POLYETHYLENIMINE AND ITS DERIVATIVES:

INVESTIGATION OF *IN VIVO* FATE, SUBCELLULAR TRAFFICKING AND DEVELOPMENT OF NOVEL VECTOR SYSTEMS

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1

INTRODUCTION

AIMS OF THIS DISSERTATION

The aim of this dissertation was to gain insight into crucial steps involved in polymeric gene delivery and to construct novel vectors with advantageous properties.

A major goal was to clarify crucial steps in the subcellular trafficking of polyethylenimine/nucleic acid complexes. Several publications have dealt with this issue, however, conclusive data describing mechanisms of cellular uptake and especially modalities of endosomal or lysosomal release are limited. To achieve this, it was necessary to monitor complex uptake and to identify the major subcellular compartment(s) of accumulation. Furthermore, we were interested in visualizing complex release from vesicular structures in order to estimate the extent to which this event happens and acquire insights into the mechanism. These issues were investigated by living cell confocal laser scanning microscopy and inhibitor experiments.

A major shortcoming of polymeric gene delivery is the low specificity of polymer/nucleic acid complexes. Consequently, our aim was to develop a gene delivery system with a high specificity for target cells combined with a very low unspecific transfection of non target cells. We chose an ovarian carcinoma model for these studies and used an antibody directed against an epitope expressed by most ovarian carcinoma cells. We constructed a vector system by coupling the antigen binding fragment (Fab´) of this antibody to pegylated polyethylenimine. Our hypothesis was that the positive charge of the poly-ethylenimine could be efficiently shielded by the linear PEG chains thus leading to a decrease in unspecific binding to non target cells, as well as increased specificity of the vector.

Currently, only rudimentary knowledge of the stability of polyethylene-imine/DNA and pegylated polyethlenimine/DNA complexes in the bloodstream is available. To address this issue, we performed pharmacokinetic studies in mice, in which polymer and DNA were labelled with two different radioactive

tracers. Comparison of polymer and DNA profiles allowed us to form conclusions about complex stability. Furthermore, in order to get an insight into the mechanisms of complex destabilization, we performed *in vitro* stability investigations, in which we tried to simulate certain aspects of an *in vivo* environment.

A further issue addressed in this thesis was the development of a new vector system with favourable properties, such as a high transfection efficiency, high stability and a low toxicity. To achieve this, novel shielding strategies were developed, which exhibited an onion-like structure when complexed with DNA. The inner core of the system consisted of polyethylenimine and DNA surrounded by a hydrophobic shell and an outer hydrophilic corona. Further, a triblock polymer was developed consisting of polyethylenimine, polycaprolactone and polyethylenglycol. In order to enhance the solubility of this compound, cyclodextrin was threaded over the linear polyethylenglycol and polycaprolactone chains. Our hypothesis was that, due to the hydrophobic chains surrounding the central core of the vector, complexes should become more stable, as a result of the decreased penetration of e.g. plasmaproteins into the core. The *in vivo* properties of these novel vectors were investigated and pharmacokinetic studies in mice were performed.

PROSPECTS FOR CATIONIC POLYMERS IN GENE AND OLIGONUCLEOTIDE THERAPY AGAINST CANCER

SUMMARY

Gene and antisense/ribozyme therapy possess a tremendous potential for the successful treatment of genetically based diseases, such as cancer. Several cancer gene therapy strategies have already been realized *in vitro*, as well as *in vivo*. A few have even reached the stage of clinical trials, most of them phase I, whereas some antisense strategies have advanced to phase II and III studies. Despite this progress, a major problem in exploiting the full potential of cancer gene therapy is the lack of a safe and efficient delivery system for nucleic acids. As viral vectors possess toxicity and immunogenicity, non viral strategies are becoming more and more attractive. They demonstrate adequate safety profiles, however their rather low transfection efficiency remains a major drawback. This review will introduce the most important cationic polymers used as non viral vectors for gene and oligonucleotide delivery and will summarize strategies for the targeting of these agents to cancer tissues. Since the low efficiency of this group of vectors can be attributed to specific systemic and subcellular obstacles, these hurdles, as well as strategies to circumvent them, will be discussed. Local delivery approaches of vector/DNA complexes will be summarized and an overview of the principles of anticancer gene and antisense/ribozyme therapy as well as an outline of ongoing clinical trials will be presented.

1. INTRODUCTION

Since progress in the therapy of cardiovascular diseases is improving rapidly, cancer is on the way to become leading cause of death in industrialized countries. Due to its invasive, aggressive growth profile as well as the complex mechanisms involved in cancer development and propagation, an efficient, specific therapy is not yet available. If a tumor is discovered at a defined, well localized stage, surgery, chemo- and/or radiation therapy may represent promising curative strategies. However, at latter stages, especially when

metastases have already spread to remote sites of the body, such approaches are less successful. In this case, chemotherapy is the most frequently applied treatment. However, it exhibits general limitations, such as a poor specificity of most chemotherapeutic drugs resulting in suppression of the bone marrow and other fast dividing tissues as well as potential genesis of secondary cancer. Another drawback of chemotherapy is the development of resistant phenotypes, which no longer respond to chemotherapy thus making the treatment inefficient. Therefore, an urgent need is evident for new anticancer strategies exhibiting high specificity, low toxicity and lack of resistant phenotypes. Conventional drugs to specifically block molecular pathways responsible for uncontrolled cancer growth [1], angiogenesis [2] or metastases genesis [3] are currently under investigation, however success is still limited.

The human genome project has substantially increased our knowledge about molecular mechanisms of cancer during the last decade, thus opening up new possibilities for cancer gene therapy. In contrast to classic cancer treatments, this approach in theory represents a much more efficient and specific therapy, due to its ability to directly influence the defected genes responsible for cancer. Several strategies have been considered for manipulation of gene expression either on the transcriptional or on the translational level and have been performed successfully *in vitro* and *in vivo*. A deficient gene can either be replaced or the effect of an unwanted gene can be blocked by introduction of a counteracting one. Suicide gene therapy offers the perspective to kill cancer cells selectively by using prodrug-converting enzymes and tumor specific promoters. Furthermore antisense and ribozyme strategies offer the potential to selectively downregulate the expression of specific genes mainly on the translational level predominantly by sequence specific interaction with messenger RNAs.

Despite the enormous potential of gene therapy, there are still numerous difficulties to be overcome before efficient clinical application is attempted.

Although remarkable advances have been made in identifying target structures for cancer gene therapy and synthesis or biotechnological production of nucleic acids has become feasible in larger quantities, progress is mainly hampered by the lack of a safe and efficient delivery system.

Generally, two different approaches have been utilized for the delivery of nucleic acids in gene therapy, namely that of viral vectors and non viral delivery systems mainly using cationic polymers or lipids. Viral vectors including retroviruses, adenoviruses and adeno-associated viruses impress by their high efficiency in introducing their genetic material into host cells. Researchers thought that it would be feasible to domesticate viruses and to take advantage of them for therapeutic needs. However, in the last few years a reappraisal has occurred due to serious safety risks. Viral vectors show excellent transfection efficiencies. However, they develop a high immunogenicity after repeated administration since the mammalian immune system has developed strategies to eliminate viral invaders as well. Other problems associated with viral vectors are their potential oncogenicity due to insertional mutagenesis and the limited size of DNA that can be carried. Furthermore the inclusion of a targeting moiety in order to transfect specific cell types or tissues is problematic. Despite these problems, still more than two thirds of clinical gene therapy trials use viral vectors. Because of these concerns non viral vectors are emerging as a viable alternative. Non viral systems, especially polymers, show significantly lower safety risks and can be tailored to specific therapeutic needs. They are capable of carrying large DNA molecules and can be produced in large quantities easily and inexpensively. The major disadvantage of these non viral vectors is their low transfection efficiency. Great efforts have been undertaken to improve the efficiency, however we are still far away from a system that could be considered as satisfactory. In the past much work went into the improvement of transfection efficiency under *in vitro* conditions with little attention of vectors for *in vivo* use. However most applications especially in the context of cancer

gene therapy require an *in vivo* application. A thorough investigation of the behavior of vector/nucleic acid complexes *in vivo* and key obstacles to their effectiveness is essential for further rational advances.

The first section of this review provides an overview of the most frequently used cationic polymers in non viral gene and oligonucleotide delivery. Since the inefficiency of these vectors in (cancer) gene therapy can be attributed to specific hurdles on a systemic, as well as subcellular level, strategies to surmount these will be discussed. The second section provides an overview of gene therapy and antisense/ribozyme strategies, as well as clinical trials using these approaches.

Looking for efficient and safe vector systems for cancer gene therapy raises the question: What is easier, taming viruses or making synthetic vectors more intelligent and efficient? A paradigm shift is currently underway, as it is questionable if viral vectors will ever be considered safe. Therefore the development of non viral systems, which may be safer and more versatile, is warranted. The goal must be to create systems that act as targeted synthetic viruses displaying high specificity for cancer tissue, high transfection efficiencies and controllable safety risks. Vectors that are currently used in non viral gene therapy are far from optimal, however studying their biological and physicochemical properties will provide valuable knowledge for the future design of more sophisticated systems. Regarding the advantages of non viral vectors compared to viruses it is obvious that they have a great perspective in (cancer) gene therapy, although more research is needed for optimisation of these systems. A combination of non viral vectors with viral moieties like fusogenic peptides or protein transduction domains may represent additional promising approaches.

2. NON VIRAL VECTORS

Cationic polymers and cationic lipids are by far the most widely used vectors in non viral gene and oligonucleotide delivery. Other strategies including particle bombardment, ultrasound transfection [4] or the application of naked DNA have been realized as well, however their applicability is restricted to specific circumstances. This review focuses on cationic polymers. For a recent review about gene delivery using cationic lipids we refer to reference [5].

Polymers display striking advantages as vectors for gene delivery. They can be specifically tailored for the proposed application by choosing appropriate molecular weights, coupling of cell or tissue specific targeting moieties and/or performing other modifications that confer upon them specific physiological or physicochemical properties. After identifying a suitable polymer structure a scale up to the production of large quantities is rather easy as well. A weakness of gene therapy with cationic polymers is our limited knowledge regarding the formation of electrostatic complexes with DNA and their biological effects. For example when forming complexes with polyethylenimine (PEI) the addition of polymer to DNA, rather than the opposite results in higher transfection efficiency [6]. Many other factors play crucial roles in this context, e.g. concentration of the polymer and DNA solutions, ionic strength of the solvents [7] and speed of mixing. Thus, gene therapy with cationic polymers is still rudimentarily understood and ongoing investigations will provide avenues to more sophisticated approaches. Figure 1 gives an overview over frequently used cationic polymers for non viral nucleic acid delivery.

2.1. Polyethylenimine (PEI)

PEI polymers have become the gold standard of non viral gene delivery. Polymers with different molecular weights and degrees of branching have been synthesized and evaluated *in vitro* as well as *in vivo*. Highly branched polymers

such as the 25 kDa PEI (Aldrich) and the 800 kDa PEI (Fluka) are most frequently used as well as polymers with lower degrees of branching [8]. PEI polymers are able to effectively complex even large DNA molecules [9,10], leading to homogeneous spherical particles with a size of around 100 nm or less that are capable of transfecting cells efficiently *in vitro* as well as *in vivo*. They offer a significantly more efficient protection against nuclease degradation than other polycations, e.g. poly(L-lysine) possibly due to their higher charge density and more efficient complexation. The huge amount of positive charges, however, results in a rather high toxicity of PEI polymers which is one of the major limiting factors especially for its *in vivo* use. The high density of primary, secondary and tertiary amino groups exhibiting protonation only on every 3rd or 4th nitrogen at pH 7 confers significant buffering capacity to the polymers over a wide pH range. This property, known as ´proton sponge property´ [11] is likely one of the crucial factors for the high transfection efficiencies obtained with these polymers. Despite this recognized association, knowledge about relationships between polymer structure and important biological properties such as toxicity or transfection efficiency is rather limited. Polymers with high molecular weight, e.g. the highly branched 25 kDa or 800 kDa PEI exhibit high transfection efficiencies, however toxicity is rather high as well [8]. Polymers with low molecular weight, e.g. with a molecular weight of 800 Da, display low toxicity yet transfection efficiency is very low as well [own unpublished data]. An approach to combine the advantages of high and low molecular weight PEI has been realized recently by crosslinking small PEIs via biodegradable disulfide bonds. A greatly enhanced transfection efficiency of crosslinked small PEIs could be observed with only a moderate increase in toxicity [12].

Not only the size, also the degree of branching plays an important role for biological properties of complexes with nucleic acids. Linear PEIs [13] have been synthesized and investigated [14] and it could be demonstrated that linear PEI 22 kDa, e.g. ExGen^{TM} 500 (Euromedex, France), displays excellent

transfection efficiency with a rather low toxicity [13-15]. Linear PEI has recently been reported to mediate a cell cycle independent nuclear entry of plasmid DNA [16] This finding is of particular importance in the therapy of slowly dividing tissues.

2.2. Poly(L-lysine)

Poly(L-lysine) was one of the first polymers used in non viral gene delivery and a large variety of polymers with different molecular weights have been utilized in physicochemical and biological experiments [17]. Due to its peptide structure poly(L-lysine) is biodegradable, a property that makes it especially suitable for *in vivo* use, however the polymer exhibits modest to high toxicity. If prepared with poly(L-lysine) of suitable molecular weights and at suitable N/P ratios, complexes with plasmid DNA display a size of around 100 nm [17] and are taken up into cells as efficiently as PEI complexes (own unpublished data), however transfection efficiencies remain several orders of magnitude lower. A potential reason for this is the lack of amino groups with a pKa between 5 and 7, thus allowing no endosomolysis and low levels of transgene expression [18]. The inclusion of targeting moieties or co-application of endosomolytic agents like chloroquine [19] or fusogenic peptides [20] may improve reporter gene expression.

2.3. Imidazole containing polymers

Polymers containing the heterocycle imidazole have shown promising transfection capabilities. In several approaches modification of ε-amino groups of poly(L-lysine) using histidine or other imidazole containing structures showed a significant enhancement of reporter gene expression compared to poly(L-lysine) [21-23]. The imidazole heterocycle displays a pKa around 6 thus possessing a buffering capacity in the endolysosomal pH range, and possibly mediating vesicular escape by a ´proton sponge´ mechanism. Supports for this

assumption is the fact that endosomal acidification is required for the efficiency of these polymers in gene transfer [23]. A sophisticated approach is the optimization of the balance between free ε-amino groups of L-lysine moieties that enable effective complex formation with DNA and the amount of imidazole heterocycles responsible for endolysosomal escape of the complexes. With this approach a polymer has been developed that mediates a transfection efficiency equal to PEI and demonstrates little toxicity due to its optimized charge density [24]. The incorporation of imidazole moieties represents a promising option for the improvement of endolysosomal escape and enhancement of the efficiency of polymers without increasing toxicity.
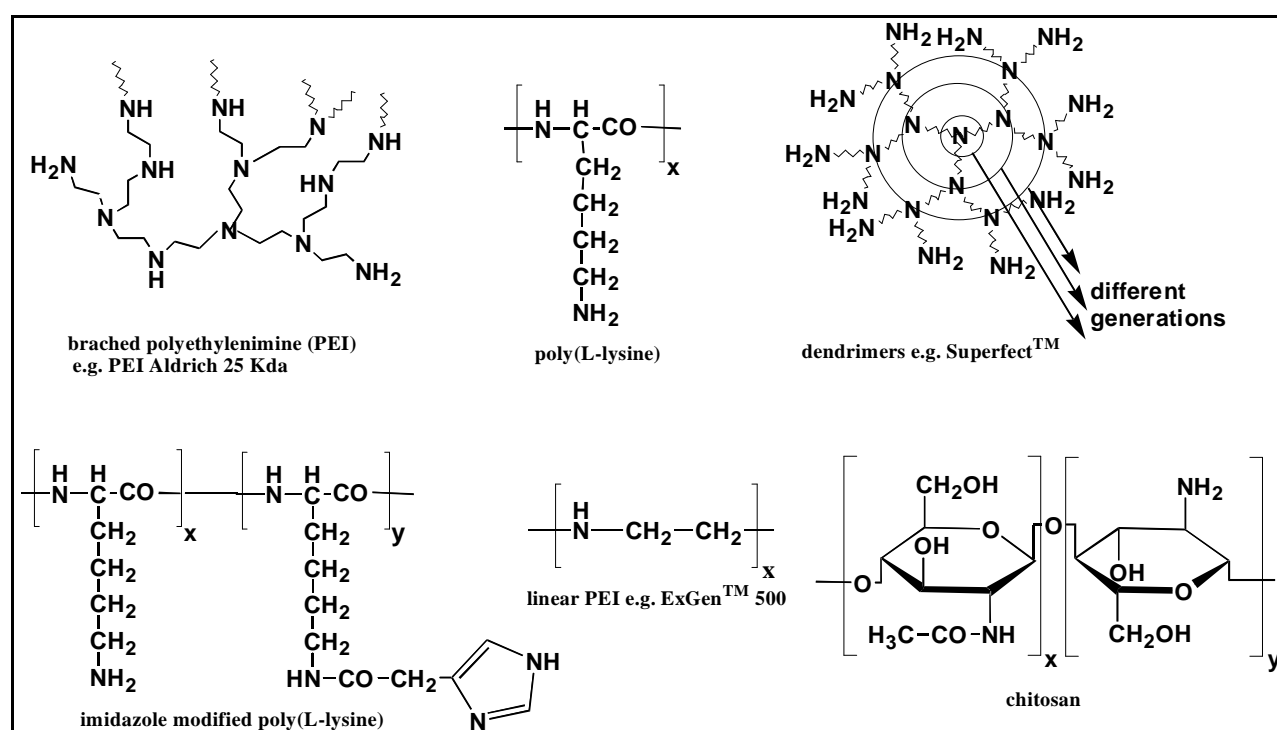
Figure 1
Cationic polymers most frequently used for nucleic acid delivery

2.4. Chitosans

Chitosans are biodegradable linear aminopolysaccharides with randomly distributed beta$_{1-4}$ linked N-acetyl-D-glucosamine and D-glucosamine, derived from the common biopolymer chitin. They have been utilized as a food additive for some time and display a significantly better biocompatibility than PEI, thus

they represent interesting candidates as gene transfer agents. Depending on the molecular weight and the degree of deacetylation, chitosans are capable of forming small (<100 nm), rather stable, toroidal complexes with plasmid DNA [25,26] and provide protection for the complexed DNA against DNase degradation that is comparable to PEI [26]. Structure-property relationships show that the percentage of positively charged monomer units must be greater than 65 % in order to obtain stable complexes capable of transfecting cells *in vitro* [26]. Small (1.2 kDa) and very large chitosan polymers lead to minimal levels of reporter gene expression [27]. However, in the molecular weight range between 30-170 kDa chitosan polymers provide levels of gene expression that are comparable to PEI [26]. When a pH-sensitive endosomolytic peptide is incorporated into chitosan/plasmid complexes, significantly increased levels of reporter gene expression can be observed [25] indicating that endolysosomal escape may be a major limiting factor in gene delivery with chitosans, probably due to the poor buffering capacity of this polymer around pH 5.5.

Remarkably the kinetics of gene expression seem to be different for chitosans compared to PEI. While gene expression using PEI reaches maximum levels at 24-72 h post incubation depending on the kind of plasmid and cell line, it reaches its highest levels for chitosans significantly later [26,28]. The reason for these different kinetics is unclear. While for PEI the polymer itself displays buffering capacity, resulting in rapid escape, it is possible that in the case of chitosans degradation of the polymer is crucial. Lysosomal enzymes may degrade the polymer into small molecules, thus leading to increased osmolarity and eventually to subsequent release by rupture of lysosomes [26]. This however is speculation.

Intratracheal instillation of chitosan and PEI (25 kDa) polyplexes revealed similar distribution patterns, however levels of gene expression were approximately 10fold lower for chitosan [26].

2.5. Dendrimers

Dendrimers are spherical, highly branched polymers prepared either by divergent (starting from a central core molecule) or convergent (starting with what will become the periphery of the molecule building inwards) synthesis strategies. The degree of branching is expressed in the generation of the dendrimer. Most commonly used dendrimers for non viral nucleic acid delivery are synthesized via the divergent strategy [29] and represent 6th generation Starburst^{TM} polyamidoamine (PAMAM) dendrimers either in intact (Polyfect^R) or fractured (Superfect^R) form. Intact dendrimers bear two new polymer arms at each point of branching, whereas in fractured polymers either one or two arms originate or the polymer is terminated at this point. Similar to PEI the structures of these polymers show high densities of amines in the periphery of the molecule. These outer amines enable efficient condensation of nucleic acids, leaving the inner amine functions available for neutralization during endolysosomal acidification, thus enabling more efficient endosomal escape. Concerning 6th generation PAMAM dendrimers the fractured dendrimers show significantly enhanced (>50-fold) levels of reporter gene expression compared to the intact polymer. The reason for this finding is unclear yet, however an increased flexibility of the polymer with a better ability to complex DNA might play a crucial role [29].

2.6. Comparison of transfection agents

Generally the *in vitro* transfection efficiency of a polymer or lipid formulation depends on a large variety of factors including dose, N/P ratio, confluency of cells, composition of incubation medium, mode of complex formation and time of incubation. Therefore, optimal formulation techniques would be established by considering each of these factors and their influence on complex properties, cellular uptake, toxicity and transfection efficiency.

Only a few studies comparing efficiency and toxicity of several transfection reagents under similar experimental conditions have been performed. Table 1 shows results of a study using several commercially available transfection agents (Data from [30]) at optimized N/P ratios on Cos-7 cells. It is obvious that lipid formulations overall showed a higher toxicity than polymers tested, although doses were only slightly more than half than when using polymers. Among the latter linear PEI ExGen^{TM} 500 and the dendrimer formulation Superfect^{TM} show superior transfection efficiency compared to PEI 25 kDa (Aldrich) with moderate toxicities. Among lipids only LipofectAMINE^{TM} exhibits high transfection efficiency (higher than any of the tested polymers). Since not all factors mentioned above have been optimized for each formulation in this study, changes in experimental conditions may lead to significantly different results.

Transfection system	DNA dose (μg/well)	Luciferase expression % of ExGen^{TM} 500	cellular protein % of control
POLYMERS			
ExGen^{TM} 500	0,75	100	79
(linear PEI 22 Kda)			
PEI (25 Kda), Aldrich	0,75	39	85
Superfect^{TM}	0,75	134	84
(dendrimer formulation)			
LIPID FORMULATIONS			
Lipofectin^{TM}	0,4	40	63
LipofectAMINE^{TM}	0,4	176	61
Cellfectin^{TM}	0,4	8	67

Table 1
Comparison of reporter gene expression and toxicity of different cationic polymers and lipids at optimized N/P ratios on Cos-7 cells (Data from [30]).

3. HURDLES IN NUCLEIC ACID DELIVERY

Ideally, a vector/nucleic acid complex should be delivered exclusively to target tissue where it may be subsequently taken up and further processed on the cellular level. To accomplish this, however, the complexes must first overcome numerous obstacles, such as misrouted deposition or complex destabilization with subsequent degradation in the bloodstream. On the cellular level great amounts of complexes are buried in the endolysosomal compartment or degraded in the cytoplasm. In the end, only a very small fraction of the applied dose has been observed to exhibit the desired effect, whereas the remaining portion of the dose is either inactivated or shows gene expression elsewhere.

3.1. Hurdles on the systemic level

3.1.1. The endothelial barrier

One major problem for systemic gene therapy is the delivery of agents beyond the endothelial barrier. Extravasation of complexes is highly dependent on their size and the permeability of the endothelia at specific sites. In most tissues the structure of the endothelia is tight. Only organs and tissues with an irregular fenestration, such as the liver, spleen, bone marrow and certain tumors (see below), have endothelia with large meshes, which allow extravasation of molecules ranging up to $0.1 - 1$ μm. Thus, in most tissues the access of polyplexes to parenchymal cells is denied, a fact that hampers the efficacy of gene therapy dramatically.

3.1.2. Problems resulting from cationic surface charge

Unmodified polyplexes exhibit numerous problems when applied systemically. The major problem associated with this type of vectors is their cationic surface charge, which leads to numerous unspecific interactions with e.g. cellular blood components, vessel endothelia and plasma proteins such as albumin, fibronectin,

immunoglobulines, complement factors or fibrinogen [31]. These interactions lead to very short plasma half lives for complexes with a high cationic surface charge [32].

Due to their size and the high number of cationic charges, polyplexes can activate the complement system [33] in a manner that suggests a correlation between the density of accessible positive surface charges and the extent of complement activation. Activation occurs via attachment of complement components, i.e. factor C3, to the complex surface and eventually leads to complex removal by the reticular endothelial system (RES). Large cationic polymers, such as long chain poly(L-lysine), PEI 25 kDa or high generation dendrimers, have enormous potential in activating the complement system, while small polyamines, i.e. oligo(L-lysine), exhibit this effect to a much lesser extent [33]. Complex formation of such polymers with DNA reduces the overall charge and as a consequence also reduces complement activation significantly. A decrease in the nitrogen to phosphate ratio leads to lower extents of activation and electrostatic neutral complexes only exhibit very low levels of complement activation.

Interactions with plasma proteins are omnipresent *in vivo* and, therefore, play a major role in determining circulation time and cellular uptake. The major component, albumin, is primarily responsible for the rapid clearance of complexes from the bloodstream [34]. It has been demonstrated that interaction of albumin with polyplexes leads to the formation of ternary complexes with a reversed surface charge [34,35], resulting in the formation of large aggregates [32,35]. These associates are removed rapidly from the bloodstream, presumably via phagocytic capture by scavenger receptors of phagocytic liver cells or via accumulation in fine capillary beds.

The administration of highly charged complexes leads to aggregate formation with cellular blood components, especially with erythrocytes. A subsequent obstruction of blood vessels accompanied by undesired consequences, such as

pulmonary embolism [31] may be the result. The complex-mediated aggregation of erythrocytes also influences the biodistribution and gene expression patterns of polyplexes *in vivo*, resulting in enhanced accumulation in the lung, due to a certain sieve effect of the pulmonary capillaries.

3.1.3. Biodistribution and gene expression after i.v. application

The biodistribution of unmodified polyplexes using different cationic polymers is quite similar. Organ distribution of the complexes immediately after injection shows a high accumulation in the lung, approximately 80-90 % of the injected dose, possibly resulting from aggregate formation with blood cells or plasma proteins and subsequent filtration in fine lung capillaries [35]. Due to the low stability of such aggregates, complexes are often released into the circulation again, leading to a secondary redistribution with high concentrations found in Kupffer cells of the liver. The endothelial tissue of other organs and tissues, e.g. spleen, kidney and especially endothelia close to the site of injection, accumulate significant levels of complexes, as well [36]. After 30 minutes post injection, usually less than 5 % of the injected dose is detectable [31] and the majority of the complexes remaining in circulation are attached to cellular blood components. A typical biodistribution pattern after i.v. administration of poly(L-lysine)/DNA complexes is shown in Figure 2.

Gene expression patterns after i.v. administration are rather similar to those of organ distribution, whereas phagocytic capture by the RES does not lead to high levels of reporter gene expression. The highest levels of gene expression are found in the lung, not only due to enhanced deposition, but also due to a more efficient gene expression in this organ [37]. Although the lung capillaries possess a tight endothelia, reporter gene expression after i.v. application has been measured not only in endothelial, but also in interstitial cells [38,39]. Some studies have even discovered a rapid crossing of the endothelial barrier by polyplexes [39], although the mechanism of this transport has yet to be

elucidated. It has been suggested that at sites where the vasculature is fragile, as in the alveoli, small complexes may be able to pass the endothelial barrier, due to vascular leakage. This theory is favored by experimental findings, which showed that only PEI/DNA complexes with a size of around 60 nm are able to exhibit this effect [40]. Other mechanisms, such as transport via a form of transcytosis or the existence of transport systems for polyamines, have also been postulated. Support for the latter theory has been shown in studies demonstrating that polyamines, such as putrescine, spermine or spermidine, are taken up into arterial endothelial cells via specific polyamine carrier systems [41,42]. Overall the lung represents an attractive organ for non viral gene delivery for the treatment of e.g. cystic fibrosis or lung cancer.
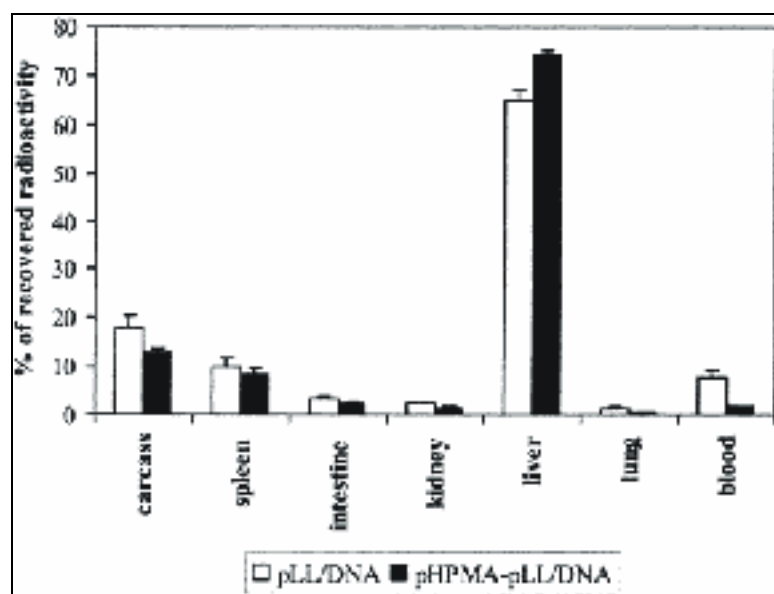
Figure 2
Typical organ distribution 30 min after i.v. administration of cationic polymer/DNA complexes. Empty columns show distribution of unmodified complexes, filled columns of pHPMA-modified complexes (Figure from [48]).

3.1.4. Steric stabilization of complexes

The pattern of organ distribution showing initial deposition in the lung and subsequent rapid uptake predominantly into Kupffer cells of the liver is of limited value for therapeutic application. Hence, strategies must be developed to

change this biodistribution pattern and to prolong circulation, hereby enabling the targeting of specific tissues.

Steric stabilization involves the attachment of hydrophilic polymers to complexes, thus shielding positive surface charges and creating a steric barrier against aggregation with e.g. albumin, complement factors or cellular components in the bloodstream. The modification of such complexes may reduce the potential of nonspecific interactions, such as opsonization or deposition due to cationic surface charges. Several strategies have been tested for their ability to shield the cationic surface charge of polyplexes.

Polyethylenglycol: Two different general strategies using PEG for steric stabilization of polyplexes have been developed. The first strategy is based on the formation of copolymers from cationic polymers and PEG [36,43-45], whereas the second approach relies on the initial formation of polymer/DNA complexes with subsequent attachment of PEG to free amino groups [31]. It could be demonstrated that small particles with a size of approximately 100 nm and surface charges close to neutrality could be obtained under appropriate conditions with both strategies [31,36,44]. The *in vivo* application of both construct types displayed a slightly prolonged circulation time compared to unmodified complexes. However, the circulation half-lives were still rather short compared to e.g. stealth liposome formulations. A decrease in gene expression in the lung and a lower initial toxicity was observed in both cases, when compared to unmodified complexes, most likely due to decreased interactions with blood constituents and, therefore, a lower rate of deposition in lung capillaries via filtration. Significant gene expression was also detected close to the site of injection for both approaches, suggesting a still significant rate of nonspecific electrostatic interactions. Overall however both PEG coating approaches were of limited use.

Transferrin not only represents a possible targeting moiety for tumor cells and brain endothelia (see below), but has also been demonstrated to effectively

shield the surface charges of polyplexes [46]. When incorporated at appropriate densities it leads to a significant decrease of non-specific interactions with erythrocytes. *In vivo* studies using charge neutral transferrin-PEI/DNA complexes displayed the accumulation of DNA primarily in the liver and tumor tissues, whereas liver uptake mainly occurred into Kupffer cells, resulting in DNA degradation without significant gene expression. In tumor tissue, on the other hand, a remarkable 100-500 fold higher reporter gene expression was detected compared to other major organs, including the lung [46], thus making this strategy an interesting approach for further research.

Poly(N-(2-hydroxypropyl)methacrylamide) (pHPMA): HPMA polymers have displayed versatile properties as polymeric carriers for a large variety of drugs with an excellent biocompatibility [47]. Hence, this group of hydrophilic polymers represents an interesting candidate for surface charge shielding of polyplexes. The formation of electrostatic complexes consisting of poly(L-lysine) and plasmid DNA with the subsequent attachment of semitelechelic pHPMA to uncomplexed ε-aminogroups led to decreased interactions with albumin and reduced association with macrophages *in vitro* [48]. *In vivo* experiments, however, did not display a prolonged circulation time in this study and liver uptake was even higher than for uncoated complexes (Figure 2). The detailed reason for these poor *in vivo* results remain as of yet unclear.

Innovative shielding strategies: Shielding by simple attachment of PEG or pHPMA has not shown satisfying results. Therefore more sophisticated strategies have been developed, in order to obtain vectors that show sufficient extracellular stability in combination with triggered intracellular release of the complexed DNA. Crosslinking of primary amines of poly(L-lysine)/DNA complexes via disulfide bonds has shown promising results [49]. In circulation those complexes are stable thus offering efficient stabilization of the DNA, however when taken up into cells disulfide bonds are reductively cleaved and DNA is released. Such crosslinked complexes showed 10 fold increased plasma

circulation after i.v. administration compared to unmodified complexes with similar levels of reporter gene expression *in vitro*.

Another approach uses plasmid DNA complexed with a reducible linear polycation and subsequent steric and lateral stabilization by multivalent HPMA copolymers [50]. In circulation these complexes provide efficient protection of DNA against degradation and furthermore shielding of the positive surface charge leads to a decrease of unspecific interactions. Transfer to a reducing environment (cellular uptake) again results in degradation, in this case of the polycation, leading to lower molecular weight compounds and hereby DNA release (see Figure 3). The exploitation of bioreversible bonds represents a very promising strategy.
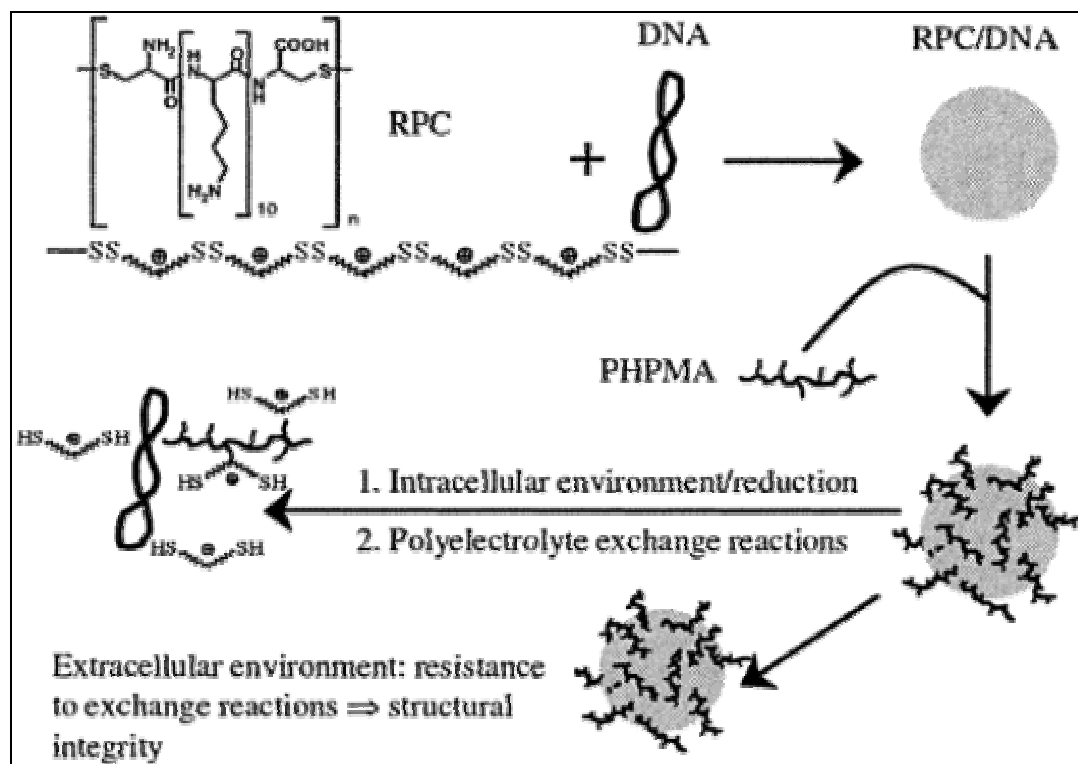
Figure 3
Example for a polyplex modification with trigerred intracellular DNA release.
RPC=reductively cleavable linear polycations (data from [50]).

3.1.5. Local application

If a particular tissue or organ needs to be targeted in non viral gene or oligonucleotide therapy, local application may in many cases represent a more promising approach, since several barriers of systemic application can be avoided. This approach has been utilized for a variety of cases, including tumors [51-54], kidney [55], lung [56], brain [57-60], heart [61,62], skin [63], muscle [64], and arterial blood vessels [65,66]. Here we will discuss some important applications employing non viral vectors.

Lung: Due to its direct accessibility, local gene therapy to the lung by direct airway application is an attractive approach. However, access is impeded by several obstacles. The first obstacle of gene delivery to and across the epithelial cells of the lung is a mucus layer secreted by goblet cells, which creates a mechanical barrier against access to the plasma membrane of the epithelial cells. Furthermore, the epithelium itself hinders the uptake of particulate structures, due to its dense structure with actin strengthened apical surfaces and characteristic tight junctions between cells inhibiting intercellular transport. Additionally, countless alveolar macrophages constantly patrol the lung removing particles from the deeper airway via phagocytosis.

The method of application greatly influences the extent of gene expression in the lung [67,68]. For example, the instillation of poly- or lipoplexes in the respiratory airways was shown to lead to significant gene expression levels that could be significantly enhanced by the coadministration of penetration enhancers. However, the use of penetration enhancers increased toxicity [69]. A further problem encountered using instillation strategies is the insufficient spreading of vector solutions on the lung surface and the channeling of airway administered solutions [70]. These factors led to unequal distribution and inhomogeneous gene expression patterns showing maximum levels in epithelial cells lining the bronchioles and in distal airways [70,71]. The even distribution

of preparations may be improved by the use of artificial surfactants, resulting in increased levels of gene expression [72].

A more convenient way of application for local gene therapy to the lung is aerosol delivery, a method that has been used for lipo-, as well as polyplexes [67,73,74]. In contrast to instillation, this method of application enables a more uniform distribution of transgene expression along the airways. In a study targeting lung metastases, a p53 tumor suppressor gene complexed with PEI was able to significantly inhibit growth of tumors, as well as to prolong survival time of laboratory animals [75].

Tumor: To reach cancer cells in a tumor, a blood-borne polyplex must enter the tumor supplying blood vessels, extravasate into the interstitium and finally migrate through the tumor tissue [76].

Two general means of application are most frequently used to treat tumor tissue: The first method is a direct injection of complexes into arterial blood vessels that supply the tumor. As previously mentioned, tumors often show irregular endothelial fenestration, which makes it possible to reach the interstitial tissue via afferent blood vessels. Furthermore, complexes may even accumulate at this site, due to the enhanced permeation and retention (EPR) effect and/or electrostatic interactions [77,78], thus, enabling passive tumor targeting. However the problem using this technique is the poor distribution of polyplexes throughout tumor tissue and thus a limited transfection efficiency towards the mass of the tumor.

Another method of application is the direct injection of the complexes into the tumor. In this case, however, the low extent of complex diffusion throughout the tissue limits its applicability. When a direct injection into tumor tissue is performed, complex size, charge and concentration are of utmost importance for mobility within the tissue and, ultimately, the overall efficiency of the complexes. Even the speed of injection seems to play a crucial role for efficiency of intratumoral (i.t.) gene delivery [52]. A more detailed

characterization of complex properties with regard to their diffusibility in tumor tissue would be helpful for optimization of this approach.

A method to enhance transfection efficiencies after i.t. injection has been demonstrated in mammary tumors. In this study *in vivo* electroporation lead to a significant increase of reporter gene expression [54]. Although this technique is limited to certain applications it represents an interesting method to enhance treatment efficiency.

Intraperitoneal application of complexes has been performed successfully in several studies in animals exhibiting intraperitoneal disseminated tumors [51] whereas a certain specificity of gene expression could be achieved in tumors, due to their high mitotic activity. In this case complexes consisting of linear PEI and DNA displayed a higher efficiency than several lipoplex formulations without showing significant levels of toxicity [51].

Brain: The brain endothelium is composed of non fenestrated cells surrounded by tight junctions that block the exchange of proteins, hydrophilic molecules, and ionic diffusion. Furthermore, transcellular movement via non-specific fluid phase endocytosis does not occur in these cells and only small lipophilic molecules are capable of surmounting this barrier. However, there are several receptor mediated transport systems for specific molecules, such as transferrin. A successful approach of exploiting this transferrin receptor to cross the blood brain barrier after i.v. application has been described recently [79].

Local approaches for non viral gene therapy to the brain have also been developed. The injection of complexes into the internal carotid artery supplying the brain displayed significant gene expression in experimental brain tumors and other tissues. Approaches using direct intraventricular application or injection of polyplexes into specific structures of the brain have also been performed [58,60]. In all these cases significant, and in several cases long lasting, levels of gene expression could be observed, however, distribution was highly dependent on mobility and stability of complexes [57].

An inventive, non invasive approach for gene delivery to the brain stem was developed based on the observation that neurons are capable of taking up exogenous particles from the muscles they innervate. Thus, the injection of PEI/plasmid complexes into the tongue led to retrograde axonal transport to hypoglossal motoneurons of the brain stem and significant levels of gene expression in this area [59]. More detailed studies about the applicability of this technique are necessary to estimate their potential.

3.2. Hurdles on the cellular level

Reaching the cell membrane of a targeted cell is only half the way to efficient gene/antisense/ribozyme therapy. The nucleic acid has to be carried across several cellular hurdles in order to reach its desired site of action and to display the desired therapeutic effect.

3.2.1. Across the cellular membrane

The first obstacle to be surmounted in gene delivery is traversing the cellular membrane. Composed of a lipid bilayer and containing various integral proteins, it acts as a gatekeeper, selectively screening all foreign matter entering the cell. It has been shown that polyplexes are able to pass the cellular membrane via endocytosis [18,80], as long as they exhibit suitable surface properties and are of the appropriate size range. Endocytotic uptake, however, does have its disadvantages. Not only may complexes be exposed to enzymatic degradation in the lysosomes, but they also may be entrapped and never released.

Strategies to circumvent such difficulties have become the object of intense investigations. A new and most promising approach includes the use of viral protein transduction domains, such as the HIV-TAT protein from HIV-1 virus. These proteins are capable of mediating the entry of large biomolecules directly into the cytoplasm without the use of endocytotic mechanisms. Some even promote transport across the nuclear envelope.

Uptake of polyplexes: Uptake into the cells may occur in various ways, including adsorptive or fluid phase endocytosis, receptor mediated endocytosis, macropinocytosis or phagocytosis.

The predominant, if not the only route of entry for polyplexes, is fluid phase or adsorptive endocytosis [80,81] following the clathrin coated pit mechanism [81]. Due to their content of glycoproteins, proteoglycans and glycerolphosphates [82] cell surfaces are negatively charged. Thus, adsorptive endocytosis is a plausible mechanism of entry for vectors with positive surface charges. The importance of proteoglycans for efficient uptake has been demonstrated experimentally [83], since removal of these compounds leads to a significant decrease in cellular uptake. Confocal microscopy studies have demonstrated that complexes adhere to the cell membrane in clumps and migrate to specific areas, most likely coated pits [81], within 30 minutes. Such findings support the theory of an adsorptive endocytotic mechanism. Additionally, uncomplexed polyethylenimine has been shown to deposit on the cell surface and subsequently enter the cell via endocytosis. [84].

In contrast studies using L929 fibroblasts demonstrated that no aggregation on the cell membrane could be observed and a rapid uptake occurred within 10 minutes, thus favoring a fluid phase mechanism of entry [80]. An explanation for this apparent discrepancy may be that, depending upon the cell line, adsorptive and fluid-phase endocytosis superpose. Therefore, factors such as the composition of the cell membrane or surface charge of complexes may influence the balance in favor of either one or the other route.

Polyplexes coupled to targeting moieties are taken up by receptor mediated endocytosis (see below). At the early endosome level the receptor is recycled back to the cell membrane, while the receptor ligand attached to the vector is processed to late endosomes and lysosomes [85]. Targeted complexes featuring targeting moieties with a positive surface charge may also enter cells via adsorptive endocytosis and, in this case, both routes of entry may be observed.

3.2.2. Improving unspecific cellular uptake

Cationization of complexes: One way to achieve improved uptake into cells is by increasing the surface charge of complexes [86]. This modification results in a higher affinity to negatively charged membrane constituents and subsequently to a higher rate of uptake. At the same time, an increase in positive surface charges also results in an increased toxicity both on the cellular and systemic level. The *in vivo* applicability of such complexes becomes increasingly problematic, since a higher surface charge leads to increased nonspecific interactions resulting in a rapid removal from the blood stream as well as unwanted events such as lung embolism due to aggregate formation with erythrocytes. Therefore, the therapeutic use of this strategy is limited to local administrations and for improving transfection efficiencies *in vitro*.

Protein transduction domains: An alternative route into the cell may be achieved with the help of so-called protein transduction domains (PTDs). Although the number of publications dealing with nucleic acid delivery in conjunction with PTDs is currently limited, this group of compounds will be discussed here to some extent, as they may hold the potency to open up a whole new branch in gene/ODN delivery. Since their discovery more than a decade ago [87,88] it has been demonstrated that PTDs mediate an endocytosis independent cellular uptake [89] of proteins, as well as other large molecules. Physiological membranes do not seem to hinder the PTDs, due to the fact that they are able to accumulate in cells in a concentration dependent manner. The overwhelming advantage of this type of cell entry is the circumvention of the harmful endolysosomal compartment with its acidic environment resulting in DNA/RNA degradation, as well as an inefficient release. All cell types used to date are susceptible to transduction which means that even cell lines with low transfection efficiencies via the classical route should be transfectable via PTDs and the percentage of transfected cells should increase. *In vivo* studies with PTDs have shown very encouraging results. The intraperitoneal injection of a

120-kilodalton beta-galactosidase protein coupled to the HIV-1 TAT protein led to a significant delivery in virtually all tissues [90] including the brain.

Several sequences have been identified to possess transducing efficiency: The TAT protein derived from the HIV-1 virus [88], the drosophila antennapedia transcription factor ANTP [91] and the herpes simplex virus type-1 VP22 transcription factor [89] are three examples possessing the highest efficiencies. Minimal sequences mediating a transducing efficiency are shown in Table 2. It should be noted that the 11 amino acid sequence derived from the TAT protein also bears a potential nuclear localization sequence, making it even more attractive for gene delivery purposes.

A characteristic feature of PTD sequences is the presence of a high number of basic amino acids, such as arginine and lysine (see Table 2), which may be necessary for interactions with negatively charged constituents of cell membranes. Indeed, it can be demonstrated experimentally that membrane bound heparan sulfate proteoglycans are required for an efficient internalization of TAT [92]. Interestingly, the maximal size of the cargo molecule gaining entry to the cell varies depending upon the PTD: While TAT and VP22 are capable of mediating the cellular entry of peptides consisting of more than 1000 amino acids [90] and even 4 µm iron nanoparticles [93], ANTP mediated delivery is limited to peptides with less than 100 amino acids [94]. Mechanistic knowledge as to how PTDs mediate cell entry is currently limited, however we do know that no classical routes, such as receptor- or transporter-mediated mechanisms, as well as adsorptive endocytosis are involved [89,95-97]. Furthermore uptake is ATP independent and cannot be inhibited by cooling to 4°C.

It is unclear if all PTDs mediate cell entry via the same mechanism. However direct interactions with the cell membrane seem to occur in all cases. A disruption of the membrane bilayer can also be ruled out, as no studies have reported leaking of cell content during transport.

Sequences of frequently used protein transduction domains

HIV-1 TAT	Y**GRKKRR**QRRR (**bold**:a potential nuclear localization sequence)
HSV VP22	DAATATRGRSAASRPTERPRAPARSASRPRRPVE
ANTP	RQIKIWFQNRRMKWKK

Table 2
Sequences of most frequently used protein transduction domains

The major physiological task of all three peptides is the interaction with nucleic acids during transcription. Their transducing property is not necessary to fulfill this function, thus the evolutionary purpose remains as of yet unclear. This, however, could also mean that PTDs may have not undergone a selection process for their transducing potential during evolution and, therefore, it might be possible to improve this property by modifying the sequences. This has been done recently with success [98].

Making use of PTDs: A major difficulty of PTD utilization in DNA/RNA delivery is the positive charge of the peptides, which leads to electrostatic interactions. As a result, the peptide is not in its maximally active conformation and, thus, a reduced transducing efficiency is observed. An additional problem is the lack of specificity, so that combinations of PTD and targeting moieties will be required.

The formation of ternary complexes using TAT peptide together with PEI and plasmid DNA has led to approximately a 10fold increase in reporter gene expression, as compared to regular PEI/plasmid complexes and an increased nuclear accumulation could be observed [99].

Furthermore it has been demonstrated that liposomes with a size of 200 nm can be delivered directly into the cytosol without causing major damage of the vesicles by attaching TAT peptides to their surface [100]. ntisense oligonucleotides have been coupled to the ANTP peptide as well resulting in an efficient and specific downregulation of the target gene product using concentrations much lower than with regular ODNs [101].

3.2.3. Strategies to improve uptake into specific cell types

Especially for *in vivo* therapy it is necessary to address gene delivery vehicles to specific cell types in order to avoid unwanted effects in non target cells. Targeting can be achieved actively by incorporating structures, which facilitate the exclusive uptake of the vector in certain tissues or cell types. Furthermore, in some cases, it can be achieved passively by taking advantage of particular physiological conditions of the target tissue such as irregular endothelial fenestration in tumors in conjunction with certain complex properties. A third method of achieving gene expression in specific tissues is the use of cell type specific promoters or enhancers, which can be activated by induction factors (e.g. hormones, growth factors, cytokines etc.) via responsive elements. This strategy has been pursued with success in several targets and represents a very helpful tool for increasing cell specific efficiency [102-106].

Active targeting: To achieve an efficient active targeting *in vivo* the vector must fulfill two requirements: On the one hand, unspecific interactions must be reduced by shielding positive surface charges of the complexes and by using rather low nitrogen to phosphate ratios. On the other hand, a targeting moiety, enabling uptake into a specific cell type, needs to be incorporated (see table 3).

Target structure	Targeting moiety	cell/tumor type	Reference
folate receptor	folate	various	[107]
integrines	RGD peptides	tumor endothelia	[108]
transferrin receptor	transferrin	rapidly dividing tissues	[109]
asialoglycoprotein	lactose	hepathocytes	[110]
receptor	galactose	hepathocytes	[111]
	mannose	dendritic cells/(hepathocytes)	[111]
	asialoglycoprotein	hepathocytes	[112]
LDL receptor	LDL	various	[113]
FGF receptor	FGF	various	[114]
EGF receptor	EGF	various	[115]

Table 3
Active targeting strategies realized with polyplexes. For targeting with antibodies see Table 4

Integrins: Integrins are heterodimeric membrane receptors that are involved in interactions between cells and the extracellular matrix. Furthermore several bacterial, viral and eukaryotic pathogens use it as a receptor to efficiently bind to and enter the cell. Peptides containing the highly conserved arginine-glycine-aspartic acid (RGD) motif, such as fibronectin [116], kistrin [117], and several viral capsid proteins [118] have been identified as candidates for specific interactions with integrins. Since integrins are highly overexpressed in tumor endothelia they represent an interesting approach for the improvement of nucleic acid delivery to tumor tissue. Several strategies have been realized to make use of this target, such as varying the carrier polymer. The size of the RGD containing peptide has been shown to influence integrin binding and the use of cyclic RGD containing oligopeptides has resulted in superior receptor affinities [119]. RGD peptides attached to polyplexes [108,120,121] and combinations of polyplex and liposomal approaches [122] have been shown to associate with cells much readily than those without a targeting moiety, resulting in an increased reporter gene expression of up to 200fold.

Transferrin: The transferrin receptor was one of the first targeting structures used in non-viral gene delivery. It is physiologically necessary for the uptake of transferrin-iron complexes into all actively proliferating cells and highly overexpressed in rapidly dividing tissues such as tumors. Cerebral endothelial cells and hepatocytes possess significant levels of transferrin receptors, a fact that makes transferrin an attractive target for systemic gene therapy. Transferrin has been incorporated into polyplexes [123-126] via covalent or electrostatic attachment with and without PEG-spacers, resulting in a significant increase in reporter gene expression when compared to complexes without a targeting moiety. Several formulations have also been evaluated *in vivo* via regional [124] administration, whereby a significant increase in transfection activity in tumors was measured.

As described above the inclusion of high amounts of transferrin into polyplexes leads to both, effective shielding of surface charge and tumor targeting [46]. Interestingly, it has been demonstrated by an *in vitro* study with lipoplexes that apo-transferrin is as efficient as transferrin in enhancing reporter gene expression. Furthermore, inhibition studies using an excess of free ligand displayed no decrease in effectiveness [127]. These results are contradictory to previous results and suggest that attachment of transferrin might also improve gene therapy e.g. by facilitating endolysosomal escape. More detailed studies are underway.

Folate: The folate receptor represents an attractive structure to target cancer cells. This issue of Advanced Drug Delivery Reviews contains a detailed review about cancer targeting with folate [128].

Glucosylated vehicles: The so-called asialoglycoprotein receptor (ASGPr) is expressed abundantly in hepatocytes and selectively binds to galactose-terminated glycoproteins. Various saccharide ligands have shown affinity to the ASGPr, including mono- or oligosaccharides, such as lactose, galactose, mannose, fucose, as well as larger carbohydrates like asialo-oromucoid. The receptor is well characterized and ligand binding leads to receptor mediated endocytosis. The high level of receptor expression on hepatocytes in addition to the rather simple coupling chemistry of the sugars, has stimulated research on gene delivery vehicles incorporating ASGPr targeting moieties. The low immunogenicity of these conjugates compared to antibody- or peptide-conjugates makes them attractive for *in vivo* use.

Several approaches have been developed to couple the above mentioned sugars to polyplexes and to evaluate their efficiency in the selective transfection of hepatocytes. Successful *in vitro* targeting has been reported in various cases using charge-neutral complexes containing poly(L-lysine) and polyethylenimine. A 10-1000fold increase of gene expression can be achieved in targeted cells when compared to complexes without a targeting moiety [129-33].

The attachment of sugars not only leads to a targeting effect, if incorporated at sufficient amounts it also increases the physical stability of complexes and enables the formation of small associates with neutral zeta potential, thus making them suitable for *in vivo* use. Efficient *in vivo* targeting of hepatocytes has been described in several studies using asialoglycoprotein-, asialo-oromucoid-, mannose-, fucose- or galactose-conjugates [111,112,134-136]. Incorporation of a sugar moiety has increased uptake into liver cells significantly, however, the ratio of uptake into parenchymal or non-parenchymal (endothelial and Kupffer cells) liver cells differs depending on the type of sugar attached. Since hepatic disorders, such as cirrhosis or liver cancer, affect predominantly parenchymal cells this cell type should be the predominant target. Galactosylated polyplexes have shown a higher affinity to parenchymal cells than non parenchymal cells, while mannosylation or fucosylation leads to a lower ratio of distribution between both cell types [111,137]. A possible explanation for this preference is the fact that the asialoglycoprotein receptor, which is present on parenchymal cells, recognizes all three sugars, while the non parenchymal cells such as Kupffer cells, also exhibit mannose and fucose receptors, that take up additional conjugate concentrations [137]. A galactose-receptor has been identified on Kupffer cells, however, uptake efficiency is much lower when compared to the asialoglycoprotein receptor on hepatocytes. Promising hepatocyte targeting results *in vivo* have been achieved by intravenous injection of a vector consisting of poly(L-ornithine) modified with galactose and a fusogenic peptide. The use of this vector led to highly enhanced uptake into parenchymal liver cells and 95 % of total reporter gene expression was observed in the liver [138]. Hepatic uptake could be inhibited by prior administration of BSA conjugates with galactose or mannose, a fact that provides evidence for a predominantly receptor mediated uptake [111,138].

Mannose conjugates are most frequently used as for enhancing the uptake into dendritic cells [139].

Conjugation of antibodies: Due to the high specificity of binding to their target structures antibodies or antibody fragments represent very promising candidates for targeted gene and oligonucleotide delivery. Several types of antibodies or their fragments have been conjugated to polyplexes via different conjugation strategies in order to achieve a targeting of specific cell types (see table 4)

Target	Antibody	Polymer	Reference
lung endothelia	anti platelet endothelial cell adhesion molecule (anti PECAM)	PEI	[140]
leukemia T-cells	anti-JL1	poly(L-lysine)	[141]
neuroblastoma	anti-RET receptor-tyrosine kinase	poly(L-lysine)	[142]
neuroblastoma	anti chCE7	poly(L-lysine)	[143]
squamous carcinoma	anti erythrocyte growth factor	poly(L-lysine)	[144]
epithelial cells	antibody fragments against polymeric immunglobulin receptor	poly(L-lysine)	[145]
peripheral blood mononuclear cells	anti-CD3 antibody	PEI	[146]
T lymphocytes	T101	poly(L-lysine)	[147]

Table 4
Examples for antibodies used as targeting moieties in polyplex gene delivery

All immunologically targeted conjugates summarized in Table 4 have shown a significant increase in transfection efficiencies, as compared to complexes without targeting moiety. Enhancement of gene expression is usually greater than obtained by most other targeting moieties. However, a potential disadvantage of the *in vivo* application of antibody conjugates is the possibility of immunogenicity after repeated administration and their huge size, which might affect complex size and stability.

Low density lipoprotein (LDL) receptor: Effective targeting of cells displaying the LDL receptor on their surface like hepathocytes or artery wall cells can be achieved by using complexes carrying LDL on their surface. A sophisticated strategy in this context is the so-called terplex system. It consists of stearyl-

poly(L-lysine), low density lipoprotein (LDL) and DNA wherein the ratio between the charged stearyl-poly(L-lysine) and the hydrophobic moiety of LDL is optimised for obtaining particles that are suitable in size and charge for an efficient cellular uptake. This system is capable of mediating a receptor dependent uptake into artery wall cells *in vitro* [113] as well as the efficient and long lasting transfection of myocardial cells *in vivo* [62].

Growth factors: Growth factor receptors, such as the epidermal growth factor (EGF) or fibroblast growth factor (FGF) receptors are attractive targets in cancer gene therapy, since they are highly overexpressed in a large variety of cancer tissues, including lung, head, neck, bladder, liver and breast cancers (EGF-receptor) [148]. These receptors bind to their target specifically and with high affinity, whereby upon binding the receptors dimerize and are internalized together with their bound target. Recently, EGF has been covalently coupled to PEI [115]. This modification led to a 300fold increase of reporter gene expression, when compared to the unmodified polymer. Similar observations were made when EGF-poly(L-lysine) [149], EGF-PEI [150] or FGF–poly(L-lysine) [151] conjugates where employed.

Passive targeting: Complexes or macromolecules may accumulate passively in solid tumors, due to an increased permeability of tumor endothelia and lack of normal lymphatic drainage in connection with hypervasculature of the tissue [77]. This effect is known as the enhanced permeability and retention (EPR) effect and leads to the capture of complexes in the tumor interstitium. It has been exploited successfully to passively target polymers [152] or liposomes [153] to tumors without incorporating a more specific targeting moiety. The extent of accumulation depends on size and charge of the compound. It has been demonstrated that cationic lipoplexes or cationic dextran derivatives accumulate in regions of high angiogenic proliferation, most likely due to electrostatic interactions [78] and this effect may be applicable to polyplexes, as well.
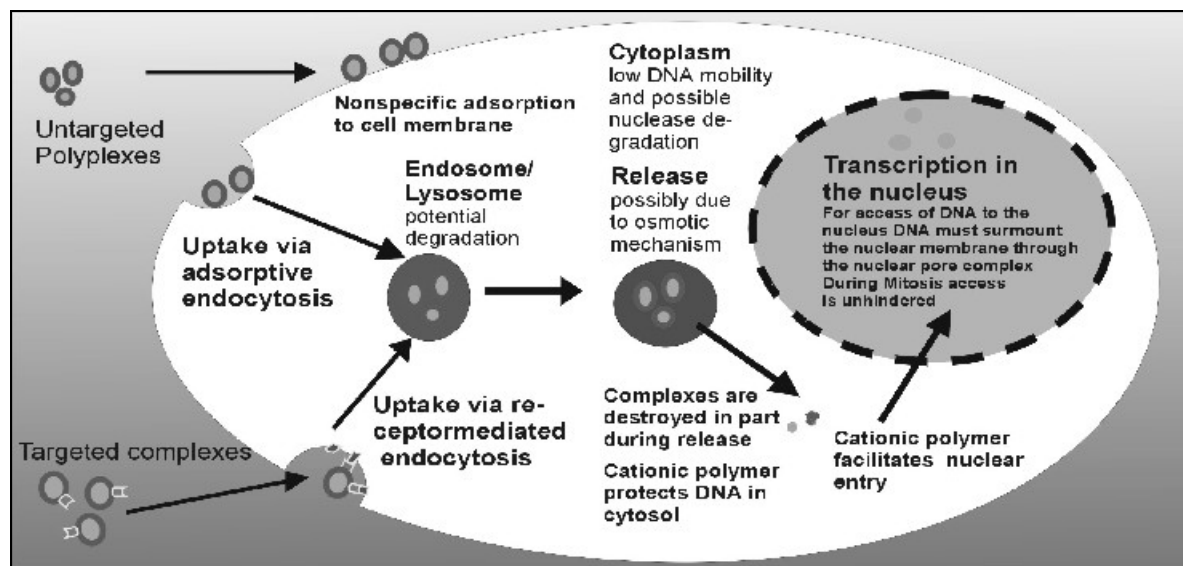
Figure 4
Scheme of subcellular trafficking of polyplexes

3.2.4. Escape from the endosome

Independent of the mechanism of endocytotic uptake, polyplexes invariably follow the scheme of the endolysosomal pathway, leading from the early to late endosomes, and ultimately ending in the lysosomal compartment. The lysosomal vesicles fuse and assemble in the perinuclear region [80]. Here the majority of complexes remains without significant changes in distribution patterns [18]. In order to be effective, a small fraction of complexes or at least their nucleic acid component must escape from this route since the lysosomal environment, with its aggressive nucleases and acidic pH of approximately 5, eventually destroys the potential efficacy of the entrapped complexes. Release has to take place rapidly, otherwise degradation occurs. Since endosomal escape is one of the major bottlenecks in non viral nucleic acid delivery, researchers are seeking to improve nucleic acid delivery at this level. They have attempted to mimic viral strategies by using peptides with pH triggered fusogenic activity, which are capable of destabilizing lysosomal vesicles. Another method uses specific polymer properties, which enable the polyplexes to exit from ensosomes/lysosomes more efficiently.

Mechanism of endosomal escape: Vesicular escape of polyplexes most likely occurs via a pH dependent process. It is obvious that the efficiency of the polymers used for non viral nucleic acid delivery is narrowly connected to their buffering capacity in the lysosomal pH range of 5-7. Polymers that exhibit high transfection efficiencies, such as polyethylenimine [8], Starburst Dendrimers[TM] [29], or imidazole-containing polymers [21-24,154] exhibit this property. The fact that the latter enhance gene expression is a rather strong evidence for the importance of buffering capacity, since their structure is unrelated to PEI or dendrimers. In contrast, cationic polymers with strong basic residues, e.g. poly(L-lysine) or polymers without titrable groups, such as quaternary amines, exhibit very low transfection efficiencies [18]. This may very well be due to the lack of protonable groups between pH 5-7. A plausible explanation for the mechanism of polyplex release, the so called ´proton sponge hypothesis´ has been developed [11]. According to this hypothesis the buffering capacity of the polymer leads to increased influx of protons and chloride ions during endolysosomal acidification, which results in an increased osmotic pressure in the vesicle. As a consequence, the passive diffusion of water into the vesicle increases, thus leading to swelling and eventually rupture or leakage of the vesicle. The expansion of the polymer structure, due to repulsion of positive charges may contribute to the vesicle destabilization. An indication for the validity of this hypothesis is shown by the fact that reporter gene expression is reduced dramatically when lysosomal acidification is abolished by the selective inhibitor bafilomycin A1. This has been demonstrated for PEI [18,155] and imidazole-containing polymers [23]. Recently more experimental evidence was obtained for a pH dependent release of PEI-polyplexes possibly due to vesicle bursting. Living cell confocal laser scanning microscopy showed that release of PEI/ribozyme complexes occurs as a sudden event and that this process could be abolished by inhibiting endolysosomal acidification [18]. Another finding supporting the ´proton sponge hypothesis´ is that complexes with improved

packaging and thus a higher density of amine groups possesses a greater buffering potential, which results in higher transfection efficiencies [156].

It has been demonstrated that high generation PAMAM dendrimers and PEI possess a high membrane destabilizing potential compared to low generation dendrimers or poly(L-lysine) [157,158]. This may be a result of electrostatic interactions between the negatively charged membrane constituents and cationic polymers, a process that may cause disturbances in the curvature of the vesicles and finally lead to leakage [157] or bursting. In the case of dendrimers, these destabilizing effects seem to be more prominent for high rather than for low generation polymers, possibly resulting from the more rigid structure of the former. The results of these studies were obtained using anionic vesicles incubated with the cationic polymers, a system in which the described interactions occurred on the outside of the vesicles and not from within, as is found *in vivo*. This mechanism seems to play a role in gene delivery as well, since there seems to be a correlation between efficient membrane disruption *in vitro* and reporter gene expression.

Strategies to enhance endolysosomal escape/ to prevent lysosomal degradation: Great efforts have been undertaken to abolish the endosomal bottleneck. The most important strategy is adapted from viruses, which have evolved sophisticated methods to escape the harsh environment of this compartment and deliver their genetic information safely into the nucleus.

One approach is the use of short amino acid sequences derived from the N-terminus of Haemophilus Influenza Haemagglutinin-2. These amino acid sequences are responsible for enabling endolysosomal escape during the acidification process. Several sequences with approximately 20 amino acids have been identified and used successfully. It has been discovered that all sequences follow a similar scheme [20]. For example, the INF7 peptide with the sequence GLFGAIAGFIENGWEGMIDGGGC exhibits a hydrophobic side chain, such as leucine, isoleucine or tryptophan (underlined) at nearly every

fourth amino acid. The rest of the sequence consists of variable amino acids, although it is important that no helix breaking amino acids are present and several glutamic acids are included. The ?-carboxyl groups of these glutamic acids play the key role in the activation of the fusogenic activity of the peptide. At neutral pH the ?-carboxyl groups are deprotonated, thus, leading to electrostatic repulsion and thereby conferring the sequence a random coil structure without fusogenic activity. When the pH drops, however, the carboxyl groups are protonated and the charge repulsions diminish. This process leads to a transition from the random coil to an a-helical structure, which displays the above mentioned hydrophobic residues on one side, thus making the helix amphipathic. The hydrophobic side of the helix may be able to insert itself into the vesicular membrane and disturb the membrane geometry, eventually leading to release of the vesicle contents. Furthermore, inserted helices may aggregate and form pores, which result in vesicle leakage.

Experimental evidence has been gathered implying that this is not the only mechanism of transfection enhancement, at least for the INF7 peptide. It was discovered that this peptide also effects transfection at later stages, e.g. that it promotes dissociation of DNA from polyplexes in the nucleus [159] or acts as a nuclear localization sequence.

Several synthetic peptides displaying pH dependent fusogenic properties have been synthesized successfully following similar structure principles [160,161]. The synthetic peptide GALA WEAALAEALAEALAEHLAEALAEALEALAA has been demonstrated to bind to phospholipid membranes at acidic pH [160,161] and to form aggregates within the membrane. These aggregates form membrane pores, although they are too small to enable plasmid DNA to escape from vesicles [162,163]. Therefore, other mechanisms must contribute to the transfection enhancement of GALA. Simoes suggested that in the case of lipoplexes the conformational change of the peptide could promote deaggregation of complexes, hereby releasing larger amounts of cationic lipids.

These could lead to a flip flop of anionic lipids from the cytoplasmic leaflet of the membrane into the region of pores formed by GALA peptides and subsequently to extensive membrane destabilization [164].

Another example of a synthetic fusogenic peptide following the above described rationale is KALA WEAKLAKALAKALAKHLAKALAKALKACEA [165]. This peptide also mediates acid dependent increased vesicular escape and achieves transfection enhancement [166-68].

Disadvantages of using protein structures like fusogenic peptides are low stability of the peptides, high costs for peptide synthesis and an immunogenic potential of these structures. They also do not enhance transfection efficiencies of polymers that already display rather high levels of reporter gene expression.

 Treatment with the lysosomotropic agent chloroquine has been used to enhance transfection efficiencies of cationic polymers without proton sponge properties, e.g. poly(L-lysine) *in vitro*. Chloroquine is thought to protect internalized material from degradation by preventing pH decrease [169,170] or acting as an endosomolytic agent [19,171]. The dimension of enhancement, however, is even lower than for fusogenic peptides and shows great variations between cell lines. The overall transfection efficiencies remain comparably low. Due to toxicity problems with chloroquine *in vivo*, the applicability of this compound is limited to the enhancement of transfection *in vitro*.

A method developed to disrupt the membranes of endosomes and lysosomes precisely in a desired location, such as in tumor tissue, is the so-called photochemical transfection. In this technique photosensitizing compounds selectively accumulate in endosomal or lysosomal membranes, which upon illumination disrupt the vesicle. This method, in conjunction with regular poly(L-lysine) mediated transfection, led to a 20fold higher reporter gene expression, as well as a significant increase in the number of transfected cells [172].

3.2.5. Through the cytoplasm to the nucleus

After release from endosomes/lysosomes the DNA/RNA component of polyplexes has to cross the cytoplasm in order to reach its site of action. The mobility of large molecules, such as plasmid DNA, is extremely low in the cytoplasm, making them an easy target for cytoplasmic nucleases. While ribozymes and antisense oligonucleotides may already be active in the cytosol, plasmid DNA has to be transported into the nucleus, in order to exhibit the desired gene expression. Thus, the major challenge is to modify the nucleic acid or complex in such a way as to protect it from degradation, however enable release from carrier and facilitate transportation into the nucleus.

Stability and mobility of DNA in the cytoplasm: The cytoplasm is a critical place with respect to stability of DNA and RNA, due to the presence of nucleases that reduce their half life dramatically. Naked plasmid DNA, for example, exhibits a half-life as short as 50-90 minutes, which is likely due to degradation by cytoplasmic nucleases [173,174]. The importance of this fact becomes even more obvious when one recognizes that the majority of plasmid DNA enters the nucleus during cell division and, therefore, must remain stable until the next disassembly of the nuclear envelope. Accordingly, the role of the cell cycle length in this process must also be stressed. It is recognized that rapidly dividing cell lines show more efficient gene expression than slowly dividing ones, which is attributed to the short intervals of nuclear disassembly.

Another factor that plays an important role in nucleic acid transit through the cytoplasm is the rate of mobility, which depends on size and spherical structure of the molecule. Low mobility means a longer trafficking time to the nucleus and thus a prolonged exposure to aggressive nucleases. While the diffusion rate for a small 100 bp DNA fragment is only ~5 times slower than in water, it is reduced dramatically with increasing size. Large DNA molecules, such as plasmids exhibit an extremely low mobility [175]. Several factors are responsible for the decrease in cytosolic diffusion rate. The higher viscosity of

the cytosol and sievelike effects of the cytoskeleton, forming meshes of ~10 nm that sterically hinder diffusion of large molecules contribute to this effect. Interactions of the negatively charged DNA with cytosolic constituents may also play an important role [176]. Microinjection studies have shown that the majority of the injected plasmid remains at its site of injection. Injections farther from the nucleus (60-90 μm) lead to a significant decrease in gene expression, as compared to injections performed in close proximity to the nucleus [176]. Therefore the site, where plasmid DNA is released into the cytosol is of great importance for efficient nuclear delivery.

Effect of delivery agent in the cytoplasm: The above mentioned microinjection studies of naked DNA may not accurately display the situation for plasmid DNA or oligonucleotides during non viral nucleic acid delivery, since the mode of cytosolic transit for these molecules is not currently known. They may be complexed to some extent either with the transfection agent or with other cell constituents such as membrane lipids [81], and thus in a compacted state. This compaction could lead to an increased cytosolic mobility, as compared to naked DNA, or could offer an increased stability against cytoplasmic nucleases. Evidence for these assumptions has been gathered experimentally by cytosolic microinjection of polyplexes [177]. The microinjection of PEI/plasmid complexes resulted in a 10fold higher level of gene expression, as compared to naked DNA. This effect was independent of the polymer-nitrogen to DNA-phosphate ratio, suggesting that the compaction mediated by the polymer may be of greater importance than the positive net charge of the complexes. In other words, the enhanced gene expression may be a consequence of increased cytoplasmic mobility, due to the smaller size of compacted DNA.

Alternatively the cationic polymer could alter the structure of the cytoskeleton by widening the ´meshes´ and, thus, enhancing the permeability for large molecules. *In vitro* studies reported interactions between cationic polymers and

f-actin fibres leading to the formation of bundles and thus disturbing their natural assembly [178].

A further reason for the higher level of gene expression could be the protection of DNA from cytoplasmic nuclease degradation afforded by the complex formation with cationic polymers demonstrated *in vitro*. Several studies have evaluated the stability of naked and complexed DNA/RNA in the presence of DNases or RNases, whereas excellent stabilization was observed using cationic polymers such as PEI even using very high enzyme concentrations [179,180].

3.2.6. Into the nucleus

The final barrier in non viral gene delivery is the nucleus. Access to this compartment enables the expression of the delivered gene and the desired therapeutic effect. The nuclear compartment is surrounded by a formidable, but not impenetratable barrier, the nuclear envelope, which consists of a double membrane interrupted by large integral protein structures, the so called nuclear pore complexes (NPC). Two paths lead into the nucleus: During mitosis the nuclear membrane disassembles and, thus, even large molecules, such as plasmids are able to gain access. In contrast, during interphases, the only way to enter the nucleus is through the NPC. This entry may occur via one of two different routes: While small molecules or ions are able to diffuse passively through the NPC, larger molecules, such as proteins or RNA, require something similar to an identification tag that is recognized by receptors and, thus, enables translocation into and out of the nucleus. These "tags" are termed nuclear localization sequences (NLS) if they mediate transport into the nucleus and nuclear export signals (NES) if they enable exit from the nucleus.

DNA, however, does not move into the nuclear compartment under physiological conditions and, therefore, does not interact with NPCs [181]. Hence, the rate of DNA entry into the nucleus is very low and highly

sophisticated strategies are required to increase entry to levels necessary for efficient gene therapy.

For therapeutic purposes nuclear translocation through the NPC seems to be only important for slowly dividing tumors. Fast dividing tissues have a sufficient frequency of cell divisions, thus nuclear entry occurs during disassembly of the nuclear membrane.

The nuclear pore complex (NPC): NPCs are large, nuclear membrane protein structures of ~120 million Daltons in vertebrates [182]. They contain 50-100 functionally distinct proteins [183], the so-called nucleoporins, which are involved in transport processes or form the structure of the NPC. During mitosis in vertebrates these proteins are fragmented into approximately 12 subcomplexes, which then are reassembled at the end of the telophase to form the structure of the pore complex again [183].

Transport through the NPC: Molecules which are trafficked through the NPC can be classified into molecules with or without affinity to components of the NPC. The entry of molecules with no affinity occurs via passive diffusion and seems to be controlled only by size and steric properties [184,185]. The upper size limit for this form of nuclear entry is approximately 50 kDa [186], corresponding to a molecule diameter of approx. 10 nm. The velocity of this process is, as in every diffusion process, inversely proportional to the size of the molecule.

On the other hand, a variety of molecules larger than 50 kDa are able to enter the nucleus via a different route. This process, however, requires not only energy, but also specific interactions with components of the NPC. Generally, a series of association/dissociation processes is thought to mediate nuclear entry of the substrate [182]. Feldherr et al. determined the upper size limit for this form of entry using gold particles of various sizes coupled to nucleoplasmin (see below) [187], and discovered that it is approximately 26 nm, which corresponds to a molecular mass of ~ 8 million Dalton. This size limit varies not only

between species, but also within the same cell line depending on the confluency or energy status of the cells [187]. The diameter of the substrate seems to be the most important property for passage across the NPC, as no limitation for the length of a substrate was found.

Nuclear localization sequences (NLS): Protein sequences that mediate nuclear import are so called nuclear localization sequences (NLS). A NLS is a short amino acid sequence that enables the active transport of proteins or viral DNA into the nucleus. Although more than 100 NLS have been identified [188], several aspects regarding their function remain yet to be investigated. One of the most intriguing questions still unanswered is: To what extent do homologies exist among NLS? NLS do not conform to a specific consensus sequence, very likely because they interact with different receptors. However, they can be classified into monopartite and bipartite motifs [189]. In these sequences, the positively charged amino acids, lysine and arginine, are typical [190]. Usually, the monopartite motif consists of several positively charged amino acids, accompanied by a helix breaking residue, for example, the 92 kDa SV40 large T-antigen NLS, PKKKRKV [191]. For this example it has been demonstrated that phosphorylation and dephosphorylation of the SV40 large T-antigen protein upstream from this sequence is involved in the regulation of affinity to proteins interacting with the NPC machinery [192]. Bipartite NLS usually consist of two sets of positively charged residues with a small upstream cluster of two arginine or lysine residues separated by a 9-12 amino acid point mutation tolerant linker from a downstream cluster similar to a monopartite NLS [193]. Examples for bipartite NLS are the nucleoplasmin NLS, KRPAATKKAGQAKKKK [194] or the nucleolin NLS, KRKKEMANKSAPEAKKKK [195]. It should be noted that not all experimentally identified NLS fit into the above mentioned scheme and also many cytosolic, non-nuclear proteins show sequences similar to the above mentioned properties. Interestingly, if a protein contains both a NLS and a DNA binding site these regions overlap in ~90 % of the cases, indicating that during

evolution the principle of DNA binding via electrostatic interactions was used for targeting DNA binding proteins to the nuclear compartment.

Examples for NLS functions differing from the classical ones described above are the arginine-rich sequences, Tat and Rev, from the human immunodeficiency virus type 1 (HIV-1). These sequences do not only act as protein transduction domains, mediating access to the cell in an endocytosis-independent way, but also enhance nuclear uptake [196].

It has been thought that poly(L-lysine) acts as a NLS, due to the large density of positive charges similar to those found in mono- and bipartite NLS [197]. This assumption, however, has been refuted. By using polylysine carrying peptides it could be demonstrated that no significant affinity to NLS-binding subunits of the nuclear import machinery exists and no rapid accumulation in the nucleus occurred [197].

Specific DNA sequences have also been observed that mediate increased nuclear import. Plasmids containing a region of the SV-40 early promoter and enhancer have been shown to cross the nuclear envelope much more efficiently than those without this specific sequence [198]. Studies investigating the mechanism of nuclear uptake have pointed out that NLS harbouring cytosolic transcription factors selectively bind to specific DNA sequences on the plasmid thus forming a DNA/peptide complex that is able to enter the nucleus [199]. The incorporation of such a viral nucleotide sequences in order to enhance nuclear uptake is a conceivable strategy to improve nuclear uptake of plasmid DNA. Modifying the binding site of these sequences for transcription factors that particularly occur in specific cell types enables the selective targeting to the nucleus in these cells [199].

Nuclear entry of DNA: In contrast to protein or viral DNA entry into the nucleus, little is currently known about the mechanism of DNA entry. What we do know is that, although the transport of DNA from the cytosol into the nucleus does not occur under physiological conditions, the NPC does not seem to be an

impenetratable barrier to passive diffusion. Unfortunately, the passive entry of DNA becomes less efficient with increasing size. Small nucleic acid molecules with a size of less than 300 bp have little difficulty entering the nucleus via free diffusion [200]. For larger DNA molecules >300 bp the rate of transfer through the intact nuclear envelope decreases with the size of the molecule.

The mode of nuclear entry of plasmid DNA in non dividing cells occurs independently of NLS-bearing proteins, as it cannot be inhibited by cooling down to 4 °C or ATP depletion, which inhibit both active protein uptake [181]. Evidence suggests that two different mechanisms of nuclear entry are likely for plasmid DNA. On one hand a rather inefficient passive diffusion through the aqueous pores of the NPC is postulated, whereas, on the other hand, entry may occur at the time of nuclear membrane disassembly during cell division. The former path has been demonstrated by several groups via microinjection studies. Pollard [177] found that no more than 0.1 % of the plasmid copies microinjected into the cytoplasm reached the nucleus. This result was improved by 10fold when plasmid DNA was complexed to PEI. Mirzayans [201], in contrast could not detect any transient gene expression after cytosolic microinjection. An explanation for this inefficient nuclear entry could be the low velocity of the translocation process in conjunction with the short half life [173] and low mobility [175] of the plasmid DNA in the cytosol.

Evidence for the latter route is supported by the fact that gene expression is generally much higher in rapidly dividing, than in slowly dividing cell lines. If cells are arrested in the G1 phase and, thus, unable to perform mitosis, reporter gene expression is up to 500 times lower than in cells that undergo mitosis starting from the S or G2 phase [202].

Nuclear localization sequences have been used in various ways to improve the uptake of plasmid DNA into the nucleus.

Non-covalent attachment of NLS: Electrostatic complexes between negatively charged DNA and positively charged SV40 large T-antigen NLS have been

utilized. Cytoplasmic microinjection of complexes into zebrafish embryos have shown a significant increase in nuclear uptake in comparison to naked DNA [203-205], which increased with higher NLS:plasmid ratios. Complexes with a nuclear import deficient reverse sequence and plasmid DNA did not show nuclear accumulation or increased gene expression under the same experimental conditions. These findings indicate that if plasmid DNA and NLS reach the NPC simultaneously, electrostatic interactions can promote the nuclear uptake of DNA.

Another approach using non-covalent interactions between DNA and NLS bearing proteins is their complexation to histones. These relatively small proteins are synthesized in the cytosol and imported into the nucleus via the NLS/NPC machinery. The use of a recombinant histone containing the carboxyterminal domain of the human histone, H1, complexed to plasmid DNA and a lipid formulation resulted in a 20fold higher luciferase expression than plasmid-lipofectin complexes alone [206,207].

The Tat sequence has also been used in electrostatic complexes with PEI. In this study an enhanced nuclear uptake was observed microscopically and a more than 10fold increase in reporter gene expression was detected [99].

Strategies exploiting complementary base pairing between DNA strands to non covalently attach a NLS to a specific site of a DNA molecule have been realized as well. For example Neves et al. [208] attached a NLS specifically to plasmid DNA by forming a triple helix with an oligonucleotide-NLS peptide conjugate complementary to a specific sequence on the plasmid. These complexes were able to interact specifically with proteins involved in nuclear uptake. Microinjection of the complexes into cells, however, did not lead to nuclear accumulation, as no diffusion from the site of injection could be observed.

Another elegant example of a complementary approach is the use of a peptide nucleic acid (PNA) sequence conjugated to a NLS. PNA contains the same bases as DNA, however, the desoxyribose-phosphate backbone is replaced by a

peptide structure that can be functionalized with a NLS. PNA can form double or triple strands with complementary DNA. The use of PNA-NLS conjugates have lead to an increased nuclear uptake of oligonucleotides and enhanced transfection efficacy of plasmid DNA, which could be even further improved by the use of PEI as delivery agent [209].

Recently it has been reported that linear 22 kDa PEI is able to mediate efficient and cell cycle independent nuclear uptake of plasmid DNA [16]. The detailed mechanism how transport through the NPC occurs, however, remains unelucidated and needs further investigation.

Covalent coupling of NLS: A different way to take advantage of NLS is to synthesize covalent conjugates with DNA. This strategy has been realized by several groups using various coupling methods and different evaluation techniques [200, 210-12]. Zanta et al. [210] synthesized a capped CMVLuciferase-NLS gene containing a single SV-40 large T-antigen NLS per DNA molecule. They found a 1000fold enhancement of the transfection efficiency for this conjugate when compared to unmodified DNA. This effect was reduced to normal levels after the replacement of a single lysine residue with a threonin in the NLS, suggesting that the enhancement of transfection was the result of an increased nuclear uptake. Ludtke et al. [200] synthesized NLS-DNA conjugates with various sizes of DNA. They observed that the coupling of NLS to DNA significantly enhanced DNA uptake into the nucleus, whereas the efficiency of uptake was dependent upon the size of the DNA.

Although the non covalent strategy for binding NLS to DNA has been tested *in vivo* with some success [213] the use of a covalently linked NLS seems to be more promising, due to the much higher stability of these conjugates.

4. GENE THERAPY STRATEGIES TOWARDS CANCER

Progress of molecular biology has provided a remarkable increase in our knowledge about cancer and its molecular mechanisms. The introduction of genes and nucleic acids into mammalian cells has become feasible during this information explosion. These advances have enabled cancer gene therapy to step from a diffuse vision into laboratory routine and even into clinical trials. Specific forms of cancer are especially suitable for gene therapy since cancer development has its origin in genetic mechanisms. At least one mutation from a protooncogene to an oncogene in conjunction with an aberration of a tumor suppressor gene leads to cancer genesis and proliferation. In contrast to other strategies, a therapy on this level is capable of providing high efficiency and specificity. Several strategies have been pursued to exploit the potential of this new field. Antisense- and ribozyme therapy provide the possibility of specifically downregulating the expression of particular genes predominantly by interactions with mRNA. In gene therapy new DNA material is introduced into the nucleus of the target cell. Different strategies such as knockout gene therapy, gene replacement, suicide gene therapy and immunomodulatory strategies have been performed successfully. The principles of these approaches will be introduced briefly, however non viral strategies most often use reporter genes which have no cancer relevance. Furthermore the vast majority of the studies have been accomplished with viruses.

4.1. Antisense oligonucleotides and ribozymes

Antisense oligonucleotides and ribozymes possess the potential to downregulate the expression of particular genes in a highly specific manner. In contrast to knockout gene therapy where a gene coding for an antisense RNA

oligonucleotide or ribozyme is introduced into the target cell, this method employs synthetic oligonucleotides.

Antisense approaches: Antisense strategies make use of a highly specific binding of an oligonucleotide (in most cases DNA) to its complementary sequence on a target mRNA and hence the subsequent inactivation of the target. To achieve sufficient specificity, the sequence must consist of at least 12 nucleotides, however, most antisense oligonucleotides contain 16-24 nucleotides.

The mechanisms of antisense action are complex and not fully understood, however, a major one appears to be RNA cleavage after hybridization of the DNA antisense oligonucleotide with its target mRNA. This reaction is catalyzed by the ubiquitous enzyme Ribonuclease H (RNase H). RNase H is an endoribonuclease that specifically degrades the RNA strand in a RNA-DNA hybrid and has its evolutionary roots in the defense of mammalian cells against retroviruses. The RNase H mechanism is by far the most important due to the fact that the antisense oligonucleotide is not destroyed or permanently occupied and therefore only catalytic amounts are necessary. Several other mechanisms such as transcriptional arrest, inhibition of splicing and inhibition of posttranslational modifications are involved as well (see Figure 7), however, they require stoichiometric amounts of oligonucleotide and are probably less important.

Since regular phosphodiester oligonucleotides are inactivated by serum nucleases rather rapidly, chemical modifications especially on the phosphodeoxyribosyl-backbone were investigated to achieve higher stability. Phosphorothioate oligonucleotides (SODN) with one of the oxygen atoms in the phosphate group replaced with sulfur have a significantly increased resistance to nuclease inactivation, however, their binding affinity to the target mRNA and their affinity to RNase H is slightly reduced. In total however the higher resistance leads to an enhanced efficiency and therefore SODN have become the

standard type of oligonucleotide for antisense therapy and several SODN targeting cancer are in clinical trials [214]. A problem associated with this type of oligonucleotides is their susceptibility towards unspecific effects due to their interactions with biomacromolecules such as proteins. Therefore a careful evaluation of experimental data should be performed in order to attribute therapeutic effects exclusively to an antisense mechanism.

Another example for a stabilizing modification on oligonucleotides is the introduction of a methoxyethoxy group at the 2' position of the ribose ring [215], which leads to significant stabilization and displays synergistic effects with phosphorothioate modifications. For a deeper insight into antisense mechanisms and chemistry we refer the reader to an extensive review for details [216].

Figure 5
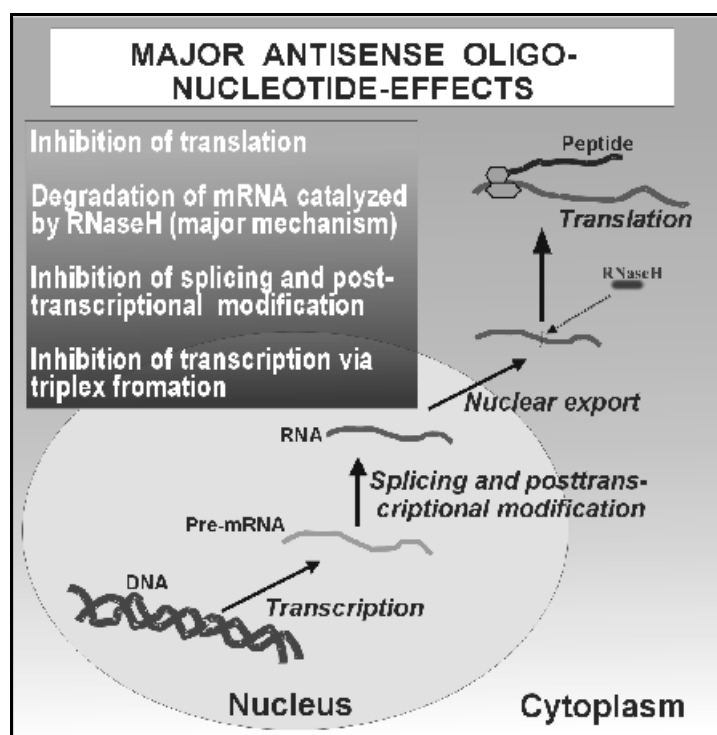**Major sites of action of antisense oligonucleotides**

Ribozymes: Ribozymes are RNA molecules endowed with catalytic activity and capable of cleaving mRNA molecules in a sequence specific, catalytic manner. They contain sequences for selective ligation with target mRNAs which confers upon them high specificity. They also contain sequences that perform cleavage

reactions with the target mRNA. By modifying the substrate recognizing sequences, ribozymes can be specifically tailored for the suppression of particular genes. A large variety of gene products have been targeted successfully using this strategy (see Table 5 for cancer specific targets).

Two types of ribozymes, the hammerhead and hairpin ribozymes have been extensively studied due to their small size and high cleavage efficiency.

One problem using these structures is their low stability against ubiquitous RNases. Therefore strategies have been developed that enable efficient protection of ribozymes without loss of catalytic activity. Modifications of the chemical structure of the phosphoribosyl backbone have been performed, including the use of 2´-O-methyl nucleotides instead of 2´-hydroxyls throughout the sequence. The addition of an inverted deoxyabasic sugar residue on the 3´ end or the introduction of phosphorothioate nucleotides on the 5´ end of the ribozyme have also been performed [217]. The sum of these modifications leads to a decreased recognition by nucleases and an increased biological activity. Complexation with cationic polymers or application of DNA/RNA chimeras have shown promising results for stabilization as well (own unpublished data). A major advantage of this strategy is the high efficiency of stabilization provided by these agents and the possibility of attaching targeting moieties to polymers in order to target specific cells.

A problem when using hammerhead ribozymes is their dependence on magnesium ion concentration for catalytic activity. Physiological concentrations of this ion are 5-10fold lower than in optimized cell culture conditions [218]. Therefore correlation from *in vitro* to *in vivo* activity is problematic.

For reviews about hammerhead and hairpin ribozymes see references [219,220].

To date the major site of action for either antisense oligonucleotides or ribozymes on the subcellular level has not been determined and it remains unclear if actual delivery into the nucleus is required for effectivity.

Target genes for antisense/ribozyme strategies: Several antisense and ribozyme strategies have been pursued with some success in cancer therapy *in vitro* as well as *in vivo*. One approach is to make cancer cells more sensitive to chemotherapy by blocking the expression of genes that are involved in multidrug resistance. A target that has been investigated intensively in this context is the Multidrug resistance gene (*MDR1*) and its product P-glycoprotein that removes a large variety of chemotherapeutic agents from the cell. Treatment with anti-*MDR1* oligonucleotides or ribozymes led to a significant reduction of *MDR1* expression (see Table 5) and subsequently to a higher sensitivity of the treated cells against a variety of cytostatic substances.

For further examples see Table 5.

Target Gene	Biological effect of ODN/ribozyme	(a)ntisense / (r)ibozyme	Reference
MDR1	reverse multidrugresistance	a,r	[215,222]
BCL2	apoptosis	a,r	[223,224]
MDM2	inhibition of proliferation	a	[225]
c-ERBB-2	inhibition of proliferation	a,r	[226,227]
c-Myc	apoptosis	a,r	[228,229]
Ras	inhibition of proliferation and CAM expression	a	[230,231]
Raf	inhibition of proliferation and CAM expression	a	[231]
AKT1	apoptosis	a	[232]
EGFR	inhibition of cell prolifration	a,r	[233,234]
FGF	antiangiogenesis	a	[235]
angiogenin	antiangiogenesis	a	[236]
telomerase	cell death	a,r	[237,238]
survivin	cell death	a	[239]

Table 5
Examples for cancer related targets in antisense and ribozyme therapy

Antisense treatment towards these targets has been investigated intensively *in vitro* and in clinical trials at several stages. Early clinical trials show only modest efficiency in some patients. At present, antisense therapy in combination

with classic chemotherapy seems to be the more favorable regimen [221]. More research is necessary to optimize such combinations and antisense therapy itself.

Target	Effector structure (a)ntisense/(r)ibozyme	Reference
k-ras	r	[240,241]
Epidermal growth factor (EGF)	r	[242]
bcl-2	r	[243]
Alpha1-antitrypsin	r	[244,245]
HER2/neu	r	[246]
mutant p53	r	[247]
BCRABL	r	[248]
Interleukin-8	a	[249]
Cyclin D1	a	[250]
VEGF	a	[251]
c-myc	a	[252]
Basic fibroblast growth factor	a	[253]
High mobility group I proteins	a	[254]
hNr-CAM	a	[255]
Urokinase-type plasminogen activator receptor	a	[256]

Table 6
Cancer related targets used for knockout gene therapy

4.2. Knockout gene therapy

Knockout gene therapy aims to inactivate or attenuate the expression of oncogenes and hereby to inhibit uncontrolled cell proliferation, angiogenesis, drug resistance or metastasis formation. This can be achieved by the introduction of a gene coding for an antisense RNA which hybridizes with the target mRNA of the desired oncogene. Since duplexed mRNA cannot be translated, it is degraded quickly and the expression of the targeted oncogene is inhibited specifically. Furthermore genes can be introduced coding for ribozyme molecules that reduce the expression of an aberrant gene by specific cleavage of its mRNA. Since decoding of the human genome has provided knowledge about

a large variety of oncogene sequences, this approach can be utilized in a variety of clinical situations and has been realized mainly using viral vectors (Table 6).

A second strategy to ´knock out´ the effect of a cancer gene is the introduction of a modified oncogene that acts in a dominant manner towards the cancer causing gene and thereby attenuates or even inhibits its expression. This method is less versatile due to difficulties in finding genes with counteracting properties and its application is limited to very specific targets.

4.3. Gene replacement/gene augmentation

This strategy tends to replace either a missing or a mutated/defective gene, in most cases tumor suppressor or other apoptosis-inducing genes. The difficulty with this approach is that this type of gene therapy replaces only one gene and is thus less effective if the cancer is oligo- or multigenic in origin. There are numerous diseases that are based on monogenic defects for example cystic fibrosis, hemophilia IX or familial hypercholesterolemia. In these situations gene replacement may be suitable. Cancer, however, is usually caused by multiple gene defects, so that either more than one gene has to be introduced or a combination with other forms of therapy must be performed. In fact the combination with chemo/radiotherapy showed synergistic effects and represents a valuable augmentation of classical cancer therapy [257].

P53 in cancer gene therapy: P53 is the best investigated tumor suppressor and mutations within the p53 gene are involved in a large portion of human tumors. It acts as a transcriptional factor and is involved in the regulation of apoptosis, cell growth arrest and senescence. Its malfunction is a common mechanism for tumorigenesis leading to cell immortalization, multidrug resistance and uncontrolled cell division. One approach in selective treatment of p53 aberrations is to reactivate its expression with low molecular weight drugs like actinomycin D or leptomycin B [258]. This has been done with success in several cell lines, however, it requires a residual level of p53 expression and is

therefore not suitable in all situations. Furthermore, toxicological problems make this approach applicable only for local administration and is therefore limited to easily accessible tumors.

Gene replacement represents a direct approach towards the restoration of wild type p53 function. It has been demonstrated *in vitro* that introduction of wild type p53 gene into human carcinoma cells induces apoptosis of cancer cells [259] and may induce remission of drug resistance connected with mutant p53 variants [260]. Most forms of lung cancer are p53 deficient, therefore this approach has been investigated in detail for this target. Delivery of wild type p53 to the lung either by systemic or local application via inhalation of aerosolized polyplexes led to a significant suppression of tumor growth in both cases and a prolonged survival time of laboratory animals [56,261].

Delivery to breast cancer cells *in vivo* using immunolipoplexes including anti-transferrin receptor antibody as targeting moiety has been reported recently [262]. This treatment led to a high level of p53 gene expression in the targeted cells and a combination treatment with docetaxel resulted in significantly improved efficacy of the drug with prolonged survival of laboratory animals. For a review concerning non-viral p53 delivery see reference [263].

4.4. Suicide gene therapy

A special form of gene augmentation therapy is the introduction of prodrug-converting enzyme genes, better known as suicide genes. A suicide gene encodes for an enzyme usually of viral or prokaryotic origin that performs the conversion of a non-toxic prodrug into an active toxin that causes cell death. The gene for this enzyme is either under the control of a tumor specific promoter, thus limiting the cytotoxic effect to specific tissues or injection directly into target tissue is performed in order to limit nonspecific toxicity. Table 7 gives an overview of frequently used suicide genes and their substrates. This strategy recently was effective in an *in vivo* model using PEI as non viral vector in

hepatocellular carcinoma [264]. Transfection with herpes simplex thymidine kinase (*HSV1-tk*) gene and subsequent ganciclovir treatment, one of the most common suicide gene systems, was used in this study. HSV1-tk converts the rather non-toxic drug ganciclovir into its triphosphate that interferes with DNA synthesis and leads to death of transfected cells. This treatment leads to a suppression of tumor growth and a significantly prolonged survival time of laboratory animals. Many suicide gene therapy approaches mainly with viral vectors as well as some liposomal formulations have been performed and resulted in tumor and metastasis remission *in vivo* [265,266]. Furthermore the HSV1-tk/ganciclovir and cytosine deaminase/5-Fluorocytosine system are currently being evaluated in clinical trials [267-69]. A major disadvantage of this strategy is that gene expression must occur specifically in tumor tissue in order to spare healthy cells. Therefore highly tumor specific promoters need to be developed in order to minimize toxic effects. Targeting moieties specific for cancer cells may also help to limit impact of non target cells. For a review about prodrug activation enzymes in cancer gene therapy see reference [270].

Enzyme	Prodrug	Active drug	Reference
Thymidine kinase (HSVtk)	ganciclovir	ganciclovir triphosphate	[271]
Cytosine deaminase	5-fluorocytosine	5-fluorouracil	[272]
Purine nucleoside phosphorylase	6-methylpurine-2´deoxyribonucleoside	6-methylpurine	[273]
Guanosine-xanthine Phosphoribosyl transferrase	6-Thioguanine	6-thioguanine triphosphate	[274]
Varizella zoster virus thymidine Kinase	araM	araM-MTP	[275]
E. coli nitroreductase	CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide)	(5-(aziridin-1-yl)-4-hydroxyl amino-2-nitrobenzamide)	[276]

Table 7
Enzyme/Substrate systems used in suicide gene therapy

4.5. Cancer vaccination using gene therapy approaches

Vaccination approaches have been performed successfully for a large variety of cancers and they represent promising strategies for future advances especially due to their rather low toxicity and their ability to reach multifocal targets throughout the body [277,278]. In fact most of the clinical trials of cancer gene therapy employ such immunological strategies, using the transfer of genes for cytokines, costimulatory factors or tumor associated antigens into a variety of cellular targets.

Malignant cells do show antigenic differences to normal cells, however their immunogenicity is not recognized by the host´s immune system. The goal of immunological strategies is either to boost the immune system in a way that enables it to identify and efficiently eliminate tumor cells or to enhance the immunogenicity of tumor cells so they can no longer remain undiscovered.

One strategy relies on augmentation of the conspicuousness of tumor cells to the immune system by explantation of cancer cells from a patient´s body and transfection ex vivo with a cytokine gene such as interferon-?, tumor necrosis factor-a and interleukin 12 or genes of costimulatory factors such as B7 (CD80) or intercellular adhesion molecule-1 (CD54) [279,280]. Cells are then transplanted back into the patient where the expression of cytokines and costimulatory factors in proximity to tumor antigens presented on MHC class I proteins on the surface of tumor cells draws attention of the host´s immune system to the tumor.

An alternative approach towards cancer vaccination is a modification in the way a tumor associated antigen is presented to the host´s immune system. If antigens are only weakly expressed via MHC class I proteins on tumor cells they are not recognized under normal conditions and the tumor remains unmolested. If, however, they are presented with professional antigen presenting cells (APC) of the immune system, especially dendritic cells in association with costimulatory

factors, an immune reaction is initiated. This strategy is most effective when combined with the introduction of cytokine genes.

One approach to achieve the presentation of tumor antigens on dendritic cells is based on the insertion of the DNA sequence encoding the desired antigen into a plasmid vector, capable of expressing exogenous proteins in mammalian cells. These plasmids are then introduced into skeletal muscle cells or subcutaneous tissue via gene gun or injection of naked DNA with the intention that these cells will express the protein and export it into the surrounding tissue interstitium. The antigen is captured by antigen presenting cells, especially dendritic cells (DC) and presented after intracellular processing on major histocompatibility complex (MHC) class I and II, thus leading to cellular and humoral immune response against the presented antigen.

A second strategy aims to directly introduce the vector containing the tumor antigen sequence into DC via a targeted vector resulting in similar effects as described above [139,281]. Introduction of the tumor antigen can be performed ex vivo as well, with subsequent reimplantation into the patients [282].

4.6. Bystander effect

One problem concerning knockout and suicide gene therapy is the fact that in theory the whole cell population of a tumor has to be transfected in order to achieve tumor eradication. Otherwise non-transfected cells survive and proliferate, thus leading to survival of the cancer. A helpful phenomenon in this context is the so called bystander effect, which means that the introduced gene can affect cells in which the gene is not expressed itself. The mechanisms of action is not fully understood, however, cell-cell exchange of soluble factors through gap junctions may be involved. Using suicide gene therapy strategies with herpes simplex virus thymidine kinase/ganciclovir treatment for example, a transfer of toxic metabolites of ganciclovir from transfected to non-transfected cells through cell-cell contacts can be observed, thus augmenting efficiency of

the treatment [283]. A mechanism involved in bystander effects in distant cells and/or anatomically separated tumors is the activation of innate and adaptive immunological antitumor responses against cancer cells [284] This effect is probably mediated by significant increase of CD4 positive, CD8 positive and natural killer cells.

A more detailed knowledge about mechanisms of action and methods to augment the bystander effect will be very valuable to future cancer gene therapy regimens.

4.7. Clinical trials

Cancer gene therapy: Cancer treatment is by far the most important proposed application of gene and antisense therapy and many clinical trials are underway (Figure 6). Approximately 50 percent of all cancer gene therapy trials use various forms of immunemodulatory strategies via systemic or local administration. Another significant contribution (15%) to the studies is represented by suicide gene therapy strategies. Further approaches like insertion of multidrug resistance genes in stem cells or tumor suppressor gene therapy represent approximately five percent. The vast majority of gene therapy clinical trials are in phase I and only a very few have progressed to phase II studies. The majority of the studies have demonstrated that gene therapy is generally feasible using both, viral as well as non viral strategies, however at present cancer gene therapy has not fulfilled the high expectations initially predicted. No one has been cured through the use of gene therapy and the therapeutic efficiency monitored in clinical trials is disappointing.

More than two thirds of gene therapy clinical trials use viral vectors, only 13 % employ lipid vectors and ~ 10 % use naked DNA (Figure 7). Cationic polymers have only been used in animal models and have not advanced into clinical trials due to the various problems described in this review. Some of the clinical trials using lipid vectors are summarized in Table 8. The majority of these studies

employ immunological strategies against cancer and another significant portion aims the transcriptional repression of HER-2/neu gene by introduction of the adenovirus type 5 E1A gene via local application. Her-2/neu is a growth factor receptor overexpressed in multiple human cancers, especially breast and ovarian cancer. Several studies demonstrated E1A gene expression in target tissue, accompanied by HER-2/neu downregulation, increased apoptosis, and reduced proliferation.

Antisense therapy: Antisense therapy is clinically far ahead of actual gene therapy and remarkably the FDA has already approved one drug. This drug is named Fomivirsen (Vitravene[R]), a 21mer phosphorothioate oligonucleotide that is being used against infections of the eye caused by human cytomegalovirus (HCMV). It inhibits the expression of proteins necessary for virus replication through an antisense mechanism and needs to be administered via intravitreal (eye) injections.

Target	Vector	Phase	Reference
Metastatic melanoma	DMRIE/DOPE	II	[285]
Breast and ovarian cancer	DC-Chol	I	[286]
Brest and head and neck cancer	tgDCC	I	[287]
Peripheral neuroektodermal malignancy	cationic lipid (ex vivo)	preliminary clinical trial	[288]
Cutaneous metastases	DC-Chol/DOPE	I	[289]
Hepathic metastases of colorectal carcinoma	DMRIE/DOPE	I	[290]
Melanoma	DMRIE/DOPE	I	[291]

Table 8
Examples for clinical trials with cationic lipids (Abbreviations: DMRIE=1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide, DOPE=dioleoyl phosphatidylethanolamine, DC-Chol=3-beta(N-(N',N'-dimethylaminoethane)carbamoyl) Cholesterol, tgDCC=3 beta[N-(n',n'-dimethylaminoethane)-carbamoyl] cholesterol/ dioleoylphosphatidyl-ethanolamine).
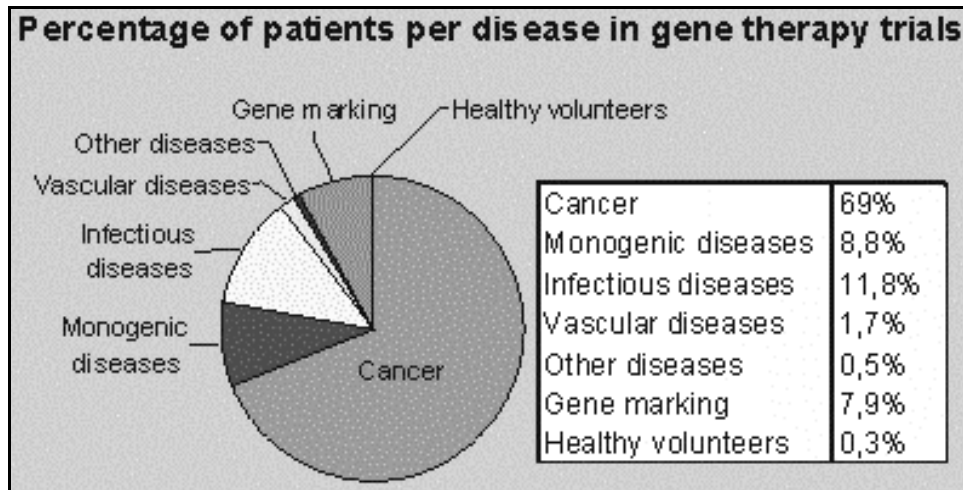
Figure 6
Percentage of diseases treated in clinical trials using cancer gene therapy
(data from http://www.wiley.co.uk/genetherapy/clinical/).
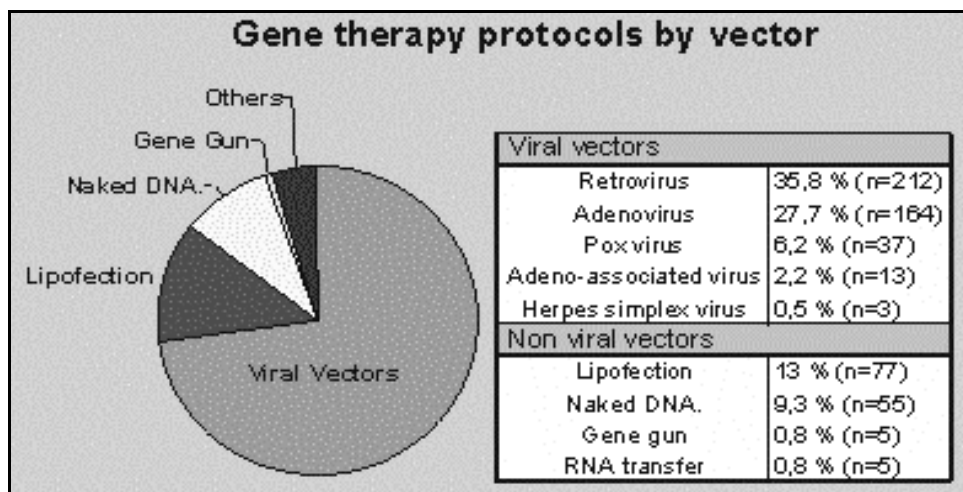
Figure 7
Overview about vectors used in clinical trials (data from
http://www.wiley.co.uk/genetherapy/clinical/).

With regard to cancer therapy a variety of phase I studies are underway. An antisense oligonucleotide for the treatment of melanoma and B-cell lymphoma targeting bcl-2 and an antisense inhibitor of protein kinase C alpha in solid tumors are being investigated in phase III trials [214]. Phase II clinical trials are in progress against raf-kinase, Hras, R2-protein and DNA methyltransferase (see table 9 and [214]).

Name	Company	Application	Disease	Target	Stage
ISIS 3521	ISIS/Lilly	parenteral	non-small cell lung cancer and others	PKC-a	phase III
G3139	Genta	subcutaneous	melanoma	bcl-2	phase III
ISIS 2503	ISIS	parenteral	cancer, pancreatic and others	H-ras	phase II
ISIS 5132	ISIS	parenteral	cancer, ovarian and others	raf	phase II
GTI-2040	Lorus	parenteral	advanced or metastatic renal cell carcinoma	R2-protein of ribo-nucleotid reductase	Phase II
GEM 231	Hybridon	parenteral	various cancers	PKA	Phase II

Table 9
Antisense ODN against cancer in higher stages of clinical trials

Numerous other antisense approaches against cancer related targets are in clinical trials and the interest in antisense strategies is expected to increase as it displays several advantages. It is easier to synthesize large amounts of pure antisense oligonucleotides as compared to plasmids as the antisense sequences used are much shorter. Furthermore, the antisense mechanism is applicable to a wide range of conditions and antisense oligonucleotides are physiologically well tolerated.

5. CONCLUSIONS

Progress in molecular biology has provided the opportunity for the introduction or replacement of missing, as well as malfunctioning genes. Using these tools, cancer cells may be selectively destroyed via suicide strategies or the expression of undesired genes may be blocked by antisense or ribozyme strategies. Immunological approaches have displayed promising therapeutic effects, as well. The best clinical results, however, have been achieved by local administration or the use of antisense oligonucleotides, as is indicated by the high number of clinical trials currently taking place. These new techniques, however, lack efficient and specifically targeted delivery systems for nucleic

acids and raise toxicity concerns. No one system meets all needs; instead different therapeutic circumstances will require different vectors. Remarkable efforts have been made to optimize gene delivery systems *in vitro*; *in vivo* application, however, requires different vector features. Properties of non viral vectors determined *in vitro* are insufficient for predicting their behavior *in vivo* and the development of a set of *in vitro* assays that allow prediction of *in vivo* behavior of the vectors would be a valuable tool for further advances. Additional research, which elucidates the factors influencing opsonization and other mechanisms leading to RES uptake and other forms of elimination *in vivo*, is necessary.

A useful strategy to reduce unspecific interactions of polyplexes after systemic administration is the steric stabilization of complexes via the attachment of hydrophilic polymers. The poor *in vivo* results displayed by the first generation of these complexes using simple attachment of PEG or pHPMA occurred probably due to ineffective shielding. Strategies developed by Oupicky et al. [49,50] using bioreversible crosslinking of surface modifying structures or DNA compacting polymers lead to higher stability in circulation and efficient intracellular release. Research employing other bioreversible complex modifications and the use of more efficient shielding strategies represent interesting approaches to improve *in vivo* vectors.

Another major problem occurring *in vivo* is gene expression in sites other than the target tissue. A number of targeting moieties for cancer tissues have been identified and these structures were effective *in vitro* and, in some cases, even after systemic application. Incorporation of tissue specific promoters, in order to limit gene expression exclusively to the target tissue, will provide synergistic effects with active targeting strategies and should be addressed in particular therapeutic circumstances.

Local gene therapy approaches, at this time, hold more promise than systemic application, because the danger of misrouted deposition and unwanted gene

expression in non target tissues can be partly avoided. Local strategies using immunological approaches have shown most promising results, due to strong bystander effects.

Delivery at the subcellular level remains inefficient as well. While endocytotic uptake of polyplexes occurs rather efficiently, the release from endosome is still one of the primary reasons for the low efficiency of non-viral gene and oligonucleotide therapy. For polyplexes, there is some evidence that the majority of the nucleic acid is entrapped in such vesicles [80]. Although some success has been obtained with fusogenic peptides or other endosomolytic agents, such as chloroquine, the applicability of these structures *in vivo* is limited. Subcellular trafficking at this stage probably bears great potential for further advances in non viral vectors. However, it should be noted that the release mechanism, as far as it is known to date, leads to the release of endosomal or lysosomal content into the cytosol. This event represents a perturbation of subcellular compartmentalization and, therefore, stress for the cell. The stage of the pathway at which the release occurs is of great importance since hydrolytic enzymes and other lysosomal content may cause major damage in the cell finally leading to apoptosis or necrosis [292]. Further improvements at the stage of vesicular escape will result in increased reporter gene expression, however, it may lead to a higher toxicity of the method, as well. More research about the release mechanism, especially for polyplexes is required for a better understanding of this problem.

Transfer of DNA, once released into the cytosol, to the nucleus and across the nuclear envelope is very inefficient. It is still unclear how plasmid DNA reaches the nucleus, if via free diffusion or via a specific cellular transport system, e.g. dynein. The complexing agent plays an important role [177] for nuclear delivery, by either helping to reach the nuclear envelope through the cytoplasm or facilitating nuclear entry. Nuclear uptake is inefficient and highly connected to the mitotic activity of the cells. This may help to target fast growing tumors,

however, there are applications that require uptake in slower dividing cells. Here some improvements have been achieved using nuclear localization sequences, however, increase in reporter gene expression is still moderate and there is probably more potential for optimization.

To summarize, cancer gene therapy still represents a highly promising research area that bears a great potential for future cancer therapy, however, to achieve this goal numerous hurdles have to be surmounted.

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2

Intracellular processing of polyethylenimine/ribozyme complexes can be observed in living cells using confocal laser scanning microscopy and inhibitor experiments.

SUMMARY

Purpose: Critical steps regarding the subcellular processing of polyethylenimine/nucleic acid complexes, especially endosomal/lysosomal escape, were visualized using living cell confocal laser scanning microscopy (CSLM) to obtain an insight into their mechanism.

Methods: Living cell confocal microscopy was used to examine the intracellular fate of polyethylenimine/ribozyme and poly(L-lysine)/ribozyme complexes over time, in the presence of and without bafilomycin A1, a selective inhibitor of endosomal/lysosomal acidification. The compartment of complex accumulation was identified by confocal microscopy with a fluorescent acidotropic dye. To confirm microscopic data, luciferase reporter gene expression was determined under similar experimental conditions.

Results: Polyethylenimine/ribozyme complexes accumulate in acidic vesicles, most probably lysosomes. Release of complexes occurs in a sudden event, very likely due to bursting of these organelles. After release, polyethylenimine and ribozyme spread throughout the cell, during which slight differences in distribution between cytosol and nucleus are visible. No lysosomal escape was observed with poly(L-lysine)/ribozyme complexes, or when polyethylene-imine/ribozyme complexes were applied together with bafilomycin A1. Polyethylenimine/plasmid complexes exhibited a high luciferase expression, which was reduced approximately 200-fold when lysosomal acidification was suppressed with bafilomycin A1.

Conclusions: Our data provide, for the first time, direct experimental evidence for the escape of polyethylenimine/nucleic acid complexes from the endosomal/lysosomal compartment. CLSM, in conjunction with living cell microscopy, is a promising tool for studying the subcellular fate of polyplexes in nucleic acid/gene delivery.

Supplementary material for this publication can be accessed under:

http://www.uni-marburg.de/iptb/institut/akkissel/motherpage.htm

INTRODUCTION

Polyethylenimine [PEI] is one of the most commonly used nonviral vectors based on polycations for DNA/RNA delivery both *in vitro* and *in vivo* [1]. In spite of numerous publications dealing with the mechanisms of uptake and subcellular processing of PEI/plasmid complexes, current understanding of the release mechanism from the endosomal/lysosomal compartment is still limited [1,2,3]. New insights could facilitate the design of new polymers for nonviral gene delivery systems. It has been demonstrated by several groups that PEI polyplexes are taken up via endocytosis [4,5], however, theories regarding intracellular processing of these complexes remain controversial. Using confocal microscopy studies Godbey et al. [3] found that PEI/plasmid complexes access cells in culture via endocytosis. According to Godbey et al. [2] these endocytotic vesicles do not seem to fuse with lysosomes, but rather proceed directly to the perinuclear region where PEI and DNA are released and taken up into the nucleus [2,3]. Further Lecocq et al. [4] examined uptake and subcellular distribution of radioactively labelled PEI/plasmid complexes *in vivo* using differential and isopycnic centrifugation methods. They observed that PEI was localized in plasma membrane fractions 5 minutes after administration and in lysosomal fractions after 4 hours. According to this study PEI persists in the lysosomal compartment without significant release for days. Another unclear, yet very important question, is how PEI/nucleic acid complexes escape from the endosomal/lysosomal compartment. Behr [6] proposed the so-called ´proton-sponge hypothesis´ explaining the lysosomal release of PEI polyplexes by their buffering capacity. During acidification of endosomes/lysosomes this property leads to increased influx of protons, chloride ions, and water, thus eventually causing rupture of the vesicles due to the high osmotic pressure. Although this hypothesis was developed some years ago, no convincing experimental verification   has   been   provided   by   anyone   so   far.

Another intensively discussed issue is the mode of entry that DNA/RNA and PEI, once released from lysosomes, use to gain access into the nucleus. There has been some preliminary work done in this field, however much still remains to be discovered [7].

In contrast to previous reports we attempted to gain insight into these subcellular distribution processes using living cell confocal laser scanning microscopy. This method is unique, as it allows a direct observation of PEI/nucleic acid complexes in the intact subcellular environment over a long time period. More specifically, it may prove to be a valuable tool in elucidating the release mechanism of PEI polyplexes from the endosomal/lysosomal compartment.

PEI/ribozyme complexes were used in the presence of and without bafilomycin A1, a selective inhibitor of the vacuolar-type H(+)-ATPase, which prevents acidification of endosomes/lysosomes. We compared the findings of these CLSM experiments to those obtained with poly(L-lysine)/ribozyme complexes under identical conditions. To confirm our observations we also performed transfection experiments with a luciferase plasmid under similar experimental conditions. Our aim was to gain a mechanistic insight in subcellular processing of dually labelled PEI/ribozyme complexes by observing fluorescence distribution over time.

MATERIALS AND METHODS

Chemicals

Polyethylenimine (Mw 25,000), poly(L-lysine) (Mw 34,300), chloroquine and bafilomycin A1 were purchased from Sigma-Aldrich.

Labelling of PEI and poly(L-lysine) with Oregon Green 488

Polymer (20 mg) was dissolved in 2 mL 0.1 M sodium bicarbonate solution with pH 9. Oregon Green carboxylic acid succinimidyl ester (1 mg, Molecular Probes) was dissolved in 200 μL dimethylsulfoxide and added dropwise under

stirring to the polymer solution. The mixture was stirred in the dark for 3 hours at room temperature before the labelled polymer was purified by ultrafiltration in an Amicon cell (regenerated cellulose membrane, molecular weight cut off 10,000) and washed with 0.1 M borate / 1.0 M sodium chloride solution pH 7.5. The washing procedure was performed until no absorption was detectable at 488 nm in the cell outflow. As a final step, the buffer was exchanged with distilled water.

Ribozyme and plasmid

A 5-carboxytetramethylrhodamine labelled 37-mer ribozyme with the sequence 3´-UCUCUCAAAGCAGGAUUGCCUGAGUAGUCAUAACCUU-5´   was purchased from MWG-Biotech. A luciferase plasmid (pGL3-control, Promega) was used for transfection experiments. The plasmid was amplified in JM109 competent cells (Promega) and purified with QIAfilter plasmid Kits (Qiagen) according to the manufacturers protocol.

Complex formation

All ribozyme/PEI or plasmid/PEI complexes were prepared at a PEI-nitrogen to DNA/RNA- phosphate ratio (N/P ratio) of 8. For microscopic experiments ribozyme (2 μg) and PEI (2.2 μg) were each dissolved in 100 μL of 0.9 % sodium chloride solution pH 7. The two solutions were mixed by gentle pipetting. Complexes were allowed to interact for 10 minutes before use. Ribozyme/poly(L-lysine) and plasmid/poly(L-lysine) complexes were prepared at an N/P ratio of 2. In this case 2 μg of ribozyme and 1.6 μg poly(L-lysine) were dissolved in 100 μL 0.9 % sodium chloride solution pH 7. For transfection experiments using pGL3-control luciferase plasmid, complexes were prepared in a similar manner with 4 μg plasmid and 4.4 μg PEI or 3.2 μg poly(L-lysine) dissolved in double the volume of sodium chloride solution 0.9 % pH 7.

Cell culture

All experiments were performed with the SW13 adrenal gland carcinoma cell line (ATCC number CCL-105) which was a kind gift from Dr. A. Aigner (Philipps University, Marburg, Germany). Cells were cultured in IMDM medium (PAA) containing glutamine (584 mg/l) and 25 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), supplemented with 10 % fetal bovine serum (HyClone). For transfection experiments cells were grown at 37 °C in a humidified atmosphere containing 10 % CO_2 (V/V).

Confocal laser scanning microscopy with Lyso Tracker Blue

A Zeiss Axiovert 100 M microscope coupled to a Zeiss LSM 510 scanning device was used for all confocal microscopy experiments.

Cells were seeded at a density of 20,000 cells per well in 8 well chamber slides (LabTek, Nunc). After 24 hours, medium was removed and complexes of Oregon Green-labelled PEI and non-labelled pGL3-control plasmid were added in new medium containing 100 nM Lyso Tracker Blue (Molecular Probes). After 1h the incubation medium was aspirated, cells were washed three times with phosphate buffered saline (PBS) pH 7, and observed in PBS with the confocal microscope. For excitation of the blue Lyso Tracker an Enterprise UV laser with an excitation wavelength of 364 nm was used. For excitation of green fluorescence (labelled PEI) an argon laser with an excitation wavelength of 488 nm was used. This experiment was performed using a longpass filter of 385 nm for blue fluorescence and a longpass filter of 505 nm for green fluorescence. All images were recorded using the Zeiss LSM 510 Multitracking Mode in which each fluorescence channel was scanned individually.

Living cell confocal laser scanning microscopy

An argon laser was used to excite green fluorescence at 488 nm and a helium-neon laser was used for red fluorescence at 543 nm. Images were taken using a band pass filter of 505-530 nm for green fluorescence and a long pass filter of

560 nm for red fluorescence. Detector sensitivity was set relatively high in order to be able to visualize faint fluorescence, however was kept constant during experiments. Thickness of the optical sections was between 0.5 and 1 μm. Stacks of 15-35 images were recorded every 3-9 minutes to obtain information over time (setting descriptions for the individual figure are listed below each figure). The different layers were overlaid for each time point by Zeiss LSM 510 software. For creating movies the overlaid images of the single time points were animated over time. Total thickness of stacks ranged between 10–20 μm. Temperature was maintained at 37 °C for the duration of the experiment. A CO_2 atmosphere was not necessary, as the cell culture medium contained 25 mM HEPES. For living cell microscopy 30,000 cells were seeded on 2 cm^2 self-made chamber dishes containing a total of 500 μL medium. Bafilomycin A1 was added to the media at a concentration of 300 nM, where indicated.

Transfection experiments with luciferase plasmid

Transfection experiments with pGL3-control luciferase plasmid were performed with poly(l-lysine)/plasmid complexes in addition to PEI/plasmid complexes in the presence of, as well as without bafilomycin A1. Cells were seeded in 12 well plates at a density of 50,000 cells per well. After 24 hours medium was removed and complexes were added to fresh medium containing 300 nM bafilomycin A1, when applicable. Media was exchanged again after four hours and cells were incubated for 44 hours. Luciferase gene expression was quantified using a commercial kit (Promega) and photon counting with a luminometer (Sirius, Berthold).

Results in relative light units per second (RLU/s) were converted into ng luciferase by creating a calibration curve with recombinant luciferase (Promega). Protein concentration in each sample was determined using a BCA assay [10]. All experiments were performed in quintuplet and data were expressed in ng luciferase per mg protein.

RESULTS

Confocal laser scanning microscopy using Lyso Tracker Blue

In Figure 1, colocalization of PEI/DNA complexes (green) and Lyso Tracker Blue is shown approximately 1 hour after transfection (turquoise vesicles). Several predominantly green vesicles are also present in this image. These could either represent PEI in nonacidic prelysosomal compartments or in lysosome remnants, suggesting that in this case the lysosomes had already burst. This strongly indicates that complexes accumulate in the lysosomal compartment after one hour. We obtained similar results with OVCAR-3 (ATCC number HTB-161) cells (data not shown).
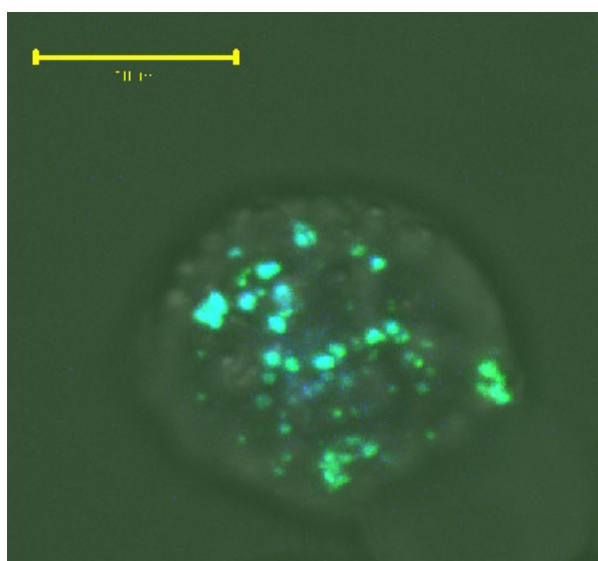

Figure 1
PEI/plasmid complexes colocalize with Lyso Tracker Blue ~ 1 hour post incubation (turquoise vesicles). In addition, vesicles containing predominantly PEI are visible (green vesicles). The image shows an overlay of light microscopic and confocal fluorescence image. (Lyso Tracker is blue in this image, PEI is green)

Living cell confocal laser scanning microscopy

Five short movies have been chosen to demonstrate the results of living cell confocal laser scanning microscopy [see Table 1]. Movies #1 and #2 were obtained with PEI/ribozyme complexes. Movie #3 shows PEI/ribozyme complexes in the presence of 300 nM bafilomycin A1 and Movie #4 was recorded using poly(L-lysine)/ribozyme complexes. Movie #5 shows an

overview of approximately 20 cells incubated with PEI/ribozyme complexes, whereas only the green fluorescence of PEI is shown. All movies can be accessed as supplementary material for this publication on our website "http://www.uni-marburg.de/iptb/institut/akkissel/motherpage.htm". Essential excerpts from Movies #1 and #2 are shown in Fig. 2 and 3. It should be noted that the vesicle sizes in Movies #1-5 do not accurately display actual sizes, due to fluorescence outshine, however relative changes in localization and size over time can be observed.
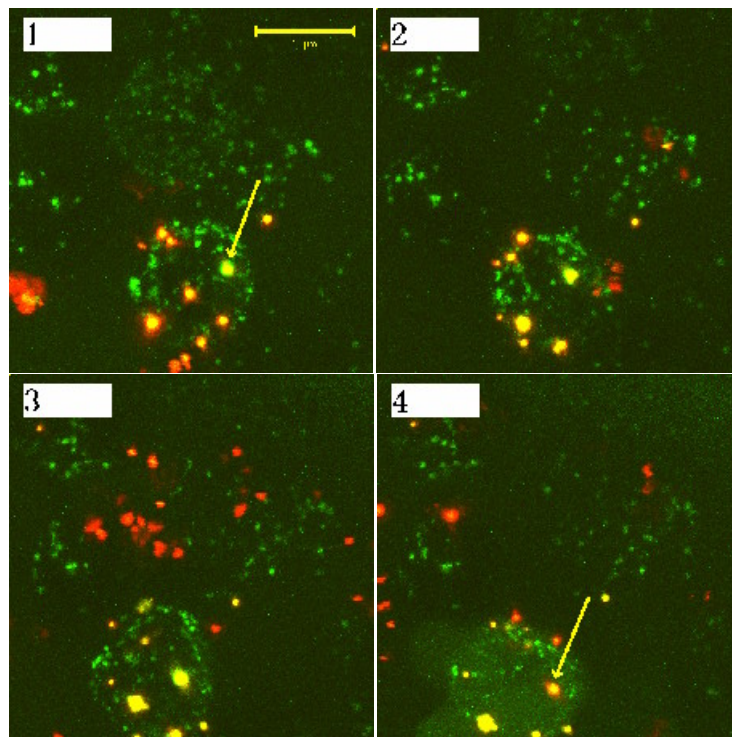

Figure 2a
Living cell microscopic visualization of swelling and, finally, bursting of a single vesicle (yellow arrow). PEI is green and ribozyme is red in this sequence. Before burst, the characteristic appearance of the vesicle with a yellow core and green PEI-corona can be seen. Afterwards the faint green-yellow fluorescence can be seen evenly distributed throughout the entire cell. The remnant of the vesicle is significantly smaller and deeper red. Images were recorded 28, 31, 37 and 40 minutes after incubation. They display computer overlaid projections of the different confocal slices for each time point. The whole movie can be accessed on our website [Movie #1]. The yellow mark displays the size of 10 μm.
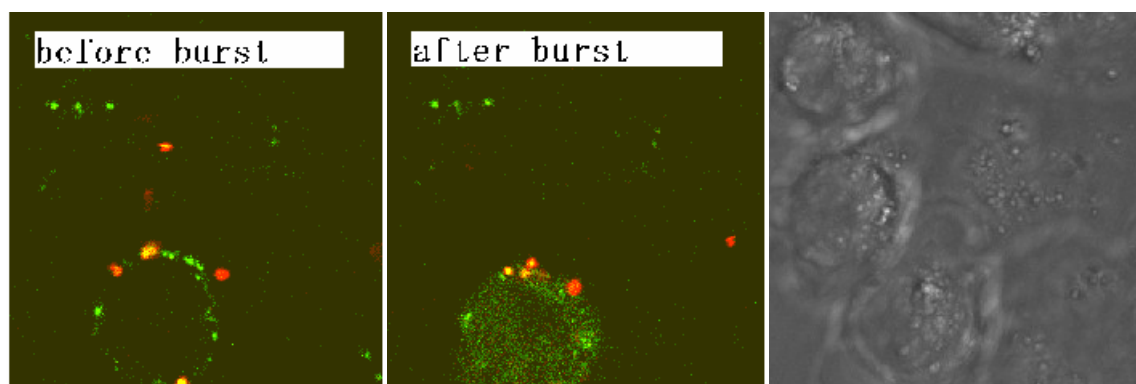
Figure 2b
The same confocal layer of 1 μm thickness before and after lysosomal burst (37 and 40 minutes after incubation). It may be observed that fluorescence intensity increases throughout the whole cell after burst whereas there seems to be an area where it is slightly weaker. This area might display the nucleus. The right image shows a light microscopic image. The complete sectioning of this cell for the 37 and 40 minutes time point can be accessed on our website.

In Fig. 2 and 3 (Movies #1 and #2) it is shown that PEI/ribozyme complexes and also some free PEI, resulting from an N/P ratio of 8, adhere to the cellular membrane within a few minutes after incubation, possibly due to interactions between positively charged complexes and the negative charges of certain membrane constituents. Time of uptake into cells is rather heterogeneous. In several cells uptake is very rapid, occurring approximately 10-15 minutes after incubation [e.g. Fig. 3, Movie #2]. In other cells internalization seems to be much slower, e.g. several cells shown in Fig. 2 [Movie #1]. Under the conditions used in this experiment (i.e. microscope settings), some PEI containing vesicles display a very typical appearance (Fig. 2, Image 1,vesicle marked with yellow arrow; Fig. 3, Images #1-7). The inner core of these vesicles is yellow, indicating that ribozyme and PEI are colocalized here, whereas a very small surrounding ´corona´ is green, which implies that PEI is predominantly located in this area. This ´corona´ might represent interactions between PEI and components of the lysosomal membrane and/or extensive swelling of the polymer matrix in proximity to the vesicular membrane. Separation of PEI and ribozyme close to the membrane might be another possible explanation for this appearance. The size of the vesicle in Fig. 2, Image 1 (marked with the yellow

arrow) seems to increase within the 9 minutes between Images 1 and 3, possibly due to osmotic swelling or fusion with other PEI containing vesicles. Finally it releases its content into the cytoplasm (Fig. 2a, Image 4). After this event the whole cell, including the nuclear compartment, is filled with a faint green-yellow fluorescence, resulting from the mixture of green fluorescence from PEI (predominantly) and the red fluorescence from ribozyme (very faint). This fluorescence can be seen only with sensitive instrument settings, but is significantly brighter than background fluorescence. If distribution of fluorescence in the different confocal layers is regarded before and after endosomal/lysosomal burst one finds a significant increase of fluorescence throughout the whole cell after burst. In Fig. 2b it can be seen that the distribution of fluorescence is not totally homogeneous after burst. These images represent the fluorescence in a single confocal layer of 1 µm thickness before and after burst. The area exhibiting slightly less intense fluorescence may likely display the nuclear compartment.

However, it should be noted that both green and red fluorescence also increase significantly in this area, thus implying that PEI and ribozyme are present in the nucleus a maximum of 3 minutes after burst. The complete sectioning of the whole cell can be accessed on our supplementary material website. The remnant of the burst vesicle is predominantly red and seems to consist of ribozyme and PEI whereas relatively more ribozyme seems to be present than before burst. It persists in the cytosol and only changes its size and color marginally. The further fate of these remnants is unclear and requires a more thorough investigation. In most of the cells only a small percentage of vesicles burst. The majority of the PEI/ribozyme complexes persists in the endosomal/lysosomal compartment for a minimum of several hours or longer [4].
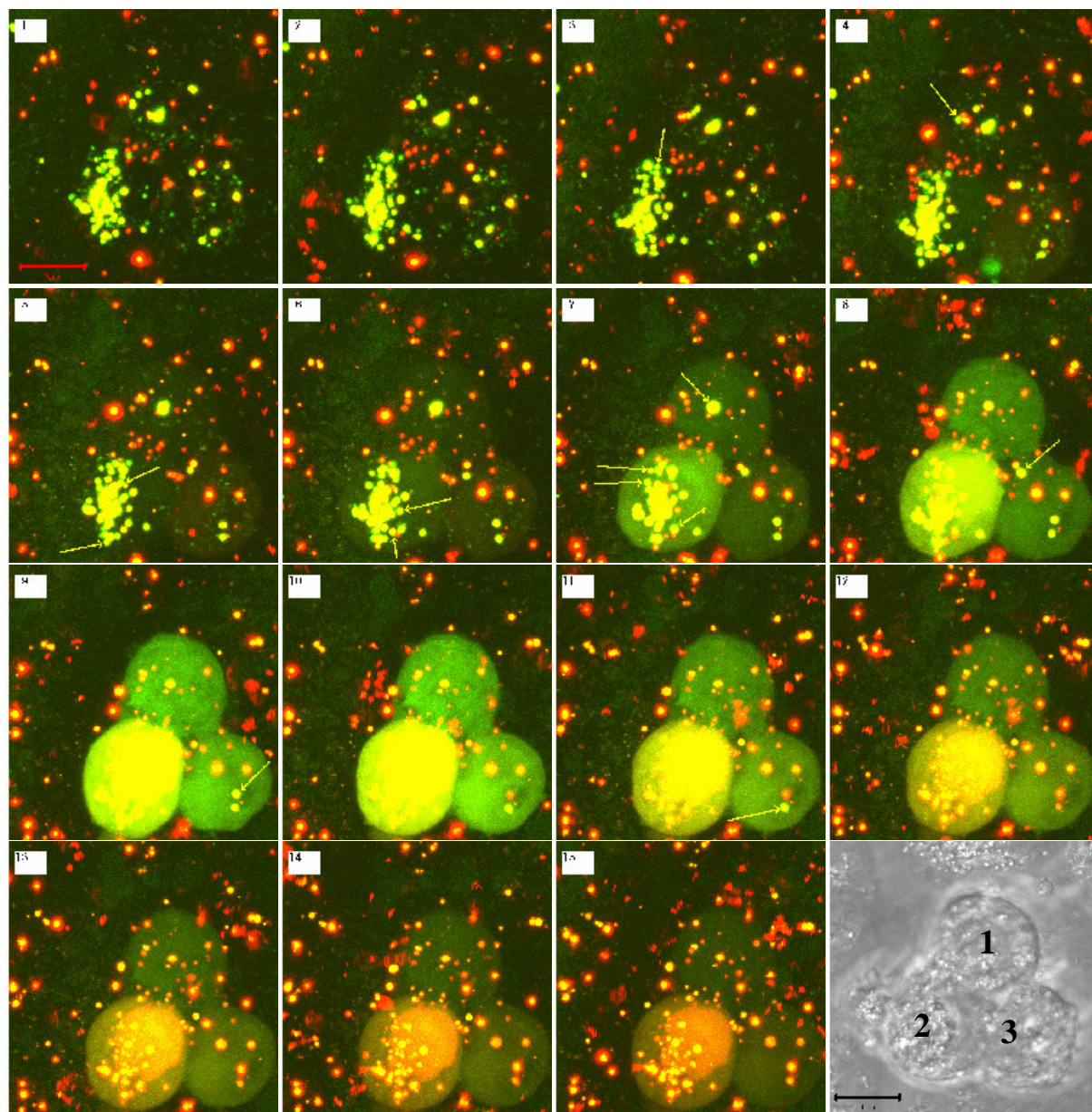
Figure 3

Living cell microscopic images of three cells loaded with enormous amounts of PEI/ribozyme complexes. PEI is green and ribozyme is red in this sequence. Vesicles start bursting between Images 3 and 4. In the next ~ 20 minutes all vesicles release their content. Yellow arrows point out vesicles which have already burst in the next image. For example, Image 8 shows the evenly distributed fluorescence throughout cells # 1 and # 3. In cell 2, obvious interactions between PEI and ribozyme with nuclear constituents occur and in this case a fluorescence remains in the nucleus. Images were recorded every 3 minutes starting 15 minutes after incubation. Images 1-15 are duo fluorescence images derived from the computer aided overlay of different confocal layers for each time point. Image 16 is a light microscopy image to show positions and numbers of the cells. The whole movie can be accessed on our website [Movie #2].

Movie #	Type of Experiment	Excerpts in Figure	Colors
1	PEI/ribozyme complexes	Figure 2	PEI green, ribozyme red
2	PEI/ribozyme complexes	Figure 3	PEI green, ribozyme red
3	PEI/ribozyme complexes with bafilomycin A1		PEI green, ribozyme red
4	Poly(L-lysine)/ribozyme complexes		Poly(L-lysine) green, ribozyme red
5	PEI/ribozyme complexes overview		PEI green

Table 1
Characteristics of movies accessible on our website. Additionally the complete sections of Images 3 and 4 from Figure 2 can be regarded. The website is optimized for Microsoft Internet Explorer and a resolution of 1024 x 768 pixels.

In Fig. 3 (Movie #2) three cells can be seen which contain enormous amounts of PEI/ribozyme complexes. Although under average conditions vesicle burst occurs only once or twice per cell, this example is suitable to demonstrate the mechanism of escape. In this case, a clear sequential increase in fluorescence in the cells after burst of individual vesicles can be readily observed. Vesicles move in a random fashion within the cells and also fuse to create larger vesicles (in Fig. 3 Images 1-4). Between Images 3 and 4 some vesicles seem to burst (arrow in Image 3), thus initiating a disastrous process for these cells. Within the next ~ 20 minutes a chain reaction takes place during which all vesicles in these three cells burst and release their contents into the cytosol (yellow arrows in images of Fig. 3 mark vesicles which have burst in the next image). A possible explanation for this phenomenon could be the destabilizing effect of PEI on the lysosomal membrane not only from the inner compartment, but from the cytosolic side, as well [5]. Once one lysosome releases its content, PEI is present in the cytosol and can destabilize other vesicles that are still intact. The more endosomes/lysosomes that rupture, the more PEI is released and the higher is the destabilizing potential present in the cytosol. This process may progress until all vesicles have burst, as shown in this example. The distribution of fluorescence throughout cells after destabilization of vesicles can be observed very well in this sequence. Interestingly, there seems to be an accumulation of green and red

fluorescence in the nucleus of cell number 2 in Fig. 3. This accumulation might display interactions of the high PEI and ribozyme concentrations present in this cell with DNA in the nucleus.

It is important to note that the time scale for uptake and lysosomal release of the PEI/ribozyme complexes is very heterogeneous, occurring after 20 minutes in some cells, whereas in others the process may take hours for unknown reasons.

Movie #5 on our supplementary material website shows an overview of approximately 20 cells, thus demonstrating that data shown here is representative. In this movie the bursting of several endosomes/lysosomes can be observed as a sudden increase of green fluorescence (PEI) within cells indicated by yellow arrows. Also the sequential increase in fluorescence within one particular cell can be observed.

When the acidification of lysosomes was prevented by using 300 nM bafilomycin A1, no bursting of lysosomes could be observed over a period of 4 hours [data shown in Movie #3 on our supplementary material website]. Cells examined under these conditions take up complexes as efficiently as without bafilomycin A1. The vesicles move within the cells in a disorganized manner, however no escape of PEI or ribozyme can be observed.

Using poly(L-lysine)/ribozyme complexes under similar experimental conditions showed that complexes were taken up into cells efficiently, however, there was no bursting of lysosomes or other visible escape route from this compartment [Movie #4 on our internet pages].

Transfection experiments with luciferase plasmid

Transfection experiments with PEI/luciferase-plasmid complexes at N/P=8 led to a high reporter gene expression [Fig. 4]. In the presence of bafilomycin A1, however, expression was decreased more than 200-fold. This stresses the great importance of endosomal/lysosomal acidification for the release of complexes from this compartment. With poly(L-lysine)/plasmid complexes only a very low

transfection efficiency could be observed, suggesting that these complexes are not as readily able to leave endosomes/lysosomes. These data are in good agreement with our microscopic observations.
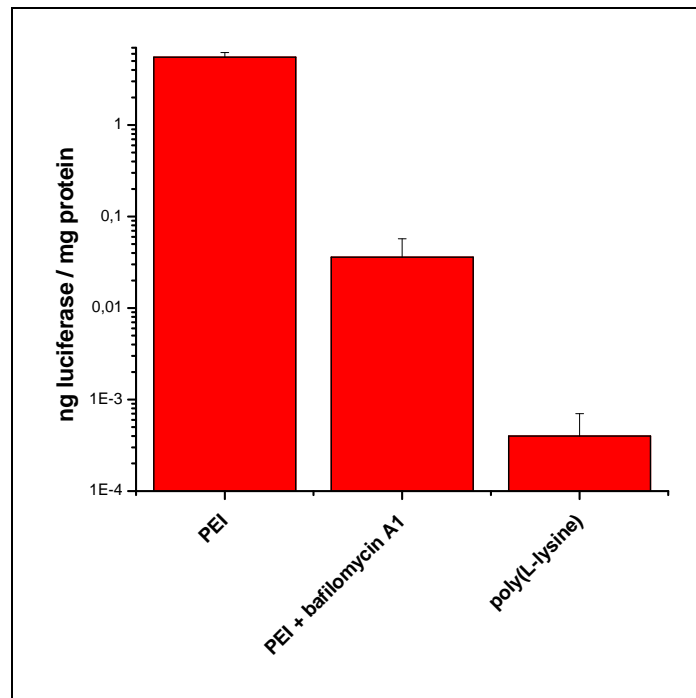

Figure 4
Luciferase reporter gene expression of PEI in the presence of and without bafilomycin A1 and using poly(L-lysine).

DISCUSSION

The subcellular trafficking of PEI/ribozyme complexes could be followed using living cell confocal microscopy. Especially the escape from the endosomal/lysosomal compartment, as well as the distribution of PEI and RNA after this event could be visualized for the first time. We also demonstrated that acidification is equally essential for the release of PEI/RNA polyplexes, as for efficient gene expression after application of PEI/luciferase-plasmid complexes. Complexes of PEI and DNA/RNA reach the lysosomal compartment. This may be concluded from our microscopic data with the acidotropic Lyso Tracker, which is in agreement with the results of subcellular fractionation experiments [4]. In contrast, Godbey et al. [2] found PEI located in non-acidic structures using confocal laser scanning microscopy. A possible explanation for this

discrepancy could be that, in this case, non-intact endosomes or lysosomes were observed. Instead, remnants of lysosomes, such as those in the last images of Figures 2 and 3 could have been observed. These compartments are no longer acidic and, therefore, do not accumulate acidotropic agents, such as Lyso Tracker Blue.

Concerning the escape mechanism of PEI/RNA complexes from endosomes/lysosomes, our results demonstrate that release occurs as a sudden event, such as the bursting or rupture of endosomes or lysosomes. No release of PEI-polyplexes from endosomes/lysosomes was observed, however, when acidification of these vesicles was prevented. In addition, there was a dramatic reduction of luciferase reporter gene expression in the presence of bafilomycin A1, a finding which is consistent with recent results of Kichler et al. [24]. The manifest importance of endosomal/lysosomal acidification suggests that the buffering capacity of PEI is one of the crucial properties of the polymer for its high efficiency in DNA/RNA delivery. There is evidence in the literature that this is one of the key features which cationic polymers require to obtain high transfection efficiencies. Cationic polymers with strong basic groups (e.g. poly(L-lysine) or quaternary amines with poor to no buffering properties possess low transfection efficiencies when used without chloroquine [11, 12, 13] or other lysosome destabilizing agents [14]. In contrast, polymers with a significant buffering capacity in the lysosomal pH range show a much higher transfection capability. For example imidazole containing polymers [16] exhibit a high reporter gene expression, very likely due to the pKa of imidazole (~ 6). Midoux et al. [16] performed transfection experiments in the presence of bafilomycin A1 and, similar to our results, reporter gene expression was reduced more than 100-fold compared to experiments without inhibition of endosomal/lysosomal acidification. Other examples of polymers supporting these findings include polyamidoamine dendrimers [17, 19] or fractured dendrimers [18]. These groups of polymers are quite similar to PEI as they also exhibit a large number of

terminal amino groups on their surface and higher order amines in their interior, whereas not all amino-groups are protonated at physiological pH. Transfection efficiencies with these polymers are significantly higher than those obtained, for example, with poly(L-lysine) and it is likely that efficient endosomal/lysosomal release is at least part of the reason for this. Further examples in literature of effective nucleic acid delivery agents with titratable groups between pH 5-7 include lipospermines [20] and poly[2-(N,N-dimethylamino)ethyl methacrylate] [21] which both exhibit good transfection efficiencies.

These similar findings with structurally unrelated agents suggest that several requirements must be fulfilled for an efficient transfection with cationic polymers. The polymer must be of low toxicity and, because of its three dimensional structure, capable to effectively complex with DNA/RNA at physiological pH. It also needs to possess a certain buffering capacity between pH 5-7. These factors enable effective uptake and a sufficient release from the endosomal/lysosomal compartment, thus leading to the desired gene expression or ribozyme action.

As suggested by Behr [6], the buffering capacity of the polymer could lead to lysosomal swelling based on two possible mechanisms: An increased H^+/Cl^-/water influx and possible swelling of the polymer network as a result of the increasing electrostatic repulsion of charged groups. Both effects eventually lead to rupture of vesicles and release of their contents into the cytosol. In our study it is remarkable that PEI and ribozyme are distributed throughout the cytoplasm and nucleus minutes after endosomal/lysosomal burst, due to the fact that PEI/ribozyme complexes are rather stable. The cause of this could be the extension of the polymer network during acidification or interactions of PEI with the lysosomal membrane or cytoplasm constituents. Both effects could lead to destabilization of the complexes and enhanced dissociation after release from vesicles. Interactions of cationic polymers and negatively charged lipids have been previously demonstrated [22]. Also other pH dependent interactions of

polyplexes with the endosomal/lysosomal membrane could play an important role for efficient escape from vesicles.

A very important question that remains to be answered is: At what stage of the endosomal/lysosomal pathway does bursting of the vesicles occur? Although our data and the findings of Lecocq et al. [4] suggest that the majority of complexes accumulate in lysosomes, release could also occur from late endosomes. If release takes place from lysosomes the simultaneous release of lysosomal content, such as hydrolytic enzymes, might be harmful for cells [25]. This could be one mechanism of toxicity of PEI/nucleic acid complexes and might be a limiting factor for this branch of nonviral nucleic acid delivery.

Another intensively discussed point is the mode of entry of PEI and DNA/RNA into the nucleus. Our results indicate that no cell division is necessary for the entry of PEI and ribozyme, a finding that has also been shown for plasmids by Pollard et al. [7]. The entry of PEI and ribozyme into the nucleus in our experiments could occur via diffusion, as implied by the high speed of the process. This is a reasonable assumption, based upon the fact that ribozyme and PEI have molecular weights of ~ 11 kD and ~ 25 kD, respectively, and therefore could be small enough to diffuse through the nuclear pore complex. The maximum size for this form of nuclear entry has been determined as ~ 50 kD [23]. PEI could promote this process by compacting the ribozyme in such a way as to facilitate nuclear entry. In addition, negatively charged phospholipids might coat these structures, such as suggested by Godbey et al. [3] to allow for an easier nuclear entry. This mechanism, however, is speculative and requires a more detailed investigation. For plasmids, on the other hand, the free diffusion or coating mechanism is unlikely, due to their large size. In this case, a condensation caused by PEI may also play a crucial role, although the nuclear transfer is much more inefficient [7]. Interactions between plasmid or plasmid/PEI and the nuclear pore complex might be considered. Experiments investigating the nuclear entry of plasmids are in progress at this time.

In summary, CSLM in living cells allows visualization of subcellular trafficking of PEI/RNA complexes and sheds some light on the mechanism, as well as kinetics of their endosomal/lysosomal escape. Our data are compatible with the 'proton sponge hypothesis´ [6], however other pH dependent membrane interactions of the polycation may play a significant role for the escape of polyplexes into the cytoplasm as well. CSLM in living cells may be a valuable tool for the design of more efficient non-viral vectors based on polycations in gene delivery.

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3

Pegylated polyethylenimine – Fab' antibody fragment conjugates for targeted gene delivery to human ovarian carcinoma cells

SUMMARY

Specific targeting of ovarian carcinoma cells using pegylated polyethylenimine (PEG-PEI) conjugated to the antigen binding fragment (Fab´) of the OV-TL16 antibody, which is directed to the OA3 surface antigen, was the objective of this study. OA3 is expressed by a majority of human ovarian carcinoma cell lines. To demonstrate the ability of the PEG-PEI-Fab´ to efficiently complex DNA, an ethidium bromide exclusion assay was performed. Comparison with PEG-PEI or PEI 25 kDa showed only minor differences in the ability to condense DNA. Since conjugation of Fab´ to PEG-PEI might influence complex stability, this issue was addressed by incubating the complexes with increasing amounts of heparin. This assay revealed stability similar to that of unmodified PEG-PEI/DNA or PEI 25 kDa/DNA complexes. Complexes displayed a size of approximately 150 nm with a zeta potential close to neutral. The latter property is of particular interest for potential *in vivo* use, since a neutral surface charge reduces nonspecific interactions. Binding studies using flow cytometry and fluorescently labeled DNA revealed a more than 6 fold higher degree of binding of PEG-PEI-Fab´/DNA complexes to epitope-expressing cell lines compared to unmodified PEG-PEI/DNA complexes. In OA3-expressing OVCAR-3 cells, luciferase reporter gene expression was elevated up to 80 fold compared to PEG-PEI and was even higher than that of PEI 25 kDa. The advantage of this system is its specificity, which was demonstrated by competition experiments with free Fab´ in the cell culture media during transfection experiments and by using OA3-negative cells. In the latter case, only a low level of reporter gene expression could be achieved with PEG-PEI-Fab´.

INTRODUCTION

The general feasibility of gene delivery using synthetic polymers has been demonstrated in numerous studies [1-5]. Cationic polymers, such as linear or branched polyethylenimine (PEI) [2,3], starburst dendrimers [4] or imidazole containing polymers [5], were found to be suitable for in vitro applications. Unmodified cationic polymers lead to complexes with DNA displaying a relatively high cationic surface charge. Therefore, unspecific binding, as well as a fairly high toxicity due to electrostatic interactions with the negatively charged components of cellular membranes, e.g. sialic acid, remain a serious limitation for *in vivo* gene delivery [3,6]. After intravenous injection of these polyplexes serious damage of body tissues [7] and a rather high rate of mortality [8] was observed in laboratory animals.

In order to progress from *in vitro* gene delivery towards *in vivo* gene therapy, vectors are needed that exhibit high stability in body fluids, minimal nonspecific interactions, low toxicity and a targeting moiety that provides selective uptake into target cells. Since an excess of cationic polymer is necessary for complexation of DNA, the cationic surface charge needs to be shielded to reduce nonspecific interactions with blood components in non target tissues.

A possible strategy towards achieving an optimized polymeric gene delivery system is schematically shown in Figure 1. Polyplexes prepared with unmodified cationic polymer yield a rather high level of reporter gene expression under *in vitro* cell culture conditions (Figure 1a). Yet, due to the high cationic surface charge of these vectors, this effect is nonspecific. Pegylation [8-10] or attachment of other hydrophilic polymers, such as pHPMA [11], to cationic polymers leads to vectors with a surface charge close to neutrality (Figure 1b). Lower levels of nonspecific interactions have been demonstrated for these systems and some have been used successfully *in vivo*. These studies have shown that such shielded gene delivery systems offer the advantage of lower toxicities and prolonged circulation times *in vivo* [8,10].

However, the resulting complexes with DNA display rather low levels of reporter gene expression. Although this property might be less favorable *in vitro*, it would be valuable for *in vivo* application because it may minimize gene expression in non-target cells. If a targeting ligand, for example Fab´, is conjugated to these polymers, the resulting complexes could then be capable of targeting specific cell types with only low levels of reporter gene expression in non-target cells (Figure 1c).
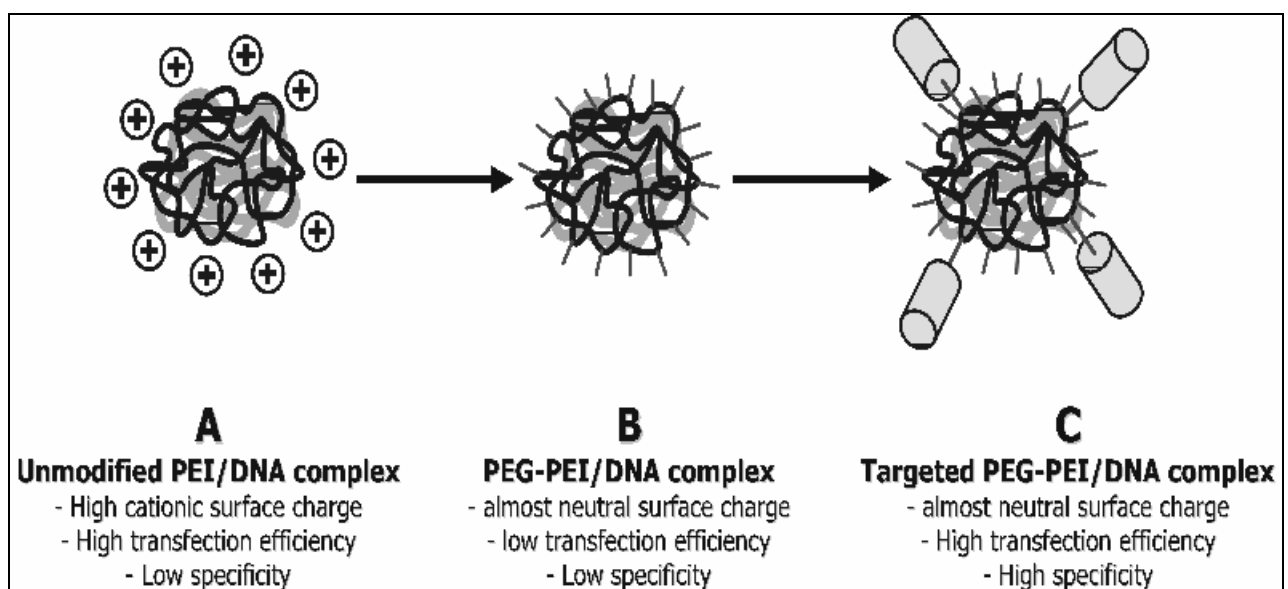

A
Unmodified PEI/DNA complex
- High cationic surface charge
- High transfection efficiency
- Low specificity

B
PEG-PEI/DNA complex
- almost neutral surface charge
- low transfection efficiency
- Low specificity

C
Targeted PEG-PEI/DNA complex
- almost neutral surface charge
- High transfection efficiency
- High specificity

Figure 1
Rationale behind polymer modifications described in this publication. Unmodified complexes (A) displayed high levels of transfection efficiency, due solely to unspecific electrostatic interactions with cellular membranes. Pegylation of PEI (B) led to complexes with an almost neutral zeta-potential, however, reporter gene expression remained at a low level. Yet, when targeting moiety was conjugated to PEG-PEI (C), the resulting PEG-PEI-Fab'complexes showed lower levels of unspecific interactions, combined with a highly specific uptake and transfection of target cells.

A variety of targeting moieties, such as transferrin [12], folate [13,14], RGD-peptides [15,16], different types of saccharides [17,18] antibodies or antibody fragments [19,20] have been described for active gene delivery. Most studies used ligands conjugated directly to unmodified cationic polymers, such as PEI or poly(L-lysine) resulting in polymer/DNA complexes with a high cationic surface charge. Of the various targeting moieties already under investigation, antibodies and their fragments are of particular interest, due to their high

specificity for their target epitopes, as well as the wide variety of possible target structures.

In this publication, we describe the use of a pegylated PEI to generate polymer/DNA complexes with a nearly neutral surface charge. We then synthesized a conjugate consisting of pegylated PEI and an antigen binding antibody fragment (Fab´) that provided specific binding and efficient reporter gene expression coupled with a high specificity for target cells. Fab´ was used instead of the whole monoclonal antibody due to its lower molecular weight and therefore a lower level of steric hindrance during complex formation with DNA. As a model, we used OVCAR-3 human ovarian carcinoma cells that express high levels of the OA3 surface antigen, a structure that is expressed on most human ovarian carcinomas [21,22]. The OV-TL16 antibody specifically binds to the OA3 antigen triggering subsequent internalization [22]. Conjugation of OV-TL16 Fab´ to PEG-PEI produced conjugates that efficiently complexed DNA. These complexes were stable and showed a very high specificity for OVCAR-3 cells as demonstrated by their enhanced binding capabilities and strongly elevated levels of reporter gene expression *in vitro*. Furthermore, only a low level of gene expression occurred in OA3-negative cells.

MATERIALS AND METHODS

Chemicals and plasmid

PEI 25 kDa, hexamethylene diisocyanate (HMDI), PEG monomethylether (2 kDa), salmon testes DNA and N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) were purchased from Sigma-Aldrich, Taufkirchen, Germany. Luciferase plasmid (pCMV-luc) was produced by Plasmid Factory, Bielefeld, Germany.

Antibody and (Fab´)$_2$ production

OV-TL16 IgG directed against the surface antigen OA3 [21] was produced by *in vitro* cartridge bioreactor culture of the OV-TL16 hybridoma cell line (Cellco, Spectrum Labs, Pancho Dominguez, CA). IgG purification and pepsin digestion to produce (Fab')$_2$ antibody fragments were performed as previously described [23].

Cell culture

NIH/3T3 (Swiss mouse embryo) cell line was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). NIH:OVCAR-3 cells were purchased from the American Type Culture Collection (ATCC), Teddington, UK. Cells were cultured according to the protocols suggested by the supplier.

Synthesis of PEG-PEI

PEI(25k)-g-PEG(2k)$_{10}$ was synthesized via a strategy which was described previously [24]. Briefly, PEG monomethylether (2 kDa) was dissolved in anhydrous chloroform (200 g/L) and activated with a 10-fold excess of hexamethylene diisocyanate, HMDI (60 °C, 24 h). Unreacted HMDI was carefully removed by repetitive extraction with light petrol. The reaction of activated PEG with the amino groups of PEI was carried out in anhydrous chloroform at 60 °C for 24 h. The reaction solution was precipitated in diethyl ether and the product was dried *in vacuo*.

Synthesis of PEG-PEI-Fab' conjugate

PEG-PEI (5 mg total polymer corresponding to 2,8 mg PEI) was dissolved in a 1 mL reaction buffer containing 150 mM sodium chloride and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) pH 7.5. SPDP (80 µg) was added to this solution in 80 µL 100% ethanol while stirring and the reaction was allowed to continue for 90 min. SPDP-activated PEI was then purified by

gel filtration on a PD-10 column. The amount of SPDP coupled to PEI was determined by pyridine-2-thione release upon addition of a 20-fold excess of dithiothreitol (DTT) and by measuring the absorption at 343 nm against a standard curve of pyridine-2-thione. PEG-PEI content was measured by a copper complexation assay [25].

In the second step, a 1.4 fold molar excess of freshly reduced Fab' was added in the same buffer. The reaction proceeded for 12 h at room temperature and purification was performed by ion exchange chromatography as described earlier using 0.9 % NaCl, 10 mM HEPES pH 7.4 as buffer A and 3 M NaCl, 10 mM HEPES pH 7.4 as buffer B [12]. The solvent was exchanged with 150 mM NaCl solution pH 7.4 via a further gel filtration step and the PEI concentration was determined by a copper complexation assay [25]. The amount of Fab' per PEG-PEI was determined by UV-absorption at 280 nm with background correction using a solution of PEG-PEI with the same concentration.

Complex formation

Luciferase plasmid (pCMV-luc) and the appropriate amount of polymer were dissolved separately in 0.9 % sodium chloride solution, pH 7. The two solutions were mixed by vigorous pipetting and complexes were allowed to interact for 10 min before use. Complexes were prepared for transfection experiments with either 0.5 or 4 µg plasmid in 45 or 300 µL 0.9 % NaCl and the appropriate amount of polymer or PEG-PEI-Fab´ in 45 or 300 µL 0.9 % sodium chloride solution pH 7. Flow cytometry experiments required 10 µg of plasmid dissolved in 300 µL sodium chloride solution, into which the appropriate amount of polymer in 300 µL 0.9 % NaCl was added. Complexes for the complex stability assay were formed with 1 µg plasmid in 50 µL sodium chloride solution and the appropriate amount of polymer in the same volume.

Ethidium bromide exclusion assay

DNA condensation was measured by the decrease in ethidium bromide fluorescence, as described earlier [26]. The assay was performed in 96 well plates in triplicate. Eight µg of salmon testes DNA was dissolved in 79 µL water and 50 µL of 60 mM Tris buffer pH 7.4 were added to each well. Volumes were equalized to 300 µL with water. Subsequently, appropriate volumes of 0.05 mg/mL polymer solutions were added to produce N/P ratios between 0.2 and 4. These were incubated for 10 min and then 20 µL of a 0.1 mg/mL ethidium bromide solution were added. Wells were mixed thoroughly and the fluorescence was measured using a fluorescence plate reader with excitation wavelength at 518 nm and an emission wavelength of 605 nm.

Photon correlation spectroscopy

Hydrodynamic diameters of the polymer/DNA complexes were determined by photon correlation spectroscopy. Plasmid (0.5 µg pCMV-Luc) in 25 µL 0.9 % NaCl were complexed with the appropriate amount of polymer in 25 µL NaCl each, as described above. Measurements were performed on a Zetasizer 3000 HS from Malvern Instruments, Herrenberg, Germany (10 mW HeNe laser, 633 nm). Scattering light was detected at 90° angle through a 400 µm pin hole. For data analysis, the viscosity (0.88 mPa s) and the refractive index (1.33) of distilled water at 25 °C were used. The instrument was routinely calibrated using Standard Reference latex particles (AZ 55 Electrophoresis Standard Kit, Malvern Instruments). Values given are the mean of 5 measurements.

Measurement of zeta potential

Zeta-potential measurements were carried out in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at position 17.0. Measurements were performed in 0.9 % NaCl and average values were calculated with the data obtained from 5 runs.

Complex stability against heparin

Complexes were formed in septuplet, as described above, in a total volume of 100 µL sodium chloride solution at an N/P ratio of 7. To these solutions, 0.01, 0.03, 0.05, 0.1, 0.2, 0.5 and 1 International Units heparin were added in 10 µL 0.9 % sodium chloride. These solutions were mixed well and incubated for 10 min before they were applied to a 1 % agarose gel containing ethidium bromide. In the eighth lane of each gel, plasmid was applied as a reference. Gels were run for 50 min at 100 V, and then scanned using a Biometra gel analysing system.

Atomic force microscopy (AFM)

The polymer-DNA complexes were prepared as described above and diluted in milliQ water (pH 5.5, 18.2 MΩ). The formulations were directly transferred onto a silicon chip by dipping into the polyplex solution for 10 min. Atomic force microscopy was performed on a Digital Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA), as described elsewhere [27]. The microscope was vibration-damped. Commercial pyramidal Si3N4 tips (NCH-W, Veeco Instruments, Santa Barbara, CA) on a I-type cantilever with a length of 125 µm, a resonance frequency of about 220 kHz and a nominal force constant of 36N/m were used. All measurements were performed in Tapping mode™ to avoid damage to the sample surface. The scan speed was proportional to the scan size. The scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals were simultaneously recorded. The results were visualized in amplitude mode.

Flow cytometry

Flow cytometry was performed with plasmid DNA labeled with the intercalating dye YOYO-1 (Molecular Probes, Leiden, The Netherlands), as described by Ogris et al. [28]. Briefly, cells were grown for 24 h in six-well plates at a

density of 400,000 cells per well. Complexes were prepared as described above and applied in fresh media with a total volume of 3 mL. After 10 min, media was aspirated and cells were washed two times with cold (4 °C) PBS pH 7.3. Cells were suspended in PBS after detachment via trypsin incubation for 1 min. Cell suspensions were kept on ice until analysis. Flow cytometry was performed using a Becton Dickinson FACS Scan equipped with an argon laser with an excitation wavelength of 488 nm. The filter settings for emission was 530/30 nm bandpass.

Transfection experiments

Transfection experiments with pCMV-Luc luciferase plasmid were performed with PEI 25 kDa, PEG-PEI and PEG-PEI-Fab´ at N/P ratios of 2.5, 3.5, 7 and 10 using 0.5 and 4 µg of plasmid. OVCAR-3 or NIH/3T3 (negative control) cells, were seeded in 12 well plates at a density of 50,000 cells per well. After 24 h, the media was removed and the complexes were added in 1.5 mL fresh media containing different amounts of free Fab´ (where applicable). Media was exchanged again after four hours and the cells were incubated for an additional 44 h. Luciferase gene expression was quantified using a commercial kit (Promega) and photon counting on a luminometer (Sirius, Berthold). Results were measured in relative light units per second (RLU/s) which were then converted into ng luciferase by creating a calibration curve with recombinant luciferase (Promega). Protein concentration in each sample was determined using a BCA assay [29]. All experiments were performed in triplicate and data were expressed in ng luciferase per mg protein.

RESULTS AND DISCUSSION

Synthesis of PEG-PEI and PEG-PEI-Fab' conjugates

Polymer synthesis resulted in a pegylated polyethylenimine consisting of 10 PEG chains (2 kDa) grafted to one PEI 25 kDa molecule as determined by ^{1}H-NMR and FT-IR spectroscopy [24]. Previous investigations suggest that this degree of pegylation is suitable for obtaining vectors with a suitable size and neutral surface charge [10].

Using SPDP as a linker for coupling OV-TL16 Fab´ to PEG-PEI led to efficient conjugation. SPDP modification of PEG-PEI produced a polymer containing approximately 0.5 PDP linkers per polymer. In the second step, OV-TL16 Fab´ was coupled to SPDP-modified PEG-PEI. The progress and the end point of the reaction were followed by measuring pyridine-2-thione release using absorption at 343 nm (data not shown). Purification of PEG-PEI-Fab´ conjugate via ion exchange chromatography displayed one peak containing free Fab´ shortly after application to the column in buffer A and one peak containing PEG-PEI-Fab´ after changing to buffer B. UV measurement at 280 nm revealed that approximately every other polymer molecule carried one OV-TL16 Fab´. The overall polymer yield after purification was about 70 %, as determined by the copper complexation assay [25].

Ethidium bromide exclusion assay

Since ethidium bromide shows fluorescence only when intercalated with DNA, the reduction of fluorescence after the addition of polymer to DNA/ethidium bromide complexes can be regarded as a measurement for the efficiency of complex formation of the polymer.

Monitoring complex formation via decrease of ethidium bromide fluorescence revealed efficient DNA complexation using the PEG-PEI-Fab´ conjugate (Figure 2). The shape and position of the curve were only slightly

different when compared to that of PEG-PEI or PEI 25 kDa, suggesting a similar DNA condensing ability.
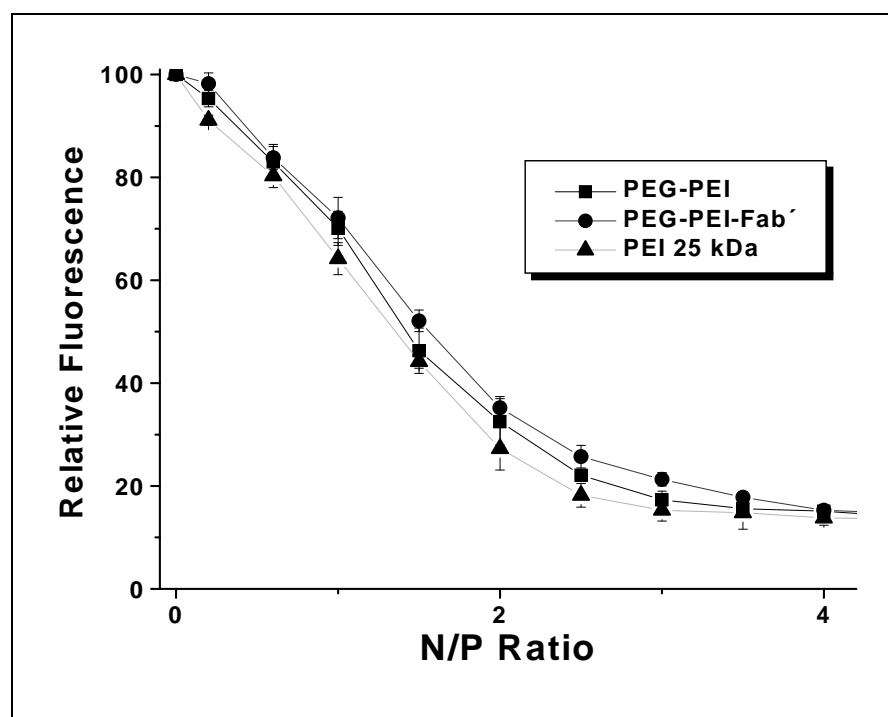

Figure 2
Ethidium bromide exclusion assay using PEI 25 kDa, PEG-PEI and PEG-PEI-Fab'. The ability to complex DNA was only marginally hindered by pegylation and Fab´ conjugation to PEI 25 kDa.

Photon correlation spectroscopy

The sizes of complexes with PEI, PEG-PEI and PEG-PEI-Fab' at different N/P ratios are shown in Figure 3a. Complexes containing PEG displayed a similar size over the N/P ratio of 3.5 to 10. Unmodified PEI 25 kDa/DNA complexes were significantly smaller at higher N/P ratios. It is very important to note that complexes of PEI 25 kDa tend to aggregate especially at low N/P ratios, with sizes increasing from about 300 to 900 nm in the first 10 min after formation (large error bar). However, those containing PEG exhibit an increase of size of less than 20 nm, probably due to efficient shielding of cationic surface charge, hereby abolishing aggregation (data not shown). This effect has been demonstrated for pegylated PEIs in several studies [8,24].

Furthermore, it can be summarized that all complex sizes except the one of PEI 25 kDa at N/P=3.5 are in a range that is suitable for endocytic uptake into cells [30]. Remarkably, sizes of DNA complexes with PEG-PEI-Fab' are only marginally different than those of PEG-PEI/DNA complexes. Obviously, the inclusion of Fab´ only slightly influences complex properties.
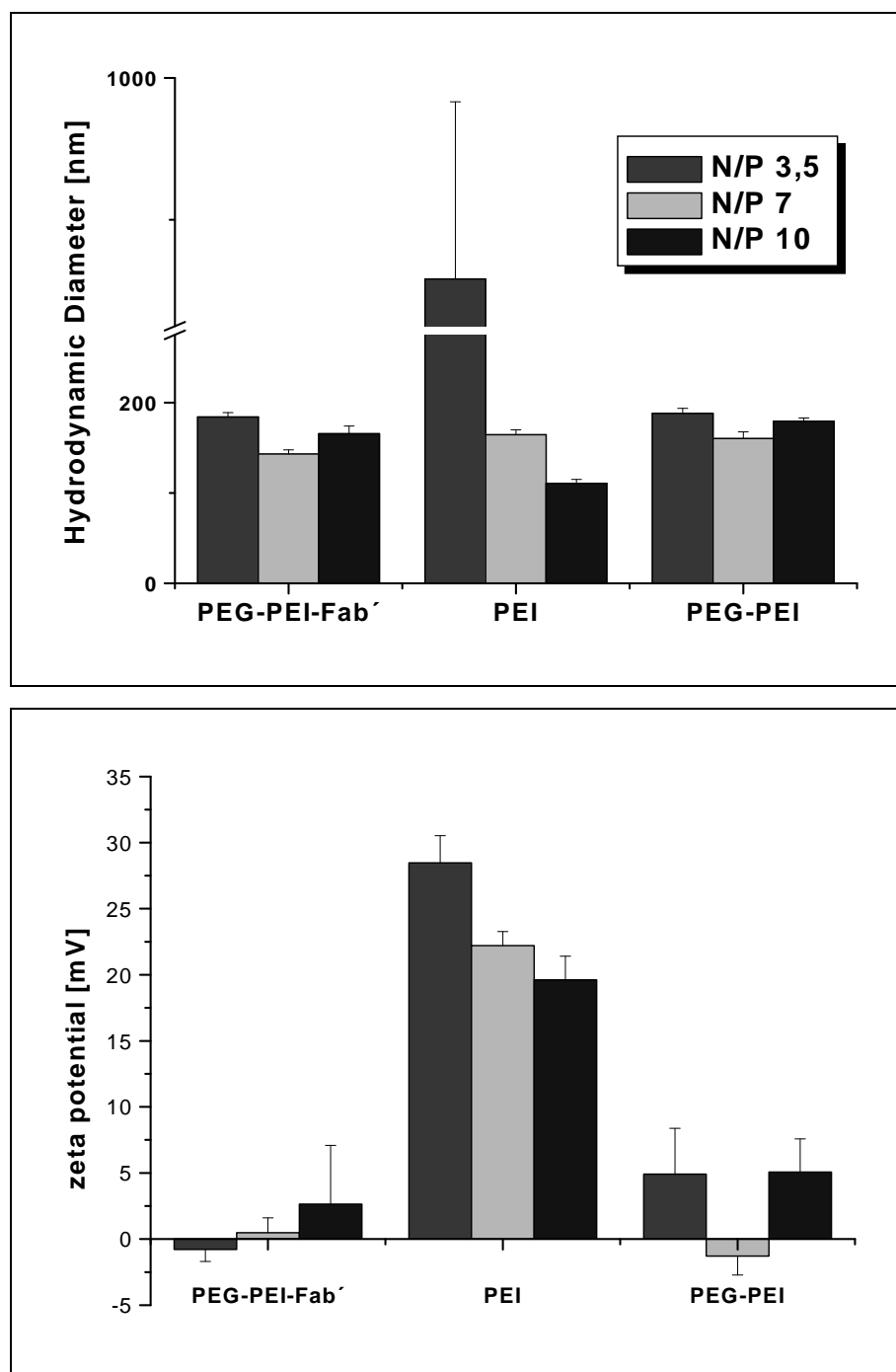

Figure 3
Comparison of hydrodynamic diameters and zeta potentials of complexes at three different N/P ratios

Measurement of zeta potential

Zeta potentials of complexes with PEI, PEG-PEI and PEG-PEI-Fab´ are shown in Figure 3b. Zeta potentials of PEG-PEI/DNA complexes are close to neutral throughout the whole N/P range tested and differences are rather small. This data suggests that the shielding effect of PEG is rather efficient, an observation that is in good agreement with previous studies [9,24,31]. Conjugation of Fab´ to PEG-PEI did not lead to significant changes in surface charge of complexes at N/P ratios from 2.5 to 10. As expected the zeta potentials of complexes with unmodified PEI were rather high. Interestingly, surface charge of these complexes decreased for N/P ratios from 3.5 and 10 although the amount of cationic polymer increased. A possible explanation for this property could be differences in complex shape and size and therefore a different exposure of cationic polymer to the surrounding media.

A neutral zeta potential is of major importance since it has been demonstrated that surface charge plays an important role for the *in vivo* fate of complexes. For example, Plank et al. found a direct relationship between opsonization of complexes via attachment of complement factors and accessible cationic surface charge [32]. Furthermore, interactions of cationic polyplexes with albumin may result in the formation of large aggregates with a reversed surface charge leading to a rapid clearance by the reticulo-endothelial system [33]. For complexes with a neutral surface charge reduced interactions with plasma proteins, vessel endothelia or cellular blood components have been demonstrated [8,11].

Complex stability against heparin.

To evaluate complex stability, we compared levels of DNA release after incubation of PEI 25 kDa/DNA, PEG-PEI/DNA and PEG-PEI-Fab'/DNA complexes with increasing amounts of heparin (Figure 4). Heparin, a polyanion capable of displacing DNA from polycation/DNA complexes, was chosen as a model substance for this assay. The lowest concentration of heparin where

displacement occurs provides an estimation of the complex stability against polyanions. In this case, no striking differences could be detected when PEG-PEI-Fab'/DNA complexes were compared to native PEG-PEI/DNA or PEI 25 kDa/DNA complexes. In all three gels shown in Figure 4, DNA release started at the same concentration, suggesting no major influence of Fab´ conjugation on complex stability. This data is in good agreement with the DNA complexing profiles obtained in the ethidium bromide exclusion assay.
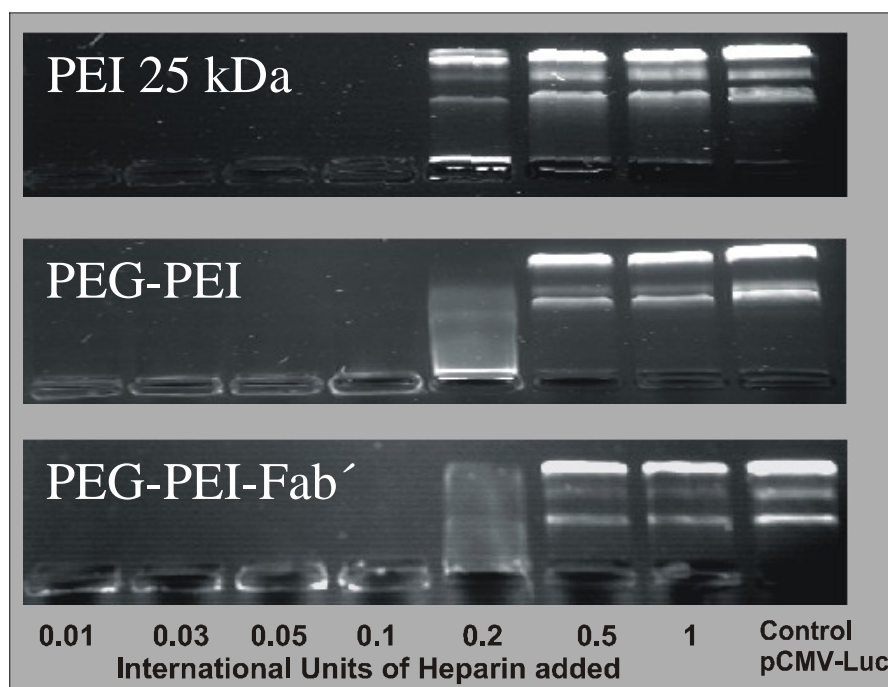

Figure 4
Determination of complex stability using increasing amounts of heparin. In the first seven lanes of each gel increasing amounts of heparin were added in order to determine the threshold where DNA release occurs. This concentration was similar in all gels

Atomic force microscopy

Images of complexes recorded under the conditions described above are shown in Figure 5. The polymers and PEG-PEI-Fab´ have been shown to form defined, toroidal complexes with plasmid DNA. The shape and size of complexes prepared with PEI, PEG-PEI and PEG-PEI-Fab´ were only slightly different. Size measurements from the AFM images were in good agreement with those obtained by photon correlation spectroscopy. The data indicated complex diameters around 140 nm.
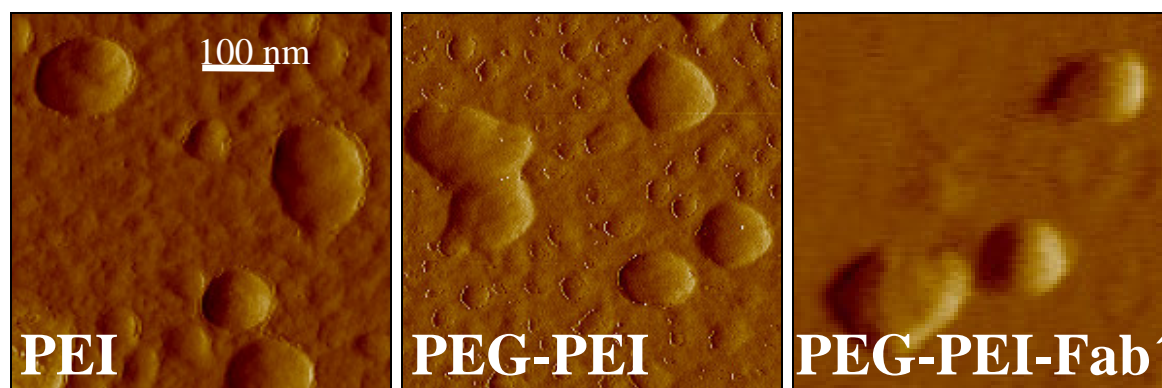
Figure 5
Atomic force microscopy images from PEI 25 kDa/plasmid, PEG-PEI/plasmid and PEG-PEI-Fab'/plasmid complexes

Flow cytometry

Flow cytometry data revealed a moderate degree of cell binding of the unmodified PEI 25 kDa/DNA complexes (Figure 6). The binding efficiency of complexes prepared with PEG-PEI is only slightly less for both cell types investigated in this study. This is somewhat surprising since surface charges of PEI/DNA complexes are about 20 mV higher than that prepared with PEG-PEI. Obviously charge is only one factor that influences cell binding. As expected, PEG-PEI-Fab´/DNA complexes show an about 6-fold higher degree of binding to OVCAR-3 cells compared to those prepared with PEI 25 kDa and PEG-PEI. However, using OA3-negative NIH/3T3 cells, PEG-PEI-Fab´/DNA complexes displayed least efficient cell binding of all three polymers. The data strongly suggests a specific binding of PEG-PEI-Fab´/DNA complexes to OA3 antigen expressing cell lines. This property is of great importance for the transfection of particular cell types *in vivo*.
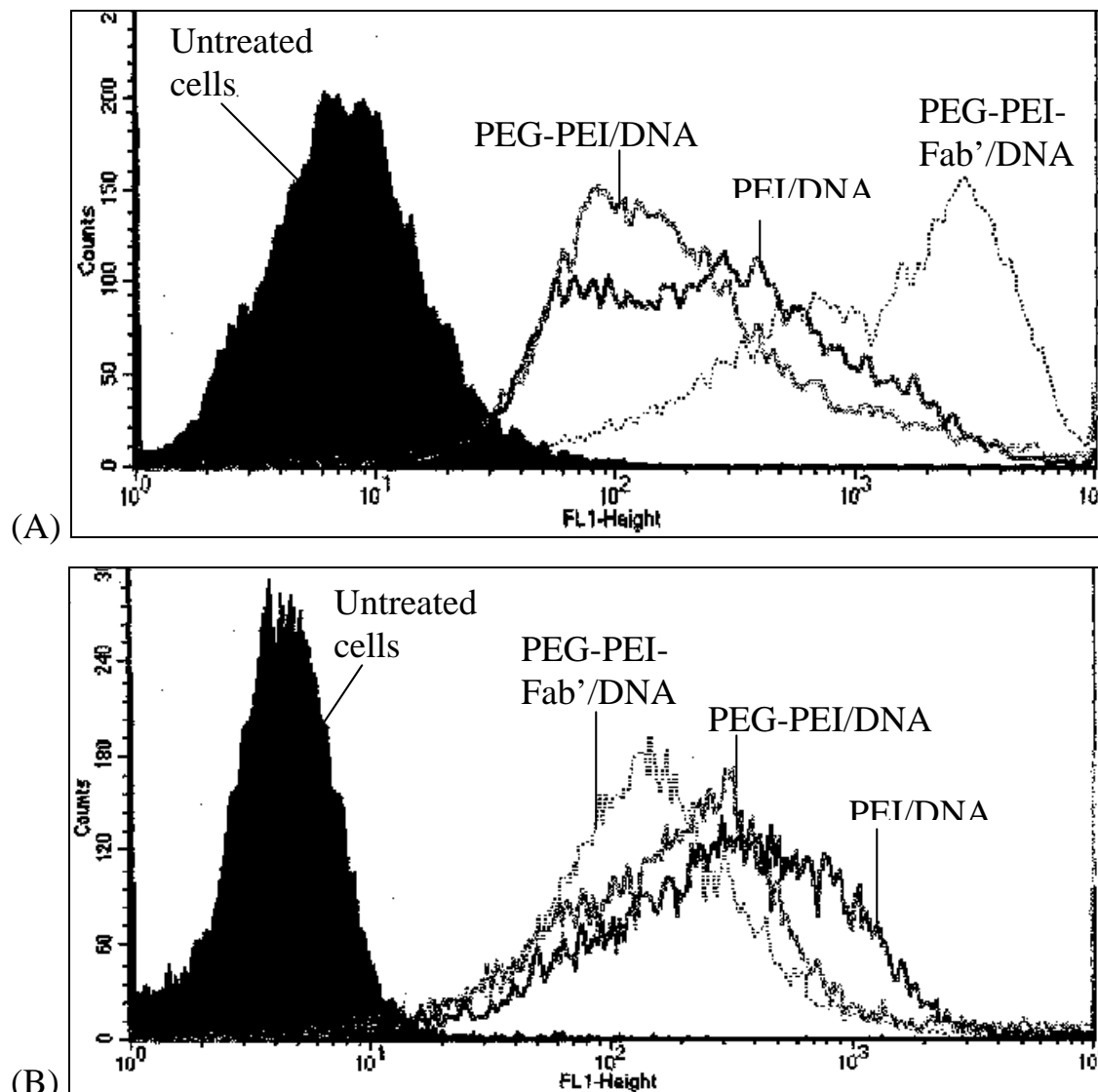
Figure 6
Investigation of cell binding using flow cytometry. In (A), a strongly enhanced binding of PEG-PEI-Fab'/DNA complexes to OA3-expressing OVCAR-3 cells can be observed when compared to PEG-PEI/DNA and PEI/DNA complexes at an N/P ratio 3.5. (B) shows binding to NIH/3T3 cells, which do not express OA3 antigen. PEI/DNA display the strongest binding compared to PEG-PEI/DNA and PEG-PEI-Fab'/DNA complexes. The latter exhibit the least efficient binding to this non OA3-expressing cell line at an N/P ratio of 3.5

Transfection experiments

Data from transfection experiments using luciferase as a reporter gene are shown in Figure 7. When experiments were performed using OA3 antigen expressing OVCAR-3 cells PEG-PEI-Fab´/plasmid complexes yielded fairly high levels of reporter gene expression between N/P 2.5 and 10 using either 0.5 or 4 µg plasmid per well (Figure 7a and b). Expression levels were 10 to 80-

fold higher than those of complexes prepared with PEG-PEI or unmodified PEI. In OA3-negative NIH/3T3 cells, however, reporter gene expression remained at a rather low level (Figure 7c), probably due to a lower level of cellular association as determined by flow cytometry.
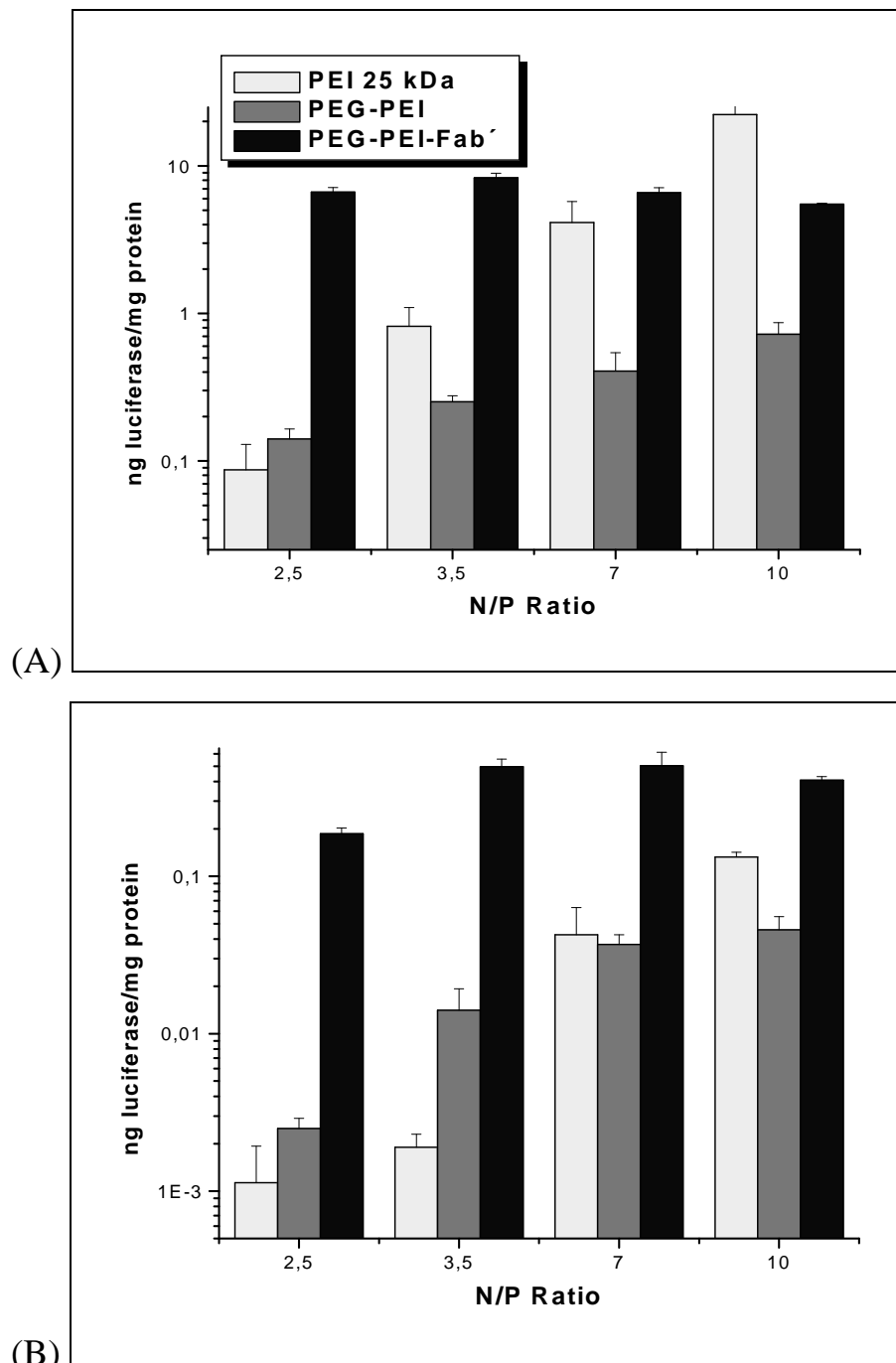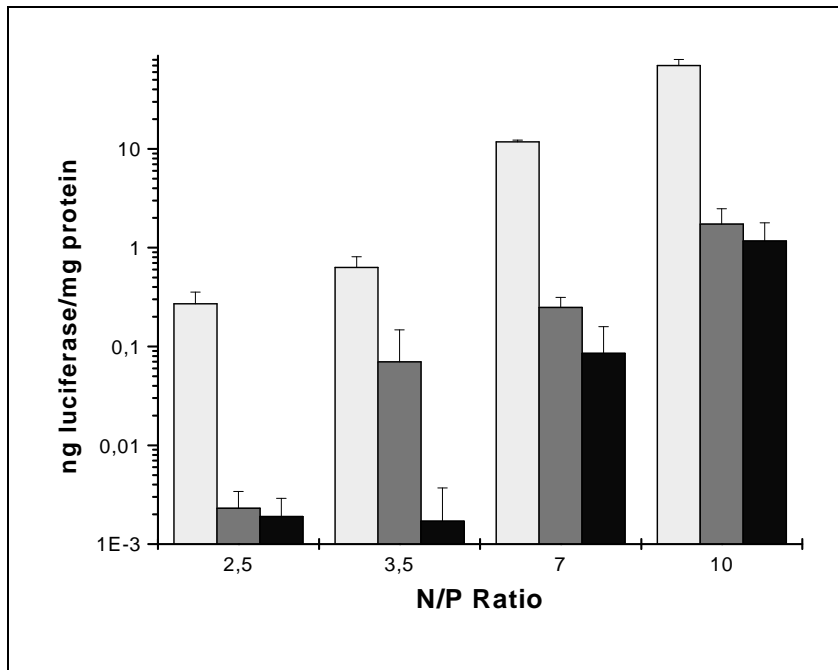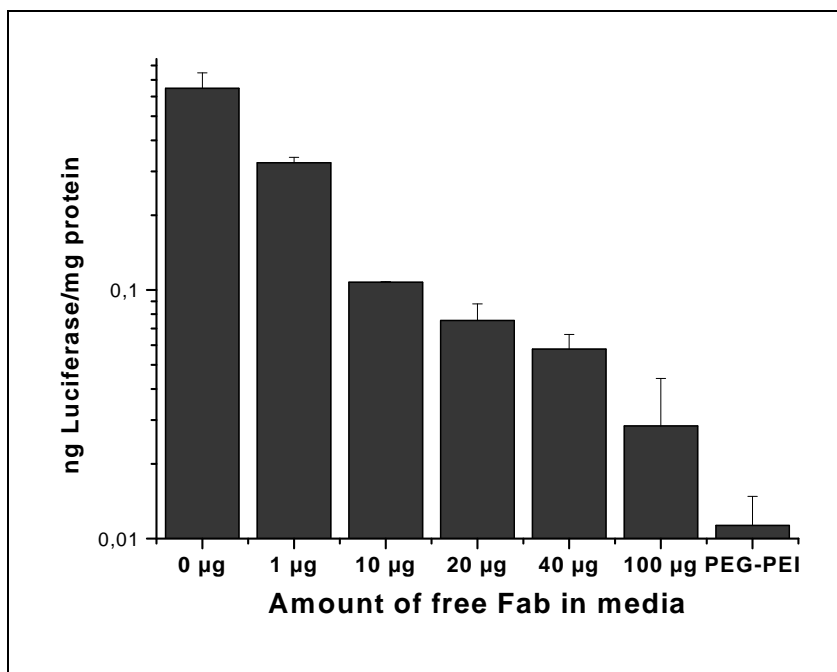

(A)

(B)

Figure 7 (A) and (B)
Levels of reporter gene expression in OA3-positive OVCAR-3 cells using either 4 μg (A) or 0.5 μg (B) of luciferase plasmid per well are shown. Strongly enhanced levels of reporter gene expression can be observed compared to PEG-PEI which is especially distinct at low N/P ratios.

(C)

(D)

Figure 7 (C) and (D)
In (C) luciferase expression in OA3-negative NIH/3T3 cells using 4 µg pCMV-Luc per well is depicted. Here PEG-PEI-Fab´/plasmid complexes led to only low levels of reporter gene expression, stressing the high specificity of this system. (D) Shows levels of reporter gene expression in OVCAR-3 cells in the presence of different amounts of free Fab´ in the incubation media. The higher the Fab' concentration, the lower the gene expression.

In both cell lines, PEG-PEI/DNA complexes showed rather low levels of luciferase expression and only a moderate increase from N/P 2.5 to 10, probably

due to the low surface charge of complexes and, therefore, a reduced uptake. Unmodified PEI, however, displayed rather high levels of reporter gene expression at all N/P ratios with an increase of more than two orders of magnitude. This effect cannot be attributed to the zeta potential of complexes since it decreases with increasing N/P ratio. Increase in reporter gene expression occurs probably due to a more efficient complexation of DNA and a reduced size of complexes. This may lead to a more efficient endocytic cellular uptake.

Competition experiments with increasing amounts of Fab´ in the cell culture media showed a constant decrease in reporter gene expression in the case of PEG-PEI-Fab´/DNA complexes (Figure 7d). This together with the low transfection efficiency in OA3-negative cell lines (Figure 7c) demonstrated a high degree of specificity for this gene delivery system.

Cytotoxicity of either PEG-PEI/DNA or PEG-PEI-Fab´/DNA complexes remained on a low level. At N/P=10 using 4 µg of DNA per well, protein content was as high as that of an untreated control containing the same amount of cells. The reason for this reduced *in vitro* toxicity is probably a reduced surface charge and hereby a reduced membrane disruptive potential of complexes as described earlier in the literature [24,34]. Unmodified PEI, however, caused a rather high cytotoxicity, leading to a protein content of as low as ~ 50 % at this N/P ratio.

In summary it can be stated that complexes formed with plasmid DNA and PEG-PEI-Fab´ display only minor differences concerning physicochemical complex properties compared to PEG-PEI/DNA and PEI/DNA complexes. A possible disadvantage of the method we described here may be a reduced ability of PEG-PEI-Fab´ or PEG-PEI to complex DNA, or a reduced complex stability due to steric hindrance by PEG chains or the targeting moiety. However, for our system, we have been able to demonstrate that neither the grafting of ten 2 kDa PEG chains, nor the conjugation of Fab´ leads to significant changes in complex

properties. An explanation could be that in the complex cationic PEI moiety is oriented towards the core where it efficiently interacts with DNA, leading to similar curves in the ethidium bromide exclusion assay. Hydrophilic PEG-chains, as well as Fab´, most likely form a shell around this core, which accounts for the efficient shielding properties and proper presentation of the targeting ligand. Consequently, complexes show a neutral zeta potential and a high level of reporter gene expression on cells expressing the OA3 antigen. The attachment of the targeting ligand to the end of a PEG-spacer like described by Woodle et al. for RGD-peptides [35] and Blessing et al. for EGF (epidermal growth factor) [36] could lead to further improvements of vector efficiency. This work is currently in progress.

Interestingly, the differences of reporter gene expression obtained with PEG-PEI-Fab´/DNA complexes were minor in the N/P range from 2.5 to 10. This strongly suggests an internalization or cell binding mechanism that is not related to charge. Flow cytometry data suggest that the increase in reporter gene expression with PEG-PEI-Fab´/DNA complexes when compared to PEG-PEI/DNA complexes is at least partially due to enhanced binding to OA3-positive cells. These results are in good agreement with previous studies where a variety of PEG-PEI copolymers and PEIs containing a targeting moiety have been investigated [16,24]. Advantages of the strategy described here are the possibility to produce conjugates in larger quantities and an easy one-step complex formation. Several steps are required in other strategies [8].

Due to their high specificity and low toxicity targeted vectors with PEG-shielding are attractive constructs for *in vivo* use. Three general approaches have been described in the literature. The first approach is the one we described here using conjugates of block polymers and a targeting moiety. In the second strategy pegylation *and* conjugation of a targeting moiety were performed after complex formation with unmodified PEI [36]. This method was developed using EGF as a targeting moiety. Complexes prepared via this strategy showed

significantly enhanced levels of reporter gene expression in target cells and almost neutral surface charges. The major disadvantage of this method compared to our procedure is that the reaction products or the reaction solvent (e.g. DMSO) may remain in the complex solution and, therefore, influence their properties. In addition, the means of characterization the true composition of the resulting complexes with regard to the degree of pegylation, for example, are limited. Furthermore, the preparation of targeted vectors via this strategy is much more complicated than in the case of simple electrostatic vectors since several sequential steps are required.

The third approach used to obtain a targeted and pegylated vector starts with the conjugation of a targeting moiety followed by complexation with DNA. Pegylation is carried out as the last step. This strategy has been performed using EGF [36] and transferrin [8], and showed rather high levels of reporter gene expression in target cells. The advantage of this approach is the possibility of purification and characterization of the intermediate PEI conjugate and targeting moiety. The disadvantage is the difficulty in characterizing the PEG-content of the complex, the high operating expense of the pegylation process and the possibility that reaction products may remain in the complex solution.

In conclusion, the strategy described in this publication represents a straightforward and efficient method to achieve active targeting using non-viral gene delivery systems with a high specificity and a low cytotoxicity. These investigations suggest that the PEG-PEI-Fab´ conjugates might be promising candidates for *in vivo* applications.

ACKNOWLEDGEMENTS

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4

Comparison of *in vitro* and *in vivo* properties of electrostatic complexes prepared with either polyethylenimine or pegylated polyethylenimine and plasmid DNA

SUMMARY

Differences between polyethylenimine (PEI)/plasmid and pegylated polyethylenimine (PEG-PEI)/plasmid complexes were investigated especially with regard to *in vivo* application. Size and zetapotential of complexes were measured and complex structures were visualized by atomic force microscopy. Furthermore, the stability of complexes against anionic exchange reactions was investigated, as well as the protection of complexed DNA against digestion by DNase I. To obtain an insight into the stability of complexes in blood we performed *in vitro* transfection experiments with polymer/DNA complexes in blood and investigated plasmid integrity after incubation with serum. Finally, to characterize the *in vivo* stability of complexes the fate of both types of complexes was investigated *in vivo* in mice after intravenous injection with radioactive labels attached to the polymer and incorporated into the DNA.

Our data indicate that both types of complexes are spherical in shape with a size of approximately 100-150 nm. PEI/DNA complexes display a highly positive zeta potential, while PEG-PEI/DNA complexes possess a nearly neutral surface charge. Complex stability against polyanions does not show striking differences. A significant discrepancy was found with respect to the ability of polymers to protect against DNase digestion. While PEI offers effective protection against degradation, this effect is less pronounced for PEG-PEI. Transfection experiments in blood revealed that both types of complexes were at least partly stable in blood. *In vivo* investigations showed similar pharmacokinetic profiles and similar levels of organ accumulation for both the polymer and DNA from PEI/plasmid complexes indicating a considerable *in vivo* stability of these structures. PEG-PEI/DNA complexes, in contrast, showed obvious differences between polymer and DNA profiles. Kinetic curves and organ accumulation of DNA applied as PEG-PEI/DNA complexes were similar to those obtained with naked DNA. This data provides evidence for a rapid complex separation after injection with subsequent DNA degradation by serum nucleases. Our data

indicate that simple electrostatic vectors prepared with pegylated PEI and DNA are, despite favorable *in vitro* properties, not suitable for systemic gene delivery, at least under the conditions used in this study.

INTRODUCTION

Gene delivery has proven its efficiency in numerous *in vitro* studies [1]. The use of genes as therapeutic agents offers a tremendous potential for the causal treatment of yet incurable diseases, especially viral infections or cancer [2]. However, if this technique is to be applied systemically for the treatment of, e.g. malignant metastases, it is necessary to develop a vector system with sufficient stability and circulation time in the bloodstream in order to reach remote sites of the body. Cationic polymers, such as linear PEI [3,4] or methacrylates [5], have been frequently used for *in vivo* transfection experiments with primarily luciferase as a reporter gene. Some pharmacokinetic and organ distribution data has been obtained for different vectors [6-8]. In order to extend the circulation time of these vectors, hydrophilic polymers, such as PEG or pHPMA have been incorporated into the vector structure [9-11] with the intent to obtain a stealth-like effect. Similar to that described for liposomes [12]. Several of these PEG-modified cationic polymers display promising *in vitro* properties, such as a neutral zeta potential, very low toxicity and little to no aggregation [9,11,13]. Remarkable efforts have gone into the investigation of the *in vitro* stability of polyplexes [11,14], however, it is still unclear which factors are crucial for the *in vivo* stability of these systems. Several promising *in vivo* results have been obtained with local application of PEG-modified polymer/DNA complexes [15,16]. In a recent study, for example, it was demonstrated that the repeated administration of PEG-PEI/DNA complexes led to a prolonged transgene expression in the spinal cord as compared to unmodified PEI/DNA complexes [17]. However, little is known about the stability of simple electrostatic complexes in the bloodstream after intravenous injection and how the

incorporation of hydrophilic polymers, such as PEG, influences this stability. A study comparing poly(L-Lysine) and pegylated poly(L-Lysine)/DNA complexes revealed less efficient DNA protection *in vivo*, when using the pegylated polymer [7]. Further studies have investigated the pharmakokinetics and organ distribution of either DNA *or* polymer after intravenous injection [9,11], however, it still remains unclear if and to what extent complexes stay together in the bloodstream.

The adequate dose for *in vivo* gene delivery is controversial. Some *in vivo* studies have used enormous amounts of DNA with up to 100 μg of plasmid per mouse [18]. However, the high levels of gene expression in lung and liver in such studies were also accompanied by a substantial *in vivo* toxicity leading to a high mortality among laboratory animals, due to lung embolism [19], and serious tissue damage [20]. If highly efficient plasmids encoding, e.g. suicide genes [21], are used for *in vivo* gene therapy, unspecific gene expression in the lung or liver is highly undesirable.

In the present study complexes were characterized with regard to size, zeta potential and complex shape. *In vitro* stability of complexes was assessed by incubation with increasing amounts of anions, such as heparin and dextran sulfate. Furthermore we investigated complex stability by performing transfection experiments in blood and analyzed plasmid integrity by extracting DNA after incubation with serum. In order to assess the *in vivo* stability of PEI/plasmid and PEG-PEI/plasmid complexes after intravenous injection, we investigated the fate of polymer and DNA using two different radioactive labels. In these experiments, a dose of 2 μg plasmid per mouse were chosen to avoid tissue damage, lung embolism or other toxic side effects.

MATERIALS AND METHODS

Materials and animals

Luciferase plasmid (pCMV-luc) was purchased from Plasmid Factory, Bielefeld, Germany. PEI 25 kDa, PEG monomethylether (2 kDa) and hexamethylene diisocyanate (HMDI) were purchased from Sigma-Aldrich, Taufkirchen, Germany. Male balb/c mice as well as citrate mouse blood were purchased from Charles River, Sulzfeld, Germany.

Polymer synthesis

PEI(25k)-g-PEG(2k)$_{10}$ was synthesized as previously described [22]. Briefly, PEG monomethylether (2 kDa) was dissolved in anhydrous chloroform and activated with hexamethylene diisocyanate (HMDI). Unreacted HMDI was carefully removed by extraction with light petrol. The reaction of activated PEG with the amino groups of PEI was carried out in anhydrous chloroform. The reaction solution was precipitated in diethyl ether and the product was dried *in vacuo*.

Preparation of complexes

Polymer solutions for complex formation were prepared in 79 μl of 150 mM NaCl with 10 mM Hepes buffer pH 7.4 and 36 μl glucose 5 % pH 7.4. These mixtures were added to solutions of plasmid in 115 μl glucose 5 % pH 7.4. Complexes were prepared at an N/P ratio of 6.

Photon correlation spectroscopy

Hydrodynamic diameters of the polymer/DNA complexes were determined by photon correlation spectroscopy. Plasmid (0.5 μg pCMV-Luc) in 25 μL glucose 5 % were complexed with the appropriate amount of polymer in 25 μL glucose 5 % each, as described above. Measurements were performed on a Zetasizer 3000 HS from Malvern Instruments, Herrenberg, Germany (10 mW HeNe laser, 633 nm). Scattering light was detected at 90° angle through a 400 μm pin hole.

For data analysis, the viscosity (0.88 mPa s) and the refractive index (1.33) of distilled water at 25 °C were used. The instrument was routinely calibrated using Standard Reference latex particles (AZ 55 Electrophoresis Standard Kit, Malvern Instruments). Values given are the mean of 5 measurements.

Measurement of zeta potential

Zeta-potential measurements were carried out in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at position 17.0. Measurements were performed in glucose 5 % and average values were calculated with the data obtained from 5 runs.

Complex stability against exchange reactions with albumin and anions

This assay was performed using a procedure described earlier [23] using a more narrow range of concentrations. Briefly: Complexes were prepared from 1 µg of pDNA and the corresponding amount of polymer in a total volume of 50 µl glucose 5 %. 0.075, 0.1, 0.125, 0.15, 0,175, 0.2 and 0.225 international units of heparin were added to these complex solutions in 10 µl glucose 5 %. The solutions were mixed well and incubated for 10 min before application to a 1 % agarose gel containing ethidium bromide. In the eighth lane of each gel, plasmid was applied as a reference. Gels were run for 50 min at 100 V and then scanned using a Biometra gel analyzing system.

Studies with dextran sulfate were performed in the same manner. Increasing amounts of dextran sulfate (0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10 µg) were incubated with the complex solutions in 10 µl glucose 5 %.

Stability against DNase digestion

Complex stability was investigated according to a method described earlier [24]. Briefly, complexes were prepared at N/P=6 in glucose 5 % using 5 µg of pCMV-luc in a total volume of 25 µl. Aliquots of 5 µl corresponding to 1 µg of plasmid were incubated with 0.0001, 0.01, 0.1, 1 and 5 international units (I.U.)

of DNase I in digestion buffer (0.1 M sodium acetate, 5 mM $MgSO_4$ pH 7.4) for 15 minutes at 37 °C. Subsequently, 6 µl termination buffer (equal volumes of 0.5 M EDTA, 2 M NaOH and 0.5 M NaCl) were added, as well as 2 µl of a heparin solution containing 1000 I.U. per ml. A positive control reaction containing naked DNA was carried out under the same conditions using 5 I.U. DNase I. Resulting mixtures were applied to a 1 % agarose gel and electrophoresed at 100 V for 1 hour. The resulting gel was visualized and photographed on a BioRad transilluminator.

Atomic force microscopy

The polymer-DNA complexes were prepared as described above and diluted in milliQ water (pH 5.5, 18.2 MΩ). The formulations were directly transferred onto a silicon chip by dipping the chip into the polyplex solution for 10 min. Atomic force microscopy was performed on a Digital Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA) as described elsewhere [25]. The microscope was vibration-damped. Commercial pyramidal Si3N4 tips (NCH-W, Veeco Instruments, Santa Barbara, CA) on a I-type cantilever with a length of 125 µm, a resonance frequency of about 220 kHz and a nominal force constant of 36N/m were used. All measurements were performed in Tapping mode™ to avoid damage to the sample surface. The scan speed was proportional to the scan size. The scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals were simultaneously recorded. The results were visualized in amplitude mode.

Transfection in blood

NIH/3T3 cells were seeded in 12 well plates at a density of 150,000 cells per well and grown for 48 hours. Complexes were prepared as described above using 4 µg of DNA in a total volume of 100 µl sodium chloride 150 mM and added either to 1000 µl mouse blood or 1000 µl sodium chloride 150 mM, pH 7.

These mixtures were shaken for 1 hour at 37 °C. After that media was aspirated, complex/blood, as well as complex/sodium chloride solutions, were added directly to cells and plates were shaken carefully. After 15 minutes, mixtures were removed and cells were washed 5 times very carefully to remove all cellular blood components. Cells were incubated for a further 24 hours and luciferase expression, as well as protein content were determined as described earlier [26].

Stability of DNA in serum

Complexes were prepared using PEI and PEG-PEI in glucose 5 % at a N/P ratio of 6 in a total volume of 200 µl and a plasmid content of 10 µg. Complexes and a control sample containing 10 µg of pCMV-Luc in 200 µl glucose 5 % were incubated with 800 µl human serum for 30 minutes at 37 °C. Subsequently, pDNA was extracted from samples. This was carried out using a commercial kit (DNeasy, Qiagen, Hilden, Germany), whereas, as an additional step, pDNA was released from polymers using 60 µl of heparin solution (1000 I.U./ml) after inactivation of DNases. The volume of eluted DNA was reduced to 20 µl using Microcon 10 ultracentrifugation units (Amicon, Bedford, USA) and applied to a 1 % agarose gel. Electrophoresis was carried out at 100 V for 50 minutes and the resulting gel was visualized and photographed on a BioRad transilluminator.

Radioactive labeling of polymers

Polymers were labeled employing N-succinimidyl-3-(4-hydroxy-3-[^{125}I]iodo-phenyl)propionate (Amersham Pharmacia Biotech, Freiburg, Germany) according to the method of Bolton and Hunter as described earlier [9,27]. Briefly: Polymers were dissolved in 0.1 M borate buffer pH 8.5 and the Bolton Hunter reagent was dissolved in DMSO. The polymer solution was added to the Bolton Hunter reagent solution and the reaction was carried out for 60 minutes at room temperature. Purification was performed on a Sephadex G-25 column

(PD10, Pharmacia), using an elution buffer containing 150 mM NaCl and 10 mM HEPES pH 7.4.

Radioactive labeling of DNA

Plasmid (pCMV-Luc) was radioactively labeled by incorporation of ^{32}P-dCTP (Readivue, Amersham Pharmacia, Freiburg Germany) using a Nicktranslation Kit (Amersham Pharmacia, Freiburg, Germany) following a protocol provided by the manufacturer. Unincorporated nucleotides were carefully removed using Autoseq spin columns containing Sephadex G50 (Amersham Pharmacia, Freiburg, Germany) in two subsequent steps. Plasmid purity was verified via size exclusion chromatography using a PD-10 column and via ultracentrifugation using Microcon 10 spin columns (Amicon, Beverly, USA). No significant amounts of free ^{32}P-dCTP were detected.

Pharmacokinetic analysis and organ distribution

All animal experiments followed the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and were approved by an external review committee for laboratory animal care. Male balb/c mice with a body weight of 20-25 g were anaesthetized using Ketamine (Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and Xylazine (Rompun, Bayer AG, Leverkusen, Germany). Complexes of either ^{125}I-poylmer/plasmid or polymer/^{32}P-plasmid were injected as a bolus of approximately 100µL through the jugular vein. In the case of naked DNA, the appropriate amount of DNA was injected as a bolus of 100 µl in the same buffer mixture as complex solutions. Blood samples were obtained via a catheter in the common carotid artery and urine was sampled by flushing the bladder with sodium chloride solution through a 2-way catheter. After 120 minutes, mice were sacrificed and organs (cortex, liver, kidneys, heart, lungs, spleen, fat tissue) were weighed and assayed for radioactivity. Radioactivity of the ^{125}I-polymer was measured on a 1277 Gammamaster (Perkin Elmer Wallac, Freiburg, Germany). To assess the radioactivity from ^{32}P-

pCMV-Luc, organs and blood samples were dissolved in 1 ml of Soluene 350 (Amersham Pharmacia, Freiburg, Germany). Subsequently, 200 µl of 30 % sodium peroxide were incubated with the mixture for 30 minutes, and then added to 15 ml HionicFluor (Perkin Elmer, Dreieich, Germany). Measurements were performed using a TriCarb liquid scintillation counter (Perkin Elmer, Dreieich, Germany) with a counting time of 15 minutes and 1 minute precount delay. Measurements of complex solutions were used for both tracers to determine the injected dose of radioactivity. In the case of PEI/DNA complexes concentration time curves from polymer and pDNA were fitted to a two compartmental model with the Software Kinetica 1.1 from Simed. The model used was $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ and the weighting applied was $1/(c_{calc})^2$. Polymer concentrations in the samples were then calculated as percent of injected dose (%ID) or %ID/mL, respectively. Unpaired t-test was performed using Microcal™ Origin to compare blood levels of different polymers at corresponding time points. Differences were considered significant if two-tail P = 0.05.

RESULTS AND DISCUSSION

Size and zeta potential measurements

At an N/P ratio of 6, both PEI 25 kDa and PEI(25k)-g-PEG(2k)$_{10}$ are capable of forming complexes with plasmid DNA. Complexes prepared with PEI 25 kDa display a size of approximately 100 nm and a zeta potential of about + 25 mV. In contrast, PEG-PEI/DNA complexes display a size of approximately 145 nm and a neutral zeta potential, indicating an efficient shielding of the cationic charge by linear PEG molecules. Compared to measurements obtained in sodium chloride 150 mM [23] complexes are significantly smaller when prepared in glucose 5 %.

Complex stability against exchange reactions with anions

The gels in Figure 1 were obtained with heparin (A) and dextran sulfate (B). Both show a similar stability of PEI/DNA and PEG-PEI/DNA complexes against exchange reactions with polyanions. Initial release being at the same concentrations, however, in the case of PEG-PEI, total release occurs at higher concentrations than that of PEI. This can be observed in lane 6 of the heparin gels (0.2 international units added) and lane 4 of the dextran sulfate gels (2.5 µg added). The differences in release profiles may reflect a DNA complexing ability from PEG as described earlier in the literature [28]. Complex stability against serum was investigated as well, however in this case no release of DNA could be observed (data not shown).
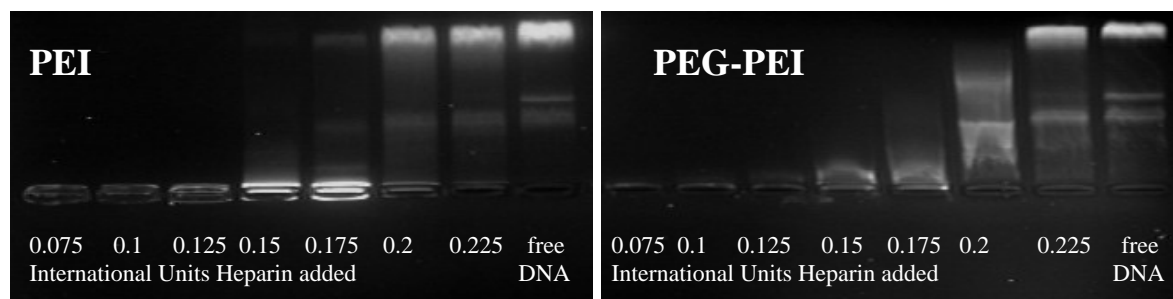

Figure 1a
Investigation of complex stability against exchange reactions with anions. Gels were obtained by incubating PEI/DNA and PEG-PEI/DNA complexes with increasing concentrations of heparin. Obviously release starts at the same concentration, however in the case of PEG-PEI total release occurs at higher concentrations as when using PEI.
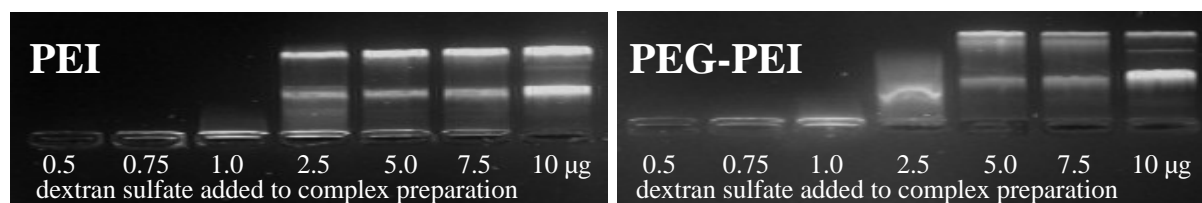

Figure 1b
The same experiment with increasing amount of dextran sulfate

Stability against DNase digestion

Gels in Figure 2 show that if complexes of PEI/DNA and PEG-PEI/DNA are incubated with increasing concentrations of DNase I for 15 minutes, digestion of pDNA starts at an enzyme concentration of 1 I.U. DNase I per complex preparation. However, the degree of degradation is different. While complete degradation can be observed for DNA from PEG-PEI/DNA complexes, PEI/DNA shows only partial degradation with the majority of pDNA remaining intact. These results suggest a significantly greater ability of PEI 25 kDa to protect pDNA as compared to PEG-PEI. An explanation for this finding may be the denser complex structure of PEI, which provides a more efficient steric hindrance against DNase attack. Similar results have been obtained by Mullen et al. in a study where the ability of poly(L-lysine) and pegylated poly(L-lysine) to protect plasmid DNA against DNase was compared and the unmodified polymer achieved a better protection, as well [7].
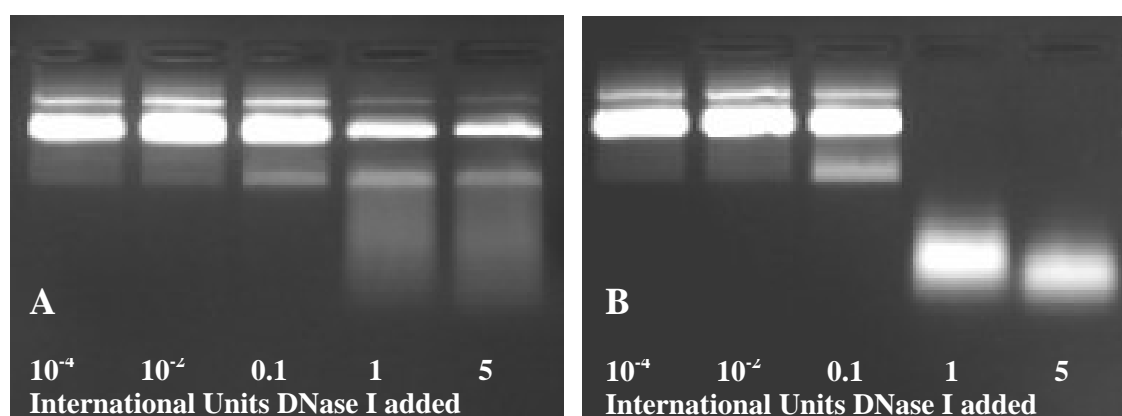

Figure 2
Investigation of DNA protection against DNase digestion using PEI (A) and PEG-PEI (B). Complexes were incubated with increasing amounts of DNase for 15 minutes and, after enzyme inactivation, DNA was released from complexes. Data suggests that PEI offers a significantly higher protection against digestion than PEG-PEI, indicating a denser complex structure.

Atomic force microscopy (AFM)

AFM images shown in Figure 3 as three dimensional plots illustrate that PEI and PEG-PEI are capable of forming defined, spherical complexes with plasmid DNA. Shape and size for both complex types appear similar. However, PEI/DNA complexes seem to be rounder, more compact and smoother. PEG-PEI/DNA complexes lie flatter on the surface of the silicon chip. Furthermore, they seem to have a slightly more irregular shape. However, no free or only loosely complexed DNA can be observed. We have put a significant effort into the confirmation of the absence of free DNA in several AFM preparations. However no free DNA could be observed, as described in earlier studies [22].

Transfection in blood

A comparison of reporter gene expression in sodium chloride 150 mM and mouse blood are shown in Figure 4. Obviously, both PEI/DNA and PEG-PEI/DNA complexes are capable of mediating gene expression when applied in blood. In the mouse blood used for this study, blood clotting was prevented by addition of citrate. This multivalent anion complexes not only calcium, but also magnesium ions, thus inhibiting serum nucleases. Since nuclease degradation is abolished in this experiment we can assume that the reduction of gene expression observed in this study is due to interactions with plasma proteins or cellular blood components. Probably a variety of serum proteins attach to complexes as described earlier [14,19]. If the degree of reduction of gene expression between sodium chloride 150 mM and blood are compared, a reduction of reporter gene expression of approximately 50 % can be observed for PEI/DNA complexes. When regarding PEG-PEI/DNA complexes, on the other hand, reduction goes down to less than 20 % when complexes are applied in blood, indicating a higher level of interactions with blood components. This is surprising, because one would expect less interactions with blood components as a result of the stealth-like effect and the neutral zeta potential.
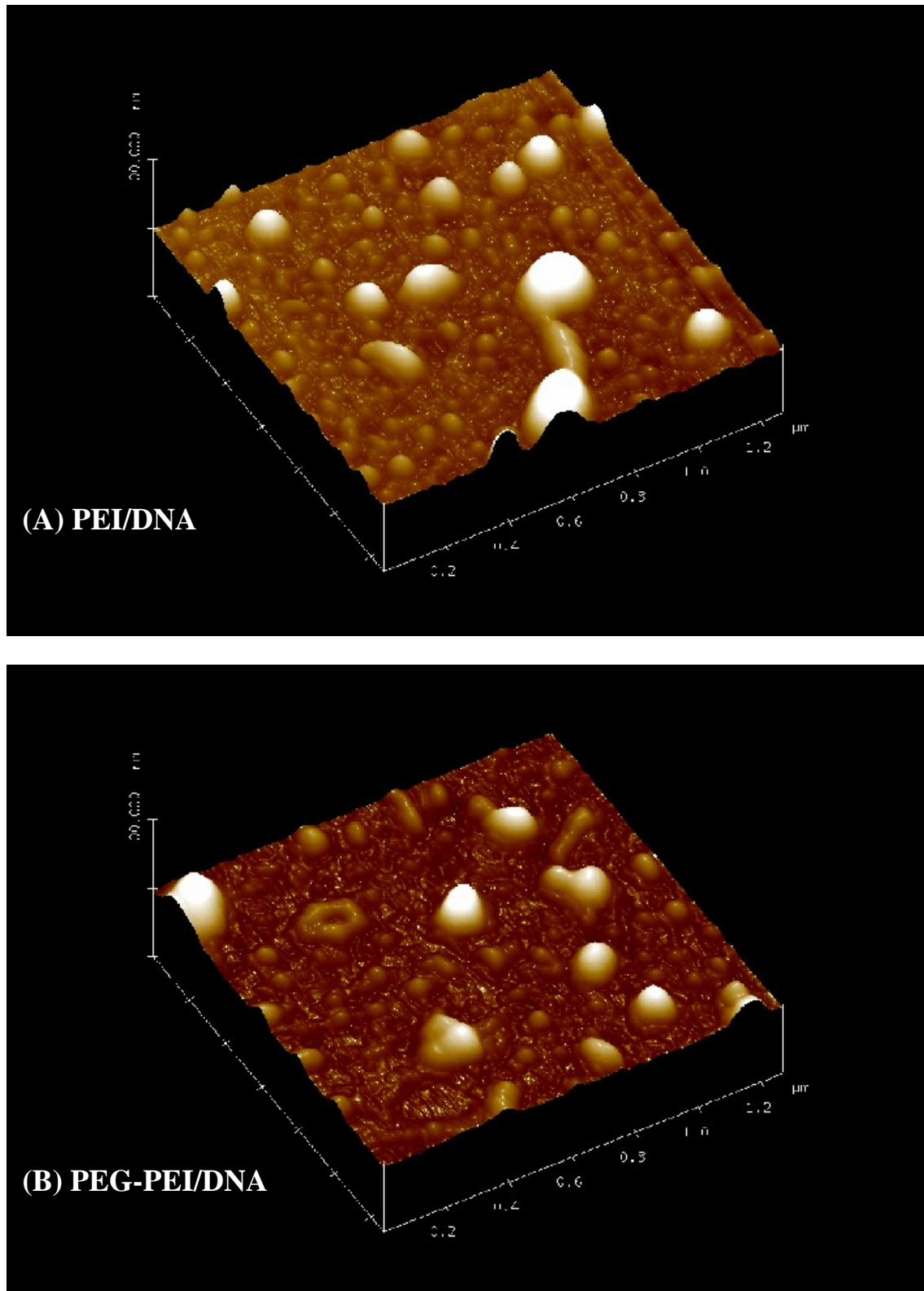
Figure 3
Complexes of PEI/DNA and PEG-PEI/DNA visualized by atomic force microscopy at N/P=6 in glucose 5 %. Images display a similar appearance of complexes, however PEI/DNA complexes seem to be more uniform and show a more regular structure than that prepared with PEG-PEI.

Our data does provide evidence of a certain degree of complex stability after incubation with blood for one hour, since the transfection levels of PEG-PEI/DNA are about one order of magnitude higher than that of naked DNA.
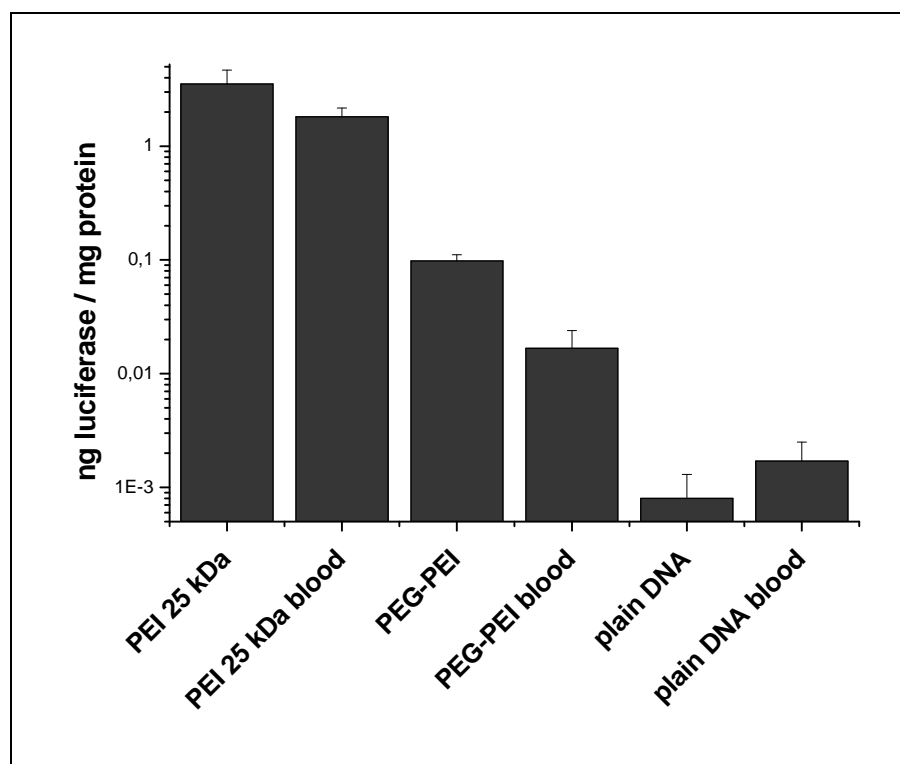
Figure 4
Comparison of luciferase reporter gene expression when complexes are applied to cells in in sodium chloride 150 mM and in blood.

Stability of DNA in serum

Results from plasmid extraction after incubation of complexes and naked DNA with human serum are shown in Figure 5. While naked DNA is almost totally degraded after 30 minutes of incubation, PEG-PEI does offer a certain protection against degradation by serum nucleases. However this effect is less pronounced than in the case of PEI 25 kDa. These results are in good agreement with data from Figure 2 and similar results have been published using poly(L-lysine) [7].

PEI/   PEG-PEI/   Naked   untreated
plasmid   plasmid   plasmi   pCMV-Luc

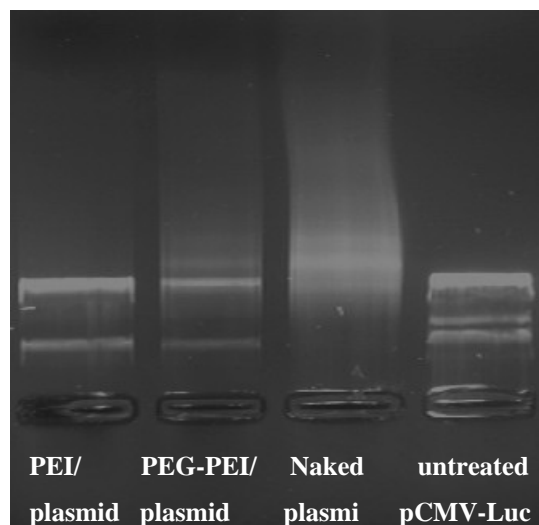**Figure 5**
Investigation of plasmid integrity after incubation with human serum for 30 minutes. This assay reveals that PEG-PEI does protect plasmid DNA from degradation in serum, however this effect is less pronounced than when using PEI 25 kDa. Naked DNA is almost totally degraded after 30 minutes.

Pharmakokinetic analysis and organ distribution

Pharmacokinetic profiles of polymers and DNA are depicted in Figures 6-8. None of the animals used in this study died during the experiments and no signs of acute toxicity were observed. The stability of the label attached to the polymer has been proven by previous studies [9]. ^{32}P labeled plasmid DNA is subject to degradation when injected intravenously [7,29,30] and, as a result, organ distributions detected after 2 hours do not contain the entire amount of intact plasmid. However, despite the evidence of a certain degree of degradation our data does show differences between PEI and PEG-PEI that allows valuable conclusions about protection against degradation.

PEI/plasmid complexes (Figure 6). Pharmacokinetic profiles obtained with labeled PEI and plasmid DNA display a very rapid clearance from the bloodstream. Curves fitted to a biexponential disposition equation by non-linear curve fitting (Figure 6) exhibit very steep alpha phases followed by very flat beta elimination phases for both, the polymer and pDNA. After 30 minutes, less than 3 % of the injected dose (polymer, as well as pDNA) per milliliter blood is still circulating in the bloodstream, approaching levels of approximately 1 %

ID/ml blood after 2 hours. Differences between the curves, especially in the first 30 minutes are only marginal. These similarities suggest that PEI/DNA complexes remain stable to a substantial degree in the bloodstream.

The organ distribution of both PEI and pDNA after two hours reveals similar levels of radioactivity in important organs, such as liver, kidney and lung. This great similarity provides additional evidence for a certain degree of complex stability in the bloodstream with joint uptake into tissues. Uptake into the liver probably occurs due to opsonization of complexes as demonstrated by Planck et al. [31] with a subsequent rapid capture by mononuclear phagocytic cells. A remarkable difference in organ deposition can be observed in the spleen, displaying an approximately threefold higher accumulation of ^{125}I compared to ^{32}P. This fairly high accumulation in the spleen has been reported earlier for PEI 25 kDa [9] and probably occurs due to free polymer present at the N/P ratio used or a minor degree of complex separation in the bloodstream. A significantly higher amount of radioactivity from ^{32}P was detected in the urine, as compared to ^{125}I from the polymer. This difference most likely occurs as a result of a minor degree of complex separation within 2 hours accompanied by a rapid degradation by serum nucleases as described earlier [29].

Remarkably, the uptake into the lung is fairly low in this study, despite the fact that the lung is the main organ of gene expression after systemic administration of PEI/DNA complexes [3,4,32]. The reason for this is very likely a result of the low dose of only 2 µg pDNA used for the present investigation. At higher doses of PEI/DNA complexes a substantial accumulation is detectable in the lung even after 2 hours following injection (data not shown). Furthermore, deposition of pDNA and gene expression do not necessarily correlate as described ealier by Liu et al. [33]. Other tissues investigated in this study, such as heart, cortex, fatty tissue or the injection site in the jugular vein, did not exhibit significant levels of radioactivity from either polymer or pDNA after application of both PEI/DNA and PEG-PEI/DNA complexes.
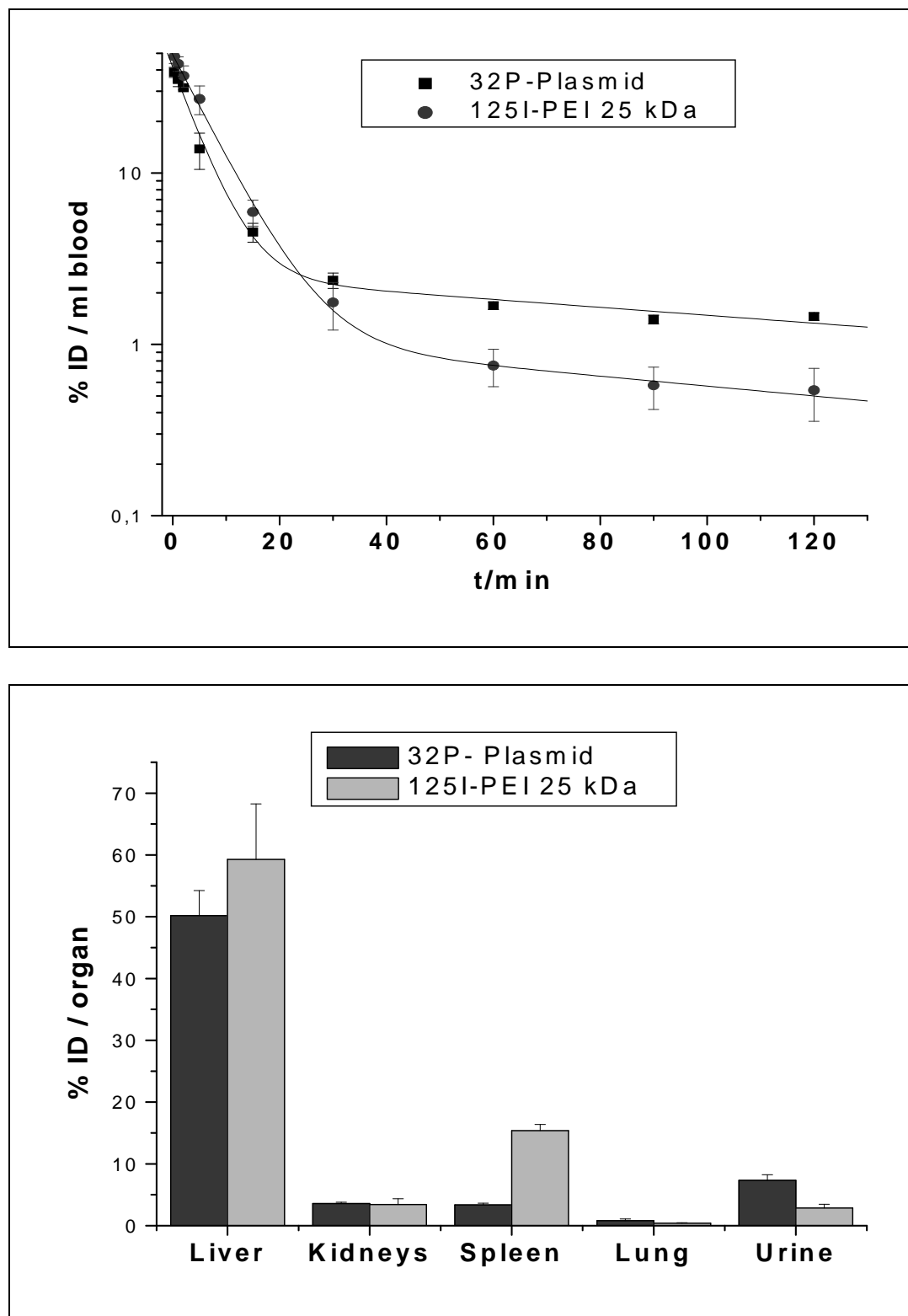
Figure 6
Pharmacokinetic profile and organ distribution after 2 hours of PEI 25 kDa / pCMV-Luc complexes. A very rapid clearance from the bloodstream of both, DNA and polymer can be observed as well as a similar organ distribution.

PEG-PEI/plasmid complexes (Figure 7). Pharmacokinetic data from PEG-PEI/DNA complexes reveals major differences between polymer and plasmid DNA. Similar to PEI 25 kDa, PEG-PEI displays a rapid initial elimination phase, however, after approx. 5 minutes a plateau can be observed at ~25 % of the injected dose per milliliter blood. The blood level decreases only marginally over the following two hours. A plausible reason for this long circulation of the polymer could be an efficient steric barrier created by PEG chains against opsonization and thus less uptake into the reticuloendothelial system of the liver. Another explanation could be the attachment of the polymer to e.g. cellular blood components. While the pharmacokinetic profile of the polymer seems very promising at the outset, it soon becomes clear that the polymer rapidly loses its cargo. The kinetic profile from ^{32}P labeled pCMV-Luc complexed with PEG-PEI displays an initial decrease over the first two minutes with a subsequent maximum at 5 minutes of approximately 25 % of the injected dose per milliliter blood. The reason for this maximum could be an initial entrapment of DNA or PEG-PEI/DNA complexes in the lung microvaculature with a subsequent time-delayed redistribution into the bloodstream as described earlier [34]. After crossing of this maximum, however, the blood level of DNA decreases rapidly reaching levels of less than two percent after 120 minutes. The organ distribution after 2 hours shows great differences between the polymer and DNA, as well. While radioactivity from the polymer accumulates predominantly in the liver, reaching approximately 40 % of injected dose, radioactivity from the DNA appears in the liver at a lower level, remarkably even significantly lower than that of naked DNA (Figure 8). Furthermore, a high level of radioactivity from ^{32}P was found in the urine after two hours, most likely present as pDNA degradation products.
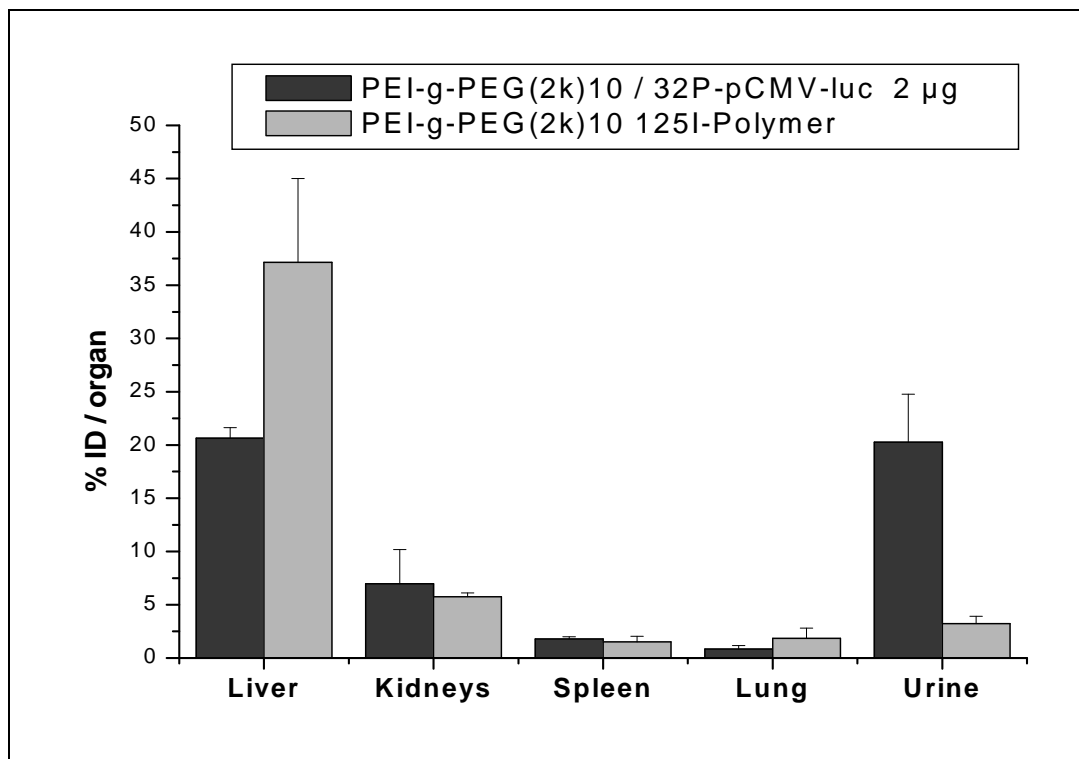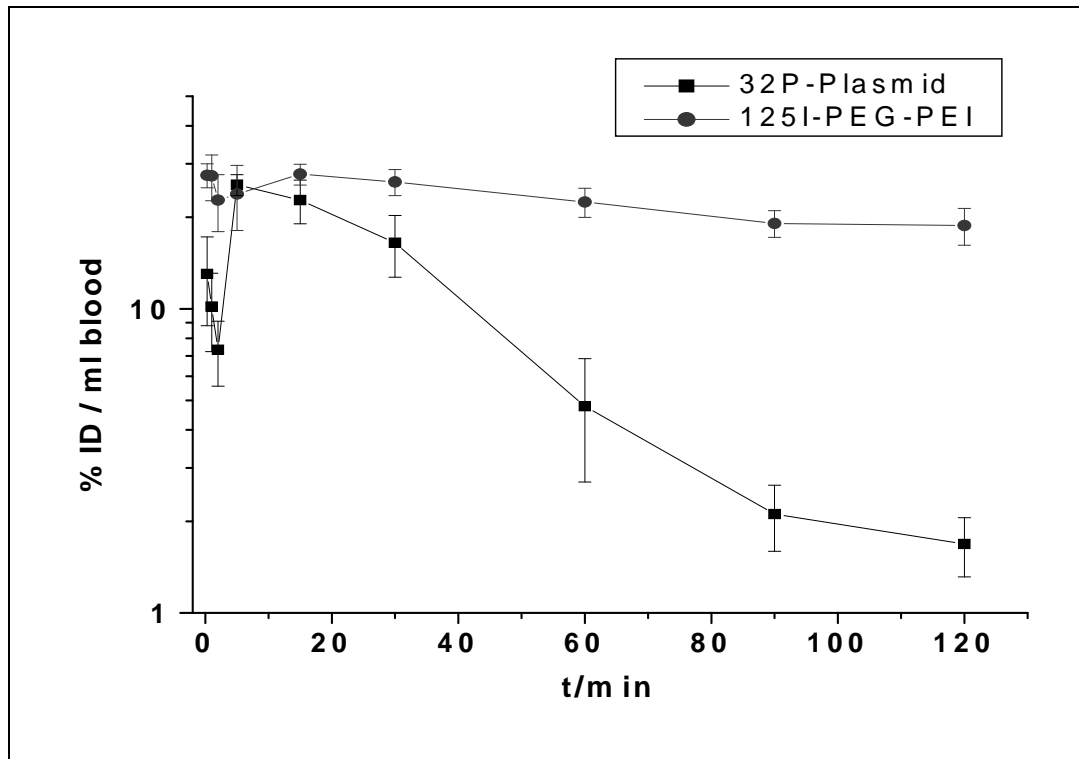
Figure 7
Pharmacokinetic profile and organ distribution (after 2 h) of PEI(25k)-g-PEG(2k)$_{10}$ / pCMV-Luc complexes. Blood levels as well as organ distribution show great differences between polymer and DNA.

From pharmacokinetic profiles and organ accumulation data it can be concluded that PEG-PEI/DNA complexes very likely undergo a rapid separation of polymer and DNA after injection into the bloodstream. After separation, the PEG-PEI is taken up by the liver and a substantial amount of the injected dose circulates in the blood, possibly attached to cellular blood components. Plasmid DNA, however, is probably degraded by serum nucleases rapidly after separation leading to a high renal excretion of degradation products. Similar findings have been obtained by Mullen et al. [7]. They reported that pegylated poly(L-lysine)/plasmid complexes very likely separate rapidly after intravenous injection.

Naked DNA (Fig. 8). The pharmacokinetic profile and organ distribution of naked pCMV-Luc was investigated under our experimental conditions and used as a reference. Interestingly, a maximum was obtained with naked DNA after 15 minutes, as well, probably again due to accumulation in the lung with subsequent release. The curve progression following this maximum shows a rapid clearance from the bloodstream. The organ distribution after 2 h shows a substantial accumulation in the liver and excretion via the urine. The high renal excretion is probably a result of degradation of unprotected pDNA by serum nucleases [7,29].

When the pharmacokinetic profile and organ distribution of DNA complexed to PEI is compared to that of naked pDNA, the impact of the polymer becomes obvious. The blood level curve for PEI/pDNA is similar to that obtained for the labeled polymer alone, while naked DNA shows a different behavior. This finding indicates that the polymer dictates the fate of the DNA and this observation can be explained by the assumption that the complexes remain stable in the bloodstream. Organ distributions of PEI complexed pDNA compared to naked pDNA shows great differences as well. In the case of PEI/pDNA, a much higher DNA accumulation in the liver and a lower excretion via the urine were measured. The threefold decrease in urine excretion is
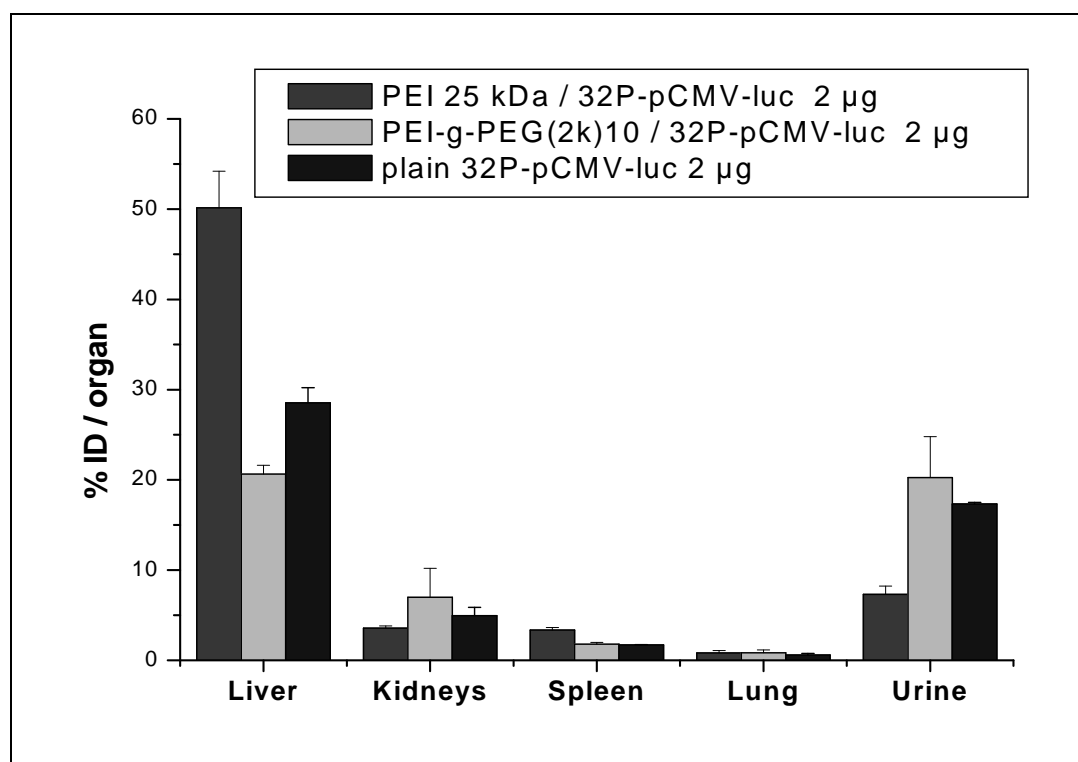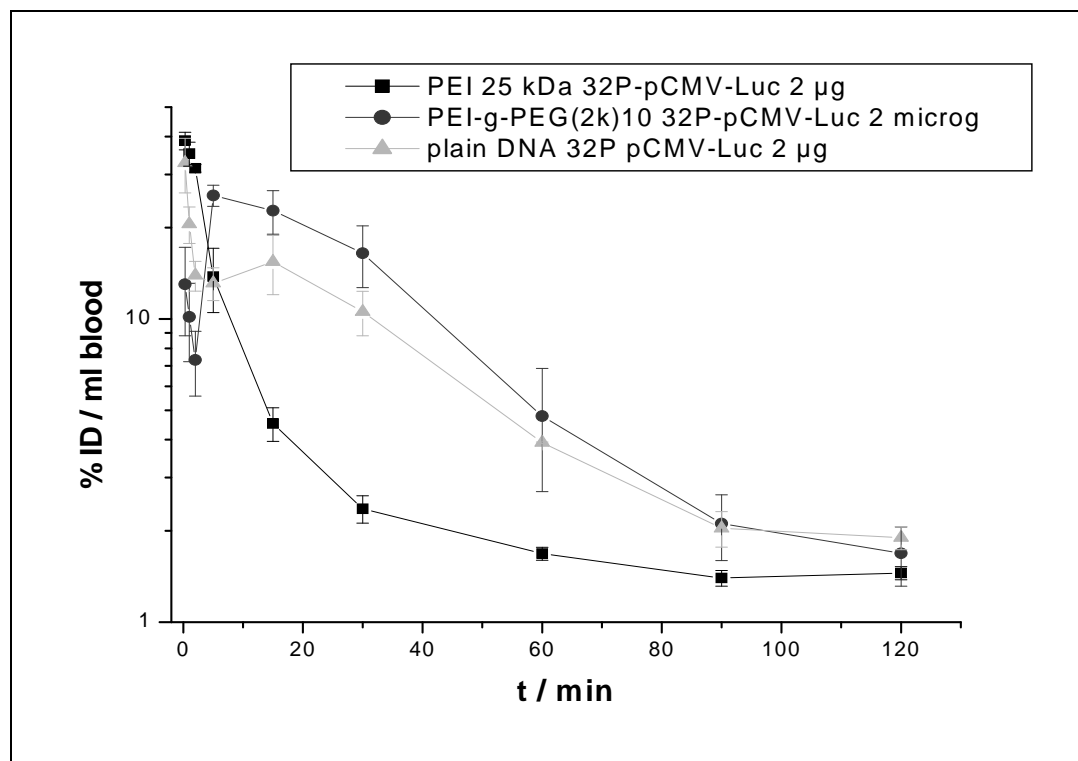
Figure 8
Comparison of pharmacokinetics and organ distribution of ^{32}P labeled pCMV-Luc complexed to PEI 25 kDa, PEG-PEI and plain DNA. Complexation with PEI 25 kDa leads to significant changes in organ distribution and blood levels over time, whereas remarkable similarities can be observed for free DNA and PEI(25k)-g-PEG(2k)$_{10}$ / DNA complexes.

evidence that efficient protection of pDNA from degradation in the bloodstream is provided by PEI 25 kDa.

Comparison of pDNA profiles from PEG-PEI/DNA complexes, however, shows similarities to that of naked DNA. While blood concentration levels are different at early time points, they become very similar after 15 minutes. Furthermore, organ distributions after 2 hours are similar, as well. Both show a moderate accumulation in the liver and a substantial renal excretion. These similarities support the assumption that PEG-PEI/DNA complexes separate rapidly after injection and that DNA is subject to degradation.

In conclusion, we can summarize that under the conditions described in this study, complexes of unmodified PEI 25 kDa and pCMV-Luc exhibit a certain stability in the bloodstream. Complexes prepared with PEI(25k)-g-PEG(2k)$_{10}$ and pCMV-luc, however, very likely separate rapidly after injection. The reason for this rapid *in vivo* separation of PEG-PEI/DNA complexes is not yet clear, especially since *in vitro* studies revealed a similar capability of both polymers to condense DNA [23]. Furthermore, complex stability against heparin and dextran sulfate were very similar, as well. A possible explanation for this reduced stability in blood could be the higher susceptibility of PEG-PEI/DNA complexes to enzymatic degradation via DNase I and serum nucleases as described in this study. This may lead to degradation of DNA in the complexes and thus destabilization. Similar findings are described for pegylated poly(L-lysine)/plasmid complexes [7]. Furthermore, AFM images showed flatter and more irregular structures indicating less stable complexes. Transfection data in nuclease inactivated blood revealed a lower stability of PEG-PEI/DNA complexes *in vitro*. This lower stability together with a degradation of complexed DNA by serum nucleases may lead to separation of complexes. Especially in fine capillary beds of the lung complexes are exposed to vigorous shear stress. Furthermore negatively charged endothelia or blood components, as suggested in the literature [14] may contribute to complex destabilization. The

sum of these factors may lead to penetration of proteins or polyelectrolytes into the core of complexes [18]. This process could be facilitated by the hydrophilic PEG-chains incorporated into the inner layers of the complexes. A more detailed investigation concerning this issue is currently underway.

It is yet unclear if complex separation of PEG-PEI/DNA complexes occurs to the same extent at higher doses as well. In this study only approximately 2 µg of plasmid DNA per mouse were used and it is conceivable that there is a saturable mechanism of complex destabilization that can be overcome or at least delayed with higher doses. Furthermore it is unclear if and to what extent the size of the complexed DNA influences complex stability. It is conceivable that complexes formed with small nucleic acids, such as antisense oligonucleotides or ribozymes, show a higher stability. Currently PEG-PEIs with different polymer structures complexed with nucleic acids of various sizes are under investigation concerning their *in vivo* properties. If a rapid destabilization occurs with other PEG-PEIs or at higher doses, it will be necessary to create new methods to stabilize the electrostatic complexes. For example, lateral stabilization using bioreversible disulfide bonds has shown promising properties [18].

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5

Supramolecular vehicles based on *hy*-PEI-*g*-PCL-*b*-PEGs and α-cyclodextrin showing unexpectedly high gene transfection efficiency and good biocompatibility

SUMMARY

Major problems using cationic polymers for nonviral gene delivery stem from fairly high positive surface charges of the resulting polyplexes with DNA and consequently high toxicity under *in vitro* and *in vivo* conditions. Attachment of hydrophilic polymers such as polyethylenglycol (PEG) has reduced toxicity of formulations. However most of these systems are not biodegradable at all. We therefore developed a biodegradable gene delivery system showing high transfection efficiency while displaying a low toxicity.

A new type of copolymer was synthesized by grafting a diblock copolymer consisting of PEG and polycaprolactone (PCL) onto hyperbranched polyethylenimine (*hy*-PEI). Since the solubility of these compounds is only very poor, we formed supramolecular inclusion complexes by threading a-cyclodextrin (a-CD) over PEG and PCL chains. The successful threading of a-CD onto PCL blocks was investigated by FTIR and UV spectroscopy as well as ^{1}H NMR. Furthermore we investigated the ability of the resulting compounds to complex DNA by ethidium bromide fluorescence quenching. Hydrodynamic diameters of complexes and zeta potentials were assessed using a Zetasizer.

Furthermore, cellular uptake was evaluated by confocal laser scanning microscopy and flow cytometry. Transfection efficiencies were evaluated employing a luciferase reporter gene assay and toxicity was estimated by determination of protein content.

Characteristic shifts of PCL in FTIR, ^{1}H NMR and UV spectra strongly suggest that α-CD is threaded over PEG and PCL. Due to the reduced hydrophobic interaction between PCL blocks the resulting supramolecular complexes displayed a dramatically increased solubility. Their ability to complex DNA was almost as efficient as that of PEI 25 kDa. Resulting complexes show a size of approximately 200 nm and a neutral surface charge. Cellular uptake was increased as well when the inclusion complex is compared to the α-CD-free triblock copolymer. Subcellular localization suggests an uptake in distinct

structures, probably by endocytosis with subsequent trafficking to lysosomes. Transfection efficiencies of inclusion complexes were in the same order of magnitude as PEI, however, a significantly lower toxicity was observed allowing the administration of nitrogen to phosphate ratios of up to 20.

The supramolecular vesicles combine a very low toxicity with high levels of reporter gene expression, which is comparable to that of unmodified PEI 25 kDa. Due to its adequate biocompatibility this system is especially promising for *in vivo* use.

INTRODUCTION

A host of nonviral gene delivery systems consisting of DNA complexes with various polycations has been investigated [1-3]. Among synthetic cationic polymers, polyethylenimine (PEI) is known to offer the highest positive charge density leading to strong DNA binding. Another positive feature of PEI is the "proton-sponge effect" over a broad pH range, which allows endosomal/ lysosomal escape without the use of disruptive agents [4]. Only high molecular weight PEIs have been reported to be very effective for gene delivery [5-7]. However, high molecular weight PEI was found to be cytotoxic due to its interaction with negatively charged cell membranes.

Therefore, the use of PEI-based copolymers, instead of PEIs, to form DNA polyplexes has been extensively investigated recently [8-12]. Most studies utilize nonionic hydrophilic polyethers, e.g. polyethylenglycol (PEG), as a building block to obtain block or graft copolymers with linear or hyperbranched PEIs. When these copolymers were mixed with pDNA in aqueous solution, the cationic PEI segments of the copolymers were first bound to DNA *via* electrostatic interactions and then the neutralized polyions aggregated to form an insoluble core, while the nonionic water-soluble PEG chains served as a hydrophilic shell stabilizing the resultant nanoscale particles [8]. Complexes

thus prepared show a lower positive surface charge compared to polyplexes from homo PEIs, and are therefore less cytotoxic.

A drawback for the *in vivo* application of these copolymers is their lack of biodegradability, since PEI and PEG were linked by chemical bonds stable under physiological conditions. Therefore, very recently we synthesized a new type of biodegradable copolymers by grafting diblock copolymers of PEG and PCL onto hyperbranched polyethylenimines (*hy*-PEIs). Strong hydrogen bonding between PCL and PEI blocks was observed in the bulk materials, and the solubility of these copolymers were strongly dependent on their compositions, i.e. block length and graft density. Further, DNA polyplexes formed with soluble copolymers or copolymers possessing high critical micelle concentration (cmc) showed considerably increased gene transfection efficiency. By contrast, those copolymers having high graft density and long PCL blocks showed very poor transgene expression. In the latter case, the access and binding of DNA to the PEI block were seriously hindered by the dense hydrophobic PCL chains surrounding the hydrophilic PEI head. Hydrogen bonding between PCL and PEI blocks is also believed to be an impediment for the DNA complexation with PEI [13]. Therefore, breaking of hydrogen bonding between PCL and PEI as well as the dissolution of PCL block in aqueous media seem to be key factors for improving gene transfection efficiency of these copolymers.

In this paper, we present an unusual way to dissolve the PCL blocks of *hy*-PEI-g-PCL-*b*-lPEGs in aqueous solution by including them inside the α-cyclodextrin (α-CD) cavities. α-CD is a cyclic oligosaccharide consisting of 6 glucose units [15]. One of its distinctive properties is the amphiphathy, i.e. it possesses a hydrophobic cavity of 4.5 Å in diameter with a hydrophilic outer layer carrying many hydroxyl groups [15-16]. Supramolecular inclusion complexes (ICs), organized by noncovalent interactions, can be formed by threading α-CD molecules onto various polymer chains [17-23]. The driving

force for the threading process is probably due to intermolecular hydrogen bonding between neighboring CDs, as well as steric compatibility and hydrophobic interactions between host and guest molecules. Thus far, it has been demonstrated that α-CD may form inclusion complexes with both polyethylenglycol (PEG) [17-18] and PCL [24-28] chains. In most cases, a full coverage of individual polymer chains with α-CD bracelets leads to a crystalline water-insoluble inclusion complex. However, if insufficient α-CD was used and the polymer solution is dilute, a partial coverage of polymer chain with α-CD can also be achieved. In this case, the resultant inclusion complexes are completely soluble in water. For instance, selective inclusion of the octane-1,8-dicarboxylate segment of a PEG octane-1,8-dicarboxylate polyester with α- or β-CD showed significantly improved water solubility [29].

MATERIALS AND METHODS

Materials

Synthesis and physicochemical properties of graft copolymers, *hy*-PEI-g-PCL-b-PEGs, were reported recently [13]. To prepare the inclusion complexes, a predetermined amount of α-CD aqueous solution (e.g. 5 mg/mL, 20mL) was added stepwise to the copolymer micelle solution (e.g. 4.2 mg/mL, 15mL) in a glass vial immersed in an ultrasonic water bath at room temperature. Ultrasonic agitation was applied for 30 minutes during and after the addition of the α-CD solution. The mixed solution was then stirred magnetically overnight at room temperature, frozen at ~-40°C, and then freeze-dried to obtain a powder sample [30].

As a reference polymer for all studies we used PEI 25 kDa (*hy*-PEI) from Sigma-Aldrich, Taufkirchen, Germany.

pCMV-Luc encoding for luciferase as a reporter gene was purchased from Plasmid Factory, Bielefeld, Germany. NIH/3T3 (Swiss mouse embryo) cell line

was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured according to the protocols suggested by the supplier. Polyplex formation at N/P 3, 7 and 20 were carried out as described previously [13].

Ethidium bromide exclusion assay

DNA condensation was measured by the decrease in ethidium bromide fluorescence, as described earlier [31-32]. The assay was performed in 96 well plates in triplicate. Eight µg of salmon testes DNA were dissolved in 79 µL water and 50 µL of 60 mM Tris buffer pH 7.4 were added to each well. Volumes were equalized to 300 µL with water. Subsequently, appropriate volumes of 0.05 mg/mL polymer solutions were added to produce N/P ratios between 0.2 and 4. These were incubated for 10 min and then 20 µL of a 0.1 mg/mL ethidium bromide solution were added. Wells were mixed thoroughly and the fluorescence was measured using a fluorescence plate reader with excitation wavelength at 518 nm and an emission wavelength of 605 nm.

Characterization of inclusion complexes and polyplex nanoparticles

FTIR, ^{1}H NMR and DSC measurements of inclusion complexes were performed as reported earlier [13]. UV spectra were recorded on a Shimadzu UV-160 UV-visible recording spectrophotometer.

Photon correlation spectroscopy

Hydrodynamic diameters of the polymer/DNA complexes were determined by photon correlation spectroscopy. Plasmid (0.5 µg pCMV-Luc) in 25 µL 0.9 % NaCl were complexed with the appropriate amount of polymer in 25 µL NaCl each, as described above. Measurements were performed on a Zetasizer 3000 HS from Malvern Instruments, Herrenberg, Germany (10 mW HeNe laser, 633 nm). Scattering light was detected at 90° angle through a 400 µm pin hole. For data analysis, the viscosity (0.88 mPa s) and the refractive index (1.33) of

distilled water at 25 °C were used. The instrument was routinely calibrated using Standard Reference latex particles (AZ 55 Electrophoresis Standard Kit, Malvern Instruments). Values given are the mean of 5 measurements.

Measurement of zeta potential

Zeta-potential measurements were carried out in the standard capillary electrophoresis cell of the Zetasizer 3000 HS from Malvern Instruments at position 17.0. Measurements were performed in 0.9 % NaCl and average values were calculated with the data obtained from 5 runs.

Transfection experiments

Transfection experiments were performed at N/P ratios of 3, 7 and 20. NIH/3T3 cells were seeded in 12 well plates at a density of 50,000 cells per well. After 24 h, the media was removed and the complexes were added in 2 ml fresh media. Media was exchanged again after four hours and the cells were incubated for an additional 44 h. Luciferase gene expression was quantified using a commercial kit (Promega, Mannheim, Germany) and photon counting on a luminometer (Sirius, Berthold, Bundoora, Australia). Results were measured in relative light units per second (RLU/s) which were then converted into ng luciferase by creating a calibration curve with recombinant luciferase (Promega, Mannheim, Germany). Protein concentration in each sample was determined. All experiments were performed in triplicate and data were expressed in ng luciferase per mg protein.

Fluorescent Labeling of PEI and DNA for Confocal Laser Scanning Microscopy Experiments

Polymer (20 mg) was dissolved in 2 mL 0.1 M sodium bicarbonate solution at pH 9. Oregon Green 488 carboxylic acid succinimidyl ester (1 mg, Molecular Probes, Leiden, The Netherlands) was dissolved in 200 μL dimethylsulfoxide and added dropwise under stirring to the polymer solution. The mixture was

stirred in the dark for 3 hours at room temperature before the labelled polymer was purified by ultrafiltration in an Amicon cell (regenerated cellulose membrane, molecular weight cut off 10,000) and washed with 0.1 M borate / 1.0 M sodium chloride solution pH 7.5. The washing procedure was performed until no absorption was detectable at 488 nm in the cell outflow. As a final step, the buffer was exchanged with distilled water. Plasmid (pCMV-Luc) was labeled with Cy-3 using a commercial kit (Mirrus Label It, Mobitec, Goettingen, Germany). Procedure was performed according to the manufacturer's manual.

Confocal Laser Scanning Microscopy Experiments

A Zeiss Axiovert 100M microscope coupled to a Zeiss LSM 510 scan module was used. NIH/3T3 cells were seeded at a density of 50,000 cells per well in 8 well chamber slides (Lab Tek, Nunc, Wiesbaden, Germany). After 24 h the media was removed and complexes of 0.5 mg Oregon Green-labeled PEI and 0.5 mg Cy3 labeled plasmid were added in new media. After an additional 4 h media was removed again and cells were washed 4 times with phosphorus buffered saline (PBS). Fixation of cells was done by incubation with 400 mL paraformaldehyde solution 3 % in PBS for 20 min. Cells were washed again for 4 times and incubated for an additional 20 min with a 0,1 mg/mL DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (Molecular Probes, Leiden, The Netherlands) solution in PBS. For excitation of DAPI fluorescence, an Enterprise UV laser with an excitation wavelength 364 nm was used. Excitation of Oregon Green was performed using an argon laser with an excitation wavelength of 488 nm and for excitation of Cy3 a Helium-Neon laser with an excitation wavelength of 543 nm was used. Images were recorded in the multitracking mode using a longpass filter of 385 nm for DAPI, a longpass filter of 505 nm for Oregon Green and a longpass filter of 560 nm for rhodamine.

Flow cytometry

Flow cytometry was performed with plasmid DNA labelled with the intercalating dye YOYO-1 (Molecular Probes, Leiden, The Netherlands), as described by Ogris et al [33]. Briefly, cells were grown for 24 h in six-well plates at a density of 400,000 cells per well. Complexes were prepared as described above and applied in fresh media with a total volume of 3 ml. After 30 min, media was aspirated and cells were washed twice with cold (4 °C) PBS pH 7.3 and once with 1 M NaCl to remove complexes attached to the cell membrane as described earlier [34]. Cells were suspended in PBS after detachment via trypsin incubation for 1 min. Cell suspensions were kept on ice until analysis. Flow cytometry was performed using a Becton Dickinson FACS Scan equipped with an argon laser with an excitation wavelength of 488 nm. The filter setting for emission was 530/30 nm bandpass.

RESULTS AND DISCUSSION

Formation of soluble inclusion complexes

Except for *hy*-PEI-*g*-PCL-*b*-PEG copolymers with very short PCL block length and low graft density which are completely soluble in a wide concentration range, most copolymers showed a distinct critical micellization concentration (cmc) in water [13]. To form inclusion complexes, the α-CD solution of predetermined concentration was added stepwise to the dilute micellar solution of the ternary block copolymer under ultrasonic agitation. During this process, dynamic light scattering (DLS) measurements were employed to monitor the micelle collapse upon dilution and addition of α-CD. When the turbid solution became transparent upon addition of an aqueous α-CD solution, nanoparticles were no longer detectable by DLS measurement, suggesting the dissolution of micelles. The copolymer concentration calculated at this point was much higher

than its initial cmc, suggesting solubilization of the hydrophobic blocks upon threading by α-CD.

It is worth noting that copolymer solutions must be dilute in order to obtain soluble inclusion complexes. Otherwise, precipitation from the solutions of copolymers of low graft density (< 3) or gelation for the solutions of highly grafted copolymers will occur, indicating the formation of insoluble crystals of inclusion complexes. In solutions of highly grafted copolymers, the graft copolymer was physically crosslinked by the insoluble crystals of inclusion complex formed between α-CD and the copolymer side chain [35]. Our objective was to dissolve PCL blocks while avoiding the occurrence of either precipitation or gelation of copolymer solution. Therefore, the control of copolymer concentration is very crucial.

It was found that the effect of adding α-CD on the solubility of the copolymers depends much on the copolymer compositions. For instance, we failed to dissolve the copolymers containing long PCL block (e.g. 3800) [13] and PEG5000 regardless of varying the graft density from 1.4 to 4.5.   When attempting to dissolve these copolymers upon addition of α-CD solutions, clear solutions were not obtained, and eventually *hy*-PEI25K-*g*-(PCL3800-*b*-PEG5000)$_{1.4}$ solution formed a precipitate while gelation occurred in *hy*-PEI25K-*g*-(PCL3800-*b*-PEG5000)$_{2.6}$ and   *hy*-PEI25K-*g*-(PCL3800-*b*-PEG5000)$_{4.5}$ solutions. For other copolymers, we successfully obtained soluble inclusion complexes. Table 1 (page 197) summarizes four typical soluble inclusion complexes selected for the gene delivery experiments.

It is well known that the cavity depth of α-CD molecules is 7 Å, which is equal to the length of approximately 0.86 repeat units of PCL. Presuming that only PCL block of copolymer had been fully and selectively covered by α-CD molecules, the PEI contents in *hy*-PEI25k-*g*-(PCL580-*b*-PEG5k)$_{2.9}$, *hy*-PEI25k-*g*-(PCL1.2k-*b*-PEG2k)$_{5.1}$ and *hy*-PEI25k-*g*-(PCL2k-*b*-PEG2k)$_{2.8}$ should be

approximately 43%, 24% and 27% respectively. The actual PEI content of complexes shown in Table 1 is close to or higher than that of the complex compositions described above (40% *vs* 43%, 34% *vs* 24%, and 26% *vs* 27%). Since PEG can also form an inclusion complex with α-CD, apparently the copolymer side chains (PCL-b-PEGs) were only partially covered by α-CD in these soluble inclusion complexes.

The threading of α-CD molecules onto the copolymer PCL blocks was demonstrated spectroscopically. Figure 1 shows an expansion of FTIR spectra covering the carbonyl bands of a diblock prepolymer MPEG-*b*-PCL, its corresponding ternary copolymer *hy*-PEI-*g*-PCL-*b*-PEG and a typical inclusion complex with α-CD.

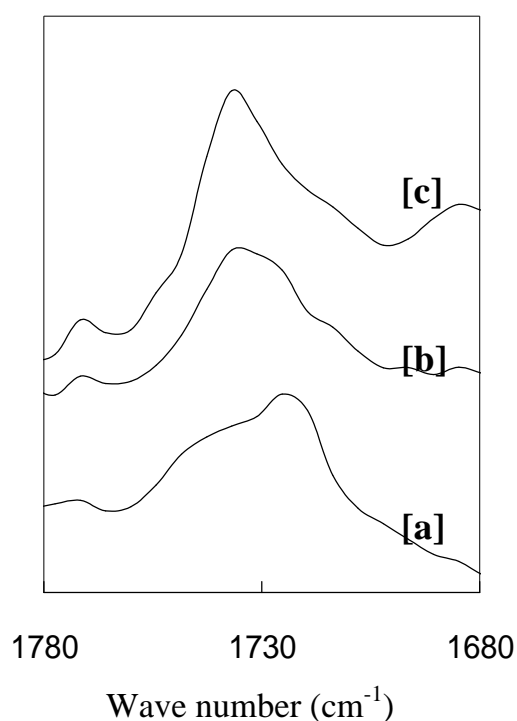**Figure 1**
FTIR spectra of HO-terminated PCL2k-b-MPEG2k (a), *hy*-PEI25k-g-(PCL2k-b-PEG2k)$_{2.8}$ (b), inclusion complex of α-CD and *hy*-PEI25k-g-(PCL2k-b-PEG2k)$_{2.8}$ (c)

The complexed powder sample was obtained by freeze-drying of an aqueous sample solution and was allowed to crystallize at room temperature for one month before FTIR measurements. It is known that the expanded $\nu_{c=o}$

absorption band of the semicrystalline PCL is very indicative of the PCL phase structure in FTIR measurements [27,28]. In agreement with these reports, the PCL carbonyl absorption band (s, $\nu_{C=O}$) of PCL-b-MPEG can be well resolved into a peak at 1726cm^{-1} and a prominent shoulder at 1736cm^{-1}, corresponding to the carbonyl absorption of the crystalline and the amorphous PCL phases, respectively. The carbonyl absorption of the crystalline phase (1726cm^{-1}) is much stronger than that of the amorphous one, suggesting high PCL crystallinity in the diblock prepolymer. Upon grafting onto *hy*-PEIs, the absorption of crystalline PCL at 1725 cm^{-1} becomes weak, while the absorption of the amorphous PCL at 1736 cm^{-1} appears as the main peak, as shown in spectrum (b). These results indicate that the crystallization of PCL blocks has been suppressed but not completely eliminated in the *hy*-PEI-*g*-PCL-*b*-PEG copolymers. By contrast, as shown in spectrum (c), the C=O absorption of crystalline PCL regions completely disappears, and only the C=O absorption of noncrystalline PCL blocks is detected in the spectrum of the ICs. These results suggest that PCL chains are individually inserted in α-CD channels and hence were not able to fold and aggregate to form any PCL crystalline domains.

^{1}H NMR measurements further demonstrated the preferential complexation of PCL block with α-CD. ^{1}H NMR spectra of copolymer micelles in D$_2$O do not show detectable resonance signals of PCL blocks due to suppressed chain mobility (data not shown). When the micelles were dissociated upon addition of α-CD, clear PCL resonance signals (see ref [13] for detailed peak assignments) were observed in the ^{1}H NMR measurements. Moreover, as shown in Figure 2, the ^{1}H NMR resonance signals of PCL blocks show significant down-field shift upon forming the inclusion complex with α-CD. These results are consistent with previous reports in which similar down-field shifts were observed when polymer chains were included inside CD cavities [29,36].

It was reported that protonated polyiminooligomethylenes may form soluble inclusion complexes with α-CD and result in the similar down-field chemical shift of oligomethylene signals. However the threading process was very slow at room temperature. In our work, the PEI block signal did not show down-field shifts upon mixing with α-CD even after several days at room temperature. A model study with homo-PEI showed the same results. Thus apparently, PEI does not form an inclusion complex with α-CD at room temperature. The different behavior of PEI and polyiminooligomethylenes may be due to the fact that the reported polyiminooligomethylenes contain long oligomethylene chains which are known to form an inclusion complex with α-CD. The PEI we used, however, is branched and therefore probably the formation of inclusion complexes is abolished.

Indeed, polyethylene is known to form an inclusion complex with α-CD [37]. It is well known that PEG, similar to PCL, can form inclusion complexes with α-CD. However, ¹H NMR signals of the PEG blocks of the copolymer complexes listed in Table 1 did not show any significant down-field shifts. These results suggest that the α-CD molecules have preferably formed an inclusion complex with the PCL block in these complexes, in case that the α-CD molar amount was stochiometrically insufficient for the whole side chain (MPEG-*b*-PCL-) inclusion. It is noteworthy that a precipitate or a gel was usually formed if a large amount of α-CD was used, indicating that whole side chains including the PEG blocks were included inside α-CD and new insoluble IC crystals were formed.

Based on these results, we concluded that there are two preconditions for copolymer solubilization resulting from complexation with α-CD. First, the amount of α-CD should be stochiometrically insufficient to include both side chain blocks.

Second, copolymer PCL blocks need to be sufficiently isolated by the other two soluble components, i.e. the long PEG and bulky *hy*-PEI blocks. Under these two conditions, the newly formed complexes between PCL blocks and α-CD are not able to aggregate and form insoluble inclusion complex crystals. This is probably the reason, why we were not able to dissolve the copolymers containing PCL3800.

Formation of inclusion complex between α-CD and PCL block was also confirmed by UV measurements. As shown in Figure 3, upon adding α-CD, the UV absorption peak of the PCL carbonyl showed a clear red-shift from 216 nm to 226 nm due to the interaction between α-CD and the included PCL chain.
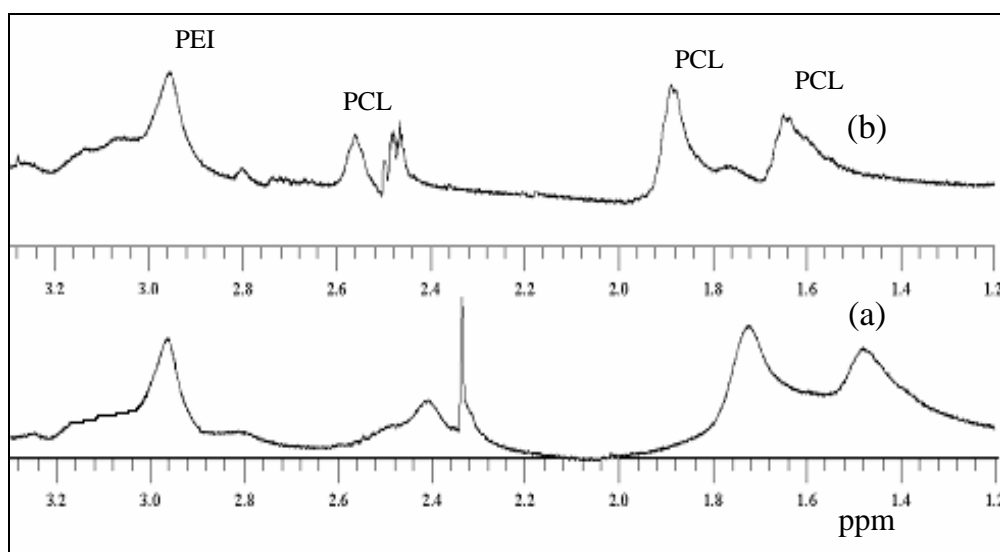

Figure 2
^{1}H NMR spectra of *hy*-PEI25k-g-(PCL580-b-PEG5k)$_{2.9}$ (a), inclusion complex of α-CD and *hy*-PEI25k-*g*-(PCL2k-*b*-PEG2k)$_{2.8}$, in D$_2$O prepared under its cmc (b).
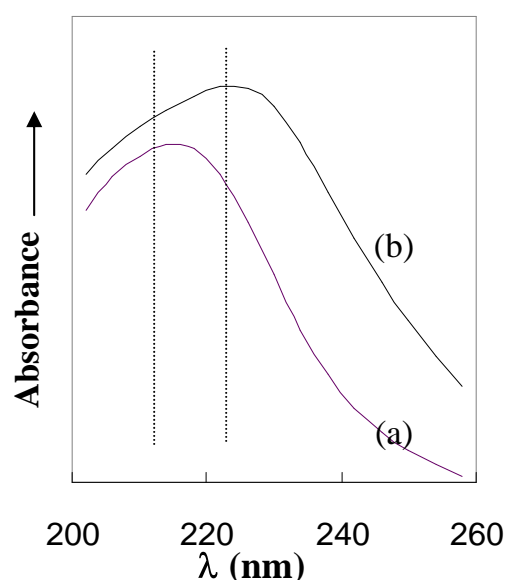
Figure 3
UV spectra of *hy*-PEI25k-*g*-(PCL580-*b*-PEG5k)$_{2.9}$ (a), and its inclusion complex (b), in water.

Ethidium bromide (EtBr) exclusion assay

Fluorescence of ethidium bromide added to polyplex solutions was determined to monitor DNA condensation. While free ethidium bromide in solution only shows weak fluorescence, fluorescence intensity strongly increases when it intercalates with DNA. If the DNA is condensed by a polycation, EtBr cannot intercalate with DNA. Therefore, condensation of DNA by polycations can be monitored by the decrease of EtBr fluorescence. As shown in Figure 4, increasing amount of PEI 25 kDa and inclusion complex both led to a significant decrease in relative fluorescence intensity from 100% to 26% until N/P ratio reached 2. By contrast, the relative fluorescence intensity was only slightly decreased to 90% at this N/P scale when DNA was condensed with *hy*-PEI25k-*g*-(PCL1.2k-*b*-PEG2k)$_{5.1}$. Apparently, PEI 25kDa and the inclusion complexes are much more efficient for DNA condensation, in comparison with the non-α-CD-complexed copolymers.
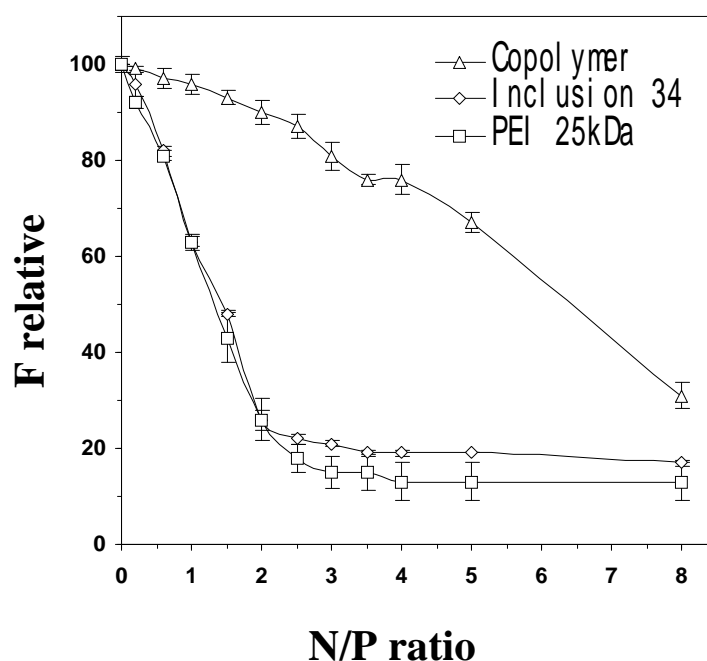
Figure 4
Condensation potential of *hy*-PEI 25kDa, *hy*-PEI25k-*g*-(PCL1.2k-*b*-PEG2k)$_{5.1}$ and its inclusion complex with α-CD (Inclusion 34), as determined by EtBr assay

Size, zeta-potential and biocompatibility of polyplex polyplexes

The particle size and ξ-potential of various pDNA/copolymer complexes formed at an N/P ratio of 7 are summarized in Table 1. Homo PEI 25kDa formed the smallest polyplexes of approximately 160 nm in diameter indicating the best compaction and condensation of DNA molecules with the high molecular weight PEI. For our polymers it is noteworthy that threading of α-CD does not increase the particle size of polyplexes, i.e. polyplex sizes detected are all around 200 nm no matter whether α-CD was incorporated or not. Since all polyplexes were prepared by controlling the copolymer concentration below its critical micellization concentration, the particle size should be mainly affected by complexation of DNA and copolymer. This means that the particle size is affected not only by the DNA loading but also by the particle density. The insoluble micelle core rather than the soluble micelle shell should contribute to the micelle size.

Composition	Copolymer			Inclusion complex		
	PEI (%)	Size (nm) (± SD)	Zeta potential (mV)	PEI (%)	Size (nm) (± SD)	Zeta potential (mV)
PEI25k	100	164.6 ± 5.4	20.9 ± 2.7	nc[b]	nc[b]	nc[b]
PEI25k-g-(PCL580PEG5k)$_{2.9}$	61	207.8 ± 6.2	-0.4 ± 1.9	40	208.5 ± 7.2	0.3 ± 1.8
PEI25k-g-(PCL2kPEG2k)$_{2.8}$	69	234.4 ± 9.8	-3.5 ± 0.3	26	217.5 ± 9.6	-1.5 ± 1.9
PEI25k-g-(PCL1.2kPEG2k)$_{5.1}$	61	208.4 ± 8.6	-3.0 ± 0.9	34	222.5 ± 12.7	1.4 ± 1.4

Table 1
DNA complexes formed respectively with net copolymers and copolymer/α-CD complexes at N/P 7[a]
[a]Detected with dynamic light scattering. [b]No detectable inclusion with α-CD.

As expected, the ξ–potential of the polyplex with *hy*-PEI 25kDa is relatively high (+ 21mV) suggesting high positive charge on the particle surface. In contrast, polyplexes based on both copolymer and inclusion complexes apparently possess approximately neutral surfaces (-3.5 to +1.4 mV ξ–potential). Since it is well known that low ξ–potential usually correlates with low cytotoxicity, polyplexes based on copolymer and their inclusion complexes with α-CD should be much more biocompatible than polyplexes with high molecular weight PEI alone. This notion was also supported by the viability of NIH/3T3 cells observed during the transfection experiments, as shown in Table 2. At N/P ratios of 3 and 7, cells remained viable when transfected with complexes based on the copolymers and their inclusion complexes. By contrast, cell viability dropped to 80 % and 69 % respectively when transfected with the PEI 25kDa-based polyplexes. As the N/P ratio reached 20, all cells were killed. However, cells retained about 50% of their viability when transfected with

copolymer-based polyplexes. Furthermore, cell viability is even higher ($> 80\%$) when cells were transfected with inclusion complex-based polyplexes at N/P 20, suggesting that incorporation of α-CD can further improve the biocompatibility of these gene delivery systems.

Gene transfection efficiency of polyplexes

The transfection efficiencies in NIH/3T3 cells with polyplexes based on three copolymers and their inclusion complexes are shown in Figure 5. All inclusion complexes showed a significant increase in transfection efficiency compared to their copolymer counterparts. Although *hy*-PEI25k-*g*-(PCL580-*b*-PEG5000)$_{2.9}$ was soluble in a wide concentration range and showed quite good performance in DNA condensation and transgene expression [13], threading of α-CD onto the PCL blocks may further enhance the gene transfection of this copolymer-based polyplex by further reducing the hydrophobic hindrance of DNA binding to PEI. Effect of incorporating α-CD on the transgene transfection of polyplexes of the two other copolymers is even much more significant. Surprisingly, at N/P 3 and 7, although *hy*-PEI25k-*g*-(PCL1200-*b*-PEG2000)$_{5.1}$ did not transfect the cells at all presumably due to its insufficient loading of DNA [13], the polyplex based on the inclusion complex of this copolymer exhibited excellent transfection efficiency. In Figure 4, it is especially noteworthy that biocompatible inclusion complexes formed polyplexes showing a transfection efficiency in the same range than that of more cytotoxic PEI 25 kDa when N/P ratios of 7 and 20 are regarded.
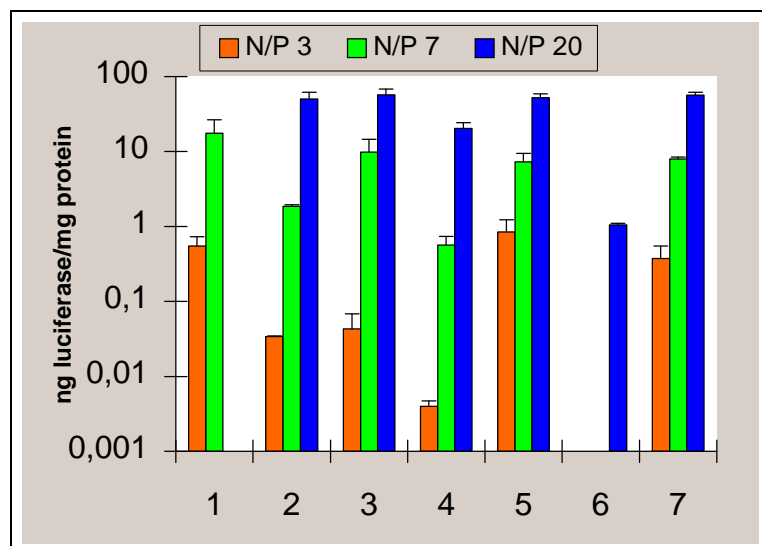
Figure 5
Transfection efficiency of polyplexes based on: (1) *hy*-PEI 25kDa, (2) *hy*-PEI25k-*g*-(PCL580-*b*-PEG5k)$_{2.9}$, (3) inclusion complex of PEI25k-*g*-(PCL580-*b*-PEG5k)$_{2.9}$, (4) *hy*-PEI25k-g-(PCL2k-*b*-PEG2k)$_{2.8}$, (5) inclusion complex of *hy*-PEI25k-*g*-(PCL2k-*b*-PEG2k)$_{2.8}$, (6) *hy*-PEI25k-*g*-(PCL1.2k-*b*-PEG2k)$_{5.1}$, and (7) inclusion complex of *hy*-PEI25k-*g*-(PCL1.2k-*b*-PEG2k)$_{5..1}$

	Cell viability* (%)					
POLYMER	**N/P = 3**		**N/P = 7**		**N/P = 20**	
	Polymer	**IC^b**	**Polymer**	**IC**	**Polymer**	**IC**
PEI25kDa	80		69		0	
PEI25k-g-(PCL580PEG5000)$_{2.9}$	99	100	94	91	46	83
PEI25k-g-(PCL1200PEG2000)$_{5.1}$	100	100	95	100	45	97
PEI25k-g-(PCL2000PEG2000)$_{2.8}$	91	100	90	88	41	84

*calculated based on the protein level detected. ^{b}DNA complexes prepared with inclusion complexes.
Table 2
Cell viability in transfection experiments with polymers and inclusion complexes

Intracellular uptake of polyplexes

To study the intracellular uptake and distribution of DNA incorporated into polyplexes, DNA was labeled with YOYO-1 and Cy3, respectively, for flow cytometry analysis and confocal laser scanning microscopy observation.

Fluorescence of cells treated for 30 min with copolymer- and its inclusion complex-based polyplexes was analyzed by flow cytometry and compared with that of the untreated cells. As shown in Figure 6, incorporation of α-CD to the

polyplex led to a approximately 10-fold enhancement of cellular fluorescence compared with cells identically treated with the α-CD-free polyplex. As it is well known that particle size is a critical factor affecting cellular uptake, and DLS measurements have already demonstrated the sizes of α-CD-containing and free polyplexes are almost the same. The enhanced intracellular uptake with the inclusion complex-based polyplexes should be mainly due to the enhanced DNA condensation, as already revealed by the EtBr exclusion assay. Further investigations are carried out to determine whether the incorporated α-CD itself has any positive effects on the cellular uptake of polyplexes. We are especially interested whether the incorporated α-CD molecules are helpful for cell targeting, cell membrane binding and permeation of gene delivery vectors. Similar differences for cellular association were obtained for the the other inclusion complexes and their a-CD-free counterparts (data not shown).

After incubating NIH/3T3 fibroblasts for 3 h, a typical confocal laser scanning microscope image of a polyplex is shown in Figure 7. The microscopic image demonstrates the efficient internalization of polyplexes, in spite of their neutral surface charge. Both DNA and *hy*-PEI-*g*-PCL-*b*-PEG colocalize in punctuate vesicles, probably in the lysosomal compartment. The mechanism by which the polyplexes escape from the endo/lysosome is presently under investigation. Most likely, the *hy*-PEI component of the copolymer acts as proton sponge, while inclusion complex formation modulates complex solubility and cytotoxicity.
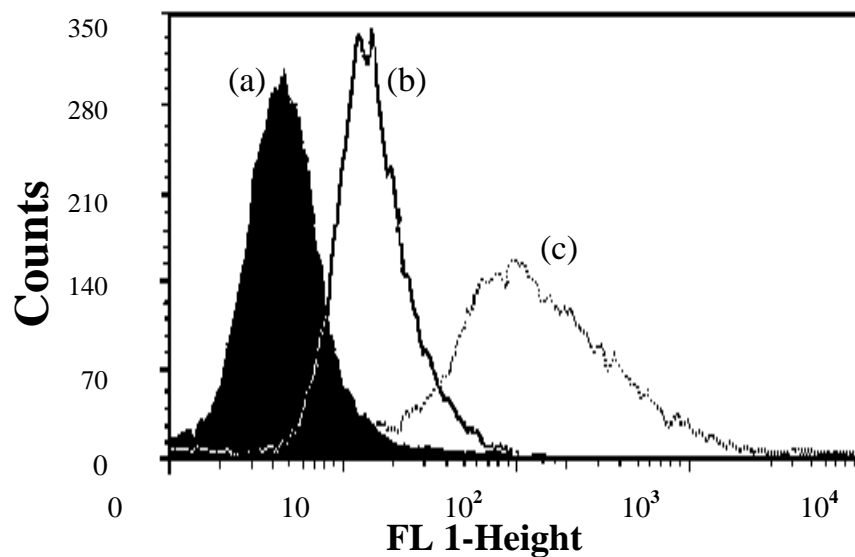
Figure 6
Flow cytometry of untreated cells (a), cells treated for 30 min with the YOYO-1-labeled pDNA/*hy*-PEI25k-*g*-(PCL1.2k-*b*-PEG2k)$_{5.1}$ (b) and the corresponding inclusion complex with α-CD (c)
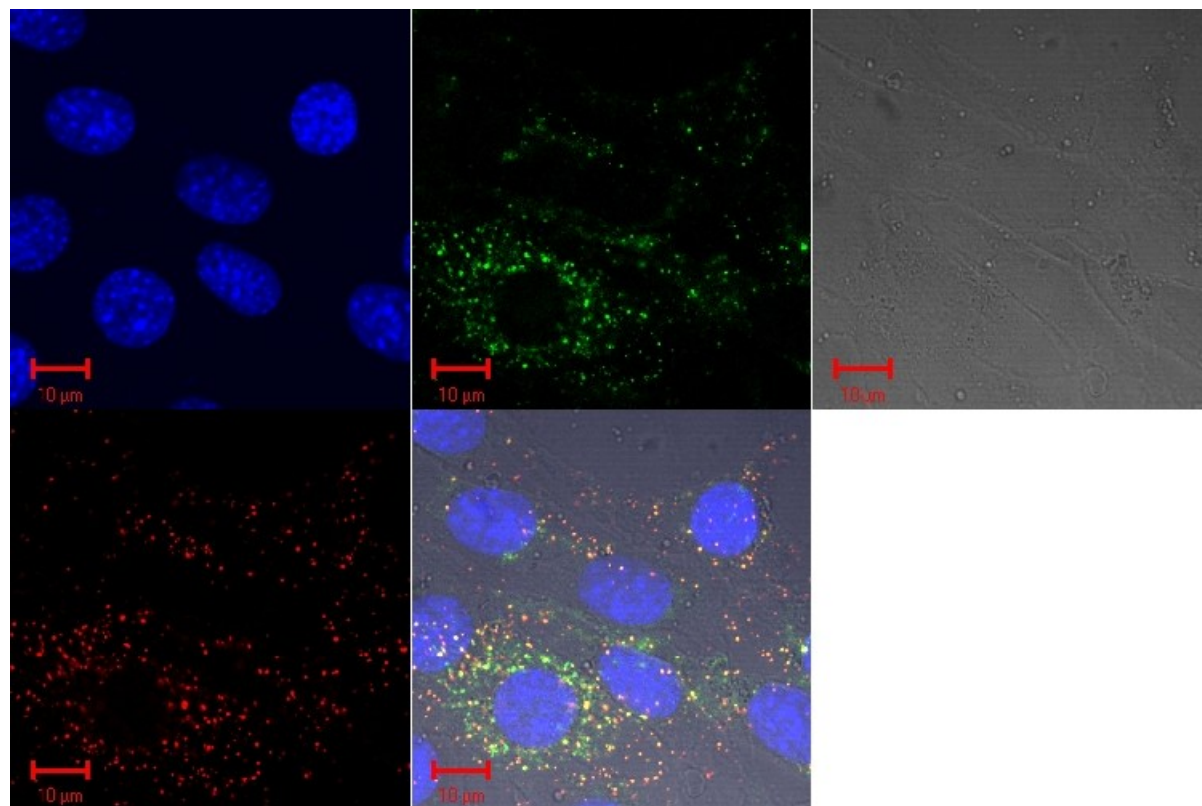
Figure 7
CLSM micrographs of internalization of fluorescently-labeled polyplex based on inclusion complex of *hy*-PEI25k-*g*-(PCL2k-*b*-PEG5k)$_{2.8}$ into 3T3 fibroblasts after 3 h of incubation. Images assignment: Red: DNA, green: inclusion complex, and blue: nucleus

CONCLUSIONS

The transfection efficiency of polyplexes based on *hy*-PEI25k-*g*-PCL-*b*-PEGs depends on composition, i.e. graft density and block length, of the copolymers. The PCL chains connected to the PEI blocks of *hy*-PEI25k-*g*-PCL-*b*-PEGs may impede the access and binding of hydrophilic DNA to PEI. Consequently, DNA compaction and condensation are poor. In addition, polyplexes based on copolymers were probably loaded with very low content of DNA because the copolymers possess low values for cmc that tends to further decrease upon complexation of DNA. Therefore, polyplexes based on *hy*-PEI25k-*g*-PCL-*b*-PEGs showed very poor gene transfection efficiency, especially when the PCL blocks are long and/or the graft density is high. Upon formation of inclusion complexes between α-CD and preferably the PCL blocks of *hy*-PEI25k-*g*-PCL-*b*-PEGs, the copolymers lost their amphiphathy and became more hydrophilic. This change on one hand favored the DNA access and binding to PEI. On another hand, when the solubility of a copolymer increased, it probably tended to complex more DNA molecules until a new critical micellization concentration is reached due to the electrostatic neutralization of the PEI head and DNA. Both effects led to the enhancement of DNA complexation and condensation resulting in very high gene transfection efficiency.

To further evaluate the high gene transfection efficiency of these novel delivery systems, we are carrying out *in vivo* gene transfection studies.

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6

Investigation of *in vitro* and *in vivo* properties of electrostatic complexes prepared with novel *hy*-PEI-*g*-PCL-*b*-PEG polymers with α-cyclodextrin inclusion and plasmid DNA

SUMMARY

We investigated several properties of electrostatic complexes prepared with plasmid DNA and novel *hy*-PEI-*g*-PCL-*b*-PEG polymers with α-cyclodextrin threaded over PEG and PCL chains.

The ability of these polymers to complex DNA was investigated by an ethidium bromide exclusion assay and atomic force microscopy was performed to obtain insights into complex size and shape. Complex stability against heparin was investigated, as well as protection of complexed plasmid DNA against degradation by DNase I. Furthermore, we investigated the pharmacokinetic profile of plasmid DNA complexed to these polymers. This data was compared to profiles gathered from PEI 25 kDa after intravenous injection in mice. Organ accumulation of both types of complexes was assessed after 30 minutes.

Our data reveal an efficient complexation of DNA by inclusion compounds resulting in the formation of defined, spherical particles. DNA release after incubation with polyanions was less efficient and commenced at higher anion concentrations than in the case of PEI 25 kDa indicating a greater stability against exchange reactions with anions. Protection of plasmid DNA against degradation by DNase I is as efficient as that of PEI 25 kDa for one inclusion compound tested in this study. Pharmacokinetic profiles of DNA complexed with either PEI or Inclusion compounds reveal an elevated area under the curve for the latter compared to PEI 25 kDa. Organ distribution after 30 minutes revealed a negligible accumulation in the lung, when inclusion complexes were used together with a very low *in vivo* toxicity. PEI, in contrast, was characterized by a high level of lung accumulation as well as a high rate of mortality among laboratory animals.

In conclusion this study provides evidence for high stability of inclusion compound/DNA complexes combined with elevated *in vivo* circulation times and an improved biocompatibility.

INTRODUCTION

Gene therapy is an evolving field with a tremendous potential for the causal treatment of yet incurable diseases, especially cancer or viral infections [1]. A variety of functional gene constructs have been designed which pursue different therapeutic strategies, e.g. cancer vaccination [2], suicide gene therapy [3] or downregulation of gene expression via ribozyme or siRNA constructs [4-6]. However, before the full potential of these techniques can be exploited, a safe *and* efficient delivery system must be developed. Viruses exhibit a very high efficiency, however their major disadvantage is an oncogenic and especially an immunogenic potential after repeated administration, as has been demonstrated by several lethal incidents in gene therapy clinical trials [7]. To circumvent this problem, alternatives must be found which also possess the capability to deliver DNA across systemic and cellular barriers. Cationic polymers, such as PEI [8], poly(L-lysine) [9] or starburst dendrimers [10], have been frequently used to deliver DNA, as well as RNA into cells. Among these polymers, different polyethylenimines have attracted particular attention, due to their enhanced ability to complex DNA and protect it from degradation [11] as well as their built-in endosomolytic activity [12] both of which lead to high levels of reporter gene expression *in vitro*. One major problem of these systems, however, is a limited *in vivo* applicability resulting from the highly cationic surface charge. The charge of complexes leads to incalculable interactions with vessel endothelia, plasma proteins and cellular blood components when injected intraveneously [13,14]. Furthermore, the circulation time of these vector systems are fairly short [15] and, therefore, are not suitable for a therapeutic systemic application. Several strategies have been developed to shield the cationic surface charge of the complexes using hydrophilic polymers, such as PEG or pHPMA [13,15-17], which aim to achieve a similar stealth-like effect as has been described for liposomes [18]. Complexes with these modified polymers exhibited favorable *in vitro* properties, such as a neutral surface

charge, lower toxicity and less interactions with blood components [13,15,17,19]. *In vivo* evaluations displayed elevated circulation time for these systems in some cases [13,15,20]. However, a delivery system that combines a high transfection efficiency with a high stability, as well as long circulation times and a high level of biocompatibility, is still not available.

Recently, we described the synthesis and *in vitro* evaluation of novel, biodegradable *hy*-PEI-*g*-PCL-*b*-PEG polymers with α-cyclodextrin inclusion [21,22]. A scheme of the formation of inclusion polymers is depicted in Figure 1. The α-cyclodextrin is threaded over PEG and PCL chains in these polymers by applying ultrasonic agitation. Since the inner part of cyclodextrin is hydrophobic, cyclodextrin molecules slide over the PEG chains and attach to polycaprolactone via hydrophobic interactions. Successful threading and attachment of cyclodextrin predominantly to polycaprolactone was verified by ^{1}H NMR, UV and FTIR spectroscopy [22,23].

COMPOSITION OF BASIS POLYMER	Name	PEI content [%]
PEI25k-g-(PCL580PEG5k)$_{2.9}$	"Inclusion 40"	40
PEI25k-g-(PCL2kPEG2k)$_{2.8}$	"Inclusion 26"	26
PEI25k-g-(PCL1.2kPEG2k)$_{5.1}$	"Inclusion 34"	34

Table 1
Composition of polymers investigated in this study.

The resulting hypermolecular inclusion polymers are capable of forming micellar complexes with plamid DNA. When complexed, the micellar structures displayed high levels of reporter gene expression combined with a neutral surface charge and low toxicities. In the present publication, we aimed to gain insight into the stability of this novel type of complexes species and to estimate if these systems possess potential for *in vivo* application. We investigated these novel vector systems with regard to their ability to complex DNA in

comparison to PEI 25 kDa. We also characterized complex size and shape using atomic force microscopy. Furthermore, we assessed complex stability against polyanions and determined whether inclusion polymers are able to protect pDNA against DNase I degradation. Finally, we performed *in vivo* studies investigating pharmacokinetic profiles and organ distribution after 30 minutes. A list of polymers used in this study and their structures is given in Table 1.
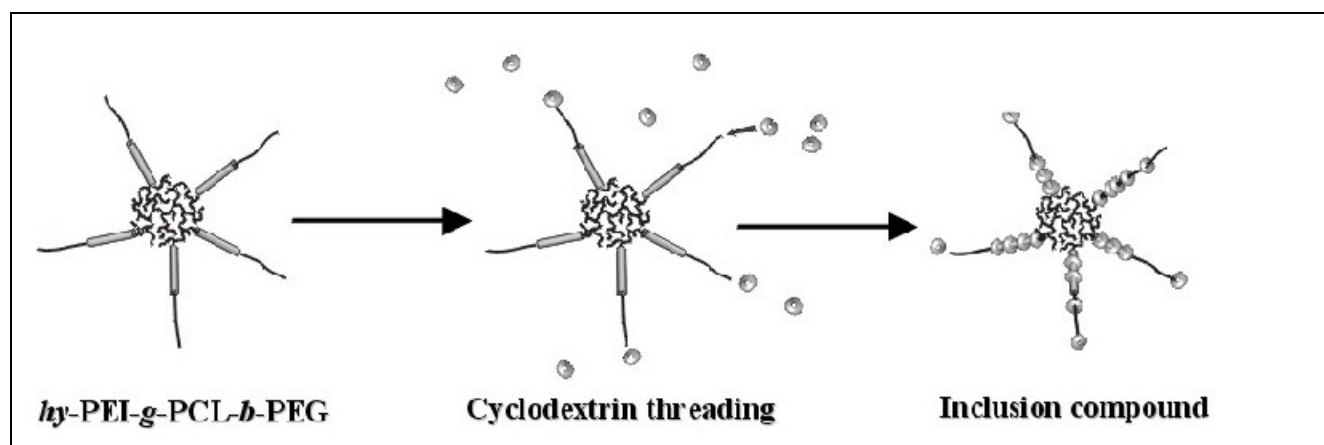
Figure 1
Scheme of inclusion compound formation: a-cyclodextrin threads over linear PEG and locates predominantly on PCL chains due to hydrophobic interactions. Primary localization on PCl chains was demonstrated by spectroscopic methods as described in chapter 5 of this dissertation.

MATERIALS AND METHODS

Polymer synthesis and threading of α-cyclodextrin

hy-PEI-*g*-PCL-*b*-PEG polymers were synthesized as described previously [21]. Subsequently, Inclusion compounds were prepared by stepwise addition of a-cyclodextrin and ultrasonic agitation [22]. Successful a-cyclodextrin threading was verified by FTIR, UV, and ^{1}H-NMR spectroscopy as described earlier [22,23].

Complex formation

All complexes were prepared in glucose 5 % pH 7.3. For the determination of complex stability against heparin 1 µg pCMV-Luc was diluted in 50 µl glucose 5 % and added to the corresponding amount of polymer in the same volume.

For atomic force microscopy, complexes were prepared using 2 µg of pCMV-Luc in 50 µl glucose 5 % and the corresponding amount of polymer in the same volume. For animal experiments, complexes were prepared using 50 µg of pCMV-Luc in 125 µl and the corresponding amount of polymer in 125 µl glucose 5 %, as well. From this solution 120-130 µl were injected into mice corresponding to approximately 25 µg of pDNA per mouse.

Fluorescence quenching assay

DNA condensation was measured by the decrease in ethidium bromide fluorescence, as described earlier [24]. The assay was performed in 96 well plates in triplicate. Eight µg of salmon testes DNA was dissolved in 79 µL water and added to 50 µL of 60 mM Tris buffer pH 7.4. Volumes were equalized to 300 µL with water. Subsequently, appropriate volumes of 0.05 mg/mL polymer solutions were added to each well to produce N/P ratios between 0.2 and 8. These were incubated for 10 min and then 20 µL of a 0.1 mg/mL ethidium bromide solution were added. Wells were mixed thoroughly and the fluorescence was measured using a fluorescence plate reader with excitation wavelength of 518 nm and an emission wavelength of 605 nm.

Atomic force microscopy

A Multimode™ AFM (Veeco Instruments ™, Santa Barbara, USA) operating with a NanoScope IIIa™ controller and a size E scanner was used throughout this study. Silicon nitride, oxide sharpened, triangular cantilevers (Veeco Instruments™) were selected, operating at resonant frequencies of approximately 8 kHz. All experiments were conducted in tapping mode, with either 256 x 256 or 512 x 512 pixel resolution. Scan speeds varied between applications. To reduce sample distortion, all data were acquired using optimum set-points and drive amplitudes aimed at minimising tip-sample interaction. Gains and scan-rate were adjusted to optimize the images obtained. Post-imaging analysis was carried out on NanoScope IIIa™ software, version

5.12b48. The background slope was removed using a first or second order polynomial function.

Complex stability against exchange reactions with anions

Complexes were formed in septuplet, as described above, in a total volume of 100 µL sodium chloride solution at an N/P ratio of 7. To these solutions, 0.01, 0.03, 0.05, 0.1, 0.2, 0.5 and 1 International Units of heparin were added in 10 µL 0.9 % sodium chloride. These solutions were mixed well and incubated for 10 min before approximately 45 µl were applied to a 1 % agarose gel containing ethidium bromide. In the eighth lane of each gel, plasmid was applied as a reference. Gels were run for 50 min at 100 V and then scanned using a Biometra gel analyzing system.

Stability against DNase digestion

Complex stability was investigated according to a method described earlier [11]. Briefly, complexes were prepared at N/P=7 in glucose 5 % using 5 µg of pCMV-luc in a total volume of 25 µl. Aliquots of 5 µl corresponding to 1 µg of plasmid were incubated with 0.0001, 0.01, 0.1, 1 and 5 international units (I.U.) of DNase I in digestion buffer (0.1 M sodium acetate, 5 mM $MgSO_4$ pH 7.4) for 15 minutes at 37 °C. Subsequently, 6 µl termination buffer (equal volumes of 0.5 M EDTA, 2 M NaOH and 0.5 M NaCl) were added, as well as 2 µl of a heparin solution containing 1000 I.U. per ml. A positive control reaction containing naked DNA was carried out under the same conditions using 5 I.U. DNase I. Resulting mixtures were applied to a 1 % agarose gel and electrophoresed at 100 V for 1 hour. The resulting gel was imaged on a BioRad transilluminator.

Radioactive labeling of plasmid

pCMV-Luc was radioactively labeled by incorporation of ^{32}P-dCTP (Redivue, Amersham Pharmacia, Freiburg, Germany) using a Nicktranslation Kit

(Amersham Pharmacia, Freiburg, Germany) following a protocol provided by the manufacturer. Unincorporated nucleotides were carefully removed using Autoseq spin columns (Amersham Pharmacia, Freiburg, Germany) in two subsequent steps. Plasmid purity was verified via size exclusion chromatography using PD-10 columns and via ultracentrifugation using Microcon 10 spin columns (Amicon, Beverly, USA). No significant amounts of free ^{32}P-dCTP were detected.

Investigation of pharmakokinetics and organ distribution in mice

All animal experiments followed the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and were approved by external review committee for laboratory animal care. Male balb/c mice with a body weight of approximately 25 g were anaesthetized by injection of ketamine (Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and xylazine (Rompun, Bayer AG, Leverkusen, Germany). Complexes were injected as a bolus of approximately 120 µL through the jugular vein. Blood samples were obtained through a catheter in the common carotid artery and urine was sampled by flushing the bladder with sodium chloride solution through a 2-way catheter. After 120 minutes mice were sacrificed by decapitation and organs (liver, kidneys, heart, lungs, spleen, fatty tissue and vena jugularis) were weighted and sampled.

Radioactivity of all samples was measured on a 1277 Gammamaster (Perkin Elmer Wallac, Freiburg, Germany). Measurements of complex solutions were used to determine the injected dose of radioactivity. DNA concentrations in the samples were then calculated as the percent of injected dose (%ID), %ID/mL or %ID/g, respectively. An unpaired t-test was performed using Microcal Origin Version 6.0 to compare blood levels of different copolymers at corresponding time points. Differences were considered significant if two-tail $P = 0.05$.

Non-linear curve fitting

Concentration time curves were fitted to a two compartmental model with the Software Kinetica 1.1 from Simed. The model used was $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ and the weighting applied was $1/(c_{calc})^2$. Each concentration time curve was fitted individually for all samples, as well as the mean of the concentrations for each time point. Pharmacokinetic parameters provided are composed of the mean of the parameters calculated from individual fits. a and ß values were transformed to half-life periods. Plots shown in the results and discussion section are fits for the mean concentrations with the standard deviation for each time point shown.

RESULTS AND DISCUSSION

Fluorescence quenching assay

Ethidium bromide exhibits fluorescence only when intercalated with DNA. When polymer is added to DNA/ethidium bromide it excludes ethidium bromide from DNA thus reducing the fluorescence signal. The degree of reduction of fluorescence can be regarded as a measure for the ardency with which the polymer complexes DNA. Fluorescence exclusion curves obtained with the three inclusion compounds and PEI 25 kDa are depicted in Figure 2. This assay revealed a very efficient complexation of DNA by inclusion 34 which was almost similar to that of PEI 25 kDa. Inclusion 26 and Inclusion 40 display a less efficient DNA condensation. It seems that this implies that a higher number of short PCL-PEG chains with a-cyclodextrin threading, as in the case of Inclusion 34, leads to more efficient interactions with DNA than a smaller number of long chains. It is astonishing that Inclusion 26 is more efficient in DNA complexation than Inclusion 40, although the latter has a higher PEI content. The reason for this finding is probably the smaller size of linear PEG chains of Inclusion 26 and therefore less steric hindrance.
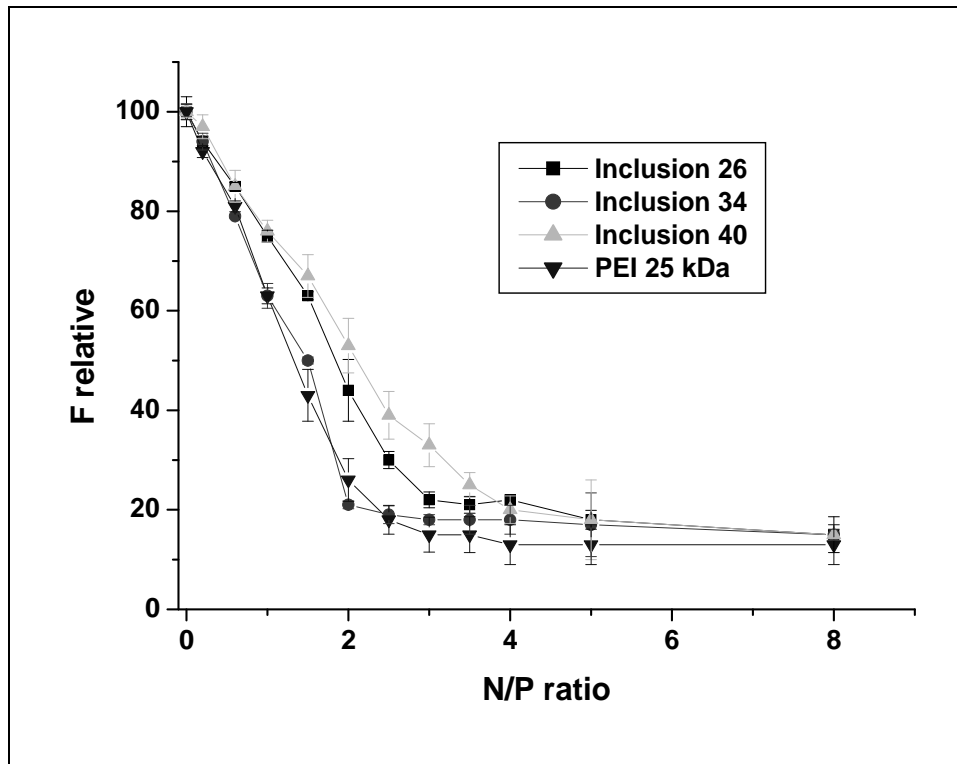
Figure 2
Ethidium bromide exclusion assay of inclusion compounds and PEI 25 kDa as a reference.

Atomic force microscopy

Images of complexes between inclusion compounds and DNA, as well as PEI/DNA complexes, are shown in Figure 3. All polymers formed defined, spherical complexes with plasmid DNA. The shape and size of complexes prepared with PEI 25 kDa and inclusion compounds were only slightly different. Sizes ranged from about 80 to 120 nm, which is suitable for endocytic cellular uptake. Sizes determined via AFM are significantly smaller than those measured via photon correlation spectroscopy [22]. A likely explanation for this finding is that complexes were prepared in glucose in this study, whereas they were prepared in sodium chloride 150 mM in the previous study. A size reduction of complexes prepared in glucose 5 % as compared to sodium chloride 150 mM has been observed earlier ([25] and own unpublished data).
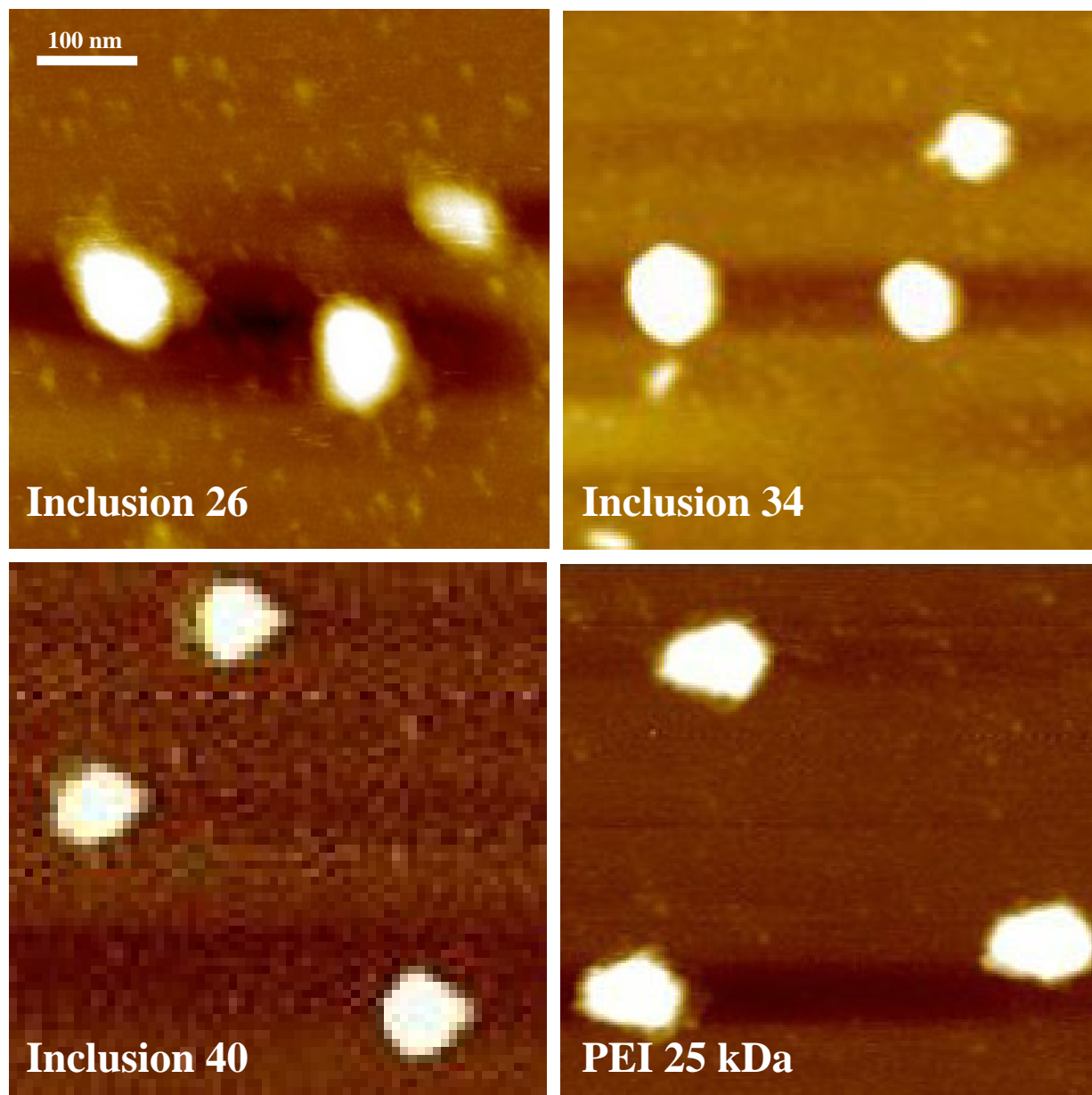
Figure 3
Atomic force microscopy images of inclusion compound/DNA complexes and PEI/DNA at N/P ratio 7. All polymers are capable of forming defined, spherical particles.

Complex stability against polyanions

Complex stability was determined by adding increasing amounts heparin. Heparin is a highly polyanionic polymer capable of displacing DNA from polymer/DNA complexes at a certain concentration. In the case of PEI/DNA complexes, total DNA release occurred at a defined concentration. Inclusion compound/DNA complexes, however, showed an incomplete release at all concentrations tested and smeared bands can be observed. Remarkably, DNA

release from the inclusion compound 34 starts at a twofold higher concentration than PEI 25 kDa, indicating a substantially higher stability of these complexes compared to PEI/DNA. The reason for this higher stability is presumably a hindered penetration of the highly anionic and thus highly hydrophilic heparin molecules through the PEG-PCL/cyclodextrin layer surrounding the core of complexes. Since Inclusion 34 is the polymer with the highest graft density, it can be assumed that a higher amount of PCL-PEG chains leads to enhanced stability of complexes.
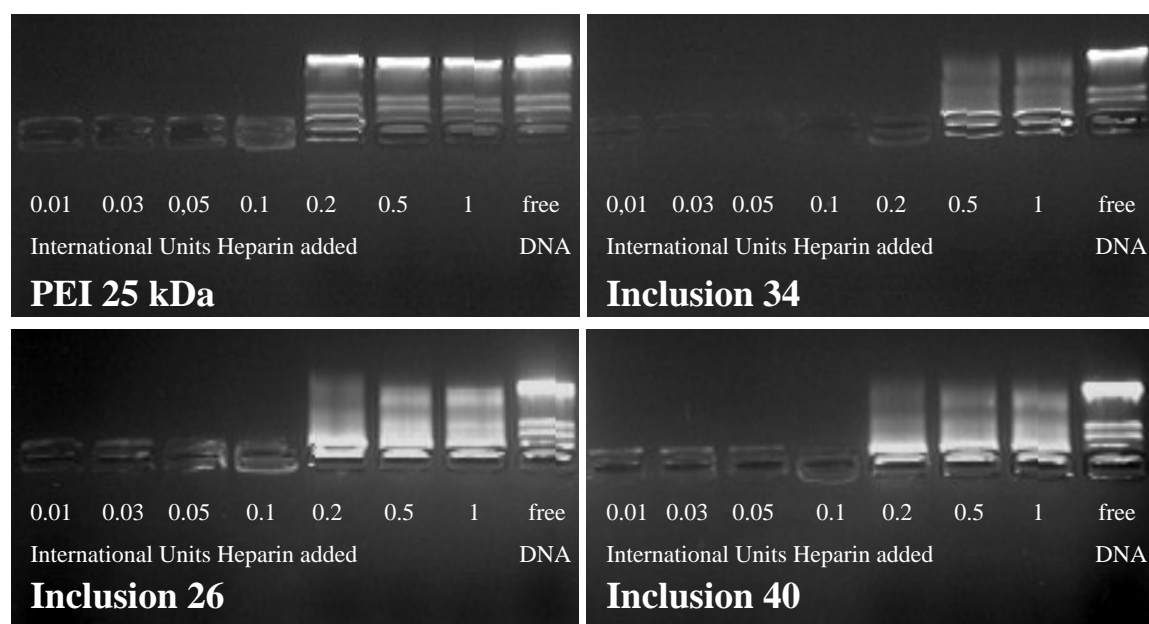
Figure 4
Investigation of complex stability against polyanionic exchange reactions. Complexes prepared in an identical way were incubated with increasing amounts of heparin. Obviously Inclusion 34 forms the most stable complexes with DNA, since release in this case takes place at a higher concentration compared to all other polymers investigated. All gels with inclusion polymers suggest that release of DNA is less complete than when regarding PEI 25 kDa.

DNA protection against DNase I digestion

Gels containing DNA from complexes incubated with increasing amounts of DNase I are shown in Figure 5. Digestion of pDNA starts at the same DNase I concentration of one international unit in all gels. However, analysis of the degree of digestion shows that complete degradation can be only observed with Inclusion 26 and Inclusion 40. Inclusion 34 and PEI 25 kDa, in contrast, exhibit

very little degradation with a substantial amount of pCMV-Luc remaining intact. This data suggests that, depending on the polymer architecture, inclusion compounds can provide very efficient protection of complexed DNA against DNase attack. It is probable that the PCL-PEG chains with cyclodextrin threading erect an effective steric barrier against enzymatic degradation, especially when polymers feature a higher graft density, as with inclusion 34.
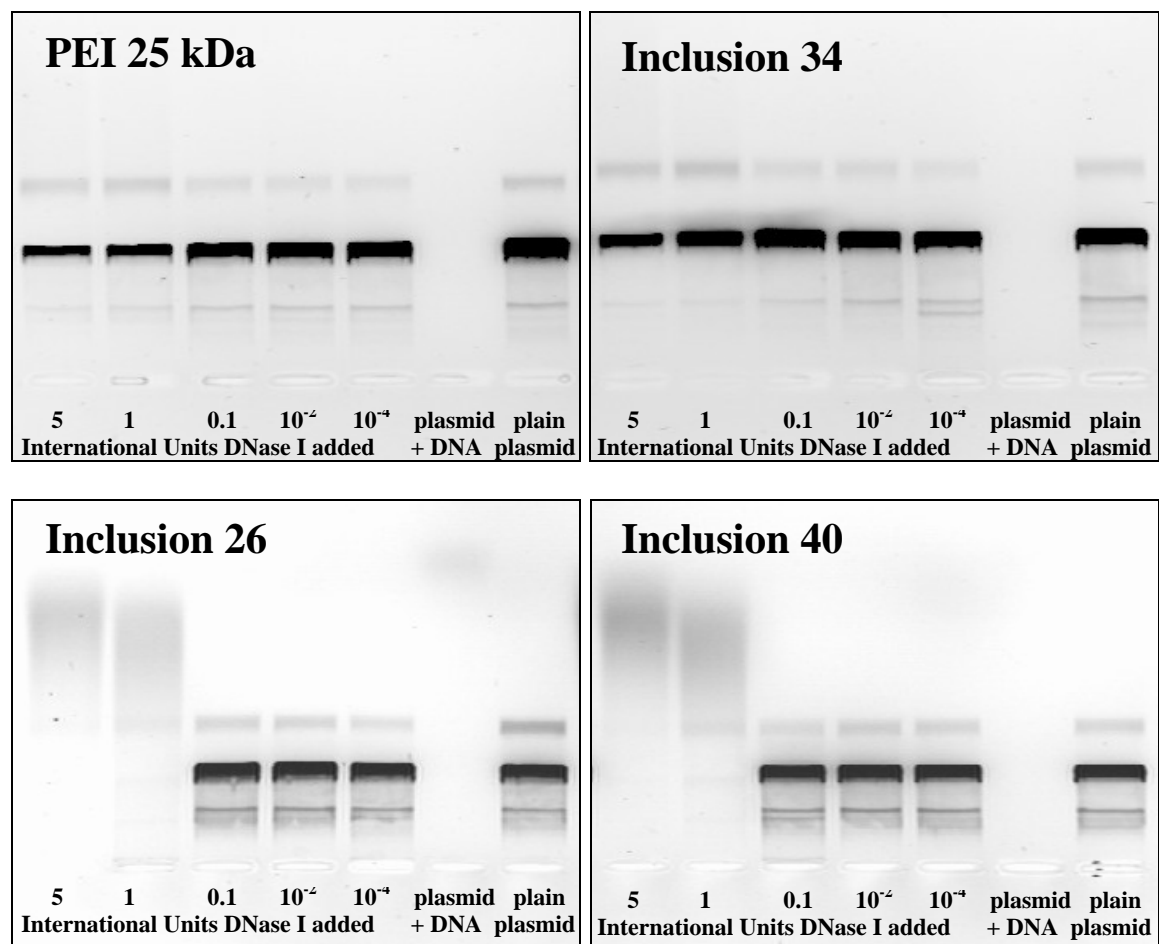
Figure 5
Investigation of DNA protection against DNase digestion using PEI 25 kDa (A) and inclusion polymers (B-D). Complexes were incubated with increasing amounts of DNase for 15 minutes and, after enzyme inactivation, DNA was released from complexes. Data suggests that Inclusion 34 offers a similar protection against DNase digestion than PEI 25 kDa. Inclusion 26 and 40 are less efficient and here no intact plasmid is existent any more at high enzyme concentrations.

Pharmacokinetic profiles over 30 minutes

Pharmacokinetic profiles from ^{32}P-labeled pDNA complexed with PEI 25 kDa and inclusion compounds are shown in Figure 6. Pharmacokinetic parameters for all polymers investigated are shown in Table 2. All graphs could be fitted to

a biexponential disposition equation by non linear curve fitting using Simed Kinetica. A very steep a-phase and a rather slow ß-elimination could be observed for all inclusion compounds and for PEI. Compared to PEI 25 kDa, all inclusion compound/DNA complexes show elevated AUCs, mainly arrising from a slower ß-elimination compared to PEI 25 kDa. Especially Inclusion 34 exhibits an area under the curve that is more than twofold higher than that of PEI 25 kDa. After 30 minutes approximately 10 % of the injected dose is still in circulation compared to approximately 4 % for PEI 25 kDa (if the total blood volume is assumed to be 1.7 ml). After 5 minutes Inclusion 34 and after 15 minutes Inclusion 26 and 40 show significantly enhanced blood levels. The reason for the prolonged circulation of these vector systems is probably their neutral zeta potential as described in an earlier publication [22]. The efficient shielding of the positive charge from polyethylenimine is provided by PCL-PEG chains with cyclodextrin inclusion. This presumably reduces opsonization of complexes and leads to a less pronounced uptake into the reticuloendothelial system (RES) of the liver, as described earlier [15].

Organ distribution after 30 minutes

Results from organ distribution assessed after 30 minutes are shown in Figure 6. Other tissues and organs investigated like heart, fatty tissue and the vena jugularis (site of injection) did not accumulate weighty amounts of radioactivity neither for PEI nor for any of the inclusion compounds. Data from PEI 25 kDa revealed a rather high and variable uptake into the lung. This accumulation in the lung is very likely caused by aggregate formation of the highly cationic PEI/DNA complexes with erythrocytes or plasma proteins and subsequent filtration of these associates in fine lung capillaries. This phenomenon is probably responsible for the high mortality of laboratory animals when using PEI 25 kDa as described earlier in the literature [13]. In the present study, about 25 % of laboratory animals died within 30 minutes after injection of PEI/DNA

(A)

(B)
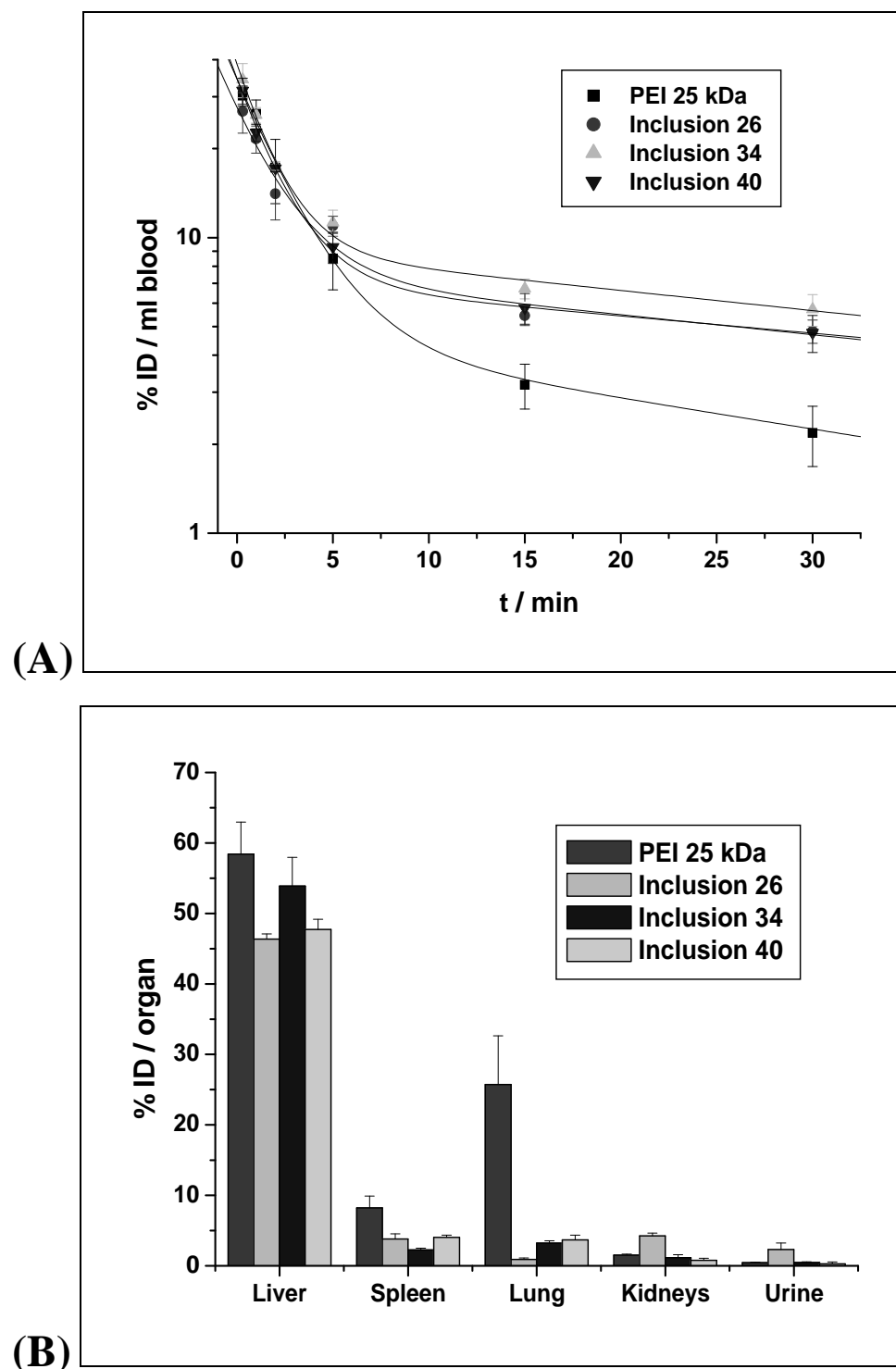
Figure 6
(A) Pharmacokinetic profile of ^{32}P labelled pDNA complexed to PEI 25 kDa and inclusion compounds. All inclusion compounds exhibit a significantly elevated AUC.
(B) Organ distribution of ^{32}P labelled pDNA complexed to PEI 25 kDa and inclusion compounds 30 minutes after injection. Most remarkable is the high and variable accumulation of ^{32}P labelled DNA complexed with PEI 25 kDa in the lung, something that could not be observed with inclusion compounds.

Polymer	AUC	A	$t_{1/2,alpha}$	B	$t_{1/2,beta}$
	[%ID*mL^{-1}*min]	[%ID/mL]	[min^{-1}]	[%ID/mL]	[min^{-1}]
PEI 25 kDa	300 ± 84	29.8 ± 2.3	1.8 ± 0.1	4.7 ± 1.7	38.2 ± 21
Inclusion 26	564 ± 122	20.1 ± 3.5	1.8 ± 0.4	7.5 ± 1.7	50.8 ± 20
Inclusion 34	699 ± 188	29.5 ± 4.1	1.4 ± 0.5	9.1 ± 1.8	53 ± 24
Inclusion 40	594 ± 72.9	27.4 ± 4.1	1.5 ± 0.4	7.1 ± 1.1	53 ± 9

Table 2
Pharmacokinetic parameters of ^{32}P labelled DNA complexed with inclusion compounds and PEI 25 kDa. Values were obtained by fitting concentration time curves to a biexponential disposition model using Simed Kinetika version 1.1. Figures shown were calculated from the pharmacokinetic data obtained from four animals.

complexes. An assessment of organ accumulation revealed very high ^{32}P values in the lung. This finding indicates that lung embolism may be the likely cause of death, as has been described earlier [13]. Lung accumulation of inclusion compound/DNA complexes on the other hand, was very low and none of the animals died during experiments. This difference is probably due to the neutral surface charge of inclusion complexes and, therefore, a reduced aggregate formation with cellular blood components or plasma proteins, preventing entrapment in fine lung capillaries. The negligible accumulation of complexes in the lung is a key advantage of such inclusion compounds. The largest fraction of radioactivity is found in the liver. PEI exhibits the highest hepatic values, whereas those of the inclusion compounds are slightly lower. The discovery of the liver as the main organ of complex deposition is not surprising and this finding is in good agreement with various studies using cationic polymers published earlier [15]. A fairly high amount of radioactivity from PEI complexes is found in the spleen. The reason for a higher deposition of PEI/DNA complexes in organs of the reticuloendothelial system is probably the result of a higher degree of opsonization of these highly cationic structures.

Several strategies have been pursued in order to obtain a better shielding of cationic surface charges and an extended circulation in the bloodstream. One frequently applied strategy is the attachment of hydrophilic polymers, such as polyethylenglycol or pHPMA, to polyethylenimine via different strategies [13,15-17]. The attachment of PEG to preformed PEI/DNA complexes led to increased circulation times and to reduced interactions with plasma proteins [13,17]. The major disadvantage of this system is a rather high operating expense for complex preparation and a fairly difficult characterization of the resulting structures. When PEI or poly(L-lysine) were pegylated prior to complex formation, neutral surface charges and enhanced circulation times were obtained as well [15]. However, these systems exhibited a reduced stability and evidence exists that suggests that complexes of this type separate shortly after injection into the bloodstream [26,27]. Another very promising group of vectors consists of electrostatic complexes stabilized via bioreversible crosslinking agents. The most frequently used system is that of disulfide bridges which are stable in the bloodstream [20,28]. On the subcellular level, however, the DNA can be released due to the reductive environment created by e.g. glutathion. Several of these vectors have shown greatly enhanced circulation times and a high degree of stability in the bloodstream. The major problem of these systems is the rather time consuming preparation of complexes on the one hand and a rather low or even entirely abolished reporter gene expression on the other [29]. When using this approach, a compromise between the degree of crosslinking required for stabilization in the bloodstream and that which is required for possible release at the subcellular level has to be found.

The system we describe in this publication can be used to prepare complexes with plasmid DNA in a simple one step procedure and polymers can be produced in bulk. As described above these new vectors are more stable against exchange reactions than those prepared with PEI 25 kDa and exhibit an excellent stability against DNase I digestion. The reason for this enhanced

stability, despite a rather low PEI content of 34 %, is probably due to a micellar assembly of the complexes with pDNA. A micellar complex structure has been suggested previously for PEG-PEI/oligonucleotide complexes, whereas in these structures PEI is believed to neutralize the anionic charge of DNA forming an insoluble complex core with PEG chains directed to the surface of the complexes [30]. This hydrophilic PEG corona leads to an efficient charge shielding and, thus, to reduced charge interactions and toxicity [19]. Furthermore, complexes are stabilized, due to a reduction in complex aggregation [19]. In the system described in this publication, a further stabilizing factor has been incorporated into the complex architecture, PCL-PEG. The hydrophobic polycaprolactone becomes partially hydrophilic when α-cyclodextrin is threaded over its linear chains. However, because these chains are not totally covered with α-cyclodextrin [22], hydrophobic regions still exist, which can interact with each other via hydrophobic interactions and, thus, provide a certain lateral stabilization of the micellar structure of complexes.

From our data, we can state that a higher number of shorter PCL-PEG chains leads to more highly stable vector systems. Furthermore, the shielding effect of these shorter chains is more effective than the longer chains, which is demonstrated by an increased circulation time of Inclusion 34, presumably due to reduced opsonization.

In conclusion, we can state that the approach described in this and in the previous chapter represents an easy, efficient method to obtain fairly stable vector systems. Furthermore they exhibit a high *in vitro* and *in vivo* biocompatibility, a high transfection efficiency, and elevated blood levels after intravenous injection. These properties make the hy-PEI-g-PCL-b-PEG/cyclodextrin vectors very attractive for further investigations. We are currently conducting more comprehensive studies with these vectors with the aim to optimize polymer properties, especially with regard to graft density and

chain length of PEG and PCL, as well as the amount of α-cyclodextrin threading.

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7

SUMMARY AND OUTLOOK

SUMMARY

In this dissertation several aspects of polymer based gene delivery were investigated. First, key issues in subcellular processing of electrostatic polymer/nucleic acid complexes were investigated and new insights into mechanisms involved in these processes were gained.

Secondly, a targeted gene delivery system was developed for the specific transfection of ovarian carcinoma cells. The resulting vector exhibited a high specificity for target cells combined with low unspecific transfection and toxicity. Furthermore, a novel type of gene delivery system was synthesized. This vector exhibited a high *in vitro* transfection efficiency and a very low *in vitro* toxicity as well as favourable *in vivo* properties, such as reduced toxicities. Another aspect that was studied in depth was the investigation of the stability of several electrostatic vectors *in vitro* and when applied intravenously.

Chapter 1 gives a detailed overview of cationic polymers as delivery systems for nucleic acid therapeutics against cancer. The current status quo concerning polymeric gene delivery is presented. Systemic and subcellular hurdles, as well as the most important strategies for cancer gene therapy, are described in depth.

In **Chapter 2,** key steps of the subcellular trafficking of PEI/nucleic acid complexes were investigated by confocal laser scanning microscopy and inhibitor experiments. Microscopic studies suggest that complexes are taken up into cells via adsorptive endocytosis. The major site of complex accumulation was identified as the lysosomal compartment by using an acidotropic dye. For the first time it was possible to monitor the release of PEI/nucleic acid complexes from vesicular compartments by using living cell microscopy. Obviously, only a very small fraction of the complex population is able to leave the endosomal or lysosomal compartment, whereas the vast majority remains within the vesicles for days at least. The application of an endosomal acidification inhibitor demonstrated the importance of vesicle acidification for

release. These findings were confirmed by performing transfection experiments in the presence of and without the acidification inhibitor. Results from these experiments demonstrated that pH decrease within the vesicle is essential for obtaining efficient reporter gene expression with PEI as a gene delivery agent.

In **Chapter 3**, the development of a highly efficient gene delivery system for the specific targeting of ovarian carcinoma cells expressing a particular epitope is described. An antibody with a high specificity for the target epitope was chosen as a targeting moiety. In order to reduce the size of the targeting moiety, antigen binding fragments (Fab´) were produced by digestion of the crystallizable fragment of the antibody. Conjugates of pegylated polyethylenimine and Fab´ were synthesized. It was postulated that the linear polyethylenglycol chains could be able to shield the cationic charge of polyethylenimine, thus decreasing the degree of unspecific interactions. The conjugate was capable of complexing DNA as efficiently as unmodified PEI. Complex stability against the polyanion heparin was similar, as well. Cell binding studies using flow cytometry revealed a high affinity binding of conjugate/DNA complexes to epitope expressing cell lines and a significantly lower degree of binding to a reference cell line. Transfection experiments in epitope expressing cell lines exhibited up to approximately 100fold higher gene expression compared to PEI or pegylated PEI. In non epitope expressing cell lines, however, gene expression obtained with conjugate/DNA complexes remained low. In order to further confirm the specificity of conjugate/DNA complexes transfection experiments were performed with free antigen binding fragments in the cell culture media. A concentration dependent decrease in reporter gene expression could be observed indicating that the specificity of conjugate/DNA complexes is due to Fab´-epitope interactions.

In **Chapter 4**, the *in vitro* and *in vivo* stability as well as the organ deposition of PEI/plasmid and PEG-PEI/plasmid complexes were investigated. While the stability of both types of complexes against polyanions was similar, striking

differences were found when the protective properties of complexed DNA against DNase digestion was investigated. Unmodified PEI 25 kDa was more effective in protecting DNA from degradation than PEG-PEI. Atomic force microscopy revealed a slightly different shape of complexes; PEG-PEI/DNA complexes exhibited a slightly irregular and rougher structure. However, no uncomplexed DNA was observed after complexation with both polymers. *In vitro* transfection experiments in blood revealed significant stability for complexes prepared with both polymers. However, the stability of pegylated polyethylenimine/DNA complexes was lower indicated by a more pronounced decrease of reporter gene expression when transfection efficiencies in blood were compared to that in sodium chloride solution. When the *in vivo* fate of complexes was investigated with different radioactive labels attached to polymer, as well as incorporated into DNA, significant differences were observed between PEI/DNA and PEG-PEI/DNA complexes. In the case of PEI/DNA, a similar pharmacokinetic profile was obtained for both polymer and DNA with a similar organ distribution after 2 h. Data for PEG-PEI/DNA, in contrast, revealed differences in blood levels and a different organ accumulation. In this case, the pharmacokinetic and organ distribution data of the complexed DNA was similar to that obtained with naked DNA, thus indicating a rapid separation of complexes. This study provided valuable information for the further development of polymeric vectors for *in vivo* use.

In **Chapter 5**, the development of a novel class of gene delivery systems is described. These vector systems are composed of triblock polymers containing hyperbranched PEI 25 kDa, polycaprolactone and polyethylenglycol. Due to the poor solubility of these compounds, supramolecular inclusion complexes were formed by threading a-cyclodextrin over the polyethylenglycol and polycaprolactone chains. Since the inner region of cyclodextrin is hydrophobic, it is a reasonable assumption that the cyclodextrin molecules were able to slide over the PEG chains and attach themselves to the polycaprolactone via

hydrophobic interactions. Successful threading and attachment of the cyclodextrin to the polycaprolactone was verified by ^{1}H NMR, UV and FTIR spectroscopy. The resulting inclusion polymers were capable of efficient DNA complexation. Complexes displayed a neutral zeta potential as well as a size suitable for endocytic uptake into cells. Cellular uptake was monitored by confocal laser scanning microscopy, in which images showing a rapid trafficking to vesicular organelles, such as endosomes or lysosomes, could be gathered. Transfection efficiencies were in the same order of magnitude as PEI 25 kDa, however, toxicities were very low, even at high nitrogen to phosphate ratios. These unique efficiency-toxicity properties made this system very attractive for further investigations.

In **Chapter 6**, several triblock polymers with cyclodextrin inclusions, such as those described in Chapter 5, were investigated with regard to properties crucial for an *in vivo* application. Complex stability against polyanions was found to be higher than that of PEI 25 kDa. Protection of DNA against DNase digestion was efficient as well. Visualization of the complex structure via AFM revealed a spherical morphology with a size similar to that of PEI 25 kDa. The *in vivo* evaluation of polymer/DNA complexes exhibited a slightly enhanced circulation time of complexes with cyclodextrin inclusion, as compared to PEI 25 kDa. The major advantage of the inclusion compounds, however, was a negligible accumulation in the lung and a reduced *in vivo* toxicity compared to PEI 25 kDa.

OUTLOOK

The ultimate goal of polymeric gene delivery is the development of a safe vector system with low *in vitro* and *in vivo* toxicity exhibiting a high stability in the bloodstream, efficient and exclusive uptake into target cells and high levels of gene expression. However, at this time we are far away from such a system.

Mechanistic knowledge about the subcellular trafficking of PEI/nucleic acid complexes is of major importance for further advances in this field. Although insight into this topic is deepening, numerous unanswered questions remain, especially concerning how exactly release from the endosome or lysosome occurs and which polymer properties are crucial for efficient release. Further, more knowledge is required concerning the mode by which plasmid DNA crosses the cytoplasm and enters the nucleus, as well as how the efficiency of this process can be improved.

The reduction of *in vitro* and *in vivo* toxicity of polymer based gene delivery systems is of crucial importance for further advances in this field. Important progress has been made, for example, by coupling hydrophilic polymers, such as PEG or pHPMA, via different strategies to polymeric gene carriers. These modifications have led to a dramatic decrease in toxicity. However a deeper insight into the behavior of polymer/nucleic acid complexes in the bloodstream is necessary for the rational development of *in vivo* gene delivery systems. Initial studies of the *in vivo* stability of such vector systems, as described in this dissertation, have demonstrated a rapid separation of PEG-PEI/DNA complexes at low doses. However, in order to make conclusive statements about the applicability of these vector systems, a systematic evaluation of different polymers, varying complex doses, and alternative modes of application is required. The new generation of triblock polymers with cyclodextrin threading described in Chapter 5 and 6 have shown several advantageous properties, such as high levels of reporter gene expression, low *in vitro* and *in vivo* toxicities, and a low accumulation in the lung. Furthermore, circulation times were slightly enhanced compared to PEI 25 kDa. However the effects are not very pronounced and therefore, further studies are required to be able to fully evaluate the potential of this approach.

The use of reversibly crosslinked complexes may show favourable properties and stimulate progress in this field. This approach involves the formation of

complexes following the regular protocol, followed by subsequent lateral stabilization using bifunctional linkers containing e.g. disulfide bonds. Such systems are potentially more stable in the bloodstream, yet when internalized into cells, the DNA can be released as a result of the reductive environment created by a high concentration of e.g. glutathion. Of course, the degree of crosslinking needs to manipulated in such a way that sufficient stability in the bloodstream is, on the one hand, guaranteed and on the other an efficient release of DNA at the subcellular level is possible. Currently, optimization of this type of system is under intensive investigation.

This dissertation and numerous other publications have described gene delivery systems containing targeting vectors with a high specificity for particular target cells *in vitro*. The challenge, however, is to develop vector systems that work *in vivo*, although the results in this field are still discouraging. Most of the systems that are in use display a low specificity and only a marginal gene expression in target tissues *in vivo.* Currently, the antibody fragment targeted vector system described in this dissertation are being tested *in vivo* in a nude mice tumor model and preliminary results look promising.

Generally, it can be stated that significant progress has been made during the last years and that polymer based gene delivery is a promising approach for future advances in gene therapy.

ZUSAMMENFASSUNG

In der vorliegenden Dissertation wurden verschiedene Aspekte des polymerbasierten Gentransfers eingehend untersucht. Zunächst wurden Schlüsselereignisse der subzellulären Prozessierung von elektrostatischen Polymer/Nukleinsäure-Komplexen erforscht und neue Erkenntnisse über Mechanismen, insbesondere von zellulärer Aufnahme und subzellulärer Freisetzung aufgedeckt. Ein weiterer Schwerpunkt lag auf der Entwicklung eines zielgerichteten Gentransfersystems für die selektive Transfektion von Ovarialkarzinomzellen. Das resultierende Vektorkonstrukt zeigte bei sehr geringer unspezifischer Transfektion und Toxizität eine sehr hohe Spezifität für Zielzellen. Da eine intravenöse Anwendbarkeit von Gentransfersystemen von großer Wichtigkeit ist, wurde die Stabilität elektrostatischer Vektoren *in vitro* und *in vivo* untersucht. Weiterhin war die Entwicklung einer neuen Klasse von Transfektionsagentien Gegenstand dieser Arbeit. Diese Systeme weisen eine hohe *in vitro* Transfektionseffizienz kombiniert mit einer sehr niedrigen Toxizität auf. Außerdem zeigten sie nach intravenöser Gabe eine verlängerte Zirkulationszeit im Blut.

Kapitel 1 gibt als Einleitung einen detaillierten Überblick über kationische Polymere als Transportsysteme für Nukleinsäuren gegen verschiedene Krebsarten. Der aktuelle Stand der Wissenschaft bezüglich kationischer Polymere als Genfähren wird dargestellt. Hierbei wird insbesondere auf systemische und subzelluläre Hindernisse sowie Strategien zur Überwindung derselben abgehoben. Weiterhin wird eine Einführung in die wichtigsten gentherapeutischen Strategien gegen Krebs gegeben.

In Kapitel 2 wurden Schlüsselereignisse der subzellulären Prozessierung von Polyethylenimin/Nukleinsäure-Komplexen mittels konfokaler Laser Scanning Mikroskopie und Inhibitorexperimenten untersucht. Wie mikroskopische Untersuchungen zeigten, findet die zelluläre Aufnahme höchstwahrscheinlich via adsorptiver Endozytose statt. Als Hauptort der Komplexakkumulation auf

subzellulärer Ebene wurde das lysosomale Kompartiment mittels eines Markerfarbstoffes identifiziert. Desweiteren konnte, zum ersten Mal überhaupt, die Freisetzung von PEI/Nukleinsäure-Komplexen aus einem vesikulären Kompartiment, wahrscheinlich Endosom oder Lysosom, mittels konfokaler Lebendzellmikroskopie visualisiert werden. Diese Freisetzung findet offensichtlich nur zu einem sehr geringen Teil statt, wobei der bei weitem größte Teil der Komplexe in vesikulären Strukturen verbleibt. Unter Verwendung eines Inhibitors der endosomalen Azidifizierung konnte die Wichtigkeit dieses Vorganges für die Freisetzung gezeigt werden. Diese Erkenntnisse wurden durch Transfektionsexperimente in Gegenwart und in Abwesenheit des Inhibitors untermauert. Auch diese Experimente zeigten, dass Azidifizierung notwendig ist, um eine hohe Reportergenexpression mit PEI als Transfektionsagens zu erhalten.

In Kapitel 3 ist die Entwicklung eines zielgerichteten Gentransfersystems für die spezifische Transfektion von Ovarialkarzinomzellen beschrieben. Als Targeting-Struktur wurde ein Antikörper ausgewählt, der eine hohe Spezifität für ein Epitop aufweist, das von den meisten Ovarialkarzinomzellinien exprimiert wird. Um die Größe der Targeting-Einheit zu reduzieren, wurden Antikörperfragmente (Fab´), welche die Antigenbindungsstellen beinhalten, durch Verdau des kristallisierbaren Teils des Antikörpers hergestellt. Anschließend wurden Konjugate aus einem pegylierten Polyethylenimin und Fab´ synthetisiert und sowohl physikochemisch als auch biologisch untersucht. Die linearen Polyethylenglykol-Ketten sollten die positive Ladung des Polyethylenimins abschirmen, um so die Spezifität des Konjugates zu erhöhen. Das PEG-PEI-Fab´-Konjugat war in der Lage, DNA genauso effektiv zu kondensieren wie unmodifiziertes PEI, außerdem war die Komplexstabilität gegenüber dem Polyanion Heparin sehr ähnlich. Zellassoziationsstudien wurden mittels Flow Cytometry durchgeführt und zeigten eine sehr effektive Bindung von Konjugat/DNA-Komplexen an Epitop-exprimierende Zellinien, jedoch eine

wesentlich niedrigere Bindung an Epitop-negative Referenzzellen. Transfektionsexperimente offenbarten eine bis zu etwa 100fach höhere Reportergenexpression in Epitop-exprimierenden Zellen verglichen mit PEI oder PEG-PEI. In Epitop-negativen Referenzzellen jedoch befanden sich die Transfektionsraten mit Konjugat/DNA-Komplexen auf sehr niedrigen Niveau. Um die Spezifität von Konjugat/DNA-Komplexen weiter zu verifizieren, wurden kompetitive Transfektionsexperimente mit verschiedenen Konzentrationen an freiem Fab´ im Zellkulturmedium durchgeführt. Hierbei konnte eine Reduzierung der Reportergenexpression mit steigender Konzentration an freiem Fab´ beobachtet werden. Die so erhaltenen Daten legen nahe, dass die hohe Spezifität der Konjugat/DNA-Komplexe auf spezifische Wechselwirkungen zwischen dem zellständigen Epitop und den antigenbindenden Fragmenten auf den Konjugat/DNA-Komplexen zurückzuführen sind.

In Kapitel 4 wurde die *in vitro* und *in vivo* Stabilität sowie die Organdeposition von PEI/Plasmid und PEG-PEI/Plasmid-Komplexen untersucht. Während die Stabilität beider Komplexarten bei Verdrängungsexperimenten gegenüber Polyanionen sehr ähnlich war, wurden signifikante Unterschiede beim Schutz der DNA vor Abbau durch DNase I gefunden. PEI 25 kDa war hier effektiver als pegyliertes PEI. Rasterkraftmikroskopie zeigte eine leicht unterschiedliche Komplex-Form, wobei die PEG-PEI/DNA-Komplexe etwas gedrungener und unregelmäßiger wirkten. In keinem Fall jedoch konnte freie DNA beobachtet werden. Bei der Untersuchung der Transfektionseffizienz in Blut zeigte sich, dass beide Komplexarten in Blut eine gewisse Stabilität aufweisen. Betrachtet man jedoch den Grad der Reduzierung der Transfektionseffizienz in Blut, zeigt sich, dass PEG-PEI/DNA-Komplexe weniger stabil sind. Bei der *in vivo* Untersuchung von Pharmakokinetik und Organdeposition mit zwei verschiedenen radioaktiven Markern an Polymer bzw. inkorporiert in Plasmid-DNA zeigten sich signifikante Unterschiede zwischen PEI/DNA und PEG-

PEI/DNA-Komplexen. Während im Fall von PEI/DNA-Komplexen ein ähnliches pharmakokinetisches Profil für Polymer und DNA sowie eine ähnliche Organverteilung nach 2 Stunden beobachtet wurde, zeigten PEG-PEI/DNA-Komplexe starke Unterschiede zwischen Polymer und DNA. In diesem Fall waren Pharmakokinetik und Organverteilung ähnlich der von nackter DNA. Diese Daten legen nahe, dass sich elektrostatische PEG-PEI/DNA-Komplexe bereits kurz nach intravenöser Injektion trennen, während PEI/DNA-Komplexe zumindest eine gewisse Stabilität aufweisen.

Kapitel 5 beschreibt die Entwicklung einer neuen Klasse von nichtviralen Transfektionsagentien. Diese Vektoren sind Triblock-Polymere bestehend aus PEI 25 kDa, Polycaprolacton (PCL) und Polyethylenglykol (PEG). Da die Wasserlöslichkeit solcher Strukturen sehr begrenzt ist, wurde diese durch Überfädeln von Cyclodextrin über PEG- und PCL-Ketten erhöht. Da das Innere des Cyclodextrins hydrophob ist, liegt die Vermutung nahe, dass die Cyclodextrin-Ringe über das hydrophile PEG hinweggleiten und schließlich durch hydrophobe Wechselwirkungen auf dem PCL-Bereich des Blockpolymers haften bleiben. Der Beweis für das erfolgreiche Einfädeln von Cyclodextrin und die vornehmliche Arretierung auf den PCL-Ketten wurde mittels ^{1}H-NMR, UV- und FTIR-Spektroskopie erbracht. Die resultierenden Cyclodextrin-Inklusions-Polymere waren in der Lage, DNA effektiv zu kondensieren. Die resultierenden elektrostatischen Komplexe hatten ein neutrales Zetapotential und eine Größe, die eine endozytotische Aufnahme in Zellen erlaubt. Zelluläre Aufnahme konnte mit Hilfe der konfokalen Laser Scanning Mikroskopie gezeigt werden, wobei die Bilder eine schnelle Aufnahme in vesikuläre Organellen wie Endosomen oder Lysosomen zeigten. Transfektionsexperimente ergaben eine Reportergenexpression in der selben Größenordnung wie die von PEI 25 kDa. Ein Vergleich der Proteinkonzentrationen im Zell-Lysat zeigte jedoch, dass die Toxizität der Inklusionskomplexe sehr gering war im Vergleich zu PEI. Sogar bei sehr hohem Stickstoff/Phosphat-Verhältnis konnte keine signifikante

Toxizität nachgewiesen werden. Diese einzigartigen Eigenschaften machten das Vektorsystem interessant für weitere Untersuchungen.

In **Kapitel 6** wurden ausgewählte Triblock-Polymere mit Cyclodextrin-Inklusion aus Kapitel 5 eingehender bezüglich der Eigenschaften untersucht, die für eine *in vivo* Anwendung entscheidend sind. Die Komplexstabilität aller Polymer/DNA-Komplexe gegen Polyanionen war höher als die von PEI 25 kDa. Außerdem waren die Polymere in der Lage, komplexierte DNA effektiv gegen enzymatischen Abbau durch DNase I zu schützen. Die Untersuchung der Komplexstruktur mittels Rasterkraftmikroskopie zeigte eine sphärische Struktur der Komplexe mit einer Größe ähnlich der von PEI/DNA. Eine *in vivo* Untersuchung von Polymer/DNA-Komplexen mit radioaktiv markierter DNA ergab eine leicht verlängerte Zirkulationszeit für DNA, die mit Inklusions-Polymeren komplexiert war. Der große Vorteil der Inklusionskomplexe jedoch war die vernachlässigbare Akkumulation in der Lunge und daraus resultierend eine sehr geringe *in vivo* Toxizität verglichen mit PEI 25 kDa und PEG-PEI.

AUSBLICK

Ziel des nichtviralen Gentransfers ist die Entwicklung eines Vektorsystems, das eine niedrige *in vitro* und *in vivo* Toxizität, eine hohe Stabilität im Blutstrom, eine effiziente und exklusive Aufnahme in Zielzellen und eine hohe Genexpression in sich vereint. Zur Zeit jedoch sind wir weit entfernt von einem solchen System.

Mechanistisches Wissen über den subzellulären Transport von PEI/Nukleinsäure-Komplexen ist von größter Wichtigkeit für den weiteren Fortschritt auf diesem Gebiet. Obwohl es in den letzten Jahren einige wegweisende Erkenntnisse gab, sind immer noch viele Fragen unbeantwortet. So ist zum Beispiel der genaue Mechanismus der Freisetzung aus Endosom oder Lysosom noch nicht vollständig geklärt. Außerdem ist weiterhin unklar, welche Vektoreigenschaften genau entscheidend sind für ein forciertes Ablaufen dieses

Vorganges. Der weitere Weg der Nukleinsäuren auf dem Weg zum Zellkern liegt ebenfalls noch weitgehend im Dunkeln. Mechanistisches Wissen auf diesem Gebiet würde eine effektiveres Vektordesign erlauben.

Die Reduzierung der *in vitro* und *in vivo* Toxizität von Polymeren als Gentransferagentien ist ebenfalls von großer Wichtigkeit für weitere Fortschritte auf diesem Gebiet. Hier konnten durch verschiedene Polymermodifikationen, zum Beispiel durch die Kopplung der hydrophilen Polymere PEG oder pHPMA Fortschritte erzielt werden. Die Inkorporierung dieser Strukturen führte zu einer stark verminderten Toxizität der entsprechenden Polymer/DNA-Komplexe. Erste Untersuchungen der *in vivo* Stabilität dieser Systeme im Rahmen dieser Arbeit zeigten eine schnelle Trennung der Komplexe im Blutstrom bei den für diese Studie gewählten niedrigen Dosierungen. Jedoch ist eine genaue Untersuchung des *in vivo* Verhaltens dieser Vektoren bei unterschiedlichen Applikationsarten und Dosierungen unerlässlich für die abschließende Beurteilung dieser Systeme. Die beschriebenen Triblockpolymere mit Cyclodextrin-Inklusion zeigten exzellente *in vitro* Eigenschaften wie hohe Transfektionseffizienz und sehr niedrige *in vitro* und *in vivo* Toxizität verbunden mit niedrigen Akkumulationsraten in der Lunge. Außerdem war die Zirkulationszeit im Vergleich zu unmodifiziertem PEI 25 kDa etwas erhöht. Ob hier noch Potential für Verbesserungen liegt, müssen weitere Untersuchungen zeigen. Ein sehr vielversprechender Ansatz für das Erzielen von Fortschritten in der *in vivo* Gentherapie ist der, elektrostatische Komplexe aus PEI und Nukleinsäuren zunächst nach standardisiertem Protokoll herzustellen und anschließend über bioreversible Bindungen wie z.B. Disulfidbrücken mittels bifunktioneller Linker zu stabilisieren. Die Idee hinter dieser Strategie ist eine hohe Stabilität der resultierenden lateral stabilisierten Komplexe im Blutstrom. Auf subzellulärer Ebene jedoch sorgt das reduzierende Milieu für eine Spaltung der Disulfidbindungen und damit für die Möglichkeit der DNA-Freisetzung. Bei diesen Systemen muß selbstverständlich der Grad der Quervernetzung so

gewählt werden, dass auf der einen Seite die Komplexstabilität ausreichend ist, auf der anderen Seite aber auf zellulärer Ebene eine Freisetzung in ausreichend schneller Zeit stattfinden kann. An der Optimierung dieser Systeme wird zur Zeit intensiv geforscht.

Bezüglich des zielgerichteten Gentransfers in bestimmte Zell- und Gewebetypen zeigten die Resultate dieser Dissertation sowie einige andere Veröffentlichungen eine hohe Spezifität der Vektorkonstrukte in der Zellkultur. Die große Herausforderung jedoch besteht in der Entwicklung eines zielgerichteten Vektorsystems, das auch *in vivo* selektiv Zielzellen bzw. Zielgewebe transfiziert. Die Ergebnisse auf diesem Gebiet sind zur Zeit noch sehr unbefriedigend. Die meisten zielgerichteten Systeme zeigen nur eine niedrige Genexpression im Zielgewebe *in vivo*. Zur Zeit wird das PEG-PEI-Fab´-Konjugat, welches in dieser Dissertation beschrieben wurde, in einem Nacktmaus-Tumormodell untersucht und erste Resultate sehen vielversprechend aus.

Abschließend kann man sagen, dass in den letzten Jahren beachtliche Fortschritte auf dem Gebiet des polymerbasierten Gentransfers gemacht wurden. Dieses Feld stellt daher einen vielversprechenden Ansatz für die Zukunft der Gentherapie dar.

Appendices

ABBREVIATIONS

AUC	area under the curve
CD	alpha-cyclodextrin
CLSM	confocal laser scanning microscopy
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
EGF	epidermal growth factor
EtBr	ethidium bromide
Fab´	antibody fragment (antigen binding part)
Fc	crystallizable part of antibody
FACS	fluorescence assisted cell sorting
HBS	HEPES buffered saline
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
hy-PEI	hyperbranched PEI (PEI 25 kDa, Sigma-Aldrich)
kb	kilobase
Mab	monoclonal antibody
Mw	molecular weight
N/P	nitrogen to phosphate ratio
NHS	N-hydroxysuccinimide
OG	Oregon Green
PBS	phosphate buffered saline
PCL	polycaprolactone
pDNA	plasmid DNA
PEG	polyethylenglycol
PEG-PEI	pegylated polyethylenimine
PEG-PEI-Fab´	conjugate of pegylated polyethylenimine and Fab´
PEI	polyethylenimine
pHPMA	poly(N-(2-hydroxypropyl)methacrylamide)
PLL	poly(L-lysine)
RGD	arginine-glycine-aspartic acid
Rh	rhodamine
SEC	size exclusion chromatography
SPDP	N-succinimidyl 3-(2-pyridyldithio)propinate

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