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Dynamic and effective gene vectors via pH-sensitive PEG-shielding

vorgelegt von

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Erklärung

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Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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Für meine Eltern

1 INTRODUCTION

1.1 Gene therapy

Although gene therapy is a relatively new research area, the first approach that went into clinic was in 1989, already more than 1300 clinical trials have been completed or are still ongoing (1). Its original intention was the correction of genetic disorders by delivering therapeutic genes into the target cells of patients to compensate for the lacking or defective genetic information. Further on, delivery of genes encoding for therapeutic proteins or the switching-on of gene expression are commonly performed methods. Nowadays this is by far not the only existing toehold. Antisense oligonucleotides, ribozymes and small interfering RNA are designated as “loss of function” approaches that aim at suppressing specific gene functions. Apart from providing or switching-off special genetic information, genes that are delivered from outside can also lead to controlled cell death or activation of prodrugs.

In 2000 a gene therapy trial from France gained massive attention with the first report of successful treatment of severe combined immunodeficiency disorder (SCID) in children. Only 2 years later, the excitement suffered a set-back when two of the ten children were diagnosed with leukaemia caused by integration of the retroviral vector in the host genome. By 2008, 5 of approximately 25 treated children developed leukaemia. On one hand this points out clearly the enormous potential of this new and promising field of therapy, on the other hand the missing of outstanding follow up successes is clear evidence that gene delivery systems still need to be further improved.

Gene therapy embraces numerous different therapeutic approaches with the ambition to prevent, treat or cure diseases. Cancer found its place in gene therapy as in most of the cases it is a disease with genetic alterations that finally result in genetic defects and unregulated cell augmentation. Due to high incidence combined with a high mortality of cancer diseases most of the clinical trials regarding gene therapy are carried out in the field of cancer research. Besides inherited or acquired diseases based on genetic defects, vascular and infectious diseases as well as DNA vaccination are topics of major interest.

1.1.1 Gene delivery

Delivering genes to specific cells is a process including many obstacles. Nucleic acids are negatively charged, hydrophilic and large macromolecules that cannot penetrate through lipid membranes. Additionally they are quickly eliminated in the organism by nucleases, mainly before they reach their target cell. These properties are not very suitable for their use as therapeutics. *In vivo* experiments already showed that the intravenous application of naked DNA does not lead to high transfection levels. If naked DNA is injected locally it has to be in large amounts to be efficient. Therefore gene vectors that are useful to protect DNA and that are able to deliver it successfully to its target cell are examined intensely. There are many different gene delivery approaches existing in the viral as well in the non-viral field. For example cationic lipids (2) and cationic polymers (PLL, PEI) that both complex DNA and result in so called lipo- or polyplexes or liposomes that envelope DNA solutions with a lipid bilayer system or combinations of both. Further on different viral vectors, like adeno-, retro- or lentiviral vectors are employed. Following, two of the main important systems are listed that are additionally directly related to this work.

1.1.2 Adenoviral vectors

Most of the clinical trials carried out in gene therapy studies use viral vectors. Since now, they are the most efficient gene vectors as their natural setup is perfectly concerted to invade and infect their target cell (3; 4). Adenoviruses belong to class I (DNA) viruses, have an icosaedric capsid and contain a large DNA genome of 30-38 kbp. They can infect dividing and non-dividing cells and do not cause insertion mutagenesis as their genome does not become integrated into the host´s DNA. Consequently expression of genes delivered by adenoviral vectors lasts only temporarily. More than 51 serotypes exist which can induce inflammatory responses in the host tissue which additionally reduce duration of gene expression and limit repeated administration.

In order to avoid replication of therapeutically applied viruses they need to be specially modified. Genes for viral replication are removed and replaced by the therapeutic gene (5). A major problem using viral vectors for gene transfer is their limited capacity to incorporate the additional nucleic acids that should be therapeutically delivered. Besides the capacity, activation of the immune system and

the possibility of mutagenesis, for example caused by retro-viral vectors, through gene insertion into the host's genome are severe disadvantages of viral vectors. Additionally, handling is delicate and the production process is complex and expensive.

1.1.3 Synthetic, PEI based vectors

Non-viral gene delivery has been considered as an alternative to the intensely researched viral systems and increasing interest is focused on the development of new non-viral gene delivery systems. Chemically defined DNA carriers offer enhanced biosafety compared to viral vectors. Furthermore, they offer an easier synthesis and production as a pharmaceutical product. They are well accessible to a diversity of chemical modifications whereas viruses are restricted to a proteinaceous composition. Synthetic vectors can either be generated protein-free or by using non-immunogenic proteins and peptides with plasmids containing no immunostimulatory motifs. Accordingly, non-viral vectors are less immunogenic and therefore can be repeatedly applied to the patient without leading to severe immune responses (6). Another major advantage is that there is nearly no limitation to the size of the nucleic acid that can be delivered. Beside all these positive aspects the main disadvantage of non-viral vectors is their low efficiency regarding gene transfer and a significant cytotoxicity depending on the used polymer.

Till now, the most commonly used and best examined polycation (PC) for non-viral gene delivery is polyethylenimine (PEI). Polyethylenimine is a linear or branched polymer, available at different sizes (e.g. 2, 22, 25, 750 kDa) that condenses DNA by electrostatic interactions. At physiological pH, PEI exhibits a high cationic charge density with approximately every fifth to sixth nitrogen atom being protonated (7). This protonation profile is slightly shifted after DNA binding. In ratios of polymer nitrogens to DNA phosphates that are suitable to form small and homogenous transfection complexes in virus-like dimensions, the resulting particles exhibit a positive surface charge due to an excess amount of polymer. This positive surface charge facilitates interaction with negatively charged cell membranes hence leading to improved cellular uptake. Another important characteristic property of PEI is its huge buffering capacity which significantly contributes to its high transfection activity. After uptake of the vector into the cell via endocytosis acidification of the endosome begins and PEI induces a massive accumulation of protons leading to a passive

influx of chloride-ions accompanied by water. This property is called “proton sponge effect” (8; 9). The increase of osmotic pressure results in swelling and finally in rupture of the endosomolytic vesicle thus leading to improved release of PEI and the genetic information.

1.2 Polyethyleneglycol (PEG): a commonly used shielding polymer

PEG is a basically linear, uncharged, hydrophilic and non-immunogenic polymer with low toxicity covering sizes ranging from 200–40000 Da. Besides, branched so called multiarm PEGs exist. Polyethylenglycol is a polyether with repetitive -CH_2-CH_2-O- units exhibiting hydroxy groups at the end. Before coupling, they have to be chemically activated with a suitable leaving group that can be displaced by nucleophilic attack. For example by the epsilon amino group of lysine residues, by a protein’s N-terminus or by the amino group of a cationic polymer. Due to its versatile application possibilities combined with low toxicity PEG was approved by the Food and Drug Administration (FDA) for use in cosmetics, foods and drugs (10).

1.2.1 PEGylation of proteins

PEGylation, originally defined as attachment of polyethyleneglycol to drug molecules, was first introduced by Davis et al. in the 1970s (11). Nowadays PEGylation of therapeutic peptides and proteins is a well established approach (see review. (12)). When PEG is covalently linked to proteins it alters their properties extensively. PEGylation of proteins maintains their bioactivity *in vivo*, increases their solubility and reduces antigenicity and immunogenicity. PEGylated proteins are protected against proteolytic degradation and show increased serum half-lives due to decreased efficiency of drug elimination processes such as renal excretion and opsonization (10). Due to reduced opsonization they are less phagocyted by natural particle elimination mechanisms, mainly organs of the RES (reticulo endothelial system).

Already in 1977, Abuchowski et al. (13) examined the effect of PEGylation on immunogenicity and circulation time of bovine liver catalase. In one of the later experiments Nandini v. Katre tested the influence of PEGylation on IL-2, which was tested in a clinical study as an anticancer therapeutic agent and induced the

development of antibodies in some of the patients (14). Nowadays a couple of PEGylated proteins are applied successfully in clinic such as PEG-adenosine deaminase, PEG-Interleukin-2 and PEG-α-Interferon. The range of molecular PEGylation is of course much bigger with antibodies, antigens, growth factors, receptors (e.g. TNFR) and blood constituents, to only name a few (10).

1.2.2 PEGylation of adenoviruses

As the PEGylation of proteins and peptides was a well examined and successful research area, O´Riordan et al. started with the PEGylation of capsid proteins of adenoviruses in 1999 (15). Up to 18000 PEG molecules could be attached to the capsid surface of one single Ad serotype 5 (Ad5) particle. PEG molecules were mainly attached to hexon, fiber and penton base, the best accessible outer proteins of the particle as shown in figure 1.

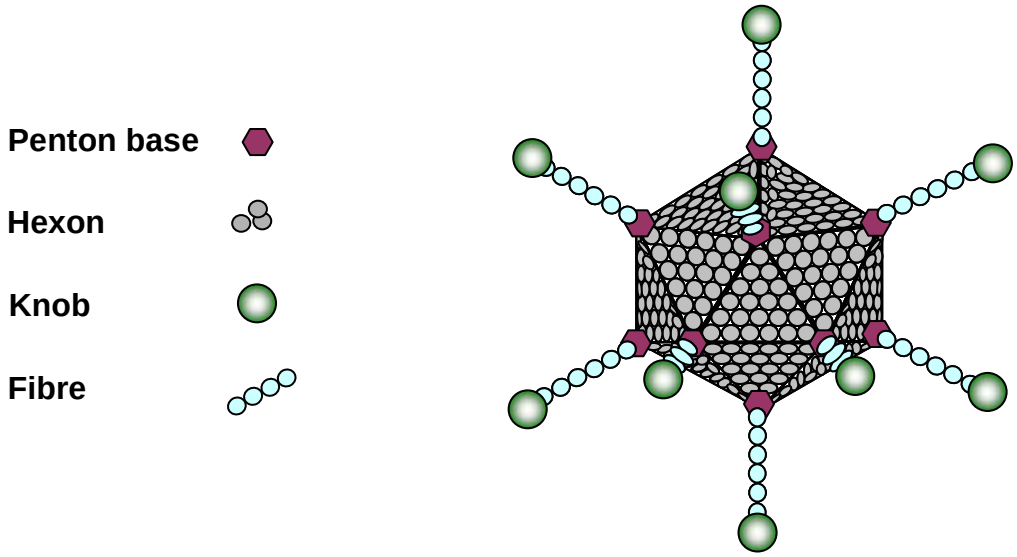


Figure 1) Illustration of an icosaedric adenoviral particle highlighting the most important capsid proteins in colour. Genetic information is packed inside the capsid.

Importantly, O´Riordan et al. could demonstrate in the same experiment that core proteins were not PEGylated indicating that PEGylation of Ad-viruses under the chosen conditions does not compromise the integrity of the vector capsid.

As the proteins of hexon, fiber and penton base are known to be the main targets for neutralizing antibodies, their PEGylation mask them in a way that they are protected from being opsonized. O´Riordan et al. could show *in vitro* and *in vivo* that PEGylated

adenoviruses could effectively escape neutralizing anti-capsid antibodies thus overcoming one of the most important hurdles for Ad5 mediated gene transfer.

The degree of PEGylation that still retains most of the Ad vectors´ biological activity (e.g. infectivity) was analyzed by Croyle et al. (16) by using different PEG-linkers (tresyl-, succinimidylester-, disulfide- or cyanuric acid- PEG). Depending on the linker, 70–100% of capsid protein PEGylation was achieved. Interestingly, the repeated application of PEGylated viruses allowed only for significant transgene expression when different functional PEG linkers were applied in the individual dosing. They assumed that due to PEGylation of the vector new antigenic epitopes appear that cause the production of antibodies only when applied repeatedly with viruses PEGylated with the same linker.

PEGylation of Ad-viruses decreases their zeta potential from -48 mV to -16 to –27 mV. It also stabilises virus particles at 37 and 42 °C and is helpful as a cryoprotectant at –20 °C. Cellular and humoral immune responses were both reduced but PEGylated Ad viruses only led to slightly prolonged gene expression. The reason for this finding might be the production of new viral proteins and/or the immunogenicity of the transgene product itself in the transducted cell.

PEGylation decreased association with kupffer cells and uptake by macrophages and also the interaction with platelets is reduced. As the injection of non-PEGylated viruses led to a drop in platelet count, unchanged platelet levels found in the case of PEGylated viruses indicate a prevention of intravascular coagulation.

The incomplete ablation of innate immune response, which was observed with PEGylated virus particles let to the idea to use these vectors in combination with immune suppressive drugs. PEGylated viruses together with glucocorticoids led to complete ablation of innate immunity.

By genetic modification of the capsid proteins fiber, hexon, penton base and pIX it is possible to introduce peptide ligand motifs onto the virion´s surface. However this often occurs with limited success since modification can alter folding of the modified capsid protein and/or assembly of intact capsids. Romanczuk et al. (17) developed a targeting approach based on the PEGylation of Ad vectors with bifunctional PEGs. This approach permits the use of full-length protein ligands that are too large to be incorporated genetically. Lancotti et al. (18) attached fibroblast growth factor 2 (FGF2) to PEG and used this reagent in an *in vivo* experiment. FGF2 decorated adenoviral particles showed a significantly higher transduction efficiency of tumour

cells as PEGylated particles did. PEGylation commonly leads to lower transduction compared to unmodified particles. Several research groups have demonstrated that PEGylation with NHS (N-hydroxy succinimidyl ester) based PEGs can efficiently disrupt binding to CAR (coxsackie adenovirus receptor) of such PEGylated viruses. Of course the degree of PEGylation plays a crucial role whether CAR can be bound or not. As with the NHS-PEG methods the capsid is PEGylated on many different proteins (hexon, penton base etc.) there are hints that ligand attachment to such a variety of locations might interact with the correct trafficking of vectors at the cell surface or inside the target cell. Kreppel et al. (19) developed a so called “geneti-chemical” approach where they genetically modified the fibre loop by introduction of cystein. By PEGylation with SPA-PEG (succinimidyl propionic acid- PEG) the vector’s native tropism was ablated but cystein at the tip of the fibre protein was accessible for ligand attachment. The tip of the fibre is evolutionary designed to bind to a receptor and the fibre protein is the first protein shed from the particle upon cell entry and therefore intracellular trafficking might be unhindered when the ligand is only bound to the fibre tip. This is still under investigation and also the pH-sensitive PEG reagents described here, might be taken into consideration. It seems that *in vivo,* PEGylated viruses still have limited access to CAR. Mok et al. (20) found that the heparin binding motif KKTK (amino acids 91 – 94) in the fibre shaft was still accessible to KKTK antibody even after heavy PEGylation and that additionally the RGD (amino acids arginin, glycin and asparagin) -integrin binding motif in the penton base was partially accessible among these conditions. This might play a role in the transduction of liver cells. They speculated that the same forces that let the virions pass through the fenestrae in liver sinusoids might be responsible for bending PEG chains and thereby giving access to KKTK and RGD motif. PEG chain length might also play a role. PEG prolongs circulation time and therefore uptake possibilities, as intravenously administrated non-PEGylated viruses are eliminated very fast. For detailed information see review Kreppel et al (21).

1.2.3 PEGylation of synthetic, PEI based vectors

One reason for the success of positively charged PEI based gene delivery vectors is their membrane interaction with negatively charged heparansulfat-proteoglycans on the cell surface (including transmembrane syndecans), that leads to increased uptake compared to neutral particles (22). These beneficial *in vitro* effects cause

most of the undesired side effects after *in vivo* application due to unspecific interactions with negatively charged serum proteins, membrane proteins or with erythrocytes. When applying PEI based polyplexes via the bloodstream the negative side effect of this electrostatical interaction becomes apparent. Unspecific binding of positively charged PEI particles to blood components leads to subsequent elimination by the reticulo endothelial system and can cause clotting. Additionally, binding to non-target cells and tissues can lead to undesired and potentially toxic side-effects. Taken together this results in reduced circulation times and targeting ability (23).

Klibanov et al. (24) reported prolonged and less hindered blood circulation for PEGylated liposomes in 1990. Since PEI was introduced in 1995 by the group of J.P. Behr as a potent gene vector (8), PEGylation of PEI based gene delivery vectors was only a matter of time. During extracellular transport its DNA load should be delivered unhampered, minimizing unspecific interactions of the positively charged polyplex with the biological environment. Prolonged circulation times, reduction of interactions and inhibition of activation of the complement system (25) can be achieved by shielding the polyplex with an inert PEG layer on its surface. PEG shielding of polyplexes leads to improved stability even after freeze-thawing cycles (26). Additionally PEGylation of PEI based polyplexes increases the solubility of the complexes. The use of plain PEI as gene vector is limited by the poor solubility of the DNA complexes. Complex solutions become turbid and precipitates appear at DNA concentrations that are much lower as when PEI–PEG/DNA polyplexes are generated (27). However, surface charge shielding can lead to a significantly reduced gene expression activity since not only undesired interactions are reduced (27; 28).

1.2.3.1 Pre-PEGylation

The positive effect of polyethyleneglycol modification of drugs, peptides, proteins and liposomes probably gave rise to investigations concerning polymer based delivery systems. Wolfert et al. (29) and Toncheva et al (30) developed novel vectors for gene delivery by self-assembly of DNA with PLL grafted to PEG. They were able to form discrete particles, around 100 nm in size that successfully transfected cells. Surface charge and also cytotoxicity of the polyplexes were decreased due to the shielding effect of the PEG chains. As PEI was the more efficient gene delivery vector compared to PLL groups started to synthesize the first PEI-PEG copolymers with Erbacher (31) and Kichler (27) being one of the first in 1999 and 2002 respectively.

Pre-PEGylation is easy to perform as it consists of simply mixing the polymer containing solution to that containing the DNA by pipetting. Although it is a very easy and quick method to generate PEGylated particles it has to be considered that only approximately 20% of the used polymer is PEGylated otherwise the hydrophilic parts seem to hinder proper DNA condensation and particle formation (32). Figure 2) illustrates the process of pre-PEGylation.

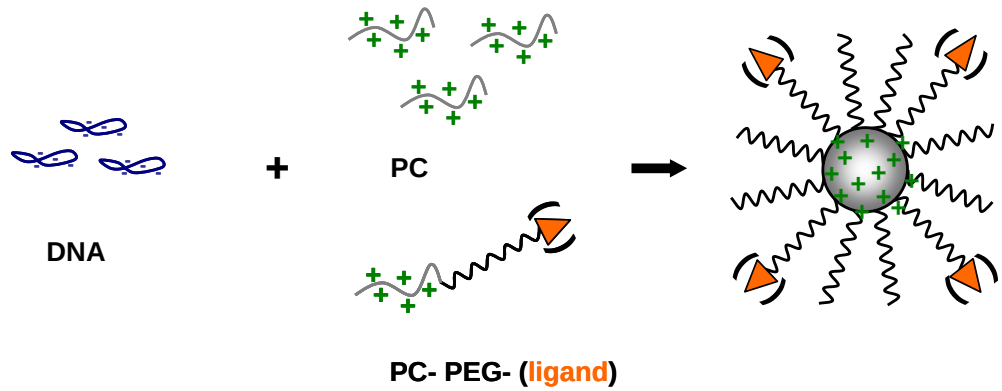


Figure 2) Schematic illustration of pre-PEGylation. Polyplexes with or without targeting ligand are prepared in a one step reaction by mixing plasmid DNA with polycation and polycation coupled to PEG or to PEG-ligand.

1.2.3.2 Post-PEGylation

Another possibility to receive PEG-shielded polyplexes is the so called post-PEGylation approach. In this method DNA is first complexed by the polycation and when particles have formed, the PEG reagent is added. PEG coupling to the polyplex surface can for example occur via active ester- (NHS) or tresyl-activated PEGs. All of them form stable amide bonds on the particle surface. PEI-ligand or PEI-PEG-ligand can also be incorporated when particles with receptor affinity are needed. These ligand containing conjugates are introduced into the polyplex by the pre-PEGylation approach mentioned above or via post-targeting as performed by Blessing et al. (33). The advantage of post-PEGylation is that the step by step procedure prevents any undesired effect of PEG-modified polycations on the DNA condensation process (34) and, what can be regarded as most important, that it is not restricted to non-viral vectors. The major drawback of post-PEGylation is that due to 1 h PEGylation time it is a more time consuming procedure and that the amount of PEG on the particle surface is not very well defined. Figure 3) illustrates the process of post-PEGylation.

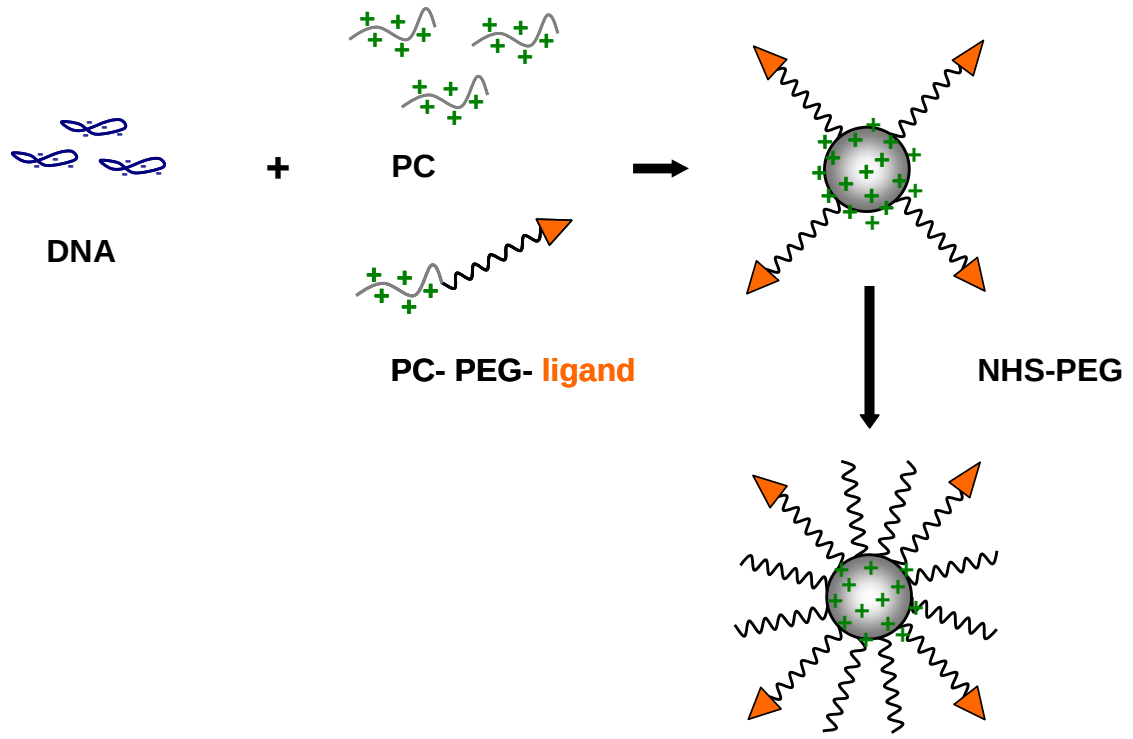


Figure 3) Illustration of polyplex formation via post-PEGylation. DNA is first condensed by PC or PC-PEG-ligand for receptor mediated uptake. Then post-PEGylation reagent is added to the reaction to couple to the surface amines.

1.3 Design of dynamic, bioresponsive gene vectors

Despite considerable activity in transfecting cells, the properties of PEI need to be further improved. It is not sufficient to compact and protect DNA; a modern gene vector should fulfil a number of other requirements. These aspects can not be operated by one single polymer and therefore it is necessary to provide the basic polyplex with various functional domains. The idea is to exploit the different local conditions and appearances that a gene vector has to pass or interact with on its way to or inside the cell in a way that an increased transfection rate can be obtained. The most striking advantage of viral particles over non-viral gene delivery vectors is that they are not static but react dynamically in response to the cellular environment they are exposed to. Therefore barriers like the endosome or the nuclear envelope are overcome with high efficiency. This exploitation of local conditions inspired many research groups to work on more dynamic, bioresponsive synthetic gene delivery vectors. However, with substantial efforts in the development of new transfection

systems and better understanding of the barriers, transfection efficiency and targeting of non-viral systems have improved significantly.

1.3.1 Application of ligands

The introduction of targeting ligands into gene delivery vectors represented a denoted progress in the development of more efficient transfection systems. If such a modified vector reaches an area with cells expressing the corresponding receptor, its ligand moiety can interact specifically and receptor mediated endocytosis causes increased particle uptake. This kind of targeting strategy that triggers receptor-mediated endocytosis of the DNA complexes requires the knowledge of specific surface receptors of different cell types. The extensive search of differences in the appearance of tumour and normal cells, for example in regard of surface proteins, already showed a lot of success. Therefore gene vectors provided with special targeting ligands were developed. Wu and Wu were the first who used gene vectors provided with a targeting ligand (35). They harnessed the knowledge of hepatocytes expressing special asialoglycoprotein receptors and achieved enhanced gene expression when they applied asialoorosomucoid modified PLL as DNA compacting domain compared to plain PLL polyplexes. Among the first successfully introduced ligands to gene transfer polyplexes were transferrin (36) and insulin (37), anti-CD3 gene transfer complexes for T cell specific uptake (38), folate (39) and EGF (40).

1.3.2 Enzyme mediated targeting

Another form of side specific structural changes of non-viral vectors can occur through an enzyme. With detailed analysis of tumour invasion and metastasis, an enzyme was found that is specially expressed on tumour cells. Matrix metalloproteinases (MMPs) are a family of endopeptidases containing a zinc-ion at their catalytic site. All members of the family are able to degrade extracellular matrix. The proteolytic breakdown of tissue barriers seems to promote intravasation and thus connection to the circulation via blood vessels (41). Gene vectors were designed that can react specifically with this enzyme that is secreted into the extracellular space. Hatakeyama et al. tested an approach with multifunctional envelope-typed nano device (MEND; condensed DNA enveloped by lipid bilayers) where they used a PEG – MMP substrate peptide- lipid conjugate (42). Cleavage and transfection efficiency is dependent on the level of enzyme expressed in the host cell. With this approach they

revealed stabilized MEND whilst systemic circulation, facilitating tumour accumulation but upon arrival at the tumour side the secreted MMPs lead to PEG removal through enzymatic cleavage and therefore to higher tumour cell transfection.

1.3.3 Incorporation of endosomolytic active peptides/proteins

Many of the commonly used gene vectors end up entrapped in the endosome as they do not possess any endosomolytic properties themselves and therefore efficient gene transfer is restricted. Escape of the entrapped particle from the endosome can be forced by incorporation of endosomal releasing compounds such as mellitin or viral based peptides (for reviews see Wagner, Meyer (43; 44)).

A series of natural membrane destabilizing agents exist, some of them belong to defense mechanisms, others to affection mechanisms. For example adenoviruses can cause endosomal rupture, rhinoviruses can form pores in membranes, and some bacterial proteins lead to vesicle disrupture. A variety of viral as well as synthetic peptides, with or without pH specifity, cause vesicle leakage by pore formation. By harnessing such natural mechanisms it became possible to develop more effective gene delivery systems. The success of membrane active substances depends on the underlying polymer in the individual case. When polymers without endosomal escape properties, like for example PLL, are combined with such membrane active compounds the efficiency can raise dramatically. Whereas there is only a slight increase when polymers, like PEI, are used that already exhibit high efficiencies by themselves. Plank et al. investigated the influence of different peptides on PLL/ PLL-Tf based gene transfer. The negatively charged peptides were bound to the positively charged DNA complex by electrostatic interactions. Using for example the dimeric peptide INF3DI they could enhance gene transfer efficiency over 5000 fold compared to absence of peptide. INF3D contains the same 20 N-terminal amino acids as the hemagglutinin subunit HA-2 of influenza virus X-31 apart from two amino acids that were exchanged by glutamic acids to achieve a more pH specific peptide derivative. As a result of the pH sensitivity, the structure of the peptide is changed in acidic surroundings offering a site specific membrane lytic activity (45). Saito G. et al. used the bacterial pore forming protein listeriolysin in a gene delivery approach with protamine as DNA compacting domain. Endosomal cleavage of comprised disulfide bonds revealed the active form of the protein and led to pore formation and enhanced cytosolic delivery of the gene vector (46).

1.3.4 Reductive cleavage by high redox potential differences

Another possibility to change the appearance of the gene vector intracellular is to exploit the differences in the redox potential between oxidizing extracellular environment and the reducing intracellular compartment of cells. These differences allow for transient stability of disulfide bonds during transport in plasma but enables environment specific cleavage intracellular (46). Knowledge of this difference led to synthesis of disulfide linked polymers. On the one hand, smaller polymers exhibit lower cytotoxicity but also lower transfection efficiency and on the other hand large polymers show high cytotoxicity but high transfection efficiency (47), (48), (49), (50). Therefore the idea came up to use large disulfide cross linked polymers for high transfection activity that should be degradable in the reductive environment of the cytosol to small fragments with lower toxicity (51; 52). Incorporated DNA can be freed from the polyplex due to vector unpacking, for example initiated through RNA exchange (53), which occurs easier when shorter polymers are used (54). The principle of intracellular polymer cleavage for facilitated DNA release was investigated by Oupicky et al. (55). They compacted DNA with a linear PLL derivative, containing of short lysine subunits with cystein moieties on each end that were coupled by disulfide bond formation. They could demonstrate intracellular reduction of the disulfide bonds but achieved only poor transfection efficiencies. This was probably due to PLL which is not offering any intrinsic endosomolytic properties. Based on these findings Carlisle et al. (56) developed another strategy employing the reversibility of disulfide bonds. They generated a PEI based gene delivery system with a hydrophilic coat connected to the polyplex surface by reducible disulfide bonds. It was shown that removing the hydrophilic surface coat of PEI polyplexes intracellular by disulfide cleavage increased gene transfer efficiency up to 100 fold in comparison to analogue particles with stable thioether linked shielding polymer.

1.3.5 pH-sensitive shielding

One approach to receive more dynamic and virus like particles that are able to react to specific changes in the microenvironment, is the synthesis of a pH-labile PEG shield as it is performed in this work. While PEG shielding is very advantageous for long systemic circulation and thus tumour accumulation, it unfortunately reduces the cell transfecting ability of the corresponding polyplex. Introduction of pH-sensitive moieties in the shielding reagent should not affect the stability of the PEG shield

upon systemic circulation at physiological pH. Upon acidification of the endosome the pH-sensitive linkage should be cleaved shedding the PEG coat and re-exposing again its initial positive surface charge for enhanced membrane interaction. Elicitor of this strategy was the poor transfection efficiency of PEGylated particles compared to their non-PEGylated counterparts and the fact that incorporation of targeting ligands for increased uptake did not restore the transfection efficiencies to values comparable to those achieved by unshielded particles. It suggests itself that PEG shielding also affects the intracellular processing of the particle. This reduction in gene transfer efficiency might be explained by depleted vector–cell membrane interactions in the endosomal vesicle due to the PEG coat of the particle that results in hampered endosomal release. The combination of endosomal swelling and positively charged particle surface might be necessary for improved release from the compartment (28).

Chemical linkages that might be suitable for pH dependent hydrolytic degradation in endosomal/lysosomal compartments include acetals (57-59), ortho esters (60), vinyl ether (61) and hydrazones (28; 62; 63). Besides late endosomes also some tumour tissues exhibit an acidic environment that can be utilized for local PEG shield removal (64).

In the present work the influence of a hydrazone bond (HZN) in a post-PEGylation approach was investigated. Walker et al. (28) have previously demonstrated successfully that hydrazone linkage in a pre-PEGylation experiment resulted in improved transfection efficiencies. The following scheme depicts the underlying idea of pH-sensitive PEGylation. Shielded and targeted polyplexes, taken up by receptor mediated endocytosis become deshielded upon acidification of the endosome, re-expose their net positive surface charge and thus result in enhanced endosomal release and improved gene transfer efficiency.

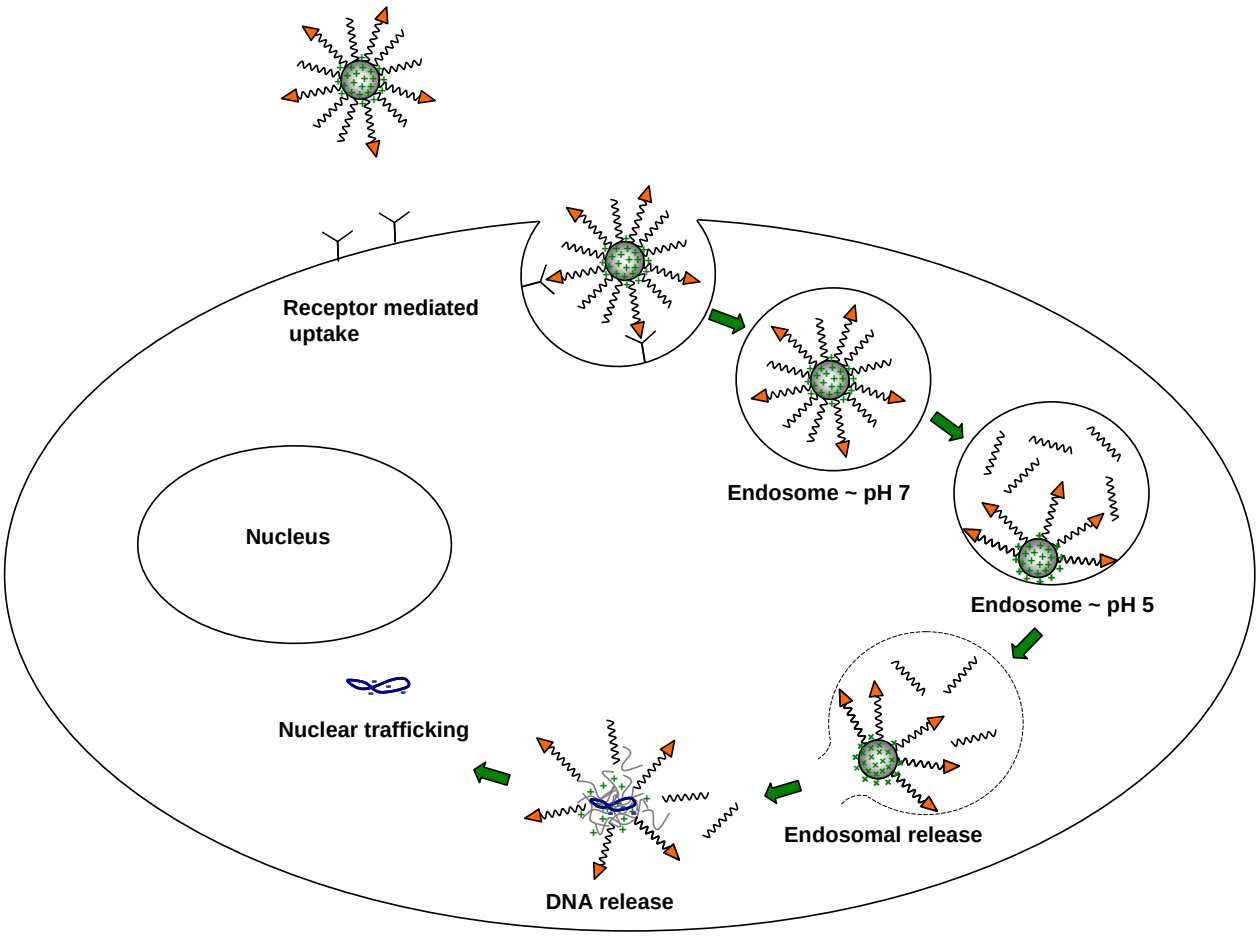


Figure 4) Illustration of the transfection process by receptor mediated uptake mechanism for targeted and PEGylated polyplexes. Shield removal upon acidification, hypothesised membrane interaction and enhanced particle release are depicted.

1.4 Purification of polyplexes

An important step towards more biocompatible PEI based gene vectors was the development of purification methods for these polyplexes. When polyplexes with PEI are prepared at an N/P ratio of 6 the amount of unbound PEI is considerable high with 60–80% (65). From literature it is known that this amount of free PEI contributes to the good transfection efficiency of PEI based delivery systems but that it also correlates with the toxicity of these formulations, especially *in vivo.* Boeckle et al. (66) developed a purification method where unbound PEI was removed via a size exclusion approach resulting in reduced *in vitro* cytotoxicity. In 2007 Fahrmeir et al. (67) further developed this purification process by the use of an electrophoresis apparatus that allowed for purification of also bigger polyplexes including their PEGylated and/or their Tf-coupled PEI derivatives. By removing the free polymer the

N/P ratio of the remaining polyplex and the transfection efficiency decrease significantly. This reduction can be partially overcome by application of higher DNA concentrations.

1.5 Aim of the thesis

1.5.1 Synthesis of pH-labile post-PEGylation reagents

The aim of this thesis was the development of a pH-sensitive post-PEGylation reagent that is not as restricted to polyplex shielding as pre-PEGylation approaches are. This new pH-labile post-PEGylation reagent can theoretically be used for shielding of any amine group bearing gene delivery vectors.

As already mentioned above, PEGylation of polyplexes results in desired and undesired effects. On the one hand the introduction of a hydrophilic coat surrounding the polyplex results in longer circulation times and reduced toxicity due to less unspecific interactions with blood components and proteins but on the other hand these reduced interactions are finally responsible for lower gene transfer efficiency. As the introduction of targeting ligands for enhanced cellular uptake did not restore the transfection efficiency to values comparable to non-PEGylated particles the idea came up to synthesize pH-sensitive post-PEGylation reagents that allow for environment-sensing modulations. Upon circulation the particle is protected from unspecific interactions but when reaching the endosome the decrease of pH triggers the removal of the PEG shield presenting the positive surface charge that was hidden before. This allows for charge interactions with endosomal membranes and facilitates release of the entrapped vector (28). For closer characterisation two variants of pH-labile shielding PEG reagents had to be synthesized namely the monofunctional NHS-HZN-PEG and the bifunctional OPSS-PEG-HZN-NHS. The latter provides a platform for PEG chain modification with a fluorescent dye to visualise deshielding of such Alexa-PEGylated (Alexa Fluor® 488) polyplexes among acidic conditions. Additionally, this pH-labile bifunctional PEG could function as a starting base for future post-targeting studies on polyplexes that have been PEGylated with the bifunctional pH-labile PEG linker.

1.5.2 Post-PEGylation of polyplexes and their biophysical characterisation

NHS-HZN-PEG had to be tested on its shielding and deshielding ability by size and zeta potential measurements while the other pH-sensitive reagent, based on the bifunctional PEG linker, with an additional fluorescent dye molecule at one end of the PEG chain, should allow for observation of PEG chain cleavage from the polyplex surface via FCS (Flourescence Correlation Spectroscopy) measurements. For the latter experiment it was vitally important that particles were pure and defined. The dye labelled PEG reagent should provide the accessory possibility to shed light on the PEGylation degree and detailed composition of the final polyplex.

1.5.3 *In vitro* and *in vivo* characterisation of novel polyplexes

Monofunctional pH-labile PEG reagent had to be analyzed for its assignment on PEI based polyplexes in *in vitro* cell culture studies and in an *in vivo* tumour mouse model. Therefore PEI based polyplexes with or without targeting ligand had to be shielded with appropriate amounts of the novel shielding reagent and afterwards the resulting gene vectors should become tested on their transfection ability and efficiency. A pH-stable comparable PEG reagent had to be used as control in these experiments.

Finally, PEI polyplexes with targeting ligand and stable or labile PEG shield should be applied intravenously into mice bearing a subcutaneous HuH7 tumour to clarify whether the new pH-sensitive PEG reagent is a promising candidate under *in vivo* conditions.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Dimethylformamide (DMF) and 1-amino-3,3-diethoxypropane (ADEP) were purchased from Sigma-Aldrich (Steinheim, Germany), ethanolamine, cysteamine and picrylsulfonic acid (trinitrobenzenesulfonic acid TNBS) from Fluka were also acquired over Sigma-Aldrich. Methoxypoly-(ethyleneglycol)-butyraldehyde (ALD-PEG; 20 kDa) and mPEG-propionic acid succinimidyl ester (mPEG-SPA 20 kDa) were purchased from Nektar Therapeutics (Birmingham, AL). These reagents are now available from Sunbio (Orinda, CA) and Chemos GmbH (Regenstauf, Germany). α-succinimidylester-ω-2-pyridyldisulfid polyethylene glycol (OPSS-PEG; 10 kDa) was synthesised from IRIS Biotech GmbH (Marktredwitz, Germany). Succinimidyl-6-hydraziniumnicotinatehydrocloride (SHNH) was purchased from Solulink Inc. (San Diego, CA). Benzyloxybenzaldehyde polystyrene beads (Novabiochem) were obtained from Merck Biosciences AG (Läufelfingen, Schweiz) and L-glycine from Fisher Scientific GmbH (Nidderau, Germany). Cell culture media, antibiotics, trypsin/EDTA solution and fetal calf serum (FCS) was purchased from Life Technologies, Alexa Fluor® 488 carboxylic acid, succinimidyl ester from Molecular probes, all by Invitrogen (Karlsruhe, Germany).

2.2 Polyethylenimine

Branched PEI with an average molecular weight of 25 kDa (BPEI) was purchased from Sigma-Aldrich (Steinheim, Germany). LPEI with an average molecular weight of 22 kDa was synthesized by acid-catalysed deprotection of poly(2-ethyl-2-oxazoline) (50 kDa, Aldrich, Munich, Germany) in analogous form as described (68). It is also commercially available from Polyplus (Strassbourg, France). Polymers were dissolved in water and then neutralized with HCl. For purification they were applied on a gelfiltration column with Sephadex G-25 superfine using 20 mM HEPES with 0.25 M NaCl at pH 7.4. For polyplex preparation LPEI was used as 1 mg/ml stock solution in HBG and BPEI in HBS 0.5x.

2.3 mEGF-PEG-BPEI

Epidermal growth factor (EGF) from PeproTech, Inc. (NJ, USA) linked with BPEI by a heterobifunctional 3.4 kDa PEG derivative was synthesized as described previously (40) with a molar ratio of EGF to BPEI of approximately 1:1.

2.4 Plasmid DNA

For *in vitro* cell culture experiments plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) described in Plank *et al*. (69) was purified with the EndoFree Plasmid Kit from Qiagen (Hilden, Germany). For *in vivo* experiments endotoxin free pCMVLuc was purchased from PlasmidFactory (PlasmidFactory GmbH & Co. KG, Bielefeld, Germany). For polyplex preparation with LPEI 1mg/ml stock solution in HBG and with BPEI in HBS 0.5x was used.

2.5 Syntheses of PEGylation reagents and PEG conjugate

2.5.1 Synthesis of monofunctional N-hydroxysuccinimidyl-hydrazone-poly(ethylene) glycol (NHS-HZN-PEG)

Monofunctional hydrazone-mediated reagent (NHS-HZN-PEG) was formed with a high molecular weight PEG (20 kDa). ALD-PEG 20 kDa (1 µmol) in 0.8 ml of anhydrous DMF was added to SHNH (4 µmol) in 0.2 ml anhydrous DMF. The reaction was mixed for 2 h at room temperature under argon in a 2 ml reaction tube. For removal of unreacted SHNH the reaction mixture was subsequently incubated at room temperature for 4 h on a rotation wheel with 0.4 mmol benzyloxybenzaldehyde polystyrene-beads, pre-soaked for 20 minutes in DMF, offering a 100 fold molar excess of bead-aldehyde groups over SHNH. After removal of the beads by centrifugation the reagent was tested on absence of SHNH and on ester activity by TNBS assay and was then subsequently stored frozen at –80 °C in aliquots of 100 µl.

2.5.1.1 Reaction efficiency between aldehyde and SHNH

The efficiency of the reaction between PEG-aldehyde and SHNH was analysed by determination of the decrease of NH_2 groups. 0.5 µmol PEG-CHO dissolved in 0.4 ml DMF were reacted with 2 µmol of SHNH dissolved in 0.1 ml of anhydrous DMF. The

second approach contained 1 µmol PEG-CHO and 0.25 µmol SHNH in 0.8 respectively 0.2 ml of DMF. Samples were analysed via TNBS assay right after mixing and after 0.5, 2, 6 and 20 hours. For reagent synthesis SHNH was used in 4 molar excess over PEG-aldehyde.

2.5.1.2 Removal of excess SHNH

Unreacted SHNH (applied in an excess over PEG-aldehyde) was removed by immobilization onto benzyloxybenzaldehyde (polystyrene-CHO) beads. Completeness of SHNH removal was monitored by TNBS assay on free, primary amino groups. To test what molar excess of bead –CHO groups was necessary for the complete removal of unreacted SHNH a defined amount of SHNH in DMF was implemented with benzyloxybenzaldehyde beads pre-soaked in DMF offering a 10, 50, 100, 150 or 200 fold molar excess of –CHO groups over SHNH. TNBS assay was performed before and after incubation with polystyrene beads and the remaining amount of SHNH was determined.

2.5.1.3 Ester reactivity

Each 2 µmol of NHS-HZN-PEG and NHS-PEG were separately dissolved in 1 ml of DMF and were implemented with equimolar amounts of ethanolamine. After one hour incubation time at room temperature the degree of conversion was determined by monitoring the remaining amount of primary amines by TNBS assay.

2.5.2 Synthesis and stability of BPEI-(HZN)-PEG

On the basis of an experiment from Kursa et al. (32) a gel-filtered solution of BPEI-HCl (0.12 µmol), at pH 6.0 in 1 ml of 0.25 M NaCl, was mixed with 0.36 µmol of NHS-PEG or NHS-HZN-PEG dissolved in anhydrous DMF. The pH of the solution was adjusted to 7.2. After 1 h, 2 M NaCl solution was added to the batch to give a final concentration of 0.5 M NaCl and a total volume of 2 ml. The reaction mixture was loaded on a cation-exchange column (Macro-prep High S; HR 10/10, BioRad, München, Germany) and fractionated with a salt gradient (see 2.21). Fractions were collected and analysed via PEG- (see 2.17) and TNBS-assay (see 2.18). Fractions containing the conjugate PEI-(HZN)-PEG (positive for PEI and PEG) were pooled and PEI content was determined. The pooled fractions were divided into aliquots containing 30 nmol PEI and were subsequently frozen. For analysis of the stability of

these conjugates they were thawed and reapplied onto the column immediately or after 1 h respectively 4 h of incubation at 37 °C.

2.5.3 Synthesis of N-hydroxysuccinimidyl-hydrazone- poly(ethylene) glycol pyridyldisulfide (NHS-HZN-PEG-OPSS)

To 1 ml OPSS-PEG-NHS 10 kDa (1 µmol) in anhydrous DMF, 5 µmol of ADEP were added. The reaction was mixed under argon in a 1 ml tube for 2 h at room temperature. Excess ADEP was removed as described in 2.5.3.1. The pool containing the PEG reagent was acidified with 1 M HCl to a 0.1 M solution to deprotect the acetal whilst incubating for 1 h at room temperature. To receive an anhydrous sample and for HCl removal the product was freeze dried over night. The product was dissolved in 0.8 ml DMF. To 0.8 ml sample containing 1 µmol of OPPS-PEG-aldehyde, 0.2 ml SHNH solution (4 µmoles in DMF) were added and mixed under argon. Reagents were allowed to react for 2 h at room temperature and were subsequently incubated with DMF soaked benzyloxybenzaldehyde polystyrene-beads to remove unreacted SHNH. Therefore a 100 fold molar excess of bead-aldehyde groups over SHNH was applied. 4 h later the beads were removed by centrifugation and the reagent was tested on absence of SHNH and on ester activity by TNBS assay (similar as described under 2.5.1.2&3) and was subsequently frozen at –80 °C.

2.5.3.1 Removal of excess ADEP

Excess of unreacted ADEP was removed by gelfiltration on a 10/30 Sephadex G25 column. The sample was diluted with water to a final volume of 2 ml and the run was performed in water as well. Fractions were tested on PEG content via PEG-assay and positive fractions were pooled. For determination of reaction efficiency fractions were additionally tested via TNBS assay on ADEP. A preliminary run with plain ADEP revealed a recovery of approximately 100% and thus the pool of retained ADEP allows for calculation of the coupling ratio.

2.5.4 Synthesis of Alexa488-disulfide poly(ethylene) glycol (hydrazone-) N-hydroxysuccinimidylester (Alexa-SS-PEG-(HZN)-NHS)

Alexa488-succinimidylester was mixed in DMF under argon and light protected for 1 h in a 1:1 ratio with cysteamine to form Alexa488-(2-mercaptoethyl)-amide. The efficiency of this reaction was determined via TNBS assay detecting the free amines of unreacted cysteamine. For synthesis of the Alexa labelled PEG-reagents, the pH-sensitive or the stable one, reagents were mixed with Alexa488-(2-mercaptoethyl)-amide in DMF with an excess of PEG over cysteamine modified Alexa488-succinimidylester of 25%. Progress of the reaction could be monitored by measuring the release of 2-pyridinethione at 343 nm (see 2.19). Afterwards the conjugate was snap frozen and stored at –80 °C.

2.6 Formation of polyplexes

DNA complexes were prepared by first diluting and mixing unmodified polycation (PC) in 0.5x HBS (10 mM HEPES pH 7.4, 75 mM NaCl) for BPEI or HBG (20 mM HEPES pH 7.4, 5% glucose) for LPEI. When particles with receptor mediated uptake capability were required polyplexes were prepared by first diluting and mixing EGF-PEG-BPEI with free PEI at a (w/w) ratio of 1 to 9. The PC buffer solution was rapidly mixed with plasmid DNA, diluted in either 0.5x HBS pH 7.4 (BPEI) or HBG pH 7.4 (LPEI) by pipetting. In standard PEI polyplexes, a molar ratio of PEI nitrogen atoms to DNA phosphates (N/P) of 6 was applied. Complexes were incubated for 20 min at room temperature before indicated molar (PEG/PEI) equivalents of the activated ester PEG reagents were added in DMF. After 1 h, unreacted reagent was quenched by adding equal molar amounts of an aqueous solution of glycine (1 µmol/ml) in regard to PEG. Afterwards particles were ready to be used. Final DNA concentration for *in vitro* application was 20 µg/ml and 200 µg/ml for *in vivo* experiments.

2.7 Particle size and zeta potential

Particle size of DNA complexes was measured by laser-light scattering using a Malvern Zeta Sizer 3000 HS and/or a Zeta Sizer Nano series ZEN 3600 (Malvern Instruments, Worcestershire, UK). For particle sizing complexes were formed at 100 µg/ml and were allowed to stand for 20 minutes. Before measuring they were

diluted to a final DNA concentration of 10 µg/ml (total volume 1 ml) with the buffer they were prepared in. BPEI particles were formed in 0,5x HBS whereas LPEI particles were formed in HBG. For estimation of surface charge, transfection complexes were diluted five fold in 1 mM NaCl to give a final DNA concentration of 2 µg/ml and zeta potential was measured as previously described (15). Average conductivity observed in these measurements was approximately 1 mS.

2.7.1 Shielding ability of PEG reagents

Therefore different polyplexes (LPEI/BPEI, +/- EGF) were prepared as described in 2.6. These complexes were incubated for 20 min at room temperature before increasing amounts of molar equivalents (PEG/PEI) of the activated ester PEG reagents (NHS-HZN-PEG or NHS-PEG of 2 and 20 kDa or OPSS-PEG-HZN-NHS or OPSS-PEG-NHS of 10 kDa) were added in DMF. After 1 h incubation time at room temperature, corresponding size and zeta potential was measured.

2.7.2 Deshielding ability of NHS-HZN-PEG shielded particles

The hydrolysis of the labile HZN-bond was monitored indirectly by zeta potential and size measurements of polyplexes PEGylated with NHS-HZN-PEG 20 kDa. Particles were prepared as described above. 100 µl of the PEGylated particles (100 µg/ml DNA, stable or labile PEG shielded with 30 fold molar excess over BPEI and 150 fold over LPEI) were then incubated with either 0.9 ml sodium acetate buffer pH 5.0 (20 mM NaOAc buffer and 75 mM NaCl) or 0.9 ml HBS 0.5x at pH 7.4 and 37°C. Size and corresponding zeta potential were measured after 0.5, 1, 2 and 4 h. T_0 indicates zeta potential of particles measured in the buffer they were prepared in right after 1 h of PEGylation time.

2.8 Cell culture

HuH7 hepatocellular carcinoma cells (JCRB 0403; Tokyo, Japan) and Renca-EGFR mouse renal carcinoma cells (stably transfected with pLTR-EGFR and pSV2neo kindly provided by Winfried Wels, Georg-Speyer-Haus, Frankfurt am Main Germany) were grown at 37 °C in a 5% CO_2 humidified atmosphere. HuH7 cells were grown in DMEM high glucose/F12 medium and Renca-EGFR cells in RPMI 1640 with Glutamax I both media supplemented with 10% FCS.

2.9 Gene expression *in vitro*

For transfection experiments HuH7 and Renca-EGFR cells were seeded in 96 well plates with $1x10^4$ respectively $7x10^3$ cells per well 24 h prior to transfection. During transfection and for the following incubation time before analysis, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen GmbH) were present in the medium. Transfection complexes with 200 ng of pCMVLuc plasmid DNA were added per well of cells. After 4 h incubation at 37 °C, transfection medium was replaced by fresh culture medium. Luciferase gene expression was measured after 24 h.

2.10 Luciferase reporter gene expression

Transfection complexes containing 200 ng DNA (pCMVLuc) were added to the cells in 100 µl fresh culture medium containing 10% FCS in a 96 well plate. Complex containing medium was removed 4 h after transfection and 200 µl of fresh medium were added. After 24 h cell culture medium was removed and cells were washed once with phosphate-buffered saline (PBS) and were subsequently lyzed with 50 µl of reporter lysis buffer (Promega, Mannheim, Germany). Detection of luciferase activity was performed with a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) and luciferase activity was determined as described by Ogris et al. (70). Values are given as relative light units (RLU) and represent luciferase activity per $1x10^4$ cells as mean $\pm$ standard deviations of at least triplicates. Two ng of recombinant luciferase (Promega, Mannheim, Germany) correspond to 10^7 light units.

2.11 Cell viability assay (MTT assay)

In parallel to transfection experiments, metabolic activity was determined using a 3-(4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) reduction assay. Cells were seeded in 96 well plates and treated with DNA-polyplexes and/or with additional used agents such as glycine or DMF in indicated amounts. After 24 h 10 µl of a 5 mg/ml MTT solution in sterile PBS were added to each well and the plate incubated at 37 °C. After 2 h, medium was removed and the samples were frozen at -80 °C for at least 1 h. After thawing 100 µl DMSO were 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 and cell viability was expressed as percentage relative to untreated control cells.

2.12 Gene expression *in vivo*

SCID mice (8 weeks of age, male and female) were housed in individually vented cages under specific pathogen-free conditions; food and water were provided *ad libitum*. They were inoculated subcutaneously in the flank with $5x10^6$ HuH7 cells in 100 µl PBS as described (40). When tumours reached a size of 6-10 mm polyplex formulation were prepared and injected intravenously. Most of the DMF in PEG reagents was removed by vacuum centrifugation before the polyplexes were added directly onto the pellet containing the appropriate amount of shielding reagent. After 1 h reaction time, size and corresponding zeta potential were controlled. Particles containing 50 µg pCMVLuc per 20 g body weight with a concentration of 0.2 mg/ml DNA in HBS 0.5x (BPEI) or HBG (LPEI) were injected into the tail vein. 48 h after injection animals were sacrificed and indicated tissues were resected and stored at -80 °C. Tissues were homogenized in 1x Promega lysis buffer, pH 7.5, using an IKA-Ultra-Turrax. Samples were centrifuged at 4 °C and luciferase activity of the tissue lysates was determined as described above. Luciferase background (approximately 200 RLU) was subtracted from each value and transfection efficiency was expressed as RLU per organ (mean +/- SD, n=3). 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 Purification of Alexa-SS-PEG-(HZN)-NHS for FCS measurement and determination of Alexa:PEG ratio

Reagents stored in DMF were diluted 1:10 in running buffer (20 mM HEPES/ 0.5 M NaCl) and were allowed to abreact for 30 minutes before they were injected onto a 10/30 column filled with Sephadex G25 column material. Fractions were analysed spectrometrically at 488 nm and via PEG assay. Distribution of Alexa488 over the two detected peaks can be used to calculate the PEG:Alexa ratio via Beer Lambert´s law with ε_{488} = 71000 $l*mol^{-1}*cm^{-1}$.

2.14 Preparation of stable and labile Alexa488-PEGylated BPEI particles for FCS measurement

2.14.1 Size exclusion chromatography (SEC)

BPEI particles were prepared as described above at an N/P ratio of 6 but for this experiment they were prepared in HBG. For removal of unbound BPEI the particles were purified on Sephacryl 200 column material (Sephacryl® S 200 HR, molecular weight exclusion limit 250 kDa for globular proteins; Pharmacia Biotech, Uppsala, Sweden) in a standard-size Pasteur pipette. The tip of the pipette was provided with glass wool and the column material, diluted 1:1 in water was filled to a height of approximately 4-5 cm. Afterwards the column was equilibrated with HBG (pH 7.4) and pre-conditioned with 600 µg of BPEI to reduce unspecific binding of polyplexes and BPEI to the column material. The column was subsequently washed with HBG (see also (67)). 600 µl of polyplex solution (100 µg DNA/ml) were applied onto the column and fractions of approximately 250 µl were collected. Fractions were analysed for DNA content by UV measurement respectively by TNBS assay for BPEI content. Polyplex containing fractions were pooled and additionally analysed for their DNA and polymer content (DNA concentration was calculated by 1 OD_{260} = 50 µg/ml or 45 µg/ml for DNA condensed with PEI, respectively. These values have been calculated after comparing plain plasmid DNA solutions with standard BPEI polyplexes). Out of these data final N/P ratio after purification were calculated.

The purified particles were subsequently post-PEGylated for 1 h at room temperature with a 30 fold molar excess of either Alexa-SS-PEG-HZN-NHS or Alexa-SS-PEG-NHS. To obtain Alexa-PEGylated particles with a minimum of free dye an additional size exclusion chromatography was performed. A Waters 996 HPLC set up equipped with a HR 10/30 column was used. The column was packed with Sephacryl S 200 HR material, equilibrated in HBG (pH 7.4) and pre-conditioned with 15 mg of BPEI. The column was washed with HEPES buffer (20 mM pH 7.4 containing 0.5 M NaCl). Polyplexes were filled up to the injection volume of 1 ml with as much of a 5 M NaCl stock solution to give a final concentration of 0.5 M salt. SEC was performed at a flow rate of 0.5 ml/min; fractions of 1 ml were collected and elution of DNA-polyplexes was monitored at 260 nm. Afterwards fractions were analyzed via PEG assay and DNA and Alexa content were determined by additional UV/VIS measurements. For

analysis of FCS experiments only the fractions up to and including the peak maximum were pooled.

2.14.2 Dialysis

For dialysis, a variety of molecules is placed into a semipermeable dialysis bag with pores and afterwards the sealed bag is put into a container which contains a huge volume of water or buffer. Small molecules can then pass through the tubing whereas larger molecules with dimensions significantly greater than the pore diameter cannot. Dialysis was performed using regenerated cellulose dialysis tubes Visking® with a MWCO of 14000 or ZelluTrans® V Serie with 25000 as MWCO both from Carl Roth GmbH & Co KG (Karlsruhe, Germany). Tubes were washed with water and allowed to incubate in the dialysis buffer at room temperature for about 30 min. Purified BPEI polyplexes were Alexa-PEGylated and afterwards diluted with HBG containing 0.5 M NaCl to 10 µg/ml DNA and were then filled into the tubes. Samples were dialysed over night under light protection against 5 litres of 20 mM HEPES pH 7.4; buffer was changed twice. Alexa and PEG content were analysed before and after dialysis.

2.14.3 Electrophoresis with ElectroPrep® System

Electrophoresis for polyplex purification was performed using AmiKa´s ElectroPrep™ System (available from Harvard Apparatus, Göttingen, Germany). Amika´s Electro Prep® system describes a combination of dialysis and targeted movement of charged particles underlying a certain electric field. As sample compartment, a 1.5 ml Teflon chamber was used with polycarbonate membranes on each side (pore size 50 and 100 nm). Prior to use, membranes were equilibrated in buffer solution (5 mM NaCl, 20 mM HEPES, pH 7.4). Alexa-PEGylated polyplexes were mixed with NaCl solution to obtain a final salt concentration of 0.5 M. Electrophoresis was performed with 200 V for 20, 30 and 60 minutes and Alexa and PEG content was determined before and after each run.

2.14.4 Ultra filtration

Vivaspin® 6 ml ultra filtration devices with 100, 300 and 1000 kDa cut-off (Sartorius AG, Göttingen, Germany) were used for separation of unbound PEG. BPEI polyplexes prepared in HBG and post-PEGylated with a 60 fold molar excess of

Alexa-SS-PEG were centrifuged four times at 4000 g at room temperature for 5 minutes using a Heraeus Megafuge 1.0 R. Prior to use, ultra filtration devices were centrifuged once with HBG for membrane equilibration. After each centrifugation step, polyplex solution was re-diluted to its original volume and briefly mixed by up- and down pipetting in order to minimize adsorption of particles on the membrane surface. Filtrate and supernatant were analysed for PEG (as described in 2.17), DNA and Alexa (UV/VIS) content.

2.14.5 Separation via density centrifugation

600 µl of Alexa-PEGylated (60x molar excess) purified BPEI polyplexes (100 µg DNA/ml 0.5x HBS) were pipetted into Eppendorf tubes that were pre-filled with 250 µl of a thick dextran solution (1.5 g/ml water). Tubes were centrifuged at 4000 g for 5 minutes at room temperature using a Sigma 2 K15 centrifuge. Afterwards supernatant was carefully removed and analysed for PEG content. As a washing step 500 µl of HBG were added onto the dextran cushion and removed subsequently. Then, the upper dextran layer was picked up with a spatula (~100 µl) and resolved in HBG to a final volume of 1 ml. The obtained polyplexes were analyzed via DLS, PEG, Alexa and DNA content were determined as described before.

2.14.6 Anion exchange chromatography

A 10/30 column was packed with DEAE Sepharose ^{TM} Fast Flow and equilibrated in 20 mM HEPES pH 7.4. Injected Alexa-PEGylated BPEI polyplexes (in 0.5 M NaCl) were eluted with a salt gradient starting at 30 minutes and reaching 0.5 molar salt at the 90 minute time point. Anion exchange chromatography was performed at a flow rate of 0.5 ml/min; fractions of 1 ml were collected and elution of DNA polyplexes was monitored at 260 nm. Afterwards fractions were analyzed via PEG assay additionally DNA and Alexa content were determined by UV measurement.

2.15 Fluorescence correlation spectroscopy (FCS)

FCS detects spontaneous intensity fluctuations resulting from fluorescently labelled molecules diffusing through a highly focused laser illuminated volume within a sample. The size of this volume is fixed by the confocal detection optics and the excitation profile of the focused laser beam and characterized by calibration

measurements against a standard of a known diffusion constant. The raw signal, the time dependent fluorescence intensity fluctuations, is time-autocorrelated to obtain dynamic information on the fluorescently labelled molecules. For Brownian diffusion of one identical species of particles, the autocorrelation function is given by:

$$G(\tau) = 1 + \frac{1}{N} \frac{1}{1+\frac{\tau}{\tau_D}} \frac{1}{\sqrt{1+\frac{\tau}{S^2 \tau_D}}} \quad (1)$$

With N representing the number of particles in the illuminated volume and $S = \frac{z_0}{r_{xy}}$ the structure parameter, the ratio of axial to radial dimensions of the focused laser beam which is a measure for the dimension of the focal volume. The diffusion time τ_D of a particle through the illuminated focal volume with radius r_{xy} is related to the translational diffusion coefficient *D* by:

$$\tau_D = \frac{r_{xy}^{\ 2}}{4D} \quad (2)$$

Additional intensity fluctuations can occur resulting from intramolecular processes like transitions to triplet states. If these processes are well separated in the time domain the autocorrelation function can be written as

$$G(\tau) = 1 + g_{triplett}(\tau) \times \frac{1}{N} \frac{1}{1+\frac{\tau}{\tau_D}} \frac{1}{\sqrt{1+\frac{\tau}{S^2 \tau_D}}} \quad (3)$$

with $g_{triplett}(\tau) = 1 + \frac{T_{triplett}}{1-T_{triplett}} \exp\left(-\frac{\tau}{\tau_{triplett}}\right)$

where $T_{triplett}$ denotes the triplett fraction and τ_{triplett} the characteristic triplett decay time.

A standard FCS setup from Carl Zeiss (Jena, Germany) was used including a ConfoCor2 module and an Axiovert200 microscope with a Zeiss C-Apochromat (40x, NA 1,2) water immersion objective. The 488 nm line of an Argon laser was utilized for fluorescent excitation. Emission light was split by a dichroic mirror and was collected by an Avalanche Photodiode (APD) with a 505-530 nm filter and a pinhole of 78 µm.

Purified BPEI polyplexes N/P = 6 were post-PEGylated with either the stable or the labile Alexa-PEG conjugate. To study the cleavage of PEG chains from polyplexes

by FCS, purified Alexa-PEGylated polyplexes were preincubated at pH 7.4 or 5.0 for 4 h at 37 °C in the dark, and FCS measurements were performed at room temperature as described in Meyer et al (71). Samples were pipetted directly into eight-well Lab-Tek chambers (Nalge Nunc International, Rochester, NY). Determination of the focal volume was established via calibration against an aqueous solution of 10 nM Alexa488-SH before each data acquisition. For each sample 10 measurements with sampling time of 30 s were performed. The measured autocorrelation curves were fitted (Zeiss ConfoCor2 software package) with equation (3) providing the diffusion time τ_D.

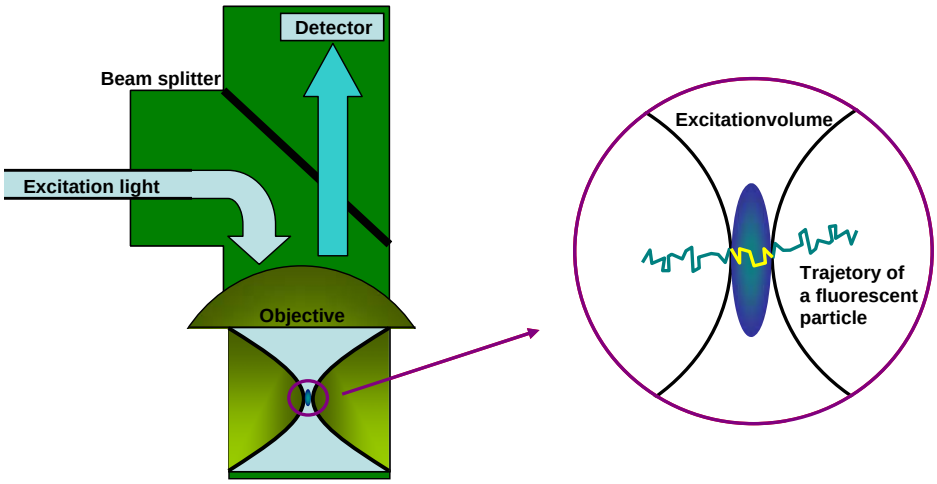


Figure 4) Schematic illustration of the instrumental set up of FCS experiments. Excitation light passes through the tiny sample volume where existing fluorescent molecules are excited and their emitted light is analysed by a detector.

2.16 Determination of PEGylation degree

Alexa-PEG-NHS 10 kDa was applied in a 30 molar excess over plain BPEI polymer. 1.4 nmol BPEI in 100 µl HBG were reacted with 42 nmol Alexa-SS-PEG-NHS in 50 µl DMF. After 1 h the volume was filled up to 1 ml with salt to reach a final concentration of 1.5 M NaCl.

When Alexa-SS-PEG-NHS was coupled to BPEI polyplexes, these particles were purified in advance as described under 2.14.1. Purified polyplexes containing 1.6 nmol BPEI were post-PEGylated adjacent with 48 nmoles of dye labelled PEG. After 1 h PEGylation time NaCl was added to the sample batch to reach a final

concentration of 1.5 M salt. Dissociation of polyplexes was followed via size measurements. Both Alexa-PEGylated polymer and Alexa-PEGylated polymer originally bound in a DNA complex were applied onto a cation exchange chromatography column (see 2.21). Eluted fractions were analyzed for BPEI by TNBS assay, for Alexa by VIS absorbance, for DNA by UV absorbance (both at Genesys 10-S spectrophotometer, Thermo Spectronic, Rochester, USA) and for PEG via PEG assay. The ratio dye to PEG of the applied Alexa-PEG conjugate was determined in advance as described in 2.13.

2.17 PEG assay

PEG was determined via complex formation with Ba^{2+} and iodine, which can be measured at 590 nm. Barium chloride solution was prepared by dissolving $BaCl_2$ in 0.1 M HCl to form a 5% (w/v) solution. Iodine solution was prepared by dissolving 1.27 g iodine in 100 ml water containing 2 g of potassium iodide (72). The reaction is dependent on the concentration of the solutions. First a red charge transfer complex arises with triiodide ions that turns gradually into a nearly water insoluble iodine complex. Performing this assay with a PEG stock solution from 0 to 75 µg/ml (same buffer as samples) reveals a standard curve that allows for comparison with the unknown samples. Absorbance at 590 nm was measured using a microplate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria).

2.18 TNBS assay

Concentration of reagents containing primary amines was measured by trinitrobenzenesulfonic acid (TNBS) assay as described in (73).

Standard amine solutions with a known amount of reagent and the test solutions containing unknown amounts of the primary amine derivative were serially diluted in duplicates with 0.1 M sodium tetraborate to give a final volume of 100 µl in a 96 well plate. Concentrations of approximately 17.5 to 210 nmol/ml amine reagent were achieved and then 2.5 µl of TNBS (75 nmol) in water were added to each well. After 5-20 minutes incubation time at room temperature (depending on the strength of the developed colour) the absorption was measured at 405 nm using a microplate reader.

2.19 DTT cleavage assay

To determine the efficiency of the reaction between OPSS-PEG-(HZN)-NHS and Alexa-SH in DMF a DTT cleavage assay was employed. By implementation with disulfide bond containing pyridyldithio molecules, DTT provokes the release of 2-pyridinethione. Measuring this chromophore at 343 nm the process of the reaction can be determined quantitatively. In this present experiment the absorbance of the dye molecule at the indicated wavelength has to be taken into account before the data can be calculated correctly. 100 µl of a 25 µmol/ml DTT solution in water served as blank. To this, 40 µl of sample were pipetted, mixed thoroughly and subsequently values were read off when no more increase in absorbance was detectable. Calculation via Lambert Beer with ε = 8080 $l*mol^{-1}*cm^{-1}$.

2.20 Ellman´s assay

This assay is used to quantitatively determine the amount of free thiol groups in chemical samples. 0.4 mg Ellman´s reagent 5,5´-Dithio-bis(2-nitro-benzoic acid) (DTNB) dissolved in 1 ml of the corresponding Ellman´s buffer (0.2 M Na_2HPO_4 with 1 mM EDTA at pH 8.0) were used as stock solution. For VIS measurement Ellman´s stock diluted 1:10 in Ellman´s buffer was taken as blank. Samples were mixed with buffer and 10% (v/v) of the stock solution. After 20 minutes the solutions were measured at 412 nm and concentration of –SH group calculated via Beer Lambert's law with ε = 13600 $^{-1}*cm^{-1}$.

2.21 Cation exchange chromatography

Cation exchange chromatography was performed on a HR 10/10 column (BioRad Munich, Germany) packed with Macro-prep High S.

For the experiment described in 2.5.2 BPEI-PEG conjugates were applied to the cation-exchange column, which was equilibrated in 20 mM HEPES buffer pH 7.4 with 0.5 M NaCl. For analysis of the samples the following program was applied: after 5 minutes, salt concentration was increased with a linear gradient from 0.5 M to 3 M NaCl at 15 min where it maintained isocratic for another 15 minutes. Flow rate was 1 ml/min.

For the experiment described in 2.16 the set up of the program was slightly different. The cation-exchange column was also equilibrated in 20 mM HEPES buffer pH 7.4 with 0.5 M NaCl but the final run was with 0.5 ml/min flow rate and a gradient starting from 10 minutes reaching 3 M salt at 30 minutes, remaining there until 65 minutes.

2.22 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 statistical software package, WinSTAT® 2003 for Excel (R. Fitch Software) was used.

3 RESULTS

3.1 Monofunctional PEG reagents

3.1.1 Synthesis of the post-PEGylation reagent N-hydroxysuccinimidyl-hydrazone poly(ethylene) glycol (NHS-HZN-PEG)

N-hydroxylsuccinimidyl-hydrazone-PEG (NHS-HZN-PEG) was synthesized via conjugation of the hydrazine residues of succinimidyl 6-hydraziniumnicotinate (SHNH) to the ω-aldehyde group of a 2 or 20 kDa PEG (Figure 5(A)). The reaction was performed water free in DMF under argon to minimise hydrolysis of the active ester. Since SHNH was applied in a 4 fold molar excess over PEG-aldehyde, unreacted compounds were removed afterwards. For this purpose a solid-phase approach was used by immobilization of non-reacted SHNH onto benzyloxybenzaldehyde (polystyrene-CHO) beads. After 4 h incubation the beads were centrifuged by a quick spin and the reagent obtained in the supernatant was tested on completeness of SHNH removal by TNBS assay for detection of free, primary amino groups. No more SHNH was detectable after incubation with the solid phase approach. As control for direct comparison the stable PEG reagent mPEG-SPA (methoxypoly(ethyleneglycol) succinimdyl propionic acid Figure 5(B)) was applied next to the pH sensitive reagent in biophysical and biological characterisation experiments.

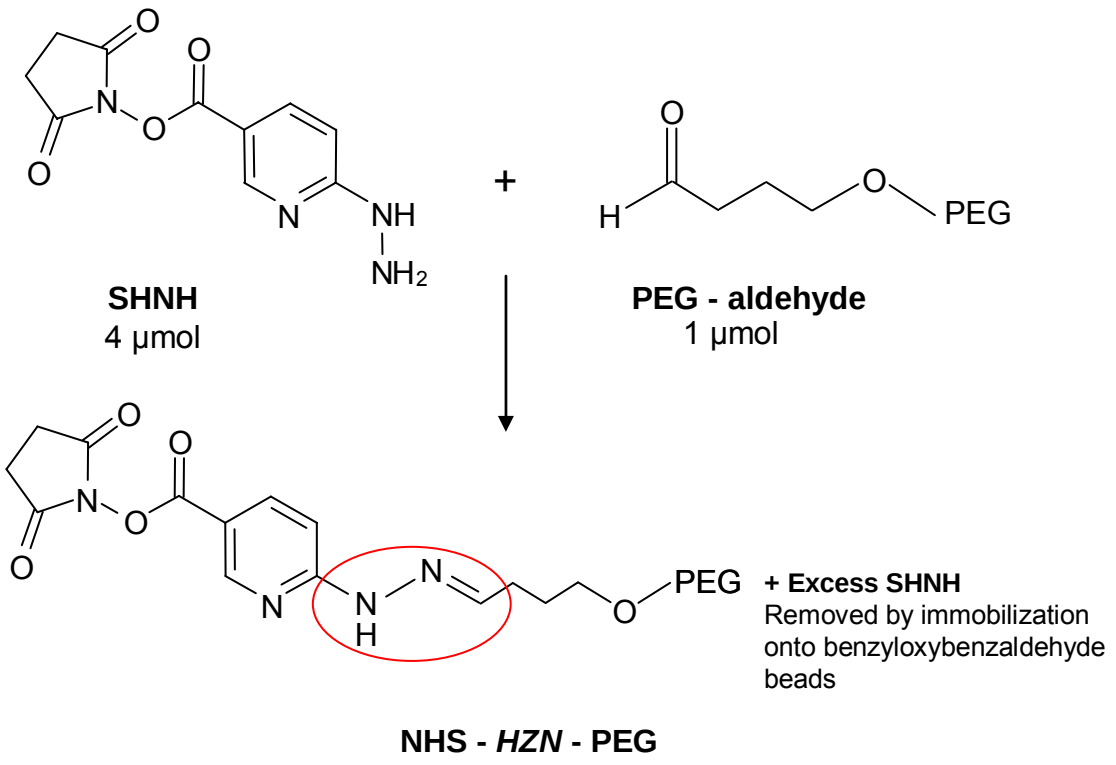


Figure 5 (A) Schematic illustration of the synthesis of monofunctional post- PEGylation reagent NHS-HZN-PEG (2 or 20 kDa). 4 µmol of SHNH were reacted with 1 µmol of PEG aldehyde in anhydrous DMF. Excess SHNH was removed by incubation with polystyrene –CHO beads.

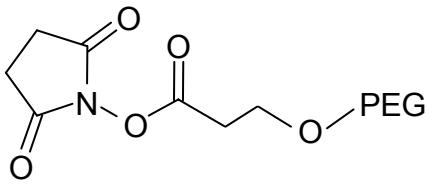


Figure 5 (B) Stable control mPEG-SPA (2 or 20 kDa)

3.1.1.1 Reaction efficiency between the aldehyde and SHNH

The efficiency of the reaction between PEG-aldehyde and SHNH was determined by the decrease of NH_2-groups of SHNH that was detected by reaction with TNBS and measurement of the absorbance at 405 nm after 5 minutes. The reaction was found to proceed very fast as measurement right after compound mixing (0), already showed a pronounced decrease in SHNH amino groups. When SHNH was used in a 4 fold molar excess over aldehyde 75% of the originally applied SHNH remained. When PEG-CHO was used in a 4 fold molar excess over SHNH, no more SHNH was

detectable after 2 h. Both reactions showed a reaction efficiency of 100% and completeness of reaction after 2 h.

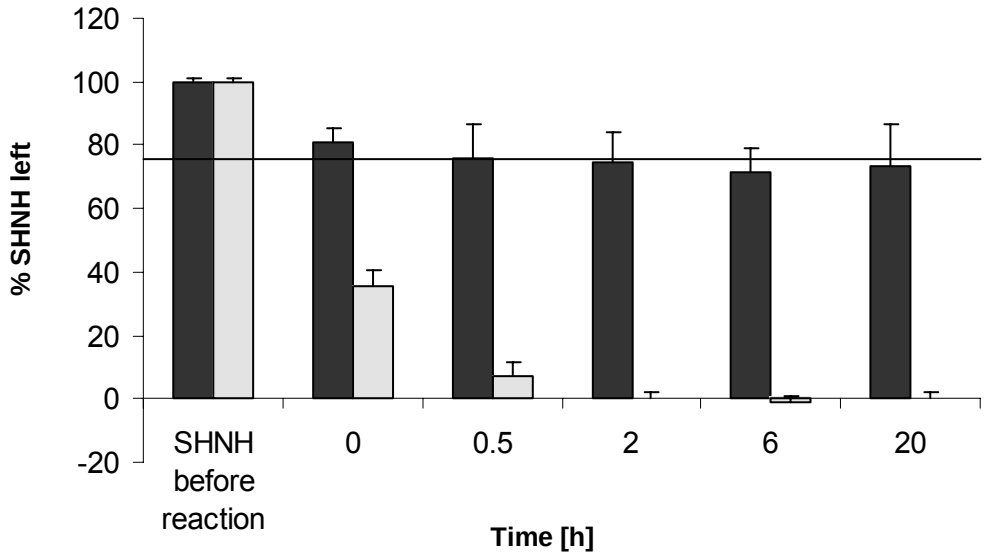


Figure 6) Estimation of reaction time between the hydrazine group of SHNH and the PEG aldehyde group. SHNH used in 4 fold molar excess over PEG aldehyde (black bars) or PEG used in 4 molar excess over SHNH (dotted bars). Reaction was carried out in DMF. Horizontal line at 75%.

3.1.1.2 Removal of excess SHNH

No TNBS-reactive SHNH was detectable for the 50 fold excess of bead aldehyde groups over SHNH already after 2 h incubation at room temperature with the solid phase approach. For reagent synthesis a 100 fold excess and 4 h incubation time at room temperature were chosen. In an earlier attempt formyl-polystyrene beads were shrink-wrapped into polypropylene foil that served as a semi-permeable membrane and were added to the reaction mixture in order to remove the excess of SHNH. This so called “teabag method” did not allow for sufficient removal of excess SHNH even after over night incubation.

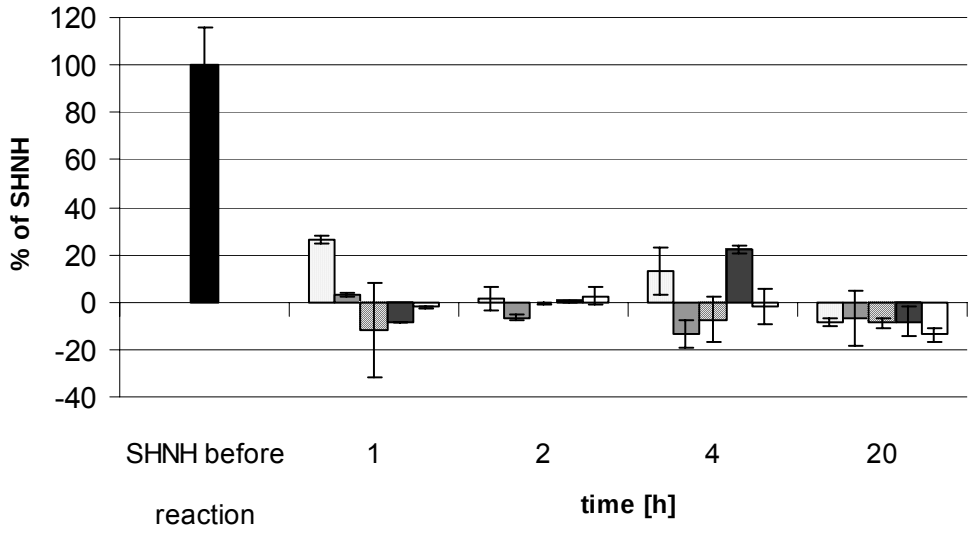


Figure 7) Removal of excess SHNH. DMF containing SHNH was incubated with different amounts of –CHO bearing polystyrene beads in water free DMF. With 10 (black dots), 50 (grey), 100 (stripes), 150 (white dots) and 200 (white) fold excess of aldehyde groups over SHNH.

3.1.1.3 Ester reactivity

To check whether NHS-HZN-PEG owns similar ester reactivity towards amines as its stable counterpart NHS-PEG, both reagents were reacted with defined amounts of ethanolamine. The remaining amount of primary amines was determined by TNBS assay. With an ester reactivity in the PEG reagent of 100% almost no ethanolamine would be detectable after the reaction. As approximately 20% of ethanolamine remained unreacted both reagents exhibited an ester reactivity of > 80% and thus a comparable reactivity towards amines.

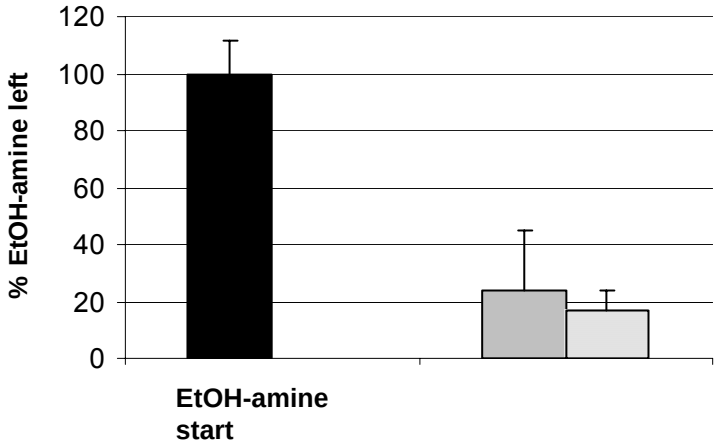


Figure 8) Determination of ester reactivity of NHS-PEG ▢ and NHS-HZN-PEG ▨ after implementation with ethanolamine. PEG reagents were used in equimolar amounts and remaining ethanolamine was determined via TNBS assay and measurement at 405 nm.

3.1.2 Synthesis of the pre-PEGylation reagent BPEI-HZN-PEG

For direct comparison of pre- and post-PEGylation analogues, control conjugates were synthesized. PEG-PEI shielding conjugates were generated by modifying amino groups of BPEI with *N*-hydroxysuccinimide ester derivatives of PEG 20 kDa (NHS-PEG and NHS-HZN-PEG). Conjugates were purified by cation exchange chromatography and stability of the PEG modified BPEI was tested by incubation and re-application of the PEI-PEG conjugates onto the column. This experiment clearly pointed out that it is possible to form a BPEI-PEG and additionally a BPEI-HZN-PEG conjugate. However, following stability analysis revealed that the labile BPEI-PEG conjugate is only temporarily stable as it broke down already after thawing. In contrast the stable BPEI-PEG conjugate stayed intact also after an incubation time of 4 h at 37 °C. Among these conditions it was not possible to form the corresponding pH sensitive pre-PEGylation conjugate. Thus, the post-PEGylation reagent could not be directly compared to its pre-PEGylation analogue. Figure 9 shows the elution profiles from the cation exchange chromatography. The first peak in figure 9 -1 A) and B) represents unbound PEG. Stable and labile BPEI-PEG conjugates show similar profiles initially. Reinjection of the BPEI-PEG fractions after 4 hours incubation at 37 °C gave a single BPEI-PEG peak for the stable conjugate and 2 peaks for the pH-sensitive one, indicating hydrolysis of the latter into a PEG and a BPEI-PEG peak. Figure 10 depicts the remaining amount of intact PEG-(HZN)-BPEI after different incubation times. Whereas the BPEI-PEG conjugate remains stable over the whole period, the conjugate containing the pH-sensitive hydrazone bond became degraded already after thawing.

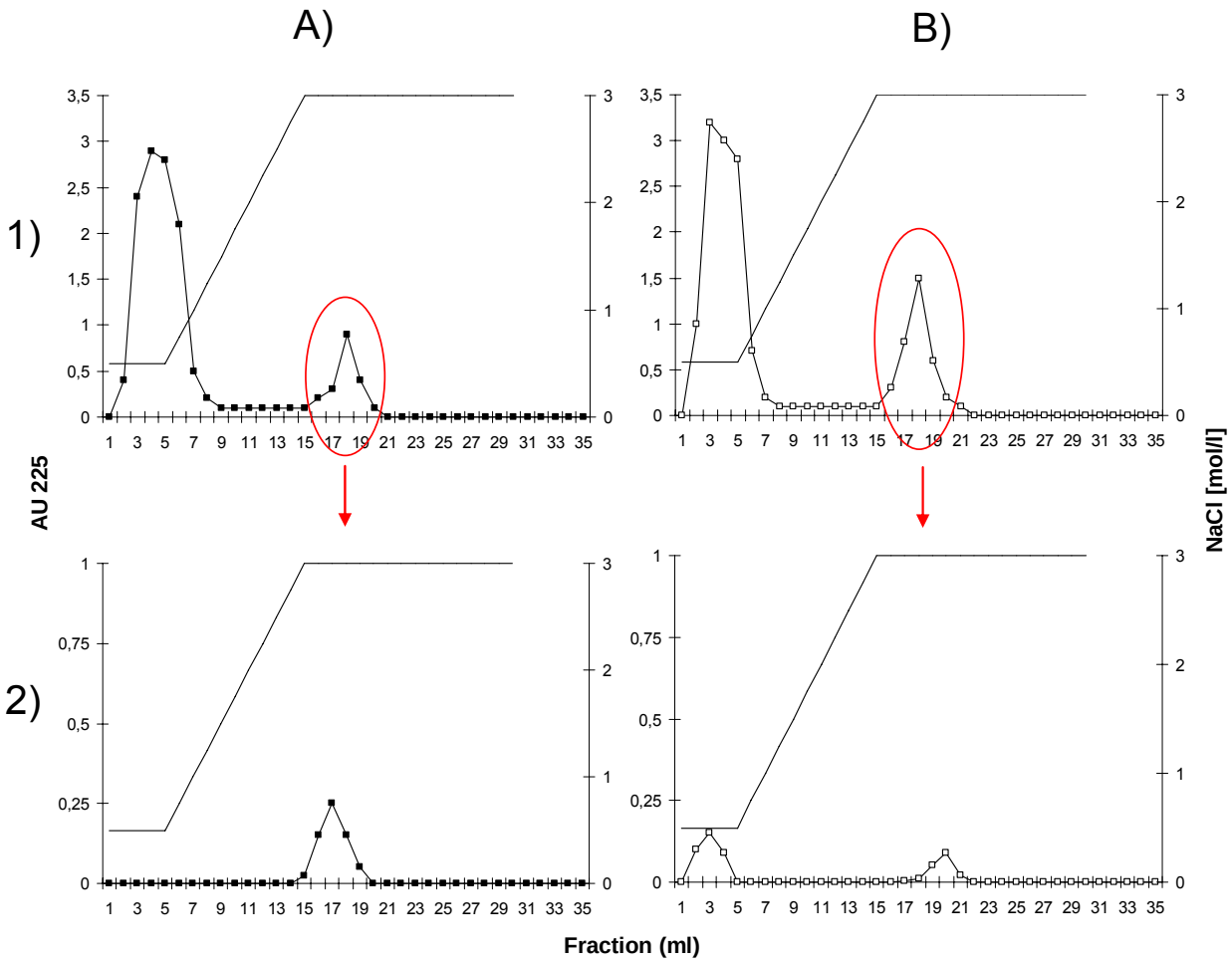


Figure 9) Elutionprofile from cation exchange chromatography (Macro-Prep High S). 20 mM HEPES/0.5 M NaCl with linear salt gradient starting at 5 minutes and reaching 3 M salt after 15 minutes. A) PEG-BPEI and B) PEG-HZN-BPEI. 1) Analysis direct after synthesis and 2) after 4 h incubation at 37 °C. PEG positive fractions were determined by PEG assay and those containing BPEI by TNBS assay.

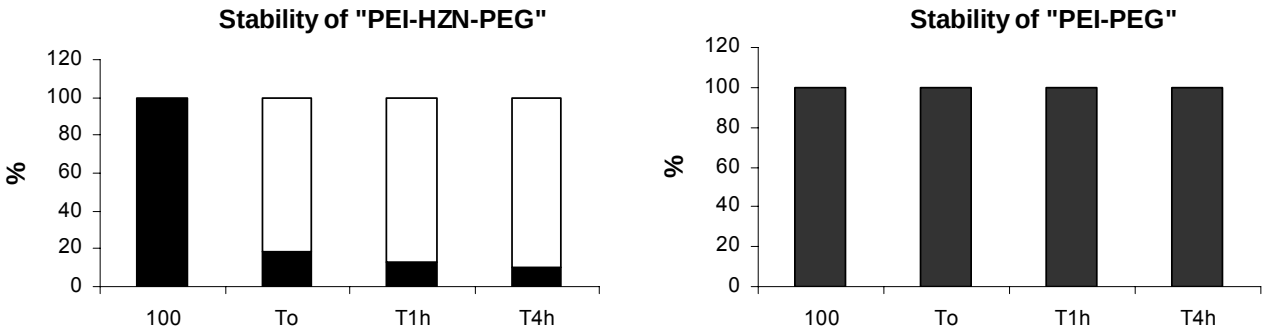


Figure 10) Remaining amount of intact conjugate in percent ■ after thawing and after incubation at 37 °C over different time periods.

3.1.3 Biophysical characterisation of NHS-(HZN)-PEG shielded polyplexes

3.1.3.1 Shielding of polyplexes by post-PEGylation with monofunctional PEG reagents

Figures 11 A) and B) show the titration curves for determination of the amount of post-PEGylation reagent necessary to achieve shielded polyplexes with a close to neutral surface charge. Therefore different polyplexes (LPEI/BPEI, +/- EGF) were mixed with increasing amounts of the activated ester PEG reagents NHS-HZN-PEG and NHS-PEG of 2 and 20 kDa. PEG content of the BPEI-PEG-EGF conjugate led to a partial shielding effect with the starting zeta potential of such particles being lower than that of untargeted polyplexes. For BPEI particles a molar excess of PEG molecules over PEI molecules of approximately 30 fold generated shielded particles with a zeta potential below 5 mV. Shielding of LPEI particles was performed in low salt containing buffer (HBG) to minimise salt induced aggregation. Here, results differed to those obtained with BPEI polyplexes, since a much higher molar ratio of 150 fold PEG was necessary. Shielding with the 2 kDa version of the PEG reagents did also reduce zeta potential. However, with the applied PEG excess it was not possible to achieve particles with a zeta potential below 5 mV. Due to these findings, 20 kDa PEG reagents were used in following *in vitro* and *in vivo* experiments. Only a small difference was observed in the molar excess required for shielding by either the stable or the reversible PEG reagent. This demonstrates that the activated ester of the synthesized pH-labile mediated PEG reagent has a similar activity towards amines as the commercially available stable NHS-PEG.

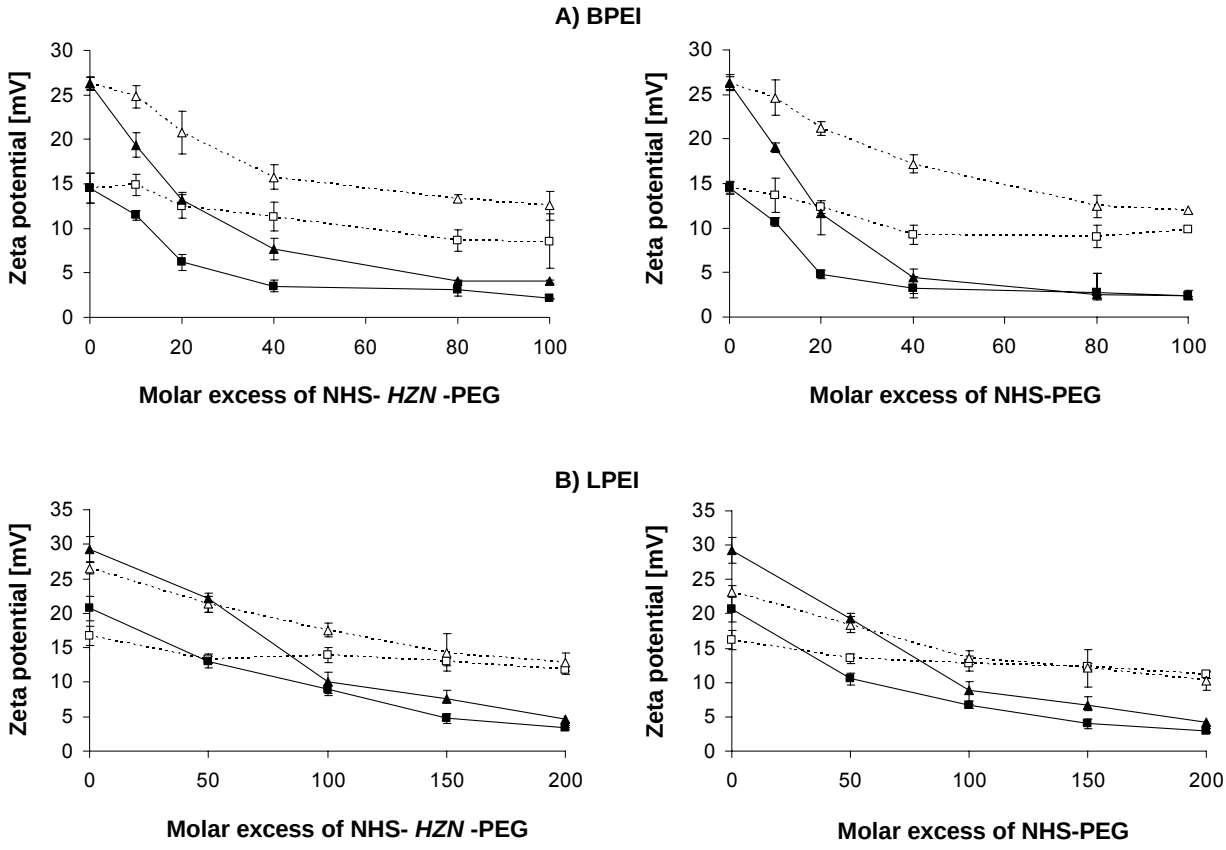


Figure 11 A) BPEI polyplexes, N/P = 6 prepared in HBS 0.5x and B) LPEI polyplexes prepared in HBG were post-PEGylated with increasing amounts of indicated PEG-reagents and corresponding zeta potential was measured. Dotted lines 2 kDa, black lines 20 kDa PEG, triangles – EGF (without BPEI-PEG-EGF), squares + EGF (with BPEI-PEG-EGF)

Further on zeta potential and size measurements of indicated polyplexes in different buffers were performed to find the formulations best suitable for shielding experiments. Table 1) summarises the data achieved from linear or branched PEI polyplexes, with or without targeting ligand and the required amount of shielding reagent either in HBS 0.5x or in HBG. LPEI polyplexes started to aggregate in salt-containing buffer whereas BPEI polyplexes only showed a slight increase in size under these conditions. LPEI particles can be stabilized by PEGylation and thus the aggregation process can be stopped. As already mentioned, the zeta potential of formulations containing the targeting conjugate is significantly reduced compared to formulations without such modification. After PEGylation with indicated amounts of shielding polymer zeta potential is reduced to values near neutral. For *in vitro* and *in vivo* experiments BPEI polyplexes were prepared in HBS 0.5x whereas LPEI polyplexes were prepared in HBG to minimize salt induced aggregation.

	A) HBG		B) HBS 0,5x	
	size [nm]	zeta [mV]	size [nm]	zeta [mV]
LPEI 100%	**112** +/- 4	**23** +/- 1	> 1 µm	**24** +/- 1
LPEI 90%				
BPEI-PEG-EGF 10%	**131** +/- 4	**15** +/- 2	> 1 µm	**14** +/- 2
+150x PEG stable	**247** +/- 23	**4** +/- 1	**332** +/- 20	**4** +/- 1
+150x PEG labile	**204** +/- 7	**3** +/- 1	**321** +/- 18	**3** +/- 2
BPEI 100%	**94** +/- 2	**27** +/- 1	**121** +/- 6	**26** +/- 2
BPEI 90%				
BPEI-PEG-EGF 10%	**106** +/- 4	**21** +/- 1	**128** +/- 15	**20** +/- 2
+30x PEG stable	**188** +/- 3	**3** +/- 2	**197** +/- 11	**3** +/- 2
+30x PEG labile	**191** +/- 11	**3** +/- 1	**213** +/- 27	**2** +/- 0

Table 1) Size and zeta potential of indicated polyplexes prepared in different buffers. Values of at least triplicates +/- standard deviation are shown. For PEGylated formulations size and corresponding zeta potential were measured after 1 h PEGylation time. A) polyplexes prepared and measured in HBG and B) in HBS 0.5x (only PEGylated LPEI polyplexes were prepared in HBG before being transferred into HBS 0.5x)

3.1.3.2 Deshielding of NHS-HZN-PEG shielded particles at pH 5.0

Stability of PEG shielded polyplexes was determined by zeta potential with (12 A) or without (12 B) targeting ligand or by size measurements (12 C) over time in salt containing buffer at pH 7.4 or 5.0. NHS-HZN-PEG shielded BPEI polyplexes incubated at pH 5.0 exhibited an increase in zeta potential with time whereas at pH 7.4 no significant change could be observed. The increase in zeta potential indicated removal of the PEG shield and re-exposure of the positively charged particle surface. As the partial shielding effect of the stable incorporated targeting conjugate remained, zeta potential increased to approximately 13 mV compared to ~ 30 mV for non-targeted polyplexes. In contrast, the zeta potential of NHS-PEG shielded polyplexes did not change after 4 h of incubation neither at pH 5.0 nor at pH 7.4.

Targeted polyplexes based on BPEI remained stable in size (~ 200 nm) under both conditions. In contrast, LPEI polyplexes shielded with NHS-HZN-PEG underwent particle aggregation in the presence of salt (particle size > 1 µm) upon de-shielding at pH 5.0, whereas at pH 7.4 no aggregation was observed for at least 4 h.

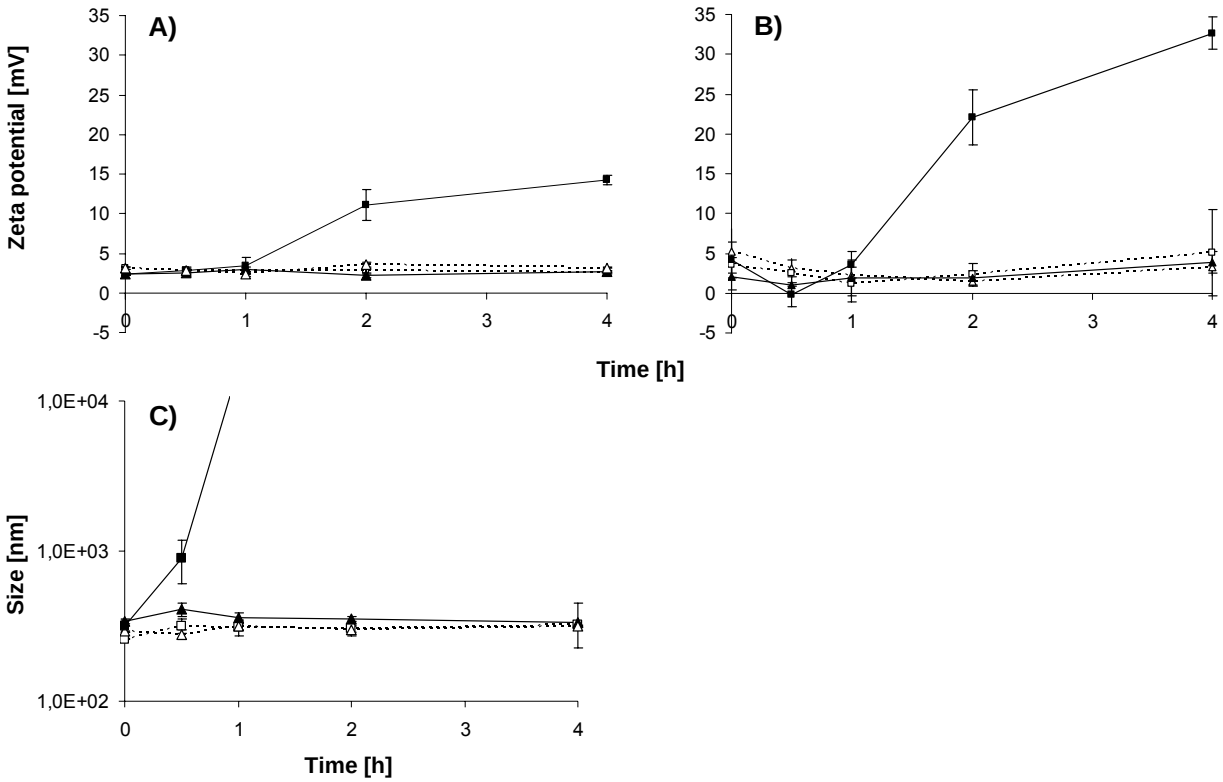


Figure 12 A) Behaviour of BPEI particles with targeting ligand BPEI-PEG-EGF stable or labile PEGylated with a 30 fold molar excess of NHS-HZN-PEG ■ □ or NHS-PEG ▲△ over BPEI at pH 5.0 (black lines) and 7.4 (dotted lines). B) Same as in A) but without targeting conjugate BPEI-PEG-EGF. Deshielding in acidic environment (pH 5.0) represented by the increase of zeta potential can only be seen for NHS-HZN-PEG shielded particles ■. C) Size over time of targeted LPEI polyplexes shielded with a molar excess of NHS-HZN-PEG and NHS-PEG of 150 fold over LPEI. Deshielding in acidic environment (pH 5.0) is represented by the increase of size only detected for NHS-HZN-PEG shielded polyplexes. (Size increase and aggregation are independent of BPEI-PEG-EGF presence).

A summary of the most important differences in appearance of LPEI and BPEI particles in terms of size and zeta potential in neutral or acidic environments is listed in Table 2). LPEI based polyplexes are slightly bigger as their BPEI based counterparts and change their size after deshielding in acidic surrounding due to aggregation, whereas BPEI polyplexes keep a constant size of approximately 200 nm. Monitoring the zeta potential of LPEI based polyplexes results in values with high standard deviation due to the starting aggregation of the deshielded particles in the salt containing buffer. BPEI polyplexes showed increasing zeta potentials up to the value of non-shielded targeted particles of approximately 14 mV and 22 mV for non-targeted particles, respectively.

	A) pH 7.4				B) pH 5.0	
	T_0		T_{2h}		T_{2h}	
	size [nm]	zeta [mV]	size [nm]	zeta [mV]	size [nm]	zeta [mV]
LPEI 90%						
BPEI-PEG-EGF 10%						
+150x PEG stable	**318** +/- 13	**3** +/- 2	**341** +/- 32	**3** +/- 2	**361** +/- 17	**4** +/- 1
+150x PEG labile	**309** +/- 23	**4** +/- 1	**327** +/- 17	**3** +/- 2	**< 1**µm	**7** +/- 5
BPEI 100%						
+30x PEG stable	**180** +/- 28	**5** +/- 3	**172** +/- 16	**2** +/- 1	**182** +/- 18	**2** +/- 1
+30x PEG labile	**180** +/- 23	**4** +/- 1	**183** +/- 19	**2** +/- 2	**169** +/- 14	**22** +/- 4
BPEI 90%						
BPEI-PEG-EGF 10%						
+30x PEG stable	**197** +/- 22	**5** +/- 2	**206** +/- 38	**4** +/- 1	**213** +/- 21	**3** +/- 1
+30x PEG labile	**202** +/- 6	**4** +/- 1	**205** +/- 24	**3** +/- 0	**211** +/- 21	**14** +/- 2

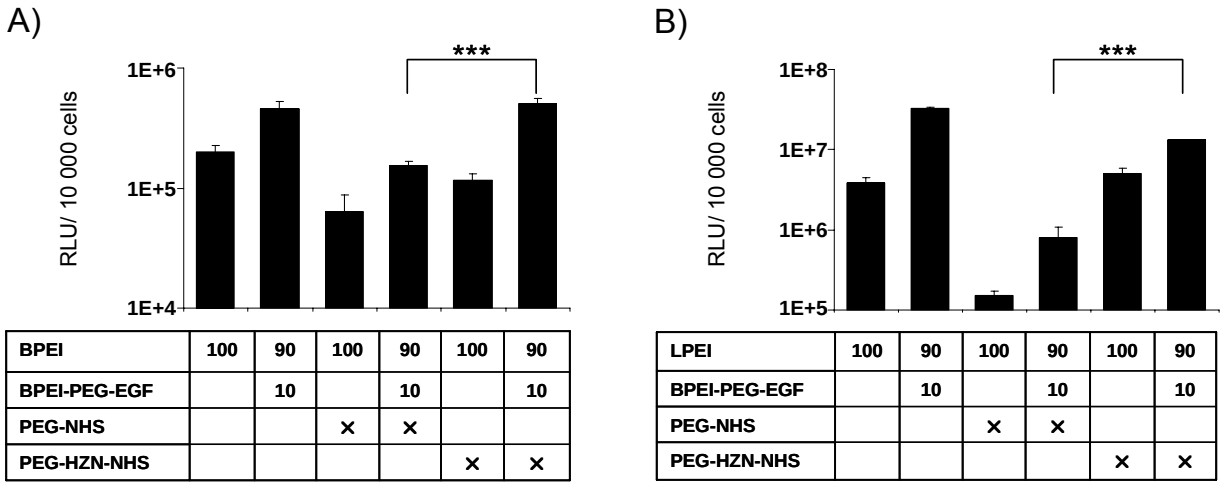
Table 2) Size and corresponding zeta potential of stable or labile PEGylated polyplexes directly after 1 h PEGylation time and after 2 h incubation at 37 °C either in HBS 0.5x pH 7.4 or in NaOAc buffer at pH 5.0.

3.1.4 Biological characterisation of NHS-(HZN)-PEG shielded polyplexes

3.1.4.1 In vitro transfection

After biophysical characterisation, the influence of the pH-sensitive PEG on levels of gene transfer efficiency *in vitro* was examined. pCMV-Luc plasmid containing polyplexes either based on BPEI (A) or LPEI (B) were used for transfection studies on HuH7 human hepatocellular (Figure 13) and Renca-EGFR mouse renal carcinoma cells (Figure 14). Both cell lines are known to overexpress the EGF receptor. After 1 h PEGylation was quenched with gycine and the formulations were applied to the cells in medium containing 10% FCS. After 4 h of incubation at 37 °C, transfection medium was replaced by fresh medium. BPEI and LPEI based particles showed similar transfection profiles whereas for LPEI values were considerably higher. For targeted polyplexes, the reversible shielded polyplexes showed a significantly higher gene transfer efficiency of 5- to 40 fold compared to the non-reversible shielded polyplexes thus showing the advantage of the reversible acid-triggered de-shielding. Standard non-reversible PEG shielding led to a decrease in gene transfer that has previously been shown for many of such formulations.

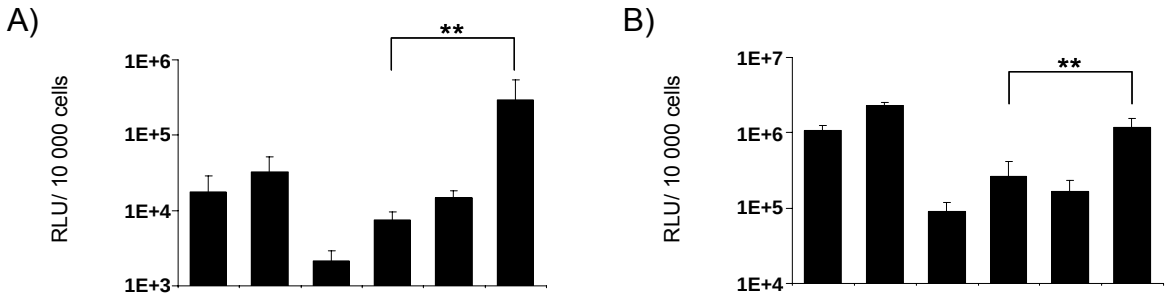
Comparing the targeting effects of PEGylated particles in combination with the acid triggered deshielding a synergistic effect can be observed.



BPEI	100	90	100	90	100	90
BPEI-PEG-EGF		10		10		10
PEG-NHS			×	×		
PEG-HZN-NHS					×	×

LPEI	100	90	100	90	100	90
BPEI-PEG-EGF		10		10		10
PEG-NHS			×	×		
PEG-HZN-NHS					×	×

Figure 13 A) In vitro transfection efficiency in HuH7 cells of EGF targeted and non-targeted polyplexes post-PEGylated with 20 kDa stable or acid labile shielding reagent (30 fold molar excess of PEG over BPEI and 150 fold over LPEI) or without shielding reagent. Each data point represents the mean +/- standard deviation (n=3). Complexes were prepared at a DNA concentration of 20 µg/ml, N/P ratio 6. Luciferase expression of A) BPEI polyplexes mixed in HBS 0.5x and (B) linear LPEI core particles prepared in HBG.



BPEI	100	90	100	90	100	90
BPEI-PEG-EGF		10		10		10
PEG-NHS			×	×		
PEG-HZN-NHS					×	×

LPEI	100	90	100	90	100	90
BPEI-PEG-EGF		10		10		10
PEG-NHS			×	×		
PEG-HZN-NHS					×	×

Figure 14 B) In vitro transfection efficiency in Renca-EGFR cells of EGF targeted and non-targeted polyplexes post-PEGylated with 20 kDa stable or acid labile shielding reagent (30 fold molar excess of PEG over BPEI and 150 fold over LPEI) or without shielding reagent. Each data point represents the mean +/- standard deviation (n=3). Complexes were prepared at a DNA concentration of 20 µg/ml, N/P ratio 6. Luciferase expression of A) BPEI polyplexes mixed in HBS 0.5x and (B) LPEI core particles prepared in HBG.

3.1.4.2 In vitro cytotoxicity studies

In order to exclude any toxic effects of employed agents, the metabolic activity of treated cells as a measure of cell viability was determined by MTT assay. As incorporation of PEG is known to reduce the cytotoxicity of PEI formulations these MTT assays concentrated on additionally utilized agents. Whilst the 4 h incubation time in transfection approaches cells are in direct contact with the used substances and therefore the assay was performed in parallel and analogue to transfection experiments. Figure 15 pictures cell viability of two standard transfection experiments. In A) transfection was performed with BPEI as basic polymer and in B) with LPEI. The patterned bars show indicated formulations in presence of glycine which was added as a quencher of PEGylation. Presence of glycine slightly altered cell viability with big standard deviations in all cases. MTT assay on cells treated with DMF (Figure 15 C) showed that higher amounts of DMF lead to a significant decrease of cell viability. For transfection experiments the amount of applied DMF did not exceed 1 µl and therefore was not influencing the cell viability to a significant degree.

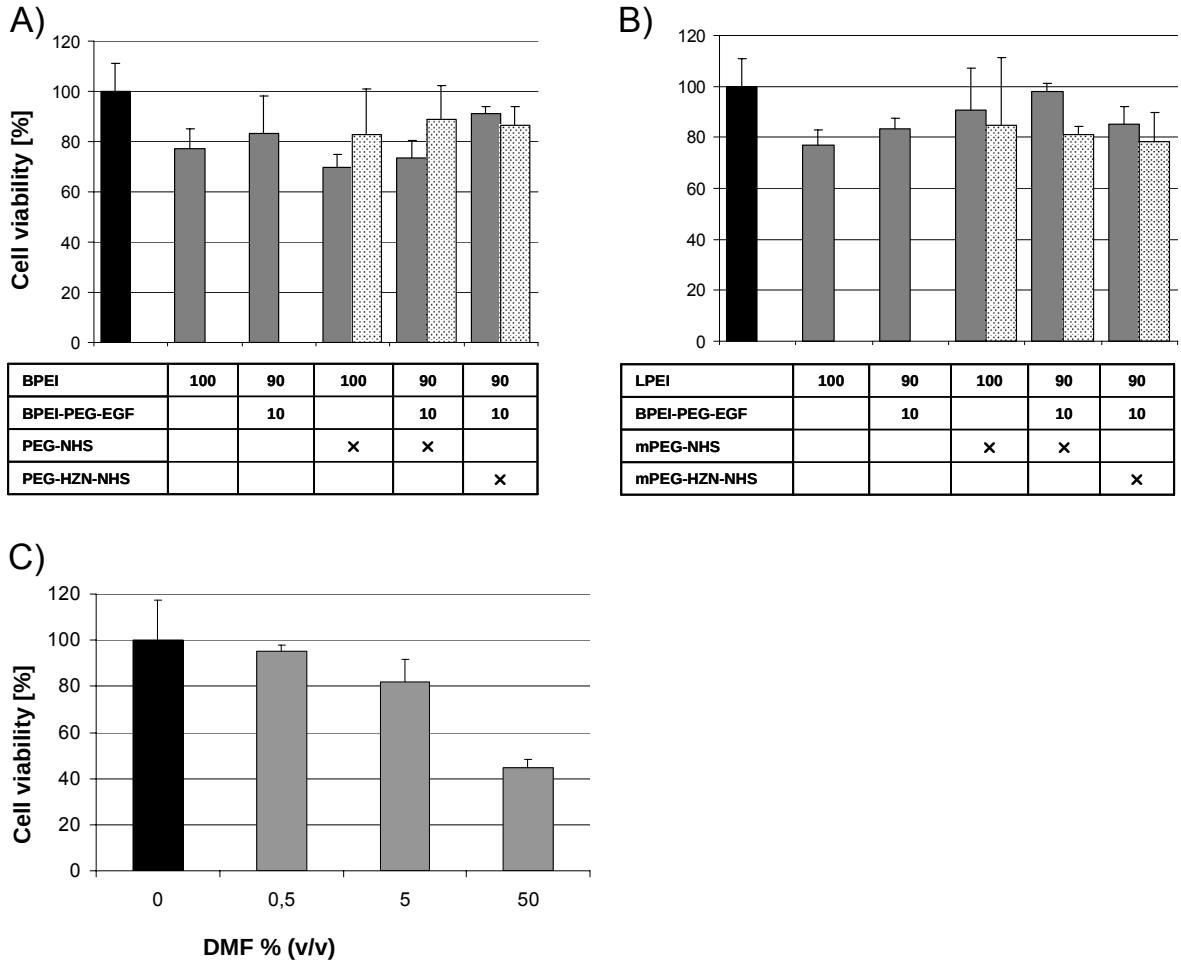


Figure 15 A) and B) MTT assay of a transfection of HuH7 cells with indicated polyplex formulations containing 200 ng pCMV Luc DNA per 10000 cells and a molar excess of PEG over BPEI of 30 respectively 40 (- EGF) and 150 respectively 200 (- EGF) over LPEI. Black bars represent untreated control cells and patterned bars the indicated formulation in presence of glycine. C) Cell viability after 4 h incubation with indicated amounts of DMF. Black bar represents untreated control cells. All assays were performed 24 h after treatment.

3.1.4.3 *In vivo* transfection

As the bioreversible shielded particles were successful *in vitro*, they were tested in an *in vivo* model of mice bearing a subcutaneous HuH7 tumour. Therefore, acid labile- and stable shielded EGF-targeted LPEI and BPEI polyplexes were injected into the tail vein of tumour bearing SCID mice. Figure 16 shows that the bioreversible shielded polyplexes resulted in a more than 14 fold higher luciferase gene expression for LPEI (2.5 fold for BPEI) polyplexes compared to the stable shielded particles with a predominant gene expression found in the subcutaneous HuH7 tumour. All other organs showed weak luciferase expression with slightly higher values in lung and liver.

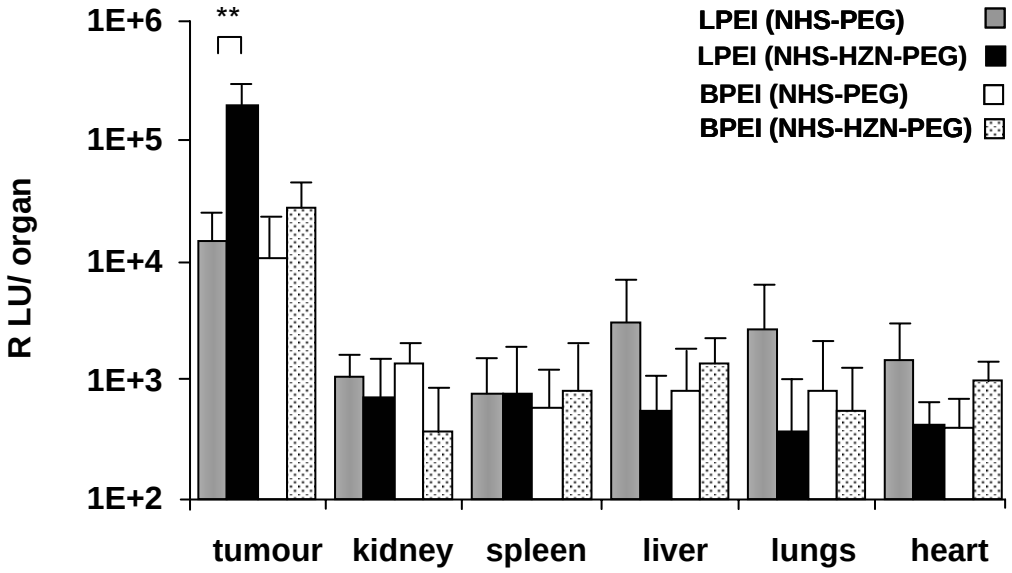


Figure 16) Luciferase expression of in vivo gene transfer into SCID mice bearing subcutaneous HuH7 hepatocellular carcinoma. All polyplexes contained 10% BPEI-PEG-EGF as targeting ligand. Polyplexes shielded either with stable NHS-PEG or labile NHS-HZN-PEG as shielding reagent (30 fold over BPEI, 150 fold over LPEI). LPEI polyplexes prepared in HBG and BPEI in HBS 0.5x.

3.2 Bifunctional PEG reagents

3.2.1 Synthesis of the post-PEGylation reagent N-hydroxysuccinimidyl-hydrazone poly (ethylene) glycol pyridyldisulfide (NHS-HZN-PEG-OPSS) 10 kDa

This bifunctional and pH-sensitive reagent enabled further modifications at the ω-end of the PEG chain. Beside an activated ester for amine binding at one end of the PEG chain (α) the new reagent contained a pyridyldisulfide as the second reactive group at the other end (ω).

First the disulfide content of the commercially available OPSS-PEG-NHS was determined by DTT cleavage assay. The percentage of arisen pyridine-2-thione was found to be 77 +/- 7% indicating that the content of disulfide bonds in the original PEG reagent was significant different from 100%. The conjugate was generated in anhydrous DMF by first reacting the amino group of 1-amino-3,3-diethoxypropane (ADEP) with the activated ester of bifunctional PEG-reagent OPSS-PEG-NHS to form a stable amide bond. The following figure 17 illustrates the individual reaction steps towards the pH-labile OPSS-PEG-HZN-NHS reagent.

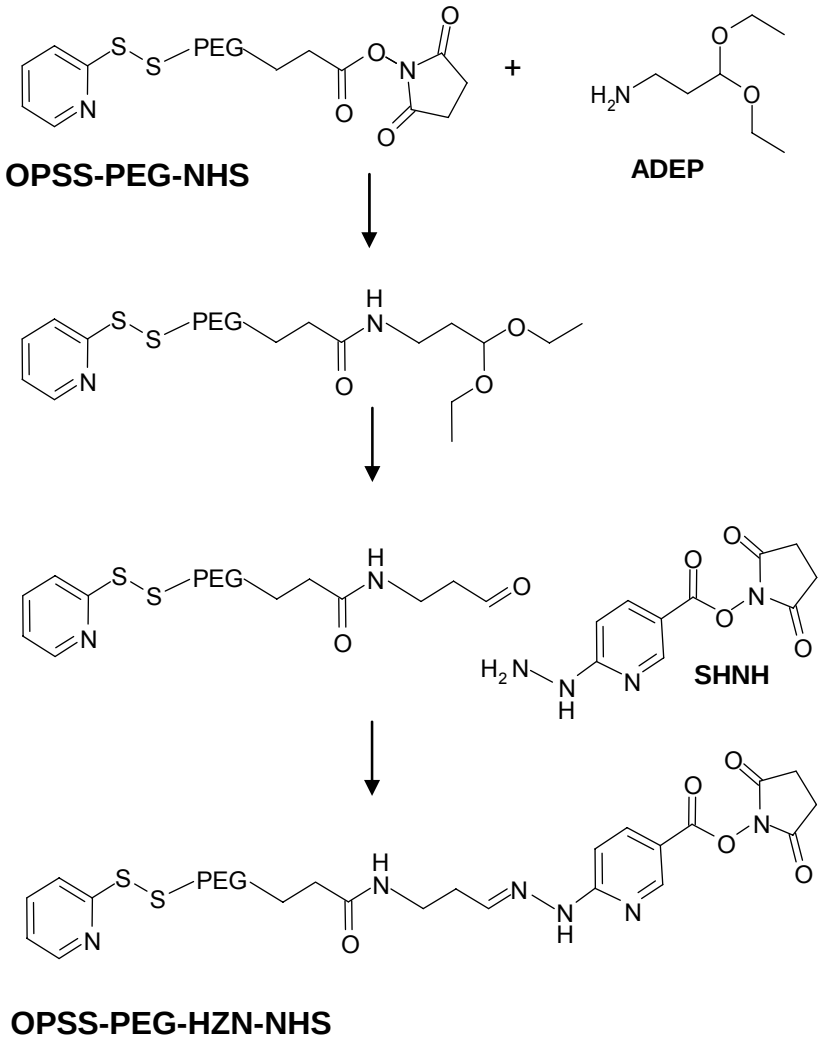


Figure 17) Synthesis of bifunctional post-PEGylation reagent by implementation of ADEP with OPSS-PEG in a 5 to 1 molar ratio. Reaction performed in water free DMF, excess ADEP was removed by size exclusion chromatography (Sephadex G25). After deprotection with HCl, the aldehyde was reacted with SHNH as described before for the monofunctional PEG reagent.

Excess of unreacted ADEP was removed by gelfiltration on a 10/30 Sephadex G25 column. As ADEP was used in a 5 fold excess, 20 % of ADEP could couple at most. Analysis by TNBS assay showed a reaction efficiency of almost 100% as approximately 80% ADEP remained unreacted after the incubation period (see figure 18).

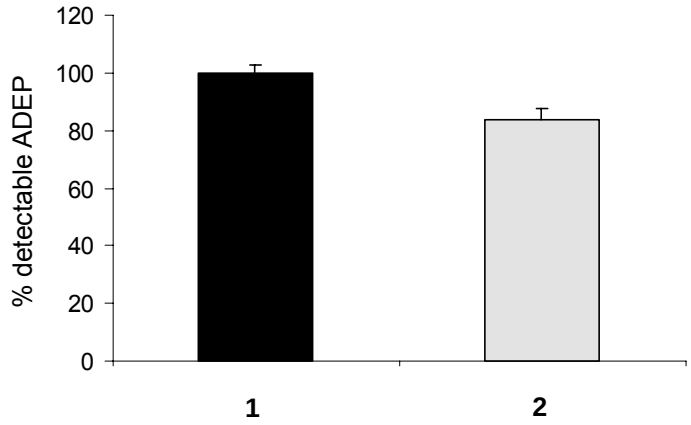


Figure 18) Amount of ADEP detectable after gelfiltration on Sephadex G25. 1) 5 µmol of ADEP and 2) 5 µmol ADEP incubated with 1 µmol of OPSS-PEG before application onto the column.

The protecting acetal group of the emerged reagent was transformed into the corresponding aldehyde by incubation in 0.1 M HCl for 1 h at RT. The product was subsequently freeze dried and resolved in DMF. Similar as for NHS-HZN-PEG, the introduced aldehyde was coupled to SHNH in a molar ratio of 1:4 to form OPSS-PEG-HZN-NHS. The reaction efficiency between SHNH and the aldehyde was found to be practically 100% and was therefore comparable to the reaction of the monofunctional reagent. Excess of SHNH was completely removed by benzyloxybenzaldehyde beads with a 100 fold molar excess of bead -CHO groups over SHNH as it was already used before for the monofunctional reagent.

3.2.2 Synthesis of Alexa488-disulfide poly(ethylene) glycol (hydrazone-) N-hydroxysuccinimidylester (Alexa-SS-PEG-(HZN)-NHS)

For visualisation of the deshielding ability of the PEG coat a hydrazone containing PEG conjugate with a fluorescent dye molecule (Alexa488) on one end was generated. The bifunctional pH-sensitive PEG derivative, Alexa-SS-PEG-HZN-NHS and its stable control counterpart Alexa-SS-PEG-NHS (Figure 19) were prepared by mixing OPSS-PEG-(HZN)-NHS in water-free DMF with Alexa488-SH.

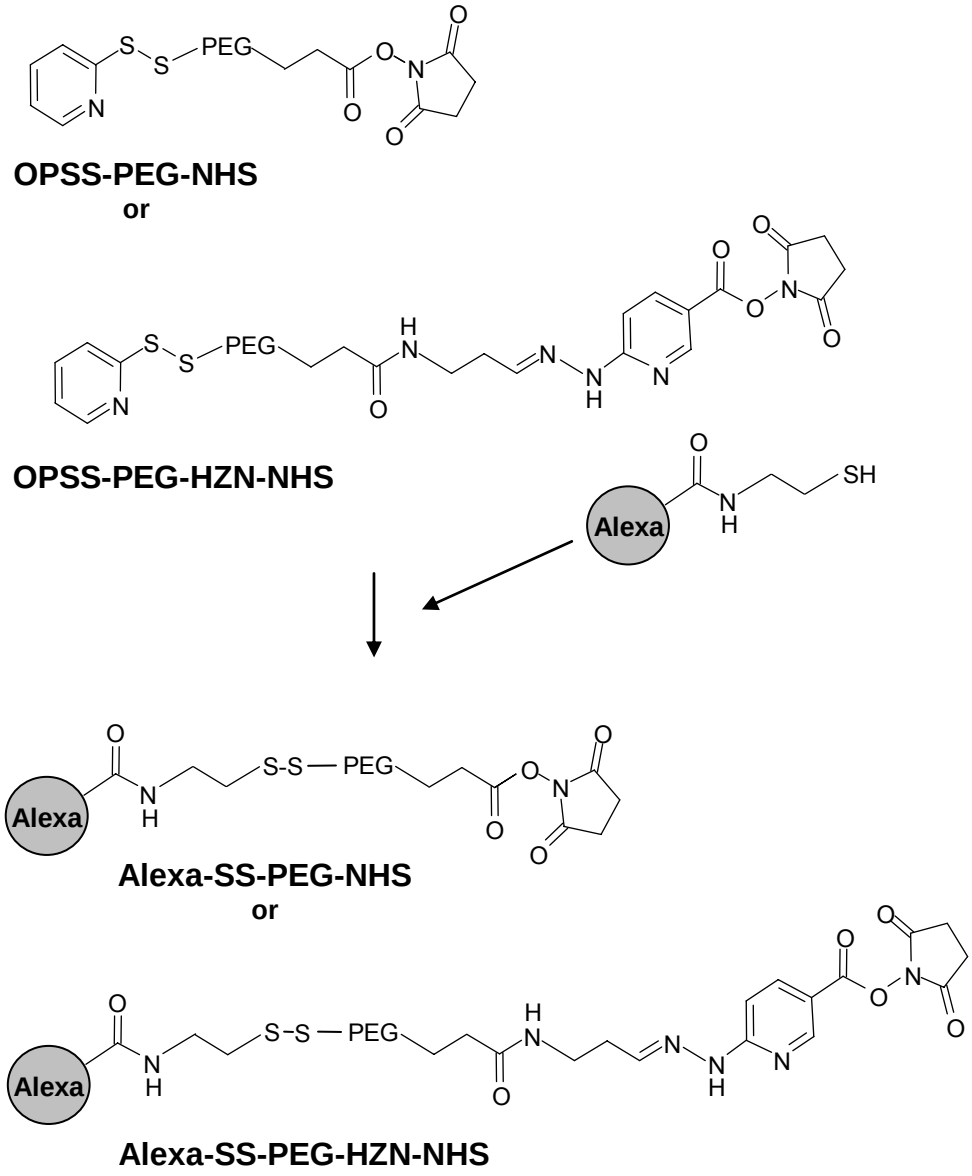


Figure 19) Synthesis of the corresponding Alexa-labelled post-PEGylation reagent. Reagents were mixed with Alexa488-SH in a molar ratio of 1:1 in waterfree DMF. Process of the reaction can be monitored by measuring the release of pyridine-2-thione at 343 nm.

The accordant Alexa with a free thiol group (Alexa-SH) was synthesized by reaction of Alexa Fluor® 488 carboxylic acid, succinimidyl ester with cysteamine.

Before mixing the components, cysteamine was analyzed for its –SH content via Ellman´s assay and was found to be ~ 95 of declared 98%. This value decreases rapidly when the substance is not handled with care and not stored under argon. Reaction efficiency between cysteamine and the active ester of Alexa was determined via TNBS assay on decrease of free amino groups (as described before) and was found to be approximately 90%. The efficiency of the coupling reaction between cysteamine modified Alexa-succinimidylester and OPSS-PEG-(HZN)-NHS

could be monitored by the release of pyridine-2-thione at 343 nm. For the stable PEG reagent 69% and for the labile one 67% coupling of Alexa-SH could be measured.

Finally, all synthesized conjugates and the stable OPSS-PEG control were analyzed for their ester reactivity and thus their ability to PEGylate amino groups. For all tested reagents ester reactivity was found to be at least 80%.

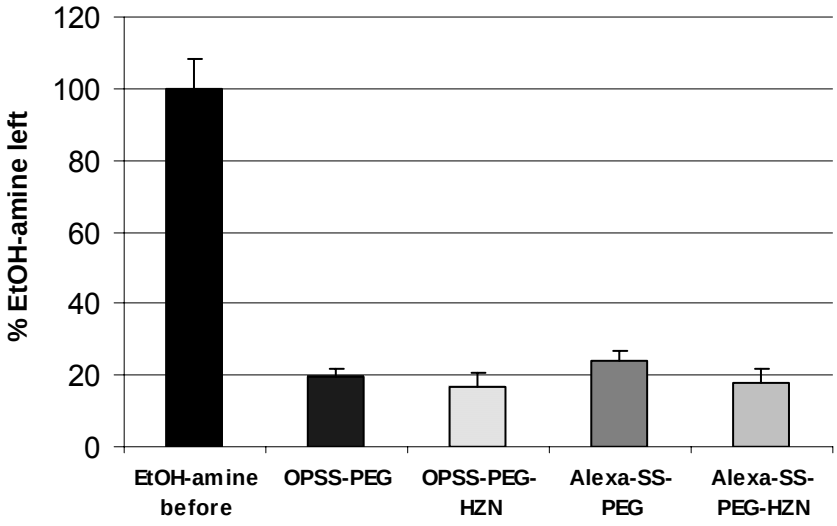


Figure 20) Ester reactivity of the final PEG reagents was tested by incubation with ethanolamine for 1 h at room temperature and subsequent analysis of remaining –NH_2 groups by TNBS assay.

3.2.3 Shielding of polyplexes with bifunctional PEG reagents

Figure 21 shows the titration curves for determination of the amount of post-PEGylation reagent necessary to achieve polyplexes with a near neutral surface charge shielded with the bifunctional PEG reagents. Therefore, polyplexes were mixed with increasing amounts of the activated ester PEG reagents OPSS-PEG-HZN-NHS and OPSS-PEG-NHS of 10 kDa. The PEG content of the targeting conjugate led to a partial shielding effect. Thus, the starting zeta potential of BPEI-PEG-EGF containing particles was lower than that of untargeted polyplexes. For BPEI particles a molar excess of PEG molecules over BPEI molecules of approximately 30 fold generated shielded particles with a zeta potential below 5 mV. Whereas shielding of LPEI particles, performed in low salt containing buffer (HBG), was different to shielding of BPEI polyplexes, as even with a much higher molar ratio of up to 180 fold excess of PEG the zeta potential could not be reduced to values near neutral. Due to these findings 10 kDa PEG reagents were used on BPEI polyplexes in later experiments. It appeared to be only a small difference in the molar

excess required for shielding by either the stable or reversible PEG reagent. This demonstrates that the activated ester of the synthesized hydrazone mediated PEG reagent has a similar activity towards amines as the commercially available stable NHS-PEG.

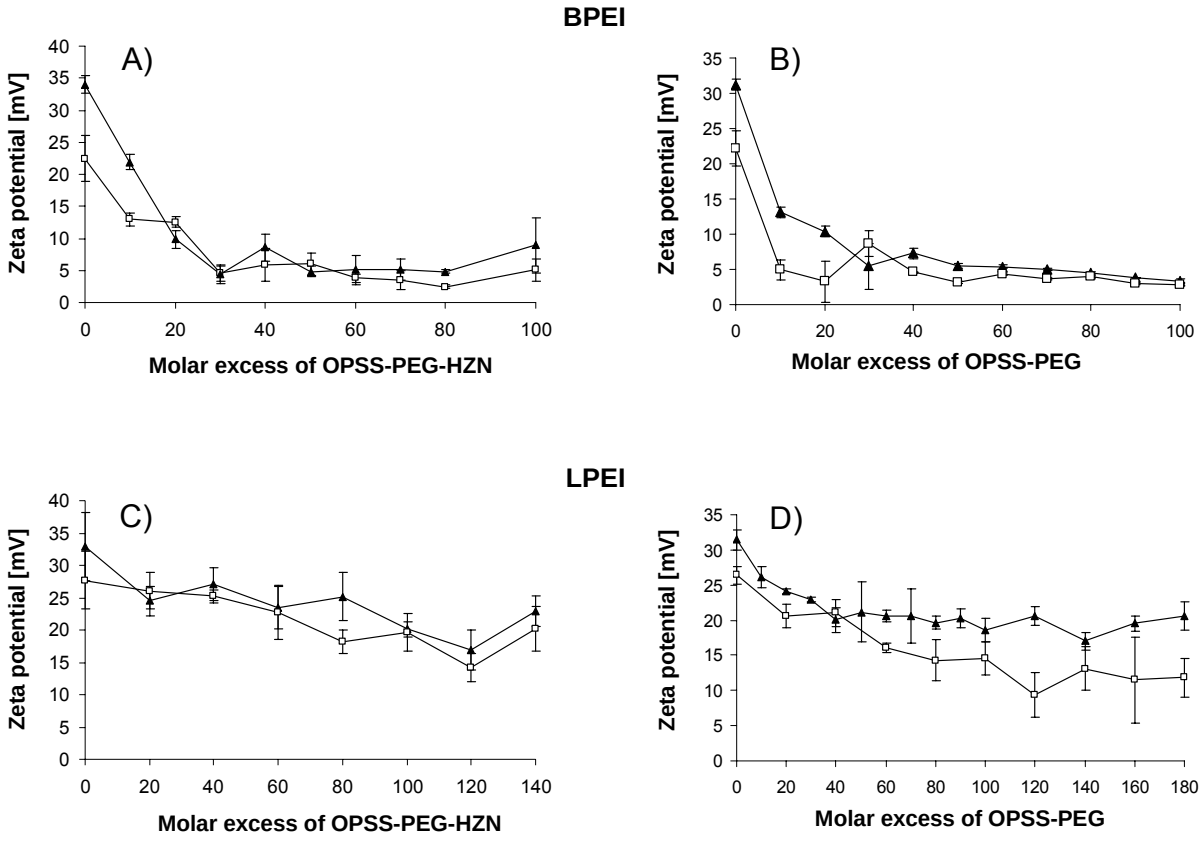


Figure 21 A and B) BPEI polyplexes, N/P = 6 prepared in HBS 0.5x and C) and D) LPEI polyplexes, N/P = 6 prepared in HBG post-PEGylated with increasing amounts of indicated PEG-reagents (10 kDa) and corresponding zeta potential. Black triangles: -10% EGF and white squares: +10% EGF

3.2.4 Fluorescence correlation spectroscopy (FCS) of Alexa-PEGylated polyplexes

For direct visualisation of PEG deshielding the fluorescent dye Alexa488 was coupled to the distal end of a NHS-containing, heterobifunctional PEG molecule. Cleavage of the molecule from the polyplex was monitored by fluorescence correlation spectroscopy (FCS). By comparing the diffusion velocities of Alexa, Alexa-PEG and Alexa-PEGylated polyplexes it was possible to determine whether the PEG-shield is still bound to the polyplex or not.

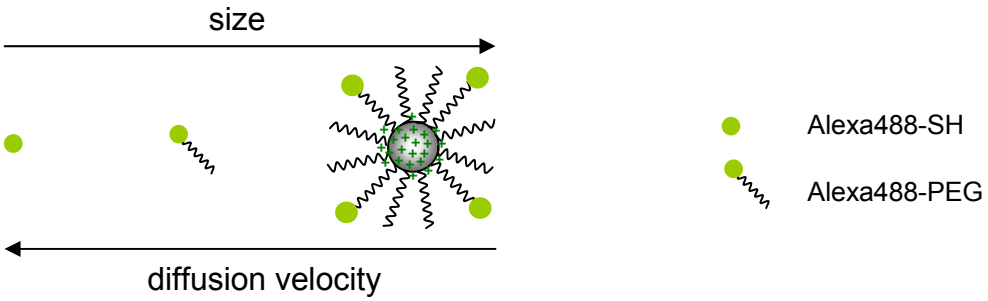


Figure 22) Illustration of the measure principle of conducted FCS experiments. With increasing size of the molecule the dye is connected to the diffusion velocity decreases.

To minimize background noise caused by free Alexa, PEGylated polyplexes were purified prior to FCS measurements. Additionally, stable and labile Alexa-PEG reagents had to be purified from free Alexa molecules as they were used for control measurements.

3.2.4.1 Purification of Alexa-SS-PEG-(HZN)-NHS for FCS measurement

Alexa-Succinimidylester and Alexa-SS-PEG conjugates, stable and labile, were measured as controls in the FCS experiment. Plain cysteamine modified dye was diluted to a 10 nM solution and PEG conjugates were purified from excess unbound dye molecules by size exclusion chromatography on a Sephadex G25 material on a 10/30 column (Figure 23).

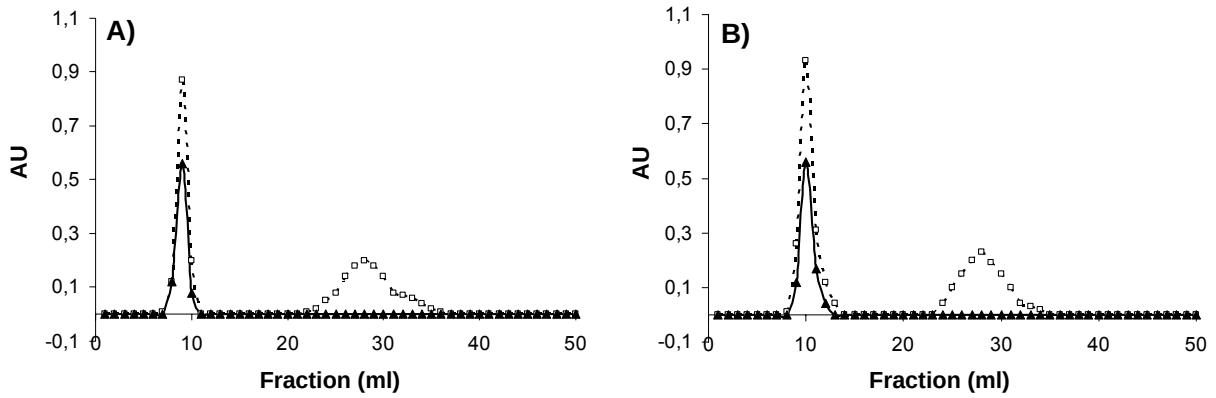


Figure 23) Elution profile of A) Alexa-SS-PEG-NHS and B) Alexa-SS-PEG-HZN-NHS from Sephadex G25 superfine column 10/30 with 20 mM HEPES/ 0.5 M NaCl and a flow rate of 1ml/min. Fractions were analysed by absorbance at 488 nm and by PEG assay at 590 nm. Black line: PEG, dotted line: Alexa

Spectrometrical analysis of collected fractions revealed that the first peak contained Alexa labelled PEG conjugates and the second peak unreacted dye. Determination of the distribution of Alexa in those two peaks allowed for calculating the percentage of Alexa coupled to PEG. The results showed slight batch to batch variations with an average of 55-65% of Alexa bound to PEG and 35-45% free.

3.2.4.2 Different approaches for purification of stable or labile Alexa-PEGylated polyplexes for FCS measurement

First PEGylation approaches with purified BPEI polyplexes and Alexa-SS-PEG or Alexa-SS-PEG-HZN contained about 70% free Alexa-PEG conjugates and unbound Alexa dye and did not allow for valid FCS measurements. To achieve maximum pure polyplexes for FCS measurements different methods were investigated for their success in removing excess unbound Alexa–PEG conjugates, unlabelled PEG-reagents and free dye molecules.

3.2.4.2.1 Purification of stable and labile Alexa-PEGylated BPEI particles for FCS measurement via SEC

As BPEI polyplexes were prepared at an N/P ratio of 6 in HBG, > 50% of BPEI is free in solution (66). To achieve as much as possible defined Alexa-labelled particles, the underlying BPEI polyplexes had to be purified from excess free BPEI in order to avoid PEGylation of free PEI polymer. This was achieved by SEC with a self made Pasteur pipette (see 2.14 (67)). The first peak (fraction 3 to 7) contained ~ 40% and the second (fractions 16 to 25) ~ 60% of BPEI and was determined via TNBS assay. DNA was detected spectrometrically at 260 nm and was found exclusively in the first

peak (see figure 24). Total recovery of BPEI was 86% and 88% for DNA. N/P ratio was reduced from 6 of unpurified to 2.35 of purified polyplexes which is in accordance with data from literature (66).

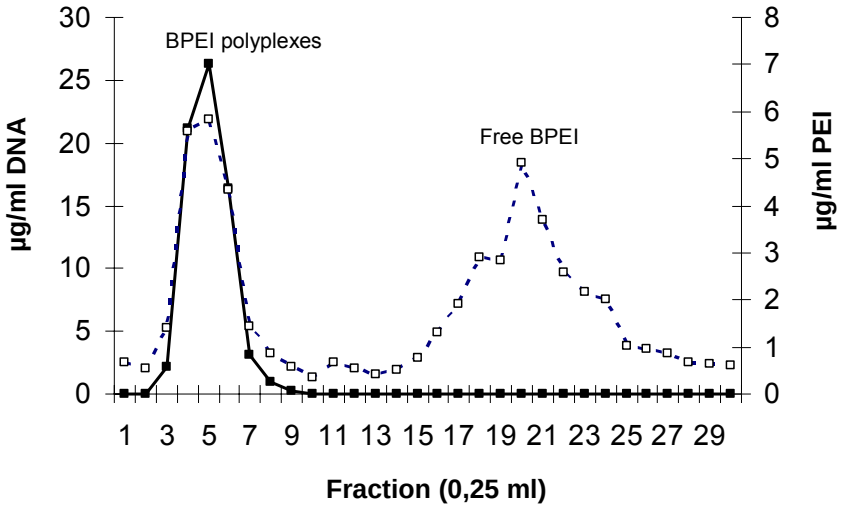


Figure 24) Elution profile of BPEI polyplexes prepared in HBG at an N/P ratio of 6 eluted from Sephacryl S200 column material in a Pasteur pipette (custom made). BPEI was detected by TNBS assay□ and DNA content by UV measurement at 260 nm■.

Afterwards purified polyplexes were post-PEGylated with either Alexa-SS-PEG-HZN-NHS or Alexa-SS-PEG-NHS. A second purification step was employed to remove unreacted Alexa-SH and Alexa-PEG conjugates (Figure 25). Similar elution profiles monitoring UV/VIS (260/488 nm) detection and PEG content of the individual fractions were obtained for stable and labile PEGylated polyplexes. PEG assay showed two peak values but no base line separation. Detection of Alexa gave 3 peaks, with the first two of them showing no baseline separation. Besides PEG and Alexa, peak 1 contained DNA, indicating elution of Alexa-PEGylated polyplexes whereas peak 2 contained only unbound Alexa-PEG conjugate. DLS measurements showed particles with a hydrodynamic diameter of around 150 nm in the pool belonging to peak 1 but no particles could be detected in the fractions of the second peak. Finally, at fraction 50 free Alexa-dye molecules were eluted.

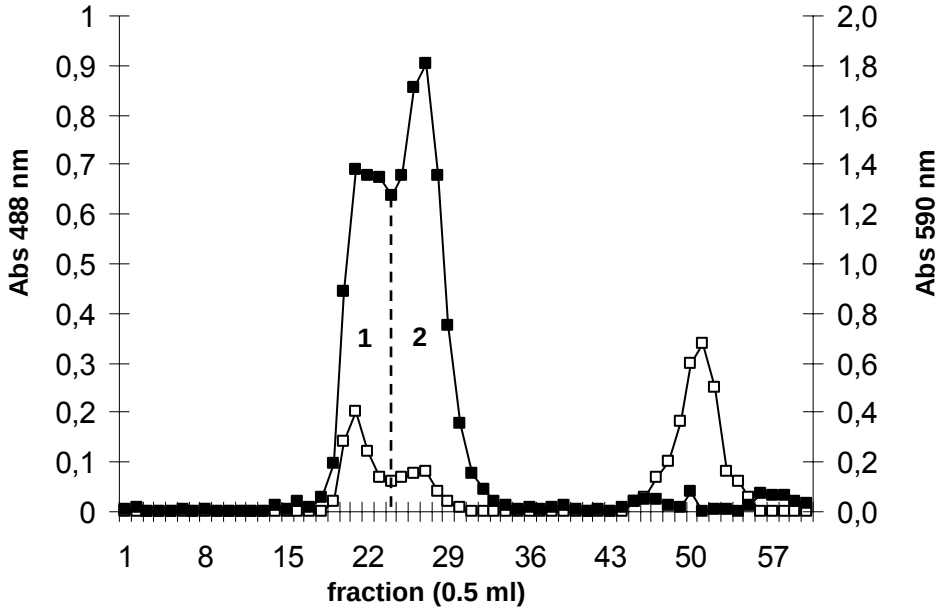


Figure 25) Purified BPEI polyplexes were Alexa-PEGylated and applied onto Sephacryl 200 column 10/30 in HBG/0.5 M NaCl. Absorbance of Alexa dye at 488 nm □and absorbance of PEG at 590 nm, determined by PEG assay ■.

3.2.4.2.2 Purification of Alexa-PEGylated polyplexes: Dialysis

One attempt to generate pure Alexa-PEGylated BEI polyplexes was the performance of a dialysis. There was no difference detectable whether the membrane with 14000 or 25000 Da MWCO was used. For both membranes the amount of removed PEG varied between 5 and 15%. In parallel, spectrometric analysis of Alexa at 488 nm showed that only ~10% of totally applied Alexa dye molecules were removed. Hence, measurement of dialysed particles in FCS was not possible as too much of free dye was still present.

3.2.4.2.3 Purification of Alexa-PEGylated polyplexes: Electrophoresis

Another purification attempt was performed with the so called Electro Prep® system. Although Alexa as well as unbound and hydrolysed PEG-conjugates exhibit negative charge moieties, analysis of samples taken before and after electrophoresis showed that neither PEG nor Alexa content was reduced after 20, 30 or 60 minutes.

3.2.4.2.4 Purification of Alexa-PEGylated polyplexes: Vivaspin®

For ultraconcentration, PEGylated complexes were applied on Vivaspin® 6 microconcentrators. Approximately 100% of PEGylated complexes (generated in HBG) could be recovered in the supernatant as UV measurements at 260 nm revealed. Independent of the used molecular weight cut-off, ~80-90% of PEG was

found in the filtrate. Similar results were measured for Alexa (see table 3). The ultracentrifugation devices of different molecular weight cut-offs showed an efficient removal of undesired molecules. PEG as well as Alexa was removed (= found in the filtrate) in similar amounts from the polyplexes which remained mainly in the supernatant. Further centrifugation steps could not increase the separation of conjugates from the polyplexes anymore (data not shown). Such purified particles were then applied for FCS measurements; however, they did not allow for valid interpretation as the amount of free dye in the samples obviously exceeded a tolerable amount.

MWCO	100 000				300 000				1 000 000			
Centrifugation step	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
% PEG removal	56	76	78	78	61	88	89	89	76	87	89	90
% Alexa removal	60	71	78	82	69	77	83	86	74	85	91	93
% DNA *remaining*	98	94	92	90	93	86	84	83	98	90	86	85

Table 3) Summarisation of polyplex purification using Vivaspin® ultracentrifugation. Stable Alexa-PEGylated BPEI polyplexes N/P = 6 in HBG were centrifuged in microconcentrator tubes of indicated MWCO at 4000 g for 5 min. Supernatant and filtrate were analysed spectrometrically for DNA and Alexa content and the amount of PEG was determined by PEG assay.

3.2.4.2.5 Purification of Alexa-PEGylated polyplexes: Dextran-cushions

In a preliminary experiment, BPEI polyplexes were centrifuged at 16000 g, afterwards the obtained pellet was resuspended in HBG in an ultrasonic bath and was filtrated through a paper filter. Size measurement of these particles revealed aggregates with high polydispersity. When in contrast, such polyplexes were centrifuged onto dextran cushions using only 4000 g, no proper pellet occurred. After solving the upper dextran layer in HBS 0.5x, particles with approximately 300 nm could be detected via DLS. When Alexa-PEGylated particles were centrifuged onto this dextran cushion the recovery was approximately 70%. Size and zeta potential measurements of the resuspended PEGylated polyplexes showed particles of 160 nm with a zeta potential of approximately 4 mV. However, measurement of such purified Alexa-PEGylated

polyplexes in the FCS set-up was not possible as still too much free dye molecules were present.

3.2.4.2.6 Purification of Alexa-PEGylated polyplexes: Anion exchange chromatography

Application of Alexa-PEGylated polyplexes on a DEAE anion exchange chromatography column and elution with 0.5 M salt containing 20 mM HEPES buffer revealed an elution profile with no separation since almost all PEG containing components were eluted immediately, free 488 was eluted at last.

Subsequent analysis in FCS showed high standard deviations between the single measurement results representing values of free dye molecules as well as PEG-bound Alexa-dye molecules.

3.2.4.3 FCS results

All Alexa-PEGylated polyplexes purified by the methods described above were analysed in the FCS experiment but interestingly only those achieved from the SEC experiment were pure enough to give clear signals.

Alexa-PEGylated particles eluted in the first peak were initially analysed by DLS. Zeta potential was found to be +2 mV and the size approximately 150 nm. The time needed for passing the detection window correlates with the size of the molecule the dye is connected to. Plain cysteamine modified Alexa-succinimidylester molecules showed the shortest diffusion time of ~30 µs. Coupling of Alexa to either stable or labile bifunctional PEG reagent revealed diffusion times of around 200 µs. The longest diffusion times from 2000 to 6000 µs were found for Alexa-PEGylated particles with no significant difference detectable between stable and labile Alexa-PEGylated polyplexes. After incubating the polyplexes for 4 h at pH 5.0 stable shielded polyplexes exhibited an unchanged diffusion time. In clear contrast, the sample with the pH-labile Alexa-PEGylated polyplexes exhibited a much faster movement of ~ 200 µs, similar to the diffusion time of Alexa-PEG conjugates. These data indicate clearly the pH induced cleavage of the dye labelled PEG molecules from the polyplex. Results are summarised in table 4.

	Diffusion Time [µs]
Alexa-Succinimidylester (cysteamine modified)	**31 +/- 5**
Alexa-SS-PEG-NHS (stable shielding)	**193 +/- 18**
Alexa-SS-PEG-HZN-NHS (pH labile shielding)	**196 +/- 21**
Polyplex stable shielded	
4 h pH 7.4 / 37°C	**1826 +/- 560**
4 h pH 5.0 / 37°C	**1988 +/- 1209**
Polyplex labile shielded	
4 h pH 7.4 / 37°C	**1713 +/- 314**
4 h pH 5.0 / 37°C	**192 +/- 19**

Table 4) Summary of the results of the FCS analysis of Alexa-PEGylated complexes. For each sample 10 measurements with sampling time of 30s were performed. The 488 nm line of an argon laser was utilized for fluorescent excitation. Emission light was split by a dichroic mirror and was collected by an Avalanche Photodiode (APD) with a 505-530 nm filter and a pinhole of 78 µm.

3.2.5 Determination of polyplex composition

In this additional experiment it was shown how much Alexa-SS-PEG conjugate can bind to BPEI integrated in a polyplex and, in comparison, how much can bind to free BPEI polymer. In a preliminary experiment BPEI was reacted with a 30 fold molar excess of Alexa-SS-PEG-NHS and after 1 h reaction time a cation-exchange chromatography was performed. In figure 26 A) the corresponding elution profile is shown. PEG and Alexa are eluted simultaneously with one higher peak at the very beginning in low salt containing buffer (0,5 M) and a second peak that is much smaller which is eluted only at high salt concentrations (3 M). Analysis via TNBS assay revealed that BPEI is eluted only in fractions with 3 M salt. Taken together one can see that first the bigger part of Alexa-PEG was eluted followed by a small fraction that has obviously coupled to BPEI as it is retained and is not eluted until a high amount of salt is present on the column.

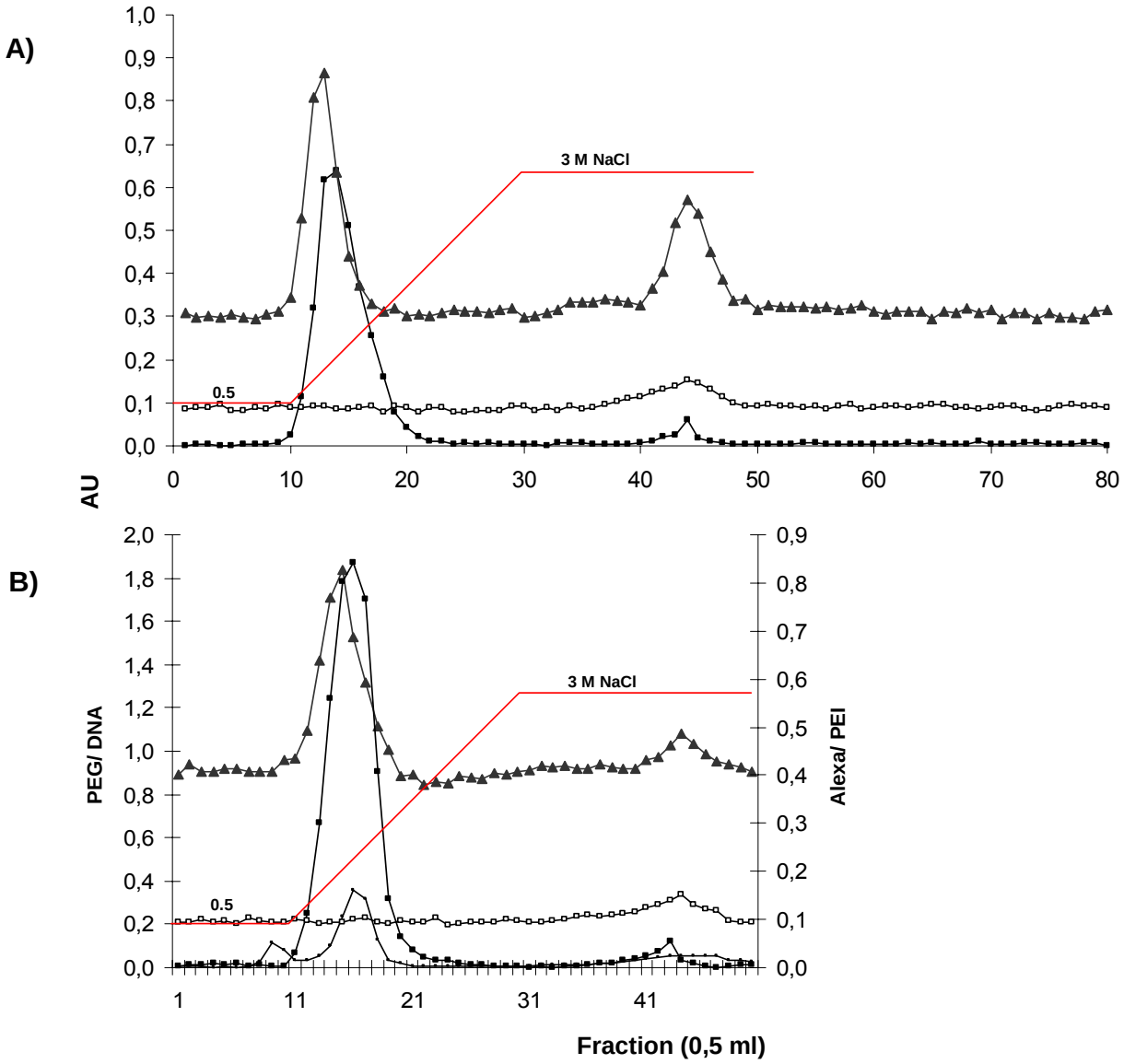


Figure 26) Elution profiles of Alexa-PEG (30 fold molar excess) coupled to A) BPEI and B) purified BPEI polyplexes N/P = 6. Grey triangles PEG via PEG assay, black squares Alexa, white squares BPEI via TNBS assay and black line in B) absorbance at 260 nm (AU = absorbance unit)

Polyplexes purified by the method described under *2.14.1* used for FCS experiments were analysed for their detailed composition. In Figure 26 B), the same experiment as described above but based on BPEI polyplexes is shown. In order to determine the amount of Alexa-PEG bound to BPEI chains on the particle's surface the whole complex was dissociated by addition of high NaCl concentration and was applied onto the cation exchange column.

Before disintegration these purified BPEI polyplexes had a size of ~ 100 nm before and ~ 150 nm after Alexa-PEGylation. Measurement after adjusting the polyplex solution to 1.5 M NaCl revealed that no discrete particles were present anymore. The polydispersity index, measure of size distribution, was found to be 1 indicating that no

homogenous particle population was present anymore with data reaching from a few to a thousand nm.

Apart from the additional line, UV absorbance at 260 nm, the profile is similar to that of plain polymer. Fractions being positive in TNBS assay were pooled and further analysed for their absorbance at 488 nm. Before calculating the amount of PEG the ratio Alexa to PEG of the utilized Alexa-SS-PEG-NHS batch was determined as described under 2.13 and was found to be 1 µmol PEG to 0.45 µmoles of Alexa resembling a ratio of 2.2 to 1. This ratio was then used to determine the amount of PEG corresponding to the measured Alexa absorbance in the BPEI fraction. The calculated ratios are shown in the following table 5. When Alexa-SS-PEG-NHS was reacted with free BPEI polymer the ratio PEG to PEI was found to be approximately 3 compared to 1.3 when coupling was performed to purified BPEI polyplexes. Calculating the ratio by PEG assay instead via the Alexa absorbance lead to very high values. These increased values are typically when PEI and PEG coexist in the analysed solution and demonstrate that PEI interferes with the PEG assay. Therefore no useful values were obtained in the second assay. Nevertheless, for both ways of calculation, values for PEGylation of free polymer are higher than those achieved for PEGylation of PEI within purified polyplexes.

Alexa-PEG-NHS coupled to:	**PEI polymer**	**PEI polyplex**
PEG: BPEI (Abs. 488)	**3,3 +/- 1 : 1**	**1,3 +/- 0,2 : 1**
PEG: LPEI (Abs. 488)	**2 +/- 0,17 : 1**	**0.9 +/- 0,16 : 1**

Table 5) Ratio of PEG to BPEI either coupled to free polymer or to DNA bound polymer calculated by absorbance of Alexa488 in comparison to values achieved by PEG assay analysis. The latter values are not considered as real due to an interference of PEI with the PEG assay under the given conditions (diluted solutions, high salt).

4 DISCUSSION

Designing chemically dynamic structures for non-viral delivery of nucleic acids into cells is a novel approach to mimic the intracellular dynamics of viruses which have naturally evolved for optimal delivery of their genetic payload. Several strategies like enzymatic activation or redox- and pH- responsive approaches have already been applied (for recent review see (74)).

PEI and PEI derived polymers are examined intensely in the area of non-viral gene delivery. One major advantage of synthetic gene vectors is that in comparison to viral vectors they can quite easily be chemically modified and therefore allow for adaption to a variety of local conditions they are exposed to on their way to the target cell, upon arrival at the target cell and finally inside the target cell. A negative side effect of PEI based gene delivery systems is that they exhibit a dose dependent articulate cytotoxicity. This cytotoxicity occurs mainly due to the interaction of the positively charged polymer with negatively charged moieties like membranes and proteins or other blood components. Introduction of the hydrophilic polymer PEG to PEI reduced its cytotoxicity, increased the circulation time of PEI based polyplexes and their solubility. These positive effects were accompanied by the fact that the transfection efficiency, already much lower than that of viral vectors, was reduced severely. Even though the incorporation of targeting ligands increased the transfection efficiency it was not restored to that of non-PEGylated polyplexes.

Important steps during transfection are particle association with the cell membrane, uptake by endocytosis followed by endosomal release, nuclear trafficking, import of the gene into the nucleus and finally its transcription. Each of these steps can potentially be influenced by PEGylation of the vector. Polyplexes that are taken up by receptor mediated endocytosis are at first localized in endosomal vesicles. Escape from such vesicles is a well-known bottleneck for successful gene delivery. Therefore, the idea came up that it might be advantageous if the PEG shield could be removed in the endosome, re-exposing the positive surface charge of the polyplex and thus allowing for increased membrane interactions and therefore increased endosomal escape.

Recently Walker et al. demonstrated that hydrazone based pH-sensitive shielding of PEI polyplexes successfully improved the transfection ability of such PEGylated polyplexes *in vitro* and *in vivo*. This approach used the so called pre-PEGylation method (as described in 1.2.3.1) for generation of shielded polyplexes. pH-sensitive PEGylation utilizes the advantages of PEGylation, like extended circulation time, reduced cytotoxicity and less unspecific interactions (25) and simultaneously overcomes its appertaining disadvantage of reduced endosomal release (27; 28) through the specific reversibility of the PEG-shield. A couple of research groups have already successfully applied pH-sensitive PEGylation but so far always in a pre-PEGylation approach where PEG is coupled in a pH-labile manner to a polycation or a lipid before DNA is added (59; 60; 75). The question was, whether it would be possible to synthesize a similar PEG reagent but in form of a post-PEGylation reagent (described under 1.2.3.2). One of the main advantages of a pH-labile post-PEGylation reagent is that it can be applied more flexible to almost any system bearing amine groups. An interesting target could be the application onto viral systems as PEGylation of viral capsid proteins is feasible and has already been shown to improve the practicability of viral particles (21) in terms of *in vivo* applications. Furthermore the addition of the hydrophilic polymer PEG after DNA condensation cannot interfere with particle formation. Finally, post-PEGylation in combination with the described purification method turned out to allow for generation of better characterized and well defined polyplexes. Removal of free PEI and other unbound molecules also results in reduced cytotoxicity (67)

4.1 Synthesis of monofunctional NHS-HZN-PEG

Numerous pH-sensitive reagents for drug delivery have been developed after it became evident that these reagents, once internalized into endosomes, encounter an increasingly acidic environment that can be used for triggering cargo release. Many research groups have contributed to the "triggered" approach to drug delivery by preparation and characterization of acid-sensitive modified drugs (76), cationic lipids (77), liposomes (64) or polyplexes (28). Whereas the last three references mentioned, applied hydrazone based chemistry. Other approaches utilized ortho esters (75; 78) or acetals (59; 79) for pH triggered release. The aim of this study was to build on previous experiences with acid labile PEG shielding of polyplexes by

preparing a novel hydrazone-based PEG reagent suitable for post-PEGylation of gene delivery systems. The post-PEGylation strategy was chosen due to the reasons mentioned above with the ulterior motive to generate a pH-sensitive PEGylation reagent that could also be tested for its applicability on viral vectors. This has been examined in cooperation with the group of Prof. Kochanek at the University of Ulm (unpublished data).

Direct coupling of PEG to PEI via hydrazone bonding to form a control pre-PEGylation reagent was not possible due to autocatalytic activity of PEI which led to spontaneous cleavage of the PEG-HZN-PEI conjugate already after thawing. These findings are consistent with data published by Walker et al. (28) who showed that it is not possible to form a pre-PEGylation reagent by coupling PEI to a hydrazone bearing PEG whereas this linkage was feasible when PLL was used as the corresponding cationic polymer. Post-PEGylation approaches have already been conducted for several years (23; 33). Here it was analyzed, whether a PEG reagent that is coupled via a pH-sensitive linker improves gene transfer efficiency in comparison to a comparable stable coupled PEG reagent. Recently Knorr et al. (59) were able to generate such acid labile conjugates, but by bridging PEG to PEI via an acetal residue and using it as a pre-PEGylation reagent.

To generate PEG with a terminal hydrazone group hydrolysable at low pH, SHNH was coupled to aldehyde functionalized PEG. The neighbouring pyridyl group in this reagent was intended to ensure stability at neutral pH and hydrolysis at pH 5, as such a behaviour was observed with a 2-pyridylhydrazone derivate in the PEG-HZN-polylysine conjugate mentioned above (28). Reaction between PEG-aldehyde and the hydrazine group of excessively applied SHNH occurred almost completely. Difficulties were merely caused by unreacted SHNH as its activity towards amine groups competes with that of the ultimate reagent. Removal of excess SHNH had to occur under water-free conditions and was finally achieved by immobilisation onto –CHO groups bearing polystyrene beads.

4.1.1 Shielding and deshielding of polyplexes

NHS-HZN-PEG synthesized for acid triggered deshielding of gene vectors was first tested on its shielding ability and thereby compared to a stable control reagent. PEI polyplexes above a molar ratio of PEI nitrogen to DNA phosphate of 3 bear a net positive surface charge (23; 80). Nevertheless, to achieve sufficient transgene

expression, polyplexes with N/P ratios of 6 or higher are commonly used (66; 67). In this case, the zeta potential of polyplexes is usually in the range of +30 mV, which triggers activation of complement and plasma protein binding after systemic application (23). Initial studies with post-PEGylation of BPEI based polyplexes revealed that a 10/1 PEG/PEI (w/w) ratio of PEG 5 kDa enabled significant shielding. This is in good correlation with the data obtained in figure 11A, where a molar ratio of PEG (20 kDa) to BPEI (25 kDa) of 20/1 (corresponding to a PEG/PEI (w/w) ratio of 16/1) reduced the surface charge of BPEI polyplexes by almost 50%. For LPEI polyplexes a significantly higher molar excess was necessary to achieve surface shielding. This can be explained by the fact that in contrast to BPEI, where on average every third nitrogen is a primary amine, LPEI only contains one primary amine per molecule with the remaining being secondary ones. It was observed that the reactivity of the PEG-succinimidyl ester in aqueous solution is much lower towards LPEI (32), which is due to the differences in the pKa value between primary and secondary amines. The incorporation of the EGF-PEG-BPEI conjugate into the polyplex already reduced the initial surface charge of the polyplex, which is most probably due to the presence of PEG-EGF, as similarly observed with transferrin containing PEI polyplexes (23; 81). There was no significant difference whether stable or labile PEG reagent was applied in the shielding experiment. This was expected as both of them offered similar ester activity in a preliminary experiment.

By measuring size and corresponding zeta potential of such pH-labile PEGylated polyplexes among conditions resembling those found whilst blood circulation (pH 7.4) and inside the endosome (~ pH 5.0) it was shown how shield removal at acidic pH changes the biophysical properties of the particles. The dynamic of acid triggered deshielding of HZN-PEG modified polyplexes was monitored by zeta potential measurements over time at pH 5.0. Complete deshielding was obtained after 2 h, whereas the HZN-PEG shield remained stable at pH 7.4 for at least 4 h. Additionally, it was observed that LPEI based polyplexes aggregated after PEG cleavage, which can also be beneficial for its endosomolytic properties (see below). This confirms the usefulness of the PEG linking strategy chosen to stabilise polyplexes at neutral pH and enable their deshielding at acidic pH. Stability of the PEG shield for at least several hours seems sufficient, as the half life of post-PEGylated polyplexes in the bloodstream of mice after intravenous injection is approximately 30 minutes (23; 81-83). Additionally it has been shown by de Bruin et al. (84) that uptake of PEGylated

and targeted polyplexes *in vitro* occurs within minutes therefore the stability of pH labile PEGylated particles over 4 h offers by far enough time to be enriched in a certain tissue and to be taken up in form of small and discrete particles.

4.1.2 *In vitro* transfection efficiency

Transfection efficiency was mainly influenced by the used polymer, whether the polyplex was PEGylated or not, whether EGF targeting ligand was incorporated or not and finally whether stable or labile PEGylation was chosen.

As already shown in many other examinations the LPEI based polyplexes led to higher transfection values as BPEI polyplexes did (85; 86). LPEI is known for a strong tendency to aggregate on and also inside the target cells (7), which leads to enhanced endosomolytic activity due to higher amount of PEI present in the endosome (87). Further on LPEI polyplexes form looser condensates with plasmid DNA compared to BPEI polyplexes (88) and are therefore supposed to allow for facilitated DNA release during the transfection process.

For both BPEI and LPEI polyplexes, PEGylation strongly reduced luciferase gene expression. This is at least partially due to reduced cellular binding and uptake. Similar observations were made on EGF-receptor expressing HuH7 cells using the pre-PEGylation strategy (84).

In the case of EGF-containing polyplexes PEGylation does not significantly hamper cellular binding and internalisation (84; 89). Incorporation of EGF as targeting ligand led to increased gene transfer into HuH7 and Renca-EGFR cells but transfection ability was not restored to the values achieved by non-PEGylated polyplexes which is in accordance with literature (90; 91). Apparently intracellular events like hampered endosomal release are supposed to be responsible for this effect (28). PEGylation can negatively affect the transfection process by several mechanisms. In the case of targeted polyplexes PEG can negatively interfere by hiding the cell targeting ligand or, in the case of a post-PEGylation strategy, can covalently modify the ligand when using amine reactive PEGylation reagents and a ligand containing primary amines in the side chain, e.g. transferrin (89). Post-PEGylation of polyplexes containing murine EGF has the advantage of not interfering with EGF binding activity, as murine EGF does not contain lysine residues. To prevent obstruction of EGF within the PEG layer EGF was coupled via a PEG spacer of sufficient length (see also Blessing 2001 (33)).

In these experiments a clear benefit of the labile PEG shield can be observed compared to stable PEG shielded polyplexes. Even with untargeted LPEI polyplexes labile PEG shielding led to higher transgene expression indicating that PEGylated polyplexes internalised by adsorptive endocytosis can benefit from this principle.

4.1.3 *In vivo* transfection efficiency

Finally, the potential of labile PEG shield to enhance efficiency and selectivity of transgene delivery to tumour tissue after systemic application was evaluated. From literature it is known that besides the size, biodistribution of polyplexes after intravenous administration is modulated by the surface charge of the particle (23). If their surface is hydrophilic and uncharged they are less taken up by phagocytic cells of the RES, aggregation can be avoided and the circulation time is significantly prolonged. Unfortunately, coating of the positive surface charge with PEG leads to comparably low cell transfection efficiency at the same time (26; 31; 92). For improved targeting of DNA complexes to EGFR overexpressing cancer cells, polyplexes have to be small enough to pass through the fenestrations in the tumour vasculature and should be provided with a suitable targeting ligand, like EGF in the present case. This was achieved by post-PEGylation of BPEI or LPEI particles containing 10% of BPEI-PEG-EGF. PEGylation protects the polyplexes from aggregation and therefore they maintain their size. The data clearly show that both the labile and stable PEG shield provide sufficient protection within the bloodstream, as the lung expression levels observed are very low. This indicated that no gross deshielding and aggregation took place in the blood, as usually lung expression with unshielded and aggregating BPEI and LPEI polyplexes is several orders of magnitude higher (see e.g. (7)). In the tumour tissue the labile PEG shield led indeed to a beneficial effect, most probably on the cellular level, as EGF-LPEI polyplexes with labile PEG shield were significantly more efficient than the corresponding formulation with a stable shield. With BPEI polyplexes the advantage of the pH-labile PEG-shield was also detectable but as already shown in the *in vitro* experiment the values were lower and less significant as with LPEI as basic polymer.

4.2 Synthesis of bifunctional NHS-HZN-PEG-OPSS

A bifunctional PEG reagent offers the opportunity to further modify the PEGylated polyplex for example by introducing a targeting ligand to the PEG reagent. Post-targeting after PEGylation of polyplexes has already been successfully performed by Blessing et al. (33) or Rao et al. (93). One advantage using a pH-labile post-PEGylation reagent containing a targeting moiety is that the complete PEG shield could be removed inside the endosome. In the approach with monofunctional PEG the incorporated PEI-PEG-EGF conjugate cannot be cleaved among acidic conditions and thus hypothesized membrane interactions are constricted. Endosomally cleaved targeting ligand also cannot interfere with intracellular trafficking.

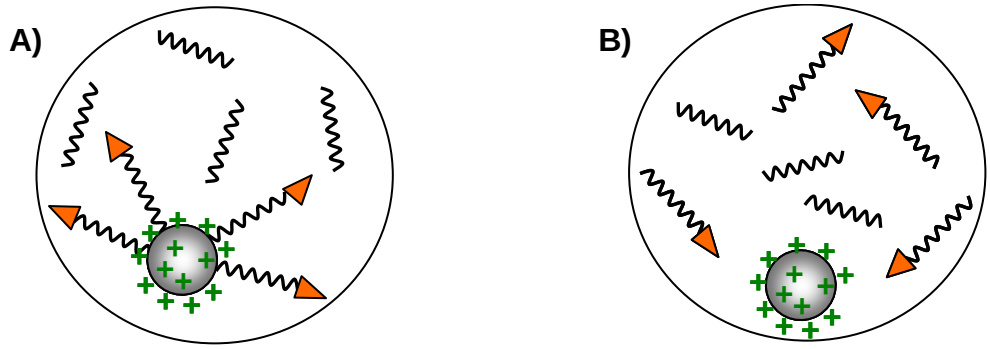


Figure 27) Hypothesized occurrences of pH-labile PEGylated polyplexes in acidified endosomes. A) Polyplex containing BPEI-PEG-EGF introduced by pre-PEGylation and cleaved PEG chains. B) Polyplex with cleaved PEG-EGF and PEG chains allowing for increased membrane interaction

Further on, post-targeting of PEGylated polyplexes could assure that the targeting ligand is best accessible to react with its suitable counterpart and is not hidden inside the PEG layer. Another problem with PEGylation of targeted polyplexes, PEGylation of the targeting ligand itself, can be easily avoided when the targeting ligand is not added until the polyplex is readily PEGylated. Future analysis of polyplexes that have been PEGylated with this bifunctional pH-labile PEG reagent followed by addition of –SH modified targeting ligands will show whether this approach brings additional advantages through removal of the whole PEG envelope inside the endosome compared to only partial removal.

After the basic bifunctional acid labile PEG reagent was synthesized and tested for its shielding ability on LPEI and BPEI polyplexes its pH sensitivity was examined closer.

It was intended to visualise the pH induced cleavage of the PEG chains from the polyplex by FCS measurements with fluorescent dye labelled pH-sensitive PEG conjugates. For this purpose, the commercially available heterobifunctional OPSS-PEG-NHS derivate was modified to harbour a hydrazone bonded succinimidylester and thus ensuring amine reactivity. Alexa488 fluorophore was modified with a thiol group by implementation with cysteamine. To prevent subsequent purification steps, where the presence of water would hydrolyse the succicimidyl ester, Alexa-succinimidyl ester and cysteamine were added in stochiometric deficiency. This –SH modified Alexa allowed for carrying out defined coupling to OPSS-PEG-(HZN)-NHS by following the reaction by the release of the UV absorbing pyridine-2-thione at 343 nm. As purification of Alexa-PEGylated polyplexes was required anyway, OPSS-PEG-(HZN)-NHS was used in an excess over Alexa-SH for increased reaction efficiency. Even without following purification unlabelled PEG does not disturb the sensitive FCS set-up like free Alexa dye molecules would.

4.2.1 Purification of BPEI polyplexes

In this work two purification steps were conducted after particle formation. The first step removed unbound PEI from the polyplex and the second one was performed to remove unbound PEG and Alexa molecules.

PEI is known for its cellular toxicity and severe systemic side effects. A standard PEI polyplex generated at an N/P ratio above 5 can contain up to 80% of free PEI which is not complexed with DNA (65; 94). Increasing N/P ratios are directly correlated to increased cellular toxicity (95); (96). A purification method via size exclusion chromatography has been established by Boeckle et al. (66) and indicated lower toxicity of purified polyplexes but simultaneously led to lower reporter gene expression. The main reason for purification lies in improvement of systemic applications of gene vectors with increased biocompatibility. Regarding the use of non-viral vectors in future gene therapy trials, it is of major importance to use purified and chemically well defined vector systems.

In the present work Alexa-PEGylated polyplexes had to be analysed for shield removal in a FCS experiment. Any accessorily present PEI of an unpurified polyplex solution would become Alexa-PEGylated additionally and thus the background noise of the measurement would be too high. Purification of BPEI polyplexes can be achieved for smaller amounts by custom made Sephacryl S 200 HR filled Pasteur

pipettes or for larger amounts in 10/30 standard HPLC columns offering additionally a good possibility to determine the amount of DNA and PEI the polyplex is composed of before Alexa-PEG conjugates are added.

4.2.2 Purification of Alexa-PEGylated polyplexes

Post-PEGylation was carried out with a 30 fold molar excess of Alexa-SS-PEG-(HZN)-NHS over BPEI. After Alexa-PEGylation of polyplexes different unreacted molecules are present in the reaction approach. Besides N-hydroxy-succinimide these are mainly negatively charged carboxylates, the hydrolysis-products of Alexa-NHS, PEG-NHS and Alexa-SS-PEG-NHS.

First FCS measurements of Alexa-PEGylated polyplexes revealed more than 70% of free Alexa and Alexa-PEG conjugates and thus did not allow for valid interpretation of the obtained data. Therefore a suitable purification method was searched for. Purification via size exclusion chromatography on Sephacryl 200 column material did not lead to baseline separation between the peak of Alexa-PEGylated polyplexes and the one containing free Alexa-PEG conjugates. Thus, it did not seem to be the purification method of choice. A number of different strategies to remove unbound conjugates was tested additionally.

For technical reasons PEI polyplexes were always purified prior to Alexa-PEGylation in all the conducted experiments. BPEI was used for DNA condensation, as it is less responsive to the presence of salt than LPEI is and most of the purification approaches were performed in HEPES buffer with 0.5 M salt.

As already mentioned size exclusion chromatography with Sephacryl 200 did not lead to baseline separation of Alexa-PEGylated particles and free PEG reagents but FCS analysis finally demonstrated that samples taken out of the first fractions of the polyplex containing peak were well applicable for this kind of analysis.

If the other approaches, like Dialysis, Electrophoresis (with the ElectroPrep® System), density centrifugation (into dextran cushions), anion exchange chromatography and ultra centrifugation (with Vivaspin® columns) would be further optimized, probably another purification method would be available additionally.

The importance of generating well defined and purified non-viral gene carriers has already been demonstrated by Boeckle et al. and Fahrmeir et al. (66; 67) and *in vivo* applications of such purified polyplexes clearly indicated their improved compatibility.

In this present work, purification was only performed to enable FCS measurements but self-evidently the described method could also be used to generate purified post-PEGylated polyplexes for biological characterization. Purification methods identical to those applied in former investigations regarding pre-PEGylated polyplex formulations could not be used analogously as the focus of this work lies on post-PEGylation. Considering pre-PEGylation approaches, shielding compounds are always coupled to positively charged polycations and therefore require a different strategy for successful removal. Removal of excess polymer and other components leads to well defined polyplexes. This displays two big advantages. First, most of the toxic side effects are caused by free polymers and/or side products and thus purified polyplexes are much better tolerated. Second, thinking about GMP production it is absolutely mandatory that the employed substances are exactly defined. This can be achieved with purification methods and determination of compounds being included in or separated from the ultimate product.

4.2.3 Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) allows to measure the diffusion velocity of fluorescently labelled molecules and is therefore a valuable tool for analysing dynamic processes related with assembly and disassembly of macromolecular structures like polyplexes (97; 98). Rusu et al. followed the binding of fluorescent labelled peptides to lipid vesicles via FCS measurements. The large unilamellar vesicles were significantly larger than the peptide and therefore the correlation times for the free and bound peptide could be distinguished using single colour autocorrelation measurements (99). Here, this method was utilized to unambiguously show the cleavage of PEG from the polyplex' surface at acidic pH. FCS measurements of Alexa-PEG exhibited a very narrow distribution of individual measurements with a standard deviation of five percent, which indicated that i) no significant amounts of free Alexa dye are present in the Alexa-PEG conjugates and ii) the PEG 10 kDa has a narrow size distribution.

Alexa-PEG modified BPEI polyplexes had a rather broad size distribution at neutral pH, which can be explained by certain heterogeneity in size commonly observed for such preparations. In contrast, incubation at acidic pH apparently led to complete cleavage of the Alexa-PEG shield, as the signal obtained was similar to that of free Alexa-PEG. FCS measurements of polyplexes shielded with the dye labelled

bifunctional pH-labile PEG conjugate represent a nice way to visualise the cleavage of the PEG chain amongst acidic conditions.

4.2.4 Determination of polyplex composition

As already mentioned before, it is of great importance to get insights into the detailed composition of a certain polyplex in order to assure reproducibility. For this experiment purified polyplexes were first Alexa-PEGylated and afterwards completely disintegrated by the addition of salt to a final concentration of 1.5 M NaCl. Adding such a high salt concentration leads to abolition of the electrostatic interaction between negatively charged DNA and positively charged polymer (100; 101). Therefore this demonstrates a good starting base for analysis of accurate polyplex composition. The data provide information on final percentages of BPEI polymer and DNA and therefore the N/P ratio of the purified core particles. Additionally, the PEGylation degree and the amount of Alexa-PEG conjugates in the final particles are clarified. The composition of such purified particles was analysed after disassembly in high salt containing HBG. Polyplexes that are applied to increasing amounts of NaCl become separated into polymer and DNA at a salt concentration of approximately 1.1 M as demonstrated in the following figure.

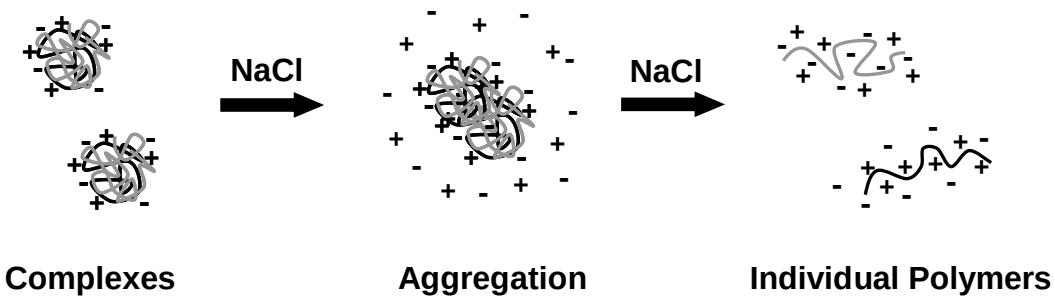


Figure 28) A schematic presentation of complex behaviour when ionic strength is increased by addition of salt.

Cation exchange chromatography as described in chapter 2.21 was chosen to separate DNA, unbound PEG, Alexa and Alexa-PEG from PEI and from Alexa-PEGylated purified BPEI polyplexes after the whole complex has been disassembled into its original compounds. Highly positively charged BPEI and BPEI that was (Alexa)-PEGylated when it was still complexed with DNA in the polyplex should be

retained by the negatively charged sulfonyl groups of the column material whereas DNA, unbound (Alexa)-PEG and Alexa molecules should be separated as they do not bind and are eluted first. The idea was to use the absorbance of Alexa488 in order to indirectly calculate the corresponding amount of PEG coupled to BPEI. The ratio Alexa to PEG can be determined in advance by separating unbound Alexa from (Alexa)-PEG conjugate via gelfiltration on Sephadex G25. As expected more PEG could bind per BPEI molecule when the reaction was performed with free BPEI polymer (3.3 PEG per PEI) compared to polymer bound to DNA (1.3 PEG per BPEI).

5 SUMMARY

Gene transfer efficiency of current non-viral vectors is by far not as efficient as that of their viral analogues. Natural viruses are perfectly attuned to invade cells and to deliver their genetic load. Therefore they represent a convenient example to teach us how to further optimize polycationic gene carriers in order to overcome most of the typical hurdles like specific cell targeting, endosomal release, vector unpacking and nuclear import that restrict the success of non-viral vectors.

The fact that viruses can exploit the decreasing pH of endosomal vesicles to change their appearance in a way that they can escape these “traps” was taken as a model for this thesis to design non-viral gene vectors that are likewise subjected to structural transformation upon acidification. Recent publications gave hints that PEGylation of PEI based polyplexes might hamper their endosomal escape properties and thus contribute to the decreased transfection ability of such PEGylated polyplexes.

The aim of the thesis was to create a pH-responsive PEGylation reagent which can overcome this hurdle and which is not restricted to non-viral vectors like the pre-PEGylation approach is.

A 20 kDa post-PEGylation reagent was synthesized containing a hydrazone moiety for pH-sensitive cleavage and an activated ester for polyplex coupling. This reagent showed similar shielding ability on PEI based polyplexes as the commercially available stable PEG-NHS ester. Depending on the underlying polymer, branched or linear PEI, deshielding of the PEG shield under acidic conditions could be shown indirectly by zeta or size measurements. Due to deshielding, BPEI polyplexes showed an increasing zeta potential after incubation in salt containing buffer at pH 5.0 whereas LPEI polyplexes started to aggregate under the same conditions making reliable zeta potential measurements impossible. For biological characterisation PEI based polyplexes with or without EGF as targeting ligand were PEGylated with the pH-labile PEG reagent and tested on HuH7 and Renca-EGFR cells, both overexpressing the EGF receptor. Branched and linear PEI based polyplexes showed significant improvement in gene transfer efficiency with the pH-labile PEG reagent compared to the stable analogue. These promising *in vitro* data gave rise to

an *in vivo* experiment where stable shielded branched or linear, EGFR targeted PEI polyplexes were compared to those shielded with the acid cleavable PEG reagent. A clear advantage of the pH-sensitive PEG shield could be observed.

Additionally, a bifunctional pH-sensitive PEGylation reagent was synthesised which offers a starting base for retargeting experiments by ligand-coupling via disulfide bond formation to PEGylated gene carriers. This reagent was applied for coupling the fluorescent dye Alexa488. The Alexa488 containing pH-sensitive PEG reagent was used for shielding of BPEI polyplexes and for visualisation of the pH dependent cleavage process. Diffusion velocities of these particles were determined by FCS measurements and cleavage of the dye labelled PEG chains from the particle was found only for the labile Alexa conjugate indicated by a significant increase of velocity after incubation at pH 5.0. The need to purify the dye labelled polyplexes used for the delicate FCS measurement led to the development of a purification method that allowed for calculation of the final composition of the PEGylated polyplex and thus offering a possibility to verify the reproducibility of each sample batch.

6 APPENDIX

6.1 Abbreviations

Abs.	Absorbance
Ad	Adenovirus
ADEP	1-Amino-3,3-diethoxypropane
ALD	Aldehyde
Alexa-NHS	Alexa Fluor® 488 carboxylic acid, succinimidyl ester
Alexa-SH	Alexa Fluor® 488 carboxylic acid, succinimidyl ester modified with cysteamine
AU	Absorbance unit
BPEI	Branched polyethylenimine 25 kDa
CAR	Coxsackie adenovirus receptor
CEC	Cation exchange chromatography
-CHO	Aldehyde group
CMV	Cytomegalovirus
Cya	Cysteamine
DEAE	Diethylaminoethylene
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamid
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-PEG-PEI25	EGF covalently linked to BPEI25 with a heterobifunctional 3.4 kDa PEG spacer

EGFR	Epidermal growth factor receptor
EPH	Electrophoresis
ε	Extinction coefficient
FCS	Fetal calf serum
HBG	HEPES-buffered glucose
HBS	HEPES-buffered saline
HBS 0.5x	HEPES-buffered glucose and HEPES-buffered saline 1/1 (v/v)
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethansulfonic acid)
HPLC	High pressure liquid chromatography
HR	High resolution
HZN	Hydrazone
LPEI	Linear polyethylenimine 22 kDa
m-PEG	Methoxy polyethyleneglycol
MTT	Methylthiazoletetrazolium
Mw	Molecular weight
MWCO	Molecular weight cut-off
MQ	De-ionized water (Millipore)
NA	Nucleic acid
NA 1,2	Numeric aperture of 1,2
NaOAc	Sodium acetate
NHS	N-hydroxy succinimidyl ester
N/P-ratio	Molar ratio of PEI nitrogen to DNA phosphate
OD	Optical density
OPSS	Orthopyridyldisulfide
PBS	Phosphate-buffered saline
PC	Polycation
pCMVLuc	Plasmid encoding for luciferase under control of the CMV promoter/enhancer
PEG	Polyethylene glycol

PLL	Poly-L-lysine
RES	Reticulo Endothelial System
RNA	Ribonucleic acid
RLU	Relative light units
RT	Room temperature
SEC	Size exclusion chromatography
Sephadex G25	Dextran-gel cross linked with epichlorhydrin, fractionation range 0.1-5 kDa for dextrans
Sepacryl S200	Allyldextran-gel cross linked by bisacrylamide, fractionation range 1-80 kDa for dextrans
-SH	Thiol group
SHNH	Succinimidyl-6-hydraziniumnicotinatehydrochloride
SPA	Succinimidylpropionic acid
siRNA	Small interfering RNA
SS	Disulfide
SEC	Size exclusion chromatography
SD	Standard deviation
TNBS	Trinitrobenzenesulfonic acid
UF	Ultrafiltration
UV/VIS	Ultraviolet/ visible light spectroscopy
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

- K.G. de Bruin, C. Fella, M. Ogris, E. Wagner, N. Ruthardt, C. Bräuchle (2008) “Dynamics of photoinduced endosomal release of polyplexes”, J Control Release 2008

- C. Fella, Greg F. Walker, M. Ogris and E. Wagner (2008) “Amine-reactive pyridylhydrazone-based PEG N-hydroxysuccinimide esters for pH-reversible modification of targeted PEI polyplexes, Eur. J. Pharm. Sci 2008

6.2.2 Poster presentations

- Walker, G.F., Fella, C., Fahrmeir, J. and Wagner, E., 2005 “Bio-reversible PostPEGylation of targeted polyplexes enhances gene transfer in vitro” European Society Of Gene Therapy, Annual Meeting, Prague, Czech Republic

- Bio-reversible Post-PEGylation of Targeted Polyplexes Enhances Gene Transfer *in vitro* Fella, C., Walker, G.F., Ogris M. and Wagner, E., CeNS Workshop, Venice International University , San Servolo, Venice, Italy

6.2.3 Oral presentation

Fella, C. (2006) Development of novel non-viral vectors for tumour-targeted gene delivery – towards an ‘artificial virus’ Scientific colloquium, GIANT European Commission Funded Research , Munich, Germany

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