *(± 6.0)*
ED E	894 *(± 78)*	- 8.8 *(± 5.6)*
ED Sp	549 *(± 246)*	1.8 *(± 1.8)*
ED S	252 *(± 46)*	9.9 *(± 1.3)*
ED O	181 *(± 48)*	24.0 *(± 3.1)*

Table 4: Mean hydrodynamic diameters and zeta-potentials of pseudodendrimers. Complex sizes were determined at c/p-ratio 2 in HBG using dynamic light scattering 20 min after polyplex formation at a DNA concentration of 10 µg/mL. For zeta-potential measurements polyplexes were diluted 1:5 with HBG to a final DNA concentration of 2 µg/mL. Mean values (± SD) out of three independent measurements are shown.

At a c/p-ratio of 2, all conjugates based on the three different pseudodendritic cores exhibited stepwise increasing zeta-potentials with an increasing number of nitrogens per coupled oligocation (**Table 4**). Hence, measured zeta-potentials of HD core conjugates were ranging from +2.7 mV (HD E) to +29 mV (HD O), BD core conjugates from +6.8 mV (BD E) to +25.1 mV (BD O) and ED core conjugates from -8.8 mV (ED E) to +24.0 mV (ED O).

3.1.4 Influences of pseudodendrimers on cytotoxicity

To investigate the influences of core characteristics and the surface modification on cytotoxicity, a lactate dehydrogenase (LDH) assay and an ATP assay were performed in parallel. This allows analogous monitoring of cell membrane damage (LDH assay) versus metabolic activity of cells (ATP assay) after treatment with various concentrations of pseudodendrimer.

Fig. 10 shows the influence of the hydrocarbon spacer in the coupled dioldiacrylate, demonstrating that pseudodendritic core hydrophobicity correlates with cytotoxicity.

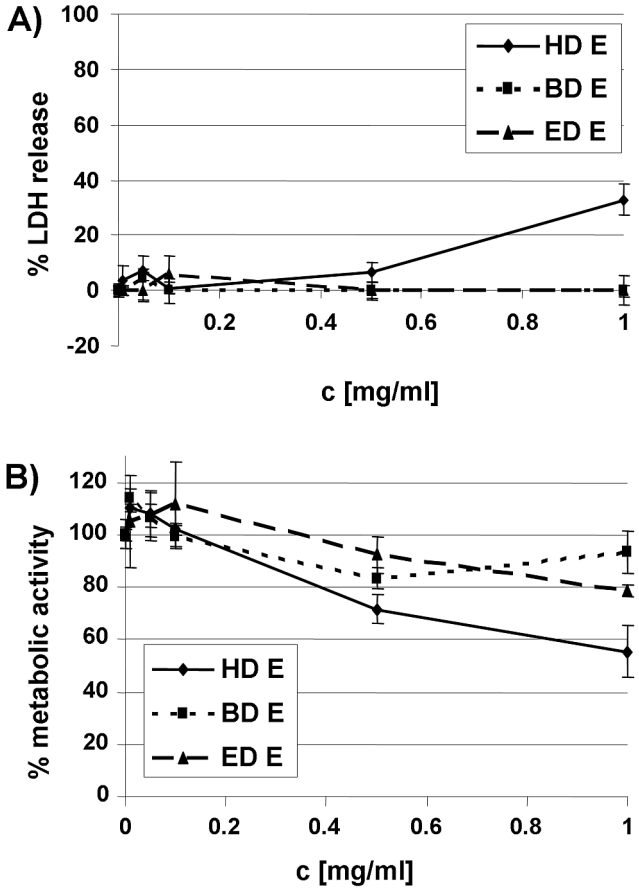

Fig. 10: Influences of pseudodendritic core characteristics of plain pseudodendrimers on cytotoxicity. The LDH release assay (A) monitors cell membrane damage and the ATP assay metabolic activity of cells (B) after treatment with pseudodendrimers as a function of the polymer concentration. Cytotoxicity increases with an increasing aliphatic spacer in the coupled dioldiacrylate, i.e. increasing core hydrophobicity enhances toxic effects. All experiments were performed in Neuro2a cells in triplicates.

In the particular example shown in the figure, ethanolamine was applied as representative surface amine. While BD and ED cores did not show any LDH release from the cytoplasm of treated cells, the HD core bearing a C6 spacer exhibited membrane damage in a dose dependent manner (**Fig. 10A**). In parallel, metabolic activity of treated cells decreased with the length of the C-spacer, shown by the rapid decrease of cell viability after HD E treatment (**Fig. 10B**). BD E and ED E exhibited rather low or no decrease of metabolic activity in cells after conjugate treatment also not at the highest tested concentration of 1 mg/mL.

Investigating the influence of the surface modification on cytotoxicity, it was found that with a rising number of nitrogens per coupled oligoamine a stepwise increased cytotoxic effect was detectable. This can be seen best in the ED core series (**Fig. 11**).

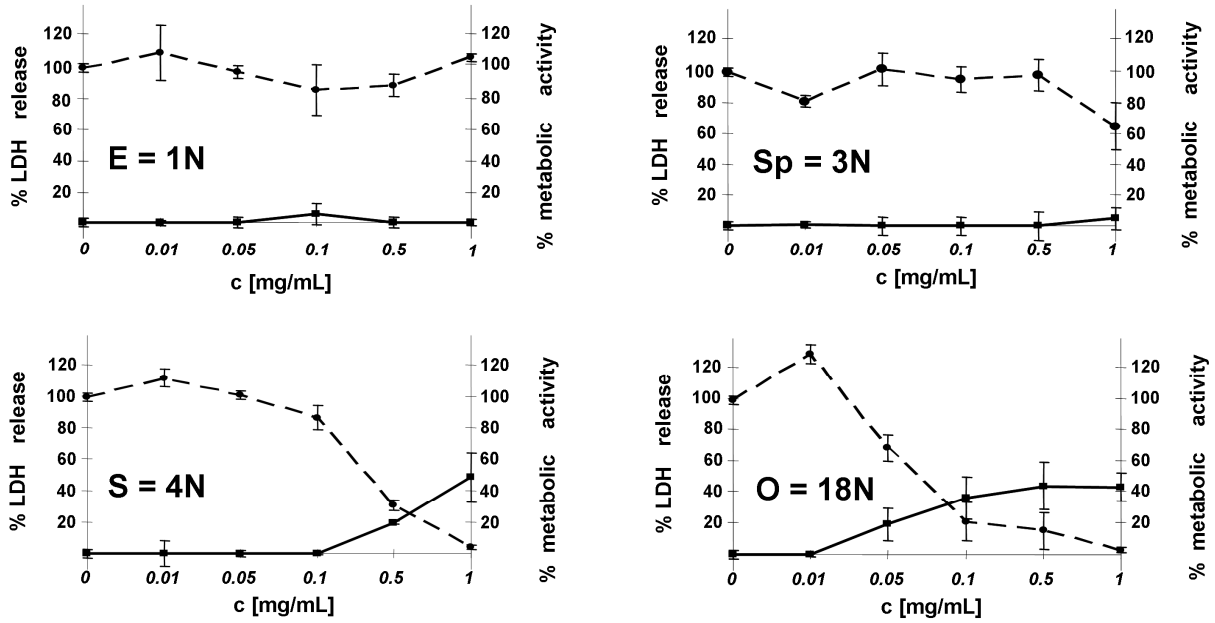

Fig. 11: Influences of pseudodendritic surface modification of plain pseudodendrimers on cytotoxicity. The LDH release assay (solid lines) monitors cell membrane damage and the ATP assay the metabolic activity of cells (dashed lines) after treatment with pseudodendrimers. Cytotoxicity increases with an increasing number of nitrogen per surface modification unit. All experiments were performed in Neuro2a cells in triplicates.

Hence, the ethanolamine conjugate bearing one nitrogen per surface modification unit (E = 1N) showed neither a decrease of metabolic activity nor an increase of LDH release whereas the spermidine surface modification (Sp = 3N) with a concentration above 0.5 mg/mL resulted in decreased cell viability while no membrane damage was detectable. A higher number of nitrogens upon the surface obviously boosted cytotoxic effects consisting of membrane damage and decreased metabolic activity of cells after pseudodendrimer treatment as shown by spermine (S = 4N) and OEI (O = 18N) surface modifications.

3.1.5 Gene transfer activity: structure-activity relationship *in vitro*

Reporter gene expression (luciferase activity) and parallel cytotoxicity (MTT assay) studies of all pseudodendrimers were evaluated in B16F10, murine melanoma and Neuro2a, murine neuroblastoma cells in order to determine a structure-activity relationship *in vitro*. The plasmid pCMVLuc encoding for firefly luciferase was used as reporter gene at a DNA concentration of 20 µg/mL. Polyplexes were prepared in HBG and cells were incubated in the presence of 10% fetal calf serum for 4 h. For LPEI and BPEI, used as references in all experiments, a c/p-ratio of 0.8 (i.e. nitrogen/phosphate N/P-ratio of 6) was used. This was optimized in preliminary studies in both cell lines (data not shown).[18]

In first screening studies the best c/p-ratios (weight ratios of conjugate to pDNA) for HD core, BD core and ED core pseudodendrimers were determined in both, B16F10 and Neuro2a cells (see **Fig. 12, Fig. 13** and **Fig. 14**).

For most of the conjugates optimized transfection conditions, i.e. highest reporter gene expression at lowest cytotoxicity levels (> 75% metabolic activity of cells 24 h post transfection), were found at a c/p-ratio of 2.

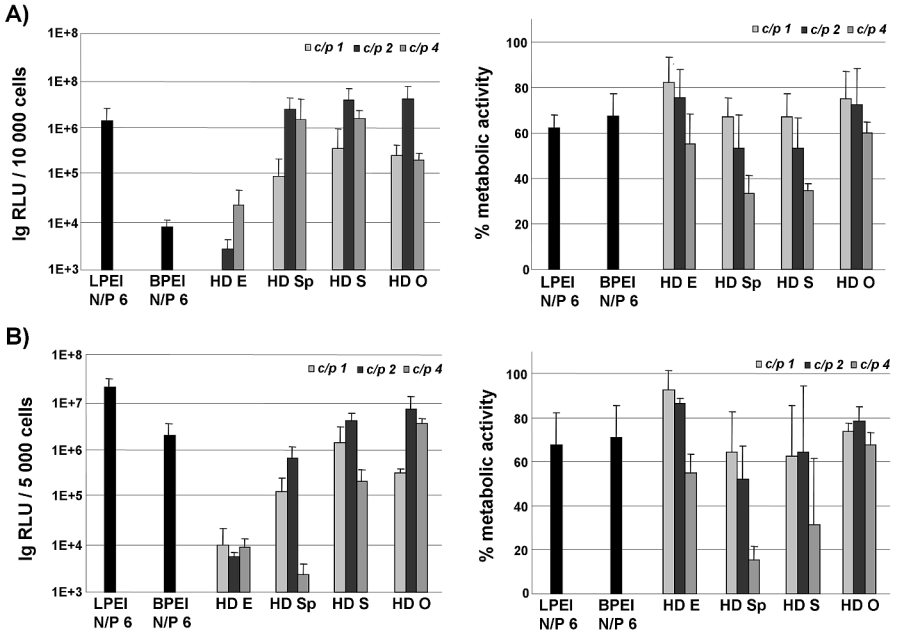

Fig. 12: Optimized c/p-ratio finding of HD core conjugates. In Neuro2a cells (A) and B16F10 cells (B) in the presence of 10% serum. All polyplexes were prepared at indicated c/p-ratios in HBG. LPEI and BPEI were applied at optimized N/P-ratio of 6 which represents a c/p-ratio of 0.8. Data is expressed as mean values (± SD) out of three independent experiments each performed in triplicates. RLU / number of cells represents the measured relative light units referred to the number of cells at the time point of cell plating.

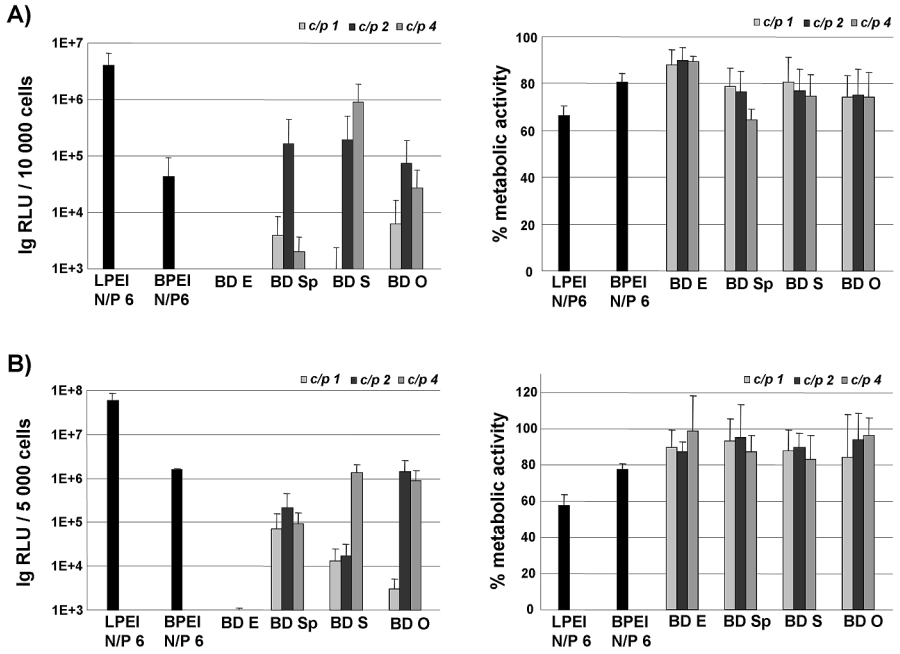

Fig. 13: Optimized c/p-ratio finding of BD core conjugates. In Neuro2a cells (A) and B16F10 cells (B) in the presence of 10% serum. All polyplexes were prepared at indicated c/p-ratios in HBG. LPEI and BPEI were applied at optimized N/P-ratio of 6 which represents a c/p-ratio of 0.8. Data expressed as mean values (± SD) out of three independent experiments each performed in triplicates. RLU / number of cells represents the measured relative light units referred to the number of cells at the time point of cell plating.

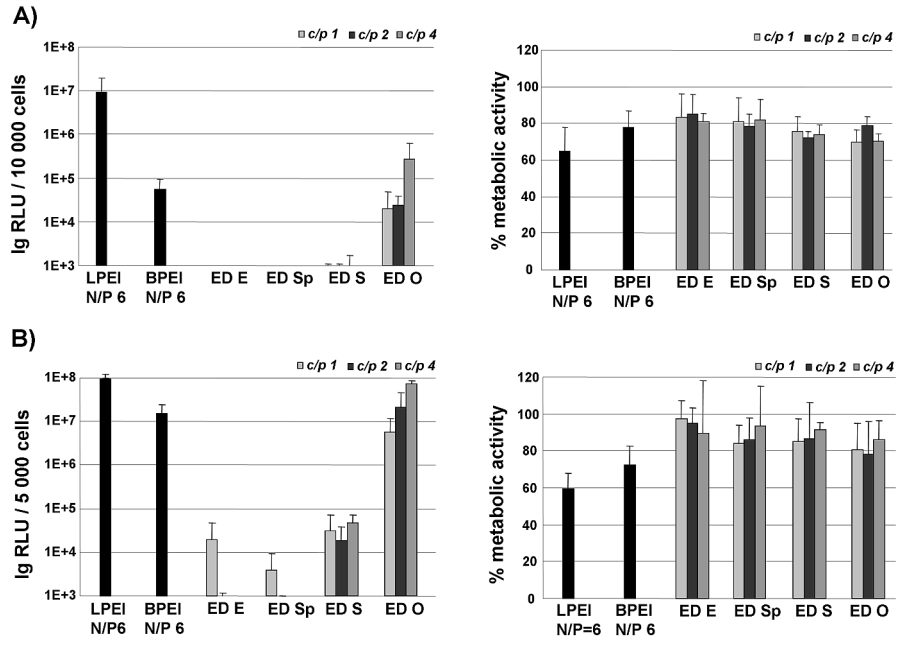

Fig. 14: Optimized c/p-ratio finding of ED core conjugates. In Neuro2a cells (A) and B16F10 cells (B) in the presence of 10% serum. All polyplexes were prepared at indicated c/p-ratios in HBG. LPEI and BPEI were applied at optimized N/P-ratio of 6 which represents a c/p-ratio of 0.8. Data expressed as mean values (± SD) out of three independent experiments each performed in triplicates. RLU / number of cells represents the measured relative light units referred to the number of cells at the time point of cell plating.

To investigate both the effects of the different pseudodendritic core characteristics and the impacts of the various surface modifications on transfection efficiency and cytotoxicity all compounds were studied in parallel. For direct comparison **Fig. 15** shows the influences in both Neuro2a and B16F10 cells.

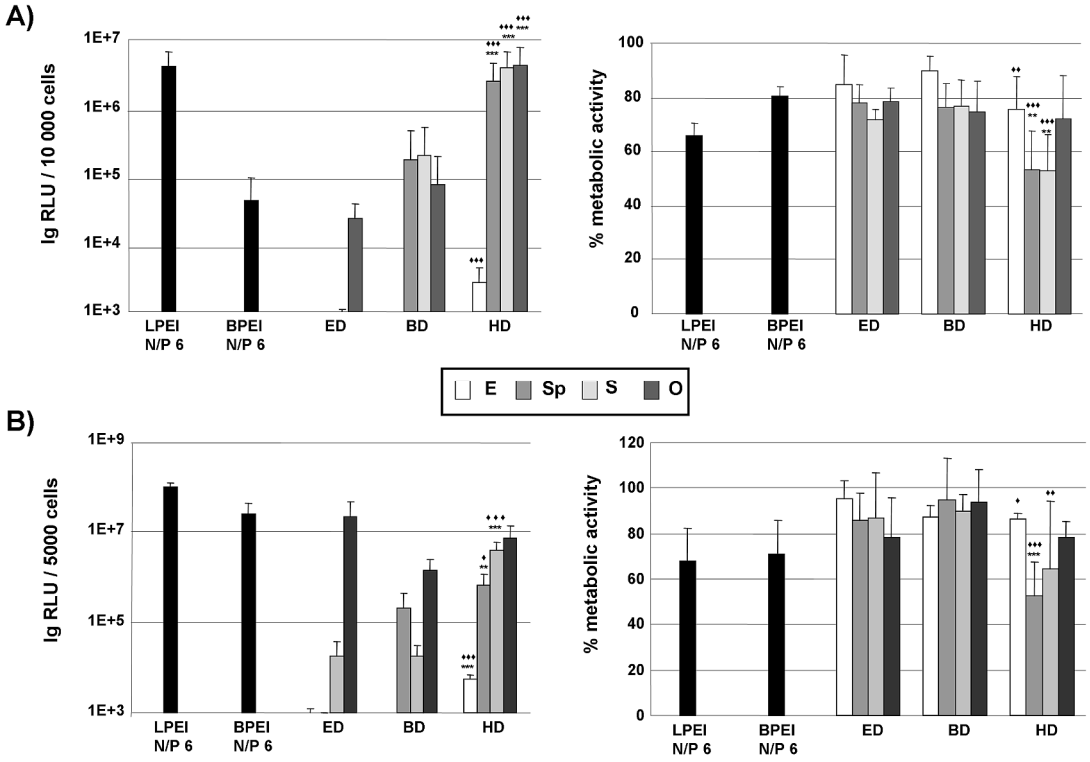

Fig. 15: Influence of the hydrocarbon spacer in dioldiacrylates and the influence of the surface modification unit in pseudodendrimers on transfection efficiency (left, luciferase assay) and metabolic activity (right, MTT assay). In Neuro2a cells (A) and B16F10 cells (B) in the presence of 10% serum. All polyplexes were prepared at c/p-ratio of 2 in HBG. LPEI and BPEI were applied at optimized N/P-ratio of 6 which represents a c/p-ratio of 0.8. Data expressed as mean values (± SD) out of three independent experiments each performed in triplicates. RLU / number of cells represents the measured relative light units referred to the number of cells at the time point of cell plating.
(ANOVA, Duncan's test, * $p < 0.05$, *** $p < 0.001$ ED vs. HD, ♦ $p < 0.05$, ♦♦♦ $p < 0.001$ BD vs. HD)

Regarding the effects of the pseudodendritic core characteristics on reporter gene expression levels, significantly enhanced transfection efficiency was detected for the HD core polymers over their BD and ED core counterparts. The improved transfection levels from ED to BD core conjugates were not significant. Only one exception was found for ED O that showed highest reporter gene expression levels in B16F10 cells, which was a cell-type specific effect (**Fig. 15B, left**). According to the influences of the surface modification on transfection efficiency it was found that within the HD and the ED core conjugates a step by step rise of the transfection level occurred by increasing the number of nitrogens per coupled oligocation (E < Sp < S < O). This effect was almost independent of all investigated c/p-

ratios. BD core conjugates, however, did not show such a direct correlation and mediated moderate transfection in both cell lines.

Furthermore, in cytotoxicity studies with BD and ED core conjugates no significant cytotoxic differences for all surface modifications (**Fig. 15, right**) were detected. Nevertheless, increasing the core hydrophobicity decreased metabolic activity at the same time i.e. increased cytotoxicity. Thus, after HD E, HD Sp and HD S polyplex treatment metabolic activity of cells significantly decreased as compared to their ED and BD core counterparts. Interestingly however, the best transfection conjugate HD O complexed with DNA did not exhibit such a decreased metabolic activity and cell viability was maintained high.

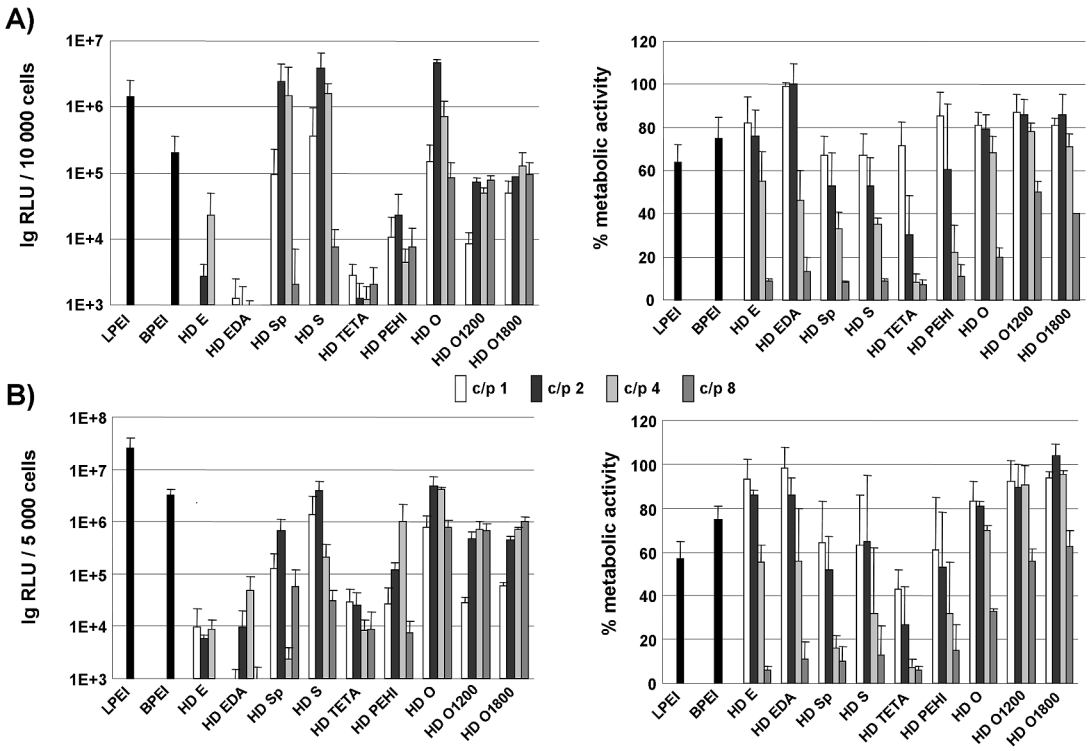

Fig. 16: *In vitro* transfection efficiency (left, Luciferase assay) and metabolic activity (right, MTT assay) of HD core pseudodendrimers. Transfection was carried out in Neuro2a cells (A) and in B16F10 cells (B) in the presence of 10% serum. Polyplexes were formed at indicated c/p-ratios in HBG; luciferase activity and metabolic activity were determined 24 h post transfection. LPEI (22 kDa) and BPEI (25 kDa) were applied at optimized N/P-ratio of 6 which represents a c/p-ratio of 0.8. Data expressed as mean values (± SD) out of three independent measurements each performed in triplicates. RLU / number of cells represents the measured relative light units referred to the number of cells at the time point of cell plating. Surface modifications upon the HD core: ethanolamine (E), ethylenediamine (EDA), spermidine (Sp), spermine (S), triethylenetetramine (TETA), pentaethyleneheximine (PEHI), OEI 800 Da (O), OEI 1200 Da (O1200), OEI 1800 Da (O1800).

To investigate this effect in more detail, additional five surface modification units within the HD core line were studied. These modification units were ethylenediamine (EDA), triethylenetetramine (TETA), pentaethyleneheximine (PEHI), oligoethylenimine 1200 Da (O1200) and oligoethylenimine 1800 Da (O1800) (see **Fig. 7**).

Fig. 16 demonstrates that high transfection levels at low cytotoxicity apparently depend on an optimized balance of core characteristics *and* the surface amine used. This is only the fact in case of HD O, exhibiting highest transfection levels at low toxicity in both Neuro2a and B16F10 cells.

Importantly, HD O showed in both cell lines similar high reporter gene expression levels as the standard polyethylenimines LPEI and BPEI. Especially in Neuro2a cells HD O exhibited similar high transfection levels as LPEI and was about 100-fold more efficient than BPEI (all at their optimized c/p-ratios). Since polymers may differently affect the protein levels of transfected cells due to differential toxicity and cell proliferation transfection levels were quantified side-by-side as RLU / mg of protein and RLU / number of seeded cells in separate experiments analogous to **Fig. 15** and **Fig. 16**. The gene expression pattern of the different polymers in the two normalizations was almost identical, thus, ruling out significant effects on protein levels (data not shown).

Fig. 17 shows the comparison of the best transfecting conjugate HD O versus LPEI and BPEI polyplexes at EGFP expression levels in Neuro2a cells.

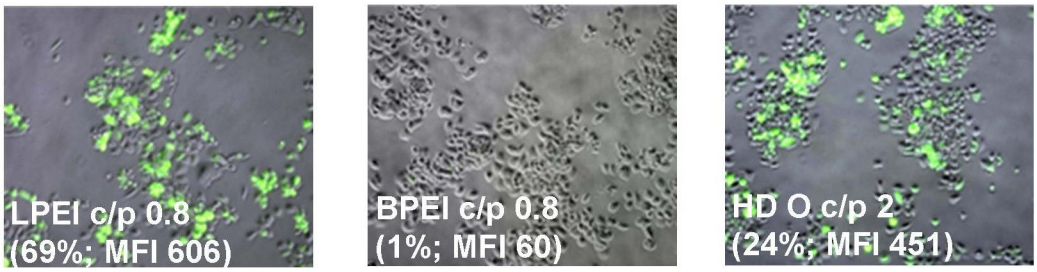

Fig. 17: EGFP expression studies of LPEI, BPEI and HD O in Neuro2a cells. All polyplexes were applied at their optimized c/p-ratios (in HBS) as indicated in the pictures. 24 h after transfection, cells were examined with a Zeiss Axiovert 200 microscope and above shown pictures were taken using an Axiocam camera using appropriate filters for EGFP excitation and emission. Further analysis of unfixed cells was performed using FACS analysis. The percentage of EGFP positive cells is indicated at brackets in the pictures. MFI represents mean fluorescence intensities of cells.

Mean fluorescence intensities (MFI) mediated by HD O and LPEI polyplexes were high, whereas BPEI in contrast showed a much lower fluorescence signal. LPEI and HD O also showed high percentage of EGFP positive cells (69% and 24%, respectively, as determined by flow cytometry), while BPEI polyplexes only transfected 1% of cells.

3.2 *In vivo* studies of promising OEI core based conjugates

Under *in vitro* conditions both compounds, HD S and HD O, resulted in high transfection efficiency levels at rather low cytotoxicity. Thus, they were further investigated in detail with regard to a potential *in vivo* application.

3.2.1 Biocompatibility studies of HD S and HD O *in vitro* and *in vivo*

After systemic application of positively charged polyplexes it is likely that undesired side effects of unbound polymer within the applied polyplexes may lead to interactions with several blood components including erythrocytes.[15] This may result in hemolysis and / or aggregated blood components e.g. due to membrane destabilizing effects of the polymers. Aggregated erythrocytes finally may cause an entrapment in small capillaries like e.g. in the lung resulting in acute toxic effects.

Thus, prior to *in vivo* application of polyplexes in mice, both the hemolytic activity of pseudodendrimers as well as their tendency to induce erythrocyte aggregation was studied using freshly isolated human erythrocytes.

Erythrocytes were incubated with different concentrations of plain polymers ranging from 50 µg/mL to 200 µg/mL at physiological pH of 7.4 for 45 min at 37 °C. The released hemoglobin concentration was determined by optical absorbance at 405 nm representing λ_{max} of hemoglobin. HD S and HD O exhibited distinct membrane destabilizing activity (up to 90% relative to Triton-X representing 100% hemolysis) if plain polymer was used. Importantly, no polymer induced erythrocyte aggregation was found under these conditions (data not shown). In order to adjust experimental conditions to the *in vivo* situation, hemolytic activity of plain HD S and HD O was studied in the presence of 10% serum. Already at this low concentration of serum, in both cases hemolytic activity was strongly reduced by the presence of serum proteins. For instance, at a concentration of 50 µg/mL, HD O induced 80% erythrocyte lysis in the absence, but only 37% lysis in the presence of serum. Again, no polymer induced erythrocyte aggregation was found under these conditions (data not shown).

Standard high molecular weight polycations used for *in vivo* application, such as LPEI or BPEI, are known to induce strong erythrocyte aggregation, even in the presence of serum, while their hemolytic activity is rather low (see also **Fig. 26**).

First studies on acute toxicity in mice revealed that at a concentration of 50 µg/mL HD S as plain conjugate was highly toxic after i.v. application resulting in lethality of mice, whereas HD O was well tolerated. The same results were found in preliminary studies using polyplexes.

3.2.2 Gene transfer studies of HD O polyplexes *in vivo*

As HD O was well tolerated by mice, it was further investigated for its *in vivo* gene transfer ability in tumor bearing mice. The experiments were conducted by intravenous injection of complexes of HD O (c/p-ratio 1 and 2) as well as LPEI and BPEI as references (c/p-ratio 0.8 = N/P-ratio 6) using pEGFPLuc as reporter gene. Complexes were prepared at a final DNA concentration of 200 µg/mL in HBG with 50 µg DNA applied per 20 g mouse. Mice receiving polyplexes showed no signs of acute toxicity. **Fig. 18** presents luciferase reporter gene expression in main organs and tumor tissue 24 h after intravenous application of polyplexes.

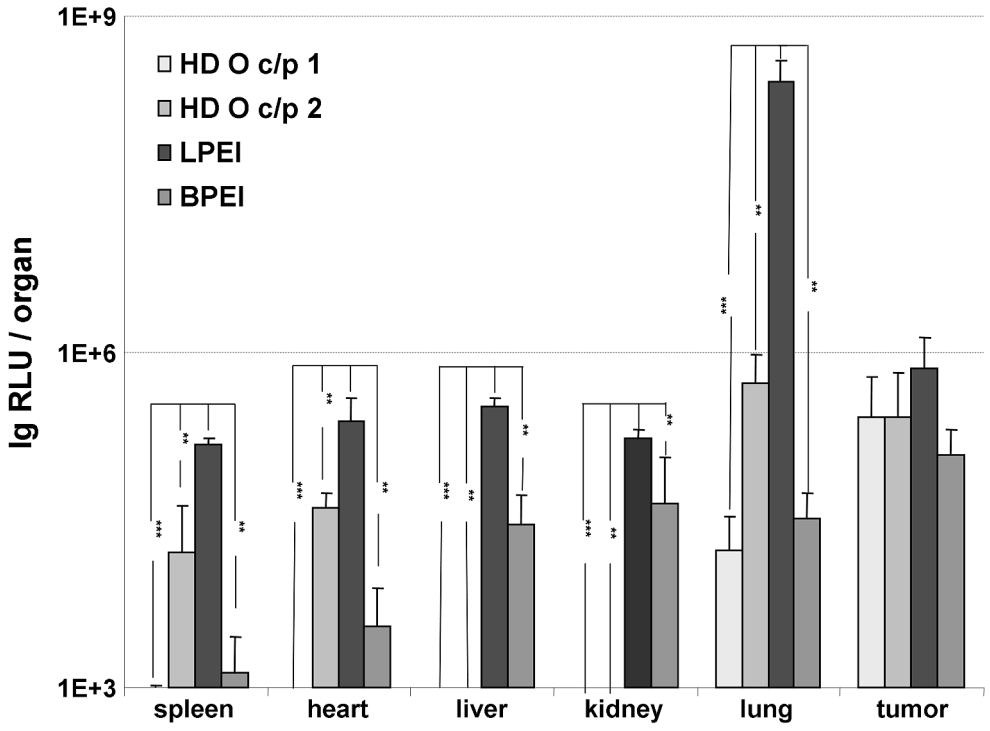

Fig. 18: Luciferase gene expression 24 h after i.v. administration of polyplexes into mice bearing s.c. Neuro2a tumors. HD O was applied at c/p-ratios of 1 and 2, LPEI and BPEI at c/p-ratio 0.8 (representing an N/P-ratio of 6). Luciferase gene expression is presented as relative light units per organ or tumor (RLU / organ) (mean ± SD, *n = 3 animals per group*). Tumor weights ranged between 250 and 300 mg. Two nanograms of recombinant luciferase correspond to 10^7 light units.
(ANOVA, Duncan's test, *** $p < 0.001$ and ** $p < 0.01$ for LPEI vs. HD O and BPEI)

Consistent with previous work, the application of LPEI polyplexes led to luciferase gene expression in all investigated organs but was predominantly found in the lung (>100-fold higher compared to all other organs and tumor tissue). BPEI exhibited reporter gene

expression mainly in tumor tissue, liver and lung at lower levels compared to LPEI. At both c/p-ratios the pseudodendrimer HD O resulted in transgene expression that was found predominantly in tumor tissue at gene expression levels comparable to LPEI. Importantly, for HD O at a c/p-ratio of 1 far lower luciferase gene expression was observed in the lung (tumor / lung ratio (T/L) ~20) and no significant transgene expression was observed in any other organ.

3.2.3 Biocompatibility of HD O polyplexes *in vivo*

A 6% loss of body weight was observed 24 h after injection of HD O polyplexes at c/p-ratio 1 whereas at c/p-ratio 2 a loss of body weight of 14% was found, showing no significant differences compared to LPEI (11%) and BPEI (14%) in both cases.

The biocompatibility of HD O (c/p-ratio 1) was further assayed in tumor-free mice. No increase of liver enzyme levels (AST, ALT, GLDH and AP) was found as compared to a buffer (HBG) treated control. In contrast, LPEI and BPEI (both at a c/p-ratio of 0.8) showed increased liver enzyme levels (for BPEI being statistically significant, * $p < 0.05$, ANOVA, Duncan's test), indicating the beginning of necrotic changes (**Fig. 19** and **Fig. 20**).

	(AST + ALT)/GLDH	AP
HBG	4.9 (± 1.4)	225 (± 37)
HD O c/p 1	6.3 (± 2.6)	190 (± 47)
LPEI c/p 0.8	11.7 (± 4.7)	189 (± 15)
BPEI c/p 0.8	17.3 (± 8.6) *	253 (± 9)

Fig. 19: Liver enzyme levels in serum 24 h after polyplex treatment. Alkaline (ALT) and aspartate (AST) aminotransaminases, glutamate dehydrogenase (GLDH) and alkaline phosphatase (AP). Data represents mean values (± SD, *n = 3 animals per group*), except buffer (*n = 4*).

Histopathologic examinations underline these findings as shown in **Fig. 20**, where no changes in liver histology were found in case of HD O compared to the buffer treated control. In contrast, LPEI led to necrotic changes in liver tissue as indicated by the arrows and BPEI resulted in microvesicular fatty liver already after 24 h shown by inclusion of lipid droplets within the hepatocytes.

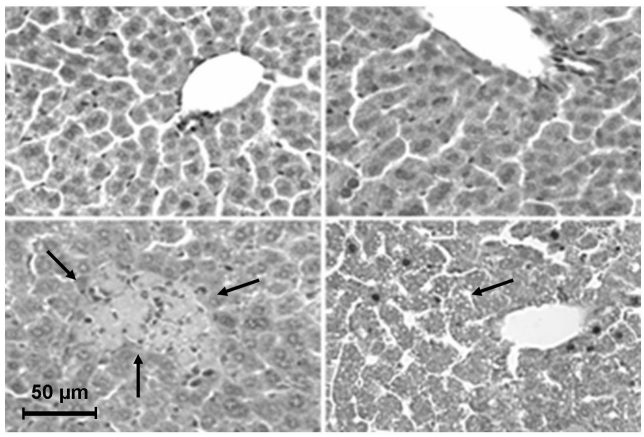

Fig. 20: Histologic examinations (H/E staining) on liver tissue. 24 h after polyplex treatment, HD O c/p-ratio 1 showed no changes in liver histology compared to HBG treated control. Necrotic areas in the liver are indicated by the arrows after LPEI treatment. Lipid droplets in hepatocytes in case of BPEI treated mice show a microvesicular fatty liver (arrows). (Upper left, control (HBG); upper right, HD O c/p-ratio 1; lower left, LPEI c/p-ratio 0.8; lower right, BPEI c/p-ratio 0.8).

3.3 Grafted PPI dendrimers: modification of G2 and G3 core derivatives

In order to obtain a better defined core unit than the randomly branched OEI, the studies were extended toward polypropylenimine (PPI) dendrimers as core moiety. To compare influences of different dendrimer generations within the core unit on physicochemical and biological properties, generation 2 (G2) and generation 3 (G3) PPI dendrimers were grafted. As grafting moieties either OEI or PPI dendrimer G2 was used in order to define differences between *ethylen*imine and *propylen*imine moieties upon the pseudodendritic surface.

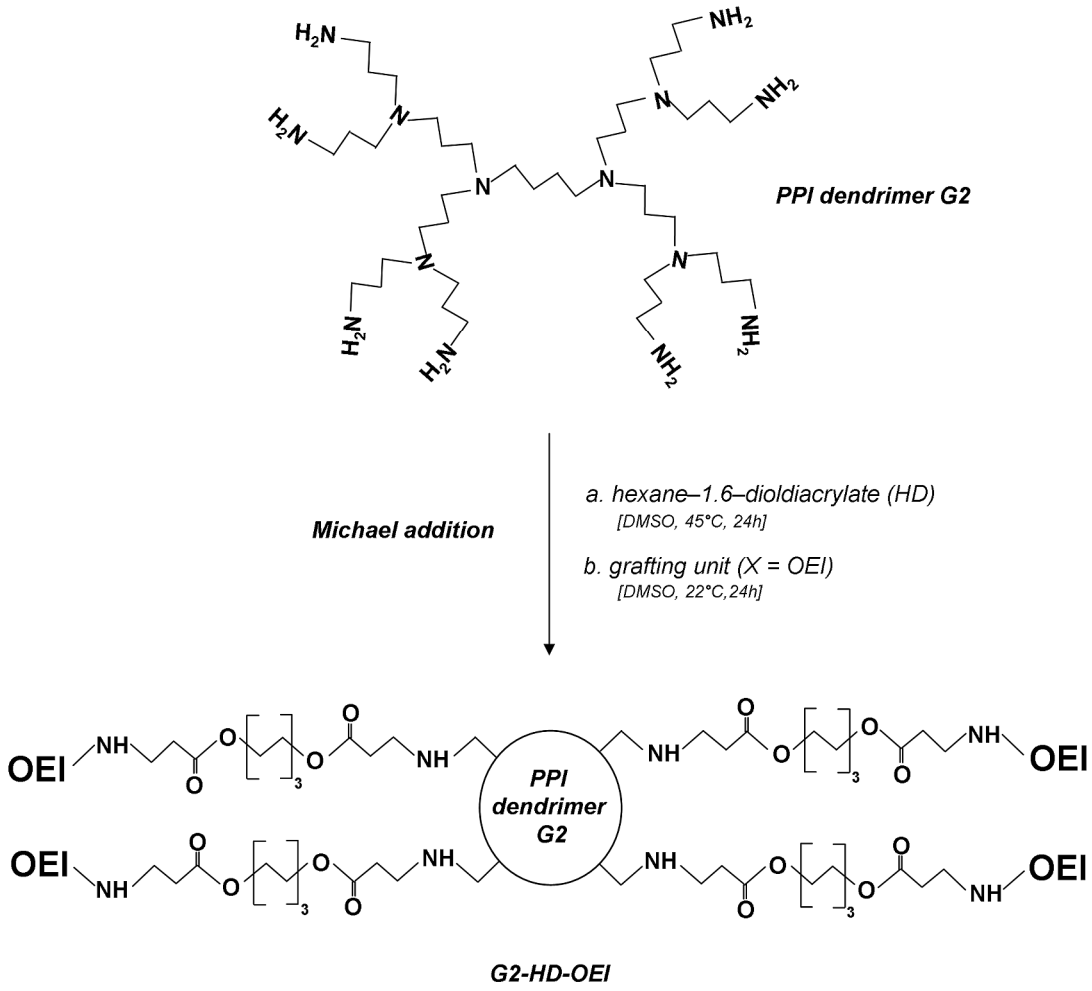

Fig. 21: Synthesis scheme of grafted polypropylenimine dendrimers. G2-HD-OEI, G2-HD-G2, G3-HD-OEI and G3-HD-G2 synthesis proceeded similarly and is exemplified here for the generation 2 (G2) core derivative G2-HD-OEI. For graphical reasons, only four (instead of eight) branches are shown.

3.3.1 Synthesis and characterization of grafted dendrimers

Grafted PPI dendrimers were synthesized in analogous fashion as described for OEI core based pseudodendrimers via Michael addition of PPI G2 or PPI G3 using hexane – 1.6 – dioldiacrylate (HD) as biodegradable linker (**Fig. 21**).

The HD linker was used since it was found in preliminary studies using PPI G2 dendrimers modified with a series of different bisacrylates that HD revealed the highest transfection levels (data not shown) which is also in accordance to the results described above. To prevent crosslinking between core units, a 20-fold (G2 HD core) or 45-fold (G3 HD core) molar excess of dioldiacrylate was used during core synthesis and a branched structure with acrylate reactive end groups was formed. In the following step, these core units (G2 HD or G3 HD) were grafted by adding an excess of either OEI or G2 as shown in **Fig. 21**, resulting in G2-HD-OEI, G2-HD-G2, G3-HD-OEI or G3-HD-G2. All products were well water-soluble and yields of obtained compounds were listed in **Table 5**.

	HD / core	GU / core	% ester	% yield	M_w	M_n	PDI	$t_{1/2}$ [d]
G2 HD OEI	9	7	79	32	5453	3305	1.6	3
G2 HD G2	1.3	n.d.	88	62	17626	7029	2.5	> 14
G3 HD OEI	16	11	83	41	7863	3118	2.5	3
G3 HD G2	1.5	n.d.	68	23	26947	2279	11.2	> 14

Table 5: Structural composition of grafted PPI dendrimers determined by ^{1}H NMR spectroscopy (D_2O). The HD to core ratio (HD / core) was determined by comparing the integrals of signals that are attributed to the methylene protons of the ester group (COOCH_2) and the ethylene protons of polypropylenimine, respectively. The grafting unit to PPI core ratio (GU / core) was calculated by the relation of the integrals which are attributed to the protons of propylenimine and of the signals specifying OEI. N.d.: not determined. The percentage of residual ester content after synthesis was determined by comparing the integrals of signals at δ 4.0 and δ 3.5 that are attributed to the methylene protons of the ester group and hydroxyl group (HOCH_2), respectively. Yields are given as weight of product / weight of educts [w/w] percent. Molecular weights were determined by GPC. M_w: weight average molecular weight; M_n: number average molecular weight; PDI: polydispersity index. $t_{1/2}$ was determined after certain intervals by comparing the integrals of signals at δ 4.0 and δ 3.5 that are attributed to the methylene protons of the ester group (COOCH_2) and hydroxyl group (HOCH_2), respectively.

The PPI derivatives were analyzed by ^{1}H NMR spectroscopy to determine their structural composition (see **Table 5**). The molar ratio of HD coupled per core unit (G2 or G3) was calculated by comparing NMR peak intensities between the protons of HD (δ 4.0 ppm) and PPI (δ 1.7 – 2 ppm). For the G2 core, 8 HD per core unit have coupled whereas for the G3 core unit 16 HD molecules have bound. This indicated that no intramolecular side reactions have occurred. The molar ratio of grafting unit (GU) / core was determined by comparing the

integrals of OEI protons (δ 2.4 – 3.4 ppm) with the core unit PPI protons. The molar ratio of grafting G2 and G3 core units with G2 cannot be determined by ^{1}H NMR spectroscopy. Generally, the GU / core ratio was lower than the HD / core ratio indicating that more than one dendritic branch per grafting unit might have reacted. Since the esters, which present the degradable bonds of the polymers, might aminolyze or hydrolyze during synthesis and purification, the ester content of all conjugates was analyzed. It ranged around 80% of the theoretical value (**Table 5**).

GPC measurements for the determination of molecular weights of the grafted dendrimers showed that G3 core dendrimers resulted in higher molecular weights than G2 core derivatives as depicted in **Table 5**. However, both conjugates that were grafted by G2 resulted in much higher molecular weights than the OEI grafted ones, which can be explained by an insufficient solubility of these polymers in the mobile phase. PDIs were ranging from 1.2 to 2.5 indicating a low- to mid-ranged polydispersity of the products.

Ester degradation of all PPI derivatives was followed by ^{1}H NMR spectroscopy at pH 7 at 37 °C in aqueous medium. The extent of degradation was determined by comparing the integrals of signals at δ 4.0 ppm and δ 3.5 ppm that are attributed to the methylene protons of the ester group (COOC*H*$_2$) and hydroxyl group (HOC*H*$_2$), respectively. **Table 5** gives $t_{1/2}$ for all grafted dendrimers. It is obvious that the OEI grafted derivatives, G2-HD-OEI and G3-HD-OEI, were degraded much faster under physiological conditions ($t_{1/2}$ = 3 days) than the G2 grafted ones, G2-HD-G2 and G3-HD-G2 ($t_{1/2}$ > 14 days). It is likely that their lower water solubility due to the more hydrophobic *propylen*imine moieties upon the surface reduced ester hydrolysis, thus increasing their $t_{1/2}$.

3.3.2 Physicochemical characterization of grafted PPI dendrimers

3.3.2.1 DNA condensing ability

The capability of grafted G2 and G3 core dendrimers to complex DNA was investigated using an ethidium bromide exclusion assay as a function of increasing c/p-ratios [w/w] (**Fig. 22**). All grafted dendrimers showed a very good DNA condensing ability already at low c/p-ratios if HBG (low ionic strength buffer) was used for complex formation. This was indicated by the very low c/p-ratios needed to obtain $FI_{50\%}$ that was found for all derivatives at c/p-ratio 0.4 (see **Fig. 22A**). Interestingly, unmodified dendrimer G3 exhibited in HBG the lowest c/p-ratio to reach $FI_{50\%}$ (c/p = 0.2) whereas the unmodified G2 dendrimer showed the highest value (c/p for $FI_{50\%}$ = 0.8), indicating its weaker capability to condense DNA. All constructs

displayed low minimum values of relative fluorescence in HBG showing a high binding strength in a low ionic strength buffer. Nevertheless, it can be clearly seen that in presence of sodium chloride (HEPES buffered saline, 0.15 M NaCl = HBS, **Fig. 22B**) the DNA binding strength within the complexes was dramatically decreased, since the obtained minimum relative fluorescence increased for all conjugates from about 3 – 10% (in HBG) to 20 – 30% (in HBS). This effect was found for either the unmodified as well as for the grafted dendrimers, notably, irrespective of the dendrimer generation within the core unit or the investigated grafting moiety used. Similar effects were also found for standard polyethylenimines like LPEI (22 kDa) or BPEI (25 kDa) and other polycationic gene delivery vectors.[37]

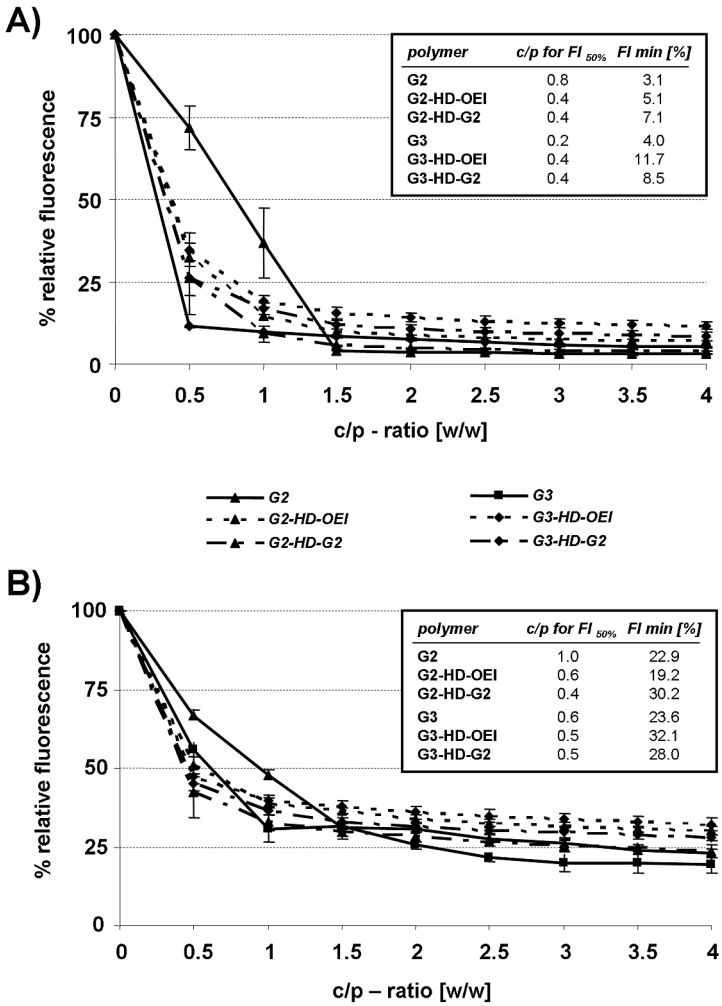

Fig. 22: DNA condensation ability of grafted PPI dendrimers studied by ethidium bromide (EtBr) exclusion assay. Experiments were carried out in HEPES buffer glucose 5% (HBG; low ionic strength condition) (A) and in HEPES buffer saline 0.15 M NaCl (HBS; physiological ionic strength condition) (B). C/p for $Fl_{50\%}$ represents the c/p-ratio to quench to 50% fluorescence of the original value. Fl_{min} indicates minimal fluorescence and represents the binding strength.

3.3.2.2 Stability of polyplexes against NaCl induced disassembly

To clarify in which extent the stability of unmodified or grafted PPI core based polyplexes was affected by salt, their colloidal stability was studied. For this the dissociation to free DNA and free polycation was investigated as a function of an increasing amount of sodium chloride via light scattering. Light scattering is a convenient procedure to measure complex formation or complex dissociation since the intensity of scattered light is dramatically increased when complexes are formed, especially if they aggregate, compared to free DNA and polycation where scattering intensity is low.[66] **Fig. 23** shows the intensity of scattered light of grafted and unmodified polyplexes (c/p-ratio 2) as a function of the sodium chloride concentration. It can be assumed that all polyplexes aggregated at 0.25 M NaCl since scattered light intensity was at maximum.

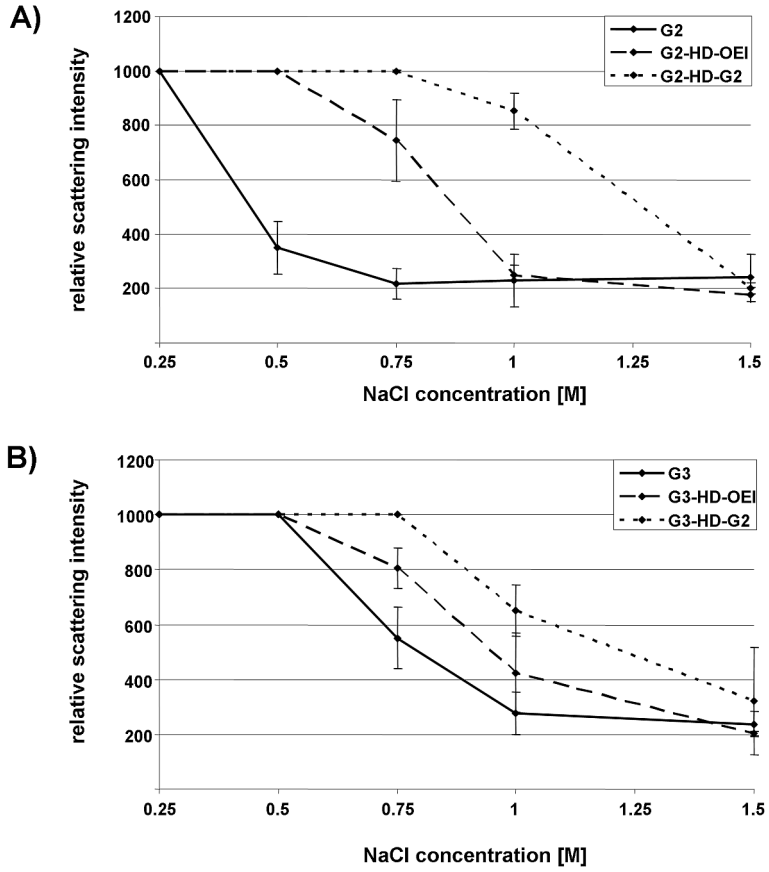

Fig. 23: Stability of polyplexes against dissociation with sodium chloride (NaCl). The colloidal stability of polyplexes against NaCl was evaluated using light scattering ($\lambda_{ex} = \lambda_{em} = 600$ nm). Stepwise addition of 5 M NaCl to the polyplex solution resulted in the decrease of scattering intensity as a function of an increasing molar NaCl concentration.

A rapidly decreasing scattering intensity was observed for unmodified G2 polyplexes immediately after salt addition at sodium chloride concentrations higher than 0.25 M and the lowest intensity occurred at 0.75 M NaCl resembling total polyplex disassembly at this point. The higher generation dendrimer G3 (unmodified) started to dissociate at 0.5 M NaCl and the minimum of scattering intensity was found at 1 M sodium chloride. With regard to the grafted dendrimers it was found that the complexes were not as susceptible to NaCl induced dissociation as compared to their unmodified counterparts. This was either demonstrated by the shift of the point of disassembly to higher salt concentrations and/or the minimum of scattering intensity. For example, the G2-HD-OEI complex started to dissociate at 0.5 M NaCl with a scattering minimum at 1 M NaCl whereas for the G3-HD-OEI polyplex a scattering minimum at 1.5 M NaCl was detected. This indicates the increased colloidal stability of the G3 core over the G2 core. Interestingly, all the derivatives that were G2 grafted were less sensitive to sodium chloride compared to the OEI grafted ones; most probably due to the higher hydrophobicity of these polyplexes resulting in lower water solubility thus minimizing the possibility of salt molecules to interfere with the electrostatic interactions between DNA and polycation.

3.3.2.3 Hydrodynamic diameter and zeta-potential of polyplexes

Hydrodynamic diameters and zeta-potentials of all compounds were examined in HBG and HBS at c/p-ratios ranging from 1 to 8 (**Table 6**).

	c/p 1		c/p 2		c/p 4		c/p 8	
	HBG	HBS	HBG	HBS	HBG	HBS	HBG	HBS
G2	298 (± 53)	agg.	247 (± 52)	agg.	314 (± 23)	agg.	255 (± 82)	agg.
G3	169 (± 18)	agg.	184 (± 43)	agg.	165 (± 30)	agg.	144 (± 51)	agg.
G2 HD OEI	187 (± 28)	281 (± 24)	187 (± 29)	294 (± 40)	197 (± 17)	203 (± 5)	194 (± 59)	202 (± 9)
G2 HD G2	186 (± 36)	297 (± 9)	217 (± 38)	336 (± 51)	192 (± 45)	214 (± 10)	159 (± 43)	209 (± 6)
G3 HD OEI	208 (± 22)	290 (± 7)	166 (± 28)	593 (±106)	225 (± 14)	200 (± 1)	246 (± 45)	158 (± 10)
G3 HD G2	231 (± 25)	656 (± 99)	200 (± 18)	496 (± 41)	208 (± 42)	313 (± 7)	182 (± 27)	332 (± 6)

Table 6: Hydrodynamic diameters of grafted PPI dendrimer polyplexes. C/p–ratios ranging from 1 to 8 were investigated in HBG (5% glucose; low ionic strength condition) or HBS (0.15 M NaCl; physiological ionic strength condition). Data expressed as mean values (± SD) out of three independent experiments.

If HBG was used as solvent, all conjugates resulted in mean diameters between 100 nm and 200 nm, except for unmodified dendrimer G2 (250 – 300 nm). Unmodified G3 compared to unmodified G2 dendrimer resulted in smaller complex sizes at all c/p-ratios. Compared to

their unmodified counterpart, both OEI and G2 grafted G2 core derivatives showed smaller polyplex sizes at all c/p-ratios. Interestingly, grafted G3 core dendrimers resulted in a slight increase of complex size that was not significant. Differences between polymers having either OEI or G2 as grafting unit were not found. In HBS (0.15 M NaCl), complex sizes of grafted dendrimers increased to mean diameters ranging from 200 nm to 650 nm while unmodified G2 and G3 dendrimers aggregated under these conditions. The polydispersity indices of all samples shown in **Table 6** ranged mostly between 0.2 and 0.3, indicating a low or mid-ranged polydispersity of polyplexes.

Regarding the surface charge, it was found that increasing the c/p-ratios resulted in rising zeta-potentials (data not shown). In general, all compounds had zeta-potentials ranging from around +25 mV (c/p-ratio 1) to +30 mV (c/p-ratio 8). Importantly, the more hydrophobic compounds G2-HD-G2 and G3-HD-G2 resulted in slightly higher zeta-potentials than the OEI grafted ones.

3.3.3 Gene transfer activity of grafted PPI dendrimers *in vitro*: the necessity of OEI

3.3.3.1 Reporter gene expression and cytotoxicity in vitro

In vitro transfection of grafted dendrimers was carried out as described above in both B16F10 cells (murine melanoma) and Neuro2a cells (murine neuroblastoma). LPEI (22 kDa) and BPEI (25 kDa) polyplexes were used as positive controls at their optimized c/p-ratio of 0.8. Complexes were prepared in HBG.

Fig. 24 shows the transfection efficiency and cytotoxicity profile of all conjugates at a c/p-ratio range of 1 to 8. The transfection results in both cell lines demonstrated that according to the unmodified dendrimers, G2 polyplexes were less efficient than G3 ones – even though polyplexes of G2 were less toxic than those of G3, which is in accordance to previous findings by Zinselmeyer et al..[69]

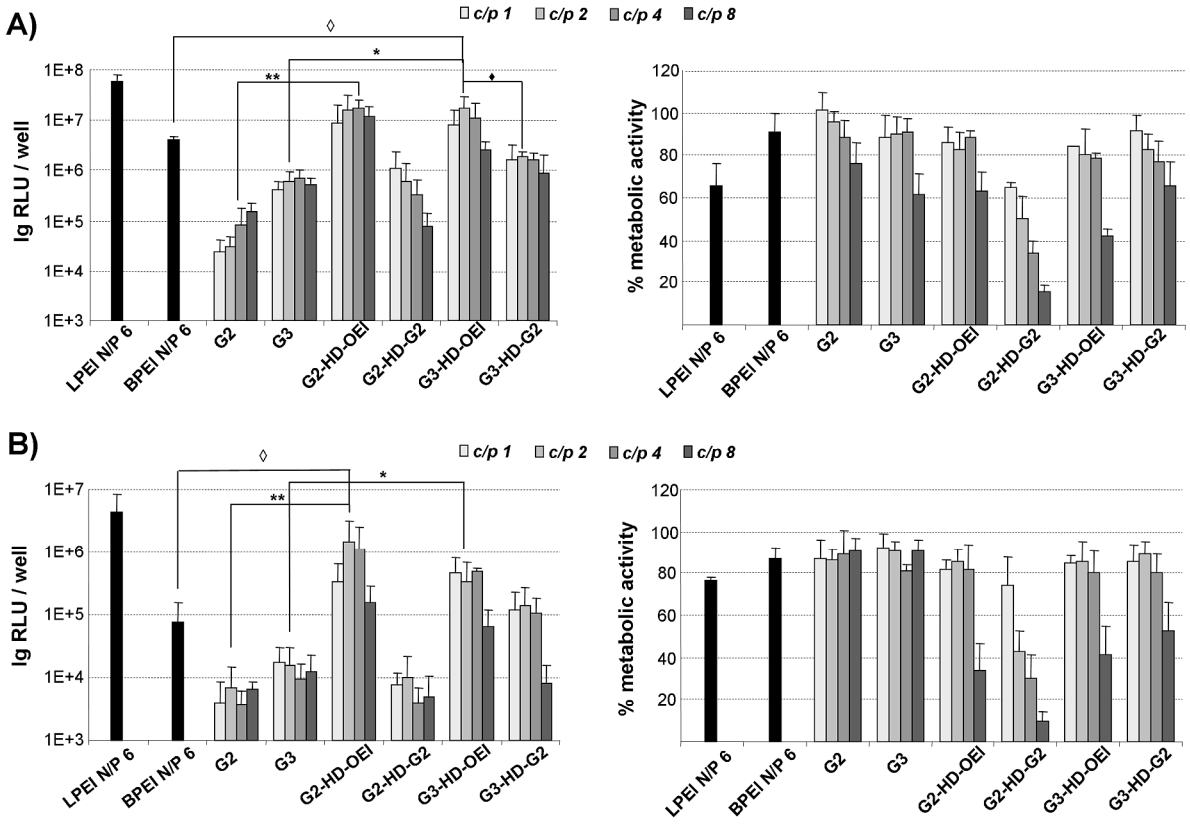

Fig. 24: Influence of grafting upon PPI dendrimers G2 and G3 and the influence of the investigated grafting moiety on transfection efficiency and metabolic activity as demonstrated in B16F10 cells (A) and Neuro2a cells (B). Transfection studies were performed in the presence of 10% serum and reporter gene expressions as well as metabolic activity of cells were evaluated 24 h post transfection. Polyplexes were formed at indicated c/p-ratios in HBG; LPEI and BPEI were applied at optimized N/P-ratio of 6 which represents a c/p-ratio of 0.8. Transfection efficiency is expressed as RLU / well and is the mean (± SD) out of three independent measurements each performed in triplicates. Two nanograms of recombinant luciferase correspond to 10^7 light units.
(ANOVA, Duncan's test, * $p < 0.05$, ** $p < 0.01$ unmodified dendrimers vs. OEI grafted ones; ♦ $p < 0.05$ G2 grafted vs. OEI grafted dendrimer; ◊ $p < 0.05$ BPEI vs. OEI grafted dendrimer)

Grafting of either G2 or G3 core units with OEI led to enhanced transfection efficiency, irrespective of the investigated core generation used. Thus, comparing the OEI grafted G2 core conjugate with its unmodified counterpart, more than 200-fold increased transfection efficiency from G2 to G2-HD-OEI ($p < 0.01$) can be detected in both tested cell lines - B16F10 and Neuro2a cells (**Fig. 24**). In this case, cell-type specific differences were found regarding the optimized conditions leading to highest transfection efficiency (B16F10 cells: c/p-ratio of 4; Neuro2a cells: c/p-ratio of 2 at metabolic activity > 80% relative to untreated cells). For G2-HD-G2, a very inconvenient toxicity profile dramatically reduced the polyplex transfection levels.

The best transfecting conjugate within the G3 line, G3-HD-OEI, resulted in enhanced transfection efficiency as compared to both the unmodified counterpart G3 as well as

G3-HD-G2. Thus, e.g. in B16F10 cells the improvement from the unmodified G3 to G3-HD-OEI was about 30-fold ($p < 0.05$) while comparing G3-HD-OEI and G3-HD-G2, both exhibiting a similar metabolic activity of around 85% post transfection, G3-HD-OEI was 9-fold ($p < 0.05$) more efficient than G3-HD-G2. Concerning the comparison of G2 and G3 core derivatives due to influences of the dendrimers´ generation on transfection efficiency, it has to be assessed that the higher generation within the core unit of the derivatives did not enhance transfection efficiency *in vitro.* However, differences according to the improved cytotoxicity profile of G3 core based derivatives were found as compared to G2 core ones causing an improved transfection/toxicity relation (G2-HD-G2 ↔ G3-HD-G2).

3.3.3.2 Bafilomycin A1 experiments

The higher potency of OEI grafted dendrimers over G2 grafted derivatives may be explained by enhanced endosomolytic activity based on the proton-sponge effect[13,70] of the oligo*ethyleni*mine moiety. To investigate this in more detail, luciferase gene transfer experiments were conducted in absence or presence of bafilomycin A1 in either B16F10 (**Fig. 25A**) and Neuro2a cells (**Fig. 25B**). Bafilomycin A1 specifically inhibits the vacuolar type of H^+ - ATPases by inhibiting the endo-/lysosomal proton pump being responsible for the reported decrease of polyethylenimine-mediated transfection.[71] **Fig. 25** illustrates the differences in observed luciferase expression levels. In these studies, all complexes were applied at their optimized c/p-ratios depending on the cell line investigated. LPEI and BPEI (tested as positive controls) showed a 20-fold decrease of transfection efficiency in presence of bafilomycin A1 in B16F10 cells ($p < 0.01$) and a 5- (LPEI) to 7-fold (BPEI) decrease in Neuro2a cells ($p < 0.05$), respectively. Similar cell-type specific effects after bafilomycin A1 treatment were previously described by Kichler et al..[71] Unmodified PPI dendrimers G2 and G3 showed no drop of luciferase expression in presence of bafilomycin A1 in both cell lines. The same result was detected for the PPI dendrimers grafted with G2. Only G3-HD-G2 in B16F10 cells displayed a minor reduction ($p < 0.05$) of luciferase expression in presence of bafilomycin A1.

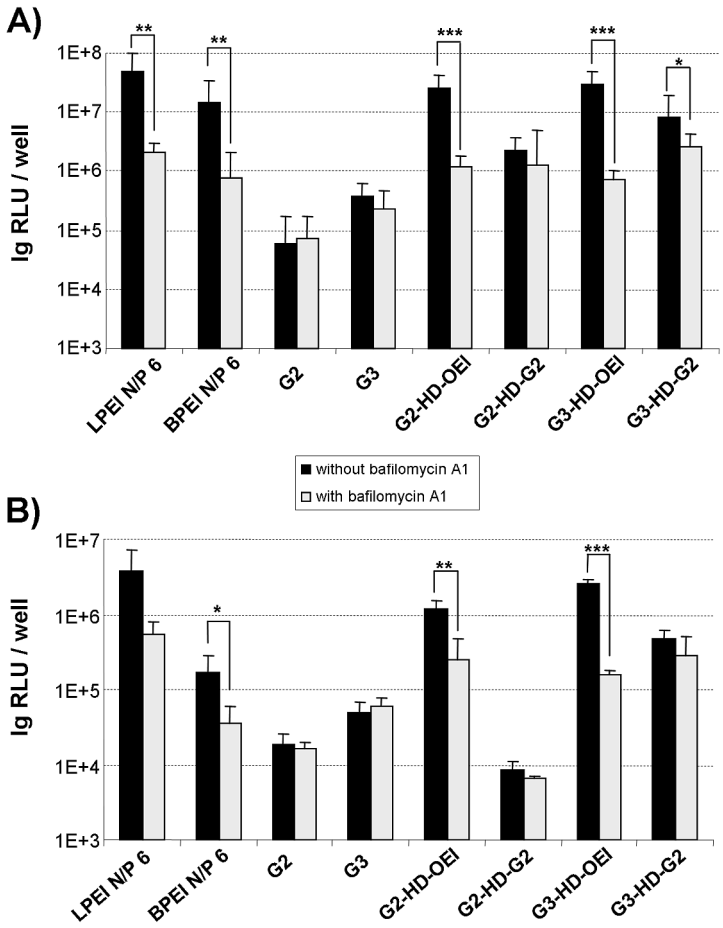

Fig. 25: Effect of bafilomycin A1 on transfection efficiency. B16F10 cells (A) or Neuro2a cells (B) were transfected with unmodified (G2 or G3) dendrimers or grafted ones (G2-HD-OEI, G2-HD-G2, G3-HD-OEI or G3-HD-G2) in absence or in presence of bafilomycin A1. LPEI and BPEI polyplexes at their optimized N/P-ratio of 6 (c/p-ratio 0.8) were included for comparison. Polyplexes were applied at c/p-ratio of 2, except for unmodified and grafted G2 dendrimers in B16F10 (c/p-ratio 4). Data expressed as mean values (± SD) out of three independent experiments, each performed in triplicates. Two nanograms of recombinant luciferase correspond to 10^7 light units.
(ANOVA, Duncan's test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ absence vs. presence of bafilomycin A1)

In sharp contrast, polyplexes with OEI grafted dendrimers G2-HD-OEI and G3-HD-OEI experienced a very significant ($p < 0.001$) 20- to 40-fold drop of luciferase activity in presence of bafilomycin A1 in B16F10 cells – very similar to LPEI or BPEI (**Fig. 25A**). In Neuro2a cells (**Fig. 25B**) this bafilomycin effect was not as pronounced, with a 5-fold ($p < 0.01$) reduction for G2-HD-OEI and 16-fold ($p < 0.001$) for G3-HD-OEI, again very similar as for PEI. No detectable cytotoxic effects of bafilomycin A1 (by measuring protein content in cell lysates using the BCA assay) were found, thereby ruling out that the decrease of luciferase expression is based on bafilomycin A1 induced toxicity (data not shown).

3.3.4 Biocompatibility and gene transfer studies *in vivo*

Both the hemolytic activity of grafted and unmodified dendrimers as well as their tendency to induce erythrocyte aggregation was studied using freshly isolated erythrocytes using the same procedure as for OEI core based pseudodendrimers. Erythrocytes were incubated with different concentrations of plain polymer ranging from 5 µg/mL to 50 µg/mL (**Fig. 26**).

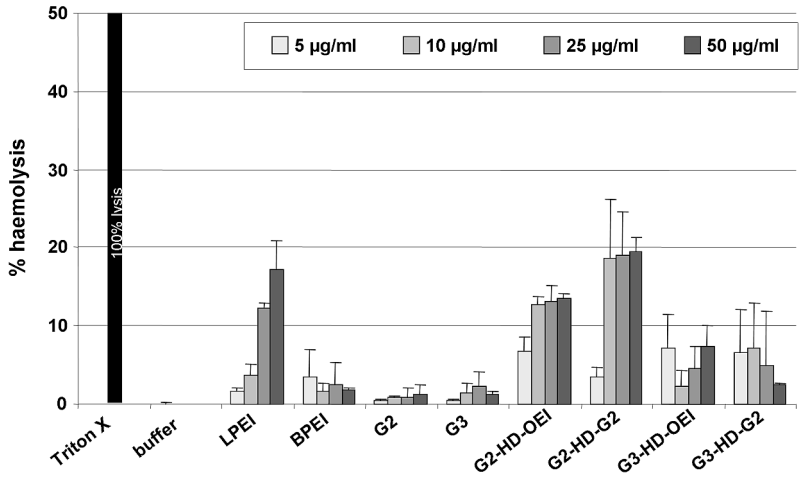

Fig. 26: Hemolytic activity of plain grafted PPI pseudodendrimers. Freshly collected human erythrocytes were incubated with increasing concentrations of polymer and hemolysis was determined by UV measurement (λ = 405 nm) relative to Triton X (100% lysis) and a buffer treated control (HBG / 10% FCS). Data expressed as means out of triplicate (± SD).

For unmodified dendrimers a very low hemolytic activity was found (around 2%). Similar results were obtained for BPEI (25 kDa) whereas LPEI (22 kDa) resulted in a dose-dependent increase of hemolytic activity from 2% to 17%. All grafted dendrimers showed an increased hemolytic activity as compared to their unmodified counterparts. Interestingly, similar toxicity profiles as found in transfection studies were also discovered here. Thus, the more hydrophobic G3 core derivatives resulted in a lower hemolytic activity ranging between 2% and 7%, whereas G2 core derivatives exhibited a higher lytic activity (7 – 12%). The higher toxicity of G2-HD-G2 over G2-HD-OEI was also detected here since the highest concentration tested (50 µg/mL) resulted in hemolysis ranging around 20% whereas G2-HD-OEI showed a lytic activity of only up to 10%.

Investigations regarding polymer induced erythrocyte aggregation showed that unmodified and all grafted dendrimers did not lead to polymer-induced erythrocyte aggregation (see **Fig. 27**). In contrast, standard PEIs – as already known – caused strong erythrocyte aggregation.[15,37]

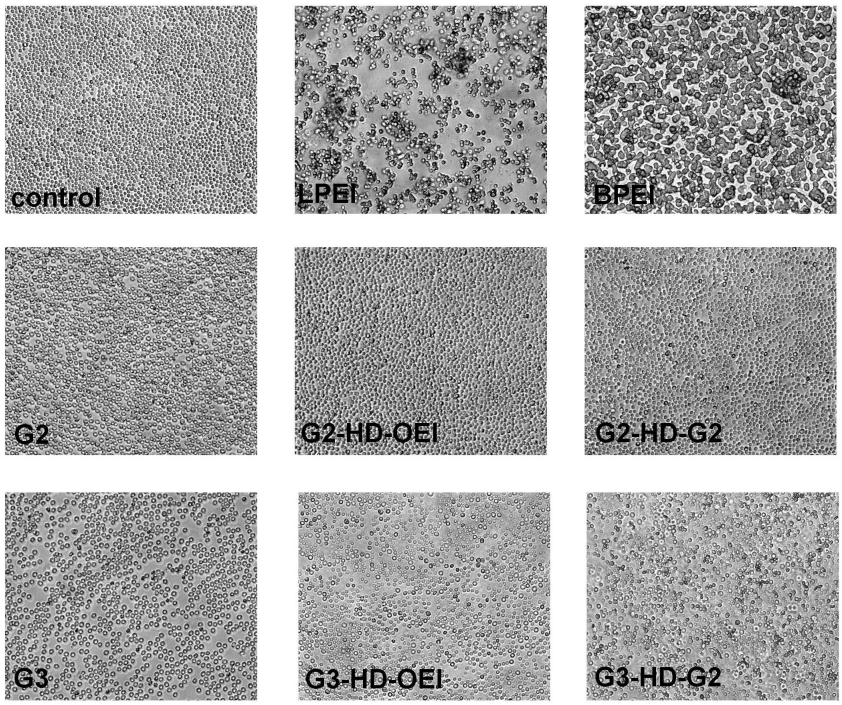

Fig. 27: Polymer induced erythrocyte aggregation. Erythrocyte aggregation after polymer treatment was determined by transmitted light microscopy (20-fold magnification). Pictures represent erythrocyte aggregation at a concentration of polymers c = 50 µg/mL.

G2-HD-OEI and G3-HD-OEI, found as best transfecting compounds in *in vitro* studies were well tolerated by mice exhibiting no acute toxic side-effects after i.v. application of plain polymers at a concentration of 400 µg/mL (in HBG) with 100 µg pseudodendrimer applied per 20 g mouse.

Both compounds were then tested for their *in vivo* gene transfer efficiency in a s.c. Neuro2a tumor model. The experiments were conducted by intravenous injection of complexes of G2-HD-OEI and G3-HD-OEI (c/p-ratio 1 and 2) using luciferase (pEGFPLuc) as reporter gene. Complexes were prepared at a final DNA concentration of 200 µg/mL in HBG with 50 µg DNA applied per 20 g mouse. Control mice were HBG buffer treated. Mice receiving polyplexes showed no signs of acute toxicity after application and polyplexes were well tolerated. 24 h after injection, no significant loss of body weight was observed for G2-HD-OEI polyplexes at a c/p-ratio of 1 whereas G3-HD-OEI polyplexes resulted in 5% loss of body weight. C/p-ratio 2 caused a body weight loss of 8 – 9% for both polymers.

Fig. 28 presents luciferase reporter gene expression in main organs and tumor tissue 24 h after i.v. application of polyplexes. All polyplexes at both c/p-ratios led to transgene expression that was predominantly found in tumor tissue. Notably, luciferase tumor levels

were as high as reported for standard polycations like e.g. LPEI in the same tumor model as depicted in **Fig. 18**.

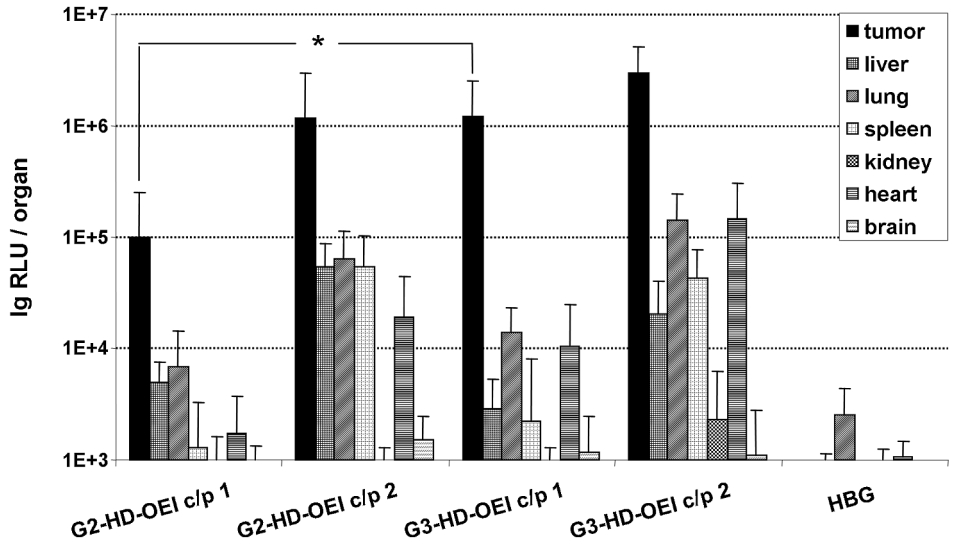

Fig. 28: Luciferase gene expression 24 h after i.v. administration of G2-HD-OEI or G3-HD-OEI in A/J mice bearing Neuro2a s.c. tumors. C/p-ratios 1 and 2 were investigated. Buffer (HBG) treated mice were used as control. Luciferase gene expression is presented as relative light units per organ or tumor, respectively (RLU / organ) (mean ± SD, n = 8 animals per group). Tumor weights ranged between 300 mg and 400 mg. Two nanograms of recombinant luciferase correspond to 10^7 light units.
(ANOVA, Duncan's test , * $p < 0.05$ for G2-HD-OEI vs. G3-HD-OEI c/p-ratio 1)

The "golden standard" LPEI is known for its highest transgene expression level (see **Fig. 18**) in mice mainly found in the lung after i.v. application, which is also responsible for toxic side-effects of this polymer (tumor / lung ratio < 0.01).[17,18,72] In contrast, G2-HD-OEI and G3-HD-OEI resulted, notably, at all investigated c/p-ratios in much lower luciferase gene expression in lung tissue. Importantly, in case of G3-HD-OEI the most favorable high tumor / lung ratio of 89 was found at c/p-ratio 1. The lower tumor / lung ratio of 15 at c/p-ratio 1 in case of G2-HD-OEI is due to the lower tumor transfection level at this c/p-ratio. At c/p-ratio 2 both compounds exhibited tumor / lung ratios of about 20 at similar high transgene expression level in tumor tissue while an increase in liver, spleen, heart and lung tissue compared to c/p-ratio 1 was observed.

Furthermore, this study demonstrated a clear influence of the dendrimer generation within the conjugates on transfection efficiency *in vivo*. For G3-HD-OEI polyplexes, gene expression levels in tumor tissue were 12-fold higher ($p < 0.05$) as compared to G2-HD-OEI at c/p-ratio 1 and 2.5-fold higher at c/p-ratio 2 (see **Fig. 28**).

The biocompatibility of all polyplexes in mice was assayed simultaneously by investigating liver enzyme levels in serum of collected blood (AST, ALT, GLDH and AP). Buffer (HBG) treated mice were used as negative control. None of the formulations induced any significant increase of liver enzyme levels (**Fig. 29**).

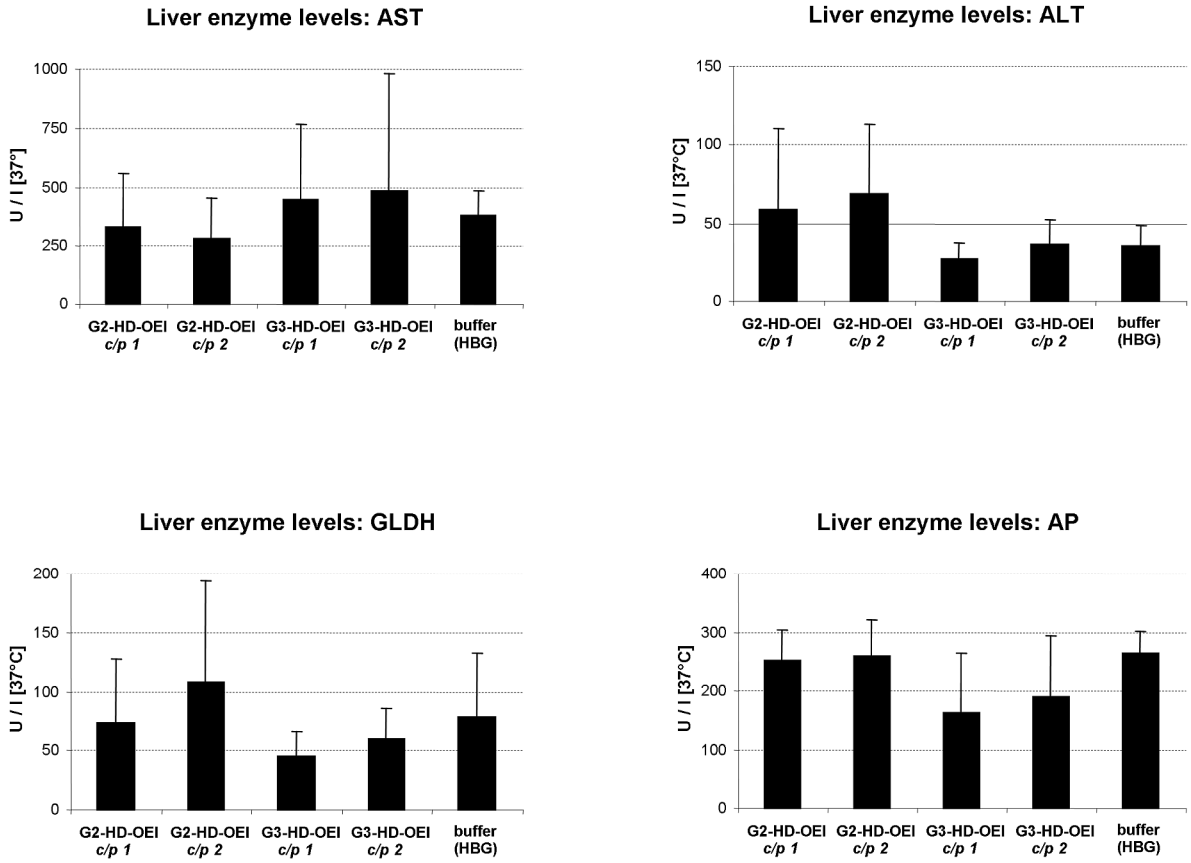

Fig. 29: Liver enzyme levels in serum 24 h after polyplex treatment. Alkaline (ALT) and aspartate (AST) aminotransaminases, glutamate dehydrogenase (GLDH) and alkaline phosphatase (AP) were determined using standard kinetic UV tests. Data represents mean values (+ SD, *n = 8 animals per group*), except for buffer treated control mice (*n = 4*). No significant differences in liver enzyme levels between control mice and polyplex treated mice were found (ANOVA, Duncan's test).

3.4 Stabilization and targeting of polyplexes

3.4.1 Lateral stabilization of HD O / DNA polyplexes

Stabilization of polyplexes using lateral reversible crosslinking strategies after complex formation could be a promising tool to overcome the undesired instability of complexes in presence of serum proteins or solutions of high ionic strength likewise under *in vivo* conditions. This conceptual approach has already been successfully shown by several researchers in case of HMW polycations like for e.g. BPEI[44,68,73] or PLL[43,66,74] polyplexes. For polyplexes of LMW oligoamines this idea might be especially beneficial as their lower charge density results in less tightened particles leading to a higher susceptibility of them against polyelectrolytes and solutions of high ionic strength. Thus, lateral stabilization to increase the colloidal stability of polyplexes was investigated here using of the LMW pseudodendritic oligoamine HD O.

3.4.1.1 Synthesis of stabilized polyplexes

As indicated in **Fig. 30**, two different crosslinkers were investigated for lateral stabilization of polyplexes: first, dithiobissuccinimidyl propionate (DSP), a homobifunctional N-hydroxysuccinimide (NHS) ester that contains a bioreducible disulfide bond, which can break down in reductive environment e.g. in the cytoplasm due to a higher gluathionine level compared to the extracellular compartment. And second, its non-reducible counterpart disuccinimidyl suberate (DSS) as stable crosslinker, used for control studies.

Both DSP and DSS reacted in an electrophilic substitution reaction with primary amines of HD O by forming stable covalent amide bonds. The crosslinking reaction proceeded under optimized conditions, as described in literature, for 30 minutes at room temperature.[44] For crosslinking, the following different molar ratios of crosslinker to amine were chosen: 0.05, 0.06 and 0.07. These ratios were used since initial physicochemical studies of laterally stabilized HD O polyplexes prepared at higher crosslinker / amine ratios demonstrated formation of undesired huge and multimodal particles, most probably due to intermolecular side-reactions. Nevertheless, one can assume that at the chosen ratios of crosslinker to HD O amine (0.05; 0.06 and 0.07) only 30 - 50% of primary amines of HD O are converted by the reaction with DSP or DSS. However, the residual primary amine content might be useful for further modification of the stabilized polyplexes with e.g. targeting ligands like transferrin (Tf) or polyethylenglycol (PEG) as shielding domain.

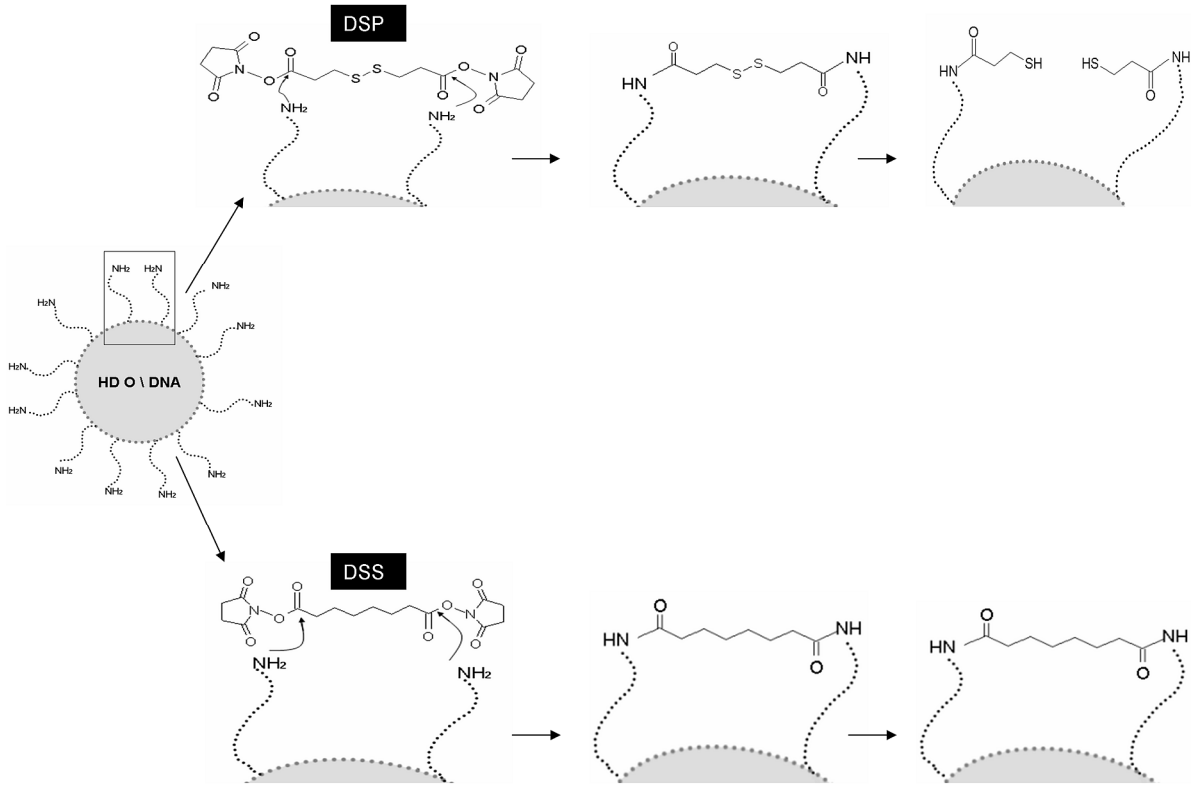

Fig. 30: Schematic illustration of HD O / DNA crosslinked polyplexes. Reaction scheme for the conversion of HD O with dithiobis (succinimidyl propionate) (DSP) or disuccinimidyl suberate (DSS). Different ratios of crosslinker to primary amine groups were investigated ranging form 0.05 to 0.07.

3.4.1.2 Physicochemical characterization of stabilized polyplexes

The first objective was to evaluate the influence of the surface crosslinking of HD O complexes on their physicochemical properties. Beside HD O / DNA polyplexes, also polyplexes of BPEI were investigated in parallel to allow a comparison to a branched, but not hydrolysable cationic structure within polyplexes.

Table 7 summarizes the basic studied biophysical characteristics of crosslinked HD O / DNA complexes formed at a c/p-ratio of 1 [w/w]. For size and zeta-potential measurements, polyplexes were prepared in HBG. Subsequently, they were crosslinked by the addition of the appropriate amount of DSP or DSS stock solution to generate the desired crosslinking degree. For the investigation of uncrosslinked HD O / DNA polyplexes HBG was added instead of the crosslinker. Uncrosslinked polyplexes resulted in a hydrodynamic diameter of about 190 nm, which is in good agreement to previous studies. The introduction of linking agents to the polyplexes – either using DSP or DSS – influenced particle sizes of HD O polyplexes only moderately; thus, hydrodynamic diameters were ranging between 150 nm and 220 nm for all degrees of crosslinker / amine. Also the surface charge was only marginally affected, retaining a comparable zeta-potential of about +30 mV.

	DSP		DSS	
ratio crosslinker / amine [mol/mol]	*size [nm]*	*ZP [mV]*	*size [nm]*	*ZP [mV]*
0	187 [± 12]	28 [± 2]	205 [± 4]	31 [± 4]
0.05	194 [± 11]	30 [± 2]	209 [± 10]	33 [± 2]
0.06	156 [± 19]	29 [± 3]	161 [± 17]	27 [± 1]
0.07	208 [± 2]	27 [± 5]	226 [± 2]	31 [± 1]

Table 7: Effect of lateral stabilization using DSP or DSS. Crosslinkers were used at indicated molar ratios upon HD O / DNA polyplexes that were prepared at a c/p-ratio of 1. All measurements were performed in three independent experiments.

In case of BPEI, used at a c/p-ratio of 0.8, similar results were found (data not shown). The original size of uncrosslinked BPEI/DNA polyplex was 120 nm (± 6nm). Polyplex sizes ranged between 100 nm and 150 nm after lateral stabilization at all crosslinking degrees using either DSP or DSS. Stabilized BPEI polyplexes resulted in zeta-potentials around +30 mV, although in case of DSP a slight decrease of the surface charge was found with an increasing ratio of crosslinker / amine (*ratio 0.00:* +31 mV; *ratio 0.05*: +30 mV; *ratio 0.06:* +26 mV, *ratio 0.07:* +23 mV).

A simple means to measure stabilization of polyplexes is to evaluate their susceptibility to disruption by polyelectrolytes such as heparin, determining the release of free DNA by electrophoresis.

Using this approach, at first the sensitivity of uncrosslinked polyplexes toward polyanions of HD O was investigated. By titration studies it was found that the use of 0.25 I.U. heparin resulted in a complete release of DNA from the HD O complex (**Fig. 31**) indicating that the non-crosslinked polyplex is completely unstable at this and higher concentrations. Similar results were also obtained for BPEI (data not shown).

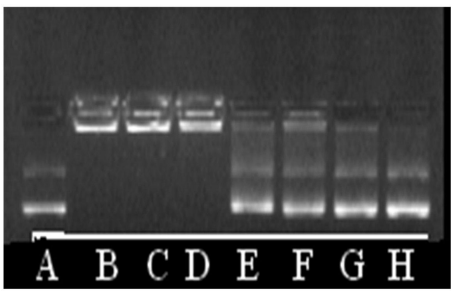

Fig. 31: DNA compacting ability of HD O after incubation with different amounts of heparin. HD O / DNA polyplexes were prepared at a c/p-ratio of 1. The release of free DNA following 0.5 h of incubation with increasing amounts of heparin was investigated by gel shift assay. (A) DNA, (B) w/o heparin, (C) 0.05 I.U., (D) 0.1 I.U., (E) 0.25 I.U., (F) 0.5 I.U., (G) 0.75 I.U., (H) 1.0 I.U..

Within the investigations on the susceptibility of laterally stabilized polyplexes against heparin, it was observed that HD O and BPEI complexes could be strengthened against polyelectrolyte exchange reactions by crosslinking with DSP or DSS. This was demonstrated in **Fig. 32** and **Fig. 33** by the retention of these complexes in the well using 0.25 I.U. heparin at all three investigated crosslinker / amine ratios (for HD O: see **Fig. 32**, lanes F, G and H; for BPEI: see **Fig. 33**, lanes C, D, E). Moreover, also an increase of the heparin concentrations up to 1.0 I.U. heparin was not able to challenge the tightened complexes resulting in no release of free DNA (data not shown).

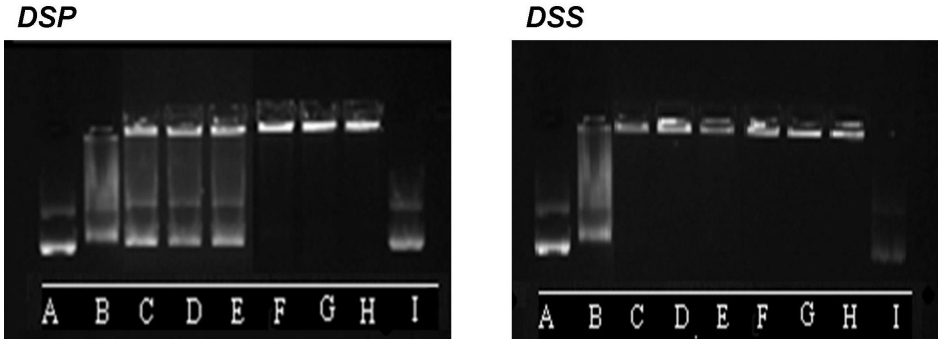

Fig. 32: Stability of crosslinked HD O polyplexes against polyanionic exchange reactions using heparin. HD O / DNA polyplexes were prepared at a c/p-ratio of 1. Polyplexes were crosslinked using DSP (left) or DSS (right) at crosslinker / amine ratios of 0.05; 0.06 and 0.07. The release of the DNA was determined by gel electrophoresis 0.5 h after incubation of polyplexes with 0.25 I.U. heparin. DTT was used to demonstrate the reversibility of lateral stabilization in case of DSP- vs. DSS-crosslinked polyplexes. For this, after 30 min DTT (25 mM) incubation, 0.25 I.U. heparin was added and proceeded as described above. Lanes A-I (in each picture) represent: DNA (A and I); without crosslinker (B); ratio 0.05 plus DTT (C); ratio 0.06 plus DTT (D); ratio 0.07 plus DTT (E); ratio 0.05 w/o DTT (F); ratio 0.06 w/o DTT (G); ratio 0.07 w/o DTT (H).

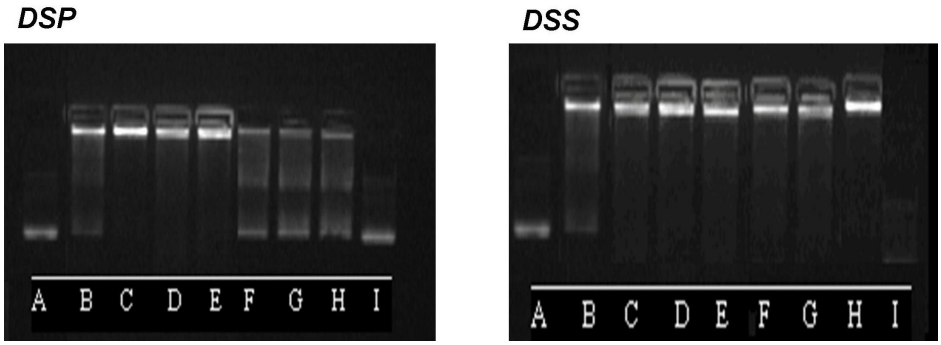

Fig. 33: Stability of crosslinked BPEI polyplexes against polyanionic exchange reactions using heparin. BPEI / DNA polyplexes were prepared at a c/p-ratio of 0.8 (representing an N/P-ratio of 6). Polyplexes were crosslinked using DSP (left) or DSS (right) at crosslinker / amine ratios of 0.05; 0.06 and 0.07. The release of the DNA was determined by gel electrophoresis 0.5 h after incubation of polyplexes with 0.25 I.U. heparin. DTT was used to demonstrate the reversibility of lateral stabilization in case of DSP- vs. DSS-crosslinked polyplexes. For this, after 30 min DTT (25 mM) incubation, 0.25 I.U. heparin was added and proceeded as described above. Lanes A-I (in each picture) represent: DNA (A and I); without crosslinker (B); ratio 0.05 w/o DTT (C); ratio 0.06 w/o DTT (D); ratio 0.07 w/o DTT (E); ratio 0.05 plus DTT (F); ratio 0.06 plus DTT (G); ratio 0.07 plus DTT (H).

The possibility to reverse the stabilization of the crosslinked polyplexes was demonstrated by treating DSP and DSS-linked complexes with the reducing agent DTT. This led, in case of DSP stabilized polyplexes due to cleavage of the disulfide bonds, to destabilized complexes where heparin caused a release of free DNA (**Fig. 32**, *for HD O*: see **Fig. 32** 'DSP' lanes C, D, E; *for BPEI*: see **Fig. 33** 'DSP' lanes F, G, H). In contrast, DSS-crosslinked polyplexes were retained stabilized as the complexes remained in the wells and no free DNA could be detected in the gel.

An alternative method to monitor the enhanced stability of complexes is to investigate their susceptibility against sodium chloride (NaCl) induced polyplex dissociation following the method as described in section 3.3.2.2.

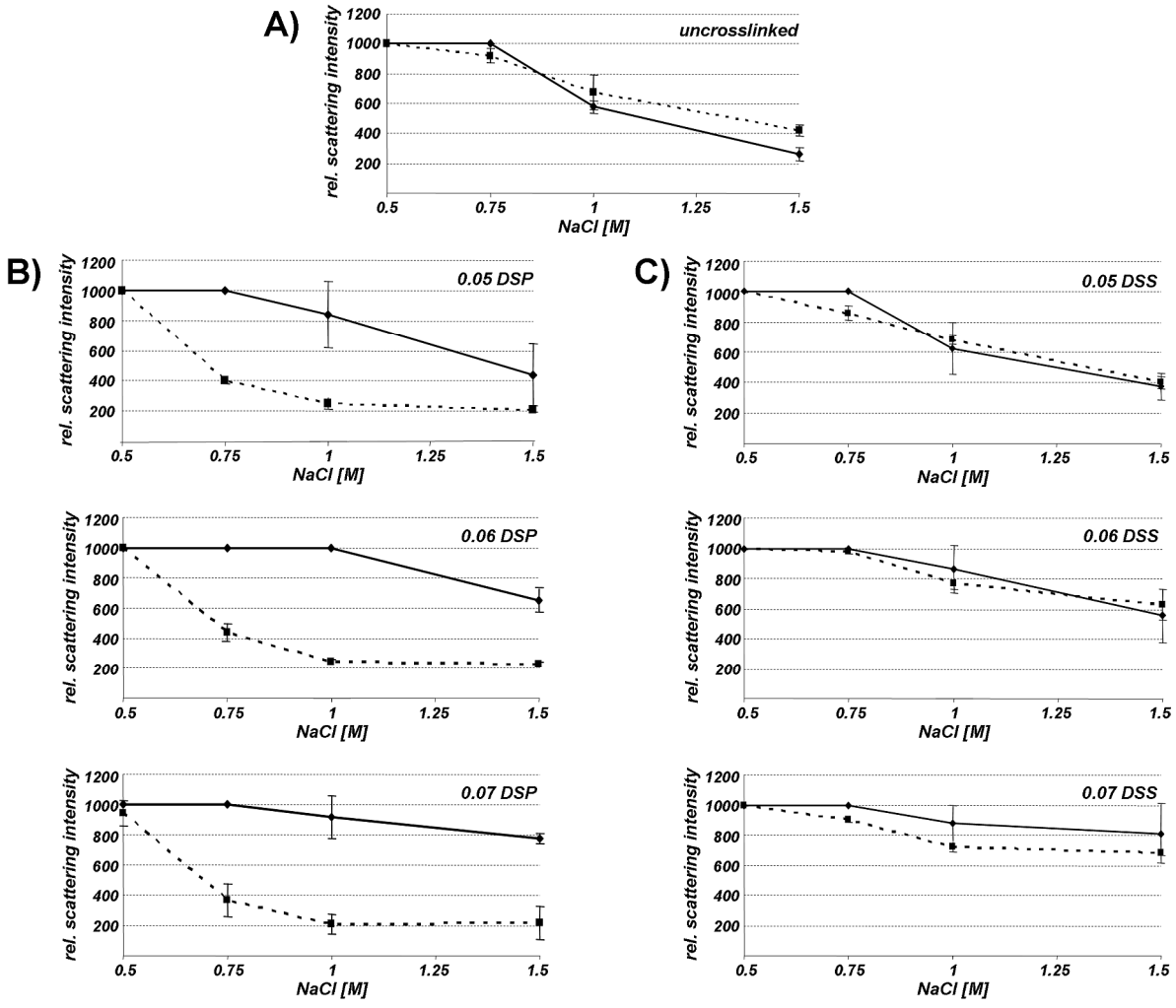

Fig. 34: Colloidal stability of DSP- or DSS-crosslinked HD O polyplexes in presence or absence of DTT. A) uncrosslinked HD O polyplex at a c/p-ratio of 1; B) DSP-crosslinked HD O polyplexes; C) DSS-crosslinked HD O polyplexes. The stability of polyplexes against dissociation with NaCl was monitored by detecting changes in the intensity of scattered light as a function of an increasing NaCl concentration. The susceptibility against NaCl in absence (solid lines) and presence of DTT (dashed lines) is presented. Values represent the mean ± SD out of three independent measurements.

Fig. 34 and **Fig. 35** show the intensities of scattered light of the polyplexes as a function of the sodium chloride concentration.

Using non-crosslinked HD O polyplexes (**Fig. 34A**) the scattering intensity decreased at a NaCl concentration of 0.75M upwards, suggesting dissociation of the complex to free DNA and polycation. But, when HD O polyplexes have reacted with either DSP or DSS at the different ratios of crosslinker / amine, a shift of the point of disassembly to higher NaCl concentrations was found. By this, it is quite obvious that due to the stabilization reaction, DNA cannot dissociate out of the polyplexes, because HD O became crosslinked upon the surface forming a continuous net surface network encaging the nucleic acid payload (**Fig. 34**).

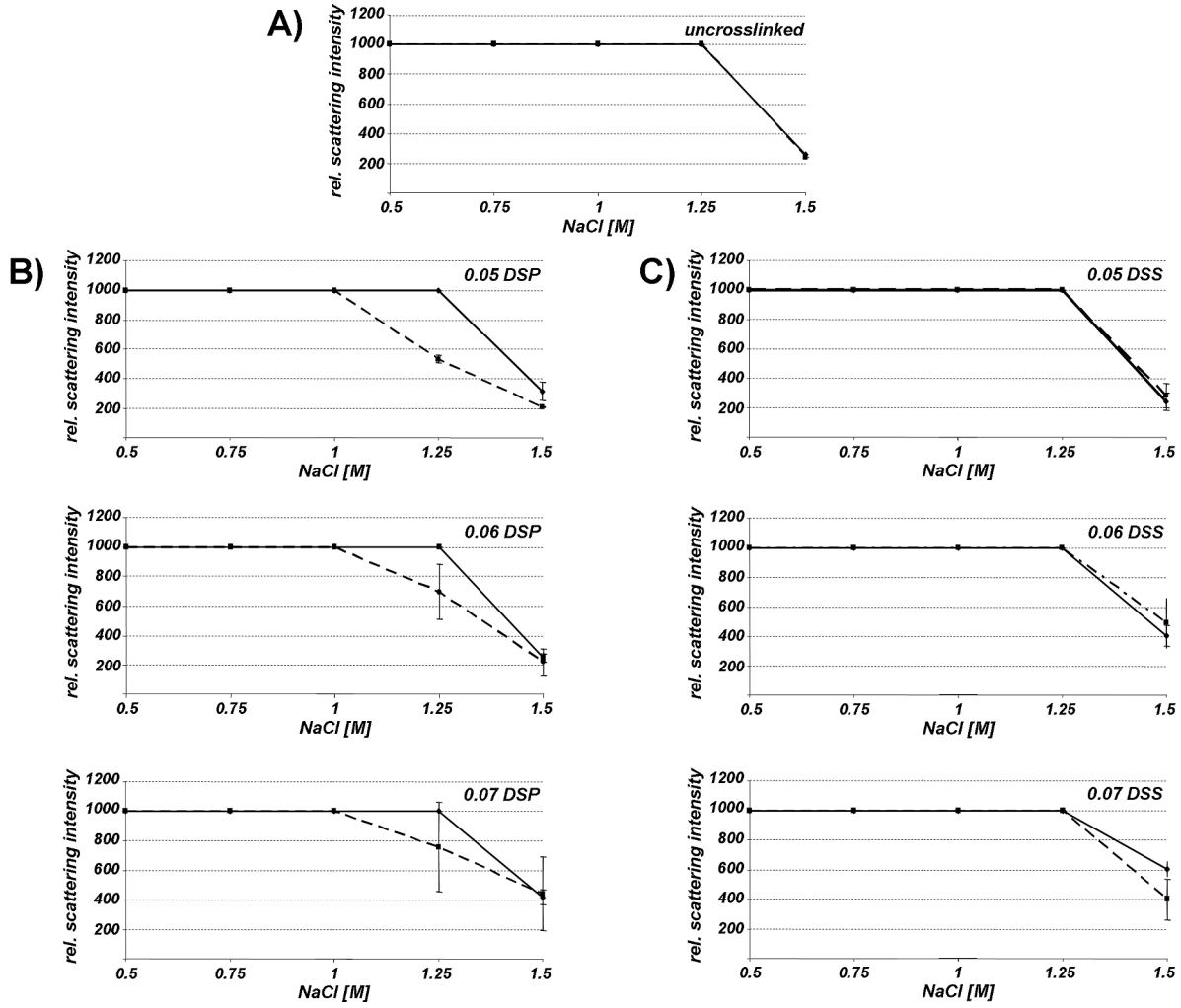

Fig. 35: Colloidal stability of DSP- or DSS-crosslinked BPEI polyplexes in presence or absence of DTT. A) Uncrosslinked BPEI polyplex at c/p-ratio of 0.8; B) DSP-crosslinked BPEI polyplexes; C) DSS-crosslinked BPEI polyplexes. The stability of polyplexes against dissociation with NaCl was monitored by detecting changes in the intensity of scattered light as a function of an increasing NaCl concentration. The susceptibility against NaCl in absence (solid lines) and presence of DTT (dashed lines) is presented. Values represent the mean ± SD out of three independent measurements.

In case of BPEI a higher colloidal stability was already found for the unstabilized complex as shown in **Fig. 35A**. Most probably this is caused by the in molecular weight larger BPEI molecule resulting in an increased strength of its electrostatic interaction with DNA and consequently tighter DNA binding. Thus, an obvious effect of crosslinking via DSP or DSS did not significantly enhance the colloidal stability of BPEI polyplexes in the investigated sodium chloride concentration range (**Fig. 35**). Although the results found after DTT incubation seemed to be contradictionary (**Fig. 35B** and **C**) since a decrease of the scattering intensity was detected, it can be assumed that during the crosslinking reaction using DSP or DSS the number of primary amine functionalities upon the surface of BPEI polyplexes was reduced. This decrease already led to a destabilizing effect of the polyplexes as electrostatic interactions for DNA compaction are diminished which gets obvious under the DTT reductive environment.

For compound HD O the reversible stability of the crosslinked polyplexes could be shown in these studies again using DTT to simulate a reducing environment. The course of both curves either in presence or absence of DTT was similar. Importantly, the incubation of DTT alone did not affect complex stability since uncrosslinked polyplexes of HD O showed the same pattern of scattering intensity in presence or absence of DTT (**Fig. 34A**). It was observed that in all cases of DSP-linked polyplexes a significant decrease of scattering intensity was found after DTT incubation (**Fig. 34B**). This can be attributed to a removal of the stabilization of the polyplexes due to the break-down of the disulfide bonds resulting in disassembly of the polyplexes. For comparison, the DSS-crosslinked polyplexes remained stable, which was indicated by the similar pattern of the curve progression in presence or absence of DTT (**Fig. 34C**). Hence, these results again strongly suggest that DSP surface stabilization can be removed under a reducing environment, and DNA could be released efficiently from the polyplex whereas DSS stabilization, in contrast, led to permanently stable polyplexes.

3.4.1.3 Effect of stabilization of polyplexes on reporter gene expression in vitro

It is important that the increased stability of the crosslinked polyplexes does not prevent transgene expression within the cells or within the target tissue. Therefore the consequences of lateral stabilization on both polyplexes, HD O versus BPEI were examined in four different cell lines. *In vitro* transfection studies were carried out as described above using pCMVLuc as reporter gene in K562 cells (human chronic myeloid leukemia), Neuro2a cells (murine neuroblastoma), B16F10 cells (murine melanoma) and PC3 cells (human prostate cancer). LPEI (22 kDa) polyplex (prepared in HBG) was used as positive control at the optimized c/p-ratio of 0.8 (corresponding to an N/P-ratio of 6).

Fig. 36 shows the transfection efficiency and cytotoxicity pattern of all DSP- or DSS-crosslinked polyplexes of both polycations. Generally, uncrosslinked HD O and BPEI particles resulted in similar good transfection efficiencies in all four investigated cell lines and exhibited reporter gene expression levels similarly high as found for the “golden standard” LPEI.

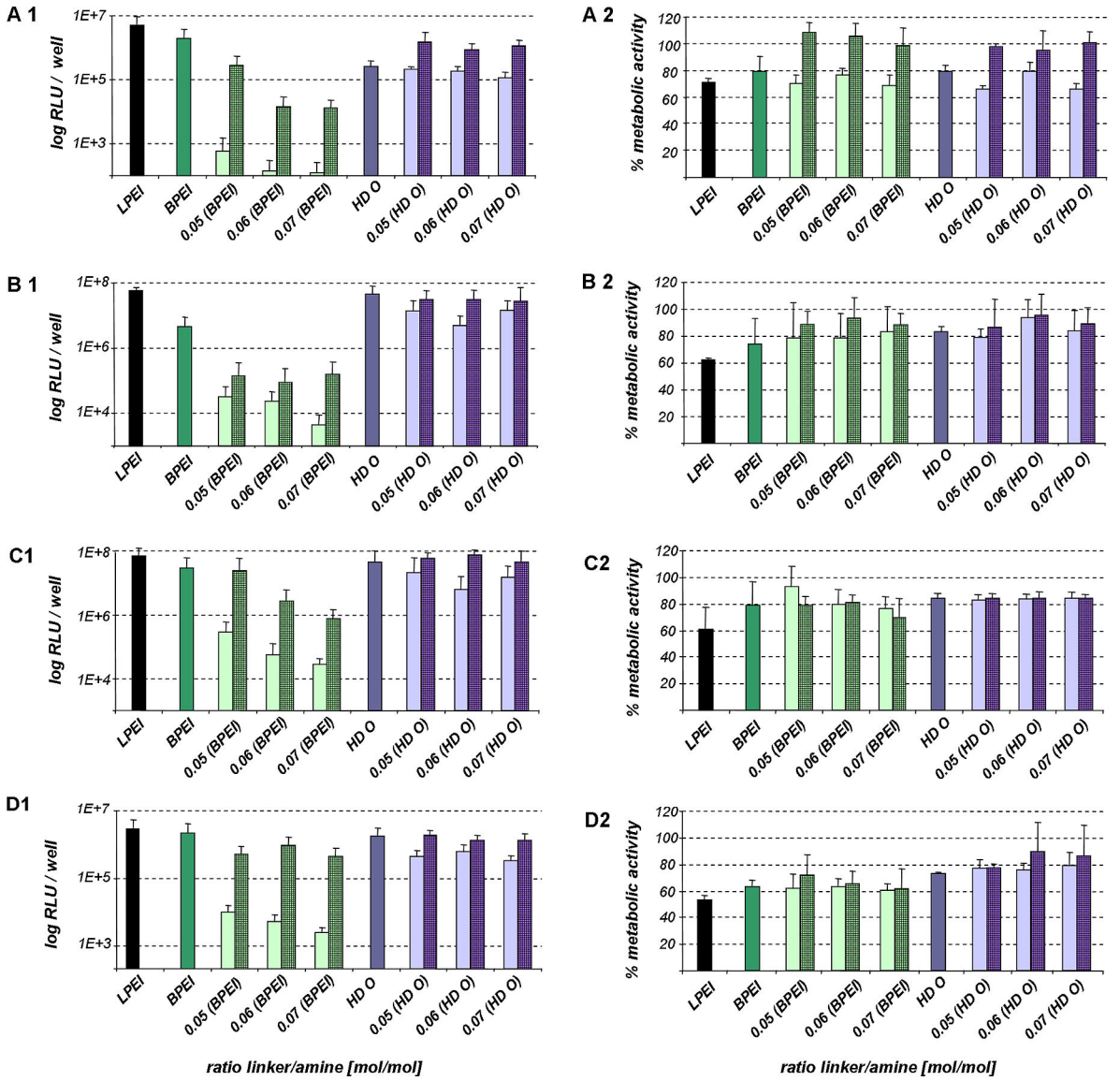

Fig. 36: Reporter gene expression of laterally stabilized BPEI (c/p-ratio 0.8) and HD O (c/p-ratio 1) polyplexes. A) K562 (125000 cells per well), B) Neuro2a (10000 cells per well), C) B16F10 (5000 cells per well) D) PC3 (5000 cells per well). Cell numbers indicated cells/well at the point in time of cell plating. Polyplexes were formed using the described procedure in HBG; LPEI was applied as positive control at the optimized N/P-ratio of 6 which represents a c/p-ratio of 0.8. Dark solid bars indicate uncrosslinked polyplexes; bright solid bars represent DSS-linked particles at indicated crosslinking degrees; crossbred bars indicate DSP-linked particles at indicated crosslinking degrees. On left side, transfection efficiency is expressed as RLU / well and shown as the mean (± SD) out of three independent measurements, each performed in triplicates. On the right side, metabolic activity of cells 24 h after transfection is demonstrated; this was performed in parallel experiments.

A distinct difference between HD O and BPEI regarding their transfection efficiency was detected in terms of the crosslinked particles: in case of BPEI, lateral stabilization of polyplexes always led to a significant reduction of transfection efficiency, whereas for HD O polyplexes lateral stabilization resulted in only marginal declines or even a slight enhancement of the transfection levels. Furthermore, for BPEI a dependency between the degree of crosslinking and the transfection level was found showing that the reporter gene expression decreased as a function of an increasing molar ratio of DSS or DSP. This is in good accordance to similar findings as previously described.[44,66,68] For HD O polyplexes such a dependency was not detected. Nevertheless, crosslinking complexes with the disulfide containing bioreducible DSP resulted in polyplexes with higher transfection activity than crosslinking them with non-degradable DSS. This finding might be explained – not only for HD O but also for BPEI – by the enhanced release of DNA due to destabilization of the polyplexes as a result of the disulfide break-down (see section 3.4.1.2). Notably, this effect was more pronounced for BPEI polyplexes showing that the DSP-crosslinked particles were between 10- to 100-fold more efficient than DSS-linked ones. In contrast, for HD O particles an only 5- to 10-fold improvement was found from DSS to DSP-crosslinked polyplexes. Importantly, in contrast to BPEI, HD O polyplexes that were crosslinked via DSP "restored" in the transfection efficiency levels to the same as found for the uncrosslinked particles all investigated cell lines. In some cases, like e.g. in K562 cells, the transfection efficacy was even around 10-fold enhanced for all three crosslinking degrees. However, it has to be considered that this can be also attributed to the improved cytotoxicity profile of the DSP-linked polyplexes compared to the DSS-linked ones in K562 cells. Generally, lateral stabilization of particles did not significantly influence the metabolic activity of cells (see **Fig. 36**, right panels).

To investigate whether the lateral stabilization of HD O polyplexes may have an influence on their transfection kinetics, luciferase gene expression levels were followed over time. **Fig. 37** shows the time course of gene expression after transfection using stabilized HD O c/p-ratio 1 polyplexes in Neuro2a cells crosslinked via DSP (**Fig. 32A**) or DSS (**Fig. 32B**) at the indicated molar ratios of crosslinker to amine. LPEI and BPEI polyplexes at their optimized c/p-ratio of 0.8 (representing an N/P-ratio of 6) were followed over time as control. The unstabilized HD O complex and also both LPEI and BPEI polyplexes resulted in a step by step decrease of reporter gene expression already 48 h post-transfection that was continued within the following days. Thus, transfection efficiency of the unstabilized HD O polyplex decreased more than 150-fold within the investigated time course of 24 h to 96 h post-transfection. In a similar manner, the transfection of LPEI and BPEI polyplexes decreased time-dependently even though the rough drop down as found for HD O between 24 h and

48 h post-transfection was not detected. This rapid decrease may be partially attributed to the ester degradability of HD O. In contrast, the stabilized HD O polyplexes, both DSP- or DSS-crosslinked, resulted in a permanent luciferase reporter gene expression over time that was kept at similar high levels as 24 h after transfection. Importantly, the bioreversibly crosslinked DSP polyplexes were more efficient and resulted in higher reporter gene expression levels than the DSS-linked compounds.

Taken together, these *in vitro* results highlighted i) the importance of using bioreversible crosslinking agents that provide an extracellular stability of the complexes followed by an enhanced release of DNA in e.g. reductive intracellular environment and ii) the advantage of lateral stabilization of the labile HD O polyplex, making this particle formulation very interesting for further investigations in terms of developing optimized polyplexes toward a "synthetic virus".

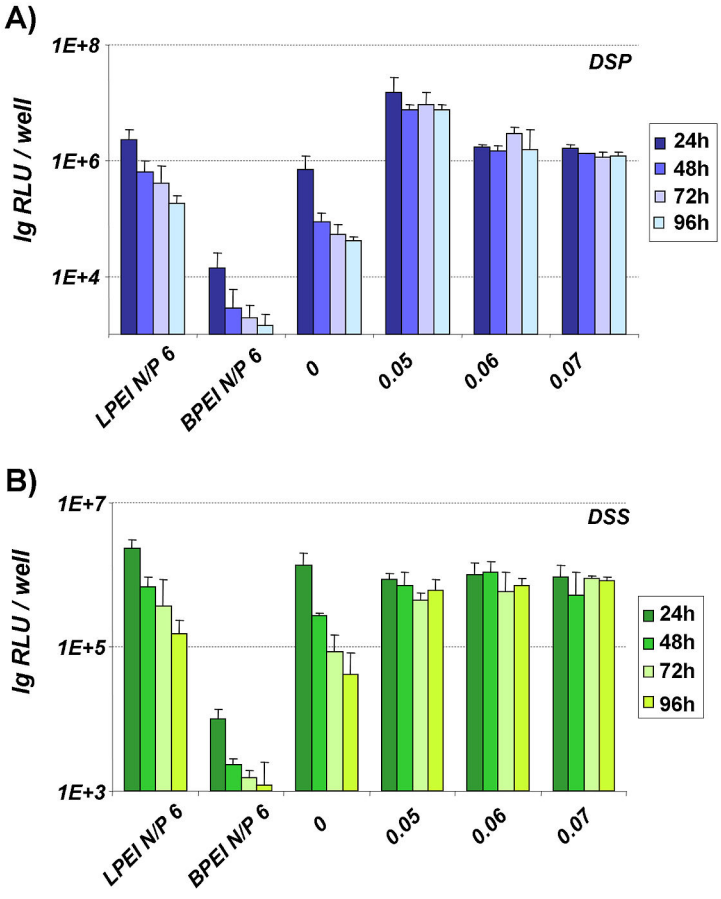

Fig. 37: Reporter gene expression levels in Neuro2a cells over time. Neuro2a cells were transfected with unmodified HD O at a c/p-ratio of 1 or stabilized polyplexes at indication crosslinker / HD O amine degree. The reporter gene expression level of DSP-crosslinked particles A) and DSS-crosslinked particles B) are shown. LPEI and BPEI polyplexes at their optimized N/P-ratio of 6 (c/p-ratio 0.8) were included for comparison. Polyplexes were prepared in HBG. Data expressed as mean values (± SD) out of three independent experiments each performed in triplicates. Two nanograms of recombinant luciferase correspond to 10^7 light units.

3.4.1.4 Effect of laterally stabilized polyplexes on gene expression after intratumoral administration

Due to the promising data found in the *in vitro* studies, the best performing laterally stabilized HD O polyplexes were further investigated for their *in vivo* gene transfer in tumor tissue. In these studies, HD O particles formed at a c/p-ratio of 1 were stabilized at crosslinking degrees of 0.05 using both DSP and DSS. The experiments were conducted by intratumoral injection into subcutaneous neuroblastoma tumors in A/J mice. Uncrosslinked HD O polyplex was used as reference for comparison. All particles were prepared at a final DNA concentration of 200 µg/mL (pEGFPLuc) in HBG. Mice receiving 100 µL polyplex solution per tumor showed no signs of toxicity.

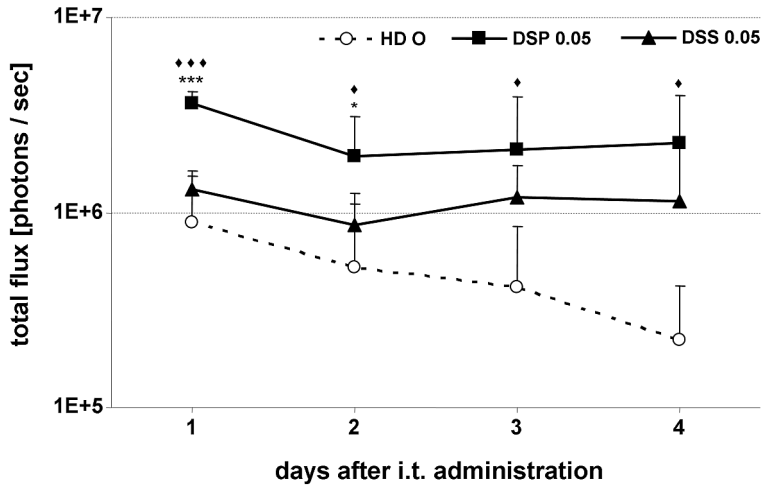

Fig. 38: Reporter gene expression of laterally stabilized and uncrosslinked HD O polyplexes *in vivo* after i.t. application. Measurements were conducted using the IVIS system for evaluation. Luciferase gene expression is presented as total flux (photons / sec) in tumor tissue (mean ± SD, n = 6 tumors per group). Tumor sizes ranged between 6 and 10 mm in diameter. HD O represents values for uncrosslinked HD O polyplexes (c/p-ratio 1); DSP 0.05 shows values for HD O (c/p-ratio 1) crosslinked via DSP at a molar ratio of DSP / HD O amine; DSS 0.05 shows values for HD O (c/p-ratio 1) crosslinked via DSS at a molar ratio of DSS / HD O amine.
(ANOVA, Duncan's test , *** $p < 0.001$ for DSS 0.05 vs. DSP 0.05; * $p < 0.05$ for DSS 0.05 vs. DSP 0.05; ♦♦♦ $p < 0.001$ for DSP 0.05 vs. HD O; ♦ $p < 0.05$ for DSP 0.05 vs. HD O)

Fig. 38 shows the reporter gene expression kinetics after intratumoral application of polyplexes. All polyplexes resulted in reasonable gene expression at day 1 post administration that was, in case of HD O (uncrosslinked), similarly high in tumor tissue as observed after intravenous injection of polyplexes into tumor bearing mice. In case of the laterally stabilized polyplexes it was found that both stabilization strategies led to elevated gene expression levels in tumor tissue, irrespective of time. Thus, while gene expression of unstabilized HD O polyplexes resulted in a half-life of about 2.5 days, both crosslinked particles showed a permanent gene expression over time being always 2- to 11-fold higher

than the unstabilized HD O polyplex ($p < 0.05$). Importantly, there was a significant difference of the gene expression pattern between DSP-, DSS-crosslinked polyplexes and unstabilized particles within the first 24 h. Thus, the bioreducible, disulfide linked polyplexes (DSP 0.05) resulted in significantly 4-fold ($p < 0.001$) higher gene expression levels compared to both DSS-linked (ratio 0.05) and uncrosslinked ones.

3.4.2 Transferrin (Tf)-targeting of lateral bioreversibly stabilized polyplexes

In previous investigations using transferrin (Tf) it was shown that the incorporation of this targeting ligand can increase the specificity of polyplexes toward tumor cells.[52,75,76] In preliminary studies following the standard protocol for preparation of targeted polyplexes by simply mixing e.g. Tf-BPEI with a polycation like LPEI, it was found in case of HD O that the resulting complexes led to huge particles that tended to aggregate in presence of salt (data not shown). This may end up in the disassembly of the polyplexes under physiological conditions, which is very undesirable with regard to both *in vitro* and *in vivo* application. To counter this, the formation of laterally stabilized HD O complexes as described above may provide a good opportunity for preparation of Tf-targeted HD O polyplexes that may remain stable under physiological conditions – being a prerequisite for systemic targeted gene delivery using HD O in terms of a "synthetic virus" system.

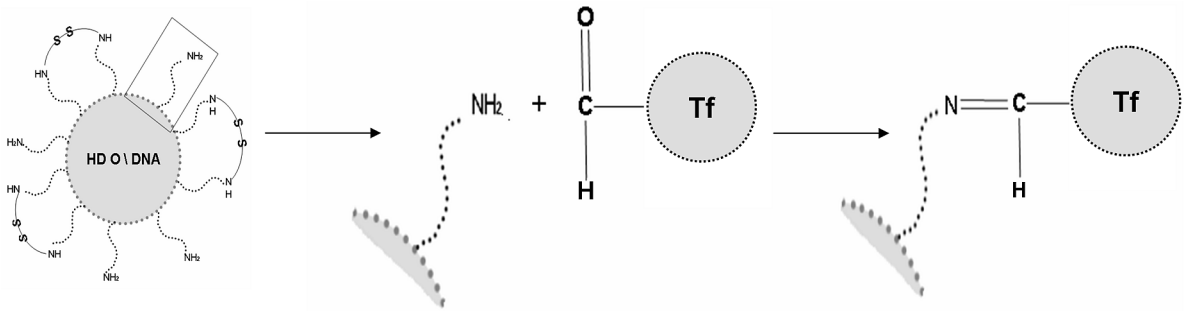

Fig. 39: Scheme for the incorporation of transferrin (Tf) as targeting ligand. DSP stabilized HDO / DNA particles are coated with oxidized transferrin. Due to an excess of primary amine bonds ever and anon meta-stable imine bonds are formed coating the particle.

3.4.2.1 Preparation and physicochemical characterization of Tf-targeted HD O polyplexes

A series of Tf-targeted HD O polyplexes was formed by addition of either oxidized or un-oxidized transferrin to stabilized HD O polyplex formulations (based on c/p-ratio 1) (see **Fig. 39**). In the following studies only DSP-crosslinked particles were investigated because of their more favorable *in vitro* characteristics (**Fig. 36**) as well as due to the promising *in vivo* data after i.t. administration (**Fig. 38**). After lateral stabilization of particles, 3.5% transferrin [w/w] (Tf / HD O) in its oxidized or unoxidized form (Tf_{ox} or Tf_{unox}) was added in order to coat the particles with the targeting ligand. In preliminary studies (data not shown) it was found that the incorporation of 3.5% transferrin resulted in optimized physicochemical properties for the investigated polyplexes. The oxidized form of transferrin contains aldehyde groups that may react with the remaining primary amino groups upon the DSP stabilized polyplexes to form imine bonds. Imine bonds are reversible in theory, but are assumed to be sufficiently stable in the current case because of the large local excess of polyplex amines. Hence, transferrin will be always “entrapped” by neighboring primary amines. In consequence, this may lead to small sized and also partially shielded particles. Unoxidized transferrin was used for control studies.

Table 8 summarizes the basic physicochemical characteristics of all studied polyplexes, based on DSP stabilized HD O polyplexes at crosslinking degrees of 0.05, 0.06 and 0.07. Interestingly, only the polyplexes that were adopted from stabilized particles in a crosslinking degree of 0.05 and 0.06 resulted in small particle sizes while a linker / amine ratio of 0.07 gave huge aggregates; Tf-coated HD O polyplexes (Tf_{ox} or Tf_{unox}) with a crosslinking degree of 0.05 and 0.06 resulted in particle sizes of 160 nm to 200 nm with a PDI ranging around 0.25. For comparison, the unstabilized HD O polyplexes resulted in all cases in aggregates, indicating the importance of lateral stabilization of LMW polyplexes prior to incorporation of the targeting ligand.

Importantly, there was a difference found regarding the measured zeta-potentials. Thus, only the oxidized form of Tf led to shielding effects (+14 mV) also indicating by this that the coating reaction using transferrin has worked, while Tf_{unox} did not reduce the detected zeta-potential as compared to non-targeted HD O complexes. In both latter cases zeta-potentials were ranging around +20 mV to +25 mV. Thus, although both forms of Tf can endow small particle sizes, shielding and steric stabilization can only be achieved using the oxidized form of transferrin.

particle formulation	mean diameter [nm]	zeta-potential [mV]
HD O / Tf_{ox}	agg.	24 *(± 1.2)*
HD O / Tf_{unox}	agg.	21 *(± 3.1)*
HD O / **0.05** / Tf_{ox}	180 *(± 45)*	14 *(± 2.2)*
HD O / **0.05** / Tf_{unox}	170 *(± 23)*	23 *(± 2.5)*
HD O / **0.06** / Tf_{ox}	200 (± 32)	14 (± 4.5)
HD O / **0.06** / Tf_{unox}	160 *(± 17)*	20 (± 2.9)
HD O / **0.07** / Tf_{ox}	agg.	24 *(± 4.3)*
HD O / **0.07** / Tf_{unox}	agg.	22 *(± 1.9)*

Table 8: Hydrodynamic diameters of Tf-targeted stabilized polyplexes. Complex sizes were determined in HBG using by dynamic light scattering at a DNA concentration of 10 µg/mL. For zeta-potential measurements polyplexes were diluted 1:5 with HBG to a final DNA concentration of 2 µg/mL. Mean values (± SD) out of three independent measurements are shown.

3.4.2.2 Biological characterization of Tf-targeted polyplexes

After determination of the transferrin receptors upon K562 and Neuro2a cells using FACS analysis (99% of Neuro2a cells and 89% of K562 cells were TfR positive), transgene expression levels using Tf-targeted HD O polyplexes were investigated in both cell lines. Transfection efficiency (luciferase assay) and cytotoxicity (MTT assay) of the formed particles were evaluated in parallel using pCMV-Luc as reporter gene (DNA concentration 20 µg/mL). Studies were performed in the presence of 10% fetal calf serum at 4 h interaction time of the polyplexes with cells. For LPEI, taken as reference in all experiments, a c/p-ratio of 0.8 was used (N/P-ratio of 6).

In these studies the effect of the incorporation of Tf (Tf_{unox} or Tf_{ox}) was investigated only using stabilized polyplexes at a crosslinking degree of 0.05 and 0.06 since the DSP / amine ratio of 0.07 resulted in aggregation after incorporation of Tf within polyplexes. Although the unstabilized HD O polyplex also “spiked” with transferrin showed aggregation these formulations were investigated for comparative studies.

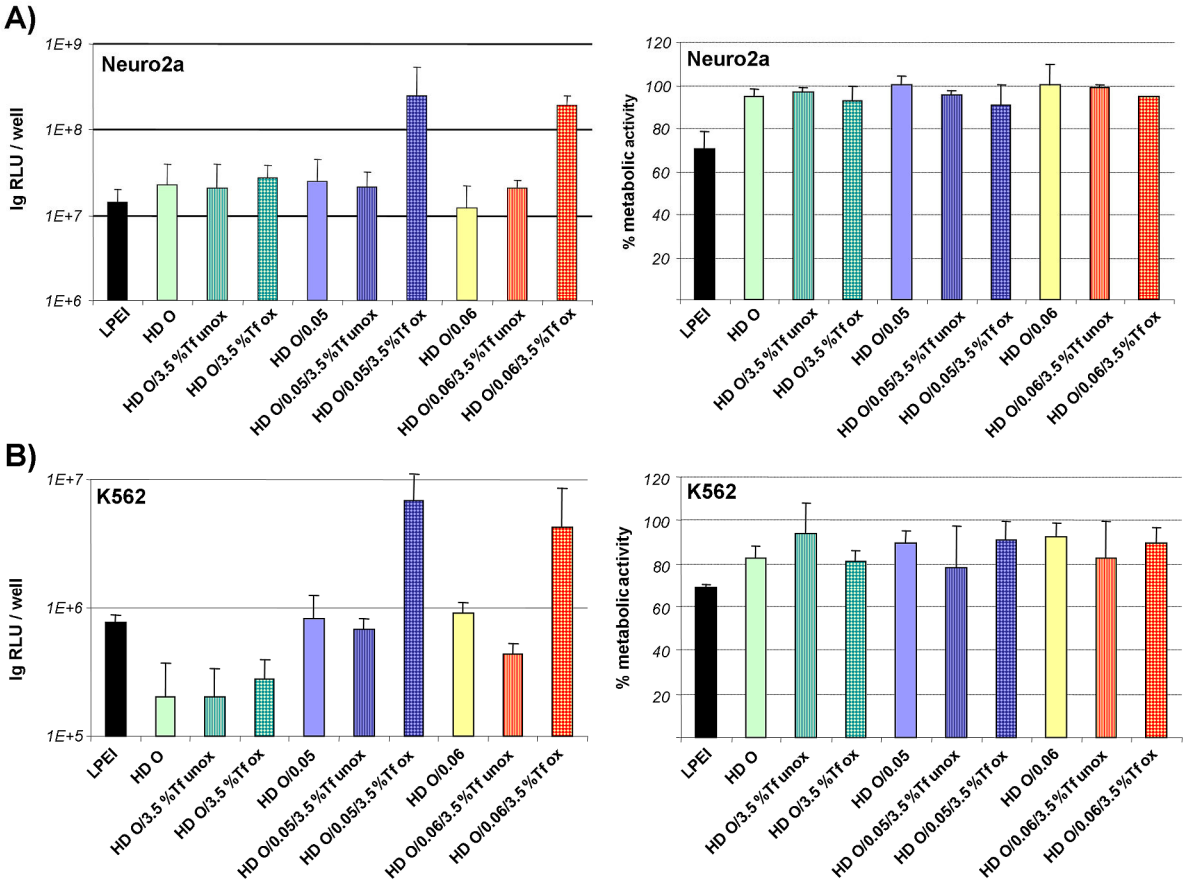

Fig. 40: TfR-targeting using different Tf-coated particles based on stabilized HD O polyplexes. Neuro2a A) and K562 B) cells were transfected with unmodified HD O or stabilized HD O polyplexes using the indicated oxidation forms of Tf. LPEI polyplexes at their optimized N/P-ratio of 6 (c/p-ratio 0.8) were included for comparison. Polyplexes were prepared in HBG. Data expressed as mean values (± SD) out of three independent experiments each performed in triplicates. Two nanograms of recombinant luciferase correspond to 10^7 light units. Metabolic activities after transfection as determined by MTT assay are shown in the right panels.

Fig. 40 depicts the reporter gene expression levels 24 h post transfection in Neuro2a and K562 cells. Transfection levels of unstabilized HD O polyplexes either un- or Tf-targeted (Tf_{unox} or Tf_{ox}) resulted in similar high reporter gene expression levels in both cell lines and thus no ligand-induced increase of transfection could have been detected; most probably this was due to aggregate formation followed by polyplex destabilization and disassembly during transfection. In case of the stabilized particles at a crosslinking degree of both 0.05 and 0.06, distinct differences with regard to the investigated Tf formulations were found. Thus, only if the oxidized form of transferrin was used for coating particles an enhanced transfection efficiency level could be found whereas the reporter gene expression level using Tf_{unox} remained similarly high as found for the untargeted stabilized polyplexes. The latter effect might have been also due to a rather unstable particle formulation upon Tf_{unox} addition leading to the same effect as described above for the unstabilized HD O particles.

Importantly, the described transfection pattern was detected in Tf-receptor expressing cell lines, Neuro2a and K562. Hence, in Neuro2a cells, an about 10-fold increase from HD O / 0.05 to HD O / 0.05 / Tf (3.5%) was found. Similarly, in K562 cells an 8-fold increase for the same formulations indicated enhanced transfection efficiency due to the transferrin receptor targeting effect. Notably, none of the investigated polyplexes induced any decrease of metabolic activity of cells after transfection making these formulations very interesting for further investigations in the context of the establishment of “synthetic viruses”.

4 Discussion

Gene therapy offers a promising tool for treating various forms of cancer and a number of inherent diseases although the clinical use is still mainly restricted due to the lack of safe *and* efficient gene delivery systems.[2] Synthetic gene carriers, including polycations, liposomes and polypeptides might be advantageous due to pharmaceutical issues such as low cost of synthesis and easy scale-up, but they show poor transfection efficiency levels in comparison to their viral counterparts.[77,78] Thus, an excellent transfection activity at low toxicity both *in vitro* and *in vivo* should be the major considerations regarding the design of novel degradable synthetic gene delivery devices. Recently, many of such polymers have been generated[35,37,79-87] aiming to reduce toxicity while maintaining transfection efficiency levels comparable to "golden standard" vectors like PAMAM dendrimers[41,88] or optimized polyethylenimines.[89,90]

4.1 Development of degradable pseudodendritic oligoamines

The aim of this thesis was to synthesize a novel class of biodegradable synthetic gene delivery carriers that show low toxic effects at gene expression levels comparable to commonly used standard BPEI and LPEI for *in vitro* and *in vivo* DNA gene delivery.

4.1.1 Synthesis and structural characterization of pseudodendrimers

Degradable random polymers based on low molecular weight (LMW) oligoamines were previously described by several researchers.[32,81,83,91-93] For example, promising results have been obtained[81,83] by coupling low molecular weight oligoethylenimine 800 Da (OEI) with short diacrylate linkages, thus combining the beneficial low cytotoxic properties of OEI with the superior transfection efficiency of higher molecular weight polyethylenimines. The hydrolysis of the ester bonds may occur under physiological conditions and the encouraging low toxicity found was correlated to this degradation behavior.[81,83] A technical weakness of this polymer approach was the polydispersity of the resulting compounds, i.e. the heterogeneity in molecular weight, which was inherent to the applied procedure in polymer synthesis. The design of dendrimers is an elegant solution to obtain well-defined polymers with narrow molecular weight distribution. As an example, PAMAM dendrimers have already successfully been applied for gene delivery.[39,41]

In the current work better defined low molecular weight pseudodendritic oligoamines were designed, starting with either oligoethylenimine 800 Da (OEI) or polypropylenimine dendrimers (PPI) as core building blocks. Different dioldiacrylates, which contain

hydrolysable ester bonds, were used to form dendritic branches and various oligoamines to modify the conjugates were added to generate a versatile family of pseudodendrimers. Although the OEI core based conjugates did not exhibit the typical dendritic structure based on a symmetric core unit, they followed the dendritic concept due to their branched organization, assembled by adding a generation of monomer onto a core unit.[38] An improvement toward better defined pseudodendrimers was achieved in the next development step by using the symmetric polypropylenimine dendrimer PPI instead of the randomly branched OEI as core building block. Synthesis procedures retained the same as for the OEI core based pseudodendrimers.

The compounds based on OEI were used for structure-activity relationship studies to define structural components within pseudodendrimers that lead to high transfection efficiency at low cytotoxicity. PPI core based pseudodendrimers were investigated to compare the influence of the dendrimer generation within the core unit on biophysical and biological properties of the resulting derivatives. Furthermore, the latter studies emphasized the importance of ethylenimine moieties within pseudodendrimers for efficient gene delivery.

All in all, 17 different OEI core based and 4 different PPI core based pseudodendrimers were synthesized. According to ^{1}H NMR analysis and GPC measurements, it was found that the applied synthesis procedure always resulted in branched, low molecular weight pseudodendrimers (4000 – 8000 Da) containing high rates of hydrolysable ester bonds (around 90% of ester bonds remained after synthesis and purification). Moreover, all conjugates showed a narrow molecular weight distribution with PDIs ranging between 1.5 and 2.5 for the most potent pseudodendrimers (see **Table 3** and **Table 5**) demonstrating the improvement toward better defined conjugates using the synthesis procedure established here.

4.1.2 Degradation behavior of pseudodendrimers

The degradability of gene delivery polymers to non-toxic monomers is desired in terms of developing suitable polyplex formulations for clinical application. Besides this, either a site-specific or time-dependent degradation leading to polyplex destabilization and an improved DNA release at the target site is worthwhile.

There is still a lack of feasible methods to proof degradation within the organism and therefore it became necessary to imitate the physiological environment (pH and temperature) *in vitro.* Several techniques to follow polymer degradation can be used like for instance size

exclusion chromatography (SEC)[83,94], mass spectroscopy (MS)[95] or nuclear magnetic resonance spectroscopy (NMR)[18,81].

HD O and the PPI core based pseudodendrimers were investigated according to their degradation behavior *in vitro* using ^{1}H NMR spectroscopy. In case of HD O, it was found that after about 3 days at physiological pH 7 and 37 °C approximately 50% degraded in aqueous medium and after about 7 days almost no residual ester bonds were found. Similarly, both oligoethylenimine grafted PPI dendrimers G2-HD-OEI and G3-HD-OEI resulted in a half-life of about 3 days. It was obvious that the OEI grafted derivatives, G2-HD-OEI and G3-HD-OEI, degraded much faster under physiological conditions than the polypropylenimine grafted ones, G2-HD-G2 and G3-HD-G2 ($t_{1/2}$ > 14 days). It is likely that the lower water solubility of the latter compounds due to the more hydrophobic G2 moieties upon the surface reduced ester hydrolysis, thus increasing their half-life. Altogether the found degradation behavior of the investigated pseudodendrimers is in good accordance to results reported by other groups concerning the degradation of poly(ester amine)s.[18,37,79,95]

4.2 Pseudodendritic oligoamines: what's needed for efficient DNA gene delivery – a study on the structure-activity relationship

In the described studies on structure-activity relationships using the various OEI core based pseudodendrimers, influences on biophysical and biological properties due to pseudodendritic core characteristics and the different surface modifications were found. Hence, these investigations gave an idea which features were essential to achieve satisfying physicochemical characteristics of polyplexes as well as of attributes needed to reveal highest transfection efficiency at lowest cytotoxicity.

4.2.1 Structural requirements of pseudodendrimers for suitable physicochemical characteristics

A key requirement for efficient gene transfer using synthetic gene delivery systems is their ability of DNA compaction to nano-sized particles suitable for efficient cell entry.

As the DNA compacting ability of polymers is strongly influenced by charge density, molecular weight[96] and hydrophobic/-philic interactions of the polycations with DNA, studies on the DNA binding ability of pseudodendrimers provided information about i) the general utilization of these conjugates as gene transfer devices and ii) the influences of both core characteristics and surface modification on DNA compaction. A suitable and fast method to determine this is the use of the ethidium bromide exclusion assay[65], which monitors

polycation/DNA interaction as a function of increasing c/p-ratios by following the decrease of fluorescence intensities.

DNA binding experiments to investigate the attributes needed for efficient nucleic acid compaction revealed that an increasing hydrophobicity of the pseudodendritic core plays an important role in DNA compaction. This is consistent with previous work by Zhong et al. who found that among their linear poly(ester amine)s investigated the most hydrophobic ones showed the best DNA binding ability.[95] Similar results were also detected by Zugates and colleagues in their studies on end-modified poly(beta-amino)esters.[97-99] Furthermore, it was obvious that an increasing number of nitrogens per coupled oligoamine on the pseudodendritic surface resulted in stepwise rising zeta-potentials, which consequently led to enhanced DNA binding and subsequent DNA compaction to nano-scaled polyplexes (see **Table 4** and **Fig. 9**). This effect was irrespective of the investigated pseudodendritic core and thus independent of core characteristics. However, these investigations also demonstrated that the surface modification of the pseudodendrimers containing more than 18 nitrogens per modification unit were able to mask the influence of core characteristics as it was demonstrated for HD O and compounds like HD O1200 and HD O1800 (data not shown).

Hydrodynamic diameters of polyplexes were determined using dynamic light scattering (DLS). All OEI core based pseudodendrimers, except for the ethanolamine surface modified ones, resulted in small complex sizes that were ranging between 100 nm and 250 nm. PDIs demonstrated a low- to mid-ranged polydispersity of the resulting complexes. Hence, all the polyplexes were small enough and suitable for efficient cellular uptake by endocytosis.[100]

4.2.2 Structural requirements of pseudodendrimers for optimized biological activity

Reduced toxicity is undoubtedly one of the most important issues with regard to *in vitro* and *in vivo* application of synthetic gene carriers, but at the same time high transfection efficiency levels should be achieved. *In vitro* studies in different tumor cell lines showed that toxic effects and transfection efficiency levels of pseudodendrimers were based on both pseudodendritic core characteristics *and* the properties of the surface oligoamine used. Hence, decreasing pseudodendritic core hydrophobicity from HD to BD to ED core led to reduced cytotoxicity (see **Fig. 10**) but in general also to significantly decreased transfection efficiency that was detected for these compounds (**Fig. 15**). Consequently, it can be concluded that for efficient DNA gene delivery using pseudodendrimers a hydrophobic core domain is essential. This probably facilitates cellular uptake due to hydrophobic interactions with the cell membrane[98] and may also be beneficial for endosomal release of the

polyplexes. Therefore, for endo-/lysosomal escape other factors besides the buffering capacity of the containing oligoethylenimine ("proton sponge")[13] that include hydrophobic interactions with endosomal membranes[101] or the cationic nature of the polymers themselves[102-104] may play an important role in mimicking the membrane-disruptive properties of viruses or natural lytic agents.[105] According to the influences of the surface modification unit on cytotoxicity, it was observed that an increasing number of nitrogens per coupled oligoamine led to an increased toxic effect if plain conjugates were used as demonstrated in **Fig. 11**. There, it was shown that with an increasing number of nitrogens per coupled surface modification unit, cytotoxicity increases which might be due to unspecific interactions of amines with cell membranes. In contrast, if complexed with DNA, pseudodendrimers showed a different behavior since direct interactions were "hampered" by the condensed DNA and the characteristics of the resulting polyplexes determined cytotoxicity and transfection efficiency (**Fig. 15**). Thus, stepwise enhanced transfection efficiency with an increase of nitrogens per surface modification unit was observed for ED and HD core conjugates whereas BD core conjugates showed no direct correlation (also not in a c/p-ratio dependent manner). Furthermore, the different surface amines of HD (but not ED or BD) core conjugates did influence cytotoxicity. All these facts indicated that both the pseudodendritic core characteristics along with the surface modification within polyplexes play a determining role for high transfection activity at low cytotoxicity as emphasized in the HD core conjugate line. Hence, for example, in Neuro2a cells, cytotoxic effects increased from E to Sp to S and then decreased again for the OEI surface modified conjugate, while transfection efficiency levels increased step by step (**Fig. 15**). Apparently, although a hydrophobic HD core, which was essential for high transfection levels displayed toxic effects, was used, cytotoxicity of the total polyplexes seemed to be "tunable" by the surface oligoamine. In order to study this phenomenon in more detail, further surface oligoamines upon the HD core were investigated (**Fig. 16**). As shown, both cytotoxicity and transfection levels depended on an optimized balance between hydrophobic and hydrophilic domains within the pseudodendrimers. On the one hand, a more hydrophobic structure like HD TETA led to very high cytotoxicity at low transfection efficiency, similarly HD S exhibited rather high cytotoxicity but interestingly also high transfection levels were found (presumably due to better protonation and DNA binding of S over TETA). On the other hand, a purely hydrophilic structure such as the hydrophilic ED core conjugates resulted in low cytotoxicity but also moderate transfection levels. However, starting within the hydrophobic HD core line by increasing the number of hydrophilic ethylenimine units in the surface modification (TETA < PEHI < O < O1200 < O1800), the toxicity was reduced stepwise while at the same time gene transfer increased to an optimum level when O (i.e. oligoethylenimine 800 Da, OEI) was used as surface modification unit.

In sum, it can be considered that for efficient DNA gene delivery using pseudodendrimers a hydrophobic core domain is needed but it has to be in an optimal balance to the hydrophilic surface modification used. Finally, “tuning” of the pseudodendritic structures toward HD O resulted in a substantial increase of transfection efficiency at low cytotoxicity, defining HD O as most potent OEI core based pseudodendrimer under *in vitro* conditions. It exhibited efficient DNA condensing ability to nano-sized polyplexes (100 – 200 nm), low toxicity and transfection levels that were similar to high molecular weight standard PEIs like BPEI and the “golden standard” LPEI. Encouraging reports within recent years highlighted the great potential of degradable OEI derivatives[35,37,79,81,106] while an improvement toward better defined structures was done by synthesizing pseudodendritic OEI containing structures.

However, OEI is a randomly branched structure and not perfectly symmetric. For this reason the next step was to further improve the structural composition of pseudodendrimers toward more symmetric, more dendrimer-like structures as it could be achieved by the synthesis of PPI core based pseudodendrimers.

4.3 PPI core based pseudodendrimers – a dendritic upgrade

The structure–activity study within the OEI core based pseudodendrimers as described above elucidated what is structurally needed to achieve high transfection levels at low toxicity using pseudodendritic oligoamines: a hydrophobic core domain that has to be in an optimized balance to the pseudodendritic surface modification. In order to obtain a better defined core unit than the randomly branched OEI, the studies were extended toward polypropylenimine (PPI) dendrimers as core moiety. To compare influences of different dendrimer generations within the core unit on physicochemical and biological properties, generation 2 (G2) and generation 3 (G3) PPI dendrimers were grafted using HD as biodegradable linker. In order to define differences in terms of biophysical or biological activities between *ethylen*imine and *propylen*imine moieties, either OEI or PPI G2 were used as surface modification units.

4.3.1 Improved physicochemical properties of grafted PPI core based pseudodendrimers

The capability of grafted G2 and G3 core dendrimers to complex DNA was investigated using an ethidium bromide exclusion assay (see **Fig. 22**). All grafted dendrimers showed a very good DNA condensing ability already at low c/p-ratios if HBG (a low ionic strength buffer) was used for complex formation. This is in good accordance to the findings made within the

OEI core based pseudodendrimers where studies showed that both the hydrophobic HD core unit as well as a high number of nitrogens upon the pseudodendritic surface is beneficial for good DNA binding. However, DNA binding studies of HD O in presence of physiological sodium chloride concentrations revealed that its DNA binding capacity was dramatically reduced in presence of salt (resulting in rel.$F._{min}$ of around 40%). Akin, but less pronounced outcomes were also found for all PPI core based pseudodendrimers (rel.$F._{min}$ of 20 – 30% in HBS) similarly it was also found for standard polyethylenimines like LPEI (22 kDa) or BPEI (25 kDa) and several other polycationic gene delivery vectors.[37] This indicates that the colloidal stability of the investigated polyplexes is obviously affected under physiological conditions, which is undesirable for future *in vivo* application of polyplexes. Some solutions regarding this problem topic have already been suggested[43,44,74] and will also be discussed for the weakest of the investigated polyplexes, the HD O polyplex at a c/p-ratio of 1, in the last section of this thesis.

To clarify in which extent the stability of grafted PPI polyplexes was improved the colloidal stability of complexes was investigated. For this, the susceptibility of polyplexes for dissociation to free DNA and polycation as a function of the amount of sodium chloride was determined via light scattering. Light scattering is a convenient procedure to measure complex formation or complex dissociation since scattered light intensity is dramatically increased by complex formation, especially if they aggregate, compared to free DNA and polycation where scattering intensity is low.[66] With regard to the grafted dendrimers it was found that the complexes were not as susceptible to sodium chloride induced dissociation as compared to their unmodified counterparts (see **Fig. 23**). This was either demonstrated by the shift of the point of disassembly to higher salt concentrations and / or the minimum of scattering intensity. However, a significant influence of the dendrimers´ generation on the colloidal stability of polyplexes could only be found with regard to the unmodified dendrimers G2 and G3 while this effect was not as pronounced in case of the grafted compounds. Importantly, all the derivatives that were grafted by polypropylenimine dendrimer G2 were found to be less sensitive to sodium chloride induced polyplex dissociation than the OEI grafted ones. It can be assumed that this was due to their higher hydrophobicity caused by the incorporation of *propylen*imine (C3) instead of *ethylen*imine (C2) moieties upon their surface, resulting in lower water solubility and therefore minimizing the possibility of salt molecules to interfere with the electrostatic interactions between DNA and polycation.

Generally, measurements of hydrodynamic diameters in HBG and HBS underlined the findings described above. With the presence of salt, complex sizes of grafted dendrimers did not change while unmodified G2 and G3 dendrimers aggregated under these conditions. This again indicated an improved colloidal stability of grafted dendrimers over unmodified

ones; hence, polyplexes of all grafted dendrimers remained stable without aggregate formation also in presence of physiological salt concentrations.

4.3.2 The necessity of ethylenimine moieties for enhanced biological activity

In vitro transfection studies of PPI core based dendrimers demonstrated that grafting of generation 2 or generation 3 core units using either polypropylenimine or oligoethylenimine moieties upon the surface obviously led to enhanced transfection efficiency. In preliminary studies (data not shown) this effect was not only found if HD was used as degradable linker but also if BD or ED were used.

Concerning the comparison of G2 and G3 core derivatives due to influences of the dendrimers´ generation on transfection efficiency it can be assessed that the higher generation within the core unit of the derivatives did not enhance transfection efficiency *in vitro.* This demonstrates that the level of transfection is obviously limited (G2-HD-OEI ↔ G3-HD-OEI). It is likely that this effect can be explained by the increased stability of G3 core over G2 core derivatives under physiological conditions leading to a reduced intracellular disassembly of polyplexes. Furthermore with regard to the comparison of the different dendrimers´ generations, differences in the resulting cytotoxicity profile of G3 core based derivatives were found as compared to G2 core ones. These might be explained by the increased overall hydrophobicity of the polyplexes from G2 core to G3 core units causing an improved transfection / toxicity relation (G2-HD-G2 ↔ G3-HD-G2) which is in line with the results described above, i.e. likewise it was found in structure-activity studies of the OEI core based pseudodendrimers.[18] There it was described that the balance of hydrophilic and hydrophobic domains within polyplexes must be optimized in order to obtain high transfection levels at low cytotoxicity.

The higher potency of OEI grafted dendrimers over G2 grafted derivatives, as e.g. demonstrated by comparing G3-HD-G2 and G3-HD-OEI (see **Fig. 24**) may be explained by an enhanced endosomolytic activity based on the proton-sponge effect[13,70] due to the incorporated oligo*ethylen*imine moieties. In order to investigate this in more detail, luciferase gene transfer experiments in the absence or presence of bafilomycin A1 were performed (see **Fig. 25**). Bafilomycin A1 specifically inhibits the vacuolar type of H^+-ATPases by blocking the endo-/lysosomal proton pump being responsible for the reported decrease of polyethylenimine-mediated transfection.[71] The results indicated that, very similar to LPEI and BPEI, the OEI grafted PPI core based compounds experienced a significant decrease of luciferase activity in presence of bafilomycin A1 in two investigated cell lines while in contrast

the polypropylenimine grafted compounds were not affected. Hence, grafting PPI dendrimers with OEI significantly improved their transfection efficiency. Importantly, reporter gene expression levels were similarly high as obtained with LPEI and BPEI. Related results based on the incorporation of oligoethylenimine moieties upon α-cyclodextrin have been recently reported by Yang et al., showing that the transfection efficiency levels of their star polymers increased with the length and the branching degree of the OEI arms. The reported best transfecting polymer was modified upon the surface with an oligoethylenimine resembling OEI 800 Da.[107]

Thus, the results found regarding PPI core based derivatives underline the importance of an optimized balance of hydrophilic and hydrophobic domains within pseudodendrimers and furthermore emphasize the necessity of ethylenimine moieties within conjugates in order to achieve highest transfection efficiency levels. Taken together, all these findings *in vitro* resulted in a couple of promising compounds for *in vivo* studies in mice.

4.4 *In vivo* reporter gene expression studies of pseudodendrimers

By structure-activity studies i*n vitro*, it was revealed that for efficient transfection an optimized balance of hydrophobic and hydrophilic domains within pseudodendritic oligoamines has to be achieved. Hence, a couple of potent DNA gene delivering agents were defined within both the OEI core and the PPI core based pseudodendrimers. These most promising conjugates were tested for i) their biocompatibility followed by ii) investigations on their *in vivo* gene transfer activity in a subcutaneous (s.c.) tumor model after intravenous (i.v.) application of the polyplexes.

4.4.1 Biocompatibility of polyplexes based on pseudodendrimers

After injection of positively charged particles into the bloodstream it is likely that interactions with several blood components including erythrocytes occur that lead to undesired side effects.[15] For instance, it was found that after i.v. application of LPEI polyplexes a polymer induced erythrocyte aggregation occurred that in some cases also resulted in lethality of mice; most probably due to inclusion of agglutinated erythrocytes in small capillaries of the lung.[15] Furthermore, as an anaemic status of the patients is also not desired, hemolytic activity of polymers or polyplexes should be avoided and should be tested prior to an *in vivo* application.

In order to circumvent unexpected lethality of mice due to acute toxic side effects, the newly designed and most promising *in vivo* candidates were tested for their hemocompatibility prior to the study in mice. To get a rough estimate, first the degree of hemolysis of erythrocytes after incubation with either the plain polymers or the polyplex formulations was tested. Remarkably, it was detected that in all cases the trends and patterns as found in cytotoxicity studies were also discovered here. Hence, similar to the findings depicted in **Fig. 10** and **Fig. 11**, pseudodendritic core characteristics and/or the surface modification influence hemolytic effects. Thus, it can be assumed that accompanied by an increasing hydrophobicity of the conjugate, its “intrinsic membrane activity” was also enhanced while in contrast more hydrophilic compounds showed no cell membrane destabilizing effect. Within the PPI core based pseudodendrimers the same trends emphasize these findings. Importantly, it was found for all pseudodendrimers that if the resulting polyplexes were investigated instead of the plain polymers the hemolytic activity was dramatically reduced.

Although a direct correlation between the cytotoxicity of pseudodendrimers and their hemolytic activity can obviously be drawn, the polymer induced erythrocyte aggregation has to be investigated separately. As it was already shown in case of LPEI or BPEI, it cannot be assumed that a low hemolytic activity is also accompanied by a missing polymer induced erythrocyte aggregation and vice versa.[15] However, according to microscopic investigations none of the newly synthesized polymers induced any erythrocyte aggregation. Notably, the investigated polymer concentrations were much higher as they would ever be applied in *in vivo* studies. Consequently, acute toxic side-effects due to agglutinated erythrocytes can rather be excluded for all pseudodendrimers.

However, in terms of investigating the biocompatibility of novel pseudodendrimers it has to be kept in mind that alongside a low acute toxicity, a negligible long-term toxicity is also desired. Therefore, degradation studies of the most promising *in vitro* candidates were performed. It was found that all pseudodendritic oligoamines are degradable with a half-life of about 3 days while the best performing OEI core based conjugate HD O was obviously completely degradable to OEI monomers and the resulting alcohol. Furthermore, all degradation products were non-toxic in cell culture and did not exhibit any toxicity of Neuro2a cells after treatment with metabolites (data not shown).

All pseudodendritic oligoamines passed the *in vitro* tests that were performed prior to a first *in vivo* administration. There, the acute toxicity of mice after application of 50 µg/mL polymer per 20 g mouse was tested. The pseudodendrimers that were interesting for further *in vivo* investigation due to encouraging *in vitro* transfection results were: HD S, HD O, G2-HD-OEI, G2-HD-G2, G3-HD-OEI and G3-HD-G2. HD O, G2-HD-OEI and G3-HD-OEI were well

tolerated after i.v. application of plain polymers and no acute toxic effects were found. In case of HD S, G2-HD-G2 and G3-HD-G2, the application of plain polymer resulted in lethality of mice and also as polyplex formulations those compounds were poorly tolerated. After i.v. administration, a pronounced vasodilatation was found in all organs, most probably due to the intrinsic membrane destabilization activity of the polymers as described above. Apparently, also if applied in a polyplex formulation, this characteristic still had a distinct impact under *in vivo* conditions in case of HD S, G2-HD-G2 and G3-HD-G2. Importantly, those effects were not found for any of the other pseudodendrimers.

Taken together, out of all performed *in vitro* studies including the first *in vivo* toxicity study, three promising pseudodendritic oligoamines remained for testing their transfection efficiency in mice using an s.c. neuroblastoma tumor model - these were: HD O, G2-HD-OEI and G3-HD-OEI.

4.4.2 Predominant gene expression in tumor tissue using pseudodendrimers

Since HD O, G2-HD-OEI and G3-HD-OEI exhibited low acute toxicity in mice, they were investigated in the following studies for their *in vivo* gene transfer ability in tumor bearing mice. As references, the well investigated “golden standard” polycations LPEI and BPEI were used. LPEI is known to reveal highest transgene expression levels in mice with gene expression mainly found in the lung after i.v. application being also responsible for acute toxic effects.[17,72] BPEI was used to compare the differences in gene expression levels between branched and linear high molecular weight structures. Transgene expression after BPEI complex application was only found in tumor, liver and lung whereas, consistent to previous work, BPEI led to a significantly lower gene expression in the lung than LPEI.[108]

Mice receiving polyplexes based on pseudodendrimers showed no signs of acute toxicity after i.v. application, thus they were all well tolerated. With regard to the resulting transfection pattern found after systemic administration it can be concluded that all pseudodendritic oligoamines, i.e. HD O, G2-HD-OEI and G3-HD-OEI, resulted in reporter gene expression which was predominantly found in tumor tissue at similar high gene expression levels as the LPEI. Importantly, it has to be emphasized that at a c/p-ratio of 1 in all cases a much lower luciferase gene expression was found in the lung than in tumor tissue and negligible gene expression could be determined in all other investigated organs. As reporter gene expression levels in tumor tissue were similarly high as those obtained by LPEI, the “golden standard”, the finding of an increased tumor-specific reporter gene expression level after i.v.

administration is very worthwhile. The reasons for the differences between the transfection pattern of LPEI and the established pseudodendrimers remain to be clarified in detail. It could be suspected that due to the lack of polymer induced erythrocyte aggregation as found for the pseudodendrimers, they do not stick in lung or liver tissue like e.g. HMW PEIs. In consequence they are able to deliver the nucleic acid payload toward the tumor site due to most probably passive tumor targeting ("EPR-effect")[47]. Nevertheless, it has to be taken into account and clarified that reporter gene expression level may not be in direct correlation to the body distribution pattern, as previously described.[51,109] Thus, in the current case, positive charges of polyplexes cannot be the sole explanation for the observed gene expression patterns since all polyplexes of pseudodendrimers at c/p-ratio 2 still showed a more favorable tumor / lung relation as compared to LPEI, although best tumor / lung ratios (T/L) were obtained when using c/p-ratio 1.

Furthermore, the study within PPI core based pseudodendrimers demonstrated a clear influence of the dendrimer generation within the conjugates upon transfection efficiency *in vivo*. For G3-HD-OEI polyplexes, gene expression levels in tumor tissue were 12-fold higher ($p < 0.05$) as compared to G2-HD-OEI at c/p-ratio 1. It can be hypothesized that this effect is either due to the better biophysical properties and improved colloidal stability of G3-HD-OEI vs. G2-HD-OEI, or due to the increase of positive charges of the polyplexes, or a combination of both factors.

HD O, G2-HD-OEI and G3-HD-OEI were all well tolerated by mice and no significant loss of body weight was observed if a c/p-ratio of 1 was applied (5 – 6% BWL after 24 h) Histopathologic investigations on liver tissue (HD O; see **Fig. 20**) underline the findings, since no changes in liver histology compared to buffer treated mice have been found as well as no increase of liver enzyme levels (AST, ALT and AP) were detectable. The same was shown after application of polyplexes using the PPI core based pseudodendrimer (see **Fig. 19** and **Fig. 29**). In contrast to this, LPEI and BPEI complexes always resulted in an increase of liver enzyme levels indicating beginning necrotic changes in liver tissue already after 24 h, which is consistent with literature and as demonstrated in **Fig. 20**.[17,110] The reasons for the better *in vivo* tolerability of the pseudodendritic polyplexes may be explained besides their degradability and good biocompatibility as described above due to obvious structural differences including the lower molecular weight compared to standard LPEI or BPEI. Within the PEI class of polymers, a lower molecular weight has previously been found to provide better biocompatibility.[111,112]

All in all, these *in vivo* studies reveal that plain pseudodendrimer / DNA polyplexes (without further functional modifications) have the potential for an increased specific transfection of

tumor tissue at levels comparable to those of LPEI and exhibit significantly reduced transgene expression in the lung (T/L-ratio (G3-HD-OEI): 89 vs. T/L-ratio (LPEI): 0.01 – 0.001). Moreover, all the investigated pseudodendrimers were well tolerated by mice exposing best biocompatibility properties.[18]

4.5 Optimization of polyplexes toward "synthetic viruses"

As the success of gene therapy is highly dependent on the delivery system, research has focused on the development of gene transfer devices suitable for highly potent systemic nucleic acid delivery. Although many polymeric carriers have been optimized *in vitro* to overcome several cellular hurdles, there are many barriers that have to be resolved in order to achieve successful, i.e. long-lasting and highly efficient, *in vivo* gene delivery. Unfortunately, most of the existing vectors are currently – independent of their toxicity profile – unsuitable for systemic administration due to rapid elimination from the bloodstream combined with unspecific interaction with and accumulation of particles in organs and tissue.[15,74,113,114] Investigations to reduce those effects, e.g. by using steric stabilization procedures (PEGylation) to create so called "stealth vectors", were extensively performed in order to achieve long-circulating polyplexes, thereby reducing additional unspecific interactions with non-target sites. However, in these studies it was also found that it is likely that this strategy in some cases also leads to labile polyplexes under physiological conditions, resulting in disruption of the polycation / DNA particles due to reorganization of the polyplexes caused by protein interaction or high salt concentrations.[42]

Several groups reported for the last years that they countered this by successfully increasing polyplex stability by e.g. lateral stabilization of particles.[43,44,68,73,74] Therefore, the surface molecules of polyplexes were crosslinked in order to prevent their dissociation. Furthermore, stabilized polyplexes may offer an attractive stable platform for subsequent modifications.

4.5.1 Lateral stabilization for improved gene expression of HD O polyplexes

Taking into account that the polymers developed in this thesis resulted in LMW polycations which also exhibited a lower charge density compared to HMW polycations like e.g. BPEI, it could be assumed that their colloidal stability under physiological conditions is reduced. Similar effects were already demonstrated for PLL polymers.[26] In fact, in pilot studies a decreased colloidal stability under physiological conditions was found for HD O / DNA complexes at a c/p-ratio of 1. Hence, as a first step toward an improved formulation for

systemic administration, the stability of HD O polyplexes was increased by employing a lateral stabilization strategy. Nevertheless, it has to be kept in mind that the gained stability of those complexes may present a double-edged sword: since the extracellular stability of the polyplexes is beneficial with regard to their pharmacokinetic properties on the one hand, the undesired rigidity in intracellular environment on the other hand may result in a decreased unpackaging ability of DNA leading to lower transfection efficiency levels. To overcome this discrepancy, the bioreducible crosslinker DSP was used for polyplex stabilization in these studies. Its stable counterpart DSS was used as control. DSP as linking reagent, but not DSS, was recently also used by Neu and colleagues[44] comparing lateral stabilization of LMW and HMW PEI polyplexes. Unlike their findings regarding stabilized LMW PEIs, HD O polyplexes did not result in aggregation after crosslinking. Presumably, a tighter polyplex structure of HD O complexes was already obtained prior to crosslinking them upon the surface. This effect might be attributed to the presence of the hydrophobic moieties within HD O, resulting in a more compact DNA binding due to an increase of hydrophobic interactions between polymer and DNA. Thereby, the possibility to destroy electrostatic interactions between DNA and polycation was minimized. Both crosslinkers (DSP and DSS) at optimized linking degrees did not affect general physicochemical properties of HD O (mean diameter and zeta-potential of particles) but showed that by lateral stabilization the susceptibility of polyplexes against either “competitive” polyanions like heparin (see **Fig. 32**) or high salt concentrations (see **Fig. 34** and **Fig. 35**) was decreased. However, unlike found for BPEI, the transfection efficiency levels under *in vitro* conditions did not significantly differ between the bioreducible DSP and the non-reducible DSS linker. As HD O, in contrast to BPEI, possesses hydrolysable ester bonds, it is likely that the bioreducibility of the disulfide bonds is largely evened out by the simultaneous degradation of the ester bonds. Furthermore, it was found in several cell lines that only if DSP was used for lateral stabilization of HD O complexes, the transfection efficiency levels were raised to the same or even higher reporter gene expression levels as detected for the unstabilized polyplexes. In comparison, the DSS-linked particles resulted in a 5- to 10-fold lower gene expression level. Hence, it can be concluded that the reversibly DSP-linked hydrolyzable HD O / DNA polyplexes exhibited an optimized balance of extracellular stability and intracellular instability maintaining high transfection efficiency while no cytotoxic effects were found.

Recent *in vivo* studies involving investigations of laterally stabilized polyplexes based on HMW polycations like e.g. BPEI or PLL revealed that after i.v. administration of those polyplexes, increased blood concentrations were found compared to unstabilized particles.[43,68,74] By this, Oupicky et al.[74] elucidated the importance of bifunctional stabilization in comparison to monovalent modification of polyplex surfaces for systemic administration.

Neu and colleagues emphasized furthermore the alteration of organ specific reporter gene expression after i.v. administration of DSP-crosslinked BPEI polyplexes.[68] However, none of the studies described an efficient *in vivo* gene delivery strategy using crosslinked biodegradable LMW polycations to date. Investigations on the effects due to lateral stabilization of the presumably rather susceptible HD O particles were important in terms of improving its *in vivo* profile for further investigations in mice. Notably, after intratumoral application of DSS, DSP or uncrosslinked HD O polyplexes two interesting and encouraging points were found: first, reporter gene expression levels of stabilized polyplexes were significantly higher compared to uncrosslinked polyplexes (4-fold; $p < 0.001$). And secondly, only for the stabilized polyplexes, a permanent and similar high reporter gene expression in tumor tissue was found over time (4 days), while DSP mediated levels were always superior to DSS ones. Presumably, polyplex destabilization by reductive cleavage of DSP was boosted by the alongside hydrolytic degradation of HD O resulting in a more efficient release of the nucleic acid payload as compared to DSS resulting in better gene expression. In contrast, uncrosslinked HD O polyplexes resulted in step by step decreasing reporter gene expression levels over time. Noteworthy, similar results were also detected in *in vitro* results. Nevertheless, it is evident that lateral stabilization of LMW polyplexes is essential for prolonged reporter gene expression.

To sum it up, it can be assessed that lateral stabilization of vectors based on LMW degradable polyplexes may provide a promising strategy to improve their pharmacokinetic properties, especially regarding further *in vivo* applications. Moreover, the previously rather susceptible HD O polyplexes resulted in more stable particles under physiological conditions and due to this they offer an interesting platform for further modifications, e.g. with targeting ligands.

4.5.2 Tf-targeting of stabilized HD O polyplexes – an outlook toward synthetic viruses

Various methods have been investigated in the past to achieve high specificity for the tumor as target as it is desired for clinical applications in cancer gene therapy. Targeting strategies include directed delivery of polyplexes to the target site as well as transductional and transcriptional targeting policies involving the intracellular fate of the particles (see **Fig. 4**). Besides biological targeting strategies also physical practices or a combination of both have been applied and showed encouraging results in several studies.[57,59,75,115-118]

Here, the transferrin receptor (TfR) was selected as cell surface target since it is over-expressed in a variety of tumor cells because of their higher demand of iron, needed for their fast growth.[49] Moreover, transferrin (Tf) as protein ligand combines both a shielding function of the polyplexes (reducing unspecific interactions e.g. with erythrocytes[15]) and a targeting moiety toward the tumor site.[50,51] The incorporation of Tf as targeting ligand into polyplexes based on LMW pseudodendritic oligoamines to increase their delivering specificity might be a further step toward the design of more virus-like and biodegradable synthetic systems. Although pseudodendritic oligoamines, as investigated here, already resulted in a predominant reporter gene expression in tumor tissue after i.v. administration, it is most likely that their biodistribution pattern differs from their gene expression pattern.[109,119] Hence, directing polyplexes, at best, exclusively toward the tumor site might be beneficial in order to reduce undesired accumulation of complexes at non-target sites.

Because HD O polyplexes at a c/p-ratio of 1 resulted, by either mixing the targeting ligand with the polyplexes or following a standard procedure for Tf incorporation by Tf-BPEI, in extensive aggregation, it was obvious that under these conditions a suitable vector formulation was not amenable. Hence, the lateral stabilization technology as established and evaluated above was essential for the incorporation of a targeting moiety. Due to their bioreducibility and most promising *in vitro* and *in vivo* results, DSP-crosslinked particles were utilized in the following studies for TfR targeting. It can be assumed that due to the deficiency of crosslinker used for efficient polyplex stabilization, no residual free linker was left while a number of primary amine bonds remained upon the surface of particles (zeta-potentials after crosslink did not significantly decrease). Those can be used for formation of Tf-coated vectors. In the described studies the oxidized form Tf_{ox} was investigated to form meta-stable imine bonds between primary amines upon the particle surface and the aldehyde functionality of Tf_{ox}. The unoxidized form of transferrin (Tf_{unox}) was studied for comparison in order to exclude unspecific effects. Generally, it is hypothesized that the efficient Tf-coating of particles using the described vector formulation only happens in case of Tf_{ox}. There, the incorporation of Tf onto polyplexes by hydrolysable imine bonds was assumed to be stable enough due to the presence of multiple amines in the polyplex.

Physicochemical examinations showed that already a very low amount of 3.5% [w/w] Tf was sufficient to form small (180 – 200 nm) complexes reducing the zeta-potential from +30 mV to +14 mV in case of Tf_{ox}. This already indicates a shielding effect due to effective coating of the particles. The decrease of the zeta-potential using Tf_{unox} was less pronounced (to +23 mV) indicating a less sufficient covering by transferrin since no imine bond can be formed. Only stabilized particles involving a DSP/HD O-ratio of 0.05 and 0.06 were suitable for Tf-coating while ratio 0.07 resulted in aggregation after addition of 3.5% Tf_{ox} or Tf_{unox}.

Investigations of the resulting Tf-polyplexes showed *in vitro* in both studied cell lines - Neuro2a and K562 cells, the same transfection pattern. Only the Tf_{ox} coated particles showed an up to 10-fold increased reporter gene expression level, whereas particles covered with Tf_{unox} remained at the same level as unstabilized, targeted HD O polyplexes. This points out that obviously only the formulation with oxidized transferrin is able to lead to enhanced reporter gene expression.

Hence, also in these studies the importance of an increased extracellular stability of the polyplex formulation, which has to be in an optimized balance to a triggered instability of the particle, was demonstrated. The combination of both characteristics (extracellular stability and triggerable destabilization) within targeted, bioresponsively stabilized HD O complexes presents a prototype for synthetic virus-like polyplexes, which undergo programmed structural changes compatible with the different gene delivery stages, “sensing” the biological (micro-) environment.

5 Summary

Gene therapy offers a promising tool for treating various diseases including cancer and a number of inherent disorders. The clinical use is still restricted mainly due to the lack of safe *and* efficient gene delivery systems. Recent investigations increasingly focus on synthetic gene carriers as alternatives to viral vectors mainly due to their better bio-safety. Furthermore, these gene transfer devices might be advantageous in terms of pharmaceutical issues such as low cost of synthesis and easy scale-up.

In the course of this thesis, the design and establishment of a novel family of hyperbranched degradable polymers, named pseudodendrimers, is reported. These structures are formed step by step in a dendritic concept by adding several monomeric moieties upon a central core unit via – in the present case – hydrolysable branches. The pseudodendritic synthesis concept, the analysis of the structural composition of the resulting polycations and the investigation of their physicochemical properties as well as biological characteristics are reported. Studies on DNA binding ability, polyplex stability, cytotoxicity and transfection efficiency demonstrated that pseudodendritic core characteristics as well as the surface modifications among the pseudodendritic core influence both transfection efficiency and cytotoxicity *in vitro*. At first, it was elucidated that a hydrophobic core moiety is essential for potent gene delivery. In the current case only HD core conjugates (C6 spacer) led to efficient gene transfer. BD and ED core compounds with a C4 and C2 spacer, respectively, resulted in stepwise decreasing transfection efficiency as the intrinsic membrane destabilizing effect was simultaneously diminished. Furthermore, in these structure-activity relationship studies it became evident that the surface modification has to be optimized relative to the core unit that should finally result in a perfect balance of hydrophilic and hydrophobic domains within the pseudodendrimer in order to obtain highest transfection efficiency at low cytotoxicity. Most importantly, in studies within the structurally better defined PPI core based pseudodendrimers it was clarified that ethylenimine moieties within the compounds are essential to improve the transfection efficiency (“proton sponge effect”). A couple of the novel gene carriers that followed the prerequisites mentioned above were successfully applied in *in vitro* transfection studies where they easily competed with the “golden standard” linear polyethylenimine 22 kDa (LPEI).

Out of the described structure-activity studies, the six most promising conjugates were investigated for their *in vivo* gene transfer efficiency in a subcutaneous tumor model in mice. Prior to systemic application, investigations regarding their biocompatibility were performed including *in vitro* studies on polymer degradation kinetics and their hemocompatibility and

first *in vivo* testings on acute toxicity after i.v. application of plain polymer in mice. All these studies revealed that only three pseudodendrimers remained for further *in vivo* administration: HD O, G2-HD-OEI and G3-HD-OEI. Notably, all these polymers had a degradation half-life of about 3 days, induced no erythrocyte aggregation and were well tolerated by mice even if applied as plain polymer.

In vivo studies in mice that were bearing subcutaneous neuroblastoma tumors exhibited that DNA polyplexes of all three compounds upon intravenous tail vein injection had the potential for transfection of tumor tissue at levels comparable to those obtained with LPEI. Importantly, all pseudodendrimers were better tolerated than LPEI while transgene expression was more tumor-specific and much lower in all other investigated organs, especially in the lung (e.g. T/L-ratio (HD O) = 20; T/L-ratio (G3-HD-OEI) = 89). A clear influence of the dendrimers´ generation used within the core unit on transfection efficiency was found resulting in significantly ($p < 0.05$) higher reporter gene expression if generation 3 was used instead of generation 2, which was demonstrated within the PPI core based pseudodendrimers.

As the success of gene therapy is highly dependent on the delivery system, research has focused on the development of gene transfer devices suitable for highly potent and specific systemic nucleic acid delivery. Compared to their viral counterparts, current synthetic vectors are still very inefficient, suffering from rapid elimination from the bloodstream combined with unspecific interaction with and accumulation of particles in organs and tissue. In order to optimize the described LMW degradable vectors toward “synthetic viruses” two approaches were followed. First, particles were laterally stabilized via bioresponsive crosslinkers in order to avoid extracellular disassembly of polyplexes followed by rapid elimination and subsequent decreased reporter gene expression levels under physiological conditions. Hence, it was demonstrated here that crosslinking polyplexes via a disulfide containing linker system (DSP) resulted in increased and prolonged transfection efficiency both under *in vitro* and *in vivo* conditions. The second optimizing step comprised the incorporation of transferrin (Tf) as site-specific targeting ligand upon the stabilized particles. *In vitro* studies demonstrated that vectors – if coated with Tf_{ox} via oxidized carbohydrate-imine linkages – resulted in up to 10-fold enhanced transfection efficiency levels. With regard to *in vivo* application, this polyplex formulation provides the opportunity to increase on the one hand the possibility of an increased site-specific delivery and on the other hand the potential to reduce unspecific interactions with non-target sites.

6 Appendix

6.1 Abbreviations

ALT	alkaline aminotransaminase
AP	alkaline phosphatase
AST	aspartate aminotransaminase
BD	1.4-butandioldiacrylate
BPEI	branched polyethylenimine of a Mw of 25 kDa
BWL	body weight loss
CMV	cytomegalovirus
c/p-ratio	weight ratio of conjugate to plasmid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSP	dithiobis (succinimidylpropionate)
DSS	disuccinimidyl suberate
DTT	dithiothreitol
E	ethanolamine
ED	1.2-ethyleneglycoldiacrylate
EDA	ethylendiamine
EPR	enhanced permeation and retention
EtBr	ethidium bromide
FCS	fetal calf serum
GPC	gel permeation chromatography
HBG	HEPES-buffered glucose
HBS	HEPES-buffered saline
HD	1.6-hexandioldiacrylate
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethansulfonic acid)
HMW	high molecular weight
GLDH	glutamate dehydrogenase
i.v.	intravenous

i.p.	intraperitoneal
i.t.	intratumoral
LMW	low molecular weight
LPEI	linear polyethylenimine of a Mw of 22 kDa
MTT	methylthiazoletetrazolium
Mw	weight average molecular weight
Mn	number average molecular weight
MQ	deionized water (Millipore)
^{1}H NMR	nuclear magnetic resonance
N/P-ratio	molar ratio of PEI nitrogen to DNA phosphate
OEI	oligoethylenimine 800 Da
PEHI	pentaethylenheximine
PDI	polydispersity index
RLU	relative light units
S	spermine
Sp	spermidine
TETA	triethylentetramine
Tf	transferrin
Tf-BPEI	transferrin covalently attached to BPEI
Tf_{ox}	oxidized transferrin
Tf_{unox}	unoxidized transferrin
T/L	tumor / lung-ratio

6.2 Publications

6.2.1 Original papers

- Russ, V., Elfberg, H., Thoma, C., Kloeckner, J., Ogris, M., Wagner, E. (2008): Novel degradable oligoethylenimine-acrylate ester-based pseudodendrimers for *in vitro* and *in vivo* gene transfer. Gene Ther. 2008 Jan; 15(1):18-29.

- Knorr, V., Russ, V., Allmendinger, L., Ogris, M., Wagner, E.: Acetal linked oligoethylenimines for use as pH-sensitive gene vectors. Bioconjug Chem. 2008 Aug; 19(8):1625-34. Epub 2008 Jul 16

- Russ, V., Günther, M., Halama, A., Ogris, M., Wagner E.: Oligoethylenimine-grafted polypropylenimine dendrimers as degradable and biocompatible synthetic vectors for *in vitro* and *in vivo* gene delivery. J Control Release. 2008 Sep 10. Epub ahead of print

6.2.2 Manuscripts in preparation

- Russ, V., Halama, A., Ogris, M., Wagner, E.: Importance of lateral bioreversible stabilization of LMW polyplexes. In preparation.

- Yu, H.*, Russ, V.*, Wagner, E.: High Efficient Synthetic Gene Vectors Based on Bioreducible PEI. In preparation.

- Klutz, K.*, Russ, V.*, Willhauck, M.J., Wunderlich, N., Zach, C., Münzing, W., Gildehaus, F.J., Göke, B., Wagner, E., Ogris, M., Spitzweg, Ch.: Tumor-specific iodide uptake activity in neuroblastoma tumors following systemic sodium iodide symporter gene transfer using synthetic gene delivery vectors. In preparation.

6.2.3 Reviews

- Russ, V., Wagner, E. (2007) Cell and tissue targeting of nucleic acids for cancer gene therapy. Pharm Res. 2007 Jun; 24(6):1047-57.

6.2.4 Poster presentations

- Verena Russ, Manfred Ogris and Ernst Wagner: *Toward artificial viruses: novel biodegradable gene carriers based on pseudodenritic oligoamines,* VIU, 2006, Venice, Italy

- Julia Fahrmeir, Anita Bogomilova, Verena Russ, Neli Koseva, Manfred Ogris, Kolio Troev and Ernst Wagner: *Novel Biodegradable Gene Carriers based on Polyphosphoramidates*, 33rd annual meeting & exposition of CRS, 2006, Vienna, Austria

- Kathrin Klutz*, Verena Russ*, Michael J Willhauck, Nathalie Wunderlich, Christian Zach, Wolfgang Münzing, Franz Josef Gildehaus, Burkhard Göke, Ernst Wagner, Manfred Ogris, Christine Spitzweg: *Tumor-specific iodide uptake activity in neuroblastoma tumors following systemic sodium iodide symporter gene transfer using synthetic gene delivery vectors,* 15th Annual Meeting of the German Society of Gene Therapy, 2008, Berlin, Germany

- Terese Magnusson, Verena Russ, Isabella Stapff, Rudolf Haase, Armin Baiker, Ernst Wagner, Manfred Ogris: *Optimising nonviral ex vivo transferrin mediated transfection of the erythroleukemia cell line K562 with episomal vectors,* 15th Annual Meeting of the German Society of Gene Therapy, 2008, Berlin, Germany

6.2.5 Oral presentations

- Kathrin Klutz, Verena Russ, Michael J Willhauck, Nathalie Wunderlich, Christian Zach, Wolfgang Münzing, Franz Josef Gildehaus, Burkhard Göke, Ernst Wagner, Manfred Ogris, Christine Spitzweg: *Tumor-specific iodide* uptake *activity in neuroblastoma tumors following systemic sodium iodide symporter gene transfer using synthetic gene delivery vectors*, ENDO 2008, San Francisco, USA

- Verena Russ, Manfred Ogris and Ernst Wagner: *Efficient gene transfer with a novel degradable pseudodendritic oligoamine: an in vitro and in vivo study;* ASGT 10th Annual Meeting, Seattle, USA, 03.06.2007

- Verena Russ, Julia *Kloeckner*, Manfred Ogris and Ernst Wagner: *Novel biodegradable gene carriers based on pseudodendritic oligoamines;* 33rd annual meeting & exposition of CRS, Vienna, Austria, 25.07.2006

7 References

1. Edelstein,M.L., Abedi,M.R., Wixon,J. & Edelstein,R.M. Gene therapy clinical trials worldwide 1989-2004-an overview. *J Gene Med* **6**, 597-602 (2004).

2. Edelstein,M.L., Abedi,M.R. & Wixon,J. Gene therapy clinical trials worldwide to 2007--an update. *J. Gene Med.* **9**, 833-842 (2007).

3. SZYBALSKA,E.H. & SZYBALSKI,W. Genetics of human cess line. IV. DNA-mediated heritable transformation of a biochemical trait. *Proc. Natl. Acad. Sci U. S. A* **48**, 2026-2034 (1962).

4. Majumdar,A. & Bose,S.K. DNA mediated genetic transformation of a human cancerous cell line cultured in vitro. *Br. J Cancer* **22**, 603-613 (1968).

5. Friedmann,T. A brief history of gene therapy. *Nat. Genet.* **2**, 93-98 (1992).

6. Cline,M.J. *et al.* Gene transfer in intact animals. *Nature* **284**, 422-425 (1980).

7. Mercola,K.E., Stang,H.D., Browne,J., Salser,W. & Cline,M.J. Insertion of a new gene of viral origin into bone marrow cells of mice. *Science* **208**, 1033-1035 (1980).

8. Kolata,G.B. & Wade,N. Human gene treatment stirs new debate. *Science* **210**, 407 (1980).

9. Wade,N. Gene therapy caught in more entanglements. *Science* **212**, 24-25 (1981).

10. Anderson,W.F. & Fletcher,J.C. Sounding boards. Gene therapy in human beings: when is it ethical to begin? *N. Engl. J Med.* **303**, 1293-1297 (1980).

11. Rosenberg,S.A. *et al.* Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J Med.* **323**, 570-578 (1990).

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

13. Sonawane,N.D., Szoka,F.C., Jr. & Verkman,A.S. Chloride Accumulation and Swelling in Endosomes Enhances DNA Transfer by Polyamine-DNA Polyplexes. *J. Biol. Chem.* **278**, 44826-44831 (2003).

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

15. Ogris,M., Brunner,S., Schuller,S., Kircheis,R. & Wagner,E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther* **6**, 595-605 (1999).

16. Brownlie,A., Uchegbu,I.F. & Schatzlein,A.G. PEI-based vesicle-polymer hybrid gene delivery system with improved biocompatibility. *Int. J Pharm.* **274**, 41-52 (2004).

17. Chollet,P., Favrot,M.C., Hurbin,A. & Coll,J.L. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J Gene Med.* **4**, 84-91 (2002).

18. Russ,V. *et al.* Novel degradable oligoethylenimine acrylate ester-based pseudodendrimers for in vitro and in vivo gene transfer. *Gene Ther* **15**, 18-29 (2008).

19. Erbacher,P., Zou,S., Bettinger,T., Steffan,A.M. & Remy,J.S. Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability 826. *Pharm. Res.* **15**, 1332-1339 (1998).

20. Park,I.K. *et al.* Galactosylated chitosan-graft-poly(ethylene glycol) as hepatocyte-targeting DNA carrier. *J Control Release* **76**, 349-362 (2001).

21. Thanou,M., Florea,B.I., Geldof,M., Junginger,H.E. & Borchard,G. Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. *Biomaterials* **23**, 153-159 (2002).

22. Sato,T., Ishii,T. & Okahata,Y. In vitro gene delivery mediated by chitosan. effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials* **22**, 2075-2080 (2001).

23. Germershaus,O., Mao,S., Sitterberg,J., Bakowsky,U. & Kissel,T. Gene delivery using chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan block copolymers: establishment of structure-activity relationships in vitro. *J Control Release* **125**, 145-154 (2008).

24. Wolfert,M.A. *et al.* Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA 937. *Bioconjug. Chem.* **10**, 993-1004 (1999).

25. Kwoh,D.Y. *et al.* Stabilization of poly-L-lysine/DNA polyplexes for in vivo gene delivery to the liver 953. *Biochim. Biophys. Acta* **1444**, 171-190 (1999).

26. Ward,C.M., Read,M.L. & Seymour,L.W. Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy 1. *Blood* **97**, 2221-2229 (2001).

27. Lim,Y.B. *et al.* Biodegradable polyester, poly[alpha-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. *Pharm Res* **17**, 811-6 (2000).

28. Koh,J.J. *et al.* Degradable polymeric carrier for the delivery of IL-10 plasmid DNA to prevent autoimmune insulitis of NOD mice. *Gene Ther* **7**, 2099-2104 (2000).

29. Ko,K.S., Lee,M., Koh,J.J. & Kim,S.W. Combined administration of plasmids encoding IL-4 and IL-10 prevents the development of autoimmune diabetes in nonobese diabetic mice. *Mol Ther* **4**, 313-316 (2001).

30. Koping-Hoggard,M. *et al.* Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo 1. *Gene Ther.* **8**, 1108-1121 (2001).

31. Gosselin,M.A., Guo,W. & Lee,R.J. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjug Chem* **12**, 989-994 (2001).

32. Ahn,C.H., Chae,S.Y., Bae,Y.H. & Kim,S.W. Biodegradable poly(ethylenimine) for plasmid DNA delivery 1. *J Control Release* **80**, 273-282 (2002).

33. Deng,H., Bloomfield,V.A., Benevides,J.M. & Thomas,G.J., Jr. Structural basis of polyamine-DNA recognition: spermidine and spermine interactions with genomic B-DNAs of different GC content probed by Raman spectroscopy. *Nucleic Acids Res.* **28**, 3379-3385 (2000).

34. Lynn,D.M., Anderson,D.G., Putnam,D. & Langer,R. Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. *J. Am. Chem. Soc.* **123**, 8155-8156 (2001).

35. Anderson,D.G., Lynn,D.M. & Langer,R. Semi-Automated Synthesis and Screening of a Large Library of Degradable Cationic Polymers for Gene Delivery. *Angew. Chem. Int. Ed Engl.* **42**, 3153-3158 (2003).

36. Anderson,D.G. *et al.* A polymer library approach to suicide gene therapy for cancer. *Proc. Natl. Acad. Sci. U. S. A* **101**, 16028-16033 (2004).

37. Kloeckner,J., Wagner,E. & Ogris,M. Degradable gene carriers based on oligomerized polyamines. *Eur. J. Pharm. Sci.* **29**, 414-425 (2006).

38. Bosman,A.W., Janssen,H.M. & Meijer,E.W. About Dendrimers: Structure, Physical Properties, and Applications. *Chem Rev.* **99**, 1665-1688 (1999).

39. Haensler,J. & Szoka,F.C., Jr. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug Chem* **4**, 372-379 (1993).

40. Kukowska-Latallo,J.F. *et al.* Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers 6 2657. *Proc. Natl. Acad. Sci. U. S. A* **93**, 4897-4902 (1996).

41. Tang,M.X., Redemann,C.T. & Szoka,F.C., Jr. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjug. Chem.* **7**, 703-714 (1996).

42. Burke,R.S. & Pun,S.H. Extracellular Barriers to in Vivo PEI and PEGylated PEI Polyplex-Mediated Gene Delivery to the Liver. *Bioconjug Chem* **19**, 693-704 (2008).

43. Oupicky,D., Parker,A.L. & Seymour,L.W. Laterally stabilized complexes of DNA with linear reducible polycations: strategy for triggered intracellular activation of DNA delivery vectors. *J. Am. Chem. Soc.* **124**, 8-9 (2002).

44. Neu,M., Sitterberg,J., Bakowsky,U. & Kissel,T. Stabilized nanocarriers for plasmids based upon cross-linked poly(ethylene imine). *Biomacromolecules.* **7**, 3428-3438 (2006).

45. Vacik,J., Dean,B.S., Zimmer,W.E. & Dean,D.A. Cell-specific nuclear import of plasmid DNA 927. *Gene Ther.* **6**, 1006-1014 (1999).

46. Russ,V. & Wagner,E. Cell and Tissue Targeting of Nucleic Acids for Cancer Gene Therapy. *Pharm. Res.* **24**, 1047-1057 (2007).

47. Maeda,H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv Enzyme Regul* **41**, 189-207 (2001).

48. Wagner,E., Culmsee,C. & Boeckle,S. Targeting of polyplexes: toward synthetic virus vector systems. *Adv Genet.* **53**, 333-354 (2005).

49. Li,H. & Qian,Z.M. Transferrin/transferrin receptor-mediated drug delivery. *Med. Res. Rev.* **22**, 225-250 (2002).

50. Xu L, Pirollo KF, Tang WH, Rait A & Chang EH. Transferrin-liposome-mediated systemic p53 gene therapy in combination with radiation results in regression of human head and neck cancer xenografts. *Hum. Gene. Ther.* **10**, 2941-2952 (1999).

51. Kircheis,R. *et al.* Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther* **8**, 28-40 (2001).

52. Wagner,E., Zenke,M., Cotten,M., Beug,H. & Birnstiel,M.L. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc. Natl. Acad. Sci. U. S. A* **87**, 3410-3414 (1990).

53. Kircheis,R. *et al.* Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Ther* **4**, 409-418 (1997).

54. Kircheis,R. *et al.* Tumor-targeted gene delivery of tumor necrosis factor-alpha induces tumor necrosis and tumor regression without systemic toxicity. *Cancer Gene Ther* **9**, 673-680 (2002).

55. Xu,L. *et al.* Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. *Mol. Cancer Ther.* **1**, 337-346 (2002).

56. Hildebrandt,I.J., Iyer,M., Wagner,E. & Gambhir,S.S. Optical imaging of transferrin targeted PEI/DNA complexes in living subjects. *Gene Ther.* **10**, 758-764 (2003).

57. da Cruz,M.T., Cardoso,A.L., de Almeida,L.P., Simoes,S. & de Lima,M.C. Tf-lipoplex-mediated NGF gene transfer to the CNS: neuronal protection and recovery in an excitotoxic model of brain injury. *Gene Ther.* **12**, 1242-1252 (2005).

58. Chen,J., Gamou,S., Takayanagi,A. & Shimizu,N. A novel gene delivery system using EGF receptor-mediated endocytosis 97. *FEBS Lett* **338**, 167-169 (1994).

59. Shir,A., Ogris,M., Wagner,E. & Levitzki,A. EGF receptor-targeted synthetic double-stranded RNA eliminates glioblastoma, breast cancer, and adenocarcinoma tumors in mice. *PLoS Med* **3**, e6 (2006).

60. Kloeckner,J., Prasmickaite,L., Hogset,A., Berg,K. & Wagner,E. Photochemically enhanced gene delivery of EGF receptor-targeted DNA polyplexes. *J Drug Target* **12**, 205-213 (2004).

61. Blessing,T., Kursa,M., Holzhauser,R., Kircheis,R. & Wagner,E. Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. *Bioconjug Chem* **12**, 529-537 (2001).

62. Moffatt,S., Wiehle,S. & Cristiano,R.J. Tumor-specific gene delivery mediated by a novel peptide-polyethylenimine-DNA polyplex targeting aminopeptidase N/CD13. *Hum. Gene Ther* **16**, 57-67 (2005).

63. Brissault,B. *et al.* Synthesis of linear polyethylenimine derivatives for DNA transfection. *Bioconjug. Chem.* **14**, 581-587 (2003).

64. Plank,C., Zatloukal,K., Cotten,M., Mechtler,K. & Wagner,E. Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjug. Chem.* **3**, 533-539 (1992).

65. Parker,A.L., Oupicky,D., Dash,P.R. & Seymour,L.W. Methodologies for monitoring nanoparticle formation by self-assembly of DNA with poly(l-lysine). *Anal. Biochem.* **302**, 75-80 (2002).

66. Oupicky,D., Carlisle,R.C. & Seymour,L.W. Triggered intracellular activation of disulfide crosslinked polyelectrolyte gene delivery complexes with extended systemic circulation in vivo. *Gene Ther* **8**, 713-724 (2001).

67. Hill,H.D. & Straka,J.G. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Anal. Biochem.* **170**, 203-208 (1988).

68. Neu,M. *et al.* Crosslinked nanocarriers based upon poly(ethylene imine) for systemic plasmid delivery: in vitro characterization and in vivo studies in mice. *J. Control Release* **118**, 370-380 (2007).

69. Zinselmeyer,B.H., Mackay,S.P., Schatzlein,A.G. & Uchegbu,I.F. The lower-generation polypropylenimine dendrimers are effective gene-transfer agents. *Pharm. Res.* **19**, 960-967 (2002).

70. Demeneix,B.A. & Behr,J.P. Artificial Self-Assembling Systems for Gene Delivery. Felgner,P.L., Heller,M.J., Lehn,P., Behr,J.P. & Szoka,F.C. (eds.), pp. 146-151 (American Chemical Society, Washington, USA,1996).

71. Kichler,A., Leborgne,C., Coeytaux,E. & Danos,O. Polyethylenimine-mediated gene delivery: a mechanistic study. *J Gene Med* **3**, 135-144 (2001).

72. Goula,D. *et al.* Polyethylenimine-based intravenous delivery of transgenes to mouse lung 246. *Gene. Ther.* **5**, 1291-1295 (1998).

73. Neu,M., Germershaus,O., Behe,M. & Kissel,T. Bioreversibly crosslinked polyplexes of PEI and high molecular weight PEG show extended circulation times in vivo. *J. Control Release* **124**, 69-80 (2007).

74. Oupicky,D. *et al.* Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol Ther* **5**, 463-472 (2002).

75. Kursa,M. *et al.* Novel Shielded Transferrin-Polyethylene Glycol-Polyethylenimine/DNA Complexes for Systemic Tumor-Targeted Gene Transfer. *Bioconjug. Chem.* **14**, 222-231 (2003).

76. Bartlett,D.W., Su,H., Hildebrandt,I.J., Weber,W.A. & Davis,M.E. Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging. *Proc. Natl. Acad. Sci U. S. A* **104**, 15549-15554 (2007).

77. Wagner,E. Strategies to improve DNA polyplexes for in vivo gene transfer: will "artificial viruses" be the answer? *Pharm. Res.* **21**, 8-14 (2004).

78. Niidome,T. & Huang,L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther* **9**, 1647-1652 (2002).

79. Lynn,D.M. & LangerR. Degradable Poly(ß-amino esters): Synthesis, Characterization, and Self-Assembly with Plasmid DNA . *J. Am. Chem. Soc.* **122**, 10761-10768 (2000).

80. Lim,Y.B., Kim,S.M., Suh,H. & Park,J.S. Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. *Bioconjug Chem* **13**, 952-957 (2002).

81. Forrest,M.L., Koerber,J.T. & Pack,D.W. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. *Bioconjug. Chem.* **14**, 934-940 (2003).

82. Zhong,Z. *et al.* A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s. *J Control Release* **109**, 317-329 (2005).

83. Kloeckner,J., Bruzzano,S., Ogris,M. & Wagner,E. Gene carriers based on hexanediol diacrylate linked oligoethylenimine: effect of chemical structure of polymer on biological properties. *Bioconjug Chem* **17**, 1339-1345 (2006).

84. Arote,R. *et al.* A biodegradable poly(ester amine) based on polycaprolactone and polyethylenimine as a gene carrier. *Biomaterials* **28**, 735-744 (2007).

85. Hoon,J.J. *et al.* Reducible poly(amido ethylenimine) directed to enhance RNA interference. *Biomaterials* **28**, 1912-1917 (2007).

86. Lee,Y. *et al.* Visualization of the degradation of a disulfide polymer, linear poly(ethylenimine sulfide), for gene delivery. *Bioconjug Chem* **18**, 13-18 (2007).

87. Gosselin,M.A., Guo,W. & Lee,R.J. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine 1032. *Bioconjug. Chem.* **12**, 989-994 (2001).

88. Haensler,J. & Szoka,F.C., Jr. Synthesis and characterization of a trigalactosylated bisacridine compound to target DNA to hepatocytes. *Bioconjug. Chem.* **4**, 85-93 (1993).

89. Ferrari,S. *et al.* ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther* **4**, 1100-6 (1997).

90. Zou,S.M., Erbacher,P., Remy,J.S. & Behr,J.P. Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J. Gene Med.* **2**, 128-134 (2000).

91. Gosselin,M.A., Guo,W. & Lee,R.J. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine 1032. *Bioconjug. Chem.* **12**, 989-994 (2001).

92. Kim,Y.H. *et al.* Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. *J Control Release* **103**, 209-219 (2005).

93. Kloeckner,J. *et al.* DNA polyplexes based on degradable oligoethylenimine-derivatives: Combination with EGF receptor targeting and endosomal release functions. *J. Control Release* **116**, 115-122 (2006).

94. Petersen,H. *et al.* Polyethylenimine-graft-Poly(ethylene glycol) Copolymers: Influence of Copolymer Block Structure on DNA Complexation and Biological Activities as Gene Delivery System. *Bioconjug. Chem.* **13**, 845-854 (2002).

95. Zhong,Z. *et al.* A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s. *J Control Release* **109**, 317-329 (2005).

96. Plank,C., Tang,M.X., Wolfe,A.R. & Szoka,F.C., Jr. Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes. *Hum. Gene Ther.* **10**, 319-332 (1999).

97. Zugates,G.T. *et al.* Gene delivery properties of end-modified poly(beta-amino ester)s. *Bioconjug Chem* **18**, 1887-1896 (2007).

98. Zugates,G.T. *et al.* Rapid optimization of gene delivery by parallel end-modification of poly(beta-amino ester)s. *Mol Ther* **15**, 1306-1312 (2007).

99. Zugates,G.T., Anderson,D.G., Little,S.R., Lawhorn,I.E. & Langer,R. Synthesis of poly(beta-amino ester)s with thiol-reactive side chains for DNA delivery. *J. Am. Chem Soc.* **128**, 12726-12734 (2006).

100. Ogris,M. *et al.* The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther* **5**, 1425-1433 (1998).

101. Murthy,N., Campbell,J., Fausto,N., Hoffman,A.S. & Stayton,P.S. Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. *J. Control Release* **89**, 365-374 (2003).

102. Hong,S. *et al.* Interaction of polycationic polymers with supported lipid bilayers and cells: nanoscale hole formation and enhanced membrane permeability. *Bioconjug Chem* **17**, 728-734 (2006).

103. Funhoff,A.M. *et al.* Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH. *Biomacromolecules.* **5**, 32-39 (2004).

104. Zhang,Z.Y. & Smith,B.D. High-generation polycationic dendrimers are unusually effective at disrupting anionic vesicles: membrane bending model. *Bioconjug. Chem.* **11**, 805-814 (2000).

105. Plank,C., Zauner,W. & Wagner,E. Application of membrane-active peptides for drug and gene delivery across cellular membranes. *Adv. Drug Deliv. Rev.* **34**, 21-35 (1998).

106. Knorr,V., Russ,V., Allmendinger,L., Ogris,M. & Wagner,E. Acetal linked oligoethylenimines for use as pH-sensitive gene carriers. *Bioconjug. Chem.* in press (2008).

107. Yang,C., Li,H., Goh,S.H. & Li,J. Cationic star polymers consisting of alpha-cyclodextrin core and oligoethylenimine arms as nonviral gene delivery vectors. *Biomaterials* **28**, 3245-3254 (2007).

108. Wightman,L. *et al.* Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J. Gene Med.* **3**, 362-372 (2001).

109. Kircheis,R. *et al.* Polycation-based DNA complexes for tumor-targeted gene delivery in vivo. *J Gene Med* **1**, 111-120 (1999).

110. Ogris,M. *et al.* Novel Biocompatible Cationic Copolymers Based on Polyaspartylhydrazide Being Potent as Gene Vector on Tumor Cells. *Pharm. Res.* in press (2007).

111. Kunath,K. *et al.* Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J Control Release* **89**, 113-125 (2003).

112. Werth,S. *et al.* A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes. *J. Control Release* **112**, 257-270 (2006).

113. Barron,L.G., Uyechi,L.S. & Szoka,F.C., Jr. Cationic lipids are essential for gene delivery mediated by intravenous administration of lipoplexes 2. *Gene Ther.* **6**, 1179-1183 (1999).

114. Dash,P.R., Read,M.L., Barrett,L.B., Wolfert,M.A. & Seymour,L.W. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery 962. *Gene Ther.* **6**, 643-650 (1999).

115. Hu-Lieskovan,S., Heidel,J.D., Bartlett,D.W., Davis,M.E. & Triche,T.J. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res.* **65**, 8984-8992 (2005).

116. Wolschek,M.F. *et al.* Specific systemic nonviral gene delivery to human hepatocellular carcinoma xenografts in SCID mice. *Hepatology* **36**, 1106-1114 (2002).

117. Ogris,M. *et al.* Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. *J. Control Release* **91**, 173-181 (2003).

118. Kunath,K., Merdan,T., Hegener,O., Haberlein,H. & Kissel,T. Integrin targeting using RGD-PEI conjugates for in vitro gene transfer. *J. Gene Med.* **5**, 588-599 (2003).

119. Kircheis,R. & Wagner,E. Polycation / DNA complexes for *in vivo* gene delivery. *Gene Therapy and Regulation* **1**, 95-114 (2000).

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9 Curriculum Vitae

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