Dissertation

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Novel Biodegradable Gene Carriers Based on Oligomerized Polyamines

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 Prof. Dr. Ernst Wagner betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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1 Introduction

1.1 Gene therapy

Gene therapy is a promising strategy to treat or prevent diseases by delivering therapeutic genes into a patient. Expression of the introduced gene can lead to a controlled cell death of tumor cells, can activate prodrugs specifically within the tumor or can substitute nucleic acids that are responsible for certain genetic disorders, like cystic fibrosis or severe combined immunodeficiency disorder (SCID).

Therapeutic nucleic acids used in gene therapy can be genes encoding therapeutic proteins or normal versions of defective or missing proteins. Furthermore, antisense oligonucleotides or double stranded small interfering RNA (siRNA) against disease causing proteins are investigated for gene therapeutic treatments. Most clinical trials in the field of gene therapy are directed towards cancer, but while first clinical success has been already achieved in the treatment of SCID, no clear benefit was obtained in gene therapy of cancer so far. Inefficient delivery to the target cells and severe side effects such as high immunogenicity still limit the therapeutic application of nucleic acid-based drugs.

An ideal gene carrier system should protect the nucleic acid against nucleases and deliver the therapeutic gene to the target cell. After interacting specifically with the cell membranes of the target cells it should enhance cellular uptake and lead to an efficient expression of the gene. Since the delivery of naked DNA in vivo is in most cases inefficient due to high nuclease activity in the serum, a broad variety of vector systems have been developed which protect the DNA, and allow efficient delivery and expression of the gene.

Generally there are two main categories of delivery systems: viral and non-viral vectors.

1.1.1 Viral vectors

Viral vectors are the most commonly used gene transfer systems in clinical trials. They derive from viruses and are modified by replacing genes required for viral replication by a therapeutic gene. Viral vectors are very potent gene delivery vehicles because of their specific machinery to enter the cell and to deliver their nucleic acids into the cell and into the nucleus (Robbins 1998, Kay 2001). However, they can cause severe immune responses and show the great disadvantages of a limited insert-size and insertional mutagenesis risk. Furthermore, the production processes are complex and expensive.

1.1.2 Nonviral vectors

Non-viral vectors are receiving increasing attention as gene delivery systems because of several favorable characteristics. They offer enhanced biosafety and biocompatibility and a high flexibility regarding the size of the delivered nucleic acid (Niidome 2002). They can be synthesized at low cost in large quantities and with high consistency. Non-viral vectors expose low immunogenicity (Zuber 2001) as they can be generated protein-free or by using non-immunogenic proteins and peptides. A weakness of non-viral vectors is their low efficiency in intracellular delivery, which currently is partly compensated by the administration of large amounts of vector.

Non-viral vectors are based on formulations with chemically defined DNA carriers such as cationic lipids (amphiphilic molecules with a hydrophilic cationic head-group and a hydrophobic tail) (Lee 1997) or polycations (e.g. polylysine or polyethylenimine) which interact electrostatically with the negatively charged nucleic acids. The resulting compact and positively charged structures protect DNA from degradation and can improve their cellular uptake by facilitating the interaction with negatively charged cell membranes.

For effective polyplex-mediated gene delivery, the gene carrier has to fulfill a series of delivery functions in order to overcome extra- and intracellular barriers ***(*Fig.1)***. The cationic polymer has to compact DNA into particles with virus-like dimensions and protect the DNA from degradation and against undesired interactions with the biological environment during the extracellular transport. After cell binding and internalization, ideally in a target cell-specific manner (Kircheis 1999, Ogris 2001,

Kursa 2003), it should promote endosomal escape (Plank 1998, Wagner 1999), vector unpacking (Schaffer 2000), and localization to the nucleus (Brunner 2002). On top of this, the polymer should be non-immunogenic, non-toxic and biodegradable. As no polymer is able to meet all these requirements in reality, it is necessary to generate an 'artificial virus' consisting of a polycationic compacting domain in combination with various functional domains.

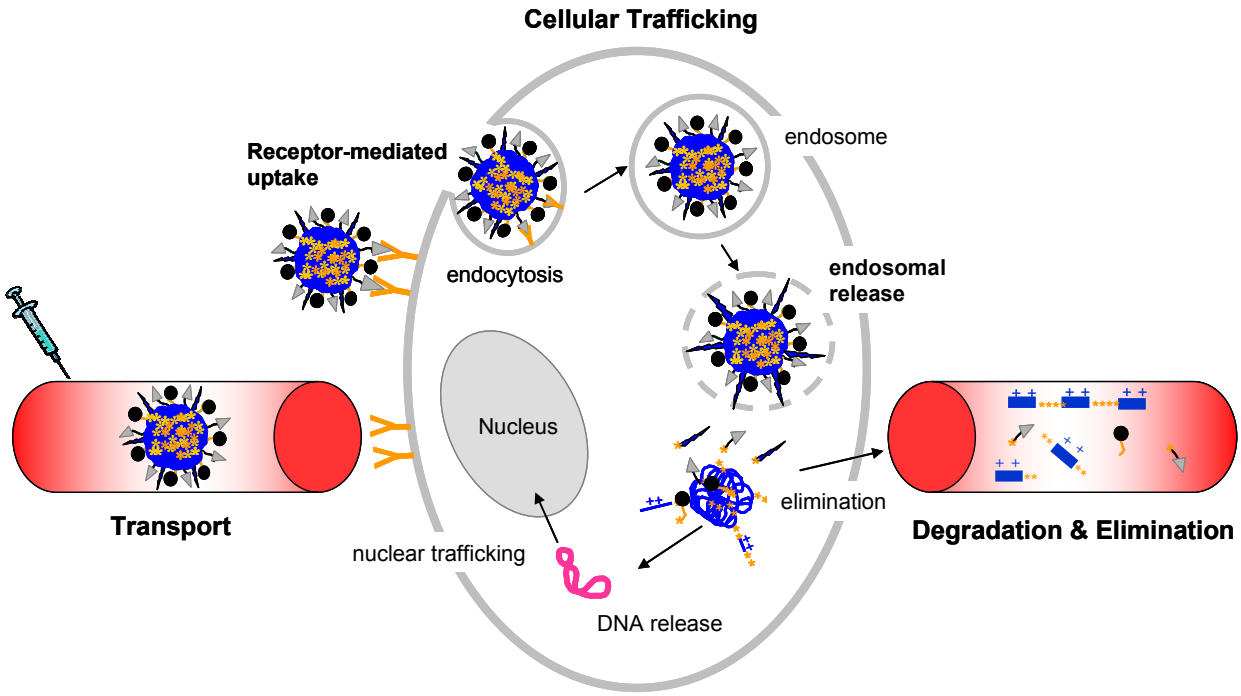

Fig.1: Barriers that have to be overcome by virus-like gene carriers.

During extracellular transport, the cationic polymer has to protect DNA against undesired interactions with the biological environment. Ideally, the polyplexes are targeted towards characteristic cell-surface receptors in order to achieve cell-specific uptake. After binding to their receptor, polyplexes are internalized by receptor-mediated endocytosis and are captured in intracellular vesicles. Degradation of macromolecules in endocytic vesicles is a major bottleneck, therefore fast and efficient endosomal escape is an urgent need for effective gene delivery. Released particles and/or the carried nucleic acid need to traffic towards the nucleus, enter the nucleus and expose the DNA to the cell's transcription machinery. After gene delivery, the polymer has to be degraded and eliminated from the organism. To overcome these barriers, different functional domains can be incorporated into the polyplex.

Over the last three decades a variety of polycations have been employed in polyplexes, including natural DNA binding proteins like histones, synthetic amino acid polymers such as polylysine (Lee 2005) and other cationic polymers, for example polyethylenimines (Kichler 1999), chitosan (Mansouri 2004), polyphosphoamidates (Wang 2004), or poly(dimethylaminoethyl) methacrylates (Verbaan 2004). Currently modifications of non-degradable cationic polymers as well as first approaches for biodegradable compacting domains are under investigation.

1.2 Polyethylenimine: a non-degradable polymer

Polyethylenimine (PEI) first introduced by Behr and his group (Boussif 1995) is the most commonly used polycation. As it forms stable polyplexes that enter the cells through adhesion to negatively charged transmembrane heparanproteoglycans (Kopatz 2004) and can promote its escape from intracellular vesicles. PEI based polyplexes have an excellent transfection efficiency in vitro and significant transfection in vivo (Zou 2000). Regarding non-viral polycationic vectors, linear PEI with an average molecular weight of about 22 kDa is the 'golden standard' for efficient gene delivery.

The problematic characteristics of PEI are a pronounced toxicity (Moghimi 2005) both in cell culture (Boeckle 2004) and *in vivo* (Chollet 2002, Merdan 2005), and a variety of undesired unspecific interactions with the biological environment. Polycationic carriers are toxic because they not only bind to negatively charged DNA but also to many other negatively charged biological materials including phospholipid bilayer cell membranes, cell membrane-integrated and circulatory proteins, or for example, mediate aggregation of erythrocytes (Ogris 1999, Brownlie 2004, de Wolf 2005). The toxic properties increase with the number of positive charges, the hydrophobicity and the molecular weight of the polycation (Fischer 1999). Neutralization of the positive polymer charges by polyplex formation reduces toxicity, both on the cellular level and in the organism, however does not eliminate the problem. In addition to acute toxicity, the long-term fate of the polymeric carrier in the host has also be taken into consideration (Lecocq 2000).

Non-degradable polymers like PEI are ‘static’ structures which often cannot be metabolized. They tend to accumulate mostly in the liver or the kidney (Takakura 1990) and lead to an uncontrollable long-term toxicity. Therefore, biocompatible polymers that are degradable by the host would be advantageous.

1.3 Biodegradable conjugates

Lack of toxicity and biocompatibility should be a major consideration in the design of new gene delivery systems. In fact many low molecular weight (LMW) polymers have been found to possess strongly reduced toxicity compared to their high molecular weight (HMW) counterparts (Ahn 2002). However they do not provide sufficient stability of the polyplexes for *in vivo* administration. Spermine for instance, is a natural LMW polyamine (MW = 202) that can be isolated from sperm in high concentrations and that is already known to have a stabilizing effect on nucleic acids. Due to its oligocationic nature it is able to condense DNA via electrostatic interaction with the negatively charged phosphate groups (Saminathan 1999, Deng 2000, Vijayanathan 2001) but it does not provide sufficient positive charge to form stable complexes under physiological conditions. Different strategies to enhance polyplex stability while maintaining the low toxicity are being explored to make use of the intrinsic DNA binding ability of e.g. spermine and other LMW polyamines (Blagbrough 2003).

One approach is the generation of biodegradable polymer conjugates where LMW oligomers with low toxicity are crosslinked to form larger polycationic carriers by conjugation with biodegradable linkers. Another possibility is the synthesis of new biodegradable polymers which have a labile bond in their repetitive monomeric unit.

Both enzymatic (e.g. by esterases or hydrolases) and chemical degradation of the labile linkages may result in a defragmentation of the polymer into LMW units.

Reversible linkages can be either disulfide bonds that are cleavable within the reducing intracellular environment or alternatively they can be hydrolytically labile groups such as esters, phosphoesters, hydrazones or acetals (for an overview see **Table 1**). Moreover, Petersen et al. reported the synthesis of a biodegradable PEI derivative based on the hydrolytic cleavage of amide bonds (Petersen 2002).

Based on this knowledge, the gene carrier can be constructed in a way that utilizes the properties of the target site to achieve site specific degradation of the polymer. Degradation of these HMW polymers results in LMW fragments with reduced charge density per molecule which are less toxic and could be eliminated from the organism.

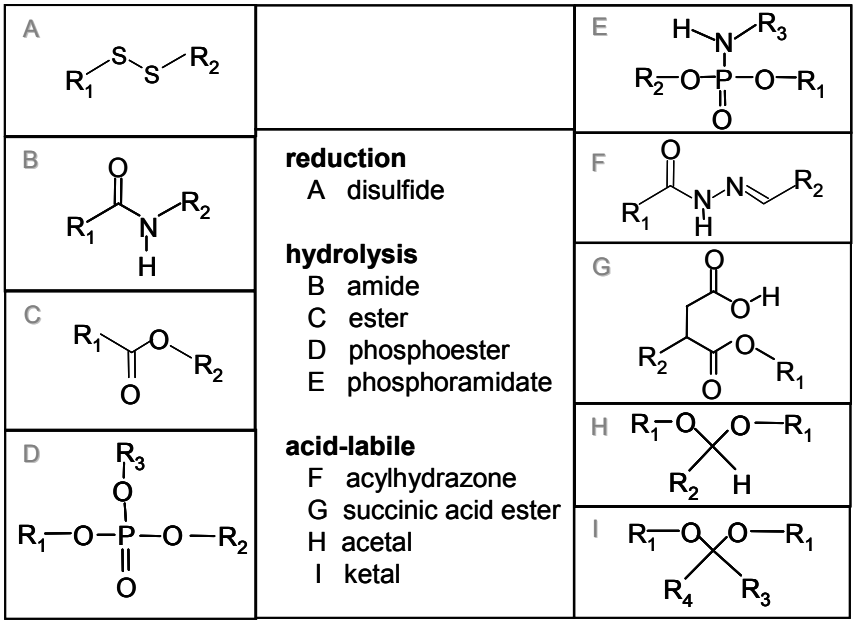

Table 1: Overview degradable linkages

1.3.1 Reductive cleavage of disulfide bonds

Disulfide bonds have been established as a useful tool for chemical modifications that allow environment-specific activation or degradation and thus improved drug delivery (Collins 1991). Characteristics that render this bond attractive are its reversibility within the cell and its transient stability in plasma (Saito 2002). The presence of a high redox potential difference between the oxidizing extracellular environment and the reducing intracellular compartment allows the design of gene delivery vectors which are highly stable during the transport in the blood stream but which break down after cellular uptake (Saito 2003).

One strategy to utilize these advantageous characteristics is the subsequent stabilization of DNA particles after polyplex formation. Intracellular reduction of the

disulfide bond will destabilize the polyplex and therefore trigger DNA release. For example, linear reducible polycations (RPCs) consisting of Cys$(Lys)_{10}$Cys form stable DNA polyplexes after oxidation of the terminal cysteinyl thiol groups (Oupický 2002). Furthermore, lipopolyplexes containing combinations of RPC with DOTAP liposomes resulted in higher gene expression levels as compared to their non-reducible counterparts (Read 2003). Similarly, McKenzie et al. generated LMW disulfide cross-linking peptides consisting of lysine and two terminal cysteine residues which polymerized by disulfide formation when bound to DNA (McKenzie 2000). 'Caged DNA polyplexes' with enhanced stability in high ionic strength were originated forming polylysine polyplex formation and subsequent introduction of disulfide crosslinks after treatment with dimethyl-3-3'-dithiobispropionimidate (DTBP) (Trubetskoy 1999).

Another approach is to oligomerize LMW polyamines with disulfide bond containing bifunctional crosslinkers (Gosselin 2001). Low molecular weight PEI with an average Mw of 800 Da was linked with either dimethyl-3-3'-dithiobispropionimidate (DTBP) or dithiobis(succinimidylpropionate) (DSP) which converted the primary amines into amidines or amides, respectively, thereby generating HMW PEI's (Mw 8 – 75 kDa) with improved transfection efficiency compared to the original LMW PEI.

1.3.2 Hydrolysis of ester bonds

Polymers with ester linkages in their backbone are characterized by an immense diversity and versatility (Edlund 2003). They offer many opportunities to tune the properties of the resulting material and, above all, esters are degradable due to their relative sensitivity to hydrolysis and have therefore gained substantial interest in the development of biocompatible and bioresponsive polymers for gene delivery.

Lim et al. synthesized a biodegradable ester analog which displayed no cytotoxicity, but only modest gene expression (Lim 2000). Another biodegradable cationic polyester, poly (4-hydroxy-L-proline ester) showed similar characteristics (Lim 1999).

Currently, a variety of other ester containing polymers such as network poly (ß-amino ester) (Lim 2002), polyphosphoesters, (Wang 2001 & 2002-1, for review see Zhao

2003) or polyphosphoamidates (Wang 2002-2) are under investigation and analyzed for further optimization.

Ongoing research is carried out in the field of diacrylate crosslinkers which react with primary and secondary amines as well as with thiol groups in a Michael addition. Langer and his group generated a library of 2350 structurally unique degradable cationic polymers by addition of various di-acrylates to different, basically mono-amine-containing monomers (Anderson 2003 & 2004, Akinc 2003). Furthermore, an ester linkage containing PEI derivative was synthesized through addition of 800 Da PEI to small diacrylate crosslinkers (Forrest 2003). Since these polymers showed better gene transfer efficiency in vitro than other ester containing polymers, diacrylate crosslinkers seem to be interesting candidates for oligomerization of LMW polyamines.

Independent of the concept for the synthesis of biodegradable polymers, these structures just represent a DNA compacting domain which should provide polyplex stability and protect the nucleic acid during transport.

1.4 Modifications of standard polyplexes

Even the most potent non-viral vectors such as polyethylenimine (PEI) can still not compete with the effective transfection machinery of natural viruses. Key issues for optimization of polyplexes include improved specificity for the target tissue, enhanced cell uptake and intracellular transport, and reduced toxicity. One strategy for advancements in non-viral gene delivery is to learn from viral cell entry mechanisms and utilize similar functional domains for further modification of polyplexes (Wagner 2004). Key steps towards an ‘artificial virus’ with enhanced specificity and optimized properties to overcome cellular barriers **(Fig.2)** are achieved by incorporation of i) shielding domains to generate neutral polyplexes, ii) targeting ligands which recognize and bind cell-specific receptors and iii) transport domains which facilitate endosomal release.

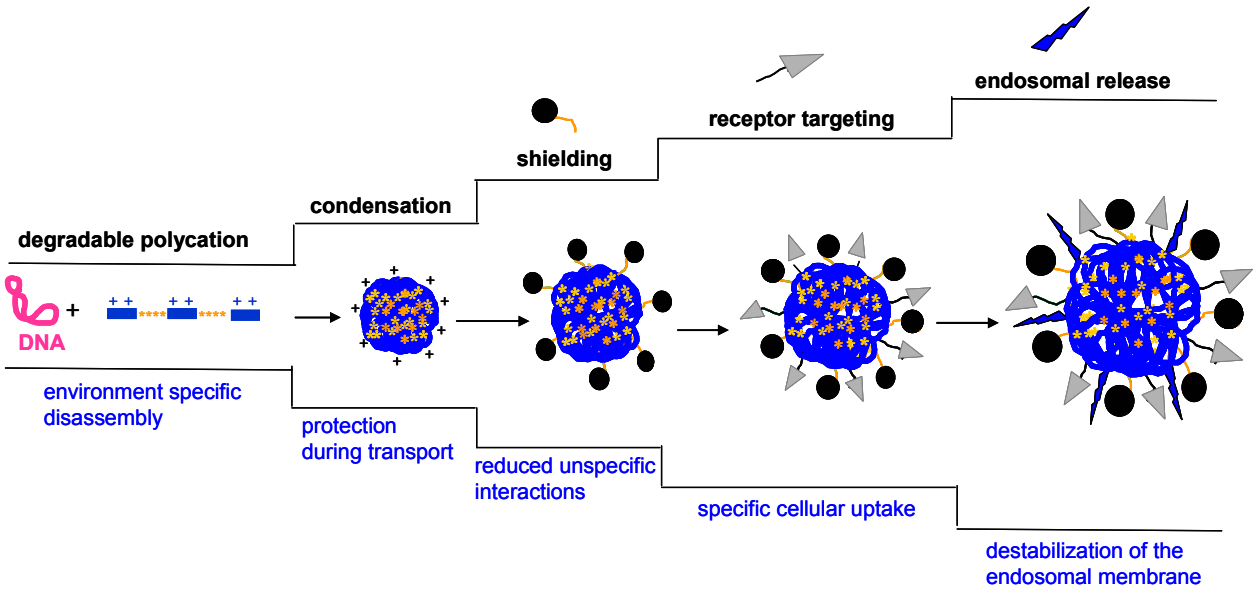

Fig.2: DNA nanoparticles with virus-like functions

For nucleic acid protection and polyplex stability the gene carrier needs a polycationic compacting domain which should be biodegradable. Incorporation of shielding domains to block undesired interactions, targeting ligands which recognize and bind cell-specific receptors, and transport domains which facilitate endosomal release are the key steps towards an 'artificial virus'. To achieve a more 'virus-like' system, these domains should be activated specifically in a defined environment within the target tissue.

1.4.1 Shielding domains

To prevent unspecific interaction of the gene carrier with negatively charged serum proteins, erythrocytes or membrane proteins and to inhibit activation of the complement system (Plank 1996), the positive surface charge of polyplexes has to be shielded by hydrophilic polymers like polyethylene glycol (PEG) (Kircheis 1999, Kursa 2003) or hydroxypropyl methacrylate (pHPMA). Furthermore, shielding provides improved stability for freeze-thawing (Ogris 2003) as well as reduced toxicity and extended circulation time in blood (Ogris 1999). However, surface charge shielding can lead to a significantly reduced gene expression activity (Kichler 2002, Walker 2005).

1.4.2 Targeting domains

Target cell specificity of polyplexes can be achieved by the incorporation of a targeting ligand into the polyplex. Numerous cell-targeting ligands such as vitamins,

carbohydrates, peptides, proteins including growth factors and antibodies have been used to enhance cell specificity and transfection efficiency of polyplexes (for review see: Wagner 2005). These ligands recognize specific receptors on the plasma membrane of the target cells. Amongst other approaches systemic targeting of PEI polyplexes towards tumors was achieved by using either the transferrin receptor (Kircheis 2002, Kursa 2003) or the epidermal growth factor receptor (EGFR) (Wolschek 2002) as targets.

Since the EGFR is overexpressed in a variety of human tumors, such as cancers of the liver, breast, lung and brain, it represents an interesting target for cancer gene therapy (Mendelsohn 2002). Epidermal growth factor (EGF) is a globular protein of 6.4 kDa that binds specifically and with a high affinity to the EGFR inducing dimerization of the receptor and subsequent internalization of the activated EGFR complex. Based on this knowledge different polycation conjugates with either EGF or EGF derivatives (Lee 2001) as targeting ligands have been studied to elucidate the mechanism of EGFR mediated polyplex uptake (von Gersdorff 2005) as well as its potential for cancer specific gene delivery (Frederiksen 2000, Wolschek 2002).

Recently, the EGFR was discovered to be the natural receptor for human cytomegalovirus (CMV) (Wang 2003). The fact that nature chose the EGF receptor for viral gene delivery makes the strategy to employ EGFR-targeted ‘artificial viruses’ even more promising.

1.4.3 Activation of the intracellular transport

1.4.3.1 Endocytosis

The different mechanisms in mammalian cells to take up extracellular material such as extracellular nutrients are summarized under the term endocytosis. Endocytosis is subdivided into several types, namely clathrin-dependent receptor-mediated endocytosis, clathrin-independent endocytosis, and phagocytosis. Both, viruses and toxins utilize the endocytotic pathway to enter the cell. Substances taken up by endocytosis become enclosed within invaginations which form free vesicles that deliver their content to early endosomes (pH~6), then to late endosomes (pH<6) and finally to lysosomes (pH 5-5.5). If not released, endocytosed nucleic acids can be

rapidly degraded by degrading enzymes within late endosomes as well as in lysosomes. Therefore, liberation from endocytic vesicles is a prerequesit step for intracellular delivery of intact nucleic acids in order to achieve efficient transgene expression.

1.4.3.2 Endosomal release

After cell binding and endocytosis, the endosomal release presents another significant bottleneck in polyplex-mediated gene delivery. Approaches to improve endosomal release include PEI itself which acts as a 'proton sponge', (Sonawane 2003), endosomolytic agents (for review see: Cho 2003), such as chloroquine (Cotten 1990), synthetic virus-derived fusion peptides (Plank 1994, Zauner 1995) or artificial membrane destabilizing peptides (Haensler 1993, Gottschalk 1996, Kichler 2003, Thoren 2005). The bee venom peptide melittin is another example which displays strong membrane destabilizating activity and strongly enhances gene transfer of PEI polyplexes (Ogris 2001, Boeckle 2005).

1.4.3.3 Photochemical intracellular release

A different way to achieve improved endosomal release is the photochemical intracellular release technology, named PCI (Berg 1999, Hogset 2000). This technology is based on the accumulation of photosensitizing compounds in endosomal membranes, followed by a light induced rupture of endocytic vesicles which triggers the release of endocytosed molecules (Prasmickaite 2000) **(Fig.3).**

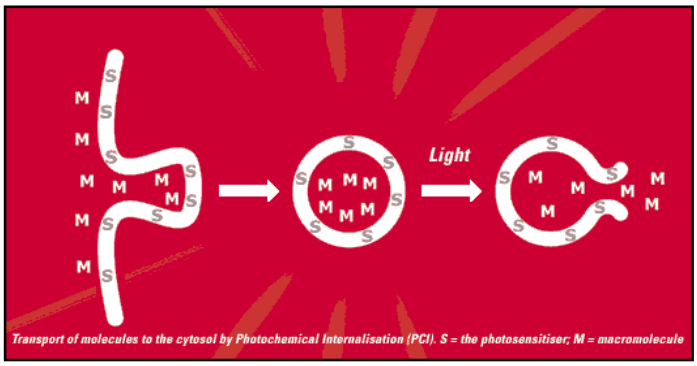

Fig.3: Light-dependent rupture of endocytic vesicles (PCI Biotech)

A photosensitizer (S) and the macromolecule of interest (M) are endocytosed and co-localize to endocytic compartments. Light exposure of the cells results in a photodynamically induced rupture of the vesicle membrane and subsequent cytosolic release of the macro-molecule.

Photosensitizers (PS) are chemical structures that become excited upon light absorption and transfer their energy to molecular oxygen, generating radical singlet oxygen ($^1O_2$) which leads to membrane destruction by oxidative processes. Amphiphilic PS such as TPPS2a and AlPcS2a **(Fig.4)** interact with membranes due to their amphiphilic properties and therefore are localized specifically in endocytic vesicles (Prasmickaite 2001; PhD thesis Lina Prasmickaite 2002). In several studies the group of Berg reported that PCI can improve polycation-mediated gene transfection, substantially increasing the fraction of transgene-expressing cells (Selbo 2002). Furthermore, they have shown that even adenovirus-mediated gene delivery could be improved by PCI (Hogset 2005). Thus, PCI represents a novel principle for the cytosolic delivery of nucleic acids which overcomes the pivotal intracellular barrier of endosomes and lysosomes.

Nevertheless, light treatment is limited to certain cell lines or easily accessible tumors; hence, for these applications direct incorporation of an endosomolytic function into DNA complexes would be preferable.

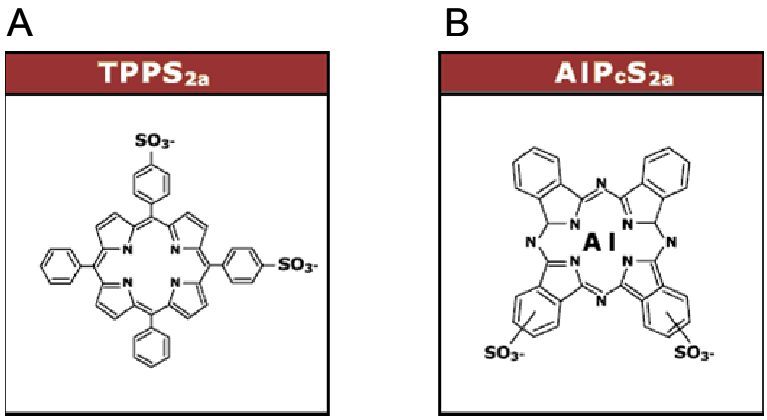

Fig.4: Structural formula of amphiphilic photosensitizers

Meso-tetraphenylporphine (A) and aluminium phthalocyanine (B) consist of a porphyrin skeleton with two sulfonate groups on adjacent phenyl rings and can be used both in vitro and in vivo.

1.5 Aim of the thesis

One major focus in the development of novel gene delivery systems is to generate highly efficient compacting domains which can be easily degraded by the host. So far a variety of non-toxic, biodegradable polymers containing either disulfide or ester bonds have been synthesized. However, they often suffered from poor transfection efficiency.

1.5.1 Design of new polycations for safe and efficient gene transfer

The first aim of this thesis was to find novel biodegradable gene carriers which are highly efficient but essentially non-toxic. The idea was to oligomerize a variety of LMW polyamines like, for example, spermine with different labile bond-containing (disulfide, ester and amide bonds, respectively) crosslinkers to originate HMW polymers which would be degraded in a specific environment **(Fig.5).**

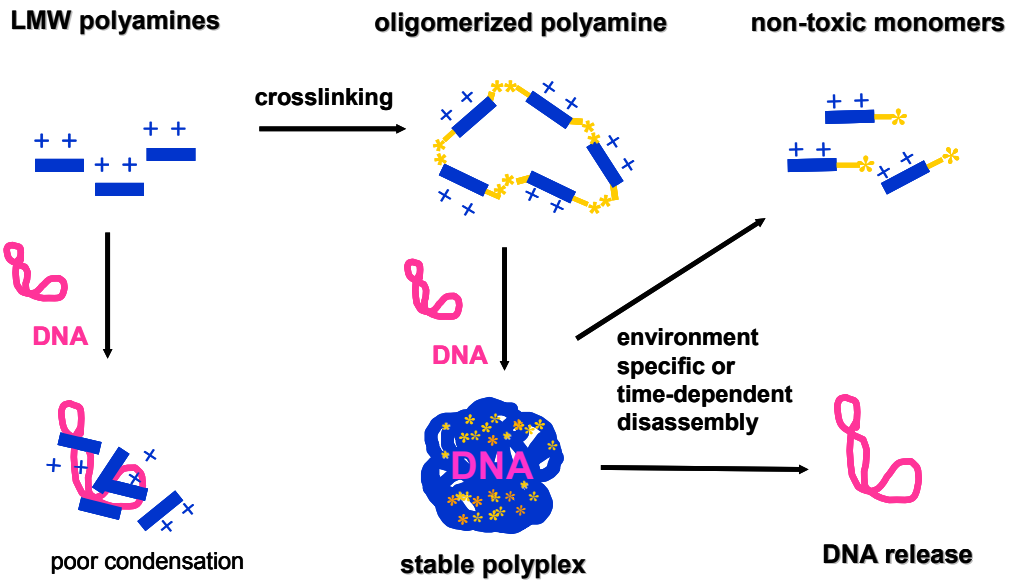

Fig.5: Oligomerization of LMW polyamines with labile bond-containing linkers (concept)

LMW polyamines are crosslinked with labile bond-containing linkers in order to generate HMW-polymers which provide increased polyplex stability in physiological environment. Polymers are degraded into non-toxic fragments after cleavage of the reversible bonds and polyplex disassembly triggers DNA release.

Biophysical (DNA binding, polyplex stability, particle size) and biological (cytotoxicity, erythrocyte aggregation, hemolytic activity) screenings, as well as evaluation of their transfection efficiency should highlight promising candidates with adequate activity versus toxicity profiles for more detailed evaluation.

1.5.2 Structure activity relationship

The next step was to analyze lead candidates in detail to elucidate the influence of chemical structure, charge density, hydrophobicity and molecular weight on biological properties. This information could provide a basis for further optimization of the synthesized polymers. The reason why some polymers result in efficient gene expression whereas others are characterized by a lack of transfection activity, even if they provide polyplex stability, might be that some polymers have their own endosomal release mechanisms like the buffering capacity of PEI or intrinsic membrane active properties. Therefore, membrane-lytic activity of the polymers should be studied in order to predict their fate in the endosome, but also to obtain information about possible cytotoxic side effects.

Polyplex-induced erythrocyte aggregation, as it is known for PEI polyplexes (Ogris 1999), can cause embolism or unspecific gene expression in the lung. This suggests that with regard to systemic *in vivo* application in the future, interaction of the polymers with blood components is an important feature that has to be analyzed.

1.5.3 Optimization towards an ‘artificial virus’

As already discussed above, no polymeric compacting domain will be able to fulfill all the criteria that are required for an ‘artificial virus’. To create a more virus-like gene delivery system, a targeting ligand for specific cell targeting should be incorporated into the polyplex, and transfection efficiency as well as target cell specificity should be compared with non-targeted polyplexes. To further improve gene transfer activity of targeted vectors, methods that trigger endosomal release should be applied. The combination of biological targeting (e.g. via the EGFR) and photochemically enhanced intracellular release using the PCI technology could be a promising step towards a ‘smart artificial virus’.

2 Materials and methods

2.1 Chemicals and reagents

Spermine (Mw 202.35), dimethyl-3.3'-dithiobispropionate x 2 HCl (DTBP; Mw 309.28), dimethylsulfoxide puriss. (DMSO) and cystamine x 2 HCl (Mw 225.2) were obtained from Fluka (Fluka Chemie GmbH, Deisenhofen, Germany).

Spermidine (Mw 145.25), pentaethylenhexamine (Mw 232.4), oligoethylenimine with an average molecular weight of 800 Da (OEI 800), 1,6-hexanediol-diacrylate (Mw 226.28) and branched PEI with an average molecular weight of 25 kDa (PEI25) were obtained from Sigma-Aldrich (Munich, Germany). Linear PEI with an average molecular weight of 22 kDa (PEI22) is available from Euromedex (Exgen, Euromedex, Souffelweyersheim, France). Linear PEI 22 was also synthesized by acid-catalyzed deprotection of poly (2-ethyl-2-oxazoline) (50 kDa, Aldrich) in analogous form as described in Brissault et al. (2003) Gelfiltrated PEI was used as a 1 mg/ml stock solution neutralized with HCl.

Dithiobis(succinimidylpropionate) (DSP; Mw 404.42) was purchased from Pierce (Perbio Science Deutschland GmbH, Bonn, Germany). Triethylentetramine (Mw 146.24) and tetraethylenpentamine (Mw 189.3) were purchased from Merck-Schuchardt OHG (Hohenbrunn, Germany). Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer / promoter) as described in Plank et al. (1992) was produced endotoxin-free by Elim Biopharmaceuticals (San Francisco, CA, USA) or Aldevron (Fargo, ND, USA).

PEI22 modified with polyethylenglycol (PEG) of 20 kDa at equimolar ratio (PEI22-PEG20) was synthesized as previously described (Kursa 2003). Conjugates of murine epidermal growth factor (mEGF) attached to PEG and branched PEI with an average molecular weight of 25 kDa (mEGF-PEG-PEI25) were synthesized as described by Wolschek et al. (2002).

Mouse anti-human EGFR (anti EGFR) clone H11 and mouse IgG1 as negative control were purchased from Dako Corporation (CA, USA). Murine EGF (lyophilized)

was obtained from Prepro Tech EC (London, Great Britain) and bovine serum albumin (BSA) from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

TPPS2a (LumiTrans®) and the corresponding light source (LumiSource®) were kindly provided by PCI Biotech AS (Oslo, Norway).

Melittin-PEI25 conjugates were synthesized and purified as described (Boeckle at al., 2005a/b). The melittin peptides containing a cysteine residue for covalent attachment to PEI had the following sequences: N-mel: CIGA VLKV LTTG LPAL ISWI KRKR QQ, NMA-3: CIGA VLKV LTTG LPAL ISWI KRKR EE, CMA-3: GIGA VLKV LTTG LPAL ISWI KRKR EEC. PEI conjugates of melittin and its analogs were synthesized as described in Boeckle 2005 (PhD thesis, chapter 2.4).

2.2 Polymer synthesis

2.2.1 Synthesis of the polymer library (JK 10/03)

Polyamine (spermidine (SD), spermine (SP), triethylentetramine (TT), tetraethylen-pentamine (TP), pentaethylenhexamine (PH) and oligoethylenimine 800 (OEI 800)) stock solutions were prepared in $DMSO_{puriss}$ at a concentration of 500 mM.

Crosslinkers were dissolved in $DMSO_{puriss}$ shortly before use.

2.2.1.1 Polyamine-Hexanediol-Diacrylate (PA-HD) batch

300 mg 1.6-hexanediol-diacrylate (HD) was dissolved in 1 ml DMSO (crosslinker stock). 0.1 mmol polyamine was mixed with 0.05 mmol (PA-HD-0.5), 0.1 mmol (PA-HD-1) or 0.2 mmol (PA-HD-2) crosslinker stock and incubated at 60 °C for 4 days under constant shaking (450 rpm, Eppendorf Thermomixer).

2.2.1.2 Polyamine-DTBP (PA-IP) batch

85 mg DTBP x 2 HCl (IP) was dissolved in 1 ml of DMSO and sonicated until it was completely dissolved (crosslinker stock). 0.1 mmol polyamine was mixed with 0.05 mmol (PA-IP-0.5), 0.1 mmol (PA-IP-1) or 0.2 mmol (PA-IP-2) crosslinker stock and incubated at 15 °C for 24 h under constant shaking (450 rpm, Eppendorf Thermomixer).

2.2.1.3 Polyamine-DSP (PA-SP) batch

250 mg DSP (SP) was dissolved in 1 ml of DMSO (crosslinker stock). 0.1 mmol polyamine was mixed with 0.05 mmol (PA-SP-0.5), 0.1 mmol (PA-SP-1) or 0.2 mmol (PA-SP-2) crosslinker stock and incubated at 15 °C for 24 h under constant shaking (450 rpm, Eppendorf Thermomixer).

All polymers were purified by size exclusion chromatography (SEC) (see 2.2.8.1).

2.2.2 Synthesis of (OEI-IP-OEI)-HD (JK 07/04)

To generate an OEI-IP-OEI dimer, 480 mg OEI 800 was dissolved in 4 ml DMSO, 85.5 mg of DTBP x 2 HCl was added directly as a powder and reacted at 15 °C for 24 h under constant shaking.

In a second step dimer OEI-IP-OEI was oligomerized with 1.6-hexanediol-diacrylate. 100 mg HD was dissolved in 1 ml DMSO. 1.8 ml of OEI-IP-OEI (= 0.142 mmol OEI-IP-OEI) was reacted with 0.142 mmol HD at 60 °C for 4 days under constant shaking.

The product was purified by SEC (see 2.2.8.1).

2.2.3 Synthesis of Cys-HD-1 (JK 09/04)

112.6 mg cystamine x 2 HCl was dissolved in 1 ml of DMSO and was mixed with 0.1 mmol HD crosslinker (300 mg/ml in DMSO). The reaction was carried out at 60 °C for 4 days under constant shaking (as described for PA-HD- batch).

The product was purified by SEC (see 2.2.8.1).

2.2.4 Synthesis of OEI-HD-Sper (JK 12/04)

a) OEI-HD core

100 mg of OEI 800 and 100 mg of HD were each dissolved in 1 ml of DMSO. 0.05 mmol OEI was mixed with 0.1 mmol HD (OEI-HD-2), 0.25 mmol HD (OEI-HD-5) or 0.5 mmol (OEI-HD-10) and reacted for 30 min at 60°C under agitation.

b) Modification with spermine

400 mg spermine was dissolved in 1 ml DMSO. 0.5 mmol spermine was added to each eppendorf tube containing the OEI-HD core and the reaction occurred at 60°C for 2 days.

All polymers were purified by size exclusion chromatography (see 2.2.8.1).

2.2.5 Synthesis of OEI-HD-1 and lt-OEI-HD-1 (JK 02/05)

2.2.5.1 OEI-HD-1

1.93 ml OEI 800 stock (400 mg/ml in DMSO) was mixed at equimolar ratio with 0.73 ml HD stock (300 mg/ml in DMSO) and reacted for 4 days at 60°C under constant shaking.

2.2.5.2 Low temperature (lt)-OEI-HD-1

4 ml OEI 800 stock (400 mg/ml in DMSO) was mixed at equimolar ratio with 1.51 ml HD stock (300 mg/ml in DMSO) and reacted for 4 days at 20°C under constant shaking.

Both polymers were purified by dialysis (see 2.2.8.2). Synthesis and purification resulted in 157 mg lyophilized OEI-HD-1 (yield 16 %) and in 224 mg lyophilized lt-OEI-HD-1 (yield 11 %).

2.2.6 Upscale of OEI polymer synthesis (JK 04/05)

2.2.6.1 OEI-HD-1

2.0 ml OEI 800 stock (400 mg/ml in DMSO) was mixed at equimolar ratio with 0.75 ml HD stock (300 mg/ml in DMSO) and reacted for 4 days at 60°C under constant shaking.

Synthesis and purification by dialysis (see 2.2.8.2) resulted in 92 mg lyophilized polymer (yield 11 %). Product that was purified by SEC (see 2.2.8.1) resulted in 86 mg lyophilized polymer (yield 46 %).

2.2.6.2 OEI-SP-0.5

2.48 ml OEI 800 stock (400 mg/ml in DMSO) was mixed 1.0 ml DSP stock (250 mg/ml in DMSO) and reacted for 24 h at 15°C under constant shaking.

Synthesis and purification by dialysis (see 2.2.8.2) resulted in 76 mg lyophilized polymer (yield 14 %). Product that was purified by SEC (see 2.2.8.1) resulted in 100 mg lyophilized polymer (yield 54 %).

2.2.6.3 OEI-IP-1

0.98 ml OEI 800 stock (400 mg/ml in DMSO) was mixed 1.8 ml DTBP stock (83 mg/ml in DMSO) and reacted for 24 h at 15°C under constant shaking.

Synthesis and purification by dialysis (see 2.2.8.2) resulted in 76 mg lyophilized polymer (yield 17 %). Product that was purified by SEC (see 2.2.8.1) resulted in 42 mg lyophilized polymer (yield 43 %).

2.2.7 OEI-HD-modifications (JK 04/05)

2.2.7.1 OEI-HD-5 core

Working solutions of OEI 800 (100 mg/ml) and HD-linker (100 mg/ml) were prepared in DMSO. 0.05 mmol OEI (400 µl) was mixed with 0.25 mmol HD (566 µl) and reacted for 30 min at 60°C under agitation (comment: longer reaction times for the core synthesis resulted in gelation of OEI-HD-5 and made modification impossible). 966 µl OEI-HD-5 core were prepared for each modification sample.

2.2.7.2 Modifications of the core

a) OEI-HD-5-control

253 µl DMSO was added to the core (OEI-HD-5), reaction at 60°C for 2 days under agitation. This reaction generated an unsoluble gel which could not be further processed.

b) OEI-HD-5-Sper

400 mg spermine was dissolved in 1 ml DMSO. 0.5 mmol spermine (253 µl) was added to the core (OEI-HD-5), reaction at 60°C for 2 days under agitation. Synthesis and purification by dialysis (see 2.2.8.2) resulted in 45 mg lyophilized polymer (yield 23 %).

c) OEI-HD-5-N-EtOH

121 mg ethanolamine was dissolved in 1 ml DMSO. 0.5 mmol ethanolamine (253 µl) was added to the core (OEI-HD-5), reaction at 60°C for 2 days under agitation. Synthesis and purification by dialysis (see 2.2.8.2) resulted in 34 mg lyophilized polymer (yield 27 %).

d) OEI-HD-5-S-EtOH

154.4 mg ß-mercaptoethanol was dissolved in 1 ml DMSO. 0.5 mmol ß-mercaptoethanol (253 µl) was added to the core (OEI-HD-5), reaction at 60°C for 2 days under agitation. Synthesis and purification by dialysis (see 2.2.8.2) resulted in 33 mg lyophilized polymer (yield 24 %).

e) OEI-HD-5-PH

459.3 mg pentaethylenhexamine was dissolved in 1 ml DMSO. 0.5 mmol pentaethylen-hexamine (253 µl) was added to the core (OEI-HD-5), reaction at 60°C for 2 days under agitation. Synthesis and purification by dialysis (see 2.2.8.2) resulted in 20 mg lyophilized polymer (yield 9 %).

f) OEI-HD-5-His

220 mg histamine was dissolved in 1 ml DMSO. 0.5 mmol histamine (253 µl) was added to the core (OEI-HD-5), reaction at 60°C for 2 days under agitation. Synthesis and purification by dialysis (see 2.2.8.2) resulted in 19 mg lyophilized polymer (yield 12 %).

g) OEI-HD-5-OEI

790.5 mg OEI 800 was dissolved in 1 ml DMSO. 0.25 mmol OEI (253 µl) was added to the core (OEI-HD-5), reaction at 60°C for 2 days under agitation. Synthesis and

purification by dialysis (see 2.2.8.2) resulted in 30 mg lyophilized polymer (yield 10 %).

h) OEI-HD-5-OEI-PEG

1/8 amount of core was synthesized (0.0063 mmol OEI + 0.0313 mmol HD).

16.9 mg PEG (2kDa)-OEI (0.7kDa) (kindly provided by R.Weberskirch, Technical University Munich) was dissolved in 50 µl DMSO. 0.0063 mmol PEG-OEI (50 µl) was added to the core (OEI-HD-5). After 20 min at 60 °C, 200µl of DMSO were added and the mixture reacted for 1 h at 60 °C. In a second modification step 25.3 µl OEI 800 (0.025 mmol) was added, reaction at 60°C for 46 h under agitation. Synthesis and purification by dialysis (see 2.2.8.2) resulted in 14 mg lyophilized polymer (yield 11 %).

2.2.8 Purification

2.2.8.1 Size Exclusion Chromatography (SEC)

Prior to purification polymer samples were diluted 1:4 with H_20_{dd} and the pH was immediately adjusted to pH 6 (with HCl conc.). Size exclusion chromatography (SEC) was performed using a Waters HPLC System equipped with a Waters 600 Controller unit, a Waters 626 pump and a Waters 996 photodiode array detector (Waters GmbH, Eschborn, Germany).

For purification the polymer was separated from unreacted crosslinker and solvent residues on a column (10/30) packed with Sephadex G25 superfine (molecular weight exclusion limit 5 kDa for globular proteins; Amersham Biosciences, Freiburg, Germany) using H_20dd as the eluent under atmospheric pressure. Volumes up to 2 ml of diluted reaction product (maximal 100 mg crude product in maximal 20 % DMSO) were loaded onto the column. Size exclusion chromatography was performed at a flow rate of 1.0 ml/min and fractions of 1.0 ml were collected. The elution of polymer was monitored at various wavelengths (210, 220, 240, 260 and 280 nm). Fractions containing the major amounts of polymer were pooled and freeze dried. The amount of lyophilized product was determined by weight analysis and working dilutions of c = 1 mg/ml and c = 5 mg/ml in H_2O_{dd} were prepared.

2.2.8.2 Dialysis

After synthesis the polymeric product was diluted 1:4 with H_2O_{dd} and the pH was immediately adjusted to pH 6 (with HCl conc.). The diluted un-purified polymer was dialyzed via a Spectra/Por® membrane (cut off 3.5 kDa; Spectrum Medical Industries, Inc.California, USA) for 48 h against 4 l of H_2O_{dd} at 4°C. After 24h, water was exchanged against 4000 ml of fresh H_20dd. The purification process was monitored by UV spectroscopy.

2.3 Polymer characterization

2.3.1 Structural analysis

2.3.1.1 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H-NMR spectra were recorded in d_3-MeOH or $CDCl_3$ on an Eclipse +500 spectrometer from JEOL, Tokyo, Japan at 500 MHz.

1.6-hexanediol diacrylate: ^{1}NMR ($CDCl_3$), δ (ppm): 6.35 (dd, 1H, J=1.5 Hz, J=17.5 Hz, cis-C*H*(H)=CH-; 6.06 (dd, 1H, J=10.5 Hz, J=17.5 Hz, CH_2=C*H*-); 5.78 (dd, 1H, J=1.5 Hz, J=10.5 Hz, tr-C*H*(H)=CH-); 4.12 (t, 2H, J=6.75 Hz, -C*H*$_2$$CH_2CH_2$-); 1.65 (q, 2H, J=6.75 Hz, -CH_2C*H*$_2$$CH_2$-); 1.38 (q, 2H, J=3.75 Hz, -CH_2CH_2C*H*$_2$-);

SD-HD-0.5: 1H NMR (500MHz, d_3-MeOH) δ = 4.16 (m, COOC*H*$_2$ ester linker, 4^{a}H, *di*-ester, 2^{b}H, *mono*-ester), 3.54 (m, 2^{b}H, HOC*H*$_2$, *mono*-ester), 2.7-3.3 (m, 8H of SD, 8H of NC*H*$_2$C*H*$_2$COO linker), 2.1 (br m, 2H, HN-CH_2-***CH*$_2$**-CH_2-NH- of SD), 1.8 (br m, 4H, -HN-CH_2-***CH*$_2$**-***CH*$_2$**-CH_2-NH- of SD), 1,67 (m, 4^{a}H, COOCH_2C*H*$_2$ *di*-ester, 2^{b}H, *mono*-ester), 1,53 (m, 2^{b}H, HOCH_2C*H*$_2$ monoester), 1,41 (m, 4^{a+b}H, -OCH_2CH_2C*H*$_2$ linker).

molar ratio (linker/SD) = 0.56 : 1; di-ester/mono-ester = 45 : 55

integrals: $^{a+b}$ (linker in total): 56% of SD protons ; ^{a} (di-ester): 25% ; ^{b} (mono-ester): 31%

SP-HD-0.5: 1H NMR (500MHz, d_3-MeOH) δ = 4.15 (m, COOC*H*$_2$ ester linker, 4^{a}H, *di*-ester, 2^{b}H, *mono*-ester), 3.54 (m, 2^{b}H, HOC*H*$_2$, *mono*-ester), 2.7-3.3 (m, 12H of SP and 8H of NC*H*$_2$C*H*$_2$COO linker), 2.1 (br m, 4H, H_2N-CH_2-***CH*$_2$**.CH_2-NH- of spermine), 1.8 (br m, 4H, -HN-CH_2-***CH*$_2$**-***CH*$_2$**.CH_2-NH- of spermine), 1,68 (m, 4^{a}H, $COOCH_2CH_2$ *di*-ester, 2^{b}H, *mono*-ester), 1,52 (m, 2^{b}H, $HOCH_2CH_2$ monoester), 1,41 (m, 4^{a+b}H, -$OCH_2CH_2CH_2$ linker).

molar ratio (linker/SP) = 0.61 : 1; di-ester/mono-ester = 40 : 60

integrals: $^{a+b}$ (linker in total): 61% of SP protons ; ^{a} (di-ester): 24 % ; ^{b} (mono-ester): 37%

TT-HD-1: ^{1}H NMR (500MHz, d_3-MeOH) δ = 4.1 (m, COOC*H*$_2$ ester linker, 4^{a}H, *di*-ester, 2^{b}H, *mono*-ester), 3.54 (m, 2^{b}H, HOC*H*$_2$, *mono*-ester), 2.6-3.4 (br m, 20H, NC*H*$_2$, of TT ethylenes and NC*H*$_2$C*H*$_2$COO linker), 1,66 (m, 4^{a}H, $COOCH_2CH_2$ *di*-ester, 2^{b}H, *mono*-ester), 1,55 (m, 2^{b}H, $HOCH_2CH_2$ monoester), 1,41 (m, 4^{a+b}H, -$OCH_2CH_2CH_2$ linker).

molar ratio (linker/TT) = 1 : 1; di-ester/mono-ester = 64 : 36

integrals: $^{a+b}$ (linker in total): 102% of TT protons ; ^{a} (di-ester): 65% ; ^{b} (mono-ester): 37%

TP-HD-1: 1H NMR (500MHz, d_3-MeOH) δ = 4.0 (m, COOC*H*$_2$ ester linker, 4^{a}H, *di*-ester, 2^{b}H, *mono*-ester), 3.46 (m, 2^{b}H, HOC*H*$_2$, *mono*-ester), 2.5-3.3 (br m, 24H, NC*H*$_2$, of TP ethylenes and NC*H*$_2$C*H*$_2$COO linker), 1,59 (m, 4^{a}H, $COOCH_2CH_2$ *di*-ester, 2^{b}H, *mono*-ester), 1,45 (m, 2^{b}H, $HOCH_2CH_2$ monoester), 1,32 (m, 4^{a+b}H, -$OCH_2CH_2CH_2$ linker).

molar ratio (linker/TP) = 0.87 : 1; di-ester/mono-ester = 68 : 32

integrals: $^{a+b}$ (linker in total): 87% of TP protons ; ^{a} (di-ester): 59% ; ^{b} (mono-ester): 28%

OEI-HD-1: ^{1}H NMR (500MHz, d_3-MeOH) δ = 4.1 (m, COOC*H*$_2$ ester linker, 4^{a}H, *di*-ester, 2^{b}H, *mono*-ester), 3.53 (m, 2^{b}H, HOC*H*$_2$, *mono*-ester), 2.6-3.5 (br m, 80H, NC*H*$_2$, of OEI ethylenes and NC*H*$_2$C*H*$_2$COO linker) 1,66 (m, 4^{a}H, $COOCH_2CH_2$ *di*-ester, 2^{b}H, *mono*-ester), 1,52 (m, 2^{b}H, $HOCH_2CH_2$ monoester), 1,38 (m, 4^{a+b}H, -$OCH_2CH_2CH_2$ linker).

molar ratio (linker/OEI) = 0.47 : 1; di-ester/mono-ester = 20 : 80

integrals: $^{a+b}$ (linker in total): 47 mol % of OEI ; ^{a} (di-ester): 9 mol % ; ^{b} (mono-ester): 38 mol %

It-OEI-HD-1: ^{1}H NMR (500MHz, d_3-MeOH) δ = 4.1 (m, COOC*H*$_2$ ester linker, 4^{a}H, *di*-ester, 2^{b}H, *mono*-ester), 3.54 (m, 2^{b}H, HOC*H*$_2$, *mono*-ester), 2.6-3.5 (br m, 80H, NC*H*$_2$, of OEI ethylenes and NC*H*$_2$C*H*$_2$COO linker), 1,66 (m, 4^{a}H, COOCH$_2$C*H*$_2$ *di*-ester, 2^{b}H, *mono*-ester), 1,54 (m, 2^{b}H, HOCH$_2$C*H*$_2$ monoester), 1,40 (m, 4^{a+b}H, -OCH$_2$CH$_2$C*H*$_2$ linker).

molar ratio (linker/OEI) = 1.24 : 1; di-ester/mono-ester = 50 : 50

integrals: $^{a+b}$ (linker in total): 124 mol % of OEI ; ^{a} (di-ester): 62 mol % ; ^{b} (mono-ester): 62 mol %

2.3.1.2 Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy was conducted with a FT-IR Paragon 1000 spectrometer from Perkin Elmer, Boston, USA using KBr pellets.

2.3.2 Molecular weight determination

2.3.2.1 Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF)

Measurements were carried out on a Bruker Autoflex II MALDI-TOF spectrometer (Bruker Daltonik GmbH, Biburg, Germany). Targets and matrices were also obtained from Bruker GmbH.

Matrix solutions of either sinnapinic acid (SA) or 2.5-dihydroxybenzic acid (DHB) were prepared at a concentration 10 mg/ml in ACN/ 0.1% TFA (1:2). KCl was dissolved in methanol at a concentration of 10 mg/ml. For MALDI analysis, monomers and polymers in water at concentrations from 0.5 to 10 mg/ml were mixed with either matrix alone at a volumetric ratio of 1:1 or with matrix and cationizing agent KCl at a volumetric ratio of matrix / sample / KCl (2:1:1). A 1 µl aliquot was applied to the MTP 384 massive aluminium target. Laser powers of 45-60 % were used to obtain spectra.

2.3.2.2 SEC/Multi Angle Laser Light Scattering (MALLS)

All SEC/MALLS measurements were performed by Dr.Bruzzano, Fraunhofer IAP, Golm.

Samples were analyzed on TSK-gel guard + G-oligo-PW column using 0.2 M Na_2SO_4 + 1 % HAc (pH 3.2) as eluent (sample loop 0.1 ml, flow rate 1 ml/min) and compared

to commercially available PEI standards. The system was calibrated with PEG standards and by online viscosimetry, respectively. Molecular weight determination and calculations were based on UV-, RI- and MALLS detection.

2.3.2.2.1 Fractionation of OEI-HD-1

Fractions of OEI-HD-1 were separated on a TSK-Gel guard + G-oligo-PW column and samples were concentrated via macro-separators (Omega 3k (PALL), 4000 rpm, 20 min). The procedure was accomplished 5 times and resulting sample solutions were analyzed repeatedly by GPC.

2.3.3 DNA-binding and condensation

DNA binding and complexation of polymers to DNA (10 µg/ml) reduces DNA intercalation of ethidium bromide (EtBr). As only intercalated EtBr fluoresces strongly (λ_{ex} = 510nm, λ_{em} = 590nm) (Parker 2002) DNA condensation can be expressed as percentage of fluorescence intensity relative to un-complexed DNA (% rel.F= $F_{complex}/F_{DNA}$).

Fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian Deutschland GmbH, Darmstadt).

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

2.3.4 Polyplex formation

Plasmid DNA encoding luciferase was condensed with standard PEI or biodegradable polymers at various conjugate/ plasmid (C/P) – ratios (w/w). DNA / polycation polyplexes were prepared at a final DNA concentration of 20µg/ml as described in Kircheis et al. 1997. Briefly, indicated amounts of plasmid DNA and polycation were each diluted in either HBS or HBG and rapidly mixed by pipetting. Polyplexes were allowed to stand for at least 20 min at RT before use.

2.3.5 Agarose gel retardation

Polyplexes were prepared as described above. 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 80 V for 1 h and then photographed under UV light.

2.3.6 Particle size measurements

Particle size of transfection complexes was measured by laser-light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). Polyplexes were generated in either HBG or HBS at DNA concentrations of 20 µg/ml and were allowed to stand 30 min prior to measurement.

2.3.7 Degradation studies

2.3.7.1 Reduction of disulfide bonds

2.3.7.1.1 Degradation of polymers

Polymer stocks (c= 1mg/ml) were treated with 50 mM dithiothreitol (DTT) at 37°C for 0-24 h. After DTT treatment, polymer/ DNA complexes were formed at C/P 4 and C/P 10 in HBG and allowed to stand for 30 min at RT. Samples were loaded on a 0.8 % agarose gel (see ‘agarose gel retardation assay’) and the gel was run at 80 V for 1 h.

2.3.7.1.2 Degradation of complexes

Polymer stocks were diluted in HBG to a final concentration of 0.4 mg/ml. A stock solution of plasmid (pCMVLuc, c= 40 µg/ml) was prepared in HBG and polyplexes were mixed at weight ratios of conjugate to plasmid (C/P) of 4 & 10. After complex formation (30min at RT) polyplexes were incubated with 50 mM DTT for 0 – 120 min at 37°C. Samples were analyzed by gel electrophoresis as described above.

Hydrolysis of ester bonds

2.3.7.1.3 Degradation of HD polymers

Polymer stocks with a concentration of 5 mg/ml were adjusted to pH 5, pH 7 and pH 9 and incubated at 37°C for 0 – 5 days. After hydrolysis, the pH was adjusted to pH 7

and polymer/ DNA complexes were formed at C/P 4 in HBG (complex formation 30 min at RT). Samples were analyzed by agarose gel electrophoresis.

2.3.7.1.4 Hydrolysis of OEI-HD-1 and lt-OEI-HD-1

Degradation was studied with polymer stocks (5 mg/ml; pH 7) incubated for 0-15 days at 37 °C in an Eppendorf Thermomixer (500 rpm). Solutions with a pH of 5 and 9 were prepared by adding the appropriate amount of HCl and NaOH, respectively. After incubation, the pH was adjusted to pH 7 and sample aliquots were analyzed by SEC on a Sephadex G25 (10/30) column with 150 mM NaCl as eluent and a flow rate of 1 ml/min. Detection was carried out at 210 nm, 217 nm and 240 nm.

2.4 Biological properties of the polymers

2.4.1 Cell culture

Cell culture media, antibiotics and fetal calf serum (FCS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). All cultured cells were grown at 37°C in 5 % CO_2 humidified atmosphere. B16-F10 murine melanoma cells (kindly provided by I. J. Fidler, Texas Medical Center, Houston, TX, USA), CT 26 murine colon carcinoma cells (ATCC CRL-2638) and murine neuroblastoma Neuro2A cells (ATCC CCI-131) were cultured in DMEM (1g glucose/l) supplemented with 10 % FCS. HUH-7 hepatocellular carcinoma cells (JCRB 0403; Tokyo, Japan) were grown in DMEM high glucose/F12 (1/1) supplemented with 10% FCS. HT-22 cells were obtained from David Schubert (Salk Institute, San Diego, CA, USA). The HT-22 line was originally selected from HT-4 cells based on glutamate sensitivity. HT-4 cells were immortalized from primary hippocampal neurons using a temperature-sensitive SV-40 T antigen (Morimoto and Koshland 1990). HT-22 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT, USA) and gentamicin (50 µg/mL).

2.4.2 Luciferase reporter gene expression

For standard transfection screenings cells were seeded in 96 well plates (TPP, Trasadingen, Switzerland) at a density of 10^4 cells (Neuro2a cells) or 5 x 10^3 cells (all other cell lines) in 100µl culture medium per well. During transfection and for the following incubation until analysis, 100U/ml penicillin and 100 µg/ml streptomycin were present in the medium. Transfection complexes with indicated amounts of DNA (pCMVLuc) were added directly to the cells. Complex-containing medium was removed 4 h after transfection, 100µl of fresh medium were added and gene expression was measured after 24 h.

Cells were washed once with phosphate-buffered saline (PBS) at 20-24 h after transfection, then lyzed with 50 µl (96 well-plate) of reporter lysis buffer (Promega, Mannheim, Germany) per well. Luciferase activity was determined from 20 µl samples of the lysate using the Luciferase Assay system (Promega); measurements were performed in a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany). Values are given as relative light units (RLU) and per seeded cells (mean +/- standard deviations of at least triplicates). Two ng of recombinant luciferase (Promega, Mannheim) correspond to 10^7 light units.

2.4.3 Metabolic activity of transfected cells

Cells were grown (96-well plate) and treated with different amounts of DNA-complexes as described in ‘cell culture’ and ‘transfection’.

Metabolic activity of each well was determined by using a MTT / thiazolyl blue 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 removed and the samples were frozen at – 80 °C for at least 2 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) and cell viability was expressed as a percent relative to untreated control cells.

2.4.4 Cytotoxicity - microscopic observations

After incubation with polymers or polyplexes, changes in morphology and detachment of cells from the well plate were observed using a Zeiss Axiovert 200 microscope (Carl Zeiss AG, Oberkochen, Germany).

2.4.5 Endosomolytic activity of free polymer

The efficiency of polycations to enhance the endosomal release of poly-L-lysine (PLL) polyplexes was analyzed similarly as previously described (Kichler 2001).

B16F10 cells were seeded at a density of $5x10^3$ cells in 96 well plates and were grown for 24 h. PLL (pLys 251, Sigma-Aldrich, Deisenhofen, Germany) polyplexes were prepared at a C/P-ratio of 1 in HBG. After an incubation for 20 min at RT, PLL-polyplexes were diluted with fresh culture medium (DMEM, + 10 % FCS) to a final concentration of 2 µg DNA/ ml medium. Medium was removed, 100 µl of transfection medium was added to each well and cells were incubated at 37 °C, 5% CO_2. Two hours later, transfection medium was exchanged against 100 µl fresh culture medium and cells were incubated for another 2 h in the presence of free polycations at various concentrations (2.5–60 µg/ml). After removal of the polymers cells were incubated for 24 h and luciferase expression was measured as described in “Luciferase reporter gene expression”. Luciferase expression levels were compared to standard PLL polyplexes in the absence of endosomolytic agents.

2.4.6 Hemolytic activity of polymers

Human erythrocytes were isolated from fresh citrate-treated blood and washed in Hepes-buffered saline (HBS) by four centrifugation cycles, each at 2200 rpm for 10 min at 4°C. The erythrocyte pellet was diluted in either HBS pH 7.1, serum containing HBS or serum containing citrate buffer pH = 5 (150 mM NaCl, 15 mM citric acid) to a final concentration of 4 % erythrocytes. Erythrocyte concentration was determined using a Fuchs-Rosenthal counting chamber. Suspension of red blood cells was always freshly prepared and used within 24 h. PEI standards and new biodegradable polymers were serially diluted in 75 µl of the appropriate buffer (pH 7.1 vs. pH 5; no serum vs. 10 % serum) using a V-bottom 96 well plate, resulting in polymer concentrations of 0.25-1.0 mg/ml. For 100 % lysis, control wells contained buffer with

1 % Triton-X-100. Erythrocyte suspension (75 µl, containing approximately 10^9 erythrocytes) was added to each well and the plates were incubated at 37°C for 45 min under constant shaking. After centrifugation at 2200 rpm for 10 min, 80 µl supernatant was analyzed for hemoglobin release at 450 nm using a microplate plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria) whereas the erythrocyte pellet was used for monitoring erythrocyte aggregation. Experiments were performed in triplicates. The percentage erythrocyte lysis was calculated according to the presented formula.

$$\% \text{ lysis} = 100\% * \frac{A_{polymer} - A_{blank}}{A_{100\%lyse} - A_{blank}}$$

2.4.7 Polymer-induced erythrocyte aggregation

The erythrocyte pellet was resuspended in 100 µl HBS and transferred to a 24-well plate (Gibco) containing 1 ml HBS per well. Erythrocyte aggregation was visualized microscopically at a 32-fold magnification.

2.5 Modification of standard polyplexes

2.5.1 EGF receptor targeting

HUH7 cells were seeded in 96-well plates at a density $5x10^3$cells per well 24 h prior to transfection. Targeted DNA complexes were prepared by first diluting and mixing unmodified polycation with various amounts of EGF-PEG-PEI conjugates in HBG. Complexes were formed at different C/P-ratios and were allowed to stand for at least 20 min at RT before use. The final concentration of DNA in EGF-targeted and non-targeted complexes was 20 µg/ml and complexes were added directly to the wells. Complex-containing medium was removed 4h after transfection, 100µl of fresh medium were added and gene expression was measured after 24h as described in ‘Luciferase reporter gene expression’.

2.5.1.1 Competitive inhibition of the EGFR

2.5.1.1.1 Competition with an excess of free murine EGF

Cells were seeded out into 24 well plates (Gibco) at a density of 2.5 x 10^4 cells per well. In case of HepG2 and HUH7 cells, the wells were previously coated with 2.5 % (w/v) collagen (in PBS). After 24 h, cells were preincubated with 500 µl of culture medium containing 12 µg murine EGF (stock solution 2 mg/ml in PBS) or 12 µg bovine serum albumine (BSA, stock solution 2 mg/ml in PBS) respectively, as previously described (Blessing 2001). After 30 min complexes were added and cells were incubated for 4 h before complex containing medium was replaced by fresh culture medium. Luciferase measurement was carried out as described in 'Luciferase reporter gene expression'.

2.5.1.1.2 Blocking the EGFR with mouse anti-human EGFR

Cells were pre-incubated with medium containing mouse anti-human EGFR (3 µg/ 500 µl medium) and mouse IgG1 (3 µg/ 500 µl medium), respectively. After 30 min complexes were added (Blessing 2001). The following procedure was identical to that described above.

2.6 Photochemical intracellular release (PCI)

Cells were seeded out into 24 well plates (Gibco) at a density of 2.5 x 10^4 cells per well. In case of HepG2 and HUH7 cells, the wells were previously coated with 2.5 % (w/v) collagen (in PBS). After approximately 6-8 h, medium was replaced by medium containing 0.2 µg /ml TPPS2a (LumiTrans®); the medium of control cells was changed with photosensitizer (PS)-free medium and all cells were incubated for 18 h at 37 °C. All the procedures after addition of the photosensitizer were carried out in subdued light. The cells were washed three times with culture medium and incubated in 0.5 ml PS-free medium containing plasmid complex. Treated cells were incubated for 4 h at 37 °C, washed once with medium and after addition of 0.5 ml culture medium, the cells were exposed to blue light (LumiSource®, 4*18 W Osram L 18/67, Blue) with a light intensity of 13.5 MW/cm^2. After a light exposure of 60 s the cells were incubated at 37 °C for 24 h before analysis for luciferase expression.

3 Results

3.1 Biodegradable polymers based on oligomerized polyamines

3.1.1 Synthesis of the polymer library

A library of 54 polycations was synthesized by oligomerization of LMW polyamines with different crosslinkers. In a combinatorial approach, six polyamines which differed in their molecular weight, their amine content and the distance between amino groups **(Fig.6a)** were crosslinked at three different molar ratios of polyamine to crosslinker in order to generate a series of degradable cationic oligoamines.

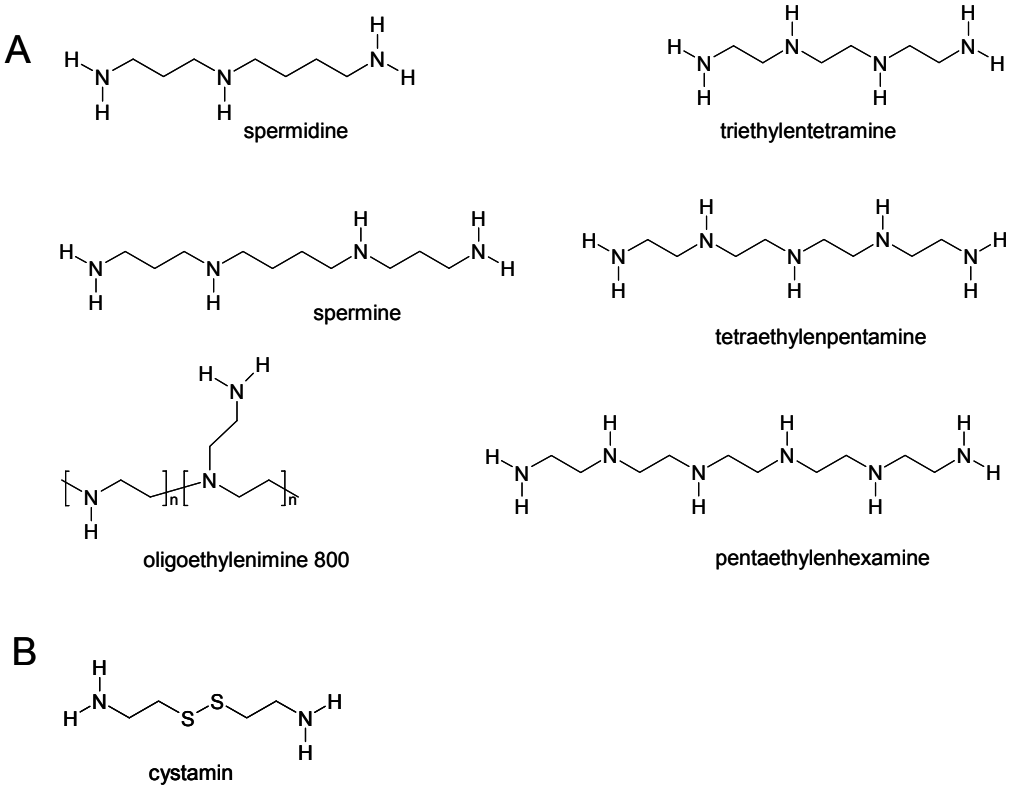

Fig.6: Chemical structures of polyamines

The absence of water during synthesis should prevent hydrolysis of reactive linker groups. Therefore, polyamines and crosslinkers were dissolved in DMSO (absolute, over molecular sieve, H_2O < 0.01 %) shortly before use.

3.1.1.1 Dithiobis(succinimidylpropionate) (DSP) crosslinking

DSP is a homobifunctional NHS (N-hydroxysuccinimide) ester-based electrophilic crosslinking reagent that reacts preferentially with primary amines to form amides. The linker is constructed around a centrally located disulfide bond, which is cleavable in a reducing environment **(Fig.7a).**

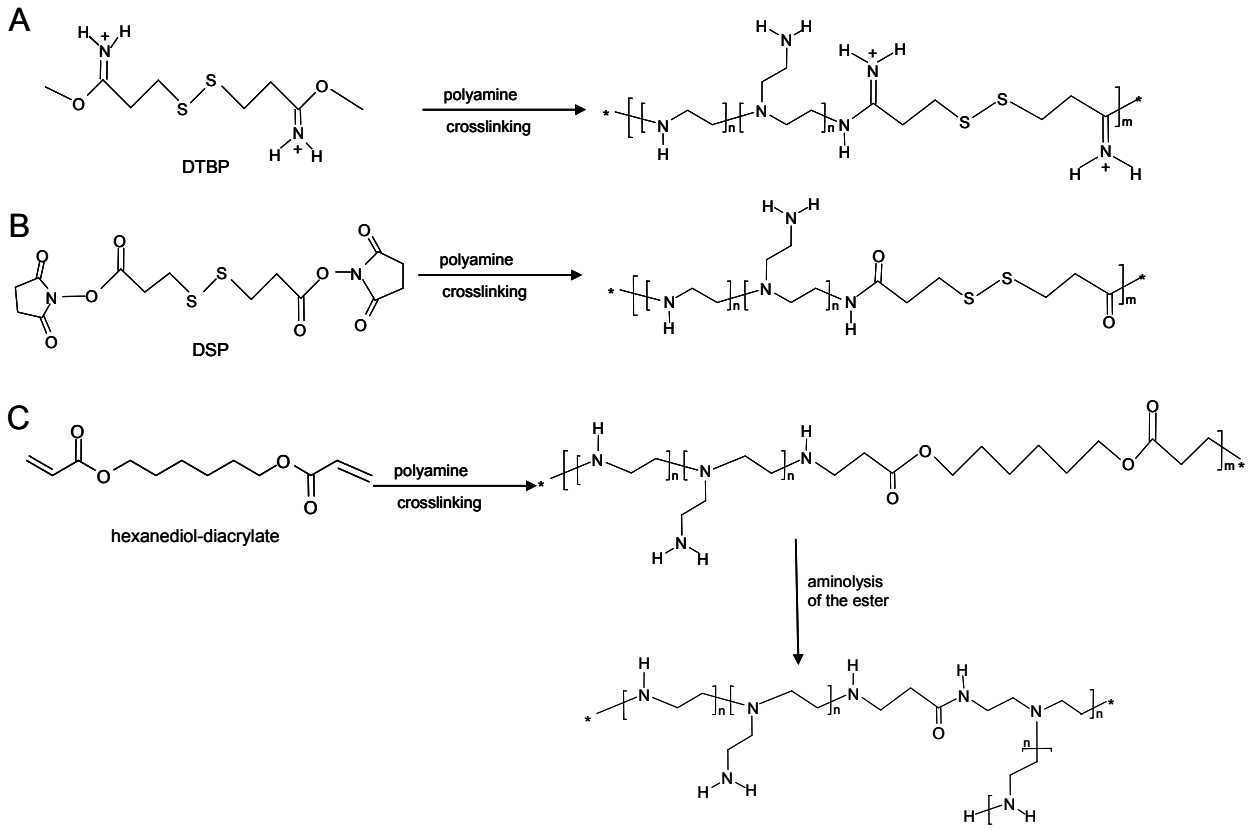

Fig.7: Synthesis of oligomerized polyamines.

Reaction with the DTBP crosslinker generates amidine-linked polymers (A). DSP crosslinking originates amide bond-containing structures (B); for DTBP and DSP polymers, degradation is based on the reduction of disulfide bonds. The HD-linker reacts with primary and secondary amines to form amine bonds. In a second reaction, esters can be converted into amides by inter- or intramolecular ester aminolysis. Thus, degradation would be based on cleavage of either ester or amide bonds (C). All reactions are illustrated for OEI 800 as model polyamine.

The reaction was carried out at 15°C for 24 hours. Use of a 2-fold molar excess of linker generated highly branched polymers which were only partly soluble in aqueous media **(Table 2)**. Whereas other polyamine-SP-2 structures could not be further processed, SP-SP-2 and OEI-SP-2 were dissolved and purified by SEC. Thus, 14 polymers of the SP series were included into the screening.

3.1.1.2 Dimethyl-3-3'-dithiobispropionimidate (DTBP) crosslinking

DTBP is very similar to DSP but it utilizes an imidoester reactive group which reacts with primary amines to form amidines, resulting in the retention of the positive charge **(Fig.7b).** As described for DSP crosslinking, the reaction was carried out at 15 °C for 24 hours. Each molar crosslinking ratio of polyamine to linker resulted in soluble polymers (altogether 18 polyamine-IP structures) which were suitable for subsequent purification and analysis **(Table 2).**

	SD	**SP**	**TT**	**TP**	**PH**	**OEI 800**
HD- 0.5	liquid	liquid	liquid	liquid	liquid	liquid
HD- 1	gel	gel	liquid	liquid	liquid	liquid
HD- 2	**solid**	**solid**	**solid**	**solid**	**solid**	**solid**
IP- 0.5	liquid	liquid	liquid	liquid	liquid	liquid
IP- 1	liquid	liquid	liquid	liquid	liquid	liquid
IP- 2	liquid	liquid	liquid	liquid	liquid	liquid
SP- 0.5	gel	gel	liquid	liquid	liquid	gel
SP- 1	clear	gel	liquid	liquid	liquid	gel
SP- 2	**gel**	gel	**gel**	**gel**	**gel**	gel

Table 2: Solubility of polymeric products after crosslinking *(fat accentuated states mean that the product could not be further processed and was therefore excluded from the following screenings)*

Abbreviations that were defined for polyamines (PA): *spermidine (SD), spermine (SP), triethylentetramine (TT), tetraethylenpentamine (TP), pentaethylenhexamine (PH) and oligoethylenimine 800 (OEI 800)* ***(first row)***
crosslinkers (CL): *hexanediol-diacrylate (HD), imidopropionate (IP) and succinimidylpropionate (SP)* ***(first column)***
and molar crosslinking ratios: *PA:CL=1:0.5* ***(-0.5),*** *PA:CL=1:1* ***(-1)****, PA:CL=1:2* ***(-2)***

3.1.1.3 Crosslinking with 1,6-hexanediol-diacrylate (HD)

The diacrylate linker reacts with polyamines by Michael addition to either primary or secondary amines, generating ester-based polymers. In a following reaction, free amino groups might convert ester-containing polymers into amide derivatives by aminolysis of the esters. This side reaction will depend on reaction temperature and the availability of supplementary amines **(Fig.7c).** For generation of the library, a reaction temperature of 60°C was applied for 4 days.

An excess of crosslinker resulted in gelation and originated highly branched caoutchuc-like polymers insoluble in aqueous media **(Table 2).** This means that only 12 polyamine-HD polymers were included into the screening.

For the following screenings, a uniform nomenclature was established. The name of each polymer consisted of three parts: first, the abbreviation for the polyamine that was oligomerized, second, the name of the linker and third, the molar ratio of polyamine to linker during the crosslinking reaction (for abbreviations see **Table 2**).

3.1.1.4 Synthesis of Cya-HD-1 and (OEI-IP-OEI)-HD

Apart from the library, two additional polymers which combined both degradation strategies (site-specific reductive cleavage of disulfide bonds and time-dependent ester hydrolysis) were synthesized and included into the screenings. The aim was to find out whether a combination of both linkages optimized polymer characteristics.

Cystamine (Cya), a disulfide-bond containing diamine **(Fig.6b)**, was oligomerized with HD-linker at a molar crosslinking ratio of 1 at 60°C and purified by SEC.

Additionally, a new OEI polymer based on OEI 800 that was dimerized in a first step by DTBP crosslinking (15°C) and then oligomerized by HD crosslinking (60°C) was synthesized (→ OEI-IP-HD-1).

Altogether, 44 soluble polymers of the library and 2 additionally synthesized structures (Cya-HD-1 and OEI-IP-HD-1) were included into the screening.

3.1.2 DNA binding activity of novel polycations

First of all it was important to investigate the ability of novel oligoamines to form polyplexes with DNA. Fluorescence of ethidium bromide (EtBr) intercalated into double-stranded DNA is strongly quenched after addition of polycations which interact electrostatically with negatively charged nucleic acids. Therefore polyplex formation as well as the density of the originated complexes can be monitored by EtBr exclusion assay.

DNA binding studies of each conjugate were performed in salt-free (HBG) and in physiological salt-containing (HBS) buffer at different conjugate/DNA ratios **(Fig.8).**

Polymers that consisted of short polyamines with few protonable amines, such as spermidine or triethylentetramine, showed weak DNA binding capacity in HBG, which was even more pronounced in the presence of 150 mM salt (HBS). At a conjugate/plasmid (w/w)-ratio (C/P-ratio) of 5, TT-IP-1 only reduced the relative fluorescence down to 60 % **(Fig.8f)** whereas the corresponding OEI-polymer, OEI-IP-1 at a C/P-ratio of 2 decreased fluorescence down to 20 % **(Fig.8l).** Comparison of different linkers elucidated that polymers that were generated by DSP crosslinking like e.g. PH-SP-0.5 **(Fig.8i&j)** were characterized by an overall weaker DNA binding efficiency. Within a polyamine group, IP-linked polymers always showed the strongest ambition to complex DNA and to exclude EtBr from the polyplex. HD-polymers, except OEI-HD's which had the same DNA binding affinity than OEI-IP, did not bind to DNA as strong as their IP counterparts, but better than their SP analogs.

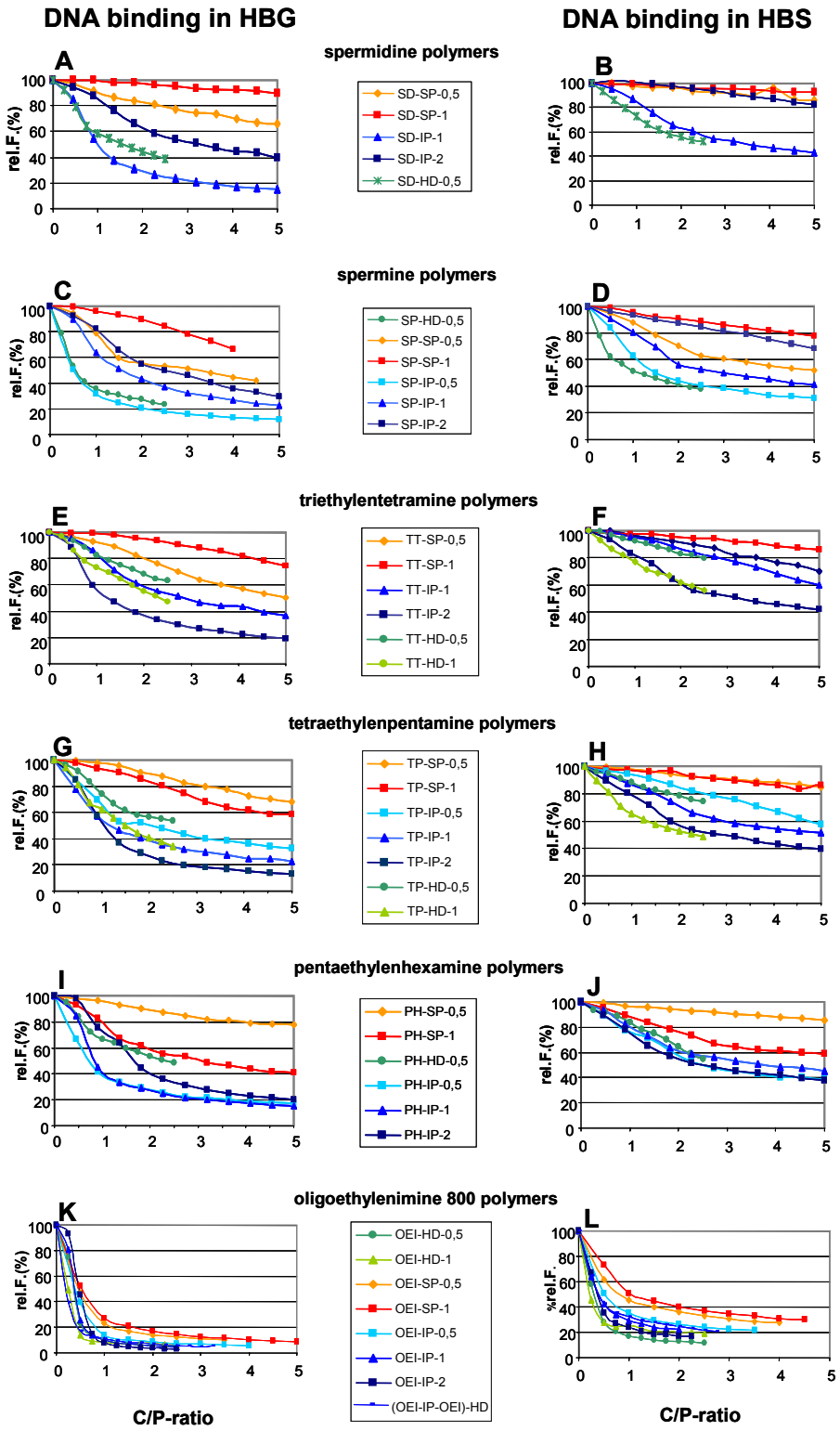

Fig.8: DNA binding and complex density *of spermidine- (A,B), spermine- (C,D), triethylentetramine- (E,F), tetraethylenpentamine- (G,H), pentaethylenhexamine- (I,J) and oligoethylenimine 800- (K,L) based polymers. Complexes were formed in the presence of EtBr in either HBG (left column) or HBS (right column) and inhibition of EtBr/DNA fluorescence was monitored as described in materials and methods. Measurements were performed in duplicates.*

To give more detailed information about the best condensing polymer out of one group, the C/P-ratio at which the fluorescence signal reached 50% of the original value (FI_{50}), and the minimal fluorescence intensity (FI_{min}) values are additionally summarized in **Table 3**.

Polymer	C/P-ratio to reach FI_{50}	FI_{min} (%)	C/P-ratio to reach FI_{50}	FI_{min} (%)
	HBG		**HBS**	
SD-HD-0.5	1.5	39	2.5	52
SP-HD-0.5	0.5	23	1.1	38
TT-HD-1	2.3	48	2.5	56
TP-HD-1	1.5	34	2.3	48
PH-HD-0.5	2.3	48	2.5	55
OEI-HD-1	0.3	6	0.3	19
SD-IP-1	1.0	13	3.4	37
SP-IP-0.5	0.5	9.4	1.5	27
TT-IP-2	1.2	14	3.2	37
TP-IP-2	1.0	12	2.7	33
PH-IP-1	0.8	14	3.6	40
OEI-IP-1	0.4	5	0.4	22
SD-SP-0.5	-	63	-	86
SP-SP-0.5	3.0	42	5.5	44
TT-SP-0.5	5.0	32	-	59
TP-SP-1	7.7	49	-	75
PH-SP-1	3.0	31	8.2	50
OEI-SP-0.5	0.5	8	1.0	30

Table 3: DNA condensation (in HBG and HBS) as studied by EtBr exclusion assay

Values represent the C/P-ratio of best condensing candidates out of one polymer group at which the fluorescence signal reaches 50% of the original value (FI_{50}) and the minimal fluorescence (FI_{min}).

DNA binding data of the additionally synthesized polymers OEI-IP-HD-1 and Cya-HD-1 was in accordance with structure/activity relationships observed for the library. OEI-IP-HD-1 had similar DNA binding affinity as OEI-HD-1 and OEI-IP-1, whereas the small polyamine-containing Cya-HD-1 was characterized by poor DNA condensation **(Fig.9).**

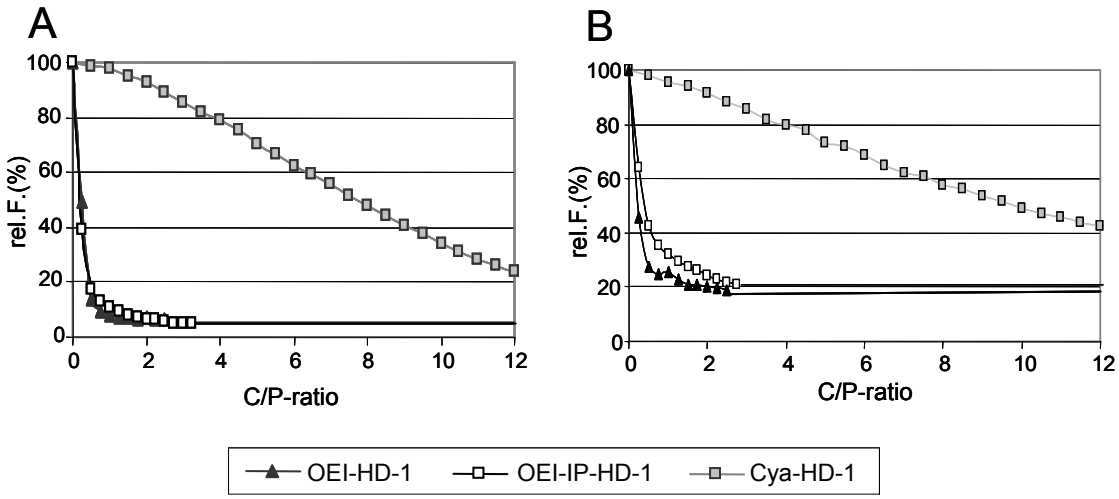

Fig.9: DNA binding and complex density of Cya-HD-1 and OEI-IP-HD-1

Complexes were formed in the presence of EtBr in either HBG (A) or HBS (B) and inhibition of EtBr/DNA fluorescence was monitored as described in materials and methods. DNA binding activity was compared to OEI-HD-1. Measurements were performed in duplicates.

Generally, efficient DNA binding correlated with the number of protonable amines provided by the polyamine and with the type of linkage generated by the crosslinker. Similar trends of complexation and polyplex stability were observed using the agarose gel retardation assay (data not shown). For example, in case of OEI-HD-1, OEI-IP-1 and OEI-IP-HD-1 DNA was completely retarded at C/P 0.5. OEI-SP-0.5 prevented DNA migration at C/P 1. All polyamine-IP polymers needed maximal C/P 4 to retard DNA, whereas small SP-crosslinked polyamines like SD-SP-0.5 did not form stable polyplexes up to a C/P-ratio of 25.

3.1.3 Reductive cleavage of disulfide linked polymers

As disulfide bonds are relatively stable in plasma and can be cleaved within the cell due to high redox potential differences outside and inside the cell, they present a useful tool for site specific drug delivery. According to previous publications, most in-vitro experiments dealing with polymer degradation based on the reduction of disulfide bonds (Read 2003 & 2005) are carried out in the presence of dithiothreitol (DTT) as reducing agent.

3.1.3.1 Degradation of polymers

First, it was important to check if SP and IP polymers were susceptible to reduction as expected. Therefore, polymer dilutions (1 mg/ml) were treated with 50 mM DTT at 37°C for 0-24 h. After reduction, polymer/DNA complexes were formed and analyzed by gel electrophoresis. Without DTT (t=0), SP-SP-0.5 and the control ester-based analog SP-HD-0.5 formed stable polyplexes which prevented electrophoretic migration of DNA **(Fig.10a).** After 2h of DTT treatment the disulfide-based polymer was partly degraded and was thereby not able anymore to provide polyplex stability, whereas polymer integrity of SP-HD-0.5 was not affected by the reducing environment **(Fig.10b-d).**

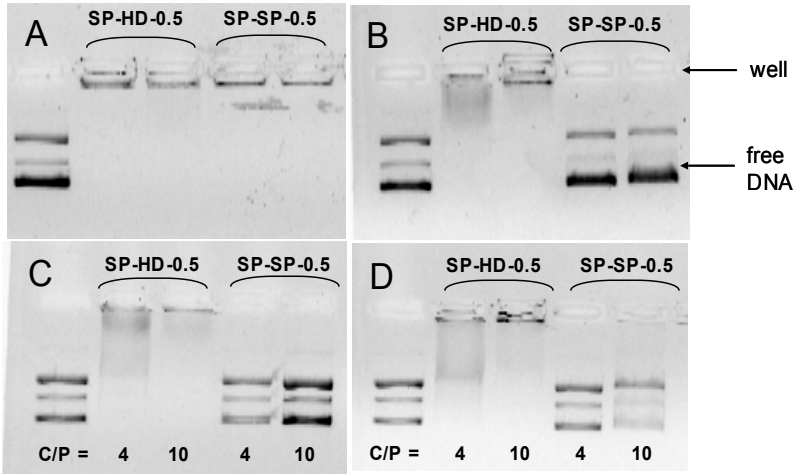

Fig.10: Reduction of disulfide bonds: polymer degradation

SP-HD-0.5 and SP-SP-0.5 were incubated with DTT at 37°C for 0 – 24 h. After DTT treatment, polymer/DNA complexes were formed at C/P 4 and C/P 10 in HBG and allowed to stand for 30 min at RT before gel electrophoresis. Incubation times t= 0 (A), t =2 h (B), t= 4h (C) and t= 24h (D) were analyzed separately. The first lane contains un-complexed DNA as a control.

3.1.3.2 Degradation of polyplexes

To determine the ability of polyplexes to be activated by reduction, SP-SP-0.5/DNA complexes with a C/P-ratio of 4 and 10 respectively, were reduced with DTT and polyplex stability was assessed by gel electrophoresis in the presence of 150 mM salt. **Fig.11** shows that a relatively low percentage of DNA migrated in the gel after complexation at C/P 4. At a C/P-ratio of 10, DNA was entirely retained in the well

under non-reducing conditions. When SP-SP-0.5 polyplexes were incubated with DTT for 15 min, particle stability decreased and DNA was completely released from the polyplex.

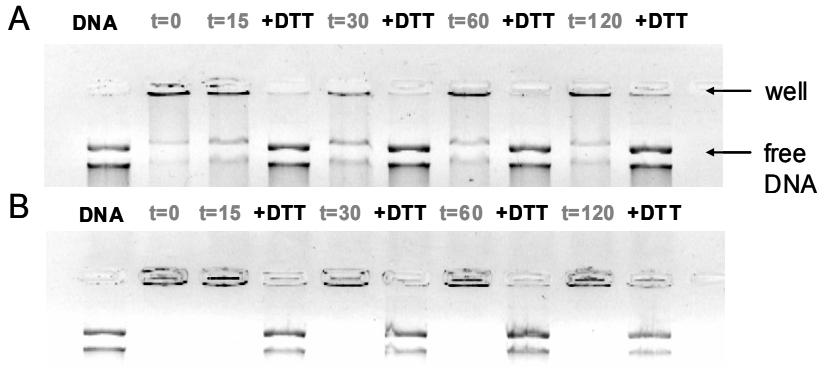

Fig.11: Reduction of disulfide bonds: polyplex degradation

SP-SP-0.5 polyplexes were prepared in HBG at C/P 4 (A) and C/P 10 (B) (complex formation at RT for 30 min) and were incubated +/- 50 mM DTT in the presence of 150 mM NaCl for 0-120 min before gel electrophoresis. The first lane contains un-complexed DNA as a control.

3.1.4 Hydrolytic degradation of HD-linked polymers

In contrast to site-specific cleavage of disulfide bonds, ester hydrolysis is pH-dependent and occurs over time. Esters are hydrolyzed in aqueous solution at basic as well as at acidic pH and should be more stable at neutral pH. Amides are less susceptible towards hydrolysis but can be degraded via base-catalyzed hydrolytic cleavage. In case of HD-linked polyamines, the resulting bonds could be either the original esters or amides (due to aminolysis of the ester) or even a mixture of both.

^{1}H-NMR analysis confirmed the presence of retained ester bonds as well as varying percentages of amide linkages for all analyzed HD polymers. Polymers containing small polyamines, for example TT-HD-1, had preserved 60 % of the hexanediol linker as diester, while in case of OEI-HD-1 80 % of the linker were hydrolyzed at one end, resulting in monoester formation. At crosslinker to polyamine ratios of 0.5, the percentage of diester relating to amount of polyamine was e.g. 14 % for SD-HD-0.5 and 24 % for SP-HD-0.5.

Degradation was studied at pH 5, 7 and 9 (incubation for several days at 37 °C) with SP-HD-0.5 as a model HD-polymer. The ability of degraded polymer fragments to form stable complexes was analyzed by gel electrophoresis. After incubation at pH 7, SP-HD-0.5 formed stable polyplexes (C/P 4 in HBG) which prevented the migration of DNA at all time points. At pH 5 and 9, polymer degradation could be observed after 24-72 h, but while hydrolysis at pH 9 resulted in complete DNA release after 5 days, the hydrolysis product of pH 5 treated polymers was still able to bind DNA to some degree **(Fig.12).**

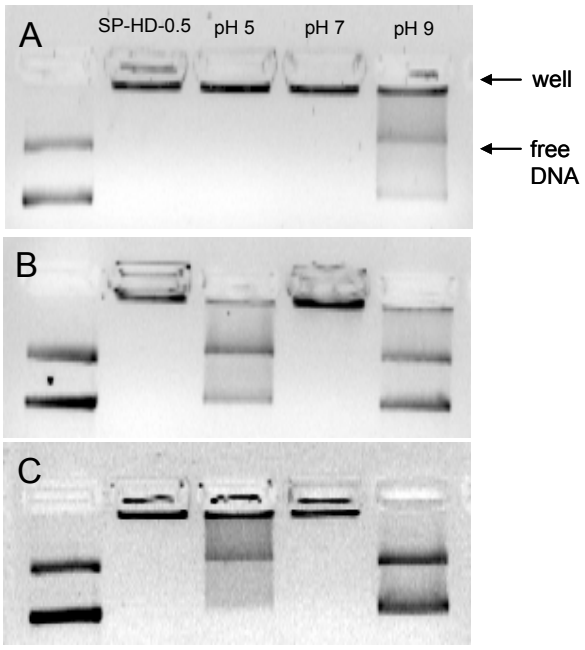

Fig.12: pH dependent hydrolysis: polymer degradation

SP-HD-0.5 was incubated at pH 5, 7 and 9 at 37°C for 1 - 5 days. After hydrolysis, polymer samples were adjusted to pH 7 and polymer/ DNA complexes were formed at C/P 4 in HBG. Complexes were allowed to stand for 30 min at RT before gel electrophoresis. Incubation times t= 24 h (A), t =3 days (B) and t= 5 days (C) were analyzed separately and compared to free DNA (lane 1) and untreated SP-HD-0.5 (lane 2).

DNA binding activity and degradation properties provided some information about the polymer and its ability to interact with nucleic acids, but for evaluation of gene delivery efficiency and toxicity, each polymer had to be screened in transfection assays using different cell lines.

3.1.5 Gene transfer activity

Reporter gene expression (luciferase activity) of the polymer library was evaluated by screening all cationic conjugates at different C/P-ratios (weight ratio of conjugate to plasmid DNA) on B16F10 murine melanoma cells. Polyplexes were prepared in HBG, a constant DNA concentration of 4 µg/ml was applied to the cells, and after 24 h luciferase expression was compared to 'golden standard' PEI22lin polyplexes (N/P 6 corresponding to C/P 0.8).

Although small non-oligomerized polyamines were able to bind weakly to DNA, they did not mediate gene transfer at all (e.g. spermine), or they were characterized by rather low expression levels (e.g. OEI 800, 10^2-10^3 RLU/5000, B16 cells; data not shown). All SP-linked polyamines needed a huge excess of polymer for efficient gene delivery **(Fig.13a)**. Whereas PH-SP-1 or OEI-SP-0.5 showed promising transfection efficiency at C/P 50 and 70 respectively, the other SP polymers were more than 10-fold less efficient than PEI22. Similarly, IP-linked polyamines lacked activity at low C/P ratios (data not shown). Increasing the amount of polymer resulted in enhanced transfection efficiency, particularly in case of TP-IP-2, PH-IP-2 and OEI-IP-1 **(Fig.13b)**. Both, SP-and IP-linked polymers were essentially non-toxic (data not shown). With regard to gene delivery, however, most of them could not compete with PEI22 under these conditions. Polymers of the HD-series exhibited stronger toxicity at C/P 50-70. Therefore, a following transfection experiment was carried out with lower C/P ratios **(Fig.13c)**. At C/P 25, SP-, SD-, TT-, TP- and PH-HD's showed maximum gene expression which varied between E+02 and 8E+07 relative light units per 5000 cells. Interestingly, OEI-HD polymers were highly efficient at C/P 2.5 and 5, achieving the same transfection activity as PEI22. Cya-HD-1 needed an immense excess of polymer to form a polyplex and was still characterized by rather poor reporter gene expression (at C/P 64 it mediated gene transfer 10,000-folds lower than PEI22lin and OEI-HD-1, data not shown). As mentioned above, OEI-IP-HD-1 had similar DNA binding affinity as OEI-HD-1 and OEI-IP-1, but transfection efficiency did not exceed that of standard OEI polymers (data not shown).

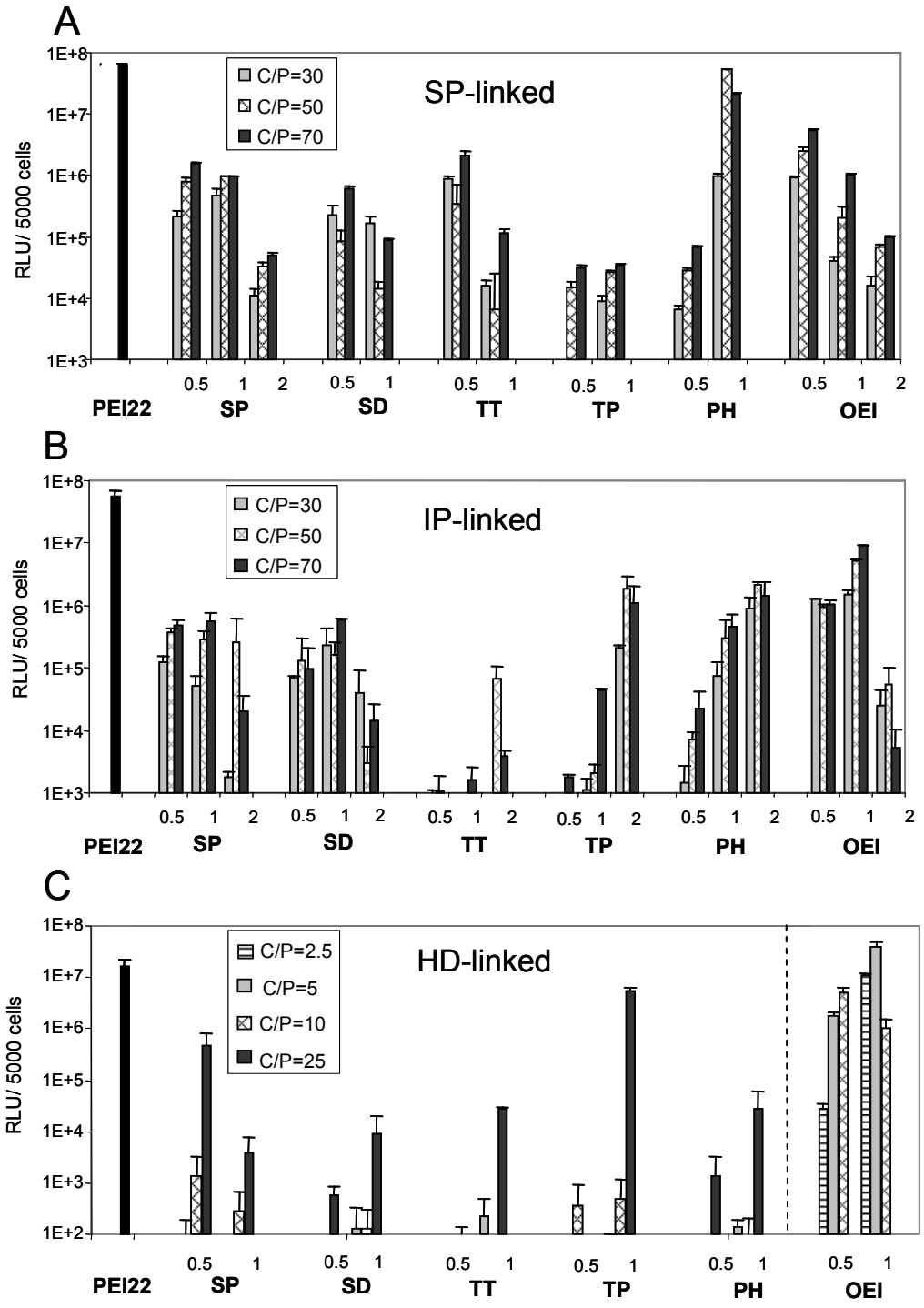

Fig.13: Reporter gene expression of B16F10 cells after transfection with novel polymers

B16F10 cells were transfected with 400ng (4 µg/ml DNA) pCMVLuc complexed with SP-linked polyamines (A), IP-linked polyamines (B) and HD-linked polyamines (C). Complexes were mixed in HBG at different C/P-ratios and compared to standard PEI polyplexes (PEI22lin at C/P 0.8). Luciferase activity is presented as mean values +SD of triplicates.

In the following screenings on different cell lines (Neuro2a, HUH-7 and HT22 cells), C/P-ratios of polymers were optimized for each cell line in order to highlight polymers with an overall promising transfection activity. Step by step, polycations with very poor gene delivery potential were extracted and the screening was continued with the best performing polymer of each group. Results are shown for the most efficient C/P-ratio of each polymer **(Fig.14).**

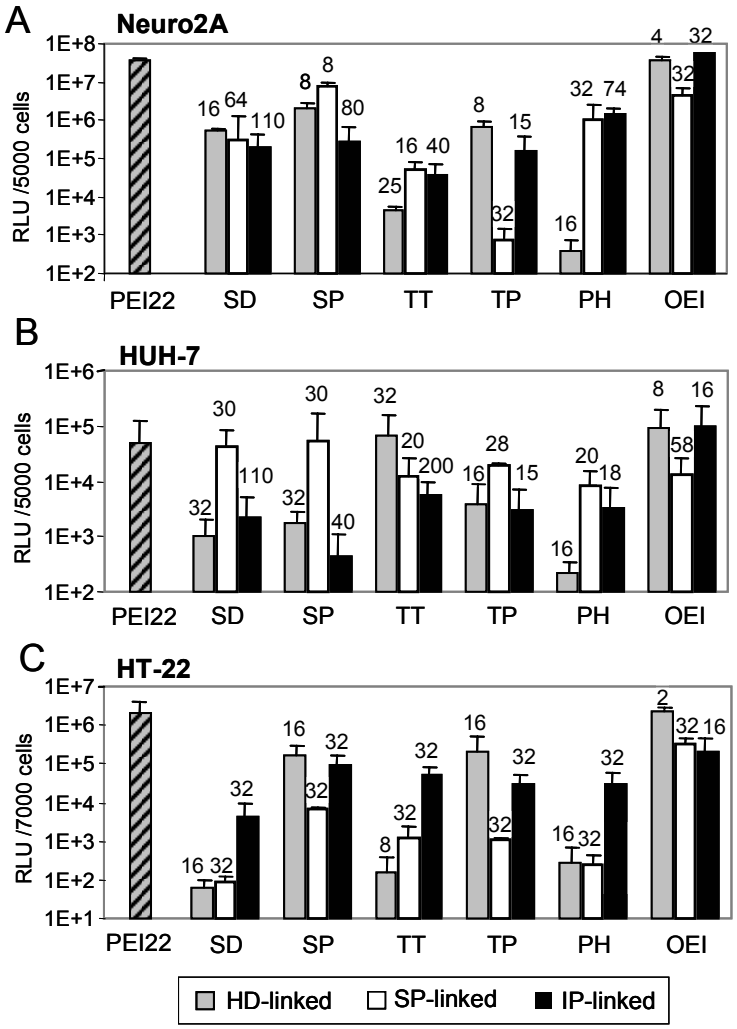

Fig.14: Transfection efficiency of novel polycations at their optimal C/P-ratio on various cell lines – screening

Neuro2a (A), HUH-7 (B) and HT-22 (C) cells were transfected with 200ng (2 µg/ml DNA) pCMVLuc complexed with SP-, IP- and HD-linked polyamines. Complexes were mixed in HBG at various C/P-ratios and compared to standard PEI polyplexes (PEI22lin at C/P 0.8). Values above the columns represent the optimal C/P-ratio for each formulation. Luciferase activity is presented as mean values of triplicates.

It became clear that some candidates performed very well in one cell type while exposing low efficiency in transfecting another one. To give an example, TT-HD-1 mediated gene transfer as efficient as PEI22lin in HUH-7 cells but had very poor transfection efficiency in Neuro2a and HT22 cells. Other polycations like e.g. SD-IP-1 needed huge amounts of polymer (C/P 32-110) to achieve fair reporter gene expression. It is important to note that OEI-HD-1 polyplexes could be applied at low C/P-ratios (2-8) and mediated gene transfer as efficient as PEI22lin in all analyzed cell lines. Cell type independent efficiency was also observed for OEI-IP-1 and OEI-SP-0.5, however, both polymers needed high C/P-ratios (C/P 16-60) for efficient gene delivery and their transfection activity was sometimes 10-fold lower than OEI-HD-1.

3.1.6 Lytic activity of novel polymers

In order to compare the membrane destabilizing activity of SP-, IP-, and HD-polymers at neutral pH, their lytic activity was tested on erythrocytes. Freshly isolated human erythrocytes were incubated with free polymers at final concentrations of 0.5 and 1.0 mg/ml in HBS, pH 7.1, for 45 min at 37°C. Whereas SP- and IP-linked polymers hardly induced any erythrocyte lysis (the maximal hemoglobin release was 10 % for OEI-SP-0.5), HD polymers exhibited distinct membrane destabilizing activity (up to 44 % for TT-HD-1) **(Fig.15a)**. Hemolytic activity of HD polymers strongly depended on the chain length of the hydrophilic polyamine unit and therefore on the overall hydrophobicity of the polymer. TT-HD-1 with a polyamine Mw of 146 Da induced 44 % erythrocyte lysis whereas OEI-HD-1 with a polyamine Mw of 800 Da resulted in 20.5 % hemoglobin release.

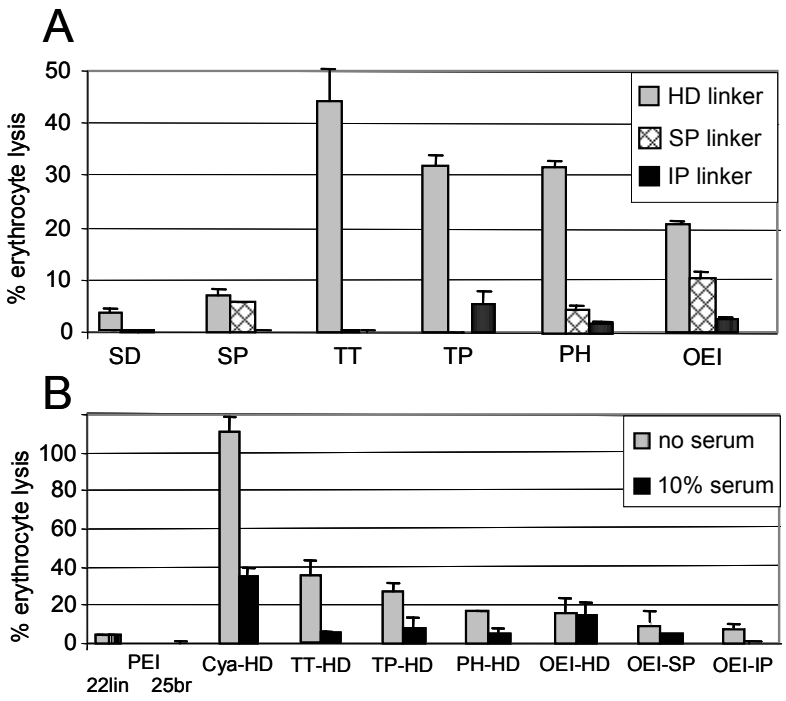

Fig.15: Influence of the crosslinker on hemolytic activity

Human erythrocytes were incubated with free polymers (final polymer concentration 1mg/ml) for 45 min at 37°C and hemoglobin release was quantified at 450nm (A). Hemolysis induced by active candidates was further studied in the presence of 10% serum (B). 100% lysis refers to TritonX treatment of erythrocytes. Percentage of erythrocyte lysis is presented as mean values +SD of triplicates.

$$\% \text{ lysis} = 100\% * \frac{A_{polymer} - A_{blank}}{A_{100\%lyse} - A_{blank}}$$

In order to better adjust experimental conditions to the in vivo situation, hemolytic activity of active candidates was studied in the presence of 10 % serum **(Fig.15b)**. In all cases hemolytic activity was strongly reduced when serum proteins were present. For instance, TT-HD-1 induced 36 % erythrocyte lysis in the absence, but only 4.5 % lysis in the presence of serum. Interestingly, Cya-HD-1 displayed a very strong hemolytic activity in the absence of serum (100 % erythrocyte lysis) which was still very significant (almost 40 % erythrocyte lysis) in the presence of 10 % serum.

3.1.7 Polymer-induced erythrocyte aggregation

Another important biological aspect for in vivo application is the influence of polymers on erythrocyte morphology. HMW polycations, such as PEI22lin, are known to induce erythrocyte aggregation which can result in occluded blood vessels and embolism. To analyze the effect on red blood cells, erythrocytes were incubated with polymers (final polymer concentration 1 mg/ml) and aggregation was visualized by microscopy. Whereas PEI22lin and PEI25br induced aggregation within 5 min, even in the presence of serum, the novel polymers (except OEI polymers) did not have any effect on erythrocytes **(Table 4)**. In the absence of serum, the number of red blood cells was extremely reduced after treatment with Cys-HD, TT-HD and TP-HD due to their hemolytic activity.

	SD	SP	CYA	TT	TP	PH	OEI
HD–0.5	-	-	-	-	-	-	+
HD–1	-	-	-	-	-	-	++
IP-0.5	-	-		-	-	-	-
IP-1	-	-		-	-	-	-
IP-2	-	-		-	-	-	-
SP-0.5	-	-		-	-	-	+
SP-1	-	-		-	-	-	+
IP-HD-1							+
PEI22lin	+++						
PEI25br	+++						

Table 4: Polymer-induced erythrocyte aggregation

Erythrocytes were incubated with free polymers at a final polymer concentration of 1mg/ml for 30 min at 37°C. Aggregation was visualized microscopically (Zeiss Axiovert 200 microscope) with a 32-fold magnification.

3.1.8 Endosomolytic activity of promising candidates

The fate of polyplexes in the endosome will strongly influence transfection efficiency; therefore, an ideal gene carrier should possess endosomolytic activity by itself. Recently, Kichler et al. reported that polycations with intrinsic lysosomotropic properties, such as PEI, can act as helper for polylysine (PLL)-mediated transfection (Kichler 2001). On the basis of these findings, cells were transfected with PLL

polyplexes (C/P 1 in HBG) and after 2 h, transfection medium was exchanged against polymer-containing medium. This experiment requires the localization of both polymers in the same vesicle and addition of the free polymer in a concentration that mediates endosomolytic activity without cytotoxic effects.

The effect of standard PEI's (PEI22lin and PEI25br) on PLL-mediated transfection was analyzed in order to establish a reliable method for our purpose. **Fig.16a** shows that luciferase activity increased 50-fold in the presence of PEI22lin and 100-fold in the presence of PEI25br (5 µg/ml free polymer). Based on these experimental conditions, new polymers were screened for their endosomolytic activity. Polymers like Cys-HD-1, TT-HD-1 and TP-HD-1 which exposed strong hemolytic activity (see 3.1.5), showed no or only weak enhancement of PLL-mediated gene delivery. OEI-HD-1, however, at a concentration of 15 µg/ml, resulted in 42-fold improved reporter gene expression compared to standard PLL polyplexes. Both, OEI-IP-1 and OEI-SP-0.5 enhanced gene transfer efficiency 9-fold at a concentration of 60 µg/ml **(Fig.16b).**

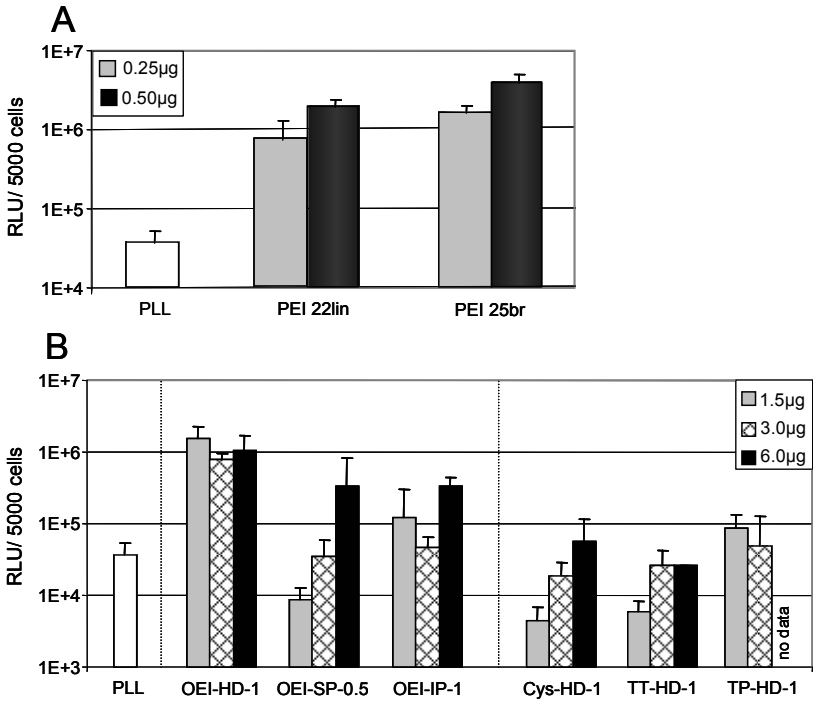

Fig.16: Polycation-mediated gene delivery of PLL polyplexes

B16F10 cells were transfected with 200ng (2 µg/ml DNA) pCMVLuc complexed to PLL at C/P 1 in HBG. After 2 hours, transfection medium was removed and fresh culture medium with 0.25 – 6.0 µg (2.5-60 µg/ml) free polycation was added. Enhancement of PLL mediated gene delivery is shown for PEI25br and PEI22lin (A) and for selected polymers of the library (B). Luciferase activity is presented as mean values +SD of triplicates.

3.2 OEI-based polymers: a comparative study

Due to their promising transfection efficiency and their interesting endosomolytic properties in the first screening experiments, the OEI polymers OEI-HD-1, OEI-IP-1 and OEI-SP-0.5 were chosen for a detailed analysis.

3.2.1 Reporter gene expression and cytotoxicity of OEI polymers

As demonstrated in **Fig.17a**, the pre-oligomer OEI 800 displayed rather poor transfection efficiency on B16 cells, but after crosslinking, all OEI polymers were able to mediate gene transfer as efficient as PEI22lin. Whereas OEI-HD-1 showed maximal gene expression at C/P 2, OEI-IP-1 and OEI-SP-0.5 needed a huge excess of polymer (C/P 64) to achieve similar luciferase activity.

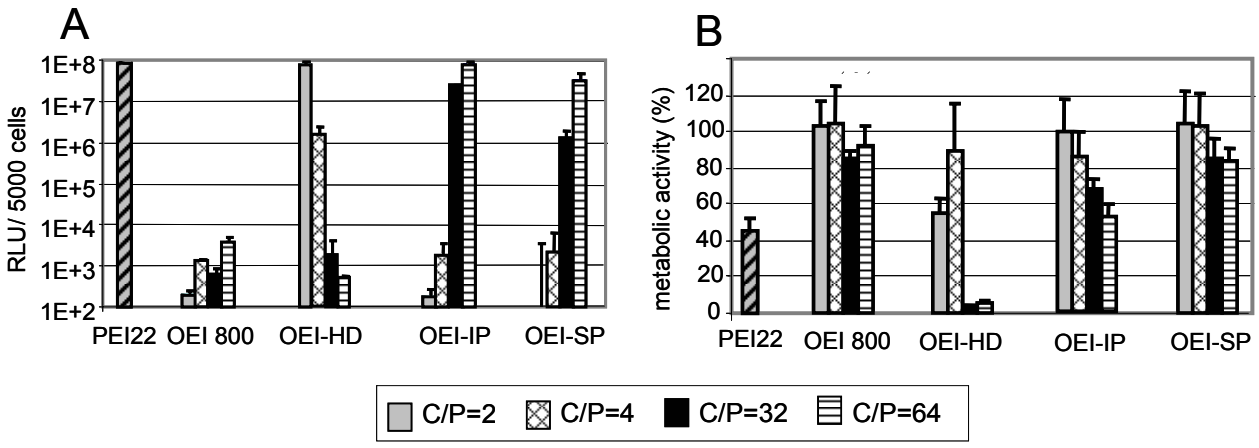

Fig.17: Reporter gene expression of B16F10 cells after transfection with OEI polymers

B16F10 cells were transfected with 200ng (2 µg/ml DNA) pCMVLuc complexed with LMW OEI, OEI-HD-1, OEI-SP-0.5 and OEI-IP-1 in HBS. Control PEI22lin polyplexes were prepared at C/P 0.8. Luciferase activity (A) and metabolic activity (MTT Assay) (B) are presented as mean values +SD of triplicates.

Twenty four hours after transfection, the metabolic activity of transfected cells was analyzed in order to evaluate toxicity profiles of OEI polyplexes. At their optimal C/P ratio all polymers were less toxic than PEI22lin **(Fig.17b)**. Nevertheless, OEI-HD-1 showed distinct toxicity at higher charge ratios. Furthermore, phase contrast microscopy revealed changes in cell morphology when treated with OEI-HD-1 and

most strikingly after PEI22lin application. In order to clarify whether these trends were only cell type specific, OEI polymers were tested in an additional cell line. Transfection of Neuro2a cells resulted in similar outputs as already described for B16 cells **(Fig.18)**. OEI-HD-1 displayed high gene transfer activity at low C/P-ratios and was less toxic (~60 % metabolic activity) than PEI22lin (~30 % metabolic activity). Again, OEI-IP-1 and OEI-SP-0.5 polyplexes required an excess of polymer (up to C/P 32) to achieve equivalent reporter gene expression.

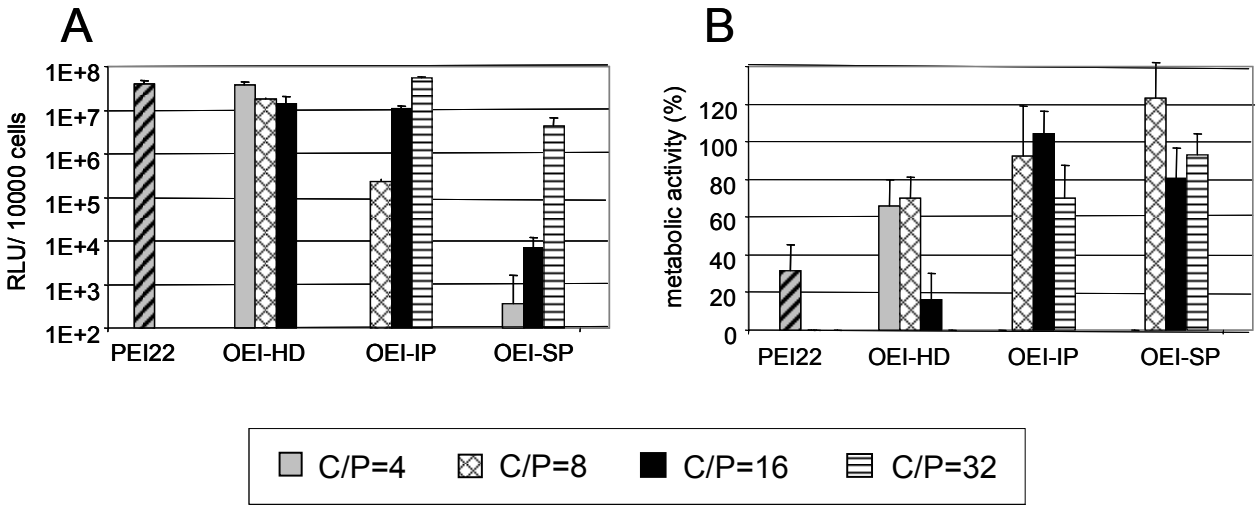

Fig.18: Reporter gene expression of Neuro2a cells after transfection with OEI polymers

Neuro2a cells were transfected with 200ng (2 µg/ml DNA) pCMVLuc complexed with OEI-HD-1, OEI-IP-1 and OEI-SP-0.5 in HBG. Control PEI22lin polyplexes were prepared at C/P 0.8. Luciferase activity (A) and metabolic activity (MTT Assay) (B) are presented as mean values +SD of triplicates.

Particle size measurements of OEI polyplexes at C/P 2 and 4 revealed that OEI-HD-1 polyplexes formed small and uniform particles < 100 nm which remained small under physiological salt concentration. In contrast, OEI-IP-1 and OEI-SP-0.5 polyplexes were about 150 nm when prepared in HBG at C/P 4, but tended to aggregate in the presence of salt. Since OEI-IP and OEI-SP polyplexes needed higher C/P-ratios for efficient gene delivery, particle size for C/P 32 polyplexes was analyzed in both buffers. Aggregation in HBS was reduced, however particles still remained big (470 and 650 nm respectively) in comparison to OEI-HD-1 polyplexes **(Table 5).**

Polyplex	C/P-ratio (w/w)	Size (nm) HBG	Size (nm) HBS
OEI-HD-1	2	61 +/- 19	114 +/- 32
	4	93 +/- 19	115 +/- 61
OEI-IP-1	2	2,554 +/- 508	2,317 +/- 436
	4	126 +/- 101	837 +/- 194
	32	105 +/- 43	469 +/- 101
OEI-SP-0.5	2	231 +/- 40	2,262 +/- 437
	4	175 +/- 35	1,948 +/- 229
	32	285 +/- 101	647 +/- 152

Table 5: Particle size of OEI polyplexes

DNA complexes with C/P-ratios of 2, 4 and 32 were prepared in either HBG or HBS at a final DNA concentration of 20 µg/ml. Particles were allowed to stand for 30 min before analysis.Polyplex formation and particle size measurement were performed as described in 'materials and methods'.

3.2.2 Erythrocyte aggregation induced by OEI polyplexes

As already shown in table 3, only OEI-based polymers induced aggregation when erythrocytes were incubated with rather high concentrations (1 mg/ml) of free polymer. Since this concentration will presumably not be reached in vivo, the amount of polymer was reduced to 20 µg/ml. Again, PEI22lin caused heavy aggregation of red blood cells resulting in 'clots'. Erythrocyte aggregation induced by OEI-HD-1 was less significant but still visible whereas OEI 800, OEI-IP-1 and OEI-SP-0.5 had no effect on the morphology of erythrocytes **(Fig.19).**

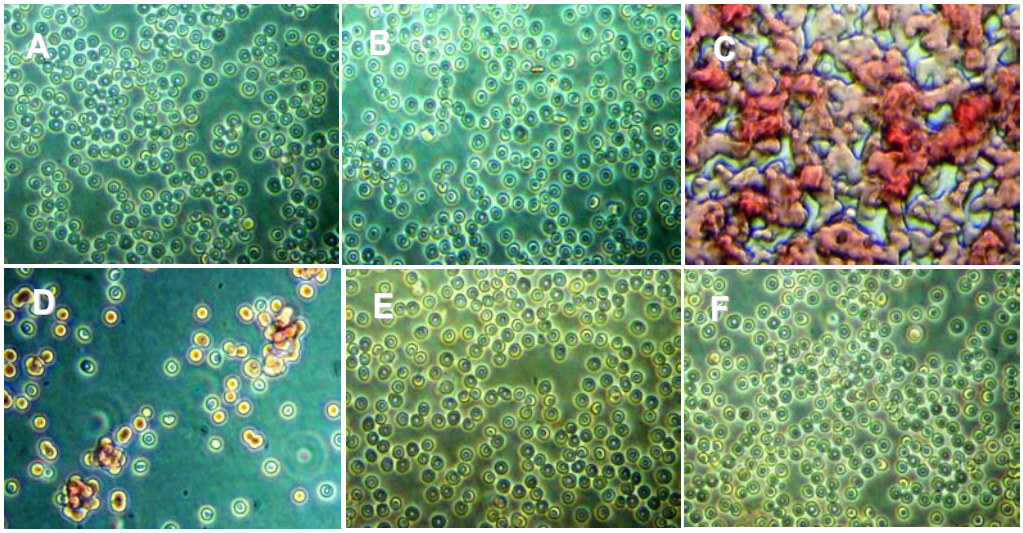

Fig.19: Erythrocyte aggregation induced by OEI polymers

Erythrocytes (2E+06) were incubated with 5 µg polymer in 220 µl HBS (+ 10 % FCS) at pH 7.1 for 20 min at 37°C. Untreated control erythrocytes (A), OEI 800 (B), PEI 22lin (C), OEI-HD-1 (D), OEI-IP-1 (E), OEI-SP-0.5 (F).

Since complexation with DNA might reduce cationic charge-dependent interaction with negatively charged membranes, the same experiment was carried out with polyplexes (containing equal amount of polymer, but complexed with DNA). Incubation with OEI-HD-1 polyplexes resulted in a drastically decreased interaction whereas PEI22 polyplexes still induced erythrocyte aggregation **(Fig.20).**

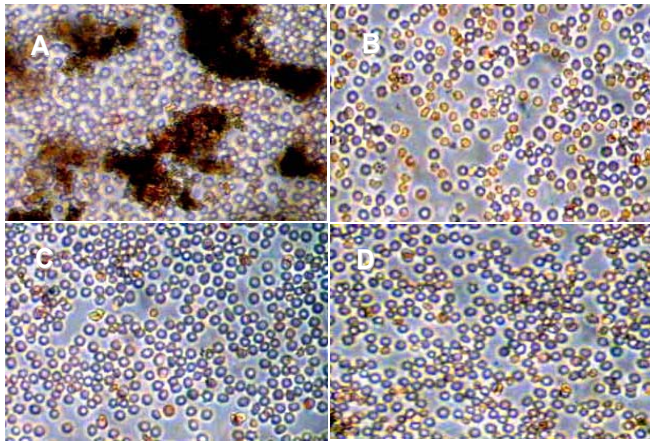

Fig.20: Erythrocyte aggregation induced by OEI polyplexes

Polyplexes were formed at C/P 2 in HBG. After complex formation, erythrocytes (2E+06) were incubated with polyplexes corresponding to 5 µg polymer as described in figure 19. PEI22lin (C/P 0.8) (A), OEI-HD-1 (B), OEI-IP-1 (C), OEI-SP-0.5 (D)

Taken together, OEI-SP-0.5 and OEI-IP-1 were characterized by negligible toxicity and efficient reporter gene delivery at high C/P-ratios. OEI-HD-1 possessed superior transfection efficiency and could compete with the 'golden standard' PEI22lin, but on the other hand exhibited distinct cytotoxicity at high doses, and free OEI-HD-1 polymer induced erythrocyte aggregation similar to PEI22lin. Since erythrocyte aggregation and cytotoxicity correlate with molecular structure, charge density and high molecular weight of a polymer (Fischer 2003), the next step was to elucidate structural properties of OEI-HD-1 which could be responsible for these toxic side effects.

3.2.3 Low temperature (lt)-OEI-HD-1: a less toxic analog of standard OEI-HD-1

The initial synthesis of OEI-HD-1, like all HD syntheses of the library, was carried out at 60°C in order to obtain a high degree of polymerization. But high molecular weight could be responsible for toxic side effects and, in addition, temperature-dependent aminolysis of esters could generate less degradable amide-based polymers.

The aminolysis of esters is a basic organic reaction considered as a model for the interaction of the carbonyl group with nucleophiles (Ilieva 2003). According to chemical theory, esters are cleaved into amides and alcohols especially at high reaction temperatures (Beyer-Walter, 23rd edition). In case of OEI-HD-1, one could predict that free amino groups of the oligoethylenimine unit can attack ester bonds, resulting in amide-based oligomers **(Fig.21)** with only few esters left. Furthermore, aminolysis of esters could finally seperate the whole hexanediol moiety from the polymer resulting in amide-derivatives of polyethylenimine and free hexanediol.

In order to circumvent this side-reaction and to generate pure ester-containing polymers, a new OEI-HD-1 derivative was synthesized at low temperature (20°C instead of 60°C). The generated polycation namely lt-OEI-HD-1 was purified and processed as described for standard OEI-HD-1.

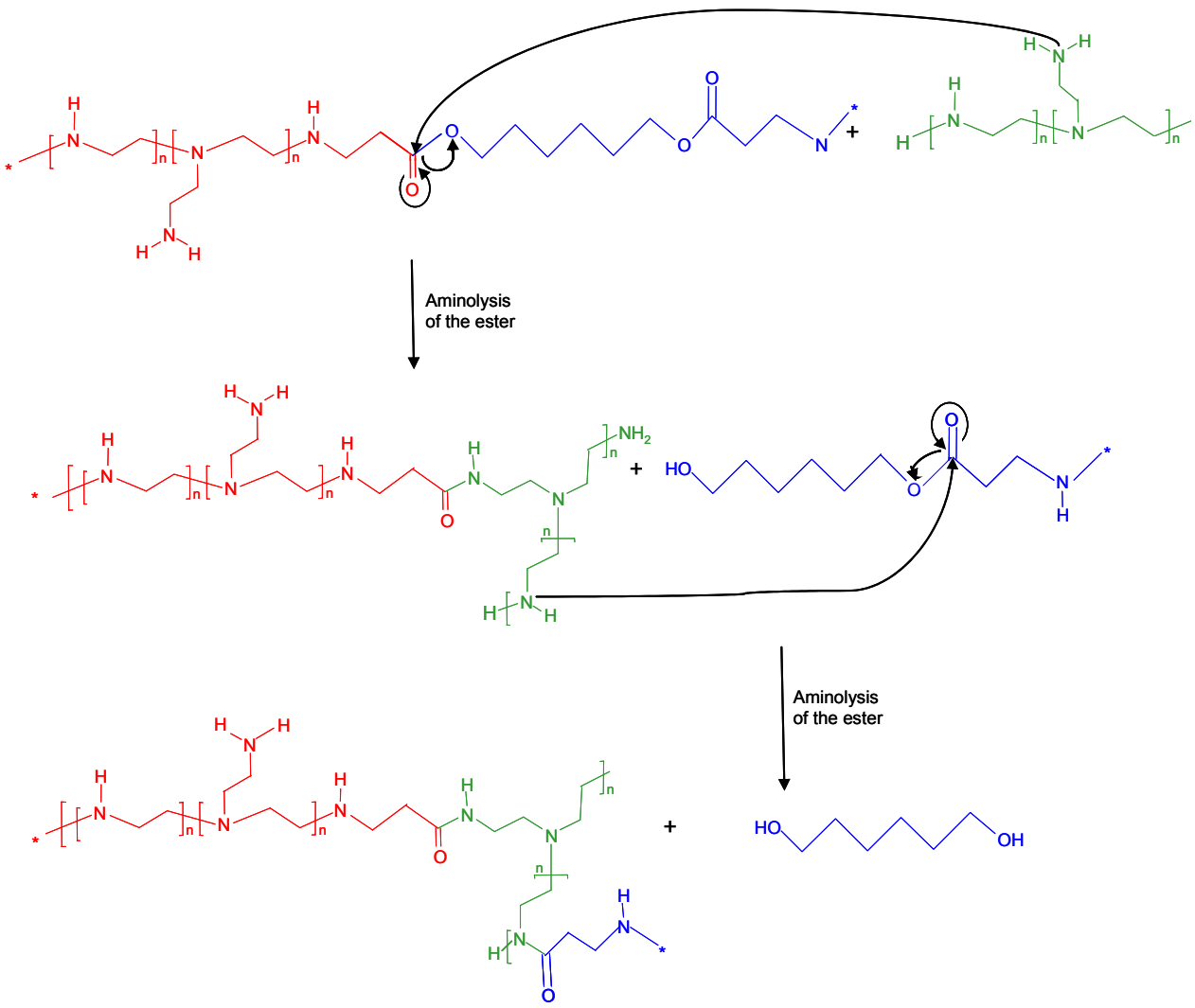

Fig.21: Mechanism of the ester aminolysis (reaction scheme)

Free amino groups of the OEI unit can attack ester bonds and thus mixed ester/amide-based oligomers are generated. In a second step, aminolysis of the ester could separate the whole hexanediol moiety from the polymer resulting in amide-derivatives of polyethylenimine and free hexanediol. This side reaction can occur inter- and intramolecular.

3.2.3.1 Structural analysis by FTIR

To confirm the original structure of 1.6-hexanediol-diacrylate, the linker was analyzed by FTIR spectroscopy. The spectrum clearly showed an ester (1731 cm^{-1}) and the alkene (1636 cm^{-1}) (data not shown). FTIR spectra of standard OEI-HD-1 verified the

formation of amide bonds (1638 cm^{-1}) and a weak signal of remaining ester bonds (1718 cm^{-1}) (signal c, d & e in **Fig.22b**).

As expected, lt-OEI-HD-1 contained ester bonds (1725 cm^{-1}) and conjugation to OEI 800 could be observed due to a broad N-H signal (3422 cm^{-1}) (signal a & b in **Fig.22a**).

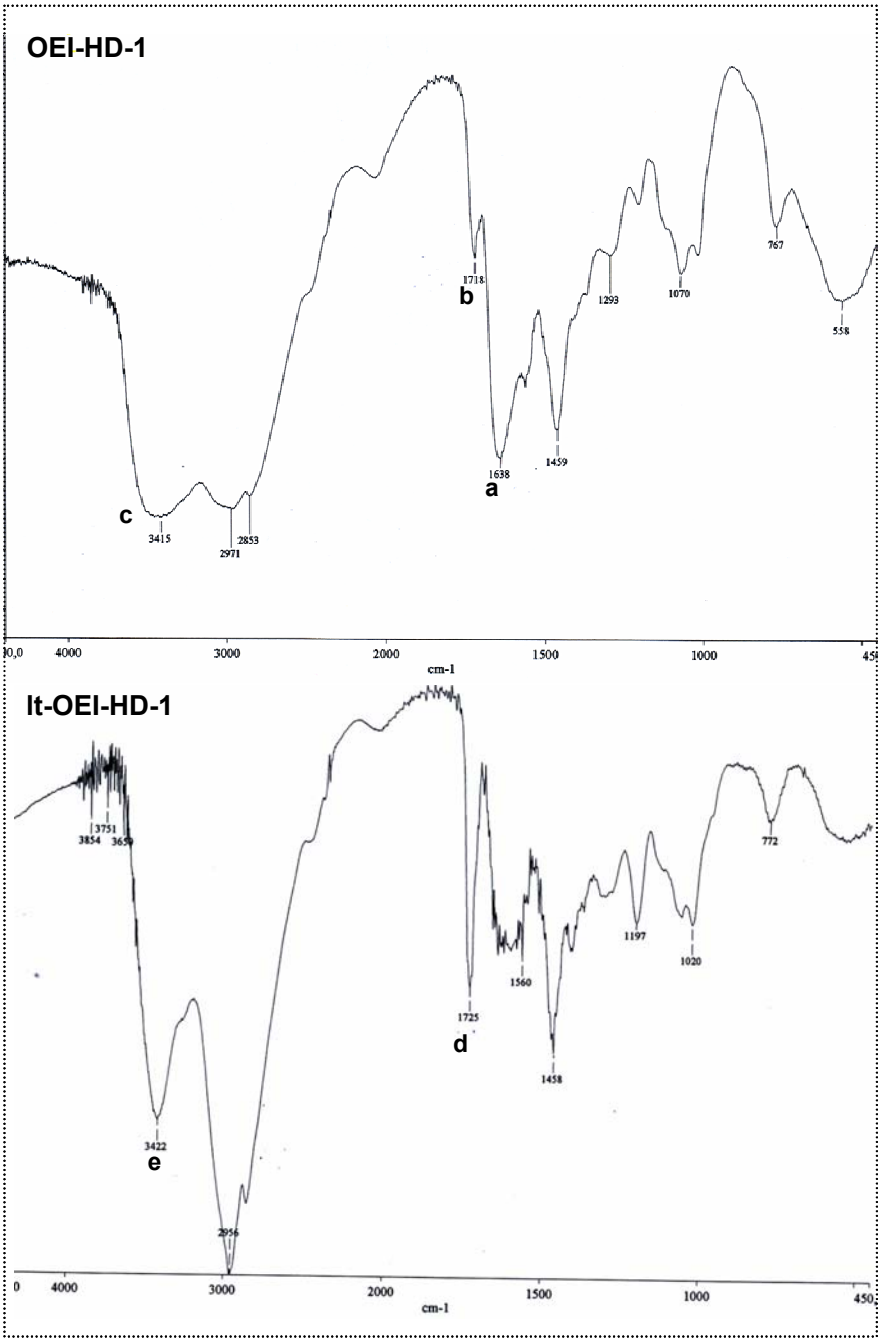

Fig.22: FT-IR analysis of OEI-HD-1 and lt-OEI-HD-1

3.2.3.2 Structural analysis by ^{1}H NMR

HD-crosslinking was confirmed by observing ester bond formation in the final product using ^{1}H NMR. Although FTIR spectra indicated extensive ester aminolysis in case of OEI-HD-1, both polymers (OEI-HD-1 and lt-OEI-HD-1) clearly showed the protons belonging to ester adjacent –CH_2 groups (signal a & b in **Fig.23**).

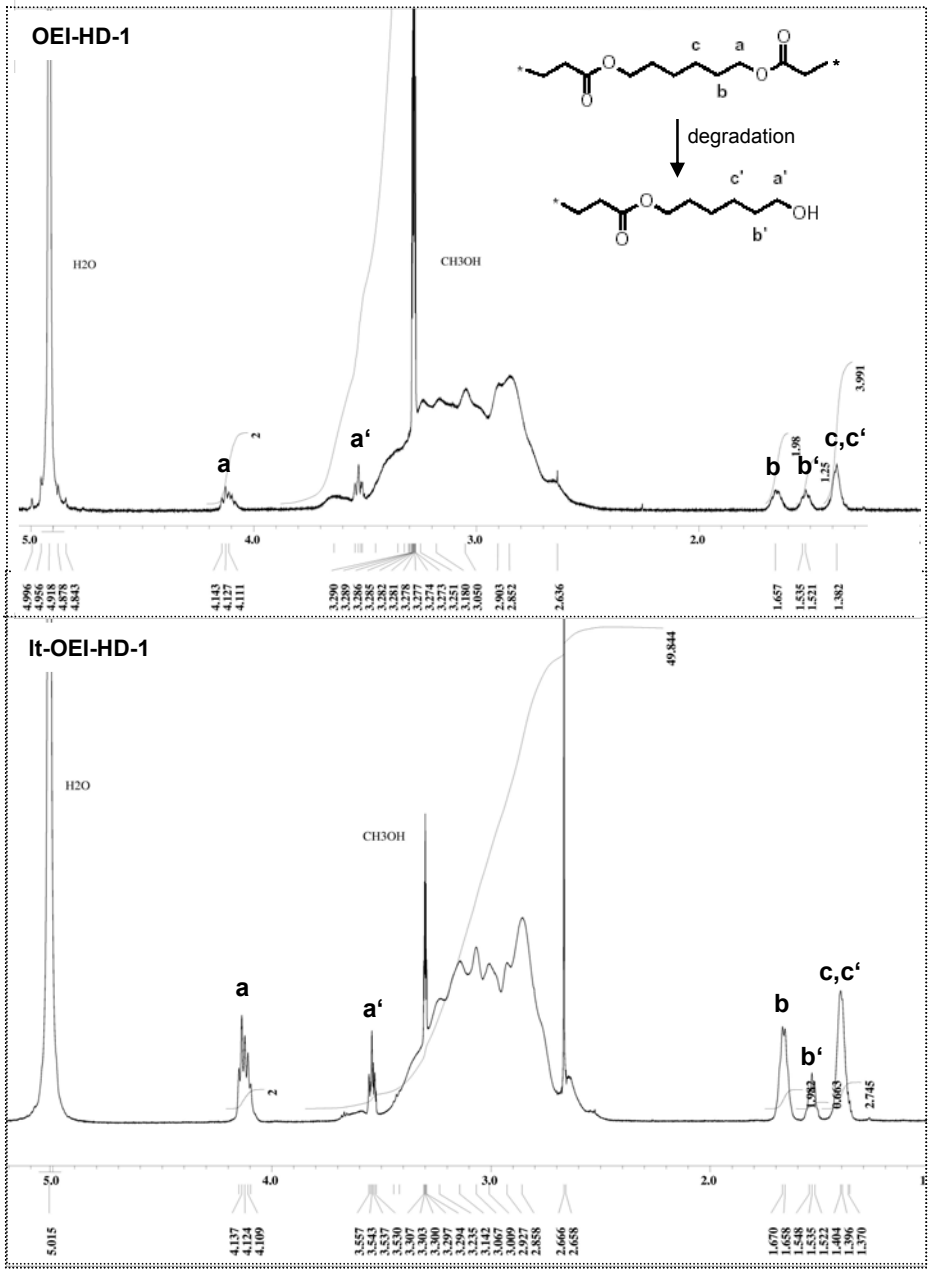

Fig.23:^{1}H-NMR analysis of OEI-HD-1 and lt-OEI-HD-1

Moreover, the hexane chain of the HD-linker was still present in both polymers. NMR spectra of OEI-HD-1 and lt-OEI-HD-1 showed the same signals, while the ratio between their integrated areas differed (signal a & b for protons belonging to ester adjacent - CH_2 groups and signal a' & b' for -CH_2 next to hydroxyl groups). Calculations based on the integration of NMR spectra resulted in a molar ratio of linker to OEI of 1.24 in case of lt-OEI-HD-1 and a ratio of only 0.47 for OEI-HD-1. Moreover, lt-OEI-HD-1 had preserved 50 % of the hexanediol linker as diester, while in the OEI-HD-1 polymer 80 % of the hexanediol diester was converted into the monoester.

3.2.3.3 Degradation studies

Degradation kinetics of both polymers were studied in aqueous solutions at pH 5, 7 and 9 at 37°C. The results of size exclusion chromatography demonstrated that molecular weight distribution of lt-OEI-HD-1 changed rapidly when incubated at pH 7 and even more pronounced after pH 9 treatments **(Fig.24a).** SEC elution profiles showed a shift from larger fractions towards oligomer structures with smaller size.

As expected, OEI-HD-1 was less susceptible to hydrolysis. After 5 days, the elution pattern of pH 9 incubated polymers changed slightly, whereas incubation at pH 5 and pH 7 had no visible effect (data not shown). Alterations in peak symmetry after 10 days indicated initial degradation processes **(Fig.24b)** however, it was not clear whether these changes would have any effect on biological activity of the polycation.

Therefore, biological activity of non-treated OEI-HD-1 and lt-OEI-HD-1 was compared to their 'degraded' analogs. Since molecular weight usually correlates with cytotoxic properties of a polycation, molecular weight decrease attributable to degradation should result in reduced toxicity of the polymer.

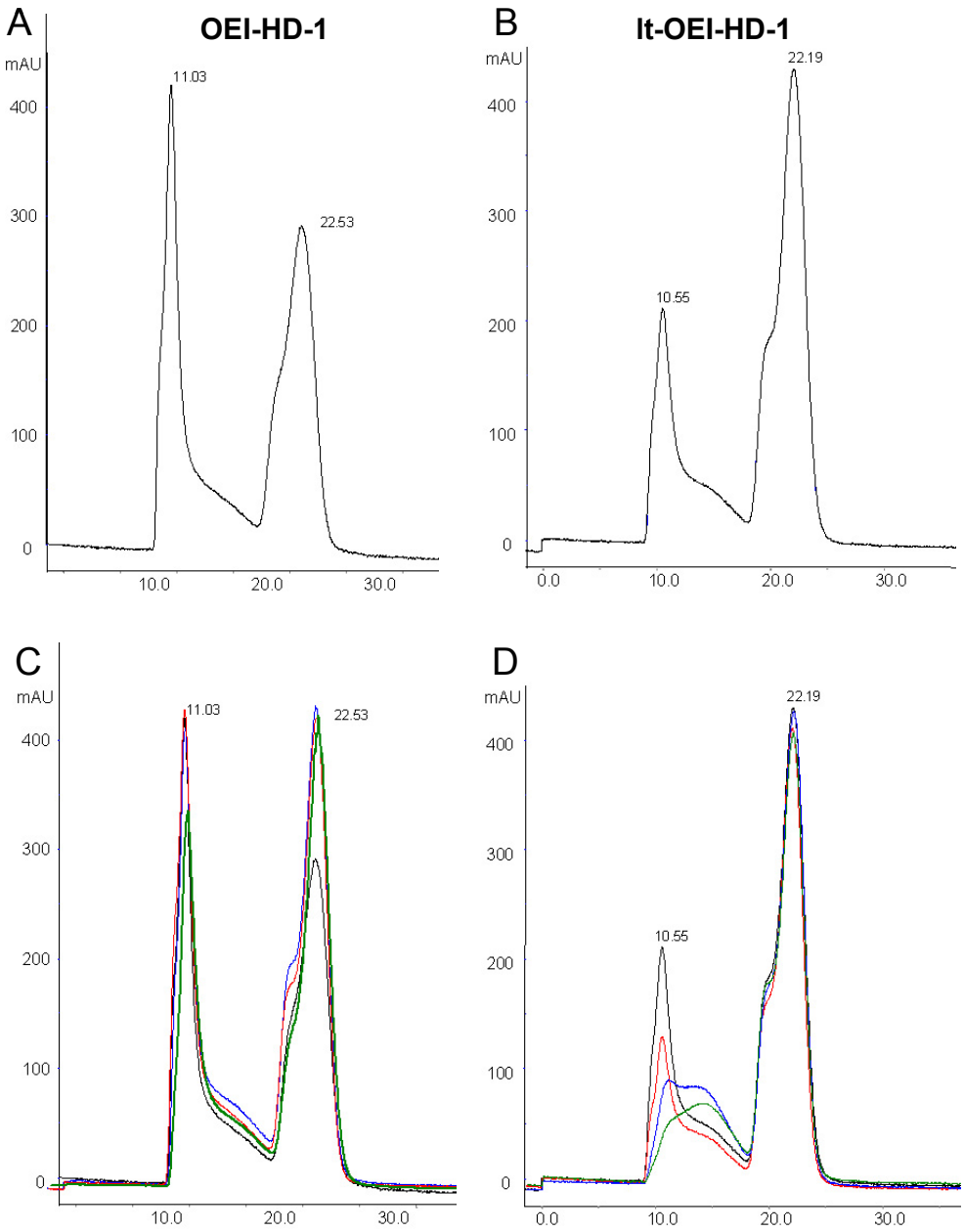

Fig.24: Degradation of OEI-HD-1 and lt-OEI-HD-1 (SEC)

Polymer samples (5 mg/ml) were analyzed by size exclusion chromatography (Sephadex G25 superfine) with 150 mM NaCl as eluent and a flow rate of 1ml/min. Black curves represent the elution profiles of untreated OEI-HD-1 (A & C) and untreated lt-OEI-HD-1 (B & D). Polymers were incubated at pH 5 (red curves), pH 7 (blue curves) and pH 9 (green curves) for 0 -15 days (37°C) and neutralized before SEC analysis. pH dependent degradation is shown for OEI-HD-1 after 10 days (C) and for lt-OEI-HD-1 after 5 days (D).

3.2.3.4 Toxicity of degraded polymers

B16F10 cells were incubated with various amounts of free polymer for 4 h. Twenty four hours after polymer treatment their metabolic activity was determined by MTT assay and compared to untreated control cells. At a concentration of 50 µg/ ml, PEI22 and OEI-HD-1 decreased metabolic activity down to 3 % (PEI22) and 15 % (OEI-HD-1) whereas cells that were treated with the same amount of lt-OEI-HD-1 retained 88 % of their metabolic activity **(Fig.25).**

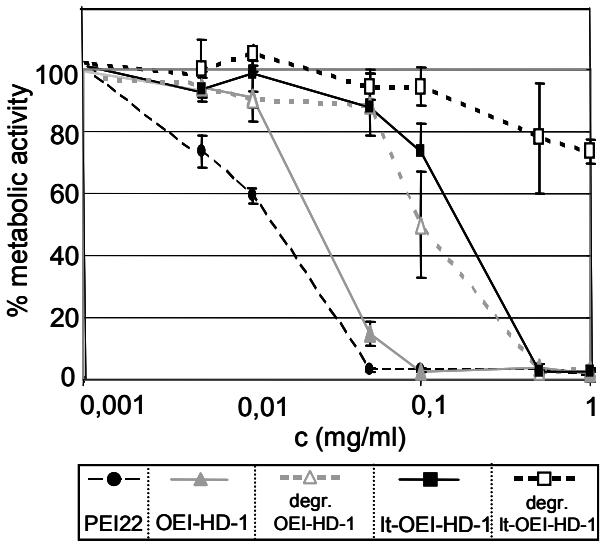

Fig.25: Cytotoxicity induced by degraded polymers

OEI-HD-1 and lt-OEI-HD-1 (5 mg/ml) were incubated at pH 9 for 5 days at 37 °C. B16F10 cells (5000 cells/well) were treated with various concentrations of hydrolyzed and non-hydrolyzed polymer. After 4 h polymer-containing medium was exchanged and 24 h later, metabolic activity was determined by MTT assay. Metabolic activity is presented as mean values +/- SD of triplicates.

Degradation of OEI-HD-1 significantly reduced its toxicity when the polymer was applied at lower doses (5 – 50 µg/ml). However, concentrations above 500 µg/ ml strongly disturbed viability and resulted in 2-3 % metabolic activity. After lt-OEI-HD-1 hydrolysis the degraded fragments exhibited negligible toxicity even at polymer concentrations up to 1000 µg/ml.

As mentioned above, high temperatures during synthesis will not only promote side reactions, but will also influence the degree of polymerization. This would suggest a higher molecular weight for standard OEI-HD-1 (60°C) and a lower degree of

polymerization in case of lt-OEI-HD-1 (20°C). Our preliminary SEC data suggested a bimodal molecular weight distribution for OEI-HD-1 with one HMW fraction eluting at the void volume. In contrast, the main peak of lt-OEI-HD-1 appeared at a later retention time which implies a lower molecular weight of this polymer.

However, our standard size exclusion chromatography did not present an accurate method for molecular weight determination and could only provide preliminary information.

3.2.3.5 Molecular weight determination

There are various methods to analyze the molecular weight of macromolecules. Depending on the analyt (size, hydrophobicity, charge, polydispersity) the molecular weight can be determined by viscosity measurement, Nuclear magnetic resonance spectroscopy (NMR), Multi Angle Laser Light Scattering (MALLS), Asymmetrical Flow Field Flow Fractionation (AF4), mass spectroscopy or size exclusion chromatography (SEC).

While reliable methods exist for the analysis of LMW substances, peptides, proteins or even hydrophobic polymers, however, molecular weight determination of cationic polymers is still a challenge.

3.2.3.5.1 Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF)

MALDI-MS is a powerful tool to determine the molecular weight of proteins but also of some polymers like e.g. polyethylenglycol (Whittal 1997). With the use of an appropriate matrix and sample preparation protocol, HMW polymers with masses up to 1.5 MDa can be studied (Schriemer 1996). Due to high sensitivity even small quantities of polymer can be analyzed. Therefore, MALDI-TOF-mass spectroscopy was a promising method to analyze the composition of educts as well as the molecular weight of novel polymers.

OEI 800 Da and OEI 2000 Da were used as standards to establish a method for the analysis of cationic oligomers. Spectra of OEI 800 showed molecular weight

distribution of the polymer and also the mass of one ethylene unity. Similar results were obtained for OEI 2000 (data not shown). Analysis of standard HMW polymers like PEI25br resulted in spectra with signals which could not be interpreted for mass determination due to high signal/noise ratios. According to the literature, molecular weight determination of strongly basic or strongly acidic components is difficult or impossible by MALDI (Juhasz 1994). One approach to solve this problem was the use of KCl as cationizing agent (as described by Lim et al. 1999) but no better spectra were obtained for HMW PEI standards or OEI-based polymers. As high polydispersity is another reason for problems in MALDI-TOF analysis, PEI25br was fractionated by SEC (Sephadex G25) and fractions were analyzed separately, but even this did not result in interpretable spectra.

MALDI-TOF mass spectroscopy did not provide reliable data, thus size exclusion chromatography should give information about the molecular weights of OEI polymers.

3.2.3.5.2 Size exclusion chromatography (SEC/MALLS)

SEC/MALLS measurements were performed by Dr. Bruzzano, Fraunhofer IAP, Golm. Polymer molecular weights were analyzed by aqueous phase gel permeation chromatography (GPC), employing 0.2 M Na_2SO_4 + 1 % formic acid as eluent. Samples of different molecular weights were successfully separated on a TSK-Gel Guard + G-Oligo-PW column. Absolute molecular weights of OEI 800 (pre-oligomer), OEI-IP-1, OEI-HD-1 and lt-OEI-HD-1 **(Fig.26a)** were determined with a MALLS detector (dn/dc (PEI) = 0.195 ml/g at 633 nm) and compared to commercially available LMW PEI's.

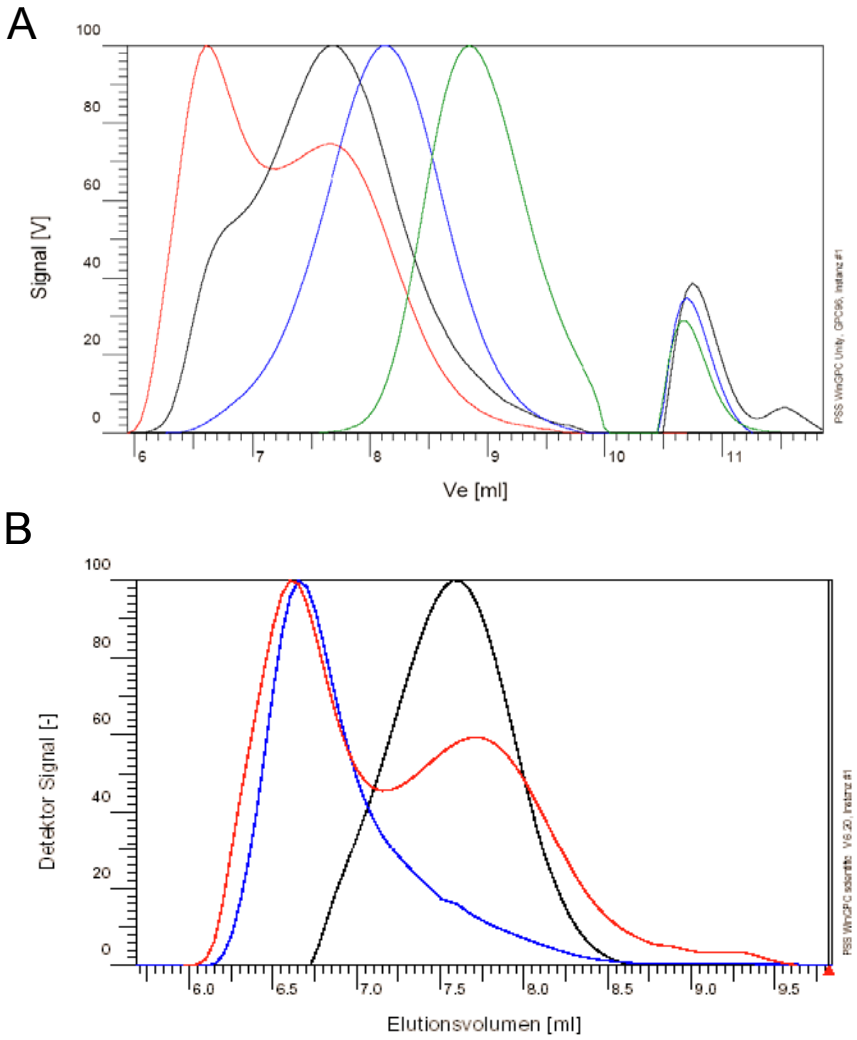

Fig.26: Molecular weight determination of OEI-based polymers by SEC/MALLS

Elution profiles of OEI 800 (green), OEI-IP-1 (blue), lt-OEI-HD-1 (black) and standard OEI-HD-1 (red) after SEC analysis (A). Fractionation of bimodal distributed OEI-HD-1 (B).

Unfortunately, molecular weight of OEI-SP-0.5 could not be analyzed properly by SEC/MALLS. OEI-IP-1 showed a monomodal distribution with an average molecular weight of 5.1 kDa. As expected, lt-OEI-HD-1 had a low degree of polymerization, resulting in a molecular weight of 8.7 kDa, whereas high molecular weight of 53.0 kDa was determined for standard OEI-HD-1. In fact, OEI-HD-1 showed a bimodal molecular weight distribution **(Fig.26b)** with a HMW fraction of 53.0 kDa and smaller fraction of 8.1 kDa **(Table 6).**

	recovery (%)	Vp (ml)	Mp^{LS} (kDa)	Mw^{LS} (kDa)
PEI 1.3 kDa	93	8.41	2.3	2.4
PEI 5.0 kDa	92	7.79	7.5	7.4
OEI 800	90	8.84	0.8	
OEI-HD-1	54 / 46	6.6 / 7.7	56.4 / 7.4	53.0 / 8.1
lt-OEI-HD-1	90	7.71	4.6	8.7

Table 6: Molecular weight determination (SEC/MALLS)

V_p: elution volume at peak maximum, M_p: molecular weight at peak maximum, M_w: average molecular weight

3.2.3.6 Reporter gene expression and toxicity of lt-OEI-HD-1

Neuro2a cells were transfected with either standard OEI-HD-1 or lt-OEI-HD-1 in order to investigate if these structural differences will influence transfection efficiency and cytotoxicity of polyplexes. At C/P 2, standard OEI-HD-1 achieved maximum reporter gene expression, which decreased with increasing amount of polymer due to cytotoxicity. Although higher charge ratios (C/P 8 and more) were needed for optimum gene delivery, lt-OEI-HD polyplexes were able to mediate gene transfer as efficient as standard OEI-HD-1 without reducing the metabolic activity of transfected cells **(Fig.27).**

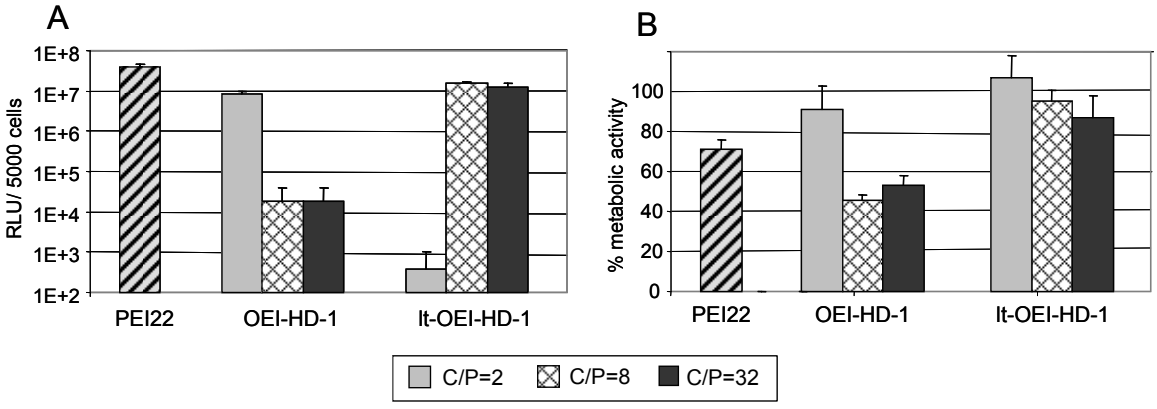

Fig.27: Reporter gene expression of Neuro2a cells after transfection with lt-OEI-HD-1

Neuro2a cells were transfected with 200ng (2 µg/ml DNA) pCMVLuc complexed with standard OEI-HD-1 or lt-OEI-HD-1 in HBS. Control PEI22lin polyplexes were prepared at C/P 0.8. Luciferase activity (A) and metabolic activity (MTT Assay) (B) are presented as mean values +SD of triplicates.

Additionally, different DNA concentrations were tested on B16F10 cells in order to verify these biological effects on a second cell line. According to previous experiments, standard OEI-HD-1 polyplexes prepared at C/P 2 were as efficient as PEI22lin. Again, lt-OEI-HD-1 needed higher polymer concentrations (C/P 4) to mediate equivalent reporter gene expression **(Fig.28a).**

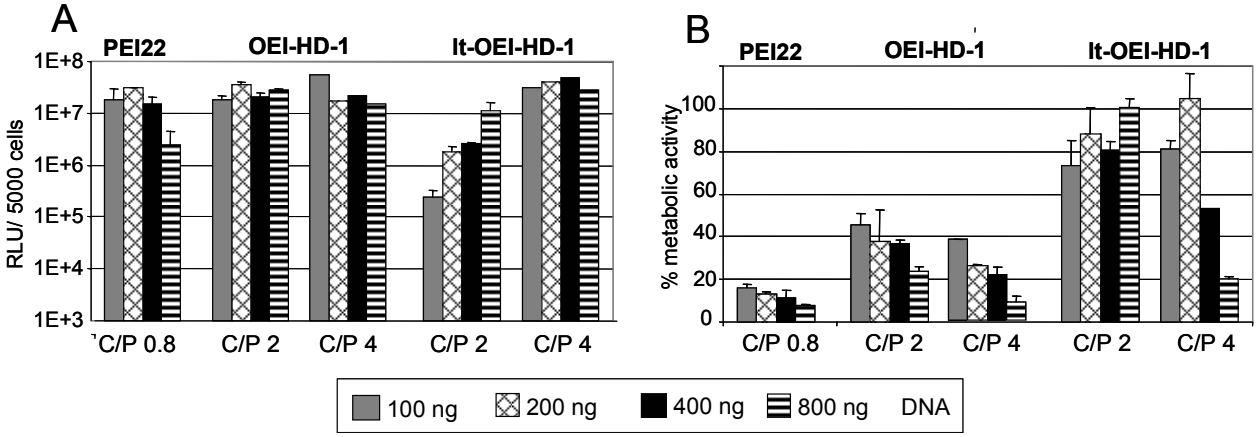

Fig.28: Reporter gene expression of B16F10 cells after transfection with lt-OEI-HD-1

B16F10 cells were transfected with 100 – 800 ng (1-8 µg/ml DNA) pCMVLuc complexed with standard OEI-HD-1 or lt-OEI-HD-1 at C/P 2 and 4 in HBS. Control PEI22lin polyplexes were prepared at C/P 0.8. Luciferase activity (A) and metabolic activity (MTT Assay) (B) are presented as mean values +SD of triplicates.

With regard to toxicity it was obvious that PEI22lin and OEI-HD-1 exhibited pronounced toxicity even at low DNA concentrations, whereas lt-OEI-HD-1 did not influence the metabolic activity of B16F10 cells at these concentrations. Generally, these cells were more affected in their viability than Neuro2a cells when treated with PEI22lin or OEI-HD-1 containing polyplexes. PEI22lin reduced metabolic activity determined 24 h after transfection to less than 20 % of the un-transfected control cells **(Fig.28b).** When cells were transfected with C/P 2 polyplexes containing 800ng DNA, OEI-HD-1 reduced metabolic activity to 22 % while cells transfected with lt-OEI-HD-1 polyplexes maintained 100 % of their metabolic activity. Cell viability was only affected by lt-OEI-HD-1 when high doses of polymer and DNA were applied. Transfection with 400ng DNA and 1.6 µg polymer (corresponding to C/P 4) resulted in 54 % metabolic activity which dropped to 20 % at the maximum DNA dose of 800 ng.

3.2.3.7 Bimodal molecular weight distribution of OEI-HD-1

According to SEC analysis, OEI-HD-1 showed a bimodal molecular weight distribution with a HMW fraction of 53.0 kDa and smaller fraction of 8.1 kDa. To find out whether one fraction is more efficient than the other or if a mixture of both is needed for optimum transfection activity, fractions were analyzed separately with regard to their gene transfer efficiency. B16F10 cells were transfected with standard OEI-HD-1 (bimodal), fraction 1 (F1), fraction 2 (F2) and lt-OEI-HD-1 **(Fig.29).** While standard OEI-HD-1 showed maximal reporter gene expression at C/P 2 and 4, both, F1 and F2 needed higher C/P-ratios for efficient gene delivery. At C/P 8, F1 resulted in luciferase expression equivalent to PEI22lin and standard OEI-HD-1. lt-OEI-HD-1-mediated efficient gene transfer at all analyzed C/P-ratios and achieved maximal reporter gene expression at C/P 8 whereas F2 was characterized by poor transfection activity at low C/P-ratios.

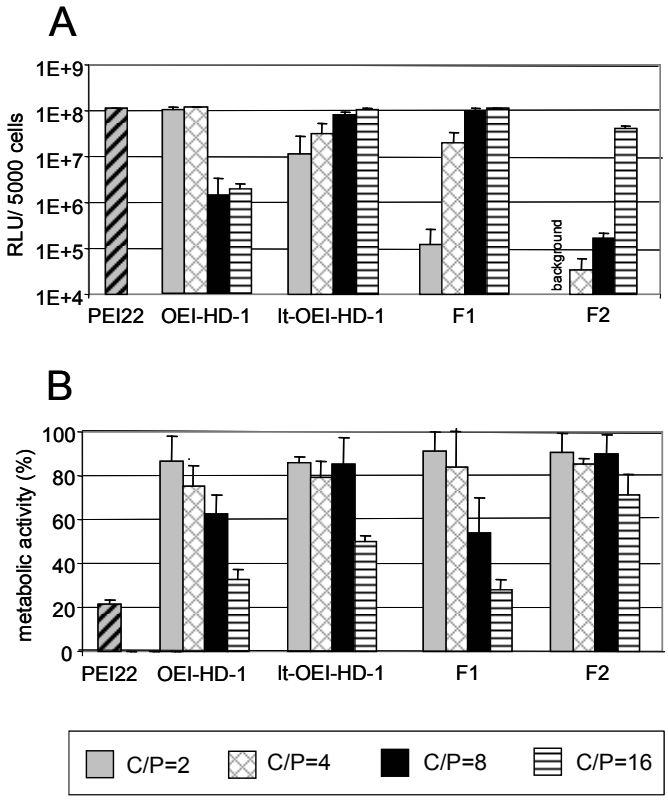

Fig.29: Reporter gene expression and toxicity of OEI-HD-1 after fractionation by SEC/MALLS

B16F10 cells were transfected with 200ng (2 µg/ml DNA) pCMVLuc complexed with standard OEI-HD-1 (bimodal), lt-OEI-HD-1 (8.7 kDa), OEI-HD-1-fraction 1 (53 kDa) and fraction 2 (8.1 kDa) in HBS. Control PEI 22lin polyplexes were prepared at C/P 0.8. Luciferase activity (A) and metabolic activity (MTT Assay) (B) are presented as mean values +SD of triplicates.

A clear correlation between molecular weight and toxicity was observed for all polymers **(Fig.29b).** Cell viability decreased rapidly when cells were transfected with standard OEI-HD-1 or F1 polyplexes, while low molecular weight polymers (lt-OEI-HD-1 and F2) were far less toxic.

Taken together, OEI polymers, particularly OEI-HD structures were characterized by efficient DNA condensation and high gene transfer activity in various cell lines; thus they presented a very promising basis for further chemical modification.

3.3 Modification of the OEI-HD core

When OEI-HD synthesis was carried out at high concentrations with crosslinking ratios above one, unsoluble network polymers with a high degree of polymerization were generated. By increasing the amount of linker and carrying out the synthesis at lower concentrations we wanted to generate low molecular weight oligomers equipped with free linker ends. These reactive OEI-HD cores could be easily modified with charge-bearing compounds (e.g. spermine), with ligands (e.g. EGF) or even with PEG chains to build up pseudo-dendritic structures.

3.3.1 Synthesis of OEI-HD-spermine pseudo-dendrimers

OEI-HD cores with different ratios of crosslinker to polyamine were synthesized in a two-step procedure. First, the core was synthesized by mixing OEI 800 and HD linker at low concentrations (in contrast to the standard OEI-HD-1 synthesis). A 2-fold, 5-fold or 10-fold molar excess of linker should prevent the formation of branched unsoluble polymers and should provide free acrylate end groups for the following modification. In this second step, spermine was linked to free linker ends in order to generate pseudo-dendritic structures **(see Fig.30).**

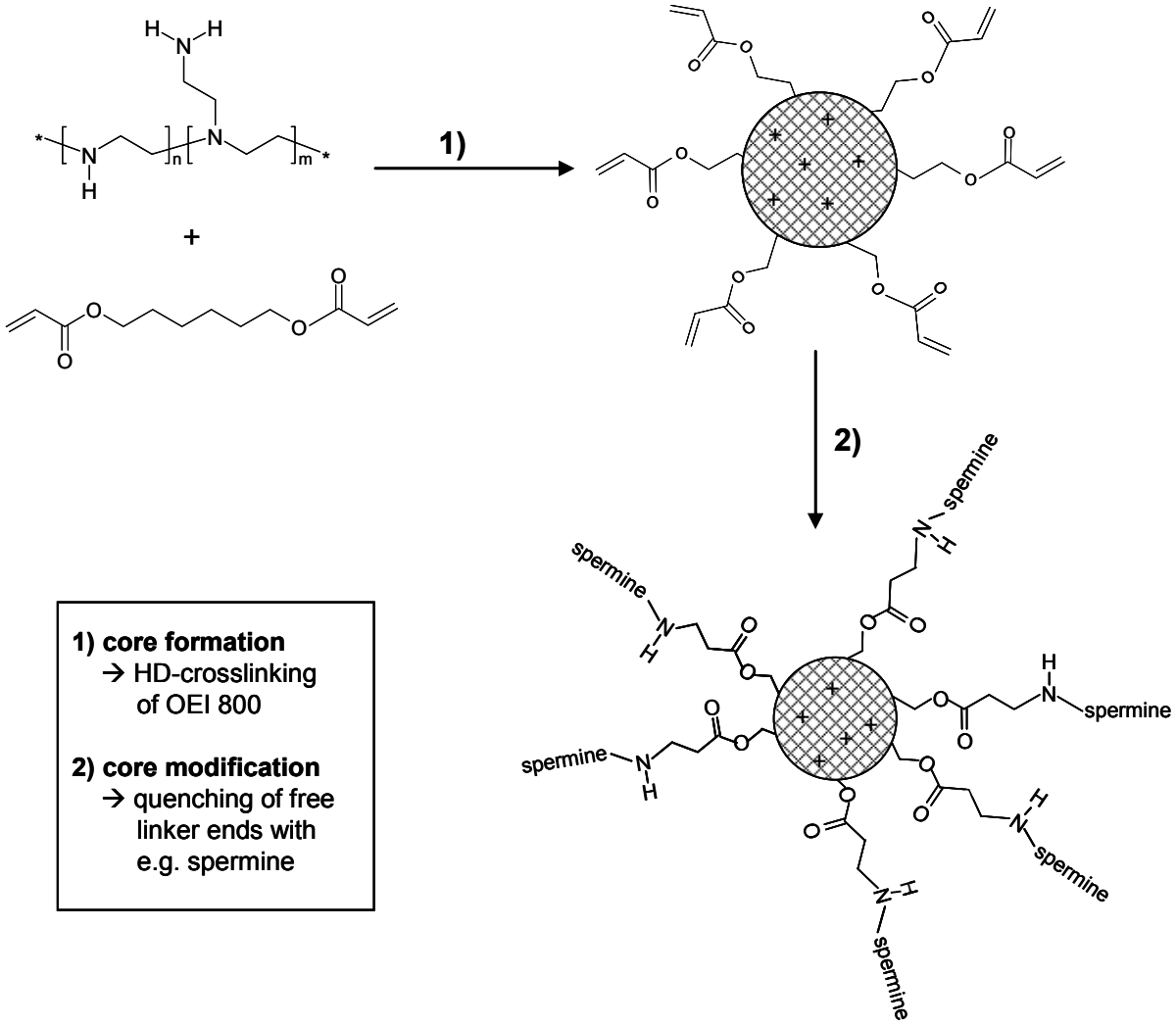

Fig.30: Concept of the pseudo-dendrimer synthesis

A highly branched core equipped with free linker ends for further modification is generated by adding an excess of HD-linker to LMW OEI. In a second step spermine is coupled to the free linker ends in order to provide additional positive charges for DNA complexation.

Polymers were purified by SEC as described in 2.3.1. In case of OEI-HD-10-Sper the polymeric product showed a bimodal distribution. Therefore, both fractions OEI-HD-10-Sper-A and OEI-HD-10-Sper-B were freeze dried and analyzed separately. All pseudo-dendritic polymers showed similar DNA binding characteristics in HBG and HBS as OEI-HD-1 (data not shown).

3.3.2 Reporter gene expression and cytotoxicity of OEI-HD pseudo-dendrimers

In a first screening in B16F10 cells, OEI-HD-Sper polymers except OEI-HD-10-Sper-B showed promising transfection efficiency but also a pronounced toxicity at high C/P-ratios (data not shown). Therefore, the polymer to DNA ratio was reduced for the following transfection experiments. At C/P 2 and 4, OEI-HD-2-Sper lacked efficient gene delivery whereas OEI-HD-5-Sper and OEI-HD-10-Sper-A mediated gene transfer as efficiently (HD-10-Sper-A) or even 3-fold better (OEI-HD-5-Sper, C/P 2) than PEI22 at all analyzed DNA concentrations **(Fig.31a).** Although pseudo-dendrimers were less toxic than PEI, OEI-HD-5-Sper and OEI-HD-10-Sper-A both exposed pronounced cytotoxicity which correlated with increasing DNA concentration as well as with increasing polymer concentration **(Fig.31b).**

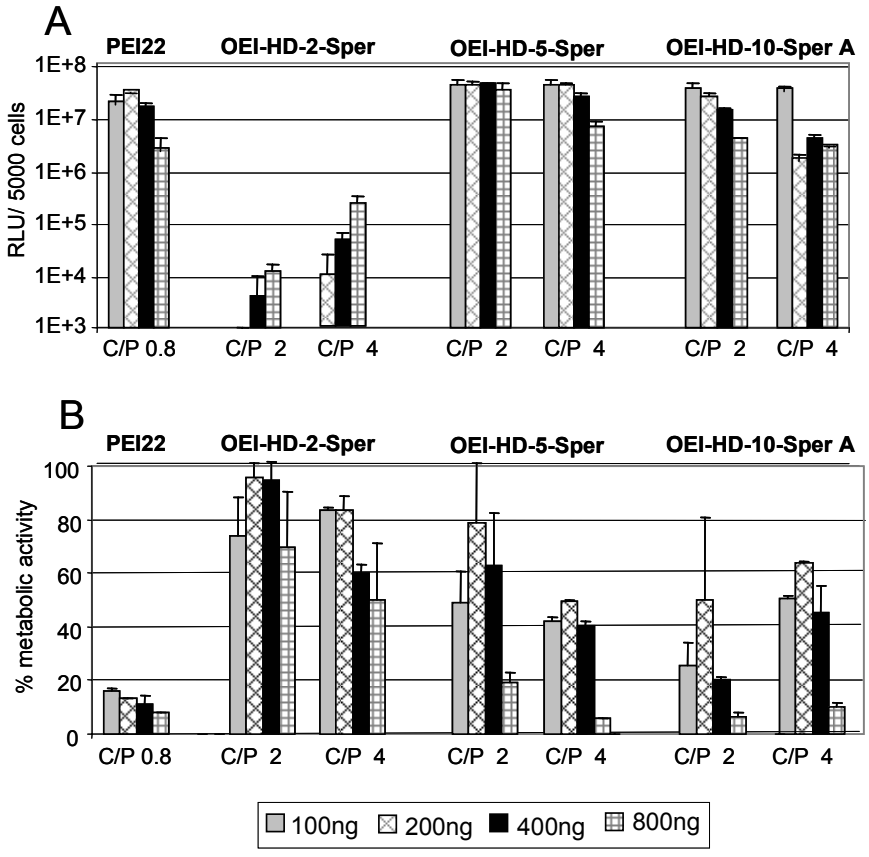

Fig.31: Reporter gene expression and cell viability after transfection with OEI-HD-Sper pseudo-dendrimers

B16F10 cells were transfected with various amounts of DNA (1.0 – 8.0 µg/ml) complexed at C/P 2 and C/P 4 respectively (HBS). Standard PEI22lin polyplexes were prepared at C/P 0.8. Luciferase activity (A) and metabolic activity (B) (MTT Assay) are presented as mean values +SD of triplicates.

3.3.3 Membrane lytic activity of OEI-HD-Sper polymers

High gene transfer efficiency but also cytotoxicity can result from intrinsic membrane activity. To find out whether OEI-HD-Sper polymers possess this membrane lytic activity which damages cell membranes but also enhances endosomal release, erythrocyte lysis assays (as described in 3.1.5) were performed in the presence and in the absence of serum.

3.3.3.1 Erythrocyte lysis induced by OEI-HD-Sper polymers

While neither standard PEI's nor any polymer of the library exhibited hemolytic activity in the presence of serum, OEI-HD-5-Sper and OEI-HD-10-Sper-A caused a very significant lysis of erythrocyte membranes (84 % lysis after incubation with OEI-HD-10-Sper-A and 56 % lysis induced by OEI-HD-5-Sper) even in the presence of 10 % serum **(Fig.32).**

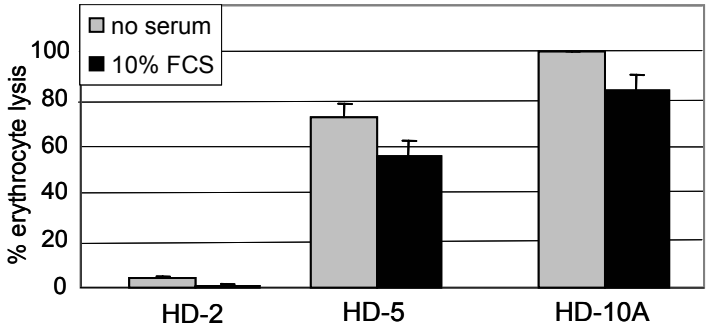

Fig.32: Hemolytic activity of OEI-HD-Sper polymers

Human erythrocytes were incubated with free polycations (final concentration 1 mg/ml) for 45 min at 37°C and hemoglobin release was quantified at 450nm. 100 % lysis refers to Triton X treatment of erythrocytes. % erythrocyte lysis is presented as mean values +SD of triplicates.

$$\% \text{ lysis} = 100\% * \frac{A_{polymer} - A_{blank}}{A_{100\%lyse} - A_{blank}}$$

With regard to endosomolytic activity it would be desirable to generate polymers that are inactive at pH 7 and that gain membrane activity at endosomal pH 5. To monitor pH-dependent hemolysis, erythrocytes were incubated with various concentrations of OEI-HD-5-Sper and OEI-HD-10-Sper-A in either HBS pH 7.1 or in citrate buffer pH 5 (both with 10 % serum). **Fig.33** shows that both polymers induce distinct erythrocyte

lysis at pH 7 (~ 60 % for OEI-HD-5-Sper and ~ 70 % for OEI-HD-10-Sper-A) whereas no hemolytic activity was detected at pH 5 at all tested concentrations.

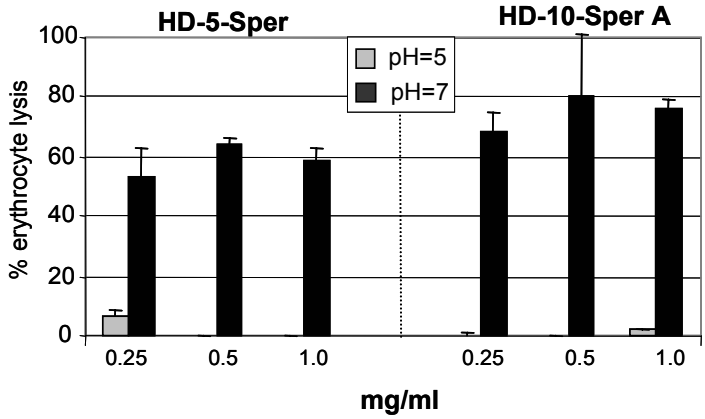

Fig.33: pH dependent hemolytic activity of OEI-HD-Sper polymers

Human erythrocytes were incubated with free polycations (final concentration 0.25 –1.0 mg/ml) for 45 min at 37°C in the presence of 10 % serum in either HBS pH 7.1 or citrate buffer pH 5 and hemoglobin release was quantified at 450nm. 100 % lysis refers to Triton X treatment of erythrocytes. % erythrocyte lysis is presented as mean values +SD of triplicates.

$$\% \text{ lysis} = 100\% * \frac{A_{polymer} - A_{blank}}{A_{100\%lyse} - A_{blank}}$$

3.3.3.2 Endosomolytic activity of OEI-HD-Sper pseudo-dendrimers

As described in 3.1.7, polycations with intrinsic endosomolytic properties, such as PEI, can act as helper for polylysine (PLL)-mediated transfection. With regard to their strong hemolytic activity, there was the need to find out whether these membrane-active properties of OEI-HD-Sper pseudo-dendrimers could also induce destabilization of endosomal membranes and thus trigger endosomal release. B16 cells were transfected with standard PLL polyplexes (C/P 1 in HBG) and 2 h later different amounts of free polymer were added **(Fig.34).**

At a concentration of 30 µg/ml, OEI-HD-10-Sper-B did not influence the transfection efficiency of PLL polyplexes whereas OEI-HD-2-Sper improved PLL-mediated gene delivery up to 5-fold. Importantly, OEI-HD-5-Sper and OEI-HD-10-Sper-A mediated more than 20-fold enhanced reporter gene expression of PLL polyplexes.

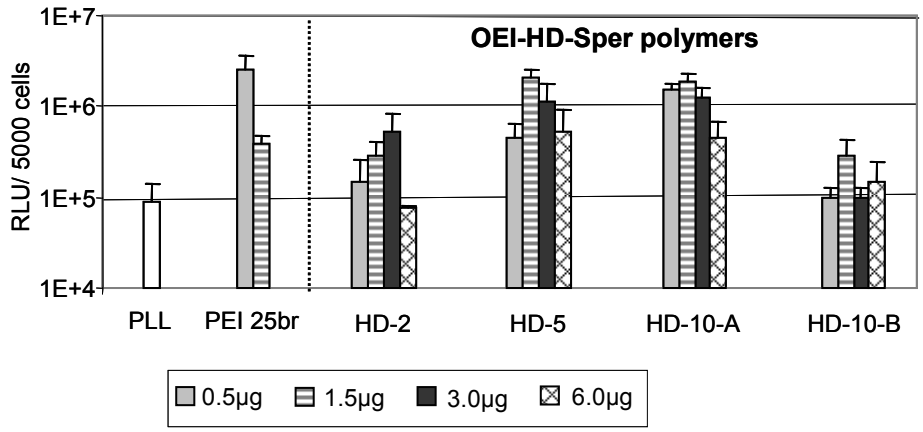

Fig.34: Polycation-mediated gene delivery of PLL polyplexes

B16F10 cells were transfected with 200ng pCMVLuc (2 µg/ml) complexed with PLL at C/P 1 in HBG. After 2 hours, transfection medium was removed and fresh culture medium with 0.5 – 6.0 µg free polycation was added. Luciferase activity is presented as mean values +SD of triplicates.

3.3.4 Designing OEI-HD-5 analogs with various functional moieties

Based on the synthesis of OEI-HD-5-Sper, preliminary experiments were performed to modify the OEI-HD-5 core in order to generate OEI-HD-based pseudo-dendrimers with various functionalities **(Fig.35).**

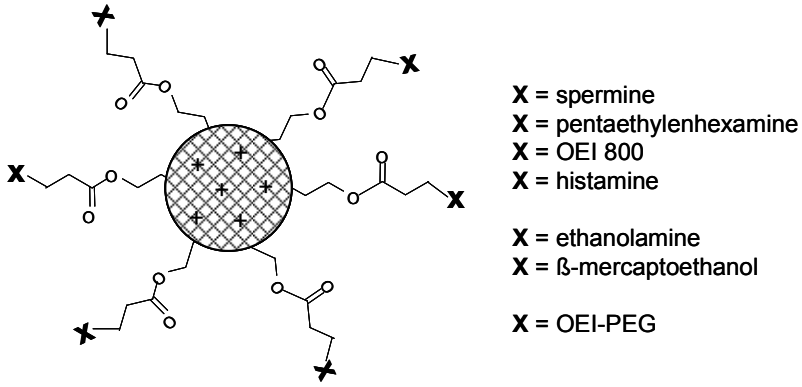

Fig.35: Modifications of the OEI-HD-5 core - concept

The first step (core synthesis) is performed as described for OEI-HD-5-Sper pseudo-dendrimers. In a second step, the OEI-HD-5 core is modified with various functional moieties which saturate the core (ethanolamine and ß-mercaptoethanol), provide additional charge-bearing groups (spermine, pentaethylenhexamine, OEI 800 and histamine) or generate partially shielded pseudo-dendrimers (OEI-PEG).

Since the OEI-HD-5 core tended to gelate after ~ 50 min reaction at 60°C, core synthesis was shortened to 30 min and modifications were performed immediately. The core structure itself (OEI-HD-5 core that was diluted with DMSO after 30 min) formed an unsoluble network polymer and could not be further processed. Reaction with ethanolamine only quenched free acrylate linker ends and was therefore similar to the OEI-HD-5 core itself. This unmodified core-like structure was synthesized in order to compare the OEI-HD-5 core with its functionalized derivatives. With regard to peptide attachment like melittin-SH, ß-mercaptoethanol was utilized as a model to monitor thiol-addition to acrylate groups. Pentaethylenhexamine, OEI 800 and histamine displayed charge-providing moieties similar to spermine and PEG 2000-OEI 700 was a first approach towards shielded OEI-HD particles.

All novel polymers showed similar DNA binding characteristics in HBG. DNA binding of OEI-HD-5-OEI (~ 25 % at C/P 3) and OEI-HD-5-OEI-PEG (~ 30 % at C/P 3) in HBS was significantly better than DNA binding activity of OEI-HD-5-His (54 % at C/P 3). Other dendrimers ranged between 35 and 43 % relative fluorescence.

A preliminary transfection experiment highlighted the potential of OEI-HD-5-Sper. Optimum reporter gene expression of B16 cells was achieved at C/P 8 (HBG; **Fig.36**) and C/P 2 (HBS; **Fig.31**), whereas OEI-HD-5-His showed more than 100-fold lower transfection efficiency.

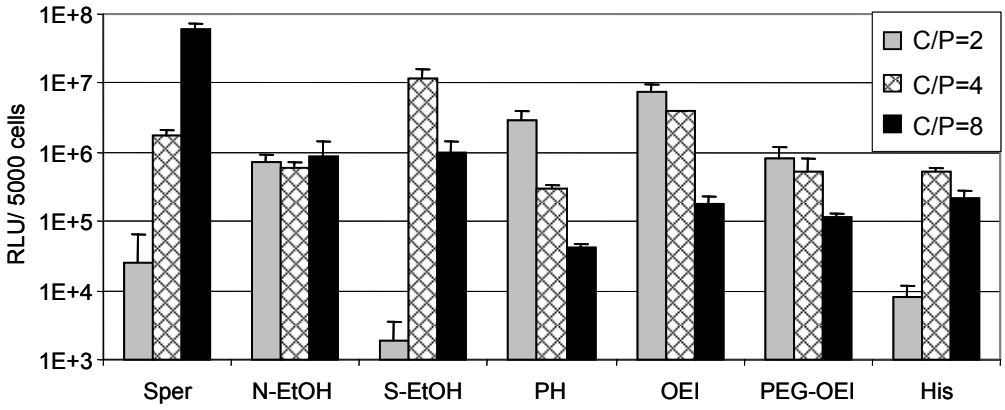

Fig.36: Preliminary transfection experiments with novel OEI-HD-5 polymers

B16F10 cells were transfected with 200ng pCMVLuc (2 µg/ml) complexed with novel pseudo-dendrimers in HBG at various C/P-ratios. Luciferase activity is presented as mean values +SD of triplicates.

However, this transfection experiment should only provide some very basic information about the potential of OEI-HD-functionalized polymers. More detailed studies will be performed by Verena Russ in the context of her PhD-thesis.

3.4 Modification of OEI-HD-1 polyplexes for virus-like gene delivery

Since no polymer was able to meet all requirements for highly effective gene delivery, it was necessary to further modify the polycationic compacting domain with virus-like functions and to find novel strategies to boost reporter gene expression. Generally, polyplexes which are positively charged can interact unspecifically with negatively charged proteins of the cell surface as well as with circulating plasma proteins. However, cellular uptake is not cell specific when polyplexes are internalized due to electrostatic interactions with membrane proteins. For cancer gene therapy it is important to specifically target the disease causing tumor cells without affecting normal tissue cells. Therefore, the aim was to incorporate epidermal growth factor (EGF) as a ligand to target EGF receptor (over)-expressing tumor cells. Furthermore, tumor cell targeting should be combined with new strategies to trigger endosomal release in order to make the whole system more efficient. Primarily, these techniques should be developed for standard PEI/DNA complexes and subsequently transferred to the novel OEI-HD polyplexes.

3.4.1 Photochemically enhanced gene transfer of EGFR-targeted PEI polyplexes

According to previous observations, our concept was based on the strategy of receptor mediated endocytosis via EGFR-targeted PEI polyplexes. After internalization into endocytic vesicles, the release into the cytosol should be triggered by light-induced rupture of endosomal membranes **(Fig.37).**

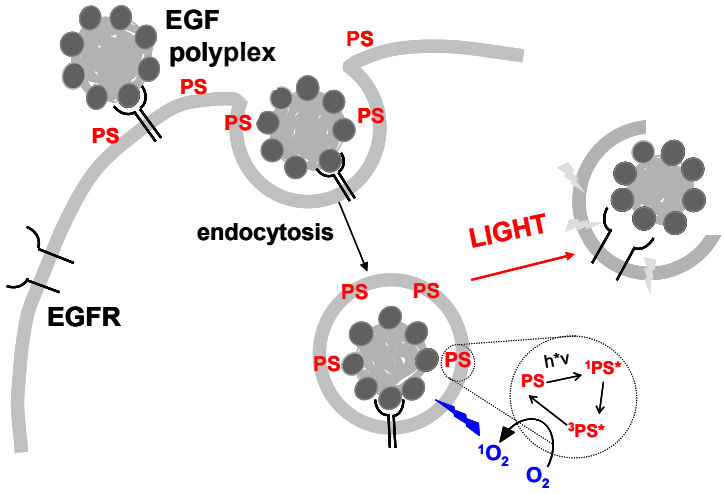

Fig.37: Photochemical intracellular release of EGF polyplexes

Amphiphilic photosensitizers (PS) are localized into membranes of the endocytic compartment. EGF polyplexes bind to the EGFR and enter the cell by receptor-mediated endocytosis. After illumination of cells, the membrane associated PS is activated and transfers its energy to molecular oxygen generating radical singlet oxygen. ($^1O_2$) $^1O_2$ leads to oxidative damage of endocytic membranes, resulting in the rupture of the vesicular membrane and therefore promotes the release of EGF polyplexes into the cytosol.

3.4.1.1 Effect of photochemical intracellular release (PCI) on PEI polyplex transfection efficiency

pCMVLuc polyplexes in various formulations of PEI with or without conjugated EGF-PEG or PEG were applied for transfection of HUH-7 hepatocellular carcinoma cells **(Fig.38),** HepG2 hepatocellular carcinoma, or A431 epidermoid carcinoma cells (data not shown; for all details see Kloeckner et al. 2004). Transfection experiments were carried out with or without photochemical treatment. This photochemical treatment termed 'PCI', resulted in enhanced gene expression in all tested cell lines. In HUH-7 cells, PCI improved PEI22 polyplexes by 600-fold at the lowest DNA concentration (0.2 µg/ml) **(Fig.38a)** and about 10-fold at the higher more effective DNA concentrations (1 µg/ml) **(Fig.38 b & c).** EGF-conjugated PEGylated PEI polyplexes showed the most pronounced (30-fold) enhancement of reporter gene expression at the lowest DNA concentration and the highest degree of PEGylation **(Fig.38c).**

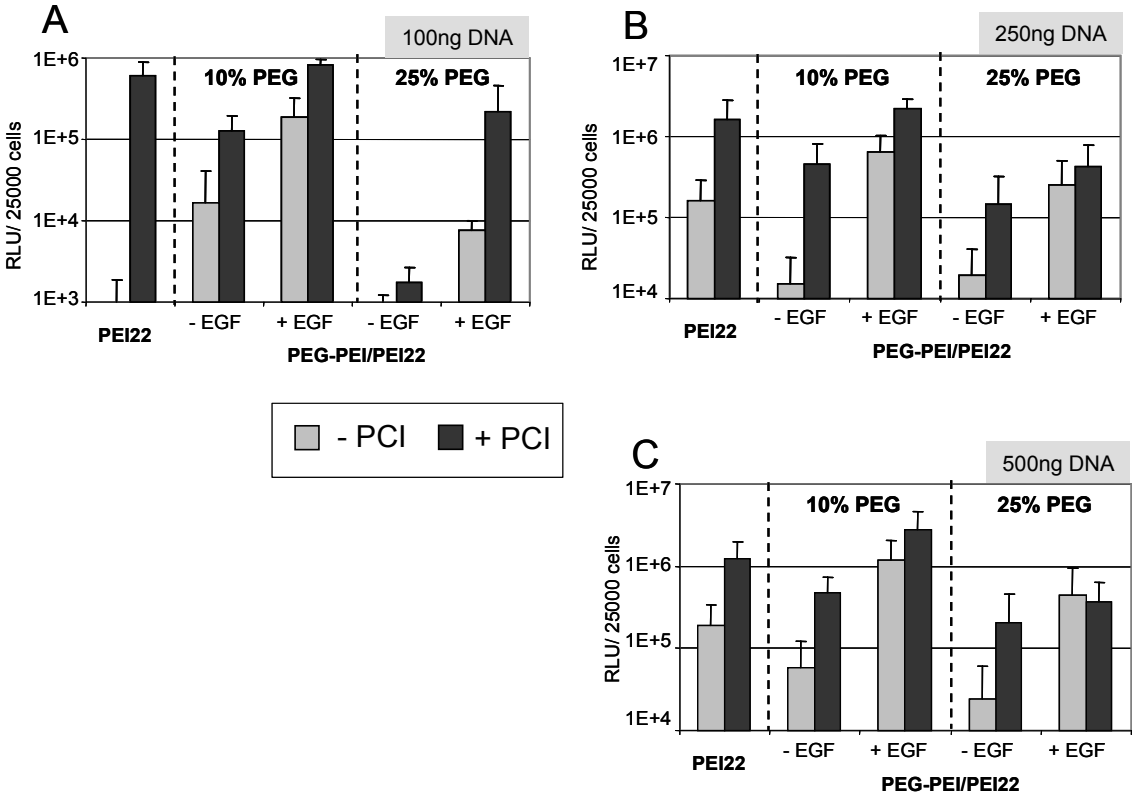

Fig. 38: EGF-mediated gene transfer enhanced by photochemical intracellular release

HUH-7 cells were transfected with 100ng (0.2 µg/ml) (A), 250ng (0.5 µg/ml) (B) or 500ng (1.0 µg/ml) (C) pCMVLuc plasmid complexed with either PEI22 alone, PEI conjugates containing 10 molar % PEG-PEI and 25 molar % PEG-PEI +/- 10 molar % EGF-PEI. All formulations were prepared at N/P 6 in HBG. Cells were treated with PS (c(TPPS2a) = 0.2 µg/ml) (dark bars) and without PS (light bars). Luciferase activity is shown as mean +SD of at least triplicates.

3.4.1.2 Effect of EGF ligand in PCI-enhanced transfection

To evaluate the contribution of EGF to gene transfer efficiency, PEGylated complexes with or without EGF ligand were compared **(Fig.38).** Without PCI, the EGF polyplexes were approximately 10-fold more effective than the corresponding ligand-free polyplexes. With PCI treatment, the EGF ligand mediated a 3-100-fold enhanced transfection (depending on the cell line) as compared to the ligand-free controls.

3.4.1.3 Competitive inhibition of EGF polyplexes by proteins blocking the EGFR

To confirm that enhanced efficiency of EGF polyplexes was mediated by attachment to the EGF receptor, competition experiments were performed by pre-treatment of the cells with free EGF **(Fig.39a)** or an anti-EGFR antibody **(Fig.39b).** Competition of EGF-containing DNA complexes with free EGF (1000-fold molar excess) decreased gene transfer efficacy down to the values found for the corresponding ligand-free PEG-PEI polyplexes. On the other hand, addition of BSA, a protein that does not interact with EGFR, did not significantly affect gene transfer activity. A strong reduction of EGF-polyplex-mediated transfection was also observed after blocking with an EGF receptor antibody, but not with an irrelevant control antibody (mouse IgG1).

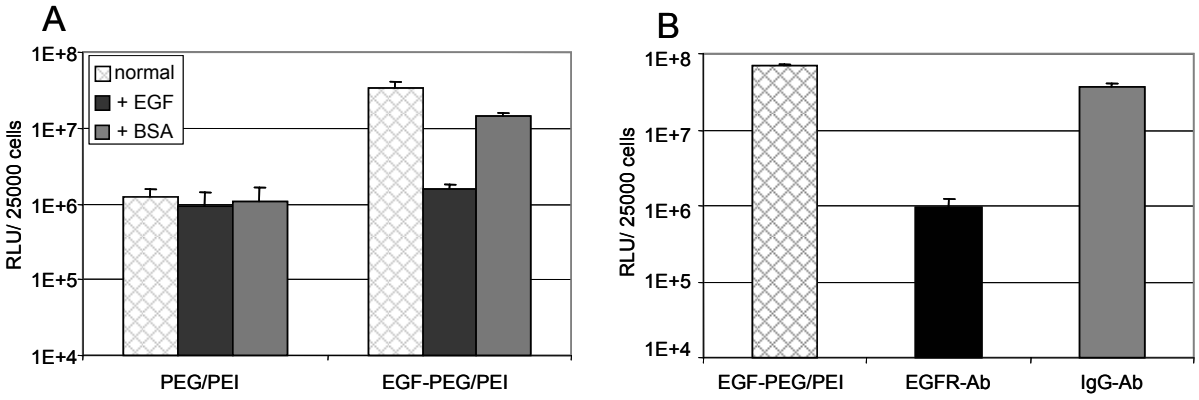

Fig. 39: Competitive inhibition of EGF receptor-mediated gene transfer

HUH-7 cells were transfected with 250ng (0.5 µg/ml) pCMVLuc plasmid complexed with PEG-PEI/PEI22 (10 molar % PEG) or EGF-PEG-PEI/PEI22 (10 molar % EGF-PEI, 10 molar % PEG-PEI) at N/P 6 (C/P 0.8) in HBG. Competition experiments were carried out in the presence of free murine EGF (12 µg/well) or BSA (12 µg/well) (A) and anti-EGFR antibody or control IgG antibody (3 µg/well) (B) respectively. All experiments were performed with photochemical enhancement. Luciferase activity is shown as mean +SD of triplicates.

Taken together, competitive inhibition experiments highlighted the role of EGFR-mediated polyplex uptake and ruled out that photochemical treatment could interfere with the ligand receptor interaction. Furthermore it was demonstrated that the combination of the targeting ligand EGF with photochemical treatment strongly enhanced gene expression.

3.4.2 EGF-receptor targeting of OEI-HD-1 polyplexes

Given that OEI-HD-1 was as efficient as the golden standard PEI22lin on many different cell lines and that it could be generated as a fast degrading ester as well as a rather slow degrading amide, it presented an interesting candidate to follow up the strategy of photochemically enhanced EGFR-targeted gene delivery.

Polyplexes consisting of different OEI-HD-1 and EGF-PEI25 ratios (5 and 10 % EGF-PEI25) were generated in order to evaluate whether OEI-HD polyplexes can be targeted specifically to EGF-receptor over-expressing cells. **Figure 40** shows the effect of 10 % EGF-PEI on OEI-HD-1 mediated gene delivery in HUH-7 cells. Incorporation of EGF resulted in 20-fold enhanced gene expression compared to standard OEI-HD polyplexes **(Fig.40a)**.

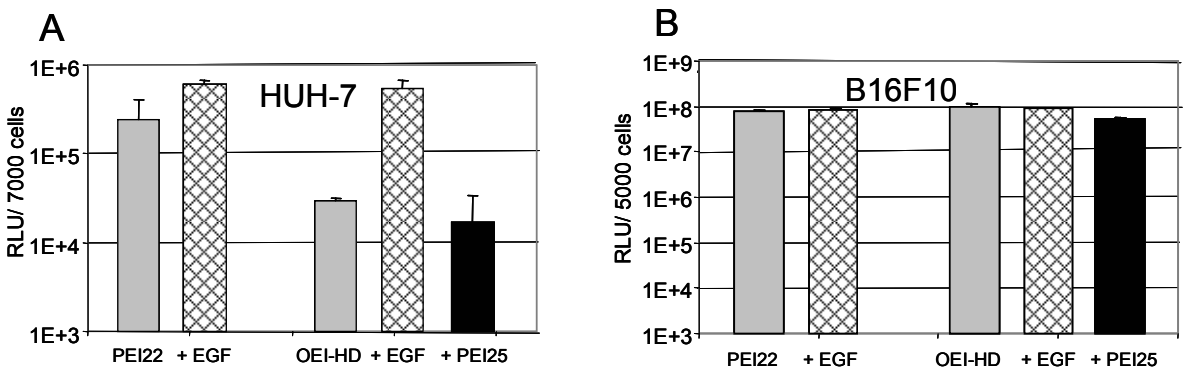

Fig. 40: EGF-mediated gene transfer of PEI22 and OEI-HD-1 polyplexes

HUH-7 cells (A) and control B16F10 cells (B) were transfected with 200ng (2 µg/ml) pCMVLuc plasmid complexed with either OEI-HD-1 or PEI22 alone, or with conjugates containing 10 molar % EGF-PEI25br and 10 molar % PEI25br respectively. All complexes of OEI-HD-1(C/P 2) and PEI22 (C/P 0.8) were prepared in HBG. Luciferase activity is shown as mean +SD of at least triplicates.

To rule out that PEI25 contributed to this improved reporter gene expression, transfection of OEI-HD/EGF polyplexes were compared with polyplexes containing equivalent amounts of PEI25 without EGF. PEI25 incorporation had no effect on OEI-HD-1-mediated gene delivery. Moreover, a control experiment was performed on B16F10 cells which do not express the EGFR, in order to test whether enhanced gene expression with EGF is actually mediated by the interaction of EGF ligand with the EGF receptor **(Fig.40b).** As expected, no EGF effect was observed.

EGFR-targeting of ester-based lt-OEI-HD-1 polyplexes was even more effective. Reporter gene expression of targeted polyplexes increased up to 940-fold (C/P 2, 50 ng DNA) after incorporation of EGF **(Fig.41b)**. According to previous experiments with PEI polyplexes, there was a clear correlation between DNA concentration and EGF receptor specificity. The EGF effect was very pronounced at low DNA doses (0.5 and 1 µg/ml) and decreased with increasing DNA concentration (2 – 4 µg/ml) **(Fig.41a&b)**. In case of standard OEI-HD-1, a DNA concentration of 4 µg/ml resulted in negligible differences of ligand-free and EGF-containing polyplexes **(Fig.41a).** Importantly, none of the applied formulations did affect the metabolic activity of target cells **(Fig.41c).**

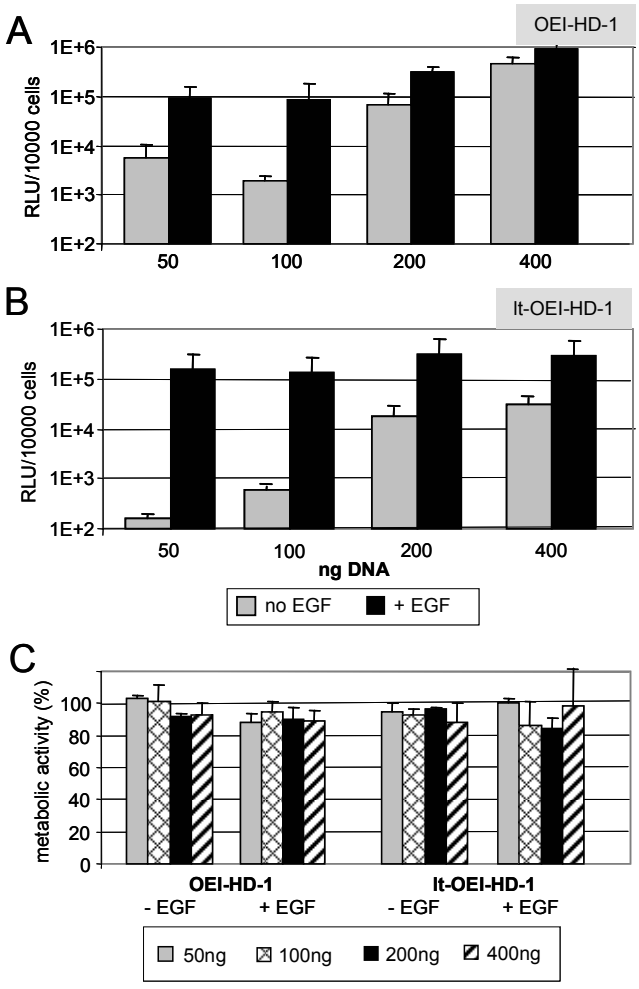

Fig. 41: EGFR-specificity of OEI-HD-1 polyplexes subject to DNA concentration

HUH-7 cells were transfected with various amounts of pCMVLuc plasmid complexed with either OEI-HD-1 (A) or lt-OEI-HD-1 (B) alone, or with conjugates containing 10 molar % EGF-PEI25br. All complexes were prepared at C/P 2 in HBG. Luciferase activity (A & B) and metabolic activity (C) are shown as mean +SD of at least triplicates.

EGFR-mediated uptake of targeted polyplexes was confirmed by competitive inhibition of the EGF receptor as described in 3.4.1.3. Pre-treatment with excessive EGF resulted in reduced transfection efficiency of EGFR-targeted polyplexes (4-fold reduction for EGF/lt-OEI-HD-1 and 4.5-fold reduction for EGF/OEI-HD-1), whereas reporter gene expression mediated by ligand-free polyplexes was not affected **(Fig.42).**

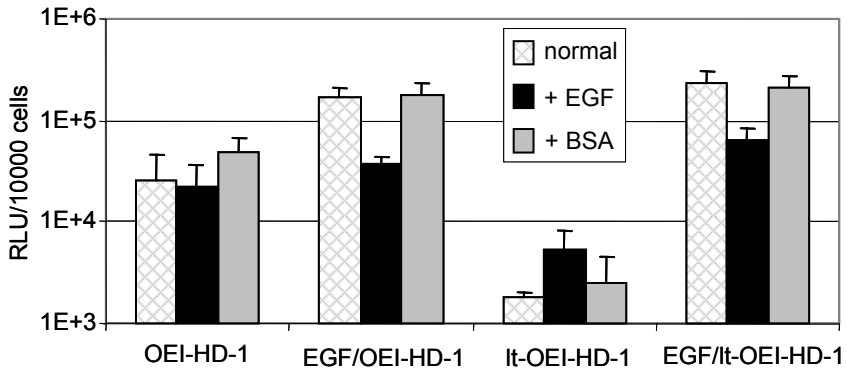

Fig. 42: Competitive inhibition of EGF receptor-mediated gene transfer

HUH-7 cells were transfected with 100ng (1.0 µg/ml) pCMVLuc plasmid complexed with lt-OEI-HD-1 and OEI-HD-1 or EGF-containing polyplexes (10 molar % EGF-PEI) at C/P 2 in HBG. Competition experiments were carried out in the presence of free murine EGF (2.4 µg/well) or BSA (2.4 µg/well). Luciferase activity is shown as mean +SD of triplicates.

3.4.3 Improved endosomal escape of OEI-HD polyplexes

After receptor-mediated endocytosis, polyplexes are entrapped within intracellular vesicles and become rapidly degraded if not released from the endosome. For this reason the endosomal membrane is the next and maybe most important barrier that has to be overcome by an ‘artificial virus’.

There are different ways to improve the release of endocytosed molecules into the cytosol. Amongst other approaches, their release can either be triggered by photochemical treatment as reported in the previous chapter or drug carriers can be combined with membrane active peptides which help to destabilize the endosome.

3.4.3.1 Photochemically triggered gene delivery of OEI-HD/EGF polyplexes

As described for EGFR targeted PEI polyplexes, the influence of photochemical treatment on OEI-HD/EGF polyplexes was studied on HUH-7 cells. The most pronounced effect of PCI (200-fold enhancement) could be observed for standard OEI-HD polyplexes at low DNA concentrations **(Fig.43).** Ligand containing polyplexes were 40-fold more effective with PCI than without PCI. Transfection efficiency of PEI polyplexes was improved up to 50-fold for standard PEI polyplexes and up to 150-fold for EGF/PEI polyplexes.

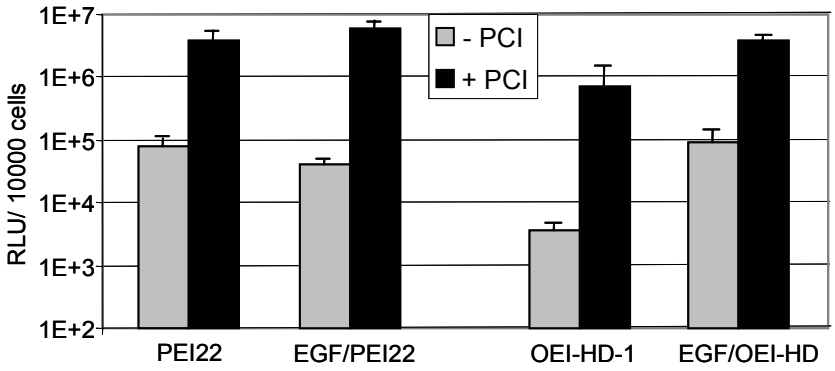

Fig. 43: EGF-mediated gene transfer enhanced by photochemical intracellular release

HUH-7 cells were transfected with 200ng (0.8 µg/ml) pCMVLuc plasmid complexed with either PEI22 or OEI-HD-1 alone, or conjugates containing 10 molar % EGF-PEI25br. All complexes of OEI-HD-1(C/P 2) and PEI22 (C/P 0.8) were prepared in HBG. Cells were treated with PS (c(TPPS2a 0.3 µg/ml) (dark bars) and without PS (light bars). Luciferase activity is shown as mean +SD of at least triplicates.

3.4.3.2 Polyplex activity after incorporation of endosomolytic melittin conjugates

Another approach to overcome the endosomal membrane barrier is the incorporation of membrane active peptides, such as melittin into the polyplex. Recently, different melittin peptide conjugates were generated and tested in combination with standard PEI22lin (Boeckle 2005, Persson 2005). First, we wanted to evaluate which conjugate performed best together with OEI-HD-1. Therefore, the peptide-PEI conjugates were incorporated into OEI-HD polyplexes and their gene transfer activity was tested in HUH-7 and CT26 cells at varying OEI-HD-1/ peptide-PEI ratios (the

melittin experiments were performed by Daniel Persson in the context of his master thesis). As shown in **Fig.44**, OEI-HD-1 polyplexes containing 10 % CMA-3-PEI and NMA-3-PEI resulted in the highest gene expression in both cell lines. Luciferase activity with CMA-3-PEI containing OEI-HD-1 polyplexes was 42-fold higher on CT26 cells compared to OEI-HD polyplexes without melittin **(Fig.44a)** and 3-fold higher in HUH-7 cells **(Fig.44b).**

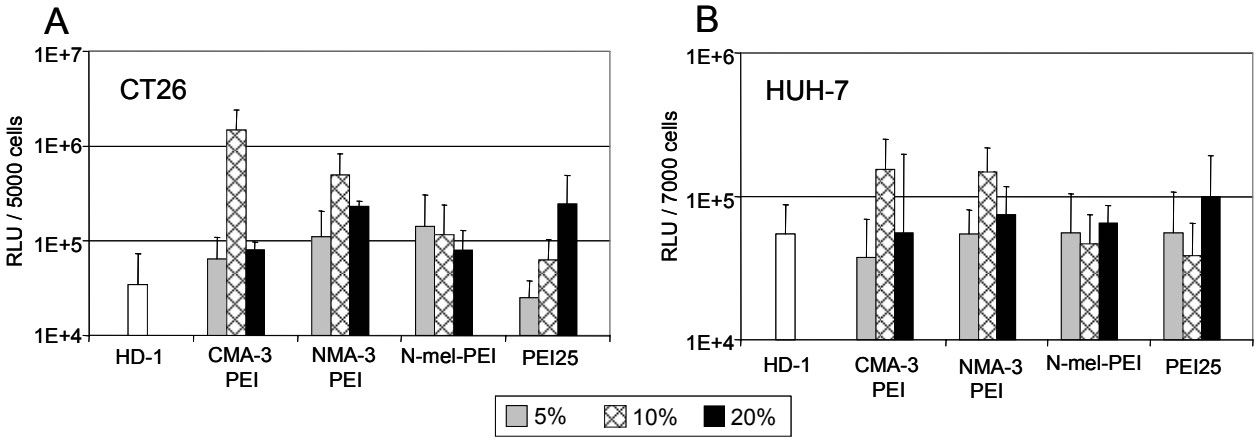

Fig.44: Combination of endosomolytic melittin peptides and OEI-HD-1

CT26 cells (A) and HUH-7 cells (B) were transfected with 100ng pCMVLuc (1 µg/ml) complexed with OEI-HD-1 at C/P 2 in HBG. Complexes contained indicated ratios of peptide-PEI conjugates. Luciferase activity is presented as mean values + SD of triplicates.

After incorporation of NMA-3-PEI, gene expression was enhanced up to 14-fold in CT26 and up to 3-fold in HUH-7 cells. Since transfection with CMA-3-PEI polyplexes was associated with higher toxicity (data not shown), the decision was to continue the studies with NMA-3-PEI.

3.4.3.3 Melittin enhanced gene delivery of OEI-HD/ EGF polyplexes

Incorporation of either EGF or NMA-3-PEI into OEI-HD-1 polyplexes resulted in enhanced reporter gene expression. Therefore, the next question was whether a combination of OEI-HD-1 with EGF as ligand and NMA-3-PEI as endosomolytic domain could further improve transfection efficiency. 5 % EGF and 10 % NMA-3-PEI were incorporated into OEI-HD-1 polyplexes (C/P 2) and compared with standard OEI-HD-1 polyplexes as well as with polyplexes containing only EGF or NMA-3-PEI. Luciferase expression of OEI-HD/EGF/NMA-3-PEI polyplexes was 2.6-fold higher

than reporter gene expression of EGF-containing polyplexes and 9-fold higher than that of NMA-3-PEI containing polyplexes **(Fig.45).**

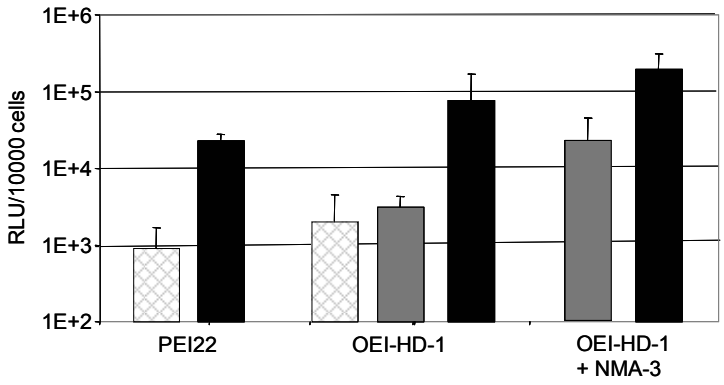

Fig. 45: Combination of NMA-3-PEI (10 %), EGF-PEI (5 %) and OEI-HD-1

HUH-7 cells were transfected with standard polyplexes containing either PEI25br (dark grey bars) or EGF (black bars) complexed with 100ng pCMV (1 µg/ml) at C/P 0.8 (PEI22) and C/P 2 (OEI-HD-1). Luciferase activity is presented as mean values + SD of triplicates.

In summary, ligand incorporation into OEI-HD polyplexes allowed cell specific and highly efficient gene transfer. Importantly, this enhanced transfection efficiency was not associated with toxic side effects. Furthermore, the combination of EGFR-targeting with a light-induced rupture of endocytic vesicles boosted gene transfer efficiency tremendously. In addition, incorporation of membrane active melittin conjugates into EGF/OEI-HD-1 polyplexes promoted their endosomal release and thus improved transfection activity.

4 Discussion

Gene therapy is a very promising approach to treat or prevent diseases. However, progress in this field is hindered by lack of suitable vectors. Nonviral vectors, including liposomes and polymers, can be readily modified by the incorporation of ligands for cell-targeting (Dash 2000) and are typically safer but much less efficient than recombinant viruses (Davies 2002, Thomas 2003, and Wagner 2004). Nevertheless, many cationic polymers exhibit significant cytotoxicity. Therefore, a major challenge is to develop novel vector systems which are highly efficient and well tolerated regarding acute toxicity and long-term effects. Since efficiency but also toxicity depend on the structure and the molecular weight of a polymer, polycations that are chosen to deliver nucleic acids have to be carefully selected for optimum gene transfer. Currently, biocompatible polymers emerge as most promising candidates for efficient gene delivery, since they could be degraded by the host, are less toxic and may also have advantages in other steps of the gene delivery pathway that further improve transfection efficiency.

4.1 Development of degradable polycations for optimized, nonviral gene delivery

The first aim of this thesis was to generate a variety of novel degradable polycations for safe and highly efficient gene delivery. In a combinatorial approach various LMW polyamines were oligomerized via different bifunctional linkers. Crosslinking of these small polycations using potentially degradable linkages was conceived as a way of increasing molecular weight and thus enhance DNA binding capacity and polyplex stability while maintaining their nontoxic nature. The polyamines used in this study were either natural polyamines like spermine and spermidine, or ethylenimine-based structures with different molecular weights. Since there are all sorts of labile linkages,

two different linkage types were chosen as basis for degradable polymers: site-specific reducible disulfide bonds and labile esters that can be hydrolyzed in a time-dependent manner.

Disulfide bonds were incorporated by DTBP and DSP crosslinking, and ester bond-containing oligoamines were obtained by the use of 1.6-hexandioldiacrylate as crosslinking moiety. For the latter case, free amino groups of crosslinked oligoamines converted the esters into amides by inter- or intramolecular aminolysis, mixed ester/amide-linked or pure amide-based polymers were generated. According to ^{1}H NMR analysis, the extent of ester-aminolysis strongly depended on the amount of amino groups provided by the polyamine. Amide bonds are enzymatically degradable and hydrolyze slowly at basic pH; notably, these polymers degrade much slower than their ester-based analogs.

Overall, a library of 46 degradable polycations was synthesized and carefully analyzed in order to investigate how structural properties impact degradability, gene transfer activity and toxicity. All polymers which could be processed and purified in aqueous media were analyzed for DNA binding activity and transfection efficiency.

4.1.1 DNA binding activity

One feature that has to be fulfilled by the compacting domain is high DNA binding affinity and, moreover, the ability to form stable DNA complexes. As DNA binding activity of polymers is strongly influenced by charge density and molecular weight (Plank 1999) this biophysical characterization provided preliminary information about the originated structures. Generally, there are two methods to examine complex formation. Ethidium bromide fluorescence induced by its intercalation into double stranded DNA is strongly reduced when polycation/ DNA interaction results in DNA condensation and complex formation. Thus, this method is useful to monitor DNA binding activity and to obtain information about the density of the originated complex (Parker 2002). Furthermore, complex formation and stability can be analyzed by agarose gel electrophoresis. As neutrally or positively charged polyplexes prevent DNA shift in an electrical field, this method is suitable to monitor complex integrity.

Non-crosslinked polyamines like e.g. spermine weakly bind to DNA, however, as a result of their low molecular weight, they do not provide sufficient charges for DNA condensation. Binding experiments and gel retardation studies revealed that small, crosslinked polyamines required large amounts of polymer were needed for polyplex formation and resulted in loose complexes. In contrast, polymers that contained polyamines with a high number of cationic groups exposed significantly higher DNA binding activity (Fig.8 and 9). With regard to the linker, it was obvious that IP-linked polymers, as a result of positively charged amidine groups, generally had the most pronounced DNA binding affinity within one polyamine group..Polycations that were generated by DSP crosslinking showed overall weak DNA binding efficiency. This can be explained by the loss of protonable amines due to amide formation. Comparison of IP-and SP-crosslinked polycations indicated that there was a clear correlation between charge density and DNA binding activity. DNA binding efficiency of HD-polymers strongly depended on the chain length of the polyamine that was used for oligomerization. Furthermore, the extent of ester aminolysis, may have influenced DNA binding since protonable nitrogens were lost after amide formation.

It was shown that oligomerization of LMW polyamines resulted in polycationic structures which efficiently bound to DNA and provided polyplex stability similar to commonly used non-degradable polymers. Since one important purpose of this study was to create biodegradable polycations which could be metabolized and eliminated by the host, the next step was to confirm their potential degradability.

4.1.2 Polymer degradation

Biodegradability of drug delivery systems does not imply breakdown of the carrier molecule immediately after application, but rather a slow degradation in order to circumvent longterm toxicity in the organism. In case of gene delivery vectors it would be desirable to achieve either site-specific degradation within the target cell or time-dependent degradation which triggers polyplex destabilization and DNA release at the target site.

First, one has to admit that the generation of polymers which are potentially degradable is one part, but the proof of such properties and therefore the confirmation of their degradability in vivo is another point. There is still a lack of

feasible methods to proof biodegradation within the cell or the organism. Therefore, it becomes necessary to imitate the environment (pH, presence of reducing agents, ionic strength and temperature) of a specific cellular compartment in vitro and to find an indirect way of monitoring degradation. Fragmentation of polymers which results in a molecular weight decrease can be followed for instance by capillary viscosity measurements (Y.H.Kim 2005), SEC/MALLS (Petersen 2002), mass spectroscopy (Zhong 2005), or NMR (Forrest 2003). In addition, the decreased ability of polymers to form stable polyplexes (Gosselin 2001, T.i.Kim 2005) is another piece of evidence for degradation.

In case of disulfide-linked polymers (SP- and IP-group), it was shown here that reducing agents like DTT mediated the formation of products which lost their ability to form stable DNA complexes. Reductive cleavage of the polymers was confirmed by gel electrophoresis which clearly demonstrated that reduction of either polymers or polyplexes facilitated DNA release. This strategy to proof the reductive cleavage confirmed previously described analysis of disulfide-based polymers (Read 2005, Pichon 2002, Gosselin 2001, Oupicky 2001).

For HD-linked polymers, the kinetics of ester hydrolysis strongly depend on ester type, adjacent groups and pH of the environment. Having in mind that HD-linked polymers contained both ester bonds and varying amounts of amides, it was even more difficult to predict degradation kinetics.

Complex formation properties of HD-linked polymers were not affected by incubation at neutral pH for 5 days. Treatment at either basic (pH 9) or acidic (pH 5) pH however, resulted in destabilization of polyplexes and DNA release. Since hydrolytic cleavage of amides would need harsh conditions (strong basic pH, high temperature, long incubation times), degradation of HD-linked polymers was likely based on ester hydrolysis. According to H-NMR spectra, HD polymers generated at 60°C contained varying ratios (~15-80 % diester relating to 100 % polyamine) of esters to amides. With regard to hydrolysis studies, one could assume that cleavage of only a few bonds resulted in fragmentation of the polymer. Apparently, this was sufficient to substantially reduce DNA binding activity of the polymer and confirmed the potential degradability of HD-linked polymers. To minimize polymer-induced toxicity in vivo, it

would not be necessary that every linkage of the polymer is cleaved. It is very probable that various enzymes like e.g. peptidases promote complete polymer degradation over time. In addition, stepwise degradation was reported to allow advantageous DNA release kinetics (Lim 2002, T.i.Kim 2005).

DNA binding activity and degradation properties indeed provided information about polymers and their ability to interact with nucleic acids, but the main goal in this thesis was the development of safe, degradable, but still efficient gene delivery vectors. Therefore, extensive transfection screenings were performed to elucidate the potential of these novel nonviral vectors.

4.1.3 Reporter gene expression in tumor cells

The novel polycations generated in this work were biodegradable and should be able to compete with the 'golden standard' PEI22lin (Boussif 1995, Zuber 2001) in terms of gene delivery. Ideally, they possess at least equivalent transfection efficiency while being less toxic than the non-degradable HMW-PEI. In order to obtain optimum transfection activity for each polymer, the whole library was screened at varying weight ratios of conjugate to plasmid (C/P-ratios) in different cell lines. Polymers based on small polyamines like e.g. spermidine or triethylentetramine were overall characterized by poor transfection activity or had to be applied at very high concentrations. These findings were in accordance with transfection studies described by Anderson et al. (2003) where weight ratios (polymer / DNA) up to 100 were needed for efficient gene delivery. Polyamines with a higher number of protonable nitrogens like pentaethylenhexamine (PH) or oligoethylenimine (OEI 800) achieved luciferase expression levels similar to PEI22lin when applied at their optimal C/P-ratios. It is important to note that differences in DNA binding activity of oligoamidines and oligoamides (IP- vs. SP-polymers) are not reflected in their gene transfer efficiency. This suggests that, depending on the polycation, effective DNA binding is important but does not necessarily correlate with high transfection activity in vitro. For example, DNA binding of polyphosphoramidates was strongly influenced by the type of charge group, however it did not correlate with transfection efficiency (Wang 2004).

Structural differences like hydrophobicity of the HD-linker, its reactivity towards primary and secondary amines and the ester/amide linkages were the reason why HD-polymers could not be compared directly with IP- and SP-polymers. While OEI-HD-1 and OEI-HD-0.5 showed very promising transfection results even at low charge ratios, HD-linked small polyamines SD-HD or TT-HD possessed poor gene transfer activity. But, exceptions confirmed the rule since, for example TT-HD-1 showed very high transfection efficiency on HUH-7 cells. This clearly demonstrated that transfection results strongly depend on the cell type and that optimal C/P-ratios have to be investigated for every cell line.

With regard to degradation and biological properties we were interested to find out whether a polymer consisting of disulfide and ester linkages had any functional advantages in comparison to standard IP-, SP-, or HD-linked polymers. Cystamine, a disulfide bond containing LMW diamine was oligomerized by HD-crosslinking and the originated polycation was included into the screening of the library. DNA binding experiments revealed that Cys-HD-1 did not form stable complexes with DNA. This lack of DNA binding affinity is in accordance with poor DNA condensation properties of other small polyamines. It is even better explicable when taking into account that each cystamine unit only provides two protonable nitrogens, whereas e.g. OEI 800 is equipped with ~16 nitrogens within one unit. Moreover, poor transfection rates even at high C/P-ratios confirmed its inability to provide enough polyplex stability for potent gene delivery.

In contrast, OEI-IP-HD-1 (via the IP-linker dimerized OEI 800 that was further oligomerized by HD-crosslinking) showed high DNA binding activity and provided polyplex stability at low C/P-ratios. With regard to transfection efficiency, this polymer had no advantages in comparison to standard OEI-HD or OEI-IP polyplexes. However, the idea of such combined polymers seemed interesting and optimization of the OEI-IP-HD-1 synthesis could result in improved biological properties.

Taken together, it became clear that some novel polycations, e.g. OEI-based polymers, possessed very promising transfection activity in all analyzed cell lines and that they provided an interesting basis for further analysis. Recently published work

highlighted the overall interest to develop degradable gene carriers. Furthermore, the strategy to oligomerize LMW amines via reversible crosslinkers as performed in this thesis turned out to be followed by many other groups **(Table 7)**.

	Polymer	Labile bond	Potential as gene carrier
Petersen 2002	Poly(ethylenimine-co-L-lactamide-co-succinamide	amide	Low toxicity; improved TE compared to PEI 1.2 kDa
Lim 2000	Poly(α-(4-aminobutyl)-L-glycolic acid (PAGA)	ester	No toxicity; 2-fold higher TE than PLL 4 kDa
Lim 2002	Network poly(amino ester)	ester	Low toxicity; similar TE as PEI25br
Jon 2003	Poly(amino alcohol esters)	ester	Less toxic than PEI; no transfection experiments
Akinc 2003	Linear poly(ß-amino esters)	ester	Higher TE than PEI25br and Lipofectamine 2000
Forrest 2003	Crosslinked 800-Da PEI	ester	Low toxicity; 2-16-fold higher TE than PEI25br
Anderson 2004	Library of linear poly(ß-amino esters)	ester	Higher TE than PEI25br and Lipofectamine 2000
Li 2004	Poly(D,L-lactide-co-4-hydroxy-L-proline)	ester	Lower toxicity than PEI25br and PLL; higher TE than PEI25br after 5 days (sustained activity)
Ahn 2004	Multi-block copolymers of PLL and PEG	ester	Low toxicity; similar TE as PLL
Kim 2005	Branched poly(ß-amino esters) mod. with amino-hexanoic acid or lysine	ester	Low toxicity; high TE in primary cells
Thomas 2005	Crosslinked 2-kDa & 423- Da PEI	ester/amide	Low toxicity; 2-fold higher TE than PEI25br; activity in vivo
Zhong 2005	Hyperbranched poly(ester amine)s	ester	Low cytotoxicity; TE comparable to that of PEI25br or pDMAEMA
Kim 2005	Crosslinked 1.8 kDa PEI	acid labile imines	Low toxicity; lower TE than PEI25br
Oupický 2001	Crosslinked 19.6 kDa PLL (crosslinking after polyplex formation)	disulfide	Similar TE for crosslinked and standard PLL polyplexes; extended circulation time in vivo
Gosselin 2001	Crosslinked 800-Da PEI via amide or amidine bonds	disulfide	Low toxicity; lower TE than PEI25br
Pichon 2002	Poly(Lys-(AEDTP))	disulfide	10-50-fold higher TE than PLL
Read 2005	Reducible polycations based on His and pLys residues	disulfide	Higher TE than PEI25br, activity in mediating mRNA and siRNA delivery

Table 7: Overview: biodegradable polymers for gene delivery

Currently developed degradable polymers are summarized in terms of degradability and their potential as nontoxic gene carriers. Abbreviation: TE → Transfection Efficiency

For example, Langer and co-workers generated a library of 2350 structurally unique degradable cationic polymers. In a combinatorial approach, various diacrylates were added to different amine-containing monomers via Michael addition, and the resulting structures were characterized in large-scale transfection screenings (Anderson 2003 & 2004). Further studies with two promising candidates were carried out to investigate the effect of end group, molecular weight and polymer/DNA ratio (w/w) on transfection efficiency and cytotoxicity (Akinc 2003). Recently, a new library of 486 second-generation poly(ß-amino esters) was synthesized and structure activity studies were performed in order to elucidate the role of chemical properties on gene transfer efficiency (Anderson 2005). High-throughput syntheses and transfection screening allowed the generation of these impressive libraries. However, transfection activity was only evaluated for a single cell line and there was a lack of detailed biocompatibility studies.

Potential degradability, efficient DNA binding and a high gene transfer activity are very important features of novel nonviral vectors. Nevertheless, it is highly desirable to develop low toxic vector systems which do not interact unspecifically with e.g. blood cells in vivo.

4.1.4 Interaction with blood components

During in vivo gene therapy, a vector system is brought into the bloodstream of a patient. It is therefore of the utmost importance that vectors are biocompatible and show a minimal interaction with blood components. Positively charged compounds with a certain molecular weight tend to induce erythrocyte or platelet aggregation because of electrostatic interaction with negatively charged membrane proteins (Peterson 2002b). These aggregates may just result in high lung expression as often caused by HMW PEI (Kircheis 2001) or can even induce vascular obliteration (Chollet 2002) and embolism. Monitoring in vitro erythrocyte aggregation is a preliminary, but easy method to analyze whether a polymer or polyplex tends to interact with red blood cells or not. However, hemocompatibility studies were only described for commonly used polycations (Ogris 1999, Dubruel 2003-part B, Verbaan 2003) and did not belong to the standard screening of novel degradable polymers up to now.

Agglutination experiments demonstrated that none of the oligomerized polyamines induced erythrocyte aggregation as pronounced as PEI22lin. Generally they had no negative effect on erythrocyte morphology although the experiment was carried out at very high, almost ‘artificial’ concentrations. This indicated that the novel polymers possessed an advantageous toxicity profile with regard to hemocompatibility.

Destabilization of the erythrocyte membrane by free polycations or polyplexes results in hemolysis (Malik 2000, Brownlie 2004), which would have severe consequences in vivo (Moreau 2002). Screening the library with regard to hemolytic activity revealed that neither IP- nor SP-polymers had any adverse effect on erythrocyte membrane integrity, whereas HD-linked polymers induced pronounced hemolysis. This membrane activity was attributed to the linker structure. As hexandiol-diacrylate consisted of a hydrophobic carbon chain, the originated oligomerized polyamines were composed of hydrophilic as well as hydrophobic moieties. Therefore, they could integrate into erythrocyte membranes resulting in membrane damage and hemoglobin release. However, hemolysis was strongly reduced when the experiment was carried out in the presence of serum.

Generally, distinct membrane activity of polymers is not desirable regarding erythrocyte lysis, but displays an interesting feature concerning the lysis of endosomal membranes. As previously described by Kichler et al. (2001), polycations with intrinsic endosomolytic properties, such as PEI, can act as helpers for PLL-mediated transfection. Assuming that endosomal release is the major bottleneck for gene transfer, polycations with intrinsic membrane activity should mediate gene expression very efficiently. In fact, OEI-based polymers which possessed high transfection activity significantly enhanced PLL-mediated gene delivery. This suggests that OEI-polymers provide polyplex stability and are able to overcome the cellular membrane. Moreover, they exposed an intrinsic endosomal release mechanism. In addition, this clear correlation between endosomolytic properties and high gene transfer activity underlined the importance of mechanistical studies in order to better understand the potential of novel gene carriers.

Recapitulating, a small library of novel degradable polycations was generated and analyzed for its potential to mediate efficient gene transfer. All polymers were

significantly less toxic than non-degradable PEI22. It is important to note that several new polymers showed interesting biological activity with reporter gene expressions comparable to the 'golden standard' PEI22. For instance, degradable PEI derivatives were highly active transfection agents and exhibited advantageous activity/toxicity profiles.

According to current research reports there is an overall tendency to oligomerize LMW PEI with different linker types. For example, a recent approach to generate biodegradable polyethylenimine was reported by Kim et al. (2005). Low molecular weight PEI (1.8 kDa) was crosslinked with glutadialdehyde resulting in polymers containing acid-labile imine linkages. The half-life of acid-labile PEI`s was 1.1 h at pH 4.5 and 118 h at pH 7.4, suggesting that it is rapidly degraded in the acidic endosome. Polyplexes exhibited low toxicity and their transfection efficiency was almost comparable to standard PEI25 kDa (Kim 2005).

Another report described the crosslinking of small polyethylenimines (branched PEI 2kDa and linear PEI 0.4kDa) with ethylene glycol bis(succinimidylsuccinate) (EGS) and disuccinimidyl suberate (DSS) which generated degradable ester- or amide-based PEI derivatives. In vitro and in vivo gene delivery efficiency of the most potent formulations exceeded that of PEI25br while being significantly less toxic (Thomas 2005).

Encouraging reports from other groups as well as results shown in this thesis highlighted the great potential of these degradable PEI derivatives. Many factors such as charge density, functional groups, molecular weight and degree of branching influence transfection activity as well as toxicity. For this reason the next aim was to design experiments which allowed to analyze and to compare directly structure-activity relationships of OEI-based polymers.

4.2 Comparison of OEI polymers: structure activity relations

Careful analysis of different LMW-PEI derivatives was performed in order to elucidate structural features that influence biophysical and biological properties of cationic polymers.

One would expect that linker structure and its specificity towards primary and/or secondary amines will control reaction kinetics and therefore have an impact on molecular weight and the degree of branching. High molecular weight polycations provide polyplex stability and exhibit in most cases higher transfection efficiency than their LMW counterparts (Dubruel 2003-Part A). On the other hand they tend to induce erythrocyte aggregation, cell membrane damage, and are hardly eliminated by the organism (Lecocq 2000). In combination with high charge density, the polymer will tightly bind to DNA and ensure polyplex stability during transport; however, it might also prevent intracellular DNA release. Moreover, structural properties like amphiphilicity can affect membrane damage on one hand, but it can also trigger endosomal release. Hence, one can often find a clear correlation between membrane activity, transfection efficiency and cytotoxicity (Boeckle 2005). Degradability in general and degradation kinetics will rather impact longterm toxicity than having an obvious influence on transfection efficiency or acute cytotoxicity. Nevertheless, in some cases (e.g. intracellular disulfide reduction) cytosolic polymer degradation might trigger DNA release and thus boost reporter gene expression (Read 2005). With this background, some functional peculiarities of OEI-based polymers were analyzed in detail.

Based on the same pre-oligomer, namely OEI 800, OEI-HD-1, OEI-IP-1 and OEI-SP-0.5 were crosslinked via different reversible linkers. Thus, varying biophysical and biological properties of the originated polycations could be directly attributed to differences in linker chemistry.

All polymers bound tightly to DNA and provided polyplex stability at low C/P-ratios. Detailed transfection screenings demonstrated that OEI-HD-1 mediated highly efficient gene delivery at low C/P-ratios whereas OEI-IP-1 and OEI-SP-0.5 needed increased C/P-ratios to achieve equivalent reporter gene expression. According to previous reports (Kunath 2003), it was possible to obtain efficient reporter gene expression when LMW polycations were applied in huge excess. In contrast, HMW analogs were inherently active at low polymer concentrations. This is in agreement with molecular weight determination of our OEI-based polymers. OEI-IP-1 and most likely OEI-SP-0.5 have significantly lower masses than OEI-HD-1 and, therefore, needed high amounts of polymer for efficient gene delivery. Moreover, a similar

correlation was observed for polymer-induced cytotoxicity. HMW polycations such as OEI-HD-1 and particularly PEI22 resulted in a rapid decrease of cell viability when applied at high polymer concentrations. OEI-based polymers with lower molecular weight did not affect metabolic activity to this extent and could therefore be utilized at high C/P-ratios. Analog results were obtained for polymer-induced erythrocyte aggregation (fig.19).

Similar to the OEI-HD-1 synthesis described in this work, Pack and co-worker previously generated ester linkage-containing PEI derivatives by addition of OEI 800 to small diacrylate crosslinkers (1,3-butanediol diacrylate and 1,6-hexanediol diacrylate)(Forrest 2003). Identical reactants were utilized, but their synthesis protocol was different in terms of solvent (methylene chloride), concentration (417 mM), reaction temperature (45°C) and reaction time (6 h). Their OEI-HD-1 analog (Mw ~30 kDa) had a half-life of 30 h and mediated gene expression two- to 16-fold more efficient than PEI25br. While OEI-HD-1 (described in this work) and their OEI-HD-1 analog possessed different molecular weights and most likely differed in ester content, their transfection efficiency appeared to be comparable.

The idea to oligomerize 800-Da OEI via reversible linkages by crosslinking with either DSP or DTBP as described in this thesis was also previously reported by Gosselin and his group. Their PEI conjugates showed improved transfection activity compared to the starting LMW OEI, but were less effective than PEI25br (Gosselin 2001). Apparently, our synthesis protocol generated different polymer structures because we needed higher polymer (OEI-IP-1 and OEI-SP-0.5) to DNA ratios for efficient gene delivery. For instance, their OEI-IP-1 analog had a molecular weight of 23 kDa (viscosity measurement) whereas OEI-IP-1 synthesized according to our protocol was smaller (Mw 8 kDa / SEC/MALLS analysis). This indicated once more that varying reaction conditions has a strong impact on molecular structure and therefore on biological activity of a polycation.

Nevertheless, our OEI-IP and OEI-SP polymers were as efficient as PEI22lin and slightly better than PEI25br while having significantly reduced cytotoxicity as compared to HMW PEI. Altogether, it became clear that apart from structural properties (Fischer 2003) the molecular weight of OEI-based polymers played an

important role for gene transfer activity, but also for toxic side effects. This was in agreement with previous reports about molecular weight-dependent properties of different PEI's (Fischer 1999).

4.2.1 Temperature-dependent ester aminolysis of OEI-HD-1

Reaction temperature does not only influence the degree of polymerization (molecular weight) and the specificity of the reaction, but also side reactions such as ester aminolysis. At high temperatures, primary or secondary amines can react in a nucleophilic substitution with esters and form amides which are enzymatically degradable too, but which are less susceptible to hydrolysis than esters (Petersen 2002a). As mentioned in chapter 3.2.3, the reason for choosing such harsh reaction conditions (4 days at 60°C) was the aim to obtain HMW polymers with high potential for efficient gene delivery.

It is important to note that Pack and co-workers synthesized their OEI-HD-1 analog at 45°C for 6 h (Forrest 2003). In order to analyze the influence of high reaction temperature on molecular structure and to elucidate properties of a faster degrading polymer, OEI-HD-1 was synthesized at 20°C and 60°C respectively. According to FT-IR analysis, lt-OEI-HD-1 (synthesized at 20°C) largely had the predicted ester structure whereas OEI-HD-1 (synthesized at 60°C) contained mainly amides with few esters left. ^{1}H NMR revealed that even OEI-HD-1 had remaining ester bonds although to a much lower extent than the "pure" ester based lt-OEI-HD-1. The observation of remaining hexane linker residues was in consistence with these results. However, partial degradation was observed by the appearance of protons belonging to -CH2 adjacent to hydroxyl groups and OEI-HD-1 lost 60 % of hexanediol residue due to ester aminolysis.

As far as presented in the publication, similar NMR results were obtained by Forrest et al. for their OEI-HD-1 analog (Forrest 2003).

In consideration of structural differences (ester content), a rapid molecular weight decrease under hydrolytic conditions was expected for lt-OEI-HD-1, whereas the amide bonds of OEI-HD-1 should be more stable. Degradation studies at pH 5, 7 and 9 revealed that hydrolysis kinetics of both polymers were accelerated with increasing

pH. This suggests enhanced polyplex stability at acidic conditions (pH 5) that are typical for where DNAses are extremely active. Moreover, during the analyzed period of time (15 days), only slight alterations (elution time, peak symmetry, AUC) were observed for OEI-HD-1. This slow amide hydrolysis of OEI-HD-1 was in accordance with results previously reported by Kissel and his group. Their copolymer poly(ethylene-co-L-lactamide-co-succinamide) degraded via base-catalyzed hydrolytic cleavage of amide bonds over months (Petersen 2002).

Hydrolysis of lt-OEI-HD-1 was confirmed by a rapid change in size distribution. After 5 days, a major part of the larger fraction was cleaved into smaller fragments when incubated at pH 7 or pH 9. Incubation at pH 5 did barely influence the molecular weight distribution of lt-OEI-HD-1 within the first 15 days. This data is in accordance with degradation profiles observed for hyperbranched poly (amino esters) (Zhong 2005). They explained rapid degradation at pH 7.4 and slow-going hydrolysis at pH 5.1 by the fact that at pH 7.4 a higher fraction of unprotonated amino groups in the polymer catalyze the hydrolysis of ester groups.

Nevertheless, it has not been shown yet whether small changes in polymer integrity would have any effect on transfection efficiency or cytotoxicity. To elucidate the impact of polymer degradation on toxicity, we analyzed metabolic activity of cells treated with either original or partially degraded polymer. In fact, changes in molecular weight distribution that seemed unimportant with regard to SEC data had significant impact on the cytotoxicity of polymers. Even OEI-HD-1-induced toxicity (which was very significant at high polymer concentrations) was strongly reduced after 5 days of hydrolysis. Degradation of originally less toxic lt-OEI-HD-1 resulted in nontoxic oligomer fragments. It should be noted that cytotoxicity induced by free polymers gives a worst case estimate for the interaction with cells and tissues. Toxicity of nonviral vectors usually decreases when polymers are complexed to DNA (Fischer 1999, Dubruel 2003-Part B); however as complex formation at high polymer to DNA ratios is accompanied with excessive polymer loosely associated to the polyplex, it is important to check toxic activities of such free polymers. In case of pronounced toxicity of free polymer, polyplexes could be purified in order to remove the unbound polycations (Boeckle 2004), especially for in vivo applications.

Apart from ester aminolysis, the chosen reaction conditions proposed a high degree of polymerization in case of standard OEI-HD-1, whereas lt-OEI-HD-1 should have a significantly lower molecular weight. Size exclusion chromatography (SEC) showed a bimodal size distribution for OEI-HD-1 as well as for lt-OEI-HD-1. A huge peak at the void volume indicated that OEI-HD-1 had an overall higher molecular weight than lt-OEI-HD-1. Since standard SEC was no appropriate procedure for absolute molecular weight analysis, we tried several methods commonly used for molecular weight determination. MALDI-TOF mass spectroscopy was only suitable for molecular weight determination of protein and PEG standards and LMW-polycations like oligoethylenimines with 0.8 and 2 kDa. Size exclusion chromatography (SEC) /MALLS analysis of OEI-HD-based polymers confirmed the influence of reaction temperature on the degree of polymerization. As expected, the molecular weight of lt-OEI-HD-1 was significantly lower than that of standard high temperature-generated OEI-HD-1. As shown here, synthesis at 20°C (4 days) generated 8.7 kDa polymers and a reaction temperature of 60°C (4 days) resulted in polymers with a molecular weight up to 53 kDa. According to the literature, synthesis at 45°C (6 hours) yielded in 30 kDa polymers (Forrest 2003).

With regard to structure, molecular weight and degradation kinetics, one would predict differences in polyplex stability, gene transfer activity and cytotoxicity. Ester conversion into amide bonds would reduce the number of potentially charge-bearing amines. On the other hand, high molecular weight and the presence of various secondary amines might provide enough positive charges for efficient DNA condensation. Whereas DNA-binding activity was similar for OEI-HD-1 and lt-OEI-HD-1, their biological activities showed significant differences. Standard OEI-HD-1 mediated high transfection levels at low C/P-ratios and was characterized by a rapid decrease in cell viability at high polymer concentrations. Furthermore, free OEI-HD-1 polymer induced erythrocyte aggregation; this can be easily explained by its high molecular weight and its overall charge density. In contrast, the lower molecular weight lt-OEI-HD-1 had no adverse effect on erythrocyte morphology and was applied at high concentrations without inducing OEI-HD-1-analog toxicity. However, more polymer was needed for efficient gene transfer. It is important to note that both

polymers exceeded the golden standard PEI22lin in terms of transfection efficiency and cytotoxicity when applied at optimal doses.

In fact, the aim of this thesis to generate novel, degradable polycations with advantageous biological activity was achieved by crosslinking LMW OEI via bifunctional reversible linkers, especially in case of HD-linking. However, the idea was to further improve transfection activity and also toxicity profiles of these polycations by modification of the synthesis protocol. As a result of the performed biophysical and biological screenings, it appeared interesting to follow the strategy of an OEI-HD-based polymer. One approach would be the design of LMW OEI-HD oligocations which could be subsequently equipped with DNA compacting moieties to generate pseudo-dendritic structures.

4.3 Novel OEI-HD based pseudo-dendrimers

It was previously reported that highly branched dendrons (Banerjee 2004) showed promising characteristics with regard to transfection efficiency and cytotoxicity. Similar results were found for dendritic polyamines with tree-like structures which could be functionalized by a two-step procedure (Krämer 2004).

4.3.1 OEI-HD-Sper pseudo-dendrimers with high potential for virus-like gene delivery

Crosslinking with a 2-fold molar excess of HD-linker at high reactant concentrations resulted in insoluble HMW polymers. As our aim was to generate LMW cores, novel syntheses were carried out at lower concentrations and in the presence of a huge excess of linker. We designed branched LMW OEI-HD cores which provided the opportunity for subsequent functionalization. The first step of the synthesis, the reaction of OEI 800 with an excess of HD-linker, generated reactive OEI-HD units with different ratios of free diacrylate end groups. In a second step, these free linker ends were saturated with spermine and the originated polymers were purified by size exclusion chromatography. All OEI-HD pseudo-dendrimers possessed high DNA binding activity, but preliminary in vitro studies revealed that a definite excess of HD-

linker was advantageous with regard to transfection activity. Furthermore, it was obvious that the larger fraction of OEI-HD-10-Sper, namely OEI-HD-10-Sper-A, was significantly more powerful in gene delivery than its LMW counterpart OEI-HD-10-Sper-B. Importantly, the novel pseudo-dendrimers OEI-HD-5-Sper and OEI-HD-10-Sper-A had an outstanding potential as gene carriers as they mediated gene delivery as efficient as PEI22lin while being less toxic. Nevertheless, OEI-HD-Sper polymers, especially OEI-HD-10-Sper-A, induced severe damage of cellular membranes when applied at DNA doses above 400ng (C/P > 2). Microscopic observations of cell membrane damage together with their overall high transfection activity indicated an intrinsic membrane activity of the novel pseudo-dendrimers.

4.3.2 Membrane activity of OEI-HD-Sper pseudo-dendrimers

Polymer-induced erythrocyte lysis is one strategy to monitor membrane activity in general and moreover, to gain information about possible toxic side effects in vivo. In comparison to PEI22lin and PEI25br, OEI-HD-5-Sper as well as OEI-HD-10-Sper-A initiated complete erythrocyte lysis which resulted in 100 % hemoglobin release. Whereas other hemolytic polymers like e.g TT-HD-1 or Cys-HD-1 lost their activity in the presence of serum, the pseudo-dendritic structures still maintained a significant membrane activity. One possible explanation for this extreme membrane lytic potential is their proposed amphiphilic micelle-like structure: a hydrophilic core of crosslinked OEI equipped with various hydrophobic HD-chains that are linked to hydrophilic spermine moieties. This design might favour membrane integration, subsequent destabilization and disruption of the membranes.

During transport and in extracellular compartments high lytic activity is considered as a toxic side effect that has to be eliminated by modification (e.g. shielding) of the gene carrier. On the other hand, such lytic activity is highly desirable within the cell when membrane destabilization is needed for efficient endosomal release (Boeckle 2005). Therefore, an optimal gene carrier should be inactive at physiological pH and should gain lytic activity in the acidic endosomal environment. To evaluate pH-specific membrane activity of OEI-HD-Sper derivatives, an erythrocyte leakage assay was performed at pH 5 as described in Plank et al. (1994). As novel pseudo-dendrimers did not show any lytic activity at pH 5, one could assume that their ability

for membrane destabilization was limited to neutral pH. This would imply unspecific damage of cellular membranes and at the same time loss of lytic activity within the endosome. Otherwise, it was possible that erythrocyte incubation at acidic pH caused protonation of membrane proteins whereupon polycations were incapacitated to interact with these artificially charged membranes. To rule out that our results were just experimental artefacts, a more practical study should elucidate the endosomolytic properties of OEI-HD-Sper pseudo-dendrimers.

As already described for the polymer library in chapter 3.1.8, endosomolytic activity of polycations can be characterized by analyzing their effect on PLL-mediated gene delivery. In fact, OEI-HD-5-Sper and OEI-HD-10-Sper-A were able to enhance the activity of PLL polyplexes more than 20-fold. This clearly confirmed their intrinsic endosomolytic properties and somehow disproved the reliability of erythrocyte leakage assays carried out at pH 5. One has to keep in mind that polymer concentrations resulting in distinct hemolysis (1 mg/ml) were 17 - 400-folds higher than concentrations needed for endosomolytic activity (2.5-60 µg/ml). Thus, for in vivo applications there would a therapeutical range of polymer doses which could potentially trigger endosomal release while being non-hemolytic. These very successful results encouraged us to utilize the core-modification strategy for novel approaches towards virus-like gene carriers.

Very recently, dendrons with spermine surface groups were reported to efficiently bind to DNA and to form concrete particles; however these novel structures lacked gene transfer in the absence and were still characterized by poor reporter gene expression in the presence of chloroquine, which promotes the escape from endocytic vesicles (Hardy 2005). Apparently their originated structures could not overcome the endosomal membrane barrier; this was rather surprising when having in mind that OEI-HD-Sper pseudo-dendrimers (based on a similar concept) as described in this thesis mediated gene transfer very efficiently and possessed pronounced membrane activity. Hence, this emphasizes again the impact of structural particularities of potential gene carriers on their biological activity.

4.3.3 Modification of the OEI-HD-5 core

Since the core-modification strategy opened up manifold variations for further developments, some preliminary studies on OEI-HD-5 modification were performed in order to complete this work.

Overall, the OEI-HD core can be modified with either supplementary charge-bearing moieties, with PEG chains, with targeting ligands or even with membrane active peptides. As primary and secondary amines or thiol groups react with free linker ends by Michael addition, potential functional domains only required one of these reactive groups. Since OEI-HD-5 gelated after approximately 1 hour reaction at 60°C it was not possible to synthesize an unmodified core. With regard to successful spermine modification, polyamines such as pentaethylenhexamine and OEI 800 were attached as DNA-binding domain. According to previous reports, polycationic gene delivery could be enhanced when PLL was partially substituted with histidyl residues which become cationic upon protonation of the imidazole groups at pH below 6 (Midoux 1999). To check whether imidazole groups favor the endosomal release and thus enhance transfection activity, we attached histamine to the OEI-HD core. The originated polymers showed weaker DNA binding activity which was in accordance with previous reports (Chen 2005). Moreover, they mediated gene transfer less efficient than other dendrimers. One could assume that core modification with imidazole-bearing histidine residues in combination with DNA binding e.g. spermine moieties would provide advantageous properties for efficient gene delivery.

Finally, a novel PEG(2k)-OEI(0.7k) oligomer was utilized to generate partially shielded OEI-HD structures. The OEI-HD-5 core was modified with 20 % OEI-PEG and subsequently saturated with 80 % OEI 800. DNA binding activity was similar to OEI-HD-5-OEI, but OEI-HD-5-OEI-PEG mediated gene delivery approximately 10-fold less efficient than its non-PEGylated analog. This is in agreement with previous reports showing that PEGylation of polyplexes generally resulted in decreased transfection efficiency in vitro (Kursa 2003); however it prolonged circulation time in vivo and reduced unspecific side effects (Ogris 1999). To evaluate the suitability of these short PEG oligomers, one will have to vary the degree of PEGylation. Furthermore, PEG-shielding and de-shielding of the polyplexes due to ester hydrolysis would have to be confirmed by zeta potential measurements. With regard

to improved transfection efficiency it would be desirable to generate reversible PEG shields which would fall apart after internalization. A very successful approach towards environment-sensitive shielding was recently described by Walker et al. (Walker 2005).

The OEI-HD core-modification strategy is a completely novel approach and presented a great step towards the development of multifunctional, bioresponsive gene carriers. Modification of the core is easy and there are many options to incorporate functional domains into the dendrimers. As further combinations would go beyond the scope of this thesis, the pseudo-dendrimer project will be continued in a successional work.

Taken together, OEI based polymers, especially OEI-HD containing structures possessed very promising biophysical and biological properties and were able to compete with the 'golden standard' PEI22lin. However, as no polycation alone is able to fulfill all requirements for virus-like gene delivery, the next step was to further modify these novel compacting domains.

4.4 Modification of OEI-HD polyplexes for virus-like gene delivery

In cancer gene therapy specificity for the tumor target can be achieved in several ways: targeted delivery of the gene into the tumor is accomplished by incorporation of targeting ligands which specifically bind to a receptor that is overexpressed in certain tumors. Other strategies are transductional targeting e.g. intracellular uptake into the nucleus of only dividing tumor target cells or transcriptional targeting which implies the use of an expression cassette that is switched on by tumor-specific transcription factor combinations (Lipinski 2001).

With regard to intracellular delivery, specificity can be obtained with different physical strategies such as electroporation (Goto 2004), gene gun (Wang 2001), magnetofection (Scherer 2002), ultrasound (Tomizawa 2001) and photodynamic therapy (Prasmickaite 2002, Hogset 2003) where either directed electric or magnetical field, light, or mechanical impact can be exploited for specific localization.

Photochemical reactions for therapeutic purposes have already been applied in patients. For example, photodynamic therapy (PDT) is a form of light-activated chemotherapy, clinically tested for treatment of cancer as well as for various non-oncological diseases (Huang 2005). Since there was already significant clinical experience utilizing photosensitizers for treatment of various tumor types, photochemically triggered gene delivery in vitro appeared to be a promising approach with high potential for in vivo application.

The combination of biological (EGFR-targeting) and physical (photochemical intracellular release) targeting was a promising step towards specific and efficient gene delivery.

4.4.1 EGFR-targeted and photochemically enhanced polyplexes

EGFR was selected as biological cell surface target, which is over-expressed in a variety of human tumors and which has been selected by nature as receptor for viral gene delivery (Wang 2003). First, EGF as ligand was incorporated into standard PEI- or PEGylated PEI polyplexes. Transfections were carried out in the presence of photosensitizers in order to investigate the effect of combined biological and physical targeting. In accordance with previous reports (Blessing 2001), transfection efficiency of small PEG-shielded polyplexes which normally gene transfer in vitro could be significantly improved by incorporation of EGF ligand. Furthermore, application of the photochemical intracelluar release technology tremendously enhanced EGF/PEG-PEI polyplex-mediated gene delivery. Specific uptake via the EGF-receptor, even in combination with PCI, was confirmed by competitive inhibition of the EGFR with either EGFR-antibody or an excess of free EGF. Receptor blockade resulted in reduced reporter gene expression, while unspecific antibodies or serum proteins had no effect on EGF/PEG-PEI-mediated gene delivery.

For commonly used PEI or PLL polymers, various approaches have been described in terms of specific tumor targeting (Wolschek 2002, Kursa 2003, Kunath 2003, Ogris 2003, for review see Lungwitz 2005) and also with regard to improved endosomal escape (Prasmickaite 2000, Boeckle 2005). Up to now, however, none of these modification strategies was performed for novel biodegradable polymers. Since the strategy to trigger endosomal release of EGFR-targeted polyplexes by PCI was really

promising, the applicability of this method was tested for our novel OEI-HD-1 polyplexes. Combination of ligand EGF-PEI with OEI-HD-1 as well as with lt-OEI-HD-1 resulted in significantly enhanced reporter gene expression in EGF-receptor rich HUH-7 cells (Fig.42). Similarly as described for standard EGF/PEG-PEI polyplexes (Kloeckner 2004), there was a clear correlation between DNA concentration and EGF-effect. This proposed that target cell receptor specificity decreased with increasing amounts of DNA-complexes. The positive effect of EGFR-targeting at low DNA doses was even more pronounced in case of lt-OEI-HD-1; ligand-free polyplexes of this LMW ester-based polymer lacked transfection activity at low C/P-ratios, whereas incorporation of EGF resulted in dramatically enhanced reporter gene expression. It seemed that lt-OEI-HD-1 provided polyplex stability, but, in contrast to standard OEI-HD-1, could not mediate cellular uptake and/or endosomal release when applied at low polymer concentrations. It is noteworthy that EGFR-targeted OEI-HD-1 and lt-OEI-HD-1 polyplexes showed almost equal reporter gene expression while being nontoxic. These results were independent from structure and molecular weight of OEI-HD polymers. Blocking the EGFR with an excess of free EGF clearly demonstrated that enhanced transfection efficiency of EGF/OEI-HD polyplexes was the result of improved receptor-mediated uptake. In addition, unspecific effects like e.g. polyplex aggregation were ruled out by particle size measurements of EGF-containing and ligand-free polyplexes. Moreover, EGF-ligand incorporation had no effect on reporter gene expression of EGFR-lacking B16F10 cells.

Incorporation of EGF ligand into OEI-HD polyplexes was the first step to design more ‘virus-like’ systems with enhanced specificity and reduced toxicity. To further improve OEI-HD-1-mediated gene delivery the maybe most crucial barrier, i.e. endosomal release, should be overcome by photochemically triggered disruption of endosomal membranes. Photochemical intracellular release significantly enhanced transfection activity of ligand-free as well as of EGF/OEI-HD-1 polyplexes. Interestingly, gene delivery mediated by standard PEI22 polyplexes, which are known to mediate intrinsic endosomal escape mechanisms (Sonawane 2003), was also dramatically increased after photosensitizer treatment. This suggested that endosomal release is still a major bottleneck even for efficient vectors like PEI22lin. A similar observation

was made by Engesaeter and his group. Even though adenoviral infection is a highly efficient process with up to 90 % of the endocytosed viral particles escaping from the endosome (Leopold 1998), they demonstrated that the PCI method increased gene expression compared to conventional adenovirus infection (Engesaeter 2005).

Taken together, the photochemical intracellular release technology was a very efficient method to trigger the endosomal release of endocytosed polyplexes and significantly enhanced EGF/OEI-HD-1 mediated gene transfer. However, application and subsequent light-induced activation of photosensitizers was somehow limited on special cell species in vitro and on the treatment of subcutaneous or easily accessible tumors in vivo. For some applications such as systemic administration, the direct incorporation of an endosomolytic function into DNA complexes would be preferable. Therefore, alternative light-independent strategies to improve the release of endosomally entrapped polyplexes were tested.

4.4.2 EGFR-targeted, melittin/OEI-HD-1 polyplexes

As described in detail by Boeckle et al. (Sabine Boeckle PhD thesis, Boeckle 2005) membrane active peptides such as the bee-venom-derived melittin and its analogs, can trigger endosomal release after destabilization of the endosomal membrane. N-mel-PEI25, with melittin coupled to PEI25 at the N-terminus and CMA-3-PEI, with a melittin analog coupled to PEI25 via the C-terminus have previously shown to be promising transfection agents (Boeckle 2005). First, studies were performed to incorporate NMA-3-PEI, a novel melittin analog coupled to PEI25 at the N-terminus and two other melittin conjugates into OEI-HD-1 polyplexes (Persson 2005). Optimal combinations of melittin conjugates with OEI-HD-1 mediated an up to 40-fold enhanced gene expression over standard OEI-HD-1 polyplexes. Finally, OEI-HD-1 was combined with the efficient and least toxic melittin analog NMA-3-PEI and the targeting conjugate EGF-PEI25. The originated more ‘virus-like’ vectors exceeded the transfection efficiency of polyplexes containing only EGF or NMA-3-PEI.

Taken together, modification of OEI-HD polyplexes by incorporation of EGF as targeting ligand in combination with membrane active moieties like NMA-PEI resulted in specific and very potent non-viral vectors. Since EGF- and melittin conjugates were covalently attached to non-degradable PEI25, the resulting polyplexes were

only partly degradable and heterogeneous regarding the structure of the binding polymers. This should be kept in mind, but nevertheless, the present results clearly demonstrate the potential of virus-like OEI-HD-1 particles. Furthermore, it was demonstrated that minor amounts of PEI25 had no influence on transfection efficiency, targeting specificity and cytotoxicity.

For future developments it would be desirable to generate conjugates with targeting moieties and endosomolytic domains covalently attached to biodegradable DNA-binding polycations. OEI-HD dendrimers showed a great potential in terms of transfection efficiency and could be easily modified with various functional moieties. The present results therefore suggest that one could utilize the OEI-HD core-modification strategy to design degradable polycationic dendrimers which are not only equipped with DNA compacting moieties like spermine, but which also possess targeting ligands and membrane active domains. This could be achieved by attaching thiol reactive EGF-SH or melittin-SH via Michael addition to the OEI-HD core. Ideally, one would employ membrane active moieties which are activated upon acidification of the endosome, since they will allow a safe transportation in the blood stream while becoming highly active within endocytic vesicles (Boeckle 2005, PhD thesis). To ensure polyplex stability and to reduce unspecific interactions with e.g. blood components, the polyplex could be reversibly shielded with bioresponsive PEG conjugates. For example, one could follow the strategy to couple PEG chains via hydrazone bonds to the polyplex in order to generate shielded polyplexes which de-shield in the acidic environment of the endosome (Walker 2005).

These virus-like particles (as described here) in combination with bioresponsives conjugates which sense their biological micro-environment and undergo programmed structural changes compatible with the different gene delivery steps present a great step towards nontoxic and efficient nonviral gene delivery systems. The whole construct exploits intelligent activation/inactivation mechanisms, thus provides a broader therapeutical range and will therefore allow safe application of nucleic acid-based drugs.

5 Summary

Gene therapy is a very promising approach to treat or to prevent diseases. However, progress in this field is hindered by lack of suitable vectors. Current research focuses on the development of novel nonviral biodegradable gene carriers with improved gene transfer activity and low toxicity.

In the course of this thesis, a library of degradable DNA compacting domains based on oligomerized polyamines was synthesized and analyzed. Degradation of the originated polymers was either based on site-specific reductive cleavage of disulfide bonds or on time-dependent ester/amide hydrolysis. DNA binding activity, polyplex stability, transfection efficiency, toxicity, and hemocompatibility studies were performed in order to identify promising candidates. Some of the novel gene carriers, especially the degradable oligoethylenimine (OEI) derivatives were successfully applied for in vitro transfection and could easily compete with the current ‘golden standard’ linear polyethylenimine with an average Mw of 22 kDa (PEI22lin). Furthermore, screening results revealed critical structure activity relationships which were very helpful for improving the polymer design. According to transfection and biocompatibility results, efficiency and toxicity correlated to some degree. Polymers with an overall high charge density and a high molecular weight like OEI-HD-1 provided polyplex stability and formed small uniform particles. On the other hand these polymers tended to induce erythrocyte aggregation and exhibited a pronounced cytotoxicity when applied at high concentrations. Polycation with a lower molecular weight (~ 10 kDa) like e.g. OEI-IP-1 were essentially nontoxic, but had to be applied at high concentrations in order to achieve efficient gene transfer. Intrinsic membrane activity of certain polymers could damage cellular membranes but may also trigger endosomal release and therefore boost transfection activity.

Crosslinking of OEI 800 with 1,6-hexanedioldiacrylate resulted in highly efficient degradable polycations. Different reaction temperatures during OEI-HD-1 synthesis had a strong impact on molecular weight and the ester/amide ratio. Despite structural differences, both OEI-HD-1 (synthesized at 60°C) and lt-OEI-HD-1 (synthesized at 20°C) possessed equal gene transfer activity as the ‘golden standard’ PEI22lin when

applied at their optimal polymer/DNA-ratio (w/w). It was important to note that lt-OEI-HD-1, the LMW-derivative which is predominantly based on ester linkages, was significantly less toxic than its HMW amide-linked counterpart. OEI-HD displayed a very promising basis for the development of further powerful gene carriers. A two-step synthesis protocol was established in order to generate OEI-HD cores bearing excessive linker which could be subsequently modified with various functionalities like spermine. OEI-HD-Sper pseudo-dendrimers were characterized by a pronounced intrinsic membrane activity and possess high transfection efficiency.

Since current nonviral vectors are still very inefficient as compared to their viral competitors, natural viruses present an ideal example educating us how to further optimize polycationic gene carriers in terms of specific cell-targeting and improved endosomal release. Modification of polyplexes towards a “smart” virus-like system was achieved in the following way. Degradable DNA compacting domains (OEI-HD-1) were utilized for complex formation. Furthermore, epidermal growth factor (EGF) was incorporated as targeting ligand into OEI-HD-1 polyplexes and thus allowed cell-specific cellular uptake via the EGF receptor (EGFR). Gene transfer potential of EGFR-targeted degradable polyplexes was further improved by applying technologies which promoted the endosomal release of endocytosed particles. Photochemical intracellular release (PCI) is based on accumulation of amphiphilic photosensitizers (PS) in endosomal membranes. Illumination of PS pre-treated transfected cells results in activation of the PS and subsequent light-induced rupture of endocytic vesicles. Combination of biological (EGFR) and physical (PCI) targeting greatly enhanced reporter gene delivery mediated by OEI-HD-1 polyplexes. Finally, the incorporation of membrane active melittin derivatives into EGF/OEI-HD-1 polyplexes was the first example of a biodegradable synthetic virus for gene delivery.

6 Appendix

6.1 Abbreviations

ACN	Acetonitril
CL	Crosslinker
CMV	Cytomegalovirus
C/P-ratio	Weight ratio of conjugate to plasmid
Cya	Cystamine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSP	Dithiobis(succinimidylpropionate)
DTBP	Dimethyl-3-3'-dithiobispropionimidate
DTT	Dithiothreitol
EDTA	Ethylendiaminetetraacetic acid
EGF	Epidermal growth factor
EGF-PEG-PEI25	EGF covalently linked to PEI25br with a heterobifunctional 3.4 kDa PEG spacer
EtBr	Ethidium bromide
FCS	Fetal calf serum
FT-IR	Fourier transformed infrared spectroscopy
GPC	Gel permeation chromatography
GSH	Glutathione

HBG	HEPES-buffered glucose
HBS	HEPES-buffered saline
HD	Hexanediol-diacrylate
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethansulfonic acid)
HMW	High molecular weight
HPLC	High pressure liquid chromatography
IP	Imidopropionate
LMW	Low molecular weight
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MTT	Methylthiazoletetrazolium
Mw	Molecular weight
MQ	De-ionized water (Millipore)
NMR	Nuclear magnetic resonance
N/P-ratio	Molar ratio of PEI nitrogen to DNA phosphate
$^1O_2$	Singlet oxygen
OEI	Oligoethylenimine 800 Da
PBS	Phosphate-buffered saline
PCI	Photochemical intracellular release
pCMVLuc	Plasmid encoding for luciferase under control of the CMV promoter/enhancer
PEG	Polyethylene glycol
PEI 25br	branched polyethylenimine 25 kDa
PEI 22lin	linear polyethylenimine 22 kDa
PH	Pentaethylenhexamine
PLL	Polylysine

PS	Photosensitizer
RNA	Ribonucleic acid
RLU	Relative light units
RT	Room temperature
SD	Spermidine
SP	Spermine
SP	Succinimidylpropionate
siRNA	small interfering RNA
SEC	Size exclusion chromatography
TBE	Tris borate EDTA
TP	Tetraethylenpentamine
TPPS2a	meso-tetraphenylporphine disulfonate on adjacent phenyl rings
TT	Triethylentetramine

6.2 Publications

6.2.1 Original papers

- Zwiorek, K., Kloeckner, J., Wagner, E., Coester, C. (2005)
  Gelatin nanoparticles as a new and simple gene delivery system
  J Pharm Pharmaceut Sci. 7(4), 22-28
- Kloeckner, J., Prasmickaite, L., Hogset, A., Berg, K., Wagner, E. (2004)
  Photochemically enhanced gene transfer of EGF receptor-targeted DNA polyplexes
  J Drug Targeting, 12(4) 205-214

Manuscript in preparation

- Kloeckner, J., Wagner, E., Ogris, M.
  Novel degradable gene carriers based on oligomerized polyamines
- Kloeckner, J., Bruzzano, S., Ogris, M., Wagner, E.
  Gene carriers based on hexanedioldiacrylate crosslinked oligoethylenimine – effect of chemical structure and size of polymer on biological properties
- Kloeckner, J., Persson, D., Boeckle, S., Roedl, W., Ogris, M., Wagner, E.
  Improved endosomal release of EGF receptor-targeted oligoethylenimine polyplexes for efficient gene delivery

6.2.2 Reviews and book chapters

- Wagner, E., Kloeckner, J. and Ogris, M. (2005) Optimizing polyplexes into synthetic viruses for tumor-targeted gene therapy in “Non-viral Gene Therapy-Gene Design and Delivery”, Eds. K.Taira, K.Kataoka, T.Niidome
  Springer-Verlag Tokyo 2005

- Wagner, E. and Kloeckner, J. (2005) Gene delivery using polymer therapeutics in “Advances in Polymer Science”
  Springer-Verlag Berlin, Heidelberg

6.2.3 Poster presentations

- Kloeckner, J., Ogris, M., Wagner, E. (2005) Biodegradable polyamines as efficient gene delivery vectors: Effects of the oligomerizing linker chemistry on biophysical and biological properties
  Controlled Release Society, Annual Meeting, Miami, USA
- Kloeckner, J., Walker, G., Wagner, E., Ogris, M. (2003) Development of an intelligent biodegradable gene carrier – 'a smart artificial virus'
  Controlled Release Society, German Chapter Annual Meeting, Munich, Germany

6.2.4 Oral presentations

- Kloeckner, J., Fahrmeir, J. (2005) Development of novel nonviral vectors for tumor-targeted gene delivery – towards an 'artificial virus'
  Scientific colloquium, Bulgarian Academy of Science, Sofia, Bulgaria
- Kloeckner, J., Walker, G., Ogris, M., Wagner, E. (2004) Development of a biodegradable gene carrier based on oligomerized polyamines, European Conference of Drug Delivery and Pharmaceutical Technology, Sevilla, Spain

7 References

A

Ahn, C.H., Chae, S.Y., Bae, Y.H., Kim, S.W.
Biodegradable poly(ethylenimine) for plasmid DNA delivery
J Control Release, 80 (1-3), 273-282, 2002

Akinc, A., Anderson, D.G., Lynn, D.M., Langer, R., Synthesis of poly (ß-amino esters) optimized for highly effective gene delivery
Bioconjug Chem. 14 (5), 2003

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 42, 3153-3158, 2003

Anderson, D.G., Peng, W., Akinc, A., Hossain, N., Kohn, A., Padera, R., Langer, R., Sawicki, J.A., A polymer library approach to suicide gene therapy
PNAS 101 (45), 16028-16033, 2004

Anderson, D.G., Akinc, A., Hossain, N., Langer, R., Structure/property studies of polymeric gene delivery using a library of poly (ß-amino esters)
Mol Ther.11(3),426-34, 2005

B

Banerjee, P., Reichardt, W., Weissleder, R., Bogdanov, A.
Novel hyperbranched dendron for gene transfer in vitro and in vivo
Bioconjug Chem. 2004

Berg, K., Selbo, P.K., Prasmickaité, L., Tjekke, T.E., Sandvig, K., Moan, J., Gaudernack, G., Fodstad, Kjolsrud, S., Amholt, H., Rodal, G.H., Rodal, S.K., Hogset, A.
Photochemical Internalisation: a novel technology for delivery of macromolecules into cytosol
Cancer Res.59, 1180-1183, 1999

Blagbrough, I.S., Geall, A.J., Neal, A.P., Polyamines and novel polyamine conjugates interact with DNA in ways that can be exploited in non-viral gene therapy
Biochemical Society Transactions 2003, 397-406, 2003

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(4), 529-37, 2001

Boeckle, S., v.Gersdorff, K., van der Piepen, S., Culmsee, C., Wagner, E., Ogris, M.
Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer
J Gene Med.6(10):1102-11, 2004

Boeckle, S., Wagner, E., Ogris, M., C- versus N-terminally linked melittin-polyethylenimine conjugates: the site of linkage strongly influences activity of DNA polyplexes
J Gene Med. 7, 1335-1347, 2005

Boeckle,S. Improved nonviral gene vectors: efficient and non-toxic polyplexes with enhanced endosomolytic activity
PhD thesis, Ludwig-Maximilians-University, Munich, 2005

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine
PNAS 92, 7297-7301, 1995

Brissault, B., Kichler, A., Guis, C., Leborgne, C., Danos, O., Cheradame, H.
Synthesis of linear polyethylenimine derivatives for DNA transfection.
Bioconjug Chem. 14(3):581-7, 2003

Brownlie, A., Uchegbu, I.F., Schatzlein, A.G.
PEI-based vesicle-polymer hybrid gene delivery system with improved biocompatibility
Int J Pharm.274(1-2):41-52, 2004

Brunner S., Furtbauer E., Sauer T., Kursa M., Wagner E.
Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation.
Mol Ther.5(1): 80-6, 2002

C

Carlisle, R.C., Etrych, T., Briggs, S.S., Preece, J.A., Ulbrich, K., Seymour, L.W.
Polymer-coated polyethylenimine/DNA complexes designed for triggered activation by intracellular reduction
J. Gene Med. 6, 337-344, 2004

Chen, D.J., Majors, B.S., Zelikin, A., Putnam, D.
Structure-function relationships of gene delivery vectors in a limited polycation library
J Control Release 103(1):273-83, 2005

Cho, Y.W., Kim, J.W., Park, K.
Polycation gene delivery systems: escape from endosomes to cytosol
J Pharm Pharmacol. 55(6):721-34, 2003

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(1):84-91, 2002

Collins, D.S., Unanue, E.R., Harding, C.V.
Reduction of disulfide bonds within lysosomes is a key step in antigen processing
J Immunol.147, 4054-4059, 1991

Cotten, F. Langle-Rouault, H. Kirlappos, E. Wagner, K. Mechtler, M. Zenke, H. Beug, and M. L. Birnstiel. Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels, *Proc. Natl. Acad. Sci. U. S. A*, 87:4033-4037, 1990

D

Dash, P.R., Read, M., L., Fischer, K., D., Howard, K.A., Wolfert, M., Oupicky, D., Subr, V., Strohalm, J., Ulbrich, K., Seymour, L., W., Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin.
J Biol Chem.11, 275(6):3793-802, 2000

Davies, M.E., Non-viral gene delivery systems
Curr Opin Biotechnol.13, 128-131, 2002

Deng, H., Bloomfield,V.A., Benevides, J.M, Thomas, G.J.
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

Dubruel, P., Christiaens, B., Vanloo, B., Bracke, K., Rosseneu, M., Vandekerckhove, J., Schacht, E., Physicochemical and biological evaluation of cationic polymethacrylates as vectors for gene delivery- Part A
Eur J Pharm Sci.18(3-4):211-20, 2003

Dubruel, P., Dekie, L., Christiaens, B., Vanloo, B., Rosseneu, M., Vandekerckhove, J., Mannisto, M., Urtti, A., Schacht, E., Poly-L-glutamic acid derivatives as multifunctional vectors for gene delivery- Part B. Biological Evaluation
Biomacromolecules 4, 1177-1183, 2003

E

Edlund, U., Albertsson, A.C., Polyesters based on diacid monomers
Adv Drug Del Rev.55, 585-609, 2003

Engesaeter, B., Bonsted, A., Berg, K., Hogset, A., Engebraten, O., Fodstad, Oystein, Curiel, D.T., Maelandsmo, G.M., PCI-enhanced adenoviral transduction employs the known uptake mechanism of adenoviral particles
Cancer Gene Ther.12, 439-448, 2005

Erbacher, P., Roche, A.C., Monsigny, M., Midoux, P., Putative role of chloroquine in gene transfer into human hepatoma cell line by DNA/ lactosylated polylysine complexes
Exp Cell Res.225, 186-194, 1996

F

Fischer, D., Bieber, T., Li, Y., Elsässer, H.-P., Kissel, T., A novel nonviral 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

Fischer, D., Youxin, L., Ahlenmeyer, B., Krieglstein, J., Kissel, T., In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis
Biomaterials 24, 1121-1131, 2003

Forrest, M.L., Koerber, J.T., Pack, D.W., A degradable polyethlyenimine derivative with low toxicity for highly efficient gene delivery
Bioconjug Chem.14 (5), 934-940, 2003

Frederiksen, K.S., Abrahamsen, N., Cristiano, R.J., Damstrup, L., Poulsen, H.S.
Gene delivery by an epidermal growth factor/DNA polyplex to small cell lung cancer cell lines expressing low levels of epidermal growth factor receptor
Cancer Gene Ther.7, 262-268, 2000

G

Gosselin, M., Guo, W., Lee, R.J.
Efficient gene transfer using reversibly crosslinked LMW-PEI
Bioconjug Chem.12, 989-994, 2001

Goto, T., Nishi, T., Kobayashi, O., Tamura, T., Dev, S.B., Takeshima, H., Kochi, M., Kuratsu, J., Sakata, T., Ushio, Y., Combination electro-gene therapy using herpes virus thymidine kinase and interleukin-12 expression plasmids is highly efficient against murine carcinomas in vivo.
Mol Ther. 10(5):929-37, 2004

H

Hardy, J., G., Kostiainen, M., A., Smith, D., K., Gabrielson, N., P., Pack, D., W.
Dendrons with spermine surface groups as potential building blocks for nonviral vectors in gene therapy
Bioconjug Chem. 2005 – ASAP article

Hogset, A., Prasmickaité, L., Tjelle, T.E., Berg, K., Photochemical Transfection: a new technology for light-induced, site-directed gene delivery
Human Gene Ther.11, 869-880, 2000

Hogset A., Prasmickaité, L., Engesaeter, B.O., Hellum, M., Selbo, P.K., Olsen, V.M., Maelandsmo, G.M., Berg, K., Light directed gene transfer by photochemical internalisation
Curr Gene Ther.3, 89-112, 2003

Huang, Z., A review of progress in clinical photodynamic therapy
Technol Cancer Res Treat. 4(3):283-93, 2005

I

Ilieva, S., Glabov, B., Musaey, D.G., Morokuma, K., Schaefer, H.F., Computational study of the aminolysis of esters. The reaction of methylformate with ammonia
J Org Chem. 68(4):1496-502, 2003

J

Jon, S., Anderson, D.G., Langer, R., Degradable poly (amino alcohol esters) as potential DNA vectors with low cytotoxicity
Biomacromolecules 4, 1759-1762, 2003

Juhasz, P., Biemann, K., Mass spectrometric molecular-weight determination of highly acidic compounds of biological significance via their complexes with basic polypeptides
PNAS 91(10), 4333-7, 1994

K

Kay M.A., Glorioso J.C., Naldini L.;
Viral vectos for gene therapy: the art of turning infectious agents into vehicles of therapeutics
Nat Med.7, 33-40, 2001

Kichler A., Behr J.P., Erbacher P.
Polyethylenimines: a family of potent polymers for nucleic acid delivery
Nonviral vectors for gene therapy, Academic Press, San Diego, Chapter 9, 1999

Kichler, A., Leborgne, C., Coeytaux, E., Danos, O.
Polyethylenimine-mediated gene delivery: a mechanistic study.
J Gene Med. 3(2):135-44, 2001

Kichler, A., Leborgne, C., Marz, J., Damps, O., Bechinger, B., Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells
PNAS 100(4), 1564-8, 2003

Kim, T., Seo, H.J., Choi, J.S., Yoon, J.K., Baek, J., Kim, K., Park, J.S.
Synthesis of biodegradable cross-linked poly(ß-amino ester) for gene delivery and its modification, inducing enhanced transfection efficiency and stepwise degradation
Bioconjug Chem.16 (5), 1140-8, 2005

Kim, Y.H., Park, J.H., Lee, M., Kim, Y.H., Park, T.G., Kim, S.W.
Polyethylenimine with acid-labile linkages as a biodegradable gene carrier
J. Control Release 103, 209-219, 2005

Kircheis, R., Kichler, A., Wallner, G., Kursa, M., Ogris, M., Felzmann, T., Buchberger, M., Wagner, E., Coupling of cell-binding ligands to polyethylenimine for targeted gene transfer
Gene Ther.4, 409-418, 1997

Kircheis R., Ogris M., Brunner S., Schüller S., Wightman L., Kursa M., Wagner E.
Transferrin-polycation DNA complexes for tumor-targeted in vivo gene transfer.
Progress in Drug Delivery Systems VIII, N. Oku, Ed., 83-94., 1999

Kircheis, R., Wightman, L., Schreiber, A., Robitza, B., Rossler, V., Kursa, M., Wagner, E.
Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application
Gene Ther.8(1):28-40, 2001

Kloeckner, J., Prasmickaite, L., Hogset, A., Berg, K., Wagner, E.
Photochemically enhanced gene delivery of EGF receptor-targeted DNA polyplexes
J Drug Target.12(4):205-13, 2004

Kopatz I., Remy J.S., Behr J.P., A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin
J Gene Med.6(7):769-76, 2004

Krämer, M., Stumbé, J.-F., Grimm, G., Kaufmann, B., Krüger, U., Weber, M., Haag, R.
Dendritic polyamines: simple access to new materials with defined treelike structures for application in nonviral gene delivery
Chembiochem.5, 1081-1087, 2004

Kunath, K., Harpe, A., Fischer, D., Peterson, H., Bickel, U., Voigt, K., Kissel, T.
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

Kunath, K., von Harpe, A., Fischer, D., Kissel, T.,
Galactose-PEI-DNA complexes for targeted gene delivery: degree of substitution affects complex size and transfection efficiency.
J Control Release 88(1):159-72, 2003

Kursa M., Walker G. F., Roessler V., Ogris M., Roedl W., Kircheis R., Wagner, E. Novel shielded Transferrin-Polyethylene Glycol-Polyethylenimine / DNA Complexes for Systemic Tumor-Targeted Gene Transfer.
Bioconjug Chem.14, 222-231, 2003

L

Leqocq, M., Wattiaux, D., Coninck, S., Laurent, N., Wattiaux, R., Jadot, M.
Uptake and intracellular fate of polyethylenimine in vivo
Biochem Biophys Res Commun.278(2):414-8, 2000

Lee R.J., Huang L., Lipidic vector systems for gene transfer
Crit Rev Ther Drug Carrier Syst.14, 173-206, 1997

Lee, T.K.W., Han, J.S., Fan, S.T., Liang, Z.D., Tian, P.K., Gu, J.R., Gene delivery using a receptor-mediated gene transfer system targeted to hepatocellular carcinoma cells
Int J Cancer 93, 393-400, 2001

Lee M., Kim S.W., Poly(L-Lysine) and copolymers for gene delivery
Polymeric gene delivery, CRC Press, Chapter 6, 2005

Leopold, P.L., Ferris, B., Grinberg, I., Fluoresecent virions: dynamic tracking of the pathway of adenoviral gene transfer vectors in living cells
Hum Gene Ther.9, 367-378, 1998

Li, Z., Huang, L., Sustained delivery and expression of plasmid DNA based on biodegradable polyester, poly (D,L-lactide-co-4-hydroxy-L-proline)
J Control Release 98, 437-446, 2004

Lim, Y.B., Choi, Y.H., Park, J.S., A self destroying polycationic polymer: biodegradable poly (4-hydroxy-L-proline ester)
J. Am. Chem. Soc, 121, 5633-5639, 1999

Lim, Y.B., Han, S.O., Kong, H.U., Lee, Y., Park, J.S, Jeong, B., Kim, S.W.,
Biodegradable Polyester, Poly (alpha-(4-aminobutyl)-L-glycolic acid), as non- toxic gene carrier
Pharm Res. 17(7): 811-6, 2000

Lim, Y.B., Kim, S., Suh, H., Park, J., Biodegradable, endosome disruptive and cationic network-type polymer as a highly efficient and nontoxic gene delivery system
Bioconjug Chem. 13(5): 952-7, 2002

Lipinski, K.S., Djeha, A.H., Ismail, T., Mountain, A., Young, L.S., Wrighton, C.J.
High-level, beta-catenin/TCF-dependent transgene expression in secondary colorectal cancer tissue
Mol Ther.4, 365-371, 2001

Lungwitz, U., Breunig, M., Blunk, T., Goepferich, A.
Polyethylenimine-based non-viral gene delivery systems
Eur J Pharm Biopharm.60 (2), 247-66, 2005

M

Malik, N., Wiwattanapatapee, R., Klopsch, R., Lorenz, K., Frey, H., Weener, J.W., Meijer, E.W., Paulus, W., Duncan, R., Dendrimers: relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo.
J Control Release 65(1-2):133-48, 2000

Mansouri S., Lavigne P., Corsi K., Benderdour M., Beaumont E., Fernandes J.C.
Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy (Review)
Eur J Pharm Biopharm.57, 1-8, 2004

McKenzie, D., Smiley, E., Kwok, K., Rice, K., Low molecular weight disulfide cross linking peptides as nonviral gene delivery carriers
Bioconjug Chem.11, 901-909, 2000

McKenzie, D., Kwok, K., Rice, K., A potent new class of reductively activated peptide gene delivery agents
J Biolog Chem.275, 9970-9977, 2000

Merdan, T., Kunath, K., Petersen, H., Bakowsky, U., Voigt, K.H., Kopecek, J., Kissel, T.
PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice
Bioconjug Chem.16(4):785-92, 2005

Midoux, P., Monsigny, M., Efficient gene transfer by histidylated polylysine/ pDNA complexes
Bioconjug Chem.10:406–411, 1999

Moghimi, S.M., Symonds, P., Murray, J.C., Hunter, A.C., Debska, G., Szewczyk, A.
A two-stage poly (ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy
Mol Ther. 11(6):990-5, 2005

Moreau, E., Domurado, M., Chapon, P., Vert, M., Domurad, D.,Biocompatibility of polycations: in vitro agglutination and lysis of red blood cells and in vivo toxicity
J Drug Target. 10(2):161-73, 2002

N

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

O

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(4):595-605, 1999

Ogris M., Steinlein P., Carotta S., Brunner S., Wagner, E., DNA/polyethylenimine transfection particles: Influence of ligands, polymer size, and PEGylation on internalisation and gene expression.
AAPS Pharm Sci. 3(3) article 21, 2001

Ogris, M., Walker, G., Blessing, T., Kircheis, R., Wolschek, M., Wagner, E.
Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes.
J Control Release, 91(1-2):173-81, 2003

Oupický, 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

Oupický, 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
JACS communications Vol.124 (1), 2002

P

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(1):75-80, 2002

Persson, D., Novel polyplexes for tumor-targeted gene delivery
Master thesis, Uppsala University and Ludwig-Maximilians-University, Munich, 2005

Petersen, H., Merdan, T., Kunath, K., Fischer, D., Kissel, T., Poly(ethylen-co-L-lactamide-co-succinamide): a biodegradable polyethylenimine derivative with an advantageous pH-dependent hydrolytic degradation for gene delivery
Bioconjug Chem. 13(4), 2002

Petersen, H., Fechner, P., M., Martin, A.,L., Kunath, K., Stolnik, S., Roberts, C.J., Fischer, D., Davies, M., C., Kissel, T., Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system
Bioconjug Chem.13(4):845-54, 2002

Pichon, C., LeCam, E., Guerin, B., Coulaud, D., Delain, E., Midoux, P.
Poly(Lys-(AEDTP)) : A cationic polymer that allows dissociation of pDNA/cationic polymer complexes in a reductive medium and enhances polyfection
Bioconjug Chem.13, 76-82, 2002

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(6):533-9, 1992

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., Wagner, E., The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems
J Biol Chem.269, 12918-12924, 1994

Plank, C., Mechtler, K., Szoka, F.J., Wagner, E., Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery
Hum Gene Ther. 7(12):1437-46, 1996

Plank, C., Tang, M.X., Wolfe, A.R., Szoka, F.C., 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(2), 319-332, 1999

Prasmickaité, L., Hogset, A., Tjelle, T.E., Oslen, V.M., Berg, K., The role of endosomes in gene transfection mediated by photochemical internalisation (PCI)
J Gene Med.2, 477-488, 2000

Prasmickaité, L., Hogset, A., Berg, K., Evaluation of different photosensitizers for use in photochemical gene transfection
Photochem Photobiol. 73(4), 388-395, 2001

Prasmickaité, L., Hogset, A., Berg, K., The role of the cell cycle on the efficiency of photochemical gene transfection
Biochim Biophys Acta 1570, 210-218, 2002

Prasmickaité, L., Hogset, Selbo, P.K., Engesaeter, B.O., Hellum, M., Berg, K.
Photochemical disruption of endocytic vesicles before delivery of drugs: a new strategy for cancer therapy
Br J Cancer 86, 652-657, 2002

R

Read, M.L., Bremner, K.H., Oupický, D., Green, N.K., Searle, P.F., Seymour, L.W.
Vectors based on reducible polycations facilitate intracellular release of nucleic acids
J Gene Med.5, 232-245, 2003

Robbins P.D., Tahara T., Ghivizzani T., Viral vectors for gene therapy;
Trends Biotechnol.16, 35-40, 1998

S

Saito, G., Swanson, J.A., Lee, K.D., Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities
Adv Drug Del Rev.1, 2002

Saito, G., Amidon, G.L., Lee, K.D. Enhanced cytosolic delivery of plasmid DNA by a sulfhydryl-activable listeriolysin O/protamine conjugate utilizing cellular reducing potential
Gene Ther.10, 72-83, 2003

Saminathan, M., Anthony, T., Shirahata, A., Sigal, L.H., Thomas, T., Thomas, T.J.
Ionic and structural specificity effects of natural and synthetic polyamines on the aggregation and resolubilization of single-, double-, and triple-stranded DNA
Biochemistry 38, 3821-3830, 1999

Schaffer D.V., Fidelmann N.A., Dan D., Lauffenburger D.A.
Vector unpacking as a potential barrier for receptor mediated polyplex delivery
Biotechnol Bioeng.67(5), 598-606, 2000

Selbo, P.K., Hogset, A., Prasmickaite, L., Berg, K.
Photochemical internalisation: a novel drug delivery system
Tumour Biol.23, 103-112, 2002

T

Takakura, Y., Fujita, T., Hashida, M., Sezaki, H., Disposition characteristics of macromolecules in tumor-bearing mice
Pharm Res.7, 339-346, 1990

Thomas, M., Klibanov, A.M., Non-viral gene therapy: polycation-mediated DNA delivery
Appl Microbiol Biotechnol. 62, 27-34, 2003

Thomas, M., Ge, Q., Lu, J.L., Chen, J., Klibanov, A.M., Cross-linked small polyethylenimines: while still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo
Pharm Res. 22(3):373-80, 2005

Thoren, P.E., Persson, D., Lincoln, P., Norden, B.
Membrane destabilizing properties of cell-penetrating peptides
Biophys Chem.114(2-3):169-79, 2005

Tomizawa, M., Ebara, M., Saisho, H., Sakiyama, S., Tagawa, M.
Irradiation with ultrasound of low output intensity increased chemosenstivity of subcutaneous solid tumors to an anti-cancer agent
Cancer Lett.173, 31-35, 2001

Trubetskoy, V., Loomis, A., Slattum, P., Hagstrom, J., Budker, V., Wolff, J.
Caged DNA does not aggregate in high ionic strength solutions
Bioconjug Chem.10, 624-628, 1999

V

Verbaan, F.J., van Dam, I., Takakura, Y., Hashida, M., Hennink, W., Storm, G., Oussoren, C.
Intravenous fate of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes.
Eur J Pharm Sci. 20(4-5):419-27, 2003

Verbaan F.J., Ousseren C., Snel C.J., Crommelin D.J.A., Hennink W.E., Storm G.
Steric stabilization of poly(2-(dimethylamino)ethylmethacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice
J. Gene Med.6, 64-75, 2004

Vijayanathan, V., Thomas, T., Shirahata, A., Thomas, T.J.,
DNA condensation by Polyamines: A laser light scattering study of structural effects
Biochemistry, 40, 13644-13651, 2001

Von Gersdorff, K., Ogris, M., Wagner, E.,
Cryoconserved shielded and EGF receptor targeted DNA polyplexes: cellular mechanisms
Eur J Pharm Biopharm. 60, 279-285, 2005

W

Wagner, E., Plank, C., Zatloukal, K., Cotton, M., Birnstiel, M.L.,
Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle.
PNAS 89(17):7934-8, 1992

Wagner, E., Application of membrane active peptides for nonviral gene delivery
Adv Drug Deliv Rev. 38(3):279-289, 1999

Wagner, E., Strategies to improve DNA polyplexes for in vivo gene transfer – will “artificial viruses” be the answer?
Pharm Res. 21, 8-14, 2004

Wagner, E., Culmsee, C., Boeckle, S., Targeting of polyplexes: towards synthetic virus vector systems
Huang, L., Hung, M.C., Wagner, E. (eds) Nonviral vectors for gene therapy, 2nd edition (Advances in Genetics, Volumes 53 and 54), 2005

Walker, G.F., Fella, C., Pelisek, J., Fahrmeir, J., Boeckle, S., Ogris, M., Wagner, E.
Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo.
Mol Ther. 11(3):418-25, 2005

Wang, J., Murakami, T., Hakamata, Y., Ajiki, T., Jinbu, Y., Akasaka, Y., Ohtsuki, M., Nakagawa, H., Kobayashi, E., Gene gun mediated oral mucosal transfer of interleukin 12cDNA coupled with an irradiated melanoma vaccine in a hamster model: successful treatment of oral melanoma and distant skin lesion
Cancer Gene Ther.8, 705-712, 2001

Wang, J., Mao, H.Q., Leong, K.W.
A novel biodegradable gene carrier based on polyphosphoester
J Am Chem Soc.123, 9480-9481, 2001

Wang, J., Zhang, P.C., Mao H.Q., Leong, K.W., Enhanced gene expression in mouse muscle by sustained release of plasmid DNA using PPE-EA as a carrier.
Gene Ther.9 (18):1254-61, 2002-1

Wang, J., Zhang, P.C., Lu, H.F., Ma, N., Wang, S., Mao H.Q., Leong, K.W.
New polyphosphoramidate with a spermidine side chain as a gene carrier
J Control Release 18;83 (1):157-68, 2002-1

Wang J., Gao S.J., Zang P.C., Wang S., Mao H.Q., Leong H.W.
Polyphosphoramidate gene carriers: effect of charge group on gene transfer efficiency.
Gene Ther. 11(12):1001-10, 2004

Wang, X., Huong, S.M., Chiu, M.L., Raab-Traub, N., Huang, E.S.
Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus
Nature 424, 456-461, 2003

De Wolf, H.K., Luten, J., Snel, C.J., Oussoren, C., Hennink, W.E., Storm, G.
In vivo tumor transfection mediated by polyplexes based on biodegradable poly(DMAEA)-phosphazene
J Control Release. [Epub ahead of print], 2005

Wolschek, M.F., Thallinger, C., Kursa, M., Rossler, V., Allen, M., Lichtenberger, C., Kircheis, R., Lucas, T., Willheim, M., Reinisch, W., Gangl, A., Wagner, E., Jansen, B., Specific systemic nonviral gene delivery to human hepatocellular carcinoma xenografts in SCID mice
Hepatology 36(5):1106-14, 2002

Z

Zauner, W., Brunner, S., Buschle, M., Ogris, M., Wagner, E.
Glycerol enhancement of ligand-polylysine/ DNA transfection
Biotechniques 20, 905-913, 1996

Zhao, Z., Wang, J., Mao, H.Q., Leong, K.W., Polyphosphoesters in drug and gene delivery
Adv Drug Del Rev.55, 483-499, 2003

Zhong, Z., Song, Y., Engbersen, F.J., Lok, M.C., Hennink, W.E., Feijen, J., A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s
J Control Release [Epub ahead of print] 2005

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(2):128-34, 2000

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

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9 Curriculum Vitae

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Education

09/2002 – to present	PhD thesis at the Department of Pharmaceutical Biology-Biotechnology, Ludwig-Maximilian-University, Munich, Germany; supervisor: Prof. Dr. Ernst Wagner
07/2002	Licensure as pharmacist
11/2001-04/2002	Internship at R&D Analytics, Pfizer GmbH, Freiburg, Germany
05/2001-10/2001	Internship at Internationale Ludwigs Apotheke, Munich, Germany
05/1997-04/2001	Studies of pharmacy, Ludwig-Maximilian-University, Munich Germany
10/1996-03/1997	Studies of French, Literature & Contemporary World History, University of Aix-en-Provence, France
09/1987-06/1996	Secondary school, Gymnasium Ottobrunn, Germany

Vocational Training

08/2005	DAAD research stay at the Institute of Polymer Chemistry, Bulgarian Academy of Science, Sofia, Bulgaria
08/2000-09/2000	Internship at the Department of Pharmaceutical Technology, University of Navarra, Pamplona, Spain: Analytical part of a study on the *role of lipases and physicochemical parameters of lipid formulations in the mechanism of oral absorption modulation*
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