Modified Poly(ethylene imines) for plasmid delivery:

Physico-chemical and in vitro/in vivo investigations

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am Institut für Pharmazeutische Technologie und Biopharmazie
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Meiner Familie

In Liebe und Dankbarkeit

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INTRODUCTION

OBJECTIVES OF THIS WORK

In this dissertation, the development of polyplexes based upon poly(ethylene imine) (PEI) and plasmid DNA for airway and intravenous application was investigated. The aim was to construct novel vectors with enhanced stability in the respective environment and advantageous properties in terms of in vivo application.

The in vivo administration of therapeutic genes is so far hampered by the lack of stable vector systems that are able to overcome the numerous hurdles on the way to their target tissue and cells. To address these issues, polyelectrolyte polyplexes between the polycationic polymer PEI and plasmid DNA, referred to as "polyplexes," were modified to circumvent the specific problems of in vivo application.

The respiratory tract presents a barrier between an organism and its environment that can be exploited for the aerosol administration of biologically active drug substances. Since polyplexes for lung administration provide an important and rapidly expanding field for the treatment of various pulmonary diseases, we attempted to design PEI conjugates for airway administration.

A new vector consisting of a protein transduction domain derived from the HIV TAT peptide coupled to PEI via a PEG linker is described in **Chapter 2**. We hypothesized that the cationic protein transduction domain would promote DNA condensation and enhance cell uptake, while PEG provided steric shielding to prevent polyplex aggregation. A broad range of physico-chemical, in vitro and in vivo studies were undertaken to assess the DNA protection capabilities and the toxicity of these conjugates. The resulting polyplexes with plasmid DNA were investigated in terms of cell uptake, biodistribution and transfection capability in vitro and in vivo.

Polyplexes are known to be rapidly cleared from the bloodstream after intravenous administration. Basically, this was believed to be due their interactions with blood components and vessel endothelia and by their rapid dissociation in the circulation with subsequent degradation of the nucleic acids. To address these issues, we constructed a stabilized vector system by crosslinking the polymer with a low molecular weight reagent. In **Chapter 3**, this strategy was systematically investigated. First, the course of the crosslinking reaction was evaluated. Different molecular weights and formulation procedures were compared in terms of their impact on polyplex size, surface charge and

stability of the resulting polyplexes. Furthermore, biocompatibility of these systems was tested.

Stability of the vectors after administration was thought to be a prerequisite to allow them to reach their target tissue. However, after cell uptake, the DNA must be released to be active. **Chapter 4** describes surface stabilized vectors of PEI/plasmid DNA that contain biodegradable groups intended to be cleaved after cell uptake. Factors influencing this unpacking and bioactivation of the DNA were systematically investigated. Then, in order to prove the feasibility of in vivo application, the stabilized polyplexes were injected into mice to determine the influence of surface crosslinking on pharmacokinetics and biodistribution as well as on in vivo transfection efficiency.

Hydrophilic polymers, such as Poly(ethylene glycol) (PEG) were believed to decrease blood clearance of PEI polyplexes via charge and steric shielding. The composition of the copolymers, i.e. the molecular weight and the grafting degree, has a great influence on polyplex properties and the in vivo behavior. We hypothesized that PEGylation using high molecular weight PEG at a low grafting degree could be promising in terms of polyplex stability in circulation. In **Chapter 5,** PEGylated PEIs were synthesized and characterized in terms of their composition and toxicity. Properties of the polyplexes with plasmid DNA were investigated for their complexation and condensation efficiency and their transfection efficiency was obtained. Furthermore, pharmacokinetic data were assessed after intravenous injection into mice. To further enhance polyplex stability, we combined polyplex surface stabilization with PEGylation and evaluated the influence on the plasmid pharmacokinetics.

Chapter 1

Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives

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Summary

The continually increasing wealth of knowledge about the role of genes involved in acquired or hereditary diseases renders the delivery of regulatory genes or nucleic acids into affected cells a potentially promising strategy. Apart from viral vectors, non-viral gene delivery systems have recently received increasing interest, due to safety concerns associated with insertional mutagenesis of retro-viral vectors. Especially cationic polymers may be particularly attractive for the delivery of nucleic acids, since they allow a vast synthetic modification of their structure enabling the investigation of structure-function relationships. Successful clinical application of synthetic polycations for gene delivery will depend primarily on three factors, namely (I) an enhancement of the transfection efficiency, (II) a reduction in toxicity and (III) an ability of the vectors to overcome numerous biological barriers after systemic or local administration. Among the polycations presently used for gene delivery, Poly(ethylene imine), PEI, takes a prominent position, due to its potential for endosomal escape. PEI as well as derivatives of PEI currently under investigation for DNA and RNA delivery will be discussed.

This review article focuses on structure-function relationships and the physicochemical aspects of polyplexes which influence basic characteristics, such as polyplex formation, stability or in vitro cytotoxicity, to provide a basis for their application under in vivo conditions. Rational design of optimized polycations is an objective for further research and may provide the basis for a successful cationic polymer-based gene delivery system in the future.

Introduction

The development of carriers for the delivery of genes or oligonucleotides, also designated as vectors, has seen considerable progress in the last three decades. Their application in gene therapy as a cure for human diseases has advanced to the stage of clinical trials, where trials in oncology take a dominant position [1]. Also, monogenic hereditary diseases, such as cystic fibrosis [2-4], adenosine deaminase deficiency [5] or infectious diseases, such as acquired immunodeficiency syndrome (AIDS) [6] are the subject of intensive research efforts. The number of clinical trials in gene therapy is steadily increasing, exceeding 900 by 2005 (data Wiley, http://www.wiley.co.uk/genmed/clinical, accessed January 2005). "Naked" plasmid DNA is unstable under in vivo conditions, due to rapid degradation by serum nucleases. Therefore, carriers or "vectors" are necessary to protect DNA or RNA from degradation, to facilitate uptake into specific cells and to transfer the DNA or RNA into the nucleus or cytoplasm, respectively.

Many of the currently used strategies for gene delivery rely upon viral vectors, because of their inherent ability to transport genetic material into cells, resulting in an efficient delivery and expression of genes. However, viral vectors may cause immunogenic and inflammatory responses, which preclude repeated administrations. Insertional mutagenesis could pose additional risks for patients undergoing gene therapy using retro-viral vectors [7, 8]. Also, the limited loading capacity and difficulties in large scale production of viral vectors have stimulated research into safe and effective non-viral vectors. Several strategies can be distinguished, among which the use of cationic lipids ("lipofection") or cationic polymers ("polyfection") has achieved some prominence [1, 9, 10].

Polyfection with cationic polymers of different structures was shown to enhance the uptake and the expression of DNA under in vitro and in vivo conditions [11]. Among polycations, PEI emerged as a very interesting candidate [12], reaching transfection efficiencies similar to viral vectors [13].

The recent years have witnessed rapid development of non-viral vectors based on PEI and derivatives which possess properties addressing delivery problems associated with gene therapy. The structure of PEI determines the physicochemical and biological

properties of the polyplexes with DNA and RNA to a large extent. It can be modified to produce new derivatives with differing architectures. The effect of the physicochemical properties of polyplexes on biological phenomena and transport processes are not yet fully understood and require more fundamental research. The intention of this review is to summarize the physicochemical characteristics of PEI-based vectors and show how structural modifications affect the behavior of the resulting polyplexes.

PEI: Polymer structure and molecular weight

PEI is a well known polymer that has been commonly used for waste water treatment and in the paper industry (Epomin®, Polymin®). PEI exists as a branched polymer, as well as in linear form. Transfection reagents based on linear PEI are already commercially available (e.g. ExGen500®, jetPEI®) [14]. An overview of the synthesis pathways of linear and branched PEI is given in Figure 1.

PEI is available in a broad range of molecular weights, from < 1000 Da to 1.6 x 10^3 kDa. It is commonly believed that the molecular weight of PEI most suitable for gene transfer ranges between 5 kDa and 25 kDa. Higher molecular weights lead to increased cytotoxicity [15], presumably due to aggregation of huge clusters of the cationic polymer on the outer cell membrane, which thereby induces necrosis [16]. Low molecular weight PEI, by contrast, has demonstrated a low toxicity in cell culture studies [27, 28]. Forrest et al. have combined the favorable low toxicity properties of the low molecular weight PEI with the higher transfection efficiency of high molecular weight PEI by coupling low molecular weight 800 Da PEIs together to form 14 kDa – 30 kDa conjugates using short diacrylate linkages. The hydrolysis of the ester bonds occurred under physiological conditions and the in vitro cytotoxicity could be correlated to the degradation behavior. The polymer with the smallest degradation half life revealed the lowest toxicity and no cytotoxic effects of degradation products were observed, but the transfection efficiency was higher for the polymer with longer degradation half-life, revealing a molecular weight effect on cell transfection similar to unmodified PEI [17].

The branched form of PEI shows a theoretical ratio of primary to secondary to tertiary nitrogen atoms of 1:2:1, based on the acid catalyzed polymerization mechanism of aziridine suggested by Dick et al. [18]. Moreover, measurements using quantitative C-13 nuclear resonance spectroscopy showed that the degree of branching was actually 1:1:1 for most commercially available PEIs, suggestive of a more branched structure [19]. The method of synthesis and the reaction conditions are likely to cause such deviations from the theoretical values. An increasing degree of branching is known to increase the in vitro cytotoxic effects, as well as the hemolysis of erythrocytes [20]. Kraemer et al. synthesized well defined pseudo dendrimers based on branched PEI and reported the lowest cytotoxicity for a degree of branching of about 60% [21]. Thus, detailed knowledge about the polymer structure is a prerequisite in order to establish clear structure-function relationships, as well as to optimize cytotoxicity and biocompatibility.

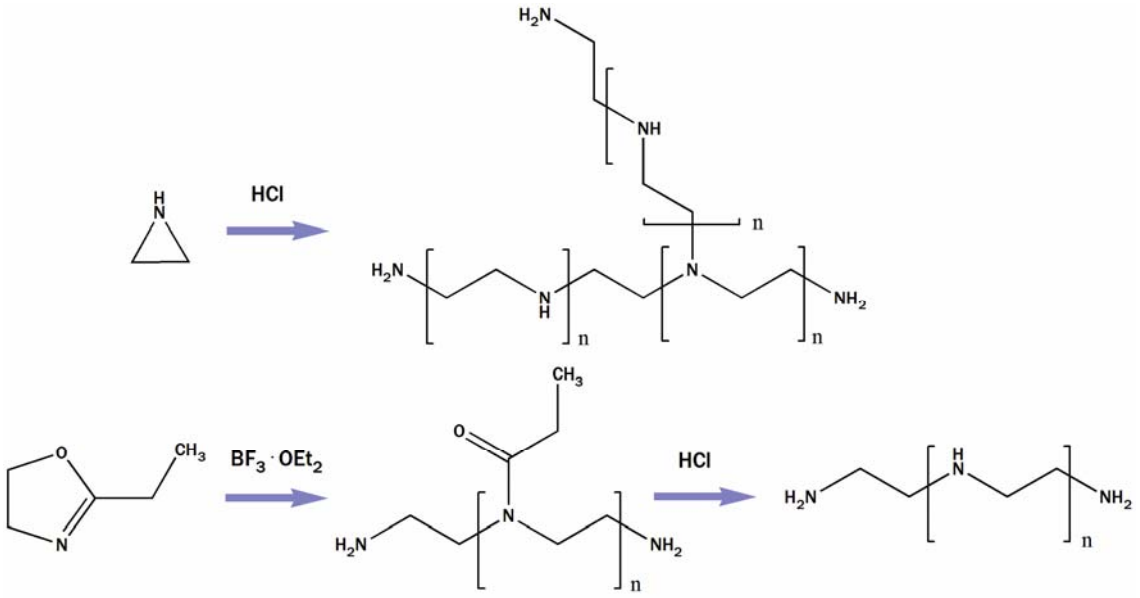

Figure 1: Acid catalyzed polymerization of aziridine leads to branched PEI, whereas ring opening polymerization of 2-ethyl-2-oxazoline leads to the N-substituted polymer, which can be transformed via hydrolysis into linear PEI [22] [23]

The most prominent feature of PEI is its high cationic charge density. Every third atom of PEI is a nitrogen atom capable of protonation. This leads to an extremely high cationic charge density of 20-25 microequivalents per gram [24]. Since PEI does not contain quarternary amines, cationic charges are generated by protonation of the amine groups in the biological environment, thus leading to a correlation between environmental pH and cationic charge density. For example, PEI shows a level of protonation of 20% at pH 7.4 compared to about 45% at pH 5 [25]. The wide range of apparent pKa values leads to a system with an effective buffer capacity.

Cytotoxicity [20] and endosomal release are a function of charge density and buffer capacity. A recent determination of buffer capacities showed that the area of highest buffer capacity lies between pH 8 and 10, which is typical for polyamines [19, 26]. Both basicity and protonation were influenced by the molecular weight and degree of branching of PEI. The pKa values (and therefore the basicity) of the polymer decreased in the pH range of 8 to 10 with an increasing molecular weight of the PEI [19]: pKa = 9 for PEI 2 kDa, 8.5 for PEI 25 kDa, and 8.3 for PEI 750 kDa [26]. The high buffering capacity above pH 7 was attributed to the secondary amines present in all PEIs, linear as well as branched [26]. Studies using a different variation of branched PEIs showed that a higher amount of primary and secondary amines could be correlated with higher pKa values, due to their higher protonation and, therefore, a higher number and density of charges [20].

Even though this region of higher buffer capacity lies above the physiological pH, a second, less distinctive maximum could be found in the pKa range between 4 and 6, where molar mass or polymer structure did not significantly influence the buffer capacity [19]. In this case, PEI would be able to buffer the interior of endosomes to some extent, thereby inducing their osmotic swelling and rupture of the endosomal membrane [12]. The so called “proton sponge” hypothesis has found wide-spread acceptance in recent years, although some publications have challenged the hypothesis [27]. Funhoff et al. suggested that the proton sponge hypothesis may not be generally applicable for polymers with a buffer capacity at low pH values of approximately 5 [28]. Others, however, have provided evidence to confirm the proton sponge hypothesis using, for example, living cell confocal microscopy [29]. Decelerated acidification, as well as elevated chloride accumulation and a 140% increase in the relative volume in

PEI containing endosomes, could be observed [30]. Additionally, the removal of protonable amine groups by quarternization decreased transfection efficiency by about 20 fold [31]. The proton sponge hypothesis alone, however, does not fully explain the prominent position of PEI or PAMAM dendrimers as transfection reagents that promote endosomal escape. More work on the elucidation of the molecular mechanism as to how polycations behave in the endosomal environment and interact with their membranes would be desirable.

Polyplexes of PEI with DNA

DNA complexation into small particles is a necessary prerequisite for the efficient delivery of the DNA into cells. Not only is endocytosis more efficient with particles < 150-200 nm, but the velocity of cytoplasmatic movement was also found to be a function of particle size [32]. The complexation of DNA with PEI protects against cleavage by nucleases. PEI is capable of condensing plasmid DNA and RNA into stable polyplexes via electrostatic interactions. The complexation and condensation behavior is dependent on several polymer characteristics, such as molecular weight, number and the density of charges, in addition to the composition of the polyplexes, e.g. the ratio of polymer to DNA. In fact, a lower charge density, as well as a lower molecular weight, might impair the condensation capability [33].

DNA-PEI condensates belong to a special class of polyelectrolyte interpenetration polyplexes. Their formation occurs in the presence of polycations [34], giving raise to spherical, globular or rod-like structures [34]. This process is supposed to rely predominantly on electrostatic interactions [35, 36], since binding of the cationic polymer and DNA occurs at a ratio of nearly 1:1 [37]. Recent FTIR data has shown a reduction in the frequency of the asymmetric phosphate stretching vibration of plasmid DNA after complexation with PEI, which may be attributed to electrostatic interactions between DNA and the polymer [17]. Additionally, microcalorimetric measurements also support polyplex formation by electrostatic interactions [36]. An increase in the salt concentration generally led to a decreased binding affinity [38], suggesting a charge shielding effect at the higher salt concentration [38]. Polyelectrolyte complexes, such as

PEI/DNA, may undergo polyion exchange and substitution reactions after formation both under in vitro and in vivo conditions.

Binding of DNA to PEI is thought to be mainly driven by entropic forces arising from the release of counter ions. However, other interactions, such as hydrogen bonds, Van der Waals forces or the removal of hydrating water molecules may also contribute to polyplex formation. Polycations with a high charge density, such as PEI or other high molecular weight polycations, can release more counter ions upon binding with DNA, thus forming more stable polyplexes [36].

The complexation of polycations with DNA was also found to be partially dependent on the DNA tertiary structure, as determined with PEI-PEG-copolymers. The polymer preferentially complexed supercoiled DNA rather than linearized DNA, especially at lower pH values around 5 [36]. The overall helical form of the DNA does not seem to be affected after complexation with PEI, since pDNA remained in its B-form, independent of the molecular weight and N/P ratio [26].

The investigation of the biological state of the DNA represents a further approach to DNA vector characterization [39], as obviously the effectiveness of the DNA transported by the carrier molecule plays a role in its therapeutic application.

Despite ongoing efforts, information on the composition and the structure of polyplexes between PEI and DNA is fragmentary, reflecting the lack of suitable, non-destructive characterization methods. Standard spectroscopic techniques can be used to determine the amount of PEI in the presence of DNA [40], however, these methods cannot distinguish between the fraction of bound polymer in polyplexes and the free polymer. Recent investigations using fluorescence correlation spectroscopy showed that polyplexes contain an average of 3.5 plasmid (5800 base pairs) and 30 PEI (25 kDa) molecules [41] when prepared at N/P ratios of 6 and 10, assuming the DNA was entirely complexed. A relatively high proportion, approximately 86% of the PEI, was found to be in a free form [41]. While these results await confirmation by independent methods, the relevance for cytotoxicity of PEI transfection reagents is obvious [16].

Purification of PEI polyplexes was recently shown to decrease the cytotoxicity as a result of the removal of excess PEI. However, this also led to a decrease in transfection efficiency. This effect was attributed to an ability of the free polymer to propagate

endosomal release, an assumption supported by the fact that the transfection efficiency was re-established after the addition of free PEI [42]. It also remains to be investigated as to how the shelf life of these purified polyplexes is affected by the removal of excess polymer, since polyplex formation is an equilibrium process.

However, an excess of polycation is essential to generate a hydrophilic cationic corona around the polyplex for sufficient solubilization [43]. Although PEI and DNA alone show excellent aqueous solubility, polyplexes of PEI and DNA become insoluble at a neutral charge.

Aside from the solubilization enhancement, the cationic surface charge is required for efficient cell transfection [44], since an interaction with anionic cell surface proteoglycans [45], presumably the transmembrane protein syndecan [46], is involved in the cell entry of PEI polyplexes.

Usually polyplexes with a positive surface charge (N/P ratios of approximately 5) are used for transfection experiments [47]. Studies with branched 25 kDa PEI polyplexes showed zeta potentials of approximately +5 mV at N/P 3.5. The zeta potential increased to about +15 mV at N/P 6 (glucose 5%/150 mM NaCl), suggesting that an excess of polycation was bound to the polyplex [48]. However, it has also been shown that PEI/DNA polyplexes with N/P ratios of 2.5 to N/P 10 exhibited a decreasing surface charge, possibly resulting from different polyplex structures and compositions [49]. These conflicting results demonstrate that the details of PEI/DNA polyplex structures and physicochemical properties, such surface charge, are still not completely understood, despite the fact that cell surface binding is a key step for polyplex gene delivery [50].

Polyplex formation protects RNA and DNA from degradation by enzymes [51]. Compared to naked DNA [52] or other cationic polymers, such as PLL (poly-L-lysine) [53], PEI has been shown to be more effective. For example, naked DNA degraded within 2 minutes after exposure to DNase I, whereas DNA complexed with PEI 25 kDa was only marginally degraded after 15 [54] and 30 minutes incubation [55], or after exposure to 25 units of DNase I for 24 hours [27]. The data from Godbey et al. implied that the protection of DNA by PEI resulted from a physical or electrostatic barrier to

enzymatic degradation with DNase I. It is also thought that additional protection occurs through inactivation of the enzyme [27].

Polymer structure influences polyplex characteristics

The molecular weight of PEI influences both the condensation behavior, as well as polyplex size. In general, an increase in the molecular weight of the PEI results in a decrease in polyplex size, although not without a limit. A molecular weight higher than 25 kDa showed no further decrease of polyplex size. Inversely, decrease in the molecular weight of PEI down to 2 kDa revealed an increasingly lower ability to form small polyplexes [56]. A further decrease of the molecular weight to 800 Da yielded huge aggregates of up to 900 nm [57]. This molecular weight dependency was observed for branched, as well as for linear PEIs [23]. These results indicate that the ability of lower molecular weight PEIs to condense DNA is so low that the resulting polyplexes are considerably larger than those of higher molecular weight species [57, 58]. The increase in condensation capacity and complexation efficiency of polymers with covalently coupled low molecular weight substructures to form higher molecular weight conjugates underlines these findings [17].

Polyplex formation is also dependent on the degree of polymer branching. Primary amines are known to condense DNA better than other amines, due to their higher protonation at a given pH [59]. Studies show that the binding capability could be correlated to the number of primary amines [37] and that polyplex stability increased with primary amine content, thus leading to a higher transfection efficiency [60]. Low branched and high branched PEI differ significantly in their polyplex forming ability [56]. Results obtained from agarose gel shift assays showed that complete complexation occurred at higher N/P ratios for low branched PEI [16]. In a further study, the content of primary amines in 2000 Da PEI--N-(2-hydroxyethyl-ethylene imine)-copolymers exhibiting degrees of branching between 0% and 23% was reduced a half with the consequence that twice the N/P ratio was needed to form small condensates [20]. Low branched PEI, therefore, again required higher N/P ratios for a complete condensation of DNA compared to highly branched derivatives [16]. An increase in the fraction of

secondary amine functions, which consequently decreased the proportion of tertiary amines, led to a higher complexation efficiency [23].

The influence of the degree of branching on gene transfer efficiency and in vitro toxicity is analogous to the complexation behavior, i.e. highly branched PEIs, which form smaller polyplexes, also achieve higher transfection efficiencies, yet simultaneously possess a higher toxicity. More flexible, hyperbranched PEI derivatives with additional secondary and tertiary amine groups show a lower toxicity in cell culture experiments along with enhanced transfection efficiency [61].

Linear PEI also possesses a lower condensation capacity, as compared to the branched forms [34]. This can be related to its decreased content in primary amines (Figure 2). Compared to linear PEI, the branched form (25 kDa) is able to retain pDNA up to 24 hours in the condensed state in cytoplasm, compared to 4 h for linear PEI (22 kDa) [62].

Keeping in mind the importance of the primary amine fraction for nucleic acid complexation, it may be useful to avoid “wasting” primary amino-groups as attachment points for ligands. It was shown that secondary amines and tertiary amines are also accessible to ligand binding, leaving, thus, primary amines free for DNA condensation, as shown for e.g. PEI-cholesterol [63] or PEI-alkyl [31] conjugates. This may become a pertinent issue if higher substitution degrees are intended than those used for the reported conjugates.

As both the transfection efficiency and cytotoxicity seem to depend on such physicochemical properties as the molecular weight [16, 64] and branching ratio [20, 56], it becomes evident that polymer structure significantly influences the efficacy of PEI based vectors [65]. Keeping in mind the different applications for PEI as a carrier system, e.g. plasmids, oligonucleotides or siRNA, the design of the proper polymer becomes a sophisticated task. The molecular weight, degree of branching or surface charge has to be adjusted to produce stable polyplexes, yet simultaneously generate systems with the desired release properties.

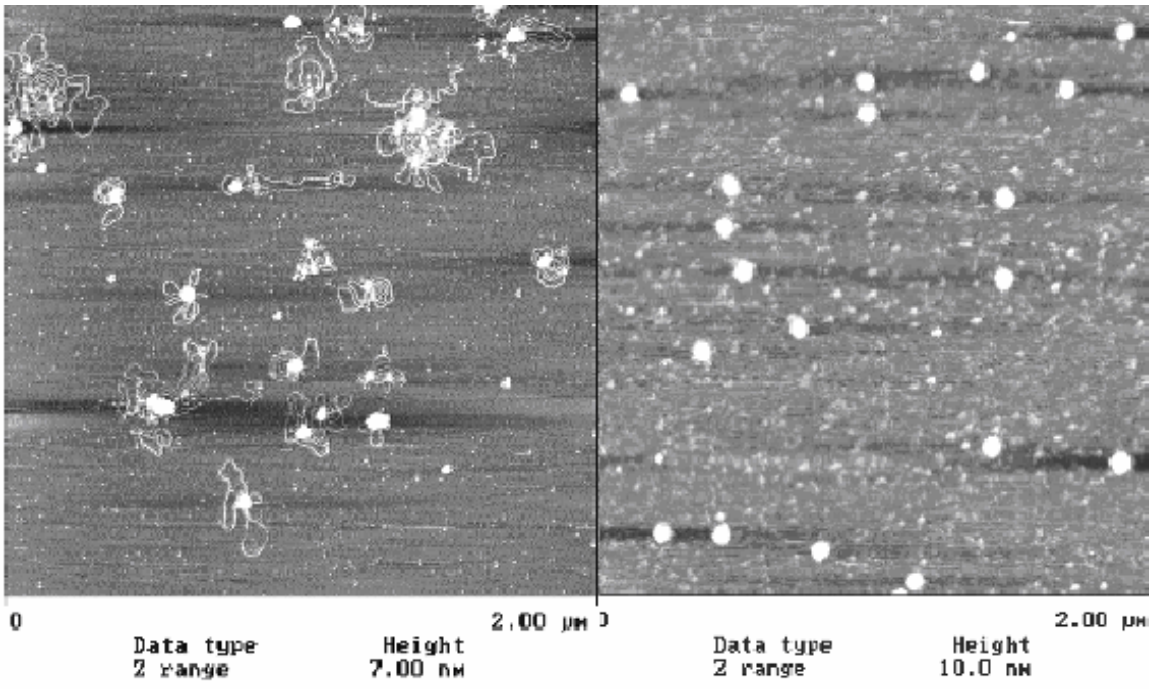

Figure 2: Loosely condensed polymer/plasmid polyplexes consisting of linear PEI 22 kDa (left) compared to polyplexes with branched PEI 25 kDa (right). (Nanoscope IIIa Multimode AFM, polyplexes prepared in isotonic glucose solution at pH 7, DNA concentration 15 µg/mL)

Formulation of PEI/DNA polyplexes

Since approximately 90% of its charged groups must be neutralized to condense DNA, a N/P ratio of about 2-3 is necessary to achieve stable polyplexes using branched [11, 66] or linear PEI [26, 67]. The complexation of DNA by PEI leads to a significant decrease in DNA size, resulting in polyplexes that require a volume 10^4 to 10^6 times smaller than that of naked DNA. Increasing the amount of polymer and thereby increasing the N/P ratio from 2 to 20 has been shown to result in a decrease in the observed particle size from > 1000 nm to 100-200 nm, accompanied by a simultaneous reduction in the polydispersity [47].

The formulation of polyplexes plays an important role in both the transfection efficiency and stability. The sequence of component addition during the complexation procedure (involving either the addition of a PEI solution to the DNA solution or vice versa) influences the resulting polyplex size, as well as the transfection efficiency [12,

68]. In part, this effect may be attributed to the respective DNA and polycation concentrations [69].

The type of medium for complexation is also an important factor. PEI/DNA polyplexes formulated in saline solution show polyplex sizes dependent on ionic strength (Table 1 provides a non-exhaustive list of polyplex sizes determined by light scattering methods). The tendency of polyplex sizes to increase with increasing saline concentration is thought to reflect a decreased binding efficiency. A drastic decrease of polyplex size with linear 22 kDa PEI was observed when comparing polyplexes prepared in physiological salt solution (> 1000 nm) with those in 5% glucose (30 – 60 nm), reaching polyplex sizes comparable (<100 nm) to branched 25 kDa PEI in ionic solutions [70]. Specifically, in the case of in vivo administration, sodium phosphate solution has shown benefits compared to NaCl or PBS as injection vehicle [71].

PEI	**DNA**	**Buffer**	**N/P**	**Polyplex size [nm]**	**Ref.**
25 kDa	1	50 mM NaCl	6	300	[72]
25 kDa	1	150 mM NaCl	4	> 1000	[72]
25 kDa	1	150 mM NaCl	6	600	[72]
25 kDa	3	10 mM NaCl	9	95	[66]
25 kDa	3	150 mM NaCl	4.5	230	[66]
25 kDa	3	150 mM NaCl	~7	156	[56]
25 kDa	3	150 mM NaCl	9	120	[66]
25 kDa	2	150 mM NaCl	10	93	[73]

Table 1: Effective diameters of polymer-DNA polyplexes as determined by dynamic light scattering in different media and with different polymer/DNA ratio (DNA type: 1: herrings testes DNA; 2: pCMV-Luc plasmid 7.2 kB; 3: pGL3 plasmid, 5.2 kB)

Furthermore, the storage conditions of the formulation may also affect transfection efficiency. For example, it was recently shown that a three week storage period of polyplexes made from highly flexible hyperbranched PEI derivatives enhanced transfection up to 8fold, presumably due to an enhancement of the electrostatic interactions resulting in more compact polyplexes [61].

Aggregation behavior of PEI/DNA polyplexes

Polyplex size plays a crucial role for biocompatibility and extravasation when targeting cells outside the vasculature [74]. Polyplex aggregation under physiological conditions is still an area of controversy and must be characterized thoroughly. Since DNA/PEI polyplexes exist as individually compact units, particles of apparently larger size are thought to consist of aggregates of these smaller units [37]. Positively charged polyplexes show a tendency to aggregate as a function of incubation time. Aggregation is also dependent on parameters such as surface charge and ionic strength of the medium. The tendency towards aggregation may be influenced by the presence of shielding components, which may decrease interactions between individual PEI polyplexes, as well as interactions between polyplexes and blood components in the systemic circulation. Such shielding components are known to inhibit the rapid elimination of these large aggregates by the RES [75].

In general, polyplexes formed at low N/P ratios in the range of 2 to 5 tend to aggregate [47], due to hydrophobic interactions, as well as van der Waals forces [76]. In contrast, higher N/P ratios reduce aggregation as a result of electrostatic repulsion of the higher positive surface potential of the polyplexes, an effect which may stabilize polyplexes under physiological salt conditions [37]. Excess PEI can associate with the condensed particles, leading to a strongly positive zeta potential of about +25 mV in 0.9% NaCl [49]. Aggregation of polycation/DNA polyplexes may also be induced by inter-particle cross-bridging of the polymer chains [77].

Time-dependence of aggregation could be observed at different ionic strengths. In 10 mM NaCl, polyplexes exceeded effective diameters of 500 nm after 30-60 min [78], whereas in 150 mM NaCl aggregates of > 900 nm were observed after 30 min [66]. During a 3 hour observation period, a rather slow growth of PEI 25 kDa polyplexes formed in 0.5x HBS from approximately 120 nm to 370 nm was observed. In contrast,

linear PEI 22 kDa polyplexes tended to aggregate much faster, reaching approximately 750 nm after only 20 min and exceeding 6 microns after 3 hours [79]. A comparison of PEI 48 kDa polyplexes with 5 kDa PEI showed that aggregation was dependent on the molecular weight of PEI [56]. While the high molecular weight polyplexes remained stable, the 5 kDa polyplexes underwent a size increase from 330 nm to 730 nm at N/P ~ 7.

Although some reports indicate that larger particles might be favorable for in vitro use [76, 79], arguing that a higher cellular uptake can be achieved due to sedimentation [76], the in vivo application of such large aggregates may not be feasible. The formulation of polyplexes in systems with a closer resemblance to the physiological environment may improve the correlation between results from cell culture experiments and the corresponding in vivo tests.

Variations of the basic structure: PEI conjugates

Strategies for PEI-copolymer synthesis

With the aim to obtain more efficient non-viral vectors for gene delivery, the structure of PEI has been extensively modified. Most notably, second-generation polymers have been developed, comprised of block and graft copolymers containing cationic and hydrophilic non-ionic components [80].

One of the first and most extensively investigated attempts to modify PEI was the covalent coupling of PEG chains to the polymer, resulting in block or graft copolymers. “PEGylation” has been widely used in gene delivery vector technologies, e.g. PEI, dendrimers [81], PLL [82], liposomes [83] and even viral vectors, such as adenoviruses [84]. Modification of PEI with PEG can be accomplished using different synthetic strategies [85]. The most common approaches rely on PEGs containing activated functionalities, which can react with amino groups. While relatively straightforward, some issues related to this type of method should be considered. For example, the activation with dimethoxytrityl chloride [43] requires careful removal of polymeric side products after PEG activation. The activation of PEG with epoxide [78] or isocyanate groups [86] leads to a simple two step synthesis. A bifunctional PEG bearing a NHS (N-hydroxy succinimide) group and a vinyl sulfone group on each opposite end has often

been used for the addition of targeting moieties to the PEI [87, 88]. This approach has the additional advantage of circumventing block-copolymer synthesis prior to polyplex formation, as will be discussed later. However, the coupling of commercial, preactivated PEG is restricted by the available molecular weight of the polymers. For a non-exhaustive summary of possible routes of synthesis see Figure 3.

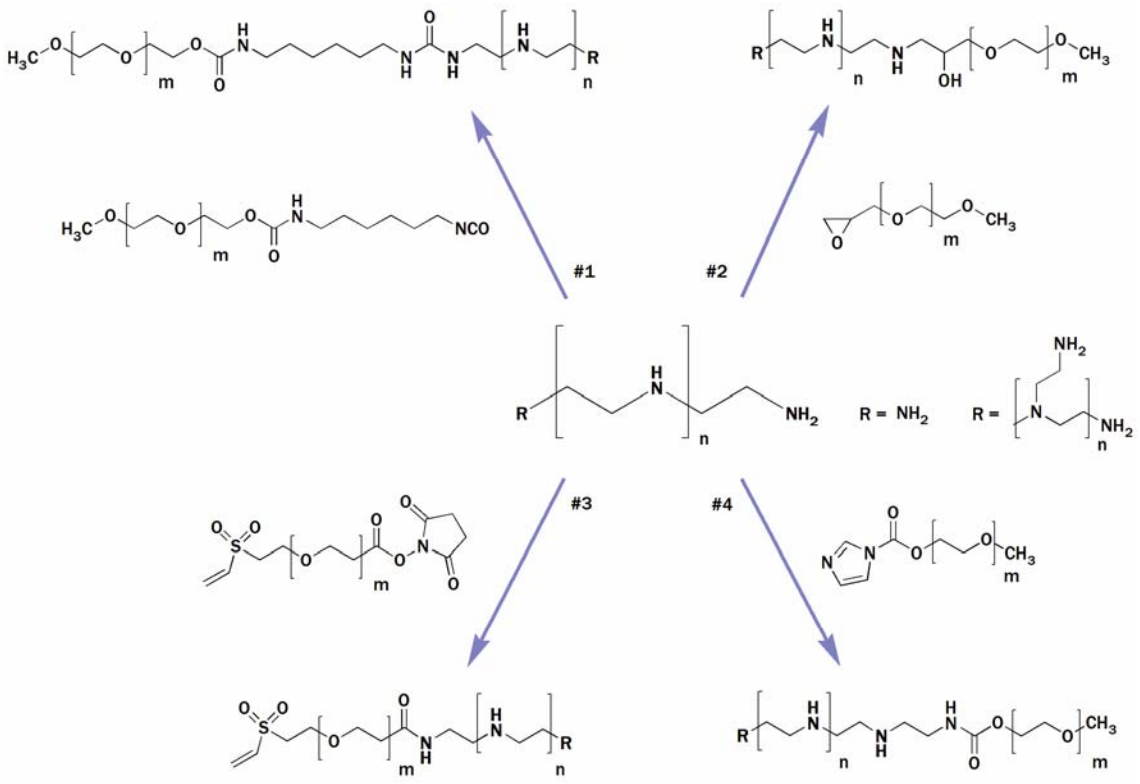

Figure 3: Different synthesis strategies of block and graft copolymers of PEG and PEI (#1 [86], #2 [78], #3 [88], #4 [89])

A new concept for the synthesis of PEI-graft-PEG (PEG-PEI)-copolymers was recently reported [90]. The addition of mono-amino-PEG as a so-called “macrostopper” was reported to lead to termination of the propagation of PEI polymerization by a direct reaction of the macrostopper PEGs with the propagating PEI chains. This prevented the formation of PEIs with multiple PEG grafts, thus leading exclusively to diblock-copolymers. Although this concept seemed to work perfectly for PEG 5000 Da, side products of free homopolymers were obtained when using higher molecular weight PEG. Consequently, a further investigation of the synthesis parameters is required for

this promising procedure. A similar method was developed using an acetale group at one end of the PEG to react with 2-methyl-2-oxazoline [91]. This led to copolymers containing a linear polyethylenimine moiety, providing an approach for the synthesis of linear PEI-copolymers.

The steric shielding of the branched PEI or the PEG chains leads to a decrease in the grafting ratio with increasing PEG molecular weight. PEG-PEI 25 kDa conjugates with approximately 57 PEG chains (350 Da) could be synthesized, but only conjugates with less than one PEG chain with a molecular weight of 40 kDa were reported (Figure 4).

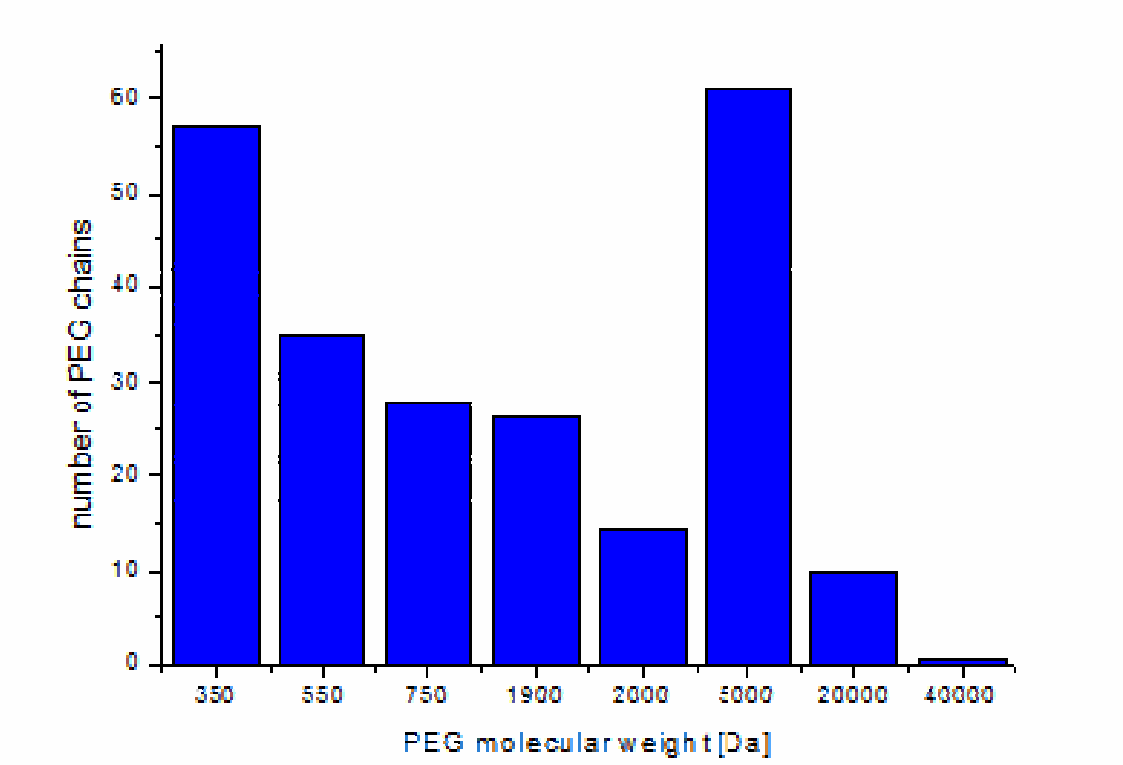

Figure 4: PEI 25 kDa grafted with PEG of different molecular weight. Higher molecular weight of the PEG chains resulted in a generally decreased substitution degree [49, 78, 86, 87, 89, 92, 93].

Biodegradable copolymers

Polymers not eliminated from the circulation may accumulate in tissues and cells, which is desirable from a gene delivery point of view. On the other hand, accumulation of non-degradable materials in tissues may pose a problem, due to unknown effects of long term toxicity. Renal elimination of water-soluble polymers is limited by the threshold

size cut-off of glomerular filtration at around 30 kDa. Higher molecular weight conjugates must contain cleavable groups to facilitate their degradation and subsequent excretion.

One drawback of most PEG coupling methods are the non-biodegradable bonds between PEI and PEG , such as urethane [43] or urea [66, 86]. These bonds are stable against hydrolysis under physiological conditions. To resolve this problem, biodegradable linkages have been introduced into the copolymers using, e.g. esters, amide bonds or reductively cleavable disulfide bridges.

The use of a bifunctional succinimidyl succinate PEG bearing two amine reactive ester groups resulted in generally non-soluble copolymers. Only strict control of concentration of the reaction mixture and temperature, as well as the use of low molecular weight PEI (< 2 kDa), resulted in the synthesis of water-soluble products [94].

A ternary copolymer consisting of diblocks made from poly-ε-capro-lactone and PEG grafted onto branched 25 kDa PEI via potentially biodegradable amide bonds bore chains with alternating hydrophilic and hydrophobic characteristics. The copolymers were able to form micelles or were found to be water-soluble, depending on the molecular weight of the poly-ε-capro-lactone and PEG [95].

Based on hydrolytically cleavable amide bonds, a biodegradable polymer was synthesized composed of several low molecular weight PEIs (1200 Da) linked together with co-L-lactamide-co-succinamide [96]. The resulting water-soluble 8 kDa copolymer showed a decreased degradation at pH 5, thus, providing protection for complexed DNA in acidic environments, such as is found in lysosomes. Additionally, this copolymer exhibited a lower toxicity as compared to commercial PEI.

Recently, Lee at al. reported the synthesis of disulfide containing, biodegradable PEG with molecular weights between 2 kDa and 20 kDa [97]. This approach may help to design PEG-PEIs that comprise the advantages of hydrophilic copolymers while having the potential to be cleaved in the reductive intracellular environment. Additionally, this may be combined with biodegradable PEI to obtain “fully” degradable PEG-PEI copolymers.

Biodegradable PEI derivatives are vital for the in vivo application over an extended period of time. Potentially, they may be able to combine the benefits of the higher molecular weight PEIs and their triggered release properties with the favorable (long-term) toxicity profiles of the lower molecular weight PEIs.

Hydrophilic copolymers: PEG-PEI-copolymers

The covalent attachment of non-ionic, water-soluble copolymers, such as PEG, is a commonly used way to improve aqueous solubility, biocompatibility and reduce the immunogenicity of drug delivery systems. PEGylation forms a hydrophilic shell that provides steric shielding of the PEI moiety, improving polyplex solubility [98] and aggregation [92]. Furthermore, PEGylated PEI polyplexes have been shown to display decreased interaction with proteins [92], a reduced activation of the complement system [99], and an enhanced circulation time in the blood [93]. PEGylated PEI tends to be less toxic than unmodified polymers in vitro and in vivo [86, 100].

From a physicochemical point of view, the polyplex formation of PEG-PEI-copolymers with DNA also appears to be an entropy-driven, spontaneous process, with the formation of ion pairs between the cationic amino groups of the co-polymer and phosphate groups of DNA resulting in polyplexes based on electrostatic interactions [36].

Galenics of PEG-PEI-copolymers

Two different approaches of introducing PEG moieties into polyplexes have been proposed. The first method involves the use of preformed PEG-PEI copolymers, which form a polyplex after the subsequent addition of DNA. The main drawback of this pre-PEGylation method is that the hydrophilic copolymer may interfere with polyplex formation [47]. In the second approach, polyplex formation is completed prior to coupling of the PEG chains. Until now, the first method was preferred [43, 47, 86, 89, 101], although recently a reverse protocol for the post-PEGylation method was reported [93, 100, 102]. When considering a possible clinical application of the polyplexes, the post-PEGylation method may show some drawbacks and the use of pre-synthesized copolymers may offer advantages, due to easier handling. For this reason, Kursa et al. have developed a method based on freeze-thaw stabilization of the components:

Plasmid DNA, linear PEI as a condensing agent, and transferrin as shielding and targeting component [103]. These formulations can generate polyplexes by simply mixing the components together similar to pre-synthesized copolymers. Ogris et al. also developed surface-shielded formulations by attaching the ligand and PEG molecules to PEI either before or after DNA polyplex formation. The polyplexes could then be ultra-concentrated, stored frozen, and applied intravenously in tumor bearing mice after thawing [104].

Influence of PEG on PEG-PEI polyplexes

The addition of a copolymer to PEI alters the complexation behavior and renders DNA condensation more problematic, due to the steric layer that shields the charged PEI. Similar effects have been reported for other polycations, e.g. PLL-g-PEG copolymers [105]. Despite intensive investigations, no consensus on the optimal degree of PEG-substitution and PEG chain length was reached, as both contribute to polyplex characteristics. Generally, the maximum substitution degree seems to be a function of the molecular weight (Figure 4); steric hindrance effects of the PEG chains may be responsible for this.

Short side chains did not show a significant effect on the complexation behavior as a function of N/P ratio for PEG molecular weights ranging from 350 to 1900 D [78]; all sizes investigated formed rather large polyplexes possessing a less compact a spherical shape. Increasing graft density with PEG 2 kDa also resulted in increasing size [88]. Complexation was slightly hindered when variations of grafted (n=2, 6, 15) PEG 5 kDa copolymers were investigated [66]. Nevertheless, increasing the number of PEG 5 kDa resulted in a significant decrease in polyplex size. Conjugates with a higher degree of grafting lost their spherical shape, with some of the polyplexes exhibiting poorly condensed DNA [66].

On the other hand, diblock copolymers containing only one 20 kDa PEG chain even enhanced DNA condensation compared to PEI forming small (51 nm, AFM) and spherical polyplexes. This is obviously contrary to the effect observed with shorter PEG chains and may be attributed to the unique AB-diblock-copolymer structure of clearly separated PEI and PEG domains [66].

Condensation	PEG 550 Da does not affect DNA condensation PEG 5 kDa slightly hinders DNA condensation PEG 20 kDa enhances condensation
Size	PEG ≥ 5 kDa reduces polyplex size PEG 550 Da enlarges polyplex size
Morphology	PEG 550 Da: large, diffuse aggregates PEG 20 kDa: small, spherical, compact aggregates
Surface Charge	PEG ≥ 5 kDa reduces zeta potential PEG 550 Da does not reduce zeta potential
Stability	PEG ≥ 5 kDa stabilizes the polyplex against aggregation PEG 550 Da does not stabilize the polyplex against aggregation

Table 2: Influence of different PEG molecular weights on the PEG-PEI/DNA polyplexes (according to [66])

PEG-PEI polyplexes are more stable with regard to aggregation of polyplexes in vitro [43, 92, 93]. Additionally, the surface charge is of major importance for the in vivo behavior of PEG-PEI/DNA polyplexes [106]. Due to complement activation or interactions with blood components, cationic polyplexes are rapidly cleared from the circulation, accumulating in the RES [75]. Ogris et al. demonstrated that polyplexes with a neutral surface charge interact only weakly with endothelia, plasma proteins or cellular blood components [93]. Masking the positive surface charges leads to longer half lives in circulation, due to reduced opsonization and RES uptake [100].

Grafting of a 25 kDa PEI with ten PEG 2 kDa chains reduced the zeta potential of PEI/DNA polyplexes to less than +5 mV in NaCl 150 mM [49], fifteen PEG 5 kDa chains could further reduce the zeta potential to less than +3 mV at an even high N/P ratio of 50. The steric stabilization provided by PEG, possibly in the form of a hydrophilic corona around the PEI/DNA core, is also important for the systemic application of polyplexes.

The use of high molecular weight PEG chains resulted in a decreased sensitivity of the polyplexes to salt induced aggregation. This effect can be attributed to the better capability of the longer side chains to cover the surface of the polyplexes, whereas shorter side chains, for example 350 Da PEG, needed a higher degree of substitution, namely 80 chains vs. 13, to achieve an effect similar to that of 1900 Da PEG [78].

Comparable results have been obtained using 8 kDa [89] and 5 kDa PEG in high ionic strength medium (150mM NaCl) [66]. A molecular weight of at least 2 - 5 kDa seems to be necessary to achieve a stabilizing effect. Low molecular weight PEG chains (550 Da) showed the opposite effect by inducing a more pronounced aggregation [66]. The poor stabilization against aggregation of these low molecular weight PEG-PEI conjugates may be attributed to the formation of a structure differing from that of the core-corona model [70]. This theory is supported by DSC (differential scanning calorimetry) data, where the miscibility of PEG and PEI segments was observed [86].

Obviously, the structure of the PEG-PEI-copolymer will drastically affect properties of the resulting polyplexes with DNA and RNA. PEGylation, therefore, might be considered as one tool for the design of custom tailored polyplexes with adjusted stabilization and release properties. However, although some ground rules have emerged from these investigations (see Table 2), clearly more work is necessary to reach a final conclusion. There is also a need for alternatives to PEG, as well as bioreversible linkages of PEG or other hydrophilic macromolecules to PEI.

New hydrophilic PEI copolymers

Research on hydrophilic copolymers of PEI and components other than PEG has not been carried out to the extent as with other polycations, e.g. polylysine [82, 105, 107-110]. The main objectives, however, remain the same, namely sufficient polyplex stabilization in vivo and an enhanced circulation half life.

The electrostatic shielding of a “copolymer“ with polyacrylic acid, when included in PEI/DNA polyplexes, showed a considerable size enlargement due to flocculation. However, this strategy seemed to achieve an effective shielding from opsonization [111].

Toncheva et al. introduced the hydrophilic polymer PHMPA (poly-[N-(2-hydroxy propyl)methacryl amide]) as a grafting agent for cationic polymers [82]. A decrease in albumin interactions and macrophage association with the PLL/DNA polyplexes could be achieved by attaching semitelechelic PHMPA to the polyplexes, however, this failed

to prolong the circulation time. Uptake in RES (liver) was even increased compared to the unmodified polymer [109].

A multivalent PHPMA bearing reactive ester groups for the covalent surface modification of PEI/DNA particles led to a laterally stabilized polyplex with an enhanced aqueous solubility [112]. The polyplexes prepared with PHPMA exhibited sizes of about 100 nm, comparable to PEGylated PEI/DNA polyplexes. However, due to a partial hydrolysis during coating, the PHMPA-coated polyplexes were negatively charged, in contrast to the favorable close to neutral of the PEG-PEI-polyplexes. The use of multivalent instead of monovalent hydrophilic polymers can lead to an enhanced resistance against salt induced aggregation, as well as a decreased susceptibility to polyanion exchange reactions, thus allowing extended systemic circulation times with α-half lives of more than 90 min [112].

Additionally, a recent report dealing with PEI-dextran polymers presented data showing an improved stability against albumin induced aggregation when using branched PEI, but not with linear PEI. Unfortunately, the dextran grafting resulted in weaker DNA compaction, due to presumably reduced charge interactions [113]. These findings were supported by results with 1500 Da dextran grafted onto PEI at different substitution degrees, which diminished the cell entry capability of the polyplexes [114].

The positive surface charge of PEI (25 kDa branched or 22 kDa linear) polyplexes could also be efficiently shielded, thereby decreasing non-specific interactions with erythrocytes, by covalently incorporating transferrin at sufficiently high densities within the polyplex [115]. This system provided a unique combination of stabilization and cell specific targeting.

Recently, a reverse approach of coupling PEI onto PEG was reported [57]. In this case, a 4-star and an 8-star PEG-core bearing PEI moieties with a molecular weight of 800 Da and 2000 Da was synthesized. This vice versa reaction scheme yielded star-shaped PEI-PEG copolymers, probably possessing a similar core-shell structure as proposed for PEGylated PEI. DNA polyplexes of these PEI-PEG copolymers had a size of approximately 100 nm in 150 mM NaCl at N/P 9, which was considerably lower than that of polyplexes made from unmodified low molecular weight PEIs of 800 Da and 2000 Da. The polyplexes showed a zeta potential of +/-5 mV and did not aggregate over a period of 20 minutes. These results are comparable with those of PEG-PEI/DNA

polyplexes, so it was assumed that after complexation the PEG moieties occupy the outer sphere of the polyplexes.

Low toxicity and the ability to modify the polyplex via inclusion polyplex formation gave rise to polyplexes between cyclodextrin and PEI. The cyclodextrin grafting level of the branched PEI 25 kDa correlated with a reduction in transfection efficiency in vitro, but also with a decrease in toxicity, both in cell culture and in vivo studies [116]. These copolymers may provide a new strategy for low toxic, modifiable and biocompatible vector systems.

Hydrophobic and amphiphilic copolymers

Amphiphilic PEI derivatives constitute a hybrid system that combines both charge interaction and self-assembly potential.

Acylation of PEI 25 kDa with palmitic acid and subsequent PEGylation created amphiphilic PEI derivatives achieving a 10fold lower toxicity in cell culture, while retaining 30% of the transfection efficiency in vitro [117]. Linkage of lipophilic chains, such as cholesterol and myristic acid, to low molecular weight PEI (1.8–2 kDa) resulted in an enhanced transfection efficiency, however, the effect on the in vitro toxicity of the conjugates remains inconsistent [63, 118, 119]. N-Dodecylation generally yielded non-toxic polycations with a 400fold transfection efficiency compared to PEI 2 kDa [31]. The incorporation of alanine in high molecular weight PEI (25 kDa) doubled the transfection efficiency and lowered toxicity when the alanine was allowed to react with the tertiary amine groups [31].

Preformed copolymers, for instance the triblock copolymer, Pluronic®, which consists of a propylene oxide block sandwiched between two PEG blocks, were grafted onto PEI using different molecular weights [80, 89, 120-122]. These water-soluble copolymers resulted in self-assembling, micelle-like particles in aqueous solution. The results are comparable with ternary block copolymers consisting of PEI, poly-ε-capro-lactone and PEG, which additionally display a lower cytotoxicity and an increased transfection efficiency [95].

Specifically, a vector based on amphiphilic Pluronic 123®-graft-PEI was recently developed [123], with the intention that the polypropylene oxide segment of the Pluronic 123® component may enhance incorporation into cell membranes, as could be

shown for Pluronic 85® [124]. Polyplexes prepared using Pluronic 123®-PEI-copolymers had larger sizes, due to lower polyplex stability as compared to PEG copolymers, resulting from the hydrophobic segments in Pluronic. In addition, the copolymers only insufficiently protected the plasmid DNA from nuclease degradation [123]. However, a mixture of Pluronic 123®-PEI(2 kDa) with free Pluronic 123® generated polyplexes with pDNA having effective diameters of approximately 110 nm and even increases transfection efficiency compared to PEI 25 kDa [89].

PEI conjugates with Pluronic 85®, P85®g-PEI(2 kDa), showed interesting properties for ODN delivery and altered the distribution in the body. While PEG(8 kDa)-PEI(2 kDa) accumulated in the kidney, P85®-g-PEI(2 kDa) was targeted to the hepatocytes of the liver avoiding the RES [121]. A possible advantage of Pluronic®-g-PEI is the increased stability of polyplexes in serum. A higher hydrophilic-lipophilic balance (Pluronic® F68 > F127 > P105 > P94 > L122 > L61) seems to be beneficial for increasing transfection efficiencies [125]. Although the actual polyplex composition remains to be elucidated, non-complexed Pluronic® chains seem to interact with hydrophobic PPO domains of polyplexes thus shielding ODNs. The protective effect is more pronounced with ODN than with plasmids, pointing to a reduced chain flexibility and polyplex stability.

A delicate balance between hydrophilic and hydrophobic components is crucial for the design of more efficient gene delivery systems based on PEI. On one hand, the cytotoxicity of polycations can be significantly altered by hydrophobic or amphiphilic modifications and, on the other hand, transfection efficiency may suffer only modestly. Incorporation of hydrophobic groups into PEI could affect interactions with endo-/lysosomal membranes resulting in a more efficient escape from these compartments.

Cross-linking of polyplexes using disulfide bonds

Cross-linking using multivalent polycations at the surface may be an alternative approach to achieving polyplex steric stabilization, preventing polyplex dissociation, and thus prolonging the circulation time of vectors in blood. Although most investigations have not been carried out with PEI, several reports have described this

approach using polycations, such as PLL [126, 127], or other vector systems, e.g. lipoplexes [128] and peptides [129, 130].

Chemical modifications of polycations based upon bioreversibly cleavable disulfide bridges are an attractive strategy [131]. Thiol groups can be either oxidized following polyplex formation [126, 129] or cross-linking is achieved through low molecular weight cross-linking reagents [127]. Disulfide bonds are known to be cleaved in the reductive environment of endo-/lysosomal compartments [132] or by glutathione [133]. This leads to a triggered release of the nucleic acids from polyplexes [133].

Gosselin et al. have synthesized a system based on 800 Da PEI cross-linked via cleavable disulfide bonds [58]. However, this modification was not intended to act as a shield on the polyplex surface, but rather as a way to build aggregates of higher molecular weight to enhance the transfection efficiency. The more effective cross-linking reagent DSP formed aggregates between 23 kDa and 75 kDa, while eliminating the positive charge of primary amine groups at the same time. In contrast, using DTBP, higher transfection efficiencies could be reached, presumably due to the preservation of positive charges.

Trubetskoy et al. were the first working with cross-linkers containing disulfide bridges to enhance the stability of PLL/DNA polyplexes against polyanionic exchange reactions and to reduce salt induced aggregation [77]. PEI/DNA polyplexes have been investigated by [134] using the same small cross-linking reagents with incorporated disulfide bonds to incorporate a trigger mechanism for activation of the polyplexes after entering the reductive environment. Their results have also shown an enhanced stability against polyanion disruption, depending on the amount of cross-linking agent. The feasibility of the reductive activation of stabilized PEI polyplexes containing disulfide bonds was recently reported. A negative control comprised of non-reducible thioether linkages revealed no activation potential [135].

However, cross-linked PEI/DNA polyplexes with low molecular weight reagents have not been characterized sufficiently and information on cross-link densities or optimal spacer lengths has not been provided. Also, the reversible cleavage of SS-bonds and the effects on transfection efficiency are somewhat controversial. Frequently a lower expression of reporter genes is observed [134, 136] or only the addition of glutathione boosted transfection [135], rendering the proposed reducible effect of cell interior

questionable. Hence, there is a need for more detailed investigations into the structure-activity relationship of stabilized polyelectrolytes.

Modification to achieve tissue specificity and enhance cellular uptake

An ideal gene delivery system should not only deliver the nucleic acid intact and without side effects, but also provide a basis for cell or tissue specific targeting.

The simplest approach is the use of the inherent, passive targeting capabilities of specific PEI or its modifications. JetPEI® achieved tumor targeting, due to passive accumulation into the permeable tumor vasculature based upon the EPR effect [137]. PEI grafted with Pluronic 123® or Pluronic 85® directed biodistribution towards hepatocytes, eight PEG chains grafted onto PEI 2 kDa targeted the kidneys [121]. Another approach relies on active targeting using receptor-mediated uptake of modified polyplexes into specific cells. These constructs have been shown to deliver DNA and RNA to specific target tissues, such as hepatocytes [87, 138-141] and dendritic cells [140, 141] via carbohydrates; tumor tissue via folate receptor [142], integrin [88] or transferrin [103] targeting; and to tissues expressing specific receptors with antibodies [143] or their fragments [49, 144] (Table 3). For example, the coupling of galactose to PEI provides a mechanism for liver specific targeting, using the asialoglycoprotein receptor, which is expressed on hepatocytes. Galactose modified PEI showed comparable transfection efficiencies at low substitution degrees (3.5%) [138], an enhancement in transfection efficiency with a higher grafting ratio of 5% [139], followed by a decrease when the amount of galactose was further increased up to 31%. The latter observation is likely due to steric shielding effects, which impaired complete DNA condensation [138]. Using the same approach for PEGylated and, therefore, sterically shielded PEI, coupling of galactose to 0.5% and 1.7% of the PEI amine functions resulted in only partially compacted structures with no hepatocyte targeting effect, presumably due to the low extent of grafting [47]. Sagara et al. reported a low enhancement of transfection comparable to PEI-gal conjugates, depending on the grafting ratio [87].

Polymer	Biodegra-dability	Steric shielding	In vitro toxicity compared to bPEI25 kDa	Ref.
Branched PEI 25 kDa	-	-	-	[12]
jetPEI®	-	-	↓	[137]
LMW-PEI 5.4 kDa	-	-	↓	[16]
Pseudodendrimeric PEI	-	-	↓	[21]
PEI-SS-PEI	+	-	n.r.	[58]
PEI-SS-PEG	+	-	↓	[101]
PEI-g-PEG	-	+	↓	[43, 86]
PEG-co-PEI	+	+	↓	[94]
PEG-g-PEI	-	+	↓	[57]
PEI-co-L-lactamide-co-succinamide	+	-	↓	[96]
PEI-co-N-(2-hydroxyethyl-ethylene imine)	-	-	↓	[20]
PEI-co-N-(2-hydroxypropyl) methacryl amide	-	+	n.r.	[112]
PEI-g-PCL-block-PEG	+	+	↓	[95]
PEI-SS-PHMPA	+	+	n.r.	[135]
PEI-g-Dextran 10000	-	+	↓	[113, 114]
PEI-g-transferrin-PEG	-	+	n.r.	[145]
Pluronic85®/ Pluronic123®-g-PEI	-	+	n.r.	[121, 123]

Table 7: Cytotoxicity, biodegradability and shielding capabilities of different PEI and PEI-Copolymers (co = Copolymer, g = grafted copolymer, block = diblock copolymer, SS = disulfide bond; n.r. = no results published)

The use of antibodies or their fragments to target tissues expressing specific receptors has led to similarly inconsistent results, although antibody-based mechanisms provide the most efficient, cell-specific targeting moieties. The coupling of a chimeric antiGD2 antibody to PEI resulted in rather homogenous polyplexes with sizes of approximately 50-100 nm, but did not reach the transfection efficiency of unmodified PEI [47].

Target structure	Receptor	Targeting moiety	Ref.
Dendritic cells	Mannose receptor	Mannose	[140, 141]
Hepatocytes/dendritic cells	Gal/GalNAc receptor	Galactose	[87, 138-141]
Tumor cells	Folate receptor	Folate	[142, 146]
Epithelial cells	Integrin receptor	RGD peptide	[88, 147]
Tumor cells	Transferrin receptor	Transferrin	[103, 145, 148, 149]
Tumor cells	EGF receptor	Epidermal growth factor	[104, 150]
Lymphocytes	CD3	Anti CD3 antibody	[144, 151]
Ovarian carcinoma cells	OA3	Anti OV-TL16 antibody fragment	[49]
Breast, ovarian cancer cells	Human epidermal growth factor receptor-2	Anti HER2 antibody	[143]
Lung endothelia	Platelet endothelial cell adhesion molecule	Anti PECAM antibody	[152]

Table 3: Active targeting strategies realized with PEI or PEI derivatives

The addition of antiCD3 antibody fragments was shown to enhance receptor mediated uptake in human peripheral blood mononuclear cells [151]. Recently, a detailed characterization of OV-TL16 fab fragment PEI conjugates showed that polyplex size (< 200 nm) and zeta potential (approx. +/-0 mV) of conjugates were comparable to PEG-PEI and, additionally, polyplex formation was only marginally hindered by antibody conjugation. The polyplexes showed a strongly enhanced transfection efficiency compared to PEG-PEI [49].

The coupling site of targeting moieties should play an additional role in effectiveness. It is assumed that coupling strategies which contain a linker between the polyplex core and the ligand [149] should provide a better accessibility of the grafted ligand to its receptor, as opposed to ligands directly coupled to the polyplex core. Only a few comparative studies have dealt with the question of site specific coupling of targeting

moieties to PEI-based vectors. However, the reported results point to a possible effect for small polyplexes [102].
Thus, effective active targeting seems to be possible in principle, but further work is necessary to define the optimal composition of the targeted vector systems.

Oligopeptides called protein transduction domains (PTD) have also stimulated increasing interest. PTDs are comprised of a fairly high number of basic amino acids. They are suggested to interact non-specifically with negative cell surface constituents, like glycosaminoglycans [153], due to arginine-rich motifs [154]. The common view that PTDs facilitate an endocytosis independent means of cell entry was recently challenged [155]. Lately, macropinocytosis, a specialized form of endocytosis that is independent of cavelae, clathrin and dynamin has been suggested to be one possible entry mechanism [156]. The mechanism of PTD translocation across the cell membrane, thus, remains to be determined. Nonetheless, this approach still represents a promising way to gain cell entry and possibly even a method for circumventing endosomal release problems. Recently, the application of PTDs in PEI based gene delivery was performed successfully. Oligomers of the HIV-1 TAT peptide were used to precompact plasmid DNA and were further complexed with common transfection agents, like PEI, resulting in a 3-fold higher transfection in nonproliferating cells compared to PEI transfection in proliferating cells [157]. The further enhancement of the natural PTDs sequences [158] may improve this approach.

Linking physicochemistry to biology

Detailed knowledge about physicochemical data can help to create gene delivery systems that overcome hurdles for the in vivo application of polyplexes. Along the delivery pathway for DNA, polyplexes are challenged by numerous biological barriers. To overcome these, several factors have to be taken into account, including (I) stability in the extracellular environment, (II) interaction with target cell surfaces and cell uptake, (III) release from endo/lysosomal vesicles and, finally, (IV) nuclear uptake, as well as vector unpacking (Table 4). For most of these barriers, the knowledge of the processes involved is still limited. The use of polyplexes based on PEI, however, seems to offer some advantages that might help overcome these hurdles.

Barrier	Strategies
(I) Extracellular stability	• Complexation with PEI homopolymer • Steric shielding by copolymerization • Crosslinking of polyplex surface
(II) Cell surface interaction and cell uptake	• Complexation with PEI homopolymer • Active/passive targeting • Protein Transduction Domains (?)
(III)Endo/lysosomal release	• Complexation with PEI homopolymer
(IV) Unpacking and nuclear uptake	• Physicochemical characteristics of PEI and PEI copolymers • Controlled degradation by environmental stimuli • Nuclear Localization Sequences

Table 4: Strategies to overcome systemic barriers with PEI based gene delivery

As discussed above, the complexation of nucleic acids with PEI provides enhanced stability against degradation in extracellular environment, improves cell uptake by electrostatic interactions of the polycation with the negatively charged cell surface, and promotes the endosomal release according to the proton sponge hypothesis.

Copolymerization leads to polyplexes that experience a decrease in interactions with blood components, due to a reduced surface charge and steric shielding capabilities, as well as an enhanced stability against shear stress through cross-linking of the polyplex surface. Depending on the copolymer, directed biodistribution could be achieved. However, PEI-copolymers often show reduced cell surface interaction, thereby limiting the cellular uptake. The effect of copolymerization on vesicular release remains to be further investigated. To what extent protection from degradation in the cytoplasm can be achieved with copolymers is still unclear. Indeed, the reduced complexation ability of most copolymers facilitates the unpacking of the vector. Since it is unclear where unpacking of the nucleic acid from the polyplex occurs, the release must be controlled by physical and/or chemical characteristics of the polymer or by environmentally controlled degradation.

Targeted polyplexes promote the biodistribution to tissues and cells of interest by specific cell surface interaction and cellular uptake, thereby compensating for the cell

uptake restrictions due to steric shielding. The attachment of targeting moieties only marginally affects the complexation ability of PEI or derivatives, and sometimes even additionally improves the polyplex stability in vivo. Again, intracellular processing is not affected by targeting moieties. However, the entry into the nucleus, which predominantly relies on nuclear envelope breakdown in dividing cells, may be stimulated by the presence of NLS [159, 160]. Finally, recent results have suggested that alternatives to endocytic uptake exist in the form of direct membrane transduction, an effect that, if feasible, would allow interesting ways to circumvent endosomal release hurdles.

Conclusion

In less than a decade, PEI has seen remarkable progress as a non-viral cationic polymer potentially useful for gene delivery [12], indeed, a successful trial in human bladder cancer therapy was recently reported [161]. In this review we attempted to highlight the advances in knowledge regarding the chemical and physicochemical properties of PEI/DNA polyplexes. This information is vital for further development of suitable non-viral vectors and may stimulate research into biological and hopefully clinical applications.

As outlined above, the ideal non-viral vector has not yet been found. Polyplexes between PEI and DNA rely on a delicate balance between DNA compaction (and thereby protection) and the necessary transport into the nucleus where DNA release must occur to achieve transfection [162]. The intracellular handling of polyplexes based upon PEI is an area where more mechanistic insight would be helpful. This could stimulate the design of improved vectors, which release DNA when triggered by environmental stimuli or via controlled degradation of the polymer.

Apart from loco-regional administration, the holy grail of gene delivery remains the development of systems with the capability of actively searching out the target tissue after systemic administration. This concept, devised by Paul Ehrlich almost a century ago, calls for gene delivery systems which are sufficiently long-lived and stable in circulation. While some progress has been made to this end using modified PEI derivatives, improvement of systemic stability is a critical issue. This includes control

over polyplex dissociation, aggregation, interaction with biomolecules and activation of the complement system. Despite significant advances, more investigations regarding the systemic stability of PEI polyplexes and their interaction with the body are needed before a clinical application can be considered. Also more detailed studies characterizing the acute and long term toxicity are required.

The problem of designing improved non-viral vectors is a challenging, multidisciplinary task, which requires knowledge from such diverse disciplines as polymer chemistry, biophysics, biochemistry, pharmaceutical sciences, biology, toxicology and medicine. It is hoped that this more chemically oriented account may serve to stimulate the interests of others to join the search for the “holy grail”.

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Polym.	Mw [kDa]	Buffer	pH	N/P	plasmid	Polyplex size [nm]	ζ-potential [mV]	Cell line	DNA [µg]	Rel. transf. eff.	Ref.
bPEI	25	10 mM Tris	7.4	6	pMB401	100	12	COS-7	-	21 [a)]	[26]
bPEI	25	10 mM Tris	7.4	6	pMB401	100	12	CHO-K1	-	0.6 [a)]	[26]
bPEI	25	150 mM NaCl	7.4	6.7	pGL3	156 ± 7	30,1 ± 3,4	3T3	4	1.1 [c)]	[56]
bPEI	25	150 mM NaCl	7.4	6.7	pGL3	156 ± 7	30,1 ± 3,4	COS-7	4	6 [c)]	[56]
bPEI	25	150 mM NaCl	7.4	6.7	pGL3	156 ± 7	30,1 ± 3,4	CHO	4	0,08 [c)]	[56]
bPEI	5.4	150 mM NaCl	7.4	67	pGL3	422 ± 131	34,9 ± 2,3	3T3	4	11 [c)]	[56]
bPEI	5.4	150 mM NaCl	7.4	67	pGL3	422 ± 131	34,9 ± 2,3	COS-7	4	10 [c)]	[56]
bPEI	5.4	150 mM NaCl	7.4	67	pGL3	422 ± 131	34,9 ± 2,3	CHO	4	12 [c)]	[56]
bPEI	25	150 mM NaCl	7.4	6.7	pGL3	156 ± 7	30.1 ± 3.4	MeWo	4	3 [c)]	[88]
bPEI	25	150 mM NaCl	7.4	6.7	pGL3	156 ± 7	30.1 ± 3.4	A549	4	2 [c)]	[88]
bPEI	25	150 mM NaCl	7.0	7	pCMV-Luc	180	23	Ovcar-3	4	4 [c)]	[49]
bPEI	25	150 mM NaCl	7.0	7	pCMV-Luc	180	23	Ovcar-3	0.5	0.04 [c)]	[49]
bPEI	25	150 mM NaCl	7.0	7	pCMV-Luc	180	23	NIH/3T3	4	10 [c)]	[49]
bPEI	25	150 mM NaCl	7.0	10	pCMV-Luc	100	20	NIH/3T3	4	15 [c)]	[49]
bPEI	25	20 mM Hepes, 5.2% gluc.	7.0	6	pEGFP-C1	109 ± 5	12.9 ± 0.2	-	-	-	[113]
bPEI	25	20 mM Hepes, 5.2% gluc.	7.0	9	pEGFP-C1	77 ± 21	16.4 ± 0.5	MDA-MB-231	2	15 [b)]	[113]
lPEI	25	20 mM Hepes, 5.2% gluc.	7.0	9	pEGFP-C1	456 ± 38	22.2 ± 4.5	-	-	-	[113]
lPEI	25	20 mM Hepes, 5.2% gluc.	7.0	9	pEGFP-C1	329 ± 137	22.1 ± 4.8	MDA-MB-231	2	8 [b)]	[113]
lPEI	25	10 mM Tris	7.4	6	pMB401	100	13	COS-7	-	5 [a)]	[26]

Table 5: Characterization of PEI polyplexes and comparison of their transfection efficiency in different cell types... The relative transfection efficiency is noted as a) ng Luc, b) GFP pos. cell %, c) ng Luc/mg protein. (bPEI/lPEI = branched/linear PEI)

Polymer	Mw PEI [kDa]	N/P	Cell line	Incubation time [h]	IC 50 [calculated for the etylenimine monomer]	Cell Viability [% of control]	Ref.
bPEI	5.4	-	L929	3/12/24	>1/0.17/0.08 mg/mL [a)]	-	[56]
bPEI	25	-	L929	3	<0.01 mg/mL [a)]	-	[56]
bPEI	600	-	L929	3/24	0.031/0.009 mg/mL [a)]	-	[218]
bPEI	25	10	COS-7	6	-	81 ± 10 [a)]	[31]
bPEI	2	20	COS-7	6	-	103 ± 3 [a)]	[31]
bPEI, permethylated	25	-	COS-7	6	-	105 ± 2 [a)]	[31]
bPEI, perethylated	25	-	COS-7	6	-	105 ±- 2 [a)]	[31]
bPEI-dodecyl	2	20	COS-7	6	-	101 ± 1 [a)]	[31]
bPEI	1.8	15	Jurkat	5	-	85 [b)]	[63]
bPEI	10	15	Jurkat	5	-	70 [b)]	[63]
bPEI	25	15	Jurkat	5	-	50 [b)]	[63]
bPEI-cholesteryl	1.8	15	Jurkat	5	-	90 [b)]	[63]
bPEI-cholesteryl	10	15	Jurkat	5	-	70 [b)]	[63]
bPEI-co-L-lactamide-co-succinamide	1.2	-	L929	3/12/24	>1/0.34/0.16 mg/mL [a)]	-	[96]
bPEI	25	-	PC3	24	0.28 mM amines [a]	-	[116]
bPEI-cyclodextrin	25	-	PC3	24	0.64-6.7 mM amines [a]	-	[116]

Table 6: Toxicity of different PEI or PEI-derivatives and their polyplexes (N/P ratio given) with pDNA in different cell types. Results have been obtained with MTT-based assays [a)] or with flow cytometry [b)]

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Chapter 2

Nanocarriers for DNA delivery to the lung based upon a TAT-derived peptide covalently coupled to PEG-PEI

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Summary

Gene therapy aimed at the respiratory epithelium holds therapeutic potential for diseases such as cystic fibrosis and lung cancer. Polyethylenimine (PEI) has been utilized for gene delivery to the airways. In this study, we describe a new modification of PEI, in which an oligopeptide related to the protein transduction domain of HIV-1 TAT was covalently coupled to 25 kDa PEI (PEI) through a heterobifunctional polyethylenglycol (PEG) spacer resulting in a TAT-PEG-PEI conjugate. Improved DNA reporter gene complexation and protection was observed for small (~ 90 nm) polyplexes as well as significantly improved stability against polyanions, Alveofact®, bronchial alveolar lining fluid and DNase I. To determine polyplex toxicity in vitro, MTT assays were performed and, for in vivo testing, the mice bronchial alveolar lavage was investigated for total cell counts, quantity of neutrophils, total protein and TNF-alpha concentration. All parameters suggest significantly lower toxicity for TAT-PEG-PEI. Transfection efficiencies of both PEI and TAT-PEG-PEI polyplexes with DNA were studied under in vitro conditions (A549) and in mice after intratracheal instillation. While luciferase expression in A 549 cells was much lower for TAT-PEG-PEI (0.2 ng/mg protein) than for PEI (2 ng/mg), significantly higher transfection efficiencies for TAT-PEG-PEI were detected in mice. Reporter gene expression was distributed through bronchial and alveolar tissue. Thus, TAT-PEG-PEI represents a new approach to non-viral gene carriers for lung therapy, comprising protection for plasmid DNA, low toxicity and significantly enhanced transfection efficiency under in vivo conditions.

Introduction

Intensive efforts have been devoted to pulmonary gene medicine trying to address diseases such as cystic fibrosis and lung cancer [1, 2]. Of crucial importance in this context are vectors or carrier systems for genetic information among which non-viral vectors based on polyethylenimine (PEI) have shown some promise due to their stability under in vivo conditions and transfection efficiency after nebulization [3]. PEG-grafted derivatives of PEI seem to be beneficial for gene therapy of the lung due to reduced interactions with proteins or cells and hence lower cytotoxicity [4, 5]. Polyplexes of DNA and branched 25 kDa PEI transfected preferably bronchial cells, a feature considered to be important in the treatment of cystic fibrosis and lung cancer [6, 7]. Normally, different sub-populations of lung cells are involved in pulmonary diseases. Thus, vectors transfecting both bronchial as well as to alveolar tissue would be of general interest.

To improve the transfection efficiency of non-viral carriers, new strategies were put forward to enhance cellular uptake of PEI based polyplexes, amongst which peptides enhancing cell adhesion and internalization reached a prominent position [8]. Peptide sequences, also designated as protein transduction domains (PTD) or membrane translocalization signals (MTS), were identified as potentially useful labels for intracellular delivery of proteins, oligonucleotides, plasmid DNA and colloidal carrier systems [9]. HIV-TAT oligopeptide sequences have been reported to show unusual translocation abilities by direct crossing biological membranes due to their strong cell surface adherence, independent from receptors and temperature [10]. Recently, however, several reports have challenged this view, suggesting a cell uptake via endocytic pathways for liposomal [11] and for cationic polymer based TAT conjugates [12]. Chloroquine influenced adsorptive endocytosis [13], as well as fluid-phase macropinocytosis [14], a receptor- and caveolar-/clathrin-independent specialized form of endocytosis, have recently been suggested as part of the translocation mechanism. In this context, cell fixation techniques need to be taken into account to avoid misinterpretation of cell uptake studies due to artifacts [15].

Apart from the uptake mechanism, sequence and the structure of the PTD have been subjects of intensive investigations. Basic amino acid sequences seem to play an

important role in translocation, as demonstrated for lysine [16, 17] and arginine [18] or guanidine rich peptides [19, 20]. Higher molecular weight peptide sequences consisting of multimers enhanced only the in vitro transfection of PEI/DNA polyplexes, but failed in increasing the in vivo expression in the mouse lung [21]. Recently, polymerization of HIV-TAT peptide resulted in high molecular weight polycations which displayed elevated DNA complexation and transfection efficiency in vitro [13].

Peptides derived from the TAT protein sequence of human immune deficiency virus type 1 have been utilized to deliver nanoparticles into cells. Particularly, TAT conjugates have been employed in cationic gene delivery vehicles, either with [12] or without [21, 22] covalent attachment to the carrier. Consequently, a TAT-derived peptide in combination with a PEG-PEI copolymer could be a promising candidate as gene delivery vehicle intended for pulmonary administration.

Here we report the synthesis and characterization of polymer conjugates in which a nonapeptide sequence derived from HIV-1 TAT modified by a C-terminal cysteine residue is covalently coupled to branched PEI (25 kDa) using a hetero-bifunctional linker based upon PEG. The aim of this study was to combine the favorable characteristics of PEG-grafted PEI 25 kDa with a TAT-related oligopeptide to yield a vector capable of mediating gene transfection after local administration to the airways. Furthermore, an ideal vector in the treatment of pulmonary diseases such as lung cancer would be able to mediate gene transfer to both bronchial and alveolar epithelial cell combined with lower toxicity than PEI. The PEG linker is intended to reduce unwanted interactions of the polyplex with protein, cell or other components of the airways, thereby providing enhanced polyplex stability and lowered toxicity. Coupling of the oligopeptide to the cationic PEI should help to overcome restrictions of pure oligopeptide based polyplexes, such as low plasmid complexation ability [23, 24] or the decrease of their potential biological effects due to interaction with the complexed DNA [25]. The PEG linker, additionally, provides a steric shielding of the cationic PEI core from the oligopeptide sequence and, thereby, facilitates the coupling reaction of the two cationic moieties. In the resulting conjugate, the TAT-derived sequence is separated from the PEI core by the hydrophilic PEG, thus enabling the presentation of the oligopeptide moieties on the surface of TAT-PEG-PEI/DNA polyplexes [26].

Experimental Section

Materials: The synthetic decapeptide sequence GRKKKRRQRC was synthesized by Bachem (Bubendorf, Switzerland), and α-vinyl sulfone-ω-N-hydroxysuccinimide ester poly(ethylene glycol) (NHS-PEG-VS) was purchased from Nektar Therapeutics (Huntsville, USA). Polyethylenimine 25 kDa (PEI) was a gift from BASF (Ludwigshafen, Germany). The plasmid pGL3 carrying luciferase coding region under the promoter control of cytomegalovirus (CMV) was kindly provided by J. Hänze (Department of Molecular Biology, University of Giessen, Germany) and was propagated in E. coli and purified by Plasmid Factory (Bielefeld, Germany). Herring testes DNA was obtained from Sigma (Seelze, Germany). The plasmid peGFP-N1 carrying green fluorescence coding region under the promoter control of cytomegalovirus (CMV-N1) was kindly provided by C. Rudolph (Department of Pediatrics, University of Munich, Germany), was propagated in E. coli and purified by Plasmid Factory (Bielefeld, Germany). The natural surfactant Alveofact® was purchased from Boehringer-Ingelheim (Germany). Bronchial alveolar lavage fluid (BALF) was freshly obtained form C57BL/6 mice via intra-tracheal instillation 1 hour prior to use. For removal of the cells, the BALF was centrifuged at 300 g at 4°C, and the pellet was discarded. All other reagents used were of analytical purity.

Conjugate synthesis:

Activation of PEI: 19.2 mg (5.65 μmol) of bifunctional PEG (3.4 kDa), containing both an α-vinyl sulfone and an ω-N-hydroxysuccinimide ester group, was weighed into a glass flask. 4.293 mL of a PEI solution (corresponding to 12.15 mg/0.486 μmol PEI; 282.4 μmol total amines) in 0.1M borate buffer at pH 5.5 were added and stirred. The activation reaction was carried out for 4 hours at room temperature, followed by pH adjustment to 7 with 1N sodium hydroxide and additionally incubated for 2 hours at room temperature.

Coupling of the oligopeptide onto activated PEG-PEI: For coupling, 2.98 mg (2.26 μmol) of the oligopeptide dissolved in 866 μL pure water were added to the activated PEG-PEI yielding a theoretical degree of substitution of approximately 1% based upon amine functions. The mixture was stirred for additional 2 hours at room temperature.

Purification of TAT-PEG-PEI: Removal of unreacted PEG and low molecular weight residues was performed with an ultrafiltration cell (Amicon, Bedford, USA) equipped with a 10 kDa molecular weight cut-off membrane (Millipore, Bedford, USA) and 0.1 M borate buffer at pH 7.5 as eluent.
Amine concentration of the conjugate was determined by a recently described copper assay [27].

Polyplex formation: The polyplexes consisting of plasmid DNA and either PEI or TAT-PEG-PEI, were prepared in sterile isotonic glucose solution at pH 7.4 as described recently [28]. Briefly, the polymer solution was added rapidly to the DNA and mixed by vigorous pipetting followed by 10-20 minutes incubation at room temperature prior to use. When various polymer nitrogen to DNA phosphate ratios (N/P) were investigated, the concentration of the PEI or TAT-PEG-PEI solution was adjusted to the amount of DNA (20 µg/mL polyplex solution for the in vitro experiments and 260 µg/mL for in vivo experiments) in order to maintain N/P ratios between 0.5 and 10.

Ethidium bromide exclusion assay: DNA condensation was measured by quenching of ethidium bromide fluorescence as described earlier [29]. Briefly, quadruplicates of 8 µg of herring testes DNA were complexed with increasing amounts of PEI or TAT-PEG-PEI in 96-well plates in 60 mM Tris buffer at pH 7.4. After 10 min incubation time, 20 µL ethidium bromide solution (0.1 mg/mL) were added. The fluorescence was measured on a Perkin Elmer LS 50 B fluorescence plate reader (Perkin Elmer, Rodgau, Germany) at λ_{ex} = 518 nm and λ_{em} = 605 nm. Results are given as relative fluorescence and the value of 100% is attributed to the fluorescence of DNA with ethidium bromide (rel. $F = F_{sample}/F_{DNA\ solution}$).

Physico-chemical properties: To study the physico-chemical properties of the polyplexes, investigations of particle charge, size and aggregation tendency were performed. The surface-charges were determined by measuring the zeta potential in the standard capillary electrophoresis cell of a Zetasizer 3000 HS (Malvern Instruments, Worchester, U.K.). The measurements were performed in isotonic glucose solution at pH 7.4 at 25 °C with automatic duration. The average values and the corresponding SD

were calculated in three independent measurements, five runs each. For dynamic light scattering, polyplexes were prepared in a total volume of 50 µL with glucose 5% or NaCl 150 mM at pH 7.5, respectively. Polyplex size measurements were carried out with a Nanosizer Lo-C from Malvern Instruments (Worchester, U.K.) at 25 °C (HeNe laser, 633 nm). For data analysis, the viscosity (0.8905 mPa*s) and refractive index (1.333) of pure water at 25 °C were used. Results are given as mean values of three runs of 120 sec duration each.

Polyplex stability in heparin, bronchial alveolar lavage fluid and Alveofact®: Increasing amounts of heparin in 10 µL pure water were added to 100 µL polyplex solution, yielding heparin concentrations of 0.1 to 2.0 IU per µg plasmid, and incubated for 10 minutes. 25 µL of this mixture was applied to a 1% agarose gel containing ethidium bromide. Gels were run for 60 minutes at 70 V, prior to the scanning with a transilluminometer (Biometra, Göttingen, Germany).

The effect of surfactant (Alveofact®) and bronchial alveolar lavage fluid (BALF) upon the polyplex stability was evaluated using a reverse fluorescence quenching [4, 30]. The fluorescence of ethidium bromide, a DNA intercalating agent, is enhanced upon binding to DNA and quenched when displaced by higher affinity compounds. For polyplex preparation, the amount of pGL3 was increased to 60 µg/mL and condensed with the appropriate amount of PEI and TAT-PEI respectively, to achieve an N/P ratio of eight. Hundred µL of the polyplex solution were placed in 96-well plate and 50 µL ethidium bromide (40 µg/mL) were added. Alveofact® was dissolved in isotonic glucose solution pH 7.4 and added to the polyplexes to achieve final concentrations of 0.002, 0.02, 0.2, 1.0, 2.0 µg/µL. When stability in BALF was tested 100 µL of freshly attained BALF instead of Alveofact® was added to the polyplexes. The fluorescence measurement (Fluorescence reader FL600, Micro plate Fluorescence BioTEK, Winooski, USA) was begun immediately and carried out over a period of 90 minutes at 37°C using λ_{ex} = 485 nm and λ_{em} = 590 nm. The fluorescence is reported relative to the value obtained with naked DNA and Ethidium bromide (= 100%), as mean ± SD of five measurements.

DNase I stability assay: Polyplexes were prepared at N/P 6 in glucose 5 % using 5 µg of pGL3 in a total volume of 25 µL, as previously described [31]. Aliquots of 5 µL corresponding to 1 µg of plasmid were incubated with 0.01, 0.1, 1, 2.5 and 5

international units (I.U.) of DNase I in 1 µL digestion buffer (0.1 M sodium acetate, 5 mM MgSO4 pH 7.4) for 15 minutes at 37 °C. To stop the DNase I digestion, 6 µL termination buffer (equal volumes of 0.5 M EDTA, 2 M NaOH and 0.5 M NaCl) were added, followed by 2 µL of a heparin solution containing 1000 I.U. per mL. To separate the polymer from DNA, the resulting mixtures were applied to a 1% agarose gel and electrophoresed at 70 V for 1 hour. The resulting gel was visualized and photographed with a transilluminometer (Biometra, Göttingen, Germany).

In vitro transfection: Human lung epithelial cell line (A549) was obtained from the German Collection of Microorganisms and Cell Cultures and was maintained according to the supplier's specifications. To evaluate the gene expression in A549, the transfection experiments were carried out as previously described in detail [28]. Briefly, cells in passages 8-15 were seeded at a density of 25,000 cells per well on 24 well cell culture plates, 24 h prior to the transfection experiments. The polyplexes were prepared as described above and 100 µL solutions were added to each well containing 900 µL fresh medium and incubated for 4 hours. The medium was replaced and the cells were allowed to grow for a further 44 hours. The luciferase expression (luciferase assay reagent Promega, Mannheim, Germany) and protein concentration (see below) were determined in the cell lysate. All experiments were performed in triplicate and data were expressed in ng luciferase per mg protein (± SD).

In vivo transfection: To study the luciferase expression in the mouse lung 150 µL polyplexes or pGL3 alone were instilled into the lung of C57BL/6 mice. The mice were purchased from Charles River Laboratories (Sulzfeld, Germany) aged 4-6 weeks, weighing 22-28 g. Prior to the treatment, the animals were hold for five days in the animal lab were they were fed regularly (Muskator GmbH, Düsseldorf, Germany). For intratracheal instillation, the mouse was lightly anesthetized with 0.06-0.08 µL mixture (1:1:3) of ketamine hydrochloride 100 mg/mL (Ketavet®, Pharmacia, Erlangen, Germany), Xylacine hydrochloride 2 % (Rompun®, Bayer, Leverkusen, Germany) and isotonic sodium chloride. Then the mouse was positioned in a vertical position and polyplexes were administered using a flexible needle (0.9 x 25 mm). Forty-eight hours after instillation the animal was sacrificed using an overdose of anesthesia. The mouse

lung was washed with isotonic sodium chloride by catheterizing the arteria pulmonalis, and then the lung was removed and weighed. Per one gram of lung tissue 2 µL of ice cold reporter lysis buffer (Promega, Mannheim, Germany) was added, the lung was immediately homogenized and frozen at -20°C. After thawing, the samples were centrifuged for 20 minutes at 4 °C and the luciferase assay was performed as described in the in vitro experiments. The transfection efficiencies and the corresponding SD were calculated as the mean value of 5 experiments and presented as pg luciferase per mg lung tissue. The use of animals in this study was approved by the local ethics committee for animal experimentation and the experiments were carried out according to the guidelines of the German law of protection of animal life.

Cytotoxic effects of the polymers on lung epithelial cells: To evaluate the toxicity of PEI and TAT-PEG-PEI, MTT assays were performed as previously reported [4, 32]. A549 cells were seeded in 96-well microtiter plates at a density of 4200 cells per well and allowed to grow for 72 hours prior to the application of the polymer solution (0.001, 0.01, 0.1, 0.5 and 1.0 mg/mL in cell media). After 4 hours of incubation, the medium was replaced with 200 µL fresh medium and 20 µL (3-(4,5-dimethylthiatol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT, Sigma, Seelze). After 4 hours, the unreacted dye was removed and 200 µL DMSO was added. The absorption was measured using the ELISA reader Titertek Plus MS 212 (ICN, Eschwege, Germany) at 570 nm, with a background correction at 690 nm. The relative cell viability (%) was related to control wells containing cell culture medium without polymer and was calculated by: absorption test x 100 % / absorption control. Data are presented as a mean of six measurements (± SD). The values of the polymers were fitted to a logistic sigmoidal function using Origin® 7.0 software (OriginLab Corporation, Northampton, MA) and IC_{50} was calculated.

Polyplex toxicity in the mouse lung: Toxic effects of the polyplexes at an N/P ratio of 10 and of the naked DNA pGL3 in the mouse airways were studied based upon the experiments of Gautam et al. [33, 34]. Controls were performed using isotonic glucose solution at pH 7.4. Animals were treated as described for in vivo transfection. Twenty-four hours post-administration mice were sacrificed and lavages of the bronchial

alveolar lining fluid (BALF) were performed with ice cold phosphate buffered saline (PBS) pH 7.4 containing 5 mM EDTA according to the following protocol: Lungs were lavaged successively with 300 µL, 300 µL, 400 µL, and 8 x 500 µL PBS to achieve a total volume of 5.0 mL. The first three lavages were pooled separately and centrifuged at 300g. The supernatant was collected and the cell pellet resuspended with cell medium. The remaining 4 mL were also centrifuged at 300g, the supernatant discarded, the cell pellet resuspended in cell medium, and added to the cells from the first three lavages. Total cells in the BALF were counted in a Neubauer hemacytometer. Polymorph-nuclear leucocytes (PMN) recruitment was assessed by preparing centrifuge smears and staining them with DiffQuick® (Dade, Munich, Germany) according to the manufacturer's protocol. The number of PMN in the BALF was expressed at the percentage of the total number of cells. A quantification of total proteins in the BALF supernatant (1 mL) was performed based on the Bradford method using a standard BCA Assay Kit from Bio-Rad Laboratories (Munich, Germany) according to the manufacturer's protocol. Protein concentrations were calculated from a standard curve made from bovine serum albumin (BSA) in concentrations ranging from 0.2 to 2.0 mg/mL. TNF-alpha quantification was performed using mouse-specific immunoassay kits for TNF-alpha (R&D systems Inc., Minneapolis, USA) according to the manufacturer's protocol. The values of the toxicity studies were calculated as mean ± SD of three experiments.

Distributions of the polyplexes in the mouse lung: Labeled DNA (pGL3-Alexa Fluor®) was prepared as described in the manufacturer's protocol for ARES® DNA labeling kit (Molecular probes, Leiden, the Netherlands). Briefly, the amine-modified dUTP was incorporated in the pGL3 plasmid strand by a Nick translation, followed by the addition of the amine-reactive dye Alexa Fluor®. Unreacted enzyme and dye were removed from the plasmid DNA using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). TAT-PEG-PEI was labeled using Oregon Green® 488 carboxyl acid (Molecular Probes, Leiden, the Netherlands) and labeling procedure was carried out as described in the manufacturer‘s protocol.

To evaluate polyplex distribution in the mouse lung, polyplexes at N/P ratio of 10 were formed between pGL3-Alexa Fluor® and TAT-PEG-PEI-Oregon Green® and applied

to the mouse lung as described above. Four hours post-administration the mouse was sacrificed, and then perfused with PBS and 4% paraformaldehyde (PFA) in PBS via the arteria pulmonalis. The lung was removed and fixation in 4% PFA was performed for 1 hour. Lung immersion in PBS at 4°C was performed overnight prior to embedding in Tissue-Tek® (Sakura Finetek, Zoeterwoude, the Netherlands) and immediately freezing in liquid nitrogen. Cryosections (50 µm) of the mouse lung were cut and embedded in FluorSave® (Merck Biosciences, Darmstadt, Germany). The distribution of the double labeled polyplexes was examined under a confocal laser scanning microscope (Axiovert™, Zeiss CLSM 501, Jena, Germany) equipped with a Zeiss Neofluor 63x/1.2 water immersion objective. Simultaneous scans for Alexa Fluor® and Oregon Green® were carried out with an excitation wavelengths of 543 nm (by pass filter: 560-615 nm) and 488 nm (by pass filter: 505-550 nm and transmission light) respectively. A gallery of optical slices (0.4 µm) was collected and xz, yz composites were processed using Zeiss LSM 510™ software.

To localize the expression of the transgene green fluorescence protein (GFP) in the mouse lung, TAT-PEG-PEI polyplexes (N/P 10) were prepared using GFP-plasmid instead of pGL3 and administered to the mouse lung as described above. Forty-eight hours after application the mice were sacrificed and their lungs were treated in the same procedure as described for the distribution studies of double labeled polyplexes. The localization of the GFP was also examined under the confocal laser scanning microscope as described above. Scans for the GFP was carried out with an excitation wavelengths 488 nm (by pass filter: 505-550 nm) and 1 % transmission light to visualize the cell membranes and nuclei. A gallery of optical slices (0.4 µm) was collected and xz, yz composites were processed using Zeiss LSM 510™ software

The distribution studies of both the double labeled polyplexes and the GFP were repeated twice to ensure reproducibility. Controls were performed with unlabeled pGL3 and TAT-PEG-PEI at an N/P ratio of 10.

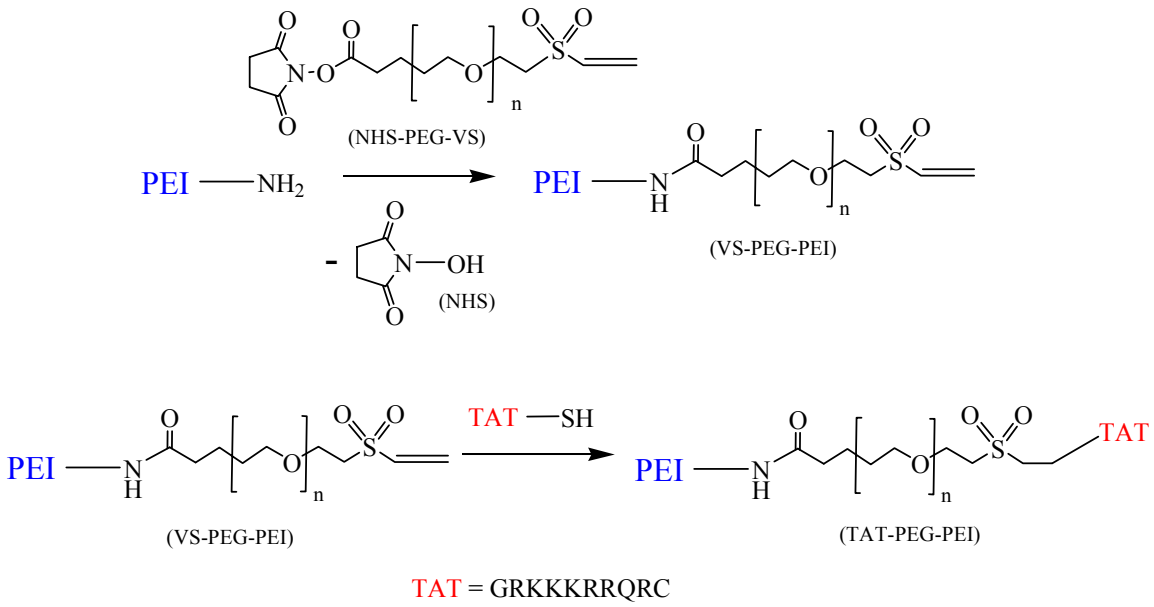
Statistical analysis: Statistical calculations were carried out using the software package GraphPad InStat v3.06 (GraphPad Software, Inc. San Diego, CA, USA). To identify statistically significant differences one-way ANOVA with Bonferroni's post test

analysis was performed. Differences were considered significant if $p < 0.05$ (*) and marked accordingly in the figures.

Results and Discussion

TAT-PEG-PEI conjugate synthesis: The TAT-PEG-PEI conjugate is composed of a 25 kDa PEI core, a 3.4 kDa PEG linker and an oligopeptide sequence. The two-step reaction was performed as shown in Scheme 1. Bifunctional NHS-PEG-VS was used to activate PEI via the primary amine reactive ω-N-hydroxysuccinimide ester moiety at pH 5.5, thus avoiding the conjugation and cross-linking of the α-vinyl sulfone groups to the amine functions of PEI, which occurs at higher pH [35]. ^{1}H-NMR was used to confirm that the vinyl sulfone protons remained intact (data not shown). Additionally, the competing hydrolysis reaction of the N-hydroxysuccinimide ester is suppressed at acidic pH [36]. The reaction was carried out for 4 h at pH 5.5. After adjusting the pH to 7, the solution was stirred for additional 2 hours to allow hydrolysis of eventually unreacted reagent.

The vinyl sulfone group has been reported to selectively react with sulfhydryl groups at neutral pH [37]. Thus, after activation of the PEI core, the oligopeptide containing a cysteine moiety at the C terminus was coupled to the α-vinyl sulfone group at pH 7. The composition of the conjugates was calculated by assuming that NHS-PEG-VS completely coupled to PEI (containing 581 amine functions) and that TAT-oligopeptide coupled to 80% of the PEG blocks (only 80% of PEG blocks of NHS-PEG-VS were substituted with vinyl sulfone as indicated by the manufacturer). The degree of PEG substitution was calculated to be 2.0% for PEG and 0.8% for TAT of all amines present in PEI.



Scheme 1: Reaction scheme for the activation of PEI with PEG vinyl sulfone followed by the addition of the oligopeptide.

Ethidium bromide exclusion assay: The quenching of ethidium bromide fluorescence was used to compare the DNA condensation ability of TAT-PEG-PEI versus PEI. While free ethidium bromide shows only weak fluorescence, its fluorescence increases strongly when intercalated into DNA [29]. As shown in Fig. 1, TAT-PEG-PEI displays an enhanced DNA condensation ability compared to the pure PEI, which becomes significant better from N/P 3. The stronger DNA condensation ability may be attributed to the enhanced cationic charge density of the conjugate due to the cationic amino acids, which exceeds the loss of positive charges due to the coupling. Additionally, these results indicate that there is no significant steric hindrance from the PEG shielding, as it was observed for similar peptide-PEG-PEI conjugates [38]. Taken together, it can be assumed that the PEI core as well as the oligopeptide plays a role in DNA compaction.

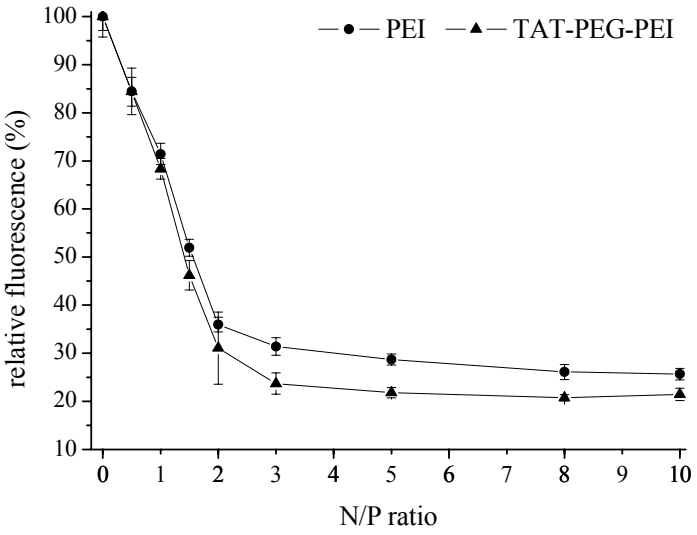


Figure 1: Ethidium bromide fluorescence quenching assay of the PEI polyplexes and TAT-PEG-PEI polyplexes respectively, formed with salmon testes DNA. Values are given as mean ± SD (n = 4).

Physico-chemical properties: To determine the surface charge of the polyplexes their corresponding zeta potentials were measured using laser Doppler anemometry. Whilst naked DNA had a highly negative zeta potential of -33 mV, the polyplexes displayed positive zeta potentials due to an excess of the polycation. For TAT-PEG-PEI polyplexes at an N/P of ratio 8 a zeta potential of 15 ± 3 mV was observed, which increased to 20 ± 3 mV at an N/P ratio 10. However, it was significantly lower than the zeta potential observed for PEI polyplexes, which displayed a potential of 32 mV for both N/P ratio 8 and 10. Investigations of TAT peptide oligomers and monomers, respectively, had shown that the complexation between TAT and plasmid DNA leads to the formation of highly positive charged (~ 30-40 mV) particles [13, 21]. These observations suggest that the reduced positive zeta potentials observed for the TAT-PEG-PEI/DNA polyplexes was neither caused by PEI or TAT alone. It is likely that both PEI and TAT are involved in the DNA condensation process and, thus, PEI as well as TAT and PEG might appear at the particle surface. Therefore, we assume that the PEG spacer in the TAT-PEG-PEI molecule contributed to surface shielding as it was described before for PEG-PEI copolymers [39, 40].

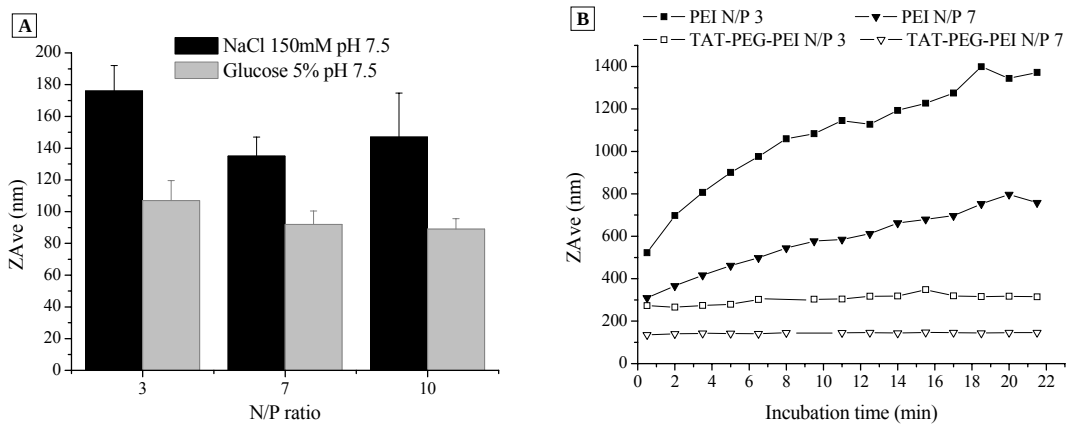


Figure 2: Particle size: A) Hydrodynamic diameters of TAT-PEG-PEI/pGL3 polyplexes in different media. B) Comparison of TAT-PEG-PEI polyplexes and PEI polyplexes in their tendency to form aggregates in high ionic strength medium (NaCl 150 mM at pH 7.5).

The size of the resulting polyplexes between TAT-PEG-PEI and plasmid DNA is shown in Fig. 2. Depending on the preparation medium and its ionic strength, the polyplex sizes ranged from 135 to 176 nm in 150 mM sodium chloride and from 89 to 107 nm in glucose 5% at pH 7.4. A decrease in the particle size was observed for TAT-PEG-PEI/DNA in glucose solution when the N/P ratio increased from 3 to 10, most likely due to the enhanced condensation of DNA. In 150 mM sodium chloride solution, the dependency of the size from the N/P ratio was less pronounced. Generally, polyplexes prepared at N/P ratio of 3 displayed the largest size, and the polydispersity index pointed to some aggregation (data not shown).

Generally, polyplexes prepared at low N/P ratios with neutral surface charges tend to aggregate. Additionally, also polyplexes prepared with excess of polymer, thus leading to particles with a net positive surface charges, display enhanced aggregation tendency in high ionic strength media [41]. To overcome both problems, a hydrophilic shielding component, such as PEG, is commonly introduced into the polyplexes to reduce aggregation [42]. It was recently shown that the PEG chains in PEG-PEI copolymers must have a minimum molecular weight of about 5 kDa to prevent polyplex aggregation [43]. In contrast, in the case of TAT-PEG-PEI, a 3.4 kDa PEG chain seems to be enough for efficient aggregation prohibition, presumably due to the unique structure of the conjugate having two cationic moieties linked together with a PEG chain. As

depicted in Fig. 2B, the TAT-PEG-PEI polyplexes prepared in high ionic strength medium (150 mM NaCl) remained stable over a period of 20 minutes, whereas PEI polyplexes prepared as a control showed a drastic increase in size due to aggregation (as confirmed by polydispersity index, data not shown).

Zeta potential and size of the polyplexes have been reported to be important factors in facilitating gene transfer in cells [44, 45]. Endocytic uptake of particles increases with increasing zeta potential, but also enhances toxic side effects due to unspecific interactions between the gene delivery systems and cell membranes. Therefore, efforts have been undertaken to shield the highly positive particle surface of PEI polyplexes to produce gene carriers with lower cytotoxicity [39, 46]. The reduced zeta potential and the absence of aggregate formation of TAT-PEG-PEI polyplexes indicate a certain particle surface shielding, providing an enhanced stability and lower cytotoxicity. Beside the complications of gene delivery to the lung cells, such as mucociliary clearance and phagocytosis by alveolar macrophages, endocytic uptake of particles is clearly affected by their size and increases with decreasing diameters [47, 48]. The TAT-PEG-PEI polyplexes obtained here were small and stable at numerous N/P ratios in several media. Therefore, prevention of mucociliary and macrophage clearance can be assumed and endocytic uptake by the epithelial cells is pronounced.

Polyplex stability: To investigate whether the enhanced condensation ability of TAT-PEG-PEI also enhances the protection of the complexed DNA against polyanion exchange and enzymatic degradation in the lung environment, the polyplexes stability were studied in the presence of heparin, Alveofact®, BALF and DNase I (Fig. 3).

The polyplexes (N/P 8) were challenged with increasing amounts of the model polyanion heparin (Fig. 3A). PEI was able to protect plasmid DNA against heparin exchange up to 0.2 IU heparin. An amount of 0.5 IU heparin resulted in a release of the DNA from the PEI polyplexes. In contrast, TAT-PEG-PEI is able to complex plasmid DNA up to 0.5 IU with only minor release and 1.0 IU heparin was necessary to completely release the DNA from these polyplexes, indicating enhanced DNA condensation ability.

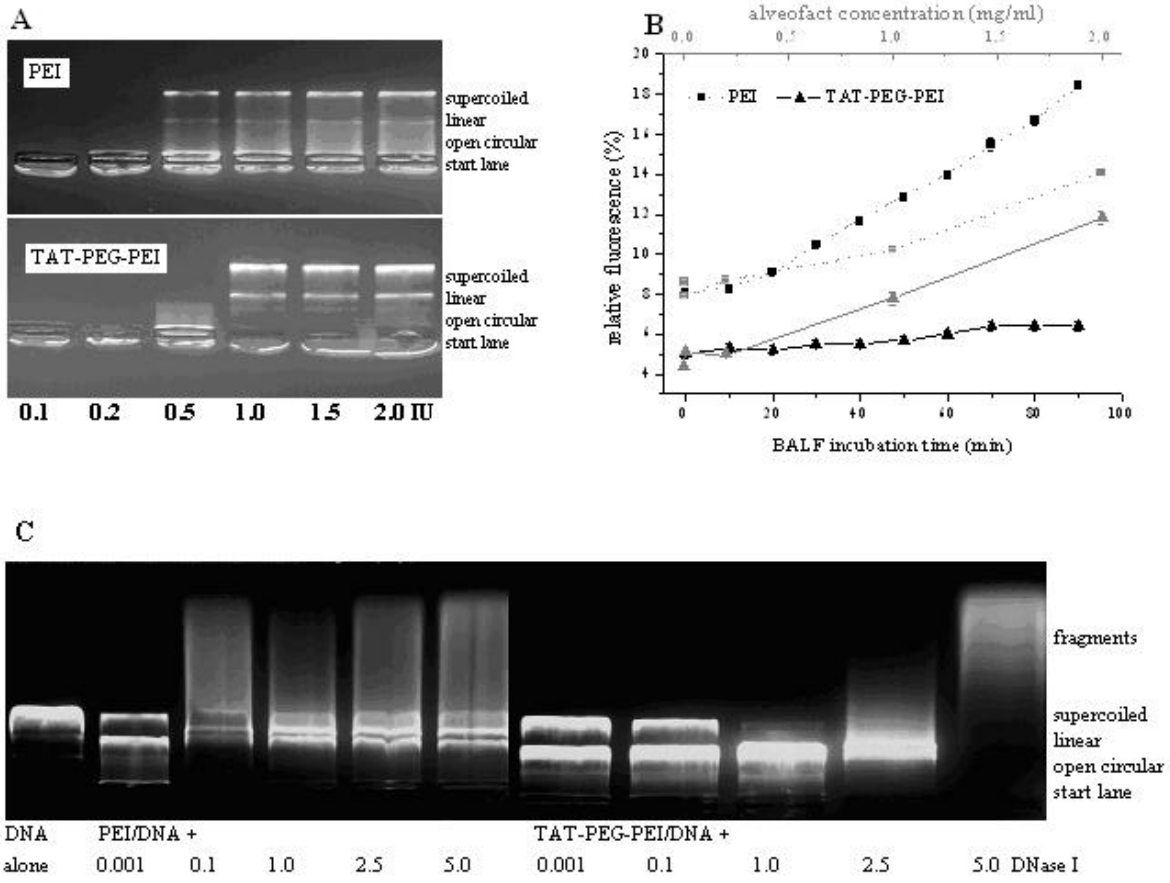


Figure 3: Polyplex stability at an N/P ratio of 8: A) PEI and TAT-PEG-PEI polyplexes challenged with increasing amounts of heparin (0.1, 0.2, 0.5, 1, 1.5, 2, IU heparin per 1 µg pGL3). B) Polyplexes were treated with increasing amounts of Alveofact® (grey scale) and BALF (black scale). Mean values ± SD were determined in three independent measurements and depicted as percentage of maximum fluorescence (pGL3 alone). C) Polyplexes (lane 2-6: PEI polyplexes and 7-11: TAT-PEG-PEI) were challenged with increasing amounts of DNase I (0.01, 0.1, 1, 2.5 and 5 IU per 1 µg pGL3). Lane 1 shows free, undigested pGL3.

Stability studies using Alveofact® and BALF were carried out with a reverse ethidium bromide exclusion assay [4] (Fig. 3B). In the absence of Alveofact® and BALF both polycations were able to condense DNA up to a residual fluorescence of approximately 8 % (PEI) and 5 % (TAT-PEG-PEI), respectively. Addition of Alveofact® increases the residual fluorescence for both polyplexes in a concentration dependent manner. PEI/DNA displayed a lower stability, with a relative fluorescence under these conditions of 10.2 % (1.0 µg/µL) and 14.1 % (2 µg/µL). The fluorescence of the TAT-

PEG-PEI polyplexes also increased (11.8 % at 2 µg Alveofact®/µL), however it was significantly lower than that of PEI polyplexes at all investigated Alveofact® concentrations. The kinetics of DNA release from the PEI polyplexes in BALF increased in a linear manner from 8 % (0 min) up to 18.5 % relative fluorescence (90 min), while DNA liberation from the TAT-PEG-PEI polyplexes was negligible (1.4 % increase over the 90 min observation). Consequently, protection of DNA against extracellular pulmonary enzymes and proteolipids was more pronounced for TAT-PEG-PEI compared to PEI.

Representative images of the DNase I digestion assay are shown in Fig. 3C. When TAT-PEG-PEI polyplexes were incubated with increasing concentrations of DNase I over a period of 15 minutes, DNA digestion occurred at a concentration of 2.5 IU DNase I per 1 µg DNA. Further increase of DNase I concentration up to 5 IU led to the complete degradation of plasmid DNA. In the case of PEI polyplexes, DNA degradation was observed at a significantly lower enzyme concentration (0.1 IU DNase I per 1 µg DNA). The denser structure of the TAT-PEG-PEI polyplexes could explain these differences. The DNA seems to be complexed more loosely in PEI polyplexes and hence DNase I partially degraded the plasmid over a vast range of enzyme concentrations, whereas TAT-PEG-PEI provides a complete protection up to more than 1 IU DNase per 1 µg DNA.

Apart from protection of DNA against intracellular enzymes (e.g. in endo-/lysosomes), also a sufficiently high stability of gene delivery systems in the extracellular environment of the lung seems to be essential for an efficient pulmonary gene delivery [1, 49]. Compared to PEI, the stability of TAT-PEG-PEI polyplexes was significantly higher in the presence of high concentration of heparin, Alveofact®, BALF as well as DNase I. This improved stability profile of TAT-PEG-PEI/DNA against anion exchange is indicative of increased complexation and condensation properties of TAT-PEG-PEI for plasmid DNA. Similarly, Soundara Manickam et al. demonstrated also an improved DNA condensation utilizing a polymerized TAT peptide in comparison to TAT monomers [13]. PEGylation of PEI has been shown to prevent interactions between the polyplexes and serum cells, proteins or enzymes [4, 43]. The modification of PEI with TAT-PEG may have contributed to the reduced zeta potential and increased polyplex stability by steric shielding through mobile PEG chains.

Transfection efficiency: Plasmid DNA was complexed with PEI or TAT-PEG-PEI at N/P ratios of 8 and 10 to examine the transfection efficiency in vitro (Fig. 4A) and in the mouse lung (Fig. 4B). While the transfection efficiency of TAT-PEG-PEI polyplexes in the lung epithelial cells A549 was shown to range far below that of PEI, an efficient gene expression in the mouse lung was achieved after administration by intratracheal instillation. At N/P ratio of 10, a significant improvement in gene expression mediated by the TAT-PEG-PEI carrier (12.6 pg luciferase/mg lung tissue) was observed in comparison to PEI (2 pg luciferase/mg lung tissue).

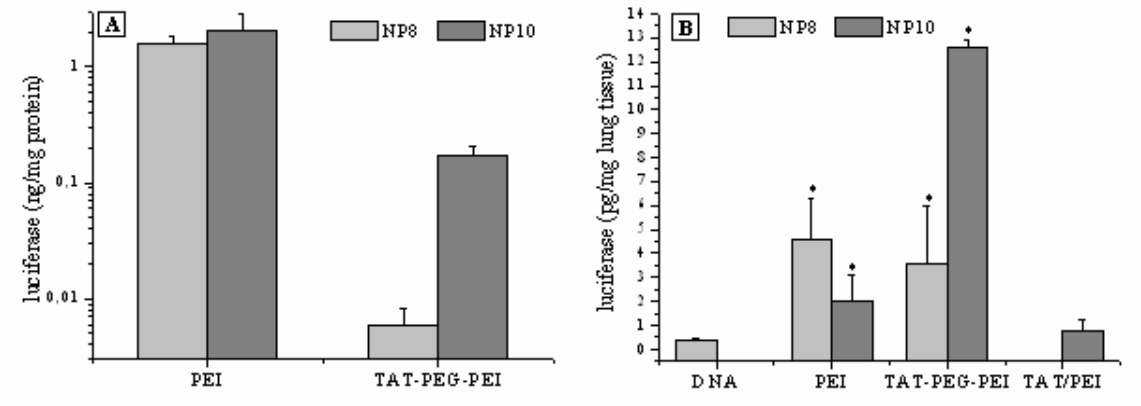


Figure 4: Transfection efficiency of the polyplexes at N/P ratio 8 and 10: A) luciferase expression in A549, B) luciferase expression in C57BL/6 mice lung. The in vivo results are provided as mean ± SD of five experiments and statistically significant differences from DNA are denoted with an asterisk ($P \leq 0.05$).

Since efficient gene transfer of non-covalently coupled TAT peptides under in vitro conditions was demonstrated in the literature [13, 21], we investigated whether the TAT derived oligopeptide also mediates efficient transfection in the mouse lung under in vivo conditions. Therefore, polyplexes between the oligopeptide and plasmid DNA were prepared and administered under the same conditions as the other polyplexes. The transfection efficiency in the mouse lung was negligible and ranged at background level (data not shown). This observation suggests that the peptide alone is not sufficient to mediate transfection of lung cells under in vivo conditions. Numerous other studies also failed to support gene transfer by TAT peptides (11- and 17 amino acid respectively) alone. Reduced stability of polyplexes and lower electrostatic interactions between the

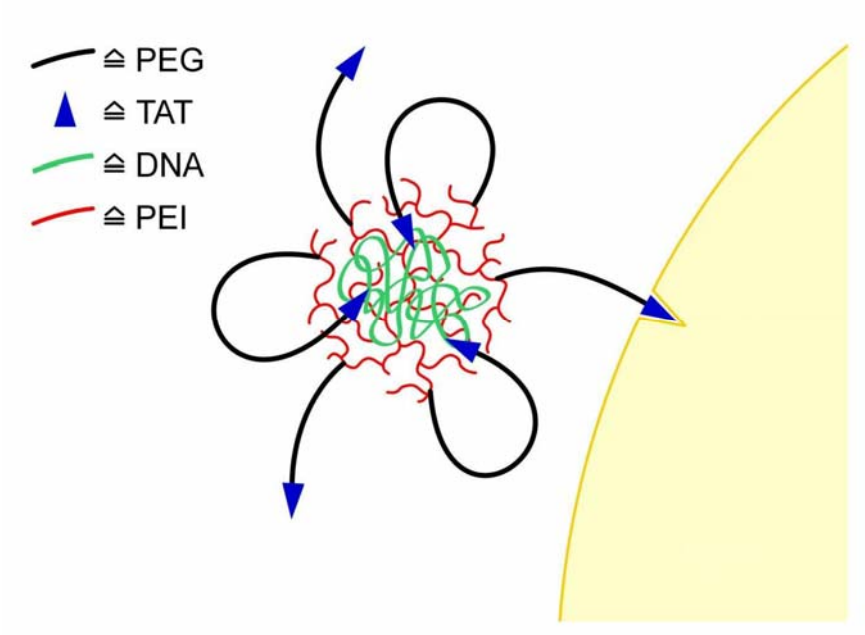
TAT peptide and DNA were put forward as explanation [22, 50]. To overcome these problems, Soundara Manickam et al. used high molecular weight polymerisates of TAT to complex plasmids [13].

To probe whether the covalent linkage between the translocating moiety and PEI is essential for improved transfection efficiency of our conjugate in the mouse lung, we prepared polyplexes between DNA and the oligopeptide followed by the addition of PEI (N/P 10), as described by Rudolph et al. [21]. We did not detect an improved transfection rate for TAT/PEI polyplexes (0.75 pg luciferase/mg lung tissue) compared with PEI polyplexes (2 pg luciferase/mg lung tissue), indicating that the PEG linker in the TAT-PEG-PEI conjugate is required to facilitate gene transfer in the mouse lung. This result is in line with the observations by Torchilin et al. who studied the transfection and cell uptake of liposomes that were covalent liked to TAT peptide and postulated that the peptide increases the cell membrane permeability only if it is covalently attached to the liposome membrane and accessible for cell surface interaction [51]. In contrast to our results, Rudolph et al. demonstrated that covalent linkage is not necessary to improve the efficiency of a TAT vector [21]. They obtained a 390-fold transfection rate increase when only a mixture of PEI with a TAT dimer was studied in vitro; however, no significant improvement could be detected in vivo. The different TAT amino acid sequences and structures used in our (GRKKKRRQRC) and Rudolph's study (dimeric $C(YGRKKRRQRRRG)_2$) might explain these conflicting results.

The in vivo transfection results obtained with TAT-PEG-PEI polyplexes demonstrated unexpected differences of a ca. 600% higher transfection efficiency compared to PEI which is not reflected in the in vitro experiments. Similar discrepancies were reported before for both TAT-DNA and chitosan-DNA polyplexes [50, 52]. The success or failure of a gene delivery system to mediate gene expression in the lung may be related to cell membrane specific differences and the extracellular environment. Thus, the multidimensional tissue structure of the lung and the extracellular protein/enzyme network reveal difficulties in extrapolation of in vitro results to successful gene delivery into the complex lung systems [53]. Furthermore, aggregated particles have been reported to increase transfection efficiency in cell culture, simply by sedimentation onto the cell surface [54]. As shown above, PEI polyplexes formed aggregates in sodium chloride and these large aggregates easily sediment on the surface of cells in culture

resulting in improved cell uptake, whereas much less of the small and stable TAT-PEG-PEI polyplexes reached the cell surfaces at the bottom of the well plates, resulting in lower in vitro gene expression compared to PEI.

A significant increase in transfection efficiency was observed for TAT-PEG-PEI polyplexes in comparison to TAT/PEI polyplexes under in vivo conditions, suggesting that covalent coupling of TAT to PEI via PEG led to significant advantages in mediating efficient gene delivery into lung epithelial cells of living mice. Recent studies also reported that in both, polyplexes and liposomes, consisting of plasmid DNA and TAT, the peptide needs to be non-shielded and freely accessible for cell membrane interactions [13, 22, 55]. It is tempting to speculate that the PEG spacer between the oligopeptide and PEI provides both, shielding of the highly cationic PEI surface charge and free, un-hindered interaction for the PTD with the cell membrane. The high gene expression in vivo and the enhanced DNA condensation as well as the protection by TAT-PEG-PEI lead us to the conclusion that the TAT derived peptide was involved in both, the DNA compaction process and the cell uptake. A schematic representation of the gene delivery system reflecting our results is depicted in Scheme 2.



Scheme 2: Schematic pattern of the interactions between TAT-PEG-PEI and plasmid DNA as well as cell membranes.

Toxicity: The metabolic and mitochondrial activity of polymer treated cells was determined using a colorimetric MTT assay [56]. As illustrated in Fig. 5A, the toxicity of the polymers was concentration dependent. Whilst PEI reduced the cell viability dramatically at concentrations of 0.1 mg/mL (~ 23 % viability), TAT-PEG-PEI showed less influence upon the cells (~ 70 % viability). Considering the IC_{50} values (PEI: 0.071 mg/mL, TAT-PEG-PEI: 0.2 mg/mL), the reduction in metabolic activity is far lower with TAT-PEG-PEI compared to PEI. Less toxic effects were also reported for both monomer and polymerized TAT peptides in comparison to PEI polyplexes [13]. In general, in vitro studies of PTDs have demonstrated their non-toxicity in numerous cell lines [57]. Furthermore, modification of PEI with PEG led to a reduction of toxic side effects in vitro as well as in vivo [39, 46, 49]. Hence, we assume that the modified TAT peptide as well as the PEG shielding together are responsible for the significantly reduced cytotoxicity when compared to the unshielded PEI at concentrations of 0.1 to 0.5 mg/mL.

The total numbers of cells in the BALF, as well as polymorph-nuclear leucocytes (PMN), are two reliable hallmarks of lung inflammation [34, 58]. Whereas the cell counts (Fig. 5B) of PEI polyplexes treated lungs was significantly higher than that of both DNA and glucose control, the cell counts increase of TAT-PEG-PEI polyplexes was less pronounced. Significant differences in the cell counts of TAT-PEG-PEI/DNA compared to DNA and glucose or to PEI/DNA could not be detected.

PMN (Fig 5B) were not observed after the application of glucose control, indicating that the isotonic solution did not provoke neutrophil infiltration into the lung and, thus, no inflammatory processes were activated. DNA and both polyplex formulations caused an increased PMN recruitment indicating a slight inflammation in the mouse lung. Whereas PEI polyplexes displayed a significantly higher PMN recruitment (66 ± 15 % of the entire cell population) compared to plasmid DNA (33 ± 11%), TAT-PEG-PEI polyplexes (47 ± 17 %) did not increase the PMN proportion significantly.

The total protein concentration (Figure 5C) in BALF characterizes general alterations of the natural composition of the lining fluid and detects enzymes, cytokines as well as other proteins. Thus, significant increases in the protein concentrations do not necessarily suggest an inflammation reaction. These results have to be considered in conjunction with PMN and cell counts. The amount of protein measured in the BALF

generally mirrored the trends observed for cell counts, although the differences between polyplexes and both DNA and glucose were more pronounced. Significant increased levels of protein were observed in the BALF for both PEI and TAT-PEG-PEI polyplexes when compared with DNA and glucose control. However, the values obtained for total cell counts and PMN indicated that the acute inflammatory reaction caused by TAT-PEG-PEI/DNA is not significantly different from DNA, in contrast to PEI/DNA. Therefore we assume that the high protein level of TAT-PEG-PEI/DNA might be the result of other mediators, such as interleukins, which were not measured here.

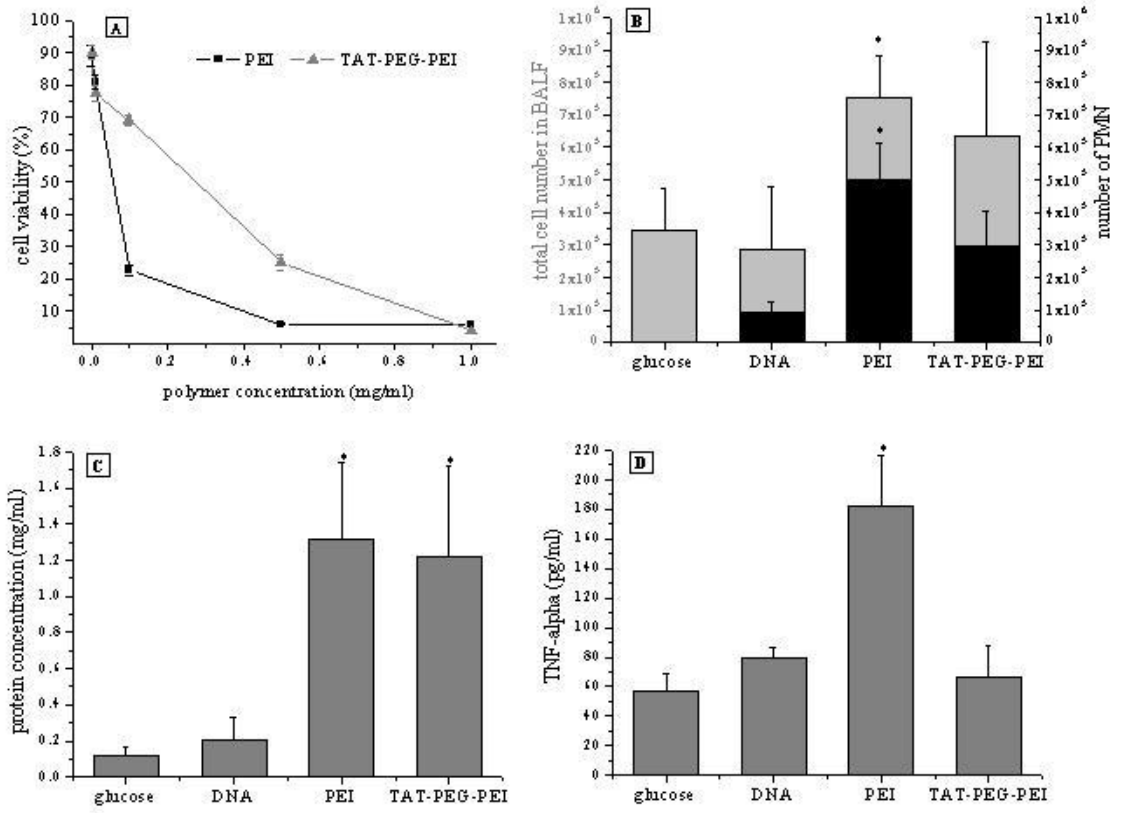


Figure 5: Toxicity studies: A) Cytotoxic effects of the polymers at concentrations of 0.001, 0.01, 0.1, 0.5, and 1.0 mg/mL on A549 cells were determined by MTT assay and presented as the relative cell viability (mean ± SD of six determinations). Pulmonary inflammation indicators (mean ± SD of three experiments) in the BALF 24 hour post-application of polyplexes (N/P 10) in the mouse lung: B) Total cell counts (grey scale) and number of PMN (black scale), C) Total protein concentration, D) TNF-alpha concentration.

Cytokine response to aerosol delivery of PEI polyplexes has also been documented [33]. Hence, our aim was to investigate whether TAT-PEG-PEI polyplexes delivered to the mouse lung also led to a tumor necrosis factor alpha (TNF-α) response, as mediator of an acute inflammatory reaction. Whilst the TNF-α level (Fig. 5D) was significantly increased after administration of PEI polyplexes, TAT-PEG-PEI polyplexes did not cause a significant increase in TNF-α.

Taken together, when TAT-PEG-PEI was administered in the mouse lung the toxicity data indicate inflammatory reactions almost at a level between naked DNA and PEI. Plasmid DNA itself, in particular under the promoter of CMV, has been reported to cause toxic side effects in numerous cells [59]. The inflammatory mediators and indicators studied here were significantly higher for PEI when compared to DNA and the glucose control. In contrast, TAT-PEG-PEI polyplexes did not cause significantly increased inflammation indicators and, in particular, only low levels of the mediator TNF-α was detected . Therefore, TAT-PEG-PEI represents a more suitable gene carrier and an improvement to the common used PEI in respect of toxicity in the lung. These in vivo results as well as the in vitro results are in line with previous reports, where low cytotoxicity for TAT peptides and TAT modified liposomes was reported [13, 51].

Distribution of the polyplexes in the mouse lung: The final aim of our study was to localize the polyplex distribution 4 hours after administration and the site of gene expression 48 hours post-administration. In this attempt, double labeled TAT-PEG-PEI/plasmid polyplexes were applied to the mouse lung and the polyplex location in the lung was evaluated. Well aware of the problems involved in cell fixation causing artifacts which have been reported to influence the cell entry of HIV-1 TAT derived conjugates [15], we used cryosections obtained by paraformaldehyde fixation via the endothelial route. Representative confocal laser scanning microscopy images are shown in Fig. 6A and B. The plasmid DNA (red) and the polymer (green) were mainly co-localized, seen as yellow overlapping. The double labeled polyplexes were localized in the bronchial epithelia cells, since those are the first cells to come to pass on the way in the deeper respiratory tract (Fig. 6A), More interesting, polyplexes could also be observed in the alveolar region (Fig. 6B). Images taken after administration of GFP coding plasmid complexed with TAT-PEG-PEI confirmed these findings, since GFP

was observed in both the bronchial as well as the alveolar epithelia cells [6C]. These findings indicate successful gene transfer followed by transgene expression in the conducting as well as respiratory airways.

Until now, it was demonstrated that PEI polyplexes are capable of targeting bronchial cells [6, 7], which could be confirmed by our studies (data not shown). In contrast, TAT-PEG-PEI was able to mediate transfection to bronchial as well as alveolar cells. It is likely that PEI/DNA polyplexes, due to their highly positive surface charge and aggregation, “stick” at the bronchial ciliated cells and are unable the reach the respiratory tract. By contrast, the small, shielded particles of TAT-PEG-PEI/DNA are able to pass the bronchial region, hence depositing also in the alveolar region. Due to the larger surface area of the alveolar sacs, a more extensive transfection of lung tissue was achieved.

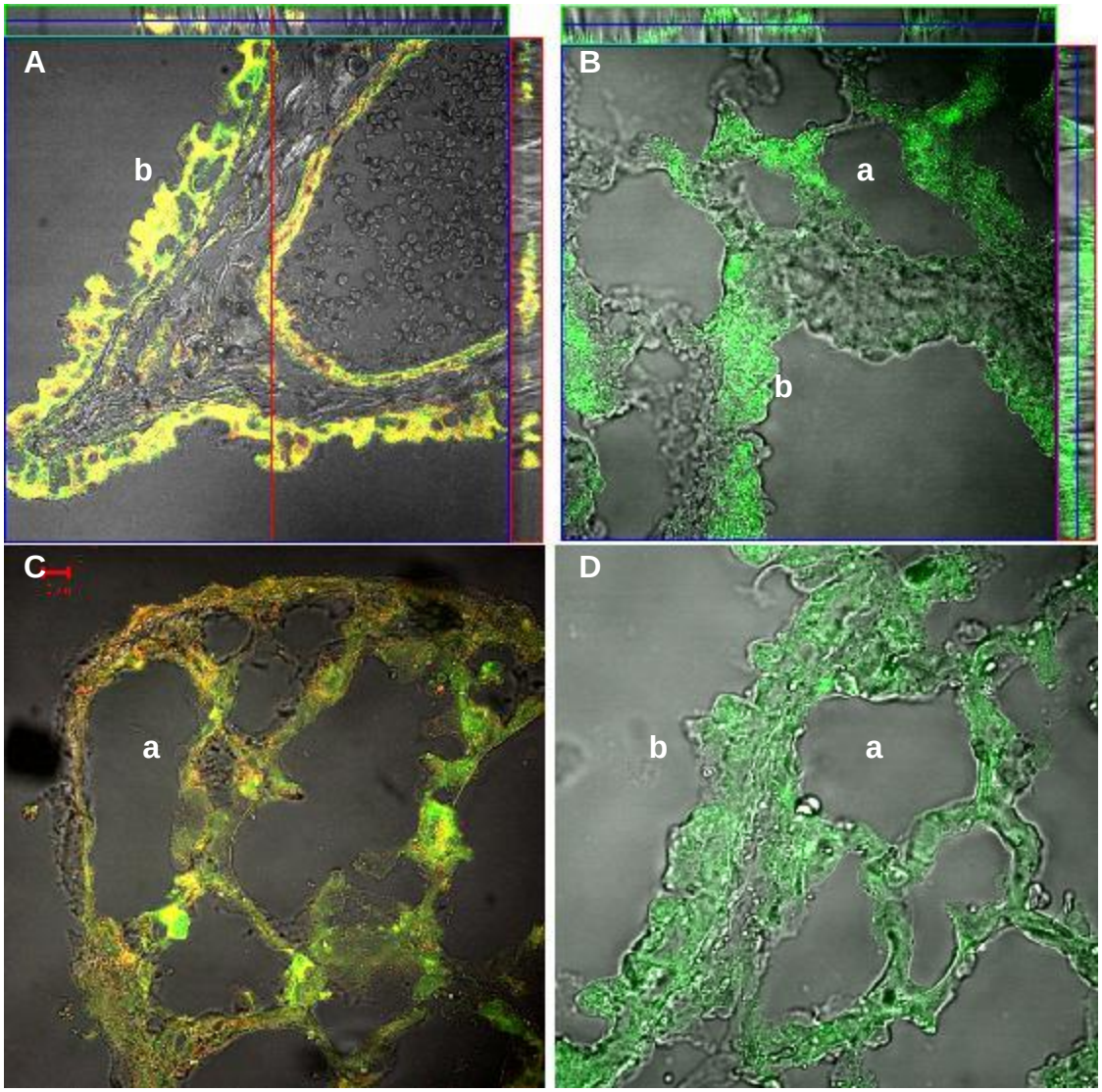


Figure 6: Polyplex distribution in the mouse lung: Localization of double labeled TAT-PEG-PEI/DNA 4 hours post-application in A) bronchial epithelia endothelia cells and C) in alveolar region. Localization of the GFP expression 48 hours post-administration in B) bronchial and D) alveolar epithelial cells. (a) indicates alveolar region, (b) indicates bronchial region.

Conclusion

In summary, TAT-PEG-PEI conjugates are a delivery system for plasmid DNA to the lung that displays several beneficial features compared to unmodified PEI: (i) TAT-PEG-PEI led to enhanced polyplex stability and DNA protection in the pulmonary environment. (ii) The zeta potential of the polyplexes formed between DNA and TAT-PEG-PEI was reduced due to PEG shielding of the surface charge, leading to decreased aggregation tendency in high ionic strength media. (iii) The lower surface charge and the PEG shielding also reduced significantly cytotoxicity in lung epithelial cells both in cell culture as well as in vivo. (iv) TAT-PEG-PEI demonstrated 600% higher transfection efficiency in vivo than PEI. (v) TAT-PEG-PEI directed plasmid DNA into the epithelial cells of the bronchi and alveoli.

The enhanced transfection efficiency under in vivo conditions is most likely due to TAT-derived oligopeptide mediated cell uptake of DNA, increased DNA condensation and polyplex stability. The PEG spacer seems to be essential for both the enhanced gene expression and the reduced toxicity. Further experiments exploring polyplex composition and the uptake mechanism are currently under way in our laboratory.

These data taken collectively suggest that TAT-PEG-PEI could be an interesting pulmonary delivery systems for DNA offering potentially new treatment modalities for different lung diseases, depending on the cell population to be targeted [1, 60]. TAT-PEG-PEI offers the possibility of transfecting both alveolar and bronchial tissue through inhalation.

Non-viral gene delivery systems, such as PEI, have not reached the same transfection efficiencies as viral vectors, but this study demonstrates that the potential of polycation modifications have not fully been exhausted. TAT-PEG-PEI might provide an interesting addition to the spectrum of polycationic delivery systems since it enhances gene expression in conducting and respiratory airways, and also improves the biocompatibility and polyplex stability in the extra- and intracellular lung environment, presenting attractive features of a gene carrier system for local therapy to the lung.

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Chapter 3

Stabilized nanocarriers for plasmids based upon crosslinked Poly(ethylene imine)

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Summary

Stabilized PEI/DNA polyplexes were generated by crosslinking PEI via biodegradable disulfide bonds. The reaction conversion of different PEIs with the amine reactive crosslinker Dithiobis(succinimidyl propionate) (DSP) was investigated and the molecular weight of the reaction products was identified. Light scattering and microelectrophoresis were employed to assess size and zeta potential of the resulting polyplexes. Polyplex morphology and mechanic stability were investigated using atomic force microscopy. Finally, stability against polyanions of PEI and DNA were prepared by two different formulation methods, either using pre-crosslinked polymers or by crosslinking polyplexes after complexation. Only the latter method yielded small (100-300 nm) polyplexes with a positive zeta potential when HMW PEI was used, whereas crosslinked LMW PEI resulted in polyplexes with increased size (>1000 nm) and zeta potentials down to -20 mV. Also, only crosslinking after polyplex formation was able to enhance resistance against polyanion exchange and high ionic strength. AFM images revealed no changes in the morphology of crosslinked HWM PEI polyplexes. Additionally, indentation force measurements using AFM revealed significant increased mechanical stability of crosslinked HMW PEI polyplexes. These polyplexes also displayed significant reduced interactions with major blood components like albumin and erythrocytes. The resulting biocompatible particles offer a means of combining enhanced polyplex stability with redox triggered activation for in vivo application.

Introduction

Systemic delivery of therapeutic genes remains a major objective for human gene therapy. While loco-regional administration of nucleic acids has shown some promise [1], intravenous gene delivery is required to reach more disseminated targets, such as metastatic cancer cells. Systemic applications of nano-scale delivery systems such as positively charged polymeric gene transfer vectors (polyplexes), liposomes or micelles are currently limited by their rapid clearance from the bloodstream following intravenous injection, with only 10-20% of the injected dose remaining in circulation after 10 min, and only 2% after 30 min [2-6]. Usually, these nanocomplexes are quickly cleared by first-pass organs of the reticuloendothelial system, such as liver or spleen, [6-8] as well as in the lung capillaries [4, 6]. The positive charge of polyplexes may lead to interactions with cellular blood components [9] and plasma proteins [4] causing aggregate formation. Additionally, polyplexes of cationic polymers and DNA may undergo exchange reactions with endogenous polyanions, such as albumin, thereby releasing DNA [10, 11]. Thus, for efficient systemic application, polyplexes should ideally bear only a low positive surface charge [12, 13].

Hydrophilic polymers, such as poly(ethylene glycol) (PEG) or poly[N-(2-hydroxypropyl) methacrylamide] (PHPMA), have been used to shield surface charges and reduce interactions of the polyplexes with blood components or endothelia, resulting in prolonged circulation times [5, 7, 14-16]. Increased blood concentrations of about 30% vs. 6% after 30 min for PEGylated PEI/transferrin polyplexes and 7-fold higher AUC for PEGylated polyplexes have been reported [9, 14]. However, even for PEGylated polyplexes, the disruption of the electrostatic polyplexes remains a challenge, as this leads to DNA degradation after systemic application. Double labeling of the DNA as well as the polymers showed differences in blood level profiles and organ distribution profiles after 2h and 12h, likely due to separation of the polyplexes and degradation of the DNA [5, 17]. Therefore, in addition to steric shielding, it is necessary to further stabilize the polyplexes by enhancing their resistance against dissociation [18].

Surface crosslinking of polyplexes has emerged as a tool to address these critical stability issues and has been investigated for different polymers (e.g. PLL, PEI) in vitro

[19, 20]. Surface coating of polyplexes with pHPMA resulted in enhanced resistance against disruption by polyanions [20], indicating minimal extracellular release of the DNA by polyelectrolyte exchange reactions, which play an essential role in the process of DNA delivery [21]. However, these constructs suffered from low surface charges of about zero or even negative values due to the shielding of the copolymers, impairing efficient cell interaction [22] and making them likely to be subject to phagocytosis via the polyanion receptors of macrophages [23]. Thus, a balance between charge shielding and positive surface charge seems to be a key factor for the stabilization of polyplexes for systemic application. Such a balance may improve target cell interactions while reducing unwanted side effects.

Our aim was to design a vector system for intravenous administration which is stabilized by the introduction of bioreversible disulfide crosslinks. Disulfide crosslinking is thought to facilitate intracellular release of the DNA, since glutathione mediated reduction will occur predominantly in the intracellular milieu [22, 24-26]. Poly(ethylene imine) (PEI) was used because it is one of the most successful polycationic carrier systems [27-29]. Crosslinks were introduced to the primary amine functions of PEI using a low molecular weight reagent, dithiobis(succinimidyl propionate) (DSP). This crosslinking method yields neutral amide bonds, which reduce the cationic charge of the PEI and show favorable transfection efficiencies compared to charge preserving reagents [24]. Besides the use of crosslinked PEI as absorbent in waste water treatment or chromatography [30, 31], crosslinking has been employed to improve DNA complexation with low molecular weight PEI [32, 33]. In this work, we evaluate the potential of two PEIs with different molecular weight in forming polyplexes with plasmid DNA prepared with two different formation strategies. Polyplexes formed with crosslinked PEI as well as surface crosslinked PEI/DNA polyplexes are compared in terms of their biophysical properties, stability and cytocompatibility.

Experimental Section

Materials: *Polymers:* Poly(ethylene imine) (25kDa, HMW (high molecular weight) PEI and 5 kDa, LMW (low molecular weight) PEI, as specified by the manufacturer) were gifts from BASF (Ludwigshafen, Germany). *DNA*: The plasmid pCMV-GL3 encoding the firefly luciferase gene was amplified in JM-109 competent cells and purified using a commercial kit (Qiagen Hilden, Germany). Salmon testes DNA (Sigma, Taufkirchen, Germany) was used for light scattering experiments. Dithiobis(succinimidyl propionate) (DSP, Lomant´s reagent), dry dimethylsulfoxide (DMSO) HPLC grade, Dithiobis(2-nitrobenzoic acid) (Ellman´s reagent) and fluorescamine were purchased from Sigma, (Taufkirchen, Germany). All other reagents used were of analytical grade. Pure water (0.22 µm filtered, 0.055 S/cm, USF Seral, Seradest BETA 25 and Serapur DELTA UV/UF) was used to prepare analytical solutions and buffers.

Polymer crosslinking: HMW PEI or LMW PEI stock solutions (1 g/L in pure water, pH 7.5) were diluted in either low ionic strength buffer (5% glucose/25 mM Hepes, pH 7.5) or high ionic strength buffer (150 mM sodium chloride, pH 7.5). Stock solutions of water insoluble DSP were prepared by dissolving DSP in water free DMSO. The primary amines of PEI were crosslinked with 0.01 M Dithiobis(succinimidyl propionate) (DSP) in DMSO. The solutions were mixed by vigorous pipetting and allowed to incubate for 30 min at room temperature. All degrees of crosslinking are reported as molar ratios between DSP and PEI amines assuming that HMW PEI (25 kDa) contains 580 amines per molecule and LMW PEI (5 kDa) contains 126 amines per molecule. Calculation of primary amines was based on ^{13}C-NMR data obtained by a recently reported method [34].

Characterization of crosslinked polymers: Solubility testing: The solubility of crosslinked polymers was tested in different media. HMW PEI and LMW PEI were diluted to 1 mg/mL and 0.1 mg/mL and crosslinked by the addition of 0.01 M DSP in DMSO. The solutions were mixed by vigorous pipetting and allowed to stand for 30 min at room temperature. The solutions were rated visually for any turbidity and precipitates.

Determination of primary amine content: Samples were prepared as described above. The colorimetric assay was performed according to the methods of Read et al. [35]. Briefly, 15 µL samples of polymer solution were added to 190 µL of 0.1M borate buffer at pH 8.0 in 96-well plates. 75 µL of freshly prepared 0.01% acetonic fluorescamine solution were added, mixed vigorously and incubated for 10 min. Fluorescence was measured with a PerkinElmer LS 50B Luminescence Spectrometer equipped with a well plate reader at λ_{ex} = 392 nm, λ_{em} = 480 nm and slit width 4 nm. Measurements were carried out in quadruplicate and the concentration of the primary amines of the crosslinked polymers was calculated using a standard curve of PEI 5kDa and PEI 25kDa standards, respectively. The primary amine content of these standards was independently determined by 13C-NMR measurements [34]. The results are expressed in mmol/L primary amines and results are given as the mean of quadruplicate experiments ± standard deviation.

Determination of thiol content: Thiol content was determined according to a method reported in the literature [36]. Briefly, 100 µL of the sample solution was added to 150 µL 0.1M phosphate buffer at pH 8.0 in 96-well plates. The color was developed by adding 50 µL per well of a solution containing 0.5 mg/mL Dithiobis(2-nitrobenzoic acid) (Ellman´s reagent, DTNB) diluted in phosphate buffer (pH 8.0) and incubated for 15 min at room temperature. Absorbance values were obtained with a microplate reader (TitertekPlus MS 212, ICN, Germany) at 405 nm and thiol content was calculated using a standard curve of cysteine and expressed as mmol/L free thiol. Results are given as the mean of quadruplicate experiments ± standard deviation.

Determination of disulfide content: A method reported in the literature was modified for microplate assays [37]. The reagent 2-nitro-5-thiosulfobenzoate (NTSB) was prepared by air oxidation of a solution of 100 mg dithiobis(2-nitrobenzoic acid) (Ellman´s reagent, DTNB) in 10 mL 0.1 M Na2SO3 at 37°, yielding a yellowish stock solution of NTSB that was stored at -20°C. The working solution was prepared by diluting the stock solution 1:100 with 50 mM glycine, 100 mM Na_2SO_3, and 3 mM EDTA buffer at pH 9.5. 190 µL of the reagent working solution were added to 10 µL sample per well and incubated in the dark for 30 min at room temperature. Absorbance

values were measured in a microplate reader (TitertekPlus MS 212, ICN, Germany) at 405 nm. The disulfide content was calculated using a DSP standard curve and expressed as mmol/L disulfides. Results are given as the mean of quadruplicate experiments ± standard deviation.

Size Exclusion Chromatography in Combination with Multiple Angle Laser Light Scattering (SEC-MALLS): The SEC setup consisted of a HPLC Pump L-6000 from Merck-Hitachi, Darmstadt, Germany, and a Merck-Hitachi autosampler AS-200A. Polymers were detected by a differential refractive index (RI) detector RI-71 from Merck and an 18 angle laser light scattering detector from Wyatt Technologies (Santa Barbara, CA, DAWN EOS, GaAs Laser 690 nm, 30 mW, K5 cell). The SEC columns Hema 40 (pre-column) and Novema 3000 were from Polymer Standard Service, Mainz, Germany.

Crosslinked polymers were prepared by reacting 10 mL of a 0.1 g/L PEI 25kDa solution in 0.1M borate buffer at pH 7.5 with increasing amounts of 0.01 M Dithiobis(succinimidyl propionate) (DSP) in DMSO for 30 min. After 30 min, the reaction solution was purified by ultrafiltration (Amicon filter membranes, 10 kDa Mw cut-off) with 0.5% formic acid. The eluent was prepared with pure water and degassed with a four-channel online vacuum degasser (DDG-75, Duratec, Reilingen, Germany). Sample size was 100µL with a 40 µL loop volume for each run. A flow rate of 1 mL/min was applied. MWs were calculated with Astra 4.73 for Windows Software.

Formation of Polymer-DNA Polyplexes and Crosslinking: Luciferase reporter gene plasmids (pGL3) and the appropriate amounts of uncrosslinked or crosslinked PEI were dissolved separately in either low ionic strength buffer (5% glucose/ 25 mM Hepes at pH 7.5) or high ionic strength buffer (150 mM sodium chloride at pH 7.5), mixed by vigorous pipetting, and incubated for 10 min to allow polyplex formation. Polyplex crosslinking was achieved by adding the necessary amount of 0.01M DSP in DMSO for the desired molar ratios of DSP/PEI amines to the preformed polyplexes. The solutions were mixed by vigorous pipetting and incubated for 30 minutes. Polyplexes were prepared at a concentration of 2µg/100µL plasmid and the appropriate amount of polymer to yield an N/P (nitrogen to phosphate) ratio of 7.

Determination of size and zeta potential of polymer-DNA polyplexes: The hydrodynamic diameters as well as the zeta potentials of freshly prepared polyplexes were measured using a Zetasizer Nano-ZS from Malvern Instruments (Herrenberg, Germany) equipped with a 4 mW He-Ne laser at a wavelength of 633 nm at 25°C. Scattered light was detected at a 173° backward scattering angle with automatic measurement position and automatic laser attenuation. The viscosity and refractive index of pure water at 25°C were used for data analysis. Hydrodynamic diameters and zeta potential were measured in folded capillary cells after 1:3 dilutions with the appropriate buffer and were calculated using DTS software v4.10. Reference measurements with Malvern size and zeta potential standards were run routinely to check for correct instrument operation. Values are given as the mean of three measurements of 10 runs each.

Atomic force microscopy: The polyplexes were prepared as described above and diluted in pure water. 10 µL of the polyplex containing solution were directly transferred onto a prewashed glass slide. Afterwards, polyplexes were allowed to immobilize on the glass slides under laminar air flow overnight. Finally, the glass slides were washed with pure water to remove buffer residues and dried with air. AFM experiments were performed using a vibration-damped NanoWizard™ instrument (JPK instruments, Berlin, Germany). Commercial pyramidal tips (Micromash, Estonia) attached to I-type cantilevers with a length of about 230 µm, a resonance frequency of about 160 kHz and a nominal force constant of 40 N/m were used. Pictures were recorded in intermittent contact mode at a scan speed of approximately 1 Hz to avoid damage of the sample surface. The acquired pictures had a resolution of 512 x 512 pixels.

Nanoindentation experiments were carried out with V-type shaped contact mode cantilevers (Micromash, Estonia). The cone angle of the tips was smaller than 10° and the cantilever had a typical length of 290 µm and a force constant of about 0.03 N/m. The deflection of the cantilever was recorded while the cantilever was extended towards the sample, indicating the force exerted on the tip of the AFM. The setup was calibrated on a precleaned glass slide as an incompressible surface. To determine sensitivity, the slope of the retracted part of the obtained spectroscopy curve was taken. Calibration of

the spring constant of the installed cantilever was carried out using a thermal noise method [38]. The height of the polyplexes was measured to ensure that the experiment was performed in the center of the polyplex and the indentation of each polyplex did not exceed 10% of the polyplex height. Force scans were performed with an extension speed of about 5 nm per second. During each experiment, 256 data pairs, deflection of the cantilever and extension of the cantilever holder were recorded. Values are reported as the mean ± standard deviation of 35-50 particles, each measured twice.

Polyanion mediated dissociation: Polyanion mediated dissociation of the polyplexes was studied using agarose gel electrophoresis as recently described [39]. Polyplex solutions were incubated for 10 min with increasing amounts of heparin. 20 µL aliquots were loaded onto a 1% agarose gel containing ethidium bromide. Gels were run for 60 min at 70 V, and then scanned with a Biometra gel analyzing system.

Albumin induced aggregation: Polyplexes were prepared at a plasmid concentration of 15 µg/mL and crosslinked as described. After the addition of 2 µL of a 40 mg/mL solution of bovine serum albumin (Behring, Germany), polyplexes were allowed to incubate for 10 min. The optical density of the polyplex solution was measured with a Shimadzu UV-160 UV/VIS spectrometer at 400 nm. Results are given as the mean of triplicate experiments ± standard deviation.

Polyplex stability against high ionic strength: The intensity of scattered light of the polyplex solutions was assessed as kilocounts per second (kcps) with a fixed pinhole (200 µm) on an Autosizer Lo-C from Malvern (Herrenberg, Germany, 90° angle, 10 mW HeNe laser, 633 nm). Polyplexes were prepared according to the procedure for size measurements using herring testes DNA in 150 mM sodium chloride. Dissociation of the polyplexes was achieved by adding aliquots of 5M sodium chloride solution. After each addition, polyplexes were incubated for 5 min and the scattered light intensity was measured as kcps. Results are given as the mean of triplicate experiments ± standard deviation.

Hemocompatibility testing: Hemolytic effects were investigated as reported earlier [40]. Briefly, fresh blood from healthy human volunteers collected in EDTA containing tubes was centrifuged at 4°C for 3 min at 3000 rpm and washed several times with phosphate buffered saline (PBS) at pH 7.4 until the supernatant was clear and colorless. 150 µL of a 2.5% (v/v) suspension of the erythrocytes was mixed with 15 µL of the polymer or the polyplex solution, respectively, prepared in 5% glucose/25 mM Hepes buffer in microcentrifuge tubes. After an incubation time of 60 min at 37°C, the blood cells were removed by centrifugation and the supernatant was transferred to 96-well plates. The supernatant was spectroscopically investigated at 570 nm with a TitertekPlus MT 212 plate reader (ICN, Germany). Pure glucose buffer and a 1% Triton X-100 solution in water were used as negative and positive controls, respectively. Hemolysis is reported as percent $(OD_{Triton} - OD_{sample} / OD_{Triton} - OD_{buffer})*100\%$. Results are given as the mean of triplicate experiments ± standard deviation.

Statistics: Experiments were performed at least in triplicate. Significance between the mean values was calculated using one-way ANOVA analysis using Origin® 7.0 software (OriginLab Corporation, Northampton, MA). Probability values < 0.05 were regarded to be significant.

Results and Discussion

Stabilization of polyplexes using crosslinking strategies could be a promising way to overcome dissociation of DNA and polycations in the blood stream after intravenous administration [18, 41]. Polyplexes containing PEI have been the subject of numerous investigations [27, 28]. Two strategies to stabilize PEI/DNA polyplexes from a low (5 kDa) as well as a high (25 kDa) molecular weight PEI were investigated here. First, PEI was crosslinked with the low molecular weight crosslinker DSP and subsequently complexed to DNA. Second, preformed PEI/DNA polyplexes were crosslinked with DSP to achieve surface stabilization.

Synthesis and Characterization of crosslinked polymers: Since crosslinked low molecular PEI has recently been used for polyplex formation, we attempted to investigated the crosslinking reaction in more detail [32, 42]. The reaction of HMW PEI and LMW PEI with the crosslinking reagent DSP was characterized by determining the amounts of primary amines of PEI and disulfide groups, which were modified by the crosslinking reagent (Figure 1). DSP is a homobifunctional N-hydroxysuccinimide (NHS) ester-based electrophilic crosslinking reagent, containing an 8-atom spacer with a length of 12 Å [43]. The amide bond formation mediated by DSP results in the elimination of two positive charges. DSP reacts with primary amines to form stable covalent bonds and its centrally located disulfide linkage is potentially cleavable after conjugation using common reducing agents. A fluorescamine based assay was used to determine the amount of primary amines in the polymers. This analysis revealed an initial decrease in primary amines after addition of the DSP, reaching an end point after 15-30 min (data not shown), suggesting successful conversion. Thus, a reaction time of 30 min was chosen for all further crosslinking experiments.

The residual primary amine content after reaction of the polymers with DSP decreased with increasing amounts of DSP. LMW PEI and HMW PEI did not differ in their reaction behavior with DSP, which can be attributed to similar degrees of branching and content of primary amines [34]. For crosslinking degrees (molar ratio of DSP/amines) of 0.15 and above, no residual primary amines were detected anymore (see Figure 2). Since the crosslinker is bifunctional, a molar ratio of 0.15 corresponds to molar ratio of

0.30 in amine reactive NHS groups. Recent reports showed that about 1/3 of all amines present in commercially available PEI were primary amines [34]. This points to a more than 90% reaction conversion of the primary amines in the polymers after reaction with DSP at a crosslinking degree of 0.15. A further increase of the DSP/amines ratio did not show further reduction of the primary amines content, suggestive of full conversion. Thus, one can assume that at least 90% of the primary amines of both LMW PEI and HMW PEI are susceptible to reaction with DSP. Because the length of the crosslinker is about 12 Å, this indicates the close vicinity of the primary amines due to the flexibility of the polymer chains. A more rigid polymer structure is obtained following the crosslinking reaction due to suppressed chain flexibility of the branched polymer structure [44].

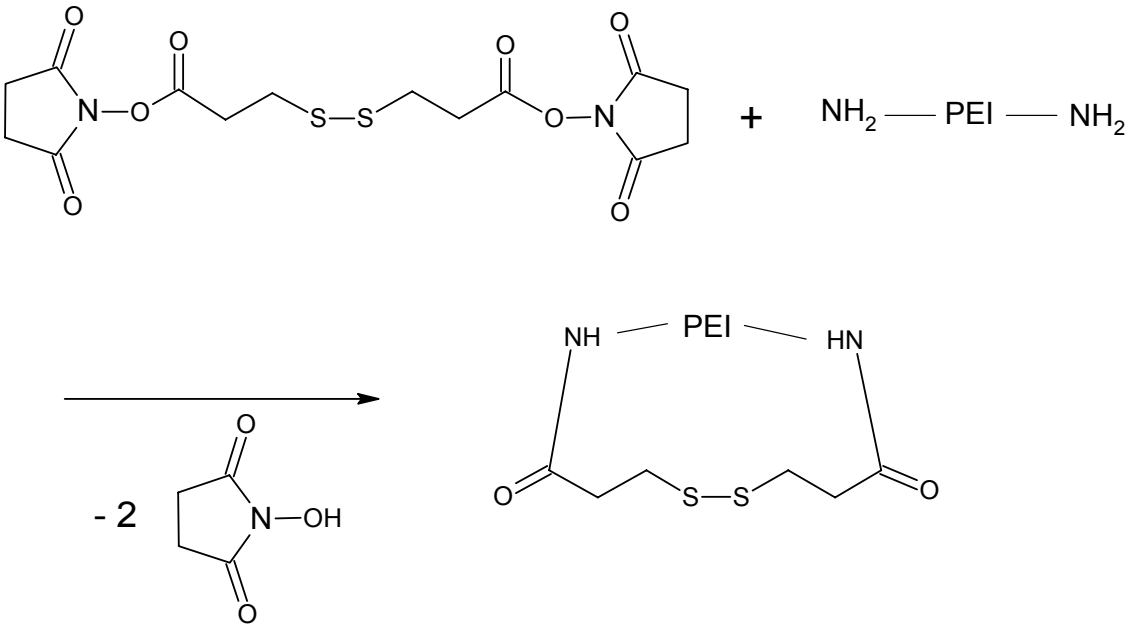


Figure 1: Reaction scheme for the conversion of PEI primary amines with Dithiobis(succinimidyl propionate) (DSP).

A recently reported method was used to determine the disulfide content of the crosslinked polymer samples [37]. The thiol content caused by a possible disulfide reduction was checked beforehand. The freshly prepared crosslinking reagent contained only a negligible amount of free thiols, 1.61 ± 1.1%. Figure 2 shows the dependency of primary amine content as a function of disulfide modification for both crosslinked PEIs.

The primary amine content of both LMW PEI and HMW PEI was observed to decrease in a linear manner with increasing disulfide, and consequently, DSP concentrations. A molar ratio of DSP to amines of 0.15 is considered to be sufficient to obtain the maximum possible crosslink density in branched PEIs of either high or low molecular weight. These measurements allow the adjustment of the desired crosslinking degrees based on the determination of reactive groups instead of by the feeding ratio alone [32, 33].

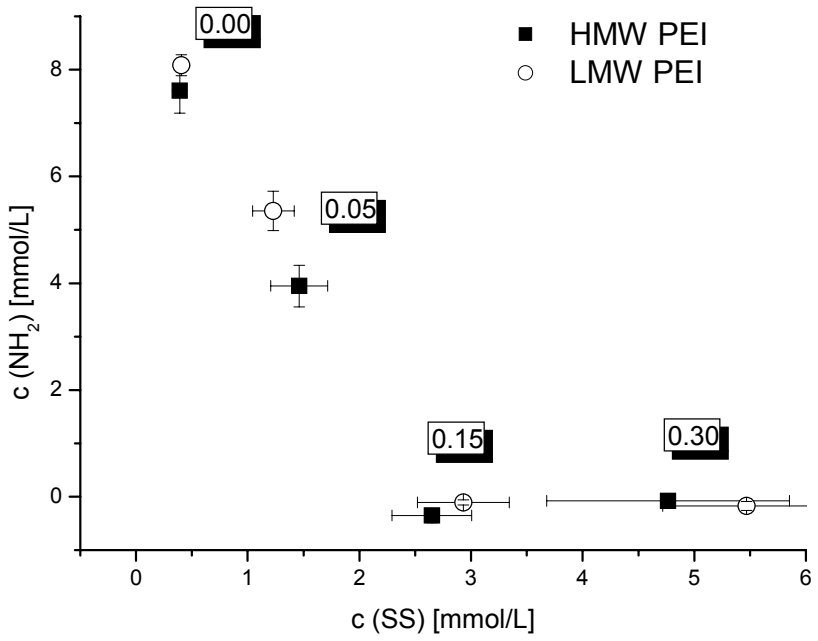


Figure 2: Loss of primary amines and introduction of disulfides due to the reaction of LMW PEI and HMW PEI with DSP (numbers in boxes indicate molar ratio of DSP to amines, n = 4)

Solubility of crosslinked polymers: The crosslinked polymers were investigated for their solubility in a broad range of aqueous buffer systems to optimize polyplex formation. Table 1 shows the results for two polymer concentrations reacted in different buffers at pH 7.5. As precipitation would impair polyplex preparation, tests were performed at a high crosslinking degree of 0.30 combined with relatively highly concentrated polymer solutions to investigate conditions that might cause precipitation. Low ionic strength media were found to be favorable in terms of preventing precipitation of the crosslinked polymers. High ionic strength sodium chloride solutions

also showed no precipitation, but high ionic strength phosphate and borate ion containing buffers led to precipitation, especially for the higher concentrated polymer solutions. Thus, low ionic strength 5% glucose/25 mM Hepes buffer and sodium chloride, both at pH 7.5, were chosen as media for complexation with DNA in further experiments.

Buffer	LMW PEI		HMW PEI	
	0.1 g/L	1 g/L	0.1 g/L	1 g/L
ultra pure water	s	s	s	s
5% glucose/25 mM Hepes	s	s	s	s
150 mM Sodium chloride	s	s	s	s
100 mM Borate buffer	t	p	t	p
100 mM Phosphate buffer	t	p	t	p
DMSO	s	s	s	s

Table 1: Influence of the buffer on the reaction of LMW PEI and HMW PEI with DSP (crosslinking degree 0.30). “s” indicates solubility, “t” is for turbid solutions, and “p” represents precipitates.

Molecular weight of crosslinked polymers: The crosslinking of polycations was recently reported to enhance the transfection efficiency of low molecular weight polycations by creating higher molecular weight conjugates due to intermolecular crosslinking [32, 33]. LMW PEI and HMW PEI solutions were found to react differently with DSP because the absolute amount of primary amines per molecule is different for the LMW and HMW PEIs used in this study (about 10 times higher per molecule for HMW PEI). The molecular weight of crosslinked PEIs was determined by size exclusion chromatography combined with multi-angle laser light scattering (Table 2). The reaction of LMW PEI with DSP resulted in generally higher molecular weight

products. Increased weight-average molecular weights were found, from an initial value of about 3.7 kDa to more than 6 kDa at a crosslinking degree of 0.15, suggesting intermolecular crosslinking. A similar rise in the molecular weight has been recently reported for a crosslinked mixture of 423 Da and 2 kDa [33], and the smaller 800 Da PEI resulted in 10-30-fold higher molecular weight after crosslinking [32]. Interestingly, in contrast to LMW PEI, HMW PEI derivatives retained their original molecular weight after crosslinking , which was found to be in the range of 45 kDa for all crosslink degrees up to a DSP/amines molar ratio of 0.15, which is in line with recently reported values [45, 46]. In fact, the chromatograms of the size exclusion chromatography revealed even higher elution volumes for the higher crosslink degrees with steady molecular weight, indicating a more dense and globular structure upon crosslink formation (data not shown) [44]. The LMW PEI eluted earlier with increasing crosslink degrees, which is in agreement with the M_w results obtained by MALLS. Comparing these data, one can assume a molecular weight dependent reaction behavior, leading from large aggregates of intermolecular crosslinked low molecular weight PEI species to intramolecular crosslinked polymer chains if higher molecular weight PEI is used.

	LMW PEI			HMW PEI		
crosslink degree	M_w [Da]	M_n [Da]	PDI	M_w [Da]	M_n [Da]	PDI
0.00	$3.8 \cdot 10^3$	$3.7 \cdot 10^3$	1.02	$5.0 \cdot 10^4$	$4.1 \cdot 10^4$	1.22
0.05	$5.2 \cdot 10^3$	$4.0 \cdot 10^3$	1.29	$4.8 \cdot 10^4$	$4.3 \cdot 10^4$	1.11
0.15	$6.2 \cdot 10^3$	$3.8 \cdot 10^3$	1.18	$4.4 \cdot 10^4$	$4.0 \cdot 10^4$	1.08

Table 2: Molecular weights of crosslinked polymers as determined by size exclusion chromatography combined with multi-angle laser light scattering.

Characterization of polyplexes: So far, either constructs generated from crosslinked low molecular weight polymers or crosslinked polyplexes have been investigated separately [22, 47, 48]. Here, we compare the two different strategies to assess differences in their polyplex formation. Polyplexes between LMW PEI as well as HMW PEI with plasmid DNA were prepared either using crosslinked polymers or polyplexes between plasmid DNA and unmodified polymers which were crosslinked after formation. All polyplexes were prepared at an N/P ratio of 7, which is commonly used for transfection experiments [45] and tested in low as well as high ionic strength medium.

Polyplex size and zeta potential: The hydrodynamic diameters (Figure 3) and the zeta potential values (Figure 4) are shown for both HMW PEI/plasmid and LMW PEI/plasmid polyplexes. Polyplexes prepared with pre-crosslinked HMW-PEI showed hydrodynamic diameters up to 500–1000 nm for the highest crosslink ratio of 0.30. In contrast, the size of HMW PEI polyplexes crosslinked after polyplex formation increased only slightly with increasing crosslink degree, ranging from 102 ± 3 nm in 5% glucose/25 mM Hepes buffer and 140 ± 2 nm in 150 mM sodium chloride to 200-300 nm for a crosslinking degree of 0.30. The sizes of polyplexes prepared with pre-crosslinked LMW PEI were larger than those of polyplexes crosslinked after formation, independent of the medium used for preparation. Pre-crosslinked LMW PEI resulted in polyplex sizes of up to 1000 nm, which is much higher than polyplex diameters of previously reported crosslinked 2 kDa and 1.8 kDa PEIs [33, 42]. Presumably, the reduction of cationic amine groups leads to decreased complexation ability. PEI resulted in generally larger hydrodynamic diameters than HMW PEI for both uncrosslinked and crosslinked forms, reflecting a lower overall complexation efficiency due to its lower molecular weight [33, 49]. Interestingly, while HMW PEI polyplexes could be successfully surface crosslinked with only negligible size increase, LMW PEI polyplexes became much larger when reacted with DSP after formation. Presumably, a looser structure is obtained due to the loss in complexation efficiency, which is not compensated by stabilizing surface crosslinks as was the case for the HMW PEI polyplexes [24].

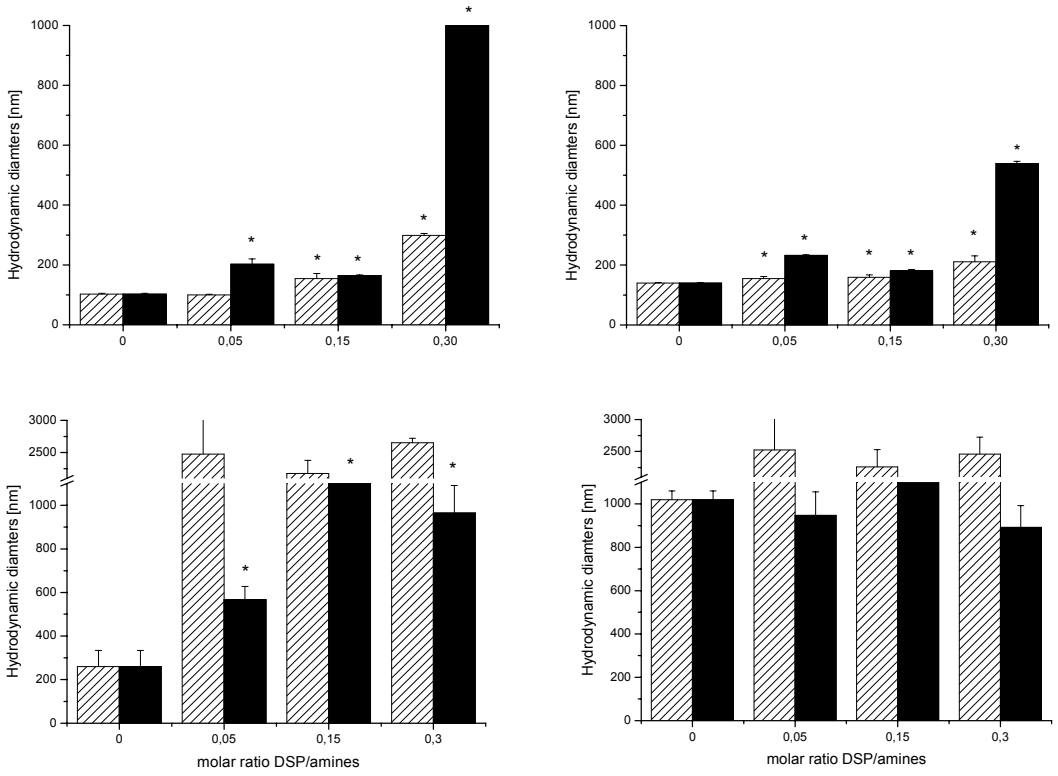


Figure 3: Hydrodynamic diameters of HMW PEI (upper) and LMW PEI (lower) polyplexes. Left: 5% glucose/25 mM Hepes buffer (pH 7.5); right: 150 mM sodium chloride (pH 7.5). Striped bars indicate polyplexes crosslinked after polyplex formation; black bars indicate polyplex formation using pre-crosslinked polymers. Crosslink degrees are reported as the molar ratio of DSP/amines (* = significant differences to unmodified polyplexes ($p<0.05$)).

The surface charge of HMW PEI polyplexes was only moderately affected by the crosslinking when prepared in 150 mM sodium chloride at pH 7.5, retaining a comparably high zeta potential of > +25 mV. A slight decrease was seen for polyplexes formed with pre-crosslinked polymers, where the surface charge decreased to +20-25 mV at high crosslink degrees. By contrast, in low ionic strength medium, high crosslink degrees led to a decrease of the surface charge for polyplexes formed via both preparation methods, yielding lowered zeta potentials of about +10-20 mV. Zeta potentials of LMW PEI polyplexes prepared according to either method decreased with increasing crosslink degrees. The surface charge was found to be in the range of +6 to +16 mV in 150mM sodium chloride at pH 7.5 and, in contrast, decreased to negative values in glucose buffer, presumably indicating a dissociation of the polyplexes. The

comparably low surface charges could also cause aggregation and, thereby, explain the resulting high hydrodynamic diameters of the crosslinked LMW PEI polyplexes.

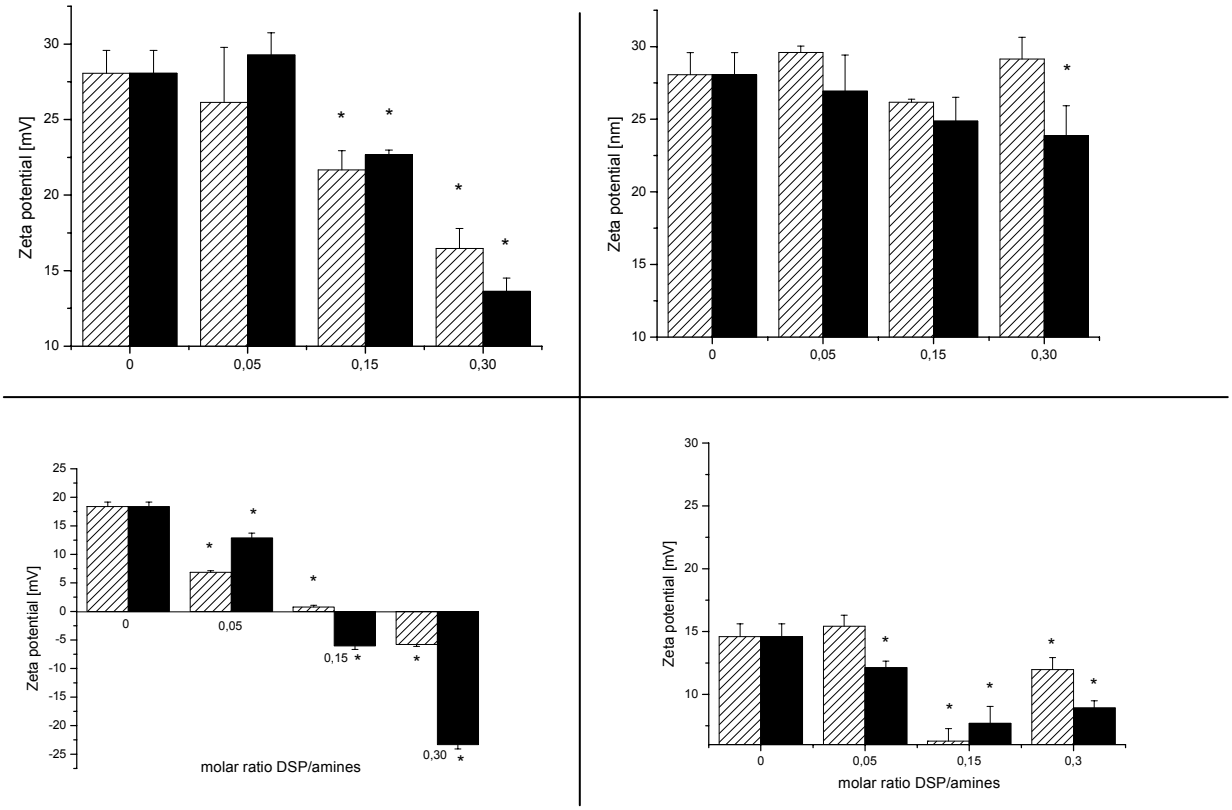


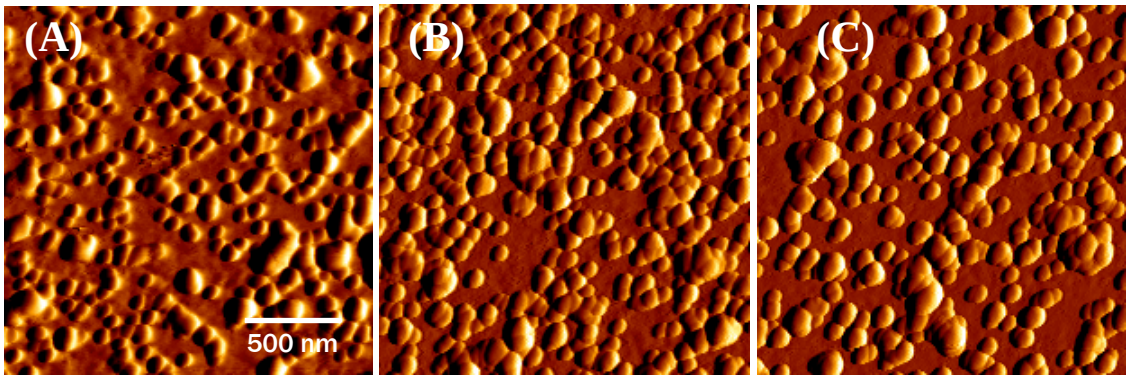
Figure 4: Surface charge of HMW PEI (upper) and LMW PEI (lower) polyplexes. Left: 5% glucose/25 mM Hepes buffer (pH 7.5); right: 150 mM sodium chloride (pH 7.5). Striped bars indicate polyplexes crosslinked after polyplex formation; black bars indicate polyplex formation using pre-crosslinked polymers. Crosslink degrees are reported as molar ratio DSP/amines (* = significant differences to unmodified polyplexes ($p < 0.05$)).

Taken together, LMW PEI seemed to be unfavorable for the formation of crosslinked polyplexes using DSP, independent of the preparation method, since hydrodynamic diameters exceeded 500 nm. HMW PEI polyplexes crosslinked after formation displayed only a slight increase in their size. For efficient endocytosis and transfection, polyplexes must be small and compact [50] and the crosslinked HMW PEI polyplexes fulfill these criteria. The low influence of crosslinking upon the polyplex sizes of the HMW PEI polyplexes can be attributed to its superior complexation efficiency

compared to the LMW PEI. This leads to more stable polyplexes and, thus, preferential surface crosslinking with only minor dislocation of the DNA. A net loss of cationic charges by crosslinking can be observed for both types of polymers. The formation of charge-lowering amide bonds instead of crosslinking with charge-preserving crosslinkers was recently reported to enhance the transfection efficiency for PEG-PLL/plasmid polyplexes, presumably by facilitating DNA escape from the carrier [24], [17]. Non-charge consuming crosslinkers, such as dimethyl-dithiobis(propione imidate) (DTBP) or ethylene glycol-bis(succinimidyl succinate) (ESS) have only been reported for crosslinking LMW PEIs to higher molecular weight conjugates, but not for surface stabilization, as no stabilization effect was intended [32, 42]. However, according to recent reports, the cationic charge of the polyplexes plays a major role in their cellular uptake. Thus, a delicate balance between charge loss and charge preservation is necessary. The surface charge of crosslinked LMW PEI polyplexes was greatly reduced and even yielded negative values in glucose buffer, rendering the cell surface interaction problematic and indicating polyplex dissociation and release of free DNA [51]. By adjusting the crosslinking degree with HMW PEI polyplexes, however, this balance can be retained with a positive surface charge of about at least +10 mV.

AFM measurements: AFM imaging of HMW PEI polyplexes crosslinked after formation were carried out to support the size measurements by DLS. Images shown in Figure 5 illustrate that non-crosslinked HMW PEI polyplexes prepared at an N/P ratio of 7 formed defined, globular polyplexes with plasmid DNA (A), in good agreement with earlier reports [17, 40]. Polyplexes crosslinked with increasing crosslinking degrees (B, C) did not show any differences in their morphology compared to uncrosslinked polyplexes, supporting the results from DLS measurements. Some larger aggregates were observed in all three cases. The introduction of the crosslinks did not seem to alter the DNA complexation properties of HMW PEI, as the polyplexes were of similar shape for all crosslink degrees and no free DNA was observed. Size measurements using AFM confirmed the results from DLS for crosslinked HMW PEI polyplexes. For crosslink degrees of 0.05 and 0.15, the size measured with AFM was similar to that of uncrosslinked polyplexes (Figure 5).

AFM has been considered as an appropriate technique to investigate mechanical properties of soft materials, such as polymer films, at a submicron scale [52-54]. A linear relationship was reported between the resistance to indentation and the amount of crosslinks for some rubber-like polymers [55]. The elastic moduli measured with AFM are comparable to those determined with macroscopic tensile tests [53]. However, macroscopic tests are not suitable in this study, as the tested structures are in the range of only 100 nm. The advantage of AFM measurements is the capability to perform local measurements with such a high lateral resolution, allowing focusing on small structures like nanoparticles or, as in this study, polyplexes. To our knowledge, no one has previously tried to determine the mechanical stability of particulate polyplexes, besides testing of polyelectrolyte multilayer films in the micrometer range [56, 57]. Crosslinked HMW PEI polyplexes were tested for their mechanical stiffness in terms of their indentation resistance by measuring the force needed to indent the polyplex surface at constant indentation depth using atomic force spectroscopy. Higher forces needed to indent a specific depth directly indicate higher stiffness of the polyplex. Uncrosslinked polyplexes showed a deflection force of about 30.9 ± 7.4 nN/µm, corresponding to an indentation force of 392.0 nN/µm (n = 35). The mechanical behavior of crosslinked HMW PEI polyplexes was tested at a crosslinking degree of 0.05. A significant increase in the force exerted on the tip was found to (35.8 +/-6.5 nN/µm, which corresponds to an indentation force of about 608.7 nN/µm, n = 50), suggesting that the network of crosslinks stabilizes the shape of the polyplexes and thereby increases the stiffness of the polyplexes. Presumably, the crosslinking not only provides higher polyplex stability in a functional sense by minimizing polyplex dissociation, but it also enhances polyplex mechanical stability, which is a prerequisite for resistance against shear stress in the blood stream. The results of the nanoindentation experiments reveal a difference in the indentation resistance between polyplexes that did not contain any crosslinks and polyplexes which had been stabilized. This suggests that AFM nanoindentation experiments may be an appropriate method to measure the mechanic properties of polyplexes. The impact of the mechanical properties of polyelectrolyte polyplexes will be investigated in our groups further.



Crosslink degree	Polyplex Size AFM [nm]	Indentation force [nN/µm]
0.00	85 +/- 16	392.0
0.05	82 +/- 15	608.7
0.15	83 +/- 15	-

Figure 5: HMW PEI polyplexes crosslinked with increasing amounts of DSP (molar ratio DSP/amines): (A) 0.00, (B) 0.05, and (C) 0.15, visualized by atomic force microscopy in amplitude mode. Polyplexes were prepared at N/P 7 in 5% glucose/25 mM Hepes buffer (pH 7.5). Images display defined polyplexes with no morphological differences at different crosslink degrees. No free DNA could be observed in any image. Some larger aggregates are visible in all formulations, independent of the crosslink degree. Size (n = 40) measurements are based on AFM images, indentation force n = 35-50.

Stability testing of polyplexes: The objective of this study was to investigate whether polyplexes of LMW PEI and HMW PEI could be efficiently stabilized with a low molecular weight crosslinker to improve their in vivo applicability. Polyplexes prepared according to both preparation methods were investigated in terms of complexation efficiency, resistance against polyanion exchange reactions, and albumin induced aggregation. Crosslinked HMW PEI polyplexes were additionally investigated by laser light scattering to examine the role of ionic strength on polyplex stability.

Stability against heparin exchange: Resistance of polyplexes to polyanion mediated dissociation is a method used to study the impact of negatively charged compounds in the blood, such as albumin [11]. A high amount of heparin (1 I.U. per 1 µg plasmid), which is able to release DNA quantitatively from unstabilized HMW PEI polyplexes at

N/P 7, was used to challenge the polyplexes and investigate highly stabilized polyplexes [39].

The use of pre-crosslinked polymers resulted in no retention of the DNA for both tested molecular weights, and the DNA was markedly shifted in the electric field (Figure 6, left hand side). The loss of charged amine functions and a more rigid polymer structure after the crosslink reaction may be responsible for this significantly reduced complexation efficiency, which resulted in polyplex dissociation for both LMW and HMW PEI. The results from both size and zeta potential measurements support these assumptions, since pre-crosslinked polymers resulted in generally larger hydrodynamic diameters with decreased surface charge, which indicate lower compaction ability. The overall lower complexation ability of LMW PEI [45] was not affected by the intermolecular crosslinking, suggesting that for precrosslinked polymers, a higher molecular weight could not compensate for the loss of charges and flexibility. In contrast, both HMW PEI (Figure 6, upper pictures) and LMW PEI (Figure 6, lower pictures) polyplexes crosslinked after formation showed increased stability after exposure to heparin, suggestive of a successful surface crosslinking of polyplexes. The reduction of the electrophoretic shift of the DNA is related to the degree of crosslinking. Heparin fully displaced DNA from non-stabilized polyplexes. Complete retention of the plasmids was achieved for HMW PEI and LMW PEI at molar ratios of DSP to amines of 0.15 and 0.20, respectively. Treating the polyplexes with less DSP resulted in some retention as well, with only limited stabilization. Even though both polymers showed protection of the DNA against dislocation by heparin, only HMW PEI at the same time retained the polyplex size. Thus, crosslinking of HMW PEI polyplexes after formation seems to be the most favorable strategy to efficiently protect plasmid DNA against polyanionic exchange reactions.

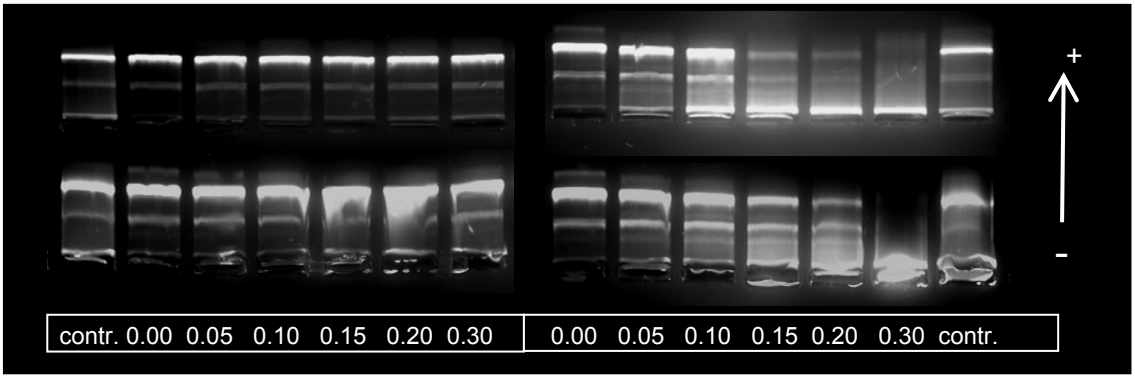


Figure 6: Stability of polyplexes against polyanion exchange. HMW PEI (upper pictures) and LMW PEI (lower pictures) polyplexes challenged with 1 I.U. heparin per 1 µg plasmid. Left: Polyplexes prepared with pre-crosslinked polymer, right: polyplexes crosslinked after polyplex formation. The numbers in each lane represent the molar ratio of DSP/amines.

Stability in high ionic strength medium: The formation of polyplexes between PEI and DNA is an electrostatic process, which is mainly driven by entropic forces arising from the exchange of sodium ions associated with DNA, thereby shifting the equilibrium towards polyplexes and releasing low molecular weight salt. Sufficiently increasing the concentration of the counter ions is known to shift the equilibrium [22]. Light scattering was used to study nanocomplex dissociation, since particulate polyplexes exhibited increased light scattering intensity compared to dissociated polyplexes [22, 58]. For example, PLL/plasmid DNA polyplexes (N/P 2) have been reported to dissociate at a sodium chloride concentration of 1.1 M by measuring the reduction of the intensity of scattered light [22]. For these reasons, the stability of surface crosslinked HMW PEI polyplexes was investigated in high salt concentrations. Enhanced stability due to crosslinking of HMW PEI polyplexes was studied by increasing the concentration of sodium chloride and measuring the resulting intensity of scattered light at a detection angle of 90°. An initial sodium chloride concentration of 150 mM was used to form the polyplexes and the scattered light intensity was calculated relative to this starting value. A decrease in the relative scattering intensity, corresponding to a dissociation of the polyplexes, was observed for uncrosslinked HMW PEI polyplexes immediately after addition of sodium chloride, leading to a relative scattering intensity of < 0.1 at a sodium chloride concentration of 1.5 M (Figure 7). Further addition of sodium chloride did not decrease the relative scattering intensity anymore, suggesting complete dissociation of the polyplexes at 1.5 M. The reaction of

the HMW PEI polyplexes with DSP at a molar ratio of 0.05 and 0.15 resulted in an increase in the relative scattered light intensity as compared to the unstabilized polyplexes. Crosslinking of the polyplexes seemed to increase their resistance against salt induced dissociation. This can be attributed to surface crosslinking, probably in combination with an apparent increase of the polymer molecular weight inside the polyplexes [22]. The higher residual intensity of the crosslinked polyplexes points to an effective crosslinking with polyplexes yielding stable systems even at high salt concentrations of more than 2.5 M.

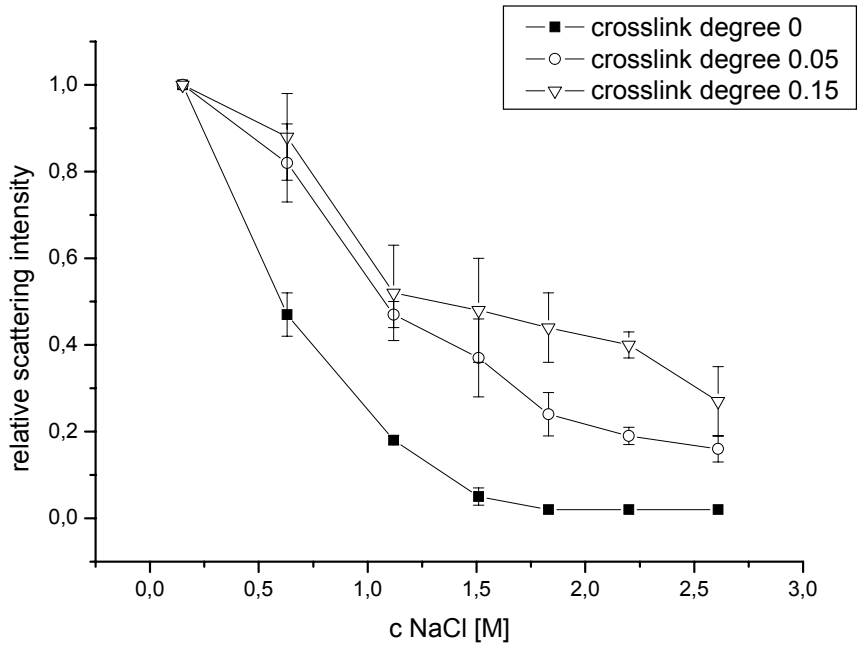


Figure 7: Stability of surface crosslinked HMW PEI polyplexes against high ionic strength. Polyplexes were challenged with increasing amounts of sodium chloride and polyplex dissociation was investigated by light scattering (n = 3).

Interactions of stabilized polyplexes with blood components: Interactions with blood components are likely to occur after injection of polyplexes in the blood stream [9, 10]. Therefore, polyplexes were incubated under in vitro conditions with two major components of the blood, namely albumin and erythrocytes.

Binding of albumin is known to lead to aggregation of colloidal positively charged colloidal particles [14]. Turbidimetric measurements were performed to assess the extent of aggregation. Whereas uncrosslinked PEI polyplexes showed severe aggregation after incubation with albumin, the introduction of crosslinks after polyplex formation was found to significantly reduce the aggregation tendency significantly (Figure 8). In fact, at a crosslinking degree of 0.15, the stabilized polyplexes were completely stable against aggregation. No differences could be found between crosslinked HMW PEI and LMW PEI polyplexes in their interactions with albumin. It is likely that the reduction of the surface charge renders the polyplexes less sensitive to aggregation. Similarly, the reduced aggregation may be caused by less crossbridging of polycationic particles with each other or with albumin, due to the surface crosslinking [59].

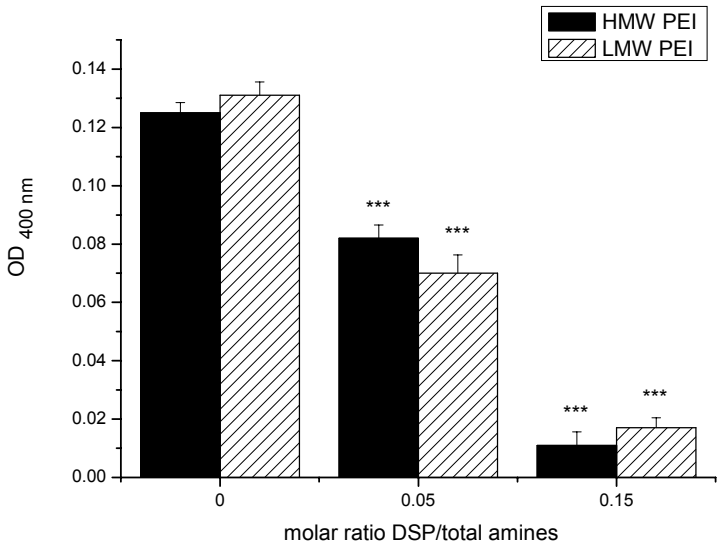


Figure 8: Turbidimetric measurement of albumin induced aggregation of polyplexes crosslinked after complexation. Optical density was measured after 10 min incubation with 0.2 mg/mL bovine serum albumin (*** $p < 0.001$, $n = 3$)

The interaction of the polymers with erythrocytes was studied using a hemolysis assay, which was performed with freshly purified human red blood cells. 5% glucose/25 mM Hepes buffer (pH 7.5) and 1% Triton X-100 was used to define the 0% and 100% values, respectively. The results of the hemolysis assay are shown in Figure 9. In general, HMW PEI polymers and polyplexes showed higher hemolysis as compared to LMW PEI. A hemolysis of 11% was found for uncrosslinked HMW PEI control, which is in good agreement with earlier reports [60]. After crosslinking of the pure polymers, an initial increase in hemolysis could be observed, reaching more than 25% for HMW PEI and about 10% for LMW PEI. The initial increase was followed by decreased hemolysis at higher crosslink degrees, presumably caused by the reduction of the cationic charges, which are known to account for the hemolytic activity of cationic polymers [61]. The hemolysis for both preparation methods was comparable for HMW PEI polyplexes. In contrast, LMW PEI polyplexes were significantly less hemolytic if prepared by crosslinking preformed polyplexes. A higher amount of free polymer in formulations with pre-crosslinked LMW PEI, as indicated by size and stability tests, may account for this observation. Substances are classified as hemolytic if the hemolytic activity is higher than 15% [62]. All stabilized polyplexes prepared with LMW PEI and surface crosslinked HMW PEI polyplexes met this requirement, except at a crosslinking degree of 0.05. Pure crosslinked HMW PEI, however, reached a hemolytic activity of up to 30% at high crosslink ratios, which decreased after complexation with DNA. Even if a certain hemolytic activity of gene transfer vectors has been claimed to be favorable due to destabilization of biological membranes [63], low hemolytic activity is critical for systemic administration.

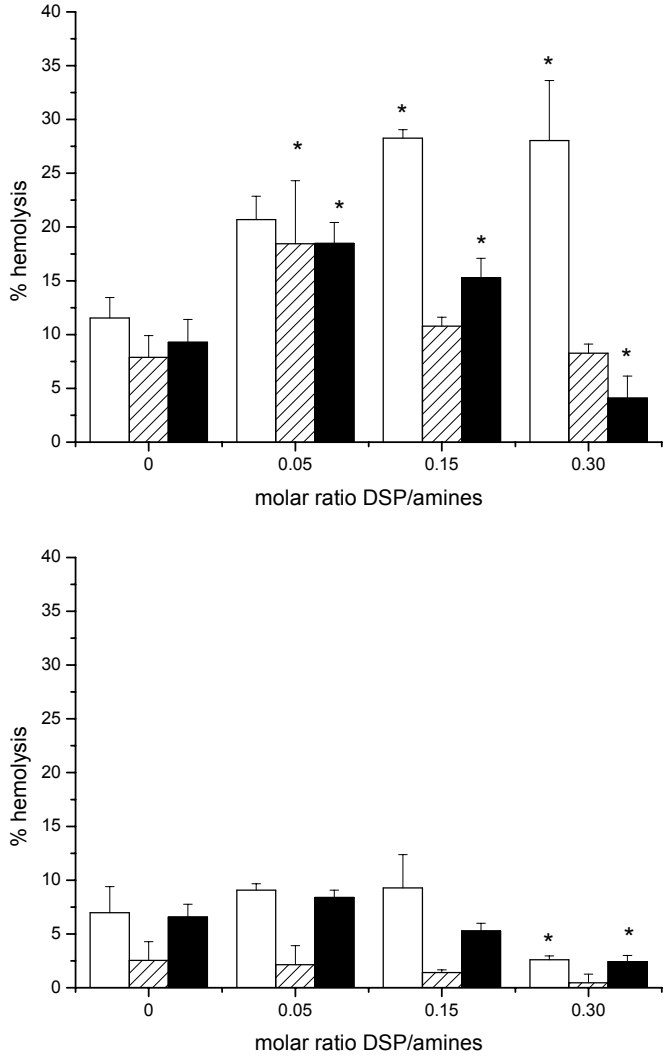


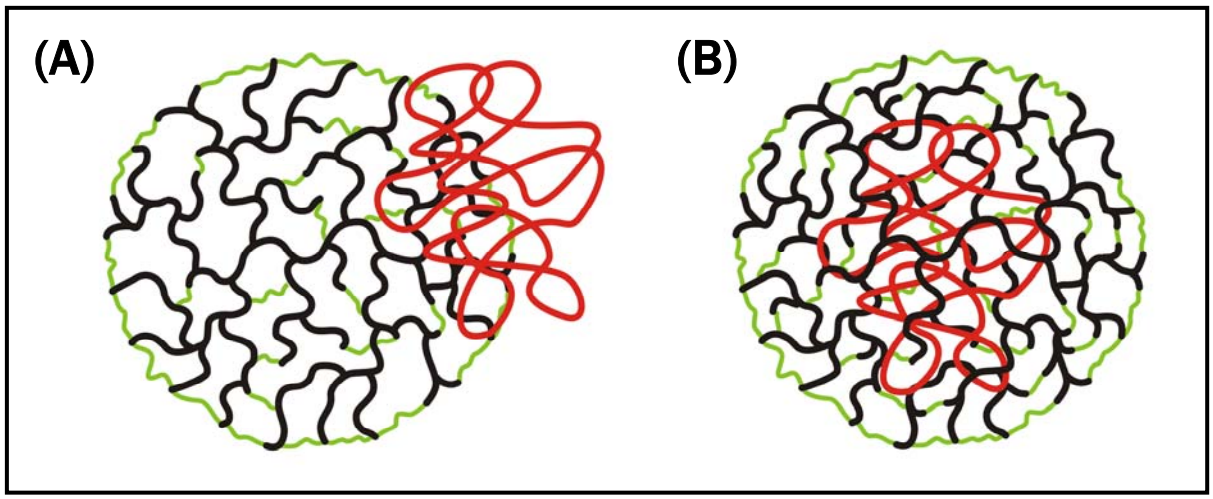
Figure 9: Hemolysis of pure pre-crosslinked polymers (white bars), polyplexes crosslinked after formation (striped bars) and polyplexes prepared with pre-crosslinked polymers (black bars) after an incubation time of 60 min at 37°C. Upper picture: HMW PEI, lower picture: LMW PEI (* = significant differences to the value of the corresponding molar ratio of 0.00, * $p < 0.05$, $n = 3$).

Conclusion

One important prerequisite for systemic gene delivery of non-viral vectors is their stability in the blood stream. Surface crosslinked PEI/plasmid polyplexes were found to show interesting properties for systemic plasmid delivery. The reaction of DSP with PEI

can easily be controlled, allowing adjusting desired degrees of disulfide crosslinking. Our study points to a remarkable influence of the molecular weight of the polymer as well as the crosslinking procedure. Pre-crosslinked PEIs showed decreased plasmid compaction and stabilization properties, suggesting incomplete caging of the DNA (Scheme 1). High molecular weight PEI in combination with surface crosslinking resulted in polyplexes with enhanced stability against dissociation by polyanions and ionic strength of the medium. In addition, the mechanical properties were improved, as shown by AFM indentation studies. Such polyplexes yielded hydrodynamic diameters and zeta potential values compatible with endocytic cellular uptake mechanisms. The resulting vector systems showed improved biocompatibility in terms of albumin and erythrocyte interactions.

Further characterization of the polyplexes in terms of their triggered activation by the cells and in vivo properties is currently under investigation. We believe that this is an advancing step towards the goal of designing stable vectors for intravenous plasmid delivery.



Scheme 1: Proposed differences in polyplex formation of pre-crosslinked polymers or crosslinking of already formed polyplexes. The DNA compaction behavior of pre-crosslinked polymers decreases presumably due to their lowered cationic charge and chain flexibility (A). Introduction of crosslinks after formation results in increased polyplex stability (B). Color scheme: black: branched PEI, red: plasmid DNA, green: crosslinks.

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Chapter 4

Bioreversibly crosslinked nanocarriers based upon Poly(ethylene imine) for systemic plasmid delivery: in vitro characterization and in vivo studies in mice

In preparation for *Journal of Controlled Release*

Summary

Cross-linked poly(ethylene imine) (PEI) polyplexes for intracellular DNA release were generated using a low molecular weight crosslinking reagent, Dithiobis(succinimidyl propionate) (DSP). Disulfide bonds of the crosslinked polyplexes were susceptible to intracellular redox conditions and DNA release was observed using an ethidium bromide exclusion assay and dynamic light scattering. Transfection experiments were performed to elucidate the effect of extra- and intracellular redox conditions. Pharmacokinetics and organ accumulation of uncrosslinked and crosslinked polyplexes were compared and gene expression patterns were measured in mice 24h after intravenous injection.

Crosslinked PEI and plasmid-DNA formed stable polyplexes in a size range of 100-300 nm, with zeta potentials between +16.4 and +26.1 mV. DNA release occurred after cleavage of the disulfide bonds. Cell culture experiments under reducing conditions as well as with GSH loaded cells confirmed the proposed intracellular activation. A significant influence of the intracellular glutathione status on the transfection efficiency was observed.

Pharmacokinetic profiles of crosslinked PEI/ DNA polyplexes in mice after intravenous administration showed higher blood levels for crosslinked polyplexes. These polyplexes accumulated mainly in the liver and the lungs. In vivo transfection data revealed significantly reduced (unwanted) lung transfection while liver transfection predominated. These studies suggest that crosslinked polyplexes are more stable in circulation and retain their transfection efficiency after intravenous administration.

Introduction

Gene therapy using non-viral vectors has received significant interest over the last decade. Despite the current interest in cationic polymer based gene delivery systems, stability of these vectors in the biological milieu as well as their circulation times after intravenous administration need to be improved significantly to allow therapeutic application [1, 2]. While local administration of nucleic acids has been successfully applied in the in the treatment of bladder cancer [3], systemic application of positively charged polyplexes is limited by their rapid clearance from the bloodstream following intravenous injection [4-7]. In fact, most of these vectors are cleared in less than 10 min by first pass organs such as the liver or the spleen [1].

Enhanced stability of polyplexes in blood circulation is thought to be an important prerequisite for successful systemic gene delivery. Also, gene delivery systems sensitive to intracellular trigger mechanisms have been subject of intensive scrutiny as they would allow formation of polyplexes stable in circulation that dissociate after intracellular uptake [8]. Various trigger mechanisms, such as sensitivity to pH changes [9, 10], temperature [11], enzymatic lability to lysosomal enzymes [12] or redox conditions [13, 14] have been put forward in this context [15].

Redox sensitive vector systems rely on the higher intracellular reduction capacity compared to the extra-cellular milieu [16]. Natural cationic polymers playing a role in DNA compaction, such as protamines, were reported to be stable at high ionic strengths due to the presence of disulfide linkages [17]. Thus, a redox triggered destabilization mechanism could be a promising strategy to design synthetic non-viral vectors with engineered intracellular release characteristic and has been. Polyplexes, that are known to be taken up by endocytic pathways [18], may undergo disulfide cleavage in the lysosomal compartments [19]. It is reasonable to assume that the glutathione pathway, which controls the intracellular redox environment [20], is significantly involved in this trigger mechanism. Large differences in the glutathione concentration can be found in different tissues (ranging from 0.5 to 11 mM in liver cells [20]) as well as between the intracellular and extracellular environment (in the cytosol about 1000 fold higher, in the nucleus approximately the same or greater than in the cytosol [16]). This difference in glutathione concentrations could potentially exploited as trigger mechanism. Moreover,

cancer cells resistant to apoptosis or to cisplatin seem to show a higher activity of GST and hence higher glutathione levels, thus displaying increased reduction capacity [21, 22].

PLL crosslinked by disulfide bonds were tested in the context of redox triggered intracellular DNA release and showed reduced transfection efficiency probably due to a failure of endosomal escape [23]. Therefore, we employed PEI as polycation since it possesses an intrinsic endosomal release capacity, known as the "proton sponge effect" [24, 25]. Additionally, polyplex stability, protection of the DNA against DNase I, and in vivo transfection capability of branched PEI have been reported to exceed those of PLL [26] [27].

Tumor tissue is known to be characterized by enhanced vascular permeability and impaired lymphatic clearance [28]. Thus, long circulating vectors are thought to accumulate in tumors due to the EPR effect [1, 29]. A combination of enhanced circulation times due to surface stabilization and redox-triggered intracellular release was believed to be a promising way for intravenous plasmid delivery. Hydrophilic copolymers are known to increase circulation times of polyplexes by steric shielding, thereby reducing unwanted interaction with endothelia or blood compounds, such as erythrocytes and proteins [30]. Redox sensitive polyplexes were obtained by coupling PEG or PHMPA to polycations. Redox sensitive PEGylated PLL polyplexes showed enhanced blood levels in mice, but the effect could be predominantly attributed to PEG [23]. Similarly, no control measurements without PEG were reported for thiolated PEG-PLL showing liver expression in mice [31]. Polyplexes formed with thiolated PEI followed by PHPMA coating enhanced the stability of the polyplexes against polyanion displacement, and plasmid release could be triggered under reducing conditions. An addition of free PEI to polyplexes was necessary to obtain transfection under in vitro conditions and no in vivo data were reported [32].

PEI polyplex stabilization using disulfide bonds without additional steric stabilization based on hydrophilic copolymers was investigated systematically utilizing a low molecular weight crosslinking reagent, DSP, as redox sensitive moiety. This procedure avoids drawbacks of a two-step crosslinking procedure, i.e. initially introducing thiol groups which were subsequently oxidized or crosslinked via thiol reactive copolymers [32, 33]. High molecular weight poly(ethylene imine) (PEI 25 kDa) was used to

complex p-DNA, followed by surface stabilization with DSP by crosslinking the primary amines in PEI. At the same time, potentially cleavable disulfide bridges are incorporated by DSP to allow the release of the DNA. Polyplexes were tested for their sensitivity to reducing environment and the factors influencing DNA release. Pharmacokinetic parameters, biodistribution of the plasmid, and the transfection efficiency in mice were studied after intravenous injection and related to the crosslinking degree of the polyplexes .

Experimental Section

Materials: *Polymers*: Poly(ethylene imine)s (25kDa, HMW PEI and 5 kDa, LMW PEI) were gifts from BASF, Ludwigshafen, Germany. *DNA*: The plasmid pCMV-GL3 encoding the firefly luciferase gene was amplified in JM-109 competent cells and purified using a commercial kit (Qiagen Hilden, Germany). pCMV-Luc plasmid was purchased from PlasmidFactory (Bielefeld, Germany). Salmon testes DNA (Sigma, Taufkirchen, Germany) was used for light scattering experiments. Dithiobis(succinimidyl propionate) (DSP), water free dimethylsulfoxide (DMSO) HPLC grade, glutathione-monoethyl ester (GSHMEE) and D, L-Buthionin-[S, R]-sulfoximin were purchased from Sigma, Taufkirchen, Germany. All other reagents used were of analytical grade.

Formation of PEI/DNA Polyplexes and Crosslinking: Polyplex formation was performed according to a recently reported procedure [34]. Luciferase reporter gene plasmids (pCMV-GL3 or pCMV-Luc) and the appropriate amounts of PEI 25 kDa were dissolved separately in low ionic strength buffer (5% glucose/ 25 mM Hepes at pH 7.5), mixed by vigorous pipetting and incubated for 10 min to allow polyplex formation. Polyplex crosslinking was achieved by adding 0.01 M DSP in DMSO to the preformed polyplexes to reach the desired degrees of crosslinking. The solutions were mixed by vigorous pipetting and incubated for 30 minutes. Polyplexes were prepared at a concentration of 2µg/100µL plasmid and the appropriate amount of polymer to yield an N/P (nitrogen to phosphate) ratio of 7, unless stated otherwise. All degrees of crosslinking are reported as molar ratios between DSP and PEI amines assuming that

PEI 25kDa contains 580 amines per molecule. Calculation of primary amine content was based on ^{13}C-NMR data obtained by a recently reported method [35].

Determination of polyplex size and zeta potential: The hydrodynamic diameters as well as the zeta potentials of freshly prepared polyplexes were measured using a Zetasizer Nano-ZS from Malvern Instruments (Herrenberg, Germany) equipped with a 4 mW He-Ne laser at a wavelength of 633 nm at 25°C. Scattered light was detected at a 173° backward scattering angle with automatic measurement position and automatic laser attenuation. The viscosity and refractive index of pure water at 25°C were used for data analysis. Hydrodynamic diameters and zeta potential were measured in folded capillary cells after 1:3 dilutions with the appropriate buffer and were calculated using the DTS software v4.10. Reference measurements using Malvern size and zeta potential standards were run routinely to verify correct instrument operation. Values are given as the mean of three measurements of 10 runs each.

DNA condensation assay: The DNA condensation was measured by quenching of ethidium bromide fluorescence as described previously [30]. Briefly, quadruplicates of 4 µg of herrings testes DNA were complexed with increasing amounts of polymer in 0.1M borate buffer at pH 7.5, using 96-well plates. After 10 min, polyplexes were crosslinked with DSP in DSMO to achieve the desired crosslinking degrees. After 30 min incubation, 20 µL of a 0.1 mg/mL ethidium bromide solution were added. To allow complete displacement of the DNA, 1 IU heparin was added to each well and incubated for 30 min. DTT solution (0.5 M in water) was added to a final concentration of 15 mM to each well to test the release of DNA after disulfide cleavage. The resulting fluorescence was measured using a Perkin-Elmer LS50 B fluorescence plate reader (Perkin-Elmer, Rodgau, Germany) with an excitation wavelength of 518 nm with a 15 nm slit, a 515 nm emission filter and an emission wavelength of 605 nm with a 20 nm slit. Results were transformed into relative fluorescence values (rel $F = F_{sample}/F_{DNA}$). A value of 1 is attributed to the fluorescence of ethidium bromide with plain DNA. Experiments were performed in quadruplicate and results are given as mean ± standard deviation.

Dynamic light scattering: The intensity of scattered light of the polyplex solutions was assessed as kilo counts per second (kcps) with a fixed pinhole (200 µm) on an Autosizer Lo-C from Malvern, Herrenberg, Germany (90° angle, 10 mW HeNe laser, 633 nm). Polyplexes were prepared according to the procedure for size measurements using herring testes DNA in 150 mM sodium chloride. Reducing conditions were obtained by adding 0.5 M DTT in water to give a final concentration of 15 mM DTT. Dissociation of the polyplexes was achieved by adding aliquots of 5 M sodium chloride solution. After each addition, polyplexes were incubated for 5 min and the scattered light intensity was measured as kcps. Results are given as the mean of triplicate experiments ± standard deviation.

In vitro transfection: 2 µg of luciferase reporter gene vector (pCMV-GL3) and the appropriate amount of polymer for N/P 7 were dissolved separately in 50 µL 5% glucose/25 mM HEPES at pH 7.5, mixed by vigorous pipetting and incubated for 10 min. 0.01 M DSP in DMSO was added to crosslinking the polyplexes (incubation 30 min). Transfection activity of the crosslinked polyplexes was studied in NIH-3T3 (NIH 3T3, Swiss mouse embryo, ATCC, Rockville, Maryland). Cells were seeded at a density of 3.0x104 cells per well on 24 well cell culture plates, 24 h prior to the transfection experiments. 100 µL of polyplex solution were added to each well containing 1 mL fresh medium and incubated for 4 hours or 8 hours, respectively. The medium was replaced and the cells were allowed to grow for an additional 44 hours or 40 hours, as indicated. The luciferase expression (luciferase assay reagent Promega, Mannheim, Germany) and protein concentration (BSA assay kit, Pierce, Rockford IL, USA) were determined in the cell lysate. As appropriate, cells were incubated with buthionin-sulfoximin (BSO, Sigma) solution (5 µM in fresh medium) for 16 hours or with glutathione-monoethyl ester (GSHMEE, Sigma) solution (5 mM in fresh medium) for 1h prior to polyplex addition. All experiments were performed in quadruplicate. Data were expressed as the means ± standard deviation in nanograms of luciferase per milligram of protein.

In vivo experiments: All animal experiments were carried out according to the German law of protection of animal life and approved by an external review committee for laboratory animal care.

Pharmacokinetic and distribution studies: Plasmids were labeled according to the manufacturer's protocol using a Nick translation kit N5000 (GE Healthcare, Germany). 100 ng pCMV-Luc were labeled with 32-P-alpha-CTP (Hartmann, Germany). The reaction mixture was purified using microspin columns (Wizard SV Gel and PCR Clean-Up System, Promega, Germany) and the labeled plasmid was used immediately after synthesis.

Polyplex formation and crosslinking were performed according to the procedure for in vitro testing. 2 µg of pCMV-Luc plasmid spiked with ^{32}P labeled plasmid and the appropriate amount of polymer were allowed to form polyplexes, which were subsequently crosslinked with 0.01 M DSP in DMSO to reach the desired crosslinking degree. 200 µL of the polyplex solution was injected in anaesthetized male balb/c mice via the tail vein. Blood samples of 25 µL were drawn from the retrobulbar plexus at the indicated time points. After 120 min, mice were sacrificed and organs were excised. Organ samples were dissolved in Soluene 350 (PerkinElmer, Germany) and blood samples were dissolved overnight in a 1:1 mixture of isopropanol/Soluene 350 at 55 °C and subsequently bleached with 200 µL hydrogen peroxide. 15 mL of scintillation cocktail (Hionic Fluor®, PerkinElmer, Germany) was then added and mixed. Activity of ^{32}P in each sample was determined using a TriCarb 2900 liquid scintillation counter (PerkinElmer, Germany) with a counting time of 10 min, active static controller and half-life correction. Disintegrations per minute (DPM) were calculated with a ^{32}P quench curve using tSIE/AEC as quench indicator. The injected dose was calculated based on activity measurements of the injected solution. All experiments were performed at least in quadruplicate and the AUC was determined using a non-compartmental logarithmic algorithm. Concentration-time data were fitted to a biexponential disposition function ($C(t) = Ae^{-\alpha t} + Be^{-\beta t}$, iterative reweighing with $1/(C_{calc})^2$, n = 3) using the software Kinetica 1.1. from Simed (Créteil Cedex, France). Akaike and Schwartz statistical criteria provided by the software were inspected. Plasmid concentrations in the samples were calculated as percent of the injected dose

per organ or as percent injected dose per mL, respectively. Results are given as mean ± standard deviation.

In vivo transfection studies: Polyplexes for in vivo gene expression studies were formed as described above, using 25 µg of pCMV-Luc and, if appropriate, crosslinked with DSP in DMSO. 200 µL of polyplex solution were injected through the jugular vein. After 48 h mice were sacrificed and the organs were removed. Liver, lung, spleen, kidneys and heart were dissolved in lysis buffer provided by Promega, Germany. After centrifugation, an aliquot of the supernatant was assayed for luciferase using a commercial kit (luciferase assay reagent Promega, Mannheim, Germany) and photon counting with a luminometer (Sirius Berthold, Germany). In vivo transfection studies were performed at least in triplicate and results are given as the average ± standard deviation.

Statistics: Experiments were performed at least in triplicate. Significance between the mean values was calculated using one-way ANOVA analysis using Origin® 7.0 software (OriginLab Corporation, Northampton, MA). Probability values < 0.05 were regarded to be significant.

Results

Characteristics of crosslinked polyplexes: Size and surface charge: Polyplexes of PEI 25 kDa and plasmid DNA were formed at an N/P ration of seven as frequently reported for in vitro as well as for in vivo studies [36, 37]. Previous studies showed that PEI polyplexes did not aggregate in Hepes buffered glucose [38]. Therefore, polyplexes were allowed to form for 10 min in 5% glucose/25mM Hepes buffer at pH 7.5 and were subsequently crosslinked by the addition of the appropriate amount of DSP stock solution to generate the desired crosslinking degrees. The resulting hydrodynamic diameters as well as the surface charge of the crosslinked polyplexes at N/P 7 are reported in Table 1. Uncrosslinked PEI 25 kDa/DNA polyplexes showed a hydrodynamic diameter of about 100 nm, which is in good agreement with earlier results [34]. The introduction of surface stabilization by crosslinking with DSP only

marginally increased the polyplex size to about 150 nm for the moderately crosslinked samples and to 300 nm for the highest crosslinking degree. The surface charge of the stabilized polyplexes decreased with increasing crosslinking degree. Zeta potential values of + 28.1 mV for the unmodified polyplexes were reduced to +21.6 mV for moderate crosslinking degree and further reduced to +16.4 mV for the highest tested crosslinking degree. Most likely, the loss of charged primary amines is due to the reaction with DSP, which resulted in neutral amide bonds [33, 39].

Crosslinking degree	Hydrodynamic diameter [nm]	Zeta potential [mV]
0.00	102 ± 3	28.1 ± 1.5
0.05	100 ± 2	26.1 ± 3.7
0.15	154 ± 17 **	21.6 ± 1.3 **
0.30	299 ± 7 ***	16.4 ± 1.3 ***

Table 1: Size and zeta potential of surface crosslinked PEI 25 kDa/plasmid DNA polyplexes. (differences between uncrosslinked and crosslinked polyplexes, ** $p < 0.01$, *** $p < 0.001$, $n = 3$)

DNA condensation: Since polyplex formation between PEI and DNA is based on electrostatic interaction, a negative effect of the lowered charge density on the condensation properties needs to be taken into account. Therefore, the DNA compaction properties after the crosslinking reaction were tested using an ethidium bromide exclusion assay. While free ethidium bromide only shows weak fluorescence, fluorescence intensity strongly increases when it intercalates with double helical polynucleotides [40, 41]. The results depicted in Figure 1A revealed that increasing degrees of crosslinking resulted in only slightly higher fluorescence intensities compared to uncrosslinked polyplexes, indicating that the crosslinking reaction does not induce a significant loss of plasmid condensation properties. Presumably, the enhanced polyplex stability compensates for the decreased cationic charge of the charge consuming reaction. A similar effect was recently observed for linear PLL polyplexes

[33] In the case of branched PEI, however, the improved DNA condensation efficiency of branched polycations [42] could also explain our results.

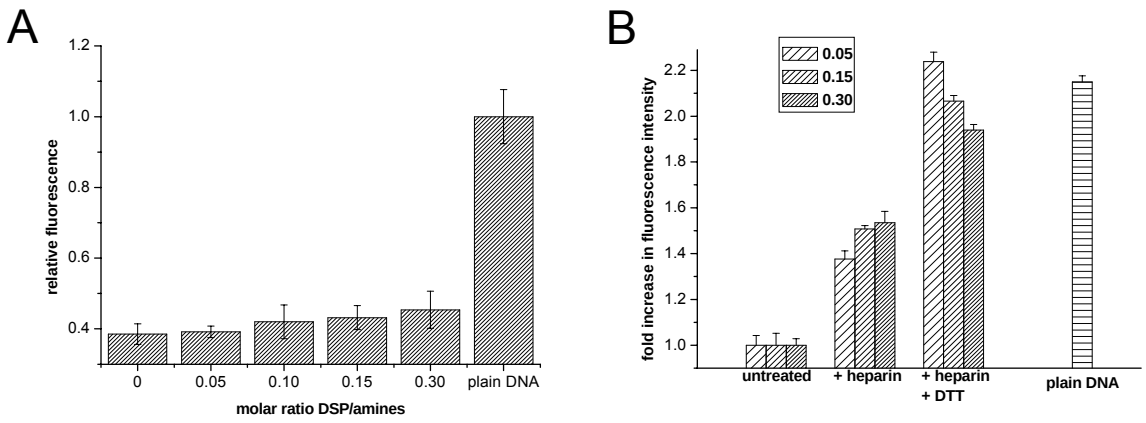

Figure 1: Ethidium bromide exclusion assay shows no significant changes in DNA condensation properties after crosslinking (A, left). Polyplexes treated with 1 IU heparin/4 µg DNA are more stable in non-reducing conditions and release DNA completely after addition of 15 mM DTT (B, left). Values were significantly different for both increasing crosslink degrees and different treatments.

Influence of reducing environment on DNA release: The stability of the crosslinked polyplexes is a major prerequisite for their application in circulation [1, 43]. DNA can be released by competitive reaction with polyanions, however, this should occur only after cellular uptake for transfection to be effective [44]. To limit DNA release to the intracellular environment, we incorporated a biodegradable disulfide bond for redox triggered activation after cell uptake [13, 14]. To test the stability of the polyplexes as well as their susceptibility to reducing conditions, a modified ethidium bromide assay was used as a marker for release of DNA [23]. Heparin was chosen as a model polyanion [45] and dissociation of the polyplexes induced by heparin was monitored. The effect of the addition of heparin and the reducing agent dithiothreitol, DTT, on the DNA release behavior of uncrosslinked polyplexes is depicted in Figure 1B. After challenging the stabilized polyplexes with only 1 IU heparin, the observed fluorescence was found to be lower than the fluorescence of the plain DNA control, indicating successful crosslinking. However, fluorescence increased compared to untreated control, indicating a certain DNA release. Interestingly, a slightly better accessibility for

ethidium bromide and, therefore, more free DNA, was found for polyplexes with the higher crosslinked polyplexes. This may be attributed to the reduced amount of cationic primary amines and is in accordance with the results depicted in Figure 1A. In contrast to the polyplex behavior under non-reducing conditions, incubation with 15 mM DTT for 30 min resulted in a remarkable release of the DNA for the crosslinked polyplexes. Since the fluorescence of the reduced polyplexes was found to be similar to the fluorescence of free DNA control, dissociation after removal of the stabilization seems to have occurred. Indeed, the polyplexes with the lowest degree of stabilization (molar ratio DSP/amines = 0.05) showed the highest DNA release. DSP crosslinking of the polyplexes resulted in increased polyplex stability against polyanion exchange, which compensates for the effect of charge loss and allows the release of the DNA under intracellular reducing conditions by exposure to polyanions.

Light scattering was used to assess the stability of nanocomplexes [23, 46]. Polyplex dissociation induced by high concentrations of sodium chloride resulted in a decrease in scattered light intensity [46, 47]. Figure 2 shows the results from experiments using non-crosslinked polyplexes, polyplexes stabilized at a crosslinking degree of 0.15 and crosslinked polyplexes after reduction with 15 mM DTT. A decrease in scattering light intensity was observed for uncrosslinked polyplexes immediately after sodium chloride addition, and the lowest scattering intensity occurred at 1.5 M sodium chloride. In contrast, stabilized polyplexes were not as susceptible to sodium chloride induced dissociation as unstabilized ones. However, after removal of the stabilization by reduction with 15 mM DTT, a decrease in scattering intensity was also observed for the crosslinked polyplexes, resulting in scattering intensities comparable to the control. These results strongly suggest that the surface stabilization can be removed by a reducing environment, and DNA can be released from the polyplex.

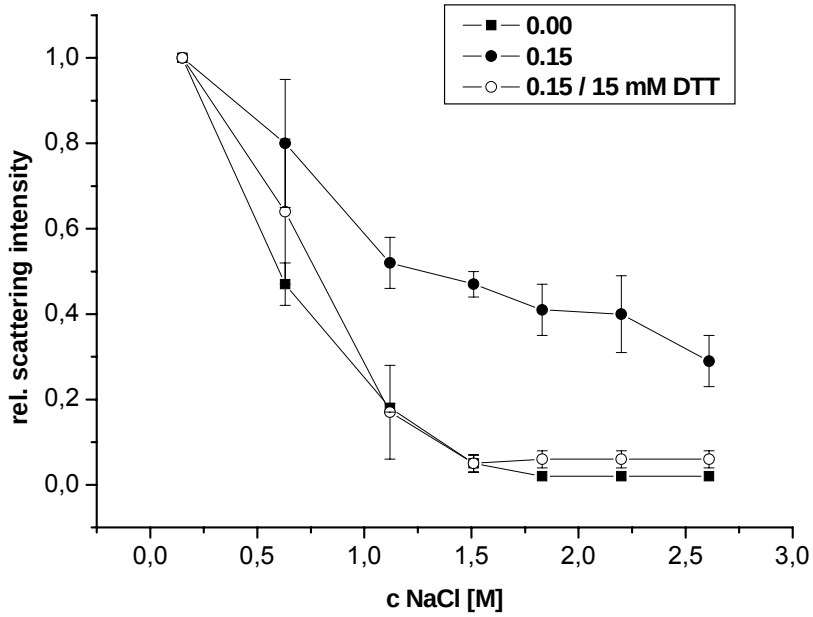

Figure 2: Increased polyplex stability against high ionic strength is completely reversible under reducing conditions.

In vitro transfection results:

Influence of environmental reducing conditions: To assess the in vitro transfection efficiency of crosslinked polyplexes, NIH 3T3 mouse fibroblasts were transfected with pGL3 luciferase coding plasmid using polyplexes prepared at different crosslinking degrees. The effect of reducing conditions in the transfection medium and the influence of the incubation time were tested.

The results of transfection experiments depending on the concentration of reducing DTT in the medium are depicted in Figure 3A. In nonreducing conditions (0 mM DTT), the relative transfection efficiency decreased with increasing crosslinking degree in a concentration dependant manner, supporting the assumption of enhanced stability of the carrier in the cytoplasm, and therefore of a decrease in the amount of released DNA. Depending on the reducing conditions, however, the negative effects of increased polyplex stability on the transfection efficiency at higher crosslinking degrees could be compensated. After the addition of DTT to the transfection medium, a recovery of the transfection efficiency of the stabilized polyplexes could be observed, even for the highest crosslinking degree tested.

These cell culture experiments corroborate the hypothesis of an efficient release of the DNA by reduction of the disulfide bonds, which has been examined in the above reported physicochemical experiments. Recently, thiol modified polycations were used to complex DNA and polyplexes were crosslinked by oxidizing the thiols after polyplex formation [33, 48, 49]. By contrast, the carrier systems presented here introduce the stabilization in a one-step procedure. Therefore, the slightly reduced condensation efficiency is immediately compensated by surface stabilization of the polyplexes. A charge lowering crosslinking strategy was reported to enhance DNA release and thereby transfection [33]. Indeed, polyplexes treated with the highest amount of DTT showed up to 500% increased transfection efficiency compared to uncrosslinked ones. Thus, lowered charge density and reduced stabilization in a reducing intracellular environment may lead to synergistic DNA release from the polyplexes, supporting this hypothesis.

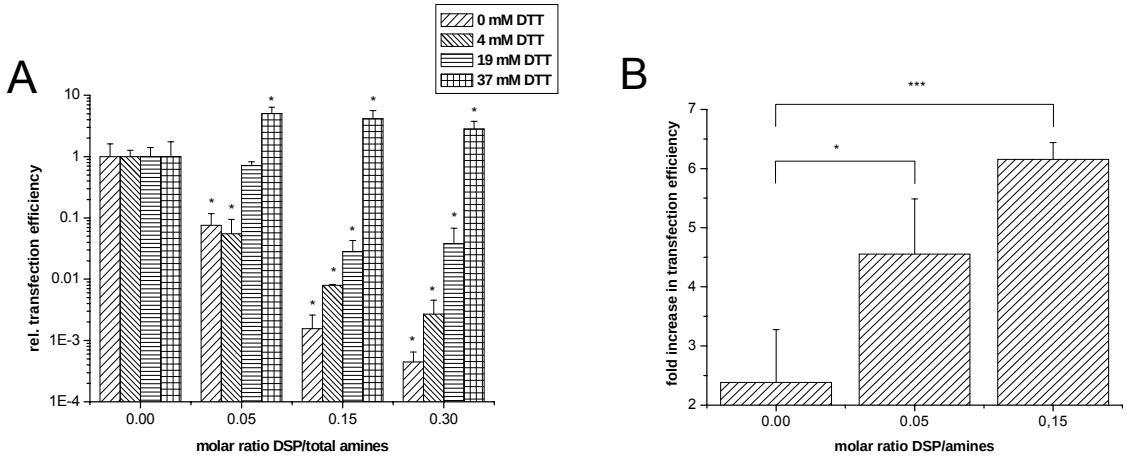

Figure 3: Transfection efficiency in NIH-3T3 fibroblasts for different crosslinking degrees and reconstitution of the transfection by reduction with DTT (A). Prolonged incubation times significantly enhanced the transfection efficiency of crosslinked polyplexes (B) (differences between uncrosslinked and corresponding crosslinked polyplexes are marked with an asterisk, * $p < 0.05$, *** $p < 0.001$, $n = 4$).

Influence of incubation time: Any additional step involved in the release of DNA from the particles, such as unshielding by removal of the crosslinks in reducing conditions, might be expected to change the transfection efficiency in a time dependant manner [50]. In this case, the release kinetics should be influenced in a way comparable to the

studies of the DNA release after disulfide cleavage. To test this hypothesis, the transfection efficiency was compared at two different exposition times of the cells to the polyplexes. The uptake of polycationic polyplexes is believed to generally take place within 4 h [51-53] and the disulfide cleavage takes place in the range of 30 min (see above). Therefore, incubation times of the cells with the polyplexes for 4 h and for 8 h were compared (Figure 3B). A general increase in transfection efficiency was observed for all polyplexes incubated at the longer incubation interval of 8 h, suggesting that a certain amount of polyplexes may also be internalized in the time range between four and eight hours, as a recent report suggested [41]. However, the increase was found to be far more pronounced for the crosslinked polyplexes than for the uncrosslinked ones. Hence, one can assume that the crosslinked polyplexes undergo an additional release step after being taken up by the cells, resulting in higher DNA release and transfection. Presumably, the reduction of the disulfides accounts for this additional step. Again, higher crosslinking degrees showed an even more pronounced increase in transfection, similar to the transfection results shown above, supporting the assumption of enhanced DNA release due to charge loss after removal of the stabilization.

Influence of intracellular reducing conditions: Transfection activities of crosslinked polyplexes were investigated in relation to the redox status of the transfected cells. Glutathione, which controls the intracellular redox environment [20], was tested for its influence on the transfection efficiency of crosslinked and uncrosslinked polyplexes. BSO, which is known to block glutamyl-s-transferase, a key enzyme in the glutathione synthesis, was used to reduce the amount of glutathione in the cytosol. In a separate experiment, we increased the concentration of intracellular glutathione by preincubating the cells with a membrane crossing precursor of glutathione, glutathione-monoethylester (GSHMEE).

The results obtained using cells with increased glutathione concentration after preincubation with GSHMEE are depicted in Figure 4A. Uncrosslinked polyplexes and polyplexes stabilized at a crosslinking degree of 0.05 were tested here. When studying the transfection efficiency between cells not preincubated and preincubated cells, a 2.5 fold increase was measured only for the stabilized polyplexes. In contrast, unstabilized polyplexes were not sensitive to the amount of GSHMEE in the medium, and therefore,

no differences could be found between transfection in GSHMEE treated and untreated cells.

Cells with depleted glutathione after incubation with BSO showed a similar trend. Generally, reduced transfection efficiency was found for all polyplexes at increased crosslinking degrees, as expected.

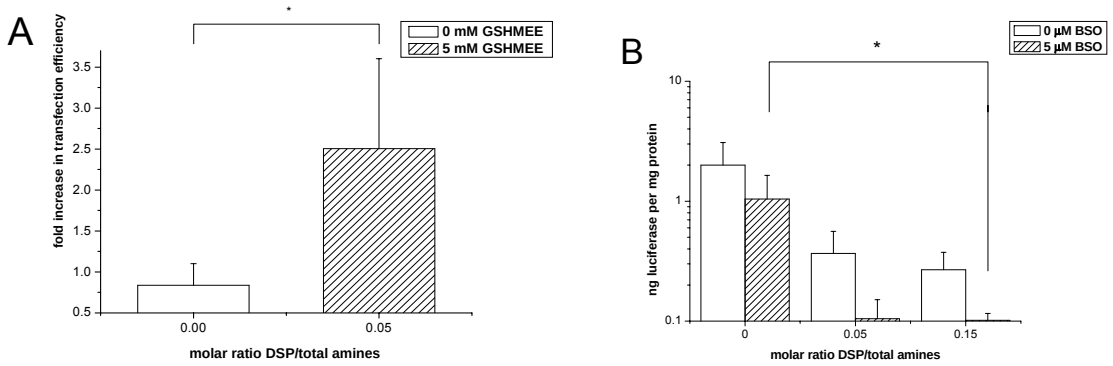

Figure 4: Influence of the intracellular GSH status on the transfection efficiency of crosslinked polyplexes. Increasing the intracellular GSH concentration after incubation with GSHMEE (A) and GSH depletion after incubation with BSO (B) affects only redox sensitive crosslinked polyplexes.

However, only the redox sensitive, crosslinked polyplexes tested with BSO preincubated cells displayed significantly reduced transfection efficiency, whereas uncrosslinked polyplexes again were not influenced by the glutathione status of the cells (Figure 4B). Both complementary tests point to glutathione as a major factor contributing to the selective bioreversibility of the surface stabilization due to disulfide reduction. Results from Carlisle et al. also point to the possibility of redox activation. However, the reported PHPMA stabilized PEI-SH polyplexes only produced certain levels of in the presence of 80% free PEI [32]. Here, crosslinked polyplexes also showed reduced transfection, reflecting the effective polyplex stabilization. However, the transfection potential could be reestablished without free PEI. Moderately crosslinked polyplexes without DTT treatment even reached about 5-10% of the transfection efficiency of unmodified ones, which emphasizes the capability of the cells to remove this kind of stabilization.

In vivo results:

Pharmacokinetics and biodistribution: In order to determine the effect of polyplex stabilization in vivo, we evaluated the surface crosslinked polyplexes in terms of their blood level profiles as well as for their organ deposition. For in vivo testing of the crosslinked polyplexes, pCMV-Luc plasmid was labeled via nick translation with ^{32}P-CTP. To ensure the integrity of the label, all in vivo experiments were performed immediately after plasmid labeling and the pharmacokinetics and biodistribution were tested in a time range of 60 min and 120 min, respectively. Polyplexes were prepared with 2 µg unlabeled plasmid spiked with ^{32}P-plasmid and injected into the tail vein of anaesthetized male balb/c mice. Blood and organs samples were processed for scintillation counting. Pure, uncomplexed plasmid was quickly removed from the bloodstream, with plasma levels of about 1% of the injected dose/mL of blood after 60 min, which is similar to earlier reports [54]. Generally, complexation to PEI 25 kDa decreased the clearance of 32P plasmid from the circulation and significantly enhanced plasma levels were observed (Figure 5), in good agreement with earlier reports [2].

After surface crosslinking of the polyplexes, the blood levels after 60 min increased for the lower tested crosslinking degree of 0.05, reaching levels of 4.65 ± 0.76% of injected dose/mL of blood 60 min after the injection compared to 3.97 ± 0.27% for the non-treated polyplexes. Presumably, a minimum crosslinking degree needs to be achieved to enhance the stability in the bloodstream and, thereby, reduce plasmid degradation. Indeed, after enhancing the polyplex stability with a crosslinking degree of 0.15, significantly higher blood levels of 5.28 ± 0.82% of injected dose/mL of blood were found. A maximum increase in the blood levels of 73% between uncrosslinked polyplexes and polyplexes with a crosslinking degree of 0.15 was found 15 min after injection, and a 36% increase in blood levels persisted up to 60 min after injection.

The area under the curve (AUC) for 0-60 min was calculated using a non-compartmental logarithmic function, and additional pharmacokinetic parameters were obtained by fitting the concentration-time data to a two-compartment model. The area under the curve (AUC 0-60 min) of the crosslinked polyplexes was found to rise with increasing crosslinking degree (Table 2). AUC values for the crosslinking degree of 0.15 were significantly higher than those for uncrosslinked polyplexes, indicating a higher amount of DNA available for tissue uptake. Additionally, most animal data could

be fitted to a two-compartment model, as recently suggested [7, 55]. Values obtained for AUC using this biexponential equation also revealed a significant increase in polyplex availability, increasing form 766% ID/mL*min to 2435% ID/mL*min (Table 2). The slower clearance of the crosslinked polyplexes from the circulation could be ascribed to both a longer alpha phase ($t_{1/2\ alpha}$ increased from 2.04 min to 5.77 min) as well as a longer beta phase ($t_{1/2\ beta}$ increased from 76.25 min to 261.73 min). These longer circulation times are believed to be due the hindered dissociation of the DNA from the polyplex after stabilization, as suggested by the ethidium bromide assay reported above. Another explanation could be the reduced surface charge of the polyplexes, leading to a lower interaction with blood components and, subsequently, less deposition in the lungs [56].

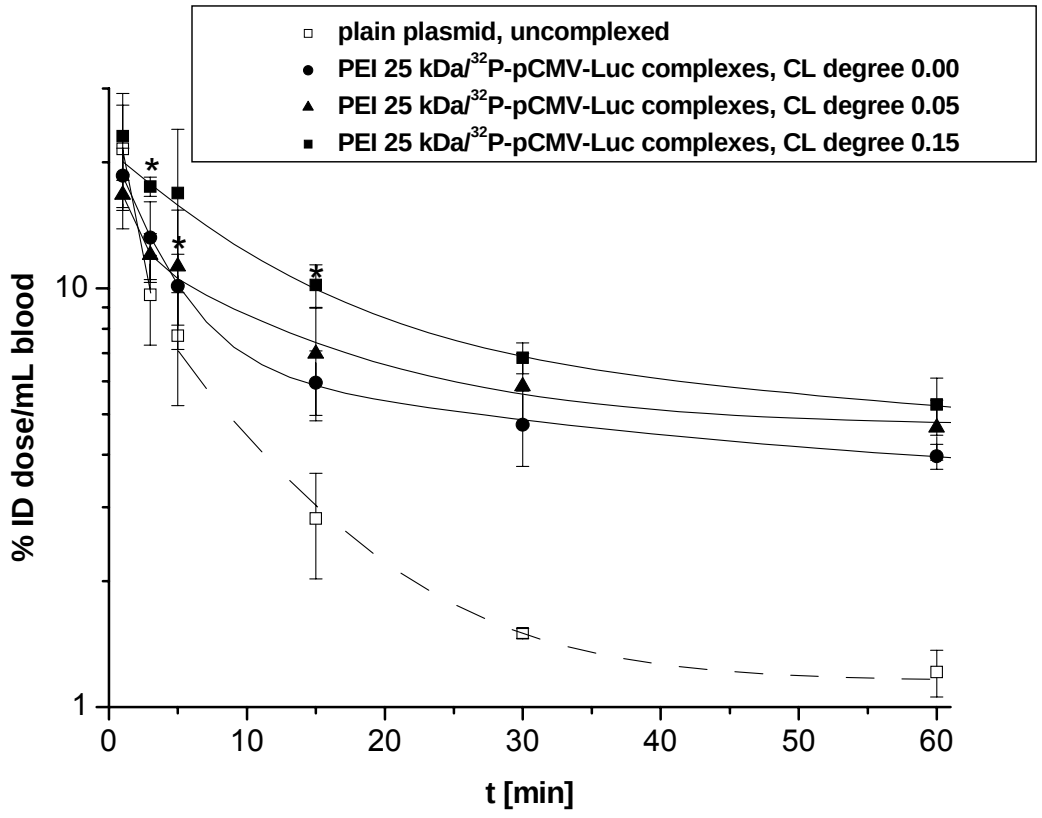

Figure 5: Blood level profiles of 2 µg 32P-pCMV-Luc/PEI 25 kDa polyplexes in male balb/c mice. Plain plasmid is eliminated rapidly, whereas plasmid concentration increases significantly with increasing crosslinking degree.

To our knowledge, these data suggest for the first time that polyplex stabilization using low molecular weight crosslinkers can improve systemic availability of polyplexes after intravenous administration. Polycationic polyplexes formulated with hydrophilic copolymers were also reported to improve circulation times by reducing the surface charge of the polyplexes and the interactions with blood components [29, 57, 58]. However, the elevated blood levels of the surface crosslinked polyplexes here were obtained without additional steric shielding. In fact, it can be anticipated that the combination of surface stabilization with steric shielding could lead to synergistic effects. This hypothesis is currently under investigation in our laboratories.

Crosslink degree	AUC [% ID/mL * min]		
0.00	two-compartment	766 ± 112	*
0.05	two-compartment	2027 ± 1811	
0.15	two-compartment	2435 ± 1253	
0.00	non-compartment	361 ± 24	**
0.05	non-compartment	410 ± 82	
0.15	non-compartment	535 ± 45	

Crosslink degree	C max [% ID]	A [% ID/mL]	$T_{1/2\ alpha}$ [min]	B [% ID/mL]	$T_{1/2\ beta}$ [min]
0.00	23..39 ± 6.70	16.79 ± 6.57	2.04 ± 0.94	6.60 ± 0.32	76.25 ± 13.39
0.05	23.15 ± 0.41	17.47 ± 0.13	1.97 ± 0.83	5.68 ± 0.54	252.85 ± 242.50
0.15	27.90 ± 6.90	20.85 ± 5.64	5.77 ± 3.79	7.04 ± 2.83	261.73 ± 162.51

Table 2: Pharmacokinetic data for 2 µg ^{32}P-pCMV-Luc/PEI 25 kDa polyplexes in male balb/c mice. AUC and plasmid half life increase with increasing crosslinking degree (differences between uncrosslinked and crosslinked polyplexes: * $p < 0.01$, ** $p < 0.001$).

Biodistribution: Figure 6 represents the tissue distribution data at 120 min after intravenous injection of polyplexes prepared with 2 μg of plasmid. Plain plasmid was quickly degraded, therefore, only negligible amounts of radioactivity were found in the tissue after 120 min. Uncrosslinked PEI polyplexes were substantially located in the liver. Spleen and kidney were also identified as major organs of plasmid accumulation, in good agreement with earlier results [2]. Surface crosslinking of the polyplexes was observed to enhance their accumulation in the liver from 15.9 to 25.7% ID/organ. For the highest crosslinking degree, a slightly higher lung deposition accompanied by a reduction in kidney deposition was also found. Interestingly, the longer circulating polyplexes, as indicated by the blood level profiles, were primarily deposited in the highly perfused organs [7]. Nishikawa et al. reported organs such as the kidneys and liver to have a higher blood flow, and therefore, polyplex clearance in these organs was higher [59]. In addition, the presence of discontinuous or fenestrated endothelia in the vascularisation of the liver and spleen may facilitate the accumulation in these tissues [56]. Indeed, our results are in agreement with these considerations. This indicates that polyplex crosslinking seems to be favorable for deposition in these organs. Presumably, polyplexes intended to passively target other tissues with highly fenestrated endothelia, such as tumor tissues, could also benefit from this type of stabilization. Tumor accumulation was recently related to longer circulation times of stabilized polycationic polyplexes [1, 29, 58].

Due to higher concentration of glutathione in liver tissue compared to lung tissue [20], a favorable release of the DNA from the crosslinked polyplexes with redox sensitive disulfide bonds in the liver tissue can be anticipated.

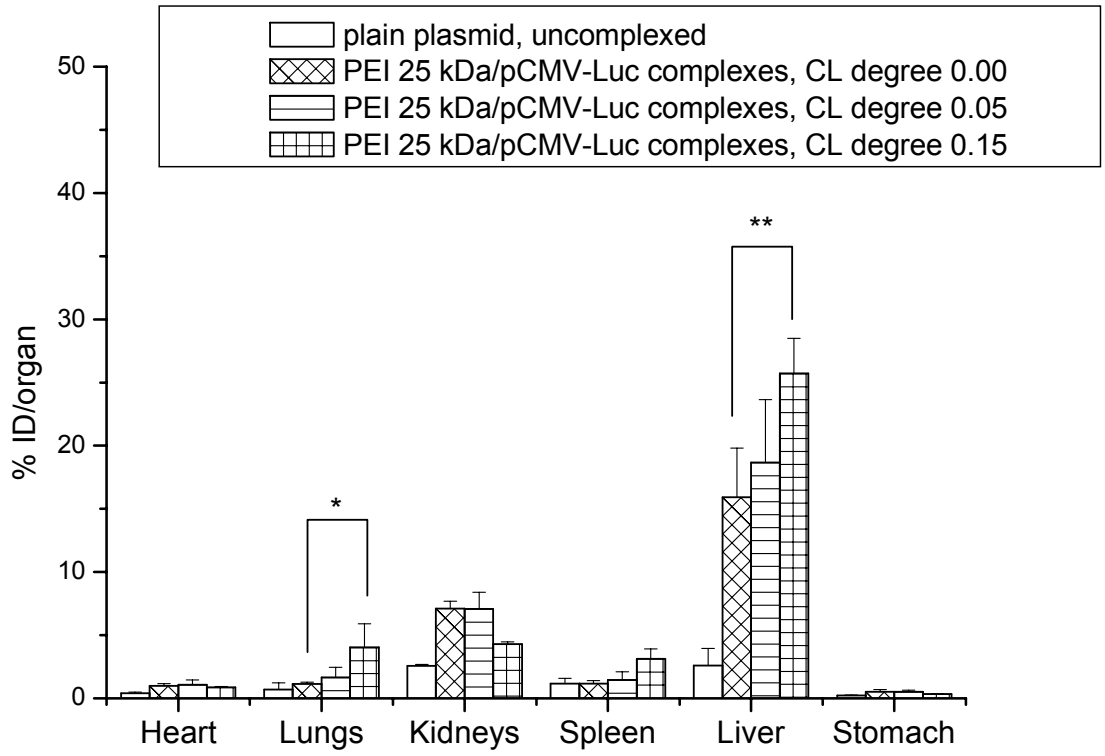

Figure 6: ^{32}P-pCMV-Luc/PEI 25 kDa polyplexes were primarily deposited in the liver. Increased crosslinking degrees lead to predominant deposition in the liver, lungs, spleen and kidneys. (differences between uncrosslinked and crosslinked polyplexes: * $p < 0.05$, ** $p < 0.01$)

In vivo transfection: To test this hypothesis, luciferase expression patterns were tested 24 h after administration of 25 µg of pCMV-Luc polyplexes (Figure 7). Polyplexes with PEI 25 kDa were injected into the tail vein of male balb/c mice and resulted in considerable expression in the liver as well as in the lungs, in accordance with earlier reports for polycationic polyplexes [2, 6]. Other organs such as the heart and kidneys were only marginally transfected. Increasing crosslinking degrees changed the luciferase expression pattern in an organ dependant manner. A significant decrease in lung transfection was observed for both crosslinking degrees, resulting in a decrease of about two orders of magnitude for a crosslinking degree of 0.15. No significant differences in liver transfection could be observed, but spleen transfection tended to increase with increasing crosslinking degrees, in agreement with the organ deposition patterns.

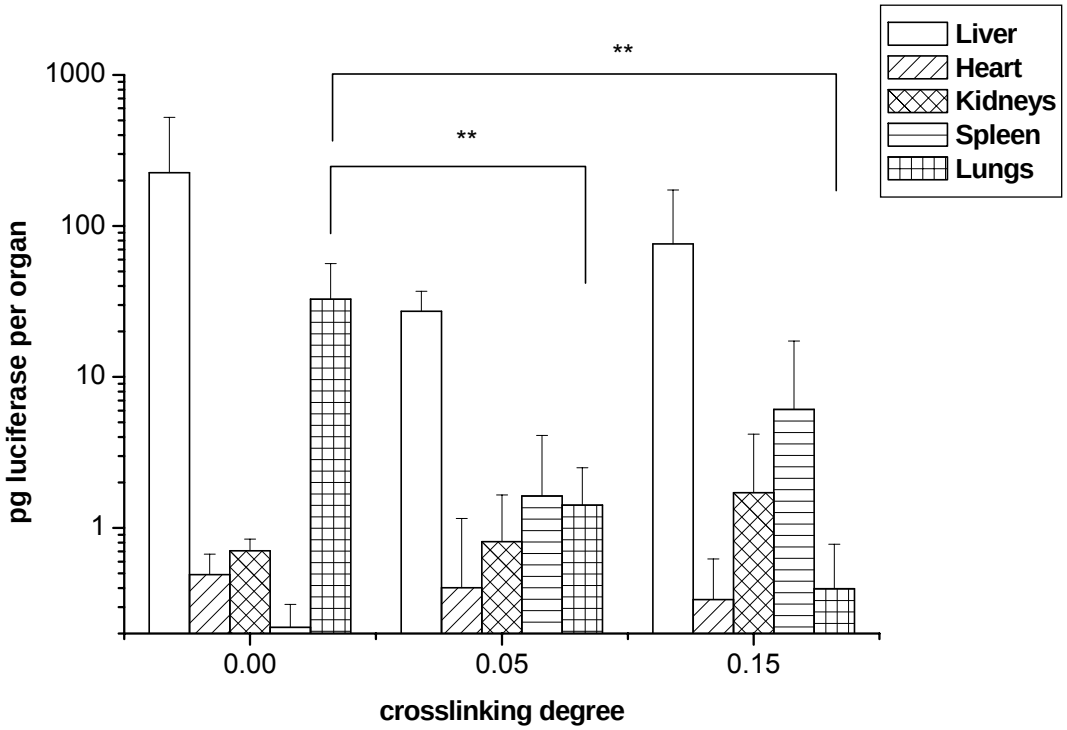

Figure 7: Gene expression pattern of pCMV-Luc/PEI 25 kDa polyplexes at different crosslinking degrees. No significant change was observed for liver transfection, whereas lung transfection was remarkably reduced. ** $p < 0.02$)

Interestingly, the lung transfection does not agree with the lung deposition, which was found to be slightly higher for the highest crosslinking degree. However, it is known that biodistribution and gene expression do not necessarily coincide [60]. In addition, it is possible that the slight increase in lung deposition may be attributed to a small amount of larger polyplexes trapped in the lung capillaries after crosslinking [2, 56]. Since both GSH and glutathione-S-transferase levels were reported to be 5-60 fold lower in lung tissue than in the liver [20, 61], a reduced plasmid release from crosslinked polyplexes is likely to be a cause of the reduced gene expression found in the lungs. By contrast, the higher redox potential of the liver compared to the lungs might explain the higher levels of liver transfection, which were less affected by the crosslinking degree, thus leading to an enhanced DNA release. The significant reduction of unwanted lung transfection displays a major advantage of the crosslinked polyplexes.

Conclusion

The aim of this study was to design stabilized polyplexes using redox sensitive disulfide bonds to prolong circulation times while maintaining transfection efficiency. It was shown that polyplex surface stabilization using a low molecular weight crosslinker met these requirements. PEI/plasmid was crosslinked to yield polyplexes comparable to unmodified ones in terms of size and DNA condensation properties. The DNA release properties of these polyplexes were sensitive to reducing conditions similar to the intracellular environment. Polyplexes were efficiently taken up by cells and DNA release could be triggered depending on extra- as well as intra-cellular redox status. Transfection experiments point to an effective destabilization of the polyplexes after cell uptake. In vivo investigations of the stabilized polyplexes in mice revealed increased blood concentrations depending on the stabilization degree and altered organ accumulation. Most importantly luciferase expression patterns demonstrated enhanced liver expression while unwanted lung transfection could be reduced. These results cumulatively suggest that surface stabilized PEI polyplexes stabilized using redox sensitive disulfide bonds could become a viable strategy for in vivo plasmid delivery.

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Chapter 5

Block-copolymers of PEI and high molecular weight PEG with extended circulation in blood

In preparation for *Journal of Controlled Release (*2006)

Summary

A set of copolymers consisting of branched PEI 25 kDa grafted with high molecular PEG at a low degree of substitution was successfully synthesized using a simple two-step procedure. The resulting AB-type and ABA-type copolymers were tested for their cytotoxicity and their DNA condensation and complexation properties. Their polyplexes with plasmid DNA were characterized in terms of DNA size and surface charge, transfection efficiency and blood compatibility. The pharmacokinetic profiles of the complexes containing ^{32}P-labeled plasmid were assessed before and after crosslinking with disulfide bonds.

A set of four copolymers containing one or two PEG 20 or PEG 30 kDa chains were obtained. The cytotoxicity of PEI was strongly reduced after copolymerization. The copolymer polyplexes showed hydrodynamic diameters of less than 200 nm, comparable to PEI 25. Similarly, no reduction in DNA condensation and complexation was found, in fact, PEI-PEG(30k) copolymers exhibited better condensation and complexation properties than PEI 25. The transfection efficiency of copolymer polyplexes was up to 10-fold higher than the PEI 25 control and the hemolytic activity could be markedly reduced. After intravenous injection into mice, the plasmids complexed to PEI-PEG(30k) copolymers revealed significantly increased circulation times. Additionally, after stabilizing the polyplexes using a redox sensitive, biodegradable crosslinker, blood levels of plasmid could be further increased up to 125% compared to PEI. These results demonstrate that polyplexes prepared using a combined strategy of surface crosslinking and PEGylation show interesting properties as stable, long circulating vectors.

Introduction

Developing non-viral vectors capable of treating diseases such as genetic disorder, cancer inflammation or acquired diseases remains an important goal for gene therapy. Especially disseminated targets, which cannot be reached by direct loco-regional injection, require vectors that can deliver therapeutic nucleic acids to cells via the intravenous route. Viral vectors, although they are able to deliver plasmids efficient into mammalian cells, are limited in their usefulness due to immunogenicity and low loading capacity [1]. Polycations have emerged as effective transfection vectors and are able to condense DNA in nanoparticles suitable for endocytic uptake [2]. Poly(ethylene imine) (PEI) has been extensively studied as efficient non-viral gene delivery system [1, 3]. The toxicity of PEI is directly related to its molecular weight and branching, however, also DNA condensation and transfection efficiency are enhanced with increasing molecular weight [4-6]. To improve its biocompatibility, PEI was modified by human serum albumin [7], dextran [8] or hydrophilic copolymers such as poly[N-(2-hydroxypropyl) methacrylamide] (PHMPA) [9, 10] and poly(ethylene glycol) (PEG) [11-14]. PEGylation of PEI offered several advantages, such as reduced toxicity, hemolytic activity and improved solubility [12, 15, 16]. However, PEGylation was also correlated with lowered complexation efficiency and reduced cell uptake due to steric hindrance [17].

Despite these efforts in investigating PEGylated PEIs, up to now there is no agreement on the optimal degree of PEG grafting and molecular weight [1]. While a higher amount of PEG is generally believed to be beneficial in terms of reduced toxicity and colloidal stability of the polyplexes, it is not yet clear whether this should be achieved by high grafting with low molecular weight PEI or by low grafting with high molecular weight PEG. An increasing degree of PEG grafting impeded complexation of DNA and a minimum PEG molecular weight of 5 kDa was necessary to shield the positive surface charge of PEI [18]. Whereas PEI-PEG containing low molecular weight PEG 550 Da resulted in large and diffuse polyplexes, high molecular weight PEG 20 kDa copolymers reduced the polyplex size and yielded compact polyplexes without interfering with the condensation process [18]. Similarly, Desphanade et al. reported linear AB copolymers of DMAEMA–ethylene glycol copolymers to form compact

complexes and exhibit higher cell association for than for statistical comb-type copolymers [19, 20]. These results suggest that a low grafting ratio with high molecular PEG may be suitable to achieve a steric shielding effect. High flexibility and hydrophilicity of outer-shell PEG surrounding the polyplexes seem to contribute to the colloidal stability [21]. A low surface coverage with PEG chains allows a greater range of motion, leading in average to a closer location to the surface. In contrast, a high surface coverage decreases the chain mobility, and therefore also decreases the steric shielding [22].

Stable vectors with reduced interaction potential are a prerequisite for intravenous plasmid delivery. Polyplexes of PEGylated PEIs with DNA displayed enhanced colloidal stability and interactions with erythrocytes, opsonization as well as uptake by the cells of the RES was reduced [18, 23, 24]. In addition, PEG with a chain length of 20 kDa was reported to be reduce immunogenicity compared to PEG 5 kDa [25]. However, polyplexes of PEGylated PEI are susceptible to complex dissociation after systemic administration due [26, 27].

To create vectors with improved stability in circulation, we synthesized block-copolymers of branched PEI 25 kDa and high molecular weight PEG 20 kDa and 30 kDa. The branched form of PEI is characterized by higher complexation efficiency as compared to the linear form [5]. Additionally, the higher content of primary amines in branched PEI is favorable for the reaction conversion with long PEG chains containing only one reactive site [12]. The resulting polyplexes were investigated for their physicochemical properties and transfection efficiency in vitro as well as for their pharmacokinetic properties. In addition, polyplexes of PEI-PEG copolymers were crosslinked with an amine reactive crosslinker, dithiobis(succinimidyl propionate) (DSP), to further improve their systemic stability and the pharmacokinetic profiles in mice were obtained.

Experimental Section

Materials: Branched poly (ethylene imine) with a molecular weight of 25 kDa (Polymin, waterfree, 99%) was a gift from BASF (Ludwigshafen, Germany). Monoamino-poly(ethylene glycol) mono ethylether (mPEG-NH_2) with a molecular weight of 20 kDa and 30 kDa were purchased from Rapp Polymere (Tübingen, Germany) and Nektar (Alabama, USA), respectively. Copolymers of PEI and mPEG-NH_2 were synthesized according to a recently reported procedure [12]. Polymers were stored as aqueous stock solutions (1 mg/mL) at pH 7.5. The graft copolymers were designated using the following nomenclature: PEI-PEG$(x)_n$, where x is the molecular weight of the PEG and the index n represents the average number of PEG chains per PEI molecule. This number was calculated based on ^{1}H-NMR spectra as described previously [28]. The plasmid pCMV-GL3 encoding the firefly luciferase gene was amplified in JM-109 competent cells and purified using a commercial kit (Qiagen Hilden, Germany). pCMV-Luc plasmid was purchased from PlasmidFactory (Bielefeld, Germany). Salmon testes DNA (Sigma, Taufkirchen, Germany) was used for DNA condensation experiments. All other materials obtained in analytical quality. Pure water (0.22 µm filtered, 0.055 S/cm, USF Seral, Seradest BETA 25 and Serapur DELTA UV/UF) was used to prepare analytical solutions and buffers.

Copolymer synthesis:

Activation of mPEG-NH_2 20 kDa: In a 250 mL flask fitted with a reflux condenser and an oil bubbler, 2.12 g of mPEG-NH_2 20 kDa were dissolved in 50 mL of $CHCl_3$. 15 mL of hexamethylene diisocyanate (HMDI, Fluka, Germany) was added and the mixture was heated under reflux for 16 h. The polymer was washed by precipitation in 400 mL of petrol ether and the precipitate was redissolved in 10 mL $CHCl_3$. The reprecipitation and washing steps were repeated three times before the polymer was isolated and residual solvents were removed at reduced pressure. 1.46 g of a white solid were obtained with a 68% yield.

Activation of mPEG-NH_2 30 kDa: Typically, according to the procedure described above, 0.4 g mPEG-NH_2 30 kDa were reacted in 100 mL $CHCl_3$ with 5 mL HMDI overnight and purified by four precipitating steps in 400 mL petrol ether each. The

precipitate was dissolved in $CHCl_3$ and solvents were removed under reduced pressure. 0.33 g of a white solid were obtained with 76% yield.

Synthesis of PEI-PEG(20)$_{1.1}$: 0.78 of PEI 25 kDa were dried under vacuo at 60°C for 1 hour and then dissolved in 100 mL of freshly distilled $CHCl_3$ in a 500 mL flask. 0.70 g of activated mPEG 20 kDa were dissolved in 50 mL of freshly distilled $CHCl_3$ and added dropwise to the PEI solution. The flask was fitted with a reflux condenser and an oil bubbler and the mixture was heated under reflux for 65 h. The clear light yellow solution was concentrated to 30 mL volume and the polymer was purified by four precipitating steps in 400 mL petrol ether each and redissolved in 10 mL $CHCl_3$. Solvents were removed under reduced pressure and 0.86 g of a white solid were obtained with 58% yield.

^{1}H-NMR (D_2O): δ = 0.82 (m, -NHC(O)-NH$(CH_2)_2$C*H*$_2$-), δ = 1.08 (m, -NHC(O)-NH$(CH_2)_3$C*H*$_2$-), δ = 1.24 (m, -NHC(O)-NH(CH_2)C*H*$_2$-), δ = 1.40 (m. NHC(O)-NH$(CH_2)4_3$C*H*$_2$-), δ = 1.96-2.28 (m. -NC*H*$_2$C*H*$_2$N-), δ = 2.89 (s, -NHC(O)NHCH_2C*H*$_2$-), δ = 3.06 (m, -NHC(O)-NH$(CH_2)_{0and5}$C*H*$_2$-), δ = 3.29 (s, OC*H*$_3$-), δ = 3.37 (m, -NHC(O)NHC*H*$_2$-), δ = 3.41 (m, -NHC(O)CH_2C*H*$_2$O-), δ = 3.60 (s. -OC*H*$_2$C*H*$_2$O-), δ = 4.07 (s, -NHC(O)C*H*$_2$-).

Synthesis of PEI-PEG(20)$_{2.4}$: Similar to the procedure described above, 0.40 g of PEI 25 kDa were dried under vacuo at 60°C for 1 hour and then dissolved in 50 mL of freshly distilled $CHCl_3$ in a 500 mL flask. 0.71 g of activated mPEG 20 kDa were dissolved in 50 mL of freshly distilled $CHCl_3$ and added dropwise to the PEI solution. The flask was fitted with a reflux condenser and an oil bubbler and the mixture was heated under reflux for 93 h. Purification was performed as described above by precipitation in petrol ether and redissolving in $CHCl_3$. 1.06 g of a white powder were obtained with 95% yield.

Synthesis of PEI-PEG(30)$_{1.1}$: 0.45 of PEI 25 kDa were dried under vacuo at 60°C for 1 hour and then dissolved in 100 mL of freshly distilled $CHCl_3$ in a 500 mL flask. 0.51 g of activated mPEG 30 kDa were dissolved in 100 mL of freshly distilled $CHCl_3$ and added dropwise to the PEI solution. The flask was fitted with a reflux condenser and an oil bubbler and the mixture was heated under reflux for 89 h. Purification was performed as described above by precipitation in petrol ether and redissolving in $CHCl_3$. 0.58 g of a yellowish solid were obtained with 60% yield.

Synthesis of PEI-PEG(30)$_{2.4}$: 0.10 of PEI 25 kDa were dried under vacuo at 60°C for 1 hour and then dissolved in 50 mL of freshly distilled $CHCl_3$ in a 500 mL flask. 0.33 g of activated mPEG 30 kDa were dissolved in 50 mL of freshly distilled $CHCl_3$ and added dropwise to the PEI solution. The flask was fitted with a reflux condenser and an oil bubbler and the mixture was heated under reflux for 62 h. Purification was performed as described above by precipitation in petrol ether and redissolving in $CHCl_3$. 0.31 g of a yellowish solid were obtained with 72% yield.

Nuclear Magnetic Resonance Spectroscopy (NMR): ^{1}H-NMR spectra were recorded in D_2O (Merck) on a Eclipse+ 500 spectrometer from JEOL (Tokyo, Japan) at 500 MHz. Spectra were evaluated with the NMR data processing program MestRe-C Version 4.7. Integration of the signals in ^{1}H-NMR spectra for -CH_2-CH_2-O- (δ = 3.6) and for - CH_2-CH_2-ND- (δ = 2.3 – 2.9) yielded the composition of the copolymers. Indices in the nomenclature of the copolymers are calculated from this integration.

Thermogravimetric Analysis (TGA): TGA was performed on a thermogravimetric analyzer TGA 7 with a thermal analysis controller TAC 7/DX from Perkin-Elmer using an approximately 10 mg polymer sample. The scanning rate was 20 K/min, and thermograms were recorded within the temperature range 25-700 °C. Analysis was performed under a nitrogen gas atmosphere in platinum crucibles.

In vitro cytotoxicity of the copolymers: The cytotoxicity of the copolymers was studied in L929 mouse fibroblasts as reported previously [6, 29] Briefly, L929 fibroblasts were seeded in 96- well microtiter plates at a density of 8000 cells/well and allowed to grow for 24 hours prior to the application of serial solutions of the copolymers (0.15-5000 µg/mL in cell media). After 4 hours of incubation, the medium was replaced with 200 µL fresh medium and 20 µL (3-(4,5-dimethylthiatol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT, Sigma, Seelze). After 4 hours, the unreacted dye was removed and 200 µL DMSO was added. The absorption was measured using the ELISA reader Titertek Plus MS 212 (ICN, Eschwege, Germany) at 570 nm, with a background correction at 690 nm. The relative cell viability (%) was related to control wells containing cell culture medium without polymer and was calculated by:

absorption $_{test}$ / absorption $_{control}$ x 100 %. Data are presented as the mean of six measurements (± SD). The values of the polymers were fitted to a logistical sigmoidal function using Origin® v 7.0 (OriginLab, Northampton, MA) and IC_{50} values were calculated as recently reported [29].

Formation and characterization of uncrosslinked and crosslinked polyplexes: Polyplex formation was performed according to a recently reported procedure [30]. Luciferase reporter gene plasmids (pCMV-GL3 or pCMV-Luc) and the appropriate amounts of polymer were dissolved separately in 5% glucose at pH 7.5, mixed by vigorous pipetting and incubated for 10 min to allow polyplex formation. As appropriate, polyplex crosslinking was achieved by adding the necessary amount of 0.01 M Dithiobis(succinimidyl propionate) (DSP) to the preformed polyplexes. The solutions were mixed by vigorous pipetting and incubated for 30 minutes. Polyplexes were prepared at a concentration of 2 µg plasmid /100 µL at an N/P (nitrogen to phosphate) ratio of 7, unless otherwise stated. All crosslink degrees are reported as molar ratios between DSP and PEI amines assuming that PEI 25 kDa contains 580 amines per molecule. Calculation of primary amine content was based on ^{13}C-NMR data obtained by a recently reported method [28].

Determination of polyplex size and zeta potential: The hydrodynamic diameters and the zeta potentials of freshly prepared polyplexes were measured using a Zetasizer Nano-ZS from Malvern Instruments (Herrenberg, Germany) equipped with a 4 mW He-Ne laser at a wavelength of 633 nm at 25°C. Scattered light was detected at a 173° backward scattering angle with automatic measurement position and automatic laser attenuation. The viscosity and refractive index of pure water at 25 °C were used for data analysis. Size and zeta potential were measured in folded capillary cells after 1:3 dilutions with the appropriate buffer and calculated using DTS software v4.10. Reference measurements using Malvern size and zeta potential standards were run routinely to check for verify correct instrument operation. Values are given as the mean of three measurements of 10 runs each.

DNA condensation assay: The DNA condensation was measured by quenching of ethidium bromide fluorescence as described previously [18]. Briefly, quadruplicates of 4 μg of herrings testes DNA were complexed with increasing amounts of polymer in 0.1M borate buffer at pH 7.5, using 96-well plates. After 10 min, 20 μL of a 0.1 mg/mL ethidium bromide solution were added. The resulting fluorescence was measured using a Perkin-Elmer LS50 B fluorescence plate reader (Perkin-Elmer, Rodgau, Germany) with an excitation wavelength of 518 nm with a 15 nm slit, a 515 nm emission filter and an emission wavelength of 605 nm with a 20 nm slit. Results were transformed into relative fluorescence values (rel $F = F_{sample}/F_{DNA}$). A value of 1 was attributed to the fluorescence of ethidium bromide with plain DNA. Experiments were performed in quadruplicate and results are given as mean ± standard deviation.

DNA complexation assay: The DNA complexation ability of the polymers was tested similar to [18]. The polyplexes were prepared as described above. Briefly, 20 μL aliquots of polyplex solution were loaded onto a 1% agarose gel containing ethidium bromide. The gels were run for 60 min at 70 V in TBE buffer solution, and then scanned with a Biometra gel analyzing system.

Hemocompatibility testing: Hemolytic effects were investigated similar to an earlier report [18]. Briefly, fresh blood from healthy human volunteers was collected in EDTA containing tubes. The blood was centrifuged at 4 °C for 3 min at 3000 rpm and washed several times with phosphate buffered saline (PBS) at pH 7.4 until the supernatant was clear and colorless. 150 μL of a 2.5% (v/v) suspension of the erythrocytes were mixed with 15 μL of the polymer or the polyplex solution prepared in 5% glucose/25 mM Hepes buffer at pH 7.5 in microcentrifuge tubes. After an incubation time of 60 min at 37 °C, the blood cells were removed by centrifugation and the supernatant was transferred to 96-well plates. The supernatant was spectroscopically investigated at 570 nm with a TitertekPlus MT 212 microplate reader (ICN, Germany). 5% glucose and a 1% Triton X-100 solution in water were used as negative and positive controls, respectively. Hemolysis is reported as percent $(OD_{Triton} - OD_{sample}/ OD_{Triton} - OD_{buffer})*100$. Results are given as the mean of triplicate experiments ± standard deviation.

In vitro transfection: 2 µg pCMV-Luc and the appropriate amount of polymer for N/P 7 were dissolved separately in 50 µL 5% glucose/25 mM HEPES at pH 7.5, mixed by vigorous pipetting and incubated for 10 min. The transfection activity of the crosslinked polyplexes was studied on NIH-3T3 fibroblasts (NIH 3T3, Swiss mouse embryo, ATCC, Rockville, Maryland). The cells were seeded at a density of $3.0*10^4$ cells per well on 24 well cell culture plates, 24 h prior to the transfection experiments. 100 µL polyplex solution were added to each well containing 1 mL fresh medium and incubated for 4 hours or 8 hours, respectively. The medium was replaced and the cells were allowed to grow for a further 44 hours. The luciferase expression was measured in the cell lysate using a commercial kit (luciferase assay reagent Promega, Mannheim, Germany) and photon counting with a luminometer (Sirius Berthold, Germany). Protein concentration was determined using a BSA assay kit (Pierce, Rockford IL, USA) and measured in a microplate reader (TitertekPlus MT 212, ICN, Germany). All experiments were performed in quadruplicate. Data are expressed as the mean ± standard deviation in nanograms of luciferase per milligram of protein.

In vivo experiments: All animal experiments were carried out according to the German law of protection of animal life and approved by an external review committee for laboratory animal care.

Plasmids were labeled according to the manufacturer's protocol using a Nick translation kit N5000 (GE Healthcare, Germany). 100 ng pCMV-Luc were labeled with ^{32}P-alpha-CTP (Hartmann, Germany). The reaction mixture was purified using microspin columns (Wizard SV Gel and PCR Clean-Up System, Promega, Germany) and the labeled plasmid was used immediately after synthesis.

2 µg pCMV-Luc plasmid spiked with ^{32}P-labeled plasmid and the appropriate amount of polymer were allowed to form polyplexes for 10 min. As appropriate, polyplexes were subsequently crosslinked with 0.01 M DSP in DMSO to reach the desired crosslink degrees. 200 µL of the complex solution was injected in the tail vein of anaesthetized male balb/c mice. Blood samples of 25 µL were drawn from the retrobulbar plexus at the indicated time points. The samples were dissolved overnight in a 1:1 mixture of isopropanol/Soluene 350 (PerkinElmer, Germany) at 55 °C and subsequently bleached with 200 µL hydrogen peroxide. 15 mL scintillation cocktail

(Hionic Fluor®, PerkinElmer, Germany) was added and mixed. Activity of ^{32}P in each sample was determined using a TriCarb 2900 liquid scintillation counter (PerkinElmer, Germany) with a counting time of 10 min, active static controller and half-life correction. Disintegrations per minute (DPM) were calculated with a ^{32}P quench curve using tSIE/AEC as quench indicator. The injected dose was calculated based on activity measurements of the injected solution. All experiments were performed at least in quadruplicate and the AUC was determined using a non-compartmental logarithmic algorithm. Concentration-time data were fitted to a biexponential disposition function ($C(t) = Ae^{-\alpha t} + Be^{-\beta t}$, iterative reweighing with $1/(C_{calc})^2$) with the software Kinetica 1.1. from Simed (Créteil Cedex, France). Akaike and Schwartz statistical criteria provided by the software were inspected. Plasmid concentrations in the samples were calculated as percent of the injected dose per mL blood, respectively. Results are given as mean ± standard deviation.

Statistics: Experiments were performed at least in triplicate, unless otherwise stated. Significance between the mean values was calculated using one-way ANOVA analysis using Origin® 7.0 software (OriginLab Corporation, Northampton, MA). Probability values < 0.05 were regarded to be significant.

Results and Discussion

Synthesis of PEI-PEG-copolymers: A series of novel PEI-PEG copolymers (Table 1) was synthesized by reacting linear, high molecular weight PEG chains with branched PEI 25 kDa as depicted in Figure 1. A recently reported method to build PEI-PEG diblock copolymers based on the "macrostopper" method is not feasible for such high molecular weight PEG [31]. Therefore, we chose to use a two-step procedure using a hexamethylene diisocynate linker [12]. After activating the monoamino-methoxy-PEG with hexamethylene diisocynate, the activated PEG was coupled onto the branched PEI 25 kDa. Excess HMDI from the activation step was carefully removed by precipitating the solutions in petrol ether to prevent crosslinking of PEI to higher molecular weight networks in the subsequent addition step. The successful PEG activation and the

coupling reaction were monitored using ^{1}H-NMR (methylene groups adjacent to urea, δ = 3.37 -NHC(O)NHCH_2-).

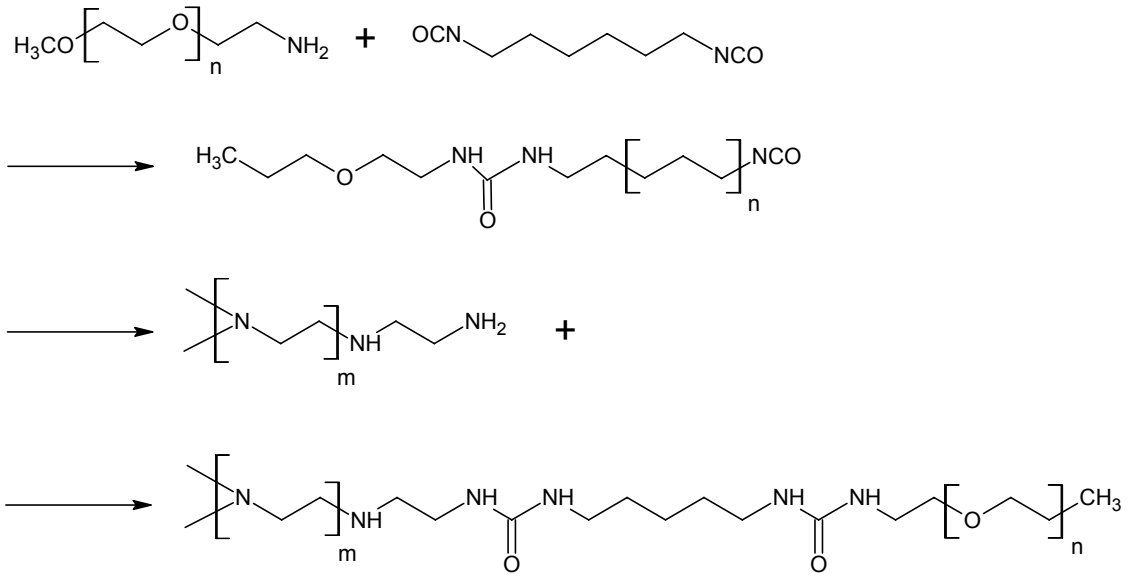


Figure 1: Synthesis scheme of the copolymers used in this study.

Table 1 gives a summary over the synthesized PEI-PEG copolymers. The composition of the copolymers was determined by integration of the appropriate signals in ^{1}H-NMR spectra. The results of PEI content calculated from the spectra was in good agreement with the calculated values. Furthermore, results from elemental analysis of the copolymers (Table 2) were also generally in line with the expected values. A decrease of nitrogen content going along with an increase in oxygen content was observed for increasing grafting ratios of PEG. Higher amounts of nitrogen found by elemental analysis went along with similar results of a higher PEI content in the NMR spectra and corroborate the NMR results with regard to the copolymer composition.

PEG-PEI	$Mw_{calc.}$ [a] [g/mol]	Mw [b] [g/mol]	Mn [b] [g/mol]	PDI [b]	% PEI in feed	% PEI found [c]
PEI-PEG(20k)$_{1.1}$	47 000	42 410	33 270	1.28	53	53
PEI-PEG(20k)$_{2.4}$	73 000	64 290	36 070	1.78	40	33
PEI-PEG(30k)$_{1.1}$	58 000	83 740	59 370	1.41	47	43
PEI-PEG(30k)$_{2.4}$	97 000	177 800	122 400	1.45	23	26

Table 1: Composition of the copolymers used in this study. [a] based on molecular weight given by supplier, [b] as determined by SEC-MALLS, [c] as determined by ^{1}H-NMR spectroscopy

PEG-PEI	C_{calc}	H_{calc}	N_{calc}	O_{calc}	C_{found}	H_{found}	N_{found}	O_{found}
PEI-PEG(20k)$_{1.1}$	55.3	10.4	17.2	17.0	51.7 ± 0.1	8.1 ± 0.8	15.4 ± 0.1	24.9 ± 0.8
PEI-PEG(20k)$_{2.4}$	55.0	10.0	11.1	23.9	48.8 ± 0.50	9.0 ± 0.1	9.7 ± 0.3	32.6 ± 0.6
PEI-PEG(30k)$_{1.1}$	55.1	10.2	14.0	20.1	51.2 ± 0.6	9.6 ± 0.4	12.8 ± 0.2	26.6 ± 1.2
PEI-PEG(30k)$_{2.4}$	55.9	9.7	8.4	27.0	51.2 ± 0.4	9.2 ± 0.1	8.8 ± 0.3	30.7 ± 0.6

Table 2: Results from elemental analysis for PEI-PEG copolymers. Calculations were based on ^{1}H-NMR data.

Additionally, the results from thermogravimetric analysis (Figure 2) showed distinct degradation patterns for the copolymers containing one or two PEG chains, respectively. The first, small mass lost step ccould be attributed to solvent residues. Since high molecular weight PEGs degrade at higher temperature than the PEI 25 kDa [12], the second mass loss step from about 400 °C could be attributed to PEI. In agreement with the proposed copolymer structure, this second step is smaller for the higher grafted PEI-PEG copolymers. The third degradation step at higher temperature, which is due to PEG degradation, is therefore smaller for the low grafted copolymers.

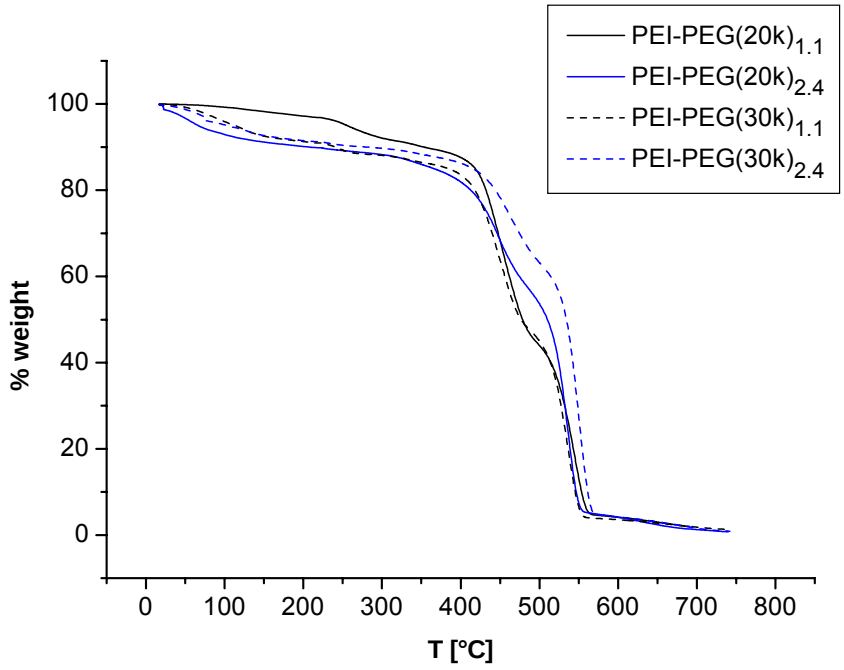
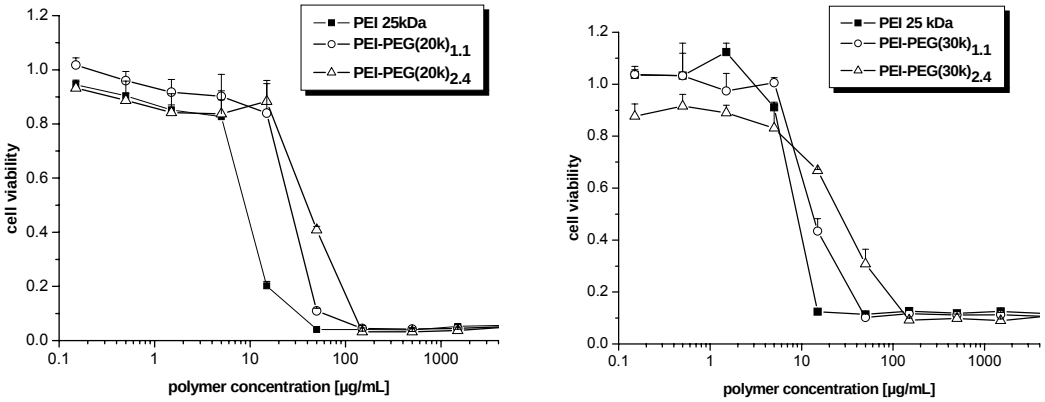


Figure 2: Thermogravimetric analysis of PEI-PEG copolymers.

Cytotoxicity: The metabolic activity of polymer treated L929 fibroblasts was tested using a colorimetric MTT assay [32]. The cytotoxicity of the copolymers was compared with the toxicity of the homopolymer PEI [6]. As illustrated in Figure 3, the toxicity of the polymers was found to be concentration dependent. Whilst PEI reduced the cell viability dramatically at concentrations of 15 µg/mL (~ 20 % viability), the copolymers showed less influence upon the cells (~ 85 % viability for those containing 20 kDa PEG and 45-70% for 30 kDa PEG). Considering the IC_{50} values, the reduction of the metabolic activity is far lower with the copolymers as compared to PEI 25 kDa. Pure PEI 25 kDa exhibited an IC50 value of about 10 µg/mL, being in agreement with previous reports [29]. By contrast, the IC_{50} values of all copolymers were significantly lower and the reduction in toxicity depended on the number of PEG chains per molecule. Generally, IC_{50} values were about two-fold higher for the higher grafted PEI-PEG.

The modification of PEI with PEG was reported to lead to a reduction of toxic side effects in vitro as well as in vivo [12, 14]. Hence, high molecular weight PEG may display sufficient shielding even at low grafting ratios due to the high flexibility of the

PEG chains allowing them to surround and cover the polyplex surface, as suggested by the MTT assay results. Interestingly, the copolymers containing the 20 kDa chains showed higher IC_{50} values than those containing the 30 kDa chains. A slight surfactant character of the copolymers due to their block-copolymer structure may play a role with high molecular weight PEG. Still, however, the steric shielding is able to compensate these effects, yielding IC_{50} values two to four times higher than unmodified PEI.



Polymer	IC_{50} [µg/mL]
PEI 25kDa	12 ± 9 (left graph) 7 ± 0.4 (right graph)
PEI-PEG(20k)$_{1.1}$	24 ± 4
PEI-PEG(20k)$_{2.4}$	49 ± 4
PEI-PEG(30k)$_{1.1}$	13 ± 1
PEI-PEG(30k)$_{2.4}$	22 ± 2

Figure 3: Cytotoxicity of PEI-PEG copolymers and related IC_{50} values in comparison to PEI 25 kDa as determined by MTT assay. All copolymers were significantly less toxic than PEI 25 kDa. Significant differences between the copolymers are marked * $p < 0.01$).

DNA condensation: The condensation of DNA into small particles is a prerequisite for their use in polycationic plasmid delivery [33]. The ability of the copolymers to

condense DNA was tested using ethidium bromide, which emits a strong fluorescence upon intercalating DNA. The results of the ethidium bromide assay are shown in Figure 4. The relative fluorescence of all samples decreased with increasing N/P ratio due to the inaccessibility of DNA for ethidium bromide when complexed to the polymers. From an N/P ratio of 3, all polymers showed no further decrease in fluorescence, indicating complete DNA condensation. However, at lower N/P ratios, differences between unmodified PEI and the copolymers were observed. The fluorescence of the PEG 20 kDa containing copolymers was generally lower at N/P > 3 compared to pure PEI, and the one with the higher grafting ratio showed continously lower values from N/P > 0.2. Similarly, the copolymers with PEG 30 kDa showed decreased fluorescence compared to PEI, however, the effect was even more pronounced. These results suggest that copolymers with long PEG chains are able to enhance the DNA condensation properties of PEI. Interestingly, PEGylation was often attributed to lower DNA condensation due to the steric hindrance of the PEG chains [24]. Our results revealed a reverse effect, possibly due to the low grafting ratio and the block-copolymer structure with clearly separated PEG and PEI moieties. This would allow the PEI moiety to effectively interact with the DNA. Additionally, some reports suggest that DNA condensation could be promoted by PEG, since PEG itself was able to condense DNA [34, 35]. This additional condensation effect seems to occur only at high molecular weights and, hence, could explain the higher condensation efficiency of.

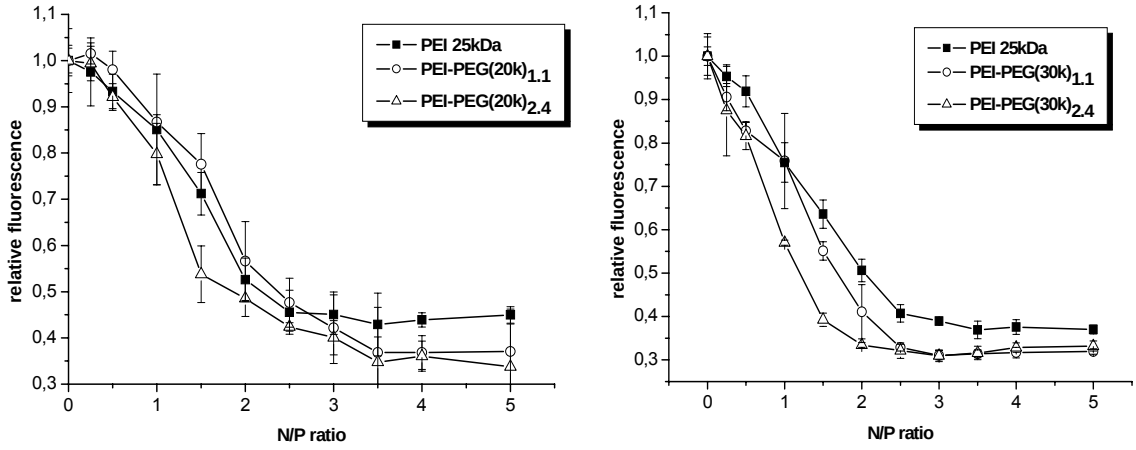


Figure 4: DNA condensation properties as determined with ethidium bromide exclusion assay.

Complexation efficiency by agarose gel electrophoresis assay:

The formation of polyelectrolyte complexes between PEI and DNA is based on electrostatic interactions between the polymer nitrogens and the phosphate groups of the nucleotides [2, 36]. To study the impact of the long PEG chains on the efficiency of these electrostatic interactions, polyplexes were prepared at different N/P ratios and were tested by gel retardation. All tested polymers completely inhibited DNA mobility at N/P > 2 (data not shown). Therefore, a narrower range of N/P ratios lower than 2 was investigated. The resulting agarose gels for N/P 0 – 1.25 are depicted in Figure 5. In the case of PEI 25 kDa, an N/P ratio of 1.25 was sufficient to completely inhibit plasmid mobility. The copolymers containing PEG 20 kDa showed slightly reduced complexation ability and complexation was completed at N/P 2. Interestingly, the higher grafted one seems to condense DNA more efficient. Moreover, PEG 30 kDa containing copolymers were able to condense DNA as good as unmodified PEI or even better. Again, a higher grafting ratio seemed to enhance DNA condensation properties, since an N/P ratio of 0.75 was enough for complete complexation with PEI-PEG(30k)$_{2.4}$. Additionally, as indicated by the complete loss of fluorescence in the pockets, an N/P ratio of 1.0 was sufficient to completely condense DNA. Generally, higher amounts of PEGylated PEI are believed to be necessary to achieve DNA complexation comparable to unmodified PEI due to steric hindrance of the PEG [37]. The results from both DNA condensation and complexation assay point to an adverse effect of low grafted copolymers with long PEG chains. These properties could be beneficial in terms of better DNA protection, and, therefore, stability in circulation

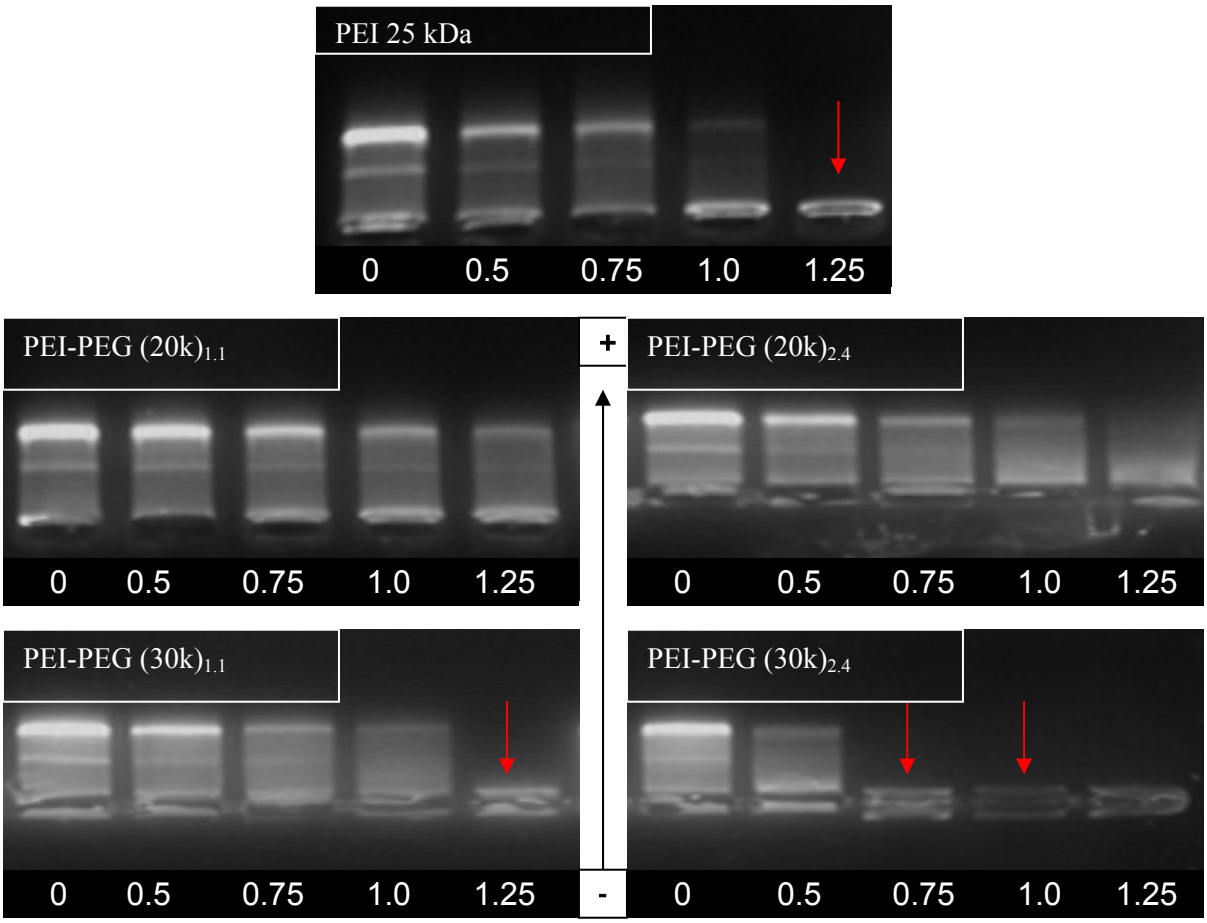


Figure 5: DNA complexation efficiency as determined by agarose gel electrophoresis. The numbers in each lane represent the N/P ratio, red arrows indicate complete complexation.

Size and surface charge of the polyplexes: To investigate the effect of PEGylation on the size and the surface charge of the polyplexes, the hydrodynamic diameters as well as the zeta potential were measured in two different media, 150 mM NaCl and 5% glucose at pH 7.5 (Table 2). PEGylation increases colloidal stability by steric shielding [38]. Therefore, size measurements were performed after an incubation time of 10 min to assess aggregate formation [30]. At low N/P ratios, PEI 25 kDa polyplexes revealed hydrodynamic diameters of several hundred nanometers. Presumably, this is due to aggregate formation, which are generally higher in high ionic strength medium, such as 150 mM NaCl [39]. Polyplexes formed in low ionic strength glucose medium were generally smaller low N/P ratios compared to 150 mM NaCl. The effect of PEG to reduce aggregation of polyplexes was, therefore, most pronounced in 150 mM NaCl. All polymers complexed DNA into nanoparticles with hydrodynamic diameters below

200 nm from N/P 7 and higher, which is a prerequisite for their endocytic uptake by the cells [17]. However, PEGylation yielded polyplexes with diameters below 200 nm also at low N/P ratios, indicating successful colloidal stabilization even with low copolymer concentrations. Only at the highest N/P ratio tested (N/P 20), PEI polyplexes were significantly smaller than the copolymer polyplexes. Presumably, the higher cationic charge density leads to a denser compaction at high N/P ratios.

Hydrodynamic diameters:

N/P	PEI-PEG(20k)$_{1.1}$	PEI-PEG(20k)$_{2.4}$	PEI-PEG(30k)$_{1.1}$	PEI-PEG(30k)$_{2.4}$	PEI 25 kDa
3	164 ± 29	178 ± 20	163 ± 7	213 ± 72	816 ± 768
7	166 ± 68	117 ± 13	158 ± 33	119 ± 11	125 ± 10
10	123 ± 12	117 ± 13	132 ± 28	136 ± 12	119 ± 2
20	156 ± 30	125 ± 6	130 ± 30	124 ± 16	115 ± 5

150mM NaCl, pH 7.5

N/P	PEI-PEG(20k)$_{1.1}$	PEI-PEG(20k)$_{2.4}$	PEI-PEG(30k)$_{1.1}$	PEI-PEG(30k)$_{2.4}$	PEI 25 kDa
3	138 ± 1	133 ± 4	131 ± 1	118 ± 2	212 ± 193
7	169 ± 3	131 ± 5	146 ± 43	120 ± 4	128 ± 41
10	127 ± 2	122 ± 3	124 ± 10	121 ± 9	112 ± 52
20	121 ± 2 *	117 ± 5 *	113 ± 6 *	116 ± 4 *	86 ± 2

5% glucose, pH 7.5

Zeta potential:

N/P	PEI-PEG(20k)$_{1.1}$	PEI-PEG(20k)$_{2.4}$	PEI-PEG(30k)$_{1.1}$	PEI-PEG(30k)$_{2.4}$	PEI 25 kDa
7	10.3 ± 1.0 *	7.7 ± 2.1 *	11.1 ± 0.5 *	8.6 ± 0.8 *	28.3 ± 1.2

5% glucose, pH 7.5

Table 1: Hydrodynamic diameter and zeta potential of copolymer polyplexes with pCMV-GL3 plasmid after 10 min incubation time (significant differences between copolymer and PEI 25 polyplexes, * $p < 0.05$).

The surface charge of copolymer polyplexes was measured in 5% glucose, pH 7.5, at an N/P ratio of 7. As expected, polyplexes of PEI 25 kDa with plasmid showed a high zeta potential of + 28.3 ± 1.2 mV, similar to earlier studies in low ionic strength media [18, 30]. Generally, PEGylation led to significantly lower surface charges compared to unmodified PEI, with zeta potentials between of + 10.3 ± 1.01 and 7.72 ± 2.12 for the PEI-PEG(20k) copolymers and 11.13 ± 0.45 and 8.63 ± 0.88 for the PEI-PEG(30k) copolymers, respectively. Whereas the length of the PEG chain did not influence the reduction in surface charge, a higher number of PEG 30 kDa reduced the zeta potential significantly ($p < 0.05$) compared to the lower grafted PEI-PEG(30k) copolymers. Since the positive surface charge of PEI polyplexes is related to their cytotoxicity [6], this aspect is of special interest for intravenous application of polyplexes. Additionally, an excess of positive surface charge is able to strongly enhance complement activation [40] and albumin or erythrocyte interactions [41], thereby reducing circulation times. Thus, the strongly reduced surface charge of the PEI-PEG-copolymer polyplexes makes them promising for in vivo administration.

In vitro transfection results:

A reduction of cell uptake due to steric hindrance by pegylation can often be observed for polycationic vectors [17, 24, 38]. Recent results suggested transfection efficiencies of PEI-PEG(20k)$_1$ copolymers comparable to PEI at N/P ratios of 20 to 50 [18]. However, the severe cytotoxicity of PEI 25 kDa at such high N/P may influence a direct comparison. Therefore, we tested the transfection efficiency of the copolymer polyplexes at N/P 3 to 15. Additionally, the condensation and complexation properties of the PEI-PEG copolymers pointed to sufficient polyplex stability even at low N/P ratios. Transfection results obtained in NIH 3T3 mouse fibroblasts are depicted in Figure 6. PEI-PEG(20k) polyplexes showed transfection efficiencies similar to PEI 25 kDa. In contrast, polyplexes based upon PEI-PEG(30k) were able to reach transfection efficiency of up to 10-fold higher than PEI 25 at N/P 3 and remained increased also at higher N/P ratios.

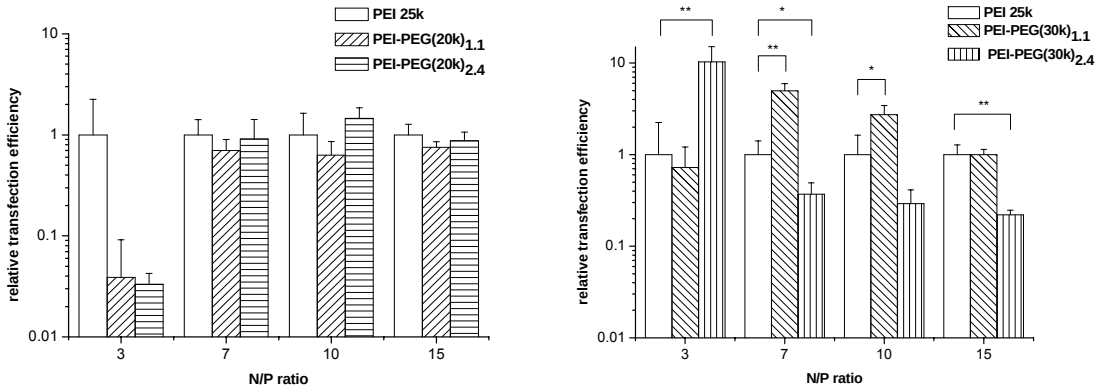


Figure 6: Transfection efficiency related to PEI 25 kDa in NIH-3T3 fibroblasts. Left: PEI-PEG(20k) copolymers, right: PEI-PEG(30k) copolymers (significant differences between copolymer and PEI 25 kDa polyplexes are marked with an asterisk, * $p < 0.05$, * $p < 0.01$).

Generally, PEGylation affects the efficiency of cell uptake and, therefore, the transfection capability of polyplexes in a negative way. Endocytic uptake into the cells is triggered by interaction of the positively charged polyplexes with proteoglycans on the cell surface [42]. In contrast, the low grafted PEI-PEG copolymers seem to be able to interact with the cells, since their surface charge is still positive, and transfection efficiency could be maintained. Similarly, Tang et al. reported higher transfection in vitro and in vivo for low grafted PEG-PEIs [23]. In addition to cell interaction, the improved DNA complexation properties, particularly for PEI-PEG(30k) copolymers, seem to contribute to the transfection results. A similar effect was recently also observed with siRNA polyplexes [43]. These results suggest that polyplex stability may also contribute to transfection efficiency in vitro and plays an opposite role to DNA release after cell uptake [44]. PEI-PEG block-copolymers with high molecular PEG provide a combination of stable polyplexes with reduced interaction potential in the extracellular environment and the intrinsic endosomal DNA release properties of PEI [3, 45].

Hemolysis: The hemocompatibility of the copolymers was tested by examining the induction of lysis of erythrocytes as a model system according to [46]. Hemolysis can serve as a marker for membrane activity [46] and displays a potential side effect of polycationic vectors for intravenous administration. Therefore, its investigation is a

prerequisite for the intravenous administration in mice [47]. The surfactant Triton-X 100 served as a positive control and 5% glucose, pH 7.5, as negative control. All values were related to these controls. The results of the hemolysis testing are shown in Figure 7. Upon complexation to DNA, the hemolytic activity of polymers is generally reduced. Hemolysis of both PEI and the copolymer polyplexes at N/P ratios from 3 to 15 was less than 10%, which can be regarded as negligible [18, 48]. PEI-PEG(20k) copolymers revealed slightly lower hemolytic activity than PEI-PEG(30k), and the AB-structures were less hemolytic than the ABA-structures. Both results point to a hemolytic effect of the PEGylation which may be attributed to surfactant properties of the block copolymers structure. However, PEGylation reduced generally hemolytic activity compared to unmodified PEI, pointing to a greater effect of the steric shielding than the adverse surfactant effect. Together, the overall low hemolytic activity of the copolymers makes them favorable candidates for in vivo tests.

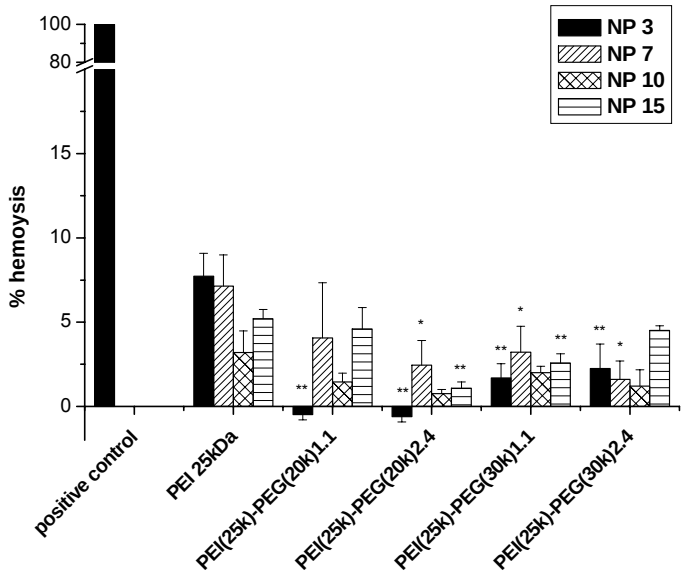
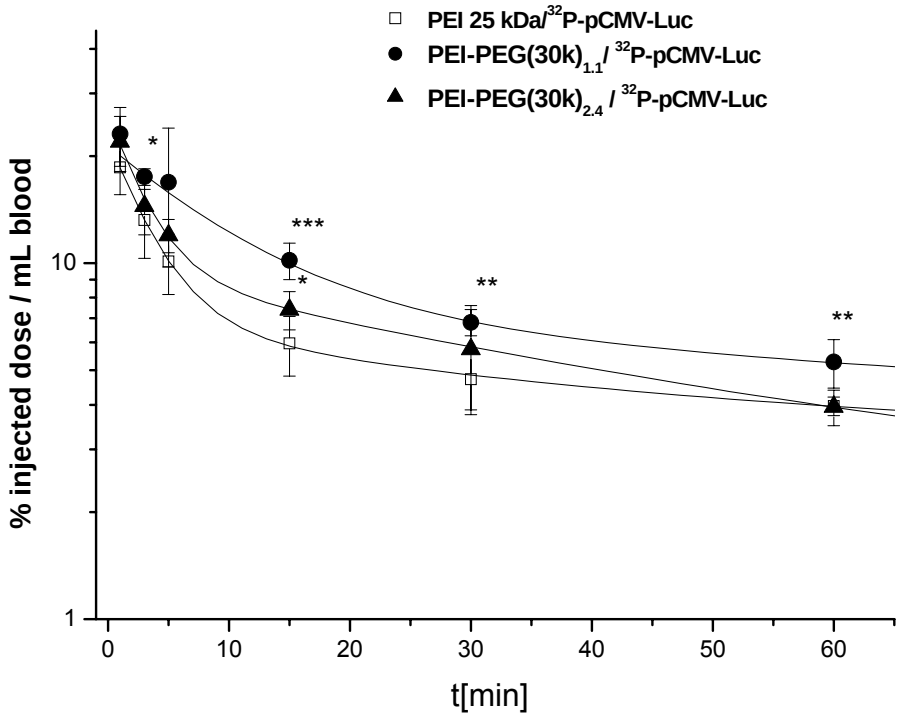


Figure 7: Hemolytic activity of the copolymers compared to PEI 25 kDa polyplexes at various N/P ratios. (* $p < 0.05$, ** $p < 0.01$)

Pharmacokinetics: In order to get appreciable amounts of an active plasmid to the tissue of desire, the carrier must be stable and long circulating. We injected polyplexes formed between pCMV-Luc plasmid and PEI-PEG(30k) copolymers, which were characterized by improved plasmid complexation and small polyplex sizes, into the tail

vein of male balb/c mice. The injection of polyplexes prepared at N/P 7 was well tolerated, in contrast to earlier reports of non-PEGylated PEI polyplexes, which often showed severe toxicity after intravenous administration [14, 49]. PEGylated polymers were recently reported to show enhanced plasma circulation times [13]. To assess the circulation time of the plasmid, polyplexes were prepared with 2 µg pCMV-Luc and spiked with ^{32}P labeled plasmid. To ensure integrity of the label, all in vivo experiments were performed immediately after plasmid labeling and pharmacokinetic studies were performed in a time range of 60 min post injection. Plain plasmid was rapidly removed from the bloodstream and less than 1% of the injected dose/mL blood could be recovered after 60 min (data not shown). Upon complexation with PEI 25 kDa, the circulation of the plasmid could be enhanced, in agreement with earlier reports (Figure 8) [50, 51]. PEGylation of PEI resulted in increased plasmid blood levels for both tested copolymers, PEI-PEG(30k)$_{1.1}$ and PEI-PEG(30k)$_{2.4}$. However, only the AB-type copolymers showed continously increased blood levels compared to PEI 25 kDa from 3 min to 60 min after injection. Blood concentrations of this copolymer up to 72% at 15 min after injection.

The area under the curve (AUC) was calculated using a non-compartmental logarithmic function, and additional pharmacokinetic parameters were obtained by fitting the concentration-time data to a two-compartment model [26, 52]. The AUC (logarithmic 0-60 min) of the AB-block-copolymer PEI-PEG(30k)$_{1.1}$ was significantly increased compared to PEI 25 kDa. Values obtained for AUC (two-compartment 0-60 min) also revealed an increase in plasmid availability, increasing form 767% ID/mL*min to 1916% ID/mL*min for the AB-type copolymer ($p = 0.1$). The slower clearance of PEI-PEG(30k)$_{1.1}$ polyplexes from the circulation could be attributed to both a longer alpha phase ($t_{1/2\ alpha}$ increased from 2.0 min to 5.3 min) as well as a longer beta phase ($t_{1/2\ beta}$ increased from 76 min to 211 min). Compared to PEI 25 kDa, PEI-PEG(30k)$_{1.1}$ enhanced the AUC of ^{32}P-pCMV-Luc 1.4-fold.

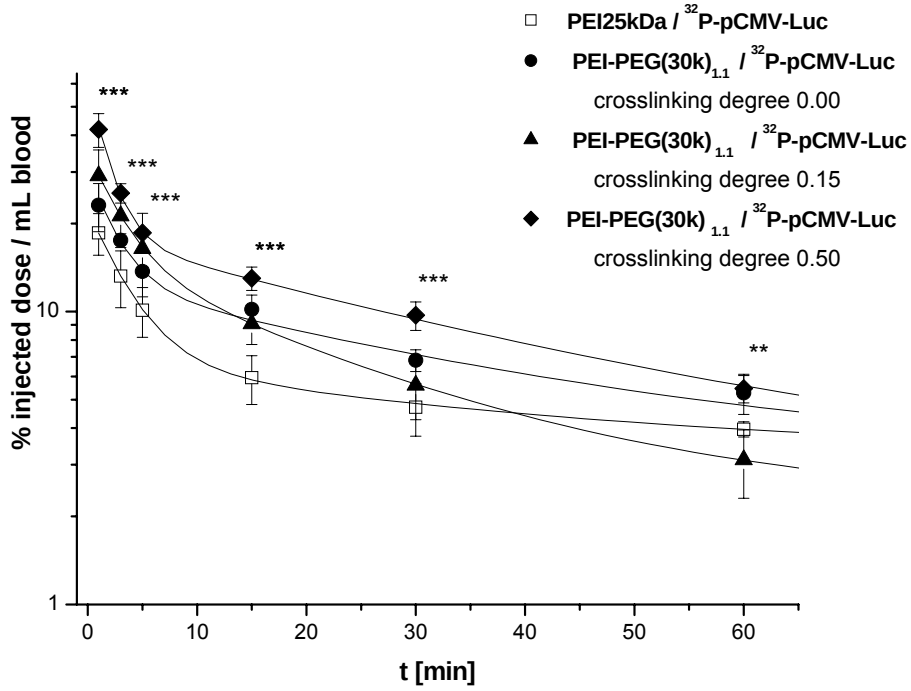


	PEI 25 kDa	PEI-PEG(30k) $_{1.1}$	PEI-PEG(30k) $_{2.4}$
AUC [% ID/mL * min]	361 ± 24	520 ± 26 ***	413 ± 60
rel. enhancement factor	1	1.4	1.1
AUC [% ID/mL * min]	767 ± 112	1916 ± 1353	704 ± 98
C max [% ID/mL]	23 ± 7	27 ± 8	29 ± 10
A [% ID/mL]	17 ± 6	20 ± 5	22 ± 6
$t_{1/2}$ alpha [min]	2.0 ± 0.9	5.3 ± 4.4	3.9 ± 3.7
B [% ID/mL]	6.6 ± 0.3	7.0 ± 2.9	7.4 ± 4.3
$t_{1/2}$ beta [min]	76 ± 13	211 ± 239	45 ± 9

Figure 8: Concentration-time profiles of PEI-PEG(30) copolymers obtained after intravenous injection of 2 µg pCMV-Luc into mice and corresponding pharmacokinetic data. (differences between modified polymers and PEI 25 kDa, *** $p < 0.001$)

Although plasma concentrations were increased after PEGylation, the circulation times are not as prolonged as reported for liposomal vectors [53] or with multivalent copolymers, which have been used to cover the surface of polycationic complexes [16, 54]. However, the high amount of plasmid used in these studies (up to 100 µg compared to 2 µg in this study) was reported to influence the concentration-time profiles. At low plasmid concentrations, the stability of PEGylated PEI in the circulation was lower than at higher concentrations [49].

Therefore, we introduced an additional stabilization to further improve the circulation times. Recently, the use of low molecular weight, amine reactive crosslinkers was reported to enhance the stability of the polyplexes [55-57]. The intrinsic disulfide bonds are intended to serve as breaking points, triggered by the lower redox potential inside the cells compared to the circulation environment [58-60]. PEI-PEG(30k)$_{1.1}$ polyplexes, which have shown significantly increased blood levels, were reacted with the homobifunctional crosslinker DSP to yield crosslinking degrees of 0.15 and 0.50 (molar ratio of DSP to amines), respectively. The stabilized PEI-PEG(30k)$_{1.1}$ polyplexes were subsequently tested for their pharmacokinetic profile. Figure 9 depicts the results in comparison with polyplexes of unmodified PEI and uncrosslinked PEI-PEG(30k)$_{1.1}$. The blood levels of plasmid were significantly influenced by the crosslinking. In addition to PEGylation, a crosslinking degree of 0.50 increased the AUC (0-60 min) to 702 ± 47 % ID/mL * min, corresponding to a relative enhancement factor of 1.9 compared to PEI 25 kDa.. However, a minimum stabilization degree seemed to be necessary, since polyplexes stabilized with a lower crosslinking degree of 0.15 only showed increased blood levels up to 15 min after injection. In contrast, PEI-PEG(30)$_{1.1}$ polyplexes at a crosslinking degree of 0.50 revealed significantly enhanced blood levels up to 60 min after injection, compared to both PEI 25 kDa and unstabilized PEI-PEG(30)$_{1.1}$ polyplexes. Blood levels were 125% higher as PEI 25 kDa directly after injection and persisted increased up to 60 min.



	PEI 25 kDa	PEI-PEG(30k) $_{1.1}$ crosslinking degree 0.00	PEI-PEG(30k)$_{1.1}$ crosslinking degree 0.50
AUC [% ID/mL * min]	361 ± 24	520 ± 26 ***	702 ± 47 ***
rel. enhancement factor	1	1.4	1.9
AUC [% ID/mL * min]	767 ± 112	1916 ± 1353	983 ± 81 *
C max [% ID/mL]	23 ± 7	27 ± 8	60 ± 13 ***
A [% ID/mL]	17 ± 6	20 ± 5	43 ± 13 **
$t_{1/2}$ alpha [min]	2.0 ± 0.9	5.3 ± 4.4	1.37 ± 0.2
B [% ID/mL]	6.6 ± 0.3	7.0 ± 2.9	17.2 ± 1.0 ***
$t_{1/2}$ beta [min]	76 ± 13	211 ± 239	36 ± 2

Figure 9: Concentration-time profiles of PEI-PEG(30)$_{1.1}$ surface stabilized by crosslinking the primary amines of the PEI with DSP obtained after intravenous injection of 2 µg pCMV-Luc into mice and corresponding pharmacokinetic data (differences between modified polymers and PEI 25 kDa, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Pharmacokinetic data analysis corroborated the results of plasmid blood level measurements. PEGylation with subsequent crosslinking of the polyplexes at a crosslinking degree of 0.50 markedly increased AUC (0-60 min) from 767 %ID/mL*min to 983 %ID/mL*min and C_{max}, from 23 ± 7 to 60 ± 13 % ID/mL.
To our best knowledge, this report is the first to deal with this special combination of stabilizing strategies.

Conclusion

Improved stability of DNA delivery vectors in the bloodstream is a prerequisite for the efficient transport of nucleic acids into cells. We synthesized a novel type of copolymers consisting of branched PEI 25 kDa low grafted with high molecular weight PEG 20 kDa and 30 kDa to obtain AB-type and ABA-block copolymer structure of low toxicity. Polyplexes between plasmid DNA and the PEI-EPG copolymers were characterized by tight DNA condensation and complexation and yielded small, biocompatible nanocomplexes with markedly reduced surface charge. In vitro experiments revealed transfection efficiency comparable to PEI or higher and N/P ratio > 7. PEI-PEG(30) copolymer polyplexes were tested in vivo and significantly increased blood concentrations were found for the AB-type block-copolymer, suggesting that low PEGylation with high molecular PEG might be beneficial in terms of increasing plasmid blood levels. Since low PEGylation spares primary amines function on PEI for further modifications, polyplex stability was additionally increased by surface crosslinking. Indeed, both modifications together synergistically improved the blood level profiles of the DNA. Due to the low grafting degree, the PEI-PEG copolymers can be further modified, e.g. with targeting moieties to improve the cell specifity. Together, the copolymers investigated here display an interesting strategy to improve intravenous application of polycationic vectors for DNA delivery.

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SUMMARY AND PERSPECTIVES

ZUSAMMENFASSUNG UND AUSBLICK

SUMMARY

This thesis describes the development of poly(ethylene imine) (PEI) conjugates as vector systems for plasmid delivery. Conjugates were synthesized and characterized regarding their suitability as non-viral vector systems for in vivo administration.

Chapter 1 gives a detailed overview of the current status of polycationic gene delivery systems based upon PEI and PEI derivatives. Basic knowledge about PEI based vectors is imparted and the range of PEI modifications currently under investigation is described in depth.

In **Chapter 2**, a novel gene delivery vector for lung administration is investigated. The conjugate is based upon a protein transduction domain, derived from the HIV TAT peptide, coupled to branched PEI via a PEG linker. The HIV TAT transduction domain is supposed to provide direct crossing over biological membranes with high translocation ability and, therefore, was hypothesized to also enhance cell uptake of plasmid DNA in the lungs. The resulting polyplexes of TAT-PEG-PEI with plasmid DNA were extensively characterized in terms of DNA condensation and complexation ability, size and surface charge, DNA protection in the intra- and extracellular lung environment and in vitro and in vivo toxicity. The transfection efficiency of the vectors was investigated in cell culture and in vivo, and related to the polyplex distribution in the mouse lung. The novel conjugate was able to form very small and stable particles with plasmid DNA, which is favorable for airway administration. A ~600% improved gene expression in the mouse lung was observed for TAT-PEG-PEI polyplexes in comparison to unmodified PEI. Furthermore, only minor effects upon lung function were observed, with no additional inflammation compared to pDNA instillation alone. A particular advantage of this carrier is its ability to transport DNA safely into the different cell types of the lung. Hence, it could be employed in the treatment of pulmonary diseases that attack the entire lung, such as lung cancer. This new carrier fulfills most of the key requirements for lung administration, namely being non-toxic and highly efficient in transfecting the epithelial cells of the conducting and respiratory airways. These results highlight that the mechanistic investigation of PEI-coupled protein transduction domains is promising in the development of stable vectors for lung administration.

In **Chapter 3**, stabilized polyplexes of HMW (high molecular weight) and LMW (low molecular weight) PEI were developed and investigated with regard to the molecular weight of the polymers and the formation procedure. It was theorized that crosslinking the primary amines of PEI would lead to enhanced polyplex stability suitable for intravenous administration. The polymers were crosslinked using a homobifunctional linker with intrinsic redox sensitive degradation properties. Two strategies to form the polyplexes were compared. First, crosslinked polymers were used to form polyplexes with plasmids. Second, polyplexes were crosslinked after formation. Only the latter method yielded small (100-300 nm) polyplexes with a positive zeta potential when HMW PEI was used, whereas crosslinked LMW PEI resulted in polyplexes with increased size (>1000 nm) and zeta potentials down to -20 mV. Only crosslinking after polyplex formation was able to enhance resistance against polyanion exchange and high ionic strength. AFM measurements showed no changes in polyplex morphology and indentation force measurements using AFM revealed significantly increased mechanical stability of crosslinked HMW PEI polyplexes. These polyplexes also displayed significant reduced interactions with major blood components like albumin and erythrocytes. These results highlight the influence of the polymer molecular weight and the formulation strategy for the formation of stable vectors.

In **Chapter 4**, the bioreversibly surface crosslinked HMW PEI polyplexes were investigated in more detail. We postulated that the intracellular redox conditions, mainly determined by the glutathione status, would influence the release properties of the DNA from the polyplex and thereby also the transfection efficiency. Indeed, the biodegradable disulfide bonds which were introduced showed a strong susceptibility to reducing conditions. Complete DNA release from the surface crosslinked polyplexes was dependent on the crosslinking degree and the redox conditions. These results were also confirmed in cell culture, where the transfection efficiency was dependent on the crosslinking degree. Increased and decreased intracellular glutathione concentration significantly influenced the transfection of the stabilized polyplexes. The in vivo behavior was also strongly influenced by the crosslinking degree. Pharmacokinetic profiles of PEI/plasmid polyplexes in mice after intravenous administration showed higher blood levels for crosslinked polyplexes, indicating successful stabilization. The liver and the lungs were identified as primary organs of polyplex deposition, with

higher crosslinking degrees leading to increases in liver deposition. Unwanted lung transfection was significantly reduced, while liver transfection remained at higher levels. These studies suggest that crosslinked polyplexes are more stable in circulation and retain their transfection efficiency after intravenous administration, but careful adjustment of the stabilization degree is required. This merits further investigation of long circulating vectors by surface stabilization.

In **Chapter 5**, the concept of surface stabilization was combined with the shielding concept using Poly(ethylene glycol) (PEG). It was hypothesized that the charge and steric shielding effect of PEG-PEI copolymers in combination with surface crosslinking would alter the stability of the polyplexes and their pharmacokinetic behavior under in vivo conditions. A set of four PEG-PEI copolymers was successfully synthesized, combining branched PEI 25 kDa with one or two high molecular weight (20 kDa and 30 kDa, respectively) PEG chains. The copolymers were generally less cytotoxic than unmodified PEI due to the shielding effect of PEG. Polyplexes of plasmid DNA with copolymers exhibited hydrodynamic diameters comparable to PEI, while the surface charge was significantly reduced. Interestingly, PEG 30 kDa containing copolymers condensed and complexed plasmid DNA even tighter than PEI. Cell culture experiments revealed high transfection efficiency of copolymer polyplexes, up to 5-fold higher than PEI for the copolymers built with 30 kDa PEG. Intravenous injection into mice revealed higher blood concentration of plasmid complexed with PEI-PEG(30k) with 1 PEG chain, indicating successful polyplex shielding. These polyplexes were further stabilized by surface crosslinking using DSP. Indeed, blood levels of plasmid could be further elevated up to 125% higher as with PEI directly after injection and persisted at higher values up to 60 min (+40%). These results highlight that a combined strategy to build stable vectors for intravenous administration is possible and promising for systemic administration.

PERSPECTIVES

The modifications of PEI presented here to yield biocompatible, stable vectors intended for systemic application have given valuable information for further development of non-viral gene delivery systems based on polycation polymers. The ultimate goal is the

development of safe and efficient vector systems for systemic delivery of nucleic acids to the tissue(s) of desire. However, several open questions still need to be addressed.

The use of protein transduction domains coupled to PEI as a vector for lung administration represents a promising new approach to in vivo application of plasmid via the airway. Still, further work in this field is necessary to obtain more information about the influence of the peptide structure on the cell transfection properties and on the influences on polyplex stability in a mucus containing environment. Currently, these issues are under investigation.

PEI 25 kDa has proven to be one of the most efficient polycationic vectors for plasmid delivery. Bioreversible stabilized PEI polyplexes were found to be susceptible to intracellular triggers like redox potential to release the DNA after cell uptake. The initial results, as described here, suggest that this surface stabilization by crosslinking might offer advantageous pharmacokinetics and biodistribution patterns of the polyplexes in mice. For instance, longer circulating vectors are necessary to achieve tumor targeting due to passive accumulation into the permeable tumor vasculature based upon the EPR effect. However, the effects are not as pronounced as for other polycationic vector systems and the transfection efficiency is very sensitive to changes in the degree of stabilization. Therefore, further studies seem to be necessary to fully evaluate the influence of the vector structure and composition on its stability and interactions on a cellular level regarding the release strategy after cell uptake.

PEGylation of PEI was also shown to increase the blood levels of polyplexes in circulation, thus representing an additional passive targeting strategy. Low grafting with high molecular weight PEG seems to be favorable in this context and further investigation of these systems would be desirable. The in vitro transfection results point to interesting transfection efficiency at low copolymer concentration, thereby reducing the amount of polymer to be applied. Since the PEI-PEG copolymers presented here are not biodegradable, this would also reduce the amount of polymer deposited in the tissues. However, further studies should investigate the feasibility of incorporation of biodegradable bonds between PEI and PEG, such as ester or disulfide bonds, to allow degradation of the vectors after cell uptake and their elimination. This would be of special interest for the treatment of chronic diseases.

The low grafting ratio of the investigated PEI-PEG copolymers allows further chemical modifications using primary amines in PEI. The combination of both passive targeting strategies, PEGylation and surface crosslinking, synergistically improved the circulation times in vivo. Interestingly, PEI-PEG copolymers possessing an AB-diblock structure were favorable in terms of prolonging the circulation times of their polyplexes with plasmids and display promising vectors for the delivery of other nucleic acid based therapeutics, such as oligonucleotides or siRNA. Currently, siRNA vector systems with AB-type PEI-PEG copolymers are under intensive investigation.

Generally, it can be stated that significant progress has been made in recent years to combine safety and efficiency of non-viral vectors with improved in vivo applicability and that polymer based gene transfer represents a promising tool for future therapeutic treatment.

ZUSAMMENFASSUNG

In der vorliegenden Dissertation wird die Entwicklung von Polyethylenimin (PEI) - Konjugate als Vektoren zur Verabreichung von Plasmiden beschrieben. Die hergestellten Konjugate wurden charakterisiert und auf ihre Eignung als nicht-virale Vektoren für die in vivo-Anwendung untersucht.

Kapitel 1 gibt eine einleitende, detaillierte Übersicht über den aktuellen Status polykationischer Gentransfersysteme basierend auf PEI und PEI-Derivaten. Grundlegendes Wissen über PEI-basierte Vektoren wird vermittelt und die Spannbreite an PEI-Modifikationen, die derzeit untersucht werden, wird beschrieben.

In **Kapitel 2** wird ein neuartiger Gentransfervektor für die Lungnenadministration untersucht. Das Konjugat basiert auf einer vom HIV TAT-Peptid abgeleiteten Proteintransduktionsdomäne, die über einen Polyethylenglykol (PEG)-Linker an verzweigtes PEI gekoppelt wurde. Von der HIV TAT-Proteintransduktionsdomäne wird vermutet, dass sie einen direkten Übergang über biologische Membranen ermöglichen kann. Wir vermuteten deshalb, dass sie ebenfalls die Zellaufnahme von Plasmid-DNA in der Lunge erhöhen könnte. Die resultierenden Polyplexe aus TAT-PEG-PEI und Plasmid-DNA wurden hinsichtlich ihrer Kondensierungs- und Komplexierungsfähigkeit für DNA, ihrer Größe und Oberflächenladung, Schutz der DNA im intra- und extrazellulären Raum sowie ihrer in vitro- und in vivo-Toxizität ausführlich charakterisiert. Die Transfektionseffizienz des Vektors wurde in Zellkultur und in vivo untersucht und mit der Polyplexverteilung in der Mauslunge verglichen. Das neue Konjugat bildete sehr kleine und stabile Partikel, günstig für die Anwendung über die Luftwege. Die Genexpression mittels TAT-PEG-PEI in der Mauslunge lag 600% über der von unmodifiziertem PEI. Weiterhin wurden nur minimale Auswirkungen auf die Lungenfunktion beobachtet, ebenso keinerlei zusätzliche entzündliche Reaktionen im Vergleich zur reinen Plasmidinstillation. Ein besonderer Vorteil dieses Trägersystems stellt seine Fähigkeit dar, DNA sicher in verschiedene Lungenzelltypen zu transportieren. Demzufolge könnte es in der Behandlung von Lungenkrankheiten, die die gesamte Lunge betreffen, wie z.B. Lungentumoren, angewendet werden. Diese Ergebnisse betonen, dass mechanistische Untersuchungen von PEI-gekoppelten

Proteintransduktionsdomäne viel versprechend für die Entwicklung stabiler Vektoren zur Lungenadministration sind.

In **Kapitel 3** wird die Entwicklung von stabilisierten Polyplexes auf Basis von hochmolekularem (HMW) und niedermolekularem (LMW) PEI beschrieben. Die Polyplexe wurden im Hinblick auf den Einfluss des Molekulargewichtes sowie der Formulierung untersucht. Theoretisch sollte die Quervernetzung der primären Amine in PEI zu einer erhöhten Polyplexstabilität führen, die sie geeignet machen würde zur intravenösen Verabreichung. Die Polymere wurden mit einem homobifunktionellen Linker quervernetzt, der eine auf das Redoxpotential ansprechende Disulfid-Gruppe enthält. Zwei unterschiedliche Formulierungsstrategien wurden untersucht. Zum einen wurden quervernetzte Polymere zur Herstellung der Polyplexe mit Plasmiden benutzt, zum anderen wurden die Polyplexe nach der Herstellung quervernetzt. Nur mit der zweiten Methode und unter Verwendung von HMW PEI konnten kleine (100-300 nm) Polyplexe mit einem positiven Zetapotential hergestellt werden. Mit LMW PEI waren die Polyplexe größer (>1000 nm) und die Oberflächenladung verringerte sich auf bis zu -20 mV. Ebenso wurde nur durch Quervernetzen der fertig gebildeten Polyplexe eine erhöhte Widerstandsfähigkeit gegen Polyanion-Austauschreaktionen und hohe Ionenstärken erreicht. Rasterkraftmikroskopische Untersuchungen zeigten keinerlei morphologische Veränderungen der Polyplexe. Bei Messungen der Eindrückkräfte bei quervernetzten HMW PEI-Polyplexe zeigte sich eine signifikant erhöhte mechanische Stabilität. Des Weiteren waren bei diesen Polyplexen Wechselwirkungen mit wichtigen Blutkomponenten wie Albumin und Erythrocyten deutlich verringert. Diese Ergebnisse zeigen den Einfluss des PEI Molekulargewichts und der Formulierungsstrategie auf die Herstellung stabilisierter Vektoren.

In **Kapitel 4** wurden die bioreversibel quervernetzten HMW PEI-Polyplexe detaillierter untersucht. Wir postulierten, dass die intrazellulären Redoxbedingungen, die hauptsächlich durch den Glutathionstatus bestimmt werden, das Freisetzungsverhalten der DNA aus den Polyplexen und damit die Transfektionseffizienz beeinflussen würden. In der Tat waren sich die eingeführten bioabbaubaren Disulfidbindungen empfindlich gegenüber reduzierenden Bedingungen. Die vollständige Freigabe der DNA aus den oberflächenquervernetzten Polyplexen war abhängig vom Vernetzungsgrad und den Redoxbedingungen. Diese Ergebnisse wurden auch in

Zellkulturtests bestätigt. Die Transfektionseffizienz war hier direkt abhängig vom Vernetzungsgrad. Auch eine Erhöhung oder Erniedrigung der intrazellulären Glutathionkonzentration beeinflusste signifikant die Transfektionseffizienz der stabilisierten Komplexe. Ihr in vivo-Verhalten wurde ebenfalls stark vom Vernetzungsgrad bestimmt. Die Pharmakokinetikprofile der PEI/Plasmid-Polyplexe in Mäusen nach intravenöser Injektion zeigten erhöhte Blutwerte für die quervernetzten Polyplexe, was auf eine erfolgreiche Stabilisierung hinweist. Leber und Lunge waren die primären Zielorgane der Polyplexe, wobei höhere Vernetzungsgrade zu einer erhöhten Polyplexdeposition in der Leber führten. Die unerwünschte Lungentransfektion wurde signifikant reduziert, während die Transfektion in der Leber auf hohen Werten erhalten blieb. Diese Untersuchungen legen nahe, dass quervernetzte Polyplexe stabiler in Zirkulation sind und ihre Transfektionseffizienz nach intravenöser Injektion erhalten. Allerdings scheint eine sorgfältige Einstellung des Stabilisierungsgrades notwendig zu sein.

In **Kapitel 5** wurden die Konzepte der Oberflächenstabilisierung sowie der Abschirmung mittels PEG kombiniert. Wir vermuteten, dass die Kombination aus Ladungs- und sterischer Abschirmung durch PEGylierung mit Oberflächenquervernetzung die Stabilität der Polyplexe und ihre Zirkulationszeiten weiter erhöhen würde. Vier PEG-PEI-Copolymere wurden durch Kopplung von verzweigtem PEI 25 kDa mit ein bzw. zwei PEG-Ketten mit hohem Molekulargewicht (20 kDa, 30 kDa) synthetisiert. Durch den abschirmenden Effekt der PEG-Ketten waren die Copolymere insgesamt untoxischer als unmodifiziertes PEI. Die Polyplexe der Copolymere mit Plasmiden hatten hydrodynamische Durchmesser vergleichbar zu PEI-Polyplexen, während die Oberflächenladung signifikant reduziert wurde. Interessanterweise zeigten die PEG 30 kDa enthaltenden Copolymere ein besseres Kondensierungs- und Komplexierungsverhalten für Plasmide als reines PEI. Zellkulturexperimente machten die hohe Transfektionseffizienz der Copolymerpolyplexe deutlich, die bis zu 5-fach höher lag als die von PEI bei den Copolymeren mit 30 kDa PEG. Nach intravenöser Injektion in Mäuse zeigten die Plasmide, die mit PEI-PEG(30k) mit nur einer PEG Kette komplexiert waren, höhere Blutwerte, was auf eine erfolgreiche Abschirmung der Polyplexe hinweist durch PEG. Diese Polyplexe wurden durch Oberflächenquervernetzung mit DSP stabilisiert.

Tatsächlich konnten so die Blutspiegel nochmals erhöht werden im Vergleich zu PEI, bis zu 125% höher direkt nach Injektion. Die Werte blieben erhöht bis 60 min nach Injektion. Diese Ergebnisse betonen, dass eine kombinierte Strategie zur Formulierung stabiler Vektorsysteme für die intravenöse Anwendung möglich und zugleich viel versprechend ist für eine systemische Anwendung.

AUSBLICK

Die hier beschriebenen PEI-Modifikationen für biokompatible, stabile Vektoren insbesondere zur systemischen Anwendung haben wertvolle Informationen geliefert für die weitere Entwicklung nicht-viraler Gentransfersysteme basierend auf polykationischen Polymeren. Dennoch verbleiben offene Fragen, wie das eigentliche Ziel, die Entwicklung sicherer und effizienter Vektoren für den systemischen Transport von Nukleinsäuren in Zielgewebe, erreicht werden kann.

An PEI gekoppelte Proteintransduktionsdomänen als Vektoren für die Lungenapplikation stellen einen viel versprechenden neuartigen Ansatz dar zur Verabreichung von Plasmiden über die Luftwege. Weitergehende Untersuchungen in diesem Bereich scheinen allerdings vonnöten, um insbesondere mehr Informationen über den Einfluss der Peptidstruktur auf die Transfektionseigenschaften und auf die Polyplexstabilität, auch vor Allem in mukushaltiger Umgebung, zu erhalten. Zurzeit werden diese Fragestellungen untersucht.

PEI 25 kDa hat sich als einer der effizientesten polykationischen Vektoren zum Plasmidtransfer herausgestellt. Bioreversibel stabilisierte PEI Polyplexe waren in der Lage, DNA nach Aufnahme in Zellen getriggert durch das intrazelluläre Redoxpotential freizusetzen. Die ersten Resultate, die in dieser Arbeit beschrieben werden, deuten darauf hin, dass Oberflächenstabilisierung durch Quervernetzung vorteilhafte Pharmakokinetiken und Bioverteilungen der Polyplexe in Mäusen ermöglichen kann. Länger zirkulierende Vektoren sind zum Beispiel nötig, um Tumortargeting durch passive Akkumulation durch die durchlässigen Gefäße in Tumoren (EPR-Effekt) zu erreichen. Allerdings haben sich die Effekte als nicht so ausgeprägt herausgestellt, wie sie für andere polykationische Vektoren beschrieben wurden. Die Transfektionseffizienz ist zudem stark abhängig von Änderungen im

Stabilisierungsgrad. Deshalb scheinen weitergehende Studien notwendig, um den Einfluss der Vektorstruktur und -zusammensetzung auf die Stabilität und die Wechselwirkungen auf zellulärem Niveau bezüglich der Freisetzungsstrategie nach Zellaufnahme vollständig abschätzen zu können.

Durch PEGylierung von PEI konnte ebenfalls die Blutspiegel der Polyplex in Zirkulation erhöht werden, was eine zusätzliche Möglichkeit des passiven Targetings eröffnet. Niedrige Substitution mit hochmolekularem PEG scheint sich dahingehend günstig auszuwirken, eine weitergehende Untersuchung dieser Systeme wäre deshalb wünschenswert. Die in vitro Ergebnisse zeigen interessante Transfektionseffizienzen schon bei niedrigen Polymerkonzentrationen, dadurch reduziert sich die Menge an zu applizierendem Polymer. Da die hier vorgestellten PEI-PEG Copolymere nicht bioabbaubar sind, würde sich dadurch auch die Menge an Polymer, das in Gewebe deponiert wird, verringern. Allerdings sollte in zukünftigen Studien die Möglichkeit bioabbaubarer Bindungen zwischen PEG und PEI, zum Beispiel Ester- oder Disulfidbindungen, untersucht werden. Das würde den Abbau des Vektors nach Zellaufnahme und seine Ausscheidung erlauben. Dies wäre insbesondere bei der Behandlung von chronischen Krankheiten von besonderem Interesse.

Der niedrige Substitutionsgrad der untersuchten PEI-PEG Copolymere erlaubt weitergehende chemische Modifikationen der primären Amine in PEI. Die Kombination beider passiver Targetingstrategien, PEGylierung und Oberflächenquervernetzung, erhöhte die Zirkulationszeiten in vivo weiterhin. Interessanterweise stellten sich die PEI-PEG Copolymere mit der AB-Diblock-Struktur in Bezug auf die Verlängerung der Zirkulationszeiten ihrer Polyplexe mit Plasmiden als vorteilhaft heraus. Damit stellen sie viel versprechende Vektoren auch für andere Nukleinsäure-Therapeutika, wie zum Beispiel Oligonukleotide oder siRNA dar. Zurzeit werden siRNA-Vektorsysteme basierend auf PEI-PEG Copolymeren des AB-Typs intensiv untersucht.

Insgesamt lässt sich sagen, dass in den letzten Jahren maßgebliche Fortschritte gemacht wurden, um Sicherheit und Leistungsfähigkeit nicht-viraler Vektoren mit verbesserter in vivo-Anwendbarkeit zu vereinen. Polymerbasierter Gentransfer stellt daher ein viel versprechendes therapeutisches Werkszeug für zukünftige Behandlungsansätze dar.

APPENDICES

ABBREVIATIONS

AUC	Area under the curve
CTP	Cytidintriphosphate
DSP	Dithiobis(succinimidyl propionate)
DTBP	Dithiobis(sulfosuccinimidyl propionate)
EPR	Enhanced vascular permeability and retention
HBS	Hepes buffer saline
Mw	Molecular weight (Da=g/mol)
NLS	Nuclear localization sequences
OD	Optical density
ODN	Oligonucleotide
PAMAM	Poly(amido amine)
PEG	Poly(ethylene glycole)
PEI	Poly(ethylene imine)
PEG-PEI	PEI-PEG copolymer
PHMPA	Poly[N-(2-hydroxypropyl) methacrylamide]
PLL	Poly(L-lysine)
PPG	Poly(propylene glycole)
NHS	N-Hydroxy-succinimide
N/P ratio	Ratio of nitrogen containing groups of the polymer to phosphate groups of the nucleic acid
RES	Reticuloendothelial System

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LIST OF PUBLICATIONS

Patents:
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