Polyethylenimine-derived Gene Carriers and their Complexes with plasmid DNA

Design, Synthesis and Characterization

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Uta Lungwitz
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To my family

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Examination board:	Chairman:	Prof. Dr. S. Elz
	1. Expert:	Prof. Dr. A. Göpferich
	2. Expert:	Prof. Dr. A. Buschauer
	3. Examiner:	Prof. Dr. J. Heilmann

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1. POLYETHYLENEIMINE-BASED NON-VIRAL GENE DELIVERY SYSTEMS

U. Lungwitz, M. Breunig, T. Blunk, A. Göpferich

Department of Pharmaceutical Technology, University of Regensburg, D-93053 Regensburg, Germany

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Abstract

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. Non-viral vectors have attracted great interest, as they are simple to prepare, rather stable, easy to modify and relatively safe, compared to viral vectors. Unfortunately, they also suffer from lower transfection efficiency, requiring additional effort for their optimization. The cationic polymer polyethylenimine (PEI) has been widely used for non-viral transfection in vitro and in vivo and has an advantage over other polycations in that it combines strong DNA compaction capacity with an intrinsic endosomolytic activity. Here we give some insight into strategies developed for PEI-based non-viral vectors to overcome intracellular obstacles, including the improvement of methods for polyplex preparation and the incorporation of endosomolytic agents or nuclear localization signals. In recent years, PEI-based non-viral vectors have been locally or systemically delivered, mostly to target gene delivery to tumor tissue, the lung or liver. This requires strategies to efficiently shield transfection polyplexes against non-specific interaction with blood components, extracellular matrix and untargeted cells and the attachment of targeting moieties, which allow for the directed gene delivery to the desired cell or tissue. In this context, materials, facilitating the design of novel PEI-based non-viral vectors are described.

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1. Introduction

In recent years the knowledge of the molecular mechanisms of many inheritable or acquired diseases has been greatly expanded, directing great attention to the field of gene therapy [1-6]. Antisense, ribozyme strategies [7] or iRNA [8] could potentially be used to downregulate or inactivate the expression of specific genes. In addition, suicide gene therapy [9] could enable the selective destruction of e.g. tumor cells using prodrug-converting enzymes and tumor specific promoters.

Viral vectors have been applied to deliver therapeutic genes into living cells, but their broad use is affected by the limited size of the genetic material that can be delivered and severe safety risks [10;11], based upon their immunogenicity and their oncogenic potential [12-14].

In light of these concerns, non-viral gene delivery has emerged as a promising alternative. Among the variety of different materials [15-18] which have been utilized in the manufacture of non-viral vectors, the use of polymers confers several advantages, due to their ease of preparation, purification and chemical modification as well as their enormous stability. The polyamine PEI has emerged as a potent candidate, even though the use of PEI-derived gene delivery vehicles is still limited by a relatively low transfection efficiency and short duration of gene expression [19;20] compared to viral transfection systems, as well as cytotoxic effects [21;22].

In view of the great diversity within the field of non-viral gene delivery, here we chose to focus on polyethylenimine-based transfection vehicles, including strategies for their optimization and the observed effects thereof.

2. Intracellular pathway

Despite the broad experimental use of PEI-based vectors, the efficiency of gene delivery remains insufficient. As understanding of the intracellular trafficking pathways has expanded, the major hurdles of gene transfer at the

cellular level have been revealed, and the first strategies to overcome these limitations have been developed.

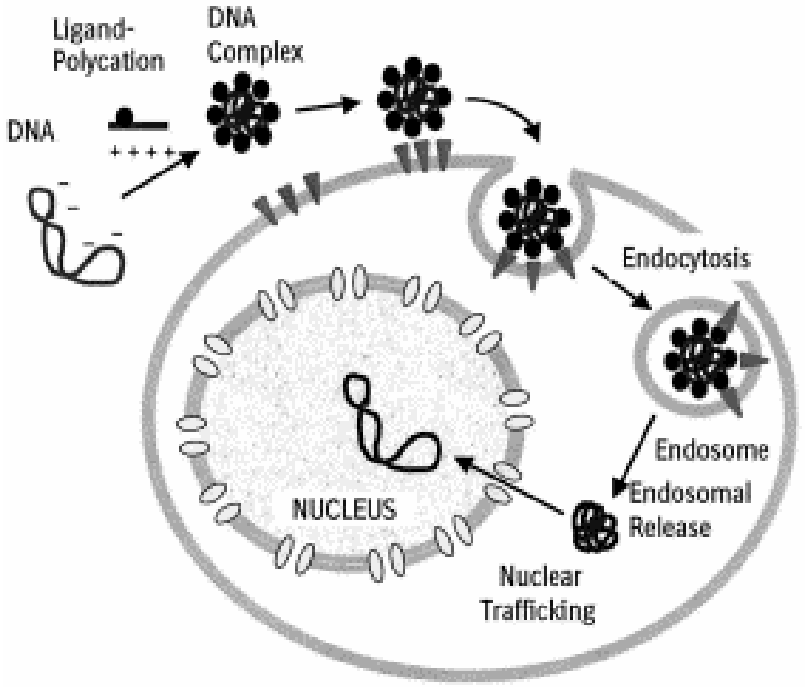


Figure 1. *General scheme of gene transfer using ligand-decorated non-viral vectors: DNA is condensed using the ligand-PEI conjugate to form ligand decorated complexes. After their cellular uptake via receptor-mediated endocytosis, the polyplexes are entrapped in endosomes. The receptor-ligand complex dissociates from the PEI/DNA complex and the receptor is recycled to the cell surface. The polyplexes escape from endosomes into the cytoplasm and DNA crosses the nuclear envelope to accumulate in the nucleus.*

[Reprinted from J. Controlled Release, 72 (2001), R. Kircheis, T. Blessing, S. Brunner, L. Wightman, E. Wagner, Tumor targeting with surface-shielded ligand-polycation DNA complexes, pp.165-170, Copyright (2004), with permission from Elsevier]

When considering the therapeutic use of non-viral gene transfer, cell internalization of exogenous DNA packed within polyplexes via receptor-

mediated endocytosis remains the most favorable mechanism and can be accomplished by labeling the polyplexes with targeting moieties (Fig. 1).

Unmodified cationic complexes interact non-specifically with negatively charged glycoproteins, proteoglycans and sulfated proteoglycans [23-26] located on the surface of cell membranes and enter the cell predominantly via adsorptive [27] or fluid-phase endocytosis [28]. The cell-polyplex association and, hence, the efficacy of cellular uptake can be greatly improved by increasing the positive net charge, prolonging the incubation time or raising the polyplex concentration [27;29;30]. Alternative paths of cell internalization include phagocytosis, particularly following particle aggregation at low N/P ratios (N/P ratio: quotient of the nitrogen atoms of PEI to DNA phosphates), and potocytosis (cellular uptake via interaction with caveolae pits [31]), but both depend largely on the cell type. Irrespective of the mechanism of endocytotic uptake, polyplexes will follow the endolysosomal pathway, remain closely attached to the vesicle membrane of early and late endosomes, and fuse with the lysosomal vesicles, assembling in the perinuclear region. The fusion of endosomes with lysosomes seems to be at least partially prevented when PEI has been used in polyplex preparation. The DNA is allowed to reside longer in the non-lysosomal compartment, thereby bypassing DNA degradation by lysosomal nucleases and promoting transfection [27;28;30]. Due to mechanisms, that are not yet fully elucidated, a sudden burst of endosomal vesicles occurs, liberating a detectable fraction of polyplexes into the cytoplasm and DNA is translocated into the nucleus [27].

It is still not clear whether or not intact polyplexes reside in the nuclear compartment. While Godbey et al. observed polyplexes within the nucleus, others could confirm these results only for necrotic cells, which lack an intact nuclear membrane [28;30]. For most non-viral vectors, a sufficient fraction of exogenous DNA reaches the transcription machinery only at the stage when the nuclear envelope breaks down [32], limiting efficient gene transfer to fast dividing cells.

One auspicious strategy uses protein transduction domains, such as the TAT protein of the HIV-1 virus [33] and herpes simplex virus type-1 VP22 transcription factor [34], to improve the efficacy of transgene delivery by simply circumventing the hurdles of the endocytotic pathway and simultaneously promoting translocation to the nucleus [35]. The positively charged protein transduction domains interact nonspecifically with cell surfaces, promoting the internalization of larger cargo proteins directly into the cytoplasm in an endocytosis-independent manner. Optimized protein sequences appear to be attractive tools for use in the design of multifunctional and effective gene delivery vehicles and may conceivably reduce any immunogenic potential.

3. Polyethylenimine (PEI)

A large variety of different polymers and copolymers of linear, branched, and dendrimeric architecture, have been tested, in terms of their efficacy and suitability for in vitro transfection. Unfortunately no morphology emerged as a general favorite [36]. The insight into the relationship between the polymer structure and their biological performance, such as the DNA compaction, toxicity and transfection efficiency is still rather limited. Hence, the discovery of new potent materials still relies on empiric approaches rather than on a rational design. Nonetheless, the results from transfection experiments with PEI were impressive from the beginning. Depending on the linkage of the repeating ethylenimine units, PEI occurs as branched or linear morphological isomers.

3.1. Branched PEI (bPEI)

bPEI-derived vectors have been used to deliver oligonucleotides [37], plasmid DNA (pDNA), and Epstein-Barr virus-based plasmid vectors [9] as well as RNA and intact ribozymes [7].

It is synthesized by acid catalyzed polymerization of aziridine either in aqueous [38-40] or alcoholic solutions (Fig. 2), where the reaction is controlled by adjusting the temperature and initiator concentration, or in a rather

vigorous bulk polymerization of anhydrous aziridine at a lower temperature [41;42].

The efficacy of bPEI-derived vectors non-viral vectors and their cytotoxic effects depend to a remarkable extent on material characteristics like the molecular weight, the degree of branching, the cationic charge density and buffer capacity [38-40], polyplex properties, such as the DNA content, particle size and zeta potential and the experimental conditions like the polyplex concentration, the presence or absence of serum during transfection, the incubation time and the transfection model chosen for the gene delivery experiment.

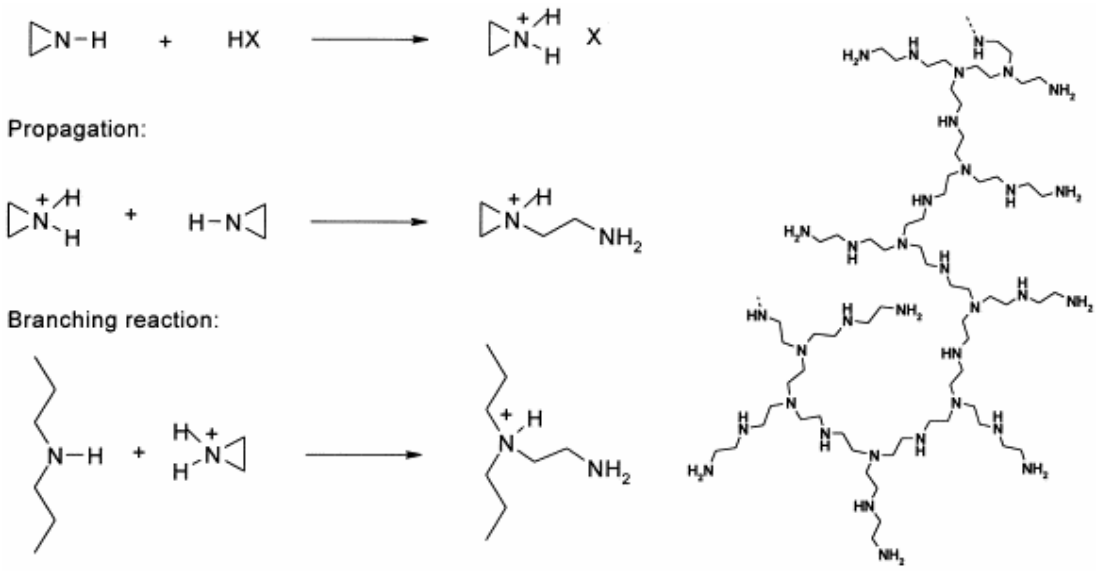


Figure 2.

Synthesis of branched polyethylenimine by acid catalyzed polymerization of aziridine in aqueous solution.

[Reprinted from J. Controlled Release, 69 (2000), A. von Harpe, H. Petersen, Y. Li, T. Kissel, Characterization of commercially available and synthesized polyethylenimine for gene delivery, pp. 309-322, Copyright (2004), with permission from Elsevier]

High molecular weight bPEI up to 800 kDa has been used for non-viral gene transfer, exhibiting a superior capability to form compact and stable

bPEI/DNA complexes and an increased transfection efficiency, compared to lower molecular weight derivatives [43].

Unfortunately, at the same time the cell viability decreased remarkably. This effect can be moderated by transfecting with low molecular mass bPEIs (5-48 kDa) [39;40;44], but amplified polymer concentrations are needed to achieve comparable efficacy. Due to their reduced cytotoxicity, the high N/P ratios were tolerated and added to the superior performance *in vitro*, irrespective of the absence or presence of serum.

The N/P ratio, and with it the zeta potential, dramatically influences the efficacy of the gene delivery system. It has been estimated that every fifth or sixth amino nitrogen of bPEI is protonated at physiological pH [45] and only these positively charged amino groups will ionically interact with the negatively charged DNA. The pK_a value of the individual nitrogen atoms within the bPEI molecules cannot be determined and so the absolute amount of positively charged amino groups is not known. Therefore, the N/P ratio refers to the ratio of the nitrogen atoms of PEI to DNA phosphates and simply describes the amount of polymer used for polyplex formation. At high N/P ratios, the positive net charge of the corresponding complexes increases, improving cell interaction and enhancing the cellular and nuclear uptake and retention [46].

It has been shown that the relative gene expression can be strongly influenced by the particle size [47], which, in turn, can be moderated by the molecular weight of the bPEI derivative, the method of particle preparation, and the N/P ratio. The polyplex size decreases with increasing molecular weight, an excess of the polyamine and in low ionic strength media, most likely due to improved DNA compaction. The addition of proteins, from serum, for example, can additionally stabilize the newly formed particles. Unfortunately, the success of bPEI-based transfection systems is derogated by the cytotoxic effects, which arise from the presence of free polymer. The cytotoxic effects correlate with the molecular weight of the polymer and intranuclear polymer concentration [48] and increase with a prolonged

incubation period. *In vivo* experiments have revealed that bPEI/DNA complexes and, to a lesser extent, free bPEI [25kDa] activates genes involved in the Th1/Th2 immune response and adaptive immune responses [49]. The systemic administration of unshielded bPEI [800 kDa] had a lethal effect in animal models [50;51], demonstrating the need for materials allowing for the design of less harmful but effective non-viral vectors.

3.2. Linear PEI (lPEI)

More recently, several *in vitro* and *in vivo* studies have investigated the potential of lPEI-derived vectors. Most of these experiments have been done in direct comparison to the corresponding bPEI/DNA complexes, revealing remarkable differences between both transfection systems in terms of DNA compaction [19], nuclear uptake [32;52], transfection efficiency and toxicity [50;51;53].

lPEI has been synthesized via cationic ring-opening polymerization of either N(2-tetrahydropyranyl)azidirine [54] or unsubstituted and 2-substituded 2-oxazolines followed by acid or base-catalyzed hydrolysis of the corresponding N-substituted polymer (Fig. 3) [46;55;56]. Potentiometric titration revealed that about 90% of the amines of the lPEI homopolymer are protonated at physiological pH. The suitability of lPEI for gene transfer was investigated and compared to random copolymers of poly(2-ethyl-2-oxazoline)-co-poly(ethylenimine) and N-alkylethylenimine, which are prepared by the partial hydrolysis or hydrogenation of the corresponding poly(2-ethyl-oxazoline) precursors, respectively [57;58]. As expected, increasing proportions of secondary amino groups within the random copolymers increased the buffer capacity and improved the DNA compaction, both contributing to the enhancement of transfection efficiency. The introduction of comparably less alkaline ternary amino groups dramatically decreased the transfection efficacy.

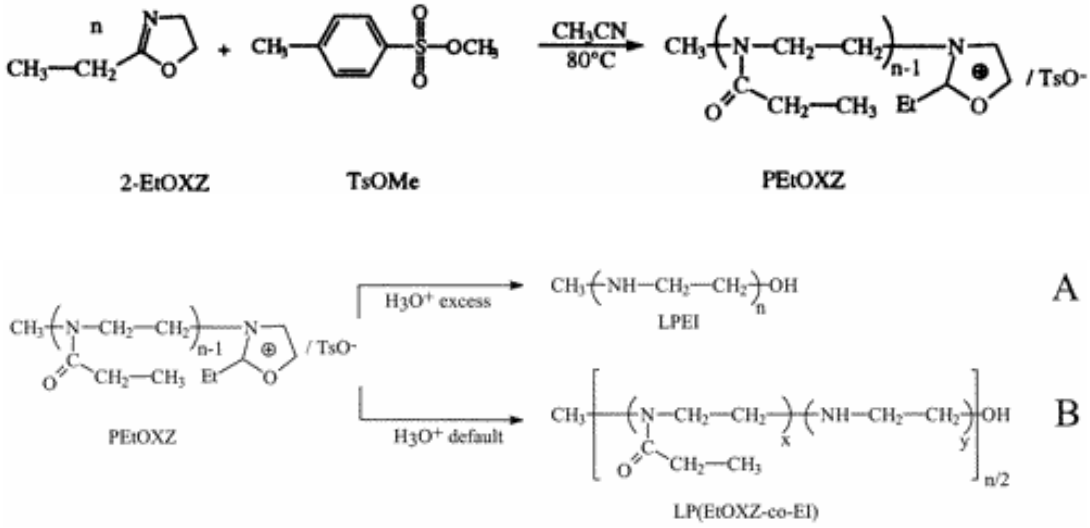


Figure 3.

Synthesis of linear polyethylenimine: The Polymerization of 2-ethyl-2-oxazoline was initiated by methyl-p-toluenesulfonate and proceeds according to a living process, yielding poly(2-ethyl-2-oxazoline)[pOXZ], capped by oxazolinium tosylate ends.

Linear polyethylenimine was synthesized by complete hydrolysis of pOXZ, using an excess of hydrochloric acid in aqueous media.

The incomplete hydrolysis of pOXZ by addition of varying equivalents of hydrochloric acid, leads to the formation of random poly (2-ethyl-2-oxazoline-co-ethylenimine) copolymers.

[Reprinted with permission from: Bioconjugate Chemistry, Vol. 14 (2003), pp.581-587, B. Brissault, A. Kichler, Ch. Guis, Ch. Leborgne, O. Danos, H. Cheradame, Synthesis of Linear Polyethylenimine Derivatives for DNA Transfection, Copyright (2004) American Chemical Society]

While bPEI/DNA-polyplexes retain a rather small size in the range up to a few hundred nanometers, which only slightly changes with respect to the complexation medium applied, lPEI mixed with pDNA forms rather large particles extending into the micrometer range in salt-containing buffers, which are prone to aggregation due to the weakened repulsion between the cationic

particles [51;59]. Only preparation under salt-free conditions with increasing N/P ratios yields small spherical or toroid shaped particles, which grow rapidly upon the addition of salt. The application of small polyplexes has been shown to improve transgene expression up to 100-fold *in vivo*, but reduces the transfection efficiency *in vitro*, compared to the larger complexes [19;47;60]. While large particles sediment rapidly and therefore interact more with cell surfaces, the mobility of small particles is dictated by Brownian motion. This difference has been abrogated by decreasing the transfection volume or increasing the incubation time, both of which increase the chances of the smaller particles coming into contact with the cell surface. Another explanation for the comparably lower transgene expression mediated by small complexes *in vitro* has been attributed to the decreased endosomolytic competence [19;47]. Irrespective of the conditions used, lPEI/DNA complexes exhibited improved cell viability, promote nuclear localization and increased transfection efficiency compared to bPEI-based vectors [61].

4. Endosomolysis

PEI/DNA polyplexes are internalized by large variety of cells, delivering polymer/DNA complexes to the endolysosomal compartment. Advantageously in comparison to other polymers like poly(L-lysine), PEI combines a high membrane destabilizing potential with a high DNA condensing activity, protecting endocytosed DNA from degradation and therefore increasing the probability that intact pDNA will reach the nucleus [62;63].

Behr postulated the so-called `proton sponge` hypothesis [25], which relates the intrinsic endosomolytic activity of PEI to its capacity to buffer the endosomal environment, prompting the osmotic swelling of the vesicle and finally its rupture, which leads to the liberation of the polyplexes into the cytoplasm. The fusion with lysosomes may thereby be prevented, circumventing DNA degradation by lysosomal DNases. In electron microscopy studies, membrane holes have been observed and were

attributed to the direct interaction of bPEI with the inside of the endosomal membrane [30]. While acidification within endosomes has been suggested to facilitate vesicle rupture [64;65], likely due to the extension of the polymer network [62;66] the membrane damage occurred in a dose-dependent manner [48] and was present even in the absence of acidification when high molecular weight bPEI derivatives were used [30]. Furthermore, *In vitro* experiments using ^{35}S-DNA/bPEI complexes have revealed that the endosomolytic activity seems to depend on the particle size, suggesting that larger polyplexes entrapped in endosomes may facilitate endosomolysis [19;47;60;67], while the efficient gene delivery mediated by small particles relies on the addition of endosomolytic compounds [60].

Viruses like the adenovirus or influenza virus achieve cellular uptake by endocytosis and posses effective tools to promote endosomal escape [68]. Therefore psoralen-inactivated adenoviruses were incorporated into bPEI/DNA polyplexes in the hopes to improving gene delivery. After systemic administration the relatively large particles were prone to phagocytosis by Kupffer cells and the subsequent immune response impeded a readministration [69;70]. These systems have been simplified by attaching virus-derived endosomolytic proteins or optimized synthetic peptide sequences onto polyplexes, greatly reducing their immunogenic potential. JTS-derived fusogenic peptides [71;72], the Haemophilus influenza hemagglutinin derived peptides [INF] [73-76] and GALA [74;77-80] all assume a random coil structure at pH 7. Acidification triggers a conformational transition, which enables the subsequent interaction with the phospholipid membranes, resulting in pore formation or the induction of membrane fusion and/or lysis [78]. The membrane-active dimeric influenza peptide INF5 ((GLF EAI EGF EIN GWEG nI DG)$_2$ K) [74] has been attached to both Transferrin (Tf)-bPEI/DNA and bPEI/DNA polyplexes, enhancing transgene expression up to 10-fold [81]. However, the optimum expression levels observed were not significantly higher than the maximum expression at high N/P ratios without INF5. KALA (WEA KLA KAL AKA LAK HLA KAL AKA LKA CEA) [82;83]

resembles the membranolytic activity of GALA (WEA ALA EAL AEA LAE HLA EAL AEA LEA LAA) [77;78] with DNA condensing properties derived from the lysine residues. [83;84]. KALA has been site-specifically mono-PEGylated in order to preserve its fusogenic potential [82] and KALA and PEG-KALA were attached to the surface of bPEI/DNA polyplexes.

The PEGylation sterically prevented particle aggregation and increasing concentrations of either bPEI or PEG-KALA led to an enhanced the transfection efficiency (Fig. 4).

However, the supplementation of membrane-destabilizing peptides, which is always inflicted with a rather low stability, the high costs of peptide synthesis and the risk of immunogenic reactions or reagents like chloroquine [85;86] failed to further enhance the transfection efficacy of PEI-based vectors, , suggesting that the DNA liberation of PEI/DNA polyplexes into the cytoplasm is sufficiently supported by its own fusogenic activity.

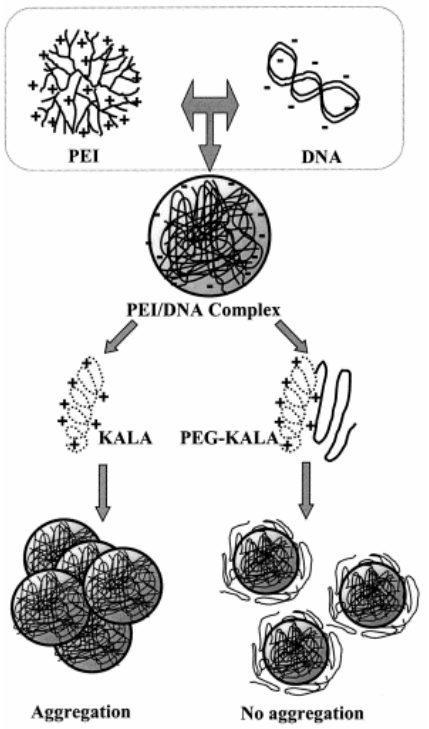


Figure 4.

The endosomolytic peptide KALA or a KALA-PEG conjugate was incorporated into PEI/DNA polyplexes by ionic interaction. While KALA-PEI/DNA increase significantly in size by inter-particle aggregation, pegylated polyplexes retain their individuality [Reprinted from J. Controlled Release, 76 (2001), H. Lee, J.H. Jeong, T. G. Park, A new gene delivery formulation of polyethylenimine/DNA complexes coated with PEG conjugated fusogenic peptide, pp.183-192, Copyright (2004), with permission from Elsevier].

5. Nuclear targeting

Successful gene therapy relies on an efficient DNA liberation from the endocytotic vesicles, DNA nuclear localization and gene expression, which proceeds in several steps, including: DNA nuclear localization, transcription factor nuclear import, transcription, mRNA processing and export and finally, translation into the therapeutic protein [87]. After the release of DNA from endocytotic vesicles, pDNA larger than 2000 bp remains nearly immobile within the viscous cytoplasm [88], and is rapidly degraded by cytosolic nucleases [89;90]. PEI has been shown to prolong the survival of pDNA in a dose-dependent manner, facilitated by the tight complexation of DNA, without affecting nuclease activity itself [90;91]. Despite the broad use of PEI-based vectors, the precise mechanisms by which pDNA reaches the nuclear compartment remain elusive.

The double membrane of the nuclear envelope is perforated by nuclear pore complexes (NPC), which assembles 8 smaller diffusion channels to allow macromolecules with an upper size limit of 50 kDa to diffuse independently, controlled only by their size [92], and a large channel for signal-mediated transport of macromolecules larger than 50 kDa [93], which can expand to an upper diameter of 26 nm (8 million Da), depending on the species and the metabolic state of the cell (Fig. 5a,b) [94].

Therefore the size [95] and copy number [96] of the pDNA largely influences the transfection efficiency. For active transport, substrates bind either directly to importin β or indirectly to importin α via nuclear localization signal-containing proteins to be carried through the nuclear pore [97-101] along the RanGDP/RanGTP gradient (Fig. 5c) [102;103].

The efficacy of transgene expression mediated by non-viral vectors, like bPEI/DNA polyplexes or, to a remarkably lesser extent, lPEI-derived systems, relies on the translocation of pDNA into the nucleus predominantly during the S/G2 phase of the cell cycle [32;52], which may explain why gene delivery is more efficient in rapidly dividing cells.

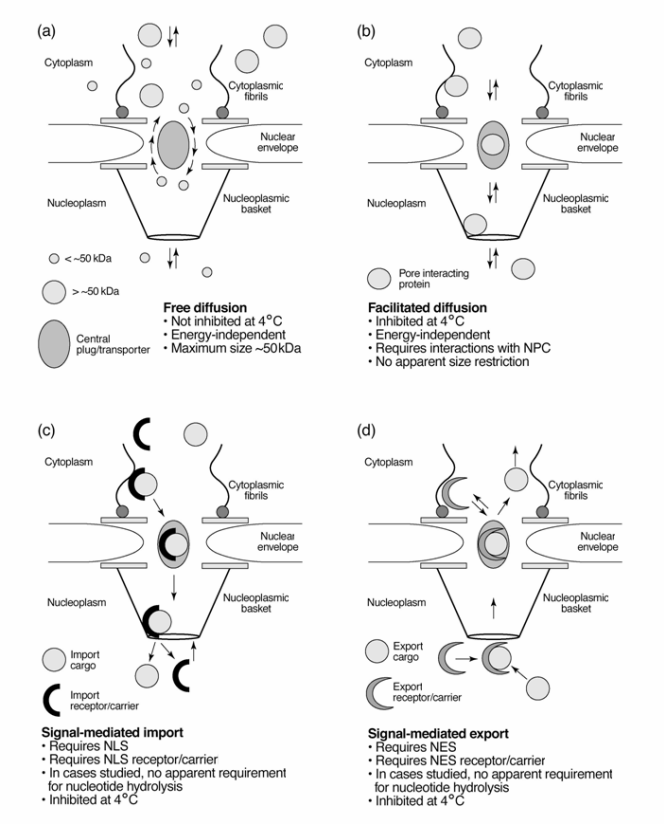


Figure 5.

Different pathways of macromolecule movement through the nuclear pore complex (NPC).

macromolecules smaller than ≈ 50 kDa are able to diffuse freely through the NPC, macromolecules larger than ≈ 50 kDa cannot

nuclear transport carriers in the absence of cargo are thought to cross the NPC by facilitated diffusion

signal mediated nuclear import: association of cargo and import carrier on the cytoplasmic side and transport through the NPC, dissociation at the nucleoplasm

signal mediated nuclear export: assembly of cargo and export carrier at the nucleus, transport across the NPC and dissembly at the cytoplasma

[Reprinted from trends in CELL BIOLOGY, 9 (1999), B. Talcott, M. S. Moore, Getting across the nuclear pore complex, pp.312-318, Copyright (2004) with permission from Elsevier]

As most cells targeted in gene therapy are non-dividing or divide very slowly, the incorporation of nuclear localization signals (NLS) that are capable of mediating nuclear import, [101;104;105] may conceivably both diminish the retention time of pDNA in the cytoplasm and facilitate the crossing of the nuclear barrier.

In several attempts, classical NLS peptides like the simian virus SV40 large T-antigen-derived peptides [106-112] and endogenous cellular proteins, such as the nucleoplasmin NLS [113], histones [93;114;115] or HMG-box proteins [93;116;117], as well as non-classical NLS, e.g. the HIV-1 virus TAT or REV peptides, the M9 sequence, derived from the heterogeneous nuclear ribonucleaprotein A1 [118;119] or SV40-DNA sequences [87;120-122], which interact with cytosolic transcription factors that harbor the desired NLS, have been applied to promote nuclear import. The latest approach is enticing because of the possibility of directing transgene expression to a specific cell [123] and then regulating it by the addition of exogenous stimulators [124]. The relative uptake and functional size of the channel can be influenced by the number of signals incorporated on each vector [125].

Other strategies have taken advantage of cytoplasmic glucocorticoid receptors, which carry cargo molecules across the nuclear envelope [126], or employed cytoplasmic transcription of the gene construct, alleviating the necessity for nuclear import [127]. In the latter, gene expression occurs immediately and proportionally to the amount of DNA released from endocytotic vesicles. Zanta et al. prepared a CMVLuciferase-NLS gene containing a single, covalently-linked SV40 large T-antigene NLS [PKKKRKVEDPYC] (Fig. 6) [128]. The luciferase reporter gene was capped on both ends with an ODN hairpin structure, increasing resistance to exonucleases up to 25-fold, and complexed to Transfectam®, bPEI 25 kDa or lPEI 22kDa.

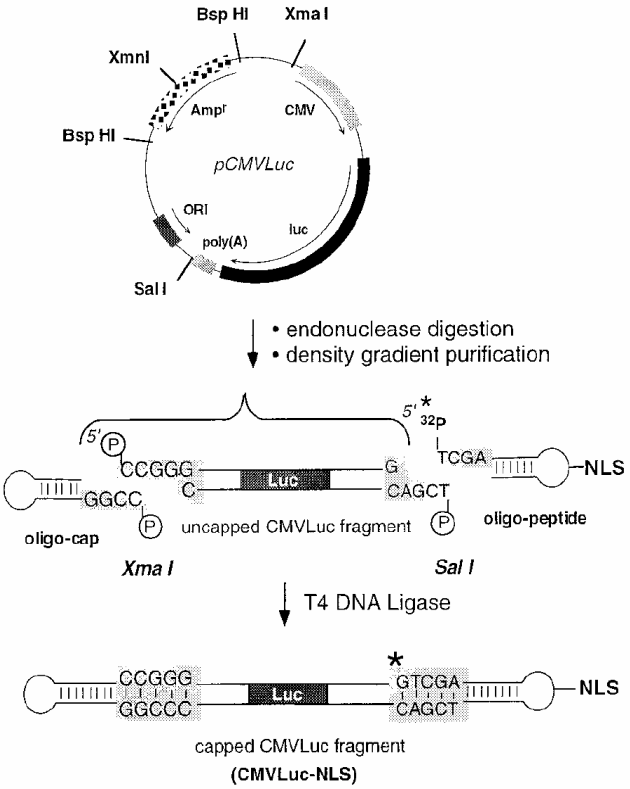


Figure 6.

Strategy for the preparation of a double-stranded DNA fragment coupled to an NLS peptide. A functional luciferase gene of 3,380 bp was cut out of pCMVLuc with the restrictive endonucleases XmaI and SalI. Further digestion with XmnI and BspHI cut the unwanted restriction fragment into small fragments (970, 875, 768, and 240 bp) that were removed by sucrose gradient centrifugation. The capped CMVLuc-NLS DNA was obtained by ligation of the ^{32}P-labeled (*) oligonucleotide-peptide and oligonucleotide-cap hairpins to the restriction fragment.

[M.A. Zanta, P. Belguise-Valladier, J.-P. Behr, Gene Delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus, PNAS USA 96 (1999) 91-96.Copyright (2004) National Acadamy of Sciences, USA]

The transfection efficiency of these vectors were enhanced 100-1000-fold even at DNA concentrations in the nanogram range, compared to polyplexes lacking the NLS peptide or prepared with a mutated NLS-peptide

[PKTKRKVEDPYC]-conjugate. Here the enhancement of transfection by nuclear targeting sequences seemed to be a general phenomenon, independent of the tissue origin or cell type.

In contrast to the concept described above, Carlisle et al. [129] attached the adenovirus major capsid protein hexon covalently to bPEI 800 kDa via a disulfide or thioether bond and formed polyplexes with comparably larger plasmid DNA. In transfection experiments performed on HepG2 cells, hexon conjugates linked with disulfide bonds yielded the highest transfection efficiency, with up to 10-20-fold amplification over the control. Compared to classical NLS bearing conjugates, the hexon protein shows the strongest stimulation of nuclear entry.

It has recently been shown that lPEI/pDNA polyplexes disintegrate during their retention time within the cytoplasm, reaching a superior transfection capacity than the corresponding bPEI/pDNA complexes, which keep pDNA in its condensed state [130]. While for bPEI-derived vectors the attachment of NLS to prefabricated polyplexes may enhance nuclear entry by improving its accessibility for importin α-binding, lPEI-based gene delivery systems may benefit from strategies similar to that of Zanta et al. [128].

So far, the improvement of transgene delivery via NLS seems to be most pronounced for short oligonucleotides, while their impact on the transfection with pDNA remains rather low. The exclusion of pDNA from preformed nuclei after mitosis [131] or the nuclear export of exogenous DNA (Fig. 5d) [132] must also be considered, as these functions may limit the efficacy of non-viral transfection systems.

6. Applications

Polycations facilitate the tight compaction of pDNA into small and positively charged complexes, which are readily internalized via non-specific adsorptive endocytosis by a large variety of cells *in vitro*. *In vivo*, however, the specific delivery of highly charged particles to the desired cell fails, due to the non-specific interaction with blood components and extracellular matrix as well as

non-targeted cells and tissues. During an incubation with human plasma, unmodified PEI/DNA polyplexes interact with IgD, IgM, proteins of the complement system C3β and C4γ, albumin, fibrinogen and apolipoproteins like apoA-I, A-II, H, C-III, and transthyretin [133;134]. The complement components adhere on the surface of complexes, triggering the activation of the complement system and removal by the reticulo-endothelial system (RES) [135]. The interaction with plasma proteins like albumin leads to the formation of ternary complexes, which tend to aggregate [47;136-139]. These large structures are rapidly cleared from the blood stream [140], most likely due to uptake by phagocytic cells or accumulation in fine capillary beds. This dramatically reduces the plasma circulation time and consequently limits the amount of therapeutic genes reaching the peripheral target cells and tissues. The aggregation with blood cells, especially erythrocytes [141;142], provokes the obstruction of blood vessels or lung capillaries [140;143], which may cause pulmonary embolism with potentially fatal consequences [47;140;143]. To suppress non-specific interactions, the cationic surface charge has been shielded by the covalent or non-covalent attachment of a hydrophilic polymer layer, using poly(ethylene glycol) (PEG), pluronic [139;144], polyacrylic acid (PAA) [145], poly (N-(2-hydroxypropyl)methacrylamide) derived copolymers [146;147], dextran [148] or dextran sulfates [149], as well as plasma proteins like transferrin [143;150] or human serum albumin [151]. It has been shown that shielded polyplexes remained soluble and small in size, and their susceptibility to salt-induced aggregation is drastically reduced. Once polyplexes extravasate into ambient tissue, they interact with the negatively charged components of the extracellular matrix, which may induce complex unraveling, exposing pDNA to degradation [152]. For PLL/DNA polyplexes, such side effects have been circumvented by crosslinking of the DNA condensing agent using intracellular reducible disulfide linkers. These nanogel-like particles, exhibited improved blood circulation times, increasing in parallel with the degree of crosslinking. Unfortunately, the efficacy of this transfection system suffered from the inefficient liberation of polyplexes into

the cytoplasm, but this strategy may promote gene delivery in the case of vectors with a high intrinsic endosomolytic capacity, such as PEI-based vectors [153].

6.1. Polyplex shielding

Unfortunately, the large majority of copolymers used produce a shielding effect that counteracts effective DNA complexation, requiring a higher molecular weight of PEI, increased N/P ratios or cross-linking of the DNA-condensing polymer. Here the most popular materials and optimized strategies for the shielding of PEI/DNA polyplexes will be described.

6.1.1. PEGylation

Linear PEG is a rather common biocompatible shielding reagent, widely used for drug delivery. The PEGylation of polyplexes has been achieved either by condensing DNA with PEG-PEI copolymers (pre-PEGylation) or coupling a PEG layer onto the surface of preformed polyplexes (post-PEGylation). Such pre- and post-PEGylated complexes can be concentrated by ultrafiltration, which is often required for the delivery of therapeutic amounts of DNA via systemic application [47;133;134;154] and can be stored at low temperatures until administration without changing the biophysical properties.

6.1.1.1. Pre-PEGylation:

Several strategies have been used to prepare PEG-PEI copolymers, most of them using homobifunctional (Table 1: A1 and A2) or heterobifunctional PEG (Table 1: A3, A4 and C1) for conjugation onto branched or linear PEI [136-139;154;155]. Preserving one active group on the PEG terminus allows for the tagging a targeting molecule to the free PEG end (Table 1: A.5, A.6, A8 and C2) [133;134;156;157]. To enable the disintegration of the corresponding polyplexes and promote the DNA release at the cellular level, the shielding agent has been conjugated to the polycation by biodegradable disulfide bonds, which can be cleaved by intracellular reduction (Table 1: A7 and A8) [133].

A major disadvantage of using copolymers for the preparation of shielded polyplexes is the reduced capacity of efficient DNA complexation [133;134;158]. This effect has been moderated by using higher molecular weight bPEI (800 kDa) [133;134] and longer PEG chains [141;142] for conjugation, by adding free bPEI or lPEI to the complexation medium [150] or using smaller ligands like EGF, instead of e.g. transferrin [133;134;158]. Higher PEG-grafting densities can prevent hemolysis or aggregation with erythrocytes [141;142], changing the biodistribution and gene expression pattern of these systems and likely aiding in an enhanced transfection efficiency *in vivo* [155].

A modified and rather elegant pre-PEGylation method led to the formation of shielded nanometer-sized polyplexes by mixing pDNA with prefabricated, purified and storable blends of ligand-PEG-PEI conjugates, PEG-PEI copolymers and PEI, forming a targeting unit, shielding agent, and DNA condensing agent, respectively. These transfection systems were based on either branched or linear PEI, shielded by linear or branched PEG derivatives of varying molecular weights. This method provides control over the particle properties, such as size and the extent of PEG shielding and ligand decoration, by the proportions of the single components within the mixture used for polyplex preparation (Fig. 7)[133;134].

6.1.1.2. Post-PEGylation:

For post-PEGylation, the PEI/DNA complexes are prepared using unmodified PEI, ensuring that neither PEG nor the ligand competes with DNA compaction. The PEI/DNA polyplexes are usually formed in a solution with low ionic strength to generate small particles and the PEG chains are only allowed to react with the particle surface to create the protective PEG-shield.

The simplest strategy uses ligand decorated PEI/DNA complexes and creates the protective layer by conjugating mono-activated PEG to the surface of the preformed polyplex (Table 1: B1) [133;140]. Here the receptor ligand seems to be hidden beneath the PEG coating, impeding the receptor binding and hence impairing cell internalization (Fig. 7).

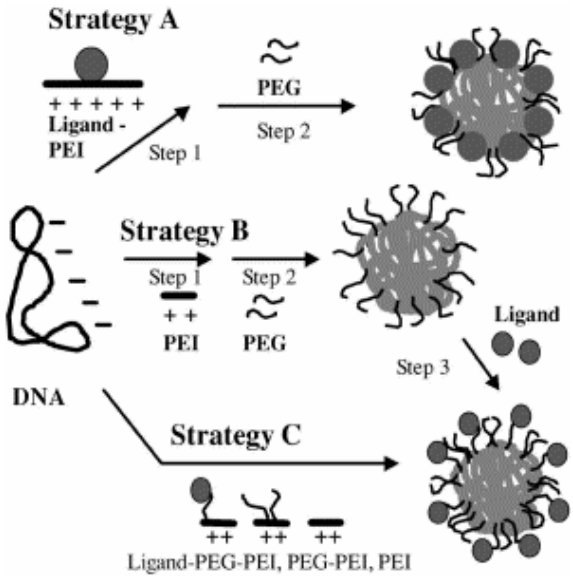


Figure 7.

Strategies for the PEGylation of PEI/DNA polyplexes:

A ligand-PEI conjugate is used to complex pDNA and the corresponding polyplex is pegylated in a second step.

PEI is used to condense pDNA. In a second step, PEG is conjugated to the polyplex surface. Finally, the ligand is attached to the distal ends of the PEG chains.

A mixture of ligand-decorated PEG-PEI copolymer, PEG-PEI copolymer and the homopolymer PEI is used to complex DNA, leading to the formation of PEG-shielded, ligand-decorated PEI/DNA polyplexes in a one step procedure.

[reprinted from J. Controlled Release, 91(2003), M. Ogris, G. Walker, T. Blessing, R. Kircheis, M. Wolschek, E. Wagner, Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes, pp.173-181, Copyright (2004) with permission from Elsevier]

In a more effective strategy, hetero-bifunctional PEG is attached to the surface amino groups of preformed unmodified PEI/DNA complexes. In the final synthesis step, a targeting moiety is conjugated to the distal ends of the

PEG chains, ensuring maximum accessibility for receptor binding (Table 1: C1 and C2) [60;133;134]. In contrast to pre-PEGylation methods, post-PEGylation suffers from a rather time consuming sequence of conjugation steps that must be performed on DNA polyplexes. Therefore the arsenal of plausible conjugation reactions and purification methods is limited and must be feasible at very low concentrations. Furthermore, the extent of PEG grafting or ligand tagging is challenging to control and characterize.

6.1.2. Pluronic

Pluronic P 123-bPEI 2 kDa copolymers have been shown to form self-assembled micelle-like aggregates with an effective diameter of 60-70 nm, used to incorporate pDNA. The delivery of transgenes depended on the addition of free pluronic, which prevented polyplex precipitation in aqueous media. These transfection systems lead to a modulated biodistribution pattern and higher expression levels, compared to similar PEG-PEI/DNA complexes, an effect which is even more pronounced for low molecular weight bPEI (Table 1: D1)[139;144].

6.1.3. Polyacrylic acid

The addition of polyacrylic acid to preformed PEI/DNA polyplexes leads to the flocculation of neutrally charged ternary complexes of several hundred nanometers and drastically improved cell viability *in vivo* [145]. Comparative experiments using other polycarboxylic acids revealed that a sufficient anionic charge density is required to achieve effective charge shielding.

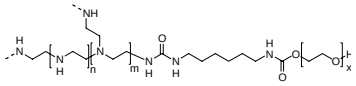
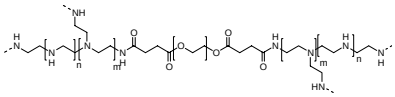
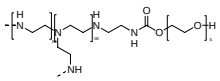
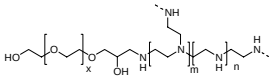
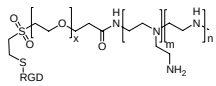
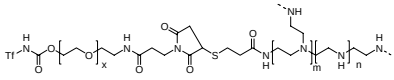
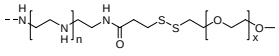
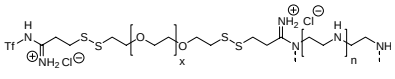
6.1.4. Poly (N-(2-hydroxypropyl)methacrylamide) [pHPMA]-derivatives

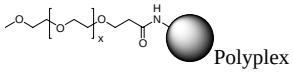
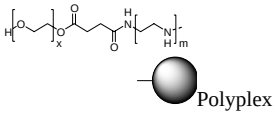
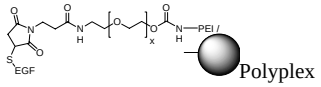
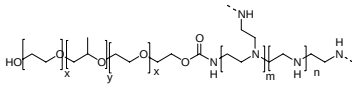
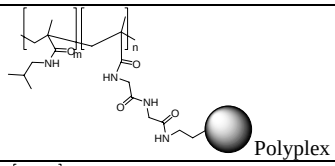
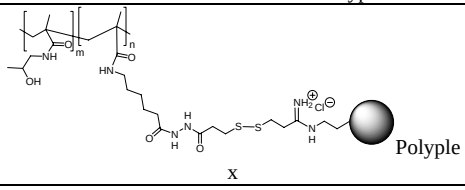
Oupicky et al. introduced pHPMA-derived copolymers for the coating of preformed bPEI25/DNA and bPEI800/DNA polyplexes. Advantageously in comparison to the rather common conjugation of monovalent PEG or pHPMA, the covering with multivalent pHPMA-derivatives protected the corresponding complexes from electrolyte exchange reactions and exhibited an improved plasma circulation performance with a significant localization in subcutaneously inoculated B16F10 tumors in C57BL/6 mice (Table 1: E1)

[146]. The use of higher molecular weight pHPMA-derivatives and a sufficient coating concentration have been shown to be the major prerequisite for an effective shielding.

Table 1.

Materials for the manufacture of PEI/DNA polyplexes by application of PEI-derived copolymers or conjugation of a hydrophilic polymer layer onto the surface of preformed complexes:

Pre-PEGylation		
Conjugation by homobifunctional linker A.1		[141,142]
A.2		[136]
Conjugation by heterobifunctional PEG A.3		[137,157]
A.4		[139]
containing a targeting moiety A.5		[156]
A.6		[134]
Copolymers cleavable by intracellular reduction A.7		
containing a targeting moiety A.8		

Post-PEGylation		
Conjugation of monofunctional PEG B.1		[134,140]
Pre/Post-PEGylation		
Conjugation by heterobifunctional PEG C.1		[154,157]
containing a targeting moiety C.2		[133,138, 155]
Pluronic P123-PEI-copolymer D.1		[139]
Coating of bPEI/pDNA polyplexes with pHPMA-derived copolymers E.1		[146]
E.2		[147]

Unfortunately, these vectors showed a comparably low transfection activity, most likely due to the lack of intracellular dissociation and DNA release, which in turn inhibits efficient DNA transcription. Using biodegradable disulfide bonds to connect the shielding polymer to the PEI25/pDNA polyplex surface allowed for the destabilization of the corresponding complexes after their reduction within the cytoplasm and nucleus. A coating extend of 20% was determined to be optimal, while higher substitution degrees reduced the transfection activity *in vitro*, most likely due to the depletion of the intracellular reduction capacity (Table 1: E2) [147].

6.1.5. Transferrin (Tf)

A very smart approach towards efficient polyplex shielding uses the plasma protein transferrin, which ideally combines both an intrinsic stealth effect and targeting towards transferrin-receptor expressing cells [143;150]. Applying blends of PEI and Tf-PEI (branched 25 kDa, 800 kDa or linear 22 kDa) yielded neutrally charged polyplexes at any N/P ratio and exhibited effective charge shielding by the incorporation of sufficient Tf ligand even in the absence of PEGylation. This gene delivery system not only exhibited decreased erythrocyte aggregation and therefore reduced toxicity even at high N/P ratios in vivo, but also shifted the biodistribution pattern from liver and lung, as usually observed for the unshielded complexes, to transgene expression predominantly in the targeted tumor.

6.2. Local application

The local application of non-viral vectors limits gene delivery to accessible target sites and depends on small, soluble and neutrally charged [59] polyplexes, that are capable of diffusing through tissue to reach their final destination. Even though the harsh environment of systemic blood circulation can be bypassed, complex unraveling due to the exposure to the extracellular matrix [152] and the risk of inflammatory and immune reactions [135] still have to be considered.

6.2.1. Lung

The mucus layers secreted by goblet cells, the almost impervious epithelium with tight junctions inhibiting the intercellular transport and the clearance by alveolar macrophages remain the major barriers for efficient PEI-based gene delivery targeted to the lung. In contrast to other non-viral transfection systems, the exposure to surfactant proteins [159] increased transgene expression by overcoming insufficient spreading of the polyplex solution on lung epithelia, while, the polyplex stability seemed to be unaffected [160]. The coadministration of penetration enhancers improved gene delivery across the dense epithelium, but unfortunately decreased the cell viability [161] as well.

Intranasal application of lPEI22/DNA polyplexes has been shown to be superior to bPEI25/DNA vectors, yielding higher transgene expression. [53]. PEG-shielding of PEI/DNA polyplexes facilitated a homogenous gene expression pattern [162], but concomitantly caused drastically reduced transfection efficiencies [154], which seemed to be recovered only by the conjugation of a targeting moiety [162].

The more convenient aerosol delivery of unmodified bPEI25-DNA polyplexes subsequently transfected most of the epithelial cells in the conducting and peripheral airways. Gene expression was detectable up to 28 days after a single aerosol dose and increased with prolonged aerosol exposure [163].

6.2.2. Tumor

The most direct approach to achieve the expression of therapeutic genes in tumor tissue may be the injection of the polyplex solution into the tumor foci [9;143] or peritoneum to reach intraperitoneal disseminating tumors [164]. Labeling of bPEI/DNA complexes with receptor-ligand transferrin could thereby enhance the gene expression in tumor cells, due to the efficient internalization of the transfecting complexes into the tumor cells via receptor-mediated endocytosis [165].

For inaccessible tumors, polyplexes could be administered into the arterial blood vessels supplying the tumor. Polyplexes reach interstitial tumor tissue through irregular endothelial fenestrations. The enhanced permeation, the lack of normal lymphatic drainage and the hypervasculature of the tissue all lead to the accumulation of the complexes in the tumor interstitium (enhanced permeation and retention [EPR] effect).

6.2.3. Brain

The blood-brain barrier tends to preclude vascular delivery, as the non-fenestrated capillary endothelial cells surrounded by tight junctions control and limit the uptake of exogenous material. The transcellular movement by fluid phase endocytosis, which otherwise aids in the extravasation and diffusion of transgenes through the tissue, does not occur here. Gene

delivery to the brain has been accomplished, however, by injection of lPEI22/DNA polyplexes into the cerebrospinal fluid or the lateral ventricle [166;167]. Polyplexes remained highly diffusible, spreading widely from the site of injection, distributing gene expression within both sides of the brain [59]. The intrathecal administration of a single dose of bPEI25/DNA into the lumbar subarachnoid space provided enhanced transgene expression in the spinal cord that was enhanced up to 40-fold, compared to naked DNA [155]. Unfortunately after the often required repeated application a up to 70% attenuation of gene expression was observed, which was associated with apoptotic cell death. By using shielded polyplexes prepared with a 1:1 PEG2-PEI25 conjugate, cell viability was improved, yielding 11-fold higher expression in the spinal cord, compared to the intrathecally injected homopolymer [138]. Another approach employed the capability of neurons to ingest exogenous material from the muscles they innervate. Hence, PEI/DNA complexes have been injected into the tongue, achieving retrograde axonal transport to hypoglossal motoneurons of the brain stem [168].

6.3. Systemic administration and receptor targeting

In order to reach distant organs or tumors, especially metastatic noodles, the systemic application of the therapeutic agent through the blood circulation should provide several advantages over the local administration. The directing of therapeutic systems to the targeted cell or tissue can be accomplished by simply taking advantage of special physiological conditions, e.g., the irregular fenestration in the liver, spleen, bone marrow or certain tumors, which facilitates passive accumulation. After intravenous [i.v.] injection, both unmodified lPEI and bPEI-derived vectors deliver the transgene into the heart, spleen, liver and kidney, with highest gene expression levels predominantly in the vascular endothelial cells of the lung, most likely due to the accumulation within the fine lung capillary beds [169;170]. But employing this organ tropism to direct gene delivery toward the lung via systemic administration seems to be only of little benefit, due to the reduced

half-life, caused by the clearance of polyplexes from the systemic circulation after i.v. injection [171]. At increasing N/P ratios, gene expression levels could be enhanced in all organs, but the biodistribution pattern largely depended on the method of polyplex administration and material characteristics. While bPEI25/DNA polyplexes promote the transfection of bronchial cells, lPEI22/DNA vectors yield the highest gene expression levels in the distal tract of the bronchial tree [172]. In contrast to lPEI/DNA complexes, with which transfection proceeded without any sign of severe toxicity, lung embolism and death of laboratory animals were observed after i.v. injection of bPEI25 and bPEI800/DNA polyplexes [51].

Receptor-targeting via the incorporation of receptor ligands like carbohydrates, transferrin, folate, growth factors, ligands for the low density lipoprotein receptor [173;174] or antibodies into the polyplex seems to be most favorable means to achieve site-directed gene delivery towards a preferred or specific cell type. An effective charge shielding is necessary to suppress competing internalization via adsorptive endocytosis, which can be more or less pronounced, due to the cell-specific presence of proteoglycans on the cell surface.

6.3.1. Glycosylated vehicles

Directing the therapeutic genes to hepatocytes or parenchymal liver cells may become a promising tool to correct genetic defects, contributing to the treatment of diseases like α1-antitrypsin deficiency, hemophilia and lipoprotein receptor deficiency, as well as cirrhosis or cancer. The asialoglycoprotein receptor (ASGPr), abundantly expressed in hepatocytes, internalizes galactose-terminated glycoproteins and N-acetylglucosamine residues via clathrin-coated pits [175]. To enhance the uptake into liver cells, mono- and oligosaccharides with an affinity for the ASGPr-receptor, like galactose [176-180], lactose [181;182] and larger carbohydrates like asialooromucoid [183;184], have been used for liver targeting, as they have a low immunogenicity compared to antibodies or peptides. The rate of cellular

uptake varies between parenchymal and non-parenchymal cells and can be directed by the type of sugar conjugated to the gene delivery vehicle.

Galactose linked to 5% of the bPEI amino groups via a four-carbon hydrophilic spacer has been shown to direct gene transfer into hepatocytes highly selectively via ASGPr-receptor-mediated endocytosis [179;180]. Higher substitution degrees failed to achieve comparable transfection efficiencies, most likely due to decreased DNA compaction and hence lower polyplex stability [180]. This effect can be moderated by applying a higher molecular weight bPEI or increasing N/P ratios [176], but may drastically reduce the selectivity and efficacy of the gene transfer, which relies on neutrally charged complexes. The conjugation of linear tetragalactose at a grafting degree of 5% resulted in effective charge shielding, even at higher N/P ratios and the efficacy of gene delivery was further enhanced, most likely due to the improved particle stability and receptor accessibility [178]. When using PEG for charge shielding and ligand presentation, a grafting degree of 1% to bPEI25 has been shown to be optimal [177]. The transgene expression levels increased compared to unmodified PEI/DNA polyplexes, irrespective of the N/P ratio.

Mannose has been used to address gene delivery to dendritic cells [185], which express high levels of mannose receptors, mediating the internalization of mannosylated antigens [186]. Dendritic cells are capable of capturing antigens and provoking antigen-specific T cell responses by presenting the processed antigens under participation of the major histocompatibility complex (MHC) class I and II molecules [187]. Since several tumor cell-specific antigens have been identified and cloned [188], the tools are available to program dendritic cells to induce strong anti-tumor immune responses. Hence, dendritic cells represent an attractive cell type for immunotherapy, for example for the treatment of cancer [189;190]. 1:1 Mannose-bPEI25 conjugates were prepared and used for DNA complexation. The gene delivery was augmented in a receptor-mediated manner, compared to unmodified bPEI/DNA polyplexes, which exhibited 50% less transgene

expression. The incorporation of inactivated adenovirus particles further improved transfection efficiency up to 100-fold [185].

6.3.2. Transferrin receptor targeting

The transferrin receptor facilitates the uptake of transferrin [Tf]-iron complexes into erythrocytes as well as actively proliferating cells and rapidly dividing tissues, such as tumors. Transferrin, a serum glycoprotein, is conjugated to polyplexes by reductive amination via its carbohydrate residue, prompting its use as a targeting moiety to direct gene transfer, especially to tumor cells. It has been shown that the incorporation of transferrin into PEI/DNA polyplexes enhances the cell internalization via receptor-mediated endocytosis [81;191] and directs gene delivery efficiently to tumor tissue *in vivo*, even at low N/P ratios [133;134;140;143;150;192]. At higher grafting densities, transferrin itself exhibits effective charge shielding and therefore provides both polyplex protection and targeting [150]. The most efficient gene delivery into tumors was achieved with vectors prepared by complexing pDNA with a blend of transferrin tagged PEG-lPEI copolymer, PEG-lPEI copolymer and lPEI. The transgene expression was enhanced over equivalent systems derived from bPEI, transferrin-shielded particles and Tf-decorated PEG-bPEI/DNA complexes [133;134].

The gene expression levels varied between different tumors, due to deviant tumor vascularization, the occurrence of necrotic tissue and the infiltration by macrophages [192]. A significant fraction of polyplexes were taken up by macrophages, leading to a significant amount of DNA-degradation within the Kupffer cells of the liver [150].

The level of the firefly luciferase gene expression and the biodistribution pattern of transferrin-tagged polyplexes after systemic administration have been determined in living mice, using a cooled charge coupled device camera (Fig. 8) [193]. To enhance the intensity of the bioluminescence signal, D-luciferin has been i.p. injected. The highest levels of transgene expression were observed in tumor tissue and the site of injection. In contrast to ex vivo

evaluations, the transfection could be monitored over longer time periods in a single animal.

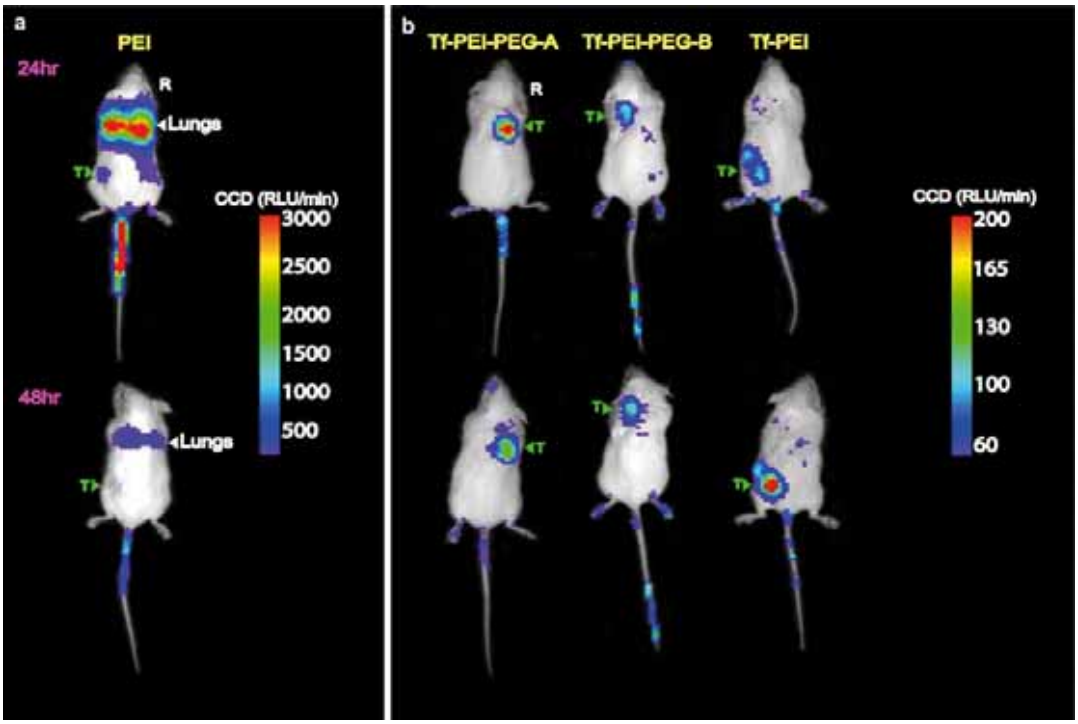


Figure 8.

Bioluminescent imaging of the optical reporter gene expression (fl: firefly luciferase gene) was determined and quantified in living, N2A tumor bearing mice using a cooled charge coupled device (CCD) camera.

LPEI22/DNA polyplexes were systemically injected into the mouse, resulting in much higher fl expression in the lung, compared with the tumor.

DNA was complexed using mixtures of transferrin labeled PEG-BPEI25 : LPEI22-PEG20 (Tf-PEI-PEGA), transferrin-PEG-BPEI25 : LPEI22-PEG40 (Tf-PEI-PEGB) or transferrin-BPEI25. The highest reporter gene expression was observed in the tumor and tail vain, without detectable signals in the lung [I.J. Hildebrandt, M. Iyer, E. Wagner, S.S. Gambhir, Optical imaging of transferrin targeted PEI/DNA complexes in living subjects, Gene Ther. 10 (2003) 758-764, Copyright (2004) Nature Publishing Group http://www.nature.com/gt/index.html].

6.3.3. Growth factors

The epithelial growth factor [EGF] receptor is highly overexpressed in a large variety of cancer cells [194] and might therefore serve as useful target for the delivery of therapeutic genes into tumor. EGF, a 53-residue polypeptide, binds to its receptor, triggering the clustering into coated pits and cell internalization. It has been shown that the tagging of EGF to bPEI25/DNA polyplexes can enhance the cellular uptake into tumor cell lines, due to receptor-mediated internalization, yielding similar transfection efficiency compared to unmodified bPEI-DNA complexes even at lower DNA doses. [60;191]. EGF was incorporated into post-PEGylated polyplexes, either directly conjugated to bPEI25 or, in the final synthesis step, to the distal ends of the PEG spacer arms. Both strategies lead to a 10-100-fold higher transfection efficiency, compared to unmodified complexes, suggesting that PEG interferes only slightly with the EGF receptor binding. To avoid the exposure of polyplexes to further chemical modifications or cleaning procedures, blends of lPEI22, bPEI25-PEG and EGF-PEG-bPEI25 have been used for DNA compaction [133] (pre-PEGylation). The transfection efficiency of this system was comparable to that of those prepared by post-PEGylation, but allowed for a defined particle composition. The pre-PEGylated EGF-labeled PEI/DNA polyplexes have been systemically injected into mice bearing a variety of subcutaneously growing human tumor xenografts. Even though the efficacy of transgene delivery differs between the individual tumors, gene delivery was successfully directed into the tumor tissue [192].

6.3.4. Membrane folate-binding protein

Quickly dividing cells like cancer cells highly overexpress the membrane folate binding protein, upregulating their folate internalization to enable an increased DNA synthesis. Folate has been tagged to several drug or gene delivery systems, all in an attempt to target cancer tissue [195]. A rather simple approach added folic acid to the medium during transfection, which inhibited

nonspecific interactions with serum components, but failed to mediate cell internalization via the folate receptor [196].

PEGylated polyplexes were tagged with folate to enhance gene delivery to malignant tissues via receptor-mediated endocytosis. Folate-PEG-bPEI [197] or bis-folate-PEG-bPEI [198] conjugates were prepared and used for DNA compaction. Derivatives of moderate substitution degrees have been shown to retain their DNA condensing capacity and endosomolytic activity. When neutrally charged pegylated complexes were used, the enhancement of *in vitro* transfection efficiency in different cell lines, as compared to either unmodified [197;198] or folate-bPEI-polyplexes [197], was attributed to the receptor-mediated endocytosis. At higher N/P ratios, adsorptive endocytosis competed with receptor-mediated internalization, reaching the highest gene expression levels using unmodified bPEI-DNA polyplexes.

6.3.5. Integrins

The integrin family includes the heterodimeric transmembrane receptors involved in cell-cell and cell-extracellular matrix interactions, which are amplified in tumor endothelia [199;200]. Several integrins recognize multiple Arg-Gly-Asp (RGD) peptide sequences presented by cell adhesion, serum or extracellular matrix proteins [201], mediate the internalization of matrix compounds [202] or are exploited for the cell entry of viruses like the adenovirus [203]. RGD peptides, like RGDC [156] and CYGGRGDTP [204], have been conjugated to bPEI25 and shown to enhance gene delivery significantly, compared to unmodified bPEI/DNA polyplexes, via ligand-receptor interaction. The selectivity and gene transfer efficiency largely depends on the cell line and appears to be a function of the RGD peptide grafting density, increasing with the number of RGD residues presented in the complex. To combine a charge shielding effect with cell targeting, RGD peptides, such as RGDC [156] or ACRGDMFGCA [157] were attached to the distal end of the PEG chain, grafted to bPEI25 and polyplexes were formed by condensing DNA with the conjugates. While complexes decorated with the ACRGDMFGCA peptide exhibited enhanced transfection efficiency above

that of unmodified bPEI/DNA polyplexes, RGDC-tagged vectors delivered DNA less efficiently than particles lacking the targeting moiety. Whether or not PEG interferes with receptor binding or may be of benefit for integrin-receptor targeting, is still controversially discussed [156].

6.3.6. Antibodies and antibody fragments

Unlike other targeting molecules, which interact with receptors expressed on the surface of a variety of cells, antibodies feature a highly selective binding to their target structure, which is exclusively presented by specific cells. The anti-CD3 antibody, a ligand for the T cell receptor-associated CD3 molecule, was conjugated to bPEI800 prior to DNA complexation. The antibody tagged polyplexes were capable of transfecting a high percentage of human T cell leukemia cells [81] or human primary lymphocytes [205] in a specific manner. In the latter, gene delivery could further be augmented by stimulation with the transcriptional activator phorbol-12-myristate 13-acetate up to 60 %.

Unfortunately, potential immunogenicity prevents the often required repeated administration and the complex structure of the antibody enlarges the polyplex size and weakens complex stability. Here the use of antibody fragments seems favorable, due to their comparably small size and reduced immunogenic potential.

The OV-TL16 antibody binds specifically to the OA3 antigen presented on OVCAR-3 human ovarian carcinoma cells. To direct gene delivery into OVCAR-3 positive cells, the OV-TL16 antibody fragment was linked to bPEI residues of a random PEG-bPEI25 copolymer. The reporter gene expression levels rose up to 80-fold above that have been achieved by unmodified bPEG-PEI/DNA and bPEI/DNA polyplexes, at least partially due to ligand-receptor binding [206].

7. Summary and outlook

In recent years, understanding of the mechanisms behind non-viral gene delivery has expanded and with it the spectrum of methods and materials available. PEI is still considered to be a gold standard compared to other

polymers, as it facilitates effective DNA binding and protection, combined with a high endosomolytic competence, which all contribute to the superior transfection efficacy of the corresponding non-viral vectors. The presence of amino groups facilitates the conjugation of targeting moieties and the attachment of charge shielding agents either to PEI itself or to the surface of prefabricated polyplexes. In comparing transfection experiments *in vitro* and *in vivo,* lPEI/pDNA polyplexes seem to have an advantage over bPEI-derived vectors, as they facilitate the translocation of exogenous DNA to the nucleus nearly independent from the cell cycle and exhibit a drastically increased cell viability and transfection efficacy.

The improvement of gene delivery by the incorporation of endosomolytic agents or NLS, however, was less than expected for pDNA/PEI polyplexes, inspiring the search for more potent molecules, and a polyplex design, orientated on the different physical properties of linear and branched PEI/DNA complexes. One very straightforward approach appears to be the use of protein transduction domains, as they mediate both, polyplex internalization independent of the endocytotic pathway and translocation of their cargo into the cell nucleus.

It has already been shown that advanced PEI/DNA polyplexes are able to deliver genes into, for example the lung or tumor tissue successfully by local or systemic application. The first attempts at developing programmable vectors appear to have advantages over other strategies. Using the multivalent but reversible attachment of the shielding component, prevents non-specific interactions at the systemic level efficiently and than, after its intracellular removal, promotes endosomolysis as well as DNA release and transcription. Further improvements may conceivable lead to convenient novel non-viral vectors, combining high efficacy, innocuousness and high stability to be widely applicable for therapeutic gene therapy.

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2. GOAL OF THE THESIS

Non-viral gene delivery concepts have gained growing interest due to its particular promise as alternative therapeutic concept for the treatment of inheritable or acquired disorders, and as tools in life sciences.

Cationic polymers, which feature the ability to form tight complexes with the polyanionic nucleic acid by spontaneous self-assembly, are most commonly used as materials for the manufacture of non-viral vectors [1;2]. Polymeric gene carriers do not share the safety risks of viral vectors; they are durable as well as inexpensive, available in large scale, and with tailored properties by chemical syntheses. From the variety of polymers applied to date, polyethylenimines (PEI) are still considered as the gold standard, due to the relatively high transfection efficacy. However, the major drawback of PEI-based non-viral gene transfer concepts remains their limited transfection efficacy and their inherent cytotoxicity.

In **chapter 1** we give a detailed introduction into the molecular mechanisms involved in PEI-mediated gene transfer, and the strategies applied for their further optimization.

In the focus of our investigations was the synthesis, modification and characterization of polyethylenimines and their DNA-complexes as novel materials for non-viral transfection agents. Unfortunately, there is only little understanding of the interplay of polymer structure and transfection performance of the corresponding polyplexes (Figure 1).

Thus, we varied the cationic “charge density”, molecular weight and branching degree of polyethylenimine and assayed the physico- and

biochemical properties, as well as the cytotoxicity, and *in vitro* transfection efficiency of the polyplexes, built from our polymers.

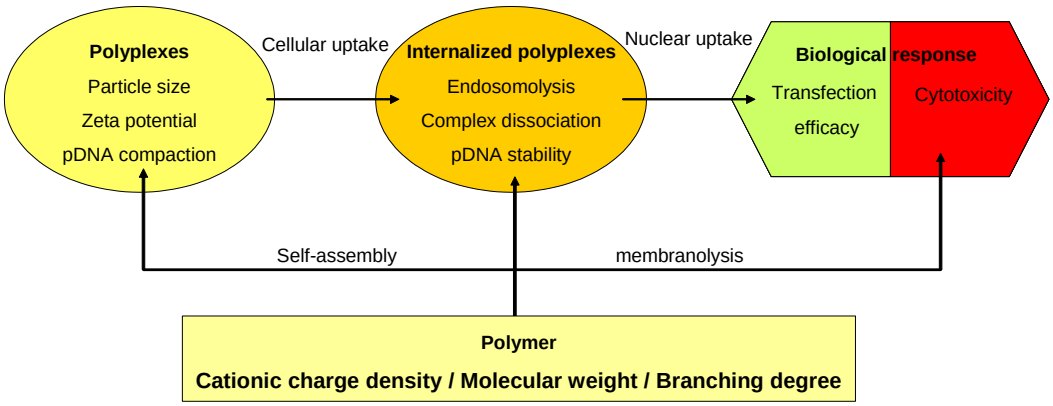

Figure 1. *The polymer characteristics like the molecular weight, the cationic charge density and the branching degree modulate the efficacy of gene transfer: directly, due to the influence on cytotoxicity of the vector, the self-assembly of polyplexes, and the particle properties itself (zeta potential, its endosomolytic activity, pDNA compaction and stability, as well as the extent of cytosolic complex dissociation and DNA release), and indirectly due to the impact of the particle properties on the rate of the particle internalization and nuclear localization of the transferred genetic material.*

It is assumed that polymers with lower protonation levels (i.e. lower “charge density” due to lower basicity) should exhibit favorable cell compatibility [3]. In **chapter 3** we describe our approach, to reduce the basicity of linear (lPEI) and branched PEI (bPEI) by complete N-methylation using formaldehyde/formic acid (Leukart-Wallach). This gave us the opportunity to investigate the impact of a modified polymer cationic charge density on the polyplex properties, in combination with the influence of the polyamine branching degree.

In recent approaches, lPEI emerged as potent alternative gene carrier to bPEI, due to improved transfection efficacy and cell compatibility [4;5]. These studies were confined to lPEI of 22 and 25 kDa, giving only limited information about the prerequisites decisive for its superior performance. We postulated that, similarly to bPEI-derived vectors, a molecular weight optimum exists, which facilitates the formation of gene carriers with a maximum transgene expression at negligible cytotoxicity. For a systematic identification of this optimum lPEI polymerization degree, we synthesized a series of twelve low molecular weight lPEIs (**Chapter 4**) differing by their molecular weights, and investigated the *in vitro* performance of the corresponding vectors (**Chapter 5**).

In the next step, we investigated whether a biodegradable PEI backbone can unite the improved extracellular stability and endosomolytic activity of high molecular weight lPEI – pDNA complexes with the enhanced cytosolic complex dissociation and reduced cytotoxicity of low molecular weight lPEI – plasmid DNA (pDNA) polyplexes. The gene carrier was synthesized by the cross-linking of non-toxic low molecular weight lPEI with disulfides that are reduced in the intracellular environment. The particle properties, the degradability of the disulfide linkages, as well as the transfection efficacy and cytotoxicity of the corresponding vectors were investigated *in vitro* (**Chapter 6**).

One of the drawbacks of polycation – pDNA complexes are their charged surfaces, which account for the tendency to form larger aggregates, as well as unspecific interactions with proteins or cells, when exposed to a biological environment. To overcome these obstacles, poly (ethylene glycol) – polyethylenimine (PEG – PEI) copolymers have been utilized, covering the polyplexes with non-charged PEG surface layers [6]. Adjusting this concept to our needs, we synthesized a series of methoxy poly (ethylene glycol) methyl (mPEG) - low molecular weight lPEI-based copolymers, to identify the

optimum composition of pDNA condensing and charge-shielding functionality, and tested the corresponding vectors *in vitro* (**Chapter 7**).

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3. SYNTHESIS OF PER-N-METHYLATED POLYETHYLENIMINE AND ITS APPLICATION FOR NON-VIRAL GENE DELIVERY

Uta Lungwitz, Miriam Breunig, Renate Liebl, Torsten Blunk, Achim Göpferich

Department of Pharmaceutical Technology, University of Regensburg, 93040 Regensburg, Germany

Abstract

We synthesized per-N-methylated linear (lPMEI) and branched polyethylenimine (bPMEI) by the complete N-methylation of the corresponding PEI 25 kDa precursors, using the Eschweiler-Clark variation of the Leuckart reaction. The tertiary polycations condensed plasmid DNA (pDNA) forming polyplexes with a zeta potential between 12 and 26 mV. While bPMEI – pDNA complexes were between 400 and 600 nm in size, lPMEI – pDNA particles formed large aggregates in the micrometer scale. The polyplexes exhibited comparable endosomolytic activity and were internalized by CHO-K1 cells to a similar degree, irrespective to the chemical modification. However, the cell viability of CHO-K1 cells *in vitro* after treatment with lPMEI- or bPMEI- derived particles was improved to one order of magnitude, while the efficacy of gene transfer was reduced to a negligible level of 0.1 % or 1 %, respectively.

Introduction

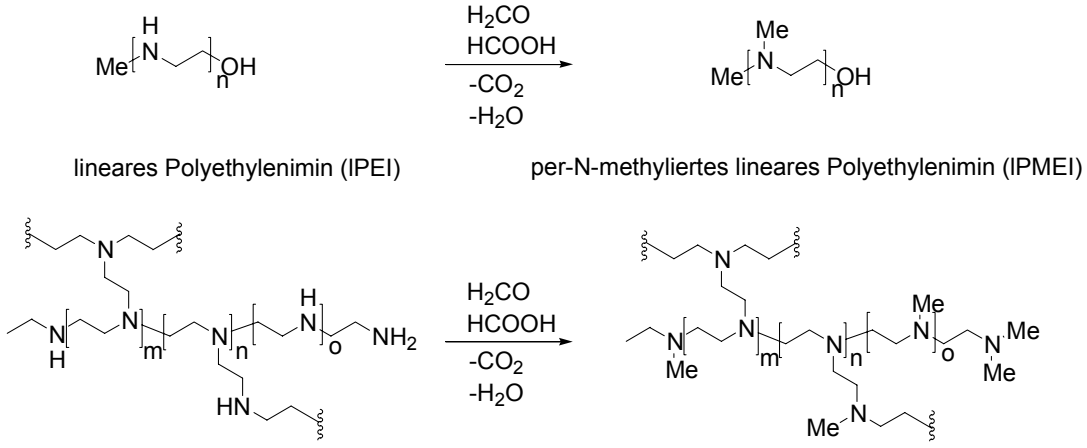
Despite the tremendous potential of viral vectors for gene delivery, they are associated with a significant risk of immunogenic and other adverse reactions [1-5]. To overcome these barriers, considerable research effort has been devoted in recent years to the development of non-viral vectors as a promising alternative. Branched polyethylenimine (bPEI) has been successfully applied for the manufacture of polymer-based non-viral vectors, as it combines efficient pDNA compaction with a high endosomolytic activity and transfer of its cargo to a large variety of cell types [6-8]. Both the strong ionic interactions with nucleic acids and the efficient endosomal escape of bPEI-derived polyplexes has been assigned to the presence of approximately 25 % primary, 50 % secondary and 25 % tertiary amines in the polymer backbone, all contributing to its high protonation level (ratio of protonated amines to unprotonated amines) at physiological pH and overall buffer capacity [7-11]. Nevertheless, the efficacy of bPEI-based vectors is still remarkably lower compared to viral ones, and accompanied by a substantial cytotoxicity resulting from membrane lysis and host DNA complexation, which limits the use of bPEI in non-viral vectors [12-14].

In order to elucidate the crucial features of gene carriers, a variety of partially or completely N-alkylated poly (2-aminoethyl methacrylate) [15], linear polyethylenimine (lPEI) [16] or bPEI [17] - derived polyplexes have been evaluated in terms of *in vitro* transfection and cell compatibility. Although the results indicate a correlation of the alkyl residue and the amine degree of substitution with the efficacy of gene transfer, the underlying principles remain elusive. The introduction of a permanent cationic charge by the quarternization of amines, intended to improve the capacity of pDNA complexation, had a detrimental effect on the cell viability.

Here we investigated the effect of a reduced overall basicity (according to the pK_a value), and in turn of a lowered protonation level on the efficacy and cytotoxicity of PEI-based vectors of different architecture. Therefore we

synthesized, exclusively tertiary polyamines by the N-methylation of lPEI or bPEI of 25 kDa, and examined the impact of the chemical modification on the polyplex properties in direct comparison with the unmodified PEI – pDNA complexes.

To avoid the formation of quarternized amines, we explored alternative routes to the classical methylating agents such as methyl iodide [17]. The Leuckart-Wallach reaction, which allows for the reductive alkylation of amines using aldehydes or ketones as an alkyl source and formic acid as reducing agent deemed thereby especially suited (Scheme 1).



Scheme 1. *Synthesis of linear and branched per-N-methylated polyethylenimine by the Eschweiler-Clark variant of the Leuckart reaction. To enable a conversion under aqueous conditions formaldehyde served as alkylating and formic acid as reducing agent.*

We decided to use highly reactive formaldehyde as the alkyl component, (Eschweiler-Clarke procedure) to further favor the complete conversion of primary and secondary amines into the corresponding tertiary amines and concomitantly minimize the creation of mono- or dialkylated amino derivatives as byproducts [18;19]. Here we describe the synthesis and characterization of lPMEI and bPMEI and report on the capacity of their

corresponding polyplexes with regard to pDNA compaction, transfection efficiency and cytotoxicity. The impact on the cell – polyplex interaction and the influence on the endosomolytic activity were investigated exemplarily for bPMEI – pDNA polyplexes in comparison to bPEI25-derived particles.

Results

We synthesized linear and branched per-N-methylated PEI by complete reductive N-methylation of commercially available branched and linear PEI of 25 kDa using the Eschweiler-Clarke variation of the Leuckard reaction (Scheme 1) [18;19]. This synthetic route allowed for the conversion under aqueous conditions into exclusively tertiary amines, while quarternization can not occur for mechanistically reasons. Furthermore, both reagents could be easily removed from the reaction mixture, which simplified the workup significantly.

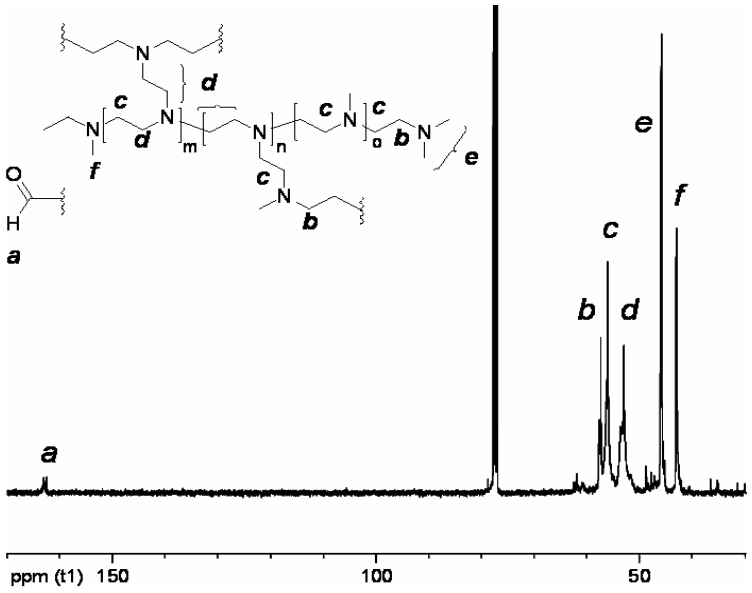


***Figure 1**. ^{13}C-NMR spectrum of bPMEI ($CDCl_3$, 600MHz, inverse gated)*

The methylation of lPEI was confirmed using ^{1}H-NMR. We observed that the two signals assigned to the protons of the methyl- and methylene group shifted to lower values. Due to the complex and dense pattern of proton signals in the corresponding bPMEI ^{1}H-NMR spectrum, which all are

detected in the range between 2 and 3 ppm, we used ^{13}C-NMR spectra and dept 134 experiments to further prove the N-methylation (Figure 1; data from ^{13}C-NMR dept 134 experiments are not shown). We calculated the approximate content of 2 % N-formyl group in bPMEI by the determination of the overall integral of the ^{13}C-NMR signal at 162 ppm. To avoid any influence of the Nuclear Overhauser effect (NOE) the ^{13}C-NMR spectra for the quantitative analysis were recorded using inverse gated decoupling pulse sequences.

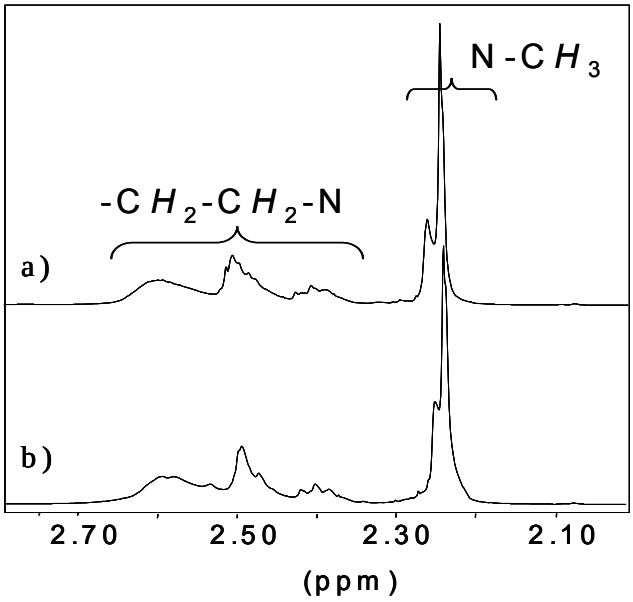


Figure 2. *1H-NMR spectra of bPMEI dissolved in $CDCl_3$ prior to (a) and after (b) the addition of D_2O.*

^{1}H-NMR measurements of PMEI in $CDCl_3$ prior to (Figure 2a) and after the addition of D_2O (Figure 2b) were performed (D_2O / proton exchange). In view of the corresponding spectra we confirmed the absence of primary and secondary amines, as both would exchange hydrogen ions with D_2O, which would lead to the disappearance of the corresponding N-H signals. As both spectra match exactly, we conclude that all primary and secondary amines underwent the conversion.

The complete N-methylation was additionally substantiated by FT-IR spectroscopy (Figure 3). The signals at 3250 cm^{-1} (υ) and 1600 cm^{-1} (δ) in the spectrum of bPEI can be assigned to N-H bending vibrations. The absence of

these signals in the spectrum for the bMPEI indicates the completeness of the conversion of primary and secondary into tertiary amines.

We assigned the signal at 1674 cm^{-1} to HCONR- bending vibrations, resulting from N-formylation of the PEI backbone, which additionally confirms the results from the ^{13}C-NMR experiments. Comparable results were obtained for lPMEI (data not shown).

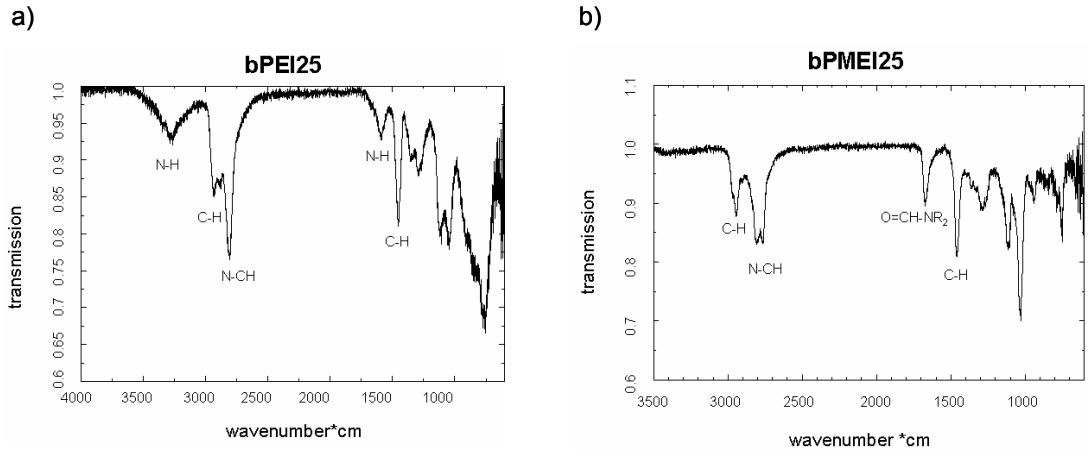


Figure 3. *The bPEI25 (a) and bPMEI (b)- FT-IR spectra were recorded to investigate the extent of N-methylation. The absence of the signals of the corresponding NH-bending vibrations at 3250 cm^{-1} and 1600 cm^{-1} indicate the absence of primary and secondary amino groups in bPMEI25.*

In order to determine the molecular weight and molecular weight distribution (PI) of the synthesized N-methylated polyamines, we chose a size exclusion chromatographic setup, described for bPEIs [20].

Unfortunately, the method was not applicable to PMEI (Figure 4a). Whether eluents of increasing acid concentrations (Figure 4b), or aqueous salt solutions (Figure 4c), nor elution with trifluoro acetic acid (TFA) in water - acetonitrile mixtures (Figure 4d) reduced hydrophobic interactions with the stationary phase, leading to numerous peaks that did not allow for the determination of the molecular weight.

The titration of bPMEI and lPMEI indicate a slight decrease of the overall basicity of the polyamine (data not shown), which has been shown to be in well accordance to the literature [16]. Nevertheless we obtained only approximate data, due to the hygroscopic nature and the statistic values for the molecular weight of both polyamines. We determined a water content of 3% using Karl-Fischer titration, even after extensive drying and handling under a nitrogen atmosphere.

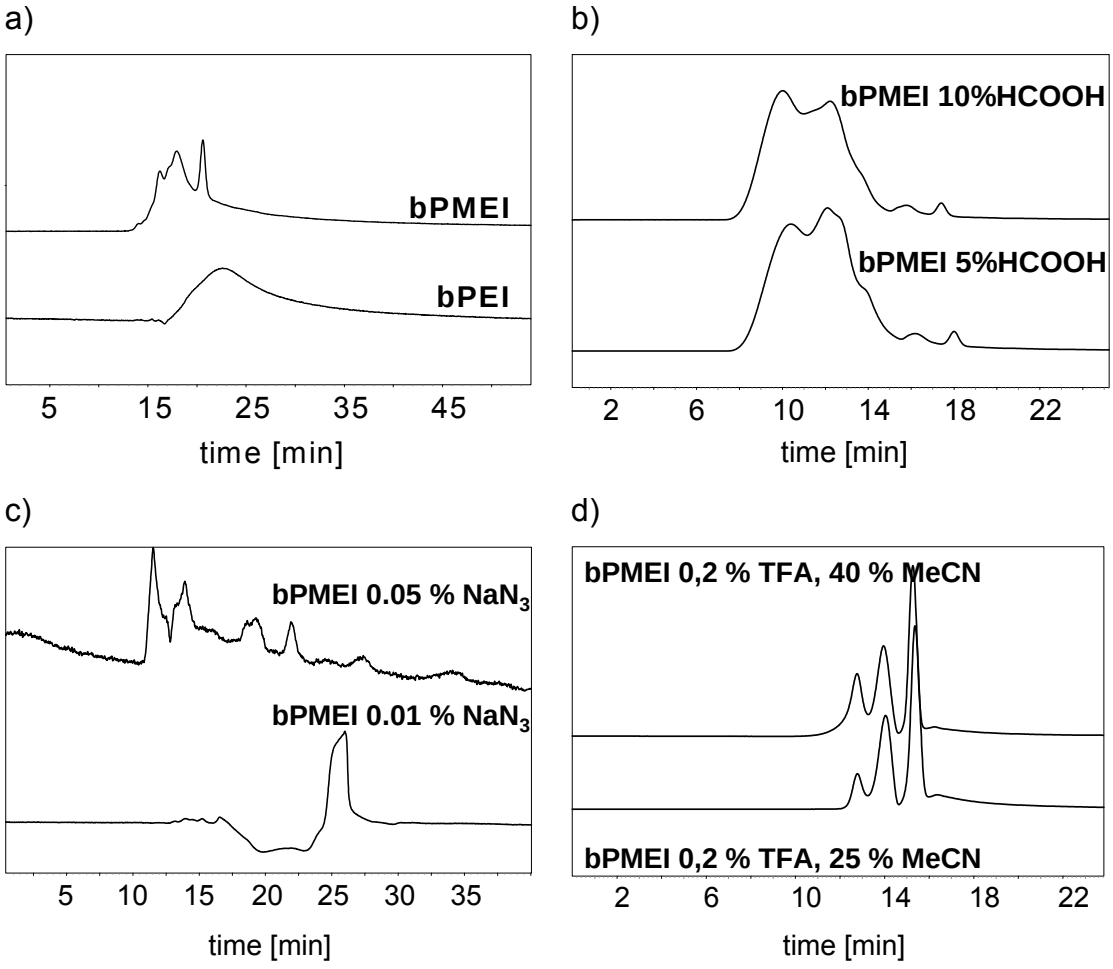


Figure 4. *Size exclusion chromatograms of bPEI25 and bPMEI using a) 1% aqueous formic acid, b) 5 % and 10 % aqueous formic acid, c) a 0.01 % and 0.05 % aqueous sodium azide solution, as well as d) a mixture of 0.2 % trifluoric acid with either 25 or 40 % acetonitrile-water mixture as eluent.*

A reduced overall basicity conceivably leads to a decreasing portion of protonated amino groups at a physiological pH. The cationic charge of the polyamine facilitates the complex formation by electrostatic interactions of the polycation with negatively charged pDNA. The interaction of the resulting netto positively charged particles with the negative cell surface [7;21-23] mediates the cellular uptake. In order to clarify the applicability of per-N-methylated PEIs as pDNA condensing agents, we investigated its capacity to form particles with pDNA and determined the polyplex surface charge.

The unmodified PEIs as well as the corresponding N-methylated polyamines condensed pDNA under the formation of polyplexes in medium of physiological salt concentration (Figure 5).

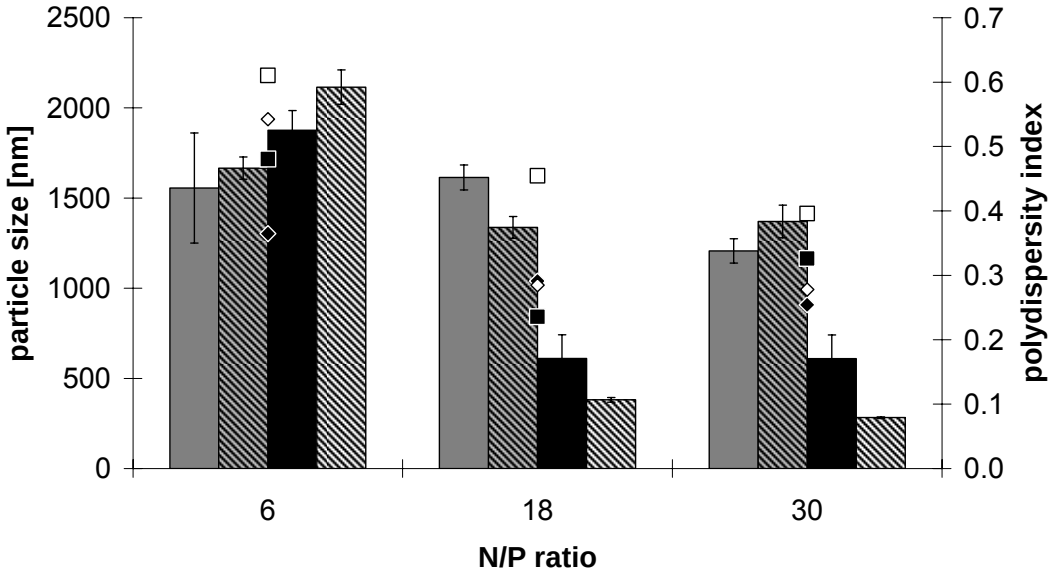


Figure 5. *The hydrodynamic diameters (m.v. + SD) of lPEI25 [■ /♦]–, lPMEI [▨ / ■]–, bPEI25 [■ / ♦]–, and bPMEI [▨ /■] – pDNA polyplexes, prepared in 0.15 M NaCl at an N/P ratio of 6, 18 and 30, were determined after a 10 minutes incubation in serum-free cell culture medium..*

The size of the corresponding polyplexes seemed thereby to be influenced by the architecture of the polycation for pDNA compaction. The branched

polyamines condensed pDNA under the formation of nanoparticles with minimum hydrodynamic diameters of 400 nm for bPMEI – pDNA complexes and 600 nm for bPEI25 – pDNA polyplexes, both obtained at an N/P ratio of 18. The linear polycations, in contrast, formed particles between 1.5 and 2.2 µm upon the addition of pDNA, irrespective to the N/P ratio applied.

An effect of the N-methylation was thereby observed only for bPEI- and bPMEI-pDNA complexes ($p < 0.01$).

All polyplexes exhibited a positive net-charge between 12 and 25 mV under the chosen conditions, irrespective of the polycation or N/P ratio applied (Figure 6).

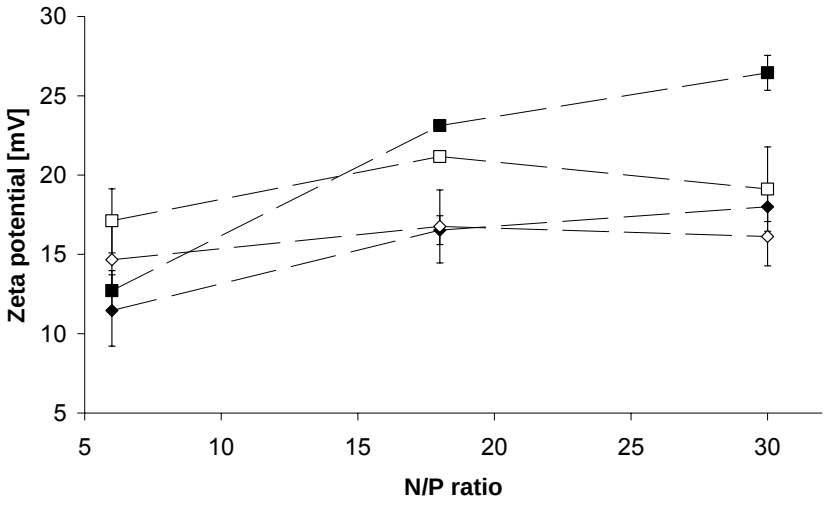


Figure 6. *Zeta potential (m.v. + SD) of lPEI25 [♦]–, lPMEI [◊]–, bPEI25 [■]–, and bPMEI [□] – pDNA polyplexes, prepared in 0.15 M NaCl at an N/P ratio of 6, 18, and 30. The surface charge was determined after incubation in serum-free cell culture medium.*

In order to investigate the capacity of per-N-methylated PEIs as gene carriers, we determined the transfection efficacy of PMEI – pEGFP-N1 complexes under serum-free cell culture conditions, using a CHO-K1 cell model. For bPEI25- and lPEI25 – pDNA polyplexes, the maximum

transfection efficacy was observed at a minimum cytotoxicity, reaching 21 % and 37 %, respectively (Figure 7a). Above the optimum N/P ratio, the cell viability decreased to minimum values of 45 % after incubation with bPEI25 – pDNA complexes or 75 % after incubation with the corresponding lPEI25 – pDNA particles (Figure 7b).

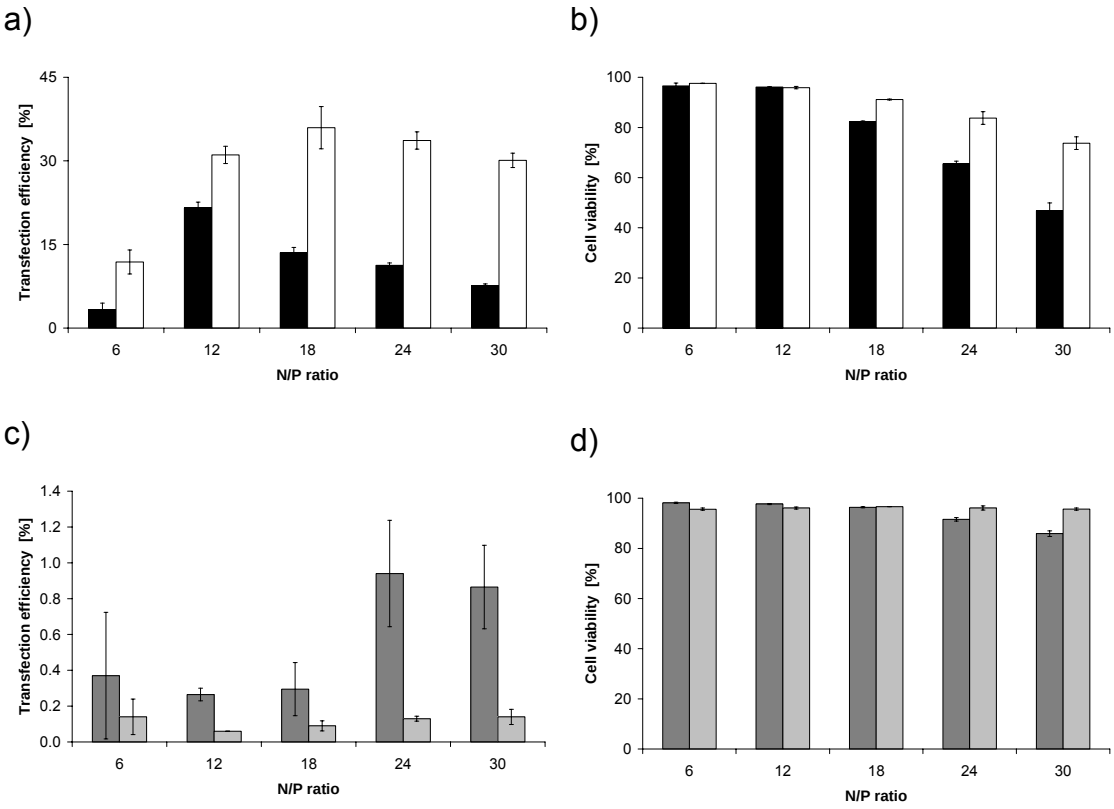


Figure 7.

a) Transfection efficacy and b) cell viability of CHO-K1 cells after incubation with lPEI25 [□]–, and bPEI25 [■] – pDNA polyplexes, as well as

c) transfection efficacy and d) cell viability after treatment of CHO-K1 cells with lPMEI [■]–, and bPMEI [■] – pDNA polyplexes, prepared in 0.15 M NaCl. The transfection experiment was performed in serum-free cell culture medium and the transfection efficiency and cell viability was determined after 48 hours by flow cytometry analysis.

The viability of CHO-K1 cells decreased with increasing polymer concentration, most pronounced for polyplexes based on the branched polyamine, concomitantly reducing the number of successfully transfected cells.

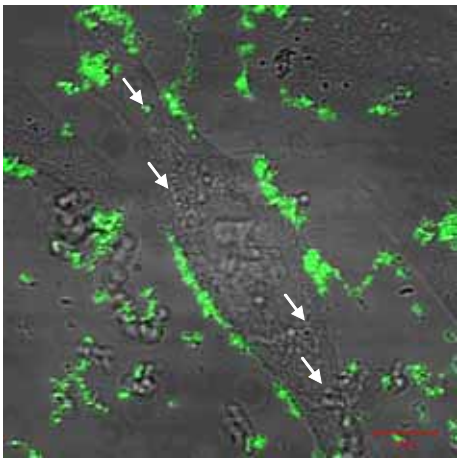
Despite the comparable particle size and surface charge, gene transfer proceeded only at a negligible level after cells were treated with polyplexes based on the per-N-methylated PEIs (Figure 7c). This effect was most pronounced for transfection vehicles, based on the linear tertiary polyamine. While the transfection efficacy of bPMEI – pDNA particles leveled at 0.9 % at an N/P ratio of 24, gene transfer with lPMEI – pDNA polyplexes was diminished to 0.1 % transfected cells, irrespective of the N/P ratio applied.

Upon the complete N-methylation the cell-compatibility of the corresponding polyplexes was remarkably improved. After treatment of CHO-K1 cells with bPMEI – pDNA complexes built at an N/P ratio of 30, we determined a minimum cell viability of 86 % (Figure 7d), while lPMEI – pDNA polyplexes exhibited no detectable cytotoxicity (cell viability of 96 %), irrespective to the N/P ratio applied.

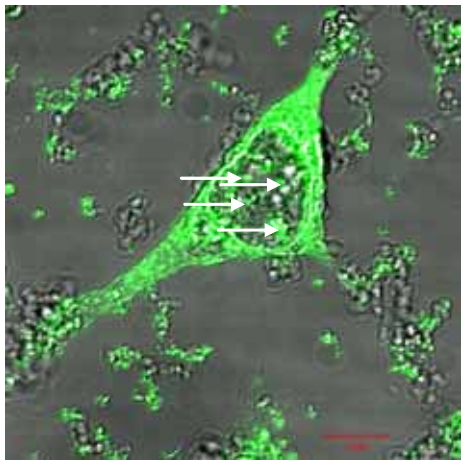
As an explanation for the significant decay of the transfection efficacy, we hypothesized that the N-methylation reduced the charge-induced cell-polyplex interactions and, thereby, the degree of polyplex internalization by CHO-K1 cells, due to a lowered protonation level of PEI. Therefore we used Confocal Laser Scanning Microscopy (CLSM) to visualize the intra- and extracellular distribution, exemplarily for YOYO-1 labeled bPEI25– and bPMEI – pDNA polyplexes (Figure 8). CHO-K1 cells were incubated with bPEI25– and bPMEI – pDNA polyplexes under serum-free cell culture conditions. The polyplexes were formed in 0.15 M NaCl at an N/P ratio of 6 and 30, according to the minimum and maximum polymer concentration used in transfection experiments.

At an N/P of 6, large aggregates of polyplexes can be observed extracellularly, irrespective of the polycations used for pDNA compaction.

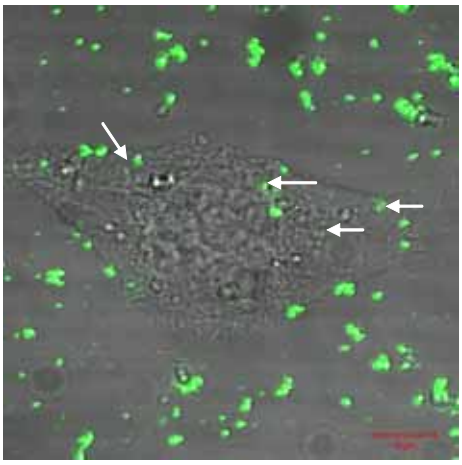
a) **bPEI25 – pDNA N/P 6**



b) **bPEI25 – pDNA N/P 30**



c) **bPMEI – pDNA N/P 6**



d) **bPMEI – pDNA N/P 30**

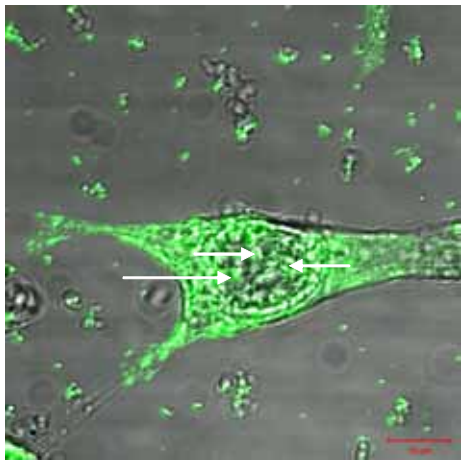
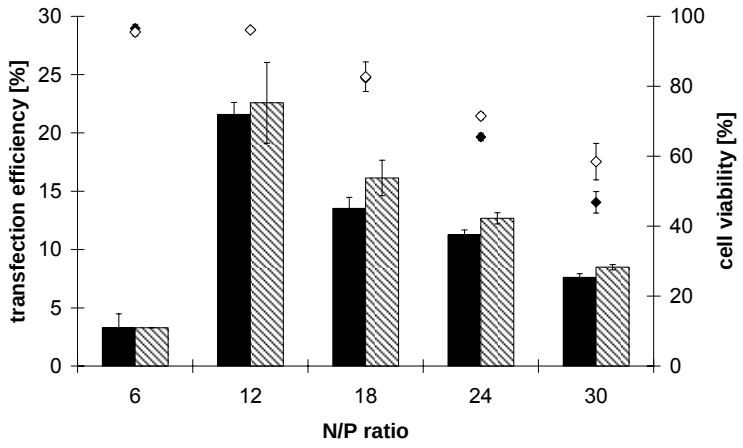


Figure 8. *CLSM images from CHO-K1 cells after 4 hours of incubation with bPEI25-or bPMEI – pDNA polyplexes in serum-free cell culture medium. The particles were prepared in 0.15 M NaCl at an N/P ratio of 6 and 30 using pDNA staining with the intercalating dye YOYO-1.*

bPEI – pDNA polyplexes seemed to align at the cell surface (Figure 8a), while the majority of bPMEI – pDNA particles are distributed extracellularly (Figure 8b). In both setups, a few polyplexes have already entered the cell interior. Increasing the polymer concentration to an N/P ratio of 30, both bPEI25- and bPMEI – pDNA polyplexes were homogenously distributed in the cytosol (Figure 8c and 8d, respectively). However, the main particle

fraction remained as large aggregates distributed in the cell culture medium. Overall, the degree of polyplex internalization seems to be comparable and may therefore not explain the differences in transfection efficacy determined for the non-methylated and methylated bPEI – pDNA complexes.

a)



b)

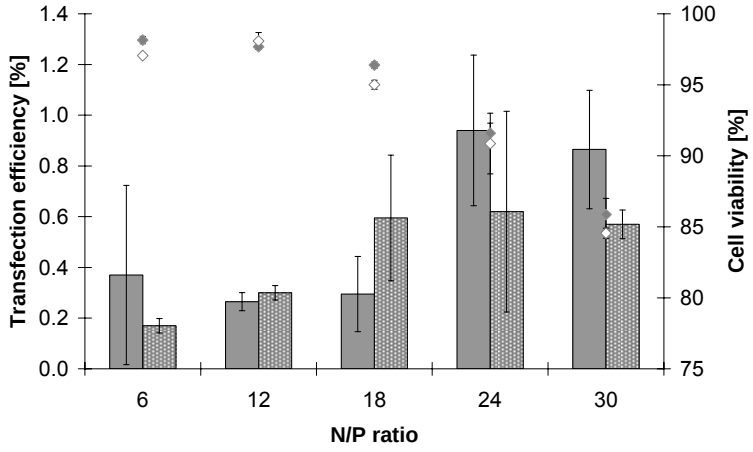


Figure 9. *Transfection efficacy / cell viability after treatment of CHO-K1 cells with a) bPEI25 – pDNA [■ / ♦] or bPEI25 – pDNA [▨ / ◊] polyplexes and 5mM saccharose, as well as b) bMPEI – pDNA [■/♦] or bMPEI – pDNA [▨ / ◊] polyplexes and 5mM saccharose. The polyplexes were fabricated in 0.15 M NaCl, added to the cells in serum-free cell culture medium for 4 hours, and the transfection efficiency and cell viability was determined after 48 hours by flow cytometry analysis.*

In advantage to other polymer-based non-viral transfection agents, PEI-derived vectors exhibit a pronounced endosomolytic capacity, which facilitates the rapid release of the polyplexes from endolysosomal vesicles [24;25]. As this is assigned to the polymer overall basicity and buffer capacity [25;26], we assumed that the decrease of the transfection efficacy determined for polyplexes derived from the per-N-methylated polyamines may be explained by the conversion into less basic tertiary amines.

Hence, we postulated that the efficacy of gene transfer would be recovered by the supplementation of endosomolytic agents, such as saccharose. The addition of 5 mM saccharose had a maximal effect on the efficacy of gene transfer to CHO-K1 cells, exemplarily tested for bPMEI – pDNA polyplexes (data not shown). Based on these results, we determined the transfection efficacy of bPMEI – pDNA polyplexes in comparison to the unmodified bPEI25 – pDNA complexes, supplementing 5 mM saccharose (Figure 9). However, the effect on transfection efficacy of both transfection agents was negligible. This suggests that both, PEI and the per-N-methylated PEIs, exhibit a comparable endosomolytic activity, or that the disruption of gene transfer can not be overcome by the addition of saccharose as endosomolytic agent.

Conclusion

We synthesized per-N-methylated linear (lPMEI) and branched polyethylenimine (bPMEI) by the N-methylation of the corresponding PEI 25 kDa, using the Eschweiler-Clark variant of the Leuckart reaction. The resulting polycations condensed pDNA under the formation of positively charged nano- or microparticles, respectively, influenced by the connectivity of the polymer. Although PMEI – pDNA polyplexes exhibited an improved cell-compatibility, compared to the unmodified PEI – pDNA complexes, gene transfer was almost aborted. As the supplementation of endosomolytic agents had no effect and the polyplex internalization by cells occurred to a

similar degree, other intracellular processes crucial for gene transfer have to be affected by the modification of the polymer properties. The reasons for ineffectiveness of the investigated tertiary polyamine-based gene delivery systems remain elusive.

Experimental section

Branched polyethylenimine (high molecular weight, water free, MW 25,000 Da (LS), M_n 10,000 Da (GPC)) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Linear polyethylenimine MW ~ 25,000 Da (7-8 % poly(2-ethyl-2-oxazoline, mp. 73-75°) was obtained from Polysciences Inc., Warrington, PA, USA. All other reagents were purchased from VWR-International, GmbH, Darmstadt, Germany. Spectra/Por® Membranes from regenerated cellulose with a molecular weight cut-off (MWCO) of 8 kDa were obtained from Carl Roth, GmbH&Co.KG, Karlsruhe, Germany).

The nutrient mixture F-12 (HAM), ethidium bromide, and kanamycin were obtained from Sigma-Aldrich. pEGFP-N1 was purchased from Clontech (Heidelberg, Germany). The *E.coli* JM109 bacterial strain was shipped from Promega. The Plasmid Maxi Kit was purchased from Qiagen (Hilden, Germany). CHO-K1 cells (ATCC# CCL-61) and fetal calf serum (FCS) were supplied by Biochrom KG Seromed (Berlin Germany). Agarose and LB broth medium were purchased from Invitrogen GmbH (Germany). Serva Blue G250 was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). For the visualization of polyplexes DNA was labeled with YOYO-1 obtained from Molecular Probes (Netherlands).

Synthesis of branched per-N-methylated polyethylenimine (bPMEI)

bPEI (0,2mmol) was dissolved in distilled water (15ml) and transferred into a round bottom flask, which was equipped with a condenser. A large excess of Formic acid (0,9mol) and formalin (0,19mol) were added and the apparatus was connected with a gas outlet that led into a concentrated solution of barium hydroxide. The reaction mixture was kept at 100°C for 5 days under

stirring and the resulting carbon dioxide was detected by the barium carbonate precipitation. The reaction was stopped when the carbon dioxide formation ceased. The volatiles were removed under reduced pressure. The residue was made alkaline with a 2N aqueous sodium hydroxide solution. Upon the addition of 25 ml ethanol, sodium formiate precipitated as white spicular crystals, which were separated by filtration (0.2µm). The solvent was removed under reduced pressure and bPMEI was collected as a dark yellow viscous material. The precipitation and drying step was repeated three times, yielding 85% bPMEI. The resulting polymers were neutralized using 0.1N hydrochloric acid and were purified from low molecular substances by dialysis over three days against double distilled water using a cellulose acetate membrane with a cutoff of 8 kDa.

^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 2.05-2.22 (m, -N(CH_3)$_2$ and -N(CH_3)-), 2.29-2.67 (m, -N(CH_2CH_2)$_3$-), 2.98-3.12 (s, HCON(CH_3)-), 2.67-2.98 and 3.24-3.58 (m, HCON-CH_2-CH_2-), 7.81-7.94 (s, *H*CON(CH$_3$)-)

To prove complete methylation by absence of a D_2O / proton exchange with NH, 150 mg of PMEI were dissolved in 1 ml $CDCl_3$ and ^{1}H-NMR spectra were recorded before and after addition of a drop of D_2O.

^{1}H-NMR (400 MHz, $CDCl_3/D_2O$): δ_H (ppm) = 2.14-2.32 (m, -N(CH_3)$_2$ and -N(CH_3)-), 2.34-2.75 (m, -N(CH_2CH_2)$_3$-), (s, HCON(CH_3)-), 2.86-2.90 and 2.95-3.00 (m, HCON-CH_2-CH_2-), 8.00-8.06 (s, *H*CON(CH$_3$)-)

^{13}C-NMR (600 MHz, inverse gated, D_2O): δ_C (ppm) = 41-42.2 (-CH$_2$-N(*C*H$_3$)-CH$_2$-), 43.7-44.9 (-N-(*C*H$_3$)$_2$), 50.1-52.3 (N-(*C*H$_2$-CH$_2$ -)$_3$, 52.8-54.8 ((N-(CH$_2$-*C*H$_2$ -)$_3$, 54.8-55.4 (-CH$_2$-*C*H$_2$-N(CH$_3$)$_2$, 164.9-165.7 (N(CH$_3$)*C*HO) 1.96%

The measurements were performed using inverse gated decoupling pulse sequences to avoid any influence of the Nuclear Overhauser effect.

Synthesis of linear per-N-methylated polyethylenimine (lPMEI)

Both, lPEI25 (0.04 mmol) dissolved in 6.5 ml formic acid (80 %) and 3.5 ml formalin were transferred into the apparatus described above. The reaction

mixture was kept at 100 °C under stirring, detecting the resulting carbon dioxide as barium carbonate precipitate. After 5 days, when the carbon dioxide formation finished, the reaction was stopped. The volatiles were removed under reduced pressure at 80 °C. The crude lPMEI was dissolved in 2 N hydrochloric acid and precipitated in 25 ml ethanol. Linear PMEI-hydrochloride was washed twice with 10 ml ethanol and was dried under vacuum, yielding 90 %.

^{1}H-NMR (300 MHz, D_2O): δ_H (ppm) = 2.05-2.92 (m, NC*H*$_3$), 3.25-3.65 (m, -N-C*H*$_2$-C*H*$_2$-)

IR-Spectra.

Adsorbed water was removed from the samples by drying at 80 °C under reduced pressure (0.01 mbar). The solid samples were transferred onto an ATR-unit, which was set in a nitrogen atmosphere. The transmittance was measured in the reflection mode over wave numbers between 4000 cm^{-1}. Spectra were recorded on a Bruker IBF 88 FTIR spectrometer.

PEI: (cm^{-1}) = 1450 (s, -*CH*$_2$-), 1600 (s, *NH*$_2$), 2800 (v, NH-(*CH*$_3$)), 2950 (v, -NH-*CH*$_2$-, -*CH*$_3$), 3250 (v, *NH*, *NH*$_2$)

bPMEI: (cm^{-1}) = 1459 (s, -CH$_2$-, -CH$_3$), 1674 (s, -*NC*HO), 2764 and 2808 (v, N-(*CH*$_3$)), 2943 (v, C-H)

lPMEI: (cm^{-1}) = 1458 (s, -CH$_2$-, -CH$_3$), 1667 (s, -*NC*HO), 2764 and 2808 (v, N-(*CH*$_3$)), 2942 (v, C-H)

Karl-Fischer titration.

bPMEI was used prior to and after drying at 60 °C for 24 hours in vacuum. Amounts of 200 - 250 mg of bPMEI were diluted in 100.0 ml of methanol. To determine the residual water content of the solvent and of the bPMEI-methanol solution, 20 ml of each sample were injected into an automated Karl-Fischer titration apparatus setup from Mettler Toledo Instruments (Giessen, Germany) and titrated three times and the perceptual water content of the bPMEI samples were calculated (n=6).

Size exclusion chromatography (SEC)

20 mg bPEI25 or bPMEI were dissolved in 1.0 ml adv. distilled water and filtered through a 0.2 µm polyethersulfonic acid membrane filter. A Novema 300 Å SEC column (10 µm, 8 x 300 mm, Polymer Standard Service, Mainz) thermostatted at 40 °C, an injection volume of 25 µl, a flow rate of 0.75 ml/min, and formic acid (1, 5, or 10 %), sodium azide (0.01 % or 0.05 %), TFA (0.2 %) supplemented with acetonitrile (25 % or 40 %) as eluents were used as chromatographic setup.
SEC was performed on Shimadzu HPLC system from Shimadzu, Germany: SIL-10ADvp auto injector, LC-10ATvp liquid chromatograph, DGU 14A degaser, CTO-0ASvp column oven, SCL-10Avp system controller; RID-10A refractive index detector thermostatted at 40 °C.

Amplification and purification of plasmid DNA [pDNA].

The pEGFP-N1 was transformed into the E.coli JM109 bacterial strain. The transformed cells were expanded in LB medium supplemented with kanamycin. The pDNA was isolated using the Quiagen Plasmid Maxi Kit according to the supplier´s protocol. The concentration and purity of the pDNA was measured by UV absorption at 260 and 280 nm.

Preparation of polyplexes

PEIs and the per-N-methylated PEIs were transformed into the corresponding hydrochloride using 2 N HCl and the nitrogen content was determined by elemental analysis. In brief: the polymers were dissolved in 150 mM NaCl and the pH was adjusted to 7.0 with 0.01 N aqueous hydrochloric acid or 0.01 N aqueous sodium hydroxide. The concentrations of the corresponding polymer solutions were adjusted to produce an N/P ratio of 1 upon the addition of 1 µl polymer solution to 2 µg pDNA. The pDNA and the appropriate volume of polymer solution were each diluted with 0.15 M NaCl to equal volumes corresponding to 25 µl per 1 µg pDNA. The pDNA

and polymer solutions were mixed by vortexing and the polyplexes were allowed to form for 20 minutes at room temperature.

Laser light scattering analysis and zeta-potential measurements.

Polyplexes prepared in 0.15 M NaCl containing a total of 10 µg pEGFP-N1 were diluted to 2.5 ml with HAM´s F12 (serum-free cell culture medium). The samples were thermostatted to 25 °C and laser light scattering analysis was performed at 25 °C with an incident laser beam of 633 nm at a scattering angle of 90° using a Malvern ZetaSizer 3000 HSA, Malvern Instruments GmbH, Germany. The count rates for all dispersants were lower than 5 Kcps, confirming their applicability in size measurements. The following parameters were used: viscosity of HAM´s F12 and 0.15 M NaCl: 0.89 mm^2/s; the refractive indices of HAM´s F12: 1.681. The sampling time was set automatically.

Six measurements each with 10 sub-runs were performed for each of three independent samples (n=3). The zeta potential measurements of the same polyplex samples were performed in the standard capillary electrophoresis cell of the ZetaSizer HSA (Malvern Instruments GmbH, Germany), measuring the electrophoretic mobility at 25 °C. The sampling time was again set automatically (n=3).

In vitro transfection and cytotoxicity experiments

For gene transfer studies, CHO-K1 cells were grown in 24-well plates at an initial density of 38,000 cells per well. 18 hours after plating, the culture media was removed; cells were washed with PBS (Invitrogen, Germany) and 900 µl serum-free media (HAM´s F12) were added. Thereafter, the prepared polyplexes were added to the cells. After 4 hours, the medium was replaced by 1 ml of culture medium. To investigate the effect of saccharose, a 1 M stock solution of saccharose dissolved in HAM´s F12 nutrient mixture was prepared and aliquots, equivalent to 0 to 10mM were supplemented to the cells. 48 hours later, cells were prepared for flow cytometry analysis. Floating

cells were collected and combined with adherent cells after trypsinization. The collected cells were washed twice with PBS, resuspended in 500 µl PBS and propidium iodide was added at a concentration of 1 µg/ml to half of the samples. Measurements were taken on a FACSCalibur (Becton Dickinson, Germany) using CellQuest Pro software (Becton Dickinson, Germany) and WinMDI 2.8 (©1993-2000 Joseph Trotter).

EGFP positive cells were detected using a 530/30 nm band-pass filter, while the propidium iodide emission was measured with a 670 nm long pass filter. Logarithmic amplification of EGFP and propidium iodide emission in green and red fluorescence was obtained with 20,000 cells counted for each sample. In a density plot representing forward scatter against sideward scatter, whole cells were gated out and depicted as a two-parameter dot plot of EGFP versus propidium iodide for analysis. The EGFP positive region, which designates transfected cells, was drawn starting above cell auto fluorescence. The geometric mean fluorescence intensity was determined from the number of EGFP positive cells. Further, the number of propidium iodide negative cells was counted as a measure of cell viability. The relative transfection efficacy and cell viability were determined by normalizing the number of EGFP-positive and propidium iodide-negative events counted in a sample to the negative control and expressed as a percent.

Confocal Laser Scanning Microscopy

A Zeiss Axiovert 200 M microscope coupled to a Zeiss LSM 510 scanning device (Carl Zeiss Co. Ltd., Germany) was used for the investigation of CHO-K1 cells. The inverted microscope was equipped with a Plan-Apochromat 63x and Plan-Neofluar 100x objective. Cells were plated in 8-well Lab-Tek™ Chambered Coverglass (Nunc GmbH & Co. KG, Germany) at an initial density of 35,000 cells/chamber in a volume of 400µl culture medium. For maintaining a pH of 7.4, 20 mM HEPES was supplied. After 18 hours, polyplexes were added and measurements were directly performed in each

well at 37 °C. The thickness of the optical section was between 0.7 and 1.2 µm.

For the visualization of polyplexes DNA was labeled with YOYO-1.The intercalate was excited with a 488 nm argon laser and the fluorescence was detected using a 505-530 nm band-pass filter.

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4. SYNTHESIS AND CHARACTERIZATION OF POLY (2-ETHYL-2-OXAZOLINE) AND LINEAR POLYETHYLENIMINE

Uta Lungwitz, Sigrid Drotleff, and Achim Göpferich

Department of Pharmaceutical Technology, University of Regensburg, 93040 Regensburg, Germany

Abstract

We synthesized a series of twelve poly (2-ethyl-2-oxazoline)s (pOXZ) by Lewis acid initiated ring-opening polymerization of 2-ethyl-2-oxazoline, using a parallel synthesis approach. The resulting polymers have been characterized by ^{1}H-NMR spectroscopy, gel permeation chromatography (GPC), and Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-ToF) mass spectroscopy. We confirmed that pOXZ batches were obtained with a relative number average molecular weight (M_n) ranging between 3.0 and 23.3 kDa and a molecular weight distribution (M_w (weight average molecular weight) / M_n, also defined as polydispersity index (PI)) between 1.02 and 1.44.

The corresponding series of twelve low molecular weight linear polyethylenimine (lPEI) was synthesized by the acid catalysed hydrolysis of pOXZ. We developed a gel filtration chromatography (GFC)- and MALDI-ToF method that allowed for the determination of the relative molecular weight and the molecular weight distribution, complementing the ^{1}H-NMR data. We obtained lPEIs with a M_n of 1.0 to 8.1 kDa and a PI between 1.07 and 2.14. The inhomogeneity of the polymer batch increased thereby with the polymerization degree.

Introduction

Polyamines have shown to efficiently deliver nucleic acid, e.g. plasmid DNA (pDNA) to a large variety of eukaryotic cells in vitro [1;2]. pDNA is thereby condensed by the polycationic carrier by the self-assembly of polyelectrolyte complexes (polyplexes), due to electrostatic interactions. The corresponding polyplexes precipitate as rather polymorphic nano- or microparticles. Polymers are, due to the comparably low cost of preparation, high chemical stability, the options of chemical modifications, and low immunogenicity, an important alternative to viral vectors for gene delivery. The design of efficient transfection systems still relies on empiric approaches, due to the limited insight into the relationship of polymer structure and the performance of the corresponding nucleic acid carrier system in an biological environment [3-5]. Summarizing the experiences with the variety of different polycations used for this purpose [6-8], the efficacy of gene transfer seems, beside other factors, to be a function of the polymer molecular weight (MW), its protonation level (ratio of protonated amines to unprotonated amines) at physiological pH, and the polymer concentration applied (N/P ratio: refers to the quotient of the nitrogen atoms of the polymer to DNA phosphates). This may in turn modulate the efficacy of pDNA compaction, pDNA stabilization and polyplex release from endocytotic vesicles, as well as its membranolytic activity.

In 1995, branched polyethylenimine (bPEI) was introduced as material for the manufacture of non-viral vectors *in vitro* and *in vivo* [9]. The polymer branching degree, which slightly differs in dependency to the synthetic route used, determines the ratio of primary, secondary and tertiary amines. All of them contribute to its high overall buffer capacity, intrinsic endosomolytic activity, and comparably high protonation level of about one third at physiological pH [10-12]. This conceivably deems it to one of the most promising gene carriers.

Unfortunately, bPEI-derived vectors also exhibit a severe cytotoxicity, mainly based on its high membranolytic capacity [13-15]. *In vitro* transfection

experiments have shown that both, the efficacy of gene transfer and the cell compatibility increase with the polymer MW, its degree of branching and the polymer concentration used for the polyplex formation [14-18]. Using low molecular weight bPEI reduced the cytotoxicity of the gene carrier, while the transfection efficacy could be at least partially recovered by increasing the N/P ratio [16].

More recently, lPEI 22 or 25 kDa – pDNA complexes were introduced as an alternative gene delivery system, due to a superior performance *in vitro and in vivo* gene transfer [19;20]. Implementing the "structure – function relationship" assumed for bPEI- mediated transfection, the improved transfection efficacy and cell-compatibility may be explained by an increased overall basicity of exclusively secondary amines (90 % protonation level at physiological pH [12]) and the absence of branching sites. Nevertheless, gene transfer with lPEI – pDNA complexes is still derogated by a reduced cell viability, which seems to be associated by its mode of action [21].

We suggested that an lPEI MW- and polymer concentration range can be found at which efficient pDNA compaction, endolysosomal escape and hence efficient gene transfer can be realized at a low level of cytotoxicity.

In order to identify the MW optimum we synthesized a series of lPEIs with gradually increasing polymerization degree and systematically investigated the effect of the polymer MW on the polymer properties and the performance of the corresponding vectors *in vitro*.

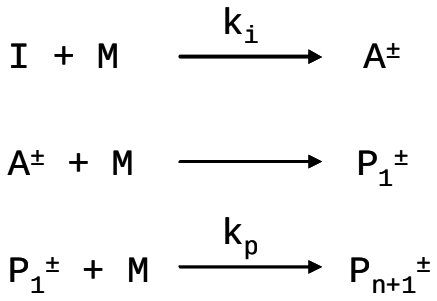
Polymer background:

Several routes for the synthesis of bPEI are described: the cationic initiated ring-opening polymerization of substituted or un-substituted aziridines, as well as the polycondensation or polyaddition of diamines with amine-reactive bifunctional compounds such as dihalogenalkanes or chlormethyloxiran (epichlorhydrine) are the most prominent ones. All of these methods lead to highly branched polymers, consisting of varying portions of primary,

secondary and ternary amines, which determinate the polymer branching degree [22;23].

A convenient route for the synthesis of strictly linear PEI is the polymerization of 2-oxazolines (IUPAC: 4,5-dihydrooxazoles) and acid- [24;25] or base-catalized hydrolysis [26] of the resulting poly (N-acylethylenimine).

The polymerization of 2-oxzolines was first reported by Kagiya et al. in 1966 [27], and follows a bimolecular reaction according to the general Scheme 1.



Scheme 1. *The polymerization of 2-oxazolines following a bimolecular reaction where I is the initiator, M is the monomer, $A^{\pm}$ is the active and $P^{\pm}$ is the propagating species. After a fast initiation (k_i) follows a slow propagation (k_p), determining the reaction rate [28].*

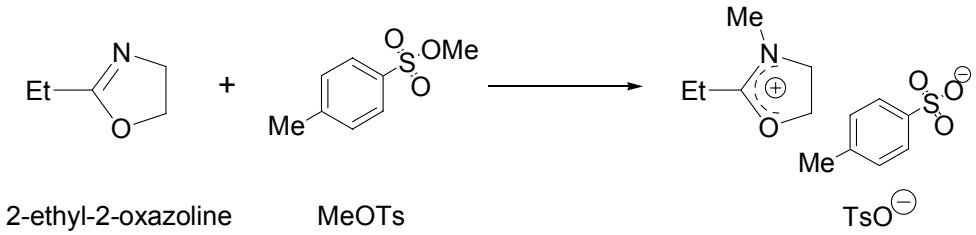
2-Oxazolines are five-membered cyclic imino ethers (imidates), which polymerize via an electrophilic growing species by a cationic ring-opening, and show all typical features of a living polymerization (Scheme 2). The propagating centers are significantly low in reactivity, which suppresses transfer or termination reactions and maintains chain propagation [29]. Most advantageous over other types of polymerization is the direct control of the chain length by the initial monomer to initiator ratio ($[M]_0$ /[I]), and a narrow distribution of the molecular weight.

The polymerizability involves both, the stabilization due to the isomerization of the imino ether monomers into the thermodynamically favorable poly (N-acylethylenimine), and factors of ring strain. By variation of the monomer, the

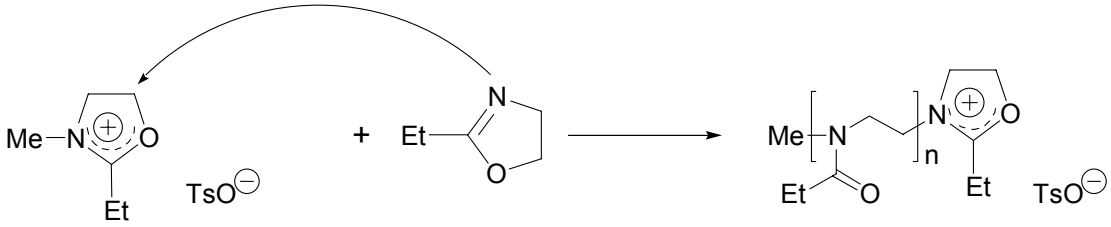
initiator and the terminating agent, a great arsenal of polymers with different chemical and structural properties is obtainable [30].

Five- to seven-membered cyclic imino ethers possess polymerizibility and can be hydrolysed to linear polyamines [31;32]. The polymerization of 2-methyl-, or 2-ethyl-2-oxazoline monomers are most commonly used and extensively studied. They allow for the synthesis of low molecular weight poly (N-acyl ethylenimine) [23], while 2-phenyl-2-oxazoline lead to the corresponding high molecular weight derivatives, still obtainable with a narrow molecular weight distribution [33].

1. Initiation



2. Propagation



Scheme 2. *Polymerization of 2-oxazolines via propagation by an ionic active species, exemplarily shown for the 2-ethyl-2-oxazoline monomer and methyl-p-toluenesulfonate (MeOTs) as cationic initiator*

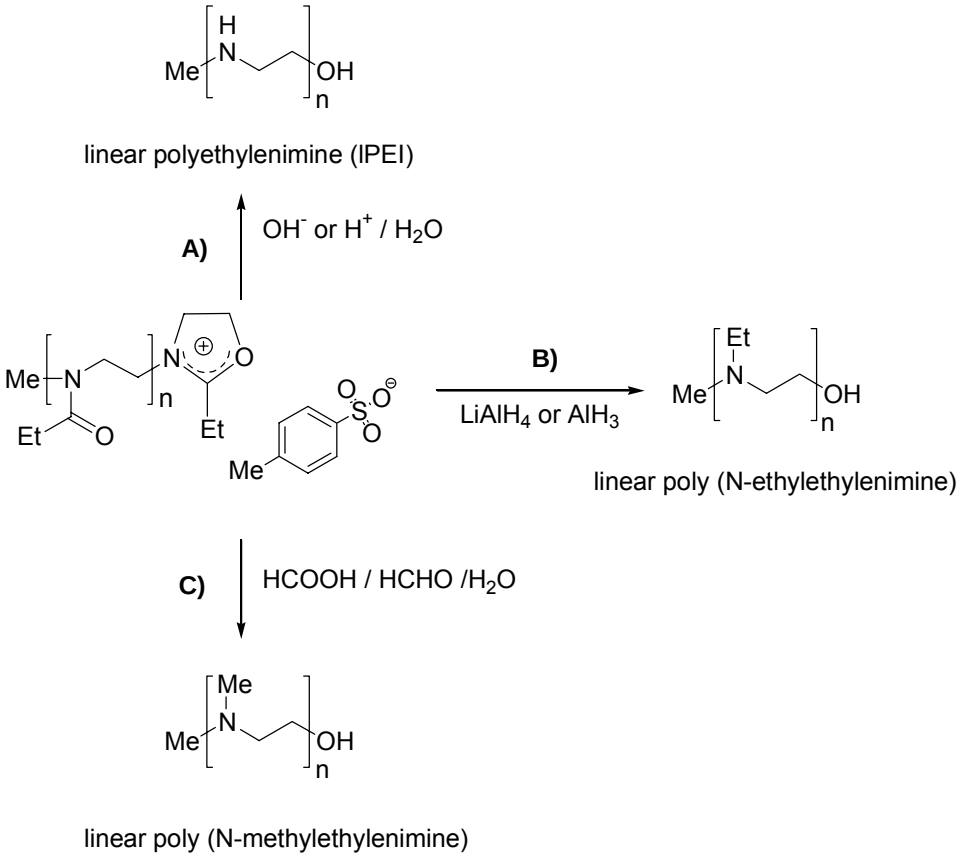
Structural diversity can also be introduced by the choice of initiator. Lewis acids (BF_3, $AlCl_3$, $TiCl_4$, PF_5, SbF_5), salts of Lewis acids ($Et_3O^+BF_4^-$), strong protic acids ($HClO_4$, CF_3SO_3H, H_2SO_4, HBr), sulfonate esters (p-

$MeC_6H_4SO_3Me$, $p\text{-}O_2NC_6H_4SO_3Me$, CF_3SO_3Me, FSO_3Me), sulfate esters ($(MeO)_2SO_2$), sulfonic anhydrides ($((MeO)_2SO_2)_2O$), alkyl halides ($PhCH_2Cl$, $PhCH_2Br$, MeI), alkyl haloformates (MeOCOCl), or photocationic compounds [34] were applied for this purpose and are summarized in [30].

By the use of bicyclic N-acetyl-D-glucosamine oxazolinium salts as initiators for the polymerization of 2-methyl-, or 2-phenyl-2-oxazoline, polymers linked to a sugar moiety as cell recognition marker at the initiation end were obtained. More complex structures, such as two- or four-armed star shaped polymers were synthesized by the 2-oxazoline polymerization initiated by allyl-type di- or tetra halides [35;36].

A covalent and an ionic mechanism for initiation and propagation are discussed, directed by the nucleophilicity of the monomer and of the counter-anion generated by the initiator [23]. It is suggested that a covalent propagating species results from a rapid ring-opening of the generated 2-oxazolinium salt by a nucleophilic attack of a counter-anion with comparably higher nucleophilicity than the monomer used [28;37;38].

The termination of the polymerization follows the same principle. A nucleophile attacks the 2-oxazolinium salt in 2-or 5-position, generating the kinetic or the thermodynamic products, respectively. The secondary cyclic amine piperidine, is most commonly used for this purpose, due to the selective termination in 5-position [39-41]. The polymerization proceeds under anhydrous conditions, starting at temperatures above 40°C.



Scheme 3.

Synthesis of linear poly-ethylenimine by the alkaline or acidic hydrolysis (A) of pOXZ. The corresponding poly (N-alkylethylenimine) derivatives will be obtained by the hydrogenolysis (B), or reductive methylation (C) of pOXZ.

In the absence of impurities the polymerization is not disturbed by chain transfer or the termination by chain combination, due to the stability of the cationic propagation species of an oxazolinium salt [23].

Saegusa et al. introduced the alkaline hydrolysis of poly (N-acylethylenimine) as synthetic route to unsubstituted linear polyethylenimine, (Scheme 3, A). This method leads to very pure crystalline di- or sesquihydrates of lPEI [26;42], which become soluble in organic solvents in its anhydrous form. The acid hydrolysis of poly (N-acylethylenimine) often remains incomplete, generating random poly (N-acylethylenimine)-polyethylenimine copolymers [24;25;43].

The metal hydride reduction (Scheme 3, B) [44] or reductive methylation (Scheme 3, C) of poly (N-acylethylenimine) have been applied to generate poly (N-alkylethylenimine).

Results and discussion

Synthesis and Characterization of poly (2-ethyl-2-oxazline)

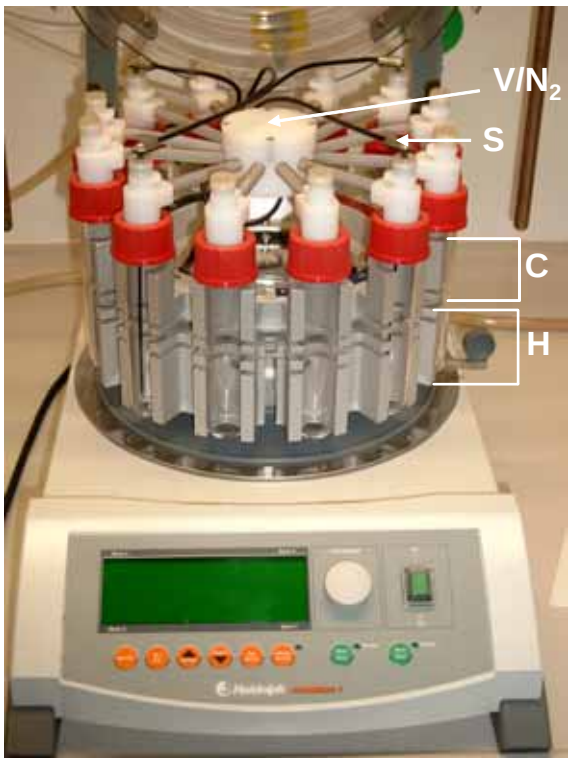


Figure 1. *The Synthesis 1 Liquid 12 parallel synthesis block from Heidolph Instruments GmbH & Co.KG (Germany) was used to perform the polymerization of 2-ethyl-2-oxazoline. The glassware was dried at 120 °C under vacuum (H: heating block; C: condenser block; V/N_2: vacuum distributer). The apparatus was set under a nitrogen atmosphere and the reactants were transferred into the tubes with a syringe. The reaction mixture was shacked for 6 days at 90 ± 2 °C, controlled by sensors placed directly in the reaction tubes.*

We chose methyl-p-toluenesulfonate as initiator for the polymerization of 2-ethyl-2-oxazoline (Scheme 1) [24], as it has been shown that it allows for the formation of polymers with a narrow size distribution [45;46].

We used acetonitrile as solvent, commonly applied for the 2-oxazoline polymerization [24;47-49]. In accordance to the literature, contact times of several days for the complete conversion to poly (2-ethyl-2-oxazline) (pOXZ) had to be considered [24]. In order to reduce time and effort, which would have to be invested in the preparation of a series of low molecular lPEIs, we adopted the described procedure for the use of a parallel synthesis block (Figure 1). This gave us the opportunity to run 12 individual batches simultaneously. A contact time of 6 days was chosen, in order to assure a complete conversion for all batches.

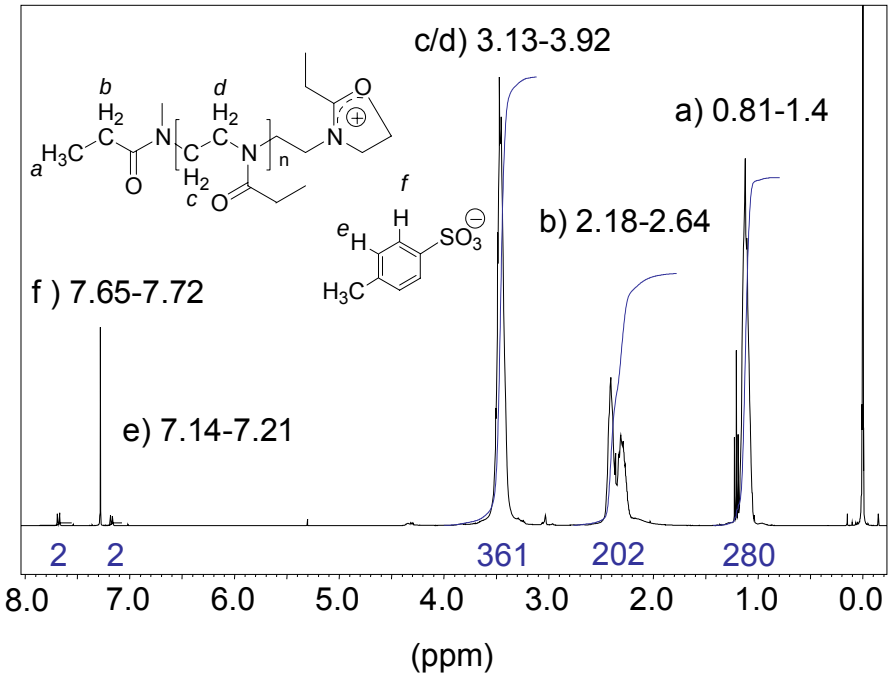


Figure 2. *^{1}H-NMR of poly (2-ethyl-2-oxazoline): ^{1}H-NMR-shifts and overall peak areas assigned to the methyl (a)- and methylene (b) protons of the propionyl side chain, the methylene protons (c, d) at the polymer backbone, and the aromatic protons of the tosylate counter-anion (e, f).*

^{1}H-NMR spectra were used to confirm the successful conversion and to approximate the number average polymerization degree, according to Brissault et al. [24]. Therefore, the peak area of the aromatic protons assigned to the tosylate counter-anion of the ionic terminations was set to 2. The overall integrals of the proton signals corresponding to the propionyl side chains (0.81 - 1.4 ppm and 2.18 - 2.64 ppm) and additionally to the methylene group of the polymer backbone (3.13 - 3.92 ppm) were determined (Figure 2). The ratios of these overall integrals to the respective number of protons of the individual group (3, 2 or 4, respectively) were calculated to estimate the average number molecular weight.

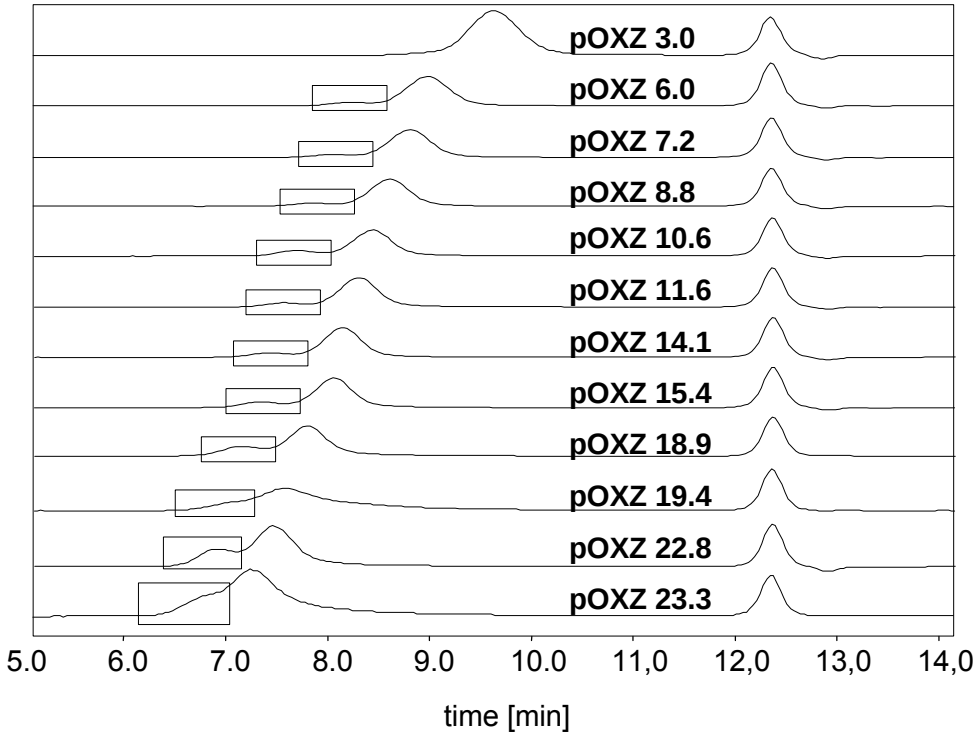
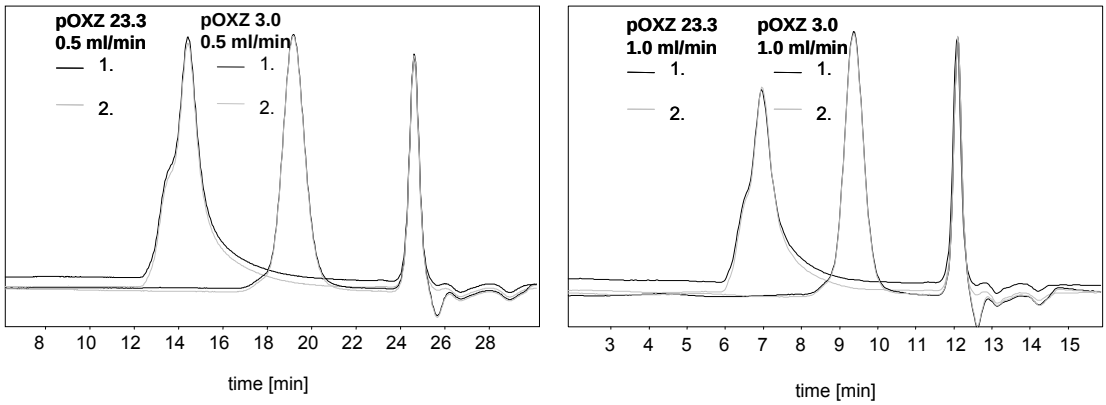


Figure 3. *Gel permeation chromatograms of the pOXZ series: the pOXZ was eluted with $CHCl_3$ at a flow rate of 1.0 ml/min. Beside the main fraction a second high MW fraction becomes more dominant with increasing MW of pOXZ (framed in the individual chromatogram).*

Unfortunately, this quantitative NMR analysis can give only approximate information.

Therefore, we analysed the series of pOXZ in gel permeation chromatography (GPC) experiments to additionally determine the M_n, the M_w, as well as the PI of the individual pOXZ batches [40]. The corresponding chromatograms indicated beside the main fraction a second high MW fraction that became more dominant with increasing MW of pOXZ (Figure 3).

We proved the applicability of the GPC method, exemplarily for a low and a high molecular weight pOXZ from the synthesized series, in order to clarify whether the second MW fraction is an artifact of the chromatographic method.



pOXZ	FR [ml/min]	area [%]	V [ml]	pOXZ	FR [ml/min]	area [%]	V [ml]
23.3	0.5	100.28	7.1222	23.2	1.0	99.6	7.0905
3.0		100.3	9.503	3.0		98.95	9.4955

Figure 4. *The elution volumes (V, depicted as m.v.) and peak areas of pOXZ 23.3 and pOXZ 3.0 in GPC performed at a flow rate (FR) of 0.5 (left) or 1.0 ml/min (right), was determined in order to trace affinity-derived interactions of the polymer with the stationary phase.*

If the elution of the samples only results from size exclusion, the elution volume will remain constant, irrespective to the flow rate applied. However,

we determined slightly increasing elution volumes at a reduced flow rate, which indicates affinity-based interactions with the stationary phase, even though only to a low extent (Figure 4).

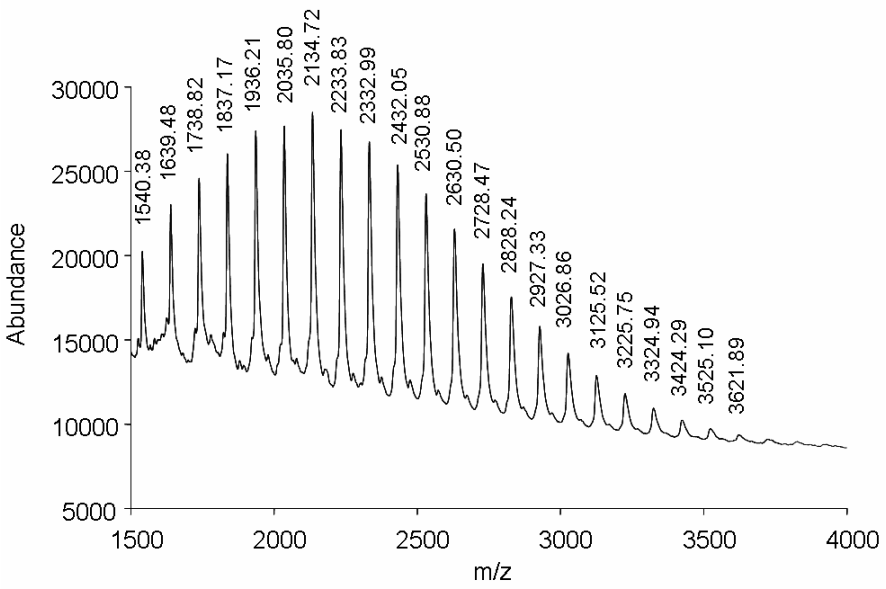
pOXZ	M_n (theor.)[1] kDa	M_n (^{1}H-NMR) kDa	M_n (GPC) kDa	M_w (GPC) kDa	M_w / M_n (PI)
23.3	22.4	21.8	23.3	34.9	1.44
22.8	19.3	20.7	22.8	32.3	1.39
19.4	15.6	14.0	19.4	28.9	1.49
18.9	13.5	13.0	18.9	24.1	1.28
15.4	11.3	11.5	15.4	17.9	1.16
14.1	10.1	10.6	14.1	16.2	1.15
11.6	9.0	8.9	11.6	13.6	1.17
10.6	7.8	8.0	10.6	12.4	1.17
8.8	6.8	6.7	8.8	9.9	1.12
7.2	5.6	5.5	7.2	8.0	1.11
6.0	4.5	4.6	6.0	6.8	1.12
3.0	2.3	2.3	3.0	3.2	1.02

Table 1. *We determined the M_n, M_w, and the PI of pOXZ by GPC, listed in comparison to the M_n estimated from the ^{1}H-NMR spectra, as well as its theoretical M_n targeted at the individual reaction batch.*

This may explain that the portion of the second, high molecular weight fraction increased with increasing chain length (MW) of the eluted polymer. The supplementation with 4 % triethylamine had no effect [40]. GPC after all revealed that a series of pOXZ with M_n between 3 and 24.2 kDa and PIs between 1.09 and 1.44, relative to poly (ethylene glycol) standards, had been obtained (Table 1). The PIs increased with the polymerization degree of the polymer. The determined values are in well accordance to the theoretical

M_n (^{1} M_n calculated from the initiator / monomer ratio), which confirms the applicability of the synthesis procedure used.

a)



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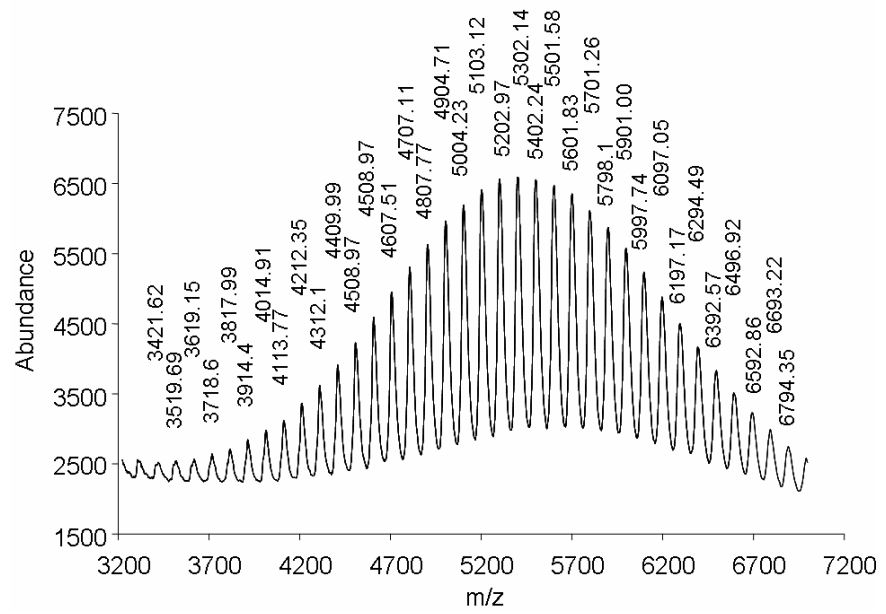


Figure 5. *MALDI-ToF spectra of a) pOXZ 3.0 (GPC) and b) pOXZ 7.2 (GPC)*

Additionally, pOXZ with a M_n between 3.0 and 7.2 kDa was characterized by MALDI-ToF [50].

The polymers with a higher molecular weight were remained not ionizable, irrespective to the sample preparation procedure, matrix or additives used. Molecular weights observed ranged from 1.5 kDa to 3.6 kDa for pOXZ 3.0 (Figure 5a), 3.1 kDa to 5.6 kDa for pOXZ 6.0 (data not shown), and 4.0 kDa to 6.9 kDa for pOXZ 7.2 (Figure 5b). The molecular weight distribution is composed of a series of individual signals, ordered in a polymer-characteristic gaussian-like curve. The signals differ by 99.0 – 100.1 kDa, correlating well with the molecular weight of the corresponding OXZ unit.

Synthesis and Characterization of linear Polyethylenimine

The corresponding low molecular weight lPEIs were synthesized by acid-catalized hydrolysis of the corresponding pOXZ derivatives (Scheme 2)[24].

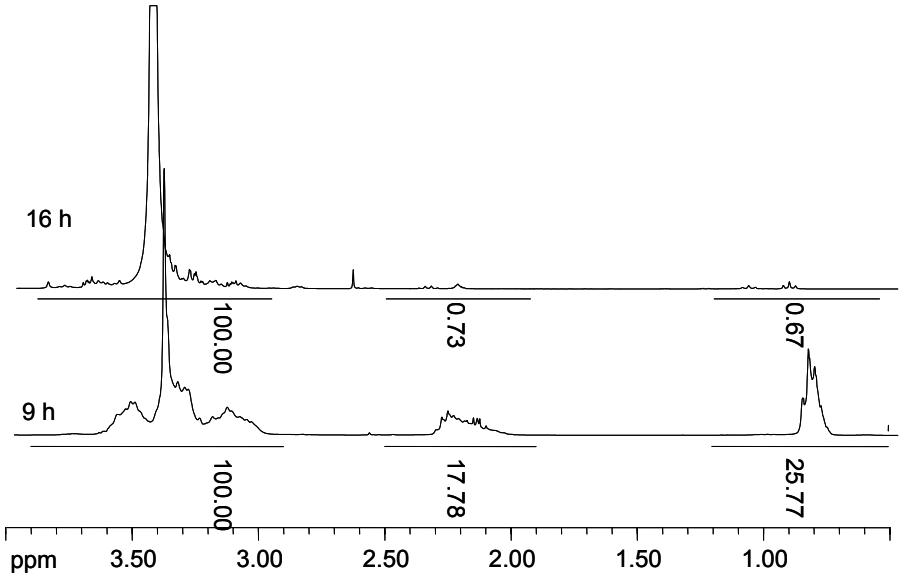


***Figure 6**. [1]H-NMR spectra of samples drawn from the pOXZ – HCl reaction mixture after 9 and 16 hours of contact time.*

In order to determine the contact time necessary for complete polymer deacylation for all pOXZ of the series, we performed the hydrolysis of pOXZ 23 kDa (NMR), and determined the remaining portion of N-propionyl groups by ^{1}H-NMR measurements.

Samples were drawn after 0.5, 1.5, 9, 16 and 24 hours contact time and the propionic acid, formed as by-product, was removed at 90 °C under reduced pressure. The samples were dissolved in D_2O and the progress of hydrolysis was determined by the overall integrals of the propionyl proton signals (0.5 – 1.2 ppm) in correlation to lPEI methylene proton signals (2.9 – 3.9 ppm) in ^{1}H-NMR spectra (Figure 6). The portion of propionyl protons steadily decreased leveling at a merely detectable level after 16 hours of contact time.

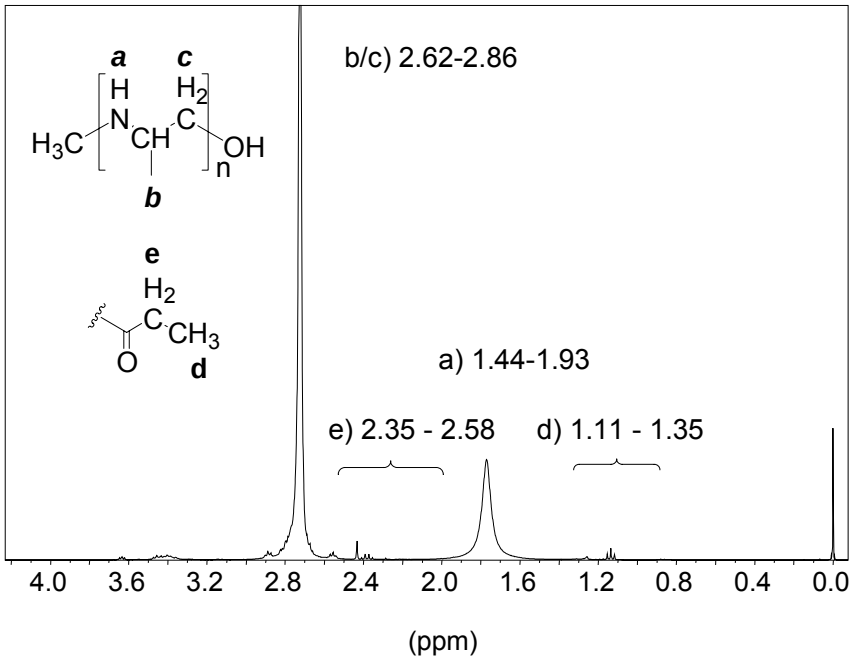


Figure 7. *^{1}H-NMR ($CDCl_3$) of the lPEI8.1- base with proton signals of NH at 1.44 - 1.93 ppm (a) and of -N-CH_2-CH_2- at 2.62-2.86 ppm (b/c). A residual amount of N-propionyl groups remain detectable at 1.11 -1.35 ppm (d) and 2.35 – 2.58 ppm (e).*

Based on these results we performed the hydrolysis using a contact time of 24 hours in order to assure a maximal deacylation for all pOXZ of the series.

The use of the parallel synthesis block allowed for the simultaneous conversion under comparable conditions into a series of lPEIs. The crude lPEIs were precipitated as amine base with sodium hydroxide. Before, the resulting propionic acid was removed under reduced pressure from the acidic reaction mixture. Residual sodium propionate was removed with water from the alkaline gel-like precipitate of the lPEI-base.

^{1}H-NMR spectra of the anhydrous lPEI-base were recorded, showing the N*H*-signal at 1.44-1.93 ppm and the methylene proton signals of the polymer backbone at 2.62-2.86 ppm (range outlined for all lPEIs of the series) (Figure 7).

So far the molecular weight of the synthesized lPEIs used in gene delivery studies, was calculated on the basis of the corresponding polymerization degrees of pOXZ, as determined by ^{1}H-NMR analysis [24].

In order to investigate the impact of M_n on the efficacy and cell compatibility of the gene carrier, and on the particle properties of the corresponding polyplexes, data to the M_n and PI data become indispensable.

Therefore, we developed a gel filtration chromatography (GFC) method, which additionally allowed us to clarify whether a second mass fraction, indicated by the pOXZ batches, is also evident in the corresponding lPEI chromatograms. A chromatographic procedure for the characterization of low molecular weight lPEIs has not been described yet. Therefore, we first orientated us on a method described for its branched isomer. An 1 % aqueous formic acid as eluent and a column with a non-polar stationary phase based on hydroxyethyl-methacrylate as non-charged stationary phase has been shown to suite best for the size-controlled separation of bPEIs [18]. A Light scattering detector was used to determine the absolute molecular weights.

However, lPEI was not separated according to its molecular weight, neither under these conditions, nor using stronger acids, like trifluoro acetic acid as

eluents. Furthermore, a determination of the absolute molecular weights by light scattering would fail, due to the low M_n expected. Therefore, we were limited to the calculation of relative molecular weights based on polymer standards, using a refractive index detector to record the signals. The supplementation with salts in order to reduce conceivably charge-induced interactions with the stationary phase diminished the refractive index detector signal.

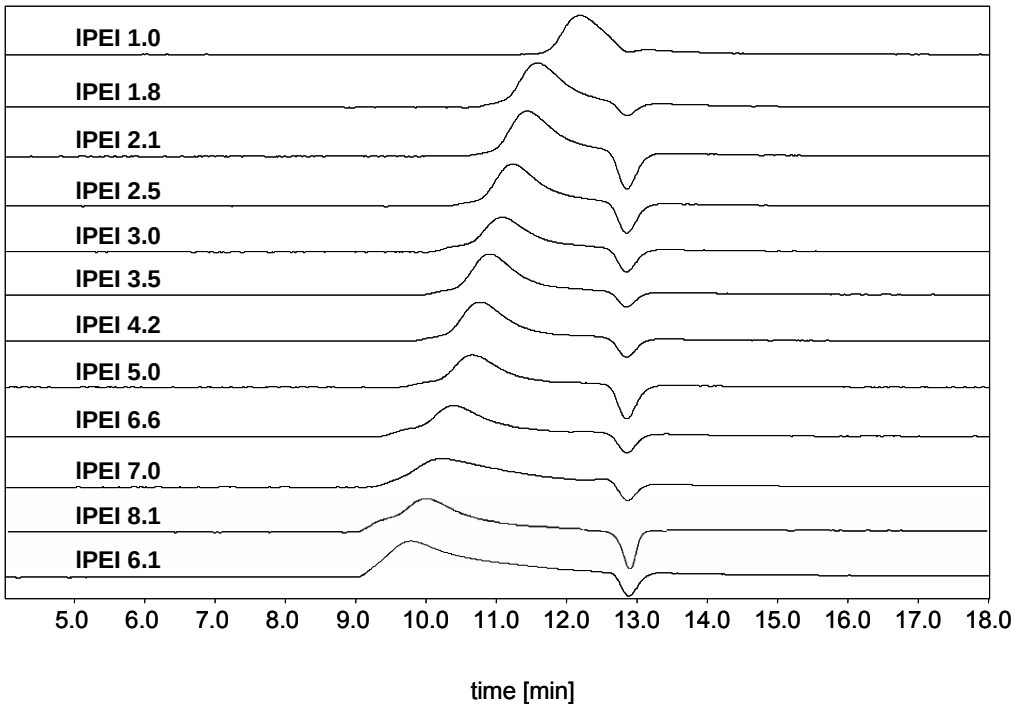


Figure 8. *Gel Filtration Chromatograms of the lPEI series, using an aqueous 0.15 M NaCl as eluent at a flow rate of 1.0 ml/min. The signals were recorded with a refractive index detector.*

After all, the elution of lPEIs according to the hydrodynamic volume, under maximal reduction of charge- or affinity-based interactions with the stationary phase was achieved using 0.15 M to 5 M aqueous sodium chloride solutions as eluents (Figure 8). The use of organic eluents ($CHCl_3$, $CHCl_3$ / 4 % of TEA or THF), which may prevent ionic interactions more efficiently than aqueous

systems, lead to the gelation of lPEI with a M_n > 5 kDa and was, therefore, not applicable. As relative molecular weights are received only in relation to a polymer standard, the choice of the reference material is crucial for the determination of meaningful values. Hence, adequate standards should resemble the physicochemical properties of the analyte.

lPEI	M_n (theor.) [kDa]	M_n (^{1}HNMR$_{pOXZ}$) [kDa]	M_n (GFC) [kDa]	M_w (GFC) [kDa]	M_w / M_n (PI)
6.1	9.8	9.3	6.1	13.1	2.14
8.1	8.5	8.9	8.1	13.6	1.68
7.0	6.8	6.0	7.0	10.0	1.43
6.6	5.9	5.6	6.6	8.9	1.35
5.0	4.9	5.0	5.0	6.3	1.27
4.2	4.4	4.6	4.2	5.3	1.25
3.5	4.0	3.9	3.5	4.5	1.27
3.0	3.4	3.5	3.0	3.9	1.27
2.5	3.0	3.0	2.5	3.0	1.19
2.1	2.5	2.4	2.1	2.4	1.15
1.8	2.0	2.0	1.8	2.0	1.16
1.0	1.0	1.0	1.0	1.0	1.07

Table 2. *We determined the M_n, M_w, and the PI of lPEI by GFC. The determined values are listed in comparison to the M_n estimated from the corresponding pOXZ ^{1}H-NMR spectra, as well as its theoretical (targeted) molecular weight.*

Unfortunately, using poly (vinyl pyridine) hydrochlorides as the only commercially available water soluble, linear and polycationic polymer standard did not lead to a analyzable detector signal, and other linear but

non-charged standards such as poly (ethylene glycol) standards were not separated by the chosen chromatographic procedure.

After all, dextranes as polysaccharides were applied for the molecular weight determination, revealing values that are in alignment with the targeted ones and those calculated on the basis of ^{1}H-NMR measurements.

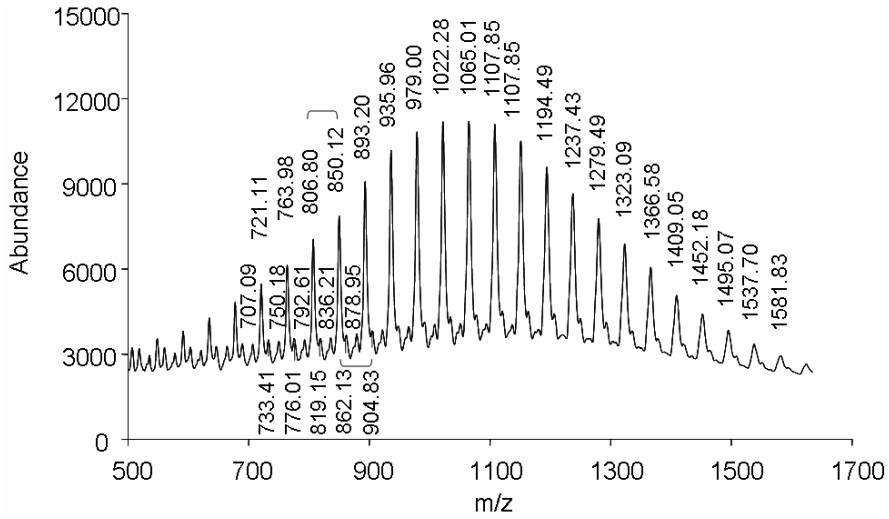
In contrast to the pOXZ chromatograms, a high molecular weight side fraction was not detected for the corresponding lPEI batches. This indicates that the bimodal mass distribution detected for the corresponding pOXZ batches was rather an artefact of the chromatographic procedure, than a side effect of the synthesis approach used.

GFC confirmed that we had synthesized a series of lPEIs with M_n between 1.0 and 8.1 kDa. lPEIs with an M_n below 5.0 were thereby obtained with a narrow molecular weight distribution, described by an PI below 1.3 (Table 2). Beyond this M_n range, the inhomogeneity of the polymer batch increased steadily.

In addition to GFC, we analyzed lPEI between 1.0 and 2.5 kDa (GFC) using MALDI-ToF. A molecular weight range between 0.7 and 1.7 kDa, 1.3 and 2.6 kDa, 1.3 and 2.8 kDa, or 2.2 and 3.6 kDa was thereby determined for lPEI 1.0 (Figure 9a), lPEI1.8, lPEI2.1, or lPEI2.5 (Figure 9b), respectively. Despite the high ionization energy applied, lPEI 3.0 only gave poor detector signals with low resolution. The molecular weight distribution, reflected by the series of individual signals, revealed differences of 42.0 – 43.1 kDa between the individual peaks (⌐¬), which we assigned to the molecular weight of the corresponding ethylenimine unit. The results were comparable irrespective to the matrix used, the supplementation with additives like LiCl or KCl, or the spotting technique applied (analyte, additive and matrix applied as mixture or drop-casted as single layers). The results obtained by MALDI-ToF spectra confirmed the data gained from ^{1}H-NMR experiments and GPC chromatograms, but it has to be considered that only a section of the whole MW range can be detected. While the low molecular weight fractions may be hidden beneath the matrix signals, remain the higher molecular weight

fraction not ionizable and thereby not analyzable by MALDI-ToF mass spectroscopy.

a)



b)

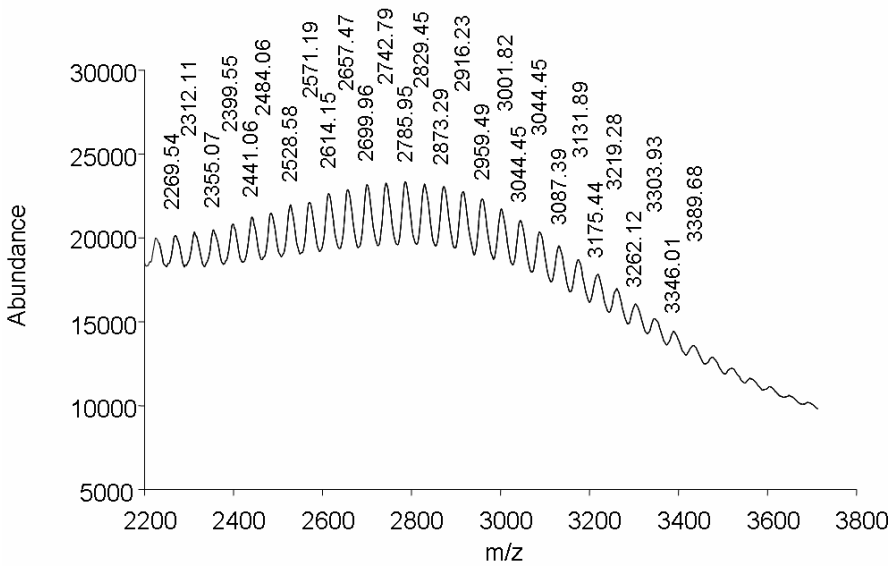


Figure 9. *MALDI-ToF spectra of a) lPEI 1.0 (GPC) and b) lPEI 2.5 (GPC). The molecular weight distribution, reflected by the series of individual signals, revealed differences of 42.0 – 43.1 kDa between the individual peaks (⌒), which we assigned to the molecular weight of the corresponding ethylenimine unit.*

Conclusion

In a parallel synthesis approach we synthesized a series of twelve pOXZ by the cationic ring-opening polymerization of 2-ethyl-2-oxazoline, using methyl-p-toluenesulfonate as initiator. We obtained pOXZ batches with relative M_n between 3.0 and 23.3 kDa and PIs between 1.02 and 1.44, as determined by GPC. On this basis we synthesized the series of twelve lPEIs by the acid-catalized hydrolysis of the corresponding pOXZ. The lPEIs were obtained with M_n between 1.0 and 8.1 kDa and PIs between 1.07 and 2.14, determined by a developed GFC method. ^{1}H-NMR spectra confirmed the conversion and MALDI-ToF measurements were used to get additional information about the composition of the polymer batches.

The series of lPEIs now gives us the opportunity to investigate the effect of the polymer molecular weight on the *in vitro* properties of the corresponding gene delivery vehicles.

Experimental section

2-ethyl-2-oxazoline (OXZ) and methyl-p-toluenesulfonate were purchased from Sigma-Aldrich. OXZ and acetonitrile were purified by distillation over calcium hydride (0.5 g/l) under nitrogen; p-toluenemethylsulfonate was dried under vacuum prior to use. Analytical grade triethylamine was obtained from VWR International, Germany, and HPLC grade chloroform was purchased from Carl Roth GmbH & Co KG, Germany. All other solvents were used in analytical grade without further purification. Sodium iodide, dithranol, and α-cyano-4-hydroxy cinnamic acid all in analytical grade were obtained from Fluka (Germany).

Polymer Synthesis

POLY (2-ETHYL-2-OXAZOLINE):

pOXZ was synthesized by the cationic ring-opening polymerization of OXZ in acetonitrile at 90 °C, initiated by methyl-p-toluenesulfonate (specific amouts listed in Table 3).

pOXZ	OXZ [ml]	OXZ [mmol]	MeOTs [mmol]	MeOTs [mg]	MeOTs - MeCN [ml]	MeCN [ml]	Yield [g]
24.2	6.25	62	0,27	50	1.8	17	6.95
23.3			0,32	58	2.1	17	7.06
19.4			0,38	71	2.6	16	6.64
18.9			0,45	83	3.0	16	6.52
15.4			0,53	99	3.6	15	6.03
14.1			0,6	110	4.0	15	5.96
11.6			0,67	124	4.5	14	6.58
10.6			0,77	142	5.2	14	6.72
8.8			0,89	165	6.0	13	6.6
7.2			1,07	199	7.2	12	6.73
6.0			1,32	245	9.0	10	6.56
3.0			2,67	496	18.0	0	6.73

***Table 3**. Reaction batches for the synthesis of the series of pOXZ*

The polymerization of OXZ was carried out in dried glassware under a nitrogen atmosphere, using the Synthesis 1 Liquid 12 parallel synthesis block (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). 18 ml of acetonitrile and 6.25 ml OXZ (0.0619 mol) were transferred into each tube with a disposable syringe. In order to synthesize twelve lPEIs of different molecular weight, a defined volume of a methyl-p-toluenesulfonate-stock solution (2.093 g in 75.0 ml acetonitrile) equivalent to a range between 0.27 and 2.67 mmol was added to the respective vial. The reaction mixture was heated to 90 °C, controlled within ± 2 °C and was kept under vigorous shaking [24]. After 6 days the mixture was cooled to room temperature, the volatiles were evaporated, the residue was dissolved in dichloromethane, and the crude polymer was precipitated in ice-cooled diethylether. The product was collected on a filter, washed twice with approximately 50 ml of ice-cooled diethylether, and dried under vacuum yielding 70 - 85 % pOXZ as white to slightly yellow powders.

^{1}H-NMR (400 MHz, $CDCl_3$/TMS): δ_H (ppm) = 0.81-1.4 (m, 3H, -NCOCH$_2$C*H$_3$*), 2.18-2.64 (m, 2H, -NCOC*H$_2$*CH$_3$), 3.13-3.92 (m, 4H, -N(COEt)C*H$_2$*C*H$_2$*-), 7.14-7.21 and 7.65-7.72 (d, 2+2, H_{Ar})

LINEAR POLYETHYLENIMINE:

The acidic hydrolysis of the corresponding pOXZ (in fractions of 1.3 g) was performed in boiling 6 N HCl (25 ml) over a period of 24 hours [24] using the parallel synthesis block. Thereafter, the mixture was allowed to cool to room temperature and the excess of hydrochloric acid was evaporated under vacuum at 60 °C. Water was added to the white residue until a clear solution was formed. A saturated aqueous sodium hydroxide solution was added until the crude polymer precipitated. The precipitate was washed with ice-cooled water by centrifugation until the supernatant became neutral. The polymer was recrystallized from boiling ethanol and dried under vacuum at 70 °C yielding 50 - 65 % lPEI.

^{1}H-NMR (400 MHz, $CDCl_3$/TMS): δ_H (ppm) = 1.44-1.93 (brs, -N*H*CH$_2$CH$_2$-), 2.62-2.86 (m, 4H, -NC*H$_2$*C*H$_2$*-), 3.61-3.67(m, 2H, -CH$_2$C*H$_2$*OH), 2.42-2.45(s, 3H, C*H$_3$*NH-)

Size exclusion chromatography (SEC)

The relative MW was determined by size exclusion chromatography (SEC) using a Shimadzu HPLC system (probe concentration: 20 mg/ml; injection volume: 25 µl; SIL-10ADvp auto injector, LC-10ATvp liquid chromatograph, DGU 14A degaser, CTO-0ASvp column oven, SCL-10Avp system controller; RID-10A refractive index detector thermostatted at 40 °C). The number average molecular weight (M_n), the weight average molecular weight (M_w) and M_w/M_n were calculated using a ClassVP 5.03 GPC software package from Shimadzu (Germany). All calculations were done without the use of the Mark-Houwink constants, determining molecular weight values only relative to the applied polymer standard.

POLY (2-ETHYL-2-OXAZOLINE) (GEL PERMEATION CHROMATOGRAPHY)
pOXZ derivatives were dissolved in chloroform, filtered (Spartan 30/0.2 RC filter, Schleicher & Schuell, Germany), and separated on a 1000 Å Phenogel column (5 µm, 300 x 7.8 mm, Phenomenex, Torrance, CA) thermostatted at 40 °C. A flow rate of 1 ml/min and chloroform or 4 % triethylamine / chloroform as eluent were used [40]. The relative M_n, M_w, and M_w/M_n of pOXZs were determined by SEC using polystyrene standards between 0.95 kDa and 68 kDa (Phenomenex, Torrance, CA; Merck, Darmstadt, Germany).

LINEAR POLYETHYLENIMINE HYDROCHLORIDE (GEL FILTRATION CHROMATOGRAPHY))
20 mg lPEI*HCl was dissolved in 1.0 ml double distilled water (ddH_2O) and filtered through a 0.2 µm polyethersulfonic acid membrane filter. A Novema 300 Å SEC column (10 µm, 8 x 300 mm, Polymer Standard Service, Mainz) thermostatted at 40 °C, a flow rate of 1 ml/min, and 0.15 M NaCl as eluent were used as chromatographic setup. The relative M_n, M_w, and M_w/M_n of lPEIs were calculated from the elution volume of dextrane standards between 1.05 kDa and 43.5 kDa (Polymer Standard Service, Mainz).

MALDI-ToF

The spectra were recorded on a HP G2030A MALDI-ToF MS system (HP G 2025A MALDI-ToF MS spectrometer from Hewlett Packard, Germany).

POLY (2-ETHYL-2-OXAZOLINE):
1 µl pOXZ dissolved in chloroform (1 mg/ml), 1 µl sodium iodide-acetone solution (43.35 µg/µl) and 1 µl of freshly prepared dithranol-chloroform solution (20 mg/ml) was mixed and dropcasted as one layer onto the MALDI-ToF sample target [50].

LINEAR POLYETHYLENIMINE:
1 µl lPEI·HCl dissolved in double distilled water (1 mg/ml) and 3 µl α-cyano-4-hydroxy cinnamic acid-methanol solution (10 mg/ml) were mixed and dropcasted onto the MALDI sample target.

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5. LOW MOLECULAR WEIGHT LPEI - PLASMID DNA POLYPLEXES: PARTICLE PROPERTIES AND TRANSFECTION

Uta Lungwitz, Miriam Breunig, Renate Liebl, Daniela Pesl, Robert Knerr, Torsten Blunk, and Achim Göpferich

Department of Pharmaceutical Technology, University of Regensburg, 93040 Regensburg, Germany

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Abstract

Recently we showed that the transfection efficacy and the cell compatibility of low molecular weight linear polyethylenimine (lmw lPEI) – plasmid DNA (pDNA) polyplexes are a function of the molecular weight (MW) and concentration (N/P ratio: refers to the quotient of the nitrogen atoms of the polymer to DNA phosphates) of the polyamine. Here we systematically investigated the impact of the lPEI MW on the particle properties of lPEI – pEGFP-N1 (reporter plasmid) polyplexes and the polymer toxicity in order to clarify whether a correlation to the transfection efficacy can be found. Therefore, lPEI – pDNA complexes were formed with lPEI of 3, 5, and 8.1 kDa at different N/P ratios in medium of low or high ionic strength. The polyplexes were characterized prior to and after exposure to serum-free (HAM´s F12) or serum-containing (HAM´s F12+10 % fetal calf serum (FCS)) cell culture conditions to access the particle changes occurring during the transfection experiment. The polyplex characterization revealed that the lmw lPEI – pDNA complexes exhibited comparable particle size, compactness, and surface charge, modulated by the ionic strength of the complexation medium. The corresponding lPEI – pDNA polyplexes were prone to particle aggregation and showed a lowered degree of pDNA compaction under cell culture conditions. Nevertheless, the complexation medium influenced the particle size of polyplexes present during the transfection experiment.

We investigated the polymer toxicity in a CHO-K1 cell culture by flow cytometry analysis, determined as CV_{50} values (polymer concentration [µg/µl] that reduces the relative cell viability of CHO-K1 cells *in vitro* to 50 %). We showed that the toxicity of the lPEI polymers and of its corresponding polyplexes was influenced by the lPEI molecular weight.

Introduction

A common goal in non-viral gene transfer is the development of efficient and cell compatible gene carriers [1;2]. Polyplexes prepared by the complexation of pDNA with linear (lPEI) or branched polyethylenimine (bPEI) were introduced as potent non-viral vectors, delivering their cargo with a relatively high transfection efficacy to a large variety of cells [3-6]. It has been shown that the polymer molecular weight, concentration and branching degree has a significant impact on the membranolytic activity [7], the cytotoxicity, the nuclear concentration [8], as well as the intracellular disintegration of the PEI – pDNA complexes [9], which interdependently influences the *in vitro* performance of the corresponding transfection agent [10]. Unfortunately, most of these studies were limited to commercially available bPEI of 25, 70 or 800 kDa (bPEI25, bPEI70 or bPEI800), and lPEI of 22 or 25 kDa (lPEI22 or lPEI25).

We hypothesized that, lPEI-derived vectors combine a higher transfection efficacy with less cytotoxicity compared to bPEI-derived vectors. This effect may be apparent at an optimum MW range that facilitates sufficient pDNA compaction and maximum transgene expression, at a polymer concentration range of negligible cytotoxicity. In order to prove the capacity of this concept we synthesized a series of twelve low molecular weight lPEIs (lmw lPEI) with number average molecular weights (M_n) between 1.8 and 8.1 kDa, and investigated the *in vitro* performance of the corresponding vectors [11;12]. We observed an improved transfection efficacy and cell compatibility of lmw lPEI – pDNA polyplexes, compared to lPEI 22 kDa equivalents, with a strong correlating to the molecular weight of the lPEI and the N/P ratio applied.

However, mechanistic studies, investigating the intracellular faith of the lmw lPEI – pDNA polyplexes in CHO-K1 cells *in vitro*, did not explain this phenomenon on the basis of their cellular uptake, intracellular stability and transport [13].

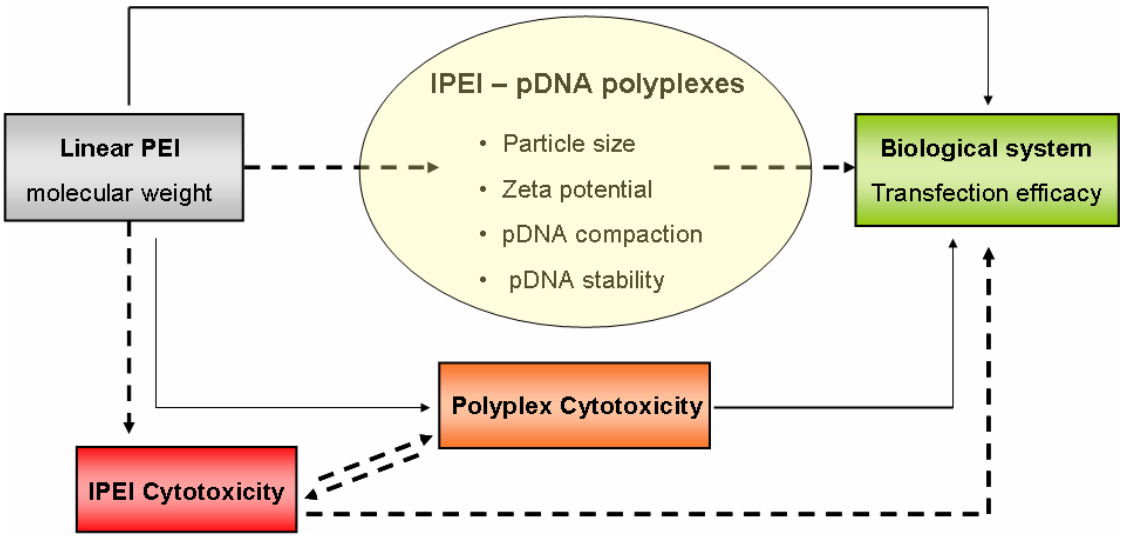


Figure 1. *This study was aimed to clarify whether the correlation of transfection efficacy and of the polyplex cytotoxicity with the M_n of lmw lPEIs (solid arrows) found in transfection experiments with CHO-K1 cells in vitro, are originated from the impact of the lPEI polymerization degree on the lPEI – pDNA complex properties as well as on the polymer cytotoxicity (M_n) (dashed arrows).*

It has been shown that the efficacy of *in vitro* gene transfer is also influenced by the particle properties [5;14;15]. Hence, based on the series of lmw lPEIs, this study was aimed to systematically investigate whether a correlation of the *in vitro* transfection efficacy with the M_n of lmw lPEIs is originated from its impact on the particle size, surface charge, or stability of lPEI – pDNA complexes (Figure 1).

For this purpose, we built polyplexes from lPEIs of 1.8, 3, 5, and 8.1 kDa (M_n (determined by Gel Filtration Chromatography (GFC)), which have been shown to exhibit significantly different transfection efficacy and cell compatibility. In view of the sensitivity of lmw lPEI polyplexes to the ionic strength of the ambient medium [5;14;15], the corresponding polyplexes were characterized prior to and after exposure to cell culture conditions. In contrast to previous work, this would allow us to detect polyplex changes, which occur

during the transfection experiment. We performed our experiments in direct comparison to bPEI 25 (bPEI25) kDa – pDNA complexes to also access differences due to the branched polyamine structure.

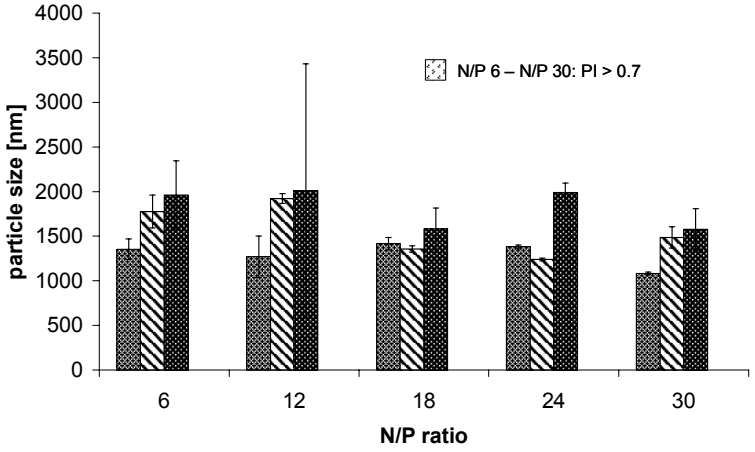
Results

In order to investigate the impact of the lPEI MW on the particle size, we determined the polyplex hydrodynamic diameters at a physiological salt concentration (0.15 M NaCl: with equivalent osmolarity to physiological fluids), as well as in 5 % glucose, which allowed to assess individual polyplexes. At physiological salt concentrations lPEI8.1, lPEI5, and lPEI3 formed polyplexes with pDNA leveling at hydrodynamic diameters between 1.1 and 2.1 µm and polydispersity indices (PI) between 0.2 and 0.7 at an N/P ratio of 18, irrespective to the lPEI M_n. A minimum lPEI M_n above 1.8 kDa was necessary to enable the formation of polyplexes with a particle size below 5 µm and a PI below 0.7 (Figure 2a) (revealing reliable particle size values by laser light scattering).

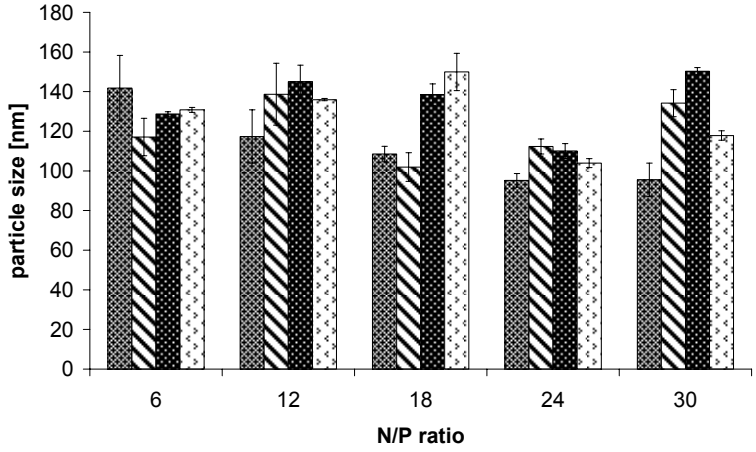
In contrast to polyplexes prepared in medium with high ionic strength, the mean hydrodynamic diameter of particles in 5 % glucose ranged from 100 nm to 150 nm (Figure 2b), irrespective to the M_n of the lPEI or N/P ratio applied. The PI values between 0.4 and 0.7 indicate a broad particle size distribution.

Atomic force microscope (AFM) imaging was performed on samples covered with medium (intermittent fluid mode) in order to detect the polyplex hydrodynamic diameters. The images show large irregular shaped structures, which conceivably result from the aggregation of lPEI – pDNA complexes in 0.15 M NaCl (Figure 2c), as well as small individual polyplexes after assembly in 5 % glucose (Figure 2d).

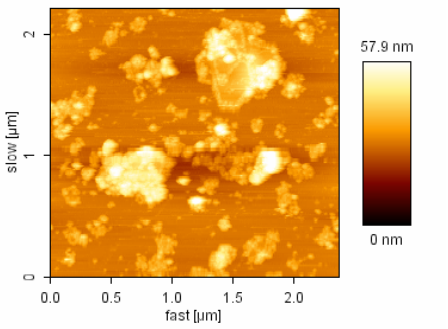
a)


4000
3500
3000
2500
2000
1500
1000
500
0
particle size [nm]
N/P 6 – N/P 30: PI > 0.7
6
12
18
24
30
N/P ratio

b)


180
160
140
120
100
80
60
40
20
0
particle size [nm]
6
12
18
24
30
N/P ratio

c)


57.9 nm
0 nm
slow [µm]
fast [µm]
0.0
0.5
1.0
1.5
2.0

d)

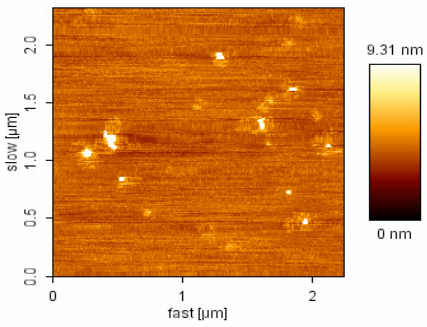
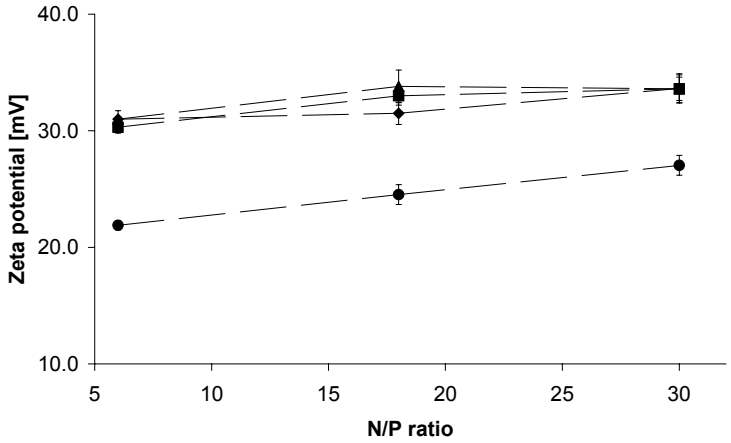

9.31 nm
0 nm
slow [µm]
fast [µm]
0.0
0.5
1.0
1.5
2.0

***Figure 2**. Particle size (m.v. + SD) of lPEI8.1[▩]-, lPEI5 [▧]-, lPEI3 [▩]-, and lPEI1.8 [⚄] – pDNA polyplexes in a) 0.15 M NaCl or b) 5 % glucose prepared at N/P ratios of 6, 12, 18, 24, and 30. AFM images were taken from lPEI8.1 – pDNA polyplexes in c) 0.15 M NaCl or b) 5 % glucose prepared at an N/P ratio of 6, using the intermittent fluid mode and scan rate of 0.3 or 1 Hz, respectively.*

lPEI8.1 –, lPEI5 –, and lPEI3 – pDNA polyplexes in 0.15 M NaCl exhibited comparable positive net charge between 30 and 34 mV (Figure 3a). Here in comparison, lPEI1.8 – pDNA adducts showed significantly reduced zeta potentials between 22 and 27 mV. The surface charge of polyplexes dispersed in 5 % glucose, reached mean values between 21 and 50 mV, which slightly increased with the polymer concentration from an N/P ratio of 6 to 30 (Figure 3b). Overall, we observed that the particle size and the zeta potential of lmw lPEI – pDNA complexes were dramatically influenced by the presence of salts in the ambient medium, while the effect of the lPEI M_n was negligible.

a)



b)

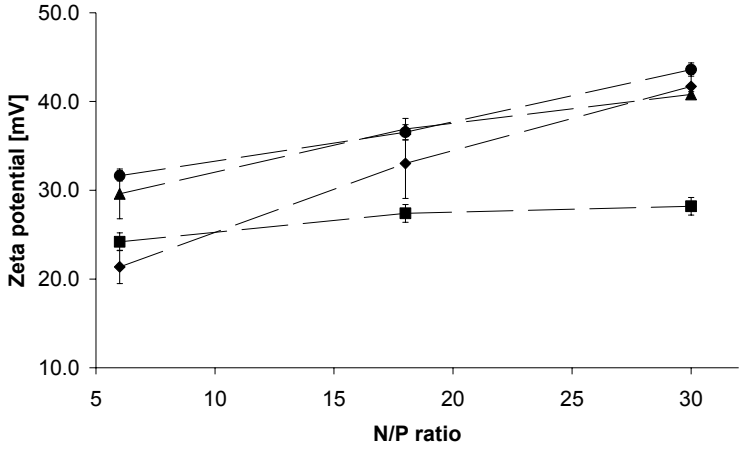


Figure 3. *Zeta potentials (m.v. + SD) of lPEI8.1 [♦] –, lPEI5 [■] –, lPEI3 [▲] –, lPEI1.8 [●] – pDNA polyplexes, prepared in either a) 0.15 M NaCl or b) 5 % glucose at an N/P ratio of 6, 18, and 30.*

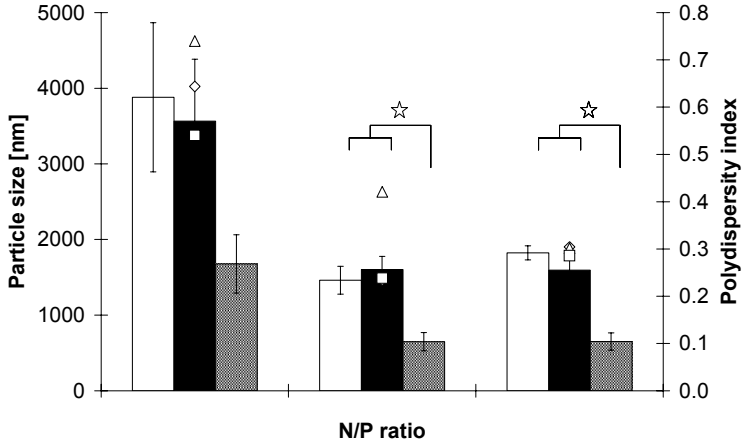
Next we investigated the effect of serum-free and serum-containing transfection medium (HAM´s F12 or HAM´s F12+10% FCS) on the particle properties, exemplarily for polyplexes formed with lPEI8.1 either in 0.15 M NaCl or 5% glucose.

In the corresponding experiments lPEI25 – and bPEI25 – pDNA complexes, which are according to the literature still considered as the polycationic gene carriers of choice, were used as positive control.

The hydrodynamic diameters of both, lPEI- and bPEI- derived polyplexes, decreased with increasing polymer concentration and leveled at an N/P ratio of 18 ($p < 0.01$) (Figure 4a). While the size of lPEI – pDNA complexes steadied at 1500 nm, bPEI25 formed significantly smaller polyplexes at the sub-micrometer scale (800 nm; $p<0.01$).

We determined the hydrodynamic diameters of polyplexes at multiple time points during about 46 minutes incubation in serum-free HAM´s F12, focusing on complexes formed at a mid N/P ratio of 18.

a)



b)

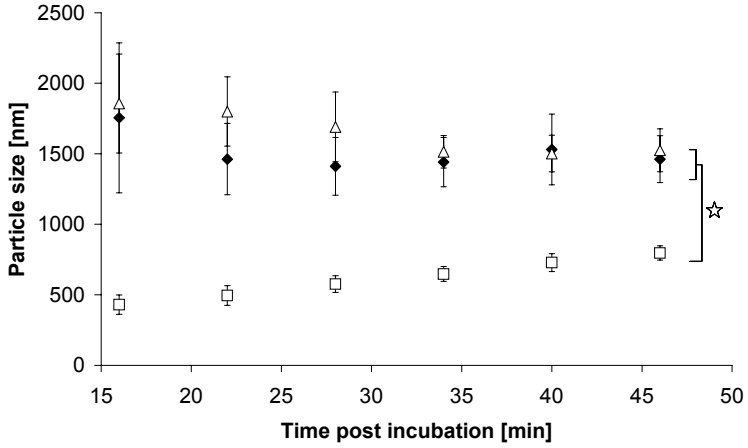


Figure 4. *a) Particle size (m.v.+SD) / PI(m.v.) of lPEI8.1 (□ / ◊)-, lPEI25 (■ / ∆)-, and bPEI25 (▩ / □) – pDNA polyplexes, prepared in 0.15 M NaCl at an N/P ratio of 6, 18 and 30 after 30 minutes incubation in serum-free cell culture medium (n=3; differences with a significance level of p < 0.01 are depicted by [☆], respectively).*

b) Particle size (m.v.+SD) of lPEI8.1 (♦)-,lPEI25 (∆)-,and bPEI25 (□) – pDNA particles, prepared in 0.15 M NaCl at an N/P ratio of 18, determined during 46 minutes incubation in serum-free cell culture medium. (n = 3, differences with a significance level of p < 0.01 are depicted by [☆], respectively)

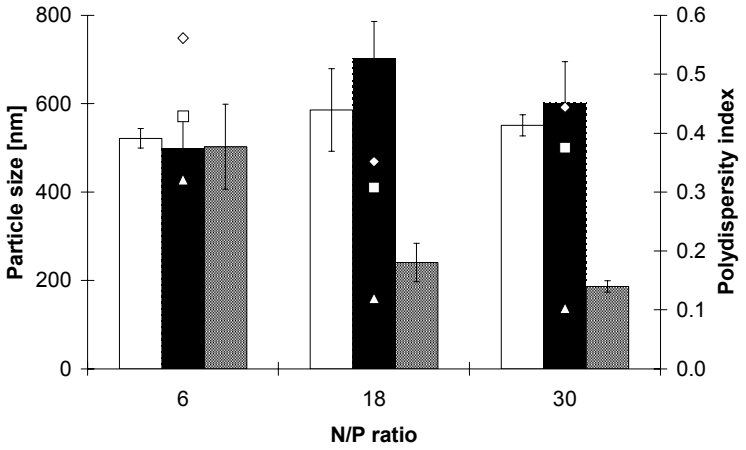
While the mean size of the corresponding particles remained almost constant, bPEI-derived polyplexes increased from 450 nm to 600 nm with time ($p < 0.01$) (Figure 4b).

In order to investigate the effect of a physiological salt concentration on the particle size of polyplexes, preformed in medium of low ionic strength, pDNA complexation was performed in 5 % glucose and the hydrodynamic diameters were determined during 35 minutes incubation in serum-free cell culture medium.

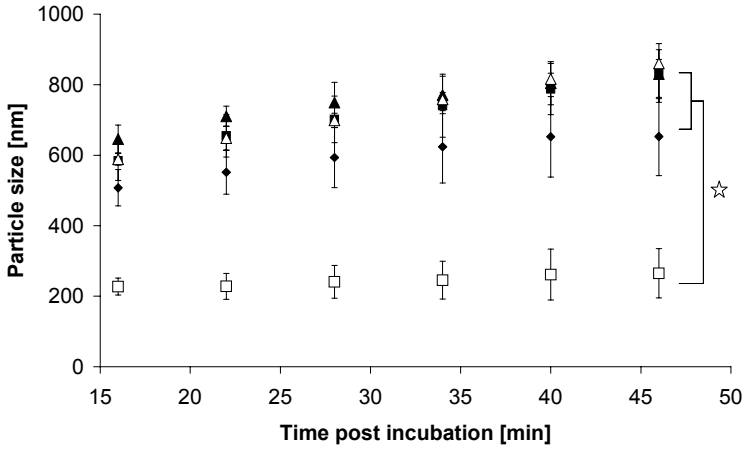
In contrast to lPEI – pDNA polyplexes, which exhibited a particle size between 500 - 850 nm, irrespective to the N/P ratio applied (Figure 5a), the hydrodynamic diameters of bPEI25 – pDNA complexes decreased with the N/P ratio from 550 nm at an N/P ratio of 6 to nearly 200 nm at an N/P ratio of 18 and 30. Similar to the particles formed in high ionic strength medium, bPEI25 compacted pDNA to significantly smaller polyplexes compared to the linear polyamine ($p < 0.01$).

In order to investigate whether particle growth proceeds differently as a function of the lPEI molecular weight, we determined the hydrodynamic diameters of lPEI8.1 –, lPEI5 –, lPEI3 –, and lPEI25 – pDNA complexes at multiple time points during approximately 46 minutes of incubation in serum-free cell culture medium. While the hydrodynamic diameter of bPEI25 – pDNA polyplexes remained constant, lPEI5 –, lPEI3 – and lPEI25 – pDNA particles grew steadily in size ($p < 0.05$), reaching maximum values of nearly 800 nm after 40 minutes (Figure 5b). For lPEI8.1 – pDNA polyplexes a significant increase of the hydrodynamic diameter was only observed after 30 minutes at an N/P ratio of 30, or 90 minutes at an N/P ratio of 18 (Figure 5c). Nevertheless, it has to be considered that during the long time particle size measurements the count rates steadily decreased to 80 % of the initial value, indicating that sedimentation of larger aggregates may have occurred (data not shown).

a)


Particle size [nm]
Polydispersity index
N/P ratio
0
200
400
600
800
0.0
0.1
0.2
0.3
0.4
0.5
0.6
6
18
30

b)


Particle size [nm]
Time post incubation [min]
0
200
400
600
800
1000
15
20
25
30
35
40
45
50

c)

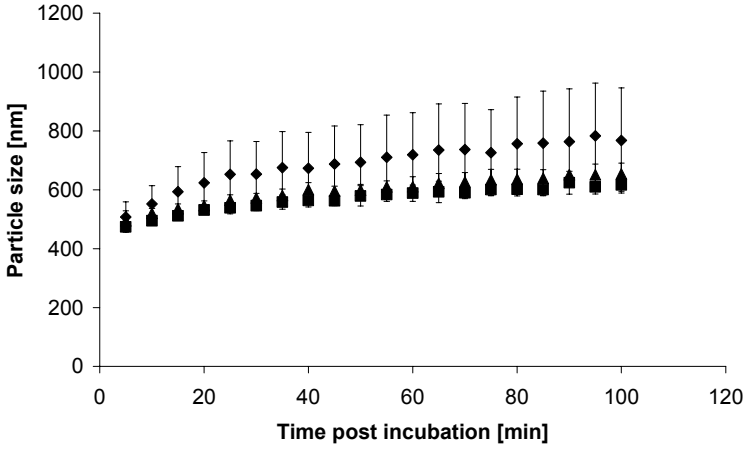

Particle size [nm]
Time post incubation [min]
0
200
400
600
800
1000
1200
0
20
40
60
80
100
120

Figure 5.

a) Paricle size (m.v. + SD) / PI (m.v.) of lPEI8.1(□ / ◊)–, lPEI25 (■ / ∆)–, and bPEI25 (▩ / □) – pDNA polyplexes, prepared in 5 % glucose at an N/P ratio of 6, 18, and 30, determined after 35 minutes of incubation in serum-free cell culture medium. (n=3; significance level of p < 0.01 are depicted by [☆]).

b) Paricle size (m.v. + SD) of lPEI8.1 (♦) –, lPEI5 (■) –, lPEI3 (▲) –, lPEI25 (∆) –, and bPEI25 (□) – pDNA polyplex prepared in 5 % glucose at an N/P ratio of 18, determined during 46 minutes of incubation in serum-free cell culture medium. (n=3; significance level of p < 0.01 are depicted by [☆]).

c) The Paricle size (m.v. + SD) of lPEI8.1 – pDNA polyplexes prepared in 5 % glucose at N/P 6 (♦)-, N/P 18 (■)-, and N/P 30 (▲) determined during 100 minutes of incubation in serum-free cell culture medium.

In accordance to the literature, size measurements of PEI-based polyplexes in the presence of serum-containing cell culture medium suggested that the particle size seemed to be conserved in the range between 150 and 350 nm [15]. However, we observed that the PIs exceeded mostly 0.7, so that data obtained by laser light scattering analysis was not meaningful (data not shown).

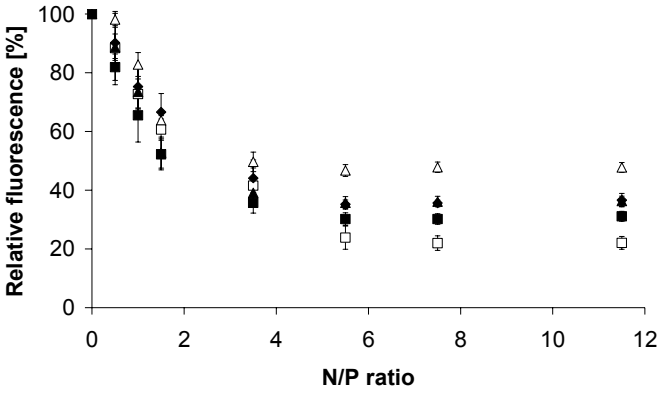
We used DNA-accessibility assays to investigate the impact of the ambient medium, of the lPEI M_n, and the PEI architecture on the efficacy of pDNA compaction. In 0.15 M NaCl the relative fluorescence values were comparable up to an N/P ratio of 3.5 (Figure 6a). At further increased polymer concentration, the relative fluorescence of the ethidium bromide – pDNA intercalate (pDNA-EB) was reduced to 25 % with bPEI25, and values between 33 and 45 % with the series of lPEIs. Overall, the pDNA compaction seemed to be most efficient in medium of low ionic strength. We observed a complete fluorescence quenching upon the addition of bPEI25 at an N/P ratio of 5.5, while the lPEIs reduced the relative pDNA-EB fluorescence to minimum values between 10 and 15 % (Figure 6b).

In order to clarify whether the exposure to serum-free or serum-containing cell culture medium alters the extent of pDNA compaction in preformed polyplexes, we exposed lPEI – and bPEI25 – pDNA complexes to HAM´s F12 or HAM´s F12+10 % FCS.

Therefore, we used particles that differed by its compactness, due to their formation either in 5 % glucose or 0.15 M NaCl.

After addition of cell culture medium to bPEI25 – and lPEI25 – pDNA-EB complexes, the relative fluorescence values remained constant (30 – 50 %).

a)



b)

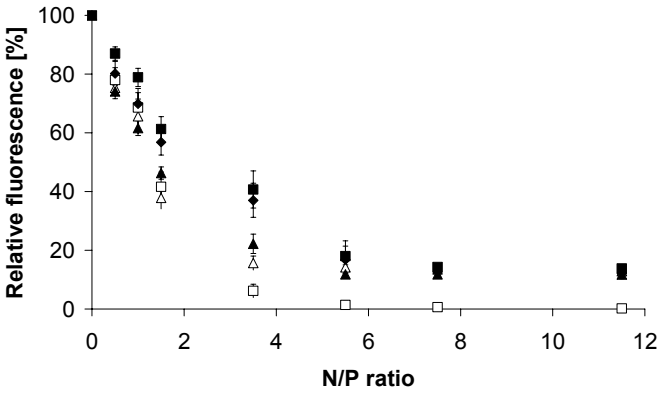


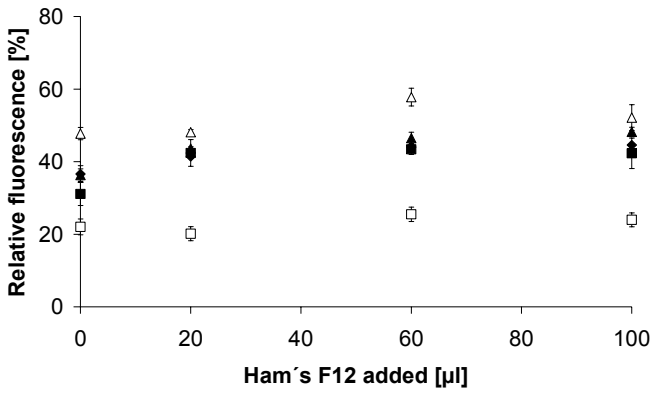
Figure 6. *The relative fluorescence intensity of the pDNA-EB after the addition of a bPEI25 [□]-, lPEI25 [∆]-, lPEI8.1 [♦]-, lPEI5 [■]-, or lPEI3 [▲]-solution*

a) in 0.15 M NaCl or

b) in 5 % glucose

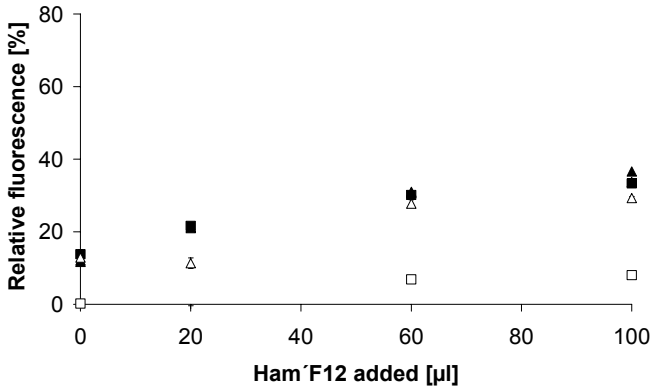
was determined at λ_{em} *= 590 nm after excitation at* λ_{ex} *= 485 nm.*

c)



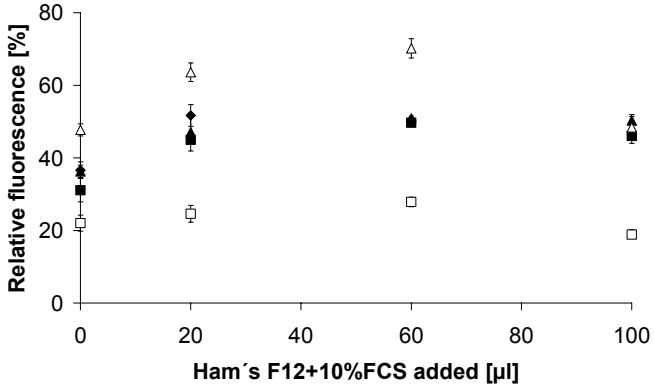
c) *The relative fluorescence intensity of preformed bPEI25 [□] –, lPEI25 [∆] –, lPEI8.1 [♦] –, lPEI5 [■] –, or lPEI3 [▲] – pDNA polyplexes after incubation in serum-free cell culture medium was determined*
c) in 0.15 M NaCl

d)



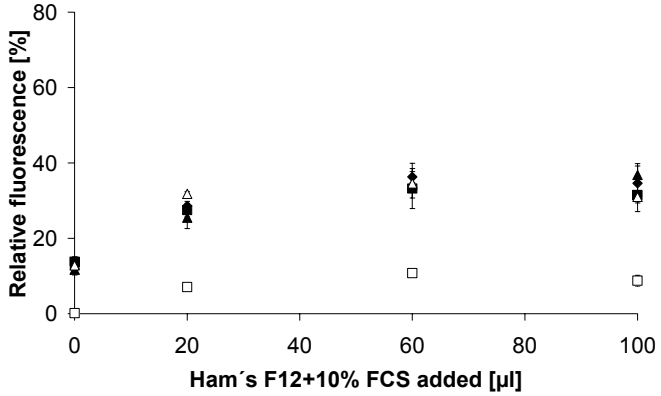
d) in 5 % glucose

e)



c) *The relative fluorescence intensity of preformed bPEI25 [□] –, lPEI25 [∆] –, lPEI8.1 [♦] –, lPEI5 [■] –, or lPEI3 [▲] – pDNA polyplexes after incubation in serum-containing cell culture medium was determined*
c) in 0.15 M NaCl

f) *f) in 5 % glucose*



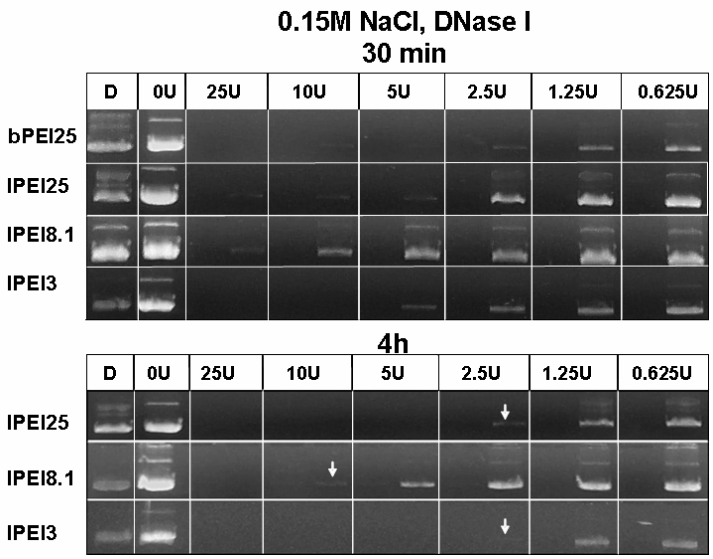
For the corresponding lmw lPEI – pDNA-EB polyplexes, we observed a slight increase to relative fluorescence intensity between 45 and 50 % leveling after 20 µl medium added ($p < 0.05$) (Figure 6c and d). This effect was most pronounced for PEI – pDNA-EB complexes, which were prepared in the absence of electrolytes (5 % glucose).

We observed up to a two-fold increase of the relative fluorescence values for lPEI-derived polyplexes, and a recovery to 10 % relative fluorescence intensity for bPEI25 – pDNA-EB complexes upon the exposure to cell culture medium ($p < 0.01$) (Figure 5e and f). bPEI exhibited superior pDNA compaction ability compared to lPEI, irrespective of the conditions applied. These effects were observed irrespective to the presence of serum proteins.

As the DNA-accessibility assay suggests, pDNA seemed to be less efficiently compacted in 0.15 M NaCl compared to complexes formed in 5 % glucose. We assumed that this may also affect the stability of pDNA upon the exposure of degrading enzymes.

Hence, we investigated the capacity of different lPEIs (lPEI25, lPEI8.1, and lPEI3) and bPEI25 to protect pDNA from degradation during incubation with DNase I or II. The corresponding polyplexes were built either in 5 % glucose or 0.15 M NaCl at an N/P ratio of 10, incubated with DNase I for 0.5 or 4 hours. The c dissociation of the complexes was induced at an alkaline pH, and the intact pDNA bands were detected by ethidium bromide staining.

a)



b)

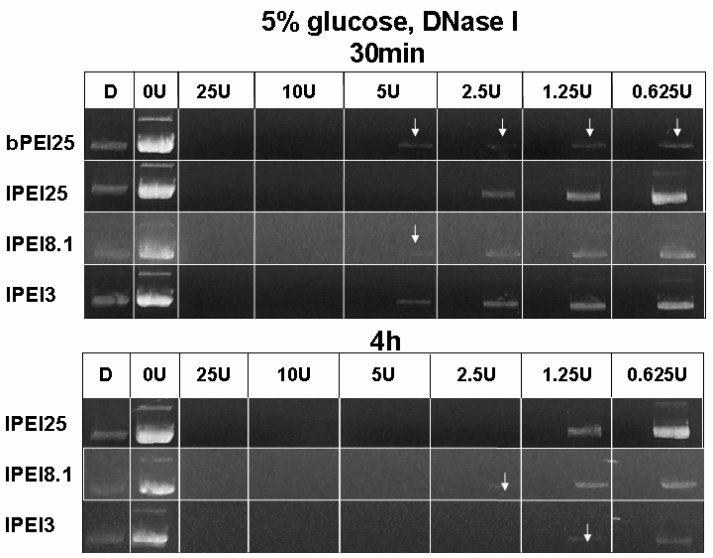


Figure 7. *pDNA was complexed by lPEI8.1, lPEI3, lPEI25 or bPEI25 in either 0.15 M NaCl (a) or 5 % glucose (b) at an N/P ratio of 10. The corresponding polyplexes were incubated with various amounts of DNase I for 30 minutes or 4 hours at 37°C. To visualize enzyme independent pDNA degradation, pDNA (D) and polyplexes (0U) were incubated in the absence of DNase I. The reaction was stopped by the addition of EDTA at elevated pH and the integrity of the pDNA was examined using gel electrophoresis.*

The increase of the incubation time led to the complete pDNA digestion at lower enzyme concentrations. pDNA of lPEI – pDNA complexes built in 0.15 M NaCl was completely digested at enzyme concentrations between 25 and 2.5 U (Figure 7a). However, pDNA was less protected by compaction with lPEI in 5 % glucose (10 - 1.25 U) (Figure 7b). bPEI25 protected pDNA to lower extent from enzymatic digestion (2.5 - <0.625 U) compared to the linear polyamines, irrespective of the complexation medium applied.

The DNase II experiments revealed no remarkable differences in the pDNA stability depending on the polycation, complexation medium or enzyme concentration applied (data not shown).

We determined the capacity of pDNA complexation for lPEIs with a molecular weight of 25, 8.1, 5, 3, and 1.8 kDa, as well as bPEI25, in order to clarify whether the efficacy of pDNA retardation by the polyamine is a function either of its molecular weight, the PEI architecture, or the presence of electrolytes in the ambient medium. We observed that, irrespective to the MW or polymer connectivity, the applied PEIs exhibited a comparable pDNA complexation capacity, retarding pDNA to the complex at N/P ratios above 2 (N/P ratio applied: 0, 1, 2, 3, 6, 12, 18, 24) (data not shown). After complete retardation to lPEI in 5 % glucose, pDNA was still stained as diffuse band mostly located in the gel pocket, while the pDNA-EB fluorescence was completely quenched in polyplexes formed in 0.15 M NaCl. This phenomenon was not observed for bPEI25 – pDNA complexes.

In order to investigate whether the exposure to serum-free cell culture medium alters the capacity of pDNA complexation in preformed polyplexes, we incubated lPEI5 – pDNA complexes, exemplarily for the series of lPEI-derived polyplexes, and bPEI25 – pDNA particles in HAM´s F12 prior to electrophoresis. We observed complete pDNA retardation at N/P ratios above 1.5, irrespective to the complexation medium or polyamine applied (Figure 8a-d). Upon the exposure of polyplexes to serum-free transfection medium, the pDNA complexation capacity of lPEI5 was reduced, leading to complete

pDNA retardation at N/P ratios above 2, while pDNA complexation by bPEI25 remained unaltered (Figure 8e-h).

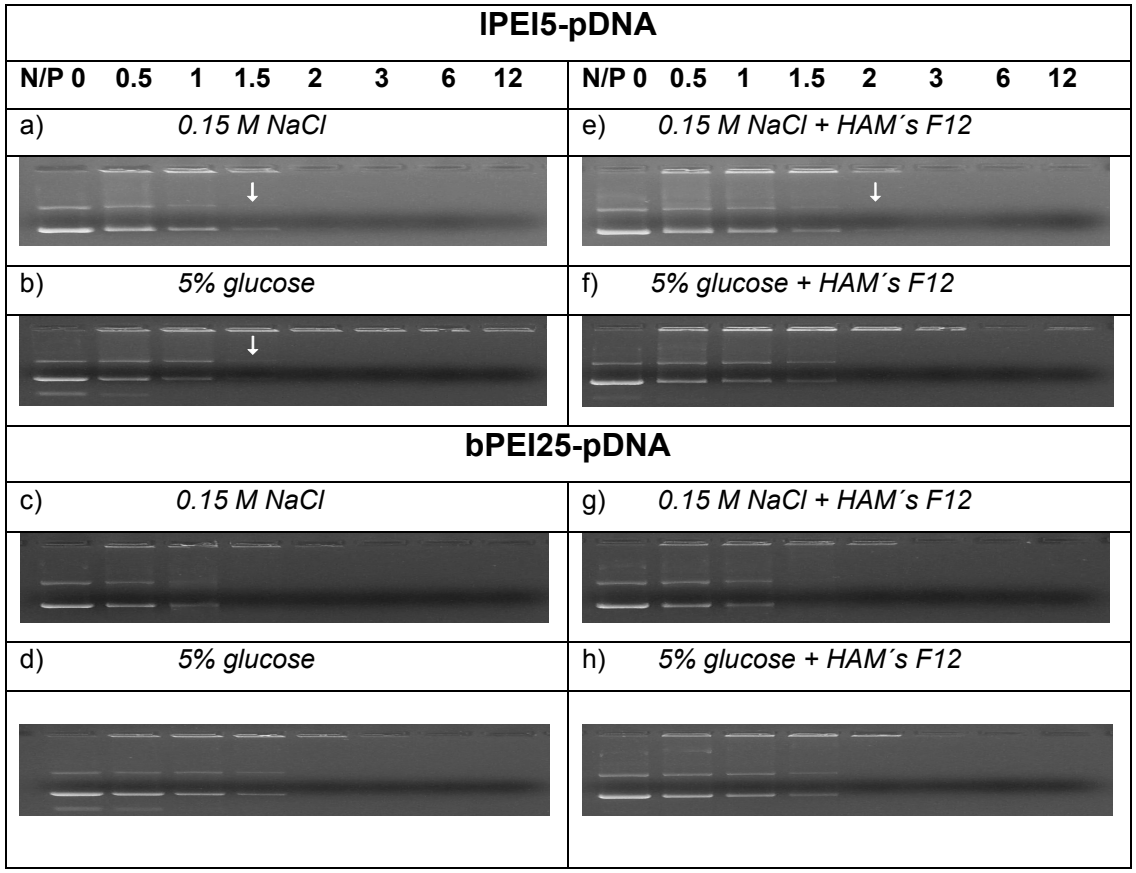
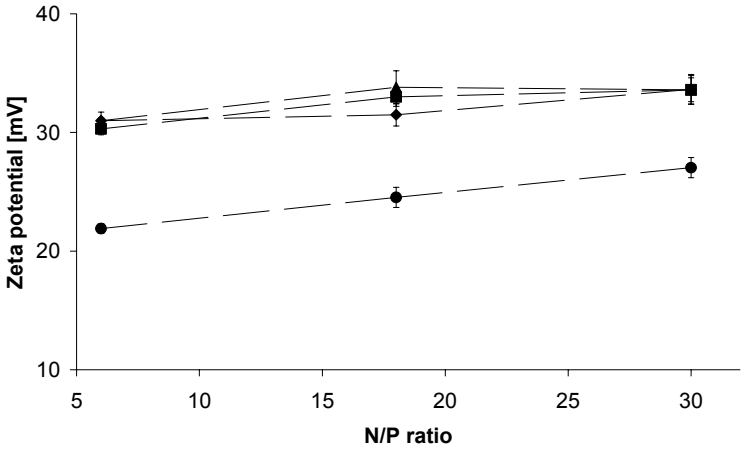


Figure 8. *lPEI5 – pDNA or bPEI25 – pDNA polyplexes were prepared either in 0.15 M NaCl (a / c) or 5 % glucose (b / d), and thereafter exposed to serum-free cell culture medium (NaCl + HAM´s F12: e / g or 5 % glucose + HAM´s F12: f / h), respectively. The electrophoretic mobility of pDNA was determined at 80 V, visualizing pDNA by ethidum bromide staining.*

In view of the content of salts, amino acids, or serum proteins in commonly used serum-free or serum-containing cell culture medium, we also determined the surface charge of PEI-derived polyplexes after the exposure to HAM´s F12 or HAM´s F12+10 % FCS.

PEI-based polyplexes prepared in 5 % glucose exhibited comparable surface charge of 5 mV to 25 mV after incubation in serum-free cell culture medium, irrespective to the polycation or N/P ratio applied ($p<0.01$) (Figure 9a).

a)



b)

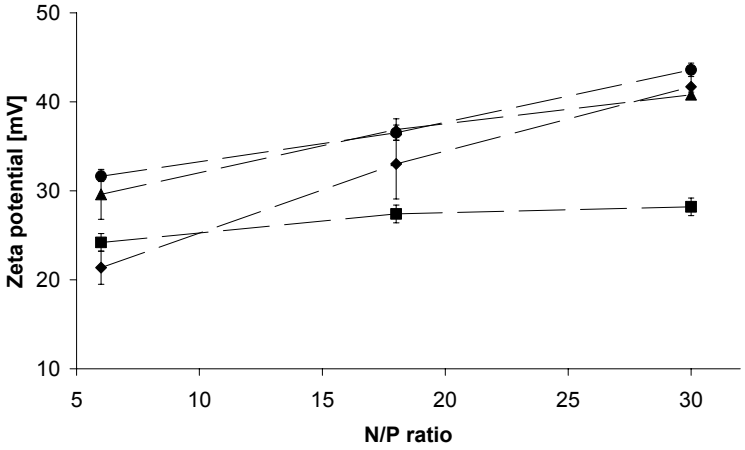


Figure 9. *Zeta potential of lPEI25 (Δ) –, bPEI25 (□) –, and lPEI8.1 (◊) – pDNA polyplexes, prepared in 5 % glucose at an N/P ratio of 6, 18, or 30, determined after 30 minutes incubation in a) serum-free or b) serum-containing cell culture medium.*

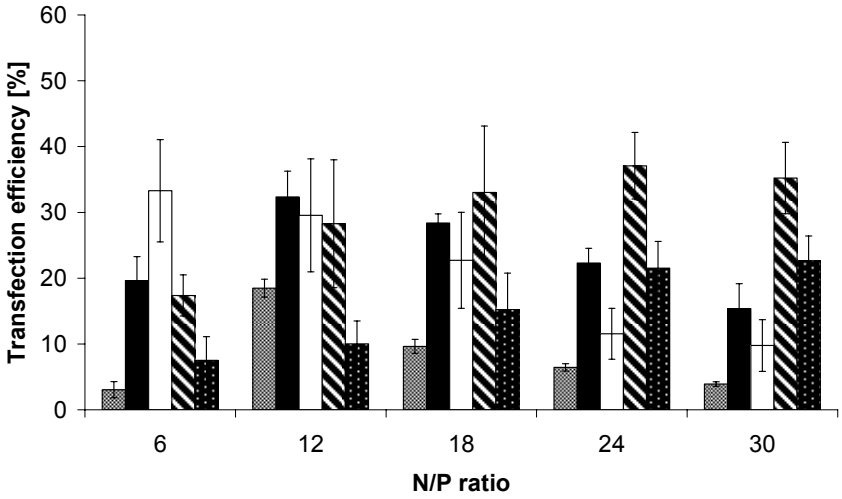
Polyplexes exposed to HAM´s F12+10 % FCS exhibited a negative net charge between -11 mV and -9 mV (m.v.). The surface charge increased thereby with the N/P ratio to -2 mV (m.v.) or -4 mV (m.v.) for lPEI8.1-, or bPEI25 – pDNA complexes, respectively, while the zeta potential of lPEI25 – pDNA polyplexes remained at similar values ($p < 0.01$) (Figure 9b). We observed a comparable surface charge for polyplexes, which have been prepared in 0.15 M NaCl (data not shown).

To investigate whether a correlation between the particle properties and the efficacy and cell-compatibility of gene transfer can be found, we determined the number of successfully transfected cells (Figure 10) and the cell viability (Figure 11) after treatment with lmw lPEI3 –, lPEI5 –, and lPEI8.1 – pDNA polyplexes, using an *in vitro* CHO-K1 cell assay. As the hydrodynamic diameters were only determinable for polyplexes in serum-free HAM´s F12, we performed the gene transfer experiments only under serum-free cell culture conditions, using either 5 % glucose or 0.15 M NaCl as medium for the polyplex preparation. In contrast to previous studies, we also determined the transfection efficacy and cell viability after incubation with bPEI25 – pDNA complexes to also enable a comparison to branched polyamine fabricated polyplexes.

Generally, the transfection efficacy increased with the N/P ratio applied, reached maximum transfection, leveled off at a plateau, and decreased at a further enhanced polymer concentration (Figure 10). lPEI25 –, lPEI8.1 –, or lPEI5 – pDNA complexes exhibited enhanced transfection efficacy compared to bPEI25 – pDNA polyplexes, irrespective of the complexation medium applied.

The efficacy of gene transfer mediated by lmw lPEI- derived vectors was additionally enhanced using particle prepared in 5 % glucose: bPEI25 – pDNA ≈ 26 %, lPEI25 – pDNA ≈ 43 %, lPEI8.1 – pDNA ≈ 47 %, and lPEI5 – pDNA ≈ 32 % (Figure 9a:); compared to the corresponding polyplexes fabricated in 0.15 M NaCl: bPEI25 – pDNA ≈ 18 %, lPEI25 – pDNA ≈ 32 %, lPEI8.1 – pDNA ≈ 33 %, and lPEI5 – pDNA ≈ 37 % (Figure 9b) ($p<0.05$).

a)



b)

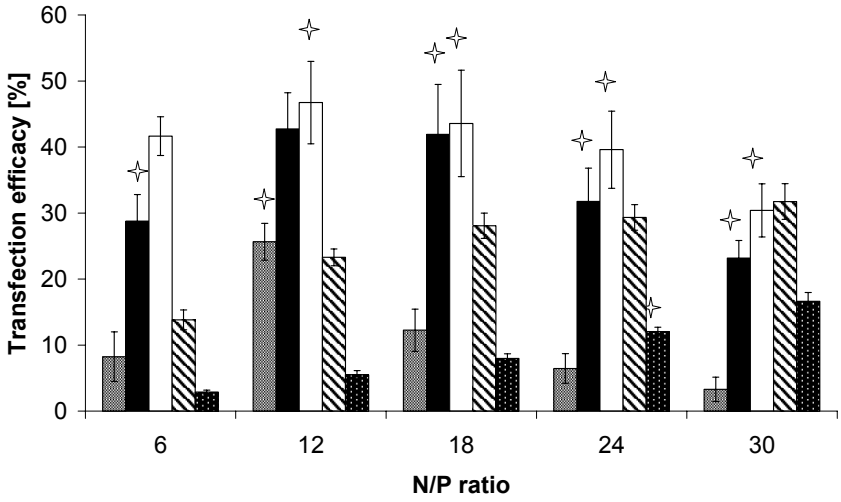
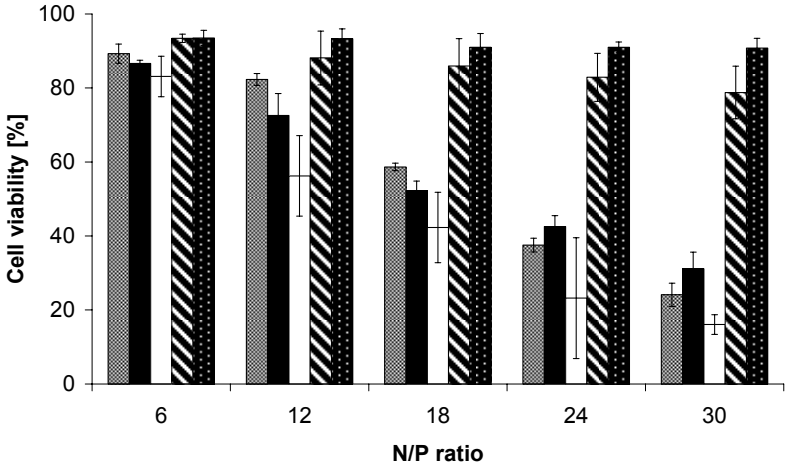


Figure 10. *Transfection efficacy (m.v.+SD) of bPEI25(▨)-, lPEI25(■)-, lPEI8.1(□)-, lPEI5(▧)-, and lPEI3(▩)-pEGFP-N1 polyplexes, prepared in a) 0.15 M NaCl or b) in 5 % glucose. The transfection experiment was carried out in serum-free cell culture medium. The transfection efficacy of polyplexes prepared at the same N/P ratio in different complexation media were proved as significantly different at a level of p < 0.05 depicted by [✧] (n=3).*

However, in contrast to the other vectors, lPEI5 – and lPEI3 – pDNA polyplexes mediated comparable transgene expression levels, irrespective of the complexation medium used. In the series of lPEI – pDNA polyplexes, we observed an increase of the transfection efficacy with the molecular weight of the polyamine. This effect was more pronounced for polyplexes build in 5 % glucose (lPEI25 – pDNA = lPEI8.1 – pDNA > lPEI5 – pDNA > lPEI3 – pDNA), compared to those formed in 0.15 M NaCl (lPEI25 – pDNA = lPEI8.1 – pDNA = lPEI5 – pDNA > lPEI3 – pDNA) ($p < 0.05$).

We investigated the impact of the lPEI M_n and of the chosen complexation medium on the cell-compatibility of the corresponding vector.

a)



b)

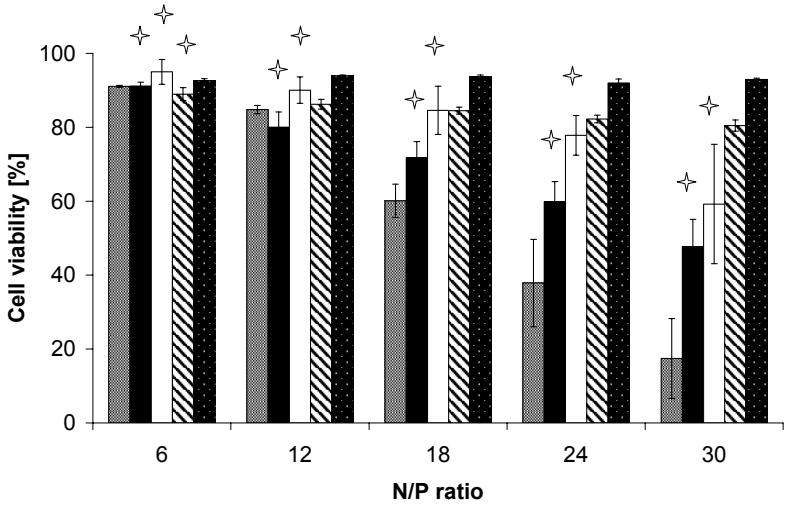


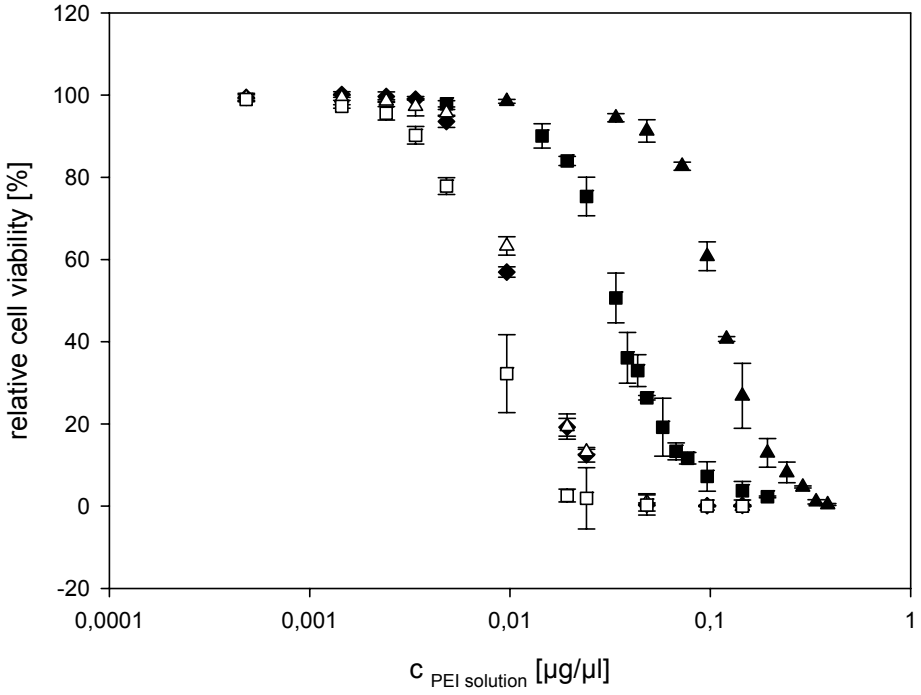
Figure 11. Cell viability of CHO-K1 cells determined by flow cytometry after treatment with bPEI25 (▨) –, lPEI25 (■) –, lPEI8.1 (□) –, lPEI5 (▧) –, or lPEI3 (▩) – pDNA polyplexes prepared in a) 0.15 M NaCl or b) 5 % glucose at N/P ratios between 6 and 30. The experiment was carried out in serum-free cell culture medium. Differences of the cell viability in respect to the complexation medium were analyzed for PEI – pDNA polyplexes, prepared at the same N/P ratio, and proved as significant at a level of $p < 0.05$ depicted by [✧] (n=3).

lPEI8.1 – pDNA (≈ 16 % N/P 24) and lPEI25 – pDNA complexes (≈ 31 % steady decrease) prepared in 0.15 M NaCl led to a sharp decline of the cell viability, compared to those formed in 5 % glucose (lPEI8.1 – pDNA ≈ 60 % N/P 24, lPEI25 – pDNA ≈ 45 % N/P 24).

However, we observed no differences depending on the complexation medium for bPEI25 – pDNA polyplexes (≈ 16 – 20 % steady decrease), or lPEI5 – pDNA (≈ 79 % - 93 %, N/P 12-18) mediated gene transfer. Transfection with lPEI3 – pDNA complexes proceeded with no detectable cytotoxicity (≈ 93-95 %). Overall, the cytotoxicity of lPEI-based gene transfer vehicles increased with the M_n of the polyamine, either steadily with the polymer concentration or at an N/P ratio level that decreases in the same manner as the lPEI MW increases.

To investigate wheter the cytotoxicity of the polyplexes is associated with the applied polycation, we determined the polymer concentration [µg/µl] at which the relative cell viability of CHO-K1 cells in vitro was reduced to 50 % compared to the control (CV_{50} values).

The relative cell viability values decreased following a sigmoid-like curve when CHO-K1 cells were incubated with increasing concentrations of lPEI (Figure. 12).



polymer	CV_{50} [µg/µl]
lPEI8.1	$1.10 \cdot 10^{-2} \pm 3.0 \cdot 10^{-4}$
lPEI5	$3.33 \cdot 10^{-2} \pm 6.0 \cdot 10^{-4}$
lPEI3	$1.11 \cdot 10^{-1} \pm 1.6 \cdot 10^{-3}$
lPEI25	$1.79 \cdot 10^{-2} \pm 5.0 \cdot 10^{-4}$
bPEI25	$7.50 \cdot 10^{-3} \pm 1.0 \cdot 10^{-4}$

Figure 12. *CHO-K1 cells in serum-free cell culture medium were incubated with increasing amounts of bPEI25 [□], lPEI25 [∆], lPEI8.1 [♦], lPEI5 [■] or lPEI3 [▲] dissolved in 0.15 M NaCl for 4 hours. After 48 hours, the cell viability was determined by flow cytometry analysis.*

We determined CV_{50} values of $1.79 \times 10^{-2} \pm 5 \times 10^{-4}$ µg/µl for lPEI25, $1.10 \times 10^{-2} \pm 3 \times 10^{-4}$ µg/µl for lPEI8.1, 3.33×10^{-2} l ± 6×10^{-4} µg/µ for lPEI5, or $1.107 \times 10^{-1} \pm 1.6 \times 10^{-3}$ µg/µl for lPEI3. For the series of lPEIs, we observed that the

polymer toxicity increased with the polymer M_n. Hence, it seems deceptive that we obtained a higher CV_{50} value for lPEI 25 kDa compared to lPEI8.1, but this may be explained by the lower relative M_n (GFC) determined for the commercially available lPEI in comparison to the synthesized lPEI8.1 [12].
bPEI25 reduced cell viability to 50 %, at the lowest concentration, compared to the other polyamines (0.0075 ± 0.0001µg/µl). In contrast to bPEI25, where a large portion of cell debris was observed after incubation with CHO-K1 cells, nonviable but individual cells were detected after treatment with the lPEI solutions.

Discussion

The results of our study did not reveal a monotone correlation between the transfection efficacy and a single particle or polymer parameter, we had investigated. The efficacy of gene transfer (Figure 9) seems to be influenced on various features of the transfection agent. To our current knowledge the optimum properties of efficient gene vectors remain elusive.
The utility of PEI-derived non-viral vectors is still limited by a severe cytotoxicity. However, we observed that a high lPEI MW led to a high polymer (CV_{50} values listed in Figure 11) as well as polyplex cytotoxicity (cell viability depicted in Figure 10), indicating that the toxicity of the vector is mediated by the polymerization degree of the polyamine. While the toxicity of PEI has been attributed to its membranolytic activity [7;16], the basis for the cytotoxic potential of the corresponding polyplexes is still a matter of discussion [17]. The determination of the CV_{50} values provides thereby the opportunity to compare polymers in terms of the cytotoxicity, irrespective to its chemical structures or architecture, and gives, thereby, valuable information to its applicability as gene carrier.
In contrast, the particle size of the polyplexes does not depend on the lPEI MW. According to the literature, the hydrodynamic diameters of lmw lPEI – pDNA complexes was mainly influenced by the ionic strength of the medium: In sodium chloride solution (physiological concentration, 0.15 M) the

formation of large aggregates beyond the micrometer scale was observed (Figure 1a), whereas in 5 % glucose defined particles of 100–150 nm size were detected (Figure 1b). Interestingly, the polyplexes pre-formed in glucose slowly aggregate, as soon as they are exposed to a medium of higher electrolyte content, like 0.15 M aq. NaCl or transfection medium (Figure 4a and b). Though, the polyplexes formed in glucose did not reach the same final (mean) particle size of that formed in 0.15 M NaCl within the time scale of the transfection experiment, transfection efficiency was not influenced by that difference (Figure 4c). While the particle size and complex stability is crucial for *in vivo* transfection, *in vitro* gene transfer seems be less limited by particle aggregation or reduced pDNA compaction, hence this may explain that we observed no consequence from particle properties to the efficacy of gene transfer [14;15].

The kind of the medium also had a substantial influence on the performance of the particles in the accessibility assay. The large lPEI – pDNA polyplexes formed in 0.15 M NaCl exhibited a higher fluorescence intensity of the DNA-ethidium bromide intercalate, indicative for a less “compact” polymer – DNA compound (Figure 5a), compared to polyplexes built in 5 % glucose (Figure 5b). Again, the smaller polyplexes formed in glucose slowly approximate the properties of the large aggregates formed in 0.15 M NaCl when given in medium of higher ionic strength (Figure 5d and 5f).

However, lmw lPEI – pDNA complexes from 5 % glucose have a minor ability to “shield” DNA against enzymatic degradation by DNase I (Figure 6).

In the gel retardation assay, no significant differences between the polyplexes, prepared in different media, were observed (Figure 7).

In conclusion, the results of this study suggest that the correlation between the lPEI MW and the transfection efficacy as well as cell viability of lmw lPEI – pDNA polyplexes is originated from the interaction between the polymer MW and the cytotoxicity of the polymer. Other factors, such as the particle size, surface charge, pDNA compaction and pDNA stability, presumed to influence the *in vitro* performance of PEI-based vectors, have been shown to

be independent from the polymer polymerization degree. An impact on the transfection efficacy and cell compatibility was not apparent.

Methods and Materials

The low molecular weight lPEIs (Table 1) were synthesized by ring-opening polymerization of 2-ethyl-2-oxazoline and acid hydrolysis of the corresponding poly (2-ethyl-2-oxazoline). The molecular weights were determined by GPC (listed in Table 1) [12].

lPEI	M_n (GPC) [kDa]	M_w (GPC) [kDa]	M_W/M_n
25	*6.8*	*15.3*	*2.25*
8.1	8.1	13.6	1.68
5.0	5.0	6.3	1.27
3.0	3.0	3.9	1.27
1.8	1.8	2.0	1.16

Table 1. *lmw lPEIs were synthesized by polymerization of 2-ethyl-2-oxazoline and acid hydrolysis of the resulting poly (N-acylethylenimine). The relative number (M_n) and weight (M_w) average molecular weights, as well as the molecular weight distribution (M_w/M_n) were determined by GFC [12].*

Branched polyethylenimine (high molecular weight, water free, M_W 25,000 Da (LS), M_n 10,000 Da (GPC)) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Linear polyethylenimine MW ~ 25,000 Da (7-8 % poly(2-ethyl-2-oxazoline, mp. 73-75°) was obtained from Polysciences Inc., Warrington, PA, USA. However, with the applied SEC method, we determined a relative M_n of 6.8 kDa and M_w of 15.3 kDa with an M_w/M_n of 2.25 for the commercially available lPEI ~ 25 kDa (Table 1).

The nutrient mixture F-12 (HAM), ethidium bromide, and kanamycin were obtained from Sigma-Aldrich. pEGFP-N1 was purchased from Clontech (Heidelberg, Germany). The *E.coli* JM109 bacterial strain was shipped from Promega. The Plasmid Maxi Kit was purchased from Qiagen (Hilden, Germany). CHO-K1 cells (ATCC# CCL-61) and fetal calf serum (FCS) were supplied by Biochrom KG Seromed (Berlin Germany). Agarose and LB broth medium were purchased from Invitrogen GmbH (Germany). Serva Blue G250 was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany).

Amplification and Purification of plasmid DNA [pDNA]

The pEGFP-N1 was transformed into the E.coli JM109 bacterial strain. The transformed cells were expanded in LB medium supplemented with kanamycin. The pDNA was isolated using the Quiagen Plasmid Maxi Kit according to the supplier´s protocol. The concentration and purity of the pDNA was measured by UV absorption at 260 and 280 nm.

Preparation of polyplexes

All PEIs were transformed into the corresponding hydrochloride using 2 N HCl and the nitrogen content was determined by elemental analysis. In brief: the polymers were dissolved in either 0.15 M NaCl or 5 % glucose and the pH was adjusted to 7.0 with 0.01 N aqueous hydrochloric acid or 0.01 N aqueous sodium hydroxide. The concentrations of the corresponding polymer solutions were adjusted to produce an N/P ratio of 1 upon the addition of 1 µl polymer solution to 2 µg pDNA. The pDNA and the appropriate volume of polymer solution were each diluted with 0.15 M NaCl or 5 % glucose to equal volumes corresponding to 25 µl per 1 µg pDNA. The pDNA and polymer solutions were mixed by vortexing and the polyplexes were allowed to form for 20 minutes at room temperature.

Laser light scattering analysis and Zeta-potential measurements

Polyplexes prepared in 0.15 M NaCl or 5 % glucose containing a total of 10 µg pEGFP-N1 were diluted to 2.5 ml with 0.15 M NaCl, 5 % glucose, serum-free or serum-containing cell culture medium, respectively. The samples were thermostated to 25 °C and laser light scattering analysis was performed at 25 °C with an incident laser beam of 633 nm at a scattering angle of 90° using a Malvern ZetaSizer 3000 HSA, Malvern Instruments GmbH, Germany. The count rates for all dispersants were lower than 5 Kcps, confirming their applicability in size measurements. The following parameters were used: viscosity of HAM´s F12, HAM´s F12 + 10 % FCS, and 0.15 M NaCl: 0.89 mm^2/s, 5 % glucose: 0.98 mm^2/s; the refractive indices of HAM´s F12 and HAM´s F12 + 10 % FCS: 1.681, 5 % glucose and 0.15 M NaCl: 1.33. The sampling time was set automatically.

At least Five measurements each with 10 sub-runs were performed for each of three independent samples (n=3).

The zeta potential measurements of the same polyplex samples were performed in the standard capillary electrophoresis cell of the ZetaSizer HSA (Malvern Instruments GmbH, Germany), measuring the electrophoretic mobility at 25 °C. The sampling time was again set automatically (n=3).

The measurements were analyzed statistically by one-way analysis of variance (ANOVA) and the Tuckey- or Dunnett´s test.

Atomic force microscope imaging

Polyplexes were prepared in an 0.15 M NaCl or 5 % glucose as described above, deposited onto freshly cleaved mica and imaged at a JPK NanoWizard scanning force microscope (JPK instruments, Berlin,Germany). Measurments were performed in 0.15 M NaCl or 5 % glucose. Surface images were obtained in the intermittent fluid mode. (silicon tips NSC 12/50, Ultrasharp, Silicon-MDT Ltd., Moscow, Russia; scan rate 0.3 - 1 Hz; duty cycle 0.7 - 0.8; IGain 30 - 90 Hz and PGain 0.004 - 0.1 Hz, setpoint 128 – 142 mV)

Gel-retardation assay

Polyplexes (0.5 µg pEGFP-N1 in 5 µl 0.15 M NaCl or 5 % glucose) were prepared with the appropriate amount of lPEI solution (always diluted to 8.5 µl) to produce N/P ratios between 0 and 12. After incubating for 20 minutes at room temperature, 5 µl serum-free cell culture medium, or 0.15 M NaCl as well as 5 % glucose as negative control, were added to the polyplexes and the solutions were incubated for another 20 minutes at room temperature. After addition of 1.5 µl loading buffer, the samples were loaded into a 1 % TAE agarose gel and run at 80 V for 40 minutes. The pDNA bands were visualized by ethidium bromide staining and detected on a Fisherbrand FT-20/312 UV transilluminator 312 (Herolab GmbH Laborgeräte, Germany).

DNA accessibility assay

The quenching of the pDNA-ethidium bromide fluorescence indicates the assembly of pDNA with the polycation by electrostatic interactions under the release of less fluorescent ethidium bromide. This effect has been used to compare the extent of DNA compaction by the different polycations.

i) 2 µg pEGFP-N1 and 0.25 µg ethidium bromide each dissolved in 100 µl 0.15 M NaCl or 5 % glucose, were mixed, and transferred into a black 96 well micro titer plate (Greiner-Bio-One, Germany). Either 1 µl of half-concentrated lPEI solutions or 1 to 2 µl of the lPEI solutions were added stepwise to the pDNA-EB to cover a series of N/P 0, 0.5, 1, 1.5, 3.5, 5.5, 7.5 and 11.5. The reaction batch was mixed thoroughly, and the fluorescence of the pDNA-EB intercalate (F_C) was measured at a emission wavelength (λ_{em}) of 590 nm after excitation at an excitation wavelength (λex) of 485 nm using a GENios Pro reader (Tecan Trading AG Switzerland).

ii) Thereafter, HAM´s F12 or HAM´s F12+10 % FCS (serum-free and serum-containing cell culture medium, respectively) were added stepwise (20 µl, 40 µl, 40 µl) to the polyplex dispersion and the fluorescence was measured again after each step (F_M). The mean fluorescence of the pDNA-EB intercalate, diluted with the equivalent volumes of 0.15M NaCl or 5 %

glucose, as well as after dilution with 20, 60 and 100 µl serum-free or serum-containing cell culture medium was set 100 % ($F_{100\%}$) . The relative fluorescence of the polyplex samples (F_{rel}) was calculated by the following equation: i) F_{rel} [%] = 100(F_C – F_{EB}) / ($F_{100\%}$ – F_{EB}), or ii) F_{rel} [%] = 100(F_M – F_{EB}) / ($F_{100\%}$ – F_{EB}).The mean value determined for the residual EB fluorescence (F_{EB}) was subtracted from all determined values. All experiments were done in sextuplicate (i) or triplicate (ii).

DNase I and II experiments

PEI-based polyplexes were prepared at an N/P ratio of 10 either in 0.15 M NaCl or 5 % glucose (2 µg pEGFP-N1 / 5 µl polyplex dispersion), allowed to form at room temperature for 20 minutes, and thereafter incubated with increasing concentrations of DNase I or II (0.625 to 25U in 5 µl) at 37 °C. The reaction compound (total volume: 13 µl) consisted of: a) polyplexes incubated in digestion buffer (20 mM MgSO4, 0.4 M NaOAc) at pH 5 or pH 8, and dilution buffer (25 mM Tris-HCl, 50 % (v/v) Glycerol, pH 7), b) pEGFP-N1 and DNase I or DNase II, incubated in digestion buffer at pH 8 or pH 5, respectively, or c) polyplexes and DNase I or DNase II, incubated in digestion buffer at pH 8 or pH 5, respectively. After 30 minutes and 4 hours incubations with DNase I or 4 hours incubation with DNase II, the digestion was halted by the addition of a stop solution (5 µl: 0.2 M EDTA pH 8, 0.7 N NaOH) and 10x loading buffer (1.5 µl) was added. The integrity of the plasmid was examined by loading the samples on a 1 % alkaline agarose gel containing ethidium bromide. The gels were run for 90 minutes at 40 V.

Transfection and Cytotoxicity experiments

CHO-K1 cells were grown in 24-well plates at an initial density of 38,000 cells per well. 18 hours after plating, the culture medium (HAM´s F12 supplemented with 10% FCS) was removed, cells were washed with PBS, 900 µl serum-free medium were supplemented and the freshly prepared polyplex dispersion was added. After 4 hours, the medium was replaced with

1 ml of culture medium and after 48 hours the cells were prepared for flow cytometry analysis. Floating cells were collected and combined with adherent cells after trypsinization. The collected cells were washed twice with PBS, resuspended in 500 µl PBS and propidium iodide was added at a concentration of 1 µg/ml to half of the samples. Measurements were taken on a FACSCalibur (Becton Dickinson, Germany) using the CellQuest Pro software (Becton Dickinson, Germany) and WinMDI 2.8 (©1993-2000 Joseph Trotter).

Logarithmic amplification of EGFP and propidium iodide emission in green and red fluorescence was obtained for 20,000 events counted for each sample. EGFP positive cells were detected using a 530/30 nm band-pass filter, whereas the propidium iodide emission was measured with a 670 nm long pass filter after excitation at 486 nm. In a density plot representing forward scatter against sideward scatter, whole cells were gated out (this process allows to distinguish between the cell population and cell fragments) and depicted as two-parameter dot plots of EGFP versus propidium iodide to analyse the measurements. The EGFP positive region, which corresponds to the transfection efficiency, was drawn starting above cell auto fluorescence, where EGFP positive cells were < 0.2 %. The geometric mean fluorescence intensity was determined from the number of EGFP-positive and propidium iodide positive cells. The propidium iodide negative cells were counted as a measure of cell viability. The relative transfection efficacy and cell viability is number of EGFP-positive or viable cells, respectively, counted in a sample normalized to the negative control and expressed as a percent.

To investigate the cytotoxicity of low molecular weight linear polyethylenimines, CHO-K1 cells were incubated with increasing volumes of polymer solutions and the cell viability was determined according to the procedure described above. The polymer concentration [µg/µl] was plotted on a logarithmic scale on the x-axes, while the y-axes displayed the cell viability [%]. The CV_{50} values (the polymer concentration that reduces the cell population to 50 % of the original value) were calculated by a non-linear

regression using the Pharmacology Standard Curve regression function (y=min+(max-min)/(1+(x/EC50)^Hill slope) of SigmaPlot 8.0. To compare CV_{50} values to the toxicity of the polyplex dispersion, we calculated the polymer concentration used to obtain polyplexes at the N/P ratio at which the corresponding polyplexes reduced the cell viability to 50 %.

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6. DEGRADABLE LOW MOLECULAR WEIGHT LINEAR POLYETHYLENIMINES FOR NON-VIRAL GENE TRANSFER

U. Lungwitz, M. Breunig, R. Liebl, D. Pesl, T. Blunk, A. Göpferich

Department of Pharmaceutical Technology, University of Regensburg, 93040 Regensburg, Germany

Manuscript in preparation

Abstract

The major drawbacks of polymer-based non-viral transfection agents remain their low transfection efficacy and detectable cytotoxicity. By cross-linking linear polyethylenimine (lPEI) of 2.6 and 4.6 kDa by disulfides linkages that will be reduced in an intracellular environment, we intended to prepare novel DNA vectors that combine the superior DNA complexation and stabilization capabilities of high molecular weight polyamines with the favourable cell compatibility of low molecular weight (lmw) lPEI. We synthesized four series of polymers differing by the lPEI molecular weight, the linker type, and the linker / lPEI ratio. The biophysical particle properties, as well as the *in vitro* transfection efficacy and cell-compatibility of the corresponding polyplexes with plasmid DNA (pDNA, pEGFP-N1) was mainly influenced by the polymer composition, N/P ratio and medium applied. Gel-retardation assays and size measurements confirmed the destabilization of the corresponding particles in the presence of dithiothreitol or glutathione, indicating the degradation of the cross-linkages. *In vitro* gene transfer experiments to CHO-K1 cells, cross-linked lPEI- derived polyplexes exhibited a several-fold improved transfection efficacy and cell-compatibility, compared to the corresponding branched (bPEI25) or linear PEI 25 kDa (lPEI25) – pDNA complexes.

Introduction

Cationic polymers are most commonly applied for the manufacture of non-viral vectors. Despite of the many advantages polymers have over other materials, especially viruses, their broad clinical or laboratory use is still limited by a comparably low efficacy of gene transfer and severe cytotoxicity [1-4]. Since its introduction in 1995, polyethylenimine is the gold standard for polymer-based gene carriers, due to the relatively high transfection efficacy of its corresponding polyplexes [5]. It has been shown that both, the efficiency and the cell compatibility of the transfection agent is a function of the molecular weight of the polyamine [6-8]. While high molecular weight polyamines exhibit improved pDNA compaction and stabilization, they also derogate transgene expression by limiting the intracellular complex dissociation, and are causative for the cytotoxicity of the vector [9-11]. Here in contrast, low molecular weight PEI-derived polyplexes exhibit a significantly improved cell compatibility [8;12;13]. Unfortunately, they also suffer from a low capacity to protect their cargo against degradation, due to the destabilization of the corresponding complexes once exposed to a biological environment [14-16]. One strategy aimed to overcome this dilemma is focused on the design of biodegradable polyamines, which ideally combine the features of high and low molecular weight PEI that are beneficial for gene transfer.

For this purpose, homobifunctional aldehydes [17], activated diesters [18], and diacrylates [19;20] were used for the polymerization with simple diamines, as well as the cross-linking of PEI. Acid-labile imine-, ester-linkages, or ß-aminoester-linked polyamines were formed, expected to degrade by the drop of the pH in endolysosomal vesicles. On the other hand, the highest enzymatic activity of DNase I is encountered in the endolysosomes. Hence, the degradation of the polymer may conceivably be accompanied by a loss of intact plasmid DNA. In literature, contradictory results on the success of this strategy are reported.

The reversible disulfide bond has been proposed as biodegradable linker, which is predominantly cleaved in the cytoplasma due to the high intracellular concentration of the disulfide-glutathione redox couple [21;22]. In advantage to the aforementioned approaches, this may facilitate high polyplex stability during its transit through the extracellular space and the DNA degrading endolysosomal compartment. Complex unravelling and release of the DNA from its polyamine carrier is induced after disulfide reduction in the presence of cytosolic glutathione [9].

Gosselin et al, first investigated the capacity of disulfide cross-linked PEI as gene carriers. bPEI of 0.8 kDa was used as polyamine component and was cross-linked with either dithiobis(succinimidylpropionate) or dimethyl-3,3´-dithiobispropionimidate [23]. However, polyplexes prepared with both polymers transfected CHO-K1 cells with less efficacy compared to bPEI25 – pDNA polyplexes.

Previously, we have shown that low molecular weight linear polyethylenimines (lmw lPEI) mediate gene transfer with improved efficacy and cell compatibility, compared to bPEI25 –, lPEI25 –, or lPEI22 – derived polyplexes [6;24]. This made them to a promising basis for further optimization.

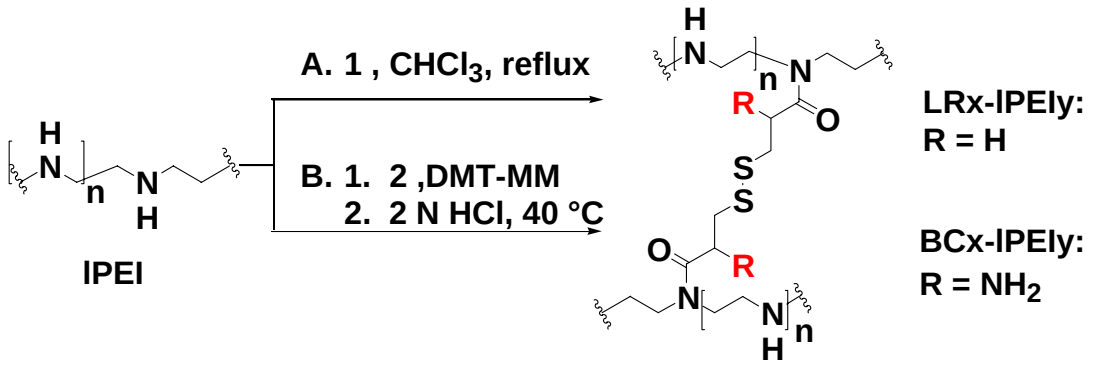
In this study, we investigated whether a biodegradable PEI backbone, synthesized by the cross-linking of non-toxic lmw lPEI (2.6 and 4.6 kDa) has the capacity to combine the high transfection efficacy of high molecular weight lPEI – pDNA complexes with the reduced cytotoxicity of low molecular weight lPEI – pDNA polyplexes.

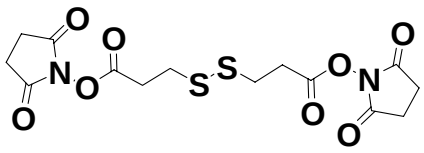
In order to approach an optimum polymer composition we varied the molecular weight of the polyamine component and the linker / lPEI monomer ratio, and used linkers with or without an additional amino group. The effectiveness of the gene carriers was proved by determining the particle properties and investigating the *in vitro* transfection performance of the corresponding vectors in comparison to bPEI25 – or lPEI25 – pDNA complexes.

Results and Discussion

Gene carriers

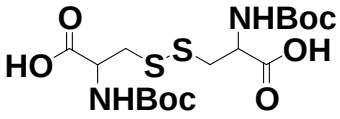
By cross-linking lmw lPEI with homobifunctional biodegradable linkers we intended to prepare novel DNA vectors that combine the superior DNA complexation and stabilization capabilities of high molecular weight polyamines with the favourable cell compatibility of lmw lPEI. In order to prove the effectiveness of this concept we chose a simple synthesis approach to randomly cross-link lmw lPEIs.





1

3,3'-Dithiodipropionsäure di(N-succinimidyl ester) Lomant's Reagenz (LR)



2

N,N'-bis-(*tert*-butoxy-carbonyl) cystin (BC)

Scheme 1.

Synthesis of LR-lPEIs (A) or BC-lPEIs (B) by cross-linking of lPEI 2.6 or 4.6 kDa with LR or a mixture of BC and DMT-MM, respectively, performed at varying molar ratios.

lPEI 2.6 and 4.6 kDa (lPEI2.6 or lPEI4.6, relative number average molecular weight (M_n) determined by Gel Filtration Chromatography (GFC) [25]), were

used as polyamine component due to its negligible toxicity and reversible cross-linked by dithiodipropionic acid (LR-lPEIs) or cystine (BC-lPEIs) linkages (Scheme 1) to enable the polymer degradation in the presence of disulfide reducing agents.

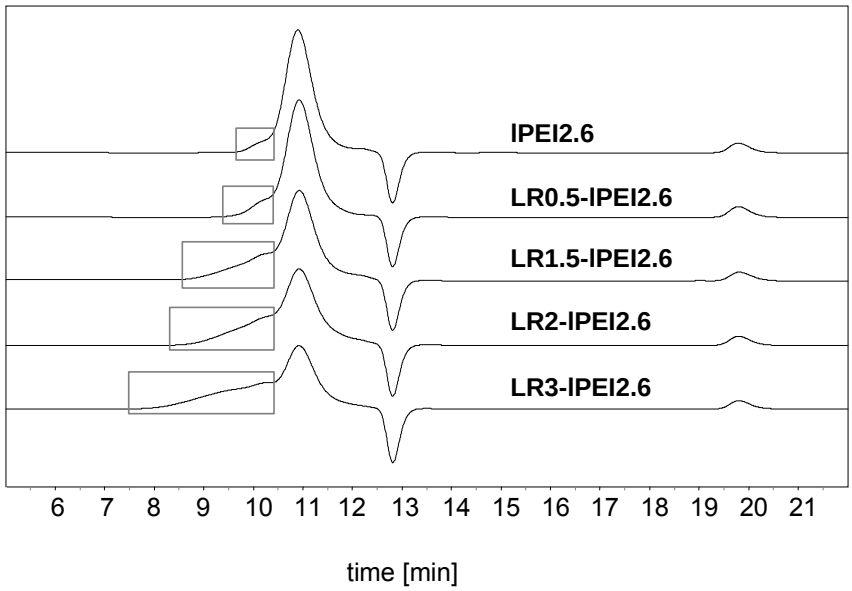
The conjugation with Lomant´s reagent (LR) or boc-cystine (BC) / 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was thereby performed in organic solvents to minimize NHS-ester hydrolysis and to enable the reaction with secondary amines, which seemed to be less reactive in water.

A problem, common to approaches using homobifunctional linkers for the cross-linking of polyamines is the formation of insoluble products [17]. After the optimization of the reaction conditions the cross-linking was performed with acceptable reproducibility. We obtained a series of LR-lPEI2.6 or LR-lPEI4.6 with a maximum lPEI / linker ratio of 3 % or 1.5 %, and a series of BC-lPEI2.6 or BC-lPEI4.6 with linker content up to 8 % or 1.5 %, respectively. All attempts to reach higher substitution degrees led to the formation of water-insoluble, gel-like solids.

On the basis of ^{1}H-NMR or ^{13}C-NMR analysis the successful conversion could not be clearly confirmed due to the low substitution degrees and the overlapping of signals assigned to the unmodified and the cross-linked lPEI.

An increase of the molecular weight in direct comparison to unmodified lPEI, due to cross-linking was confirmed by a GFC method, previously described [25]. As exemplarily shown for the series of LR and BC cross-linked lPEI2.6s, a higher molecular weight fraction, beside a fraction of unmodified lPEI, became more dominant with an increasing linker / lPEI ratio (Figure 1). Even at maximum linker / lPEI ratios a residue of lPEI remained the main component in the polymer batch.

a)



b)

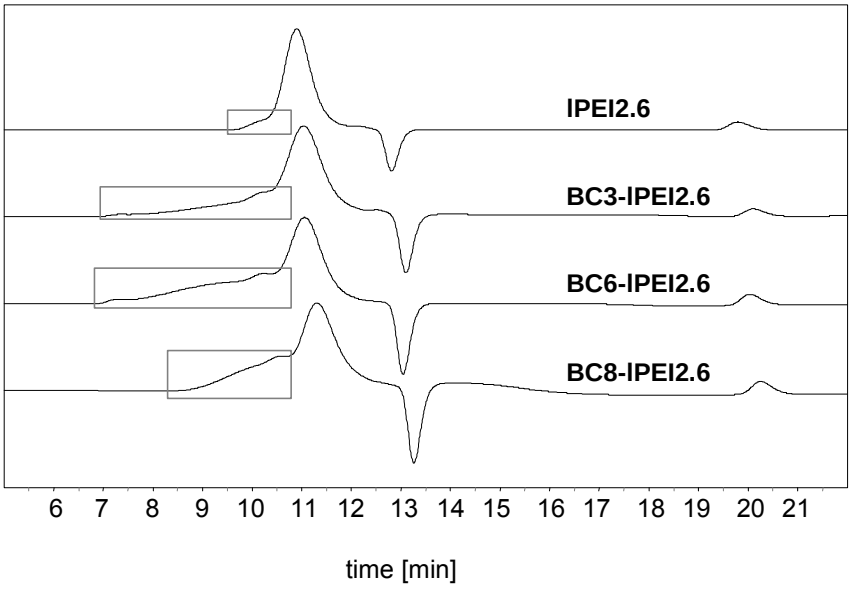


***Figure 1**. lPEI cross-linked by a) Lomant´s Reagent using 0.5 to 3% (mol/mol lPEI monomer), b) or boc-cystine / DMT-MM applying 3 to 8% (mol/mol lPEI monomer). The corresponding hydrochlorides were dissolved in water and were analyzed by GFC according to a procedure previously described [25].*

Transfection efficacy and cell viability

In order to prove the capacity of cross-linked lPEI – pDNA polyplexes as *in vitro* gene delivery agents, we determined the transfection efficacy and the cell viability, with respect to the different polymer structure and in comparison to lPEI25 – pDNA and bPEI – pDNA. We used a CHO-K1 cell model, which allows for higher levels of transgene expression, compared to other cell lines that are more difficult to transfect, and therefore enables to assess gradual, material-dependent differences between the single transfection reagents.
We observed, limited to low N/P ratios between 6 and 18 that the transfection efficacy increased with the linker content of the polyamine. This trend was at least partially inverted at higher N/P ratios, conceivably due to an arising cytotoxicity. Irrespective of the polyamine applied for pDNA compaction, the efficacy of gene transfer followed a general trend: The number of transfected cells increased with increasing N/P ratio, reached a plateau, and decreased at further enhanced N/P ratio. The maximum transfection shifted to the lowest N/P ratio for polyplexes built with cross-linked lPEIs of the highest linker / lPEI ratio or to the highest N/P ratio for the polyamine with the lowest linker content. In accordance with the results described by Gosselin et al., we observed that cross-linking with LR increased the cytotoxicity of the corresponding vector, compared to polyplexes prepared with a gene carrier cross-linked under an agreed nitrogen balance [23], such as in the BC-lPEI – pDNA complexes. This phenomenon was observed irrespective of the molecular weight of the lPEI precursor.

LR/BC-lPEI4.6 – derived polyplexes: Transfection efficacy and cell viability

LR1.5-lPEI4.6 – pDNA polyplexes exhibited superior transfection efficacy (50 %) compared to BC1.5-lPEI4.6 – pDNA (30 %) polyplexes, but a comparable efficiency to unmodified lPEI5 – pDNA polyplexes [6].

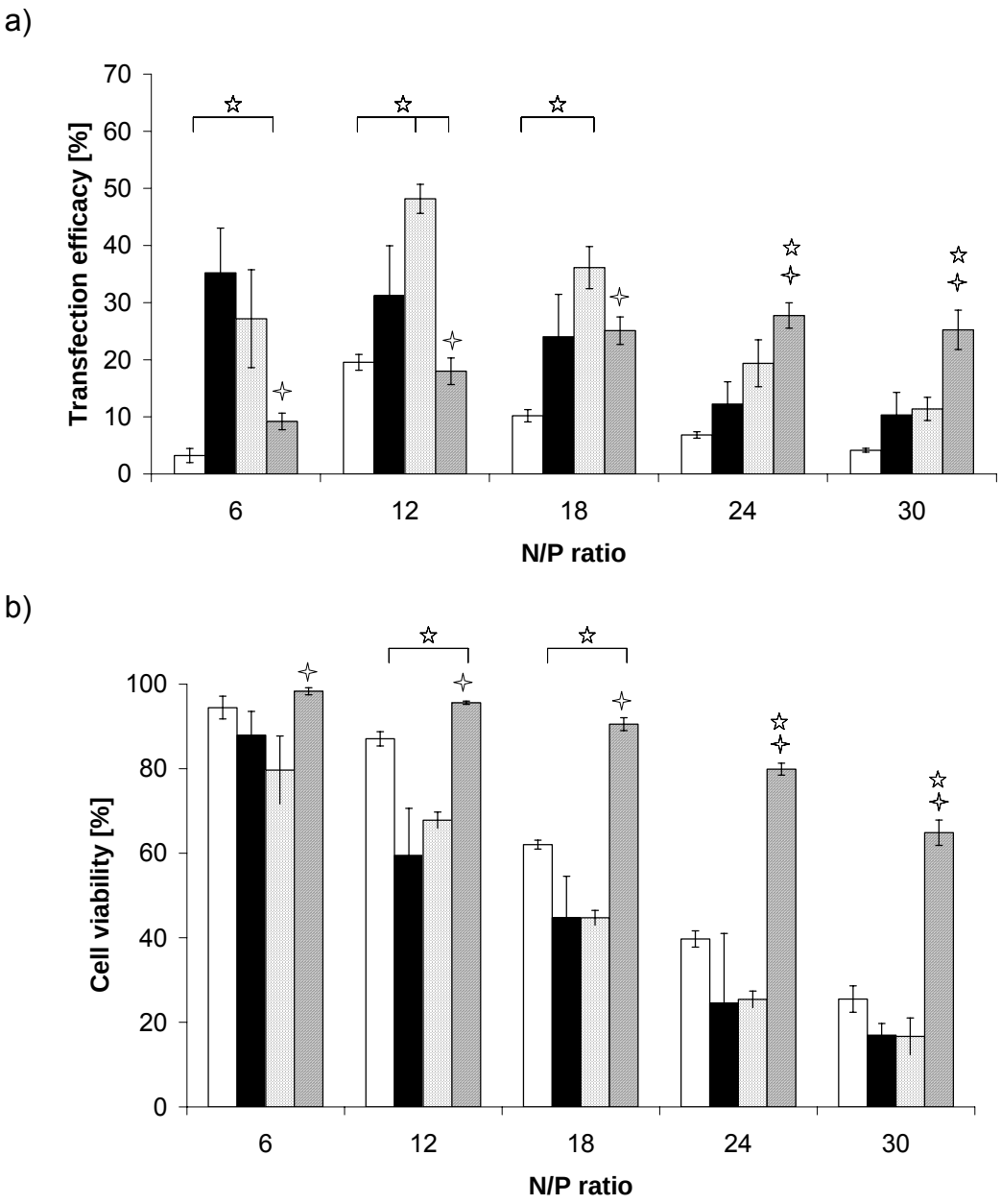


Figure 2. *a) In vitro transfection efficacy and b) cell viability (m.v. + SD, n = 3) determined in CHO-K1 cells after incubation with lPEI25 – pDNA [■], bPEI25 – pDNA [□], LR1.5-lPEI4.6 – pDNA [▨], and BC1.5-lPEI4.6 – pDNA [▩] polyplexes, prepared in 0.15 M NaCl at an N/P ratio between 6 and 30. Significant differences between lPEI25 – pDNA and the other polyamine-derived polyplexes at the same N/P ratio are denoted by [☆](p < 0.01), and between LR1.5-lPEI4.6 – pDNA and BC1.5-lPEI4.6 – pDNA are denoted by [✧] (p < 0.05).*

The transfection maximum shifted from low N/P ratio (N/P 12) for LR1.5-lPEI4.6 – pDNA polyplexes to elevated N/P ratios (N/P 30) for BC1.5-lPEI4.6 – pDNA polyplexes (Figure 2).

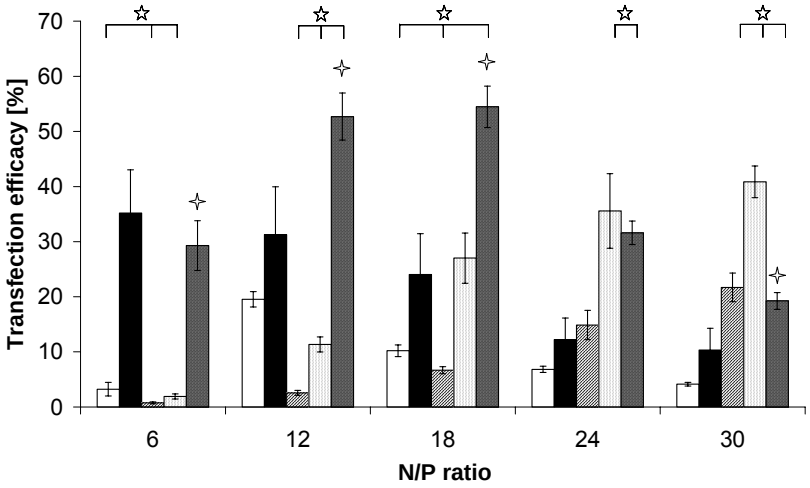
However, neither the use of LR- nor BC-cross-linked lPEI – derived polyplexes improved both the efficacy and cell viability during gene transfer, compared to the corresponding lPEI25 – and bPEI25 – pDNA complexes. While LR1.5-lPEI4.6 mediated transfection with up to 2.5-fold improved efficacy (lPEI25 – pDNA ≈ 35 %; bPEI25 – pDNA ≈ 20 % ($p < 0.05$)), it concomitantly reduced the cell viability to similar or increasing degree (60 % at an N/P ratio of 12 or nearly 20 % at an N/P ratio of 30).

The corresponding BC1.5-lPEI4.6 – pDNA complexes exhibited comparable transfection efficacy to lPEI25 – pDNA polyplexes (30 %). The cell compatibility is only significantly improved at elevated N/P ratios (N/P 30 ≈ 70 %). This becomes irrelevant, considering that the maximum transfection with lPEI25 – pDNA polyplexes is obtained already at an N/P ratio of 6 and 12.

LR/BC-lPEI2.6 – derived polyplexes: Transfection efficacy and cell viability

For the series of cross-linked lPEI2.6 – derived polyplexes the maximum transgene expression levels between 55 – 58% after application of LR3-lPEI2.6 – pDNA and BC8-lPEI2.6 – pDNA complexes built at an N/P ratio of 12 (Figure 3). The transfection efficacy of polyplexes formed with cross-linked lPEIs of lower linker content levelled between 50 % (BC6 - and BC3 - lPEI2.6 – pDNA; 40 % LR2-lPEI2.6 – pDNA), and 20 % (LR1.5-lPEI2.6 – pDNA). In comparison to the corresponding lPEI25 – pDNA, bPEI25 – pDNA, or unmodified lPEI2.6 – pDNA polyplexes [24], the efficacy of gene transfer was thereby improved up to 1.6-fold, 2.8-fold, or 11-fold, respectively ($p < 0.01$).

a)



b)

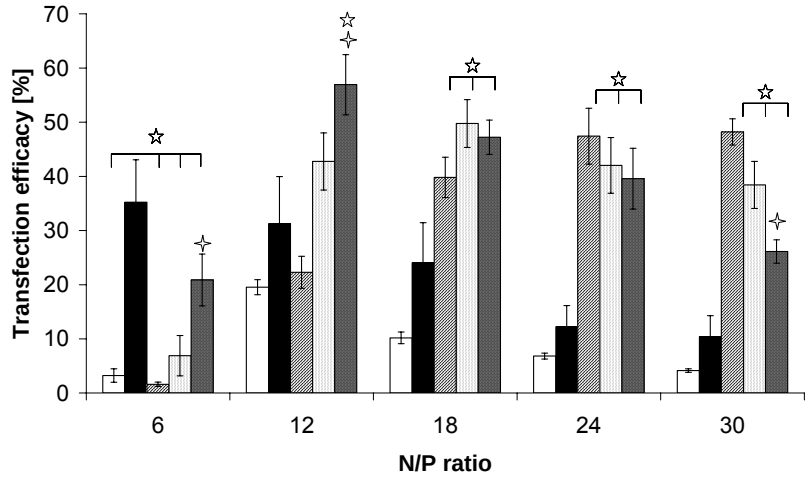
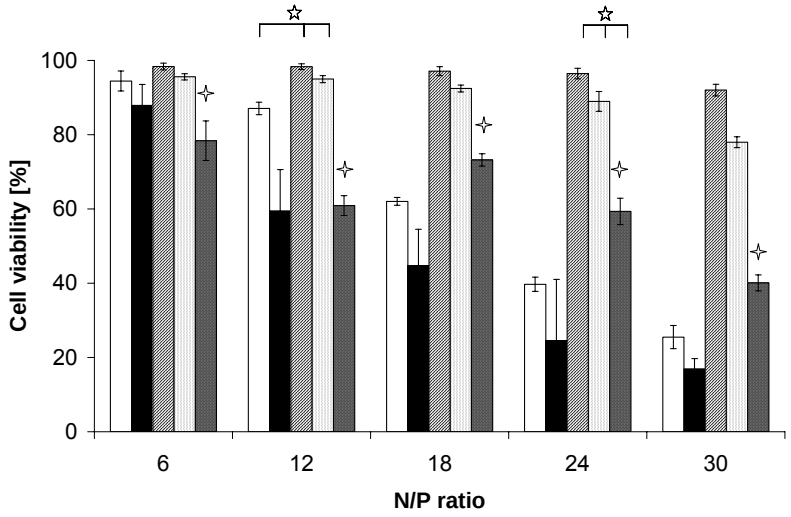


***Figure 3**. Transfection efficiency (mean ± S.D., n = 3) determined in CHO-K1 cells in vitro after treatment with a) lPEI25 – pDNA [■], bPEI25 – pDNA [□], LR1.5-lPEI2.6 – pDNA [▨], LR2-lPEI2.6 – pDNA [□], and LR3-lPEI2.6 – pDNA [■] polyplexes, or b) lPEI25 – pDNA [■], bPEI25 – pDNA [□], BC3-lPEI2.6 – pDNA [▨], BC6-lPEI2.6 – pDNA [□], and BC8-lPEI2.6 – pDNA [■] polyplexes prepared in 0.15 M NaCl at N/P ratios ranging from 6 to 30. Significant differences between lPEI25 – pDNA and the other polyamine-derived polyplexes at the same N/P ratio are denoted by [☆](p < 0.01), and between LR2-lPEI2.6 – pDNA and LR3-lPEI2.6 – pDNA as well as BC6-lPEI2.6 – pDNA and BC8-lPEI2.6 – pDNA are denoted by [✧] (p < 0.05).*

a)



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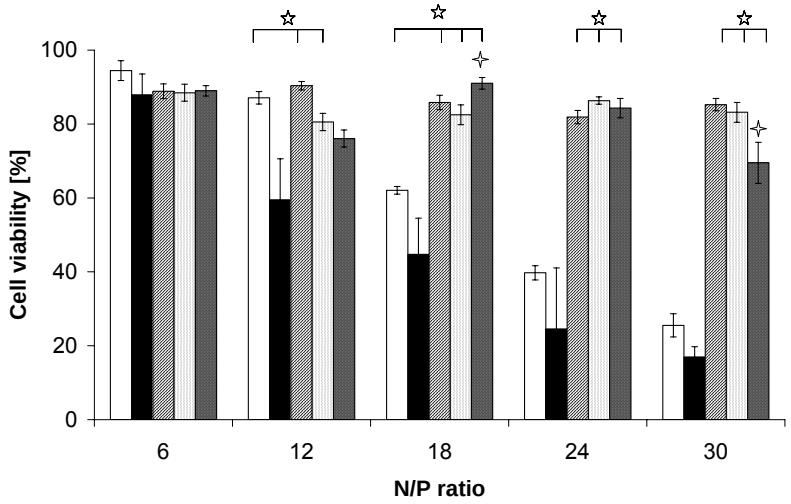


Figure 4. *Viability (m.v. + SD, n = 3) of CHO-K1 cells after incubation with a) lPEI25 – pDNA [■], bPEI25 – pDNA [□], LR1.5-lPEI2.6 – pDNA [▨], LR2-lPEI2.6 – pDNA [▨], and LR3-lPEI2.6 – pDNA [■] complexes, or b) lPEI25 – pDNA [■], bPEI25 – pDNA [□], BC3-lPEI2.6 – pDNA [▨], BC6-lPEI2.6 – pDNA [▨], and BC8-lPEI2.6 – pDNA [■] polyplexes prepared in 0.15 M NaCl at N/P ratios between 6 and 30. Significant differences to lPEI25 – pDNA polyplexes at the same N/P ratio are denoted by [☆]($p < 0.01$), and between LR2-lPEI2.6 – pDNA and LR3-lPEI2.6 – pDNA as well as BC6-lPEI2.6 – pDNA and BC8-lPEI2.6 – pDNA are denoted by [✧] ($p < 0.05$).*

The cytotoxicity of bPEI25 – pDNA, lPEI25 – pDNA, and LR-lPEI2.6 – pDNA polyplexes increased with the N/P ratio, while by contrast, the cell viability after treatment with BC-lPEI2.6 – pDNA seemed to be unaffected by the polymer concentration (Figure 4). At an N/P ratio of 12, where BC8-lPEI2.6 – and LR3-lPEI2.6 – pDNA complexes exhibited the maximal transfection efficacy, the cell viability was reduced to a similar degree by BC8-lPEI2.6 – and bPEI25 – pDNA polyplexes (80 %) or LR3-lPEI2.6 – and lPEI25 – pDNA polyplexes (60%).

Increasing the N/P ratio to 30, the LR- or BC-lPEI2.6 – pDNA polyplexes exhibited a significantly reduced cytotoxicity compared to the corresponding lPEI25 – pDNA and bPEI25 – pDNA complexes. The cell viability was thereby maximally improved to 4-fold or 5.5-fold, compared to bPEI25 – pDNA or lPEI25 – pDNA complexes, respectively.

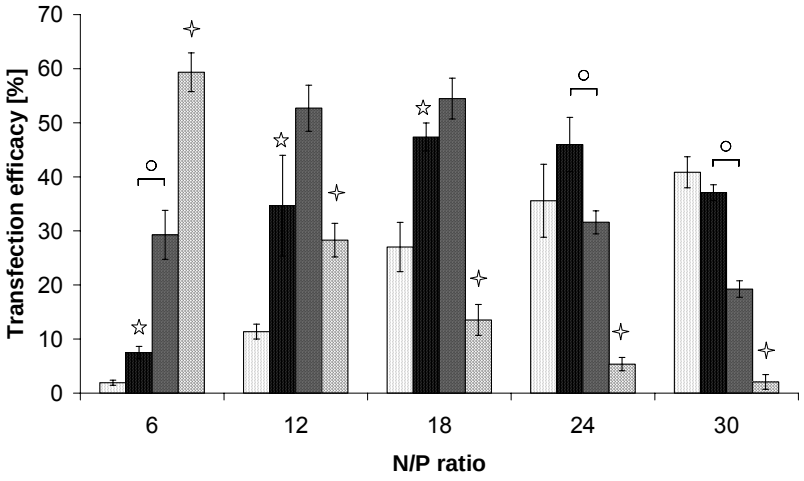
As aforementioned, it has been shown that the efficacy of PEI-based transfection systems is a function of the molecular weight of the gene carrier. By cross-linking of lmw lPEI we obtained a mixture of polymers with broad molecular weight distribution.

We used a simple size exclusion chromatography setup for the separation of the so-called S-fraction (higher molecular weigh fraction) from residual unmodified lPEI, and investigated its capacity to further enhance gene transfer.

At an N/P ratio of 6, the transfection efficacy increased significantly with the linker / lPEI ratio, and was further augmented using the higher molecular weight fraction of the corresponding cross-linked lPEI (Figure 5). However this effect was leveled at elevated N/P ratios.

Overall, comparable levels of transgene expression were determined for LR2-lPEI2.6S – pDNA, LR3-lPEI2.6 – pDNA, BC6-lPEI2.6 – pDNA, as well as BC8-lPEI2.6 – pDNA polyplexes, reaching a maximum efficacy of 60 % at similar N/P ratios (N/P 6 -18).

a)



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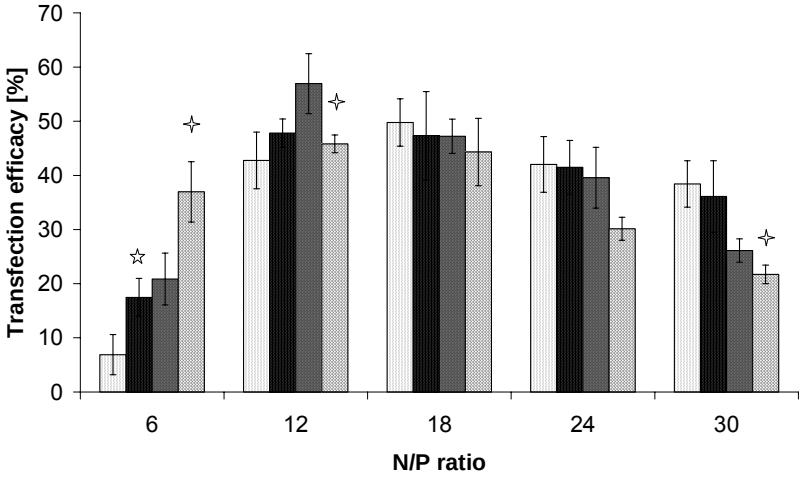
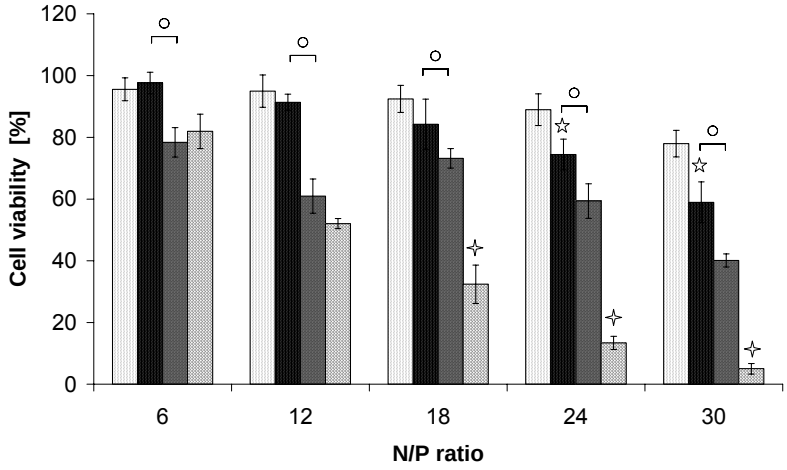


Figure 5. *Polyplexes were prepared at an N/P ratio of 6, 18 and 30 in 0.15 M NaCl. a) The transfection efficacies (m.v.+SD, n = 3) of LR2-lPEI2.6 – pDNA [□] were significantly different from LR2-lPEI2.6S – pDNA [■],denoted by [☆], of LR3-lPEI2.6S – pDNA [▨] from LR3-lPEI2.6 – pDNA [▩] denoted by [✧], and LR2-lPEI2.6S – pDNA from LR3-lPEI2.6 – pDNA polyplexes denoted by [○](p < 0.05). b) The transfection efficacies of BC6-lPEI2.6 – pDNA [□] were significantly different from BC6-lPEI2.6S – pDNA [■], denoted by [☆], BC8-lPEI2.6 – pDNA [▩] from BC8-lPEI2.6S – pDNA [▨] denoted by [✧], and BC6-lPEI2.6S – pDNA differed from BC8-lPEI2.6 – pDNA denoted by [○] (p < 0.05), always analysed for the same N/P ratio.*

a)



b)

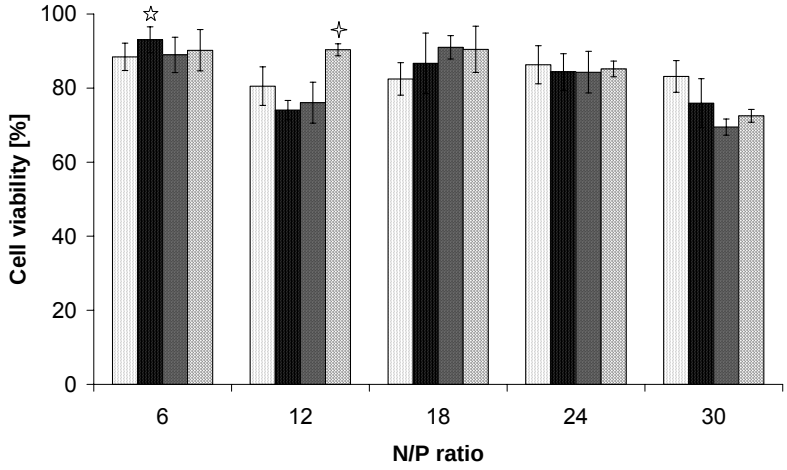


Figure 6. *Polyplexes were prepared at an N/P ratio of 6, 18 and 30 in 0.15M NaCl. a) The viability of CHO-K1 cells (m.v. ± S.D., n = 3) after incubation with of LR2-lPEI2.6 – pDNA [□] was significantly different from LR2-lPEI2.6S – pDNA [■], denoted by [☆], and LR3-lPEI2.6 – pDNA [■], from LR3-lPEI2.6S – pDNA [□], denoted by [✧], and LR2-lPEI2.6S – pDNA differed from LR3-lPEI2.6 – pDNA, denoted by [○] (p < 0.01). b) The viability of CHO-K1 cells after exposure to BC6-lPEI2.6 – pDNA [□] was significantly different from BC6-lPEI2.6S – pDNA [■], denoted by [☆], BC8-lPEI2.6 – pDNA [■] from BC8-lPEI2.6S – pDNA [□] denoted by [✧], and BC6-lPEI2.6S – pDNA from BC8-lPEI2.6 – pDNA, denoted by [○], p < 0.05, always analysed for the same N/P ratio.*

While with an increasing N/P ratio, theoretical linker content and with the lowered molecular weight distribution of the cross-linked lPEI the cytotoxicity of LR-lPEI2.6 – pDNA ($p < 0.01$) increased, remained the toxicity of BC-lPEI2.6 – pDNA polyplexes at a similar level ($p < 0.01$) (Figure 6).

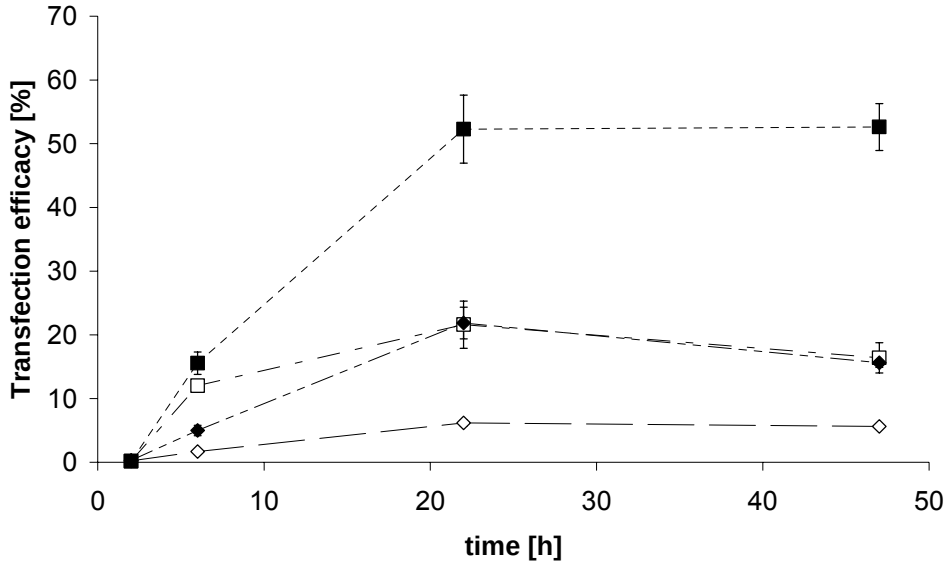
Generally, the purification of the cross-linked lPEI batches from residual lPEI seemed to have no striking advantage. While the effect on the efficacy of gene transfer was negligible, the cytotoxicity of the corresponding polyplexes was thereby comparable or even significantly enhanced.

It is discussed in literature that low molecular weight lPEI facilitate the smooth intracellular complex dissociation, concomitantly promoting pDNA transcription and transgene expression [9;10]. This may suggest that transfection and transgene expression proceeds with a different kinetic for cross-linked and unmodified lPEI-derived polyplexes. For a comparison, we chose LR3-lPEI2.6 – pDNA and lPEI5 – pDNA at an N/P ratio of 6 and 12, at which both exhibited maximum transfection, and determined the transfection efficacy and cell viability 2, 6, 22 and 47 hours after addition of the polyplexes to CHO-K1 cells *in vitro* (Figure 7).

However, the time-dependent course of transgene expression was similar for both gene carriers, while the level of transgene expression differed significantly with the linker / lPEI- and the N/P ratio ($p < 0.05$). Between 22 and 47 hours after addition of the polyplexes to the cells, a maximum transfection of 53 % was determined for LR3-lPEI2.6 – pDNA complexes, prepared at an N/P ratio of 12 ($p < 0.01$).

The cell viability was minimal at 2 hours post transfection. The cytotoxicity of polyplexes was significantly enhanced for polyplexes either with the N/P ratio or with the linker / lPEI ratio (LR3-lPEI2.6 – pDNA N/P 12 ≈ 8 %, lPEI5 – pDNA N/P 12 ≈ 30 %, LR3-lPEI2.6 – pDNA N/P 6 ≈ 37 %, lPEI5 – pDNA N/P 6 ≈ 50 %) (Figure 7b). After cells were treated with lPEI5 – pDNA as well as the LR3-lPEI2.6 – pDNA complexes built at an N/P ratio of 6, the cell viability was completely recovered after less than 10 hours.

a)



b)

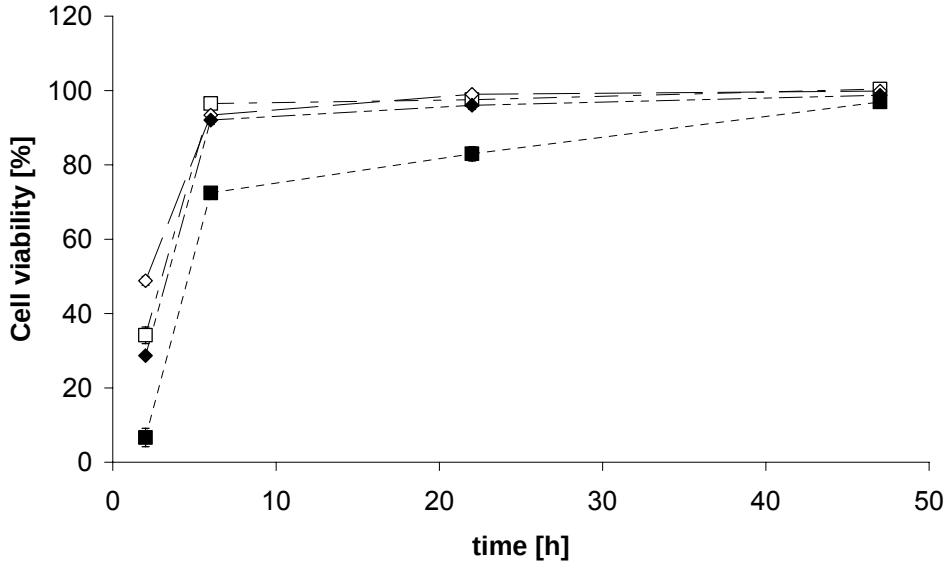


***Figure 7**. pEGFP-N1 was complexed in 0.15 M NaCl either with LR3-lPEI2.6 at an N/P ratio of 6 [□] and N/P 18 [■], or lPEI5 at an N/P ratio of 6 [◊] and 18 [♦]. 2, 6, 22, and 47 hours after addition of the polyplex dispersion to the adherent cells, a) the transfection efficacy and b) cell viability of CHO-K1 cells were determined by flow cytometry analysis. Data is represented as mean ± S.D., n = 3.*

After incubation with LR3-lPEI2.6 – pDNA polyplexes formed at an N/P ratio of 12 similar values were determined after 47 hours.

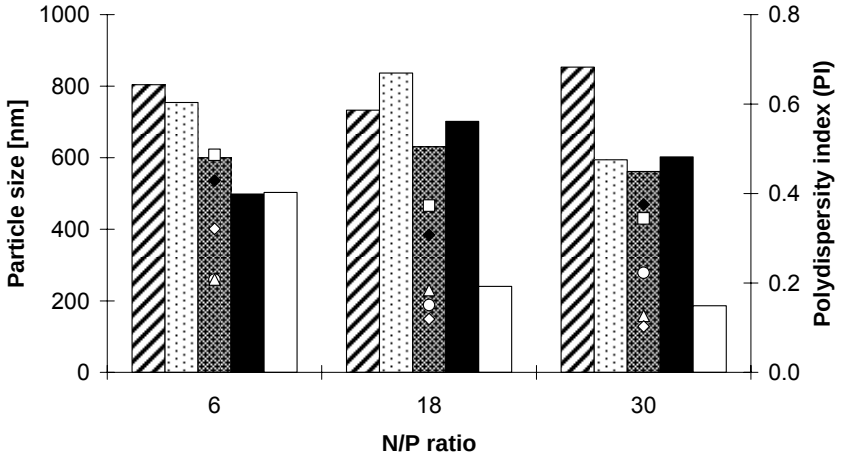
Polyplex size and stability

A major drawback of lmw lPEI-mediated gene transfer remains the low stability of the corresponding polyplexes at a physiological salt concentration [6;14;16]. The polyplex unravelling lowers the capacity to protect DNA from degradation and limits thereby the amount of intact genetic material available for transcription. We chose a cross-linking of the single lPEI chains aiming to result in a branched structure, which has been shown to be superior in the formation of small polyplexes with a narrow size distribution [6]. Here we investigated the impact of the cross-linking degree on the polyplex size and the surface charge. We further proved the accessibility of cross-linked lPEIs to the degradation in the presence of disulfide reducing agents, such as the synthetic dithiothreitol or the naturally occurring reduced form of glutathione.

Particle size and zeta potential

In medium of low ionic strength pDNA was complexed under the formation of nanoparticles, irrespective to the polymer applied. The polyplexes built with lPEI25 or the cross-linked lPEIs exhibited comparable hydrodynamic diameters between 500 and 800 nm after exposure to serum-free cell culture medium (HAM´s F12) (Figure 8), prone to particle aggregation (data not shown). In contrast to unmodified lmw lPEI – pDNA, polyplexes built with the cross-linked polymers exhibited a slightly negative zeta potential between -8 and -2 mV, which increased to positive values at further enhanced polymer concentrations (2-12 mV at an N/P ratio of 30; data not shown). The particle size of bPEI25 – pDNA complexes levelled at 200 nm with a positive net charge between 12 and 20 mV (zeta potential data not shown).

a)



b)

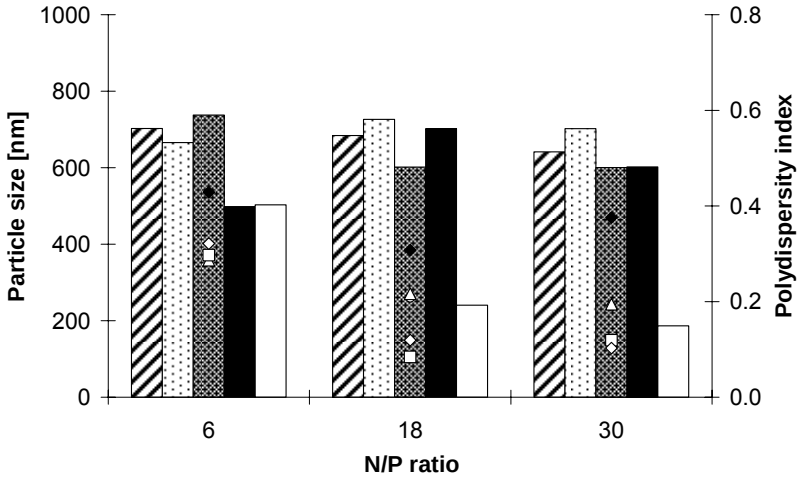
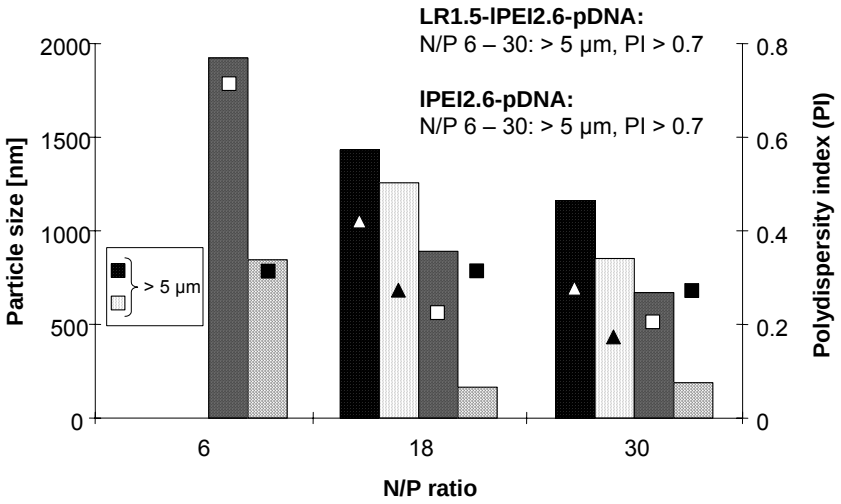


Figure 8. *The particle size / particle size distribution (m.v., n = 2) of a) LR1.5-lPEI2.6 – pDNA [▨ /○], LR2-lPEI2.6 – pDNA [□ / Δ], LR3-lPEI2.6 – pDNA [■ / □], bPEI25 – pDNA [■ / ♦], lPEI25 – pDNA [□ / ◊] polyplexes, or b) BC3-lPEI2.6 – pDNA [▨ /○], BC6-lPEI2.6 – pDNA [□ / Δ], and BC8-lPEI2.6 – pDNA [■ / □], bPEI25 – pDNA [■ / ♦], lPEI25 – pDNA [□ / ◊] polyplexes, manufactured in 5% glucose at an N/P ratio of 6, 18 and 30, determined after incubation in serum-free cell culture medium (HAM´s F12).*

Characterization of lmw lPEI – pDNA polyplexes revealed that, in contrast to pDNA complexation in medium of low ionic strength, the formation of discrete

polyplexes at a physiological salt concentration (0.15 M NaCl) relies on a minimum molecular weight of the polyamine [6].

a)



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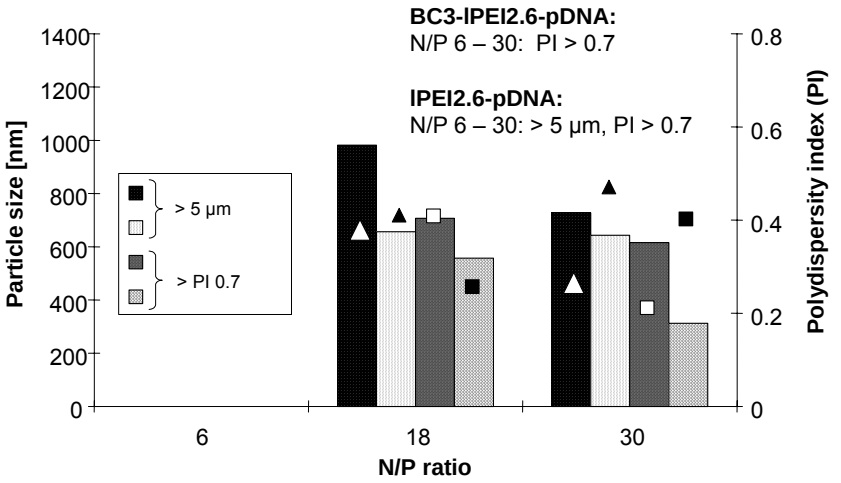


Figure 9. *The particle size and size distribution of a) LR2-lPEI2.6 – pDNA [■ / ∆], LR2-lPEI2.6S – pDNA [□ / ▲], LR3-lPEI2.6 – pDNA [■ / □], and LR3-lPEI2.6S – pDNA [□ / ■], or b) BC6-lPEI2.6 – pDNA [■ / ∆], BC6-lPEI2.6S – pDNA [□ / ▲], BC8-lPEI2.6 – pDNA [■ / □], and BC8-lPEI2.6S – pDNA [□ / ■] polyplexes prepared at an N/P ratio of 6, 18 and 30 in 0.15 M NaCl. Data represented as mean values (n = 2).*

pDNA complexation by polymers with a number average molecular weight (M_n) below 3 kDa resulted in large particle aggregates with hydrodynamic diameters above 5 µm and with a particle size distribution (polydispersity index (PI)) larger than 0.7.

We investigate the impact of the linker content and ergo of the increasing molecular weight of the polyamine, on the hydrodynamic diameters of corresponding polyplexes in 0.15 M NaCl.

In contrast to polyplexes built with the unmodified lPEI2.6, LR-lPEI2.6 or BC-lPEI2.6 condensed pDNA under the formation of discrete complexes between 250 and 2000 nm at an N/P ratio of 6 or 18, respectively (Figure 9).

The results indicate a correlation between the particle size of LR-lPEI2.6 – pDNA and less pronounced for BC-lPEI2.6 – pDNA complexes and the linker / lPEI ratio as well as to the N/P ratio applied. We determined the lowest hydrodynamic diameters and the particle size distribution for polyplexes built with the purified high molecular weight fraction of the corresponding cross-linked polymers. The LR-lPEI2.6 – pDNA or BC-lPEI2.6 complexes exhibited a positive net charge between 7 and 23 mV, irrespective to the N/P ratio applied (data not shown).

Polymer degradation

To assess the impact of the molecular weight of the cross-linked lPEI on the capacity of pDNA complexation, we performed gel retardation assays of LR1.5-lPEI2.6 – pDNA in comparison to LR3-lPEI2.6 – pDNA polyplexes and BC3-lPEI2.6 – pDNA versus BC8-lPEI2.6 – pDNA (Figure 10, left side).The cross-linked lPEIs complexed pDNA with comparable efficacy, irrespective to the molecular weight of the polyamine or the type of linker. The pDNA was thereby completely retarded to the complex at an N/P ratio between 1.5 and 2. In order to confirm that the disulfide linkages at the polymer backbone are susceptible to disulfide reducing agents and enable polymer degradation, we incubated the corresponding complexes after formation in 0.15 M NaCl

with 25 µM DTT (Figure 10 right side). After incubation of the polyplexes with DTT, the N/P ratio at which stable complexes were formed shifted to values above 2 or 2.5 for LR1.5-lPEI2.6 – and LR3-lPEI2.6 – pDNA polyplexes or BC3-lPEI2.6 – and BC8-lPEI2.6 – pDNA, respectively.

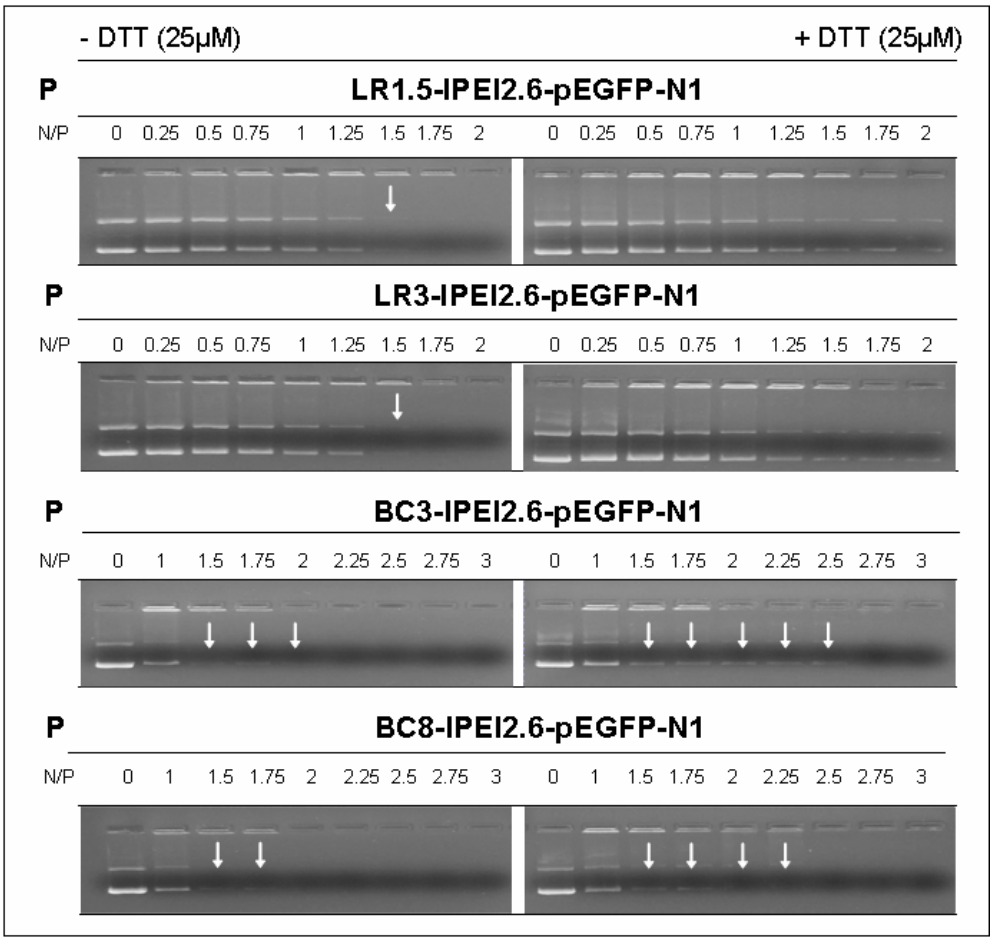


Figure 10. *Gel-retardation assays of cross-linked lPEI – pDNA polyplexes performed prior and after incubation with DTT (25 µM). The LR-lPEI-and BC-lPEI-derived polyplexes were prepared in 0.15 M NaCl at N/P ratio ranging between 1 and 2 or 1 and 3, respectively. pDNA bands either not complexed to the polyamine (N/P 0) or dissociated from polyplexes were separated by electrophoresis and visualized by ethidium bromide staining.*

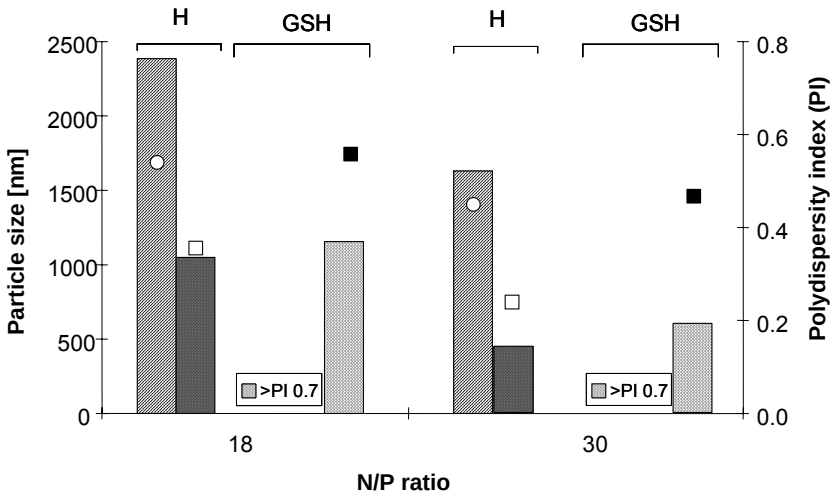
As aforementioned, we have shown that the complex size of lmw lPEI – pDNA complexes dispersed in medium of high ionic strength is influenced by the molecular weight of the polyamine [6]. Polymer degradation into short lPEI strands with an M_n below 3 kDa would result in the formation of large (> 5µm) and irregular structures (PI > 0.7). Hence, disulfide reduction would conceivably induce the disintegration of the corresponding cross-linked lPEI 2.6 – derived kDa polyplexes, detectable by an increasing polyplex size or polydispersity index.

In order to confirm that the cross-linking is reversible in the presence of disulfide reducing agents, we determined the particle size of LR1.5-lPEI2.6 – pDNA and LR3-lPEI2.6 – pDNA and BC3-lPEI2.6 – and BC8-lPEI2.6 – pDNA polyplexes prior and after 1 hour incubation with 50 mM GSH (Figure 11). The polyplexes were formed in 0.15 M NaCl and the incubation was performed at 37 °C, according to the conditions used for the transfection experiment.

Irrespective to the cross-linked lPEI used, polyplexes formed at an N/P ratio of 6 exceeded the 5 µm size scale with polydispersity indices above 0.7. At an N/P ratio of 18 and 30, LR1.5-lPEI2.6 – pDNA and LR3-lPEI2.6 – pDNA, as well as BC3-lPEI2.6 – pDNA and BC8-lPEI2.6 – pDNA exhibited hydrodynamic diameters between 300 and 2500 nm and PIs between 0.2 and 0.7, which decreasing with the linker / lPEI ratio and the N/P ratio applied.

After incubation with GSH the polydispersity indices of LR1.5-lPEI2.6-pDNA and BC3-lPEI2.6-pDNA exceeded 0.7, while LR3-lPEI2.6-pDNA increased in size and size distribution, indicating a reduction of the polyamine molecular weight. The differences between untreated polyplexes and complexes exposed to GSH seemed thereby most pronounced for BC-lPEI2.6-derived particles.

a)



b)

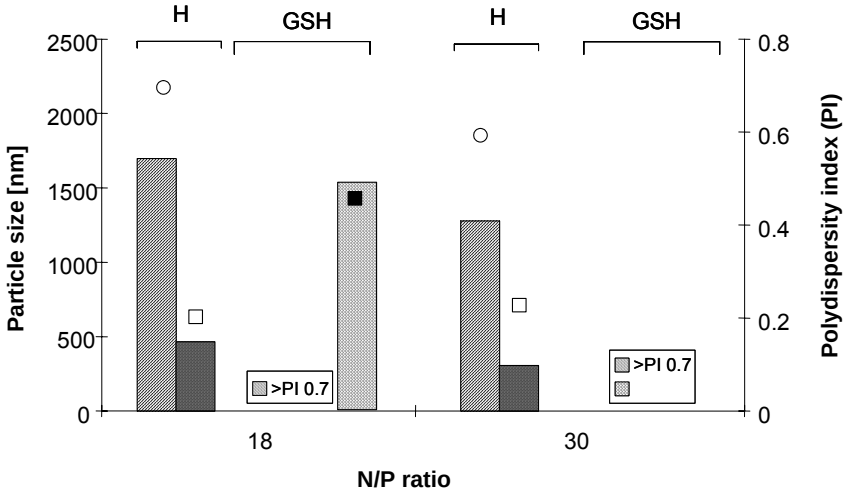


Figure 11. *Polyplexes were prepared in 0.15 M NaCl at an N/P ratio of 6, 18, and 30. The particle size / size distribution (m.v., n = 2) was determined either after 60 minutes incubation in 7.5 mM HEPES (pH 7.5) or 50 mM GSH at 37 °C:*

a) LR1.5-lPEI2.6 – pDNA [▨ /○] and LR3-lPEI2.6 – pDNA [■ / □] in HEPES, LR1.5-lPEI2.6 – pDNA [▨/●] and LR3-lPEI2.6 – pDNA [▨ / ■] in GSH, or

b) BC3-lPEI2.6 – pDNA [▨ /○] and BC8-lPEI2.6 – pDNA [■ / □] in HEPES, BC3-lPEI2.6 – pDNA [▨ / ●] and BC8-lPEI2.6 – pDNA [▨ / ■] in GSH.

Conclusion

In order to further improve lPEI-mediated gene transfer we synthesized series of degradable disulfide cross-linked low molecular weight lPEIs with varying polymer compositions as materials for the manufacture of *in vitro* pDNA transfection agents. The resulting gene carrier complexed pDNA under the formation of nanoparticles in medium of low ionic strength, as well as at physiological salt concentrations. First experiments confirmed the destabilization of the cross-linked lPEI-derived polyplexes in the presence of disulfide reducing agents *in vitro*, indicating the degradability of the polymers. We have shown that the efficacy of *in vitro* gene transfer, the cell viability and the particle size has been a function of the linker / lPEI ratio and of the N/P ratio applied. In comparison to the corresponding lPEI25 – pDNA or bPEI25 – pDNA complexes, both the transfection efficiency as well as the cell compatibility was improved to several-fold using cross-linked lPEIs as gene carriers. BC-lPEI2.6-derived polyplexes exhibited a superior *in vitro* performance, compared to LR-lPEI2.6 – pDNA complexes, reaching comparable transfection efficacy at significantly higher level of cell viability.
In advantage to the aforementioned approaches, we obtained novel polymer gene carriers that first enable gene transfer with relatively high efficacy and cell compatibility.

Experimental section

lPEIs with M_n (GFC) [25] of 2.6 and 4.6 kDa were synthesized by ring-opening polymerization of 2-ethyl-2-oxazoline and acid-catalyzed hydrolysis of the corresponding poly(2-ethyl-2-oxazoline). 3,3´-Dithiodipropionic acid di(N-succinimidyl ester) (Lomant´s reagent) was purchased from Fluka. N,N'-bis-(tert-butoxycarbonyl) cysteine (boc-cystine, BC) was shipped from Advanced Chem Tech, Louisville, KE, USA. 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), dichloromethane, ethanol, and ninhydrine spray for TLC were obtained from Acros Organics, Geel, Belgium. Branched polyethylenimine (high molecular weight, water free, M_W 25,000 Da

(LS), M_n 10,000 Da (GPC)) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Linear polyethylenimine MW ~ 25,000 Da (7-8 % poly(2-ethyl-2-oxazoline, mp. 73-75°) was obtained from Polysciences Inc., Warrington, PA, USA. PD10 prepacked columns were obtained from Amersham Biosciences, Freiburg, Germany.

The nutrient mixture F-12 (HAM), ethidium bromide, glutathione (GSH) in its reduced form, and kanamycin were obtained from Sigma-Aldrich. Dithiothreitol (DTT; Cleland´s Reagent) was purchased from Bio-Rad Laboratories, Munich, Germany. pEGFP-N1 was obtained from Clontech (Heidelberg, Germany). The *E.coli* JM109 bacterial strain was shipped from Promega. The Plasmid Maxi Kit was purchased from Qiagen (Hilden, Germany). CHO-K1 cells (ATCC# CCL-61) and fetal calf serum (FCS) were supplied by Biochrom KG Seromed (Berlin Germany). Agarose and LB broth medium were purchased from Invitrogen GmbH (Germany). Serva Blue G250 was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany).

Polymer Synthesis

Polyethylenimine cross linked with 3,3´-Dithiodipropionic acid di(N-succinimidyl ester)(Lomant´s Reagent (LR))

Anhydrous lPEI was dissolved in 12 ml dichloromethane and heated to 50 °C. LR was dissolved in 5 ml of dichloromethane (DCM) (specific amounts listed in table 1). The clear solution was added drop-wise to the lPEI solution under vigorous stirring and the mixture was kept at 50 °C over night. After the volatiles were removed under reduced pressure, the yellow waxen residue was dissolved in 2 N hydrochloric acid and the cross-linked lPEI was precipitated with a concentrated aqueous sodium hydroxide. The white gel-like residue was washed with water until the supernatant became neutral. The purified cross-linked lPEI was dried at 70 °C under reduced pressure.

Product	lPEI2.6 [mg]	Ethylenimine units [mmol]	LR [mg]	LR [mmol]	DCM [ml]
LR0.5-lPEI2.6	683.5	16.27	16.60	0.04	17
LR1.5-lPEI2.6	730.0	17.38	53.08	0.13	17
LR2-lPEI2.6	764.0	18.19	73.73	0.18	17
LR3-lPEI2.6	773.8	18.42	112.53	0.28	17
Product	lPEI4.6 [mg]	Ethylenimine units [mmol]	LR [mg]	LR [mmol]	DCM [ml]
LR1.5-lPEI4.6	723.0	17.21	52.4	0.13	20

Table 1. *Amounts and molar ratios of the reactants used for the synthesis of the LR- lPEI2.6- and LR-lPEI4.6-series.*

^{1}H-NMR (600 MHz, $CDCl_3$/TMS): δ_H (ppm) = 1.10-1.16 (m, -NCOCH$_2$C*H*$_3$), 1.40-2.1 (brs, N*H*), 2.33-2.41 (m, -NCOC*H*$_2$-CH$_2$-S- /-NCOC*H*$_2$-CH$_3$), 2.41-2.45 (brs, -NHC*H*$_3$), 2.5-3.2 (m, -C*H*$_2$-C*H*$_2$-NH- / C*H*$_3$NCO-), 3.25-3.6 (m, C*H*$_2$-C*H*$_2$-NCO-CH$_2$-CH$_3$ / -C*H*$_2$-C*H*$_2$-NCO-C*H*$_2$-C*H*$_2$- / C*H*$_3$N(CO-)- / -C*H*$_2$-S-), 3.6-3.7 (m, -CH$_2$-C*H*$_2$-OH)

Polyethylenimine cross linked with Cystine

DMT-MM (6 ml), lPEI (10 ml) and boc-cystine (BC) (4 ml) were each dissolved in ethanol (specific amounts listed in table 2). The solution of lPEI and boc-cystine were transferred into the glas tube of a parallel synthesis block, mixed by vigorous shaking, and the DMT-MM solution was then added to the clear mixture. After the mixture was shacked over night at room temperature [26], the volatiles were removed under reduced pressure. The yellow waxen residue was dissolved in 2 N hydrochloric acid and the cross-linked lPEI was precipitated with concentrated aqueous sodium hydroxide. The white gel-like residue was washed with water until the washing water became neutral. The purified cross-linked lPEI was dried at 70 °C under reduced pressure.

Product	lPEI2.6 [mg]	Ethylen-imine units [mmol]	BC [mg]	BC [mmol]	DMT-MM [mg]	DMT-MM [mmol]	EtOH [ml]
BC3-lPEI2.6	500.7	11.64	77.54	0.17	160.65	0.35	20
BC4-lPEI2.6	98.3	2.29	20.47	0.05	44.24	0.09	10
BC6-lPEI2.6	685.0	15.93	210.39	0.48	218.21	0.79	20
BC8-lPEI2.6	812.0	18.90	328.92	0.76	321.2	1.13	20
Product	lPEI4.6 [mg]	Ethylen-imine units [mmol]	BC [mg]	BC [mmol]	DMT-MM [mg]	DMT-MM [mmol]	EtOH [ml]
BC1.5-lPEI4.6	564.3	13.4	43.55	0.10	54.72	0.2	21

Table 2. *Amounts and molar ratios of the educts used for the synthesis of the BC- lPEI2.6- and BC-lPEI4.6-series.*

^{1}H-NMR (600 MHz, $CDCl_3$/TMS): δ_H (ppm) = 1.10-1.18 (t, -NCOCH$_2$C*H*$_3$), 1.42-2.25 (brs, N*H*), 2.2-2.40 (m, -NCOC*H*$_2$-CH$_3$), 2.41-2.45 (brs, -NHC*H*$_3$), 2.55-3.0 (m, -C*H*$_2$-C*H*$_2$-NH- / C*H*$_3$NCO-), 3.0-3.55 (m, -NCO-C*H*$_2$-CH$_3$ / -C*H*$_2$-C*H*$_2$-NCO-C*H*$_2$-C*H*$_2$- / C*H*$_3$N(CO-)- / -C*H*$_2$-S-), 3.6-3.8 (m, -CH$_2$-C*H*$_2$-OH / -S-CH2-C*H*(NH2)-N-)

The amine-base was transformed into the corresponding hydrochloride with 2 N HCl to facilitate water solubility. Gel-chromatography with a pre-packed Sephadex G25 column was used to separate the higher molecular weight fraction (S-fraction: e.g. LR-lPEI2.6S) from the respective polymer bulk. The column was rinsed with water (20 ml) and equilibrated with 20 ml 0.15 M NaCl. 100 mg LR/BC-lPEI dissolved in 1.0 ml 0.15 M NaCl was eluted with 0.15 M NaCl and fractions of 500 µl were collected. Probes were analyzed

using TLC, detecting thiol-containing and pure amine fractions with ninhydrine spray. The nitrogen content of the cross-linked lPEI·HCl was determined by elemental analysis.

Gel Filtration Chromatography (GFC)

GFC was performed on a Shimadzu HPLC system (SIL-10ADvp auto injector, LC-10ATvp liquid chromatograph, DGU 14A degaser, CTO-0ASvp column oven, SCL-10Avp system controller; RID-10A refractive index detector thermostatted at 40 °C).
20.0 mg LR/BC-lPEI·HCl was dissolved in 1.0 ml double distilled water (ddH2O) and filtered through a 0.2 µm polyethersulfonic acid membrane filter. A Novema 300 Å SEC column (10 µm, 8 x 300 mm, Polymer Standard Service, Mainz) thermostatted at 40 °C, a flow rate of 1 ml/min, injection volume of 25 µl and 0.15 M NaCl as eluent were used as chromatographic setup.

Amplification and Purification of plasmid DNA [pDNA]

The pEGFP-N1 was transformed into the E.coli JM109 bacterial strain. The transformed cells were expanded in LB medium supplemented with kanamycin. The pDNA was isolated using the Quiagen Plasmid Maxi Kit according to the supplier´s protocol. The concentration and purity of the pDNA was measured by UV absorption at 260 and 280 nm.

Preparation of polyplexes

The polymers were dissolved in either 0.15 M NaCl or 5 % glucose and the pH was adjusted to 7.0 with 0.01 N aqueous hydrochloric acid or 0.01 N aqueous sodium hydroxide. The concentrations of the corresponding polymer solutions were adjusted to produce an N/P ratio of 1 upon the addition of 1 µl polymer solution to 2 µg pDNA. The pDNA and the appropriate volume of polymer solution were each diluted with 0.15 M NaCl or 5 % glucose to equal volumes corresponding to 25 µl per 1 µg pDNA. The pDNA and polymer

solutions were mixed by vortexing and the polyplexes were allowed to form for 20 minutes at room temperature.

Particle size measurements

Polyplexes prepared in 0.15 M NaCl or 5 % glucose containing a total of 10 µg pEGFP-N1 were diluted to 2.5 ml with 0.15 M NaCl or HAM´s F12 (serum-free cell culture medium), respectively. The samples were thermostated to 25 °C and laser light scattering analysis was performed at 25 °C with an incident laser beam of 633 nm at a scattering angle of 90° using a Malvern ZetaSizer 3000 HSA, Malvern Instruments GmbH, Germany. The count rates for all dispersants were lower than 5 Kcps, confirming their applicability in size measurements. The following parameters were used: viscosity of HAM´s F12 and 0.15 M NaCl: 0.89 mm^2/s; the refractive indices of HAM´s F12: 1.681 and 0.15 M NaCl: 1.33. The sampling time was set automatically.
Six measurements each with 10 sub-runs were performed for each of two independent samples (n = 2). The zeta potential measurements of the same polyplex samples were performed in the standard capillary electrophoresis cell of the ZetaSizer HSA (Malvern Instruments GmbH, Germany), measuring the electrophoretic mobility at 25 °C. The sampling time was again set automatically (n = 2).
In order to investigate the polyplex destabilization in an reductive environment we formed polyplexes (10 µg pEGFP-N1) in 0.15 M NaCl, added 500 µl HEPES 7.5 mM pH 7.5 (negative control) or 500 µl GSH 50 mM in HEPES 7.5 mM pH 7.5, incubated the polyplexes for 60 minutes at 37 °C, and determined the particle size, size distribution and zeta potential as described above (seven measurements each with 10 sub-runs for each of two independent probes, n = 2).

Gel-retardation assay

Polyplexes (0.5 µg pEGFP-N1 in 5 µl 0.15 M NaCl) were prepared with the appropriate amount of LR1.5/LR3-lPEI solution 0.25, 0.5, 0.75, 1, 1.25, 1.5,

1.75, and 2 or BC3/BC8-lPEI solution (always diluted to 8.5 µl) to produce N/P ratios of 1, 1.5, 1.75, 2, 2.25, 2.5, 2.75, and 3. After incubating for 20 minutes at room temperature, 6 µl DTT (41.4 mM) was added to the polyplexes and the mixture was incubated for another 60 minutes at room temperature [27]. As negative control, pEGFP-N1 was treated according to the procedure described above (N/P 0). After addition of 1.5 µl loading buffer, the samples were loaded into a 1 % TAE agarose gel and run at 80 V for 50 minutes.

The pDNA bands were visualized by ethidium bromide staining and detected on a Fisherbrand FT-20/312 UV transilluminator 312 (Herolab GmbH Laborgeräte, Germany).

In vitro transfection and cytotoxicity experiments

For gene transfer studies, CHO-K1 cells were grown in 24-well plates at an initial density of 38,000 cells per well. 18 hours after plating, the culture media was removed; cells were washed with PBS (Invitrogen, Germany) and 900 µl serum-free media (HAM´s F12) were added. Thereafter, the prepared polyplexes were added to the cells. After 4 hours, the medium was replaced with 1 ml of culture medium. 48 hours later, cells were prepared for flow cytometry analysis. Floating cells were collected and combined with adherent cells after trypsinization. The collected cells were washed twice with PBS, resuspended in 500 µl PBS and propidium iodide was added at a concentration of 1 µg/ml to half of the samples. Measurements were taken on a FACSCalibur (Becton Dickinson, Germany) using CellQuest Pro software (Becton Dickinson, Germany) and WinMDI 2.8 (©1993-2000 Joseph Trotter).

EGFP positive cells were detected using a 530/30 nm band-pass filter, while the propidium iodide emission was measured with a 670 nm long pass filter. Logarithmic amplification of EGFP and propidium iodide emission in green and red fluorescence was obtained with 20,000 cells counted for each sample. In a density plot representing forward scatter against sideward scatter, whole cells were gated out and depicted as a two-parameter dot plot

of EGFP versus propidium iodide for analysis. The EGFP positive region, which designates transfected cells, was drawn starting above cell auto fluorescence. The geometric mean fluorescence intensity was determined from the number of EGFP positive cells. Further, the number of propidium iodide negative cells was counted as a measure of cell viability. The relative transfection efficacy and cell viability were determined by normalizing the number of EGFP-positive and propidium iodide-negative events counted in a sample to the negative control and expressed as a percent. The statistical analysis was performed by one-way analysis of variance (ANOVA) and the Tuckey- or Dunnett´s test.

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7. METHOXY POLY (ETHYLENE GLYCOL) - LOW MOLECULAR WEIGHT LPEI - DERIVED COPOLYMERS ENABLE POLYPLEX SHIELDING

U. Lungwitz, M. Breunig R., Liebl, T. Blunk, A. Göpferich

Department of Pharmaceutical Technology, University of Regensburg, 93040 Regensburg, Germany

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Abstract

We synthesized a series of copolymers by covalent linkage of activated 5 or 20 kDa linear methoxy poly(ethylene glycol) (mPEG) or 10 kDa 2-arm-mPEG to nontoxic low molecular weight (2.6 and 4.6 kDa) linear polyethylenimine (lPEI) at different molar ratios (mPEG-lPEI copolymers). All of the copolymers condensed pEGFP-N1 plasmid DNA (pDNA) to form nanoparticles with hydrodynamic diameters between 150 and 420 nm, demonstrating a resistance to polyplex aggregation. The efficacy of pDNA complexation was thereby reduced compared to the corresponding lPEI homopolymers. Copolymer – pDNA complexes exhibited a zeta potential between – 4 and 6 mV, depending on the particle environment. That the use of the PEGylated nanoparticles reduced non-specific cellular uptake compared to lPEI2.6 – or lPEI4.6 – pDNA particles, was confirmed by *in vitro* gene transfer experiments, complemented by Confocal Laser Scanning Microscopy (CLSM). After four hour incubations of CHO-K1 cells with the mPEG-lPEI – pDNA complexes under serum-free conditions, the transfection efficacy was only between 0.2 % and 2.5 %. From our experiments we conclude that these polymers sufficiently suppress non-specific polyplex internalization by cells, providing a basis for the design of *in vivo* applicable polyplexes optimized for a cell-specific gene delivery.

Introduction

Growing insight into the molecular pathology of many diseases has evoked a great interest for gene therapy using gene replacement or supplementation strategies [1;2]. Nevertheless, its clinical utility still demands the development of biocompatible and efficient gene carriers that allow for targeted gene transfer to disseminated cells or tissues after systemic administration. A class of materials showing particular promise are self-assembling polyethylenimine (PEI) – nucleic acid complexes, which have been shown to transfect a large variety of cells *in vitro* and *in vivo*, can encapsulate large amounts of DNA, and are easy to prepare and modify [3]. Unfortunately, the systemic administration of the highly cationic PEI – pDNA complexes is limited due to size-restricted diffusion and elimination of its large aggregates from the bloodstream by liver macrophages [4]. In addition, the so-called polyplexes provoke the aggregation of erythrocytes and activate the complement system. The non-specific interaction of the vector with serum proteins, non-targeted cells, or extracellular matrix additionally reduces the amount of the therapeutic gene successfully delivered to the targeted cells or tissues [5-7]. Therefore, the utility of PEI-based vectors for cell-specific gene transfer requires sufficient shielding of the PEI – pDNA complex surface charge.

In efforts to stabilize the polyplexes for a biological environment, researchers have formed complexes using PEG-PEI copolymers (pre-PEGylation) [8-12] and alternatively PEGylated preformed PEI – pDNA complexes (post-PEGylation) [5;13]. In both cases, hydrophilic PEG layers coated the particle surface, reducing particle aggregation and non-specific interactions through steric hindrance and shielding of the excess positive net charge. Unfortunately, post-PEGylation strategies must be compatible with polyamine – DNA complexes and result in non-uniform and poorly characterized polyplexes. While using PEG-PEI copolymers ensures that the particle composition is well-defined, the charge shielding effect of the PEG component often counteracts efficient pDNA complexation [5;6;8;9].

We have recently shown that the use of low molecular weight lPEI – pDNA complexes significantly improved the *in vitro* transfection efficacy and cell viability compared to commonly used vectors based on the commercially available branched (bPEI) and 25 kDa linear polyethylenimine (lPEI) [14]. Unfortunately, the low molecular weight lPEI – pDNA complexes rapidly aggregated into large clusters on the micrometer scale at physiological salt concentrations [15], conceivably due to reduced Coulombic repulsion and increased van der Waals attraction [16].

We therefore focused on the particle stabilization using a pre-PEGylation strategy. In order to identify a copolymer composition that combines adequate pDNA compaction with an efficient charge-shielding effect, we synthesized a series of methoxy-poly (ethylene glycol) (mPEG) - low molecular weight lPEI-based copolymers (mPEG-lmw lPEI). The pDNA condensing residue is based on non-toxic lPEI with a relative number average molecular weight (M_n) of 2.6 or 4.6 kDa. For charge-shielding, 5 or 20 kDa linear mPEGs, as well as a branched PEG consisting of two 5 kDa α-amino-ω-methoxy PEG chains tethered to a glycerol-butanoyl linker, have been conjugated to the lPEI unit at various stoichiometric ratios (Table 1 and 2). We investigated the capacity of mPEG-lPEI copolymers to condense pDNA and to stabilize the complex against particle aggregation.

As an indication of sufficient charge-shielding by the mPEG-lPEI copolymers, we used CLSM imaging to visualize the interaction of YOYO-labelled polyplexes with the surface of CHO-K1 cells and determined the transfection efficacy of pEGFP-N1-mPEG-lPEI – pDNA polyplexes in CHO-K1 cells *in vitro* by flow cytometry analysis. To prove the biocompatibility of the transfection vehicles, we determined the cytotoxic effect of the corresponding mPEG-lPEI-based polyplexes on CHO-K1 cells.

Methods and Materials

lPEIs with number average molecular weight of 2.6 and 4.6 kDa were synthesized by ring-opening polymerization of 2-ethyl-2-oxazoline and acid-

catalysed hydrolysis of the corresponding poly(2-ethyl-2-oxazoline) and characterized by ^{1}H-NMR and Gel Filtration Chromatography (GFC) [17].

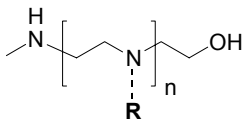
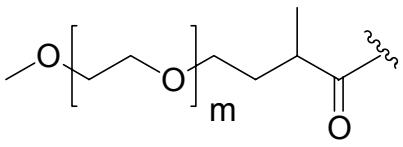
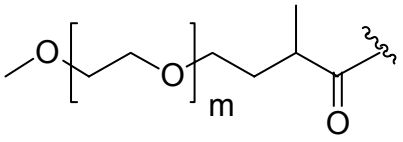
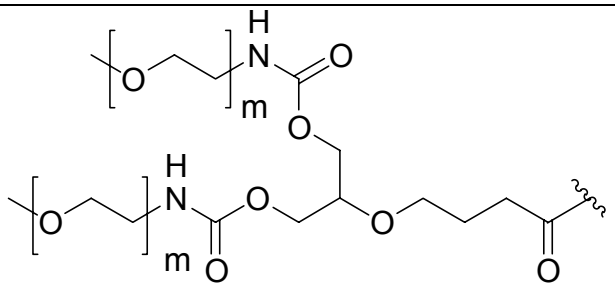
linear polyethylenimine	mPEG component	PEGylation reagent
 Linear polyethylenimine 2.6 kDa or: 4.6 kDa (lPEI2.6 or lPEI4.6; M_n (GFC))	 R_1: $M_n \approx 5$ kDa mPEG5	mPEG-SMB-5000
	 R_2: $M_n \approx 20$ kDa mPEG20	mPEG-SMB-20K
	 R_3: $M_n \approx 10$ kDa mPEG10	mPEG2-NHS-10K

Table 1. *The methoxy poly(ethylene glycol)-linear polyethylenimine (mPEG-lPEI) copolymers were synthesized by coupling of linear or branched mPEG-NHS active esters with a molecular weight of 5 kDa, 20 kDa or 10 kDa to lPEI with a number average molecular weight of 2.6 kDa or 4.6 kDa.*

The mPEG-active ester methoxy-poly(ethylene glycol) succinimidyl-α-methylbutanoate was obtained from Nektar Therapeutics (AL, USA) in the 5,000 Da (mPEG-SMB-5000) and 20 kDa (mPEG-SMB-20K) chain lengths, as was the 10 kDa 4-[1,3-bis(ω-methoxy-α-amino poly(ethylene glycol)

carbonyloxy) propan-2-yloxy]-butanolyl-N-hydroxysuccinimide (mPEG2-NHS-10K) (Table 1).

Analytical grade dichloromethane and acetone were purchased from VWR International, Germany. Branched polyethylenimine (high molecular weight, water free, M_W 25,000 Da (LS), M_n 10,000 Da (GPC)) was obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Linear polyethylenimine MW ~ 25,000 Da (7-8 % poly(2-ethyl-2-oxazoline, mp. 73-75°) was purchased from Polysciences Inc., Warrington, PA, USA.

The nutrient mixture F-12 (HAM), ethidium bromide, and kanamycin were obtained from Sigma-Aldrich. pEGFP-N1 was purchased from Clontech (Heidelberg, Germany). The *E.coli* JM109 bacterial strain was shipped from Promega. The Plasmid Maxi Kit was purchased from Qiagen (Hilden, Germany). CHO-K1 cells (ATCC# CCL-61) and fetal calf serum (FCS) were supplied by Biochrom KG Seromed (Berlin Germany). Agarose and LB broth medium were purchased from Invitrogen GmbH (Germany). Serva Blue G250 was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany).

mPEG-lPEI copolymer synthesis

Copolymer	lPEI 2.6 kDa [mg]; [mmol]	lPEI 4.6 kDa [mg]; [mmol]	PEGylation reagent [mg]; [mmol]	lPEI : mPEG (mol / mol)
mPEG5/3-lPEI2.6	75; 0.0288	–	mPEG-SMB-5000: 500; 0.0868	1 : 3
mPEG5/3-lPEI4.6	–	145; 0.0315		1 : 3
mPEG20-lPEI2.6	63; 0.0242	–	mPEG-SMB-20K: 500; 0.0243	1 : 1
mPEG20-lPEI4.6	–	121.5;		1 : 1

		0.0264		
mPEG10/2-lPEI2.6	60; 0.0231	–	mPEG2-NHS-10K: 500; 0.0462	1 : 2
mPEG10/2-lPEI4.6	–	115.5; 0.0251		1 : 2

Table 2. *Amounts and molar ratios of the starting materials used for the synthesis of the mPEG-lPEI2.6 and mPEG-lPEI4.6 copolymer series.*

The anhydrous lPEI and the mPEG-derivative (specific amounts listed in Table 2) were each dissolved in 5 ml dichloromethane. The clear solutions were mixed and stirred overnight at room temperature. The solvent was evaporated and the residue was dissolved in diluted hydrochloric acid (pH 5). The crude white gel-like mPEG-lPEI copolymer was precipitated by the addition of a concentrated sodium hydroxide solution. Precipitates containing lPEI4.6 were washed with water until the supernatant was neutral, yielding 20 to 60% of the corresponding copolymer. Because of the water-solubility of the copolymers, the crude copolymer was alternatively purified by extracting the concomitantly formed NHS with acetone at low temperature. Diluted hydrochloric acid was added to all of the derivatives to yield the hydrochlorides of the mPEG-lPEI copolymers.

mPEG5/3-lPEI2.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.28-3.31 (s, C*H*$_3$O-PEG), 3.40-3.54 (m, -$^{+}$NH$_2$-*CH*$_2$-*CH*$_2$-), and 3.56-3.71 (m, -O-*CH*$_2$-*CH*$_2$-).

mPEG5/3-lPEI5.6: ^{1}H-NMR (400 MHz, D_2O): δ_H (ppm) = 3.28-3.30 (s, C*H*$_3$O-PEG), 3.38-3.52 (m, -$^{+}$NH$_2$-*CH*$_2$-*CH*$_2$-), and 3.56-3.66 (m, -O-*CH*$_2$-*CH*$_2$-).

mPEG20-lPEI2.6 and mPEG20-lPEI4.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.28-3.29 (s, C*H*$_3$O-PEG), 3.41-3.53 (m, -$^{+}$NH$_2$-*CH*$_2$-*CH*$_2$-), and 3.53-3.70 (m, -O-*CH*$_2$-*CH*$_2$-).

mPEG10/2-lPEI2.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.23-3.27 (s, C*H*$_3$O-PEG), 3.38-3.52 (m, -$^{+}$NH$_2$-*CH*$_2$-*CH*$_2$-), and 3.52-3.66 (m, -O-*CH*$_2$-*CH*$_2$-).

mPEG10/2-lPEI4.6: ^{1}H-NMR (600 MHz, D_2O): δ_H (ppm) = 3.14-3.20 (s, C*H*$_3$O-PEG), 3.38-3.45 (m, -$^{+}$NH$_2$-*CH*$_2$-*CH*$_2$-), and 3.45-3.57 (m, -O-*CH*$_2$-*CH*$_2$-).

Amplification and Purification of plasmid DNA [pDNA]

The pEGFP-N1 was transformed into the E.coli JM109 bacterial strain. The transformed cells were expanded in LB medium supplemented with kanamycin. The pDNA was isolated using the Quiagen Plasmid Maxi Kit according to the supplier´s protocol. The concentration and purity of the pDNA was measured by UV absorption at 260 and 280 nm.

Preparation of polyplexes

Both lPEIs and all of the mPEG-lPEI copolymers were converted into the corresponding hydrochloride using 2 N HCl and the nitrogen content was determined by elemental analysis. In brief: the polymers were dissolved in either 150 mM NaCl or 5 % glucose and the pH was adjusted to 7.0 with 0.01 N aqueous hydrochloric acid or 0.01 N aqueous sodium hydroxide. The concentrations of the corresponding polymer solutions were adjusted to produce an N/P ratio of 1 upon the addition of 1 µl polymer solution to 2 µg pDNA. The pDNA and the appropriate volume of polymer solution were each diluted with 0.15 M NaCl or 5 % glucose to equal volumes corresponding to 25 µl per 1 µg pDNA. The pDNA and polymer solutions were mixed by vortexing and the polyplexes were allowed to form for 20 minutes at room temperature.

Laser light scattering analysis and zeta potential measurements

Polyplexes prepared in 0.15 M NaCl or 5 % glucose containing a total of 10 µg pEGFP-N1 were diluted to 2.5 ml with 0.15 M NaCl or HAM´s F12 (serum-free transfection medium), respectively. The samples were thermostated to 25 °C and laser light scattering analysis was performed at 25 °C with an

incident laser beam of 633 nm at a scattering angle of 90° using a Malvern ZetaSizer 3000 HSA, Malvern Instruments GmbH, Germany. The count rates for all dispersants were lower than 5 Kcps, confirming their applicability in size measurements. The following parameters were used: viscosity of HAM´s F12 and 0.15 M NaCl: 0.89 mm^2/s; the refractive indices of HAM´s F12: 1.681 and 0.15 M NaCl: 1.33. The sampling time was set automatically.

Five measurements each with 10 sub-runs were performed for each of two independent samples (n=2). Size measurements of polyplexes prepared at an N/P ratio of 18 were carried out for three independent samples (n=3).

The zeta potential measurements of the same polyplex samples were performed in the standard capillary electrophoresis cell of the ZetaSizer HSA (Malvern Instruments GmbH, Germany), measuring the electrophoretic mobility at 25 °C. The sampling time was again set automatically (n=2, polyplexes at an N/P ratio of 18: n=3).

The measurements were analyzed statistically by one-way analysis of variance (ANOVA) and the Tuckey- or Dunnett´s test.

Gel-retardation assay

Polyplexes (0.5 µg pEGFP-N1 in 5 µl 0.15 M NaCl or 5 % glucose) were prepared with the appropriate amount of lPEI or mPEG-lPEI solution (always diluted to 8.5 µl) to produce N/P ratios of 0, 1, 1.5, 2, 3, and 4. After incubating for 10 minutes at room temperature, 5 µl HAM´s F12 transfection medium was added to the polyplexes and the solutions were incubated for another 10 minutes at room temperature. After addition of 1.5 µl loading buffer, the samples were loaded into a 1 % TAE agarose gel and run at 80 V for 50 minutes. The pDNA bands were visualized by ethidium bromide staining and detected on a Fisherbrand FT-20/312 UV transilluminator 312 (Herolab GmbH Laborgeräte, Germany).

Confocal Laser Scanning Microscopy

A Zeiss Axiovert 200 M microscope coupled to a Zeiss LSM 510 scanning device (Carl Zeiss Co. Ltd., Germany) was used for imaging of the CHO-K1 cells. The inverted microscope was equipped with Plan-Apochromat 63x and Plan-Neofluar 100x objectives. Cells were plated in 8-well Lab-Tek™ Chambered Coverglass (Nunc GmbH & Co. KG, Germany) at an initial density of 35,000 cells/chamber in 400 µl culture media. To maintain a pH of 7.4, 20 mM HEPES was supplemented in the media. After 18 hours, polyplexes were added and the cells in each well were immediately imaged at 37 °C. The thickness of each optical section was between 0.7 and 1.2 µm. For the visualization of polyplexes, pDNA was labelled with YOYO-1. The intercalate was excited at 488 nm using an argon laser and the fluorescence was detected using a 505-530 nm band-pass filter.

In vitro transfection and cytotoxicity experiments

For gene transfer studies, CHO-K1 cells were grown in 24-well plates at an initial density of 38,000 cells per well. 18 hours after plating, the culture media was removed; cells were washed with PBS (Invitrogen, Germany) and 900 µl serum-free media (HAM´s F12) were added. Thereafter, the prepared polyplexes were added to the cells. After 4 hours, the medium was replaced with 1 ml of culture medium. 48 hours later, cells were prepared for flow cytometry analysis. Floating cells were collected and combined with adherent cells after trypsinization. The collected cells were washed twice with PBS, resuspended in 500 µl PBS and propidium iodide was added at a concentration of 1 µg/ml to half of the samples. Measurements were taken on a FACSCalibur (Becton Dickinson, Germany) using CellQuest Pro software (Becton Dickinson, Germany) and WinMDI 2.8 (©1993-2000 Joseph Trotter). EGFP positive cells were detected using a 530/30 nm band-pass filter, while the propidium iodide emission was measured with a 670 nm long pass filter. Logarithmic amplification of EGFP and propidium iodide emission in green

and red fluorescence was obtained with 20,000 cells counted for each sample. In a density plot representing forward scatter against sideward scatter, whole cells were gated out and depicted as a two-parameter dot plot of EGFP versus propidium iodide for analysis. The EGFP positive region, which designates transfected cells, was drawn starting above cell auto fluorescence. The geometric mean fluorescence intensity was determined from the number of EGFP positive cells. Further, the number of propidium iodide negative cells was counted as a measure of cell viability. The relative transfection efficacy and cell viability were determined by normalizing the number of EGFP-positive and propidium iodide-negative events counted in a sample to the negative control and expressed as a percent. The statistical analysis was performed by one-way analysis of variance (ANOVA) and the Tuckey- or Dunnett´s test.

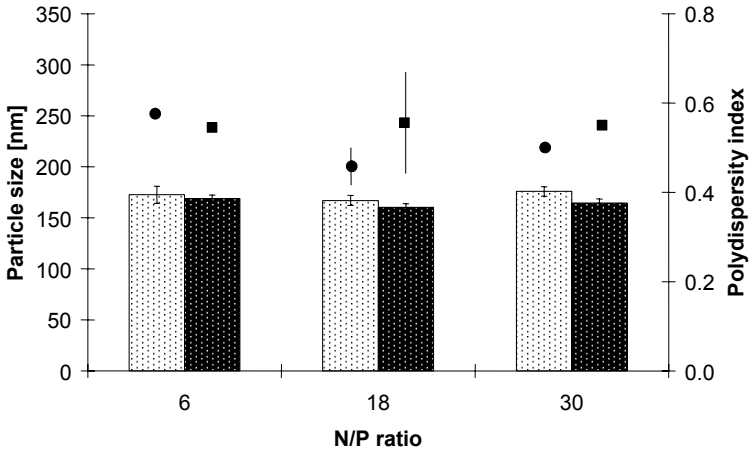
Results

Particle size, surface charge and efficacy of pDNA complexation

We used mPEG-NHS active esters with different molecular weights and architectures for conjugation with 2.6 and 4.6 kDa lPEIs (Table 1 and 2), as confirmed by ^{1}H-NMR spectroscopy. In order to prove that the synthesized copolymers are capable of condensing pDNA and to assess any particle aggregation, we determined the hydrodynamic diameters of copolymer-based polyplexes in 0.15 M NaCl in comparison to the corresponding unmodified lPEI – pDNA complexes.

While lPEI4.6 – pDNA complexes in 0.15 M NaCl grew to hydrodynamic diameters between 1500 and 2500 nm, lPEI2.6 did not form discrete polyplexes with pDNA (aggregates were larger than 5 µm with polydispersity indices (PI) larger than 0.8, data not shown). Using mPEG-lPEI copolymers as pDNA-condensing agents in 0.15 M NaCl, however, polyplexes between 150 and 300 nm with a broad particle size distribution were formed (PI between 0.4 and 0.7; Figure 1).

a)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Polydispersity index
N/P ratio
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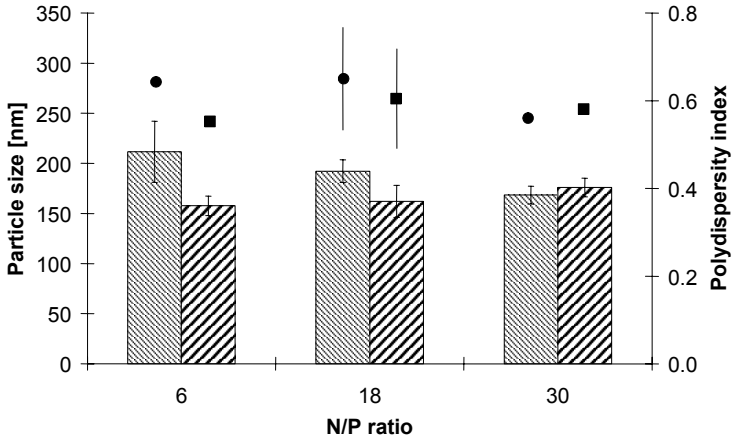
18
30

b)


Particle size [nm]
Polydispersity index
N/P ratio
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350
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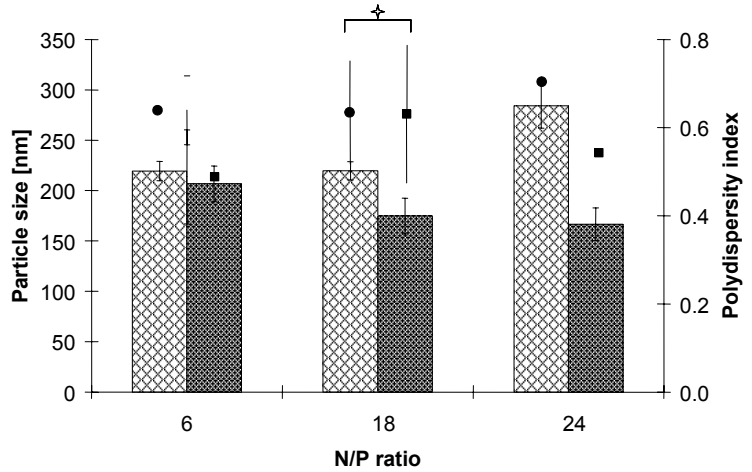
0.2
0.4
0.6
0.8
6
18
30

c)


Particle size [nm]
Polydispersity index
N/P ratio
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50
100
150
200
250
300
350
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0.2
0.4
0.6
0.8
6
18
24

Figure 1. The mean particle size / PI of
a) mPEG5/3-lPEI2.6 (⬚ / ●) –, mPEG5/3-lPEI4.6 – pDNA (▨ / ■),
b) mPEG10/2-lPEI2.6 (▧ /●) –, mPEG10/2-lPEI4.6 – pDNA (▧ /■), or
c) mPEG20-lPEI2.6 (▩ / ●)–, and mPEG20-lPEI4.6 – pDNA (▩ / ■) polyplexes prepared in 0.15 M NaCl at N/P ratios of 6, 18 and 30 after approximately 45 minutes incubation in 0.15 M NaCl. N/P 6 and 30: data represented as mean value (n=2); N/P 18: data represented as mean value ± S.D. (n = 3) Differences with a significance of p<0.05 are denoted by ✧.

Since these initial results indicated that the impact of the polymer concentration is negligible, we chose copolymer-based complexes formed at an N/P ratio of 18 to investigate whether the molecular weight of the lPEI-component or the mPEG-portion influences the particle size.

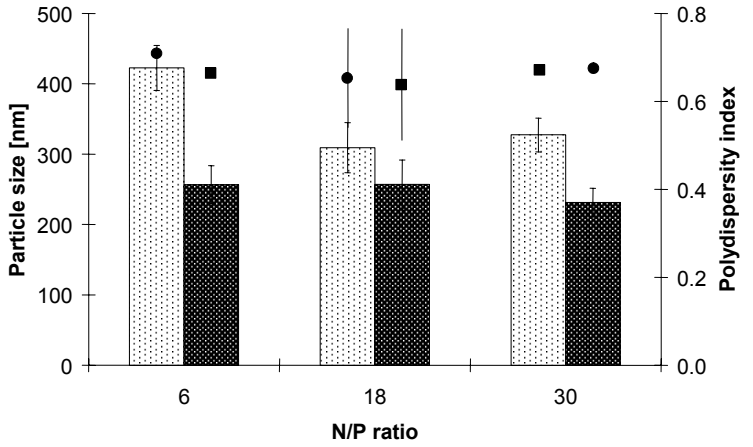
We observed a minor, but significant, reduction in the hydrodynamic diameter with an increasing molecular weight of the polycationic residue for mPEG20-lPEI – pDNA ($p<0.01$). Considering the mPEG variants used, significant differences in the particles were only observed for mPEG5/3-lPEI2.6 – pDNA and mPEG20-lPEI2.6 – pDNA complexes ($p<0.01$). mPEG-lPEI4.6- derived polyplexes all exhibited hydrodynamic diameters of about 150 nm, irrespective of the copolymer structure (Figure 1).

When using 5 % glucose as the pDNA complexation medium, we observed that polyplexes prepared with lPEIs of varying molecular weights doubled in size during less than 60 minutes incubation in serum-free cell culture medium (HAM´s F12), irrespective of the N/P ratio (data not shown).

Polyplexes formed with the synthesized copolymers exhibited, hydrodynamic diameters between 200 and 420 nm under these conditions and period of time (Figure 2). The effect of the polymer concentration was negligible.

At the mid-range N/P ratio of 18, the average size of copolymer-derived polyplexes in 0.15 M NaCl was significantly reduced, compared to the corresponding polyplexes in 5 % glucose / HAM`s F12 ($p < 0.05$).

a)


Particle size [nm]
Polydispersity index
N/P ratio
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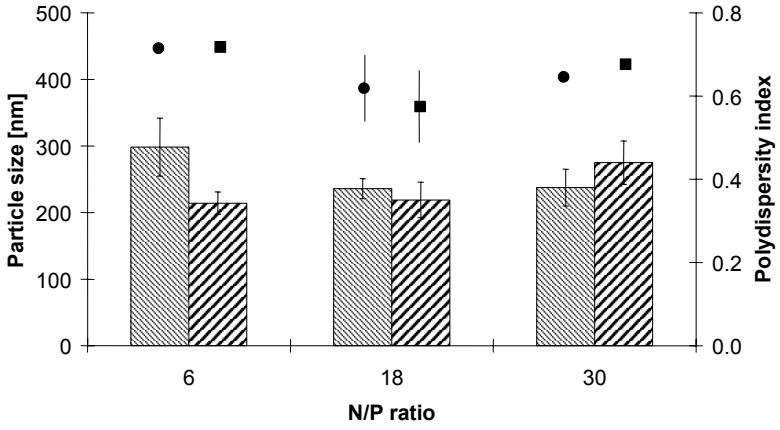
200
300
400
500
0.0
0.2
0.4
0.6
0.8
6
18
30

b)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Polydispersity index
N/P ratio
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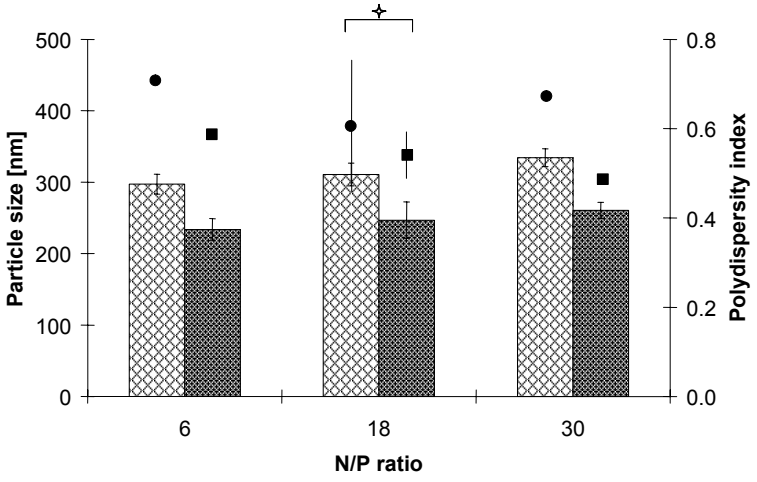
6
18
30

c)


Particle size [nm]
Polydispersity index
N/P ratio
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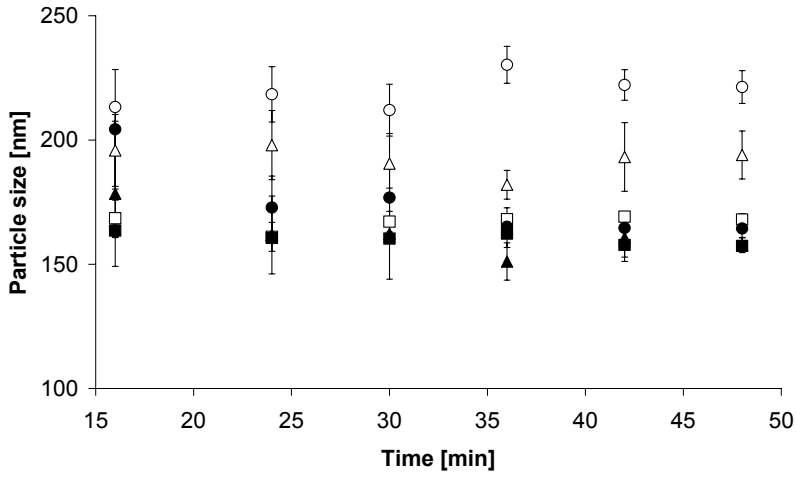
100
200
300
400
500
0.0
0.2
0.4
0.6
0.8
6
18
30

Figure 2. The mean particle size / PI of
a) mPEG5/3-lPEI2.6 (▨ / ●)–, mPEG5/3-lPEI4.6 – pDNA (▨ / ■),
b) mPEG10/2-lPEI2.6(▧ /●) –, mPEG10/2-lPEI4.6 – pDNA (▧ /■), and
c) mPEG20-lPEI2.6 (▩ / ●) –, mPEG20-lPEI4.6 – pDNA (▩ / ■) polyplexes, prepared in 5 % glucose at N/P ratios of 6, 18 and 30 after approximately 65 minutes incubation in serum-free cell culture medium. N/P 6 and 30: data represented as mean value (n=2); N/P 18: data represented as mean value ± S.D. (n = 3). Differences with a significance of p<0.05 are denoted by ✧.

Similarly to mPEG-lPEI – pDNA in high ionic strength media, we observed a reduction in the particle size with increasing molecular weight of the lPEI-component only for mPEG20-lPEI-derived polyplexes (between 220 and 250 nm).

The mPEG-content only appeared to induce a significant reduction in the particle size of the mPEG10/2-lPEI2.6 – pDNA compared to mPEG5/3-lPEI2.6 – and mPEG20-lPEI2.6 – pDNA ($p < 0.05$).

a)



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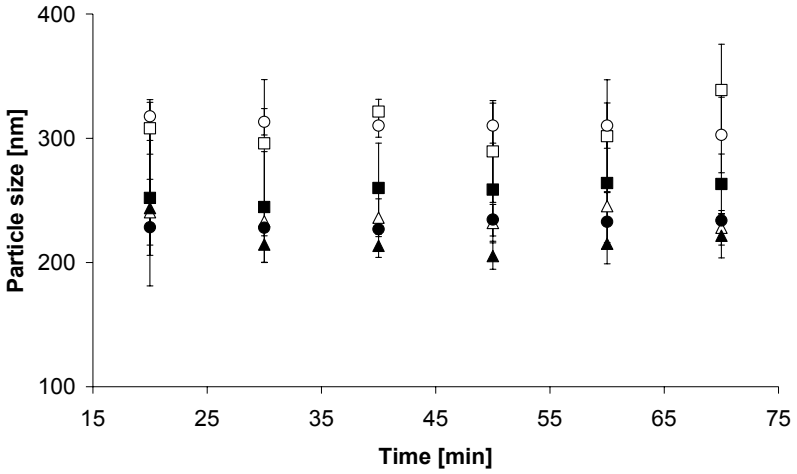


Figure 3. Mean particle size values of mPEG5/3-lPEI2.6 – pDNA(□), mPEG5/3-lPEI4.6 – pDNA(■), mPEG10/2-lPEI2.6 – pDNA(∆), mPEG10/2-lPEI4.6 – pDNA(▲), mPEG20-lPEI2.6 – pDNA (○), and mPEG20-lPEI4.6 – pDNA(●) polyplexes prepared at an N/P ratio of 18, determined
a) over 45 minutes in 0.15 M NaCl or
b) over at 65 minutes in 5 % glucose/HAM´s F12.
Data represented as mean value ± S.D (n = 3). No significance (p<0.05) was detectable.

In order to prove that polyplexes manufactured with the copolymers are stabilized against particle aggregation, we determined the polyplex size at multiple time points over at least 45 minutes in 0.15 M NaCl or 5 % glucose/HAM´s F12 (Figure 3).
The hydrodynamic diameter of the mPEG-lPEI – pDNA did not significantly increase during time ($p < 0.05$), irrespective of the copolymer used or the particle environment.
We used gel-retardation assays to investigate the efficacy of pDNA retention by the copolymers in comparison to the lPEI precursors. The capacity of mPEG5/3-lPEI- and mPEG20-lPEI- derived copolymers to complex pDNA in

5 % glucose after incubation in HAM´s F12 was remarkably reduced compared to unmodified lPEI2.6 and lPEI4.6 (Figure 4).

pDNA was completely immobilized by lPEI2.6 and lPEI4.6 at all N/P ratios above 1. The lowest pDNA complexation efficacy was observed for mPEG5/3-lPEI2.6 and increased slightly with the molecular weight of the lPEI residue of the copolymer. pDNA was completely retarded by mPEG5/3-lPEI4.6 and mPEG5/3-lPEI2.6 at N/P ratios of 3 and 6, respectively.

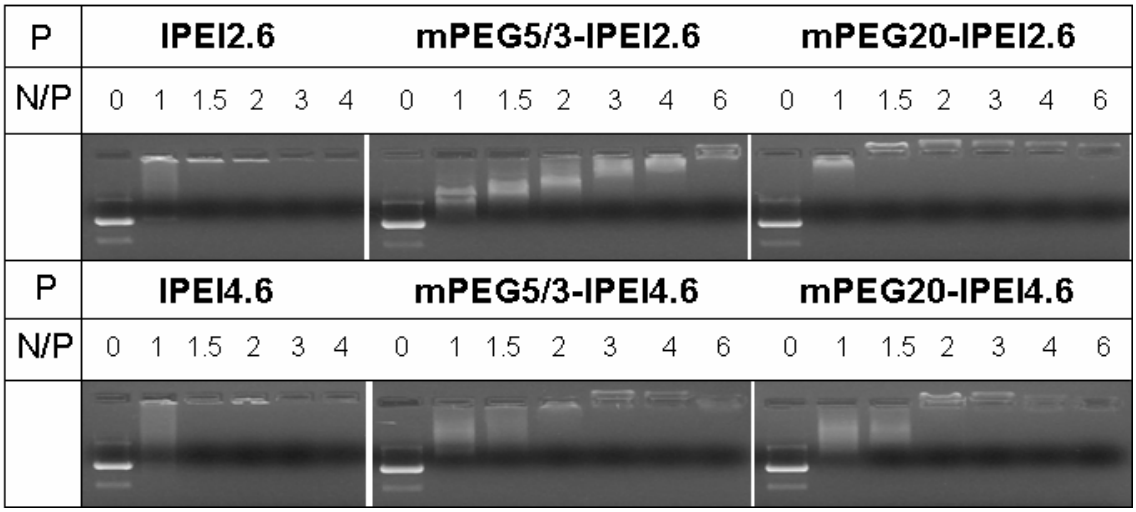
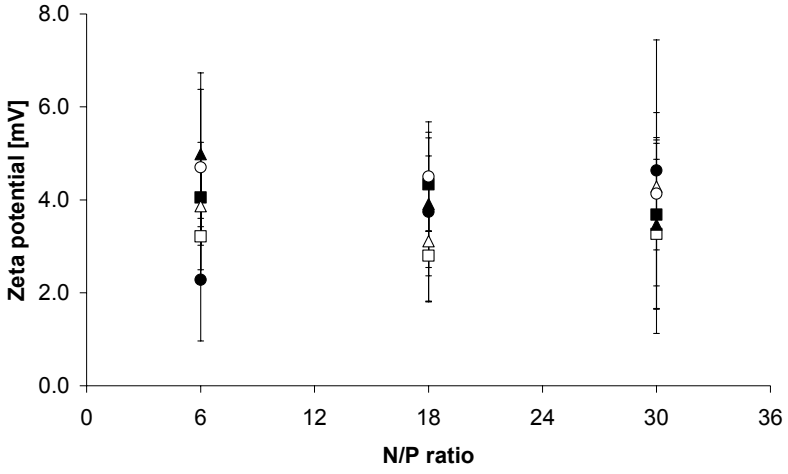


Figure 4. *Gel-retardation assay*

Polyplexes were prepared by the complexation of pDNA with lPEI2.6, lPEI4.6, mPEG5/3-lPEI2.6, mPEG5/3-lPEI4.6, mPEG20-lPEI2.6 or mPEG20-lPEI4.6 in 5 % glucose at N/P ratios between 1 and 6. The gel-retardation assays were performed after polyplexes were incubated in serum-free cell culture medium for 10 minutes at room temperature.

mPEG20-lPEI copolymers exhibited a slightly improved pDNA complexation efficiency compared to the mPEG5/3-lPEIs, retarding pDNA dissociation even at an N/P ratio of 1.5. In contrast to the unmodified lPEI – pDNA complexes, most of the copolymer-based polyplexes with elevated N/P ratios were detected as pDNA bands localized on top of the gel pocket.

a)



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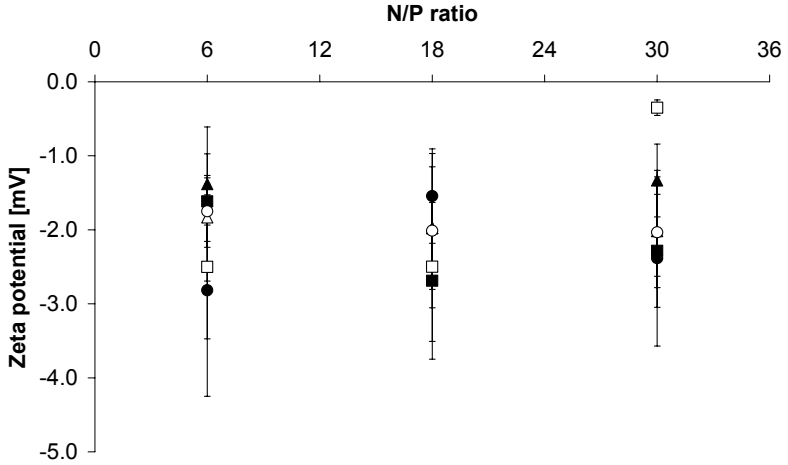


Figure 5. *Zeta potential of mPEG-lPEI-pDNA polyplexes mPEG5/3-lPEI2.6 – pDNA (□), mPEG5/3-lPEI4.6 – pDNA (■), mPEG10/2-lPEI2.6 – pDNA (∆), mPEG10/2-lPEI4.6 – pDNA (▲), mPEG20-lPEI2.6 – pDNA (○), and mPEG20-lPEI4.6 – pDNA (●) polyplexes were prepared at N/P ratios of 6, 18 and 30. The zeta potential was determined a) after polyplex formation in 0.15 M NaCl or b) for polyplexes prepared in 5 % glucose after at least 30 minutes incubation in serum-free cell culture medium. N/P 6 and 30: data represented as mean value (n=2); N/P 18: data represented as mean value ± S.D. (n = 3). No significant differences (p<0.05) were detected between the samples tested.*

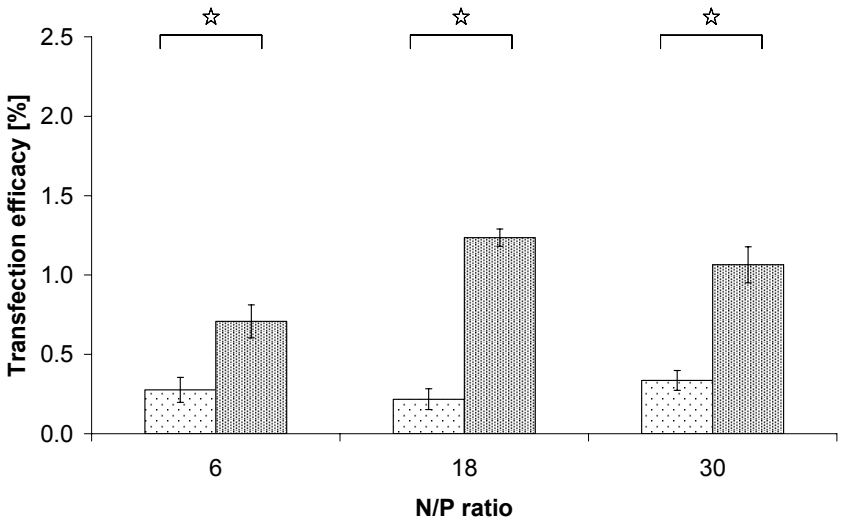
The zeta potential of the copolymer-based polyplexes spanned from low positive to slightly negative values, depending on the ambient medium used during the measurement (Figure 5).

For polyplexes in 0.15 M NaCl, a surface charge between 1 and 6 mV was determined, while mPEG-lPEI – pDNA complexes prepared in 5 % glucose and incubated in HAM´s F12 exhibited a negative net charge between -4 and -1 mV. The PEG residue, the molecular weight of the lPEI component, and the N/P ratio had no significant impact on the zeta potential of the corresponding polyplexes.

Cell – polyplex interaction and transfection efficacy of mPEG-lPEI – pDNA complexes

In order to clarify whether the synthesized copolymers are capable of reducing non-specific interactions with cells, which mediate the internalization of cationic charged polyplexes, we determined the transfection efficacy of the corresponding mPEG-lPEI – pDNA complexes (Figure 6).

a)



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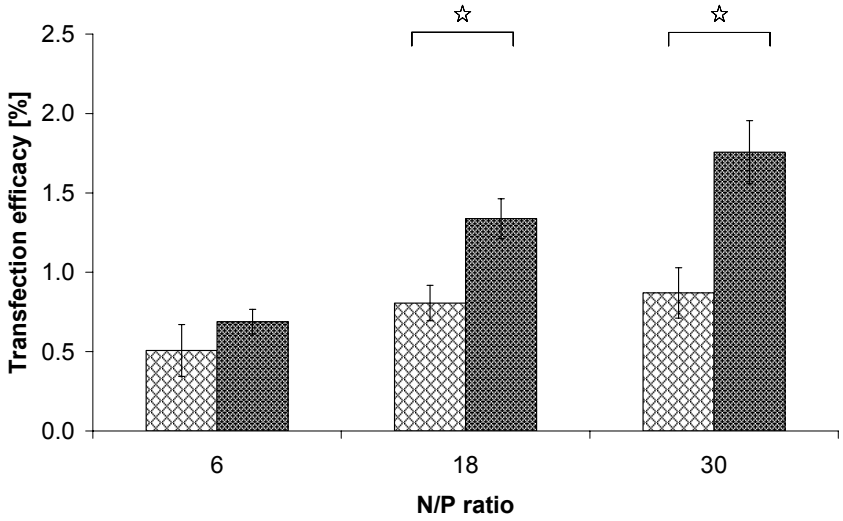

c)

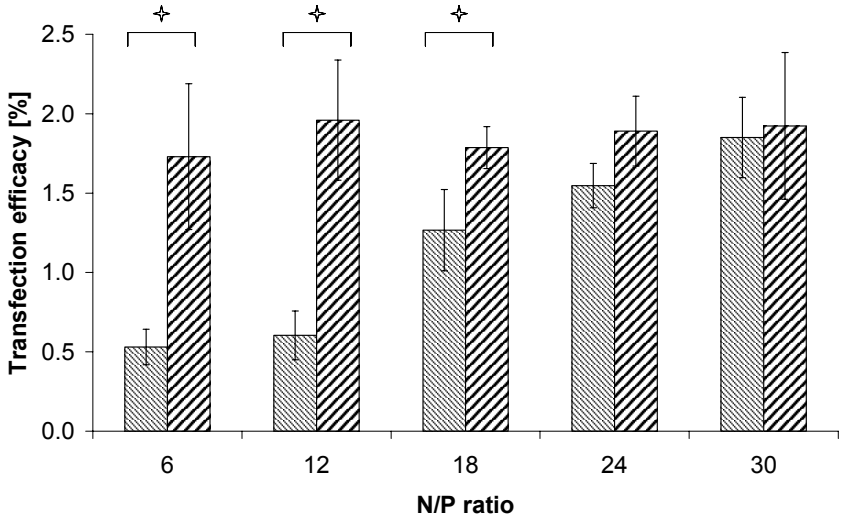

Figure 6. *CHO-K1 cells were incubated for four hours under serum-free conditions with:* a) mPEG5/3-lPEI2.6 (⬚) –, or mPEG5/3-lPEI4.6 (▒) –, b) mPEG20-lPEI2.6 (▩) – or mPEG20-lPEI4.6 (■) –, as well as c) mPEG10/2-lPEI2.6 (▨) – ,or mPEG10/2-lPEI4.6 – pDNA (▧). The polyplexes were prepared in 0.15 M NaCl at N/P ratios between 6 and 30 and the transfection efficacy was determined after 48 hours by flow cytometry analysis. Data represented as mean value ± S.D. (n = 3); differences with significance of $p < 0.05$ or $p < 0.01$ are depicted by ✧ and ☆, respectively.

Comparing the three different groups of copolymer-based polyplexes, which vary in their mPEG content, the efficacy of gene transfer seemed to follow a similar trend for both mPEG5/3- and mPEG20-lPEI-derived polyplexes. For both series we observed that the transfection efficacy was significantly increased with increasing molecular weight of the lPEI residue, at least at elevated polymer concentrations. An influence of the N/P ratio was only observed for polyplexes prepared with the mPEG-lPEI4.6 derivatives ($p < 0.01$).

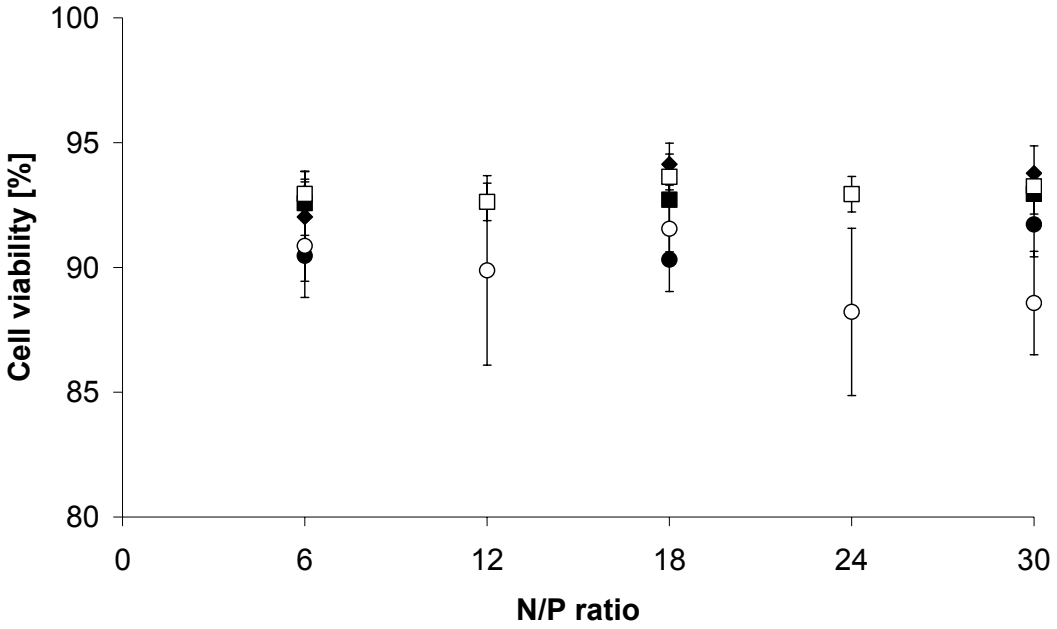

Figure 7. *mPEG5/3-lPEI2.6 – pDNA (♦), mPEG5/3-lPEI4.6 – pDNA (■), mPEG20-lPEI2.6 – pDNA (▲), mPEG20-lPEI4.6 – pDNA (●), mPEG10/2-lPEI2.6 – pDNA (□), and mPEG10/2-lPEI4.6 – pDNA (○) prepared in 0.15 M NaCl at N/P ratios between 6 and 30. The polyplexes were added to CHO-K1 cells under serum-free conditions for 4 hours and the relative cell viability was determined after 48 hours by flow cytometry analysis. Data represented as mean value ± S.D. (n = 3). Differences with significance of $p < 0.05$ or $p < 0.01$ are depicted by ✧ and ☆, respectively.*

However, this trend seemed inverted for mPEG10/2-lPEI – pDNA. While the transfection efficacy of mPEG10/2-lPEI2.6 – pDNA increased from an N/P ratio of 6 to 30 ($p < 0.01$), gene transfer seemed unaffected by the polymer concentration when mPEG10/2-lPEI4.6 – pDNA were used.

Both gene delivery vehicles reached a maximum transgene expression of only 1.8 %, showing significant differences only at low N/P ratios.

The cell viability of CHO-K1 cells after exposure to mPEG-lPEI copolymers remained between 88.5 ± 2.1 % and 94.1 ± 1.1 %. Significant differences in dependency on the copolymer occurred only sporadically at an N/P ratio of 18 or 30 ($p < 0.05$), although even there no general trends were discernable (Figure 7).

To investigate differences in the cell-polyplex interactions in more detail, we prepared polyplexes with YOYO-1 – labelled pEGFP-N1 reporter plasmid and either lPEI or mPEG-lPEI copolymers at an N/P ratio of 30. Using CLSM imaging (Figure 8), we observed large aggregates of unmodified lPEI2.6 – and lPEI4.6 – pDNA polyplexes, most of them dispersed extracellularly in the cell culture medium. The CLSM images suggest that the number of intracellular localized polyplexes increased with the molecular weight of the lPEI used for complex formation (Figure 8a and b). It seemed that lPEI4.6-pDNA complexes almost flooded the cytoplasm of CHO-K1 cells, while a few are localized in the nucleus (Figure 8b).

With the exception of the mPEG10/2-lPEI-derived polyplexes, CLSM images indicated a similar trend for mPEG-lPEI – pDNA complexes. Even though the particle size and the number of particle aggregates seemed to be dramatically reduced compared to unmodified lPEI – pDNA particles, the internalization of polyplexes into the cytoplasm was enhanced with increasing molecular weight of the lPEI residue in the copolymer. The corresponding polyplexes seemed, thereby, to be homogeneously distributed in the cytoplasm, partially assembling at the nuclear membrane.

The polyplex internalization also seemed to be drastically reduced for mPEG10/2-lPEI-based complexes compared to unmodified lPEI2.6-or

lPEI4.6 – pDNA complexes. However, the extent of the intracellular mPEG10/2-lPEI – pDNA distribution appeared to be similar, irrespective to the molecular weight of the copolymers lPEI residue.

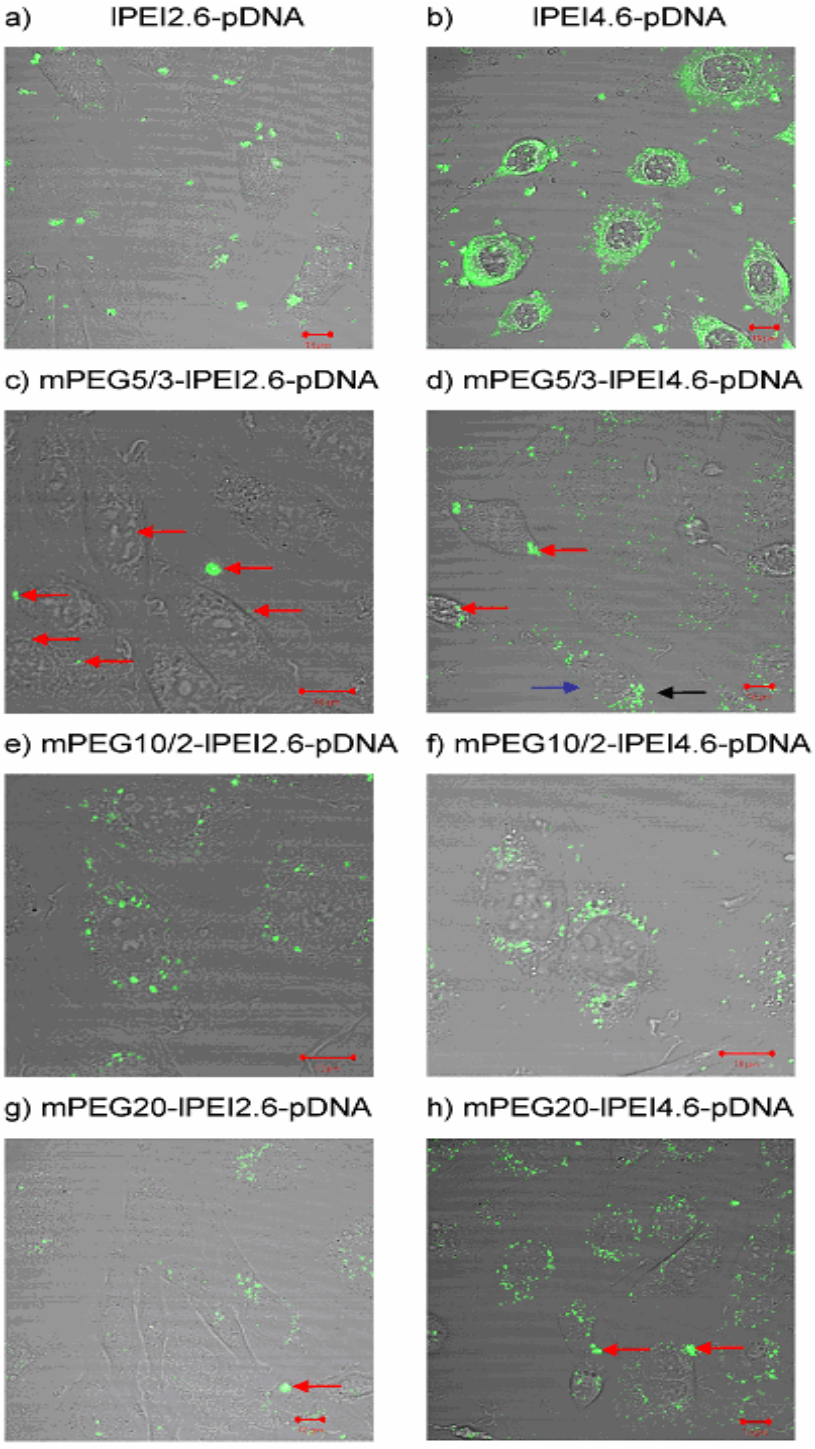

Figure 8. *Confocal miroscope images of adherent CHO-K1 cells in serum-free cell culture medium after 4 to 5 hours incubation with (a) lPEI2.6 –, (b) lPEI4.6 –, (c) mPEG5/3-lPEI2.6 –, (d) mPEG5/3-lPEI4.6 –, (e) mPEG10/2-lPEI2.6 –, (f) mPEG10/2-lPEI4.6 –, (g) mPEG20-lPEI2.6 –, and (h) mPEG20-lPEI4.6 – pDNA complexes. For visualization of the corresponding polyplexes, YOYO-1-labelled pEGFP-N1 pDNA was used for particle formation. The red arrows indicate individual polyplexes or large polyplex aggregates, the black and the blue arrows are exemplarily pointed at a cell membrane and nuclear membrane of a CHO-K1 cell, respectively.*

Discussion

In order to design PEI – pDNA complexes that resist particle aggregation and reduce non-specific cellular uptake, we implemented a concept described by Ogris et al. [5]. lPEI and PEG-PEI copolymers were synthesized to produce stable building blocks, which were then used for the manufacture of tailor-made *in vivo* applicable polyplexes. Ogris and others have investigated PEG-PEI copolymers based on 25 kDa bPEI and 22 or 25 kDa lPEI, using varying mass ratios of PEI and PEG in some studies [9;18]. Thereby, it has been shown that the use of PEG-PEI copolymers improved the water-solubility of the corresponding gene carriers and reduced their cytotoxicity.

Recently, we have shown that polyplexes based on low molecular weight lPEIs exhibited improved transfection efficacy and cell compatibility *in vitro* [14]. Therefore, we selected two non-toxic low molecular weight lPEIs (2.6 and 4.6 kDa) as the polyamine component for the mPEG-lPEI copolymer synthesis. In contrast to other approaches, we attached the mPEG residue to a comparably small polycationic chain in order to induce the maximum shielding effect (Table 2).

We performed the conjugation of mPEG in an aprotic solvent to reduce NHS-ester hydrolysis. The purification of the corresponding amine-base with water

allowed for the complete removal of unmodified mPEG, but also reduced the yield of mPEG-lPEI, due to a remarkably improved water-solubility [18;19].

Despite the dominating charge-shielding component, all of the copolymers condensed pDNA at moderate N/P ratios, resulting in the formation of nanoparticles between 150 and 420 nm (Figure 1, 2, and 3). In comparison to unmodified lPEI–pDNA, which formed aggregates between 500 and 5000 nm after less than 10 minutes in media containing physiological salt concentrations [15;17], the copolymer-based particles resisted particle aggregation under these conditions for at least 45 minutes. The reduced capacity to complex pDNA, compared to the unmodified polyamines, was compensated using the mPEG-lPEI derivatives based on the higher molecular weight lPEI (lPEI4.6) or elevated N/P ratios (Figure 4).

The zeta potential of copolymer-based polyplexes was remarkably reduced, compared to unmodified lPEI2.6-or lPEI4.6 – pDNA polyplexes. However, the zeta potential ranged from slightly positive to slightly negative values, depending on the ambient media (Figure 5).

We determined the transfection efficacy of PEGylated polyplexes in comparison to the unmodified cationic lPEI – pDNA complexes as a measure for the extent of its non-specific internalization by cells. It is expected that a sufficient charge-shielding effect lowers the positive polyplex net charge, thereby reducing the non-specific interaction of the complexes with negatively charged glycoproteins, proteoglycans, and sulfated proteoglycans located at the cell surface. This in turn decreases the amount of internalized pDNA and ergo the level of transgene expression [20-22].

In contrast to other copolymers [8;10;18;19;23], we could show that the transfection efficacy determined for mPEG-lPEI – pDNA complexes (between 0.2 and 2.5 %) was reduced by several orders of magnitude, compared to unmodified polyplexes containing only lPEI between 2 and 5 kDa (4 % to 40 %) (Figure 6) [14]. Nevertheless, the efficacy of gene transfer mediated by copolymer-based pDNA complexes still showed a correlation to the molecular weight of the lPEI component and copolymer concentration used for polyplex

formation. This may indicate that the shielding effect of the introduced PEG-layer may still be incomplete, increasing in the lPEI2.6 copolymer series from mPEG10/2-lPEI2.6 to mPEG20-lPEI2.6, reaching its maximum with mPEG5/3-lPEI2.6. For mPEG-lPEI4.6-derived polyplexes, transfection was most efficiently suppressed by mPEG5/3-lPEI4.6 – pDNA, followed by mPEG20-lPEI4.6 – pDNA and mPEG10/2-lPEI4.6 – pDNA. This effect was most pronounced at an N/P ratio of 30. At the maximum polymer concentration both mPEG20-lPEI2.6 – pDNA and mPEG10/2-lPEI4.6 – pDNA reached a comparable level of transgene expression, irrespective to the molecular weight of the lPEI component.

Additionally, we used CLSM images to complement the results from gene transfer experiments (Figure 8). This allowed us, in contrast to other approaches, to investigate if the lowered transfection efficacy was a consequence of a reduced particle uptake or of an enhanced intracellular complex stability [24].

We observed copolymer-pDNA polyplexes localized in the cytoplasm of CHO-K1 cells, but to remarkable lower degree compared to the unshielded lPEI-based particles. CLSM images suggest an increase in cell-polyplex interactions and, hence, an increase in intracellularly localized polyplexes dependent on the copolymer used for pDNA compaction in the following order: mPEG5/3-lPEI2.6 < mPEG20-lPEI2.6 < mPEG5/3-lPEI4.6 < mPEG20-lPEI4.6 ≈ mPEG10/2-lPEI2.6 ≈ mPEG10/2-lPEI4.6. This confirms the trend observed in the corresponding transfection experiments. For mPEG5/3-lPEI – and mPEG20-lPEI– derived complexes, differences in the cellular uptake dependent on the molecular weight of the lPEI component became obvious. The extent of polyplex internalization seemed thereby to increase with increasing molecular weight of the lPEI component, not exceeding a basal level, correlating with lPEI2.6 – pDNA polyplexes at an N/P ratio of 30.

Overall, copolymers of the mPEG5/3-lPEI and mPEG20-lPEI series have been shown to be the most promising candidates for the manufacture of shielded polyplexes. While the capacity to compact pDNA and the surface

charge was comparable to the other copolymer-based polyplexes, non-specific polyplex – cell interactions seemed to be maximally reduced.

Conclusion

We have shown that mPEG-derived copolymers based on nontoxic 2.6 and 4.6 kDa low molecular weight lPEI were still capable of compacting pDNA, resulting in the formation of nanoparticles between 150 and 420 nm. The efficacy of pDNA complexation of the corresponding mPEG-lPEI copolymers decreased compared to the unmodified lPEIs. mPEG-lPEI – pDNA polyplexes exhibited slightly positive to low negative zeta potentials, depending on the particle environment. Despite the different architecture, mPEG5/3-lPEI and mPEG20-lPEI copolymers showed a similar potential to impair ion-induced particle aggregation and to reduce non-specific internalization of polyplexes by CHO-K1 cells. Gene transfer mediated by the corresponding mPEG-lPEI – pDNA complexes was negligible, ranging from 0.25 to 1.8 %, compared to a maximum transgene expression of 40 % previously determined for unmodified lPEI – pDNA complexes.

From the series of mPEG-lPEI copolymers synthesized, we have now identified derivatives that complex pDNA efficiently, stabilize the corresponding polyplexes against aggregation, and sufficiently reduce unspecific particle-cell interactions. This provides the basis for the design of polymers that will facilitate the manufacture of *in vivo* applicable polyplexes for the cell-specific delivery of nucleic acids by targeting.

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8. SUMMARY AND CONCLUSION

Summary

With a growing understanding in cell biology and gene functions, gene transfer emerged as a promising technique in molecular medicine as well as in basic research. Two major concepts are used to date based on viral and non-viral transfection systems [1-3]. Viral vectors are already applied in phase II studies world-wide, due to their high efficacy in transferring the gene of interest into cells or tissues of varying origin (homepage: “Gene Therapy clinical Trials Worldwide” provided by the Journal of Gene Medicine (www.wiley.co.uk/genetherapy/clinical/)). In recent years a reappraisal has occurred, due to their high immunogenic and oncogenic potential, endowing growing attention to the development of non-viral gene transfer strategies [4;5]. Polycations have been among the most commonly used gene carriers [6]. Their major advantages are their capacity for chemical modifications, their chemical stability, the large quantity of DNA that can be encapsulated, and the improved safety profile compared to viral systems. Unfortunately, the applicability of non-viral gene carriers is often limited by a low transfection efficacy and severe cytotoxicity. Despite the great variety of polymers applied, the structural prerequisites for efficient and cell-compatible transfection remain elusive. Hence the design of non-viral vectors is still based on empiric approaches, rather than on the insight of the underlying structure – function relationships.

1995, Boussif et al. introduced branched polyethylenimine as promising gene carrier. Since then, polymers of different molecular weight and degree of branching have been evaluated in terms of transfection efficacy and cell

compatibility [7;8]. The overall buffer capacity and high protonation level (ratio of protonated amines to unprotonated amines) at physiological pH have been held in account for its high efficacy of pDNA complexation and protection, its intrinsic endosomolytic activity, the relatively high transfection efficacy, but also for its eminent cytotoxicity [9] (**Chapter 1**).

The goal of this thesis was the design, synthesis and characterization of polyamines that facilitate the manufacture of efficient and cell-compatible *in vitro* plasmid DNA (pDNA: pEGFP-N1 reporter plasmid) delivery systems. We focused on structural modifications of branched and linear polyethylenimine. The characterization of the transfection vehicles *in vitro* provided the basis for the investigation of the polymer functionality and the design of novel gene carriers.

In order to investigate the impact of a reduced overall basicity on the cytotoxicity and transfection efficacy of branched (bPEI) – and linear polyethylenimine (lPEI) – pDNA complexes, we synthesized per-N-methylated linear (lPMEI, Figure 1a) and branched polyethylenimine (bPMEI, Figure 1b) by the complete N-methylation of the corresponding PEI 25 kDa precursors.

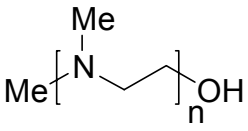


Figure 1a.
linear per-N-methylated polyethylenimine (lPMEI)

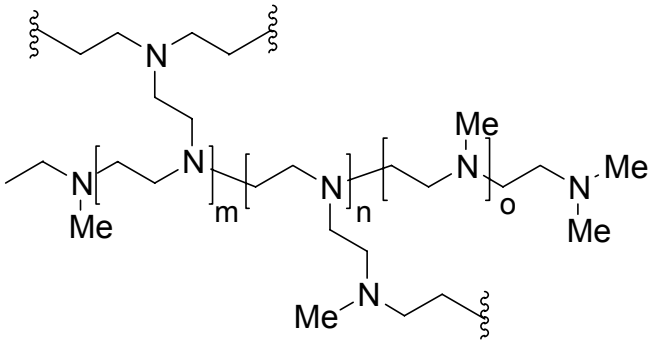
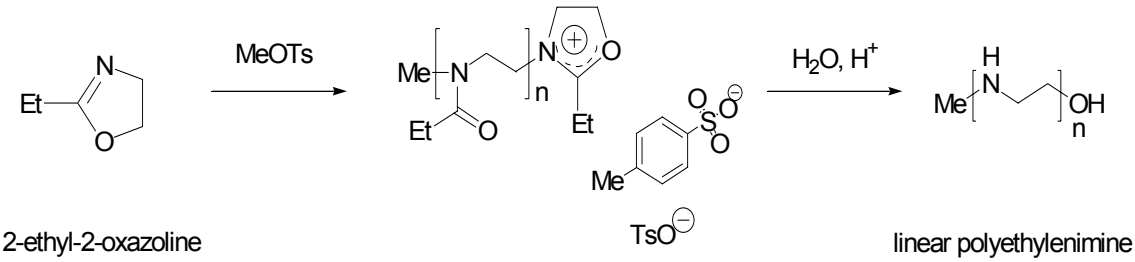


Figure 1b.
branched per-N-methylated polyethylenimine (bPMEI)

The corresponding tertiary polyamines built polyplexes with pDNA, which exhibited similar hydrodynamic diameters, surface charge and endosomolytic activity, were internalized by CHO-K1 cells to a comparable degree, and were significantly less cytotoxic, compared to unmodified PEI – pDNA complexes. However, the capacity to transfect cells *in vitro* was almost diminished (**Chapter 3**).

Based on these results, we postulated that a sufficient overall basicity and linear architecture may be favorable prerequisites for an efficient and cell-compatible gene carrier, respectively. Hence we chose lPEI, a linear polyamine composed of exclusively secondary amines with a pK_a value of 7.9 [10], as basis for further modifications. In order to identify the lPEI with the optimum properties for pDNA delivery, we synthesized a series of twelve low molecular weight lPEIs (lmw lPEI) by the cationic ring-opening polymerization of 2-ethyl-2-oxazoline and acid-catalized hydrolysis of the corresponding poly (2-ethyl-2-oxazoline) [10] (Scheme 1). The corresponding lPEIs were synthesized by a parallel synthesis approach and differed by their relative number average molecular weights (M_n) between 1 and 8.1 kDa. We established a gel filtration chromatography procedure that allowed for the determination of the relative number (M_n) and weight (M_w) average molecular weights as well as the molecular weight distribution for the corresponding polymer batches.

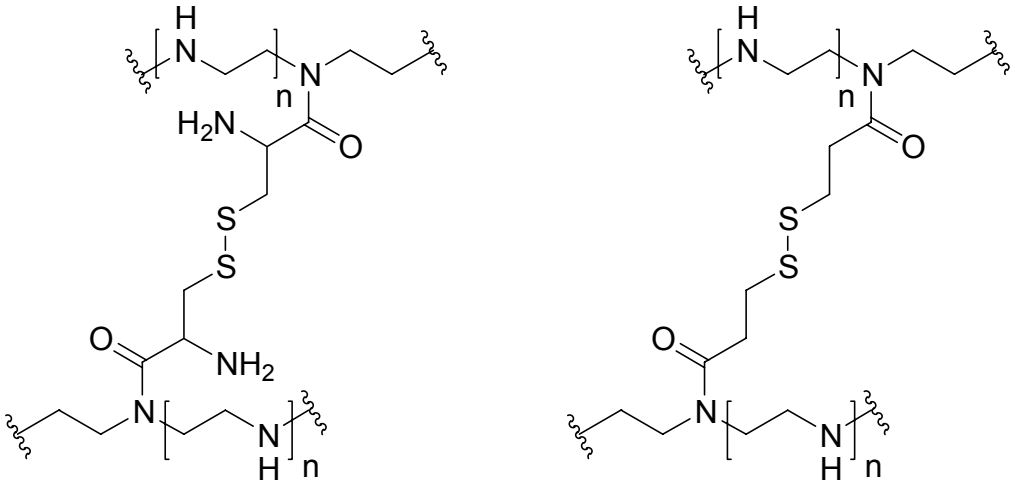


Scheme 1. *Synthesis of linear low molecular weight polyethylenimine by acid hydrolysis of the corresponding poly (2-ethyl-2-oxazoline).*

On the basis of these results, we systematically investigated the impact of the lPEI molecular weight on the *in vitro* performance of the corresponding polyplexes (**Chapter 4**).

In medium of low ionic strength (5% glucose) all lmw lPEIs condensed pDNA under the formation of nanoparticles between 100 and 150 nm, irrespective to the molecular weight of the polyamine or N/P ratio applied. However, the corresponding polyplexes were unstable at physiological salt concentration. Large particle aggregates that exceeded the nanometer scale were formed, and the efficacy of pDNA compaction was significantly reduced. lmw lPEIs with a M_n below 3 kDa even failed to form discrete particles with pDNA under these conditions. We have shown that polyplexes built with lPEI of 8.1 kDa and lPEI 5 kDa enhanced the efficacy of gene transfer and exhibited improved cell compatibility, compared to the standard lPEI 25 kDa- and bPEI 25 kDa-derived transfection systems. However, we observed that a high lPEI M_n led to a high polymer as well as polyplex cytotoxicity, indicating that the toxicity of the vector is mediated by the polymerization degree of the polyamine (**Chapter 5**).

In order to prove if the efficacy of high molecular weight lPEI – pDNA polyplexes can be combined with the low cytotoxicity of low molecular weight lPEI – pDNA complexes, we cross-linked non-toxic lmw lPEI (lPEI) by reducible disulfide linkages. We synthesized four series of polymers differing by the molecular weight of the lPEI component (2.6 and 4.6 kDa), the linker type, and linker / lPEI ratio (Figure 2). The cross-linked lPEIs condensed pDNA, forming polyplexes between 200 and 2500 nm, influenced by the polymer features and the ionic strength of the ambient medium. Gel-retardation assays and laser light scattering analysis confirmed the destabilization of the cross-linked lPEI-derived polyplexes in the presence of disulfide reducing agents and thereby indicated the degradation of the polymer.



BC-lPEI series **LR-lPEI series**

Figure 2. *degradable lPEI-derived polymers synthesize by the cross-linking of low molecular weight lPEI with boc-Cystine / DMT-MM (BC-lPEI series) or Lomant´s reagent (LR-lPEI series).*

The efficacy of gene transfer to CHO-K1 cells as well as the cell compatibility after transfection was improved several-fold compared to the corresponding linear or branched PEI 25 kDa – pDNA complexes, and were both modulated by lPEI molecular weight, the linker / lPEI ratio, and the N/P ratio applied (**Chapter 6**).

The utility of positively charged lmw lPEI – pDNA polyplexes for *in vivo* gene transfer is still limited due to particle aggregation and the unspecific interaction with a biological environment. To overcome these obstacles, we synthesized a series of methoxy poly (ethylene glycol) (mPEG) - lmw lPEI copolymers to facilitate the manufacture of charge-shielded and sterically stabilized polyplexes.

Although, all mPEG - lmw lPEI copolymers exhibited a reduced efficacy of pDNA complexation, compared to the lPEI homopolymers, they condensed

pDNA under the formation of nanoparticles between 150 and 420 nm. The different mPEG - lPEI copolymers showed a similar potential to impair ion-induced particle aggregation and to reduce non-specific internalization of polyplexes by CHO-K1 cells. The transgene expression, mediated by the corresponding mPEG - lmw lPEI – pDNA complexes was thereby reduced to a negligible level, ranging from 0.25 to 1.8 %, compared to 40 % previously determined for unmodified lPEI – pDNA complexes (**Chapter 7**).

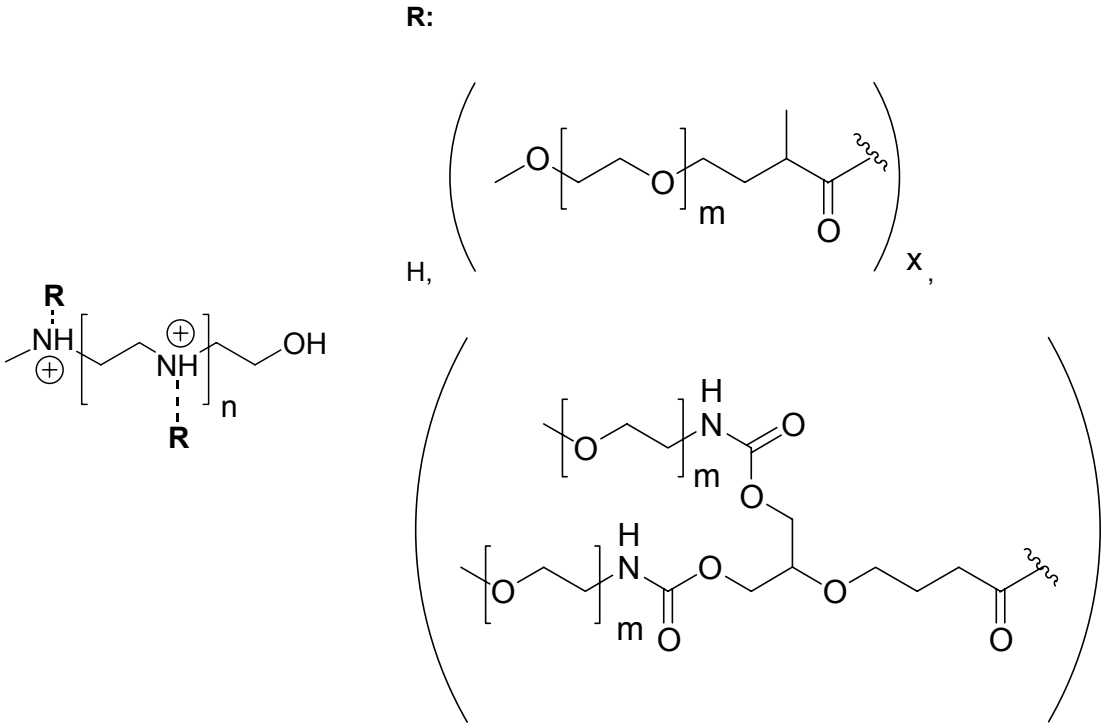


Figure 3. *mPEG - lmw lPEI copolymers synthesized by the conjugation of mPEG-active esters of different architecture to low molecular weight lPEIs at varying molar ratios.*

Conclusion

We have shown that lmw lPEIs facilitate the manufacture of efficient and cell compatible non-viral vectors for *in vitro* gene transfer, and provide a reliable basis for further optimization. New degradable gene carriers were

synthesized by the reversible cross-linking of lmw lPEIs with disulfide linkages. The corresponding polyplexes exhibited superior transfection compared to bPEI 25 kDa – and lPEI 25 kDa – pDNA complexes, the bench mark in polymer-mediated gene delivery. We successfully developed and identified an optimum mPEG - lPEI copolymer composition that facilitates the manufacture of polyplexes, that resist particle aggregation and reduced non-specific internalization by CHO-K1 cells.

Implementing a strategy described by Ogris et al, we are now able to manufacture *in vivo* applicable transfection vehicles. This concept is based on polymer blends that consist of a polyamine as pDNA condensing agent, and a PEG - PEI copolymer for charge shielding. In a next step, the synthesis of PEG - lmw lPEI copolymers linked to targeting sequences leads to the component that mediates the cell-specific gene delivery. This opportunity of free combination of the single classes of polymers will allow for the manufacture of tailor-made non-viral vectors optimized to the specific therapeutic concept or laboratory application.

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APPENDIX

Abbreviation list

BC	boc-cystine; N,N'-bis-(*tert*-butoxycarbonyl) cystine
BCx-lPEIy	lPEI cross-linked by the reaction with BC; x indicates the molar ratio of BC to ethylenimine monomers in percent (linker / lPEI ratio); y indicates the M_n of lPEI in kDa determined by GFC
BCx-lPEIy – pDNA	polyplexes formed by the complexation of pEGFP-N1 reporter plasmid with the corresponding BC-or LR-lPEI derivative
brs	broad singulett
CLSM	**C**onfocal **L**aser **S**canning **M**icroscopy
CV_{50}	polymer concentration [µg/µl] that reduces the relative cell viability of CHO-K1 cells *in vitro* to 50% of the original value
DCM	dichloromethane
DMT-MM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride
DTT	dithiothreitol
FCS	fetal calf serum
GFC	**G**el **F**iltration **C**hromatography
GPC	**G**el **P**ermeation **C**hromatography
GSH	Glutathione (reduced form)
HEPES	HEPES 7.5 mM pH 7.5
lmw lPEI	low molecular weight linear polyethylenimine
LR	Lomant´s reagent: 3,3´-Dithiodipropionic acid di(N-succinimidyl ester)
LRx-lPEIy	lPEI cross-linked by the reaction with LR; x indicates the molar ratio of LR to ethylenimine monomers in percent (linker / lPEI ratio); y

	indicates the M_n of lPEI in kDa determined by GFC
LRx-lPEIy – pDNA	polyplexes formed by the complexation of pEGFP-N1 reporter plasmid with the corresponding LR-lPEI derivative
MALDI-ToF	**M**atrix **A**ssisted **L**aser **D**esorption **I**onization-**T**ime **o**f **F**light mass spectroscopy
M_n	number average molecular weight
mPEGx/y - lPEIz	methoxy poly(ethylene glycol) - linear polyethylenimine copolymers; x indicates the M_n of mPEG in kDa defined by the supplier; y indicates the molar ratio of mPEGx to lPEIz; z indicates the M_n of lPEI in kDa determined by GFC
mPEGx/y -lPEIz-pDNA	polyplexes prepared by complexation of pEGFP-N1 with the indicated mPEG-lPEI copolymers
MW	generally for molecular weight
M_w	weight average molecular weight
M_w/M_n	ratio of the weight average molecular weight to number average molecular weight defined as molecular weight distribution or polydispersity index of the polymer batch
N/P	N/P ratio; refers to the quotient of the nitrogen atoms of PEI to DNA phosphates, and describes the polymer concentration used for pDNA complexation
OXZ	2-ethyl-2-oxazoline
pDNA	plasmid DNA
pDNA-EB	intercalate of ethidium bromide with plasmid DNA
pEGFP-N1	reporter plasmid carrying the reporter gene, which encodes for the enhanced green fluorescent protein,
PEG-PEI	generally for poly(ethylene glycol)-co-polyethylenimine copolymer

PI	polydispersity index
pOXZ	poly (2-ethyl-2-oxazoline)
PEI	polyethylenimine
b/lPEI·HCl	branched (b) or linear (l) polyethylenimine hydrochloride
bPEIz or lPEIz	b or l indicate the branched or linear conformational isomer of PEI; the number z indicates the M_n of PEI in kDa
(l or b)PEI(y) – pDNA	describes polyplexes prepared by complexation of pEGFP-N1 reporter plasmid with l/bPEI; y indicates the polymer MW in kDa)
SEC	**S**ize **E**xclusion **C**hromatography
TLC	**T**hin **L**ayer **C**hromatography

Curriculum vitae

Name:	Uta Lungwitz
Date of birth:	January 16th 1974
Place of birth:	Greifswald
Nationality:	German
Marital Status:	Married, son Jona born on July, 6th 2000

Education:

1980 – 1990	Erich-Weinert- School in Binz, Rügen
1990 – 1994	Grammer School: Kronwerk - Gymnasium in Rendsburg and Gymnasium - Heide/Ost in Heide/SH
06 / 30 / 1994	School graduation (Abitur)
1992 – 1993	Exchange student, Oak-Harbor-High School, WA, USA

Professional Training

02 / 1995 – 05 / 2000	Pharmaceutics course at the University of Regensburg
09 / 2000 – 09 / 2001	Practical education at the Rungholt-Apotheke in Heide/SH
09 / 2001	Acquisition of the license to practice as pharmacist
since 10 / 2001	PhD program at the Department of Pharmaceutical Technology of the University of Regensburg, Prof. Achim Göpferich

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