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Development of purification methods for tumor targeted polyplexes applied in vivo

vorgelegt von

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Erklärung

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

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Für meine Eltern

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1. Introduction

1.1. Gene therapy

Gene therapy is aimed to provide novel genetic information to specific cells of a patient and promises revolutionary advances in the prevention, treatment or cure of many human diseases. It was first introduced by Friedman and Roblin [1] in 1972 and was originally aimed to correct genetic disorders. For this purpose, therapeutic nucleic acids have to be delivered into the target cells to turn on or restore a certain gene function (‘gain of function’). However, the field of gene therapy has been much broadened in its scope. Antisense oligonucleotides (AS-ON) [2], ribozymes [3] or small interfering RNA (siRNA) [4] used for suppression of specific gene functions (‘loss of function’) have emerged as potential therapeutic agents. Many inherited and acquired diseases based on single genetic defects like Cystic Fibrosis or SCID (severe combined immunodeficiency disorder) and more complex disorders like vascular or infectious diseases are amendable for gene therapy as well as DNA vaccination. Most of all, cancer gene therapy has emerged as a promising field of interest since cancer is in many cases a genetic disease with genetic aberrations and subsequent gene defects. Actually, most of the clinical trials are directed to cancer gene therapy, highlighting out the potential of the treatment for this disease. However, a therapeutic benefit in larger numbers of patients was so far not obtained. The major challenges are still the improvement of efficiency, specificity and safety of the existing gene delivery systems (gene vectors).

However, since the first clinical success in the treatment of the monogenetic disease SCID has already been achieved [5], it becomes clear that gene therapy has the potential to become the foundation for a whole new class of therapeutic applications.

1.2. Gene vectors: general requirements

Like all medicines, nucleic acid (NA) based therapeutics should be in general highly specific, highly effective and well tolerated. However, unlike normal drugs, NA’s have unfavorable physical properties: they are large, hydrophilic and negatively charged. In addition, very effective defense mechanisms have been developed by the human body to recognize and degrade foreign NA’s. During the past, several tools, namely gene vectors, have been developed to ensure the stability of the delivered NA of

interest and protect it from degradation by serum nucleases. However, once applied via the bloodstream, vectors are threatened to be eliminated due to unspecific binding to blood components and/ or subsequent elimination by the reticulo endothelial system (RES). Delivery of the vector to the desired target is of crucial impact, as interaction with non-target tissues could trigger undesired and potentially toxic side-effects, leading to reduced circulation times and targeting ability (Figure 1). Once the vector has reached the target site, access can be limited by restricted diffusion within the target tissues [6] and unspecific binding to components of the extra cellular matrix. Depending on the type of the tumor, the influence of the extra cellular matrix on mobility of the vector can vary significantly. After successful internalization into the target cell, intracellular barriers (e.g. release of the vector out of the endosome into the cytoplasm, nuclear trafficking, nuclear entry and vector unpacking) are presenting major challenges.

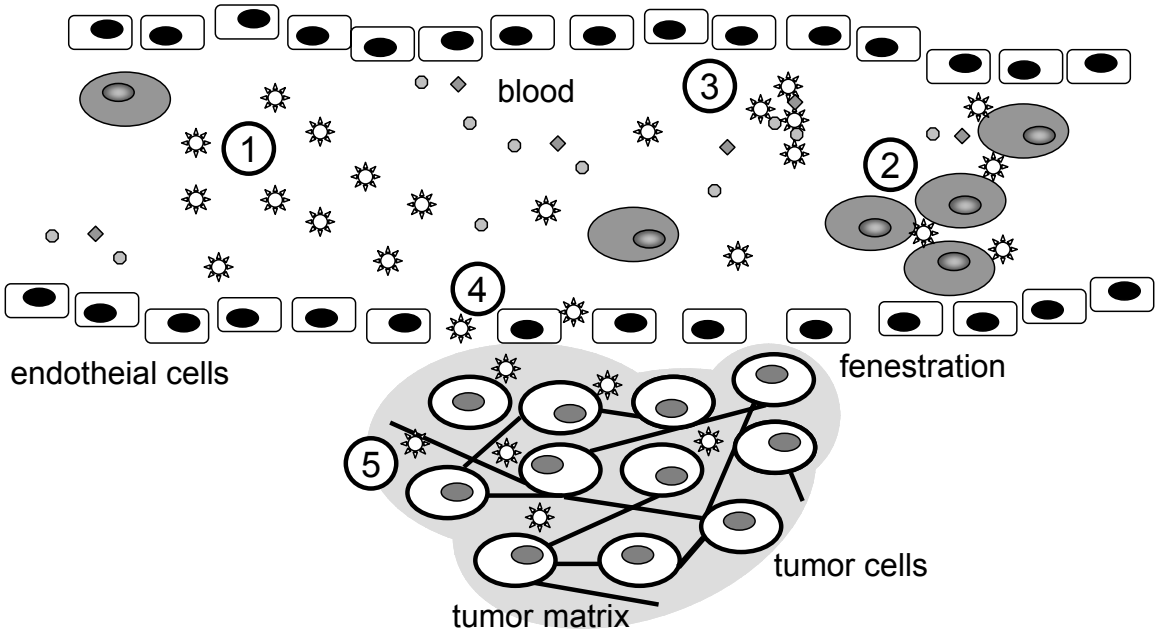


Fig. 1. Extracellular barriers for systemically applied gene delivery systems: gene vectors [1] can interact with blood components like blood cells [2] or plasma proteins [3], resulting in the formation of aggregates. They can clog blood capillaries leading to embolism or be cleared by the RES resulting in inefficient gene delivery. Incomplete or leaky vasculature can facilitate extravasations of the vector to the target tissue [4]. Once reached the target tissue, diffusion of the vector may be limited; interactions with the extra cellular matrix proteins can lead to binding, aggregation or dissociation of the gene vector [5]

Once being expressed, suboptimal or instable expression of the transgene can be caused by silencing of the gene [7], loss of gene or of transfected cells. Risk of

random, unspecific integration into the host genome might switch on oncogenes [8]., It is also of great importance that the gene transfer affects exclusively the somatic cell line of the patient and not the germ line. Once the vectors have delivered their therapeutic NA, they should be biodegradable and/ or easily being eliminated and not accumulate in any organ.

For successful gene therapies, development and choice of appropriate vectors for gene delivery will be a major challenge in future works since all of these aspects have to be taken into consideration. In the last three decades, a number of delivery methods have been developed which can be split in two separate “worlds”, namely into the field of viral and non-viral vectors. The majority of clinical approaches so far have employed retroviral or adenoviral vectors [9]. For a review on viral gene therapy see reference [10].

However, non-viral vectors are becoming more and more attractive as gene vectors since they offer some attractive characteristics ([11], [12]). They are more flexible regarding the type and size of delivered NA: a broad range from small siRNA up to large artificial chromosomes can be used. Furthermore, they offer easy synthesis and low production costs. It is described that non-viral vectors show lower immunogenicity than viral vectors since they can be generated either protein free or by using nonimmunogenic human or humanized proteins [13]. On the other side, many cationic carrier molecules utilized for non-viral NA condensation are far from being non-toxic. However, the major bottleneck of synthetic viruses is their limited efficacy compared to viral vectors which consequently requires the application of large amounts of DNA. Actually, this weakness is presenting an important opportunity since the chemical synthesis of non-viral vectors allows various modifications and easy manipulations. They can be engineered to introduce cell-specific binding thus delivering specifity which is a key issue for improved cancer therapy. Among the non-viral vectors so called ‘polyplexes’ have emerged as potent in vitro transfection agencies (for review see [14], [15], [16], [17],[18]). They are based on the condensation of negatively charged DNA by electrostatic interactions with polycationic compounds into compact particles. If polyplexes will be one day able to metamorphose into potent NA therapeutics for human applications will be dependent on their future development. For this aim, improvement of their efficacy, safety and specificity for in vivo applications are vital preconditions. Therefore, future development of NA delivery systems may overcome the historically founded

separation in viral and non-viral research fields: Viral vectors can be chemically modified to reduce immunogenicity and improve specificity while synthetic systems may be engineered to mimic the dynamic cell entry and cell trafficking processes of viruses and metamorphose into efficient “synthetic virus” delivery systems for human applications.

1.3. Gene transfer mediated by PEI polyplexes

Among the known polymeric carriers tested, polyethylenimine (PEI) has been evaluated with regard to its efficacy as a versatile, inexpensive and useful transfection system ([19], [20]) reaching in vitro gene delivery efficiencies similar to viral vectors [21]. It has been proposed that the high gene transfer efficiency of PEI is due to its capacity to facilitate endosomal release ([22], [23]). This hypothesis is based on the chemical structure of PEI: only every third nitrogen atom is protonable at physiological pH, hence the remaining amino groups can act like a proton-sponge inside the endosomes, leading to osmotic swelling followed by breaking up of the endosomal membrane [24]. Direct interaction of positively charged DNA particles with the negatively charged inner endosome membrane surface may also play a significant role in membrane disruption ([25], [26], [27]). PEI polymers can be synthesized in a linear and a branched topology (LPEI and BPEI) [28] and are available in a broad range of molecular weights. Most successful and widely studied polymers are LPEI with a molecular weight of 22kDa and BPEI with 25kDa.

Due to its high charge density of protonable amine functions, PEI is ideal to condense NAs into complex particles [29]. The size of the PEI/DNA polyplexes is strongly dependent on the present salt concentration and the molar ratio of PEI nitrogen to DNA phosphate (N/P ratio) groups ([30], [31]). For efficient complexation, an excess amount of PEI is required, leading to a net positive surface charge and leaving a considerable amount of PEI free in solution ([32], [33]). Unfortunately, these higher N/P ratios (≥ 5) leave a narrow window between transfection efficiency and severe toxicity ([34], [35]) strongly restricting the in vivo application of crude PEI/DNA polyplexes. However, irrespective of the conditions used, LPEI/ DNA complexes are generally considered as a “golden standard” for in vitro gene delivery since they exhibit an improved cell viability and increased transfection efficiency compared to BPEI complexes [36].

1.3.1. Modifications of PEI polyplexes

In the last decade, it became clear that any polyplex with “static” properties will have strong difficulties to successfully overcome all biological delivery steps, especially for systemic delivery. Figure 2 shows the most crucial barriers influencing the extra- and intracellular fate of the polyplexes. Future polyplexes will have to sense their physiological environment and undergo programmed changes upon the changes in the delivery pathways.

Fortunately, the amino groups of the PEI polymers offer a very attractive platform for easy chemical modification making the incorporation of functional domains possible.

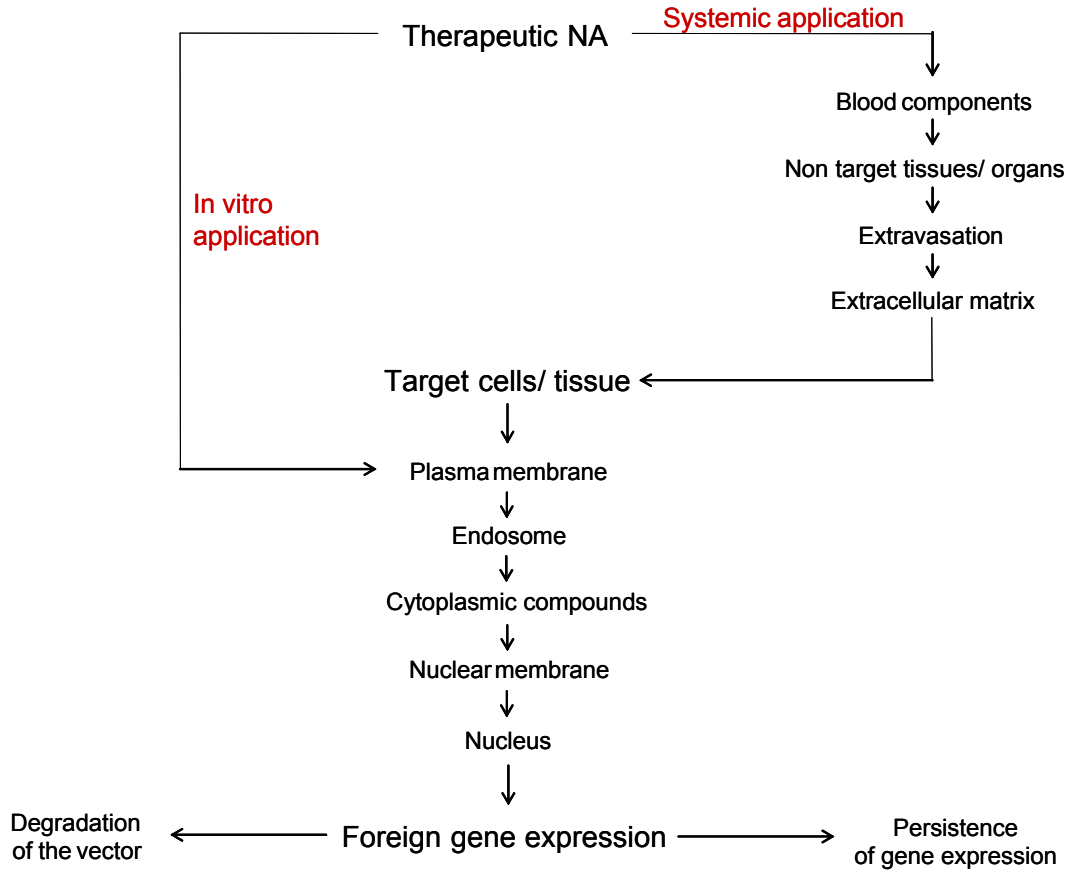


Fig. 2. Extra- and intracellular pathways for gene delivery

Since PEI/DNA polyplexes are favorable for in vitro gene transfer efficiency but problematic for systematic application many attempts have been made to improve extra- and intracellular delivery characteristics of these vectors.

A common approach to prevent non-specific trapping and to reduce toxicity of the vectors is masking the surface charges with hydrophilic polymers like polyethylene glycol (PEG) or the serum protein transferrin [37]. PEGylation can either be done prior to complex formation (“prePEGylation”) ([38], [39]) or after complex formation

("postPEGylation") ([40], [41]). Pegylation of polyplexes has been shown to prevent their aggregation as well as aggregation with blood components, to lower toxicity, and to significantly prolong circulation time ([40], [42], [35]). Unfortunately, as shielding improves polyplex properties for systemic application, at the same time gene delivery turned out to be less efficient, probably due to reduced cell surface interactions ([35], [38], [41], [43]). Additionally, differences in the mode of internalization, endosomal entrapment and intracellular trafficking account for this effect ([44], [45]). Therefore, different methods for triggered deshielding of the PEG chains have been established leading to a stable PEG coat during extracellular transport possible and triggered intercellular release of the PEG shield. They all take advantage of changes in physiological parameters within the cell like pH, redox potential or enzyme concentration. Many of them exhibit very interesting and promising results, highlighting the importance of labile PEGylation strategies for non-viral gene delivery systems (see [46] for a recent review).

To increase specificity of polyplexes, the concept of targeted delivery was developed. The covalent attachment of targeting ligands to PEI polyplexes introduced cell specificity and partly restored transfection efficiency of stable shielded particles thus presenting a very attractive concept. These targeting molecules recognize specific cell surface receptors thus directing the polyplex to the specific tissue. Numerous ligands such as small chemical compounds, peptide ligands, vitamins, carbohydrates, growth factors, antibodies and others have been investigated (for review see [47] and [48]). To date, transferrin-mediated uptake of polyplexes is one of the best-studied targeting systems. The serum protein transferrin thereby presented not only the targeting domain but also led to an efficient shielding of the polyplex ([49], [37]).

After cellular association of polyplexes to the target cell, particles are taken up by endocytosis. Irrespective of the mechanism of cellular uptake, after internalization complexes are mainly found in intracellular vesicles such as endosomes, as evidenced by fluorescence microscopy [50]. Subsequent release of the particles into the cytoplasm is crucial for efficient gene delivery since endosomal acidification (from pH ~ 7 to pH ~ 4.5 found in lysosomes), is associated with degradation of the complexes. PEI polyplexes and free PEI have considerable endosomolytic properties as explained by the proton sponge hypothesis. However, this is apparently not efficient to release the major part of the complexes into the cytoplasm. Endosomal

release is still one of the major bottlenecks for efficient gene transfer, especially with small PEI polyplexes at low concentrations [30] or purified polyplexes [33]. Approaches to improve endosomal release include endosomolytic agents like chloroquine (see [51] for a review), synthetic virus derived fusion peptides ([52], [53]) and membrane destabilizing peptides like the bee venom derived melittin ([50], [54]).

Another interesting approach to achieve endosomal release is the photochemical intracellular release technology (PCI) which is based on the accumulation of photosensitizing compounds in endosomal membranes. Light induces break-down of the endosomal membrane leading to release of trapped molecules ([55], [56]).

The schematic “artificial virus” in Figure 3 presents the most important modifications made in the development of “smarter” PEI polyplexes. Of course, many other additional functional changes in for more dynamic and “virus-like” polyplexes will be engineered in future work.

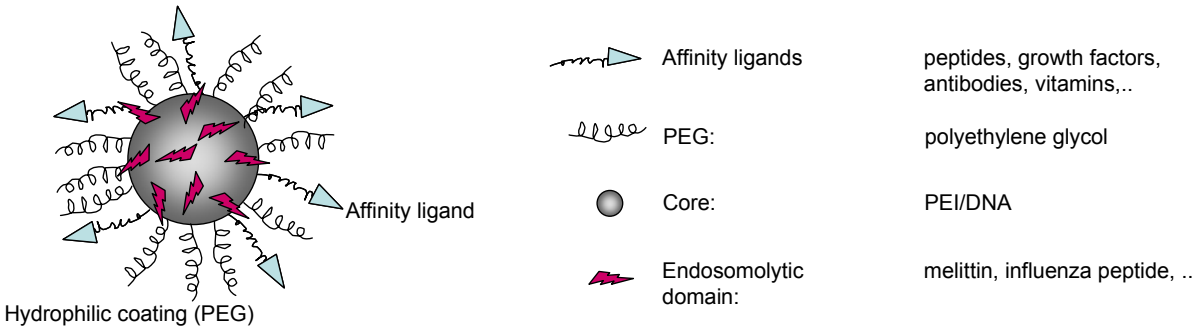


Fig 3: Important modifications of PEI polyplexes

1.3.2. Tumor targeting of PEI polyplexes

Treatment of cancer using gene therapy presents a very attractive and advantageous strategy since a relatively short expression of the therapeutic protein might be already sufficient to eradicate the tumor. Several therapeutic concepts can be utilized (see [57] for a recent review): Introduced therapeutic genes might lead to a direct killing of the tumor cells through local expression of a specific enzyme which converts an inactive prodrug into the active compound directly at the tumor site (“Gene directed enzyme prodrug therapy, GDEPT”, [58]). Expression of highly potent cytokines can be used for local killing of the tumor cells (e.g. [59], [60]). Other strategies interfere with tumor associated processes like metastasis or neoangiogenesis. Bacterial plasmid DNA containing unmethylated CpG sequences

can act like a vaccination and stimulates the expression of anti-tumor active cytokines such as interferon-γ or IL-2 [61].

A major challenge for PEI-based polyplexes is the efficient and specific targeting of the desired NAs to the tumor cells. A recent review on targeted delivery of NAs is given in [62]. A few tumor types are amenable for direct application and some PEI polyplexes have been successfully investigated for the intratumoral delivery of therapeutic NAs ([63], [64]). Nevertheless, targeting tumors via the intravenous route opens the possibility to reach and attack multiple spread metastatic tumor nodules. Metastases with a diameter ≥ 2mm depend on the constant supply of nutrients and oxygen via the blood stream and therefore induce the formation of new blood vessels. This abnormal neovascularisation leads to an imperfect and leaky tumor vasculature combined with an inadequate lymphatic drainage. Therefore, permeation and retention of nanoparticles via leaky tumor vasculatures with passive accumulation of polyplexes at the tumor site can take place. This passive targeting strategy first described by Maeda et al [65] is called the “enhanced permeability and retention (EPR) effect”. To take advantage of this effect, macromolecules have to exceed a molecular weight of 50kDa to avoid renal clearance. Additionally, in order to increase the possibility of the particles to extravasate at the tumor site [40] they have to be long circulating in the blood stream (which is realised by the hydropholic surface modification e.g. with PEG chains). However, the most attractive and successful way for directing NAs to the tumor tissue is active targeting. This approach can be realised by coupling targeting ligands to PEI polyplexes as described in 1.3.1. Since tumor cells are fast dividing cells, they typically over-express specific surface bound receptors like the growth factor receptors EGFR or HER-2 ([66], [67]). Incorporation of EGF into PEI polyplexes led to high reporter gene expression in hepatocellular carcinoma bearing SCID mice. Expression was predominantly found in the tumor with levels up to 100 fold higher than in the liver, which was the highest major expressing organ [68]. Another very successful approach was reported by Shir at al. [64]: Polyplexes containing EGF as targeting ligand and incorporation of double-stranded RNA polyIC led to complete regression of EGFR over expressing glioblastoma in mice.

Endothelial cells of the tumor vasculature also over express specific surface receptors like integrins making the neoangiogenic blood vessel a promising target ([69], [70]). Recently, RGD-PEG-PEI polyplexes delivering antiangiogenic siRNA

have been successfully tested for systemic applications exhibiting significant inhibition of neuroblastoma growth in mice [71]. Another interesting study was the inactivation of the vascular endothelial growth factor (VEGF) (which is an endogenous mediator for tumor angiogenesis) through RGD-PEG-PEI mediated delivery of its corresponding soluble receptor resulting in tumor growth inhibition [72].

A successful approach in active tumor targeting of non-viral delivery systems is the utilisation of the serum protein transferrin (Tf) for targeting Tf-receptors which are over expressed on fast growing cells due to their high demand of iron [73]. Since Tf is a large (≥80 kDa), hydrophilic plasma protein [37], it ideally combines an intrinsic stealth effect with targeting towards transferrin receptor expressing cells. Tail vain injection of Tf shielded and targeted polyplexes demonstrated excellent potential for in vivo applications: the biodistribution pattern was shifted from lung and liver to a 100 fold higher transgene expression in distant grown neuroblastoma tumors ([37], [40], [39]). High specificity of Tf targeted vectors (with and without additional PEG shield) could be confirmed by luciferase imaging in living mice [74]. Within the tumor, transgene expression was associated mainly within tumor cells next to tissue structures resembling primitive blood vessels. Expression levels varied between different tumors, due to deviant tumor vascularisation, necrotic tissue inside the tumors and infiltration by macrophages [75], leading to DNA degradation within the Kupffer cells of the liver [37]. A successful therapeutic approach performed by Kircheis et al. [59] presents the repeated administration of Tf polyplexes encoding tumor necrosis factor alpha (TNF-α) into tumor bearing mice. Inhibition of tumor growth and induction of tumor necrosis was observed whereas no systemic TNF-related toxicity occurred, emphasising the high tumor specificity of this vector system.

1.3.3. Biocompatibility of PEI and PEI polyplexes

The use of standard PEI/ DNA polyplexes for systemic applications is strongly limited since pronounced toxicity with massive damages in lung and liver tissues occurs ([20], [34], [40]). Acute toxicity is manly attributed to i) the positive surface charge of the cationic carrier and ii) the amount of free PEI being not involved in DNA complexation. In general, positively charged particles (free PEI as well as PEI polyplexes) bind to plasma proteins [40] and activate the complement system [76] triggering immune defense mechanisms. Binding to negatively charged membranes can mediate erythrocyte aggregation, leading to occlusion of lung capillaries followed

by lung embolism ([77], [40]). Opsonisation of the particles with serum proteins leads to macrophage-induced elimination and relocation of the polyplex to the liver.

Given in a sufficient large bolus, systemically delivered small LPEI/DNA polyplexes result in high gene expression in the lung and lower gene expression in other organs (liver, heart, spleen and kidneys) ([78], [79]). These particles are supposed to rapidly cross endothelial cells of the lung capillaries [80]. In contrast, BPEI-based polyplexes are less efficient while being often more toxic ([31], [81]) than LPEI particles. The biophysical reason for this differing behavior is still not completely clarified.

Masking the positive surface charge by PEGylation reduces systemic toxicity [40] but does not completely eliminate it [35]. Additionally, gene transfer efficiency of the polyplexes is in general strongly reduced which is currently partly compensated through the delivery of large amounts or multiple administrations of vectors modified with targeting ligands like Tf or EGF. However, application of high dosages and/ or repeated administrations of PEI polyplexes concomitantly increase the amount of free PEI being simultaneously applied: one of the most striking features of PEI based polyplexes is that they have to be mixed with an excess amount of polymer for successful gene delivery. This protocol generates 60 – 80% PEI remaining in a free form. Unfortunately, unbound PEI mainly attributes in a dose-dependent manner to significant cell toxicity ([32], [33], [82], [83]). *In vitro*, PEI induces membrane damage and initiates apoptosis in clinically relevant cell lines ([81], [84]). *In vivo*, massive damage in lung and liver occurred in mice models ([34], [35], [81],). Godbey and co-workers classified PEI-mediated toxicity into an immediate toxicity, associated with free PEI and a delayed form, connected with cellular processing of PEI/DNA complexes [82]. PEI may also be involved in the transport of DNA into the nucleus since naked DNA is not able to enter the nucleus efficiently [85]. Another indication that PEI/DNA complexes enter the nucleus intact is that PEI and DNA co-localize within the nucleus [86]. This drives the question of the effect of unbound polymer on the cells after nuclear entry since it might interact with host DNA or RNA [87]. In addition to acute toxicity, the long term fate of the polymeric carrier in the host has to be taken into consideration [88]. PEI is a nondegradable polymer and cannot be metabolized. Those molecules mostly tend to accumulate in the liver or the kidney leading to uncontrollable long-term toxicity [89]. Taken together, toxicity and side effects of the gene carrier have to be minimized. One reasonable approach is the synthesis of novel biodegradable polymers with the specific goal of improved

biocompatibilities while maintaining high transfection efficiency (for example see [90-93]; [94]). An alternative and additional approach is the development of efficient methods to purify a broad range of polyplexes from unbound polycations such as PEI. The first published approach for the purification of PEI particles ([95], [96]) was based on ultra filtration. Subsequently, an approach based on size exclusion chromatography (SEC) was developed which was more effective in complete separation of unbound polymer [33]. Purified polyplexes exhibited a greatly improved toxicity profile in vitro and in vivo ([33], [96])

Besides the improvement of biocompatibility, an excess charge of polycation is disadvantageous for any post-grafting strategy (for example postPEGylation) since the grafting molecule not only binds to complexed but also to unbound PEI. It may even prove inhibitory effects in combined post-grafting strategy, for example in a postPEGylation and a posttargeting approach. Additionally, excess free ligand-PEG-PEI conjugates might compete with the polyplexes for receptor binding.

In summary, further development of purification methods for polyplexes would be advantageous for the given reasons.

1.4. Aims of the thesis

Regarding future developments, one has to get straight that purification of polyplexes is a vital precondition for reasonable systemic gene transfer based on synthetic carriers.

Therefore, one major focus was the upscale of existing SEC-based method for effective polyplex purification in order to evaluate purified, targeted and shielded PEI-polyplexes for their tumor targeting efficiency and biocompatibility. Since these small, PEGylated particles might not be very efficient in gene delivery, additional endosomolytic domains should be incorporated into the polyplex in order to increase transfection potency.

The applied SEC method for purification of PEI polyplexes is limited to particles with a size below 200 nm since bigger particles get stuck within the gel beads. However, effective gene carriers that are promising in vivo candidates might possess sizes above 200 nm. It is also unclear if gel filtration can remove an excess of larger PEI conjugates containing targeting ligands or shielding molecules. However, at the target site, unbound targeting conjugate could lead to competition with the targeted polyplex for receptor binding resulting in reduced gene delivery. Additionally, a

formulation of well-defined composition is necessary for the practical administration and development of polyplex gene carriers as a potential future pharmaceutical product.

Therefore, the aim of this work was the development of a novel purification method which should be suitable for a broad range of polyplexes exhibiting virus-like dimensions with up to a few hundred nanometres. Additionally, the approach should be capable of removing unbound polymer as well as free PEI-conjugates like PEG-PEI or Tf-PEG-PEI. Medium-sized Tf-shielded and targeted polyplexes which have been previously shown to exhibit high transfection efficiency should be purified and systemically applied in tumor bearing mice in order to analyse transfection efficiency and biocompatibility.

2. Materials and methods

2.1. Chemicals and reagents

LPEI (linear PEI, 22kDa) was synthesized by acid-catalysed deprotection of poly(2-ethyl-2-oxazoline) (50 kDa, Aldrich) in analogous form as described in [28]. It is also commercially available from Polyplus (Straßbourg, France). BPEI (branched PEI) with an average molecular weight of 25 kDa was obtained from Sigma-Aldrich (Munich, Germany). PEI was used as a 1-10 mg/ml stock solution neutralized with HCl. PEG20-BPEI (with a molar ratio of 20 kDa PEG to branched 25 kDa PEI of 2:1) and PEG20-LPEI (with a molar ratio of 20 kDa PEG to linear 22 kDa of 0.9:1) conjugates, and Tf-PEG-BPEI conjugates linked with a heterobifunctional 3.4 kDa PEG derivative were synthesized and purified as previously described in [39]. Tf-BPEI conjugate (with a molar ratio of one transferrin molecule linked to PEI) was synthesized as described [37]. Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) [97] was produced by Plasmid Factory (Bielefeld, Germany). Fluorolink^{TM} Cy5 monofunctional dye was purchased from Amersham Biosciences (Freiburg, Germany). Melittin-BPEI conjugates (N-mel, C-mel and analogs CMA-3 and NMA-3) were synthesized as described in [54], [98], [93]. All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Covalent labeling of PEI and PEI-conjugates

LPEI and BPEI: One vial of Fluorolink TM Cy5 monofunctional dye was dissolved in 100 µl DMSO (Cy5 stock solution). 1 mg of PEI diluted in water (910 µl with a concentration of 1.10 mg/ml for LPEI and 925 µl with a concentration of 1.08 mg/ml for BPEI, respectively) was covalently labeled with 10 µl of Cy5 stock solution for LPEI, respectively 25 µl Cy5 stock solution for BPEI, to give a final volume of 1 ml.

Tf-PEG-BPEI and Tf-BPEI: 0.5 mg of Tf-PEG-BPEI respectively Tf-BPEI conjugate diluted in water was mixed with each 25 µl Cy5 stock solution to give a final volume of 500 µl.

PEG20-LPEI and PEG20-BPEI: 0.5 mg of PEG20-LPEI respectively PEG20-BPEI conjugate diluted in water was each mixed with 25 µl Cy5 stock solution to give a final volume of 500 µl.

475 µl of Tf- and PEG- conjugates with a concentration based on PEI content of 1.05 mg/ml was used. Reaction mixtures were left at room temperature in the dark for at least 2h. Unreacted dye was removed by size exclusion chromatography using a Sephadex G25 column (Pharmacia Biotech, Sweden) equilibrated in 75 mM NaCl, 10 mM HEPES, pH 7.4. Cy5 content was measured by absorption at 650 nm; PEI content was estimated by TNBS Assay as described in [99].

The molar ratios of BPEI / Cy5 and LPEI / Cy5 were 1 / 0.7, respectively 1 / 0.5; for Tf-PEG-PEI / Cy5 and Tf-BPEI / Cy5 1 / 1.8, respectively 1 / 1.4; for PEG20-BPEI / Cy5 a ratio of 1 / 1.5 and for PEG-LPEI/ Cy5 a ratio of 1 / 0.8 calculated.

2.3. Quantification of BPEI and LPEI

2.3.1. TNBS assay

Concentration of PEI and PEI conjugates was measured by trinitrobenzenesulfonic acid (TNBS) assay as described in [99]. Standard PEI solutions with a known amount of polymer and test solutions containing either BPEI, LPEI or their respective conjugates were serially diluted in duplicates with 0.1 M sodium tetraborate to give a final volume of 100 µl using a 96 well plate, resulting in PEI concentrations of 10 to 50 µg/ml o 20 to 100 µg/ml for BPEI or LPEI, respectively. 2.5 µl of TNBS (75 nmol) diluted in water was added to each well. Since TNBS reacts with primary amino groups of PEI to form colored trinitrophenylated derivatives, the reaction is faster for BPEI and slower for LPEI. After a reaction time of 5 minutes for BPEI respectively 30 minutes for LPEI carried out at room temperature, absorption was measured at 405 nm using a microplate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria) and a reference wavelength of 630 nm.

2.3.2. Copper complex assay

Quantification of PEI and PEI-linkers was also performed by a copper complex assay described in [100]: 50 µl of Copper-(II)-sulfate dissolved in 0.1 M sodium acetate (0.23 mg/ml), pH 5.4 were mixed with 50 µl of standard PEI samples with known polymer content or test solutions of BPEI and LPEI containing 10 to 80 µg/ml PEI diluted in water. The resulting Cu(II)/PEI complexes were quantified by measuring the absorbance at 285 nm using a Cary 3 Bio spectrophotometer (Varian, Mulgrave, Australia).

2.4. Calculation of N/P ratios in purified polyplex formulations

For naked PEI/ DNA particles polymer content was determined unless indicated otherwise by TNBS assay or copper complex assay. For the latter, the concentration of PEI in the presence of DNA was calculated by the following equation:

$C_{PEI} = (A_{tot} – A_{DNA}) / \varepsilon_{Cu(II)/PEI} * b$

A_{tot}: absorbance at 285 nm of Cu(II)/PEI in the presence of DNA

A_{DNA}: absorbance at 285 nm of the PEI polyplex

$\varepsilon_{Cu(II)/PEI}$: molar absorptivity of Cu(II)/PEI at 285 nm ($\varepsilon_{Cu(II)/BPEI} = 4.85 \times 10^5$; $\varepsilon_{Cu(II)/LPEI} = 6.31 \times 10^5$)

b: fixed optical path length (cm)

Since the Cu(II)/PEI complex is measured at 285 nm (A_{tot}), absorption substantially overlaps with the absorption of DNA (A_{DNA}) present in the solution. Hence absorption of the polyplex at 285 nm must be separately analysed and subtracted from the total absorption (A_{tot}).

For the analysis of multi-functional polyplexes quantification of PEI content either by TNBS or copper complex assay was possible but turned out to be unsteady, especially in the presence of amine-containing buffers (e.g. TBE). Hence, for exact and reproducible determination of N/P ratios in more complex DNA formulations containing well-defined mixtures of polymers and conjugates, a fluorescence-based assay for PEI and PEI conjugates was developed and used if indicated. For this purpose, PEI and PEI-conjugates were labeled with Cy5 dye as described in 2.2 and inserted into the polyplex formulations by replacing 5 % – 20 % of non-labeled material by labeled material. Fluorescence intensity was measured using a Varian Eclipse Fluorimeter (excitation wavelength 649 nm; emission wavelength 670 nm). Since labeling of PEI and PEI conjugates with Cy5 dye was performed at a low ratio of dye to polymer (for every Cy5-conjugate described in 2.2 no more than an average of 2 molecules Cy5 label per molecule PEI or PEI-conjugate), no influence on DNA-binding and polyplex formation occured.

DNA amount was quantified by measuring the absorbance at 260 nm, using a Genesys 10-S spectrophotometer (Thermo Spectronic, Rochester, USA). For exact

calculation of N/P ratios, a non-purified control polyplex generated at N/P 6 with known DNA and polymer content was always used as a standard.

2.5. Polyplex formation and composition

Plasmid DNA was condensed with PEI and PEI conjugates at a molar ratio of PEI nitrogen to DNA phosphate (N/P ratio) of 6 unless otherwise indicated.

All DNA polyplexes were prepared at final DNA concentrations of 20, 50,100, 200 or 400 µg/ ml. For naked LPEI and BPEI polyplexes, indicated amounts of plasmid DNA and PEI were diluted separately in HEPES-buffered glucose (HBG, 5% (w/w) glucose, 20 mM HEPES, pH 7.4) unless indicated else. PEGylated and targeted polyplexes were prepared by first diluting and mixing targeting ligand (Tf-PEG-BPEI), shielding domain (PEG-PEI conjugates) and PEI at given ratios (mol% PEI) in HBG or in 5 mM NaCl, 20 mM HEPES, pH 7.4 for electrophoresis experiments. The solution was rapidly mixed with plasmid DNA (diluted in HBG, respectively in 5 mM NaCl, 20 mM HEPES, pH 7.4).

To enable shielding and targeting of polyplexes, PEI was partially replaced by PEG-PEI (and Tf-PEG-BPEI) or Tf-BPEI. The amount of PEG-shield varied within the experiments between 10 % – 50 %. Assembly of 10 % targeting ligand for PEGylated particles remained constant, while PEI content diversified between 50 % and 80 %, depending on the amount of shielding domain used.

When non-PEGylated but shielded particles with receptor-mediated uptake capability were required, a mixture of 25 % Tf-BPEI and 75 % PEI was prepared prior to addition to the DNA solution (see [37]). In order to generate larger particles, Tf-BPEI conjugate, PEI as well as plasmid DNA were diluted in 0.5 HBS, pH 7.4 (10 mM HEPES, 2. 5 % (w/w) glucose, 75 mM NaCl).

All indicated ratios (mol %) given above are based on the PEI content of the different components. After mixing, polyplexes were allowed to stand for at least 20 minutes at room temperature prior to use.

For detailed composition of all different polyplex formations, tables showing the correct contents and allotments of polymer and polymer conjugates in the formulations with a reference to the used nomenclature for the respective polyplex are given in the individual chapters.

2.6. Measurement of particle size and zeta potential

Particle size of DNA complexes was measured by laser-light scattering using a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). For particle sizing complexes were measured at a final DNA concentration of 10 µg/ml (total volume 1 ml). For estimation of the surface charge, transfection complexes were diluted 5 - fold in 1 mM NaCl to give a final DNA concentration of 2 µg/ml and the ξ potential was measured as described in [39].

2.7. Purification of polyplexes

2.7.1. Cation exchange chromatography

Cation exchange chromatography for *in vitro* experiments was performed with a self made free flow column: glass wool was plugged as filter substance into a standard-sized Pasteur pipette. Column material (CM Sepharose® FF, GE Healthcare, Munich, Germany) diluted 1:1 in water was filled into the glass pipette to give a final bed volume of approximately 1.2 ml and the column equilibrated with 20 mM HEPES, 1.25 M NaCl, pH 7.4. PEGylated polyplexes with a minimum of 20 % PEG-BPEI shielding and a DNA concentration of 100 µg/ml containing up to 60 µg plasmid were loaded onto the column. Fractions of 200 – 300 µl were collected. After elution of shielded polyplexes between 1 M and 1.25 M NaCl, the salt gradient was elevated to 3 M NaCl to elute PEI and PEI conjugates. Fractions containing the major amount of DNA were pooled and additionally analyzed for their DNA and polymer content as described above.

2.7.2. Ultra filtration

For separating unbound PEI, Vivaspin6 ultra filtration devices with a 100 kDa cut-off (Sartorius AG, Göttingen, Germany) were used (see also [95], [96]). Increasing amounts of polyplex dilutions (50 µg/ml DNA) either generated in HBG or 0.5 HBS were centrifuged five times at 3000 g at 4°C for 3 minutes using a Heraeus Megafuge 1.0R. Prior to use, ultra filtration devices were washed with the respective buffer. Between centrifugation steps, polyplex solutions were re-diluted to their original volumes and briefly mixed by up- and down pipetting in order to minimize adsorption of particles on the membrane surface. The filtrate and supernatant were determined for their PEI and DNA content as described above.

2.7.3. Size exclusion chromatography (SEC)

Size exclusion chromatography for *in vitro* experiments was performed with a self made free flow column: A standard-sized Pasteur pipette was plugged with glass wool and column material (Sephacryl® S 200 HR, molecular weight exclusion limit 250 kDa for globular proteins; Pharmacia Biotech, Uppsala, Sweden) diluted 1:1 in water was filled into the glass pipette to give a final bed volume of 1.2 ml. After equilibration with HBG (pH 7.4), column material was preconditioned with a single dose of 400 µg PEI to reduce unspecific adsorption of polyplexes (see [33]) and subsequently washed with HBG. A minimum amount of 400 µl polyplex with a DNA concentration of 100 µg/ml was loaded onto the column. Fractions of 200-300 µl were collected.

For in vivo applications gel filtration was scaled up using an ÄKTA basic system (GE Healthcare, Freiburg, Germany) equipped with a HR 10/10 column packed with Sephacryl® S-200 HR, equilibrated with HBG and preconditioned with 10 mg PEI. Volumes of 5 ml with up to 400 µg/ml DNA were loaded onto the column. SEC was performed at a flow rate of 0.5 ml/ min; fractions of 0.3 ml were collected. Elution of DNA-polyplexes was monitored at 254 nm.

For both variations of SEC, fractions containing the major amount of DNA were pooled and additionally analyzed for their DNA and polymer content as described above.

2.7.4. Electrophoresis (EPH)

2.7.4.1. Standard electrophoresis

A 2% agarose gel was prepared by dissolving 0.6 g agarose (Sigma-Aldrich, Taufkirchen, Germany) in 40 ml TBE buffer [(trizma base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g (all Sigma-Aldrich, Taufkirchen, Germany) ad 1000 ml Millipore water]) and boiling everything up to 100° C. After cooling down to about 70 °C the agarose gel was casted in the electrophoresis unit. A 1 ml volume plastic syringe was cut three times breadthways to obtain the middle piece giving an open cuvette with a volume of 400µl, serving as the sample compartment. A needle with a syringe was pricked at both ends of the cuvette (syringe 1 and 2 in Figure 14) to be able to inject a sample with the first syringe (injection device, syringe 1) and to remove it with the second syringe (removal device, syringe 2). This construct was carefully placed in the agarose gel which had a temperature of about 40°C. The open

sides of the sample compartment were directed to the electrodes (see Figure 14). Since the agarose gel was almost hardened, it was ensured that the sample compartment was completely enclosed by the gel but that the latter did not enter into it. Sample preparations containing PEI only or polyplexes were loaded into the sample compartment via the first syringe. Electrophoresis was performed for 60 minutes at 50 V in TBE Subsequently, the sample was extracted via the second syringe and analyzed for PEI respectively DNA content.

2.7.4.2. ElectroPrep® System

Electrophoresis was performed using AmiKa´s ElectroPrep™ System (available from Harvard Apparatus, Göttingen, Germany). A 1.5 ml Teflon chamber was used as sample compartment. Polycarbonate membranes on each side of the teflon chamber (pore size 0.05 µm) were equilibrated in buffer solution (5 mM NaCl, 20 mM HEPES, pH 7.4) prior to use. To minimize membrane fouling of free polycations, 0.5 ml – 1.0 ml of a 3 % agarose layer was optionally added on the inner side of the membrane, thus reducing the volume of the sample compartment to 0.5 ml – 1.0 ml. Free PEI and various polyplex formulations containing 5 – 20 % of Cy5-labeled PEI respectively conjugate with DNA concentrations between 100 – 400 µg/ml were loaded into the sample unit. PEI molecule was labeled with only 1 – 2 molecules of fluorescent dye per 22 kDa or 25 kDa polymer molecule to avoid any difference with regard to DNA binding of labeled and non-labeled polymers.Electrophoresis was performed in 5 mM NaCl/ 20 mM HEPES, pH 7.4 with a constant voltage of 200 V and 10 mA current over a 20 minute time period if not indicated otherwise. After electrophoresis, samples were collected and DNA respectively PEI and PEI-conjugate concentration was measured. For reporter gene expression and viability measurements, polyplexes were further diluted to a DNA concentration of 20 µg/ml. For in vivo application of purified particles, glucose was added to give a final concentration of 5 % (w/v).

2.8. Cell culture

Cell culture media Dulbecco's modified eagle medium 1 g/l glucose (DMEM) was obtained from Invitrogen GmbH (Karlsruhe, Germany). 500 ml DMEM was supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Invitrogen, Karlsruhe, Germany) and contained 1.6 mM L-alanyl-L-glutamine (Biochrom, Berlin,

Germany) and 1 mM sodium pyruvate (Biochrom, Berlin, Germany). All cultured cells were grown at 37°C in 5 % CO_2 humidified atmosphere.

For reporter gene expression and viability measurements, cells were seeded in 96 well plates at a density of 10^4 cells in 200 µl medium per well 24 hours prior to transfection. During transfection and for the following incubation time until analysis, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen GmbH) were added to the medium.

2.9. Luciferase reporter gene expression

Transfection complexes with indicated amounts of DNA (pCMVLuc) were added to the cells in 100 µl fresh culture medium containing 10 % FBS. Complex containing medium was removed 4 hours after transfection and 100µl of fresh medium were added. 24 h after transfection, cells were washed once with phosphate-buffered saline (PBS) and lyzed with 50 µl of reporter lysis buffer (Promega, Mannheim, Germany). Detection of luciferase activity was carried out as described by Ogris et al. [50]; Measurements were performed in a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany). Values are given as relative light units (RLU) per 10000 seeded cells as mean +/- standard deviation of at least triplicates. Two ng of recombinant luciferase (Promega, Mannheim) correspond to 10^7 light units.

2.10. Metabolic activity of transfected cells

Cells were grown (96 well plate) and treated with different amounts of DNA-polyplexes as described above. Metabolic activity was determined using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay: to each well 10 µl of a 5 mg/ml MTT solution in sterile PBS was added by following incubation at 37°C. After 2 hours, medium was removed and the samples were frozen at -80° C for at least 1 h. 100 µl DMSO was added and samples were incubated at 37°C for at least 30 min under constant shaking. Optical absorbance was measured at 590 nm (reference wavelength 630 nm) using a micro plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria) and cell viability was expressed as percentage relative to untreated control cells.

2.11. Ultra concentration of purified polyplexes for in vivo applications

Purified particles were concentrated through Vivaspin20 ultra filtration devices (Sartorius AG, Göttingen, Germany) for three times at 3000 *g* at 4°C using a Heraeus Megafuge 1.0R. Prior to use, ultra filtration devices were washed with the respective buffer. Between centrifugation steps, the sample was mixed by up- and down pipetting to minimize adsorption of particles on the membrane surface. Polyplexes with a concentration of 400-800 µg/ml DNA were obtained.

2.12. In vivo gene transfer

Male and female A/J mice (8 weeks old), purchased from Harlan Winkelmann (Borchen, Germany) were injected subcutaneously with 1 x 10^6 Neuro2A cells. After 14 days, when tumors had reached approximately 7 – 9 mm in size, transfection polyplexes were applied. Unless indicated otherwise, polyplexes containing 50 µg or 100 µg pCMVLuc per 20 g body weight at a concentration of 200 µg/ml or 400 µg/ml DNA respectively, were injected into the tail vein. Animals were sacrificed 24 h respectively 48 h after application, indicated tissues were resected and stored at -80°C. Tissues were homogenized in Lysis Buffer (Promega, Mannheim) using an IKA-Ultra-Turrax and subsequently centrifuged at 4000 g, 4°C for 15 minutes to separate insoluble cell components. Luciferase activity was determined as described above. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

2.13. Blood sample analysis

To circumvent aggregation of blood samples, syringes were pre-drawn with 200µl heparin. Immediately after sacrificing the animals, blood samples were collected by heart puncture. For the determination of various blood enzymes samples were allowed to clot at 37 °C for 4 h, overnight at 4 °C, then centrifuged at 3000 *g* for 20 min at 4 °C and the supernatants were collected for serum analysis and stored at - 80°C until they were further analysed. Enzymes were quantified by the Institut für klinische Chemie at the Universitätsklinikum Großhadern. Alkaline and aspartate aminotransaminases (AST, ALT) as well as alkaline phosphatase (AP) were measured using a kinetic UV test from Olympus (Olympus Life and Material Science,

Hamburg, Germany). Glutamate Dehydrogenase (GDPH) in plasma was analyzed using a kinetic UV test from Hitado (Hitado Diagnostic Systems, Möhnesee Delecke, Germany).

2.14. Histological examination

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

2.15. Statistics

Where indicated, one-way analysis of variance (ANOVA) was conducted. A *p* value of less than 0.05 was considered to be significant. Duncan test was used as a post-hoc method. As a statistical software package, WinSTAT® 2003 for Excel (R. Fitch Software) was used.

3. Results

3.1. Cation exchange chromatography

The idea of purifying polyplexes by cation exchange is based on the concept that unbound PEI and PEI-conjugates are retained by carboxymethyl groups of the sepharose column material while efficiently shielded particles should pass the column without being retarded.

At the beginning, LPEI polyplexes were generated with 20 % PEG-LPEI shielding domain at N/P 6 in 20 mM HEPES/ 75 mM NaCl, pH 7.4 and loaded onto the column. These particles exhibited a size around 140 nm bearing a zeta potential of 2.6 mV (Table 1). Rather unexpected, the polyplexes were retarded within the given salt concentration and pH of the elution buffer (20 mM HEPES/ 75mM NaCl, pH 7.4); elution of the particles was not possible until a high salt concentration of 1.25 M NaCl was used. Increasing the salt concentration up to 3 M NaCl led to elution of PEI and

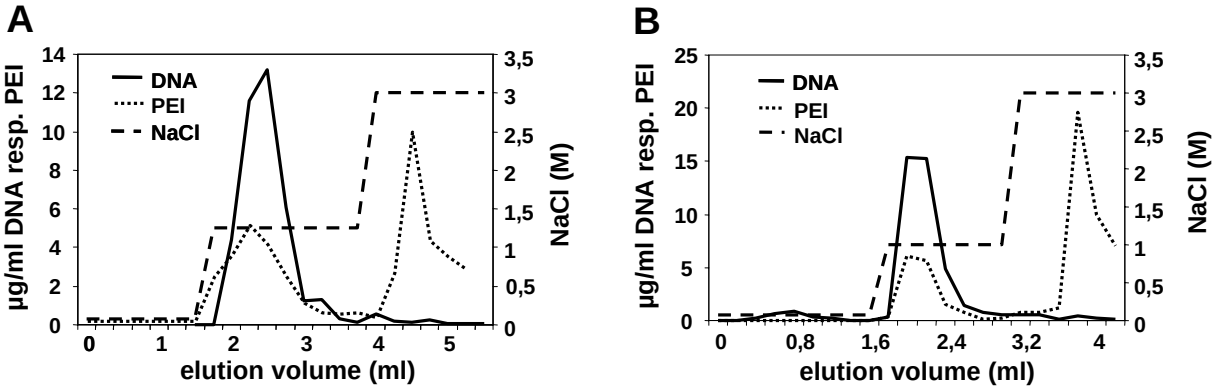


Fig. 4. Purification of shielded LPEI polyplexes by cation exchange chromatography. 20 % (w/w) PEG-LPEI containing complexes (A) were eluted at a salt concentration of 1.25 M. Increasing the shielding domain to 25 % PEG-LPEI (B) slightly reduced the salt concentration required for elution to 1M NaCl. Unbound PEI was washed from the column material with 3 M NaCl .

PEI conjugates (Figure 4A). Increasing the amount of shielding conjugate to 25% resulted in small particles with a surface charge of 1.8 mV (Table1). Still, elution of complexes needed a salt concentration of 1M (Figure 4B). Both formulations exhibited an increased size after cation exchange purification due to the high salt concentration of 1.25 respectively 1 M NaCl in which the particles were measured. Zeta potentials did not change significantly; N/P ratios could be reduced to 2.5 for 20 % PEGylation and 2.8 for 25 % PEG-LPEI, respectively. Cation exchange purification

with 50 % PEG-LPEI conjugate was performed to determine if these particles would posses enough shielding to pass the column material without being retained. Still, gene carriers had to be eluted with 1 M NaCl (data not shown). Beyond, the formulation seemed to be sheared apart as the strongly negative zeta potential of -22 mV indicated (Table 1).

Polyplex	Cation exchange	Size (nm)	Zeta (mV)
80% LPEI	-	140.3 +/- 28.8	+2.6 +/- 1.6
20% PEG-LPEI	+	327.3 +/- 18.6	+3.9 +/- 2.7
75% LPEI	-	117.5 +/- 28.1	+1.8 +/- 1.6
25% PEG-LPEI	+	320.1 +/- 73.1	-2.6 +/- 7.3
50% LPEI	-	396.1 +/- 105.9	+ 2.5 +/- 1.6
50% PEG-LPEI	+	1462 +/- 376.1	-22.5 +/- 12.5

Table 1: Biophysical properties of PEGylated LPEI polyplexes after purification by cation exchange chromatography

3.2. Ultra filtration

Table 2 gives an overview about the polyplex formulations evaluated in the following chapter and the nomenclature used.

	Ratios		
Polycation formulation	*(%)*	*N/P*	*Nomenclature*
LPEI	100	6.0	LPEI/ DNA
BPEI	100	6.0	BPEI/ DNA
Tf-PEG-BPEI/ PEG-LPEI/ LPEI	10/ 20/ 70	0.6/ 1.2/ 4.2	Tf/ PEG/ LPEI
Tf-PEG-BPEI/ PEG-LPEI/ BPEI	10/ 10/ 80	0.6/ 0.6/ 4.8	Tf/ PEG/ BPEI
Tf-BPEI/ LPEI	25/ 75	1.5/ 4.5	Tf/ LPEI
Tf-BPEI/ LPEI	25/ 75	1.5/ 4.5	Tf/ LPEI

Table 2: PEI formulations and nomenclature used in chapter 3.2 and 3.5

3.2.1. Efficiency of PEI separation

Different gene transfer formulations based on LPEI (see Table 2) generated at N/P 6 were ultra filtrated in a Vivaspin6 concentrator with increasing amounts of DNA (12.5 µg – 62.5 µg) respectively LPEI. The following small and large sized DNA formulations were compared: Naked LPEI/ DNA polyplexes mixed in HBG, resulting in the formation of 130 – 150 nm sized particles (see also [30] and [31]); shielded

PEGylated and Tf-targeted LPEI complexes (Tf/ PEG/ LPEI), also mixed in HBG generating gene carriers with sizes in the same range; Tf-shielded LPEI polyplexes (Tf/ LPEI) generated in 0.5 HBS, pH 7.4 resulted in big particles with a mean diameter between 1-2 µm, similar as previously described in [37]). In every polyplex formulation 5 % of the total LPEI content was replaced by 5 % Cy5-labeled LPEI. This allowed i) an easy and reproducible quantitative determination of the polymer in the filtrate and ii) the exact calculation of the N/P ratios after ultra filtration in the supernatant.

After ultra filtration, a clear correlation between resulting N/P ratios of filtrated particles and the amount of DNA respectively polymer present in the non-filtrated complexes was observed. For low amounts of plasmid (12.5 µg DNA), N/P ratios of all filtrated polyplexes observed decreased after five centrifugation circles to values between 2.5 and 2.8 (see Table 3).

polyplex formulation	amount DNA present in the polyplex		
	12.5µg	**37.5µg**	**62.5µg**
N/P before purifiation	**6**	**6**	**6**
LPEI	*2.50*	*3.38*	*4.79*
Tf/ PEG/ LPEI	*2.70*	*3.84*	*4.45*
Tf/ LPEI	*2.78*	*4.19*	*5.09*

Table 3. N/P ratios of ultra filtrated complexes in dependency of applied DNA amount. Filtration was performed with Vivaspin6 100000 for 5 cycles at 3000 g each lasting 3 minutes. Efficiency of purification decreases with increasing amount of DNA respectively polymer applied. Values are based on calculations for LPEI only

Increasing the amount of DNA and LPEI present as polyplex in the ultrafiltration device resulted in less pronounced decreases in N/P ratios after ultra filtration (between 3.4 and 4.2 for 37.5 µg DNA). In line, at the highest DNA amount of 50 µg, efficiency of purification was rather low (N/P ratios of filtrated particles between 4.8 and 5.1). Comparable results were obtained with BPEI-based complexes (data not shown). N/P ratios of filtrated polyplexes could not be significantly decreased by additional centrifugation steps but resulted in lower polyplex recovery and sometimes even membrane fracture. In general, particle yield determined as DNA recovery in the supernatant was similar for all formulations and for every dosages applied and varied between 65 % and 80 %. Biophysical properties of polyplexes like size and zeta potential did not change significantly after ultra filtration (data not shown). In

order to verify incomplete removal of LPEI for large amounts of polyplexes applied, the ultra filtrated PEGylated and targeted polyplex formulation as shown in Table 3 (containing 37.5 µg DNA) was gel filtrated in a subsequent step via an S-200 SEC setup. The elution profile revealed a second peak, marking free LPEI which was obviously not completely removed by ultra filtration (Figure 5).

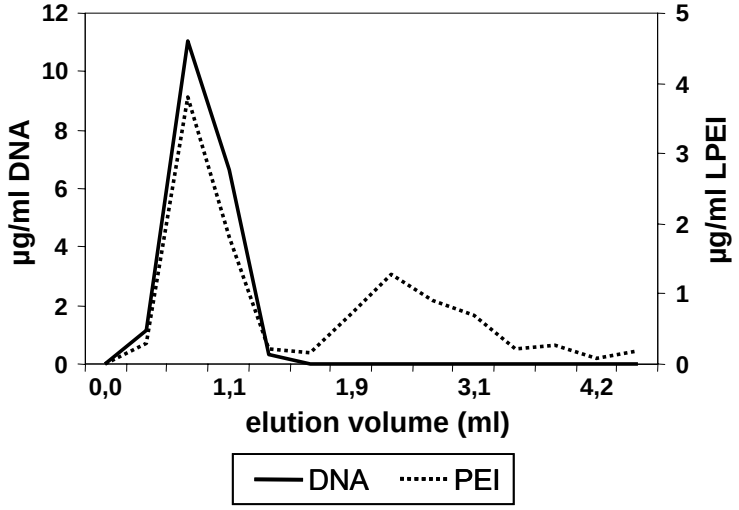


Fig. 5. Size exclusion chromatography after ultra filtration of 30 µg DNA containing Tf/ PEG/ LPEI N/P 6 polyplexes. The second peak of the SEC elution profile indicates the presence of free PEI thus demonstrating incomplete removal of unbound LPEI by ultra filtration

Efficiency of purification by ultra filtration was obviously correlated with the amount of polymer applied. For a detailed analysis of LPEI during and after ultra filtration, the permeation of 10 µg LPEI (containing 9.5 µg LPEI and 0.5 µg Cy5-LPEI) through the membrane of the Vivaspin6 concentrator was determined. The amount of polymer in the supernatant and in the filtrate was estimated by measuring the Cy5-fluorescence intensity of labeled PEI (Figure 6). Additional application of 10 µg PEI revealed that after five centrifugation rounds less than 10 % of PEI could be recovered in the supernatant (indicating an efficient separation of at least 10 µg non-bound LPEI in complex formulations). However, only 22 % of PEI was found in the filtrate. Hence, more than 70 % of polymer could not be detected and stuck most likely to the filter material.

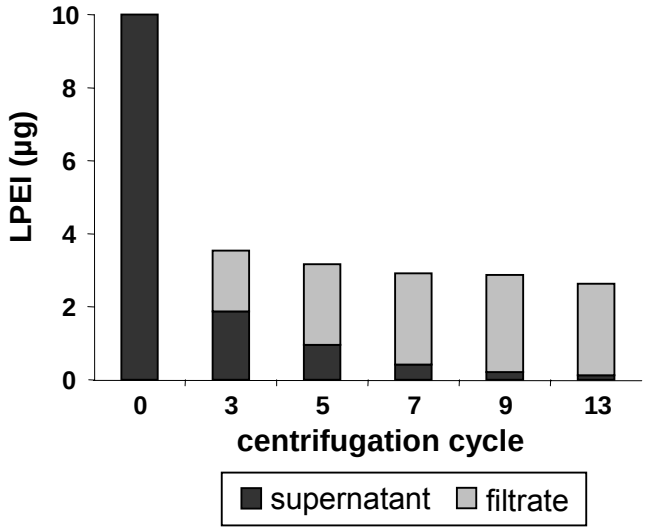


Fig. 6. LPEI content found in the supernatant and filtrate as a function of centrifugation cycles.

The amount of PEI which could not be detected slightly increased with the number of centrifugation cycles. After 13 centrifugation cycles, more than 89 % of PEI were removed from the supernatant, but only 25 % of polymer could be recovered in the filtrate. This implies that a high proportion of LPEI might irreversibly stick on the filter membrane leading to membrane blocking and therefore causing inefficient separation of polyplex and free PEI.

3.2.2. Gene expression and toxicity profiles of ultra filtrated complexes

Since it was demonstrated that polyplexes with up to 10 µg DNA can efficiently be purified by ultra filtration, gene transfer efficiencies and cytotoxicity of different LPEI- and BPEI-based crude and ultra filtrated polyplex formulations were evaluated on Neuro2A cells. All particles were generated at N/P ratio of 6.

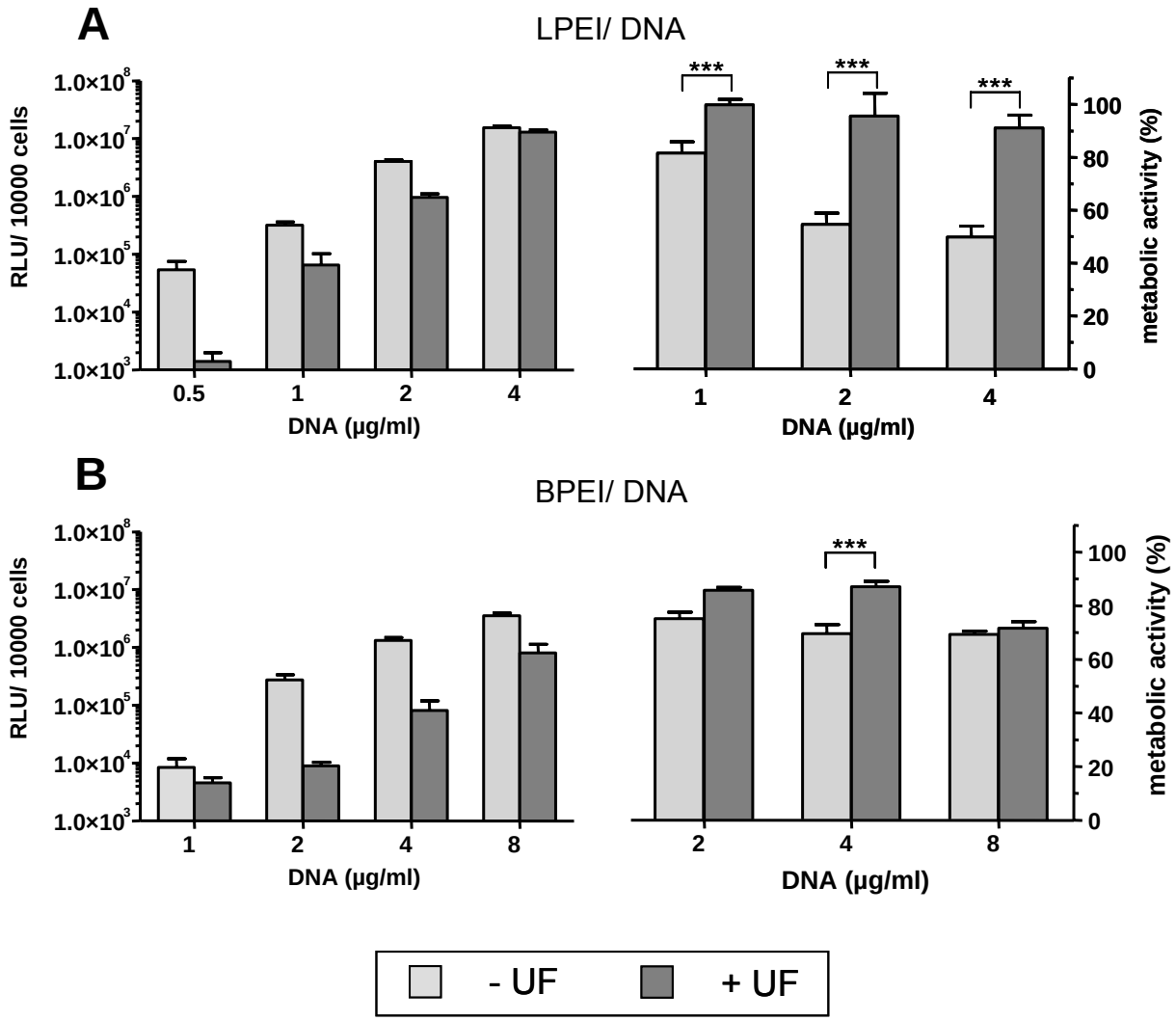


Fig. 7. Reporter gene expression and metabolic activity of PEI polyplexes in Neuro2A tumor cells. LPEI (A) and BPEI (B) polyplexes with increasing concentrations of DNA were applied. Polyplexes at N/P 6 with a maximum amount of 10 µg DNA were ultra filtrated (+UF) and compared to non-filtrated complexes (-UF) at N/P 6. Polyplexes were mixed in HBG. Luciferase activity (left side) is given in RLU per 10000 cells, metabolic activity (right side) is displayed as % of untransfected control cells. Mean values +SD of triplicates are shown.

First, naked PEI/ DNA gene carriers were investigated (Figure 7). As expected, LPEI/ DNA and BPEI/DNA polyplexes were up to 30-fold less efficient in transfection at low DNA concentration (0.5 µg/ml DNA for LPEI and 2 µg/ml DNA for BPEI). At a DNA concentration of 4 µg/ml, LPEI polyplexes showed equivalent transfection efficiency as particles containing free PEI (Figure 7A). For BPEI complexes, differences in transfection efficiency decreased at the highest DNA concentration of 8 µg/ml (Figure 7B). In general, differences between purified and non-purified gene carriers with regard to transfection levels were more pronounced for BPEI than for LPEI.

In order to evaluate the effect of ultra filtration on toxicity, metabolic activity of cells was determined 24h after transfection by measuring intracellular ATP levels. LPEI polyplexes containing free PEI exhibited high toxicity above 2 µg/ml DNA with only 40 % – 50 % vital cells whereas metabolic activity of cells treated with purified LPEI complexes could be maintained at 80 % compared to control cells (Figure 7A). In contrast to pronounced variations in transfection efficiencies, differences in metabolic activity of purified and non-purified BPEI polyplexes were less distinct. Nevertheless, at 2 and 4µg/ml DNA, purified complexes demonstrated 10 % – 15 % higher metabolic activity compared to polyplexes containing free BPEI (Figure 7B).

As demonstrated, ultra filtration allows purification of large particles (which is not possible for the method based on SEC). Therefore, transferrin-shielded BPEI and LPEI particles, generated in 0.5 HBS resulting in large gene carriers with diameters between 500 – 1500 nm were purified by ultra filtration. *In vitro* transfection potency and toxicity profile was evaluated and compared to the non-purified versions (Figure 8).

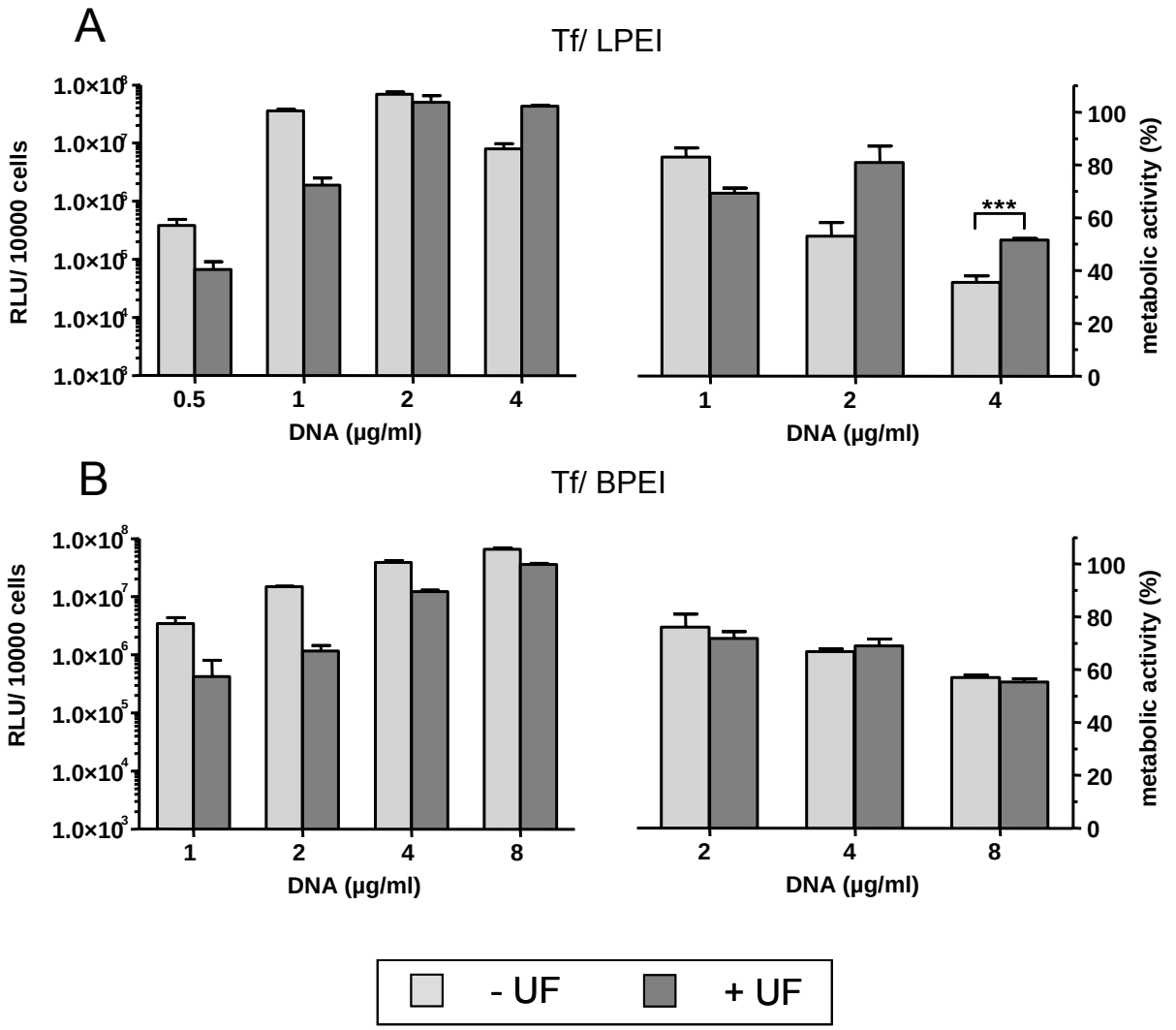


Fig. 8. Reporter gene expression and metabolic activity of Tf/ PEI polyplexes on Neuro2A cells. Transfection was carried out with Tf/ LPEI (A) and Tf/ BPEI (B) polyplexes with increasing concentrations of DNA. Polyplexes mixed in 0.5 HBS at N/P 6 with a maximum amount of 10 µg DNA were ultra filtrated (+UF) and compared to non-filtrated complexes (-UF) at N/P 6. Luciferase activity (left side) is given in RLU per 10000 cells, metabolic activity (right side) is displayed as % of untransfected control cells. Mean values +SD of triplicates are shown.

In line with the results obtained for naked PEI polyplexes, purified Tf/ LPEI polyplexes showed reduced transfection efficiency in comparison to non-purified ones at low DNA concentrations (0.5 and 1 µg/ml). In contrast, at higher DNA concentrations (≥ 2 µg/ml) purified Tf/ LPEI polyplexes exposed equivalent or even higher transfection efficiencies than polyplexes containing free PEI.

Again, transfection efficiency of Tf/ BPEI complexes was reduced for all DNA concentrations tested in comparison to non-purified polyplexes. However, this difference was decreasing with increasing DNA concentrations.

Toxicity profiles of purified Tf/ LPEI and Tf/ BPEI polyplexes were similar to the corresponding naked LPEI and BPEI particles: Again, the advantage of purification

was more pronounced for Tf/ LPEI gene carriers showing clearly reduced toxicity at higher DNA dosages in comparison to non-purified particles [at 2 µg/ml DNA 80 % metabolic activity for purified Tf/ LPEI polyplexes and 50 % metabolic activity for non-purified control complexes (see Figure 8A)]. However, for Tf/ BPEI no differences in metabolic activity for purified and non-purified complexes were observed. In general, BPEI polyplexes exhibited less toxicity than the corresponding LPEI formulations (Figure 8B).

3.3. SEC of shielded and targeted melittin-based polyplexes

3.3.1. Stable insertion of melittin

In order to enhance endosomal release of polyplexes into the cytosol, three different membrane-active melittin-PEI analogs previously described in [98] and [93] were incorporated into targeted and shielded complexes. For evaluation of their impact on gene transfer efficiency as well as on toxicity, these novel polyplex formulations were compared to standard shielded and targeted LPEI- and BPEI-based polyplexes. For detailed composition of particles and their nomenclature used, see Table 4.

	Ratios		
Polycation formulation	*(%)*	*N/P*	*Nomenclature*
Tf-PEG-BPEI/ PEG-LPEI/ LPEI	10/ 10/ 80	0.6/ 0.6/ 4.8	Tf/ PEG/ LPEI
Tf-PEG-BPEI/ PEG-BPEI/ BPEI	10/ 10/ 80	0.6/ 0.6/ 4.8	Tf/ PEG/ BPEI
Tf-PEG-BPEI/ PEG-BPEI/ N-mel-BPEI	10/ 10/ 80	0.6/ 0.6/ 4.8	Tf/ PEG/ N-mel
Tf-PEG-BPEI/ PEG-BPEI/ NMA-3-BPEI	10/ 10/ 80	0.6/ 0.6/ 4.8	Tf/ PEG/ NMA-3
Tf-PEG-BPEI/ PEG-BPEI/ CMA-3-BPEI	10/ 10/ 80	0.6/ 0.6/ 4.8	Tf/ PEG/ CMA-3

Table 4. PEI formulations and nomenclature used in chapter 3.3

The first key question was to elucidate, if the endosomolytic domain would be still active in the polyplex after removal of free melittin-PEI conjugates by size exclusion chromatography. Therefore, CMA-3-BPEI was fluorescently labeled with Cy5 dye and gel filtration was performed as describes in [98]. Incorporation of melittin-PEI conjugate into polyplexes was demonstrated (Figure 9) as upon SEC, 45 - 50 % of the melittin-PEI was recovered within the complexes, a result comparable to pure PEI polyplexes [33].

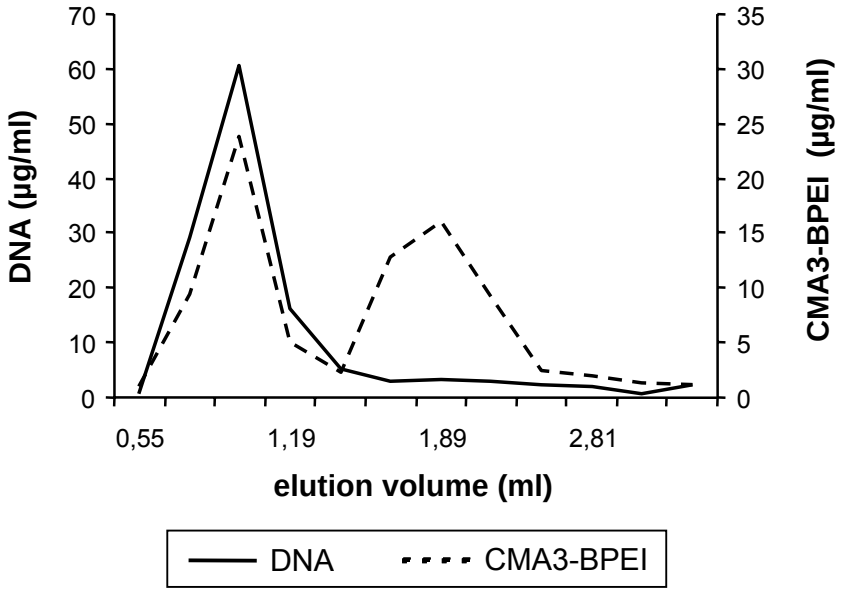


Fig. 9. Size exclusion chromatography of Tf/ PEG/ CMA3 polyplexes demonstrates stable insertion of CMA3-BPEI into the gene carrier

3.3.2. Biophysical characterization

For determination of biophysical properties, all polyplexes were generated in HBG. Both purified and non-purified polyplexes were further diluted in 0.5 HBS. As expected, particle size and zeta potential did not change significantly after purification. Quantification of separated PEI revealed that gel filtration removed 50 – 60 % of free PEI resulting in N/P ratios between 2.5 and 3.1 (see Table 5).

Polyplex formulation	*SEC*	*Size (nm)*	*Zeta potential (mV)*	*removed PEI*
Tf/ PEG/ LPEI	-	165.1 +/- 26.6	2.2 +/- 1.6	*60%*
	+	150.6 +/- 14.9	2.1 +/- 2.2	
Tf/ PEG/ BPEI	-	158.9 +/- 19.6	2.3 +/- 0.6	*48%*
	+	114.7 +/- 15.2	2.2 +/- 1.6	
Tf/ PEG/ N-mel	-	149.0 +/- 23.1	2.2 +/- 1.6	*52%*
	+	155.3 +/- 18.8	2.6 +/- 1.8	
Tf/ PEG/ CMA-3	-	142.1 +/- 17.2	3.1 +/- 2.3	*57%*
	+	150.1 +/- 15.1	2.9 +/- 0.8	
Tf/ PEG/ NMA-3	-	126.4 +/- 11.7	2.3 +/- 1.6	*45%*
	+	138.8 +/- 19.0	3.1 +/- 1.6	

Table 5. Biophysical properties of polyplexes before and after SEC purification

3.3.3. Reporter gene expression and toxicity profiles

All polyplex formulations were prepared in HBG and increasing DNA concentrations of 1 – 4 µg/ml were added to the cells. Purification was performed by SEC.

At the lowest DNA concentration of 1 µg/ml (Figure 10A), both standard polyplexes (Tf/ PEG/LPEI and Tf/ PEG/ BPEI) exhibited the lowest transfection efficiency with purified versions, resulting in negligible reporter gene expression. In contrast, non-purified melittin-containing polyplexes possessed improved transfection properties with Tf/ PEG/ CMA-3 and Tf/ PEG/ NMA-3 derivatives being the most potent ones, exhibiting a 10-fold enhancement in expression level in comparison to LPEI and BPEI containing particles. In line, purified melittin containing polyplexes led to an enhancement in expression levels when compared to the standard complexes: N-Mel and the NMA-3 derivative induced an approximately a 5-fold higher gene expression level than purified LPEI containing gene carriers. Purified CMA-3 polyplexes exhibited the highest transfection efficiency with more than 10-fold increase towards the LPEI-based complex also maintaining the same expression level as non-purified Tf/ PEG/ CMA-3 formulation containing free CMA-3.

Increasing the DNA concentration to 2 µg/ml led to a general enhancement in gene expression level. Differences in efficiency between the three non-purified melittin-containing particles and the non-purified standard Tf/ PEG/LPEI and Tf/ PEG/ BPEI complexes are still quite pronounced with a 10 fold increase in comparison to LPEI and a 15 fold increase towards BPEI containing gene carriers (Figure 10B). At this DNA concentration, all melittin-containing crude non-purified polyplexes exhibited the same expression level of approximately 2 x 10^5 RLU. In line, all three purified

counterparts were showing similar gene transfer efficiencies with expression levels ranging 3 - 4 times lower than the respective non-purified ones. On the other hand, those purified melittin-containing particles still exhibited a 3 – 4 fold enhancement in transfection efficiency in comparison to polyplexes containing unmodified L- or BPEI.

At the highest DNA amount applied (4 µg/ml, Figure 10C), all purified formulations exhibited similar expression efficiencies with values between 2 x 10^6 RLU and 4 x 10^6 RLU and reached expression levels in the range of non-purified Tf/ PEG/ CMA-3 and Tf/ PEG/ NMA-3 polyplexes.

24 hours after transfection, metabolic activity of transfected cells was analyzed in order to evaluate toxicity profiles of purified and non-purified complexes. In general, neither purified nor non-purified polyplexes caused severe signs of toxicity (Figure 10A - C, left panels). At the high concentration of 4µg/ml DNA metabolic activity of cells treated with non-purified Tf/ PEG/ CMA-3- and Tf/ PEG/ NMA-3 was reduced to 75% in comparison to control cells. However, purification of these polyplex formulations could clearly improve the toxicity profile.

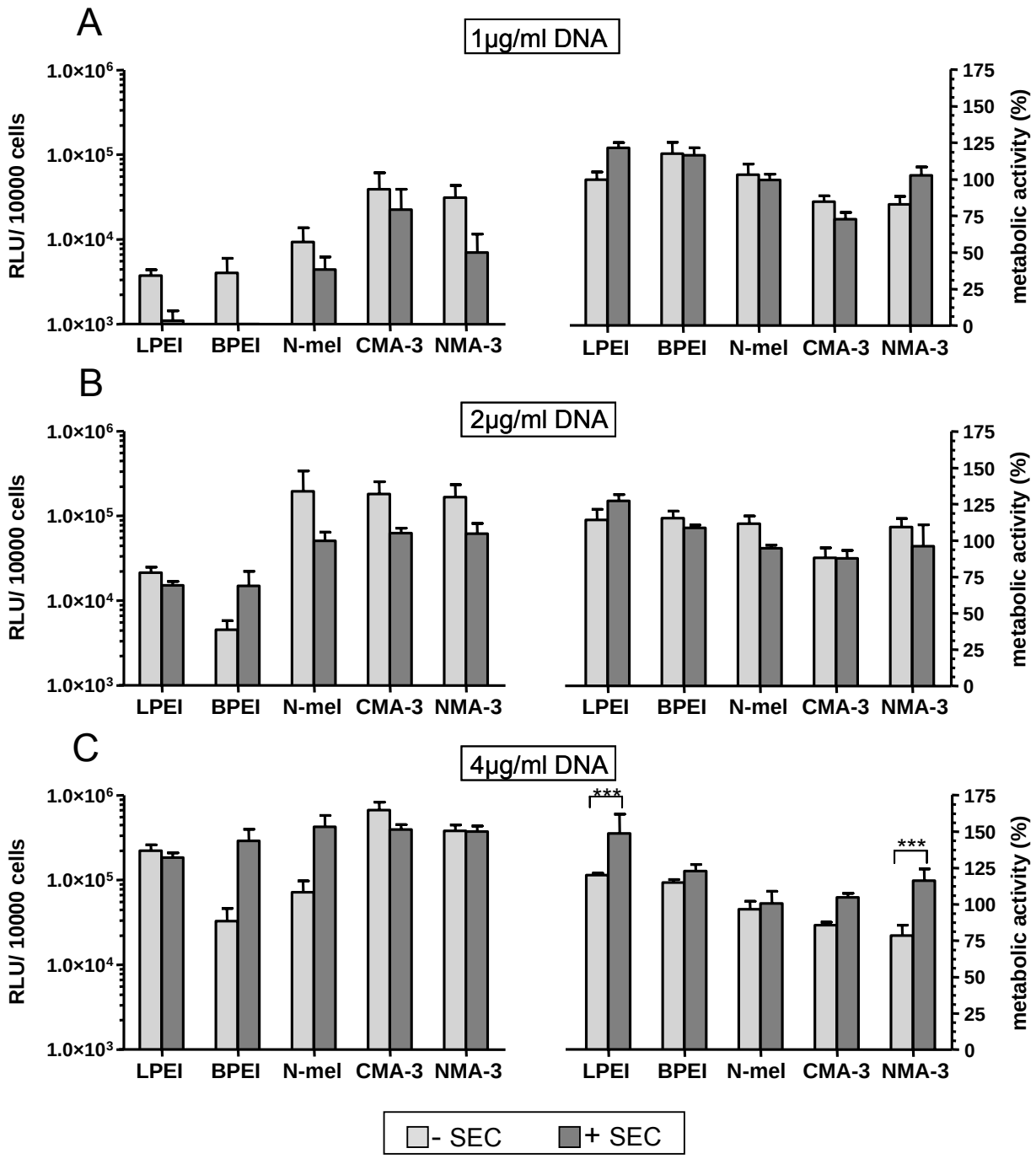


Fig. 10. Reporter gene expression and metabolic activity of Neuro2A cells transfected with various Tf-targeted and PEGylated polyplexes mixed in HBG. Transfection was carried out with increasing concentrations of DNA polyplexes (1 µg/ml DNA [A], 2 µg/ml DNA [B] and 4 µg/ml [C]). Luciferase activity (left side) is given in RLU per 10000 cells, metabolic activity (right side) is displayed as % of untransfected control cells. '+SEC': purification of complexes by SEC; '-SEC': non-purified control complexes. All complexes included 10% (w/w) Tf-PEG-BPEI and 10% (w/w) PEG-PEI. The X-axis displays the different PEI cores of compared polyplexes. Mean values +SD of triplicates are shown.

3.3.4. Up-scaling of SEC and combination with ultra filtration for in vivo applications

Generation of polyplexes, SEC and up concentration of gene carriers were performed under sterile conditions. Up to 5 ml of DNA polyplexes with a concentration of 200 µg/ml were loaded onto the column. Particles were eluted in HBG. Polyplex recovery and detection of BPEI and LPEI was performed as described in Materials and Methods. As shown in Figure 11, up-scaling still enabled a clear separation of DNA polyplex and free PEI. Polyplex recoveries between 60 and 70 % corresponding to 600 – 700 µg DNA per run were detected. During SEC a dilution of particles occurred. Therefore, an additional concentration step was necessary: gel filtrated complexes were transferred to Vivaspin20 concentrator (100000 kDa cut-off) tubes which were centrifuged 3 times at 3000 g at 4°. Thus, an up concentration to 200 – 400 µg/ml DNA was achieved. Ultra concentration of gene carriers did not lead to significant changes in particle size (mean diameters around 180 nm) were detected (data not shown).

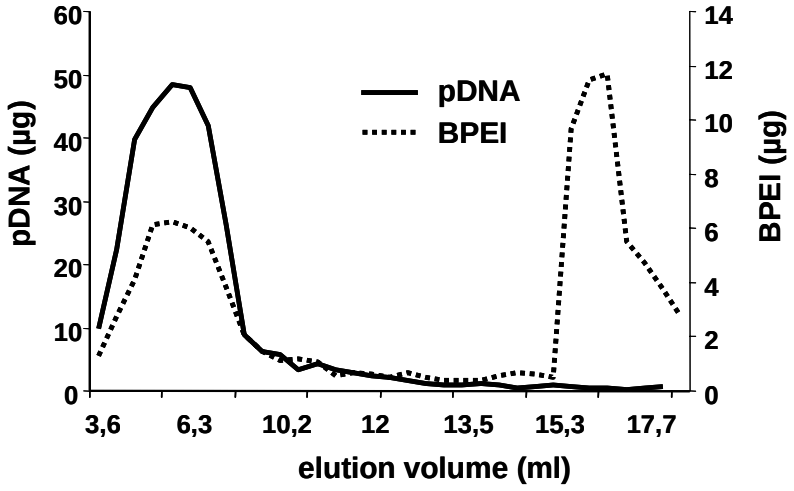


Fig. 11. Up-scaling of SEC based purification of Tf/ PEG/ BPEI polyplex. Elution profile of DNA and BPEI after purification of 5 ml Tf/ PEG/BPEI mixed at N/P 6 in HBG containing 200 µg/ml DNA.

3.3.5. In vivo administration of gel filtrated polyplexes

3.3.5.1. Low dosage administration

Systemic delivery of purified versus non-purified polyplexes in an in vivo model of A/J mice bearing subcutaneously grown Neuro2A tumors was evaluated due to previous encouraging findings [33] and the in vitro data described above. Non-purified polyplex formulations given in Table 5 with a standard DNA amount of 50µg per mouse (n = 4) were injected. Mice receiving the non-purified Tf/ PEG/ BPEI and Tf/ PEG/ LPEI formulations did not show any signs of toxicity. In contrast, all four mice obtaining the non-purified Tf/ PEG/ CMA-3 formulation suffered from severe signs of toxicity and died within 5 – 10 minutes after injection. The same could be observed with 2 mice receiving the full dosage (50 µg) of Tf/ PEG/ NMA-3: both exhibited signs of shock, reduced activity, ruffled fur accompanied with convulsions, leading to death within 30 minutes after application. In order to have at least the remaining two mice surviving the Tf/ PEG/ NMA-3 application, we decided to inject only half of the dosage (25 µg). Both of them showed again signs of toxicity which emerged to be reversible as the mice survived the treatment. Application of non-purified Tf/ PEG/ N-mel did not lead to acute toxicity within the first two hours after application; however, all four mice died within the next 20 hours. Examining the bodies of all dead mice revealed large bleedings in the intestines (mainly bowel). The liver had a sponge-like look with lamella-like structures and macroscopically visible bleedings.

Since toxicity was too pronounced with the CMA-3 core particles, the purified version was not further evaluated in vivo. All other groups (containing the LPEI, BPEI, NMA-3 and N-mel core) were purified by gel filtration and subsequently ultra concentrated. Application of 50 µg DNA per mouse demonstrated good biocompatibility of purified polyplexes. No mice did suffer from any signs of toxicity.

The remaining mice surviving the treatment with non-purified polyplexes (each four mice in the Tf/ PEG/ LPEI, the Tf/ PEG/ BPEI and the Tf/ PEG/ NMA-3 group) and all animals receiving purified particles were sacrificed 24 hours after application and organs were analysed for reporter gene expression (Figure 12A-D). In general, detected luciferase signal was rather low for all formulations with or without purification and exceeded hardly background levels of 103 – 104 light units (LU) per organ, so that only slight propositions about tendencies can be made. Expression levels of mice receiving purified as well as non-purified Tf/ PEG/ LPEI formulations (Figure 12A) were the lowest with expression levels under 104 LU, even lower than

the animals injected with Tf/ PEG/ BPEI. The non-purified version of the latter showed the highest gene expression in the tumor, followed by the liver whereas the purified BPEI core formulations exhibited very low expression values mostly found in the tumor and the lung (Figure 12B). 25 µg of Tf/ PEG/ NMA-3 containing free NMA-3-BPEI per animal did not lead to a reasonable reporter gene expression in any organ. The double amount of 50 µg purified Tf/ PEG/ NMA-3 led to a gene expression signal mainly found in the tumor and lung with values around 1.5 x 104 LU (Figure 12C). Gene expression of purified Tf/ PEG/ N-mel was mainly found in the lung (1.5 x 105 LU); tumor expression was detectable but rather low (Figure 12D) and comparable to the one found with purified BPEI core complexes (approximately 4 x 103 LU).

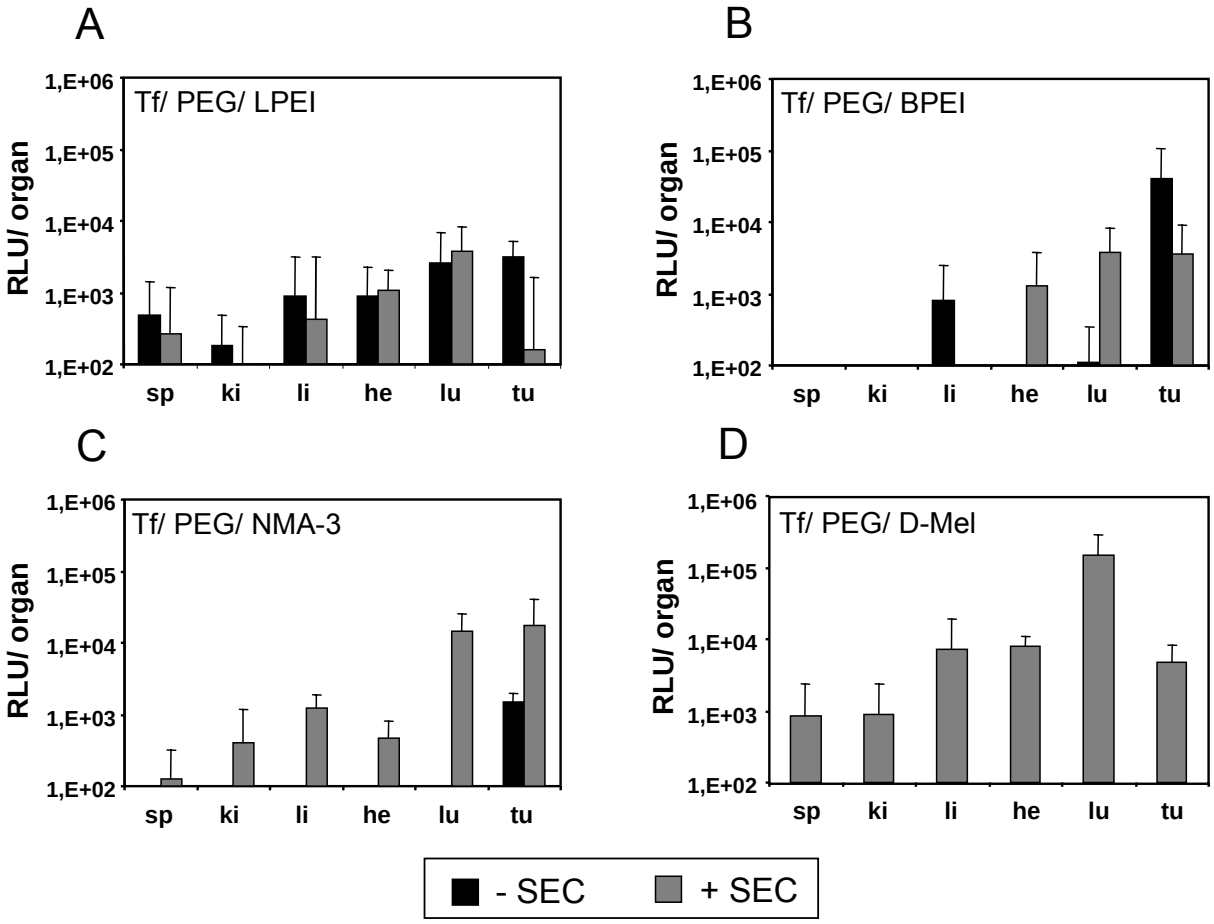


Fig. 12. Transgene expression in vivo after systemic delivery of polyplexes into A/J mice bearing subcutaneous Neuro2A tumours. Tf/ PEG/ LPEI (A), Tf/ PEG/BPEI (B), Tf/ PEG/ NMA-3 (C) and Tf/ PEG/ N-mel (D) complexes with a final concentration of 200 µg/ml DNA (50 µg per 20 g bodyweight) were injected into the tail vain. Luciferase expression was measured 24h after application and is expressed as total luciferase activity per organ. Note that all mice receiving 50 µg of non-purified Tf/ PEG/ D-mel died due to high toxicity; only 25 µg of non-purified TF/ PEG/ NMA-3 polyplexes were applied.

3.3.5.2. High dosage administration

Systemic administration of small and shielded complexes did not result in remarkable gene expression but purification of the formulations resulted in nontoxic polyplexes as no signs of toxicity occurred. Therefore, another in vivo series in male A/J mice was added with the application of the double amount of DNA (100 µg per 20 g body weight; n = 6). To maintain an injection volume of 250 µl per 20 mg bodyweight, the polyplexes had to be generated at an increased DNA concentration of 400 µg/ml. Due to high toxicity of non-purified NMA-3 containing particles, only purified and non-purified Tf/ PEG/ BPEI polyplexes and purified Tf/ PEG/ NMA-3 complexes were included in the high dosage in vivo series. Since application of Tf/ PEG/ N-mel did not lead to any advantage in comparison to the BPEI or the NMA-3 containing gene carriers, this formulation was excluded from further evaluation. Tf/ PEG/ LPEI polyplexes exhibited no significant expression levels as well and revealed to be unstable at DNA concentrations above 250 µg/ml DNA, forming big aggregates. Therefore, this polyplex formulation was not further examined during the following in vivo series. Polyplex formulations at this high DNA concentration were increased in size (190 +/- 87.6 nm for BPEI and 179.1 +/- 65.3 nm for N-mel core polyplexes) and possessed slightly higher zeta potentials (+7.8 mV for BPEI and +7.5 mV for N-mel core polyplexes).

Again, all purified polyplex formulations, including the ones containing melittin-conjugate, were well tolerated by the animals. In contrast, administration of 100 µg non-purified Tf/ PEG/ BPEI resulted in significant toxicity with three mice out of six dying within 30 minutes. They all showed the same signs of shock as described in 3.3.5.1. Livers and the small intestines of the dead mice showed strong bleedings; furthermore spleens were black-colored.

24 hours after application of polyplex formulations, surviving mice were sacrificed and organs analyzed for luciferase expression. Gene expression levels of delivered non-purified Tf/ PEG/ BPEI formulations were quite high. However, biodistribution pattern was unfavorable: indeed highest luciferase expression (2×10^5) was found in the tumor (Figure 13A) but expression levels in the liver were quite high (6×10^4 LU) followed by lung expression.

Strikingly, purified Tf/ PEG/ BPEI complexes (Figure 13B) exhibited tumor expression levels even higher (3 x 10^5) than that of the non-purified particles whereas liver expression was on a background level. Comparable high expression in the lung implicates that the polyplexes are not completely shielded at the high DNA concentration of 400 µg/ml DNA. This was confirmed by zeta potential measurements. Purified Tf/ PEG/ NMA-3 polyplexes (Figure 13C) exhibited also highest expression in the tumor tissue with values about 1.2 x 10^5 LU being slightly lower than the values obtained for the BPEI containing polyplexes. Again, lung expression reached high levels implicating only partial shielding of polyplexes.

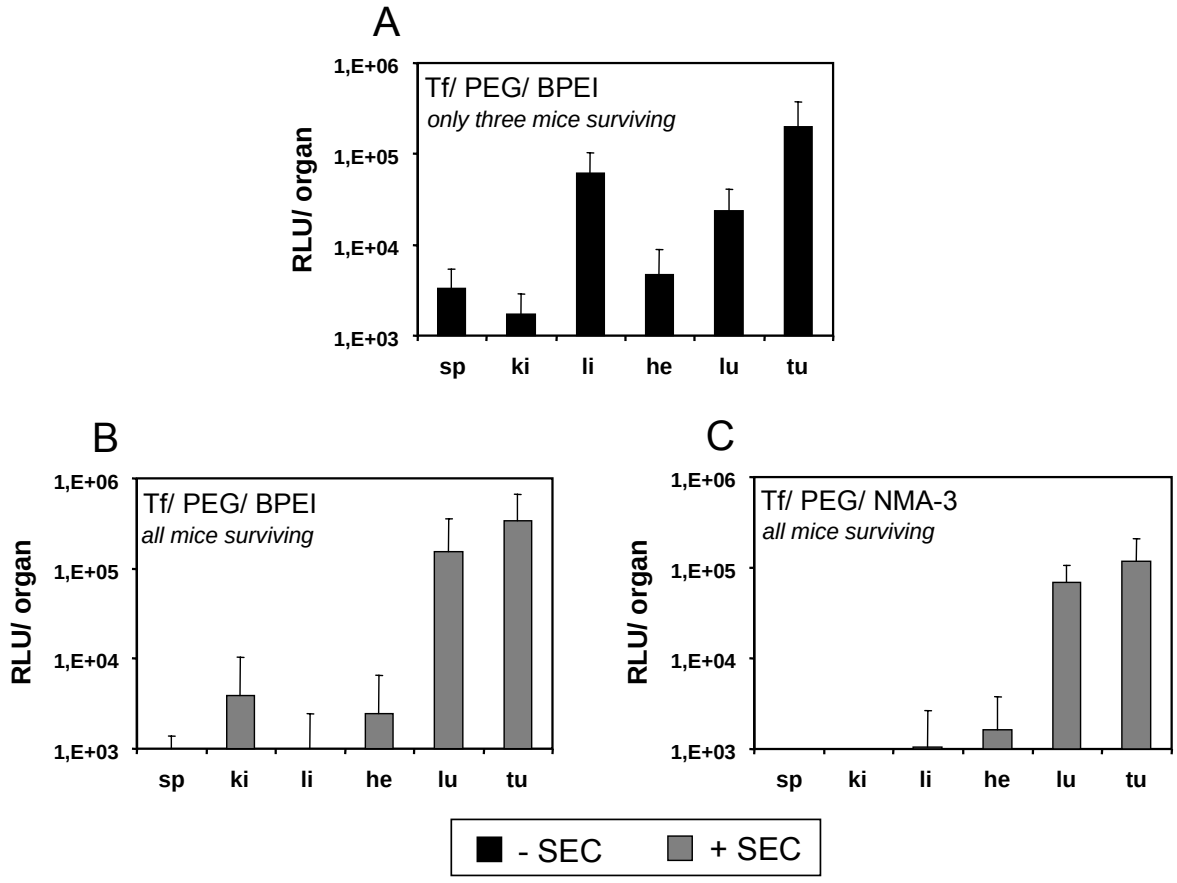


Fig. 13. In vivo gene transfer after systemic delivery of polyplexes into A/J mice bearing subcutaneous Neuro2A tumours. Tf/ PEG/ BPEI (A and B) and Tf/ PEG/ NMA-3 (C) complexes with a final concentration of 400 µg/ml DNA (100 µg per 20 g bodyweight) were injected into the tail vain. luciferase expression was measured 24h after application and is expressed as total luciferase activity per organ.

3.4. Electrophoresis (EPH)

3.4.1. Standard agarose gel electrophoresis demonstrates proof of concept

Inspired by the so called agarose gel retardation assay performed to determine complex formation of DNA polyplexes in an agarose gel, a setup was developed to separate polyplexes from unbound PEI (see Figure 14).

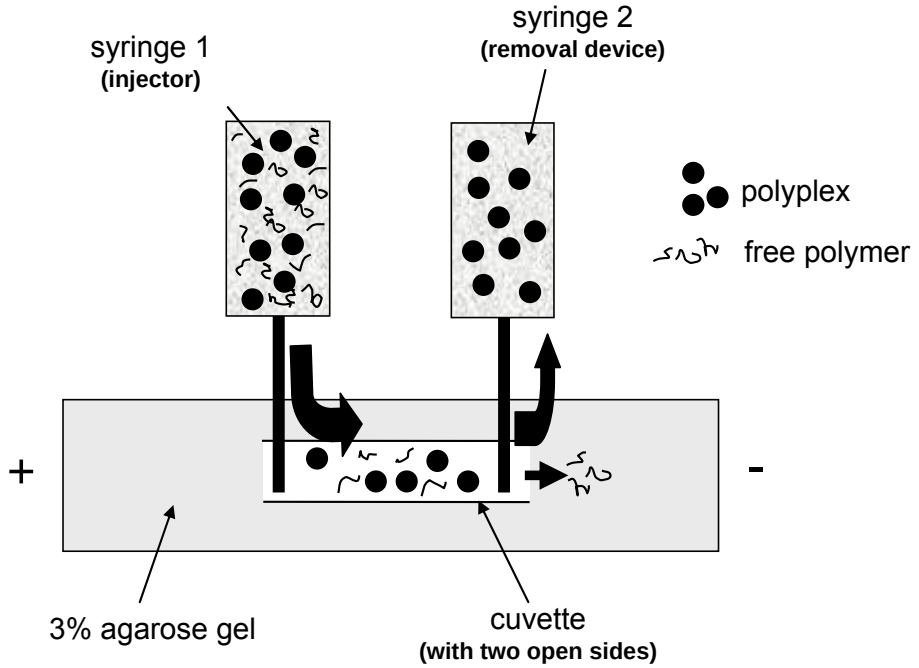


Fig. 14. Setup for purification of complexes by electrophoresis (originally developed by Michael Günther from our research group). Non-purified sample is injected with syringe 1 into a cuvette bedded into an agarose gel. The cuvette contained two open sides next to the electrodes. Separation of free polycation is realized by the application of an electric field, hence resulting in targeted movement of charged molecules like PEI to the electrodes in dependency of their size. Purified polyplex can be removed from the cuvette with syringe 2.

Monitoring the fluorescence labeled BPEI revealed that the polymer was moving fast in the applied electric field and migrated into the agarose gel (data not shown). Hence, 400 µl of polyplex dilution (generated at N/P 6 in HBG consisting of 10 % Tf-PEG-BPEI/ 10 % PEG-BPEI and 80 % BPEI) with a DNA concentration of 100 µg/ml were injected. Electrophoresis was performed at 50 V for 1 h. Samples were removed and immediately analyzed for DNA and BPEI content. N/P ratios of purified polyplexes were reduced to 2.5 +/- 0.36, corresponding to a total removal of 58 % polymer. Since the running buffer used (TBE) contained amino-groups which could possibly react either in the TNBS assay as well as in the copper sulfate assay, complete separation of BPEI was controlled by a following SEC analysis of the purified polyplex (see Figure15). Analysis of the fractions for BPEI revealed that no

detectable free BPEI could be measured since a second peak indicating BPEI in the elution profile did not occur (which was the case for a non-purified control complex mixed at N/P 6).

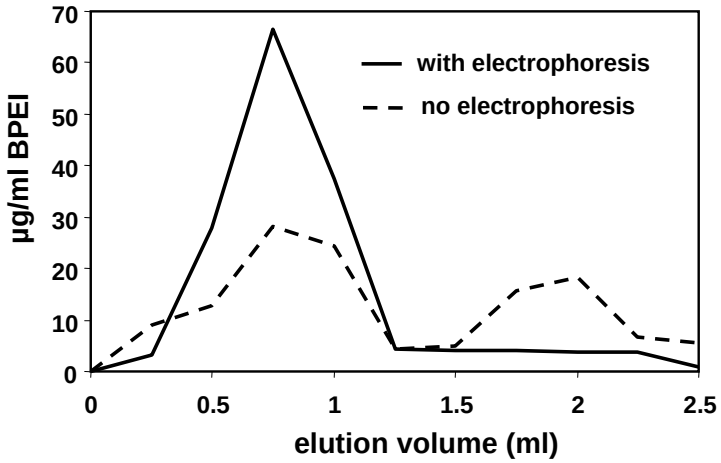


Fig. 15. Detection of BPEI after size exclusion chromatography of Tf/ PEG/ BPEI polyplexes. Complexes were mixed at N/P 6. Purification of polyplexes was performed by electrophoresis with one part of the particles. SEC was performed for both formulations in HBG. For purified polyplexes ('with electrophoresis') all detectable BPEI was recovered within the void fraction containing the polyplex. In contrast, non-purified complexes ('no electrophoresis') showed two PEI peaks, the second marking unbound BPEI. For purified polyplexes, double amount of DNA was gel filtrated to improve the detection of BPEI.

3.4.2. Electro dialysis with ElectroPrep® System

Indeed, classic gel electrophoresis experiments demonstrated proof of concept, but the setup and the whole procedure was not easy to handle. However, Amika´s Elektro Prep® system (see Figure 16) proofed to have an easier handling and exhibits a combination of dialysis and targeted movement of charged particles underlying a certain electric field.

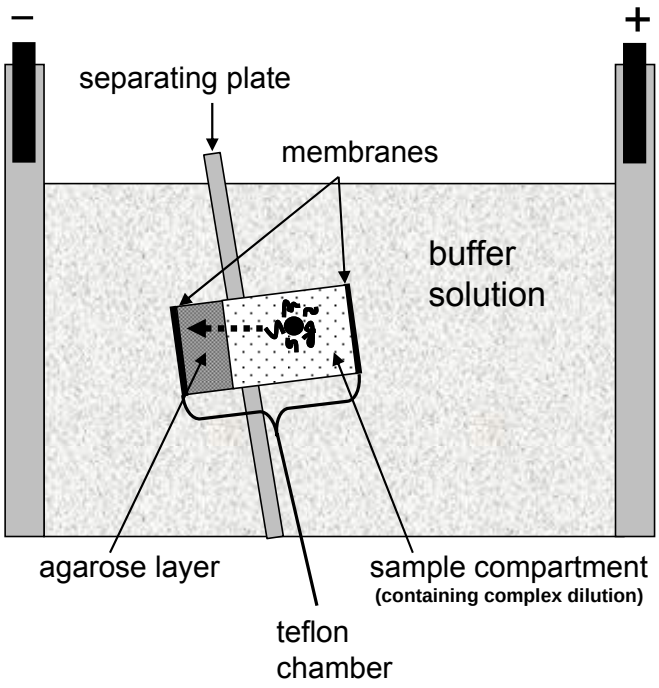


Fig. 16. Setup of Electro Prep® device used for electrophoresis of polyplexes. Polymer or complex dilution is placed in an inert Teflon chamber, fixed within a plastic plate. Two membranes are separating the sample compartment from dialysis buffer filling the Electro Prep® chamber. For improved removal of polycations, an additional agarose layer is applied on one side of the chamber. Pointed arrow inside the Teflon chamber indicates direction of targeted removal of free polymer.

3.4.2.1. Parameters for efficient and gentle purification

3.4.2.1.1. Buffer and current

The ElectroPrep® System (Figure 16) could be in principle run with a standard TRIS/ glycine puffer used for electrophoresis. Amine-containing buffers were disadvantageous for the following quantification of PEI; hence amine-free buffers were desirable. Since PBS is forming large aggregates with PEI due to the phosphate groups (data not shown), phosphate was replaced by HEPES. Starting from 75 mM NaCl and 20 mM HEPES, salt content could be reduced to 5 mM NaCl/ 20 mM HEPES. As expected, resulting current flow in the system was proportional to the conductance of the used buffer. Therefore, a clear correlation between the current necessary for purification of polyplexes and the changes in polyplex size after electrophoresis could be observed as demonstrated in Figure 17: for 80 mA, the size of PEGylated PBEI polyplexes was more than doubled. For 50 mA and 30 mA, polyplexes still grew in size, whereas for 10 mA current no significant change in diameter of PEGylated particles could be observed. Similar results were obtained for

5 mM NaCl/ 20 mM HEPES as electrophoresis buffer. Since low salt content is a vital precondition for stable non- or only partially shielded polyplex formulations, further development of electro elution with the ElectroPrep® System was carried out using 5 mM NaCl/ 20 mM HEPES, pH 7.4.

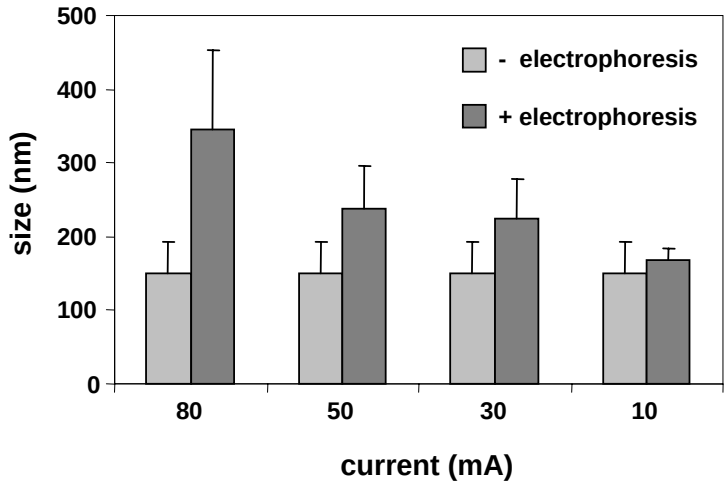


Fig. 17. Changes in sizes of polyplexes (20% PEG-BPEI/ 80% BPEI) after electrophoresis with different currents. Elution was carried out in 75mM NaCl/ 20mM Hepes pH 7.4 over a 30 minutes time periode with 0.05µm polycarbonate membranes. Only polyplexes underlying an electric field of 10mA did not grow in size significantly.

3.4.2.1.2. Insertion of an agarose layer enhances elution efficiency

For evaluation of elution efficiency of PEI, 200 µg of BPEI (10 % Cy5-BPEI, 90 % BPEI) diluted in 5 mM NaCl/ 20 mM HEPES, pH 7.4 were placed in a 1.5 ml Teflon chamber enclosed by two polycarbonate membranes attached to each side of the sample compartment (see Figure 16). Sample was electrolysed for 10, 20 and 30 minutes and the amount of remaining BPEI in the sample compartment quantified at indicated time points. Indicated amounts of BPEI in Figure 1 were determined based on copper complex assay and fluorescence analysis of Cy5-PEI. Calculated values did not differ significantly (data not shown). After 10 minutes, not more than a 25 % reduction of total polymer content could be observed (see standard setup, Figure 18). Even at the 30 minutes time point, electro elution was far not completed with 20% PEI remaining, which was at least partially attributed to fouling of BPEI onto the membrane surface. To minimize sticking of polymer to the membrane a layer of agarose was placed directly onto the membrane inside the sample chamber orientated towards the cathode. Electrophoresis could be performed maintaining the same conditions as developed for the standard setup (200 V, 10 mA in 5 mM NaCl/ 20 mM HEPES, pH 7.4). We observed a dramatic increase in elution efficiency with

90 % removal of BPEI after 10 minutes and separation of 98 % polymer within 30 minutes. Similar results were found for LPEI (data not shown). Hence, for realisation of efficient electrophoresis, an additional agarose layer containing a volume between 0.5 – 1.0 ml was attached for all other experiments.

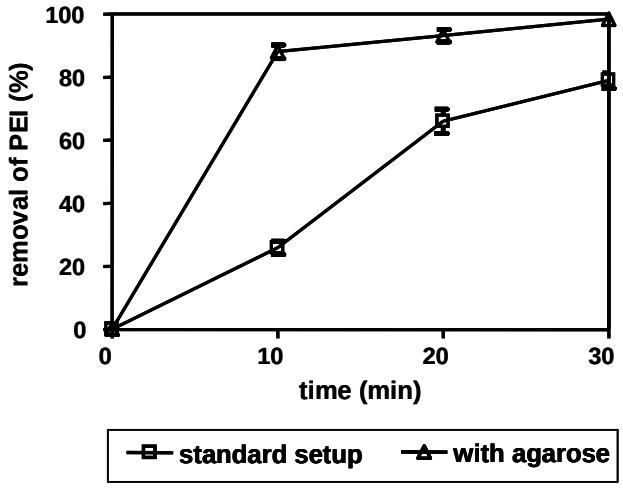


Fig. 18. Removal of 200 µg BPEI by electro dialysis over time. Polymer was diluted in dialysis buffer (5 mM NaCl/ 20 mM HEPES, pH 7.4) and applied on a 1.5 ml Teflon chamber with a standard setup (squares) or containing an additional agarose layer (triangles). BPEI content was determined based on copper complex assay and fluorescence analysis of Cy5-BPEI (see Materials and Methods)

3.4.2.2. Application of electro dialysis-based purification

3.4.2.2.1. Time dependent removal of polymer and recovery of polyplexes

To check whether a 10 minute electrophoresis would be sufficient to remove free PEI from a polyplex formulation, the same time-course experiment was repeated with polyplexes. A polyplex formulation (N/P 6) consisting of 60 % LPEI, 10 % Cy5-LPEI (to monitor LPEI removal), 20 % PEG20-LPEI and 10 % Tf-PEG-BPEI (N/P ratio 4.2/ 1.2/ 0.6) in 5 mM NaCl/ 20 mM HEPES pH 7.4, to obtain a final DNA concentration of 100 µg/ml was used (Figure 19). Separation of free PEI in PEI-based polyplexes was determined as decrease of the N/P ratios calculated at the indicated time points (calculations for the N/P ratios were based on the LPEI content as described in Materials and Methods). After 10 minutes, the initial N/P ratio of 4.2 decreased to N/P 2.2. A 20 minutes electrophoresis run could further reduce the N/P ratio to 1.7. Further increasing the electrophoresis time did not result in significant decrease of the N/P ratio, but in a high loss of recovered particles (polyplex yield decreased from 68 % after 20 minutes to 47% at 30 minutes).

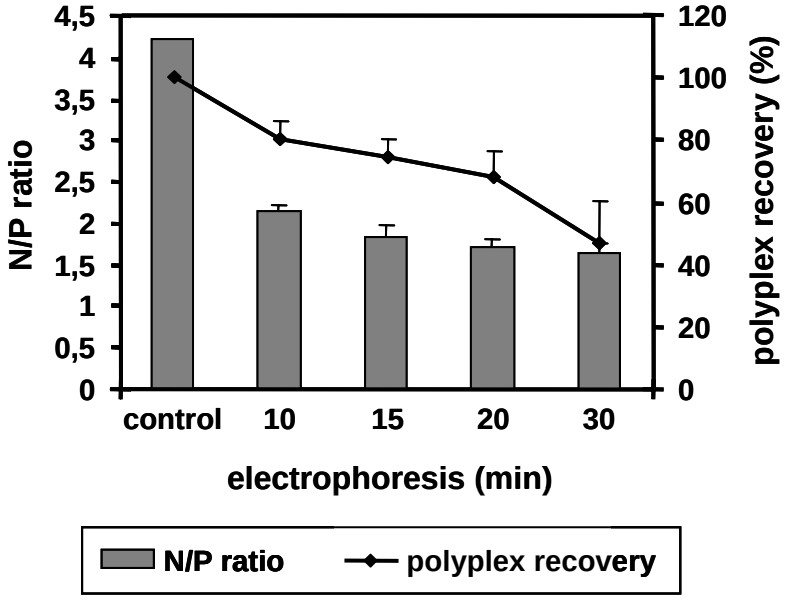


Fig. 19. Reduction of molar ratios of PEI nitrogen to DNA phosphate (N/P ratios) and polyplex recovery in dependency of dialysis time. PEI22/ PEG20-PEI22/ Tf-PEG-PEI25 complexes containing 100 µg/ml DNA (N/P = 4.2/ 1.2/ 0.6) were electrolysed in 5 mM NaCl/ 20 mM HEPES, pH 7.4 (see Materials and Methods). Removal of free polycation was calculated at the indicated time points for LPEI. Corresponding N/P ratios are shown as bars (left y-achsis); polyplex recovery in % is displayed as lines (right y-axis).

This was probably due to the migration of polyplexes into the agarose gel layer. Therefore, all further experiments were carried out with a 20 minute electrophoresis time, leading to efficient removal of polymer, concomitantly receiving good yields of purified gene carrier.

3.4.2.2.2. Influence of electrophoresis on biophysical parameters of polyplexes

Polyplex formulation	*Electro-phoresis*	*Size (nm)*	*Zetapotential (mV)*
LPEI	-	150 +/- 17	26.3 +/- 5.3
	+	992 +/-268	n.d.
BPEI	-	135 +/- 12	28.8 +/- 4.6
	+	182 +/- 30	21.8 +/- 2.0
Tf/PEG/LPEI	-	168 +/- 18	3.1 +/- 1.0
	+	173 +/- 20	2.5 +/- 0.35
Tf/PEG/BPEI	-	156 +/- 29	2.6 +/- 0.5
	+	171 +/- 36	2.2 +/- 1.1
Tf/LPEI	-	1-2 µm	6.5 +/- 0.6
	+	1-2 µm	4.1 +/- 1.6
Tf/BPEI	-	436 +/- 24	12.4 +/- 2.3
	+	481 +/- 84	9.2 +/- 3.2

Table 6. Biophysical properties of polyplexes after electrophoresis. Complexes were prepared at N/P 6 with a DNA concentration of 100 µg/ml.

Biophysical properties of different polyplexes before and after electrophoresis were determined (Table 6). To secure the analysis of a broad range of complex formulations, naked positively charged PEI particles were compared to surface shielded and targeted gene carriers, all based on LPEI or BPEI. LPEI and BPEI polyplexes were mixed in HBG, resulting in the formation of 130 – 150 nm particles (see also [30] und[31]). Electro elution resulted in aggregation of LPEI/ DNA particles which might be due to the electrophoretic stress combined with the salt content of the electrophoresis buffer (5 mM NaCl/ 20 mM HEPES pH 7.4). In contrast, BPEI based polyplexes did not aggregate in salt containing media (see [31]) and therefore exhibited a stable size after purification in NaCl containing buffer. PEG shielded and targeted LPEI respectively BPEI polyplexes were mixed in 5 mM NaCl/ 20 mM HEPES pH 7.4 resulting in gene carriers with sizes in the range of 150 – 170 nm. Tf-shielded Tf-BPEI/PEI polyplexes generated in 0.5 x HBS (20 mM HEPES, 75 mM NaCl, 2.5 % glucose w/v) exhibited a particle size of 1-2 µm (for Tf/ LPEI) respectively 400 – 600 nm (for Tf/ BPEI), similar as observed recently [37]. After electrophoresis no significant changes in size were observed for all shielded and targeted formulations. The zeta potential, reflecting the surface charge of polyplexes did not change significantly for any formulation.

3.4.2.2.3. In vitro reporter gene expression and toxicity of purified polyplexes

In order to evaluate gene transfer properties and cellular toxicity, biophysical properties of shielded and targeted polyplex formulations were characterized (Table 6), and in vitro transfections were carried out on Neuro2A neuroblastoma cells. Non-purified polyplexes generated at N/P of 6 or 2.5 were compared to polyplexes purified by electrophoresis. For all three transfection experiments shown in Figure 20, purified gene carriers were considerably less efficient at low DNA concentrations (≤ 2 µg/ml) compared to non-purified polyplexes mixed at N/P 6. These differences were most pronounced for Tf/LPEI polyplexes (350 fold difference at 1 µg/ml DNA; Figure 20B left), whereas for Tf/BPEI respectively Tf/ PEG/ LPEI polyplexes only a 10-fold difference in gene transfer efficiency was observed (Figures 20A and 20C, left). However, at higher DNA concentrations [≥ 2 µg/DNA (Figure 20A, left) and ≥ 4µg/ml DNA (Figure 20B and 20C, left)], purified formulations displayed equivalent or even slightly higher gene delivery capacity than the non-purified polyplexes generated at an N/P ratio of 6. In all transfections shown and for every DNA concentration applied, non-purified particles generated at the low N/P ratio of 2.5 exhibited by far the lowest gene transfer efficiency.

In addition, the effect of purification on polyplex mediated toxicity was evaluated as relative metabolic activity, determined 24 hours after transfection by a standard colorimetric MTT assay (Figure 20**,** right panels). Except for the PEGylated formulation (Figure 4A, right), up to a concentration of 2 µg/ml DNA no difference between purified and non-purified particles was observed. DNA concentrations ≥ 4 µg per ml resulted in more pronounced differences. At 8 µg/ml DNA, highest toxicity was observed for all non-purified polyplexes generated at N/P of 6. The lowest value of 25 % metabolic activity was observed for the PEGylated, non-purified polyplex formulation, followed by 32 % viable cells for the Tf/ LPEI N/P 6 group (Figure 20A and 20, right). However, toxicity of corresponding purified polyplexes was significantly ($p < 0.001$) reduced. Lowest toxicity with a metabolic activity of 68 % at 8 µg/ml DNA was shown for purified Tf/ BPEI particles.

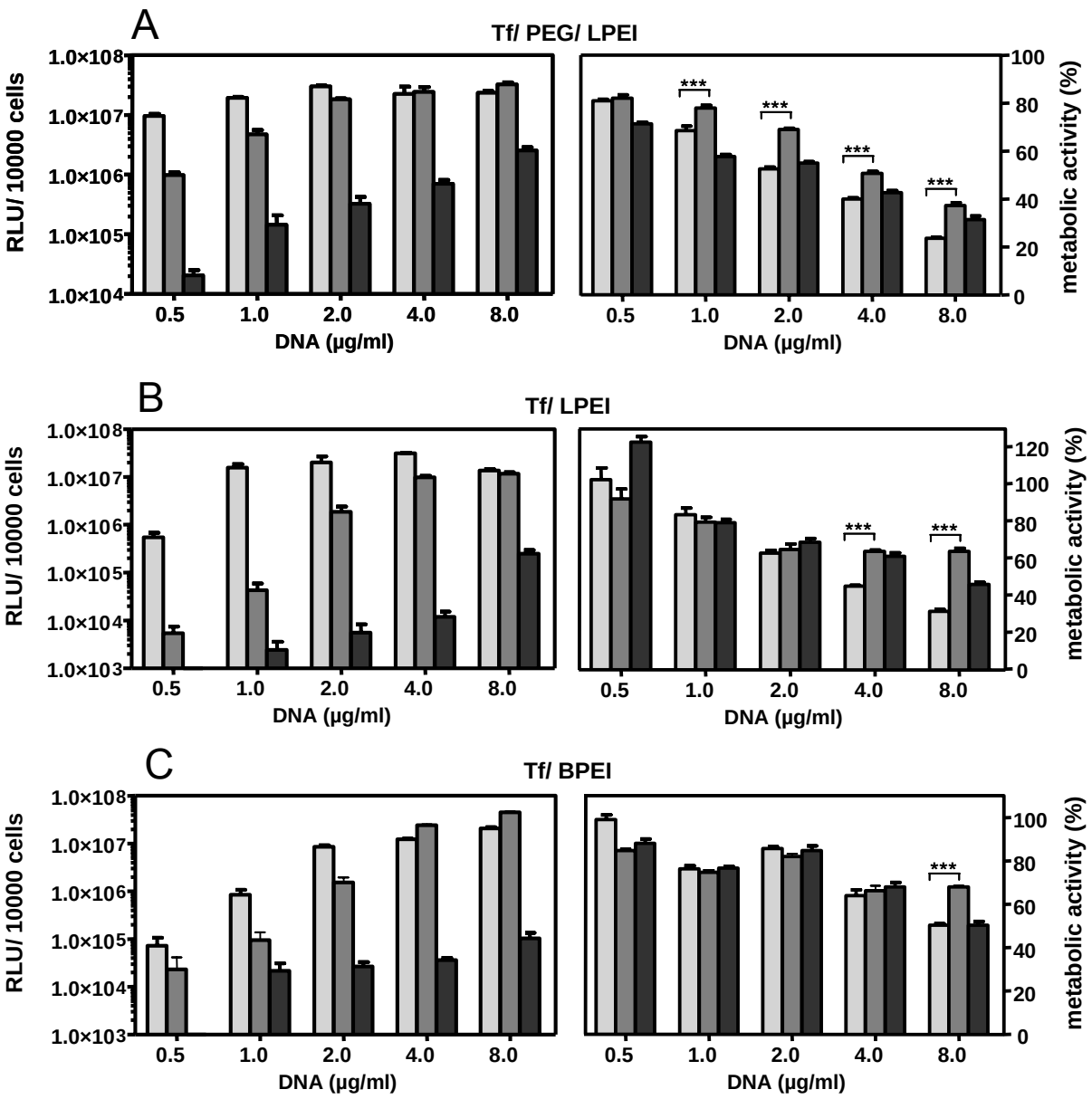


Fig. 20. Gene transfection efficiency and metabolic activity of purified and non purified complexes. Neuro 2A cells were transfected with Tf/ PEG/ LPEI (A), Tf / LPEI (B) and Tf / BPEI(C) polyplexes with increasing concentrations of DNA as described in Materials and Methods. Purification was performed by electrophoresis. Luciferase expression of transfection complexes is displayed on the left, corresponding metabolic activity is displayed on the right graph.'- EPH, N/P 6': non purified polyplexes at N/P 6; '+ EPH': purified complexes; '- EPH, N/P 2.5': non purified complexes at N/P 2.5. Shown are mean values of triplicates + SD.

3.5. Comparison of purification methods: electrophoresis (EPH) versus size exclusion chromatography (SEC)

In this chapter, the two most efficient and easiest methods to purify polyplexes were compared side by side. A compilation of polyplex formulations and the respective nomenclature used is given in Table 2, chapter 3.2

3.5.1. Composition of polyplexes after purification

For exact calculation of the N/P ratio after purification, which should not only be based on PEI content but also on the amount of the individual conjugates, PEI's and all conjugates were individually fluorescently labelled with Cy5. SEC respectively electrophoresis was performed as described. For every single run, only one of the DNA-binding partners was exchanged by the corresponding Cy5-labeled material. For instance, to determine the change of N/P ratio of PEG20-BPEI after purification, the polyplex was formed with 80 % BPEI, 10 % PEG20-BPEI-Cy5 and 10 % Tf-PEG-PEI (N/P ratio of 4.8/ 0.6/ 0.6).

For both purification methods, SEC and electrophoresis, the amount of PEI removed was similar (around 60 %, see Table 7), whereas big differences could be observed with PEI-conjugates: no significant separation of PEG-PEI conjugate was obtained by gel filtration. N/P ratios changed from 1.2 to 1.13 for PEG20-LPEI respectively from 0.6 to 0.59 for PEG20-BPEI. In contrast, efficient removal of free PEG-conjugates was achieved by electrophoresis: the N/P ratio decreased from 1.2 to 0.48 for PEG20-LPEI (equivalent to 60 % removal of total conjugate) and to 0.3 for PEG20-BPEI (50 % removal of total conjugate). The same tendency could be observed with Tf-PEG-BPEI: for both formulations, the targeting conjugate was only partially removed by SEC (initial N/P ratio of 0.6 was decreased to 0.52 for LPEI based polyplexes and to 0.42 for BPEI based polyplexes). Electrophoresis resulted in N/P reduction to 0.43 for LPEI based, and to 0.26 for BPEI based polyplexes (28 %, respectively 56 % removal of conjugate).

Another drawback of SEC based purification of polyplex formulations is that particles >200 nm cannot be purified as they are too big to pass in between the gel matrix of the column [33]. In contrast, by using the novel electrophoresis method, free LPEI and BPEI as well as unbound Tf-BPEI conjugate could be purified from the respective medium-sized polyplexes (see bottom rows in Table 7). Resulting N/P ratios after purification were ranging between 2.8 and 3.1.

Polyplex formulation	*non-purified (N/P)*	*SEC (N/P)*	*Elpho (N/P)*
LPEI	6	2.5*	2.62 +/- 0.13
BPEI	6	2.8*	2.56 +/- 0.13
LPEI	4.2	1.57 +/- 0.10	1.64 +/- 0.12
PEG20-LPEI	1.2	1.13 +/- 0.06	0.48 +/- 0.05
Tf-PEG-BPEI	0.6	0.52 +/- 0.01	0.43 +/- 0.00
BPEI	4.8	1.79 +/- 0.22	1.79 +/- 0.24
PEG20-BPEI	0.6	0.59 +/- 0.04	0.30 +/- 0.03
Tf-PEG-BPEI	0.6	0.42 +/- 0.07	0.26 +/- 0.06
LPEI	4.5		1.82 +/- 0.18
Tf-BPEI	1.5		1.25 +/- 0.17
BPEI	4.5		1.85 +/- 0.13
Tf-BPEI	1.5		0.90 +/- 0.12

Table 7. N/P ratios of different complex formulations before and after purification, comparison of particle composition after size exclusion chromatography (SEC) versus electrophoresis

3.5.2. Gene transfection and toxicity in vitro

To evaluate if the differences in complex composition of SEC- or EPH-purified polyplexes play a role for in vitro transfection efficiency and cell viability, Neuro2A cells were transfected with increasing amounts of SEC-purified and EPH-purified Tf/ PEG/ LPEI polyplexes. Figure 21 demonstrates that no obvious differences between polyplexe purified by the two distinct methods can be seen.

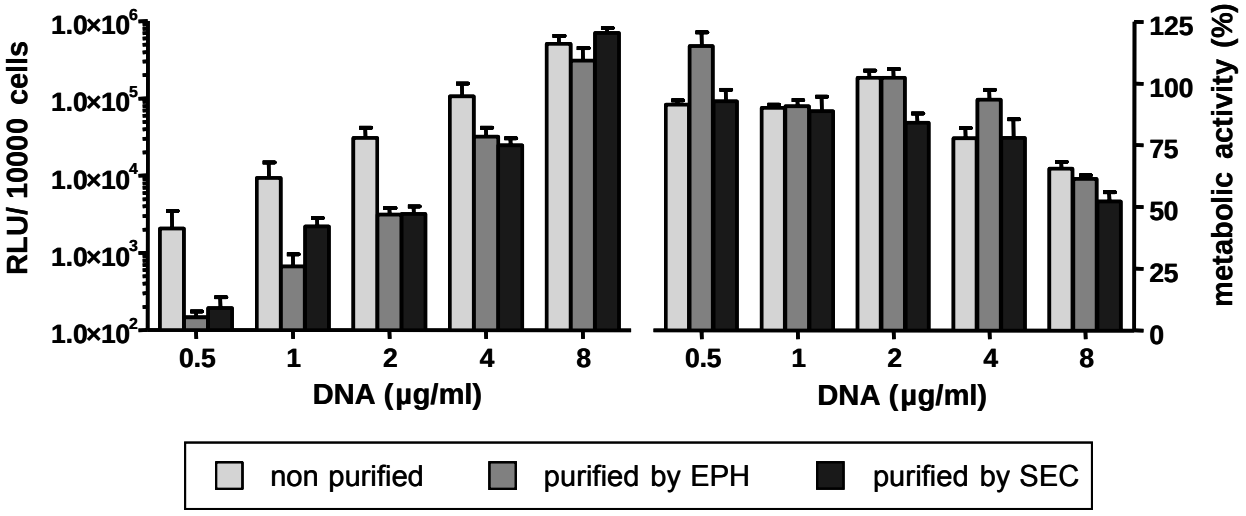


Fig. 21. Gene transfection efficiency and metabolic activity of purified and non-purified complexes. Neuro 2A cells were transfected with Tf/ PEG/ LPEI polyplexes with increasing amounts of DNA as described in Materials and Methods. Purification was performed by EPH and SEC. Luciferase expression of transfection complexes is displayed on the left, corresponding metabolic activity is displayed on the right graph. Mean values of triplicates + SD are shown.

3.5.3. Systemic application of purified PEI-based polyplexes

Finally, the key question was whether EPH-purified particles would retain their transfection efficiency for tumors after systemic application in vivo and whether they would show an improved toxicity profile. The transfection efficiency of small (200 nm), Tf targeted and PEG shielded polyplexes (mixed in 5 mM NaCl/ 20 mM HEPES) was compared with Tf targeted and shielded polyplex formulations (approx 500 nm in size, generated in 0.5 x HBS). Another approach was to analyse whether both methods for purification of small sized particles (SEC versus EPH) were comparable concerning in vivo gene expression levels. To obtain polyplexes with DNA concentrations of 400 µg/ml, an additional ultraconcentration step was performed after purification. Ultraconcentration did not lead to significant changes in biophysical properties. Mean diameters of ultraconcentrated gene carriers were only slightly increased, the zeta potential was unaltered (data not shown).

3.5.3.1. Purification significantly improves biocompatibility in vivo

To emphasize possible toxicity, polyplexes were injected at a high dose of 100 µg plasmid DNA per 20 g mouse into the tail vain of male A/J mice bearing subcutaneously grown Neuro2A tumors.

3.5.3.1.1. General observations

A significant difference in acute toxicity was observed shortly after application of non-purified and purified polyplexes. All mice receiving non-purified Tf/ PEG/ BPEI particles showed distinct signs of toxicity, finally leading to death of five out of six mice at 1 - 2 hours after i.v. application. Similar observations were made with non-purified Tf/ BPEI polyplexes: 3 out of 6 mice died within 2 hours after i.v. administration. Organs of these mice were resected immediately after death. Macroscopic tissue damage (haemorrhages) was observed in liver, spleen and small intestine, similar as described in 3.3.5.

In contrast, all mice receiving purified polyplex formulations did survive the application up to the defined end point of the experiment (48h after polyplex injection). With the exception of two animals exhibiting reduced activity after i.v. administration, mice did not show any signs of toxicity. A loss of body weight 48 hours after application was observed for all groups. However, this effect was more pronounced for of animals obtaining non-purified polyplex formulations (16 % for the one surviving mouse receiving Tf/ PEG/ BPEI/ DNA and 8 +/- 2 % for the animals receiving Tf/ BPEI polyplexes) than obtaining purified particles (Tf/ PEG/ BPEI/ DNA: 7 +/- 2 % body weight loss for purification by SEC respectively 9 +/- 1 % for purification by electrophoresis; 4 +/- 4 % weight loss for purified Tf/ BPEI polyplexes).

3.5.3.1.2. Plasma analysis

Plasma analysis of the mouse surviving the non-purified Tf/ PEG/ BPEI application revealed elevated levels of the enzyme alkaline aminotransaminases (ALT) and glutamate dehydrogenase (GLDH). In contrast, ALT and GLDH plasma levels of animals receiving purified Tf/ PEG/ BPEI were found to be at control level (Figure 22 A and B). Similar results were obtained for aspartate aminotransaminase (AST) (data not shown).

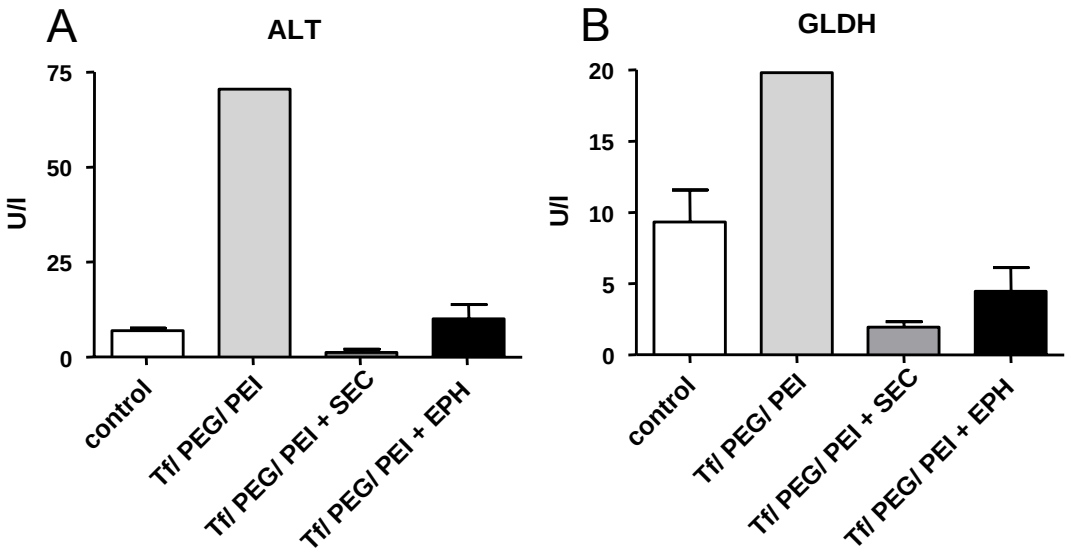


Fig. 22. ALT and GLDH plasma levels of mice treated with non-purified and EPH- or SEC-based purified Tf/ PEG/ BPEI polyplexes (100 µg DNA per 20 g bodyweight). Control: mice injected with equal volumes of buffer.

Application of non-purified Tf/ BPEI polyplexes resulted in only slightly increased ALT and GLDH levels in comparison to mice receiving purified Tf/ BPEI complexes. In contrast, AST levels of both purified and non-purified Tf/ BPEI groups were found to be on the same level (Figure 22).

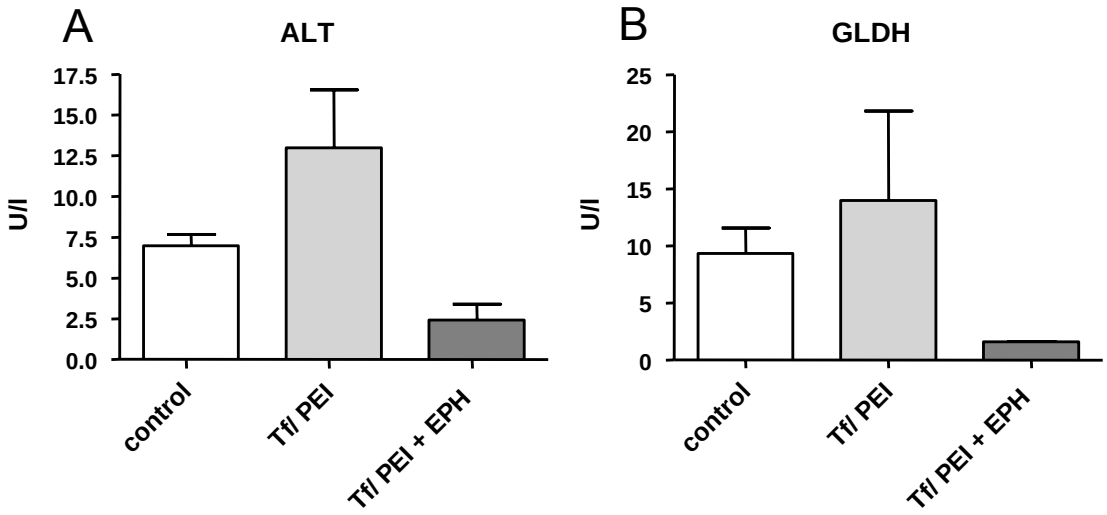
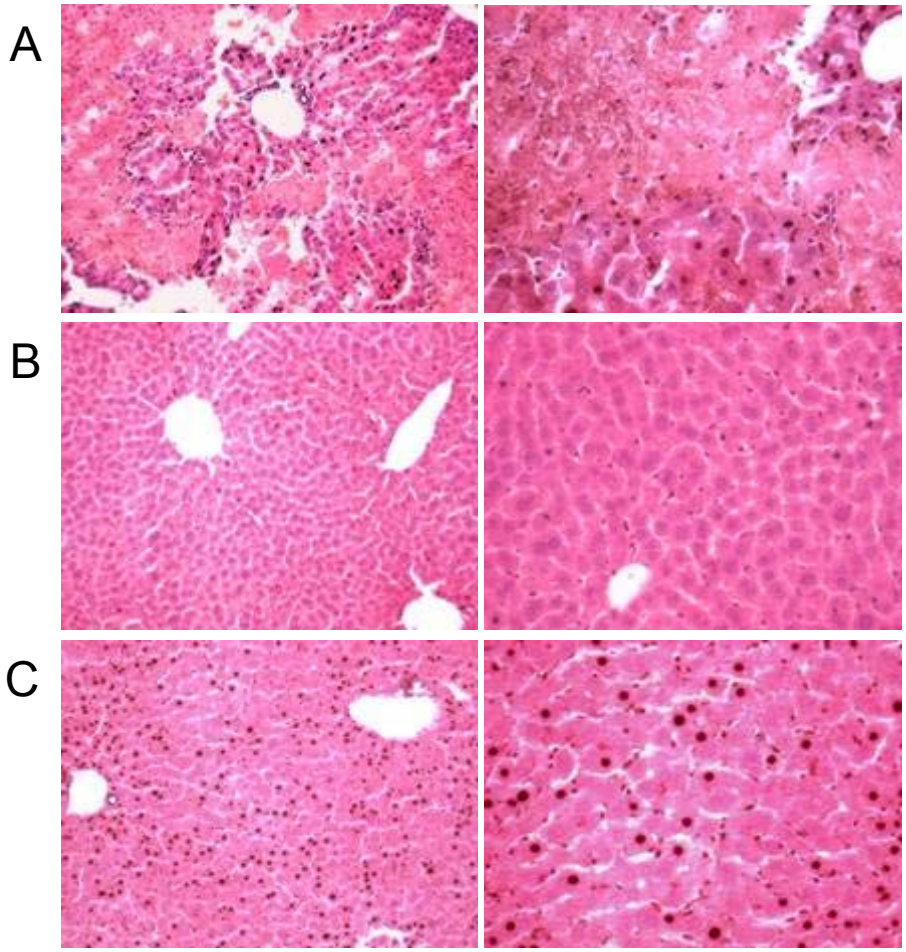


Fig. 23. ALT and GLDH plasma levels of mice treated with non-purified or purified Tf/ BPEI polyplexes (100 µg DNA per 20 g bodyweight). Control: mice injected with an equal volume of buffer.

AP levels of all non-purified and all purified groups were found to be at a normal level.

3.5.3.1.3. Histology of liver sections

Most pronounced liver tissue changes were detected in the mouse surviving the application of non-purified Tf/ PEG/ BEI polyplexes. Besides haemorrhages broad areas containing necrotic tissue were visible (see Figure 24A). Necrotic tissue appears as merged bright areas without detectable cell morphology. In contrast, mice receiving purified Tf/ PEG/ BPEI polyplexes did neither show signs of necrosis nor strong haemorrhages (Figure 24B, purification by SEC and Figure 24C, purification by EPH).



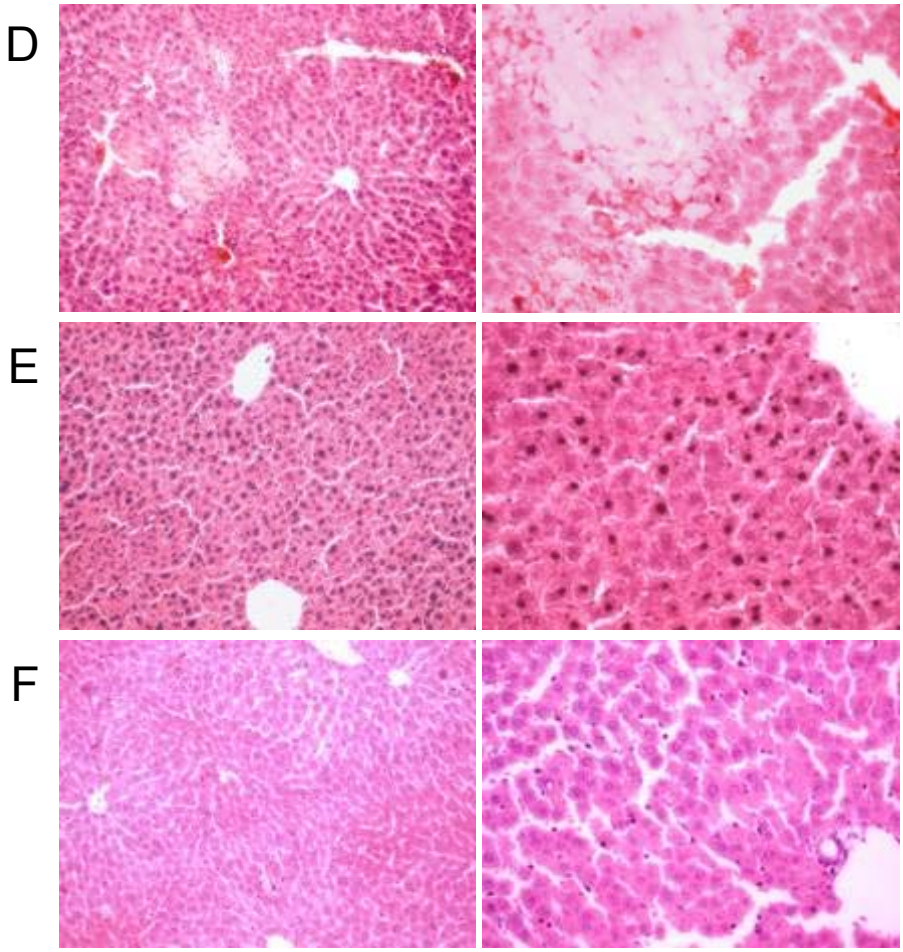


Fig 24. Liver sections of mice obtaining 100 µg DNA per 20 g bodyweight 48h after systemic application; eosin/ haematoxilyn staining; left 100-fold magnification; right 200-fold magnification. A: non-purified Tf/ PEG/ BPEI; B: SEC-based purification of Tf/ PEG/ BPEI; C: EPH-based purification of Tf/ PEG/ BPEI; D: non-purified Tf/ BPEI; E: EPH-based purification of Tf/ BPEI; F: no treatment

Mice surviving the application of non-purified Tf/ BPEI polyplexes did not suffer from pronounced liver damages as the animals obtaining the non-purified Tf/ PEG/ BPEI formulation. Still, in three out of four mice necrotic areas (Figure 24D) as well as broad haemorrhages were visible. Livers of mice receiving purified Tf/ BPEI polyplexes did not show significant tissue changes (Figure 24E). Liver sections of mice receiving only buffer served as control (Figure 24F)

3.5.3.2. Reporter gene expression of purified and non-purified complexes in vivo

Although both non-purified formulations exhibited similar high toxicity, non-purified Tf/ BPEI polyplexes (Figure 25D) showed a more tumor-targeted gene expression profile. Only moderate luciferase gene expression was found in the liver, whereas the highest expression was detected in the tumor. However, expression level in the lung was almost equal to tumor expression level, leading to an unfavourable tumor/ lung ratio hardly higher than one. In contrast, purified Tf/ BPEI polyplexes (Figure 25E) clearly targeted gene expression to the tumor with a 5-fold increase of gene expression level compared to the corresponding non-purified particles was demonstrated. Lung expression level did not increase simultaneously, leading to a favourable tumor/ lung ratio of five. Liver values of luciferase expression of mice receiving purified Tf/ BPEI polyplexes were reduced to background levels.

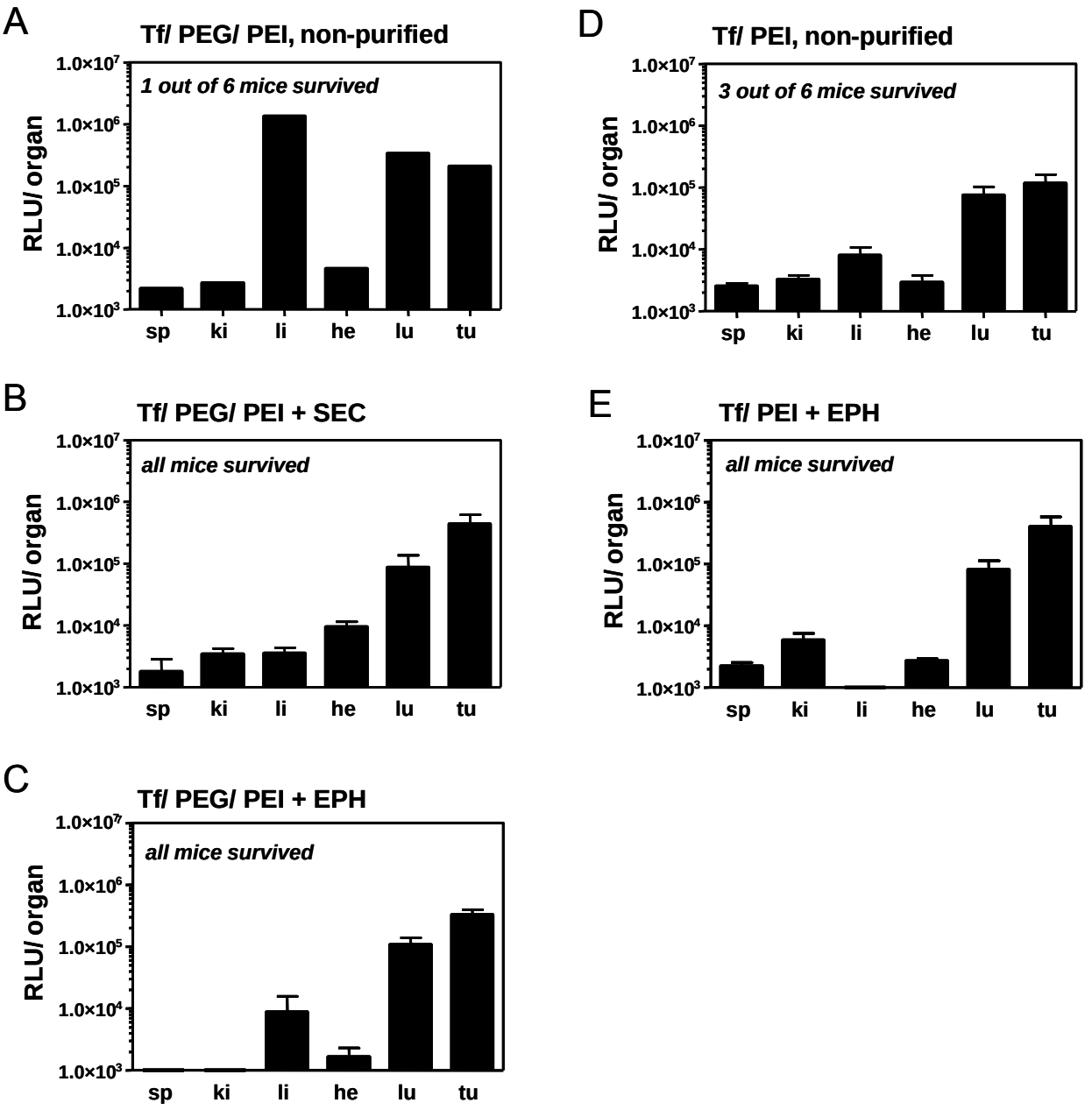


Fig. 25. In vivo gene transfer after systemic delivery of polyplexes into A/J mice bearing subcutaneous Neuro2A tumours. Different transfection complexes (see 5A – E) with a final concentration of 400 µg/ml DNA (100 µg per 20 g body weight) were injected into the tail vain. Luciferase expression was measured 48 h after application and is expressed as total luciferase activity per organ.

4. Discussion

4.1. Development of purification methods for safer non viral gene delivery

Polyethylenimine (PEI) introduced for gene transfer by the group of Jean-Paul Behr [19] is one of the most widely studied and successful polymers used for gene delivery *in vitro* and *in vivo*. However, high transfection efficiency is correlating with high toxicity, limiting the use of PEI polyplexes *in vivo*. As toxicity is considerably attributed to free PEI [33], efficient purification methods for polyplexes are desirable, especially for systemic applications. Beside the removal of free unmodified polycation, also separation of unbound functional polycation-conjugates such as shielding- or targeting conjugates should be warranted. As several efficient gene carriers based on PEI possess sizes between 0.3 and 1 µm ([39], [59], [68]) a novel purification approach should be suitable for particles of a broader size range.

4.1.1. Cation exchange chromatography

The purification method described in chapter 3.1. is based on the following concept: Highly positively charged polymers and conjugates should be retained by the carboxymethylgroups of the column material whereas particles exhibiting a neutral surface charge should pass the column unhindered (see Figure 26).

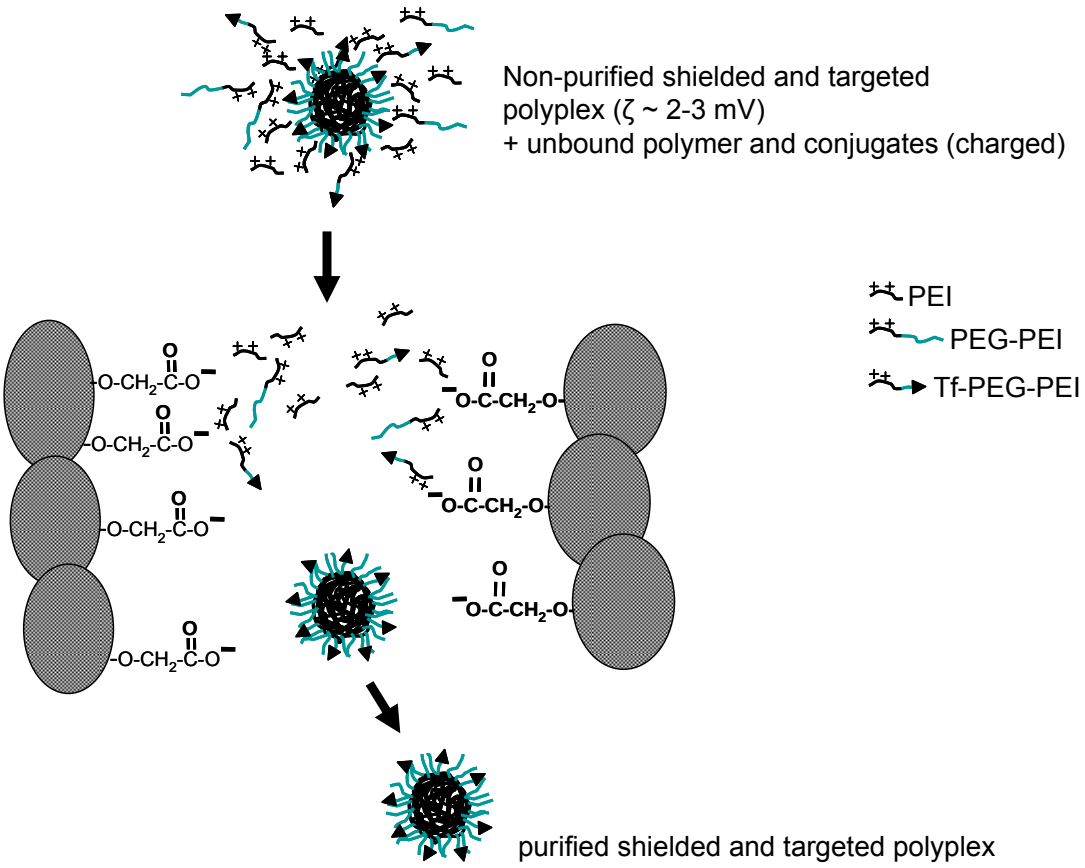


Fig. 26: Purification of polyplexes by cation exchange chromatography

Synthesis of PEI conjugates as described e.g. in [39] demonstrated that these copolymers can be separated by cation exchange chromatography from an excess of uncharged material as the polycationic part of the conjugate is kept back by the negatively charged groups of the column material. PEI conjugates were not eluted until a high salt concentration between 2.2 and 3 M NaCl was reached.

A preliminary experiment performed with PEI and PEI conjugates demonstrated retention of the polymer and copolymers for the weak cation exchange material (CM Sepharose) used (data not shown).

The question was, (i) if the surface of PEGylated polyplexes exhibiting a zeta potential between 2 – 3 mV would be neutral enough to pass the cation exchange column without retention and if so, (ii) which amount of PEG-shielding would be sufficient for a proper shielding of the strongly positively charged core of the particles.

Yet, shielding of polyplexes with 20 - 25 % PEG-PEI conjugate was apparently not enough for unhindered passing of the particles through the column material. Indeed, polyplexes were retained by the carboxymethyl groups and were not eluted until a minimum salt concentration of 1 M NaCl. Increasing the shielding amount in order to

further mask the positive surface charges resulted in instable particles which were sheared apart during cation exchange chromatography.

Nevertheless, LPEI based particles with a shielding content of 20 - 25 % PEG-LPEI could be purified from free LPEI since their N/P ratio decreased to 2.5 – 3. Particles were still shielded after purification (neutral zeta potential, see Table 1), but due to the high salt content, polyplexes doubled in their diameter. Size stayed stable for at least 4 more hours, afterwards a tendency in aggregation was observed. However, for cell culture experiments further removal of the salt content (for example via dialysis or ultra filtration) is necessary. Since an additional step would make this purification method too complicated, wasting time and material, several efforts were made to reduce the salt concentration necessary for elution of the particles.

Neither conditioning of the column with a starting salt concentration higher than 75 mM NaCl nor impregnation of the column material with free PEI in order to reduce unspecific interactions (as performed for SEC) did lead to a significant decrease of required salt concentration. Another approach to reduce the salt concentration was the deployment of $MgCl_2$ as elution buffer: since Mg^{2+} is a stronger cation as Na^+, the polyplex should theoretically be eluted at lower $MgCl_2$ concentrations. Actually, a polyplex consisting of 20 % PEG-BPEI and 80 % BPEI could be eluted at 0.4 M $MgCl_2$. However, analysis of the fractions revealed that only DNA was eluted at this $MgCl_2$ concentration but almost none of the polycations. This is most probably due to the fact that Mg^{2+} is able to complex DNA and therefore elutes the plasmid from the column.

Taken together, the PEG-shielding of the polyplexes does lead to an average neutral surface charge, but obviously particles are still too positively charged to freely pass the column material. The positive core may still be influencing the particle environment despite PEG-shield, and/ or single PEI chains being not completely complexed with DNA may form loops which stick out on the polyplex surface, therefore being able to interact with the column material. The required high salt concentrations for elution of purified particles as well as the finding that only small and tightly condensed particles were able to pass the column without being sheared apart were the main problems which occurred. Due to these findings, cation exchange chromatography was not further evaluated with regard to the biologic activity of purified vectors and their gene delivery potency.

4.1.2. Ultra filtration enables purification of various formulations but is limited to the polyplex amount

Ultra filtration is an easy and fast method to separate small and large molecules through several connected centrifugation steps. The ultra filtration device is divided by a membrane with a suitable molecular cut off. Since centrifugation leads to quick separation of small molecules passing through the filter pores and big particles staying in the supernatant, ultra filtration would be a very attractive and elegant method for the purification of polyplexes: while separating free polymer, the vector system can simultaneously be concentrated, representing a main advantage for further in vivo applications. Actually, the first published approaches for the purification of PEI particles ([40], [95], [96]) were based on ultra filtration. For reasonable PEI-mediated gene transfer *in vivo*, polyplexes have to fulfil multiple tasks. To meet all requirements, additional functional domains are conjugated to PEI leading to polyplex formulations composed of polymer and one or several PEI-bound conjugates. Regarding a potential systemic administration, this thesis focuses on the purification of more multifunctional PEI-based vectors, and therefore stands out from the already published data. Several formulations were selected for biophysical and biological evaluation after ultra filtration: i) Tf/ PEG/ LPEI and Tf/ PEG/ BPEI polyplexes which are rather small particles with 150 – 170 nm in diameter shielded with PEG-PEI conjugate and targeted by Tf-PEG-PEI conjugates [39], and ii) Tf/ LPEI respectively Tf/ BPEI polyplexes being shielded and simultaneously targeted by incorporating a high percentage of Tf directly linked to BPEI (25% of total PEI in the formulation), leading to polyplexes of up to 1 µm in size [37], and iii) simple PEI/ DNA polyplexes as ‘golden standard’.

Ultra filtration as performed in this thesis demonstrated that it is in principle possible to purify a broad range of polyplexes, regardless of size and surface charge. The apprehension that big or non-shielded particles would predominantly adsorb on the filter did not come true since polyplex recovery was around the same (70-80 %) for all formulations tested. However, complete removal of polymer was only assured when small amounts of polyplexes were applied (Table 3). Using Vivaspin6 devices, efficient removal of 55 – 60 % PEI from non-purified polyplexes (50 µg/ml DNA, N/P 6) was at least possible up to the amount of 12.5 µg DNA resulting in purified polyplex formulations with N/P ratios between 2.5 and 2.8. At higher DNA respectively PEI amounts, separation of unbound polymer got more and more inefficient: 30 – 40 % removal of PEI at 37.5 µg DNA and only 15 – 25 % removal of

polymer for polyplexes containing 62.5 µg DNA. Within the polyplex formulations the contingent of separated PEI was similar for all three different DNA amounts tested. In line with previous reports, ultra filtration did not significantly change biophysical properties [[95], [96], [40]].

Monitoring the permeability of the membrane for PEI revealed that a large fraction of polymer is not recovered neither within the supernatant nor the filtrate and will accordingly stick on the filter membrane (Figure 6). Consequently, it is most likely that above a certain amount of PEI, the membrane gets completely blocked and simply looses its filtration function. Hence, above this PEI threshold, removal of free polycation can not proceed anymore whereas under the threshold complete separation is possible. The same conclusion can be drawn for PEI conjugates like Tf-PEI or PEG-PEI which only partially pass the Viviaspin membranes (data not shown).

These findings are in line with previous ultra filtration-based PEI/DNA polyplex purifications which also suffered from incompleteness of polymer separation [32]. Membrane blocking due to a too large portion of polycation could likewise be the reason for the presented results.

Nevertheless, purification of polyplexes proofed to be efficient for DNA amounts up to 12.5 µg. Since this is still a sufficient portion for standard cell transfections, several *in vitro* experiments were performed testing ultra filtration based purification as proof of principle with respect to gene transfer capabilities and toxicity. Naked purified PEI/ DNA vectors as golden standard and big-sized, shielded particles (Tf/ LPEI and Tf/ BPEI) were analysed in order to emphasize the possible purification of nanostructures with sizes above 300nm not given by the SEC method published by Boeckle et al [33].

Transfection efficiencies of purified simple *L*PEI/ DNA polyplexes were reduced up to 40-fold in comparison to particles containing free LPEI (Figure 7). However, at high DNA concentrations, purified complexes displayed equal transfection levels. However, purified *B*PEI/DNA complexes exhibited in general lower transfection capabilities than non-purified BPEI/DNA particles for every DNA concentration observed. The effect of purification on cell viability is more pronounced for LPEI/DNA particles, which show a greatly improved toxicity profile in comparison to non purified LPEI/DNA vectors. Naked BPEI/ DNA particles are in general more toxic with no big differences between purified and non-purified polyplexes. This is in line with the SEC results previously obtained by Boeckle et al. [33], but differs with the results

presented by Erbacher et al [96]. A possible explanation for this discrepancy may be the high N/P ratios of particles used in the latter work [32].

Transfection with purified Tf/ PEI polyplexes was overall sufficient and similar effects could be observed as with naked PEI/DNA vectors: initial lower transfection efficiency of purified Tf/ LPEI complexes at low DNA concentrations was restored at higher DNA concentrations with up to 5-fold higher gene expression of purified vectors, whereas purified BPEI-containing Tf-complexes did not reach transfection levels of the formulations containing free polymer for any of the tested DNA concentrations. In line, purified Tf/ LPEI particles exhibit an improved toxicity profile.

Taken together, proof of principle for ultra-filtration based purification could be shown as well as the purification of large particles which was up to now not demonstrated before. Small amounts of PEI can be efficiently removed from polyplexes. In vitro studies, which do not require large amounts of gene vectors, can in principle be performed like the evaluation of transfection efficiencies, differences in cell association and uptake or differing intracellular behaviour of purified polyplexes. After each ultra filtration cycle, particles were concentrated 2 - 3 fold, a nice feature for in vivo applications. However, since this work demonstrates that ultra filtration is limited due to blocking of PEI on the membrane, the method will not play an important role for the purification of PEI-based polyplexes for in vivo application.

4.1.3. SEC: modification of polyplexes with endosomolytic functions for virus-like gene delivery

A successful purification method for PEI/DNA polyplexes by size exclusion chromatography with a removal of 50-60 % of the total PEI amount was developed by Boeckle et al [33]. Preliminary in vivo data of intravenously applied purified PEI/DNA particles indicated lower toxicity of purified vectors, but also a general lower reporter gene expression in all major organs [33].

Additionally to the described approach, this work focuses on the purification of shielded and Tf-targeted gene vectors with the specific aim of intravenous applications of purified polyplexes in tumor bearing mice. Tf-targeted L- and BPEI polyplexes with a sufficient amount of PEG-shield generate small particles exhibiting a neutral surface charge. Unfortunately at the same time coating of the positive charge with PEG improves properties of vectors for systemic applications, they display comparably low cell transfection activity [[38],[101],[43]], even with the additional integration of targeting ligands [[102], [103]]. Beneath reduced interaction

with cell surfaces [40], a major bottleneck seems to be endosomal escape of the PEGylated PEI polyplexes [104]. Hence, regarding the development of successful non viral vectors for in vivo applications, the membrane active peptide melittin, which is known to efficiently trigger endosomal release, was additionally inserted into the polyplexes. Boeckle et al (S. Boeckle PhD thesis, Boeckle 2005) described in detail the bee-venom-derived melittin and its analogues. N-Mel-BPEI, (with melittin covalently linked to BPEI at the N-terminus) as well as CMA-3-BPEI and NMA-3-BPEI (with the melittin derivative coupled to BPEI via the C-terminus respectively the N-terminus of the peptide) have previously been shown to be promising gene transfection vectors ([54],[98],[93]). However, the improvement in gene transfection efficiency is again paired with pronounced toxicity. Therefore, these melittin conjugates were incorporated into PEGylated and Tf-targeted polyplexes (N/P ratio of Tf/ PEG/Melittin-BPEI: 0.6/ 0.6/ 4.8) which were finally purified by SEC to improve the toxicity profile by removing unbound melittin-BPEI conjugates from the polyplex formulation.

For the most promising new melittin conjugates which are all coupled to BPEI, a direct comparison of melittin-core complexes to B- and LPEI polyplexes was performed in order to figure out the most successful purified, shielded and targeted small-sized gene delivery system regarding in vitro transfection efficiency and toxicity. The “winners” of the in vitro group should later be systemically applied in tumor bearing mice.

First of all, incorporation of melittin-conjugate into the polyplex formulations after purification was demonstrated (Figure 9): 50 – 55 % of the melittin-containing BPEI conjugate could be removed by size exclusion, according to 45 – 50 % of the conjugate being incorporated within the polyplexes.

Transfection experiments in Tf-receptor rich Neuro2A cells demonstrated the following: Firstly, at low DNA concentrations the most potent non purified polyplex formulations are in fact melittin-containing vectors (Figure 10A and B) whereas at higher DNA concentration (Figure 10C), LPEI displays equal transfection efficiency. Secondly, purified melittin-containing polyplexes exhibited again the highest transfection efficiency at low DNA concentration. However at high DNA concentration, transfection efficiency of both L- and BPEI was quite elevated and had the same expression level like melittin-containing gene carriers (Figure 10C). Concerning cell toxicity, all purified gene carriers did not show any significant

difference from untreated control cells 24 hours after transfection. Likewise, a difference in cell viability among the five different groups could hardly be observed.

Significant differences in transfection efficiency within the five groups could only be shown for low DNA concentrations indicating the melittin-analogs as most promising gene delivery vectors. However, at high DNA concentration of 4 µg/ml, all different purified polyplex formulations exhibited similar luciferase expression levels. Therefore all five groups with and without purification were included in a following in vivo study.

4.1.4. Electrophoresis allows efficient purification for polyplexes in a broader size range and exact determination of polyplex composition

The electrophoretic method as developed in chapter 3.4. is based on the following concept: highly positively charged polymers and conjugates with a high charge/ molecular weight ratio should move rapidly towards the cathode in a time period where particles exhibiting a far lower charge/ weight ratio hardly migrate.

At first, this was verified by standard agarose gel electrophoresis experiments performed with Tf/ PEG/ BPEI polyplexes which revealed that unbound PEI moves fast in the applied electric field and can be separated from rather immobile polyplexes. Completeness of separation was verified through a downstream gel filtration step (Figure 15). The appearance of a single peak in the void fraction of the SEC elution profile confirmed that no cationic carriers (PEI and PEI-conjugates) were detectable within the polyplex.

For preparative separations a commercially available electro dialysis device (see Figure 16) was adapted. Besides, polyplexes are too big to pass the membranes of the sample compartment, whereas polymers and conjugates are able to pass. Therefore a membrane with a 50 nm cut-off was chosen. Electrophoresis performed at currents above 30 mA induced growing of particles (Figure 17). This was most probably due to the strong electric field which sheared apart the polycations and the DNA. Since a gentle electrophoretic purification without changing biophysical properties of polyplexes is strongly desirable, a low current of 10 mA was employed for all relevant experiments. To hinder possible salt-induced aggregations of polyplexes, a buffer with a minimum salt amount of 20 mM HEPES and 5 mM NaCl was used. For reducing unspecific adsorption of polymer, a membrane consisting of polycarbonate was selected. Nevertheless, especially with higher amounts of PEI applied, a reduced PEI elution by time was observed (Figure 18). This was mainly due to sticking of polymer onto the membrane since we observed a blue coloration

attributed to Cy5-labeled PEI on the membrane surface. This was not surprising, since the ultra filtration experiments performed in this thesis and previous work [[33], [32]] describes the high affinity of PEI to adhering onto many kinds of surfaces like filters and membranes. According to the standard gel electrophoresis experiments performed, an agarose layer was put inside the Teflon chamber, placed between the sample solution and the membrane. As expected, removal of PEI was strongly improved with more than 98% of PEI being removed from the chamber after 30 minutes (Figure 18).

With the electrophoresis setup described in 3.4.2, we were able to efficiently purify a broad range of PEI-based polyplexes independent of their size and surface charge: naked, positively charged PEI/ DNA complexes, small shielded and targeted complexes (Tf/ PEG/ PEI) as well as big-sized vectors (Tf/ PEI), with the two latter being the more attractive formulations for future *in vivo* applications. Purification was complete after a 20 minutes electrophoresis run while achieving high polyplex recoveries (Figure 19). With the exception of plain LPEI-polyplexes, biophysical properties did not change significantly (Table 6). This is consistent with previous findings that non-shielded LPEI are very sensitive to aggregation [31], a property which gets intensified after polyplex purification [33].

In general, 60% of the total amount of PEI for particles initially generated at an N/P of 6 could be removed by electrophoresis which is in good agreement with previous reports [[33]; [32]; [98]]. For LPEI and PEGylated polyplex formulations, total N/P ratios found after electrophoresis ranged from 2.4 – 2.6. N/P ratios of particles shielded by Tf-BPEI were only slightly higher after purification with values ranging from 2.8 – 3.1 (Table 2) and were very similar to the values obtained for ultra filtration-based purification of marginal DNA-amounts (Table 3).

Importantly, electrophoresis of polyplexes allowed removing not only unmodified free PEI but also unbound PEI-conjugates (which was not able for SEC). This method therefore generates well-defined, purified particles which are regarded to be crucial for any future development as a pharmaceutical product. The data also provide information on the composition of polyplexes and final percentages of LPEI and BPEI polymers respectively conjugates therein. Table 7 shows that polyplexes composed of BPEI-based conjugates (Tf-PEG-BPEI and Tf-BPEI) and LPEI as well, LPEI was removed to a higher extent then BPEI-conjugates during purification. For instance, Tf-BPEI/ LPEI polyplexes mixed at a percentage ratio of 25/ 75 (at N/P 6) upon

purification by electrophoresis display average incorporation of 41% Tf-BPEI and only 59% LPEI (values calculated from N/P ratios in Table 7). Tf-BPEI/ BPEI particles also mixed at a 25/ 75 ratio with DNA, exhibit actually a differing 33% (for Tf-BPEI) and 66% (for BPEI) distribution in respect to DNA binding. This is in agreement for example with FRET experiments made by Ithaka et al [105] where a lower DNA binding affinity for DNA of LPEI compared to BPEI was observed.

Crucially, SEC-based purification did not separate unbound PEI conjugates as Table7 demonstrates and therefore results in small differences in complex formulation. An important question was therefore, if removal of unbound targeting ligands (possible only for EPH) would lead to improved transfection efficiency since free Tf-conjugates might compete with polyplexes incorporating Tf for the corresponding receptors on the target cell. However, *in vitro* transfection experiments performed with Tf/ PEG/ LPEI polyplexes (Figure 21) revealed no difference in gene delivery efficiency of electrophoretic-based and gel filtration based purified complexes.

For the exclusive evaluation of transfection capability of electrophoretically purified polyplexes, we compared Tf-targeted DNA polyplexes with and without PEG conjugates (A: Tf/ PEG/ LPEI; B: Tf/ LPEI and C: Tf/ BPEI) as the most promising candidates for safe and efficient gene transfer. Purified formulations were compared to non purified particles generated at N/P 6. To underline the importance of generating particles with an excess amount of polymer and its impact for gene transfer also non purified polyplexes mixed at a low N/P ratio of 2.5 were used. In vitro transfection with purified polyplexes was overall efficient with a slightly improved toxicity profile compared to non purified formulations at N/P 6. Especially at high DNA concentrations, purified gene carriers exhibited equivalent transfection efficiencies. In contrast, all particles generated at N/P 2.5 displayed significantly less gene transfer capability. This may be due to the fact that particles generated at low N/P ratios mainly form loose, non compacted aggregates [30] which are close to electro neutrality.

Taken together, purification of particles by electrophoresis fulfils several crucial requirements: i) the method is quick and easy to perform, ii) biophysical properties of virus-like particles are not changed, iii) removal of PEI can be performed independently of the polyplex size and iv) unbound PEI-conjugates can be

separated. Since purified particles maintain their transfection ability in vitro, the last, most crucial question remains: their effectiveness in systemic applications.

4.2. Purified, highly concentrated shielded and targeted polyplex formulations: significance for in vivo applications

In vitro, purification of PEI-based polyplexes proved to be important not only due to the improved toxicity profile of the polyplexes but also for mechanistic studies concerning the role of free polycations during extra- and intracellular processing steps [[33], [32], [106]]. However, the basic impact of purification lies in the improvement of systemic application of gene vectors with respect to biocompatibility and toxicity of complexes. Regarding future developments of non-viral gene transfer mediated by polyplexes, one has to get straight that purification of the carrier systems is a vital precondition for reasonable gene therapies. Since the first published results describing the in vivo application of purified PEI/DNA polyplexes have been very promising [[96], [33]] a crucial aim of the thesis was the i.v. injection of purified, shielded and targeted gene carriers into tumor-bearing mice and investigation of their transfection efficiency and biocompatibility.

4.2.1. Incorporation of melittin does not improve transfection efficiency

Unfortunately, application of 50 µg DNA per 20 g bodyweight led to an overall low gene expression both for SEC-based purified and for non-purified polyplex formulations (Figure 12). Highest tumor expression of purified complexes was found for Tf/ PEG/ BPEI and Tf/ PEG/ NMA-3 vectors. This result clearly demonstrated, that despite targeting ligand small and PEGylated particles are mainly inefficient in vivo gene vectors and purification does not contribute to an increase of transfection efficiency. We considered two general possibilities to enhance the gene transfer capabilities of these vectors: Firstly, application of a higher amount of DNA, and secondly, increasing the size of the gene carriers to the range of 500 – 1000 µm in order to exploit the enhanced permeability and retention (EPR) effect of circulating particles, also termed as ‘passive targeting’ strategy [65]. Indeed, increasing the polyplex size through adoption of high salt concentration or freeze-thaw cycles concomitantly maintaining the required low polydispersity proved to be not easy. On the other hand, purified polyplexes were all well tolerated (which was not the case for non-purified particles). Therefore we decided to simply double the amount of applied DNA to 100 µg DNA per 20 g bodyweight. To keep the injection volume small the

polyplexes were concentrated to 400 µg DNA per ml. This time, analysis of reporter gene resulted in higher gene expression: in the case of Tf/ PEG/ NMA-3 polyplexes an almost 10-fold and for Tf/ PEG/ BPEI polyplexes more than 50-fold enhancement in transfection efficiency compared to the low dosage administration was detected (Figure 13). Unfortunately, incorporation of NMA-3-BPEI into the particles did not lead to an improvement of transfection capabilities since tumor expression levels were even slightly lower than those of standard Tf/ PEG/ BPEI complexes. However, the in vitro data at the DNA concentration of 4 µg/ml performed in chapter 3.3.3 (Figure 10C) already gives a first hint for the only modest performance of melittin-containing polyplexes: no clear predominance of these vectors at high DNA concentrations could be demonstrated.

4.2.2. Purification leads to significant improvement of biocompatibility while maintaining high transfection efficiency

Indeed, delivery of both purified and non-purified targeted and shielded polyplexes with a DNA amount of 50 µg as described in 4.2.1. did not lead to significant gene expression. On the other hand, it already turned out that purification contributes to reduced in vivo toxicity: a full 50 µg dose of any melittin-containing polyplex was highly toxic since none of the animals survived the application. In sharp contrast, the analogous gel filtrated melittin-polyplexes were well tolerated in the systemic application, even at a high dosage of 100 µg DNA per 20 g body weight. For the same amount of DNA, a high toxicity for non-purified Tf/ PEG/ BPEI formulations was observed (Figure 25A and 25D) with only one out of six animals surviving the Tf/ PEG/ BPEI administration and 50 % of the animals surviving the Tf/ BPEI delivery.

Death of mice treated with non-purified particles seems to be correlated with liver failure as strong bleedings in the liver tissue were visible. Livers of mice surviving the non purified formulations showed bleedings within the sinusoids and large areas of necrosis (Figure 24). Also, especially in the case of non-purified Tf/ PEG/ BPEI polyplexes, ALT and GLDPH liver enzyme level was elevated, indicating liver damages. Additionally, for the small PEGylated BPEI formulation a comparably high amount of luciferase expression was found in the liver (Figure 13A and 25A).

Since purification of small polyplexes is possible with SEC and with EPH, a comparison regarding in vivo transgene expression profile and expression levels was meaningful. The in vivo data demonstrated that both methods of purification led to well tolerated gene carriers upon systemic application. This confirms that the major in

vivo toxicity of PEI-based polyplexes mixed at high N/P ratios correlates with unbound polymer. Comparably high lung accumulation of purified and non-purified polyplexes is most probably due to incomplete shielding of particles (for both formulations zeta potential of concentrated particles was 6 – 8mV).

Notably, high dosage of non-purified Tf/ PEG/ LPEI polyplexes did not lead to severe toxicities. On the other hand, in vivo transfection level of these vectors im the tumor tissue was lower than the ones obtained with Tf/ PEG/ BPEI polyplexes (data not shown), which was surprising and in clear contrast to the most in vitro data. However, reasons for that remain speculative.

Most interesting, highest tumor expression was found for purified BPEI gene carriers; up to 5-fold increase in gene expression levels in comparison to non-purified polyplexes was observed. Apparently free PEI does not contribute to tumor gene expression as the polycation is quickly cleared by the RES before reaching tumor tissue. An even increased tumor expression with Tf targeted polyplexes was not expected and reasons for it remain speculative. Possibly free PEI, which may aggregate with erythrocytes [[33], [40]] and may activate lung endothelium [34], enhances entrapment and subsequent uptake of polyplexes in the small capillaries mainly of the lung [[20], [34]]. This might lead to a reduced systemic circulation of polyplexes into the tumor. Conversely, removal of free PEI would enhance delivery into the tumor. Such a hypothesis however remains to be tested by polyplex distribution studies.

The in vivo study described in chapter 3.5.3. verified that both methods (electrophoresis as well as gel filtration) are resulting in similar gene expression patterns and levels, despite differences in polyplex composition of purified particles (Table 2).

Nevertheless, the electrophoresis based method also allowed the purification of polyplex formulations >250 nm in size. Hence, purified Tf/ BPEI polyplexes (400 - 600 nm) were included in the in vivo series. Since those vectors were as efficient in tumor expression level as small sized Tf/ PEG/ BPEI particles, the enhanced retention and permeation (EPR) effect as mentioned in 4.1.3. apparently did not play a significant role for a further enhancement of tumor gene delivery.

Still, this method can also be potentially utilized for a size-independent purification of other cationic DNA carriers consisting of tailor made, “smart” polymers. The electrophoretic method developed in this thesis may therefore present an important

contribution for the development of effective and safer non-viral vectors for systemic applications. Taken together, efficient gene expression of purified shielded and targeted PEI polyplexes is possible but requires a threshold amount of delivered DNA. However, the high amount of gene vector did not lead to significant toxicities.

5. Summary

Recently, two different methods based on ultra filtration and SEC describing the purification of plain PEI/DNA polyplexes were published with promising in vitro data. Also provided preliminary in vivo applications of these particles demonstrated an improved toxicity profile clearly indicating the impact of generating well defined, purified gene carriers. However, up to now, not much attention was drawn to the in vivo application neither of purified shielded and targeted polyplexes nor at the exact composition of these multi-functional vectors after their purification. This thesis focuses on the evaluation of already existing approaches and the development of alternative purification methods regarding their efficiency to remove both unbound PEI and PEI-conjugates. Additionally, a size-independent purification should be warranted and a scale up of the established methods should allow purification of large polyplex amounts to analyze gene transfer and biocompatibility after systemic application in tumor-bearing mice.

A first purification approach based on cation exchange chromatography demonstrated proof of principle. Small PEGylated complexes could be efficiently removed from unbound polymer. However, despite a neutral zeta potential the particles exhibited a strong affinity to the negatively charged carboxymethyl groups of the column material. Therefore, a high salt concentration with a minimum amount of 1 M NaCl was necessary to elute the complex. Although the purified polyplex seemed to be stable at the high salt content for a certain time, a required additional dialysis step in order to reduce the salt content was considered to be time- and material wasting. Thus, further evaluation regarding in vitro transfection experiments were not carried out.

An already published method based on ultra filtration was successfully augmented on the purification of both small and big sized, shielded as well as targeted complexes. Purification by ultra filtration proofed to be a very quick, easy and efficient method to remove PEI and PEI conjugates beneath a certain amount of polyplex. Exceeding this borderline, purification turned out to be inefficient, which was also the outcome of the first already published ultra filtration approach. Stagnating of PEI removal is most probably due to blocked membrane. Therefore, only a few in vitro transfection experiments were carried out. It was demonstrated that also big-sized polyplexes can

be purified and still maintain their transfection efficiency at high DNA concentrations while exhibiting an improved toxicity profile.

Up to now, the only efficient and complete removal of PEI in PEI/DNA polyplexes was demonstrated with SEC. This thesis describes the purification of Tf-targeted and PEG-shielded L- and BPEI polyplexes, which were optionally tuned with melittin-containing PEI as endosomolytic functions in order to create a more virus-like particle. The approach was scaled up and combined with a downstream ultra concentration step to produce a large amount of highly concentrated, purified and stable gene vectors. Being systemically applied, these formulations exhibited a high tumor expression level. However, melittin-containing complex formulations did not exhibit an increase in transfection efficiency compared to non-modified Tf/ PEG/ BPEI polyplexes. But for the first time it could be demonstrated that purified Tf/ PEG/ BPEI polyplexes show high tumor expression level and are well tolerated.

The purification approach based on electrophoresis combines the most crucial advantages. Since it allows complete separation of PEI and PEI conjugates, also a clear determination of the polyplex composition is possible. With suitable conditions applied, electrophoresis does not change biophysical properties of shielded polyplexes and most importantly, complexes of larger size ranges can be purified.

Crucially, electrophoresis-based purified polyplexes were still effective in vitro as well as in vivo. Distant tumors were targeted with equal or higher transfection efficiencies than obtained with the respective non-purified particles. Purified polyplexes were well tolerated in systemic applications even at high DNA dosages.

6. Appendix

6.1. Abbreviations

ALT	alkaline aminotransaminase
AP	alkaline phosphatase
AST	aspartate aminotransaminase
BPEI	branched PEI of 25 kDa
CMA-3-BPEI	PEI covalently attached to the C-terminus of a melittin analog
CMV	cytomegalovirus
DMEM	Dulbecco´s Modified Eagle´s Medium
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPH	electrophoresis
EPR	enhanced permeation and retention effect
GLDH	glutamate dehydogenase
FBS	fetal bovine serum
HBG	HEPES-buffered glucose
0.5 HBS	HEPES-buffered glucose and HEPES-buffered saline 1/1 (v/v)
HBS	HEPES-buffered saline
HEPES	N-(2-hydroxyethyl)piperazine-N`-2(-ethanesulfonic acid)
IL-2	interleukin-2
LPEI	linear PEI of 22 kDa
LU	light units
MTT	methylthiazoltetrazolium salt
NA	nucleic acid

NMA-3-BPEI	BPEI covalently attached to the N-terminus of a melittin analog
N-mel-BPEI	BPEI covalently attached to the N terminus of melittin
N/P ratio	molar ratios of PEI nitrogen to DNA phosphate
PBS	phosphate-buffered saline
pCMVLuc	plasmid encoding luciferase under control of the CMV promoter/ enhancer
PEG	polyethylene glycol
PEG-BPEI	PEG of 20kDa covalently attached to BPEI of 25kDa
PEG-LPEI	PEG of 20kDa covalently attached to LPEI of 22kDa
RES	reticulo endothelial system
RGD	synthetic peptide, containing arginine-glycine-aspartate as sequence motif
RLU	relative light units
RNA	riboxy nucleic acid
SD	standard deviation
SEC	size exclusion chromatography
siRNA	small interfering RNA
Tf	transferrin
Tf-BPEI	transferrin covalently attached to BPEI of 25kDa
Tf-PEG-BPEI	transferrin covalently linked to BPEI of 25kDa via a heterobifunctional 3.4 kDa PEG spacer
UF	ultra filtration
v	volume
w	weight

6.2. Publications

6.2.1. Original papers

Walker, G.F., Fella, C., Pelisek, J., Fahrmeir, J., Boeckle, S.,Ogris, M. and Wagner, E. (2005)

“Toward Synthetic Viruses: Endosomal Triggered Deshielding of Targeted Polyplexes Greatly Enhances Gene Transfer In Vitro and In Vivo”

Mol Ther 2005; 11(3):418-425

Boeckle, S., Fahrmeir, J., Roedl, W., Ogris, M. and Wagner, E. (2006)

“Melittin Analogs With High Lytic Activity At Endosomal pH Enhance Transfection With Purified Targeted PEI Polyplexes”

J Control Release 2006; 112(2):240-248

Fahrmeir, J., Guenther, M., Wagner, E., Ogris, M.

“Electrophoretic Purification Of Tumor Targeted PEI Polyplexes Reduces Toxic Side Effects In vivo”, *submitted*

6.2.2. Book chapter

Fahrmeir, J. and Ogris, M. (2006)

“Transferrin Receptor Mediated Delivery of Protein and Peptide Drugs Into Tumors For Cancer Treatments” in “Delivery Of Protein And Peptide Drugs In Cancer”, Imperial College Press, Edt V.P. Torchlin

6.2.3. Poster presentations

Walker, G.F., Fella, C., Fahrmeir, J. and Wagner, E.

“Bio-reversible PostPEGylation of targeted polyplexes enhances gene transfer in vitro”

European Society Of Gene Therapy, Annual Meeting, Prague, Chech Republic

Fahrmeir, J., Bogomilova, A., Russ, V., Koseva, N., Ogris, M. Troev, K. and Wagner, E. (2006)

“Novel gene carriers based on polyphosporamides”

Controlled Release Society, Annual Meeting, Vienna, Austria

6.2.4. Oral presentation

Fahrmeir, J., Kloeckner, J. (2005)

“Development of novel nonviral vectors for tumor-targeted gene delivery – towards an ‘artificial virus’

Scientific colloquium, Bulgarian academy of Science, Sofia, Bulgaria

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9. Curriculum Vitae

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