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Dynamic endosomolytic polymer conjugates for pDNA and siRNA delivery

vorgelegt von

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Erklärung

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

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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Meinen Eltern

The outcome of any serious research can only be to make two questions grow where only one grew before.

Thorstein Veblen (1857-1929)
amerikanischer Soziologe und Ökonom

Table of Contents

1 Introduction

1.1 Nucleic acid-based therapy

Nucleic acid-based therapies hold tremendous promise in the treatment of many genetic and acquired diseases by delivering therapeutic nucleic acids into patients. The field started exploring gene therapy, where genes are transferred to compensate a genetic defect or to indirectly mediate a therapeutic effect (“gain of function”). Although gene therapy has not yet been established as standard treatment, it was already applied in various clinical studies, e.g. in the field of cancer therapy, monogenic diseases (Hemophilia A and B, cystic fibrosis, severe combined immunodeficiency syndrome), infectious diseases, vascular diseases, or DNA vaccination [1]. Novel strategies include the use of short interfering RNAs (siRNA) for knockdown of pathogenic target genes (“loss of function”), antisense oligonucleotides which can be used both to suppress [2] or improve gene expression by specific degradation of micro RNA [3], and by triggering exon-skipping for partial repair of defective genes [4]. In addition various other nucleic acids, such as decoy oligonucleotides [5], aptamers [6] or poly inosine-cytosine RNA (polyIC) [7] are in current use. Among those, siRNA mediated RNA interference (RNAi) has attracted considerable research interest. Andrew Fire and Craig Mello won 2006 the Nobel Prize for their work on RNA interference in *Caenorhabditis elegans* which was published in the year 1998 [8]. Tuschl and his colleagues published 2001 that synthetic siRNAs can silence target genes in mammalian systems [9]. siRNAs function in the cytoplasm and are incorporated into a RNA-induced silencing complex (RISC). When the RNA in the RISC complex is complementary to a sequence in a target mRNA, the RISC complex guides mRNA cleavage in the middle of the homologous sequence [10]. The RNAi discovery is not only used as an established research tool to understand or screen for genes functioning in cellular pathways and discover new drug targets, also its potential therapeutic application as a new class of pharmaceutical drugs is promising.

Small-molecule pharmaceutical drugs in general meet Lipinski’s rule-of-five criteria, such as high lipophilicity and a molecular weight below 500 [11]. In contrast, nucleic acids naturally lack this classical drug like properties. They are negatively charged, extraordinary hydrophilic due to their sugar backbone and large macromolecules that do not cross lipid cell membranes unaided. Furthermore they are quickly eliminated in the organism by nucleases before they reach their target tissue. For their use as (intracellular working) therapeutics these properties are not very suitable. Additional many of the established transfection methods for *in vitro* applications cannot be used in most *in vivo* settings. Clearly, solving the

problem of *in vivo* delivery plays a vital role in the development of nucleic acid-based therapies.

1.2 pDNA and siRNA delivery

Genes are extensively degraded in plasma. Supercoiled DNA is converted into the open-circular form with a half-life of 1.2 min upon incubation in isolated rat plasma. The open-circular form has a half-life of 21 min; the linear form degrades with a half-life of 11 min. The clearance from plasma after intravenous administration is even more rapid [12;13]. Tail vein injection of naked plasmid DNA into mice resulted in no gene expression in major organs, because of clearance by the mononuclear phagocyte system (mainly Kupffer cells in the liver) with a disappearance half-life of less than 5 min and its rapid *in vivo* degradation by nucleases, as well as because of limited extravasation [14;15]. Although gene expression can be achieved by local delivery of naked plasmid DNA (pDNA), e.g. intramuscular [16] or intratumoral injection [17], these facts indicate the importance of a suitable carrier for efficient systemic gene delivery.

Since it is the evolutionary character of viruses to deliver their genes into host cells, they present obvious candidates for the development of effective gene delivery vectors. Replication-defective viruses, where viral genes were replaced by therapeutic genes, were applied as viral vectors in gene therapy. Retroviral vectors and adenoviral vectors have been most commonly used in clinical trials [18;19]. Other viruses that were used to develop viral vectors include adenoassociated virus, herpes virus and lentivirus [20;21]. One problem of viruses is their limited capacity to incorporate the additional therapeutic nucleic acids. Besides capacity, activation of the immune system and the possibility of mutagenesis, for example caused by retroviral vectors through gene insertion into the host's genome, are severe disadvantages of viral vectors. It's worth considering that natural evolution designed viruses for their propagation and not for safe and efficient gene transfer.

Synthetic delivery systems ("non-viral vectors") are more flexible with respect to chemistry and nucleic acid type. They can be generated by non-immunogenic peptides or protein free, hence there is only bare possibility that patients have high titers of neutralizing antibodies against them [22]. However synthetic vectors show in general lower gene transfer efficiency after systemic administration in comparison to their viral counterparts.

Cationic lipid- [23] and polymer-based systems [24;25] are the major types of non-viral carriers. Felgner *et al.* first reported in 1987 on the *in vitro* transfection of mammalian cells with a cation lipid formulation [26]. The lipid formed electrostatic complexes with pDNA, named "lipoplexes". The complex formation is due to ionic interactions between the positively charged group on the lipid molecule and the negatively charged phosphate groups on the

pDNA. Since then lipid based systems have been extensively studied as gene delivery carriers and have even been used in clinical trials [27;28].

In 1962 Smull and Ludwig published that the addition of either histone or protamine (both are cationic proteins) to poliovirus derived RNA was found to increase the infectivity of the RNA on eukaryotic tumor cells. Only when the cationic proteins were mixed with the RNA before addition to the cell culture medium, an infection enhancement could be observed [29]. The so formed transfection complexes by nucleic acid and cationic polymers were later termed “polyplexes” [30]. High molecular weight cationic polymers used as pDNA delivery systems include polysaccharides such as chitosan and cyclodextrine, polyaminoacids like poly-L-lysine (PLL) and polyarginine, polyethylenimine (PEI), dendrimers or polymeric scaffolds [31]. PLL has been widely used as a non-viral gene carrier since the formation of polyplexes between PLL and pDNA was identified (**Fig. 1**) [32]. The primary epsilone-amine groups of lysine are protonated under physiological conditions and interact electrostatically with negatively charged pDNA. Complexation into nanoparticles protects the nucleic acid from degradation. The positively charged polyplexes associate with the negatively charged cell surface (due to the presence of glycoproteins and glycolipids) and are internalized by the cells. Although PLL has some properties suitable for a gene carrier including biodegradability, the gene transfer efficiency is rather low: After endocytosis, the PLL polyplexes are trapped within the endosome which leads to lysosomal degradation and hampers gene expression.

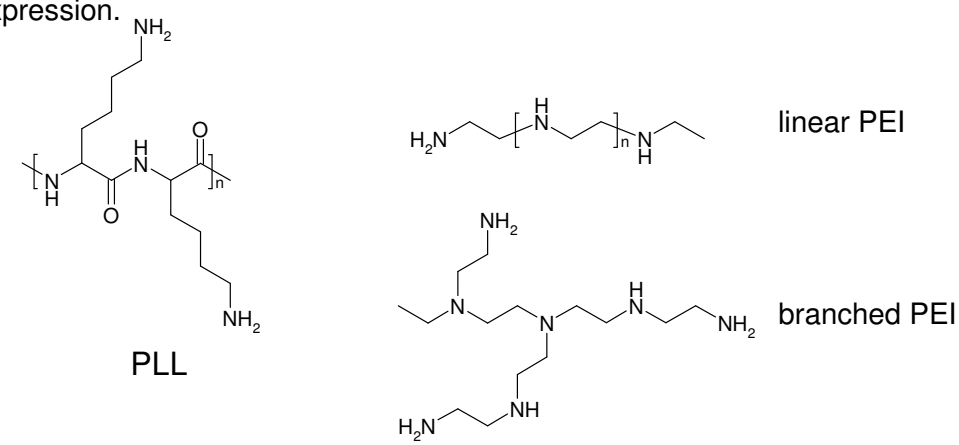


Fig. 1: Structures of polyethylenimine (PEI) and polylysine (PLL)

Over the past decade PEI polymers have evolved into currently the most frequently used polycations (**Fig. 1**) [33]. They contain primary, secondary and tertiary amines which are partially protonated in a physiological milieu [34]. The resulting high charge density is ideal to compact nucleic acids into nanosized particles [35]. To form small particles in the range of 50 to 500 nanometers, an excess of PEI nitrogens to DNA phosphates is necessary. As a consequence polyplexes have an overall net positive charge. A low PEI nitrogen to DNA phosphate ratio (N/P) below 4 leads to the formation a large aggregates [36]. PEI polymers can be synthesized with different molecular weights in a linear or branched structure. Both

paramaters have large influence on the transfection efficiency [37;38]. In contrast to PLL, PEI is not degradable but can mediate efficient gene transfer without the use of an additional endosomolytic component because it possesses inherent endosome-escape properties. PEI containing polyplexes have been used successfully *in vivo* including nucleic acid transfer in brain tumors and lung [7;39].

The 2001 / 2002 discovery that RNAi is active in mammals was the most exciting technical breakthrough in biological research of the last decade. Although non-viral gene delivery could up to now not make the breakthrough for pDNA delivery, the long experience in non-viral gene delivery helped providing several promising proof-of-concept siRNA delivery solutions in a relatively short period of time. There are two approaches for bringing siRNA to effect. One is to stably express siRNA precursors, such as shRNAs, from pDNA vectors using gene therapy; the other is to directly deliver synthetic siRNAs. The latter approach has the advantage that delivery into the nucleus is not necessary (one major unsolved problem of non-viral pDNA-based gene delivery) and that synthetic siRNAs can be stabilized by chemical modifications. siRNAs can be stabilized against nuclease degradation in the plasma by introducing phosphorothioate backbone linkages for exonuclease resistance or 2′-modifications (e.g. 2′-OMe) for endonuclease resistance [40;41]. Current formulations for siRNAs include liposomes, lipoplexes, polyplexes and siRNA-conjugates [42;43]. It has to be considered that not every system which is effective in pDNA delivery also works for siRNA delivery [44;45]. Although locally applied siRNA therapeutics for macular degeneration, hepatitis B and respiratory syncytial virus are already in clinical trials and the first phase I human trial using systemically applied transferrin-receptor targeted cyclodextrin-containing polymer siRNA formulations has been initiated in cancer patients in 2008 [46], further work on resolving the challenges of systemic siRNA delivery for all cell types and organs is necessary to provide the fullest possible clinical benefit of this technology.

1.3 Physiological barriers of nucleic acid delivery

As mentioned, nucleic acid carriers are faced with numerous extracellular and intracellular barriers. For reaching distant target sites the vectors should show an elongated plasma circulation time. However any particle will be treated like an invading micro-organism. Opsonization by serum proteins, mainly by hydrophobic interactions, and following phagocytosis by cells of the reticulo-endothelial system will lead to fast clearance. The positive charge of polyplexes not only leads to target cell attachment and internalization, it also causes unspecific charge-mediated interactions which take place with the ubiquitous occurring negatively charged membranes, e.g. of blood components, vascular endothelial cells or other non-target tissues. To avoid unspecific interactions, recognition by the immune

system and to increase circulation time, the surface of the particles can be modified with hydrophilic polymers, e.g. polyethylene glycol (PEG). PEG-lipids have been incorporated into lipoplexes [47;48] and also polyplexes have been PEGylated to reduce the surface charge [49;50]. Consequences of PEG grafting of carriers may vary for different nucleic acid types, for example siRNA or pDNA complexes. Recently the effect of different molecular weights and degrees of PEG substitution on PEI-graft-PEG polymers for pDNA and siRNA delivery was evaluated [51]. The researchers revealed that in contrast to the pDNA transfection efficiency, siRNA delivery was much less dependent on density and molecular weight of PEG-modifications of PEI.

To reach the site of action within the target cell, the nucleic acid has to stay associated with its carrier during the complete extracellular delivery process. As described, conventional polyplex and lipoplex formulations are held together electrostatically. A weak point resides in the fact that other charged molecules can disrupt such complexes before they reach the target cell. Burke *et al.* demonstrated that both the serum and the extracellular matrix can lead to vector disassembly. They observed significant vector unpackaging of systemically injected fluorescence-labeled PEI / pDNA polyplexes in the liver, where the labeled DNA was found in different areas than the labeled PEI. This obviously negatively affects gene transfer efficiency [52]. The work of Buyens *et al.* reveals the same weak point. They developed a method based on fluorescence correlation spectroscopy to study the dissociation of siRNA complexes in human serum. The work demonstrates that nonpegylated and pegylated siRNA liposome complexes undergo fast disassembly in full serum. Similar observations were made for PEI / siRNA polyplexes [53]. Probably vector unpacking is more likely a problem in the case of siRNA in comparison with pDNA, as the far larger number of negative charges of pDNA stabilizes the interelectrolyte complex [54;55]. To overcome this drawback polymers can be crosslinked to cage the nucleic acid in the extracellular environment [56;57] or the nucleic acid can be attached covalently [58].

Selective targeting to cell-type specific tissues can be achieved by incorporation of targeting ligands into the delivery vehicles recognizing cell-specific receptors expressed on cell surfaces, in order to promote cellular uptake via receptor-mediated endocytosis [59]. After cellular association of nonviral vectors, particles are internalized by receptor-mediated endocytosis, macropinocytosis, phagocytosis or related processes [60;61]. The internalization route resulting in successful gene expression may depend both on polyplex type and cell type [62;63].

For successful nucleic acid delivery, more barriers have to be overcome in the intracellular environment. The internalized nucleic acid carriers are mostly found in intracellular vesicles. The vesicles fuse to “early endosomes” which have a slight acidic pH of 5.9 – 6. Acidification is driven by ATP-dependent proton pumps in the endosomal membrane. When the pH

continues to drop to pH 5 – 6, “late endosomes” are formed. Late endosomes function to degrade many proteins and lipids but may not be able to digest all the material. Therefore, the next step is a fusion of late endosomes and lysosomes. Lysosomes contain digestive enzymes including nucleases and are considered to be the end product of endocytosis. Hence entrapment in endosomes is associated with degradation of the nucleic acids upon endosomal acidification; the release of particles into the cytoplasm represents a major bottleneck to nucleic acid delivery. Researchers have started to investigate the intracellular kinetics and fate of polyplexes in more detail by single particle tracking [64] and computational modeling (*in silico* transfection). While the internalization step seems to have only a moderate impact on the transfection efficiency, endosomal ecape was identified as significant rate limiting step [65]. After endosomal release pDNA complexes or free pDNA have to traffic through the cytoplasm towards the nucleus. The pDNA has to enter the nucleus to reach the transcription machinery. The cytoplasmic trafficking and nuclear import process is not clarified yet. Free pDNA could be degraded by nucleases in the cytoplasm, hence trafficking is supposed to happen with intact polyplexes by motor proteins [66]. Nuclear import of pDNA is another major bottleneck. Transfection of rapidly dividing cells where the nuclear envelope breaks down during mitosis is much more easier than transfection of non-dividing cells [67]. In case of siRNA, the RISC in the cytoplasm is the target location, therefore trafficking towards the nucleus and nuclear import are irrelevant processes for siRNA delivery. For siRNA delivery, release of the siRNA in the cytoplasm is required by dissociation of the nucleic acid from the electrostatically complexed cationic carrier [68].

Besides cellular membranes, CpG motifs [69] of the nucleic acid represent a further barrier to efficient and sustained gene expression. CpG motifs in the plasmid are agonists for the endosomal toll-like receptor 9 and stimulate an inflammatory immune response which reduces degree and duration of gene expression. When CpG-free plasmids were used in lipid and polymeric pDNA complexes, sustained *in vivo* gene expression in the absence of inflammation could be observed [70;71] .

In brief, an ideal vector has to fulfill a number of different functions to guide its payload to the target location which is different for pDNA and siRNA. It is hard to imagine that one simple polymer alone can get the job done.

1.4 Overcoming endosomal entrapment

1.4.1 Natural models

As pointed out, endo-lysosomal degradation represants a major threat for nucleic acid delivery. Natural cell invading organisms such as bacteria or viruses have evolved skills to

cope with this drawback. Because learning from nature has a long tradition (bionics evolved in the 1960s as a framework to pursue the development of artificial systems based on the study of biological systems), a few examples are reviewed in this section.

Influenza virus, like a number of other enveloped viruses, contains a lipid membrane that it obtains during maturation by budding from the cytoplasma membrane of an infected cell. The membrane contains cellular lipids, but the membrane proteins are coded by the virus. These proteins are inserted into the membrane during biosynthesis. Influenza virus has two membrane glycoproteins, the hemagglutinin (HA) and the neuraminidase (**Fig. 2**) [72]. The HA is synthesized as a single polypeptide chain, which is subsequently cleaved by removal of arginine 329 into two subunits, HA1 (36 kDa) and HA2 (25 kDa). The subunits are crosslinked by a disulfide-bond. HA binds to a sialic-acid containing receptor of the target cell to initiate internalization of the virus. Influenza virus enters the cell by a endocytotic process. Erythrocyte leakage experiments revealed that influenza viruses show high lytic activity at pH 5.0 to 5.8, depending on the particular virus type [73]. At low endosomal pH, a conformational transition of HA is described which results in the activation of membrane-fusion activity together with a molecular rearrangement that exposes a hydrophobic endosomolytic peptide of the HA2 subunit. The observed changes at low pH were also detected by electron-microscopy and resulted in a change of HA antigenicity and immunogenicity [74], confirming an extensive rearrangement. This triggered membrane-fusion activity at low pH mediates the endo-lysosomal escape of the virus into the cytoplasm which avoids intracellular degradation.

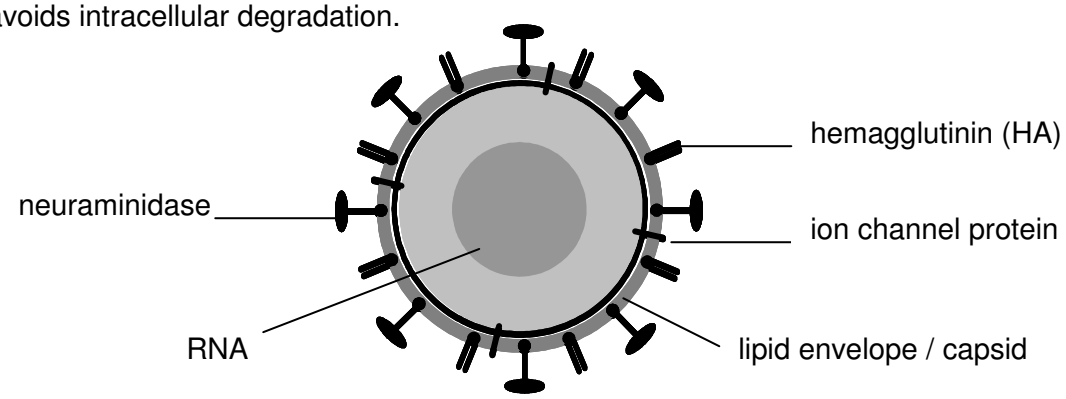


Fig. 2: Illustration of an influenza virus

Another organism with endosomolytic features is the bacterium *Listeria monocytogenes* **(Fig. 3).** The ability of *Listeria monocytogenes* to escape from vacuoles of infected cells and subsequently to replicate in the cytosol and spread from cell to cell is one of the individual features of this facultative intracellular pathogen. *Listeria monocytogenes* enters the cell via phagocytosis. The bacterium escapes into the cytosol before the phagosome fuses with a lysosome. The process of escape is mediated by several proteins. These include the pore-forming cholesterol- and pH-dependent cytolysin listeriolysin O (LLO) and a phospholipase

whose proteolytic activation is mediated by a metalloprotease. LLO is a secretory protein which is only produced when *Listeria monocytogenes* is located within a host. LLO lytic activity has a maximum at pH of 5.5 and rapidly decreases with the increase of the pH. Thus it is selectively activated in acidic phagosomes and shows a reduced activity after successful release in the neutral cytosol [75].

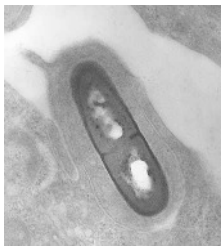
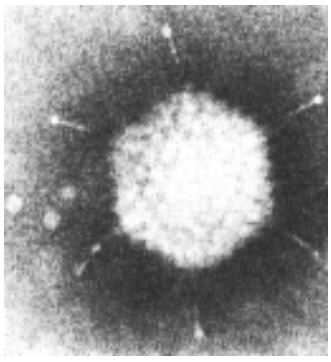


Fig. 3: Electron micrograph of *Listeria monocytogenes* bacterium in tissue

While in the first two described organisms the lytic domain works pH-dependent, the adenovirus utilizes a different pH-triggered strategy. Adenovirus is a non-enveloped virus with an icosaedric capsid (**Fig. 4**). The capsid is composed primarily of 240 homotrimeric hexon capsomers with 12 penton capsomers located at each of the 12 fivefold axes of symmetry. Each penton consists of the fiber protein protruding from the penton base. Further capsid components are thought to act primarily as cement proteins that help to stabilize the viral particle. The interaction of the penton base with cell surface integrins mediates the internalization via clathrin-coated pits [76]. Internalization is thought to trigger capsid disassembly and appears to require endosomal acidifcation. The conformational changes under acidic conditions lead to partial uncoating of the capsid in the endosome, which is necessary for release into the cytosol. During capsid disassembly at low pH, an internal capsid protein (protein VI) is exposed. Protein VI containes a N-terminal amphipathic α-helix which exhibits a pH-independent lytic activity and induces endosomal membrane penetration [77].



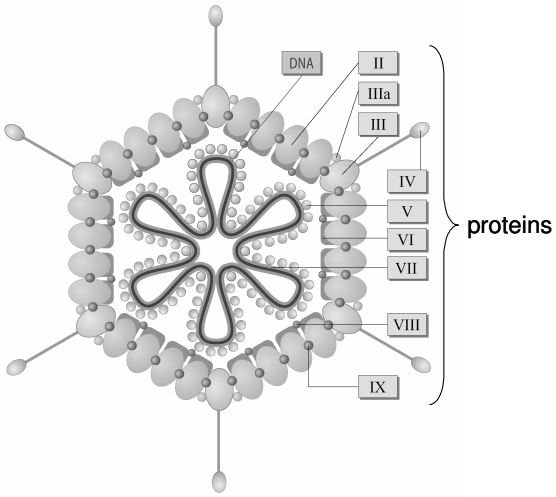


Fig. 4: Illustration of an adenovirus. Picture: Human adenovirus 2. In: ICTVdB - The Universal Virus Database, version 4. Büchen-Osmond, C. (Ed), Columbia University, New York, USA

Changes in physicochemical properties of viruses induced by slight pH differences have been also observed in many other types of viruses. Semliki Forest virus, an enveloped virus,

gains access to cytosol through reorganization and disassembly of its subunits induced by low pH within endosomes. Poliovirus and rhinovirus release its RNA into cytoplasm at acidic pH [78;79].

In summary, natural organisms consist of different functional components for different delivery steps like internalization and endosomal escape. Furthermore it is evident that the lytic activity is exhibited in a pH-specific manner which leads to triggered activation in the endosomal compartment.

1.4.2 Artificial approaches

Some polycations possess considerable buffering properties below the physiological pH, with PEI and polyamidoamine polymers leading the way [80]. These polymers are effective gene carriers without any modification, e.g. cell targeting ligands or membrane disruption domains. PEI containing formulations can promote endosomal escape to a certain degree due to the so called “proton sponge” effect. At neutral pH, PEI is only partially protonated: only approximately every third nitrogen is positively charged. At acidic pH the secondary amines are protonated and the overall protonation level increases [81;82]. During intracellular processing in the endosome, the buffering capacity of PEI will not only inhibit the activity of the lysosomal enzymes that have an acidic optimal pH, but will also increase the osmotic pressure in the vesicle [83]. The accumulation of protons pumped in by the endosomal V-ATPase is linked to an influx of hydrated chloride ions. Presumably this endosomal swelling in combination with membrane-interaction of the polycation induces rupture of the endosomal membrane leading to a release of the gene carrier into the cytoplasm. Histidine or rather imidazole containing polymers can also exhibit a proton sponge effect and mediate endosomal release and efficient gene transfer. The imidazole group of histidine has a pK around 6.0 and thus becomes protonated in the acidic endosomal environment. Because histidine is not charged at neutral pH, poly-L-histidine was combined with nucleic acid binding lysine-domains for the preparation of effective pDNA and siRNA polyplexes [84-86]. Recently PEI / pDNA polyplexes were coated with carboxymethyl poly-L-histidine to form ternary complexes with improved buffering and membrane disruption capacity. The resulting ternary complexes showed gene expression values higher than that of the PEI / pDNA binary complexes [87]. But not only polymers with buffering capacity can induce release into the cytosol, also several lipid-containing formulations possess endosomal escape properties. The membranous nature of the lipoplex seems to be important because it allows the exchange of lipids between the endosomal membrane and the lipoplex, which results in membrane pertubation [88]. A hexagonal phase formation in lipoplexes appears essential for this step, however overall limited insight is available into how endosomal membrane destabilization occurs in case of lipoplexes [89;90].

But despite the proton sponge effect, PEI and related polyplexes demonstrate only inefficient endosomal escape. Especially, endosomal escape represents a major bottleneck when only small amounts of PEI are accumulated per endosome [91]. To overcome this drawback of non-viral nucleic acid delivery, endosomolytic agents were incorporated into nucleic acid carriers [92;93]. Natural agents including adenovirus [94], rhinovirus [79], listeriolysin O [95] and virus-derived peptides [96-98] have been shown to enhance pDNA and siRNA transfection efficiency of polycations. PLL was covalently modified with the N-terminal amphiphilic anionic peptides of the influenza virus haemagglutinin subunit HA2. At neutral pH, the negatively charged peptide is present in a non-helical conformation. At low endosomal pH, protonation causes a transformation to a helical structure, inducing lytic activity. The PLL-peptide conjugate showed a higher lytic activity than the free peptide and kept its pH-dependent lytic activity. Incorporation of the peptide clearly improved gene transfection efficiency [98;99].

Melittin, a cationic lytic peptide derived from bee venom, has been shown to enhance transfection efficiency of lipoplexes and polyplexes *in vitro* [100-102]. Boeckle *et al.* found out that the site of melittin linkage strongly influenced the membrane lytic activity. PEI was covalently attached either to the C-terminus or the N-terminus of melittin, resulting in two different conjugates (C-Mel-PEI and N-Mel-PEI). C-Mel-PEI was highly lytic at neutral pH, increased doses of C-mel-PEI polyplexes induced high toxicity. In contrast, N-mel-PEI showed a reduced lytic activity at neutral pH but maintained a higher lytic activity than C-mel-PEI at endosomal pH. These differences resulted in better endosomal release of N-Mel-PEI polyplexes and efficient gene delivery in different cell lines [103]. For cytosolic delivery of cytotoxic double stranded RNA (polyIC; which triggers interferon responses and apoptosis in targeted tumor cells) a conjugate was constructed containing the lytic peptide melittin, branched PEI for nucleic acid binding and EGF as targeting ligand. The conjugate was applied *in vivo* and facilitated the intracellular delivery of polyIC to glioblastoma tumor [7]. Because melittin has inherent lytic activity at extracellular neutral pH, several melittin analogs were designed to shift the lytic activity towards the endosomal pH. Acidic modification of melittin by replacing neutral glutamines (Gln-25 and Gln-26) with glutamic acid residues improved the lytic activity of melittin PEI conjugates at endosomal pH of 5, but lytic activity at neutral pH was not reduced [104].

Synthetic artificial amphiphatic peptides that mimic natural lytic peptides were also designed. GALA contains 30 amino acids with a main repeat sequence of glutamic acid-alanine-leucine-alanine. Protonation of the glutamic acid residues at acidic pH triggers the transformation from a random coil to an amphiphatic alpha-helix. The exposed hydrophobic domain can interact with lipid bilayers and lead to membrane rupture at acidic pH [105]. Recently Harashima and collegues incorporated GALA into transferrin- and PEG-modified

liposomes which significantly increased transfection efficiency [106]. KALA is a similar basic amphipathic peptide (repeating units of lysine-alanine-leucine-alanine) intentionally designed to combine DNA-binding and membrane-destabilizing activities [107]. Self-crosslinking of KALA via terminal cysteine-residues resulted in a polypeptide which efficiently bound and delivered siRNA [108].

Beside chemical approaches, physical methods like photochemical internalization can be applied to enhance cytosolic delivery of nucleic acids and drugs [109;110]. The technology is based on the activation of photosensitizing compounds that are localized in the membranes of endocytic vesicles. Upon illumination photosensitizers induce formation of reactive oxygen species resulting in rupture of vesicular membranes and release of endocytosed complexes into the cytosol. In the initial applications, photosensitizers were applied as separate molecules. To ensure the necessary co-localization with the drug after systemic administration, recently formulations were developed where the photosensitizer is incorporated into the nucleid acid complex [111].

In summary, existing data prove that endosomal release is a major barrier of nucleic acid delivery. In the past, several chemical and physical approaches have been developed to boost the efficiency of synthetic delivery sytems.

1.5 Design of programmed nucleic acid carriers

On closer inspection of the complete process of nucleic acid delivery (**Fig. 5**), the carrier is not only faced with various delivery steps and requirements, it even has to fulfill contrary functions with time: Outside the cell the nucleic acid has to be stably associated with the carrier, but in the cytosol or nucleus the carrier has to release the nucleic acid. The endosomal membrane has to be destabilized for efficient delivery, but with regard to toxicity outside the endosome, membrane lytic activity is unfavorable. PEG shielding of carriers is beneficial for systemic circulation and reduction of undesired interactions. But the advantage of shielding is limited to the extracellular environment. Within the endosome, PEGylation may hinder lipid membrane interactions of the carrier which might be required for endosomal release. The conflicting requirements outside and inside the target cell present a dilemma: the surface charge and lytic activity must be shielded in the extracellular environment, but after arrival at the target cell and following internalization the lytic activity / the positive charge has to be present. Polymers like PEI appear to be a good compromise. They bind pDNA in a manner which is stable enough for the extracellular transport but also weak enough for intracellular release of their payload. The polymer is positively charged at neutral extracellular pH which leads to membrane interaction and internalization, but only in combination with the buffering capacity in the endosome the membranes are ruptured.

However even the effectivity of such “jack-of-all-trades“-polymers is limited [52;101], a compromise seems to be not always the ideal way.

To handle the described dilemma, the unique functions should be active only during the phases of the delivery process were they are required, e.g. the hydrophilic polymer shield should be present in the extracellular environment but be removed at the target site. Like many viruses, the nucleic acid carriers should sense the environment and dynamically alter their properties according to the required tasks [112]. As biological stimuli for programmed gene carriers, differences in the pH, the redox-gradient and varying concentrations of specific enzymes have been exploited.

1.5.1 Redox- and enzyme-sensitive systems

Disulfide bonds are stable in circulation, but are cleaved upon entry into the reductive intracellular environment, thus allowing site specific cleavage. siRNA was covalently bound to the carrier via a disulfide bond to ensure extracellular stability. The relatively high intracellular concentration of the physiological reducing agent glutathione triggers the release of the siRNA in the cytosol [58;113]. The difference in redox potential has been also used for triggered polymer biodegradation. Linear disulfide crosslinked low-molecular-weight polyethylenimine, a biodegradable pendant to linear PEI, was synthesized and displayed high efficiency but reduced cytotoxicity [114]. Takae *et al.* developed PEG-based polyplex micelles, which can detach the PEG chains in response to the intracellular reducing environment. PEG was linked to the polycation via a cleavable disulfide linkage to form the block copolymer. The copolymer formed stably dispersed polyplex micelles with pDNA. After treatment with dithiothreitol the polyplex micelles aggregated indicating disulfide cleavage followed by PEG detachment. Transfection experiments revealed a superior delivery efficiency of the biocleavable PEG-micelles in comparison to the stable PEGylated micelles [115].

Enzyme specific removal of the PEG shield has been exploited by Hatakeyama and colleagues [116]. Matrix metalloproteinases (MMPs) are a family of endopeptidases that are specially expressed on tumor cells. As MMP substrate a PEG-peptide-lipid conjugate was synthesized and incorporated into a multifunctional envelope-typed nano device. After reaching the target cell MMPs cleave the PEG shield which enhanced gene transfer in comparison to a non-cleavable PEG shield.

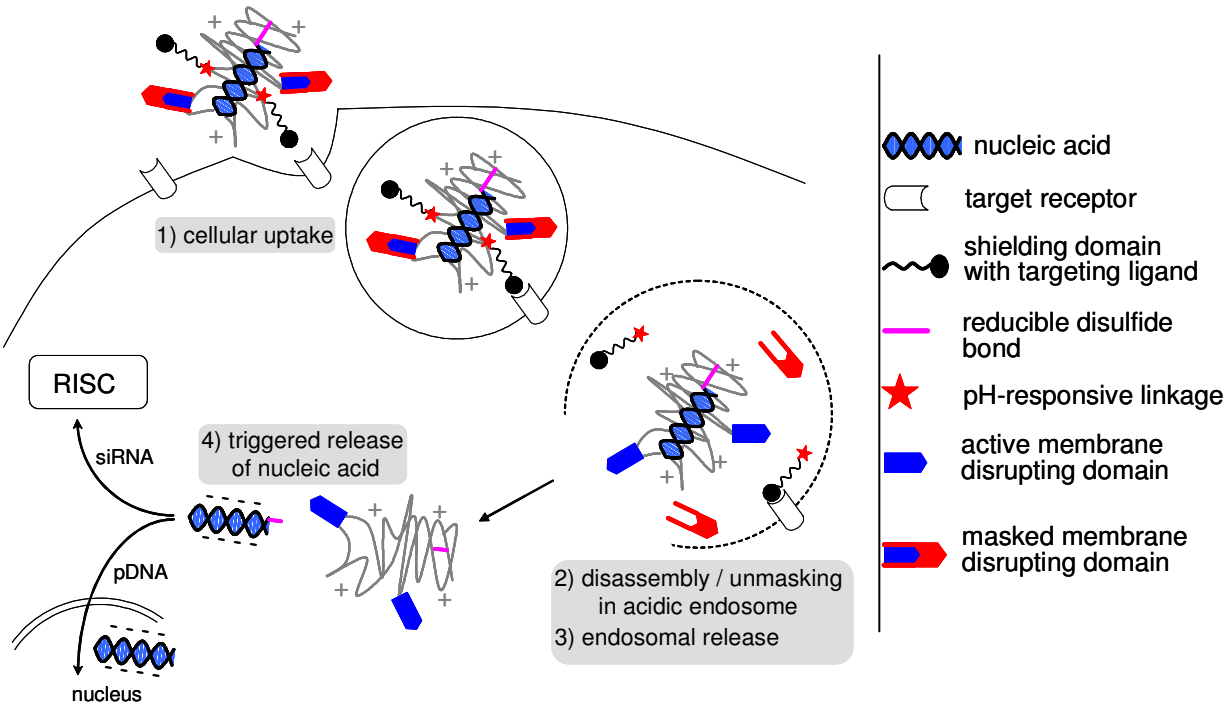


Fig. 5: Nucleic acid delivery process using a dynamic bioresponsive (polymeric) carrier
After association with the target cell via ligand-receptor interaction the carrier is internalized by receptor-mediated endocytosis. Acidification during the endo-lysosomal pathway cleaves the shielding coat (e.g. PEG) and unmasks a membrane disrupting domain. Next, the carrier is released into the cytoplasm. Nucleic acid release of the covalently bound nucleic acid or polymer degradation can be triggered by the reductive environment in the cytosol (disulfide cleavage by glutathione). Dependent on the type of nucleic acid the site of action is the cytosol (siRNA) or the nucleus (pDNA).

1.5.2 pH-sensitive systems

For triggered deshielding, also endosomal acidification can be exploited as physiological stimulus. Endosomal and lysosomal vesicles exist at pH values between 4.5 and 6.5, in contrast to the extracellular neutral pH. The acidic environment has been used to remove the PEG coating from liposomal and polymer-based gene vectors [117;118]. Acid cleavable bonds include acetals [119], hydrazones [120;121], orthoesters [122;123], thiopropionate linkers [124], dialkylmaleic acid monoamides [113] or vinyl ethers [125]. Moreover pH-triggered deshielding via diffusion could be achieved by use of non-covalently attached protective copolymers. Positively charged pDNA or siRNA complexes are ionically coated with PEG containing negatively charged molecules which act as anchors. At acidic pH in the endosomes protonation of the negatively charged PEG anchors reduces the electrostatic interactions and mediates dissociation of the shielding coat from the pDNA or siRNA complexes [126;127].

pH-responsive deshielding elements can be combined with targeting ligands. For example, EGF containing PEI-polyplexes were combined with PEG chains attached via pH-sensitive hydrazone linkers. The pH-responsive deshielding enhanced gene transfer up to 100-fold *in vitro* compared to stable shielded carriers and also improved tumor specific *in vivo* transgene

expression after intravenous injection [120;121]. Instead of hydrazone linkages also pH-responsive piperazinobenzaldehyd PEG acetal groups were applied. A novel maleimide-containing acetal-based PEG reagent was coupled to thiol-functionalized PEI and consequently incorporated into polyplexes. Both EGF- and Tf-receptor targeted polyplexes enhanced *in vitro* luciferase gene expression in receptor-expressing target cells as compared to their stably shielded analogues [119]. Cleavage of the PEG chains in the endosomal vesicles leads to an effective endosomal escape presumably due to interactions between the exposed polycation and the negatively charged endosomal membrane.

Kataoka and colleagues developed a lactosylated PEG-antisense oligonucleotide conjugate (Lac-PEG-ODN) containing an acid-labile beta-propionate linkage between the PEG and ODN segments. They combined Lac-PEG-ODN with PLL to form polyion complex micelles with targeting lactose domains on their surface. It's worth mentioning that in this case the PEG molecule is not coupled to the polycation but to the nucleic acid. The lactosylated formulations showed better uptake and antisence effect than the lactose-free formulations on hepatoma cells. Without acid-labile linkage the antisense efficiency decreased considerably, suggesting that pH-triggered disassembly of Lac-PEG-ODN in the endosome is a key issue in the delivery process [128].

As mentioned above, the lytic peptide melittin enhances the transfection efficiency. Unfortunately, melittin displays high lytic activity at neutral pH which has an unfavorable effect on the toxicity profile of the polyplexes when administered systemically because lytic activity is not restricted to the endosome. Rozema *et al.* showed how this problem may be solved: the primary amines of melittin were modified with a dimethylmaleic anhydride derivative, which masks the lytic activity at neutral pH [129]. Following acidification, the maleamate shield is cleaved and the lytic activity of melittin is restored. This approach showed good results in the delivery of phosphorodiamidate morpholino ODNs (PMOs). PMOs are uncharged antisense nucleotide analogues, which are endocytosed but are unable to escape from the endosome. The masked melittin and the PMOs were co-delivered for the transfection experiments. Co-administration resulted in an increase of antisense efficiency in comparison to PMOs alone. These results are promising but for *in vivo* delivery, the membrane-disruptive peptide has to be associated with the vector. The same group developed a bioresponsive "PolyConjugate" to deliver siRNA specifically to hepatocytes after intravenous injection [113]. The conjugate consisted of a membrane-disruptive polymer whose amine groups were modified with a maleic anhydride PEG shielding reagent, generating acid-labile maleamate linkages and thus a reversibly masked lytic activity. Additional the hepatocyte targeting ligand N-acetylgalactosamine was reversibly attached to the polymer via pH-sensitive maleamate linkages. Covalent coupling of the siRNA to the carrier via a reductive cleavable disulfide bond ensured extracellular stability and triggered

siRNA release in the cytosol. Using this conjugate effective knockdown of two endogenous genes in mouse liver (apolipoprotein B and peroxisome proliferator-activated receptor alpha) was demonstrated. Besides bioresponsive deshielding and lytic activity, the difference in pH can also be utilized to trigger the degradation of polymers for toxicity reduction. Polyplexes with acid-labile polymers showed an improved toxicity profile compared to those made with acid-stable polymer analogues [130].

In general shielded complexes with biocleavable linkages showed enhanced nucleic acid delivery activity when compared with their corresponding formulations lacking bioresponsive linkers. The reviewed approaches highlight the superiority and increasing impact of dynamic delivery systems in comparison with their static counterparts [131;132].

1.6 Aims of the thesis

The lack of efficient nucleic acid delivery systems still limits the therapeutic application of pDNA and siRNA. Poor endosomal release is one major barrier of nucleic acid delivery. Endosomolytic polyethylenimine-melittin conjugates exhibited enhanced gene transfer efficiency. However, cytotoxicity due to their general membrane destabilizing properties restricts their utilization. To overcome this drawback, melittin's lytic activity can be reversibly masked in the extracellular environment. The concept of a reversible acylation of melittin through a dimethylmaleic anhydride derivative was presented by Rozema *et al.* and generated a lytic activity triggered only upon acidification [129]. Simultaneous co-delivery of the modified peptide and oligonucleotides enhanced cytosolic drug delivery *in vitro.* However melittin does not stably associate with pDNA or siRNA under physiological conditions [102]. The first aim of this study was to covalently modify a non-endosomolytic polycation with a pH-responsive masked melittin derivate to enhance gene transfer efficiency. Dimethylmaleicanhydride modified melittin peptide (DMMAn-Mel) was grafted on PLL and the effect of the modification on lytic activity, gene transfer efficiency and cytotoxicity had to be investigated.

Hydrophilic polyethylene glycol (PEG) has been used as part of polycation conjugates to enhance solubility and stability of polyplex formulations. In addition PEG may shield the surface charge, reducing undesired nonspecific interaction and cytotoxicity. The second aim of this study was to elucidate if the DMMAn-Mel concept is transferable to PEI-PEG and PLL-PEG block copolymers. On basis of the PEG copolymers, conjugates containing the lytic peptides melittin, DMMAn-Mel and influenza peptide were synthesized and pDNA and siRNA delivery efficiencies had to be compared.

Conventional polyplex and lipoplex formulations are held together electrostatically. A weak point resides in the fact that other charged biomolecules can disrupt the complexes before they reach the site of action. Other research groups demonstrated that both serum and extracellular matrix can lead to vector disassembly [52;53]. They observed significant vector unpackaging of systemically injected PEI / pDNA polyplexes and dissociation of siRNA complexes in human serum. As the third aim of the thesis, to guarantee the association of the siRNA with the polycationic carrier, a synthetic method to covalently attach the siRNA had to be developed. Accordingly, the most promising noncovalent carrier for siRNA delivery as found in the second part of the thesis was covalently modified with siRNA. To enable release of the free siRNA in the cytosol a reducible disulfide bond was chosen as linkage. Release of the siRNA under physiological conditions had to be investigated by agarose gel electrophoresis studies and the gene silencing activity of the siRNA-conjugate had to be compared with the activity of a corresponding polyplex containing electrostatically complexed siRNA.

2 Material and methods

2.1 Chemicals and reagents

Branched polyethylenimine (PEI; average MW = 25 kDa) and poly-L-lysine-hydrobromide (PLL, MW = 32000, degree of polymerization (DP) = 153 or PLL, MW = 55000, DP = 263) were obtained from Sigma-Aldrich (Munich, Germany).

Succinimidyl 3-(2-pyridyldithio) propionate (SPDP), 2,3-dimethylmaleicanhydride (DMMAn) and succinicanhydride (Succ), 1,4-Dithiothreitol (DTT), Tris(2-carboxyethyl)phosphine (TCEP), ethidium bromide (EtBr) were obtained from Sigma-Aldrich. Succinimidyl propionate monomethoxy polyethylene glycol (SPA-mPEG, MW = 5000) was purchased from Fluka (Buchs, Switzerland). RNAse-free water, absolute ethanol, deuterium oxide (D_2O) and dimethyl sulfoxide puriss. (DMSO) were purchased from Sigma-Aldrich. Water was used as purified, deionized water.

Cysteine-modified melittin (Mel) was obtained from IRIS Biotech GmbH (Marktredwitz, Germany). Mel had the sequence CIGA VLKV LTTG LPAL ISWI KRKR QQ (all-(*D*) configuration), the C-terminal amino acid was introduced as carboxylic acid, the N-terminal amino acid as amine. All-(*D*) stereochemistry was used because it is nonimmunogenic while being as lytic as the natural peptide. Influenza peptide (INF7, sequence GLFE AIEG FIEN GWEG MIDG WYGC) was synthesized by Christian Plank, Institute of Experimental Oncology and Therapy Research, Technical University Munich.

Plasmid pCMVLuc (Photinus pyralis luciferase under control of the CMV enhancer/promoter) was produced with the Qiagen Plasmid Giga Kit (Qiagen, Hilden, Germany) according to the manufacturer recommendations. Ready to use siRNA duplexes were purchased from MWG-Biotech (Ebersberg, Germany) namely, luciferase-siRNA: GL3 luciferase duplex: 5'-CUUACGCUGAGUACUUCGA-3'; control-siRNA: non-specific control duplex IX with similar GC content as anti-luciferase-siRNA and Cy5-siRNA: Cy5-labeled GL3 luciferase duplex: 5'-Cy5-CUUACGCUGAGUACUUCGA-3'. Thiol-modified GL3 and control siRNA were purchased from Dharmacon. Thiol-modified GL3 siRNA had the sequence 5'-thiol-CUUACGCUGAGUACUUCGAdTdT-3' (sense). Thiol-modified control siRNA had the sequence 5'-thiol-AUGUAUUGGCCUGUAUUAGUU-3' (Dharmacon's non-targeting control#3).

Cell culture media, antibiotics, and fetal calf serum were purchased from Invitrogen (Karlsruhe, Germany). Formulations for nucleic acid delivery were prepared in HBG (HEPES-buffered glucose solution; 20 mM HEPES, 5% glucose, pH 7.4). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was obtained from Sigma-Aldrich. Bafilomycin A1 was purchased

from Alexis Biochemicals (Lausen, Switzerland). Luciferase cell culture lysis buffer and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany).

2.2 Conjugate synthesis

2.2.1 Synthesis of 3-(2-pyridyldithio)propionate-modified PLL

PLL (1 µmol; DP = 263) in 0.8 ml buffer (0.5 M NaCl, 20 mM HEPES, pH 7.4) was mixed with SPDP (20 µmol) dissolved in 200 µl dimethyl sulfoxide (DMSO). After 2 hours at room temperature (RT) PLL with pyridyldithiopropionate linkers (PLL-PDP) was purified by size exclusion chromatography (SEC) using an Äkta Basic high-performance liquid chromatography system (Amersham Biosciences, Freiburg, Germany) equipped with a Sephadex G25 superfine HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 0.5 M NaCl, 20 mM HEPES, pH 7.4; the flow rate was 0.5 ml/min. The void fractions containing PLL were pooled, aliquots were snap frozen in liquid nitrogen and stored at -80 °C. PLL content of the fractions was measured by trinitrobenzenesulfonic acid (TNBS) assay at 405 nm. The degree of modification with the dithiopyridine linker was determined spectrophotometrically at 343 nm by release of pyridine-2-thione: 10 µl of the sample was added to 140 µl water and blank absorbance at 343 nm was measured. Subsequently 5 µl of the reducing agent dithiothreitol (0.5 µmol, 77 µg; diluted in water) was added and release of pyridine-2-thione at 343 nm (molar absorptivity = 8080 M^{-1} cm^{-1}) was measured. For the synthesis of PLL-Mel and PLL-DMMAn-Mel, the same PLL-PDP batch was used with a molar ratio of PLL/PDP of approximately 1 to 13. The average yield was 60 % (w/w percent) based on analysis of PLL .

2.2.2 Synthesis of PEG-modified PEI and PLL

PLL (1.25 µmol; DP = 153) in 2 ml buffer (0.5 M NaCl, 20 mM HEPES, pH 7.4) was mixed with mPEG-SPA (1.6 µmol, 8 mg) dissolved in 400 µl DMSO. After 2 h at RT the reaction mixture containing modified PLL (PLL-PEG) was loaded on a cation-exchange column (MacroPrep High S; HR 10/10, BioRad, München, Germany) and fractionated with a salt gradient from 0.6 to 3.0 M NaCl in 20 mM HEPES pH 7.4. The flow rate was 0.5 ml/min. The fractions containing PLL-PEG were pooled, dialyzed against water (MWCO of 6000 - 8000) and lyophilized. The degree of modification of PLL with PEG was determined by proton NMR (PLL / PEG = 1 / 1). The PLL content was measured by TNBS assay. The average yield (after dialysis and freeze-drying) was 63% based on analysis of PLL.

Synthesis of PEI-PEG was carried out analogously as described for PEG-modified PLL, starting with 1,25 µmol PEI (31.3 mg) in 2 ml buffer (0.5 M NaCl, 20 mM HEPES, pH 7.4) and mPEG-SPA (2.5 µmol, 12.5 mg) dissolved in 400 µl DMSO. The degree of modification

of PEI with PEG was determined by proton NMR (PEI/PEG = 1/1.5). Polycation content was measured by TNBS assay. The average yield (after dialysis and freeze-drying) was 70% based on analysis of PEI.

2.2.3 Synthesis of 3-(2-pyridyldithio)propionate-modified PEI- and PLL-PEG

PLL-PEG (0.313 µmol, containing 6.45 mg PLL as free base) in 2 ml buffer (20 mM HEPES, pH 7.4) was mixed with SPDP (3.8 µmol, 1.19 mg) dissolved in 200 µl DMSO. After 2 h at RT PLL-PEG with pyridyldithio-propionate-linkers (PLL-PEG-PDP) was purified by gel filtration using an Äkta Basic HPLC System (Amersham Biosciences, Freiburg, Germany) equipped with a Sephadex G25 superfine HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 0.5 M NaCl, 20 mM HEPES, pH 7.4; the flow rate was 1 ml/min. The fractions containing PLL-PEG-PDP were pooled, aliquots were snap frozen in liquid nitrogen and stored at -80 °C. PLL content was measured by TNBS assay. The degree of modification with dithiopyridine linker was determined spectrophotometrically by release of pyridine-2-thione as described in section 2.2.1. The PLL-PEG-PDP conjugate had a molar ratio of PLL/PEG/PDP of approximately 1/1/8. The average yield was 81% based on analysis of PLL.

PEI-PEG-PDP was synthesized analogously as described for 3-(2-pyridyldithio)propionate-modified PLL-PEG. PEI (0.313 µmol) in 1.84 ml buffer was mixed with SPDP (3.8 µmol, 1.19 mg) dissolved in 200 µl DMSO. The PEI-conjugate had a molar ratio of PEI/PEG/PDP of approximately 1/1.5/11. The average yield was 74% based on analysis of PEI.

2.2.4 Synthesis of PLL-Mel

Melittin peptide (1.38 µmol) was dissolved in 400 µl of 0.5 M NaCl, 20 mM HEPES, pH 7.4, and mixed with 1000 µl PLL-PDP (71 nmol PLL with DP = 263, 0.93 µmol PDP) diluted in the same buffer under argon. After 3 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction. PLL-Mel conjugates were purified on the Äkta Basic HPLC system equipped with a Superdex 75 HR 10/30 column equilibrated in 0.5 M NaCl, 20 mM HEPES, pH 7.4. The flow rate was 0.5 ml/min. The void fractions containing PLL-Mel were pooled, aliquots were snap frozen in liquid nitrogen and stored at –80 °C. PLL content of the PLL-Mel conjugate was determined by TNBS assay. The concentration of melittin in the conjugates was measured by absorption at 280 nm (molar absorptivity of melittin = 5570 M^{-1} cm^{-1}). The average yield was 66% based on analysis of PLL.

2.2.5 Synthesis of DMMAn-Mel-modified PEI and PLL

Mel peptide (1.38 µmol) was dissolved in 400 µl of 100 mM HEPES and 125 mM NaOH and mixed with 1000 µl ethanol containing 15.8 µmol DMMAn by rapid vortexing under argon for

0.5 h following concentration and purification via ultrafiltration (Vivascience, Vivaspin 2, MWCO 2000 HY). The acylated melittin was mixed with 1000 µl PLL-PDP (71 nmol PLL with DP = 263, 0.93 µmol PDP) diluted in 1 M guanidine hydrochloride, 0.5 M NaCl, 20 mM HEPES, pH 8. After 3 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction / the modification degree. Subsequent purification was carried out analogously to the PLL-Mel conjugate procedure as described above but using pH 8 buffers. The average yield was 64% based on analyis of PLL.

PEI-DMMAn-Mel conjugate was synthesized analogously as described for PLL-DMMAn-Mel and had a molar ratio of approximately 1/8. The average yield was 54% based on analysis of PEI.

2.2.6 Synthesis DMMAn-Mel-modified PEI- and PLL-PEG

Mel peptide (1.38 µmol, 4 mg) was dissolved in 400µl of 100 mM HEPES and 125 mM NaOH and mixed with 1000 µl ethanol containing 15.8 µmol (2 mg) DMMAn under argon for 0.5 h following concentration and purification via ultrafiltration (Vivascience, Vivaspin 2, MWCO 2000 HY). 1.38 µmol of the acylated melittin was mixed under argon with 1.06 ml PLL-PEG-PDP (116 nmol PLL, 2.39 mg PLL, molar ratio of PLL/PEG/PDP of approximately 1/1/8) diluted in 2M guanidine hydrochloride, 0.5 M NaCl, 20 mM HEPES, pH 8. After 2 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction. The degree of modification was determined at 343 nm by release of pyridine-2-thione from residual PDP linkers after reduction with dithiothreitol. PLL-PEG-DMMAn-Mel conjugates were purified on the Äkta Basic HPLC System equipped with a Superdex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in 0.5 M NaCl, 20 mM HEPES, pH 8. The flow rate was 0.5 ml/min. The void fractions containing PLL-PEG-DMMAn-Mel (molar ratio of PLL/PEG/DMMAn-Mel of approximately 1/1/8) were pooled and aliquots were snap frozen in liquid nitrogen and stored at – 80 °C. The PLL content of the conjugate was determined by TNBS assay. The average yield was 60% based on analysis of PLL.

PEI-PEG-DMMAn-Mel conjugate was synthesized analogously, starting with 1.34 ml PEI-PEG-PDP (83.6 nmol PEI, 2.1 mg PEI, molar ratios PEI/PEG/PDP of approximately 1/1.5/11) yielding in PEI-PEG-DMMAn-Mel conjugate with a molar ratio of PEI/PEG/DMMAn-Mel of approximately 1/1.5/8. The average yield was 56% based on analysis of PEI.

2.2.7 Synthesis of INF-modified PEI- and PLL-PEG

INF peptide (1.38 µmol) was dissolved in 400 µl of 0.5 M NaCl, 20 mM HEPES, pH 8 and mixed with 1060 µl PLL-PEG-PDP (116 nmol PLL, 2.39 mg PLL, molar ratio of PLL/PEG/PDP of approximately 1/1/8) diluted in the presence of 1.5 M guanidine

hydrochloride. After 2 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction. Following purification was carried out as described for DMMAn-Mel-modified PLL-PEG but using pH 7.4 buffers. The average yield was 43% based on analysis of PLL:

PEI-PEG-INF conjugate was synthesized analogously starting with 1.34 ml PEI-PEG-PDP (83.6 nmol PEI, 2.1 mg PEI, molar ratio of PEI/PEG/PDP of approximately 1/1.5/11). The average yield was 50% based on analysis of PEI.

2.2.8 Synthesis of Mel-modified PEI- and PLL-PEG

Mel peptide (1.38 µmol, 4 mg) was dissolved in 400 µl of 0.5 M NaCl, 20 mM HEPES, pH 7.4 and mixed with 1060 µl PLL-PEG-PDP (116 nmol PLL, 2,39 mg PLL, molar ratio of PLL/PEG/PDP of approximately 1/1/8) diluted in the same buffer under argon. After 2 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction and degree of modification. Subsequent purification was carried out as described for DMMAn-Mel-modified PLL-PEG but using pH 7.4 buffers. The average yield was 65% based on PLL.

PEI-PEG-Mel conjugate was synthesized analogously starting with 1.34 ml PEI-PEG-PDP (83.6 nmol PEI, 2.1 mg PEI, molar ratio of PEI/PEG/PDP of approximately 1/1.5/11). The average yield was 78% based on analysis of PEI.

2.2.9 Synthesis of succinic anhydride-melittin-modified PEI-PEG

Mel peptide (1.38 µmol, 4 mg) was dissolved in 400µl of 100 mM HEPES and 125 mM NaOH and mixed with 1000µl ethanol containing 15.8 µmol (1.58 mg) succinicanhydride by rapid vortexing under argon for 0.5 h following concentration and purification via ultrafiltration (Vivascience, Vivaspin 2 HY, MWCO 2000). Subsequent coupling to PEI-PEG-PDP and purification of PEI-PEG-Succ-Mel was carried out analogously to the PLL-PEG-DMMAn-Mel conjugate procedure as described for DMMAn-Mel-modified PLL-PEG but using pH 7.4 buffer for size exclusion chromatography. The yield was 80% based on analysis of PEI.

2.2.10 Deprotection of thiol-modified siRNA

Thiol-modified siRNA (with the sense strand modified at the 5' end with a thiol-containing linker) was shipped with the thiol group in an oxidized (protected) form to prevent the formation of dimers. Prior to use, the thio groups of the modified siRNA were reduced into the free thiol form. 188 nmol of lyophilized GL3 luc siRNA was incubated for 1 hour at RT with 400 µl 0.05 M TCEP-solution under shaking. 150 µl 9.5 M ammonium acetate and 1.5 ml ice cold ethanol was added and the solution was incubated for 20 minutes at -80 °C. Sequently the sample containing precipitated siRNA was centrifuged at 13400 rpm for 20

minutes. After pouring off the supernatant the sample was air dried for 15 minutes and the pellet reconstituted in 200 µl RNAse-free water. For complete removal of TCEP the precipitating procedure was repeated. siRNA quantification was carried out by obtaining an absorbance at 260 nm. The free thiol-groups were quantified by Ellman's assay. This deprotection protocol allowed relatively high siRNA recovery rates with an approximate yield of 80%. Thiol-modified siRNA of the non-targeting control#3 sequence was prepared in analogous manner.

2.2.11 Synthesis of PLL-PEG-DMMAn-Melittin-siRNA

672 µl PLL-PEG-PDP (2.15 mg PLL; 0.84 µmol PDP) was mixed with 0.1 µmol thiol-siRNA (1.35 mg GL3 luc siRNA) and incubated for 1 h at RT under argon. Before adding siRNA, the solution was adjusted to 1.5 M NaCl. After 1 h at RT released pyridine-2-thione was measured at 343 nm to determine the extent of the reaction and degree of modification with siRNA. Subsequent DMMAn-Mel coupling was carried out analogously to DMMAn-Mel-modified PLL-PEG (2.2.6) except high salt (1.5 M NaCl) was used instead of guanidine hydrochloride. The mixture containing the siRNA-conjugate was loaded onto a Superdex 75 HR 10/30 column (eluent: 1M NaCl, 20 mM Hepes, pH 8.2; flow rate: 0.5 ml/min; 0.5 ml fractions collected). After purification the PLL-containing fractions were pooled and snap-frozen in liquid nitrogen. The yield was 71% based on analysis of PLL. This conjugate was used for the *in vitro* transfection experiments.

A second batch of PLL-PEG-DMMAn-Mel-siRNA conjugate containing GL3 luc siRNA was synthesized analogously, starting with 750 µl PLL-PEG-PDP (3.2 mg PLL; 1.05 µmol PDP). But after purification and pooling of the PLL-containing fractions, the siRNA-conjugate first desalted by dialysis. The sample was placed in a 3 ml Slide-A-Lyzer cassette (MWCO 3500) and dialyzed for 4 hours at 4 °C against 9.5 liter HBG pH 8.2. After dialysis, the siRNA-conjugate was snap-frozen in liquid nitrogen. The yield was 49% based on analysis of PLL.

The conjugate containing Dharmacon's non-targeting control #3 siRNA was synthesized analogously to the first batch of the GL3 luc siRNA – conjugate, starting with 250 µl PLL-PEG-PDP (1.07 mg PLL; 0.35 µmol PDP). The yield was 58% based on analysis of PLL.

2.2.12 Quantitative analysis of PEI and PLL (TNBS assay)

The concentration of PEI and PLL was measured by TNBS assay. Standard PLL or PEI solutions and test solutions containing polycation were serially diluted in 0.1 M sodium tetraborate to a final volume of 100 µl using a 96 well plate, resulting in e.g. PLL hydrobromid concentrations of 10 to 60 µg/ml. To each well 2.5 µl of TNBS (75 nmol, 22 µg; diluted in water) was added. TNBS reacts with primary amino groups to form colored trinitrophenylated

derivatives. After 5 - 20 minutes incubation time at RT (depending on the strength of the developed colour) the absorption was measured at 405 nm using a microplate reader (Spectraflour Plus, Tecan Austria GmbH).

2.2.13 Determination of PEG to polycation ratio

1H NMR spectra were recorded on a Jeol JNMR-GX500 (500 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) and refer to the solvent as internal standard (D_2O at 4.8 ppm). 3 mg of dialyzed and lyophylized material were dissolved in 1.2 ml D_2O.

PLL-PEG (**Fig. 6**): 1.26 – 1.82 (-CH2- ; PLL); 2.96 (-CH2-N-; PLL); 4.27 (-NCHR-COO-); 3.66 (-CH2-CH2-O-; PEG). The content of PEG was calculated from the proton integrated values of PEG (-CH2-CH2-O-, 3.66 ppm) and the polylysine backbone protons (-NCHR-COO-, 4.27 ppm) and from the molecular weight values of the polymers and PEG given by suppliers.

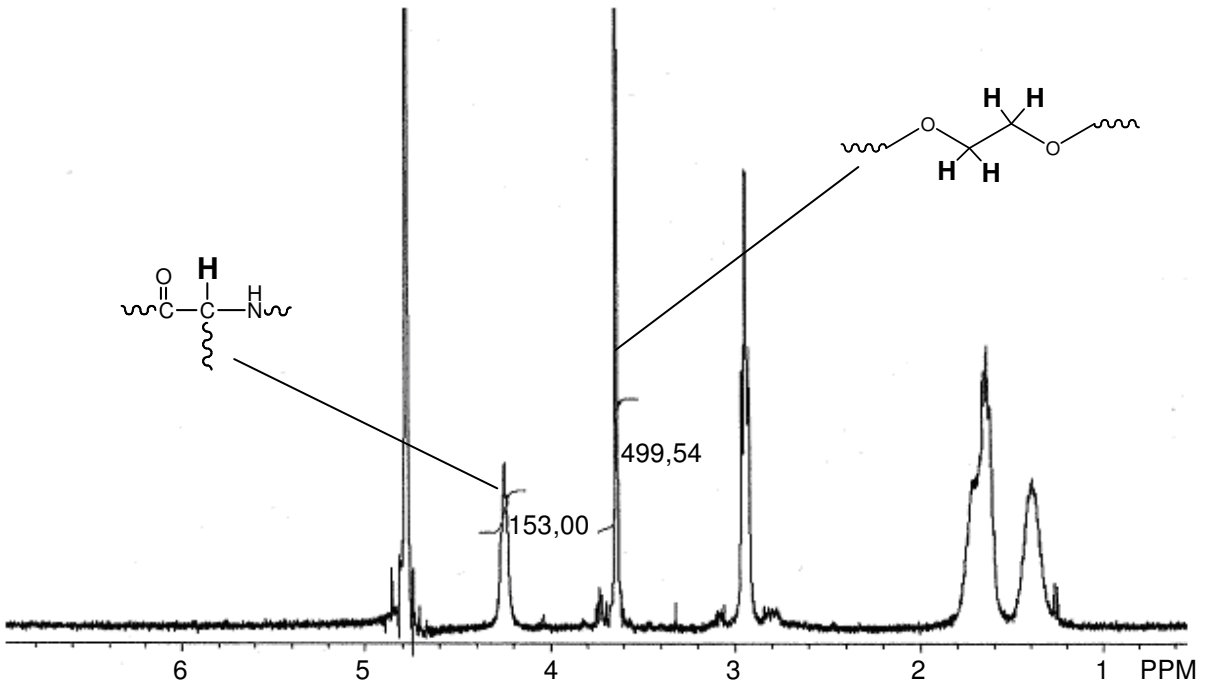


Fig. 6: H-NMR spectra of PLL-PEG. Calculation of PLL to PEG5000 ratio: ppm 4.2 = 153.00 protons (1 PLL molecule, 153 lysines per polymer); ppm 3.8 = 499.54 → 1.1 PEG5000-molecules (453 protons per PEG polymer). PLL / PEG ~ 1/1

PEI-PEG: The content of PEG was calculated (analogously to the PLL-PEG-conjugate) from the proton integrated values of PEG (-CH2-CH2-O-, 3.66 ppm) and the ethylenimine protons (-CH2-CH2-NH-, 2.5 – 3.1 ppm) and from the molecular weight values of the polymers and PEG given by suppliers.

2.2.14 Ellman's assay

This assay is a colorimetric reaction between Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB) and the free thiol groups. 0.4 mg 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) was used as stock solution. For VIS absorption measurement Ellman´s stock diluted 1:10 in Ellman´s buffer was taken as blank. Standard cystein solution and samples were serially diluted in Ellman's buffer and 10% (v/v) of the stock solution. After 20 minutes at 37 °C the solutions were measured at 412 nm and concentration of the thiol group was calculated via the standard curve.

2.3 Polyplex formation

pDNA encoding luciferase was complexed with polycations at different ratios of polycation to pDNA in HBG at a pDNA concentration of 20 µg/ml during polyplex formation. In case of PLL, PLL and PLL-conjugates were mixed at different molar ratios of PLL epsilon-amino nitrogen to DNA phosphates (N/P ratio). For instance, DNA/PLL polyplexes at N/P 2 were prepared by mixing a solution of 2 µg of pDNA in 50 µl HBG with a solution of 2.66 µg PLL in 50 µl HBG. For Tf-targeted polyplexes, polycations PLL (N/P = 1.7) / PLL-Tf (N/P = 0.3) were applied, for Tf-targeted and PEG-shielded polyplexes polycations PLL (N/P = 1.3) / PLL-Tf (N/P = 0.3) / PLL-PEG (N/P = 0.4) were applied. pDNA was condensed with PEI or PEI-conjugates at a molar ratio of PEI nitrogen to DNA phosphate (N/P ratio) of 6. Polyplexes were rapidly mixed by pipetting and allowed to stand for 20 - 30 min at RT before use.

Formulations for siRNA delivery were prepared as follows: first 2 µg siRNA and adequate amounts of the PEI- or PLL-conjugates using various polycation / siRNA ratios (w/w) were diluted in separate tubes in HBG. Then, the polycation solution was added to the siRNA, mixed by pipetting up and down and incubated for 20 – 30 min at RT to form the siRNA polyplexes that were used for transfection experiments.

2.4 Measurement of zeta-potential

Zeta-potential of nucleic acid complexes was measured using a Malvern Zetasizer 3000 HS or a Zetasizer Nano series ZEN 3600 (Malvern Instruments, Worcestershire, UK). Polyplexes were formed at a nucleic acid concentration of 20 µg/ml in HBG and were allowed to stand for 20 – 30 min. For estimation of surface charge with Malvern Zeta Sizer Nano series, transfection complexes were diluted in 1 mM NaCl to give a final nucleic acid concentration of 10 µg/ml and zeta potential was measured. In case of Malvern Zetasizer 3000 HS complexes were diluted in 1 mM NaCl to give a final volume of 5 ml and a DNA concentration of 2 µg/ml. Average values were calculated with the data of 10 runs with standard deviation.

2.5 Measurement of particle size via dynamic light scattering (DLS)

Particle size of nucleic acid complexes was measured by laser-light scattering using a Malvern Zetasizer 3000 HS or a Zetasizer Nano series ZEN 3600 (Malvern Instruments, Worcestershire, UK). Polyplexes were formed at a nucleic acid concentration of 20 µg/ml in HBG and were allowed to stand for 20 – 30 minutes. Prior to size measurement transfection complexes were diluted to give a final nucleic acid concentration of 10 µg/ml.

2.6 Measurement of particle size via fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was employed to determine the size (hydrodynamic radius) of siRNA polyplexes. For FCS experiments siRNA labeled with Cy5 was utilized. FCS detects spontaneous intensity fluctuations resulting from fluorescently labeled molecules diffusing through a highly focused laser illuminated volume within the 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 labeled molecules. For free Brownian diffusion of one identical species of particles, the autocorrelation function is given by

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

Where N represents 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 dimensions 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 $\tau_D = \frac{r_{xy}^2}{4D}$ (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 $\tau_{triplett}$ the characteristic triplett decay time.

For fluorescently labeled spherical particles with dimensions smaller than the illuminated focal volume ($R<<r_{xy}$) diffusing through a homogeneous solvent consisting of small molecules ($R>>r_{solvent}$) the hydrodynamic radius R_H can be calculated from the Stokes-Einstein relation

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (4)$$

D denotes the translational diffusion coefficient of the particle, k_B the Boltzmann constant, T the temperature and η the solvent viscosity.

FCS experiments were performed using a commercially available FCS setup (Carl Zeiss, Jena, Germany), consisting of the Confocor 2 module and an Axiovert 200 inverted microscope equipped with a Zeiss C-Apochromat 40x, N.A. 1.2 water immersion objective. The illumination source was a HeNe Laser (633nm, 5mW) with emission passed through a 650nm long-pass before detection. All FCS measurements were performed at RT. Samples were pipeted 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 Cy5 before each data acquisition. For each sample 10 measurements with sampling time of 30s were performed. The measured autocorrelation curves were fitted (Zeiss ConfoCor2 software package) with equation (3), the resulting diffusion time τ_D was used to calculate the hydrodynamic radius R_H of the siRNA polyplexes via equations (2) and (4).

2.7 Ethidium bromide exclusion assay

The binding strength of the conjugates to siRNA was compared via EtBr exclusion. The fluorescence of EtBr is significantly enhanced by nucleic acid intercalation. When a polycation binds to the nucleic acid, intercalated EtBr is displaced, and a reduction of fluorescence can be noticed. The fluorescence of a 20 µg/ml solution of nucleic acid containing 400 ng/ml of EtBr in HBG was first measured (ex 510 nm, em 590 nm, slit width =

10 nm, Varian Cary Eclipse fluorescence spectrophotometer) and fluorescence was set to 100%. Aliquots of the conjugates were added stepwise to the nucleic acid EtBr solution and the decrease of fluorescence was measured indication interaction of the conjugate with the nucleic acid.

2.8 Agarose gel electrophoresis

A 2.5% agarose gel was prepared by dissolving 1 g agarose (Sigma-Aldrich, Taufkirchen, Germany) in 40 g TBE buffer (trizma base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g ad 1000 ml Millipore water) and boiling everything up to 100 °C. After cooling down to about 50 °C the agarose gel was casted in the electrophoresis unit. Sample preparations containing 500 ng siRNA and loading buffer (12 ml glycerine, 2.4 ml 0.5 M EDTA, 5.6 ml H_2O, 0.04 g xylenecyanole) were placed into the sample pockets. For reduction of disulfides samples were treated with TCEP or glutathione. To inhibit electrostatic interactions between the nucleic acid and the polycations samples were treated with heparin. Electrophoresis was performed at 80 V for 40 minutes. Alternatively a 2.5% agarose gel with 120 g TBE buffer was prepared and settings were adapted.

2.9 Erythrocyte leakage assay

Human erythrocytes were isolated from fresh, citratetreated blood and washed in phosphate-buffered saline (PBS) by four centrifugation cycles, each at 800 g for 10 min at 4 °C. The erythrocyte pellet was diluted 10-fold in 150 mM NaCl. Melittin, DMMAn-melittin and PLL-melittin conjugates were serially diluted in 90 µl buffer (HBS, pH 7.4) using a V-bottomed 96-well plate, resulting in melittin concentrations of 0.25 - 16 µM. For 100% lysis, control wells contained buffer with 1% Triton X-100. A volume of 10 µl of erythrocyte suspension was added to each well and the plates were incubated at 37 °C for 30 min under constant shaking. After centrifugation at 300 g for 10 min, 60 µl supernatant were analyzed for hemoglobin release at 450 nm using a microplate plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria).

2.10 Cell culture

Cell culture media, antibiotics and foetal bovine serum (FBS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). Cultured cells were grown at 37 °C in 5% CO_2 humidified atmosphere. Neuro2A murine neuroblastoma cells (ATCC CCL-131) and Neuro2A-eGFPLuc cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1 g/l glucose) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. HUH-7

hepatocellular carcinoma cells (JCRB 0403; Tokyo, Japan) and HUH-7-eGFPLuc cells were cultured in DMEM high glucose / F12 (1/1) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine

2.11 Luciferase gene silencing

All experiments were performed in stably transfected Neuro2A-eGFPLuc cells. Cells were seeded in 96-well plates (TPP, Trasadingen, Switzerland) using 5000 cells per well 24 h prior to transfection. Transfection complexes containing siRNA were then added to cells in 100 µl culture medium containing 10% serum, 100 U/ml penicillin and 100 µg/ml streptomycin (final siRNA-concentration 367 nmol/l). 48 h after initial transfection medium was removed and cells were lysed in 50 µl Promega cell lysis solution to measure the gene expression as described below. Transfections were also performed with a non-specific control siRNA to distinguish between specific gene silencing and unspecific knockdown of protein expression due to carrier toxicity. Qualitative judgement on the toxicity of the conjugates was made by diminution in luciferase expression upon delivery of the non-specific control siRNA compared to the luciferase expression from the same number of cells that were not exposed to the carrier. Data were generated by Alexander Philipp as part of his PhD thesis (in preparation).

2.12 Luciferase reporter gene expression

Cells were plated in 96 well plates at a density of 10000 cells (Neuro2A or HUH7) per well 24 h prior to transfection. The polyplexes with 200 ng of pDNA (pCMVLuc) were added to the cells in 100 µl culture medium containing 10% serum, 100 U/ml penicillin and 100 µg/ml streptomycin. For investigating the effect of endosomal acidification the transfection medium was supplemented with 200 nM of the inhibitor bafilomycin A1 (Alexis Biochemicals Corporation). The transfection medium was replaced after 3 h by 100 µl fresh cell culture medium. 24 h after initial transfection medium was removed and cells were lysed in 50µl Promega cell lysis solution to measure the gene expression. Luciferase activity was measured using a Lumat LB9507 instrument (Berthold, Bad Wildbad, Germany). Luciferase light units were recorded from an 20µl aliquot of the cell lysate with 10 s integration time after automatic injection of freshly prepared luciferin using the luciferase assay system (Promega, Mannheim, Germany). Transfection efficiency was evaluated as relative light units (RLU) per number of seeded cells. Two ng of recombinant luciferase (Promega, Mannheim, Germany) corresponded to 10^7 light units.

2.13 Cell viability assay (MTT assay)

Metabolic activity of cells was determined using a methylthiazoletetrazolium (MTT) / thiazolyl blue assay as follows: 10 µl of a 5 mg/ml solution of MTT in sterile PBS buffer was added to each well of the 96 well plate. After incubation for 2 h at 37 °C, the medium was removed and cells were frozen for 2 hours (-80 °C). 100 µl of DMSO was added and samples were further incubated at 37 °C for 30 min under constant shaking. Optical absorbance was measured at 590 nm (reference wavelength 630 nm) using a microplate reader (Spectrafluor Plus) and cell viability was expressed as a percent relative to buffer (HBG)-treated control cells.

2.14 Cytotoxicity assay (LDH release assay)

Cells were seeded in 96-well plates and treated after 24 h with different amounts of conjugates ranging from from 2.4 to 14.8 µg/ml. Cytotoxicity was determined after 30 min using the lactate dehydrogenase (LDH) release assay (Promega, CytoTox 96 nonradioactive cytotoxicity assay) according to the manufacturer's instructions.

2.15 Transmission light microscopy

Transmission light microscopy of living cells was performed after 1 hour of conjugate treatment using an Axiovert 200 microscope (Carl Zeiss, Jena, Germany) equipped with a Sony DSC-S75 digital camera (Sony Corporation, Tokyo, Japan). Light was collected through a 32 x 0.25 NA objective (Zeiss), and images were captured using phase contrast.

2.16 Cy5 labeling of pDNA

Plasmid pCMVLuc was labeled with the fluorophore Cy5 using the Label IT kits (MIRUS, Madison, WI) following the manufacturer´s instructions. 20 µg of pDNA were diluted with 1 x Buffer A to a final volume of 195 µl. After addition of 5 µl reconstituted Label IT reagent, the reaction mixture was incubated for 3 h at 37 °C. To precipitate labeled DNA, 550 µl of ice-cold ethanol and 22 µl of 3 M sodium acetate were added. The solution was then mixed and placed at - 20 °C overnight. Subsequent centrifugation at 16000g for 60 min (4 °C) allowed removal of unreacted label in the supernatant. The pellet was gently washed with ice-cold 70 % ethanol, centrifuged again and all traces of ethanol were removed. Labeled DNA was allowed to dry for 5 min and finally resuspended in sterile HBG. Cy5 (ε 650nm = 250000 $l \cdot mol^{-1} \cdot cm^{-1}$) content was measured by absorption at 650 nm. DNA was quantified by measuring the absorbance at 260 nm with the ratio of 260 nm / 280 nm serving as an index

for DNA purity (≥1.8; ≤1.9). On average, one dye molecule was bound per 100 bp, approximately.

2.17 Flow cytometric analysis of cellular polyplex internalization

Neuro2A cells were seeded in 12 well plates (TPP, Trasadingen, Switzerland) at a density of 80000 cells per well 24 h prior to transfection. PLL polyplexes containing Cy5-labeled pDNA (20 % labeled pDNA / 80% unmodified pDNA) were added to the cells at a concentration of 1.5 µg DNA per well in 1 ml fresh culture medium. Polyplexes were generated at a N/P ratio of 2 in HBG. After 3 h incubation at 37° C medium was removed, cells were washed with cold PBS, treated with trypsin / EDTA solution (Invitrogen GmbH, Karlsruhe, Germany), resuspended in cold PBS and kept on ice until cell uptake of polyplexes was assayed using a Cyan MLE flow cytometer (DakoCytomation, Copenhagen, Denmark). Before measurement propidium iodide was added to distinguish dead, permeable cells from undamaged, live cells.

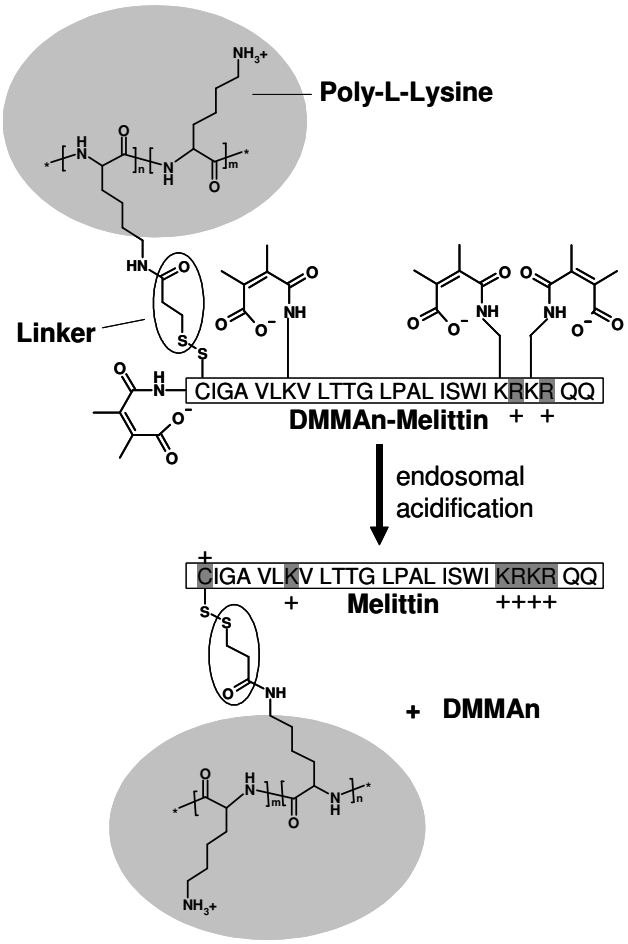
Dead cells show fluorescence caused by binding of the dye to cellular DNA and RNA that is higher staining than that of intact cells. The fluorophore Cy5 was excited at 635 nm and emission was detected at and 665 ± 20 nm. Data acquisition was performed in linear mode and data were analyzed in logarithmic mode. To discriminate between viable and dead cells, cells were appropriately gated by forward versus sideward scatter; to exclude doublets cells were gated by forward scatter versus pulse width, and 2 x 10^4 gated events per sample were collected. Experiments were performed in duplicates.

3 Results

3.1 A dimethylmaleic acid–melittin-polylysine conjugate for pDNA delivery

3.1.1 Design and synthesis of the DMMAn-modified PLL conjugate

Aim of the work was to create an endosomolytic PLL-based non-viral nucleic acid carrier. PLL belongs to the class of synthetic amino acid polycations which can be ionically complexed to nucleic acids. Due to the natural amino acid backbone it is easily metabolized and thus should possess a low long-term toxicity. Endosomolytic properties and transfection activity however are very low. To overcome this limitation, melittin was grafted onto the polycation. DMMAn modification of melittin was carried out to mask the lytic activity in the extracellular environment (and thus reduce acute toxicity of the carrier). Endosomal acidification is exploited to trigger the lytic activity in the intracellular environment. Hence a relatively low cytotoxicity and high gene transfer efficiency should characterize the pH-responsive gene carrier. The design is shown schematically in **Scheme 1**.



Scheme 1: Design of the bioresponsive melittin conjugate

For the synthesis non-immunogenic all-*(D)*-melittin was used. All-*(D)*- and all-*(L)*-melittin have the same hemolytic and antibacterial activities [133;134]. It was shown that gene transfer activity of melittin-PEI polyplexes does not depend on the enantiomeric configuration of the attached peptide [103]. In order to synthesize the endosomolytic gene carrier, DMMAn-Mel was conjugated to PDP-modified PLL by disulfide bond formation. DMMAn-Mel was produced using an excess of DMMAn. Excess of DMMAn in the reaction mixture which could cause undesired acylation of primary amines on PLL during the coupling procedure was removed by filtration through a Vivaspin ultrafiltration unit. Coupling with PLL-PDP was carried out in the presence of 1 M guanidine hydrochloride. Guanidine hydrochloride prevents aggregation of the negatively charged DMMAn-Mel and the polycation before coupling. Purification was performed by SEC with the column equilibrated at pH 8 to avoid acidic cleavage of DMMAn. Resulting conjugates had 7 to 10 molecules peptide per PLL. A schematic illustration of the synthesis is shown in **Fig. 7**.

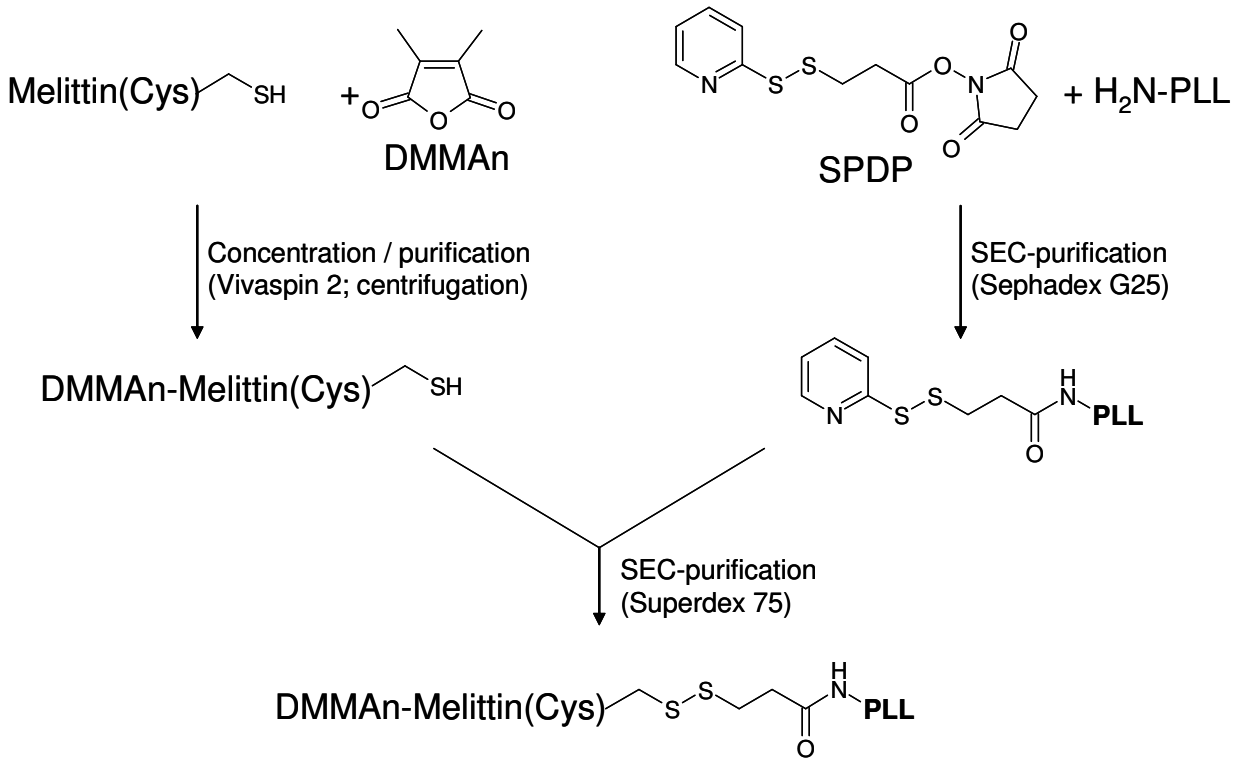


Fig. 7: Synthesis of PLL-DMMAn-Mel conjugate. The primary amino groups of PLL react with the succinimidyl group of SPDP resulting in 3-(2-pyridyldithio)propionate modified PLL. In a second synthesis step the free sulfhydryl groups of cysteine at the N-terminus of DMMAn-melittin react with dithiopyridine to the desired PLL-DMMAn-Mel conjugate.

3.1.2 pH-dependent lytic activity

To evaluate the membrane destabilizing activity of unmodified melittin in comparison with DMMAn-modified melittin, lytic activites of the peptides were investigated in an erythrocyte leakage assay (**Fig. 8**). Erythrocytes were incubated with the peptides in HBS, pH 7.4 (extracellular pH) or citrate buffer, pH 5 (endosomal pH) for 30 minutes at 37 °C. The free peptide exposed high lytic activity (> 93% of hemoglobin release) at neutral pH at concentrations of 4.5 µM and above which is consistent with previously published work [103]. In contrast, DMMAn-Mel showed relatively low lytic activity at neutral pH (< 20%). However, at pH 5 lytic activity of DMMAn-Mel increased and reached comparable levels like unmodified melittin (50 – 60% of hemoglobin release).

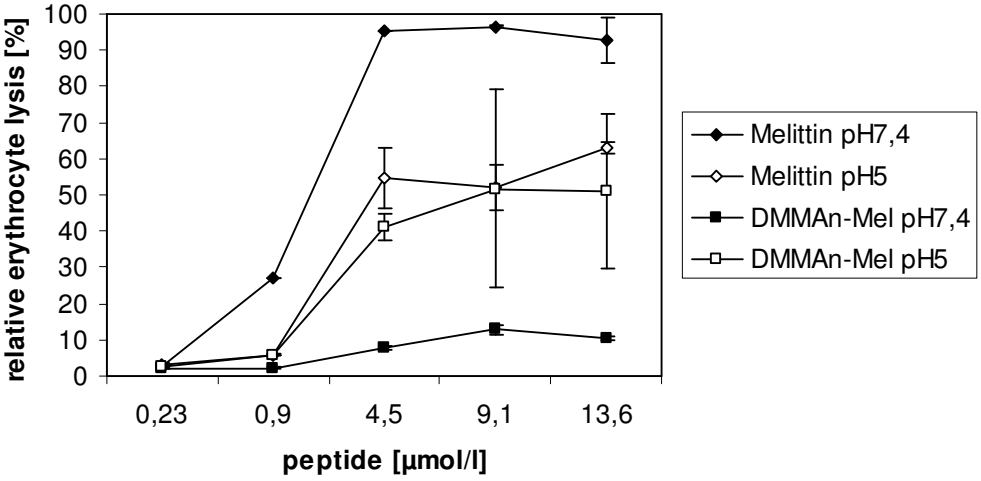


Fig. 8: Erythrocyte leakage assay. Erythrocyte lysis induced by free melittin and DMMAn-Melittin at neutral and endosomal pH. Washed human erythrocytes were incubated with melittin and DMMAn-melittin peptides at different peptide concentrations.

The lytic activity of the PLL-Mel conjugate was similar to free melittin, suggesting that covalent attachment of PLL to melittin did not significantly affect the lytic activity (**Fig. 9**). PLL-DMMAn-Mel conjugate showed relatively low lytic activity at neutral pH (< 20%). After acidic preincubation at pH 5, lytic activity was enhanced (80% hemoglobin release). At neutral pH, a longterm study (2.5 h preincubation of PLL-DMMAn-Mel at 37 °C and pH 7.4) revealed that lytic activity increased, but is still lower compared to the unmodified PLL-Mel conjugate (approximately 50% hemoglobin release at highest concentration).

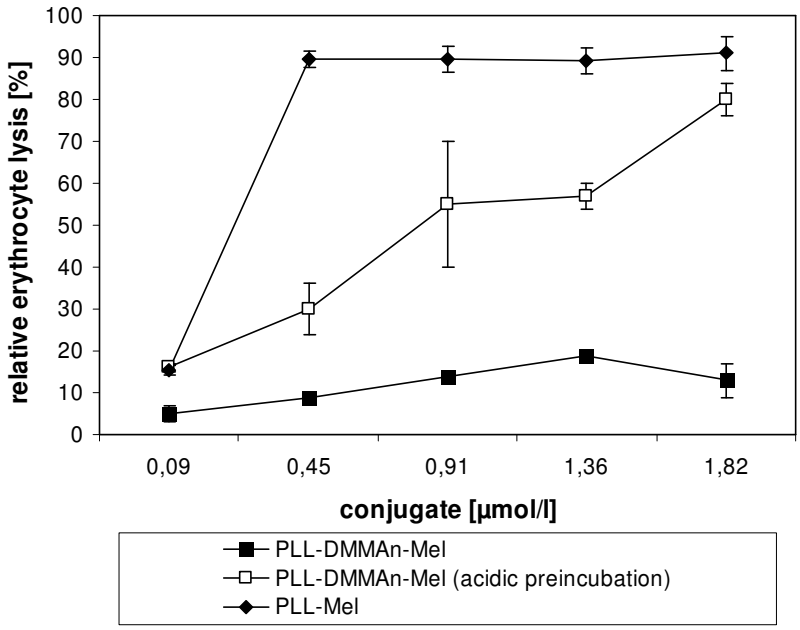


Fig. 9: Erythrocyte leakage assay. Erythrocyte lysis induced by melittin conjugates at pH 7.4. Washed human erythrocytes were incubated with increasing conjugate concentrations. The conjugate PLL-DMMAn-Mel was preincubated at pH 5 for 30 minutes at room temperature (open squares) before performing the assay.

3.1.3 Gene transfer activity

Reporter gene expression (luciferase activity) and cellular toxicity were evaluated in Neuro2A cells after the application of different polyplex formulations. For transfection experiments, polyplexes were generated in HBG at N/P ratios of 1 to 4 (**Fig. 10a**). While PLL mediated only low gene transfer, PLL-Mel showed the highest gene transfer activity at N/P 2. At higher N/P ratios the gene transfer activity of PLL-Mel decreased. In contrast PLL-DMMAn-Mel mediated high gene transfer activity at all N/P ratios (**Fig. 10a**). In parallel a cytotoxicity assay was performed to detect the amount of LDH in the medium which was released after 3 h of transfection time (**Fig. 10b**). While at N/P ratios of 1 and 2 no LDH was detectable in the case of any polyplex formulation, at higher N/P ratios the PLL and PLL-Mel but not PLL-DMMAn-Mel polyplexes induced LDH release up to 15% and 25% LDH release (100% refers to completely lysed cells). A higher toxicity of the PLL and PLL-Mel polyplex formulations as compared to PLL-DMMAn-Mel also explains the decreasing luciferase expression at higher N/P ratios.

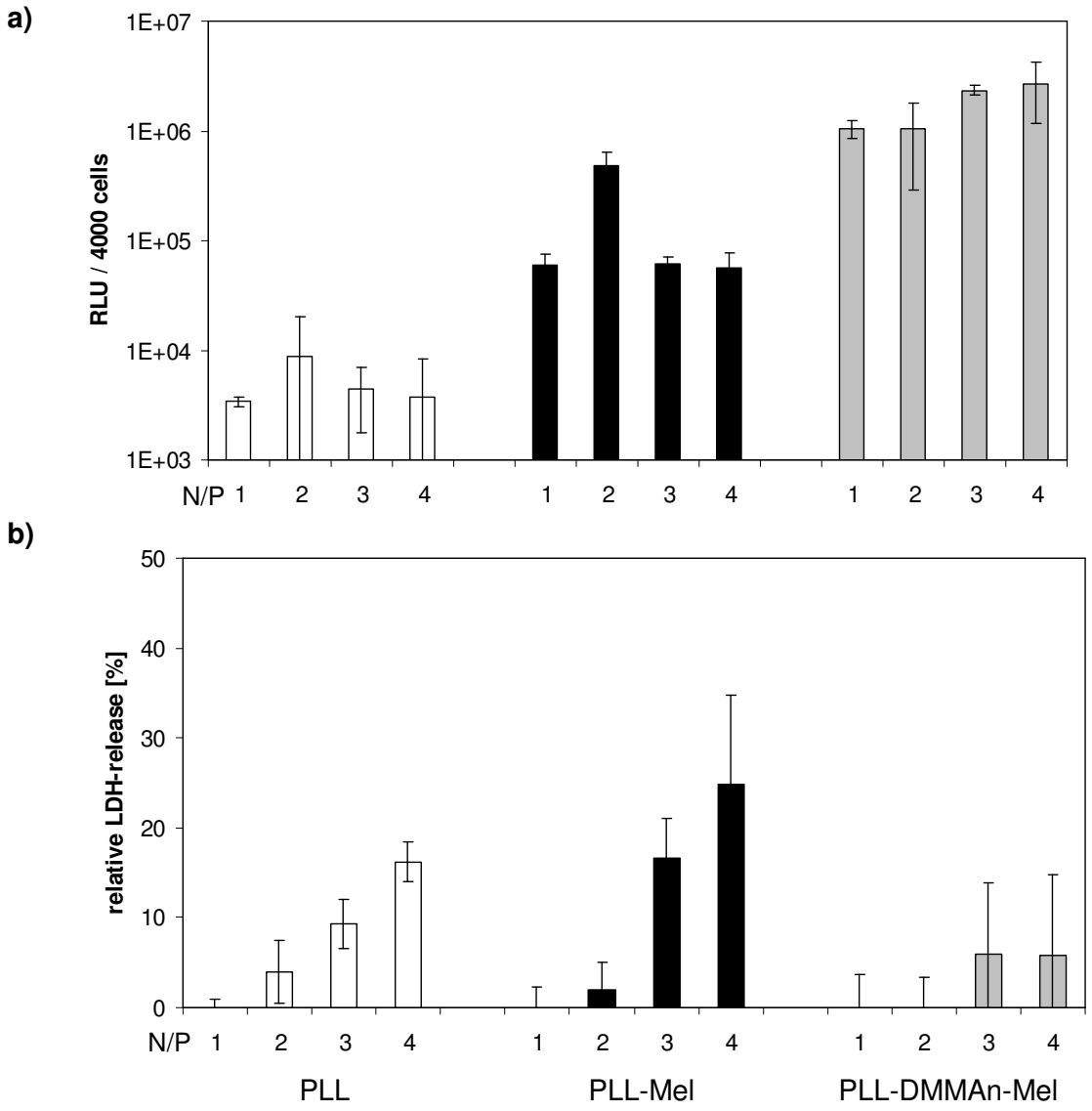


Fig. 10: **Gene transfer with PLL, PLL-Mel or PLL-DMMAn-Mel polyplexes**. Neuro2A cells were treated with pCMVLuc polyplexes at different N/P ratios (1 to 4). Luciferase gene expressions at 24 h after transfection are shown in (**a**). White bars indicate PLL, black bars PLL-Mel and grey bars PLL-DMMAn-Mel. Corresponding LDH-release values are shown in (**b**).

To investigate the effect of endosomal acidification on the gene transfer activity of the conjugates 200 nM bafilomycin A1, an inhibitor of vacuolar ATPase endosome pumps, was included in the transfection medium (**Fig. 11**). As expected, the efficiency of standard PEI polyplexes was about 7-fold reduced. Bafilomycin A1 did not alter gene transfer of PLL polyplexes, but 3- and 8-fold enhanced gene transfer activity of the PLL-Mel conjugate at N/P ratios of 2 and 1, respectively. Gene transfer by PLL-DMMAn-Mel was slightly (up to approx. 2-fold) reduced by the treatment**.**

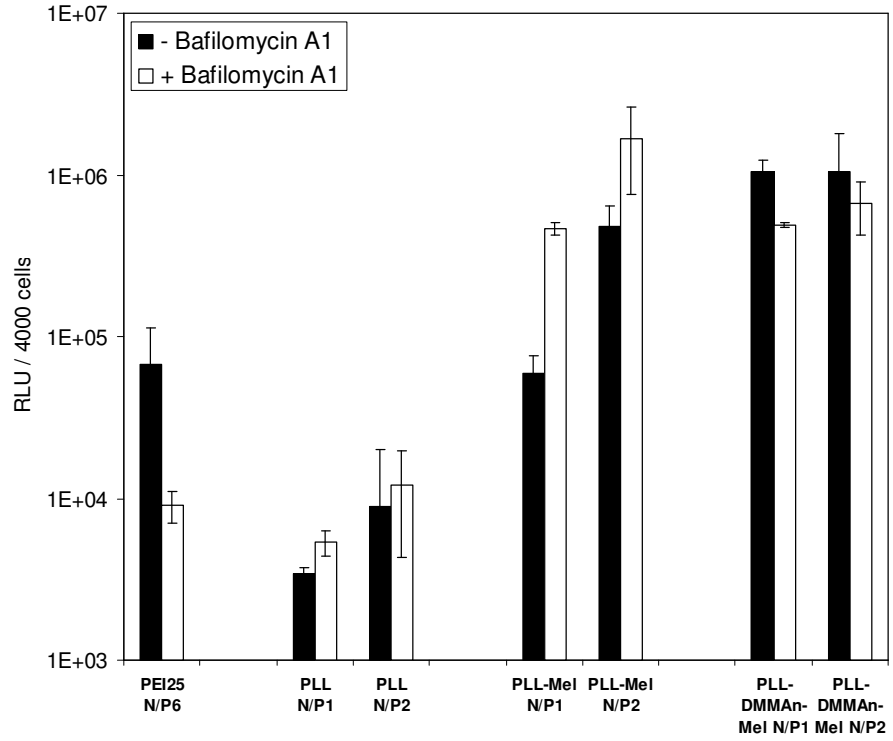


Fig. 11: Effect of bafilomycin A1 on gene transfer. Luciferase gene expression after transfection of Neuro2A cells with PLL, PLL-MeI or PLL-DMMAn-MeI polyplexes in medium supplemented with 200 nM of the inhibitor bafilomycin A1. Medium was replaced by fresh medium after 3 hours, luciferase expression was determined after 24 h.

The incorporation of targeting ligands and shielding domains into polyplexes is advantageous in terms of polyplex stability and systemic administration. Hence polyplexes containing transferrin (Tf) as targeting ligand and polyethylene glycol (PEG 20k) as shielding domain were generated at an N/P ratio of 2. Additional polyplexes containing PLL-PEG with pH-sensitive hydrazone PEG-linkers (PLL-HZN-PEG) were generated and compared with regard to reporter gene transfer. PLL-PEG and PLL-HZN-PEG were synthesized as described in [120]. In all polyplex compositions, linkage of MeI or DMMAn-MeI to PLL enhanced luciferase expression between 85-fold and more than 1000-fold (**Fig. 12**). Incorporation of PEG, either stably- or pH-reversibly-linked, into polyplexes had no considerable negative effect on transfection efficiency.

In summary, attachment of DMMAn-MeI greatly enhanced gene transfer efficiency of PLL, which for itself only revealed poor delivery activity.

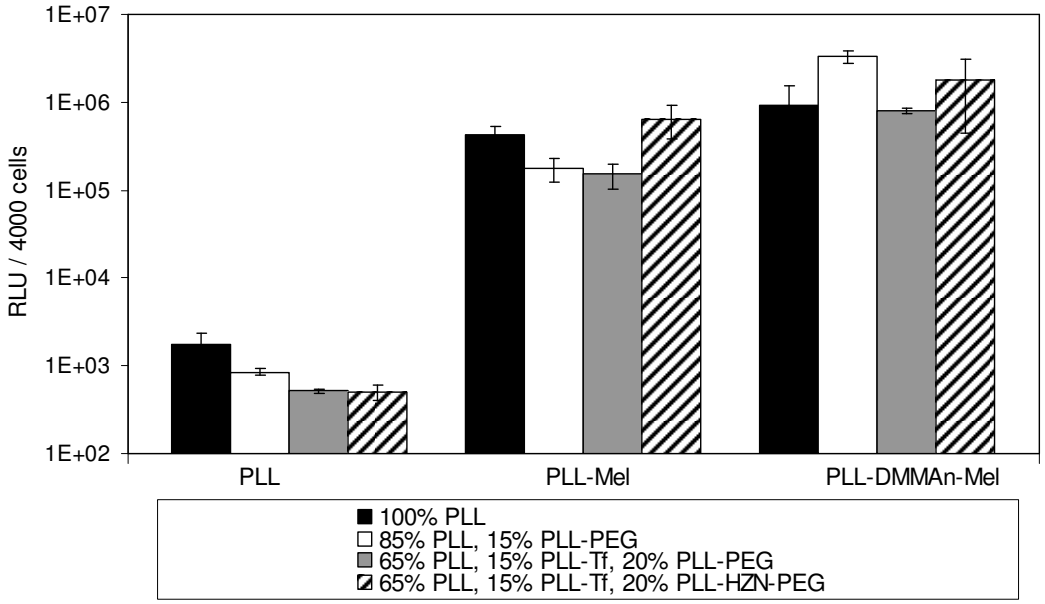


Fig. 12: Luciferase gene expression with different polyplex formulations. Luciferase gene expression after transfection of Neuro2A cells with PLL, PLL-Mel or PLL-DMMAn-Mel polyplexes generated at N/P 2. Black bars indicate 100 (molar) % indicated conjugate, white bars indicate 85% conjugate and 15% PLL-Tf, grey bars indicate 65% conjugate, 15% PLL-Tf and 20% PLL-PEG, striped bars indicate 65% conjugate, 15% PLL-Tf and 20% PLL-HZN-PEG.

3.1.4 Biophysical characterization

Determinaton of particle size is important in two kinds of aspects. On the one hand small polyplexes (< 500 nm) are desirable for *in vivo* administration because large aggregates could trigger acute toxicity after systemic administration. On the other hand information about the particle size is necessary to properly judge the *in vitro* transfection experiments in well plates: large aggregates can sedimentate on the cell surface, in contrast limited transport of small particles to the cell surfaces could lead to decreased transfection efficiency. Thus differences in transfection efficiency between varying polyplex formulations might be not only due to to the composition (e.g. incorporation of targeting ligands or lytic agent) but also due to resulting different sizes of the polyplexes. The ratios (% w/w) are based on the amount of PLL present in the PEG or transferrin conjugates or the PLL stock solutions. The PLL conjugate mixture was mixed with pDNA diluted in HBG at an N/P ratio of 2 and a final pDNA concentration of 20 µg/ml. Particles were analyzed by using a Malvern Zetasizer 3000 HS (**Table 1**).

Polyplex formulations	Particle size [nm]	Zeta potential [mV]
PLL	74 +/- 8	22,6 +/- 5,8
PLL-Mel	133 +/- 38	16,51 +/- 1,1
PLL-DMMAn-Mel	211 +/- 29	17,21 +/- 1,0
PLL 85% / 15%Tf	95 +/- 18	9,90 +/- 1,5
PLL-Mel 85% / 15%Tf	109 +/- 29	13,34 +/- 0,6
PLL-DMMAn-Mel 85% / 15% Tf	233 +/- 21	13,8 +/- 0,7
PLL 65% / 15%Tf / 20%PEG	158 +/- 79	3,7 +/- 1,3
PLL-Mel 65% / 15%Tf / 20%PEG	144 +/- 25	12,7 +/- 0,9
PLL-DMMAn-Mel 65% / 15%Tf / 20%PEG	178 +/- 38	3,7 +/- 0,6

Table 1: Particle size and zeta potential of polyplexes
pDNA complexes were prepared in HBG at a N/P ratio of 2. pDNA concentration during polyplex mixing was 20 µg/ml. Polyplexes were allowed to stand for 20 – 30 minutes before measurement.

The preparation of polyplexes under salt-free conditions resulted in overall small nanosized polyplexes. In general DMMAn-Mel containing polyplexes were slightly larger in size compared with their PLL- and PLL-Mel analogs. Incorporation of transferrin lowered the zeta-potential. To achieve effective shielding incorporation of PEG (MW 20000) was necessary. In case of PLL-Mel application of the PEG-conjugate only resulted in partial shielding (+ 12.7 mV) of the polyplex.

The binding affinity of the conjugates to pDNA was evaluated by a competitive displacement assay using fluorescent EtBr. PLL showed an efficient DNA condensation at N/P ratio of 1. In the case of PLL-Mel and PLL-DMMAn-Mel conjugates higher amounts of polymer were necessary to achieve complete reduction of EtBr fluorescence (approx. N/P 2) (**Fig. 13**).

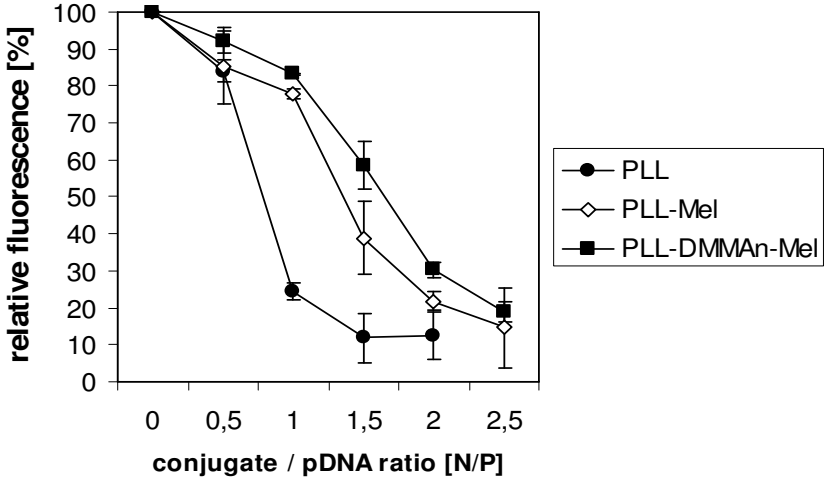


Fig. 13: pDNA binding of PLL, PLL-Mel and PLL-DMMAn-Mel conjugates. Complexes were formed in the presence of EtBr in HBG and inhibition of EtBr/DNA fluorescence was monitored.

3.1.5 Cellular toxicity

Toxicity of conjugates is of major interest and displays a limiting issue for application of polyplexes. To evaluate the differences in toxicity of PLL, PLL-Mel and PLL-DMMAn-Mel, relative metabolic activity of Neuro2A cells was determined after treatment with (free) conjugates. 3 hours after treatment, polymer containing medium was replaced by fresh medium and metabolic activity was determined after 24 hours. PLL and PLL-Mel reduced the metabolic activity of the cells to 15%, whereas, even for the highest concentration of PLL-DMMAn, almost 50% of metabolic activity remained. No significant differences were found between PLL and PLL-Mel (**Fig. 14**). However 1 hour after initial treatment differences in cell morphology between PLL- and PLL-Mel-treated cells could be detected by transmission light microscopy (**Fig. 15**, PLL-concentration 4.8 µg/ml). In contrast to PLL- and PLL-DMMAn-Mel-treated cells, PLL-Mel showed changes in cell morpology. Treatment with PLL-concentration of 23.8 µg/ml led to changes in cell morphology in case of PLL and PLL-Mel; PLL-DMMAn-Mel-treated cells were not affected in a large extent. Cells were also assayed for metabolic activity already after 1 hour of conjugate treatment (data not shown), but PLL- and PLL-Mel displayed a similar toxicity profile in contrast to the observed visible changes is cell morphology.

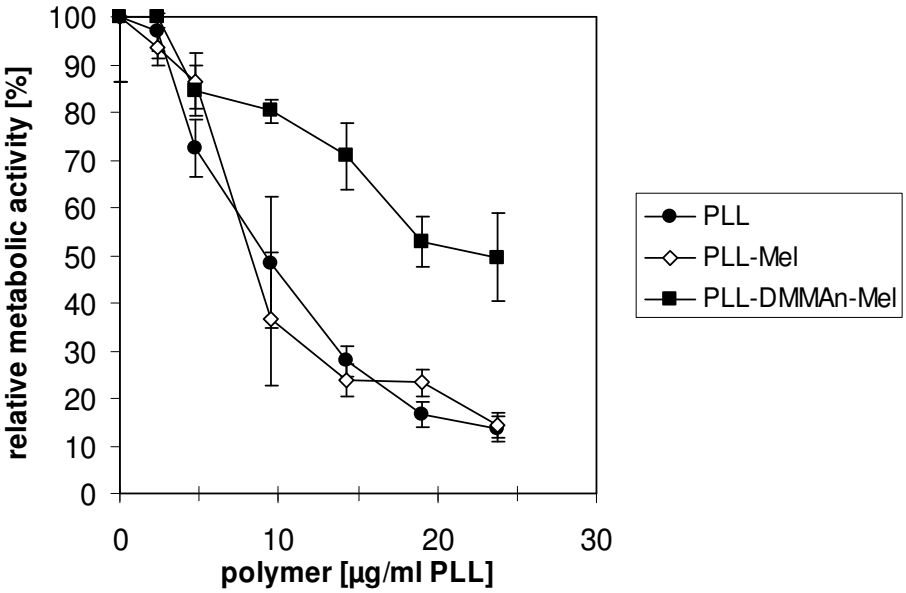


Fig. 14: Cell viability of Neuro2A cells after conjugate treatment. Neuro2A cells were treated with indicated concentrations of PLL, PLL-Mel and PLL-DMMAn-Mel conjugates. Polymer-containing medium was replaced by fresh medium after 3 hours and metabolic activity was determined by MTT assay after 24 h.

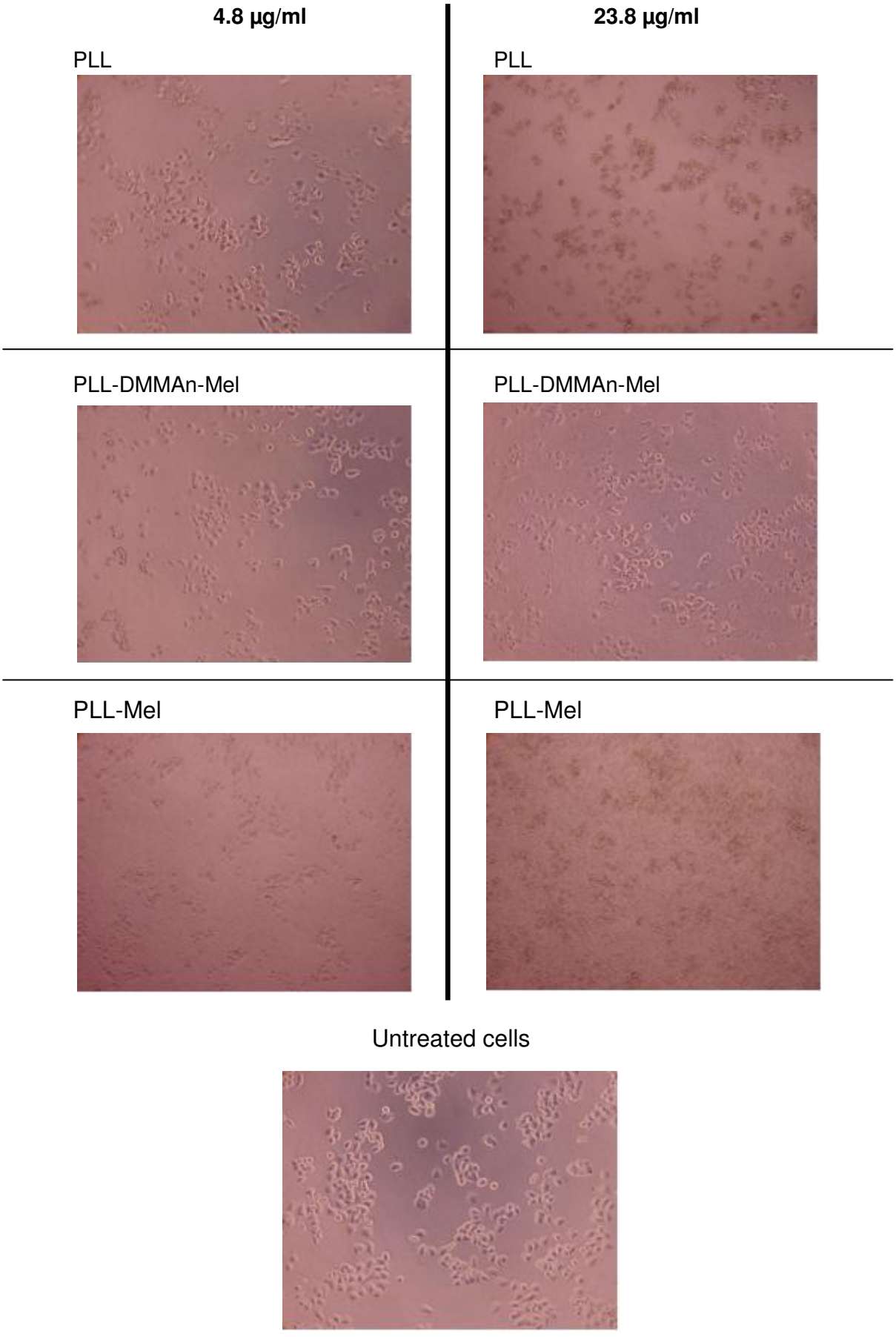


Fig. 15: Transmission light images of Neuro2A cells after conjugate treatment. Pictures were taken 1 hour after initial conjugate treatment (PLL-concentrations: 4.8 µg/ml and 23.8 µg/ml)

Hence toxicity was further investigated by an LDH release assay. While the MTT assay technically spoken measures the activity of mitochondrial enzymes that reduce MTT to formazon, an LDH release indicates membrane damage and / or cell death. The normal plasma membrane is impermeable to cytosolic LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. As illustrated in **Fig. 16**, the amount of released LDH increased with the increase in polymer concentration. The least LDH was released by PLL-DMMAn-Mel, whereas PLL-Mel induced the most LDH release, which supports the microscopy data.

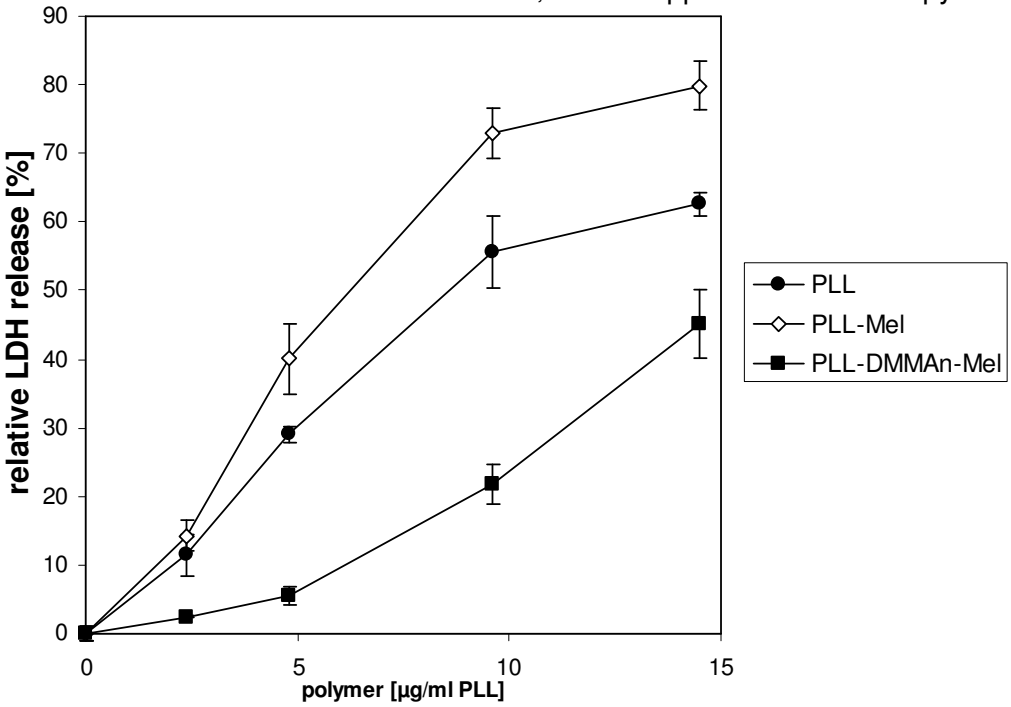


Fig. 16: Polymer-induced lactate dehydrogenase release. Neuro2A cells were treated with indicated concentrations of PLL, PLL-Mel and PLL-DMMAn-Mel conjugates. After 0.5 h incubation time the amount of released LDH was determined.

To exclude the possibility that all toxic effects are due to PLL and not melittin, a cytotoxicity assay (LDH-release) with melittin alone and PLL alone was performed. The corresponding amounts of melittin and PLL which are present in the PLL-Mel conjugate were compared at different concentrations (e.g. PLL at 2.4 µg/ml was compared with melittin 1.25 µg/µl). At the concentration range tested (PLL: 2.4 µg/ml – 19 µg/ml) both PLL and melittin exhibited a similar toxicity and induced LDH-release up to 60% (**Fig. 17**). As expected, melittin contributes to the toxicity of the PLL-Mel conjugate.

Taken together, attachment of unmodified melittin increased toxicity of the polymer whereas modification with DMMAn-Mel reduced *in vitro* cytotoxicity.

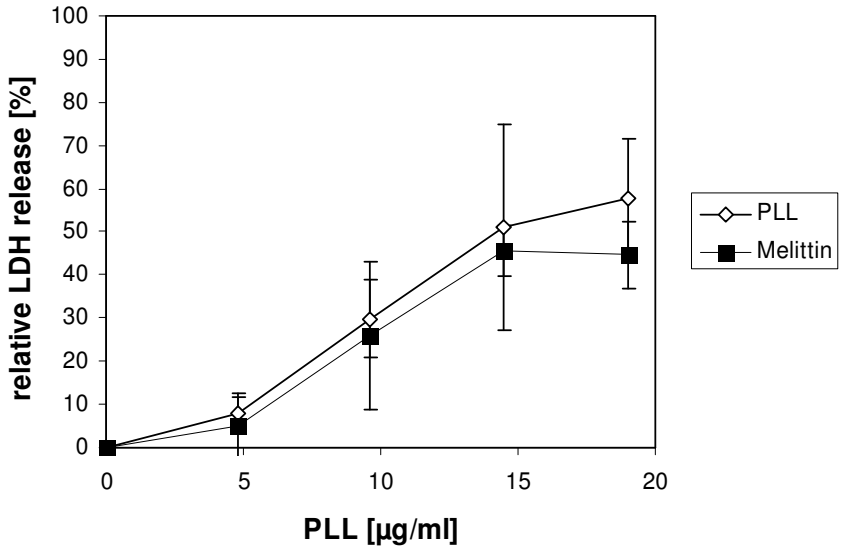


Fig. 17: Cytotoxicity (LDH-release) induced by melittin and PLL on Neuro2A cells. Corresponding amounts of melittin and PLL which are present in the PLL-Mel conjugate were compared at different concentrations.

3.2 PEGylated endosomolytic conjugates for pDNA and siRNA delivery

3.2.1 Design and synthesis of the conjugates

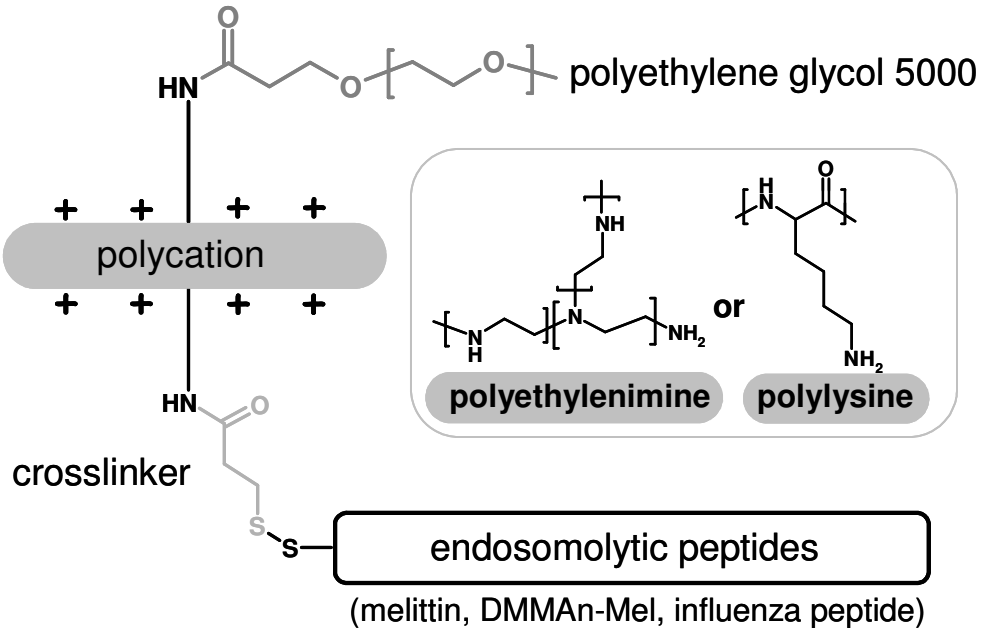
Currently polyethylenimines / oligoethylenimine-derived polymers are the most frequently used polycations for nucleic acid delivery [33]. Also in our workgroup novel ethylenimine-based carriers have been developed, including biodegradable polymers and pseudodendrimers [130;135-137]. Accordingly, the next logical step was to transfer the promising DMMAn-Mel strategy from PLL to a PEI-based system. Hence branched PEI (25 kDa) was chosen as backbone for modification with DMMAn-Mel. The synthesis was carried out analogously to PLL-DMMAn-Mel, after SPDP-modification of PEI, DMMAn-Mel was coupled via disulfide bond formation. Released pyridine-2-thione was measured at 343 nm to determine the DMMAn-Mel modification degree. Purification of the conjugate was carried out by SEC using a Superdex 75 HR 10/30 column (elution buffer: 0.5 M NaCl, 20mM Hepes, pH 8) and yielded in a PEI-DMMAn-Mel conjugate with a molar ratio of approximately 1 PEI to 8 DMMAn-Mel. The erythrocyte leakage assay showed the desired pH-dependent lytic activity similar to PLL-DMMAn-Mel (data not shown). However it was not possible to generate small nanosized nucleic acid polyplexes with PEI-DMMAn-Mel. Modification of PEI with the negatively charged DMMAn-Mel peptide induced polymer collapse and polyplex aggregation (> 1µm). In contrast modification of PEI with the unmodified positively charged melittin could be accomplished without problem [101]. As described above (section 3.1.4.) large particles are not favored in terms of systemic *in vivo* delivery and represent a serious exclusion

criterion for the development of carriers. To overcome this drawback PEI was first modified with monofunctional succinimidyl propionate polyethylene glycol (MW 5000). Size exclusion chromatography (Sephadex G25 superfine, Superdex 75, Superdex 200) and dialysis against a MWCO 6000 – 8000 membrane were not suitable to remove uncoupled PEG-reagent from the mixture, thus the conjugate was purified by cation exchange chromatography. After PEGylation, the conjugate was modified with SPDP and sequently DMMAn-Mel was coupled via the terminal cysteine residue. This prePEGylation-strategy allowed the formation of soluble conjugates and the formation of nanosized polyplexes. Encouraged from this result, the negatively charged influenza peptide (INF) also was coupled to PEI-PEG.

Finally four different peptide-conjugates were synthesized based on PEI: PEI-PEG-Mel, PEI-PEG-DMMAn-Mel, PEI-PEG-INF and PEI-PEG-Succ-Mel. For synthesis of the conjugates, PEI-PEG with a molar ratio of 1:1.5 (PEI:PEG) was used. Measurement of released pyridine-2-thione revealed that 7 to 11 peptides were coupled to PEI-PEG. After reaction of the peptides with PEI-PEG-PDP, unbound peptides and released pyridine-2-thione were removed by SEC using a Superdex 75 HR 10/30 column. In case of acid-sensitive PEI-PEG-DMMAn-Mel, the elution buffer was adjusted to pH 8.2. Succ-Mel represents a negative control peptide were melittin's lytic activity is irreversibly inhibited by stable modification of the primary amino groups with succinicanhydride (analogously to the DMMAn-modification of melittin).

PEI has been considered the gold standard for gene carriers because of the relatively high transfection efficiency. However, if the focus is laid on *in vivo* application, PEI has the disadvantage of not being biodegradable. Therefore analog conjugates were synthesized with a PLL backbone (MW 32000, as hydrobromide), resulting in PLL-PEG-Mel, PLL-PEG-DMMAn-Mel, and PLL-PEG-INF. PLL due to its peptide linkages represents a biodegradable polycation. The synthesis route of the PEI-PEG-peptides was adopted for the PLL-based conjugates. Cation exchange chomatograpy was used to remove free PEG from the PLL-PEG containing synthesis mixture. PLL-PEG-PDP with a molar ratio of 1:1:8 (PLL:PEG:PDP) was modified with peptides yielding in peptide conjugates with approximately 6 to 8 peptides coupled to 1 PLL.

A schematic structure of all conjugates is shown in **Scheme 2**.



Scheme 2: Schematic structure of the PEGylated endosomolytic conjugates

Because modification of PLL with DMMAn-Mel had an effect on the cytotoxicity of the polymer, the toxicity of the free PEI-PEG-peptide conjugates was investigated with an LDH-release cytotoxicity assay (**Fig. 18**). Modification with DMMAn-Mel lowered the acute toxicity of PEI-PEG, in contrast an increased cytotoxicity was observed when unmodified melittin was attached.

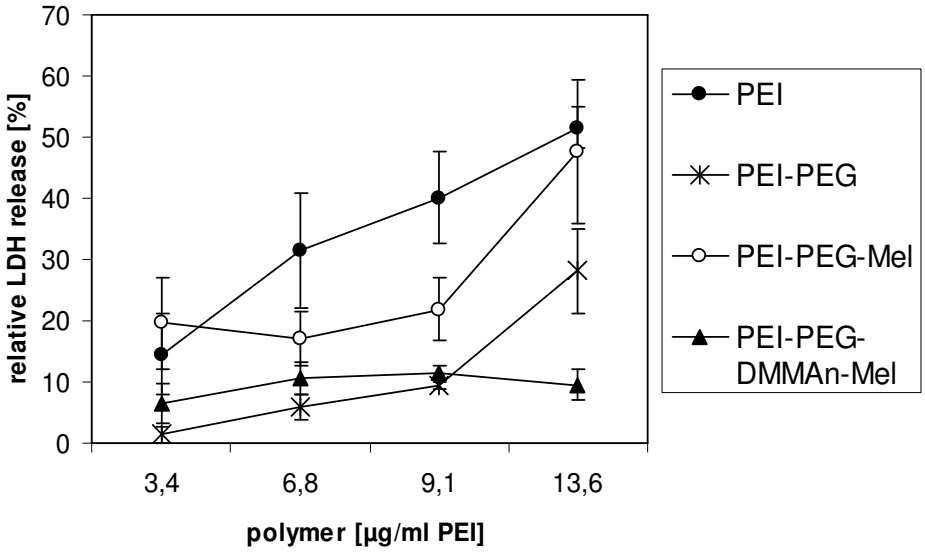


Fig. 18: LDH-release induced by PEI-PEG-conjugates on Neuro2a cells. Neuro2A cells were treated with indicated concentrations of PEI, PEI-PEG, PEI-PEG-Mel and PEI-PEG-DMMAn-Mel conjugates. After 0.5 h incubation time the amount of released LDH was determined.

3.2.2 pDNA-delivery

3.2.2.1 PEI-PEG-conjugates

First of all the binding strength of the synthesized PEI-conjugates to pDNA was compared via EtBr exclusion assay. Modification with negatively charged DMMAn-Mel could result in an decreased pDNA binding ability. But except for modification with influenza peptide the effects of peptide-modification on the pDNA-binding capability were negligible. At an N/P ratio of 3 sufficient pDNA-binding was observed (**Fig. 19**).

As previously mentioned, DMMAn-Mel modification of PEI without prePEGylation resulted in polymer collapse and the formation of large pDNA-polyplexes (> 1 µm). Thus dynamic light scattering measurements were carried out to determine the size and zeta-potential of the PEI-conjugate / pDNA-complexes (**Table 2**). In general the complexes containing peptide-modified PEI-PEG were larger compared to PEI and PEI-PEG polyplexes. However with sizes smaller then 200 nm it has been proven that the prePEGylation strategy allows the formation of nanosized polyplexes also with PEI-PEG-DMMAn-Mel conjugate. The addition of PEG 5000 resulted in partial shielding of the polyplexes. It is assumed that PEG with a higher MW (e.g. 20000) is necessary for complete shielding of surface charge.

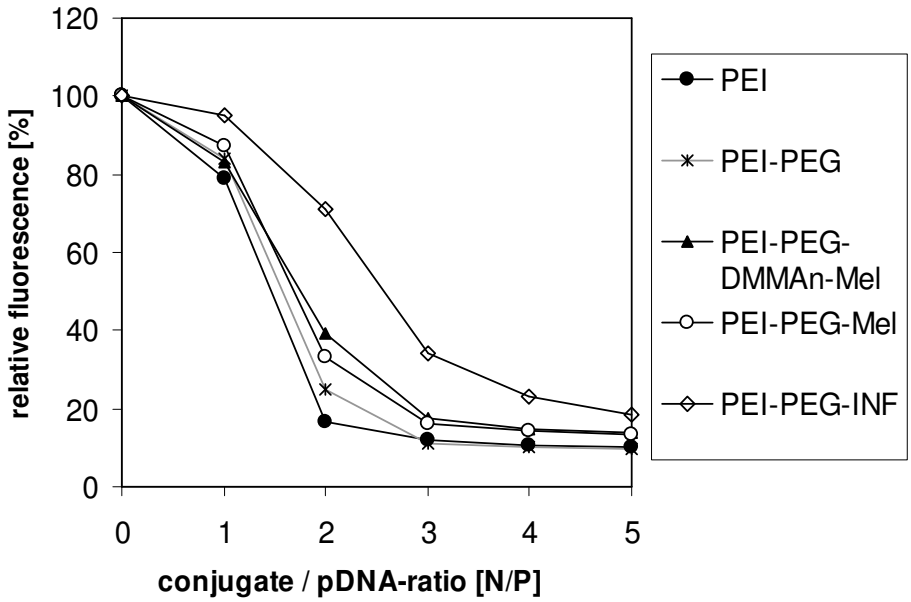


Fig. 19: DNA binding of conjugates measured by ethidium bromide exclusion assay. Complexes were formed in the presence of EtBr in HBG and inhibition of EtBr/DNA fluorescence was monitored. The data points represent the average of two measurements.

	Size [nm]	Zeta-potential [mV]
PEI	80 +/- 11	27 +/- 1
PEI-PEG	90 +/- 13	11 +/- 4
PEI-PEG-Mel	110 +/- 14	16 +/- 4
PEI-PEG-DMMAn-Mel	184 +/- 21	15 +/- 1
PEI-PEG-INF	188 +/- 12	14 +/- 3

Table 2: Particle size and zeta-potential of PEI-conjugate polyplexes. pDNA complexes were prepared in HBG at a N/P ratio of 6. pDNA concentration during polyplex mixing was 20 µg/ml. Polyplexes were allowed to stand for 20 – 30 minutes before measurement.

After biophysical characterization, the influence of PEGylation and peptide-modification on levels of gene transfer efficiency *in vitro* was examined. Reporter gene expression (luciferase activity) and cellular toxicity were evaluated in two different tumor cell lines (Neuro2A, HUH7) after the application of different polyplex preparations (**Fig. 20**). For transfection experiments, all polyplexes were generated at a N/P ratio of 6. Polyplexes were generated in HBG at a pDNA concentration of 20 µg/ml during polyplex formation and the formulations (each containing 200 ng pCMVLuc DNA) were applied to the 10000 cells in medium containing 10% FBS. After 3 h of incubation at 37 °C, transfection medium was replaced by fresh medium. 24 hours after initial transfection cells were assayed for luciferase expression.

PEI showed similar transfection profiles in both cell lines. PEGylation had a disastrous effect: PEI lost its gene transfer activity. But after attachment of endosomolytic peptides to PEI-PEG gene transfer activity could be restored. PEI-PEG-DMMAn-Mel and PEI-PEG-INF showed a slightly decreased gene transfer activity on HUH7 cells in comparison to the results obtained on Neuro2A cells. Attachment of the non-endosomolytic peptide Succ-Mel (lytic activity of melittin irreversibly masked by stable modification of melittin's primary amines with succinic anhydride) did not enhance gene transfer activity of PEI-PEG.

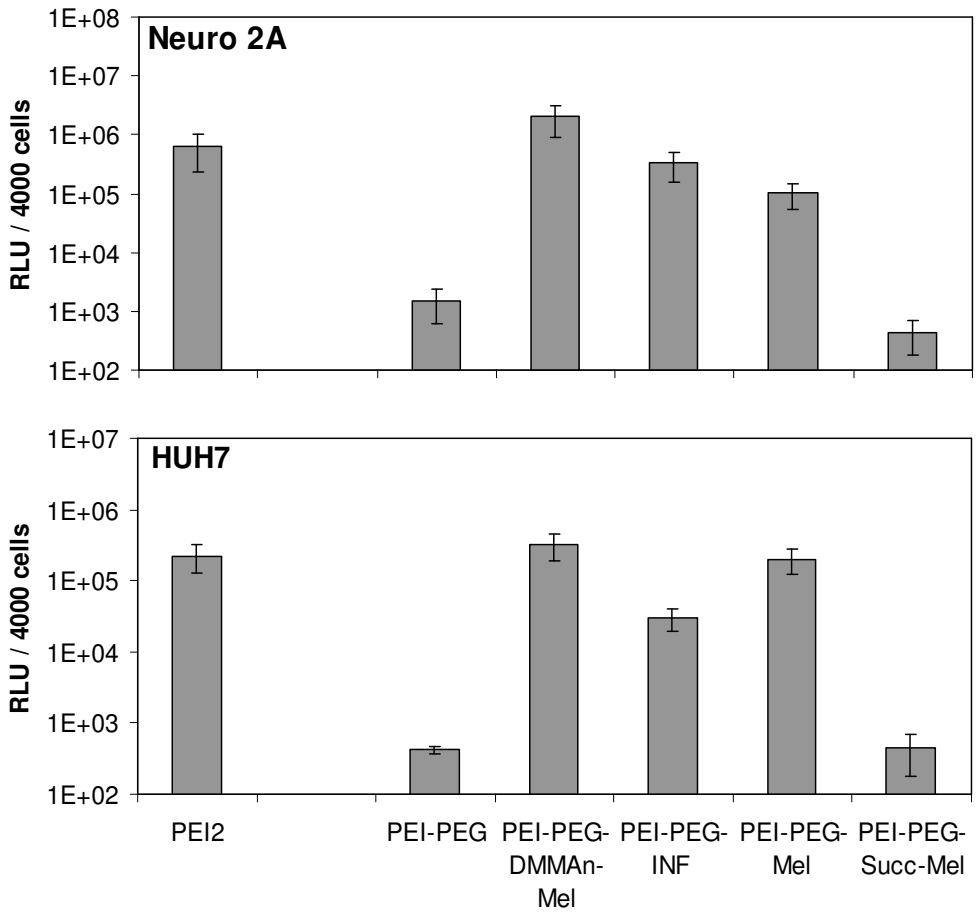


Fig. 20: Transfection efficiencies of PEI-PEG-conjugate polyplexes on Neuro2A- and HUH7-cells. Polyplex formulations with branched PEI 25 kDa, PEG 5000 and peptides were compared. Complexes were prepared in HBG at a DNA concentration of 20 µg/ml, N/P 6. 200 ng pCMVLuc DNA were applied per 10000 cells.

In order to evaluate toxic effects of the different polyplex formulations, metabolic activity of treated cells was measured by MTT assay 24 h after initial transfection. The assay was performed in parallel to transfection experiments. Overall cell viability was not affected to a large scale. The polyplex formulation which induced the highest toxicity (PEI-PEG-Mel) reduced cell viability only to as low as 70% of untreated control cells (**Fig. 21**). This indicated that differences in transfection efficiency of polyplexes were not attributed to varying toxic effects of the formulations.

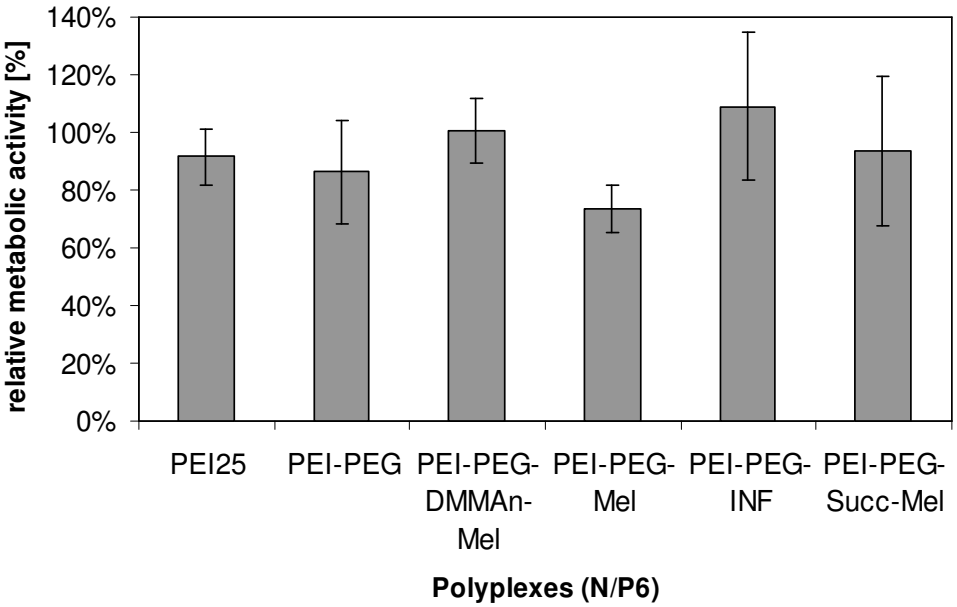


Fig. 21: Metabolic activity of Neuro2A cells after transfection with indicated polyplex formulations. Polyplexes with branched PEI 25 kDa, PEG 5000 and peptides were prepared in HBG at a DNA concentration of 20 µg/ml, N/P 6. Metabolic activity of untreated control cells was set to 100%.

3.2.2.2 PLL-PEG-conjugates

Binding strength of the PLL-conjugates to pDNA was compared via EtBr exclusion assay. The effects of PEGylation and peptide-modification on the pDNA-binding ability were negligible. In general at a PLL epsilon-amino nitrogen to pDNA phosphate ratio (N/P) of 1.5 complete pDNA-binding was observed (**Fig. 22**)

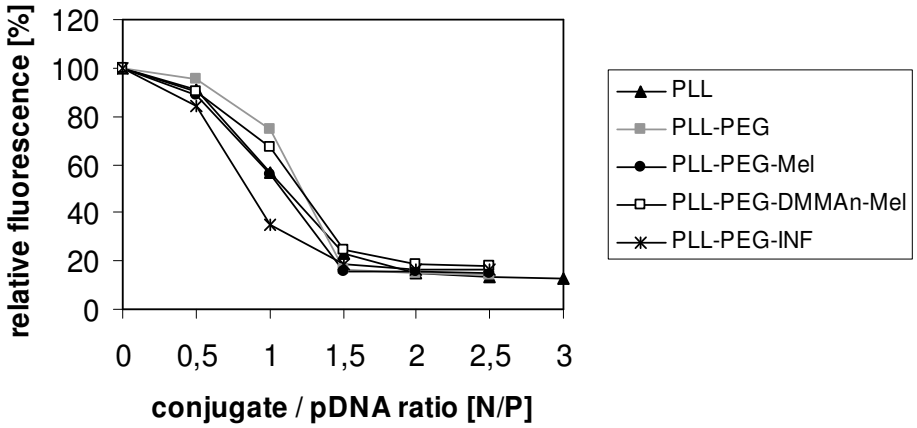


Fig. 22: DNA binding of conjugates measured by ethidium bromide exclusion assay. Complexes were formed in the presence of EtBr in HBG and inhibition of EtBr/DNA fluorescence was monitored. The data points represent the average of two measurements

Particle sizes were in the range of 80 to 200 nm with PLL-PEG-DMMAn forming the largest complexes. As observed with PEI-PEG-conjugates, the zeta-potential measurements confirm that application of PEG 5000 only leads to partial shielding of polyplexes (**Table 3**).

Polyplexes (N/P 2)	Size [nm]	Zeta-potential [mV]
PLL	83 +/- 2	34 +/- 2
PLL-PEG	87 +/- 14	22 +/- 4
PLL-PEG-Mel	111 +/- 12	17 +/- 1
PLL-PEG-DMMAn-Mel	203 +/- 15	14 +/- 1
PLL-PEG-INF	100 +/- 14	15 +/- 3

Table 3: Particle size and zeta-potential of PLL-conjugate polyplexes. Polyplexes were generated at N/P 2 in HBG. pDNA-concentration during polyplex mixing was 20 µg/ml. Polyplexes were allowed to stand for 20 – 30 minutes before measurement.

Analogous to transfection experiments with PEI-PEG-peptide-conjugates, the transfection efficiencies of PLL-PEG-conjugate-polyplexes on Neuro2A cells were examined. As expected, PLL and PLL-PEG were not able to enhance reporter gene expression (**Fig. 23**). While attachment of endosomolytic peptides (DMMAn-Mel, Mel, INF) to PEI-PEG enhanced gene transfer activity in all cases, only PLL-PEG-polyplexes containing DMMAn-Mel mediated gene transfer activity. Transfection efficiency of PLL-PEG-DMMAn-Mel polyplexes at N/P ratios of 2 and 3 were remarkably high (over 1000-fold higher efficiency than e.g. PLL-PEG-Mel).

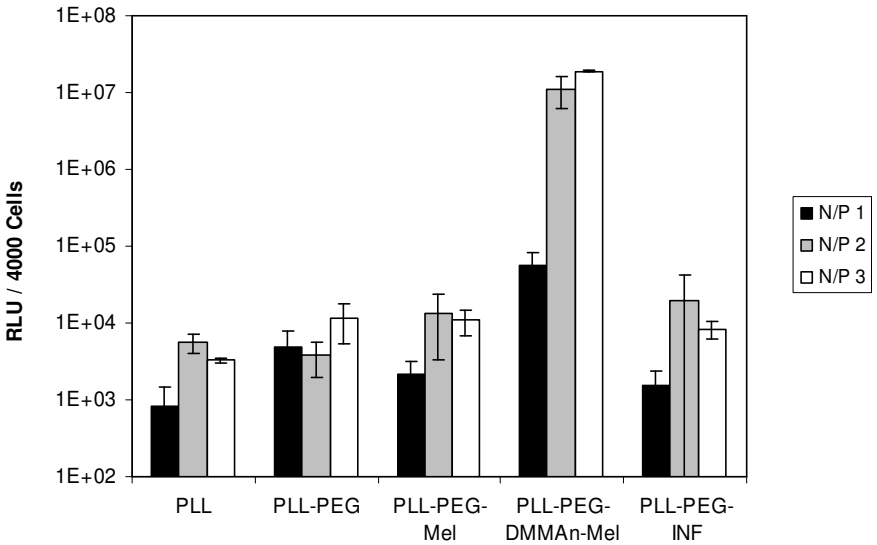


Fig. 23: Transfection efficiencies of PLL-PEG-polyplex formulations on Neuro2a cells. Polyplex formulations with branched PLL 32000, PEG 5000 and peptides were compared. Complexes were prepared in HBG at a DNA concentration of 20 µg/ml, N/P ratios of 1 to 3. 200 ng pCMVLuc DNA was applied per 10000 cells.

Metabolic activity of transfected cells was overall above 80% with the conclusion that differences in toxicity were not responsible for the great difference in gene transfer activity (**Fig. 24**).

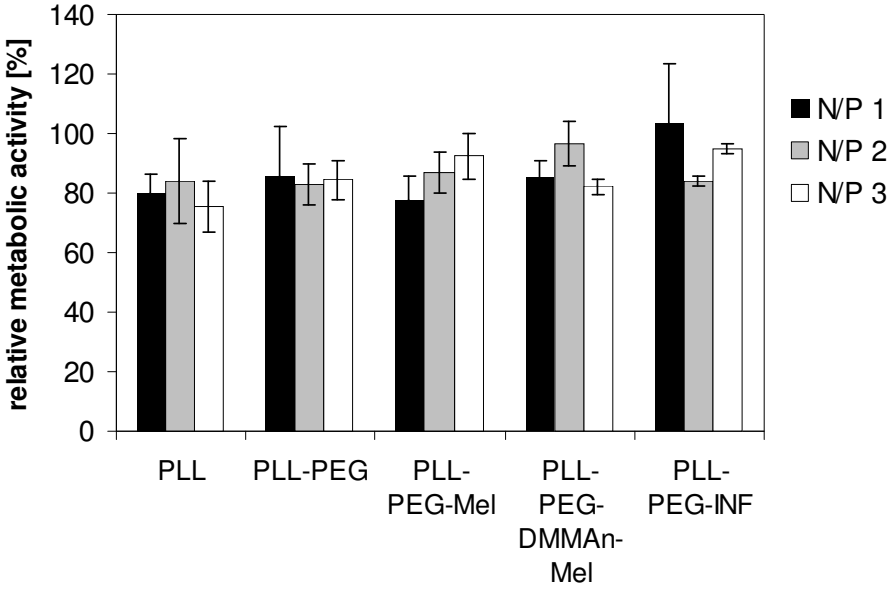


Fig. 24: Metabolic activity of Neuro2A cells after transfection with indicated polyplex formulations. Polyplexes with PLL 32000, PEG 5000 and peptides were prepared in HBG at a DNA concentration of 20 µg/ml, N/P ratio 1 to 3. Metabolic activity of untreated control cells was set to 100%.

Particle size is known to influence *in vitro* gene transfer activity [138]. Thus additional size measurements were carried out after incubation of polyplexes in serum containing medium. These experiments were performed to clarify if polyplexes aggregate in cell culture medium after adding the polyplex solution at begin of transfection (**Table 4**).

	Size [nm]	
Polyplexes (N/P 2)	**0.1 hour**	**3 hours incubation in cell culture medium**
PLL	76	179
PLL-PEG	91	187
PLL-PEG-Mel	105	105
PLL-PEG-DMMAn-Mel	190	178
PLL-PEG-INF	135	109

Table 4: Sizes of indicated PLL polyplexes after incubation in serum containing DMEM cell culture medium. Sizes represent the average of two measurements.

PLL- and PLL-PEG-polyplexes increased in size, while size of PLL-PEG-peptide polyplexes did not alter in a large extent. Based on these observations it is supposed that changes in size after incubation in cell culture medium-containing solution were not responsibly for the observed differences in transfection efficiency.

To determine if varying internalization rates led to differences in gene transfer activity, flow cytometry internalization studies were performed (**Fig. 25**). Neuro2A cells were incubated for 3 h with PLL-polyplexes at 37 °C. Polyplexes were generated in HBG at N/P ratio of 2. After incubation cell culture medium was removed and trypsin treatment of cells was performed to remove surface-associated (but not internalized) polyplexes.

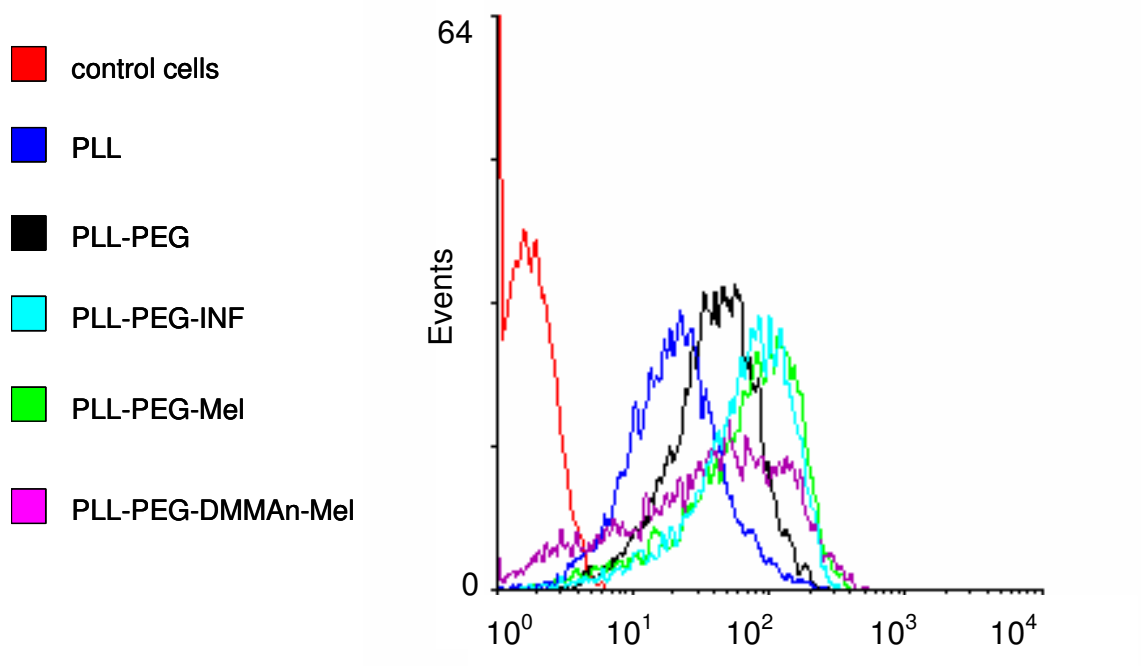


Fig. 25: Flow cytometry of Neuro2A cells incubated with PLL/DNA complexes. Neuro2A cells (80000 cells in 1 ml cell culture medium) were incubated with PLL / Cy5-labeled DNA complexes (N/P 2.0 in HBG), containing 1.5 µg of DNA for 3 h at 37 °C. Following incubation, the medium was removed, cells were washed with ice-cold PBS, treated with trypsin/EDTA, resuspended in ice-cold PBS and after addition of propidium iodide flow cytometry analysis was performed.

Comparing cells incubated with PLL, PLL-PEG and PLL-PEG-peptide polyplexes reveals the highest degree of internalization in case of PLL-PEG-peptide polyplexes (PLL-PEG-DMMAn-Mel, PLL-PEG-INF and PLL-PEG-Mel). PLL-PEG polyplexes showed a reduced and PLL-polyplexes the lowest degree of internalization.

In summary, PLL-PEG-DMMAn-Mel polyplexes displayed high gene transfer efficiency. PLL-PEG-DMMAn-Mel polyplexes were slightly larger in size, but neither polyplex aggregation in cell culture medium nor a better internalization of PLL-PEG-DMMAn-Mel explained the remarkable gene transfer activity in comparison to the other PLL-PEG formulations.

3.2.3 siRNA-delivery

RNA interference strategies using siRNA is one of the most important discoveries in biology in the past years. There is a great demand in delivery systems for this biological therapeutics. It has to be pointed out that successful pDNA formulations are not naturally

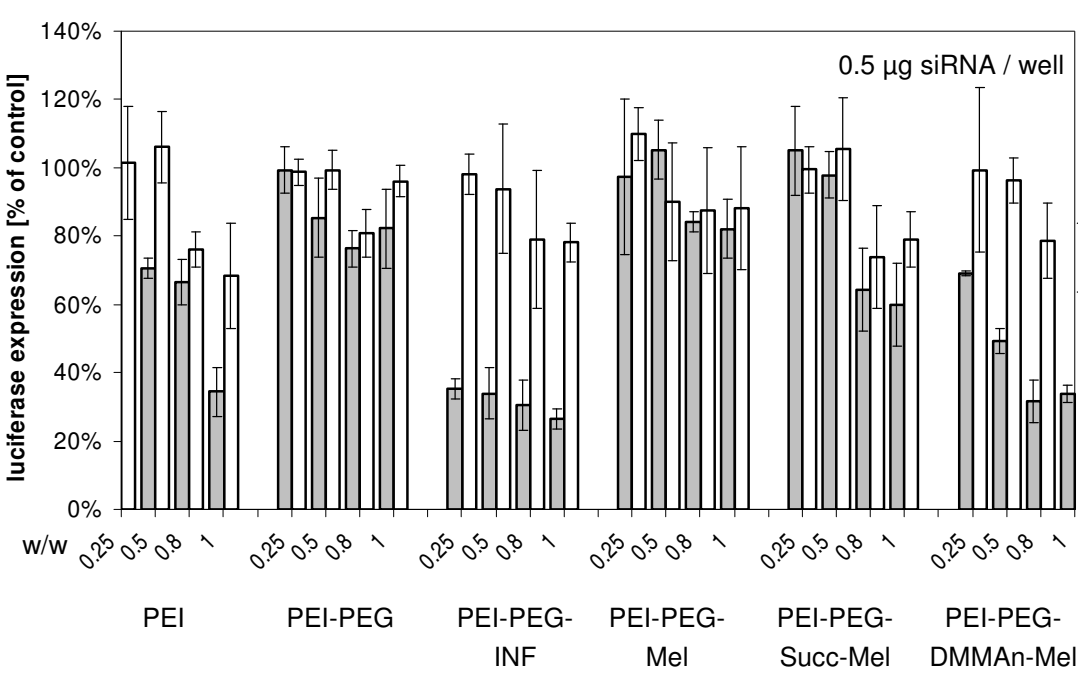
suitable for siRNA delivery. For example Mixson and coworkers demonstrated that a branched carrier consisting of lysine and histidine residues designed for pDNA could not mediate delivery of siRNA [44]. Experiments to determine the biological activity and biophysical properties were carried out to evaluate the siRNA delivery efficiency of the synthesized endosomolytic conjugates. Biological siRNA transfection data were generated in collaboration with Alexander Philipp, as part of his PhD thesis (in preparation) where details will be described. The most important biological data are also presented within the following sections, to provide a complete view on chemical and biological characteristics of the conjugates.

3.2.3.1 PEI-PEG-conjugates

siRNA delivery efficiency of the conjugates was examined using a Neuro2A-eGFPLuc- (mouse neuroblastoma stably expressing luciferase) and a HUH7-eGFPLuc-cell line (human hepatoma stably expressing luciferase). siRNA was complexed with PEI conjugates in HBG at a siRNA concentration of 20 µg/ml. The experiments were carried out with each polymer bound to siRNA at different w/w-ratios. 48 h after initial transfection of 5000 seeded cells, cells were lysed and assayed for luciferase expression.

As shown in **Fig. 26**, PEI (with or without PEG) did not mediate siRNA knockdown at both tested siRNA doses (0.25 and 0.5 µg). But after modification with DMMAn-Mel and INF-peptide siRNA delivery efficiency was greatly enhanced as demonstrated by luciferase gene knockdown (**Fig. 26a**). This effect was more pronounced on HUH7-eGFPLuc cells (**Fig. 27**).

26 a)



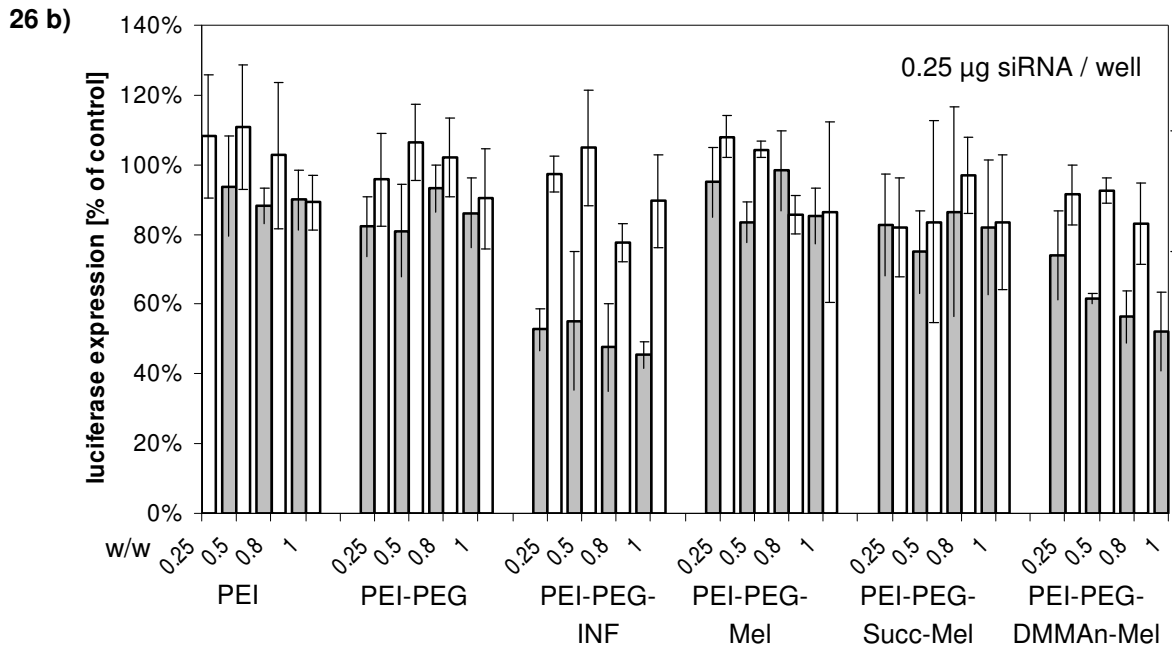


Fig. 26: siRNA gene silencing efficiency on stably luciferase expressing Neuro2A-eGFPluc cells. PEI conjugates were complexed with 500 ng (**a**) or 250 ng (**b**) siRNA at different PEI to siRNA ratios (w/w). Gray bars indicate transfection with luciferase siRNA. White bars indicate transfection with complexes containing control siRNA. Reduced gene expression with control siRNA indicates unspecific knockdown / carrier toxicity. Data were generated by Alexander Philipp as part of his PhD thesis (in preparation).

Attachment of Mel did not enhance siRNA delivery activity on Neuro2A-eGFPLuc cells; on HUH7-eGFPLuc cells a slight knockdown effect was observed. Additional a PEI-PEG-conjugate with a peptide lacking lytic activity (Succ-Mel) was tested. The activity profile was comparable to ineffective PEI-PEG-Mel. No knockdown activity was observed on Neuro2A-eGFPLuc cells, only a slight knockdown on HUH7-eGFPLuc cells. In parallel, complexes with a control siRNA sequence were applied (white bars): if luciferase expression decreases upon transfection with control siRNA, “knockdown” is mainly due to carrier toxicity and can hereby be distinguished from a real protein knockdown effect.

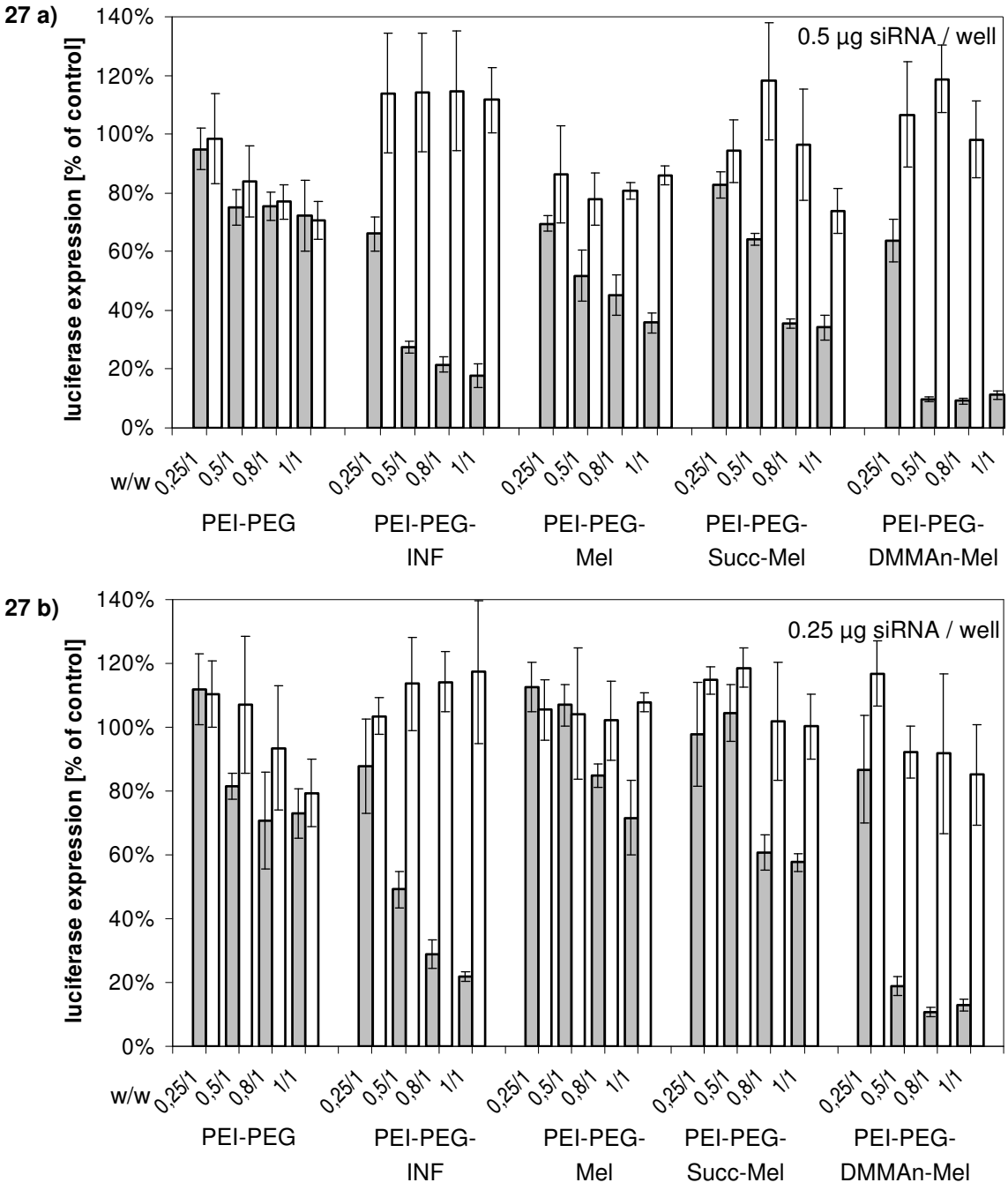


Fig. 27: siRNA gene silencing efficiency on stably luciferase expressing HUH7-eGFPluc cells. PEI conjugates were complexed with 500 ng (**a**) or 250 ng (**b**) siRNA at different PEI to siRNA ratios (w/w). Gray bars indicate transfection with luciferase siRNA. White bars indicate transfection with complexes containing control siRNA. Reduced gene expression with control siRNA indicates unspecific knockdown / carrier toxicity. Data were generated by Alexander Philipp as part of his PhD thesis (in preparation).

Interaction of PEI-conjugates with siRNA was proven by EtBr exclusion fluorescence assay (**Fig. 28**).

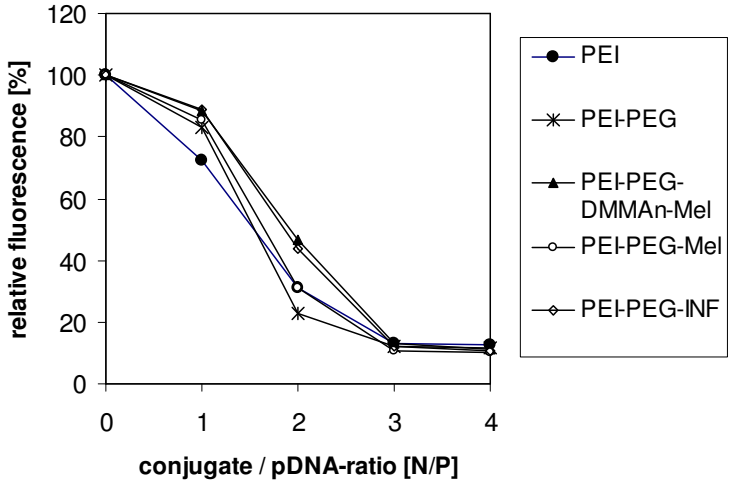


Fig. 28: siRNA binding of PEI-conjugates measured by ethidium bromide exclusion assay. Complexes were formed in the presence of EtBr in HBG and inhibition of EtBr/siRNA fluorescence was monitored. The data points represent the average of two measurements.

Particle size of PEI, PEI-PEG, PEI-PEG-Mel and PEI-PEG-DMMAn-Mel siRNA polyplexes was measured by fluorescence correlation spectroscopy (FCS). For FCS experiments Cy5 labeled siRNA was utilized. FCS detects spontaneous intensity fluctuations resulting from fluorescently labeled molecules diffusing through a highly focused laser illuminated volume within the 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 labeled molecules / polyplexes and to calculate the hydrodynamic radius. The obtained results are displayed in **Table 5**.

siRNA polyplexes	size [nm]
PEI w/w 0.5	93 – 2800 (high polydispersity)
PEI w/w 1	20 +/- 8
PEI-PEG w/w 0.5	10 +/- 3
PEI-PEG w/w 1	15 +/- 8
PEI-PEG-Mel w/w 0.5	30 +/- 2
PEI-PEG-Mel w/w 1	10 +/- 2
PEI-PEG-DMMAn-Mel w/w 0.5	89 +/- 13
PEI-PEG-DMMAn-Mel w/w 1	26 +/- 4

Table 5: Size of siRNA polyplexes. For FCS measurements Cy5 labeled siRNA was used. Polyplexes were generated in HBG at indicated polycation to siRNA ratios (w/w)

In general polyplexes prepared at w/w ratio of 1/1 were smaller than polyplexes generated at w/w 0.5. PEI-PEG formed the smallest polyplexes (10 – 15 nm). Aggregation was observed in case of PEI polyplexes generated at w/w 0.5. DMMAn-Mel polyplexes were larger in size (26 – 98 nm) than polyplexes containing unmodified melittin (10 – 30 nm).

3.2.3.2 PLL-PEG-conjugates

As expected, neither PLL- nor PLL-PEG-polyplexes showed siRNA delivery activity on Neuro2A-eGFPLuc-cells (**Fig. 29**). In contrast to modification of PEI-PEG, attachment of INF-peptide did not enhance siRNA activity of PLL-PEG. Once more linkage of DMMAn-Mel enabled gene silencing (over 80% reduction of luciferase expression compared to Neuro2A-eGFPLuc-cells treated with control siRNA containing PLL-PEG-DMMAn-Mel polyplexes). Notably PLL-PEG displayed remarkable toxicity at increasing polycation to siRNA ratios (as indicated by decrease of luciferase expression after transfection with control siRNA containing formulations).

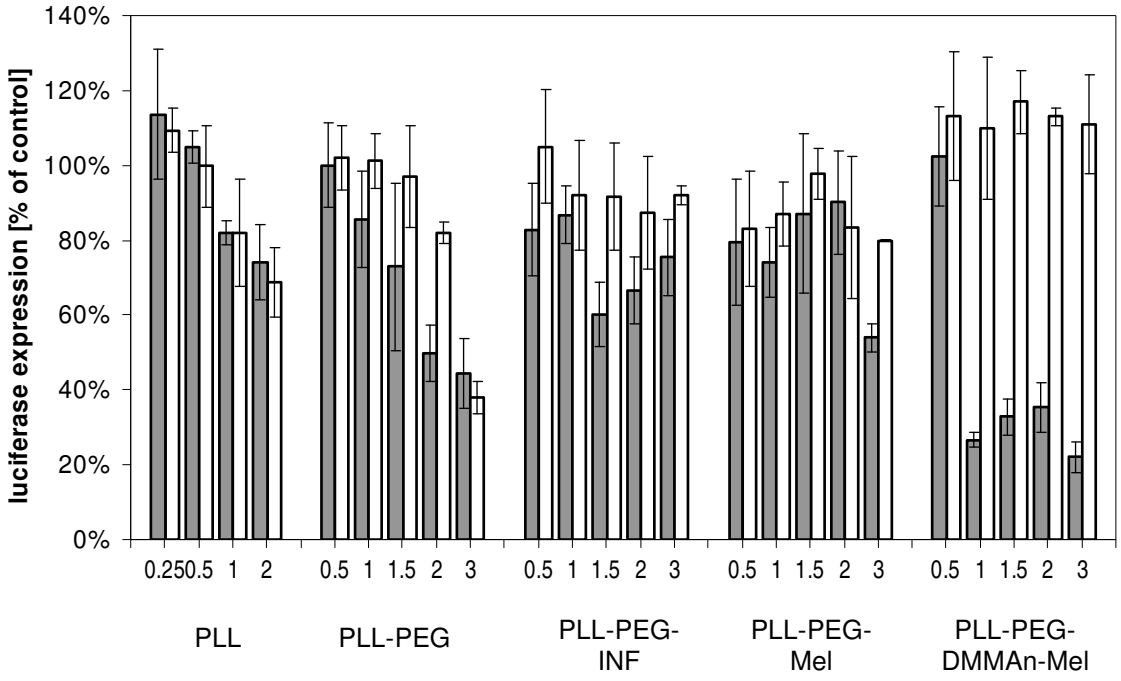


Fig. 29: siRNA gene silencing efficiency on stably luciferase expressing Neuro2A-eGFPluc cells. PLL conjugates were complexed with 250 ng siRNA at different PLL to siRNA ratios (w/w). Gray bars indicate transfection with luciferase siRNA, white bars indicate transfection with complexes containing control siRNA. Reduced gene expression with control siRNA indicates unspecific knockdown / carrier toxicity. Data were generated by Alexander Philipp as part of his PhD thesis (in preparation).

The binding strength of conjugates to siRNA was assayed by EtBr-exclusion-assay (**Fig. 25**). Modification with peptides demanded a greater amount of polycation to completely bind the nucleic acid. At w/w 0.5 (corresponds approximately N/P 0.8) PLL-PEG-DMMAn-Mel showed

no nucleic acid binding which correlated with its non-existing delivery efficiency at this w/w-ratio.

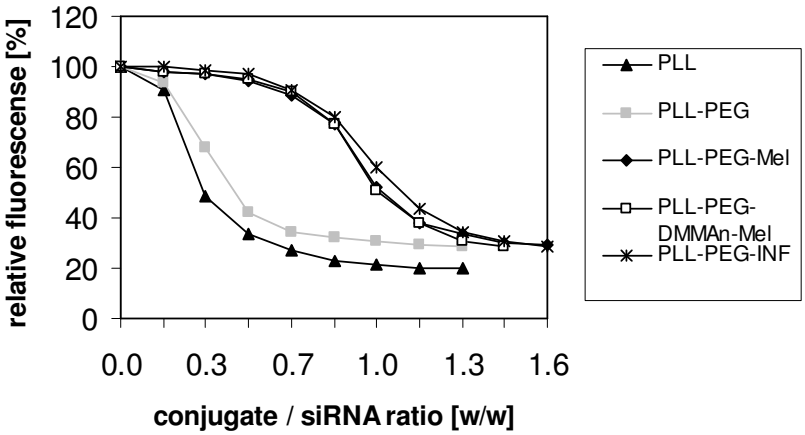


Fig. 30: siRNA binding of PEI-conjugates measured by ethidium bromide exclusion assay. Complexes were formed in the presence of EtBr in HBG and inhibition of EtBr/DNA fluorescence was monitored. Each data point represents the average of two measurements.

siRNA polyplexes	size [nm]
PLL w/w 1	29 +/- 11
PLL w/w 2	18 +/- 9
PLL-PEG w/w 1	8 +/- 1
PLL-PEG w/w 2	7 +/- 1
PLL-PEG-Mel w/w 1	8 – 430 (high polydispersity)
PLL-PEG-Mel w/w 2	12 +/- 2
PLL-PEG-DMMAn-Mel w/w 1	6 – 794 (high polydispersity)
PLL-PEG-DMMAn-Mel w/w 2	40 +/- 26

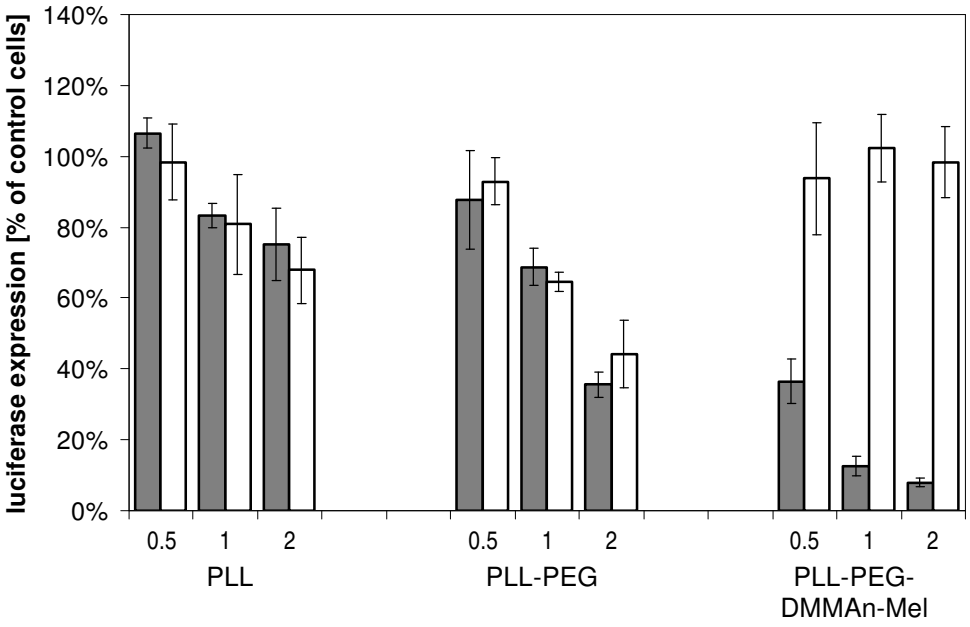
Table 6: Size of siRNA polyplexes. For FCS measurements Cy5 labeled siRNA was used. Polyplexes were generated in HBG at indicated polycation to siRNA ratios. FCS measurements were performed in cooperation with Claudia Schmidt, research group Prof. J. Rädler, Physics, LMU.

Particle size of PLL, PLL-PEG, PLL-PEG-Mel and PLL-PEG-DMMAn-Mel siRNA polyplexes was measured by FCS (**Table 6**). Consistent with the results obtained with PEI-conjugates, polyplexes prepared at the lower w/w-ratio were larger in size. Again PEGylated formulations without peptide formed the smallest complexes (8 nm). Formulations containing the lytic peptides were larger.

Combined PEGylation and DMMAn-Mel-modification of PEI and PLL enabled siRNA delivery. Hence the most promising conjugates (PEI-PEG-DMMAn-Mel and PLL-PEG-DMMAn-Mel) were compared side-by-side in transfection experiments on Neuro2A-eGFPLuc

cells (**Fig. 31**). PLL-PEG-DMMAn-Mel showed the best gene knockdown activity (up to 90% gene silencing compared to untreated control cells). Treatment with PEI-PEG-DMMAn-Mel siRNA polyplexes led to 70% protein knockdown.

31 a)



31 b)

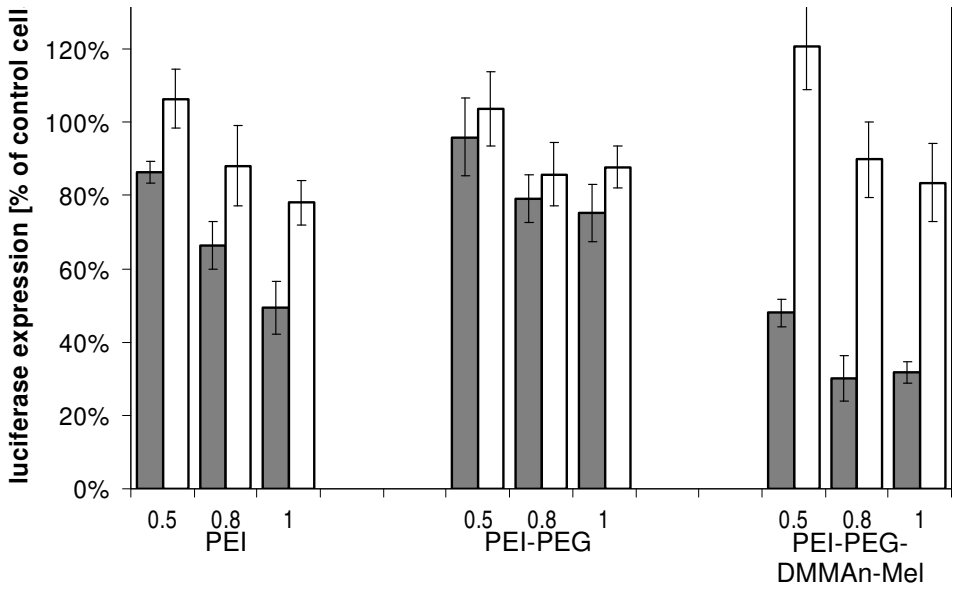


Fig. 31: siRNA gene silencing efficiency of PLL- and PEI-based conjugates. PLL- (**a**) PEI-based (**b**) conjugates on stably luciferase expressing Neuro2A-eGFPluc cells complexed with 500ng siRNA at different polycation to siRNA ratios (w/w). Gray bars indicate transfection with luciferase siRNA, white bars indicate transfection with complexes containing control siRNA. Reduced gene expression with control siRNA indicates unspecific knockdown / carrier toxicity. Data were generated by Alexander Philipp as part of his PhD thesis (in preparation).

Influence on metabolic activity was also assayed after transfection (**Fig. 32**). Treatment with PLL-PEG polyplexes at a high polycation to siRNA ratio resulted in a distinct reduction of metabolic activity, while cell viability of cells was not reduced after transfection with other tested formulations.

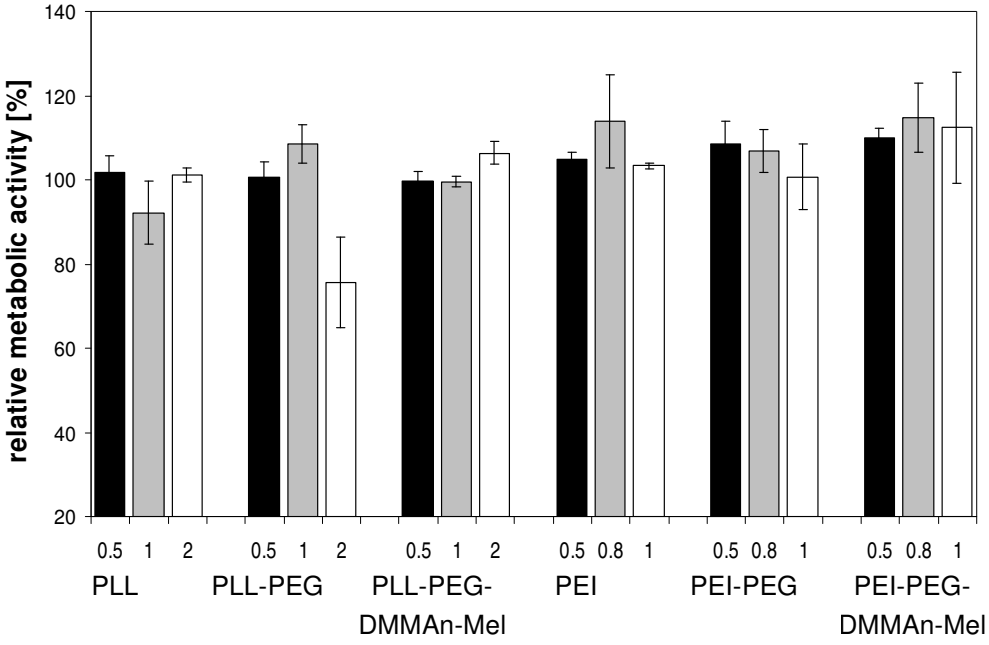
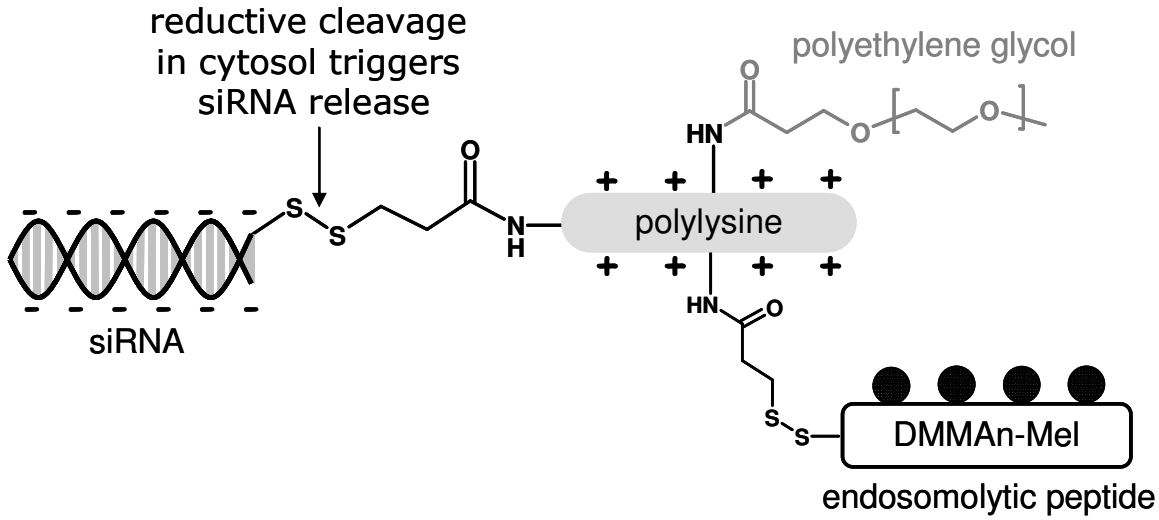


Fig. 32: Metabolic activity of Neuro2A-eGFPLuc cells 48 hours after initial siRNA transfection with indicated polyplexes. Metabolic activity of untreated control cells was set to 100%. Data were generated by Alexander Philipp as part of his PhD thesis (in preparation).

3.3 Conjugate containing covalently attached siRNA for improved delivery

3.3.1 Design and synthesis of the programmed siRNA carrier

Non-viral nucleic acid vectors are commonly generated by the complexation of negatively charged nucleic acids with cationic polymers or cationic lipids. Thus electrostatic interactions hold the polyplex together. However - especially *in vivo* - the polyplex is faced with polyanions, e.g. negatively charged plasma proteins like soluble glycosaminoglycans or heparan sulfates of the extracellular matrix. Interaction of polyplexes with this polyanions can lead to vector unpackaging before the nucleic acid carrier reaches the target cell [52]. To ensure extracellular stability a siRNA delivery system containing covalently attached siRNA was synthesized. As linkage a bioreversible disulfide bond was chosen. After reaching the cytosol the intracellular reductive environment could break the bond and thus enable release of the siRNA. The siRNA was linked to PLL-PEG-DMMAn-Mel, which in the previous *in vitro* experiments turned out to be the most promising siRNA carrier. A schematic structure is shown in **Scheme 3**.



Scheme 3: Schematic structure of PLL-PEG-DMMAn-Mel-siRNA

Synthesis of PLL-PEG-DMMAn-Mel-siRNA was carried out similarly to the PLL-PEG-DMMAn-Mel synthesis. In brief, thiol-modified luciferase siRNA (sense strand modified at the 5' end) was mixed with PLL-PEG-PDP. After reaction of thiol-siRNA with PDP-linkers, DMMAn-Mel was added to the mixture. To avoid aggregation of siRNA and DMMAn-Mel with PLL-PEG-PDP during synthesis, a high salt concentration (1.5 M NaCl) was applied. As previously mentioned PEG (MW 5000) could not be separated from branched PEI (25 kDa) by SEC. Because it was questionable if unbound siRNA (MW 13400) could be separated from the conjugate by size exclusion chromagraphy, a mock synthesis without covalent linkage was carried out. Unmodified siRNA was mixed with PLL-PEG-PDP and DMMAn-Mel.

After coupling of DMMAn-Mel the mixture containing PLL-PEG-DMMAn-Mel-PDP, free siRNA, DMMAn-Mel and low molecular weight impurities was loaded onto a Superdex 75 column. **Fig. 33** and **34** clearly show that separation of the mixture was possible.

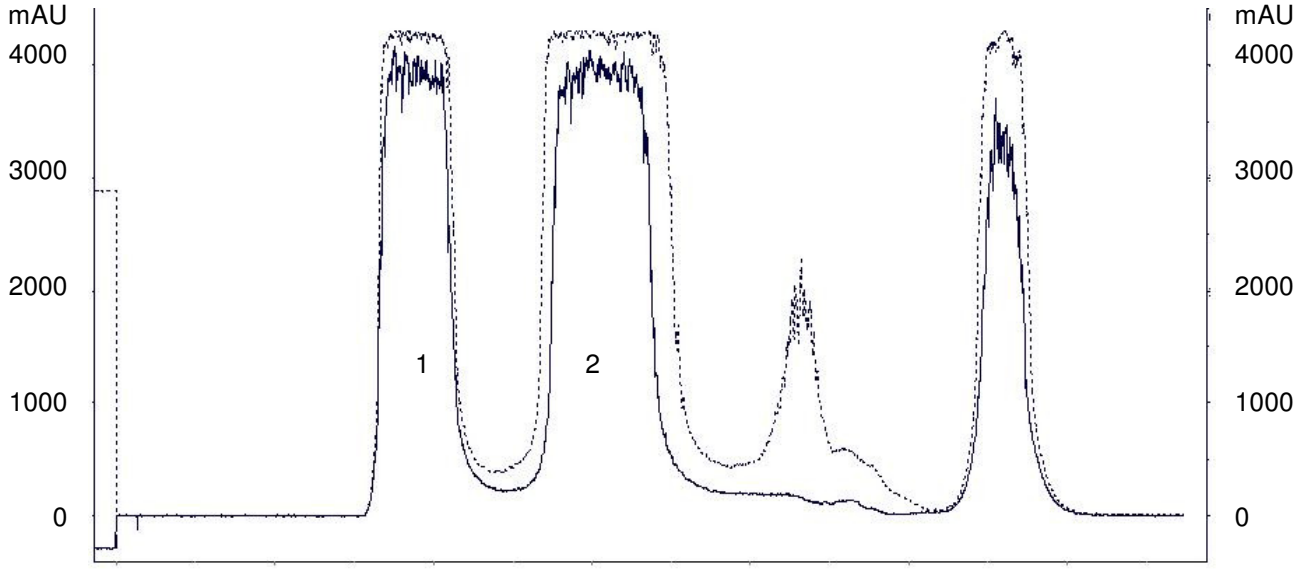


Fig. 33: Chromatogram of the PLL-PEG-DMMAn-Mel and siRNA-mixture. The mixture containing the siRNA-conjugate was loaded onto a Superdex 75 HR 10/30 column. The conjugate was eluted with 1M NaCl, 20mM Hepes, pH 8.2; flow rate: 0.5 ml/min. x-axis: elution volume; y-axis: absorption (dotted line: absorption at 260 nm. Solid line: absorption at 280nm). Peak 1: product conjugate; peak 2: unbound siRNA (see Fig. 34)

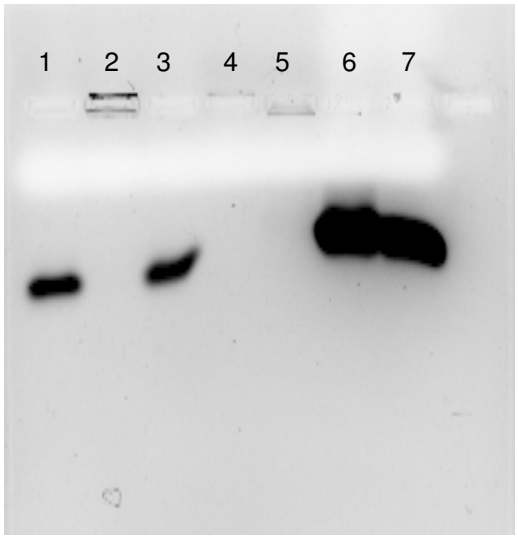


Fig. 34: Agarose gel electrophoresis of peaks 1 and 2 from Fig. 33. 1: 0.5 µg siRNA; 2: PLL-PEG-DMMAn-Mel siRNA polyplex (w/w 2); 3: like 2 plus heparin; 4: Peak 1; 5: Peak 1 plus heparin; 6: peak 2; 7: peak 2 plus heparin

As expected mixing of siRNA and PLL-PEG-DMMAn led to complex formation (Fig. 34, lane 2). However electrostatic interactions could be eliminated by heparin (Fig. 34, lane 3). Free siRNA was only present in peak 2 (Fig. 34, lanes 6 and 7) and hence could be separated from the PLL-PEG-DMMAn-Mel conjugate by SEC.

After establishing the reaction and purification protocol, synthesis was conducted analogously with the thiol-modified siRNA. First thiol-modified GL3 luc siRNA was mixed with

PLL-PEG-PDP. After 1 h released pyridine-2-thione was measured at 343 nm to determine the degree of modification with siRNA (approximately 1.5 siRNA / 1 PLL). Subsequently DMMAn-Mel was coupled to the remaining PDP-groups and the siRNA-conjugate was loaded onto a Superdex 75 size exclusion column for purification. **Fig. 35** shows the chromatogram of the SEC purification.

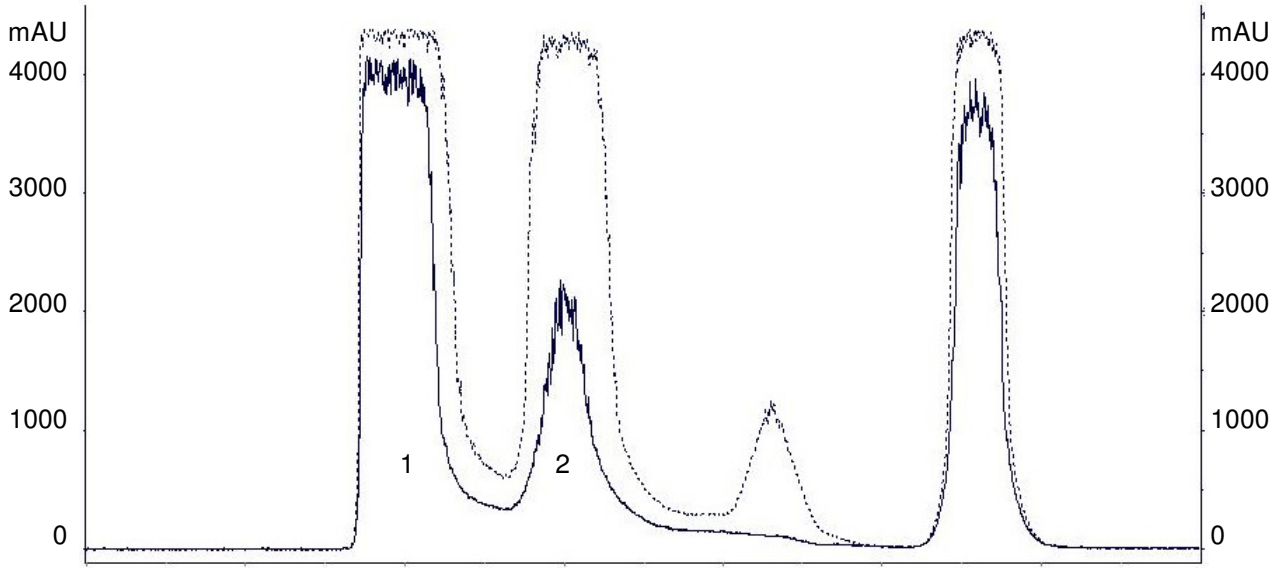


Fig. 35: Chromatogram of the PLL-PEG-DMMAn-Mel-siRNA purification. The mixture containing the siRNA-conjugate was loaded onto a Superdex 75 HR 10/30 column. The conjugate was eluted with 1M NaCl, 20mM Hepes, pH 8.2; flow rate: 0.5 ml/min. x-axis: elution volume; y-axis: absorption (dotted line: absorption at 260 nm. Solid line: absorption at 280nm). Peak 1: product conjugate; peak 2: unbound siRNA

In comparison with the chromatogram obtained after synthesis with unmodified siRNA (Fig. 33) a significant smaller amount of free siRNA (peak 2) was found, which is due to conjugation of a large part of thiol-modified siRNA to PLL-PEG-PDP. The fractions containing the conjugate were pooled and snap-frozen in liquid nitrogen. The purified PLL-PEG-DMMAn-Mel-siRNA conjugate had a molar ratio of approximately 1 / 1 / 7,5 / 1,5 (PLL / PEG / DMMAn-Mel / siRNA). This batch was used for the transfection experiments. A second batch was synthesized analogously, however after SEC-purification the conjugate was frist desalted by dialysis against HBG (pH 8.2). Not before desalting, this conjugate was snap-frozen in liquid nitrogen.

Additional, a conjugate containing Dharmacon's non-targeting control#3 siRNA was synthesized for transfection experiments. This control-conjugate was purified and handled like the first batch. Briefly, after SEC-purification it was snap-frozen in liquid nitrogen without further desalting. The composition was comparable to the GL3 luc conjugate: 1 / 1 / 6 / 1.3 (molar ratio of PLL / PEG / DMMAn-Mel / siRNA).

Covalent attachment of siRNA or rather absence of free siRNA in the conjugate were checked by agarose gel electrophoresis (**Fig. 36**).

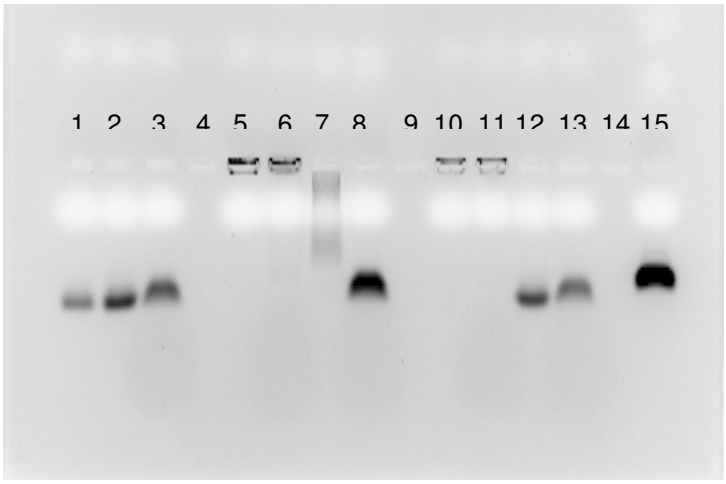


Fig. 36: Agarose gel electrophoresis of purified PLL-PEG-DMMAn-Mel-siRNA conjugate. 1: 0.25 µg siRNA; 2: 0.5 µg siRNA; 0.5 µg siRNA + heparin + TCEP; 4: free lane; 5: PLL-conjugate (2.4 µl of peak 1, Fig. 35) 6: peak 1 plus TCEP; 7: peak 1 plus heparin; 8: peak 1 plus heparin and TCEP; 9: free lane; 10: PLL/siRNA-polyplex (w/w 2); 11: PLL/siRNA-polyplex plus TCEP; 12: PLL/siRNA-polyplex plus heparin; 13: PLL/siRNA-polyplex plus heparin and TCEP; 14: free lane; 15: 20µl of peak 2, Fig. 35

From untreated conjugate (Fig. 36, lane 5) no siRNA is released during agarose gel electrophoresis. Neither TCEP nor heparin pretreatment alone could induce a release of siRNA (Fig. 36, lane 6 and 7, respectively). Only if reducing conditions and heparin treatment are combined siRNA is released from the PLL-PEG-DMMAn-Mel-siRNA conjugate (Fig. 36, lane 8). In contrast, heparin treatment alone could already unpackage a conventional electrostatic complexed PLL/siRNA polyplex (Fig, 36, lane 12).

3.3.2 siRNA delivery efficiency and cytotoxicity

siRNA delivery effficiency of the conjugate was evaluated on Neuro2A-eGFPLuc-cells and compared with an equal formulation apart from the fact that luciferase and control siRNA were electrostatically complexed (**Fig. 37**). In parallel cell viability of cells was assayed using a MTT assay (**Fig. 38**).

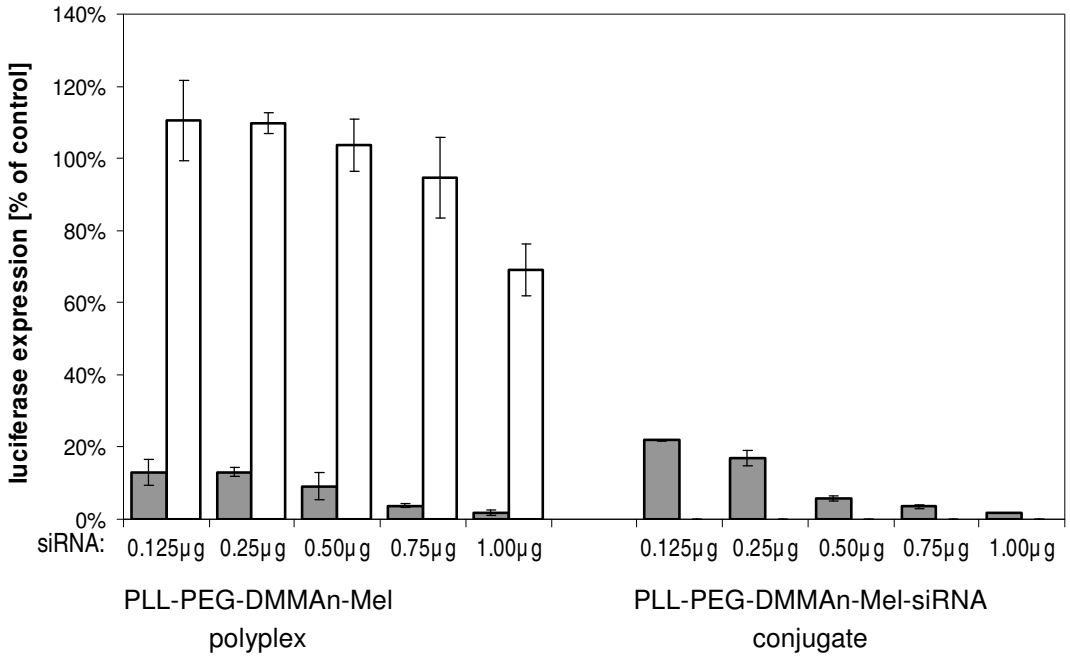


Fig. 37: siRNA gene silencing efficiency of PLL-PEG-DMMAn-Mel-siRNA. Protein knockdown of the conjugate was compared with knockdown activity of PLL-PEG-DMMAn-Mel electrostatically complexed at w/w ratio of 2 with indicated amounts of siRNA. Grey bars indicate transfection with luciferase siRNA, white bars indicate transfection with control siRNA. Data were generated by Alexander Philipp as part of his PhD thesis in preparation.

With the PLL-PEG-DMMAn-Mel / luc siRNA-polyplex (complexed at w/w ratio 2) a 90% reduction of luciferase expression could be observed. The conjugate containing covalently linked GL3 luc siRNA enabled 80% luciferase knockdown at lower siRNA doses (0.125 and 0.25 µg) and a 90% knockdown at higher siRNA amounts (0.5 – 1 µg). At the highest siRNA dose (1 µg) the luciferase knockdown effect is not only based on RNAi: also with control siRNA the luciferase expression is reduced, indicating unspecific protein knockdown caused e.g. by carrier toxicity.

Most recently, the covalent conjugate of non-targeting control#3 siRNA was also tested in the luciferase cell assay. Consistent with the concept of specific siRNA knock down, no reduction of luciferase activity was observed, unless the high 1 µg dose was applied (Alexander Philipp, unpublished results).

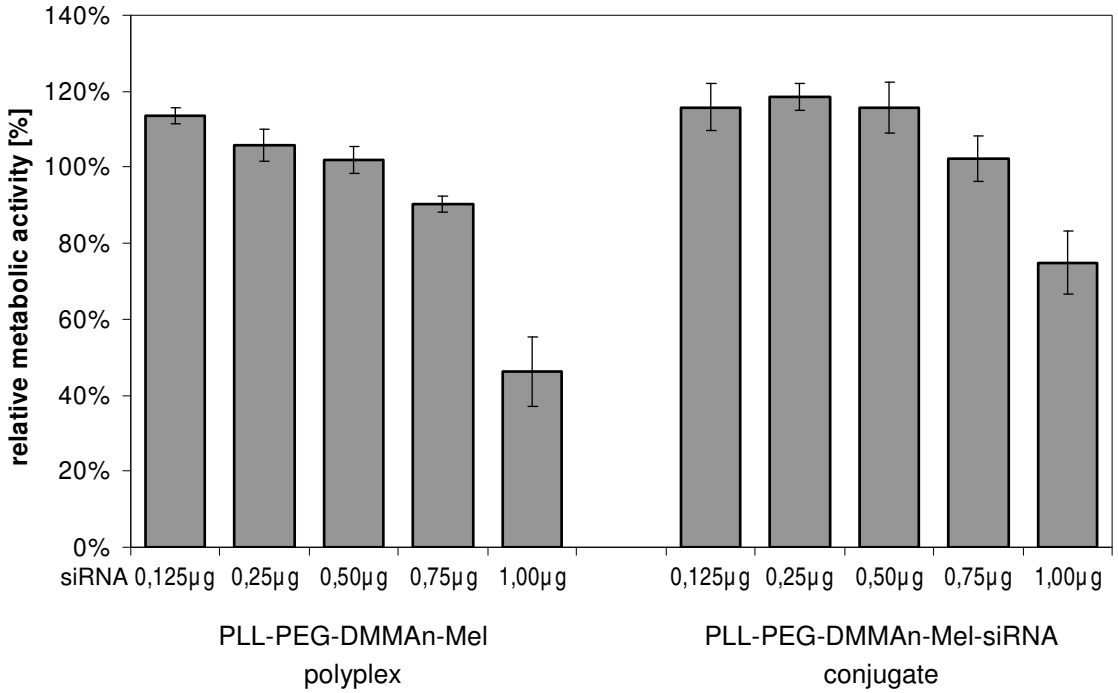


Fig. 38: Cell viabiliy after transfection with PLL-PEG-DMMAn-Mel-siRNA and the corresponding formulation containing electrostatically complexed siRNA. 5000 seeded Neuro2A-eGFPLuc-cells were treated with formulations containing indicated amounts of siRNA. 48 hours after initial transfection metabolic activity of cells was evaluated by a MTT assay. Data were generated by Alexander Philipp as part of his PhD thesis in preparation.

This observation was also reflected in the MTT assay. While cells maintened a high metabolic activity at siRNA doses from 0.125 µg to 0.75 µg, the metabolic activity was reduced significantly at the highest applied siRNA dose. In direct comparison, the PLL-siRNA-conjugate showed a reduced cytotoxicity (over 70% metabolic activity after treatment with 1 µg siRNA in contrast to 50% metabolic activity in case of the polyplex formulation).

3.3.3 Glutathione induced release of siRNA

In the above described agarose gel electrophoresis study (**Fig. 36**) TCEP was used as reducing agent. Compared to DTT, TCEP is more stable, more effective, and able to reduce disulfide bonds at lower pH. However it presents artificial, unnatural conditions. The less effective glutathione (GSH) is nature's reducing agent which is found in millimolar concentrations in the cytosol (1 – 10 mM) [139]. In the extracellular environment only micromolar concentrations are present. To clarify if release of siRNA is possible in cells, the conjugate was incubated at 37 °C with physiological glutathione concentrations and release of siRNA was monitored by agarose gel electrophoresis (**Fig. 39**).

In a side by side comparison of GSH-incubated and untreated siRNA it was found out that incubation with GSH weakened the EtBr-siRNA-fluorescence (data not shown). This can also be seen at lanes 1 (GSH-incubation of 0.5µg siRNA) and 4 (TCEP-incubation of conjugate

containing ~ 0.5 µg siRNA). Interestingly incubation with both TCEP and GSH did not weaken the fluorescence (lane 5). At GSH-concentrations of 1.25 to 5 mM released siRNA could be detected. At higher GSH – concentrations no released siRNA could be detected, remarkably after incubation of the conjugate at 200 mM GSH no fluorescence could be observed at all (lane 13). Detection of siRNA was not possible under the influence of high GSH concentrations.

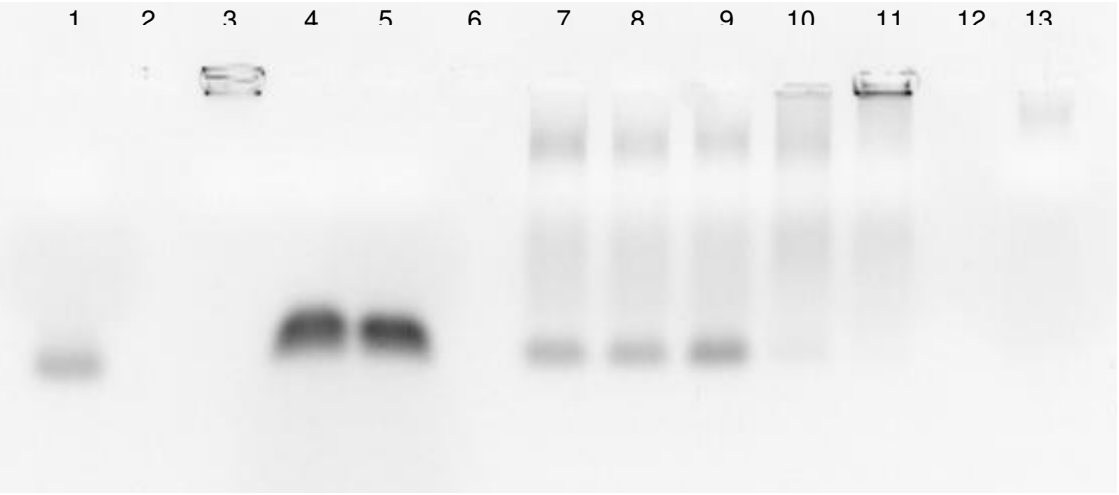


Fig. 39: Agarose gel electrophoresis of PLL-PEG-DMMAn-Mel-siRNA conjugate after glutathione treatment. The conjugate was preincubated for 75 minutes at 37 °C with different glutathione amounts (cytosolic glutathione concentration: 1 to 10 mM). 1: 0.5µg siRNA + heparin + GSH (20 mM); 2: free lane; 3: 2.4 µl conjugate; 4: 2.4µl conjugate + heparin + TCEP; 5: 2.4µl conjugate + heparin + TCEP + GSH (20 mM); 6: free lane; 7: 2.4µl conjugate + heparin + GSH (1.25 mM); 8: 2.4µl conjugate + heparin + GSH (2.5 mM); 9: 2.4µl conjugate + heparin + GSH (5 mM); 10: 2.4µl conjugate + heparin + GSH (10 mM); 11: 2.4µl conjugate + heparin + GSH (20 mM); 12: free lane; 13: 2.4µl conjugate + heparin + GSH (200 mM)

To prove the fluorescence eliminating effect, GSH was added stepwise to a fluorescent EtBr-siRNA-solution (**Fig. 40**). At GSH-concentrations above 12 mM fluorescence dropped abruptly. In a control experiment water in corresponding volume was added (data not shown).

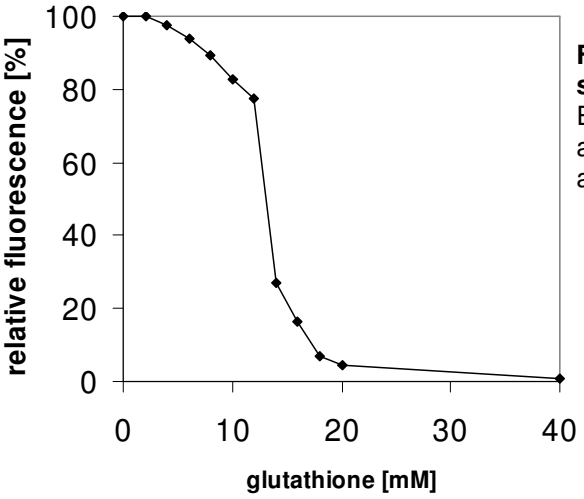


Fig. 40: Influence of GSH-concentration on EtBr-siRNA fluorescence. To 20 µg of siRNA in 2 ml EtBr-solution (0.4 µg/ml EtBr in HBG) GSH was added stepwise at RT. 30 seconds after each addition fluorescence was measured.

The same assay was performed with GelRed, a more conventient environmentally safe EtBr alternative. But GSH-addition also led to a constant decrease in fluorescence (data not shown).

In summary, glutathione treatment at 37 °C could induce release of siRNA from the PLL-PEG-DMMAn-Mel-siRNA-conjugate, however exact quantification was hardly possible due to the observed GSH-induced fluorescence variation.

3.3.4 Particle size

With respect to *in vivo* toxicity and passive targeting, size of the conjugate / particle is of considerable interest. Because size of the formulation can depend on parameters like salt-concentration and / or freeze-thaw-processes, size was determined by DLS at different formulation steps. Directly after SEC before freezing the conjugate formed 80 nm particles in 1M NaCl. After snap-freezing and thawing, size increased to 150 nm. When the unfrozen sample was diluted to 0.5 M NaCl and 0.15 M NaCl sizes grew to 300 nm and 250 nm, respectively. When 5% glucose was added to the 1 M NaCl-solution directly after SEC before freezing, the size of the main fraction did not change (80 nm), but some larger particles of different sizes were found (e.g. 600 nm). During the dialysis process against HBG pH 8.2, the conjugate is faced with salt concentrations which favor aggregation (0.15 – 0.5 M NaCl). Dialysis at 4 °C for 4 hours immediately after SEC before freezing resulted in 150 nm particles. Freezing and thawing of the conjugate in HBG did not change particle size. In summary, the PLL-PEG-DMMAn-Mel-siRNA conjugate formed particles in the range of 80 – 300 nm, monomolecular structures could not be detected in DLS experiments. This important formulation subject is currently being evaluated by the succeeding PhD thesis (Christian Dohmen, work in progress).

4 Discussion

4.1.1 DMMAn-Mel-modification enhances pDNA transfer efficiency and reduces cytotoxicity of PLL

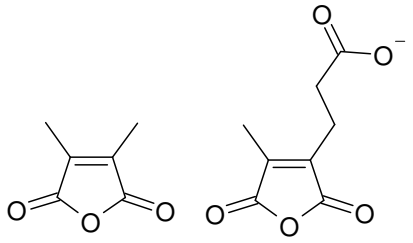
The development of dynamic formulations ('artificial viruses') is a key issue in the field of nucleic acid delivery [112;140;141]. The intracellular dynamic behaviour of viruses, which have naturally evolved for delivery of their nucleic acid payload and their replication, can serve as inspiring example for the design of gene carriers.

Poor endosomal release and toxicity of the carrier limit the application of non-viral gene transfer systems. A variety of endosomolytic peptides have been evaluated for the enhancement of nucleic acid delivery. Among those for example the acidic peptide GALA [105] possesses a most favorable pH-specific lytic activity. The acidic influenza derived peptide INF7 shows pH-specific high activity also on natural erythrocyte membranes and strongly enhances polylysine-mediated gene transfer [97]. The acidic, anionic nature of INF7 however also causes problems for formulation with DNA: PLL-INF7 conjugates similar to those described previously [99] formed DNA complexes which were unstable and resulted in strong particle aggregation (data not shown). The cationic membrane-active peptide melittin does not display this problem; covalent melittin-polycation conjugates are well soluble and mediate strongly increased gene transfer activity [102;142].

Melittin displays similar highly efficient activity on biological membranes as INF7; however, it is not pH-specific. Lytic activity in the extracellular environment is unfavorable as it mediates toxic side effects [104]. To overcome this problem, Rozema *et al*. used an amine-reactive carboxylated dialkylmaleic anhydride derivative (2-propionic-3-methylmaleic anhydride, CDM) to mask the lytic activity of melittin at neutral pH which was restored after acidic cleavage of the protecting groups [129]. As described in 1967, maleic anhydrides reversibly react with the lysine residues and the N-terminal amino group of peptides and are removed again at slightly acidic, endosomal pH [143]. CDM represents a not commercially available dialkylmaleic anhydride derivative (**Fig. 44**). Rozema *et al.* state in their published work that only CDM, but not the commercially available inexpensive DMMAn is sufficient for masking the lytic activity of melittin.

However the same group (Mirus Corporation) published the results of a hemolysis assay in US patent Nr. 6630351 where the presented data suggest that DMMAn is suitable for masking melittin's lytic activity. No pDNA delivery studies were performed, and no covalent incorporation into cationic polymer carriers were tested. Aim of the current study was (i) to evaluate whether DMMAn-shielding of melittin is effective and pH-specific and (ii) to build on

the concept by now covalently linking a masked melittin peptide to a pDNA-binding polycation for polyplex formation.



DMMAn CDM

Fig. 44: Structures of CDM and DMMAn. DMMAn: dimethylmaleic anhydride; CDM: 2-propionic-3-methylmaleic anhydride [129]

Although the developed polymers in our group were more or less all OEI- or PEI-based, PLL was chosen as backbone polymer. In contrast to ethylenimine-containing polymers, PLL possesses no innate endosomal escape mechanism and as a consequence shows a very low gene transfer efficiency. In previous works melittin and analogs were linked to PEI [101;103;104]. On the one hand this is comprehensible because PEI for itself is highly effective in gene transfer and the most-frequently used polycation. But on the other hand if the influence of endosomolytic active agents wants to be analyzed, a combination with an endosomal escape inducing polymer might lead to synergistic effects and as a consequence complicate the evaluation. Thus, to “isolate” the endosomolytic effect, DMMAn-masked melittin was linked to PLL, yielding the conjugate PLL-DMMAn-Mel. For comparison unmodified melittin was also coupled to PLL; such a conjugate also has not been described previously.

In a preliminary experiment melittin was modified with DMMAn end erytrocyte leakage activity was monitored. DMMAn-modification resulted in reduced lytic activity at neutral pH and a to unmodified melittin comparable lytic activity at pH 5. This demonstrates that DMMAn is an appropriate reagent for reversible masking of melittin’s lytic activity. In contrast to maleic and monomethylmaleic anhydrides, which produce an irreversible modification of sulfhydryl groups, dimethylmaleic anhydride does not cause a stable modification of thiol compounds. This behavior makes dimethylmaleic anhydride a specific reagent for the peptide amino groups [144]. Erythrocyte leakage assay (**Fig. 9**) demonstrated that also PLL-DMMAn-Mel (in contrast to PLL-Mel) has a relatively low lytic activity at physiological pH over the whole tested concentration range. This is not self-evident because linkage of a polycation can change the endosomolytic properties [103]. The lytic activity could be restored almost to the level of PLL-Mel after preincubation at endosomal pH. These results prove that dimethylmaleic anhydride shielding can be used to generate bioresponsive conjugates with the desired lytic activity profile.

The lack of endosomolytic properties in case of PLL correlates with its low gene transfer activity. In contrast, both melittin conjugates greatly enhanced the gene transfer efficiency. A positive effect was also observed with a targeting ligand (transferrin) and a PEG shield within the polyplex formulation (**Fig. 12**). Such targeting and shielding domains have been found advantageous for *in vivo* gene delivery. While PEG shielding is very advantageous for polyplex stability, inhibition of undesired interactions or long systemic circulation after i.v. administration, it regrettably hampers intracellular trafficking and overall can reduce the transfection efficiency of the polyplex. To overcome this problem a pH-reversible PEG shielding can be applied. After reaching the acidic intracellular environment the PEG shield is removed. This strategy has been successfully applied for PEI polyplexes *in vitro* and *in vivo,* pH-reversible linkage improved gene transfer efficieny in comparison to stable shielded polyplexes [120]. In this work polyplexes containing both pH-dependent endosomolytic peptide and pH-reversible PEG shielding were also tested, however the usage of pH-reversible in comparison with stable linked PEG did not further improve gene transfer. In consideration of the fact that stable PEG shielding did not decrease transfection efficiency of the tested polyplexes (**Fig. 12**) this finding is not surprising: pH-reversible PEGylation for itself cannot enhance luciferase gene expresssion, it just can avoid the negative effect of stable PEG shielding on gene transfer activity. Futhermore confocal laser scanning microscopy pictures of the targeted and PEGylated polyplexes containing labeled pDNA were acquired. After 3 and 24 hours polyplexes were found located in endosomal vesicles, but no spreaded fluorescence could be detected in the cytoplasm (data not shown). This indicates that endosomal escape is still a rare event even if gene transfer enhancing endosomolytic peptides are applied.

To investigate the effect of endosomal acidification on the gene transfer activity, bafilomycin A1, an inhibitor of vacuolar ATPase endosome pumps, was included in transfection experiments. The efficiency of the gene carrier PEI relies on the “proton sponge” effect upon acidification of the endosomal compartment. Consistent with published literature [145] inhibition of acidification by bafilomycin A1 strongly reduced the gene transfer activity. Bafilomycin A1 did not alter gene transfer of PLL polyplexes which lack proton sponge activity. Interestingly, bafilomycin enhanced gene transfer activity of the PLL-Mel conjugate. This can be explained by the higher lytic activity of melittin at neutral pH than at acidic pH. Gene transfer by PLL-DMMAn-Mel was only slightly reduced by bafilomycin treatment. At first sight this incomplete inhibition seems to contradict the previous findings that 30 min incubation of erythrocytes with masked melittin does not result in lysis and that bafilomycin A1 can inhibit membrane lytic activity of masked melittin when incubated with cells for 10 min [129]. However, we observed an increase in erythrocyte lytic activity of DMMAn-Mel at neutral pH after a longer incubation period (2.5 h at 37 °C). Taken all together PLL-DMMAn-

Mel data are consistent with a slow unmasking of melittin at neutral pH in the presence of bafilomycin inhibitor. The lack of fast acidic pH-triggered unmasking seems to counteract the enhancing effect of bafilomycin A1 as seen with non-masked PLL-Mel. A slow unmasking of melittin at neutral pH over a longer time period should be sufficient in terms of reduction of the acute toxicity, since polycationic gene carriers will be cleared from the blood circulation system and extracellular space within relatively short periods [146;147]. It also might ensure enhanced transfection efficiency upon intracellular trafficking of polyplexes into non-acidic intracellular vesicles.

Apart from poor endosomal release also cytotoxicity of gene vectors is one of the limiting barriers to systemic non-viral nucleic acid delivery. High lytic activity of polymers before reaching their target enforces the problem. To address this issue relative metabolic activity of conjugate treated cells was determined via MTT assay (**Fig 14**). It has to be emphasized that the transfection experiments were performed at rather low polymer concentrations (2.4 µg/ml at N/P 2). Toxicity effects might be disguised at low concentrations, and higher polymer amounts might also be necessary for *in vivo* delivery. Hence toxicity was studied over a broad concentration range. Notably, PLL-DMMAn-Mel was much better tolerated by cells resulting in higher metabolic activity than PLL and PLL-Mel. To further evaluate the differences in toxicity we performed a LDH release assay (**Fig 16**). The result confirmed that PLL-DMMAn-Mel is the conjugate with the lowest acute toxicity. As expected, PLL-Mel mediated the highest LDH release. Remarkably, PLL-DMMAn-Mel released less LDH than the non-endosomolytic PLL. In accordance with the observed visible changes in cellular morphology PLL-Mel is the most toxic conjugate. It has to be mentioned that at higher polymer concentrations LDH release might also be enhanced because of cell necrosis and not only due to conjugate induced membrane interaction and pore formation. But also at low concentrations (0 to 5 µg/ml) where cells are viable according to the metabolic activity assay differences in LDH release are detectable. The observed results are in agreement with recent work on the interactions of polycations with lipid bilayers and cell membranes [148;149]. PLL can induce lipid bilayer nanoscale pore formation and thus enhances membrane permeability due to its regular positive charges. When the polycation is grafted with DMMAn-Mel, parts of the regular polycationic positive charges are masked and acute toxicity and membrane-disruptive interactions are decreased. In contrast, standard melittin which on its own can mediate cell membrane disruption (as measured by LDH release; **Fig. 17**) upon conjugation adds lytic activity to the polymer. The data proves that DMMAn shielding of melittin not only avoids the undesired lytic activity in the extracellular environment, but also further reduces acute toxicity of the core polycation PLL which is advantageous particularly with regard to systemic *in vivo* delivery.

In summary, PLL-DMMAn-Mel represents a dynamic gene transfer system based on the polycation PLL with triggered endosomal lytic activity. Both melittin and DMMAn-Mel enhance gene transfer activity, but while melittin increases toxicity, DMMAn-Mel grafting even lowers the polycation cytotoxicity. In the following work, this promising strategy was also applied to other polymeric nucleic acid carriers.

4.1.2 PEGylation and peptide modification of polycations: impact on pDNA and siRNA delivery

PEI is one of the most frequently used polycations for non-viral gene delivery. PEI polymers can be synthesized with a branched or a linear structure and are available in a wide range of molecular weights. In this study branched PEI (25 kDa) was modified with DMMAn-Mel. However it was not possible to generate small nanosized nucleic acid polyplexes with PEI-DMMAn-Mel. Modification of PEI with the negatively charged DMMAn-Mel peptide induced polymer collapse and polyplex aggregation (> 1µm, colloidal solution after SEC). DMMAn-Mel has a net negative charge of -3 (unmodified melittin a net positive charge of +5). The negative charge favors interactions with the polycation. If peptides are grafted in a high density onto a polycation this can result in polymer aggregation. The same was observed when the negatively charged influenza peptide was grafted in high ratios on PLL (personal communication, E. Wagner); high ionic strength had to be applied during synthesis [99]. This handling difficulty was one of the reasons why Ogris *et al.* introduced the cationic peptide melittin, although it does not feature the desired pH-dependent lytic activity [101]. In general large particles are unfavored for intravenous administration. To overcome the aggregation problem, the PEI polymer was prePEGylated before peptides were attached. For polyplex shielding usually PEG with a high MW (e.g. 20 kDA) is used. The main intention of the PEGylation certainly was not shielding / reduction of surface-charge but rather solubility improvement and prevention of aggregation. For that reason PEI primarily was modified with PEG 5000. After this prePEGylation the SPDP linkers and subsequently DMMAn-Mel peptides were coupled. This strategy turned out to be successful, the PEI-PEG-DMMAn-Mel conjugate (molar ratio of PEI/PEG/DMMAn-Mel ~ 1/1.5/8) formed pDNA polyplexes in HBG with an adequate size (< 200 nm). Encouraged from this finding, the negatively charged INF peptide also was coupled successfully to PEI-PEG. The conjugates of the first series of synthesis were PEI-PEG, PEI-PEG-DMMAn-Mel, PEI-PEG-Mel and PEI-PEG-INF. With this conjugates different characteristic endosomolytic properties can be compared (e.g. pH-dependent vs. static lytic activity).

However conjugation of peptides not only changes the lytic activity of polymers, it also alters physicochemical properties like charge density or molecular weight of the conjugates which again can result in different polyplex characteristics. If worst comes to worse an improved *in*

vitro gene transfer efficiency of a polyplex containing an additional targeting peptide can be just the consequence of e.g. a different size of the polyplex and not of the introduced intrinsic targeting ability. In general this has to be kept in mind if transfection experiments are interpreted. To cope with this aspect, succinicanhydride modified melittin was linked to PEI-PEG. Succinicanhydride reacts (like DMMAn) with the primary amines of melittin and eliminates the lytic activity of melittin, but in contrast to acid-sensitive DMMAn the modification is irreversible. As a result PEI-PEG-SuccMel should possess due to its melittin-grafting similar properties like PEI-PEG-DMMAn-Mel, except for the fact that the protecting group is not cleavable. PEI-PEG-Succ-Mel was used as negative control in transfection experiments.

With regard to cytotoxicity, DMMAn-Mel modification of PEI-PEG had similar effects comparable to DMMAn-Mel modification of PLL: After incubation of Neuro2a cells with conjugates, DMMAn-Mel modified PEI-PEG induced less LDH-release than PEI-PEG-Mel or PEI-PEG (**Fig. 18**). Modification with the negatively charged and at neutral pH unlytic influenza peptide also reduced LDH-release.

pDNA transfection efficiency was evaluated on Neuro2A- and HUH7-cells. PEI polyplexes showed good transfection efficiency. PEGylation had a disastrous effect: PEI lost its gene transfer activity; PEI-PEG polyplexes only reached background levels of luciferase expression (**Fig. 20**). This phenomenon was investigated in detail by Mishra and coworkers. Both unmodified and PEGylated particles enter cells through the endocytic pathway and accumulate in a perinuclear region. The overall cellular uptake of polyplexes was similar, but when unPEGylated PEI polyplexes were applied, more PEI per endosome was found. In case of PEI-PEG polyplexes the amount of PEI per endosome was too low to mediate buffering and endosomal escape [91]. But after attachment of DMMAn-Mel, Mel and INF to PEI-PEG gene transfer activity was restored. Conjugation of Succ-Mel did not improve gene transfer activity. This indicates that the added endosomolytic properties are responsible for the gene transfer enhancement; adding a similar structured peptide without lytic activity (Succ-Mel), which might influence the biophysical properties of the polyplex, is not sufficient.

Size of the polyplexes ranged from 80 nm to 190 nm. Polyplexes containing negatively charged peptides formed the largest polyplexes (190 nm). The polyplexes were generated at N/P 6 in HBG at a pDNA-concentration of 20 µg/ml during complex formation. For transfection experiments the polyplexes were prepared under the same conditions. This is an important issue, because the conditions during complex formation can have an influence on the size of the nucleic acid formulation. PEGylation reduced the zeta-potential in comparison to PEI-polyplexes. However all PEGylated polyplexes were only partially shielded. It is known that PEGylation can almost neutralize the surface charge of polyplexes [121], but for this purpose PEG with a higher MW has to be applied (e.g. PEG 20000). The remaining positive

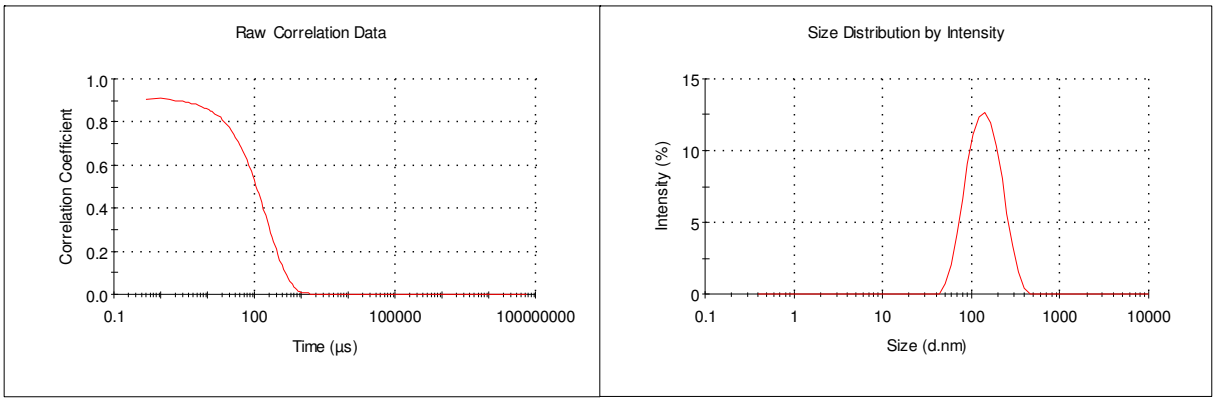
surface charge contributes to the polyplex-cell-interaction, which is important in terms of association and internalization because no additional targeting ligand is incorporated into the formulation.

Endosomal escape is not only required for pDNA delivery, also siRNA polyplexes are endocytosed and release of the endocytosed material into the cytoplasm is mandatory. For that reason, siRNA delivery efficiency of the synthesized endosomolytic conjugates was investigated. While PEI is effective in pDNA delivery, no siRNA delivery activity was observed on Neuro2A-eGFPLuc- and HUH7-eGFPLuc cells (**Fig. 26 and 27**). PEGylation alone did not improve siRNA delivery activity. But after modification of PEI-PEG with DMMAn-Mel and INF-peptide siRNA delivery efficiency was greatly enhanced as demonstrated by luciferase knockdown (~ 70% protein knockdown). As expected, modification with Succ-Mel did not improve gene silencing. Interestingly, PEI-PEG-Mel was not effective (despite the fact that pDNA-transfer efficiency was enhanced with melittin). Beside endosomal escape, polymer-siRNA complex dissociation was discussed as barrier in siRNA delivery [150;151]. A positively charged peptide (like Mel) could lead to a too strong siRNA association, while negatively charged DMMAn-Mel and INF reduce the binding strength of PEI so that dissocation in the cytoplasm can take part. However recently published results suggest that the opposite – a too low stability of the siRNA polyplex – is a major drawback [52-54]. Hence the reason for the found difference in pDNA- and siRNA-delivery activity of PEI-PEG-Mel stays unclear. In general the gene silencing effect was more pronounced in HUH7-eGFPLuc cells. 80 % protein knockdown was achieved with PEI-PEG-DMMAn-Mel and PEI-PEG-INF polyplexes. Even with PEI-PEG-Mel and -Succ-Mel a slight protein knockdown could be observed with this cell line. In both cell lines luciferase expression was not reduced when experiments were performed with non-targeting control siRNA, which demonstrates that protein knockdown of PEI-PEG-DMMAn-Mel and PEI-PEG-INF is not due to carrier toxicity. This was confirmed by a MTT assay at the end of transfection which proved that metabolic activity of Neuro2A-eGFPLuc cells was not affected in a large extent (**Fig. 31**).

Differences in polyplex size can explain differences in delivery activity. Size of selected siRNA-polyplexes was measured by fluorescence correlation spectroscopy (FCS). Usually dynamic light scattering (DLS) was used for determining the hydrodynamic radius of the polyplexes. However only poor data quality could be obtained with DLS, the results were not reliable. If the the particles are small (e.g. 10 nm) and the concentration is too low, only a low intensity / poor data quality can be obtained with DLS. **Fig. 45** shows exemplarily the DLS results of two different formulations. Panels of **(a)** display the raw correlation and size distribution data of an evaluable measurement, **(b)** shows inappropriate results obtained with small siRNA polyplexes. One feasible solution would be to use more siRNA / a higher polyplex concentration for the size measurements (in general the Zetasizer Nano ZS is

capable to measure sizes in the area of 5 nm, as demonstrated by measurement of a concentrated albumin solution), but the (low) concentration during polyplex formation limits the concentration during measurement (additional the available siRNA and conjugate amounts are limited).

45 a)



45 b)

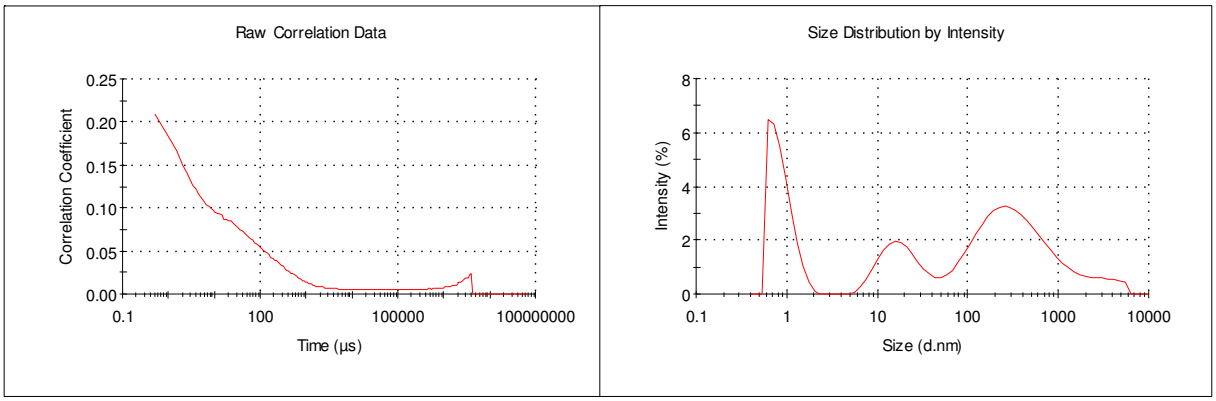


Fig. 45: Exemplary dynamic light scattering results of nucleic acid formulations. Panels of **(a)** display the raw correlation and size distribution data of a valid measurement, **(b)** shows inappropriate results obtained with small siRNA polyplexes.

Therefore fluorescence correlation spectroscopy (FCS) was chosen as method to evaluate the size of this siRNA polyplexes. FCS detects spontaneous intensity fluctuations resulting from fluorescently labeled molecules diffusing through a highly focused laser illuminated volume within the sample. Measured diffusion times (of Cy5-labeled polyplexes) were used to calculate the hydrodynamic radius. As displayed in **Table 5**, PEI-PEG formed polyplexes with 10 and 15 nm (at w/w ratios of 0.5 and 1). PEI-PEG-DMMAn-Mel polyplexes were larger. At w/w-ratio of 0.5 polyplexes had a size of 89 nm, at w/w 1 a size of 26 nm. This size difference might explain why PEI-PEG-DMMAn-Mel mediates siRNA delivery; however non-transfecting PEI polyplexes prepared at w/w 0.5 were also large in size and highly polydispers (93 – 2800 nm). Additional PEI-PEG-Mel forms particles at w/w 0.5 with a similar size like PEI-PEG-DMMAn-Mel generated at w/w 1, but PEI-PEG-Mel polyplexes did not work for siRNA delivery.

Altogether, the results suggest that differences in siRNA delivery activity are not mainly based on size differences of polyplexes.

PEI's are among the most efficient non-viral gene vectors that have been developed for *in vitro* and *in vivo* delivery because they reconcile several attributes which are necessary for efficient gene transfer, namely nucleic acid complexation, protection from nucleases, cell internalization, endosomal buffering / escape and release of pDNA. Accordingly people hazard the consequences of using a non-biodegradable polymer like PEI because the polymers show good transfection efficiencies even without extensive modification. But as demonstrated, branched PEI on its own was not effective in siRNA delivery. Now the question rised why a polycation which is both not biocompatible and not effective should be used as conjugate backbone. As a logical consequence it was decided to turn once more towards the biodegradable (on its own also ineffective) PLL, not just as proof-of-concept polymer but rather as serious backbone alternative to PEI.

Analog to PEI-PEG-conjugates, DMMAn-Mel-, Mel- and INF-peptide were linked to PLL-PEG and siRNA delivery efficiency was studied using Neuro2A-eGFPLuc cells. As expected, PLL and PLL-PEG did not mediate siRNA delivery (**Fig. 29**). In contrast to the results obtained with PEI-PEG-conjugates, INF-modified PLL-PEG was not effective in siRNA-delivery. Only PLL-PEG-DMMAn-Mel delivered successfully siRNA which resulted in a 90% reduced luciferase expression compared to untreated control cells, thus being more effective than the corresponding PEI-PEG counterpart. Remarkably PLL-PEG polyplexes displayed a considerable protein knockdown when cells were transfected with formulations containing control siRNA, indicating carrier toxicity. This observed cytotoxicity was confirmed by MTT assay were PLL-PEG polyplexes reduced the metabolic activity (**Fig. 31**). The finding is contrary to popular belief: commonly PEG is applied to reduce toxicity of formulations. But PEG improves the solubility and in this way the "bioavailability" of the (potentially toxic) polycation is raised. Viewed in this light the cytotoxicity of PLL-PEG polyplexes is understandable.

Although all three applied peptides are lytic, they develop the endosomolytic activity in several ways. Melittin is lytic both at pH 5 and 7. Before cleavage of the protecting groups DMMAn-Mel shows reduced lytic activity at neutral pH, after deshielding it is obviously lytic at both acidic and neutral pH. In comparison to unmodified Mel the lytic activity of DMMAn-Mel could be exposed at a later phase of intracellular trafficking. INF is only lytic at acidic pH. If the endosome is not acidified, endosomal escape function is not activated. Furthermore, INF might be self-limiting: after a first membrane pore formation in the acidic endosomal environment the pH can be immediately neutralized due to a fast influx of the neutral cytosol / protons through the just formed leak. This neutralization might inactivate the INF peptide before sufficient membrane perforation and escape of the polyplex can take place.

Additional, in case of PEI-conjugates a combined effect consisting of PEI's proton sponge capacity and the lytic effect of the peptide can contribute to endosomal escape. Beside differences in biophysical properties between the formulations, these mechanistic characteristics can contribute to varying delivery activities of the conjugates.

Size of selected polyplexes was measured by FCS. Related to siRNA polyplexes of PEI-conjugates, PLL-PEG formed the smallest particles (8 nm, **Table 6**). Both, PLL-PEG-DMMAn-Mel and PLL-PEG-Mel formed larger particles at w/w 1 (high polydispersity) and small particles at w/w 2 (40 nm and 12 nm, respectively). Because large PLL-PEG-Mel polyplexes did not enhance siRNA delivery, the activity of (small and large) PLL-PEG-DMMAn-Mel polyplexes cannot be explained by a size effect.

In summary PEGylation and DMMAn-Mel modification of polycations allowed the formation of nanosized polyplexes and enabled siRNA delivery despite the fact, that unmodified polycations showed no siRNA delivery at all.

pDNA-transfections were also carried out with the PLL-conjugates on Neuro2A cells (**Fig. 23**). PLL-PEG-DMMAn-Mel polyplexes showed an exceptionally high gene transfer activity while the other conjugates were ineffective. By MTT assay cytotoxicity was ruled out as reason for the great differences in transfection efficiency (**Fig. 24**).

In transfection experiments, the polyplex solution is added to the cell culture medium. It might be that polyplexes aggregate in the cell culture medium before they reach the cells, which could explain differences in transfection experiments. To investigate this effect polyplexes were incubated in cell culture medium for 3 h at 37 °C and sizes were measured. While PLL and PLL-PEG polyplexes grew in size (from ~ 90 to 180 nm), PLL-PEG-peptide polyplexes did not change significantly. Additional cellular polyplex internalization was studied using flow cytometry. PLL-PEG polyplexes showed a better uptake than PLL polyplexes, the highest degree of internalization was reached by PLL-PEG-peptide polyplexes. There is, therefore, every indication that differences in transfection efficiency between PLL-PEG-peptide polyplexes are not due to varying aggregation in cell culture medium or an extraordinary high internalization degree of PLL-PEG-DMMAn-Mel polyplexes. Murthy *et al.* designed a polymer which is degraded in the acidic environment of the endosome [152]. They claim that the decomposition induces a colloid osmotic disruption of the vesicle which delivery the content into the cytosol. The Kataoka group also argues that an acid-labile PEG-nucleic acid conjugate releases hundreds of free PEG strands in the acidic endosomal compartment which increases the osmotic pressure and eventually induces swelling and disruption of the endosome [128]. Basically this is also imaginable when DMMAn-groups are cleaved within the endosome; however this is not evidence-based. Hence, apart from differences in polyplex size, the reason for the remarkably high nucleic acid delivery activity of DMMAn-Mel-conjugates remains unsolved.

4.1.3 Programmed conjugate containing bioreversibly attached siRNA for improved delivery

To reach the site of action within the target cell, the nucleic acid has to stay associated with its carrier during the complete extracellular delivery process. Most polyplex and lipoplex formulations are held together electrostatically. A weak point resides in the fact that other charged molecules can disrupt such complexes before they reach the target cell. Burke *et al.* demonstrated that both the serum and the extracellular matrix can lead to vector disassembly. They observed significant vector unpackaging of systemically injected fluorescence-labeled PEI / pDNA polyplexes in the liver, where the labeled DNA was found in different areas than the labeled PEI. This obviously negatively affects gene transfer efficiency [52]. The work of Buyens *et al.* reveals the same weak point. They developed a method based on fluorescence correlation spectroscopy to study the dissociation of siRNA complexes in human serum. The work demonstrates that nonpegylated and pegylated siRNA liposome complexes undergo fast disassembly in full serum. Similar observations were made for PEI / siRNA polyplexes [53]. Vector unpacking is more likely a problem in case of siRNA in comparison with pDNA, as the far larger number of negative charges of pDNA stabilizes the interelectrolyte complex [54;55].

To overcome this drawback a conjugate containing covalently attached siRNA was synthesized and tested for siRNA delivery activity. Luciferase siRNA was linked via disulfide bond to PLL-PEG-DMMAn-Mel. All needful domains (PEG, pH-responsive lytic peptide, polycation, siRNA) are assembled in one single conjugate, which is an alternative to the conventional modular building set approach where e.g. 10% PLL, 10% PLL-PEG and 80% PLL-DMMAn-Mel are mixed and complexed with the appropriate amount of siRNA. The all-in-one solution guarantees optimal codelivery of all functional domains, it circumvents disintegration. Additional apparently no “free” polymer is present in the formulation. Usually polyplexes are prepared with an excess of polycation, thus containing not with nucleic acid associated polymer. Boeckle *et al.* proved that free polycations cause a higher cellular and systemic toxicity of the formulation [153]. The reverse of the medal is that changes in the carrier design (such as a higher PEG content, a varying polycation to siRNA ratio or another siRNA target sequence) can be only realized by a new resynthesis of the complete conjugate.

As demonstrated in siRNA transfection experiments, the polycation to siRNA ratio has a great impact on delivery efficiency and cytotoxicity. Because PLL-PEG-DMMAn-Mel showed good siRNA delivery activity at w/w ratio of 2, it was decided to synthesize a similar composed conjugate containing covalently attached siRNA. At w/w ratio of 2, 2 µg PLL (as hydrobromide) is conjugated with 1 µg siRNA. An overall net positive charge is guaranteed

because ~ 9.5 nmol positive charges (lysine's primary amines) come up against ~ 3 nmol negative charges (siRNA phosphates). For two principal reasons this is an important characteristic of the conjugate. On the one hand a positive charge is necessary for interaction with the negatively charged cell surface and consequently internalization. This is of particular importance because no targeting ligand is included into the conjugate. On the other hand complexation in addition to the covalent linkage is useful because a siRNA with a conventional backbone was used. Conventional siRNA is acceptable for most applications a serum-free environment, but a relatively short bioavailability of free siRNA *in vivo* or in a serum-containing environment limits the potential. Complexation protects the nucleic acid against degradation through RNAses. Instead of the conventional siRNA, a nuclease-resistant siRNA containing a phosphothioate backbone could be used, making complexation needless regarding this issue. Basically, if mentioned aspects are considered, also a higher siRNA loading degree or a smaller polycation backbone is imaginable, generating neutral or negatively charged siRNA-conjugates.

To avoid aggregation of PLL-PEG with thiol-modified siRNA, the synthesis was carried out at high NaCl concentration [55]. First siRNA was conjugated to PLL-PEG-PDP. Afterwards, without preceding purification, DMMAn-Mel was added to the mixture. Finally the conjugate was purified by SEC purification using an eluent buffer containing a high salt concentration. It has to be mentioned, that not all PDP-groups could be loaded with DMMAn-Mel peptide. This is possible due to the steric hindrance effect of the siRNA molecule in combination with the flexible PEG chain which hampers DMMAn-Mel coupling. In general determination of peptide loading ratio or PEGylation degree of the synthesized conjugate is complex: by H-NMR or pyridine-2-thione-release assay obtained information does not represent the actual composition of each single conjugate, the calculations are approximate values based on calculations with average molecular weights. For instance the starting material for itself, PLL, is not a higly defined molecule, it is a mixture of polylysine polymers with different chain lengths. Hence, the molecular weight of the starting material, which is used as basis for subsequent calculations, is already an approximate value. However the calculated theoretical compositions are suitable to compare conjugates among each other, because for all synthesized carriers the same theoretical basics were applied.

The purification performance was proven by agarose gel electrophoresis. If uncoupled siRNA could not be separated from the conjugate with SEC, it would be ionically complexed with the PLL-backbone. As a consequence uncoupled siRNA would not be released during gel electrophoresis; it would not run into the gel and stay undetected. To deal with this issue heparin was added to the conjugate. Heparin, a natural polyanion, dissasembles nucleic acid polyplexes and makes sure that free siRNA is released from the conjugate. As demonstrated purification was successful, uncoupled siRNA was only found in peak 2 (**Fig. 37**). To verify

that siRNA was linked to the PLL-PEG-conjugate and to quantitate the amount of the same, the disulfide bond has to be cleaved. Without disulfide reduction, also in the presence of heparin no siRNA would run into the gel. In other words: without reductive intracellular environment, also heparane sulfates and other negatively charged macromolecules cannot lead to vector unpackaging. Accordingly both TCEP and heparin had to be applied to release siRNA from the conjugate. In comparison, a conventional PLL / siRNA polyplex was destroyed by addition of heparin alone (**Fig. 39**).

Directly after SEC elution in 1 M NaCl buffer, conjugates or more precisely particles had a size of 80 nm. After snap-freezing and thawing size grew to ~ 150 nm. The Mirus Bio Corporation published a conjugate, named polyconjugate, containing pH-responsive PEGylation, covalently attached siRNA and targeting ligands were the main fraction (> 95%) had a size of 10 nm, making is substantially smaller than the PLL-PEG-DMMAn-Mel-siRNA conjugate [113]. While PLL-PEG-DMMAn-Mel-siRNA contained only one PEG chain per polycation, the polyconjugate had a higher degree of PEGylation. Among other things this can contribute to the observed size differences. At first appearance it is surprising that electrostatically formed PLL-PEG-DMMAn-Mel siRNA polyplexes were smaller in size (40 nm) in comparison with PLL-PEG-DMMAn-Mel-siRNA. However the polyplexes were formed at a siRNA concentration of 0.02 mg/ml. In contrast the conjugate containing covalently attached siRNA had a final siRNA concentration of ~ 0.25 mg/ml. When PLL-PEG-DMMAn-Mel siRNA polyplexes were formed at this siRNA concentration, polyplexes were also larger in size (~ 150 nm).

To evaluate the gene silencing potential of PLL-PEG-DMMAn-Mel-siRNA, transfection experiments were performed in Neuro2A cells stably transfected with the eGFPLuc vector. siRNA delivery activity was compared with electrostatic PLL-PEG-DMMAn-Mel polyplexes formed at w/w 2 with either luciferase or control siRNA (**Fig. 37**). Using the conjugate, a dose-dependent reduction of luciferase activity was observed with approximately 80% reduction at 0.125 µg siRNA and over 90% at 0.5 µg siRNA per well. The knockdown observed with the electrostatic polyplexes was similar, demonstrating that covalent attachment of siRNA did not weaken the siRNA delivery efficiency of the polycationic carrier. Transfection with control siRNA complexed to PLL-PEG-DMMAn-Mel at w/w 2 and with a conjugate containing covalently attached control siRNA did not influence luciferase expression at siRNA doses of up to 0.75 µg. Only transfection with the highest siRNA dose of 1µg resulted in a 30% reduction of luciferase expression, indicating unspecific knockdown / carrier toxicity. To clarify this finding, the cellular viabilities were determined 2 days following treatment with the siRNA formulations (**Fig. 38**). Significant toxicity began to manifest only at the highest siRNA dose with both formulations (1 µg), confirming the transfection results obtained with polyplexes containing control siRNA.

To elucidate the process of delivery with the siRNA conjugate, it was tested if physiological glutathione concentrations can induce cleavage of the disulfide bond and lead to siRNA release. To monitor the conjugate purification and to examine the siRNA content, TCEP was used in the gel electrophoresis studies to cleave the bound siRNA (**Fig. 39).** TCEP is a powerful and irreversible reductant which is stable in aqueous, acidic, and basic solutions. However in the cytoplasm only glutathione (GSH) is present as natural reducing agent. Glutathione is a tripeptide which can be synthesized from cysteine, glutamate and glycine. In cells, the majority of the total glutathione pool is in the reduced form GSH; less exists in the oxidized disulfide form (GSSG). To clarify if physiological cytoplasmatic GSH concentrations (1 – 10 mM) can cleave the siRNA-connecting disulfide bond, the conjugate was incubated for 1 hour at 37 °C with appropriate GSH concentrations. Heparin was added for polyplex disassembly and agarose gel shift using EtBr staining was performed after incubation. As shown, after incubation with GSH-concentrations of 1.25 to 5 mM, released siRNA could be detected (**Fig. 39**), suggesting that siRNA cleavage of the conjugate may occur in the cellular environment. Based on these observations, cleavage of the siRNA before RNA-inducing silencing complex formation is likely. We do not know formally whether disulfide cleavage is required for activity. Derfus *et al.* synthesized quantum dots containing covalently bound siRNA. Reversibly attached siRNA by disulfide cross-linkers showed greater silencing efficiency than siRNA attached by a nonreducible thioether linkage, which also suggests that disulfide cleavage in the intracellular takes place and that release of the siRNA may be advantageous [58;154].

In summary, the bioresponsive endosomolytic PLL-PEG-DMMAn-Mel-siRNA conjugate showed good *in vitro* protein knockdown activity. Covalent attachment of siRNA improved stability of the siRNA carrier against natural occurring polyanions like heparin. In addition to the dynamic endosomolytic activity, cytosolic glutathione levels trigger the programmed cleavage of the disulfide linkage and enable release of the siRNA.

5 Conclusion and Outlook

Overall, application of DMMAn-Mel strongly enhanced nucleic acid delivery efficiency of the polycations PLL and PEI. PrePEGylation of the polycations before peptide attachment prevented polyplex aggregation and allowed the formation of nanosized particles. Remarkably, despite big differences of the unmodified polycations PLL and PEI in pDNA transfer activity, pDNA transfer activity of PLL-PEG-DMMAn-Mel was similar to that for the PEI analogue and even better in the case of siRNA transfer. But unlike the PEI analogue, PLL has the additional advantage of being biodegradable. Conjugation of DMMAn-Mel (in contrast to unmasked melittin or PEGylation only) lowered the *in vitro* cytotoxicity of the polycations. These findings might have implication for the selection of novel polymers for siRNA delivery: polycations with favorable basic characteristics such as nucleic acid binding, compaction, protection and biocompatibility, but poor delivery activity which obviously would fail in high-throughput screenings might nevertheless be converted into highly efficient siRNA carriers.

The most promising conjugate for siRNA delivery (PLL-PEG-DMMAn-Mel-siRNA) is equipped with pathbreaking features. First of all the carrier consists of several components with specialized functions (PEG for solubilization and shielding, degradable polycation for nucleic acid compaction and protection, and reversible masked lytic domain for endosomal release). This modular design takes into account that the nucleic carrier has to fulfill various tasks during the process of nucleic acid delivery. Furthermore the siRNA is not only complexed electrostatically but also bound covalently to the carrier. This feature guarantees enhanced carrier stability on the way to its intracellular site of action. Finally the conjugate works in a dynamic virus-like manner: pH- and redox-gradients are exploited to trigger endosomal lytic activity and release of the siRNA in the cytosol. This programmed “smart” behavior allows the drug carrier to adapt to the unique needs of the different sequentiel delivery barriers, which is a key issue in the development of highly effective nucleic acid delivery devices.

Beside improvements including addition of a targeting ligand to increase the specifity or optimized shielding of the conjugate, one general issue in the field of polymer drug delivery has to be adressed in future work: improved chemistry is necessary to provide monodisperse conjugate structures with defined size, topology and functional domain location. A highly defined structure and arrangement of the carrier components (e.g. PEG chain, lytic peptides) is not only important for meaningful structure-activity-relationships, also on the way from the lab bench to the bedside pharmaceutical regulatory requirements have to be met. In combination with enhanced delivery efficiency, polymeric nucleic acid therapeutics could feed the clinical development pipelines in the future.

6 Summary

Nucleic acid-based therapies provide hope for treating various diseases including cancer and a number of inherent disorders. Many disease-causing proteins thought to be “undrugable” could now be targeted by therapeutic gene silencing. However the lack of efficient delivery systems is still limiting the full therapeutic potential of pDNA and siRNA. Poor endosomal release is one major barrier of nucleic acid delivery. Natural organisms like viruses have developed strategies to escape from the endosome and thus avoid degradation in the acidic endosomal compartment. Hence viruses can be seen as highly efficient model delivery systems.

Aim of the thesis was to improve endosomal escape and transfection efficiency of polycation-based nucleic acid carriers. Endosomolytic polyethylenimine-melittin conjugates have shown enhanced gene transfer efficiency; however, due to their general membrane destabilizing properties, cytotoxicity limits their application. To overcome this drawback a polycation was grafted with a reversibly masked pH-responsive lytic peptide. Melittin was modified with dimethylmaleic anhydride (DMMAn) and covalently coupled to polylysine (PLL). Membrane lytic activity was analyzed after incubation at neutral or endosomal pH. Transfection experiments using a luciferase reporter gene were carried out. Cellular cytotoxicity was analyzed by measurement of membrane integrity and metabolic activity. Covalent attachment of DMMAn-modified melittin to PLL resulted in the desired pH-responsive conjugate. Reduced lytic activity was observed at neutral pH; after acidic cleavage of the protecting groups at pH 5 lytic activity was restored. Acute toxicity was greatly reduced (as compared to PLL-Mel or even unmodified PLL) and high gene expression levels were obtained.

The promising concept was transferred to polyethylenimine, one of the most frequently used polycations for non-viral gene delivery. However aggregation of the conjugate required modification with polyethylene glycol (PEG) for improved solubility and stabilization. PrePEGylation allowed the formation of nanosized polyplexes. PEGylated conjugates based on PEI and PLL and the lytic peptides melittin, DMMAn-Mel and influenza-derived peptide were synthesized and pDNA and siRNA delivery activity was evaluated. In summary, application of DMMAn-Mel combined with PEG strongly enhanced pDNA and siRNA delivery efficiency of the polycations. Remarkably, despite big differences of the unmodified polycations in pDNA transfer activity, pDNA transfer activity of PLL-PEG-DMMAn-Mel was similar to that for the PEI analogue and even better in the case of siRNA transfer. But in contrast to the PEI analogue, PLL has the additional advantage of being biodegradable.

Conventional polyplex formulations are held together electrostatically. A weak point resides in the fact that other charged biomolecules can disrupt the complexes before they reach the

site of action. Recently other research groups demonstrated that both serum and extracellular matrix can lead to vector disassembly. To ensure the association of the siRNA with the polycationic carrier, a synthesis method to covalently attach the siRNA was developed. PLL-PEG-DMMAn-Mel, the most promising conjugate for siRNA delivery as found in the gene silencing studies, was covalently modified with siRNA. To enable release of the free siRNA in the cytosol a reducible disulfide bond was chosen as linkage. While the conventional polyplex could be disassembled by heparin treatment, heparin in combination with a reducing agent was necessary to induce release of the siRNA. Heparin treatment alone was not sufficient to destroy the conjugate, indicating the improved stability. Release of the siRNA under physiological intracellular reducing conditions was evaluated by agarose gel electrophoresis studies. The synthesized siRNA-conjugate mediated effective gene silencing as effective as the corresponding electrostatic polyplex.

7 Appendix

7.1 Abbreviations

ATP	Adenosine triphosphate
CMV	cytomegalovirus
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMMAn	dimethylmaleic anhydride
DMMAn-Mel	DMMAn-modified melittin
DMSO	dimethyl sulfoxide
DNTB	5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent)
DTT	dithiothreitol
EPR	enhanced permeation and retention
EtBr	ethidium bromide
FBS	foetal bovine serum
FCS	fluorescence correlation spectroscopy
GSH	glutathione
HBG	HEPES-buffered glucose
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethansulfonic acid)
INF	influenza peptide
LDH	lactate dehydrogenase
Luc	luciferase
Mel	all-(D)-melittin (with a cysteine residue at the N-terminus)
mPEG	monomethoxy PEG
MTT	methylthiazoletetrazolium
MW	molecular weight
MWCO	molecular weight cut-off
1H NMR	nuclear magnetic resonance
N/P-ratio	molar ratio of nitrogen to DNA phosphate (PLL: molar ratio of epsilon-amino nitrogen to DNA phosphate)
PBS	phosphate-buffered saline

pCMVLuc	plasmid encoding for luciferase under control of the CMV promoter / enhancer
pDNA	plasmid deoxyribonucleic acid
PEG	polyethylene glycol
PEI	branched polyethylenimine of a Mw of 25 kDa
PLL	poly-L-lysine
PLL-PDP	PLL modified with pyridyldithio-propionate-linkers
PLL-PEG	PLL modified with PEG
PLL-PEG-Mel	PLL modified with PEG and Mel
RISC	RNA-induced silencing complex
RLU	relative light units
RNA	ribonucleic acid
RT	room temperature
SEC	size exclusion chromatography
siRNA	small interfering RNA
SPA-PEG	succinimidylpropionic acid modified PEG
SPDP	succinimidyl 3-(2-pyridyldithio) propionate
Succ	succinic anhydride
TCEP	Tris (2-carboxyethyl) phosphine
Tf	transferrin
TNBS	trinitrobenzenesulfonic acid
w/w	weight to weight ratio (e.g. of conjugate to nucleic acid)

7.2 Publications

7.2.1 Original papers

- Martin Meyer, Alexander Philipp, Reza Oskuee, Claudia Schmidt and Ernst Wagner: *Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery*, J. Am. Chem. Soc. 2008 Mar
- Martin Meyer, Arkadi Zintchenko, Manfred Ogris and Ernst Wagner: *A dimethylmaleic acid-melittin-polylysine conjugate with reduced toxicity, pH-triggered endosomolytic activity and enhanced gene transfer potential*, J. Gene Med. 2007 Sep
- Martin Meyer, Christian Dohmen, Alexander Philipp, Ernst Wagner: *Dynamic siRNA – polymer conjugate* (tentative title), in preparation.
- Kevin Buyens, Martin Meyer, Ernst Wagner, Joseph Demeester, Stefaan C. De Smedt and Niek N. Sanders: *Fluorescence Fluctuation Spectroscopy: a powerful tool to monitor the disassembly of polymer based siRNA delivery systems in serum* (tentative title), in preparation.

7.2.2 Reviews

- Alexander Philipp, Martin Meyer and Ernst Wagner: *Extracellular Targeting of Synthetic Therapeutic Nucleic Acid Formulations*, Curr. Gene Ther. 2008 Oct
- Martin Meyer and Ernst Wagner: *pH-responsive shielding of non-viral gene vectors*, Expert Opin. Drug Deliv. 2006 Sep
- Martin Meyer and Ernst Wagner: *Recent developments in the application of plasmid DNA-based vectors and small interfering RNA therapeutics for cancer*, Hum. Gene Ther. 2006 Nov

7.2.3 Book chapter

- Martin Meyer, Gururaj Rao, Ke Ren and Jeffrey Hughes: *Pharmacokinetics of Viral and Non-Viral Gene Delivery Vectors*, in: Pharmacokinetics and Pharmacodynamics of Biotech Drugs, Wiley-VCH, Weinheim, 2006 Sep

7.2.4 Poster presentations

- Alexander Philipp, Martin Meyer, Arkadi Zintchenko, Manfred Ogris, Christian Plank and Ernst Wagner: *Programmed Endosomolytic Conjugates for pDNA and siRNA delivery*, ESGCT 15th Annual Congress, Rotterdam, The Netherlands, 2007 Oct

- Martin Meyer, Arkadi Zintchenko, Alexander Philipp, Manfred Ogris, Christian Plank and Ernst Wagner: *Bioresponsive Endosomolytic Conjugates for DNA and siRNA Delivery*, ASGT 10th Annual Meeting, Seattle, USA, 2007 June

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10 Curriculum vitae

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