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Novel, sequence-defined oligo (ethane amino) amides
for siRNA delivery

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1 INTRODUCTION

1.1 Nucleic acid based therapy

The idea of nucleic acid based therapy is an exciting, rational notion, tackling many serious diseases at the root of their origin, at gene level. Classic gene therapy utilizes DNA, preferentially delivered by viral vectors, to insert functional genes into the human genome for replacement of defective gene sections. This way, genetic disorders like severe combined immunodeficiency (SCID) [1], mucoviscidosis [2] or haemophilia [3], as well as acquired diseases like viral infections [4, 5] have been addressed. DNA based therapy has been extended to other fields like DNA vaccination [6], cancer therapy [7] and many more. Although remarkable success has been achieved, e.g. in treatment of SCID, considerable side effects have been reported and safety concerns about interference with the human genome remain.

In recent years, focus of nucleic acid research shifted to the exploitation of RNA for therapeutic purposes. Since the discovery of RNA interference (RNAi) by Fire et al. in 1998, significant efforts and resources have been invested in exploiting the therapeutic potential of RNA for the treatment of human diseases [8-11]. The RNAi process makes use of double stranded RNA for specific gene silencing [12, 13]. The introduction of exogenous long dsRNA (>30 nucleotides) into cells was found to inhibit cellular protein expression, provoke innate immune response by interferon activation, and induce apoptosis. A boost of attention for RNAi followed when Tuschl et al. demonstrated that gene target-specific RNA interference without significant side effects can be mediated by application of small synthetic 21-23 nucleotide interfering RNA (siRNA) duplexes [14]. As siRNAs reach the cytosol of cells, they are loaded onto a multiprotein complex called RNA induced silencing complex (RISC) (Figure 1). After incorporation into RISC the siRNA is unwound by the enzyme Argonaute 2 and the sense strand is cleaved [15, 16]. The antisense strand ('guide strand') remains incorporated and activates RISC to cleave complementary mRNA [17]. Because an activated RISC is capable of repeatedly cleaving mRNA, this process effectively promotes gene silencing over a significant period of several days. Therefore, siRNA has become an indispensable tool for studying gene function in mammalian cells and

the sequence-specific interaction on mRNA level holds great promise for a vast variety of gene-specific therapeutics.

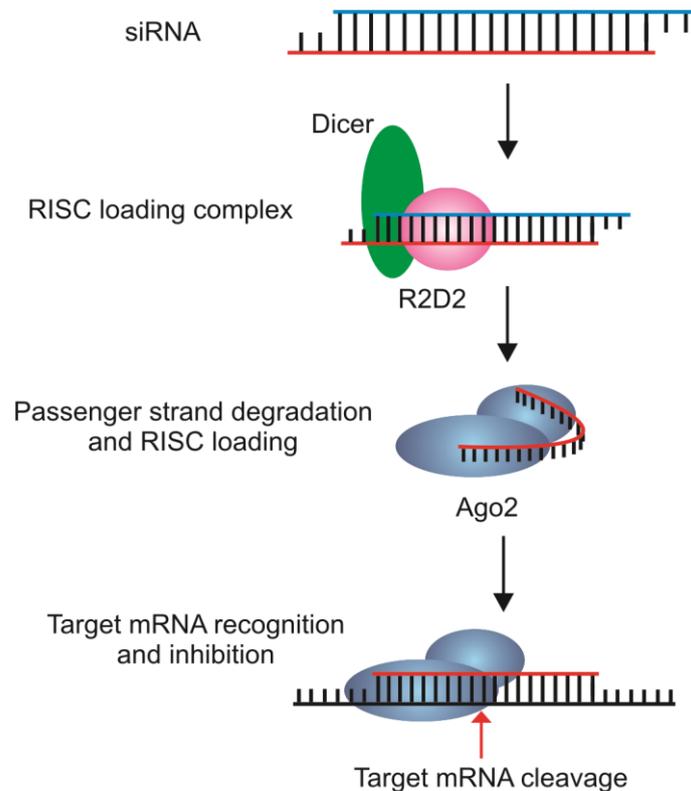


Figure 1: Mechanism of RNA interference (RNAi). After reaching the cytosol, siRNA is loaded into the RNA induced silencing complex (RISC), a multi enzyme complex. After unwinding of the double strand, the sense strand is degraded and the antisense strand ('guide strand') is loaded to the core protein Argonaute 2 (Ago2). Base pairing between the guide strand and a complementary target mRNA leads to cleavage of the mRNA strand by the activated RISC. R2D2: double-stranded RNA binding protein. Figure modified from Li et al. [18].

Besides siRNA, there are other types of therapeutic RNAs, including target-binding RNA aptamers [19], antisense RNAs, or immunostimulatory RNAs (Figure 2). The delivery of antisense RNA (single stranded RNA) presents the most straightforward way to inhibit gene expression. Antisense RNA oligonucleotides like siRNA interfere with gene expression by binding to their complementary mRNA strands [20]. Usually one antisense RNA can inhibit only one mRNA molecule and therefore is less effective than catalytically active siRNA. Novel interesting options of exploiting the

antisense mechanism are exon-skipping [21] or the targeting of micro RNAs with complementary oligoribonucleotides (anti-miRs) [22].

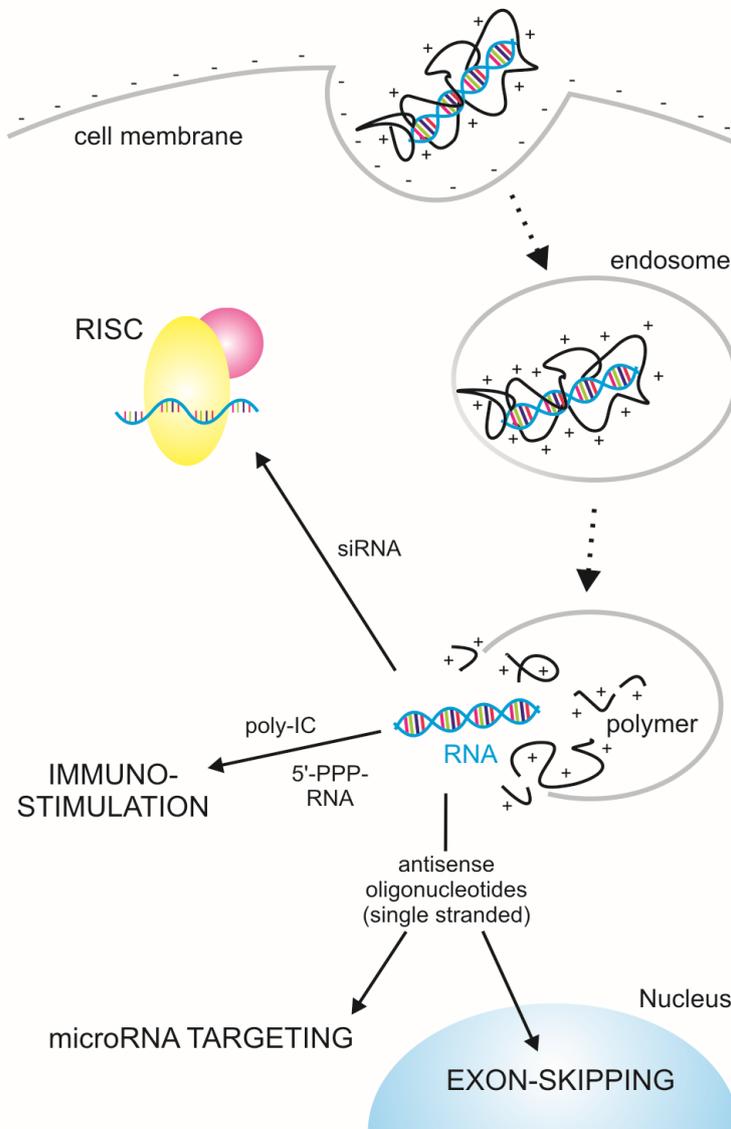


Figure 2: Cellular entry of RNA polyplexes and intracellular mechanisms of different therapeutic RNAs (double stranded as shown in figure or single stranded). The different types of RNA execute their biological function inside the cell. siRNA is loaded onto RISC for cleavage of corresponding target mRNA, in this way knocking down gene function. Poly-IC or 5'-triphosphate-modified RNA initiate distinct immune responses, whereas single stranded oligonucleotides may modulate gene functions by antisense mechanisms, including microRNA targeting or exon-skipping.

MicroRNAs regulate the expression of genes by interfering with protein translation, and the silencing of a specific microRNA by the delivery of anti-miR consequently

leads to an altered expression of the corresponding gene. Other interesting approaches apply immunostimulatory and cytotoxic RNAs. Poly-IC (poly-Inosine-Cytosine), a synthetic double stranded RNA, mimics the expression of dsRNA in virus-infected cells, which via endosomal toll-like receptor TLR3 and cytosolic mda-5 stimulation activates different pro-apoptotic processes simultaneously. This makes it an interesting tool for cancer treatment because the induced mechanisms lead to cell death and the additional expression of anti-proliferative cytokines like interferons inhibits growth of neighbouring cancer cells that have not been “infected” with poly-IC [23, 24]. Other immunostimulatory RNAs contain 5'-triphosphate ends, which activate innate immune cells and lead to direct interferon expression and apoptosis in tumor cells via recognition by the antiviral helicase Rig-I. Hartmann et al. used a combination of gene silencing and immunostimulation. Administration of siRNA, targeted against Bcl-2 and containing immunostimulatory 5'-triphosphate ends, provoked strong tumoral apoptosis in a lung metastasis model [25]. The same group recently showed that activation of Rig-I and Mda-5 helicases by RNAs with 5'-triphosphate ends also initiates an interferon independent signalling pathway in tumor cells, resulting in apoptosis [26]. Due to the dual pathway of inducing apoptosis, this may be an effective strategy of facing the frequently occurring drug resistance problems in many tumors.

Although therapeutic RNAs have the potential to revolutionize medicine, so far naked RNA formulations have only been administered successfully to local tissues by direct injection, e.g. into the eye for the treatment of adult late stage wet macular degeneration (AMD) [27]. Barriers for systemic application of RNA are the relatively large size (siRNA: ~13 kDa), the negative charge due to the phosphate backbone, both hindering crossing of cellular membranes, and their susceptibility to enzymatic degradation in the bloodstream. However, a break through of this technology in the clinic requires easy-to-handle systemic applications to address a broad range of disease indications. For this purpose, safe additional carrier systems have to be developed, which are able to protect RNA in the extracellular environment and effectively deliver it to the site of interest. Viral and non-viral delivery strategies are being pursued. Viral delivery of RNA is very effective but there are also significant disadvantages like high production costs, immune response and safety concerns. Among the non-viral delivery systems peptides, RNA-conjugates [28, 29], cationic

polymers [30-36], lipids [37, 38] and lipid-modified polymer formulations [39-42] are explored.

1.2 Optimization of the RNA payload

Besides development of appropriate carriers for efficient siRNA delivery, both potency and specificity of siRNA can be enhanced by chemical modifications [43]. Unmodified siRNAs are unstable in blood because of rapid degradation by serum nucleases. They have been found to unspecifically activate the immune system to varying extent [44, 45] and they may trigger sequence-dependent off-target effects by partial sequence complementarity in the seed region, resulting in micro RNA-type inhibition of protein translation [46]. Chemical modifications of the 2'-OH group in the ribose residues, including 2'-O-methyl [47] and 2'-fluoro [48] modifications, stabilize against endonuclease, phosphorothioate linkages at the 3'-end against exonuclease activity. Furthermore, the incorporation of locked nucleic acid (LNA) nucleotides, in which the ribose moiety is modified with an extra bridge connecting the 2' and 4' carbons, increases thermal stability of the siRNA base pairing without negative effects on RNAi efficiency [49]. Besides resistance to digestion by nucleases, specific 2'-O-methylation of the seed region (nucleotides 2 – 8 of the guide strand), the main interaction site with mRNA in RISC, was also shown to eliminate off-target effects, like activation of the immune system [50, 51]. Recently, Bramsen et al. [52] screened a large number of chemically modified siRNAs to generate design rules for high stability, high activity and low toxicity. Comparing the effects of 21 types of chemical modifications and screening 2160 siRNA duplexes they found that siRNA activity can be enhanced by favoured incorporation of the antisense strand (guide strand) into RISC. This can be triggered by chemical modifications which create a thermodynamic asymmetry of the siRNA duplex and engineering the 3'-overhangs [52]. The strand with the thermodynamically less stable 5'-end is preferred as guide strand in RISC.

The classical 21 bp double stranded siRNA molecules are not the only possible trigger for RISC induced gene silencing. Other RNA forms have also shown effective gene knockdown [18], for example dicer substrate siRNA (dsiRNA), asymmetric interfering RNA (aiRNA) or small internally segmented interfering RNA (sisiRNA).

dsiRNAs, 25 – 30 bp RNA duplexes, are substrates of the cytosolic Dicer endonuclease, which processes the dsiRNA into siRNA. By using the natural processing machinery some dsiRNAs are more potent than the corresponding conventional siRNAs [53]. aiRNA [54] consists of a short, 14 – 15 nucleotide sense strand, paired to the middle segment of a conventional 21 nucleotide guide strand. The asymmetric duplexes combine effective and durable gene silencing with reduced off-target effects by the shortened sense strand. sisiRNA [55] is composed of three RNA strands: two short 10 – 12 nucleotide sense strands, complementing one intact antisense strand (guide strand). The lack of an intact sense strand reduces potential off-target effects.

1.3 Non viral carrier systems for siRNA delivery

The most prominent non viral delivery systems for siRNA that are currently explored rely on the formation of nanoparticles. These systems are mostly built up of lipids or polymers [56-59], which package the negatively charged nucleic acids into particles of nano scale dimensions, protecting them from degradation and delivering them to the cytosol of target cells.

Representatives for lipid based siRNA formulations are liposomes and lipoplexes. Liposomes encapsulate siRNA into a lipid bilayer, formed by cationic lipids like DOTAP, DOPE, DSPC etc., representing amphiphilic molecules with a hydrophobic tail and cationic, hydrophilic head group. Internalized liposomes are able to destabilize the endosomal membrane by lipidic interaction and thereby release their payload into the cytosol [60]. Liposomal siRNA formulations are probably the most studied siRNA carriers *in vivo* [38, 61]. Within this class of carriers the formulation of Stable Nucleic Acid Lipid Particles (SNALPs), based on a mixture of cationic lipids, cholesterol and PEG lipids received special attention and demonstrated promising *in vivo* results [37, 62]. Lipoplexes represent an alternative approach of utilizing cationic lipids for siRNA delivery *in vivo*. They are produced by mixing a preformed cationic liposome with siRNA, resulting in a complex, where nucleic acid is incorporated at the outside shell of the liposome [63]. Systemic application of lipoplex formulations inhibited tumor progression in orthotopic cancer models [64, 65]. Major drawback of

lipidic systems is their preferential accumulation in the liver, often limiting their use to hepatic applications.

Cationic polymers are able to bind nucleic acids through their positive charge by ionic interaction with the negatively charged phosphate groups of the nucleic acids and have the capability to form nanoparticles with these by complexation [66]. The so called polyplexes are generated simply by mixing the RNA with polymer.

The widely used “golden standard” for gene transfection, polyethylenimine (PEI), has also been explored for RNA delivery. One of the reasons for PEI’s effectiveness is that it binds and protects the nucleic acid cargo against degradation, and promotes endosomal escape, a crucial step in the delivery process (Figure 2), through the “proton-sponge effect” [67]. Once the polyplexes have entered the cell via endocytosis, the acidification of the endosome enhances the positive charge density of the polymer. This leads to an enhanced influx of chloride and water, swelling of the endosomes which due to the cationic polymer interaction finally burst and release the payload into the cytosol. Whereas for gene delivery both linear PEI and its branched relative brPEI show high pDNA transfection results, the efficiency of PEI for siRNA delivery is less clear. Screening different PEI structures, Grayson et al. could only show *in vitro* gene silencing efficiency for brPEI formulations and here only within a narrow window of conditions [68]. Aigner et al. on the other hand successfully demonstrated *in vivo* gene silencing with linear polyethylenimine/siRNA formulations in several tumor models [69, 70]. Furthermore, local administration of linear PEI/siRNA to the lung resulted in strong gene knockdown [71]. By modifying siRNA with sticky overhangs to further stabilize the PEI polyplexes, systemic delivery to the lung was possible [72]. However, the major drawback of these simple and effective polymers is cytotoxicity. In addition, PEI is not biodegradable.

One way to cut back cytotoxicity is to create derivatives of PEI through introduction of negatively charged propionic acid or succinic acid groups and other modifications of the amines, as reported by Zintchenko et al [73]. These derivatives showed far lower cytotoxicity compared to unmodified PEI and a high efficiency in siRNA-mediated knockdown, especially the succinylated branched PEI.

Akinc et al. created a whole library of lipid-modified cationic oligomers (lipidoids). Screening of over 1.200 lipidoids, varying in alkyl chain length, linkage between alkyl and amines, different residues on the amines, identified lead structures for siRNA

delivery in cell culture experiments. Optimization yielded formulations for successful functional delivery of siRNA or 2'-methoxy RNA *in vivo* in several animal models [74]. Polycationic dendrimers like polyamidoamines (PAMAMs) are characterized by a branched spherical shape and high density surface charge. The primary amine groups on the surface of PAMAM enable RNA binding, while the tertiary amino groups inside the dendrimer are supposed to act as a proton sponge in endosomes and enhance the release into the cytoplasm [75]. Functionality of the polymers was recently demonstrated by siRNA-induced silencing of cancer relevant heat-shock protein 27 in prostate cancer cells, leading to apoptosis *in vitro* [76]. In order to reduce the cytotoxicity without losing gene silencing efficiency, surface- and core-modifications have been introduced to PAMAM dendrimers [77].

Apart from synthetic polymers, various biopolymers like atelocollagen [78, 79] and chitosan have been exploited for gene silencing. Chitosan, a linear polysaccharide composed of randomly distributed β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, has several beneficial qualities for *in vivo* use: low toxicity, low immunogenicity and biodegradability. Its high positive charge enables the biopolymer to bind RNA and form nanoparticles with it. Effective *in vitro* siRNA delivery using chitosan/siRNA nanoparticles was shown by several groups [80, 81]. *In vivo* efficacy of chitosan was demonstrated by Howard et al, amongst others, by systemic administration of chitosan/siRNA nanoparticles, in a murine arthritis model [82].

1.4 Functional domains of polymeric carriers facilitating nucleic acid delivery

1.4.1 Shielding agents

For successful *in vivo* delivery, polyplexes have often been functionalized with hydrophilic molecules like polyethylene glycol (PEG), resulting in a prolonged circulation time of the polyplexes in the bloodstream, reduced susceptibility to aggregation with serum proteins and reduced degradation via phagocytosis by cells of the reticulo-endothelial-system (RES). Different PEGylation strategies have proven successful, e.g. incorporating PEG into the polymeric carrier system [83], direct PEGylation of RNA before complexation [84, 85] or PEGylation of the polyplexes after complex formation (post PEGylation) [86]. Rädler et al. created diblock

copolymers consisting of PEG and cationic peptide for the formation of shielded siRNA complexes, containing only one single siRNA. The same work addressed the important issue of a reduced endosomal release due to the PEG coat. In one of the block copolymers, PEG was attached to the polymeric carrier via a pH-sensitive hydrazone linkage. This linkage is cleaved in the acidic environment of endosomes, in consequence the PEG shield is stripped off and endosomal release efficiency is restored [87]. The disadvantage of shielding polyplexes with PEG is that the positive surface charge of polyplexes is diminished, which is an important factor for cell adhesion and in consequence cellular uptake (Figure 2). However, this loss of efficiency, which besides would promote undesirable unspecific uptake into various cells *in vivo*, can at least be partly compensated by introducing targeting ligands into the polyplexes. Another versatile method to escape the PEG dilemma is the alternative use of biodegradable hydroxyethyl starch (HES) for shielding. The HES shield of polyplexes is slowly enzymatically degraded during blood circulation and thus leads to gradual deshielding of polyplexes, facilitating enhanced cellular internalization [88].

1.4.2 Ligands for cellular targeting

Specific tissue targeting is essential for effective *in vivo* nucleic acid delivery at low doses that would not trigger side effects at other sites. The long lasting experience in the field of targeted gene delivery of liposomes or nanoparticles serves as a basis in the development of effective RNA delivery agents. Various ligands have been incorporated into the carrier systems that are specifically recognized by receptors expressed on cell surfaces. Once the ligand has bound to its corresponding receptor, the carrier system and its payload may be taken up by the cell via receptor-mediated or related forms of endocytosis. Several ligands have shown to be suitable for targeted gene delivery, e.g. anti-receptor antibodies, fragments of antibodies, peptides, aptamers, glycoproteins etc. [89-92] and several of them have already been screened for use in RNA delivery.

For example, integrins have been studied as receptors for siRNA delivery at an early stage. They are expressed on the activated endothelial cells in tumor vasculature and it is known that the arginine-glycine-aspartic acid (RGD) motif of fibronectin is responsible for the ligand-receptor interaction [93]. Schiffelers et al. attached the

RGD motif to the end of a PEG-conjugated polyethylenimine and used the modified polymer for selective tumoral delivery of siRNA to tumor-bearing mice [33].

Wang et al. [94] selected the most effective polymer (EHCO) of their library and functionalized it with bombesin peptide analogue, which exhibits high binding affinity to bombesin (BN) receptors. BN receptors are over-expressed on the cell surface of various cancer cells and therefore ideal for targeting strategies in cancer therapy [94, 95].

The transferrin (Tf) receptor, a cell surface receptor for the uptake of the glycoprotein Tf, is also over-expressed in many types of tumor cells because of their fast growth and therefore high demand for iron, which is carried by Tf. Several research groups have utilized the Tf receptor for targeting their RNA vehicles [96-98]. Davis and colleagues [98, 99] studied the impact of incorporating Tf into a cyclodextrin-containing polycation/siRNA formulation on delivery efficiency in tumor bearing mice. The epidermal growth factor (EGF) has caught much attention as a possible target ligand because the EGF receptor is strongly expressed on many types of cancers. Apart from the advantage of a specific receptor-mediated uptake, EGF also greatly accelerates intracellular uptake of the polyplexes [100]. Synthetic double stranded RNA (polyinosine-cytosine), a potent activator of apoptosis and interferon response, was selectively delivered to cancer cells by an EGF targeted PEI-PEG conjugate [23].

Instead of incorporating the targeting moiety into the polymeric carrier, it is also possible to couple it directly to the nucleic acid. For example, a luteinizing-hormone releasing hormone (LHRH) peptide analogue was covalently attached to siRNA against VEGF via a PEG spacer. The siRNA-PEG-LHRH conjugate formed nanosized polyelectrolyte complex (PEC) micelles upon mixing with PEI and achieved enhanced VEGF gene silencing on LHRH receptor over-expressing ovarian cancer cells compared to polyplexes without LHRH [101].

1.4.3 Intracellular release functions

The major bottleneck for polymer based RNA formulations is the effective release of the payload from the endosome into the cytosol. Ways of overcoming this problem are for example making use of the proton sponge effect of some polycations or the incorporation of lytic lipid moieties into the polymer. Another versatile method is the

coupling of endosomolytic peptides to the carrier system. Lytic artificial peptide sequences or natural sequences derived from influenza virus haemagglutinin HA2, HIV gp41 envelope glycoprotein, melittin and others have been applied [102-109]. For example, Pun and colleagues covalently linked PEI with the HIV gp41-derived fusion peptide [107]. This effectively increased endosomal release and the gene silencing activity of siRNA polyplexes.

1.4.4 Dynamic functionalized RNA carriers

The described different delivery functions are required at different time points of the delivery process. In fact, some of the steps request radical changes in the carrier characteristics. For example, polymers should strongly bind and protect the RNA in the blood circulation, but efficiently release it in the cytosol. PEGylation should shield polymeric carriers against unspecific interactions with proteins and cell membranes in blood, yet after specific cellular uptake the same carrier should interact with the endosomal lipid membrane and destabilize it. Therefore RNA carriers have to be dynamic in their characteristics, like natural viruses, to be most effective at the different steps of extracellular and intracellular delivery [110-112]. The requested changes can be programmed into the carriers by introduction of bioresponsive cleavable bonds or conformations. The acidification in the endosome [113-117] or cleavage of disulfides in the cytosol [109, 118] may trigger the changes. Figure 3 shows two published examples illustrating the concept [31, 119]. In both examples siRNA was covalently coupled to the carrier polymer by bioreducible disulfide bonds. Thereby the risk of fast polyplex dissociation in the extracellular environment is eliminated. Meyer et al. [119] applied polylysine (PLL) as carrier, functionalized with PEG and a pH-responsive form of the lytic peptide melittin. At physiological pH the amines of melittin were masked with dimethylmaleic anhydride, which minimizes lytic activity. After endosomal acidification the protecting groups are cleaved and lytic activity is restored. This modification greatly enhanced gene silencing efficiency *in vitro*. Rozema et al. [31] used a different type of polymeric carrier, containing membrane-active modified vinyl ether subunits, reversibly shielded by PEG residues which are removed in a pH-sensitive fashion in the endosome. The “Dynamic Poly Conjugates” are able to deliver covalently attached siRNA to hepatocytes both *in vitro* and *in vivo* after *i.v.* injection.

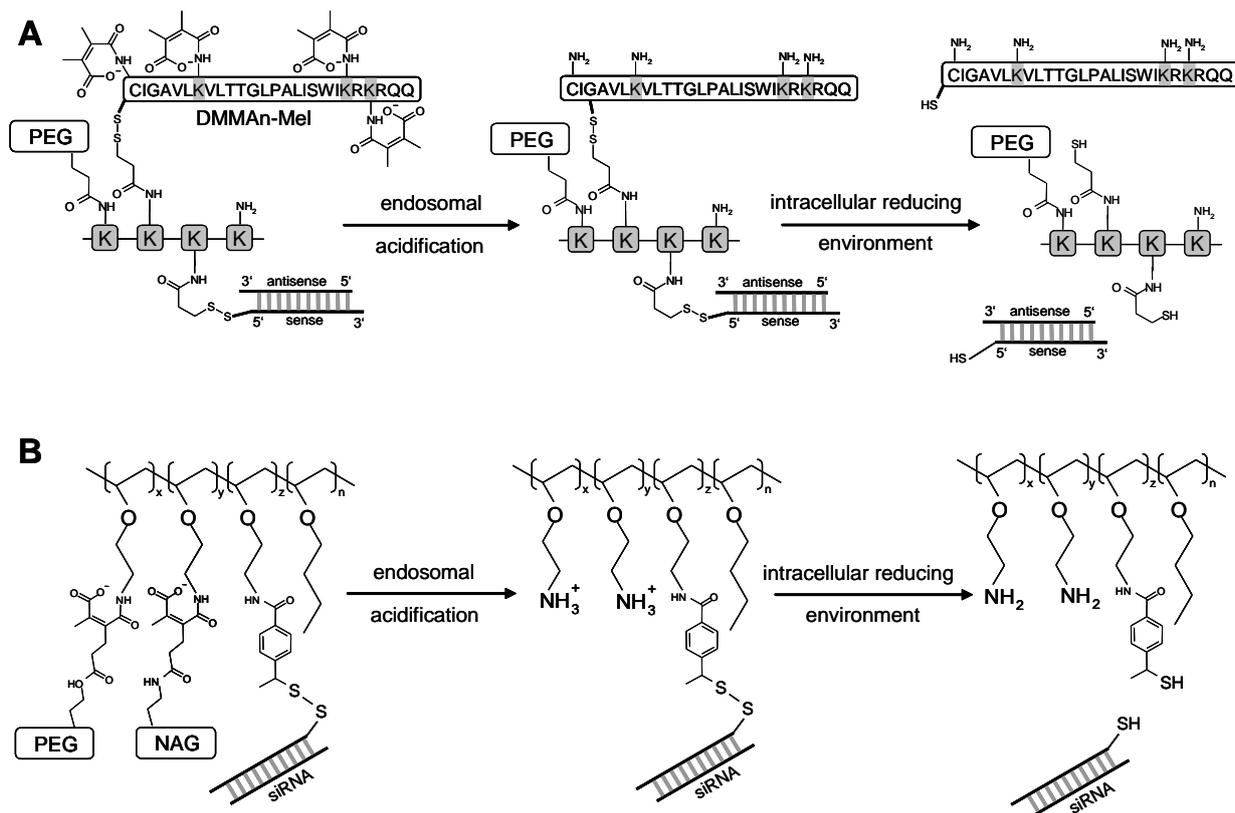


Figure 3: Bioresponsive siRNA-polymer conjugates. (A) Endosomolytic siRNA-poly-L-lysine conjugate (K represents L-lysine monomer, PEG polyethylene glycol) [119]. Cleavage of the dimethylmaleic anhydride (DMMAn) masking groups after endosomal acidification restores the lytic activity of melittin (Mel) and promotes endosomal escape; in a final step siRNA is released from the conjugate after disulfide cleavage in the reducing cytosol. (B) Dynamic PolyConjugate for hepatocyte-targeted delivery [31]. Endosomal acidification triggers cleavage of the shielding agent (PEG) and targeting ligand (NAG, N-acetyl galactosamine) and activates endosomolytic properties of the polymer backbone; subsequently the siRNA is released by cleavage of the disulfide bond.

1.5 Solid phase assisted synthesis for rational polymer design

A major drawback for most established polymeric carriers is their polydispersity, due to the applied synthesis strategies. This limits structure/efficiency correlations and batch reproducibility, especially when polymeric structures become more complex, e.g. by incorporation of functional domains. Solid phase synthesis of polymers presents an interesting alternative, leading towards precise, sequence-defined polymeric carriers. Solid phase peptide synthesis (SPPS), introduced by Merrifield in 1963 [120], allows the controlled step-by-step attachment of natural amino acids, as well as artificial amino acids with modifications suitable for nucleic acid binding. Solid

beads (resin), functionalized with linker units, form the anchor and starting point for multiple peptide coupling steps (Figure 4).

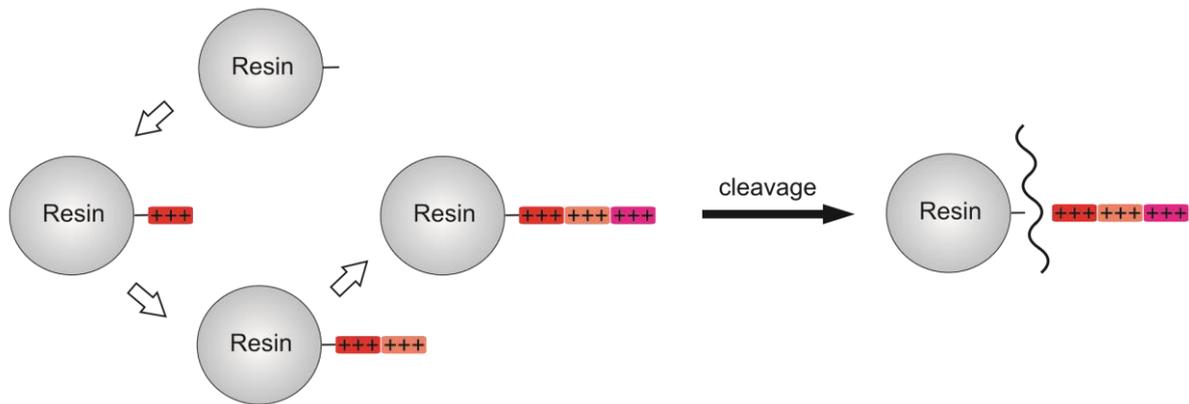


Figure 4: Solid phase peptide synthesis for assembly of defined, monodisperse polycations, suitable for nucleic acid delivery. Defined cationic building blocks are coupled to the solid phase in multiple rounds and the final structure is subsequently cleaved from the resin, yielding sequence defined polycationic carriers.

After completion of the peptide sequence, the construct is cleaved from the resin, for example by treatment with trifluoroacetic acid. Because the peptide is immobilized on the solid phase during synthesis, until it is cleaved from the resin after the last reaction step, it can be purified by filtration after each coupling step. High coupling efficiency during each synthesis step is ensured by treatment of the growing peptide chain with a major excess of the next amino acid. Combination of high coupling efficiency with purification from reagents and by-products after each coupling step yields pure and highly defined structures. Additionally, this synthesis method allows the incorporation of functional moieties like shielding domains or targeting ligands at defined positions within the structure. Hartmann and colleagues transferred this method to the assembly of nucleic acid carriers, rendering sequence defined polyamidoamines [121, 122].

Based on standard peptide synthesis, Leng et al. generated various branched carriers consisting of lysine and histidine residues (with different degrees of branching, length of terminal arms, and changed histidine–lysine sequences) and identified suitable carriers for siRNA delivery with minimal toxicity and knockdown activity *in vitro* and *in vivo* [123, 124].

Wang et al designed a small library of novel multifunctional siRNA carriers, reversibly polymerizable surfactants with pH-sensitive amphiphilicity, using combinatorial solid-phase chemistry [40]. The polymerizable surfactants are made up of two lipophilic tails, a protonatable amino head group for siRNA complexation and two cysteine residues, enabling the construct to encage siRNA by autoxidative polymerization of the dithiols.

1.6 Aims of the thesis

Nucleic acid based therapy holds great promise for the treatment of multiple serious diseases at the root of their origin. Especially the exploitation of RNAi by the use of siRNA seems a safe and direct way for therapeutic application of nucleic acids in humans. However, for similar success as other biological therapeutics like peptides and proteins, which are extensively used in extracellular pathways, nucleic acid therapeutics require intracellular delivery to be active. The current lack of efficient delivery systems for siRNA hampers its broad application in the clinic. Focus of the current thesis was laid on the exploration and characterization of novel polymeric siRNA delivery systems with high efficiency and good biocompatibility. Within this work, the systematic evaluation of structure-activity relationships received special attention, as this yields important conclusions for the optimization of siRNA carriers.

The first aim of the thesis was to physically stabilize labile siRNA polyplexes, based on a pseudodendritic, biodegradable polymer, which lacked gene silencing efficiency, for optimization of siRNA delivery. This should be achieved by crosslinking the surface of the in situ formed polyplexes with an amine reactive crosslinking agent, forming a cage around the particles. Biodegradability of the polyplexes should be maintained after chemical modification for a low toxicity profile of the siRNA nanoparticles. The optimum relation between polymer, siRNA and crosslinking agent should be determined for maximum delivery performance. Biophysical and biological properties of such stabilized particles should be examined for their potential as an effective siRNA delivery system.

The second aim of the thesis was the evaluation of a small library of novel, sequence defined oligomers, based on new oligo (ethane amino) amides and solid phase assisted peptide synthesis, for their efficiency as siRNA carriers. The defined

structure of the novel oligomers renders precise insights into structure-activity relationships of the different sequences. In this way, the new polycationic building blocks and functional groups, like lipid moieties or crosslinking domains, for the assembly of the carriers can be evaluated for their effects on binding, delivery and intracellular release of siRNA. In contrast to most reported synthesis methods of polymeric delivery systems, which result in polydisperse end products, the applied method enables the defined assembly of various different topologies and insertion of functional groups at defined positions of the cationic backbone of the carriers. The rapid and reproducible assembly of new structures enriches the understanding on requirements of siRNA carriers and accelerates the discovery of highly efficient delivery vehicles.

Third aim of the thesis was to exploit defined, multifunctional carrier systems, based on the platform of the upper mentioned monodisperse, polycationic oligomers, for enhanced siRNA delivery. The functionalization of siRNA polyplexes is essential for successful application in vivo, due to the changing biological environment during the delivery process. For this reason, domains for receptor targeting, shielding or with lytic activity were incorporated into the delivery systems, either directly linked to the nucleic acid or to a polycationic backbone. As targeting ligand, folic acid, targeting the human folic acid receptor, which is expressed on the surface of many tumor cells, was chosen. Receptor specific cellular attachment and internalization of targeted polyplexes should be demonstrated. Effective shielding should be realized by PEGylation of siRNA or carrier with defined starting products. The ligation of the lytic peptide INF7 to siRNA should facilitate enhanced endosomal release of internalized particles. The biological effect and specificity of each incorporated moiety alone and in combination with others should be examined in detail. Premise here was to maintain the defined character of all modified molecules during functionalization, rendering clear and reliable structure-activity relationships.

2 MATERIALS AND METHODS

2.1 Chemicals, reagents and polymers

HD O was synthesized as described previously using LMW polyethylenimine 800 Da and 1.6-hexanediol diacrylate (Sigma-Aldrich, Steinheim, Germany) [125]. Dimethyl sulfoxide (DMSO) was also obtained from Sigma-Aldrich. dithiobis(succinimidylpropionate) (DSP) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Cell culture 5 x lysis buffer and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany). Cell culture media, antibiotics and fetal calf serum (FCS) were purchased from Invitrogen (Karlsruhe, Germany). Cell culture lysis buffer was purchased from Promega, Mannheim, Germany.

Sequence defined oligomers, based on oligo (ethane amino) amides, were synthesized by solid phase supported synthesis as described previously [126, 127].

siRNA duplexes for the luciferase reporter gene assay were purchased from Dharmacon (Lafayette, CO, USA) or provided by Roche Kulmbach (Germany).

Dharmacon:

LucsiRNA:	GL3 luciferase 5'-CUUACGCUGAGUACUUCGA-3' (sense)
siControl:	nontargeting control 5'-AUGUAUUGGCCUGUAUUAG-3' (sense)

Roche Kulmbach:

GFP siRNA:	5'-AuAucAuGGccGAcAAGcAdTsdT-3' (sense) 5'-UGCUUGUCGGCcAUGAuAUdTsdT-3' (antisense) (small letters: 2'methoxy-RNA; s: phosphorothioate)
Control siRNA:	5'-AuGuAuuGGccuGuAuuAGdTsdT-3' (sense) 5'-CuAAuAcAGGCcAAuAcAUdTsdT-3'(antisense)
Cy5-siRNA:	(Cy5)-5'-cuuAcGcuGAGuAcuucGAdTsdT-3' (sense) 5'-UCGAAGuACUcAGCGuAAGdTsdT-3' (antisense)

AHA1 siRNA: 5'-GGAuGAAGuGGAGAuAGudTsdT-3' (sense)
5'-ACuAAUCUCcACUUC AUCCdTsdT-3' (antisense)

As buffer and solvent HBG (HEPES-buffered glucose solution: 20 mM HEPES, 5% glucose, pH 7.4) was used.

Human folate receptor (FOLR-1) antibody conjugated to Allophycocyanine was purchased from R&D Systems (U.K.).

High Pure RNA Tissue Kit™, Transcriptor High Fidelity cDNA Synthesis Kit™, UPL Probes, Probes Master and primers for quantitative real time PCR were obtained from Roche Applied Science, Germany.

2.2 Polyplex formation

Polyplex formulations were prepared as follows (unless otherwise mentioned): siRNA and the calculated amount of polymer, either w/w (weight to weight) or N/P (protonable nitrogen to phosphate) molar ratio, were diluted with HBG buffer in separate Eppendorf tubes. The polycation solution was added to the siRNA solution, rapidly mixed by pipetting up and down (at least five times) and incubated for 40 minutes at room temperature in order to form the polyplexes.

2.3 Lateral stabilization of HD-O polyplexes via crosslinking

Lateral crosslinking of the polyplexes was performed by adding indicated amounts of DSP (2 mM solution in DMSO) to the prepared polyplexes, with an additional incubation time of 30 minutes. The indicated different ratios of crosslinker are reported as a molar ratio between crosslinker (DSP) and HD-O amine groups, assuming that HD-O contains 54 amines per molecule with an average molecular weight of 4138 Da.

2.4 Biophysical characterization

2.4.1 Ethidium bromide assay

The ability of cationic polymers to compact siRNA was studied using an ethidium bromide (EtBr) assay. Intercalation of EtBr into free siRNA strongly enhances fluorescence of EtBr. Binding of siRNA to polymers displaces the intercalated EtBr, leading to a significant decrease of fluorescence intensity.

Polymer solution was added stepwise to the solution of siRNA (10 $\mu\text{g/mL}$) in 1 ml HBG containing EtBr (0.4 $\mu\text{g/mL}$). After each step, the fluorescence intensity was monitored ($\lambda_{\text{ex}}=510\text{nm}$, $\lambda_{\text{em}}=590\text{nm}$) using a Cary Eclipse fluorescence spectrophotometer (Varian GmbH, Germany). The fluorescence intensity of the EtBr solution in the presence of free nucleic acid corresponded to 0% condensation, whereas the fluorescence intensity without nucleic acid corresponded to 100% condensation.

2.4.2 Agarose gel shift assay

A 2% agarose gel was prepared by dissolving agarose in TBE buffer (trizma base 10.8 g, boric acid 5.5 g, disodium EDTA 0.75 g and 1 L of water). GelRed[®] was added for the detection of nucleic acid. Polyplexes, containing 500 ng of siRNA in 20 μL HBG and loading buffer (prepared from 6 mL of glycerine, 1.2 mL of 0.5 M EDTA, 2.8 mL of H_2O , 0.02 g xylene cyanol) were placed into the sample pockets. Electrophoresis was performed at 80 V for 40 minutes.

2.4.3 Particle size and Zeta potential measurement

Particle size and Zeta potential of siRNA formulations was measured by dynamic laser-light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). siRNA polyplexes (10 μg siRNA) were prepared in 20 mM HEPES pH 7.4 buffer and diluted in HEPES pH 7.4 buffer just before measurement.

2.4.4 Ellman`s assay

The Ellman assay is a colorimetric assay used to quantify the number or concentration of thiol groups in a sample. With the help of this assay the formation of disulfides during polyplex incubation time can be monitored. The reaction between Ellman`s reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB) and the free thiol groups present in the polymer sample results in the formation of the yellow dianion NTB²⁻, which can be quantified colorimetrically. 4 mg of DTNB dissolved in 1 mL of the corresponding Ellman`s buffer (0.2 M Na₂HPO₄ with 1 mM EDTA at pH 8.0) was used as stock solution. A DTNB working solution was produced by diluting the stock solution 1:40 in Ellman`s buffer. For UV/VIS absorption measurement this DTNB working solution was taken as blank. A standard curve with L-cysteine dilutions was created before each set of measurements. Polymer solutions or corresponding siRNA polyplexes (different N/P ratios with *in vitro* siRNA concentrations of 370 nM) were prepared in HBG pH 7.4 or pH 8.4 and incubated at room temperature or 37 °C. At indicated time points, aliquots of the solutions were diluted with the DTNB working solution. After 15 minutes at 37 °C the solutions were measured at 412 nm and the concentration of the free thiols at 0 minutes was set 100%.

2.5 Biological characterization

2.5.1 Cell culture

Mouse neuroblastoma Neuro2A cells, Neuro2A/eGFPLuc and human hepatoma HepG2/eGFPLuc cells, both stably transfected with the eGFPLuc gene, were grown in Dulbecco`s modified Eagle`s medium (DMEM). Human prostate carcinoma DU-145/eGFPLuc and human cervix carcinoma KB/eGFPLuc cells, both stably transfected with the eGFPLuc gene, were grown in RPMI-1640 medium. Human hepatoma HUH7/eGFPLuc cells, stably transfected with the eGFPLuc gene, were grown in HAM`s 12 / DMEM (50%/50%) medium. All media were supplemented with 10% FCS, 4 mM stable glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. All cultured cells were grown at 37°C in 5% CO₂ humidified atmosphere.

2.5.2 Luciferase gene silencing

Gene silencing experiments were performed using 0.5 µg/well (unless otherwise mentioned) of either GFP-siRNA or Luc-siRNA for silencing of the eGFPLuc protein, or control-siRNA as control. siRNA delivery was performed in 96-well plates with 5×10^3 cells per well in triplicate. Cells were seeded 24 hours prior to transfection and then medium was replaced with 80 µL fresh growth medium containing 10% FCS. Transfection complexes for siRNA delivery (20 µL formed in HBG) were added to each well and incubated at 37 °C for 48 hours (unless otherwise mentioned). After transfection, cells were treated with 100 µL cell lysis buffer (Promega, Mannheim, Germany). Luciferase activity in the cell lysate was measured from a 35 µL aliquot of the lysate using a luciferase assay kit (100 µL Luciferase Assay buffer, Promega, Mannheim, Germany) and a Centro LB 960 plate reader luminometer (Berthold Technologies, Germany). The relative light units (RLU) are presented as percentage of the luciferase gene expression obtained with buffer treated control cells.

2.5.3 Cell viability assay (MTT) of plain polymers

Neuro2A/eGFPLuc cells were seeded into 96-well plates at a density of 5×10^3 cells/well. After 24 hours, culture medium was replaced with 80 µL fresh growth medium containing 10% FCS. Serial dilutions of polymer stock solutions (in 20 µL HBG) were added. All studies were performed in triplicates. For Neuro2A/eGFPLuc cells, 10 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml) were added 48 hours after polymer addition. After an incubation time of two hours, unreacted dye and medium were removed. The purple formazan product was dissolved in 100 µL/well dimethyl sulfoxide and quantified by a plate reader (Tecan, Groedig, Austria) at 590 nm with background correction at 630 nm. The relative cell viability (%) relative to control wells containing cell culture medium with 20 µL buffer was calculated by $[A]_{\text{test}} / [A]_{\text{control}} \times 100$.

2.5.4 Erythrocyte leakage assay

Fresh, citrate buffered murine blood was washed with phosphate-buffered saline (PBS). The washed murine erythrocyte suspension was centrifuged and the pellet was diluted to 5×10^7 erythrocytes per ml with PBS (pH 7.4, 6.5 and 5.5). A volume

of 75 μ l of erythrocyte suspension and 75 μ l of polymer solution were added to each well of a V-bottom 96-well plate (NUNC, Denmark), resulting in a final concentration of 2.5 μ M polymer per well. The plates were incubated at 37 °C under constant shaking for one hour. After centrifugation, 80 μ l of the supernatant was analyzed for hemoglobin release at 405 nm using a microplate plate reader (Spectrafluor Plus, Tecan Austria GmbH, Grödig, Austria). Control wells contained buffer with 1 % Triton X-100 for 100% lysis.

2.5.5 Serum stability assay

A 2% agarose gel was prepared containing GelRed[®] as described above. Polyplexes (N/P 12) containing 200 μ g/ml of siRNA in 12.5 μ L 20 mM HEPES (pH 7.4) were incubated at room temperature for 30 minutes. Fetal bovine serum (FBS) in different concentrations (0, 10, 50 and 90% v/v) was added to the polyplexes for 15 minutes. An aliquot of the samples containing 500 ng siRNA and loading buffer (containing xylene cyanol) were placed into the sample pockets. Electrophoresis was performed at 80 V for 40 min.

2.5.6 Flow cytometry

2.5.6.1 Cellular uptake of untargeted polyplexes

Neuro2A or HUH-7 cells were seeded into 24-well plates at a density of 5×10^4 cells/well. After 24 hours, culture medium was replaced with 400 μ L fresh growth medium containing 10% FCS. Transfection complexes for siRNA delivery (containing 2.5 μ g Cy5-labeled siRNA) were added to each well and incubated at 37 °C for 4 hours. Subsequently, cells were washed twice with 500 μ l PBS, containing 100 I.U. of heparin per ml, for 15 minutes to detach any polyplexes sticking to the cell surface. Cells were detached with trypsin/EDTA, taken up in PBS with 10% FCS and flow cytometry was performed using a FACSCanto[™] II flow cytometer (Becton Dickinson) or a Cyan[™] ADP flow cytometer (Dako, Hamburg, Germany). Cellular uptake was assayed by excitation of Cy5 dye at 635 nm and detection of emission at 665 nm. To discriminate between viable and dead cells as well as for exclusion of doublets, cells were appropriately gated by forward/sideward scatter and pulse width,

counterstained with propidium iodide or DAPI and 1×10^4 gated events per sample were collected. Data were recorded by BD FACS Diva™ software or Summit™ software (Summit, Jamesville, NY, USA) and evaluated using FlowJo® software.

2.5.6.2 Cellular association of targeted polyplexes

KB cells were seeded into 24-well plates at a density of 5×10^4 cells/well. After 24 hours, culture medium was replaced with 400 μ L fresh growth medium containing 10% FCS and 400 μ L fresh growth medium containing 10% FCS, saturated with folic acid, respectively. Next cells were incubated on ice for 30 minutes. Transfection complexes for siRNA delivery (Cy5-labeled INF-siRNA) at an N/P ratio of 16 (100 μ L in HBG) were added to each well and incubated at 4 °C for 30 minutes (final siRNA concentration: 200 nM). Subsequently, cells were washed twice with PBS. Cells were detached with trypsin/EDTA and taken up in PBS with 10% FCS. Flow cytometry was performed as described in 2.5.6.1.

2.5.6.3 Cellular uptake of targeted polyplexes

KB or HUH-7 cells were seeded into 24-well plates at a density of 5×10^4 cells/well. After 24 hours, culture medium was replaced with 400 μ L fresh growth medium containing 10% FCS. Transfection complexes for siRNA delivery (Cy5-labeled INF-siRNA) at an N/P ratio of 16 (100 μ L in HBG) were added to each well and incubated at 37 °C for 30 minutes (final siRNA concentration: 200 nM). Subsequently, cells were washed twice with PBS. Cells were detached with trypsin/EDTA and taken up in PBS with 10% FCS. Flow cytometry was performed as described in 2.5.6.1.

2.5.6.4 Cellular uptake of targeted siRNA

KB or Neuro2A cells were seeded into 24-well plates at a density of 5×10^4 cells/well. After 24 hours, culture medium was replaced with 400 μ L fresh growth medium containing 10% FCS. Plain Cy5-labeled siRNA (2.5 μ g in 100 μ L HBG) or transfection complexes (N/P ratio of 6 and 12 in 100 μ L HBG, containing 2.5 μ g Cy5-labeled siRNA) were added to each well and incubated at 37 °C for 30 minutes. Subsequently, cells were washed twice with 500 μ L PBS. Cells were detached with

trypsin/EDTA and taken up in PBS with 10% FCS. Flow cytometry was performed as described in 2.5.6.1.

2.5.7 Fluorescence microscopy

2.5.7.1 Folic acid receptor determination

KB or HUH-7 cells were seeded into 8 well Labtek™ chamberslides at a density of 2×10^4 cells/well. 24 hours after seeding cells were exposed to 5 μ l of the FOLR-1-antibody (conjugated to Allophycocyanin) directed against the human folate receptor 1 for 30 minutes at room temperature. Unbound antibody was removed by washing the cells twice with PBS and nuclei were stained with Hoechst 33342 dye. A Zeiss Axiovert 200 fluorescence microscope was used to collect the images and data were analyzed and processed by AxioVision LE™ software.

2.5.7.2 Cellular uptake of untargeted polyplexes

Neuro2A or HUH-7 cells were seeded into 8 well Labtek™ chamber slides at a density of 2×10^4 cells/well. After 24 hours culture medium was replaced with 240 μ L fresh growth medium, containing 10% FCS. Transfection complexes for siRNA delivery (1.5 μ g siRNA including 20% Cy5-labeled siRNA) at indicated N/P ratios (60 μ l in HBG) were added to each well and incubated at 37 °C for 24 hours. Subsequently, a washing step with PBS, containing 100 I.U. heparin per ml, was performed for 20 minutes. Cell nuclei were stained with Hoechst 33342 dye and in the case of HUH-7 cells endo-/lysosomes were stained with LysoTracker™ green (50 nM) for 30 minutes. Cellular uptake was assayed by detection of Cy5 (excitation 635 nm, emission 665 nm) and LysoTracker green (excitation 504 nm, emission 511 nm). A Zeiss Axiovert 200 fluorescence microscope was used to collect the images and data were analyzed and processed by AxioVision LE™ software.

2.5.7.3 Cellular uptake of targeted polyplexes

KB or HUH-7 cells were seeded into 8 well Labtek chamber slides at a density of 2×10^4 cells/well. After 24 hours culture medium was replaced with 240 μ L fresh growth medium, containing 10% FCS. Transfection complexes for siRNA delivery (Cy5-

labeled INF-siRNA) at an N/P ratio of 16 (60 μ L in HBG) were added to each well and incubated at 37 °C for 30 minutes (final siRNA concentration: 200 nM). Subsequently, cells were washed twice with 500 μ L PBS and cell nuclei were stained with Hoechst 33342 dye. Cellular uptake was assayed by excitation of Cy5 at 635 nm and detection of emission at 665 nm. A Zeiss Laser Scanning Microscope LSM510 Meta was used to collect the images and data were analyzed and processed by AxioVision LE™ software.

2.5.7.4 Cellular uptake of targeted siRNA

KB or Neuro2A cells were seeded into 8 well Labtek chamber slides at a density of 2×10^4 cells/well. After 24 hours culture medium was replaced with 240 μ L fresh growth medium, containing 10% FCS. Cy5-labeled siRNA (1.5 μ g in 60 μ L HBG) was added to each well and incubated at 37 °C for 30 minutes. Subsequently, cells were washed twice with 500 μ L PBS and cell nuclei were stained with Hoechst 33342 dye. Cellular uptake was assayed by excitation of Cy5 at 635 nm and detection of emission at 665 nm. A Zeiss Axiovert 200 fluorescence microscope was used to collect the images and data were analyzed and processed by AxioVision LE™ software.

2.5.8 Quantitative real-time PCR (RT-qPCR)

Neuro2A/eGFPLuc cells were seeded into 24-well plates at a density of 3×10^4 cells/well. After 24 h, culture medium was replaced with 400 μ L fresh growth medium containing 10% FCS. Transfection complexes for siRNA delivery (100 μ L in HBG) were added to each well (2.5 μ g siRNA / well) and incubated at 37 °C without medium change. 48 hours after transfection total RNA was isolated using High Pure RNA Tissue Kit™ (Roche, Germany) and transcribed with the Transcriptor High Fidelity cDNA Synthesis Kit™ (Roche, Germany) according to manufacturer's protocol. Quantitative real-time PCR was performed using UPL Probes and Probes Master (both Roche, Germany) on a LightCycler 480™ system (Roche, Germany) with GAPDH as control. Primers used include ACTB (ready to use Universal Probe library assay Roche); AHA1 (UPL Probe #43) left: CAATGAATGGAGAGTCAGTAGACC, right: GCCTGGGTTTTTTGAAGGAG.

Experiments were performed in triplicates and the achieved average C_T values were normalised against control as ΔC_T . AHA1 expression pattern in AHA1-siRNA and control-siRNA treated samples were compared to HBG treated cells

2.6 Statistical analysis

Results are presented as mean \pm standard deviation (SD). Statistical significance of differences was evaluated by one-way analysis of variance (ANOVA). P-values < 0.05 were considered significant. Statistics were performed with Graph Pad Prism 5.0[®].

3 RESULTS

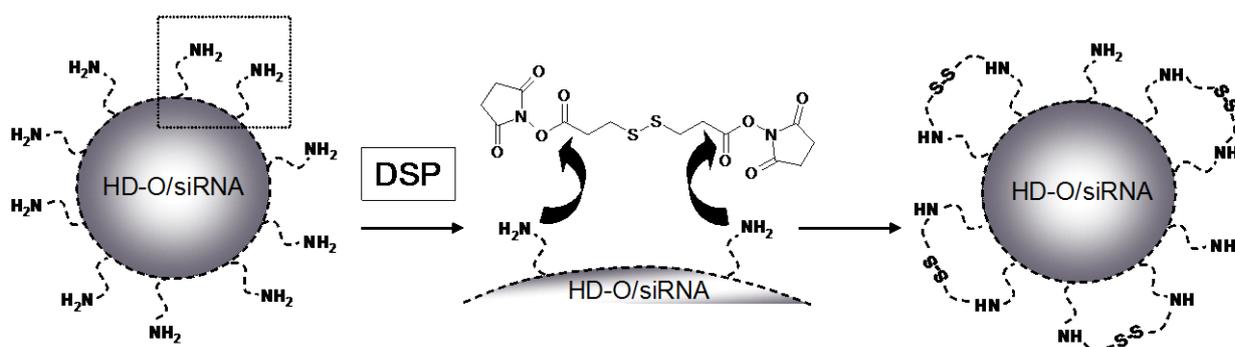
3.1 Lateral stabilization of siRNA polyplexes via DSP crosslinking for efficient siRNA delivery

3.1.1 Design of crosslinked HD-O polyplexes

The pseudodendritic, biodegradable polymer HD-O was reported to effectively transfect plasmid DNA (pDNA) *in vitro* and *in vivo* with good biocompatibility [125]. A polyplex surface crosslinking strategy with the amine reactive crosslinker dithiobis(succinimidylpropionate) (DSP) and disuccinimidyl suberate (DSS), respectively, further improved the stability of DNA polyplexes, which is particularly important for *in vivo* application, but was not strictly required for efficacy [128]. The findings for pDNA transfection cannot always be transferred to siRNA delivery [129]. Due to the much smaller size of the siRNA duplex compared to pDNA, the siRNA polyplexes lack stability in an electrolyte and protein containing environment.

Plain HD-O/siRNA polyplexes showed very moderate gene silencing ability in serum containing medium. pDNA polyplexes owe their stability largely to numerous electrostatic, entropy-driven interelectrolyte interactions which during polyplex formation condensate pDNA into a compact particle. Because of the much smaller size of siRNA (usually 21 to 23 base pairs) compared to pDNA (1 to 10 kbp) and additionally the small size of HD-O polymers (~ 4100 Da) the resulting particles are not as stable as HD-O/DNA polyplexes.

Scheme 1: Surface crosslinking reaction of HD-O polyplexes with the homobifunctional, amine reactive crosslinker DSP.



Therefore the previously applied crosslinking strategy for DNA was also applied for siRNA polyplexes (Scheme 1). The formed polyplexes of HD-O and siRNA were laterally stabilized by adding a fixed percentage of the crosslinker DSP. This homobifunctional crosslinker with reactive NHS esters at each end readily reacts with amino groups of the polymer on the surface of the polyplexes. The fixed percentage of the crosslinker was optimized to a molar ratio of 0.05/1 between crosslinker and the amines of HD-O, assuming that HD-O contains about 54 amines per molecule with an approximate molecular weight of 4100 Da.

3.1.2 Effect of lateral stabilization on siRNA delivery *in vitro*

HD-O polyplexes proved ineffective in siRNA delivery. Therefore particle optimization was performed by crosslinking the polyplexes and finding the optimum weight to weight balance between polymer and siRNA (Figure 5).

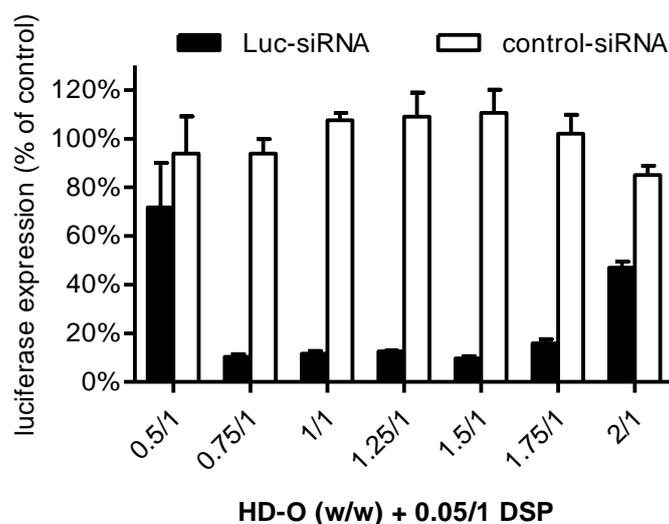


Figure 5: Gene silencing ability of HD-O polyplexes crosslinked with 0.05/1 DSP and increasing weight/weight ratios of polymer/siRNA in Neuro2A-eGFPLuc cells. Black: Luc-siRNA, white: control-siRNA.

Figure 5 illustrates that within a window between w/w 0.5/1 and 2/1 the crosslinked particles mediate effective gene silencing in the murine neuroblastoma Neuro2A cell line. Luciferase activity was determined for cells treated either with Luc-siRNA (directed against the eGFPLuc fusion protein) or with control-siRNA and compared

with the luciferase activity of buffer treated control cells. As an example, a w/w ratio of 1 plus crosslinking reproducibly resulted in more than 90% silencing of the luciferase signal without unspecific knockdown or toxicity, indicated by the absence of gene silencing in the control-siRNA treated group.

Further proof for the good biocompatibility of the linker-stabilized polyplexes was provided by a simultaneously performed MTT test (Figure 6).

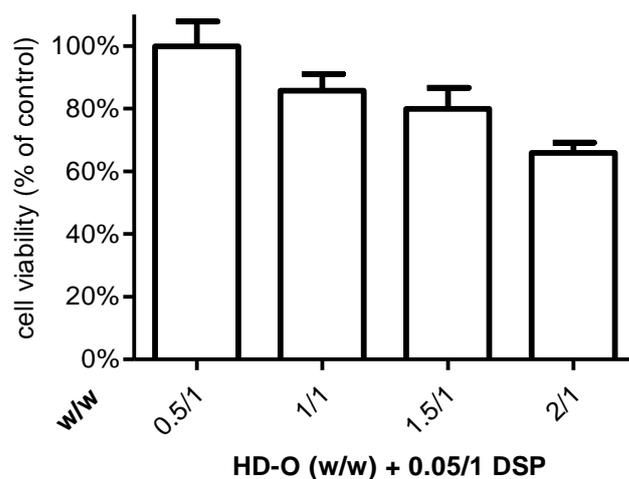


Figure 6: Cell viability of Neuro2A-eGFPLuc cells 48 hours after treatment with 0.05/1 DSP crosslinked siRNA polyplexes (at indicated w/w ratios).

A very moderate decrease of cell viability was detected for the crucial w/w ratios of 1/1 and 1.5/1. Significant signs of toxicity were observed only from a w/w ratio of 2/1 and higher (data not shown).

In order to optimize the DSP linker ratio, several DSP concentrations were reacted with HD-O polyplexes at the optimum w/w ratio of 1/1. Gene silencing efficiency of the crosslinked polyplexes was tested in the luciferase reporter assay (Figure 7). It could be shown that 0.05/1 is the most effective ratio. DSP concentrations below this ratio are insufficient and do not enhance gene silencing efficiency. Concentrations above 0.1/1 lead to aggregation of the particles, probably by increased interparticulate crosslinking between the polyplexes. An excess of linker leads to complete loss of gene silencing activity.

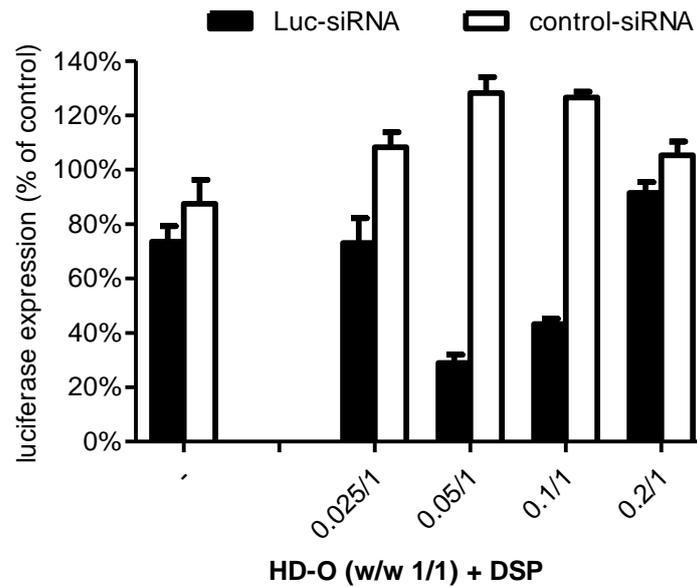


Figure 7: Effect of increasing crosslinker concentrations on gene silencing ability of HD-O polyplexes (w/w 1/1) in Neuro2A-eGFPLuc cells. Black: Luc-siRNA, white: control-siRNA.

Efficiency of the optimized crosslinked polyplexes was also determined in a different cell line, the human hepatoma HUH-7 cell line (Figure 8).

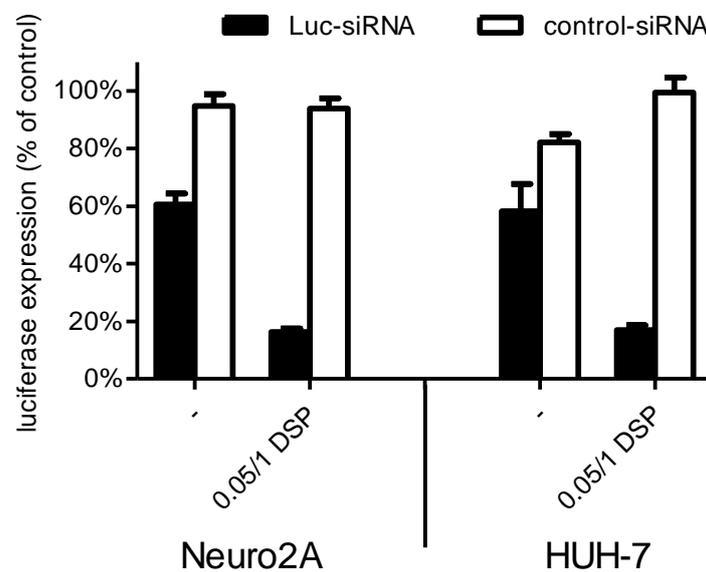


Figure 8: Comparison of the gene silencing ability of HD-O polyplexes (w/w 1/1) without and with 0.05/1 DSP crosslinking in Neuro2A-eGFPLuc and HUH7-eGFPLuc cells. Black: Luc-siRNA, white: control-siRNA.

The results of the luciferase reporter gene assay for HUH-7 cells are comparable to those obtained with Neuro2A cells. A strong increase of luciferase knockdown after lateral stabilization of the polyplexes with DSP was observed. In both cell lines, the tested ratios of polymer and crosslinker showed no signs of unspecific knockdown in the control-siRNA treated group.

3.1.3 Biophysical characterization

The optimized polyplexes with a weight to weight ratio of 1/1 and 1.5/1 and a DSP crosslinker ratio of 0.05/1 were examined for size and Zeta potential in comparison to their unmodified counterparts (Table 1).

Table 1: Particle size and Zeta potential of HD-O/siRNA polyplexes (w/w 1/1 or 1.5/1) with and without DSP crosslinking.

HD-O	w/w	Size [nm]	Zeta [mV]
-	1/1	113 ± 3	27,6 ± 0,4
+ 0.05/1 DSP	1/1	197 ± 3	20,1 ± 0,7
-	1,5/1	87 ± 1	33,1 ± 0,2
+ 0.05/1 DSP	1,5/1	127 ± 1	30,8 ± 0,5

Linker-stabilized nanoparticles showed a slightly increased size compared to nonstabilized polyplexes but were still smaller than 200 nm. The measured Zeta potential decreased for both measured weight to weight ratios after crosslinking. This decrease is caused by the loss of surface amines in the polyplex through the crosslinking reaction. Amines from the polymer HD-O on the polyplex surface react with DSP to form stable amide bonds, which cannot be protonated any more and thus cannot contribute to a positive surface charge of the particles. The decrease of the Zeta potential is an indicator for successful surface crosslinking by DSP.

Stability of the polyplexes is essential for successful delivery of siRNA into the cellular cytosol. The stability of linker-stabilized and nonstabilized polyplexes at increasing w/w ratios was determined in the agarose gel shift assay (Figure 9). The electrophoresis assay revealed that both crosslinked and uncrosslinked polyplexes

retain the majority of siRNA in the loading pocket from a weight to weight ratio of one onward.

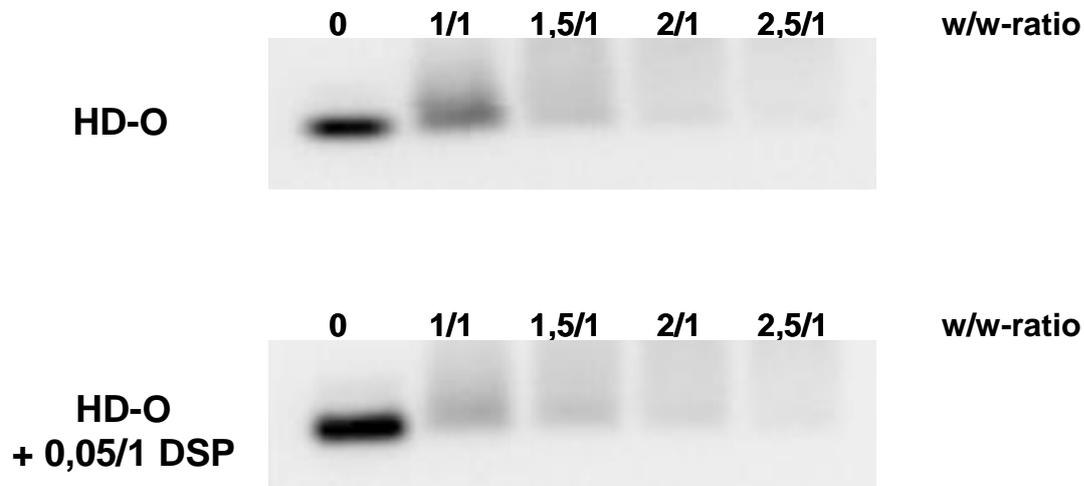


Figure 9: Agarose gel shift assay of crosslinked (0.05/1 DSP) and uncrosslinked HD-O polyplexes at rising w/w ratios.

A small difference between stabilized and unstabilized polyplexes is observable at w/w 1/1: the DSP crosslinking slightly improves siRNA retention, supporting the assumption that surface crosslinking enhances the stability of the polyplexes.

3.1.4 Cellular uptake of linker-stabilized polyplexes

The gene silencing assay demonstrated successful silencing of luciferase protein by linker-stabilized HD-O/siRNA polyplexes, targeting the eGFPLuc fusion protein. In order to support this finding, internalization of the polyplexes was determined by flow cytometry. Polyplexes were spiked with fluorescently labeled Cy5-siRNA and the amount of Cy5 positive cells compared to buffer treated control cells was detected (Figure 10 and 11). Although nonstabilized particles showed a moderate uptake (mean: 7.1% \pm 2.5% positive cells, Figure 11), a substantial shift of the Cy5 signal was detectable after cells had been treated with stabilized polyplexes (mean: 43.6% \pm 5.4% positive cells, Figure 11) already at the short 1 h incubation time.

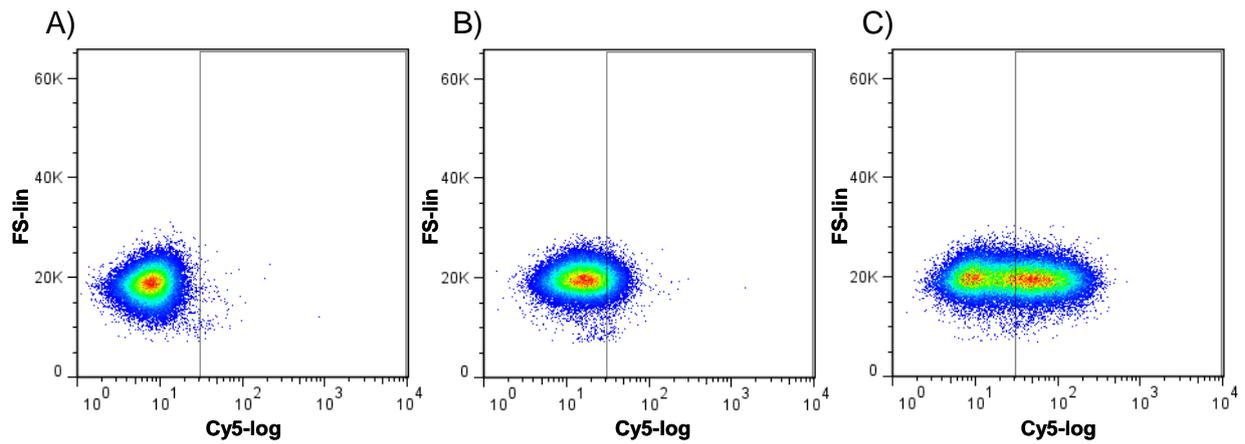


Figure 10: Flow cytometric assay of polyplex uptake at 1 hour after transfection (single measurement as representative for three individual measurements; for summary of triplicates see Figure 11). HD-O polyplexes were spiked with 20% fluorescently labeled (Cy5) siRNA. A) Buffer treated control. B) HD-O polyplexes (w/w 1/1). C) HD-O polyplexes (w/w 1/1) + 0.05/1 DSP.

The moderate uptake of nonstabilized polyplexes in this experiment is consistent with the results of the luciferase reporter gene assay (Figure 8), in which also only moderate gene silencing is detectable for both tested cell lines.

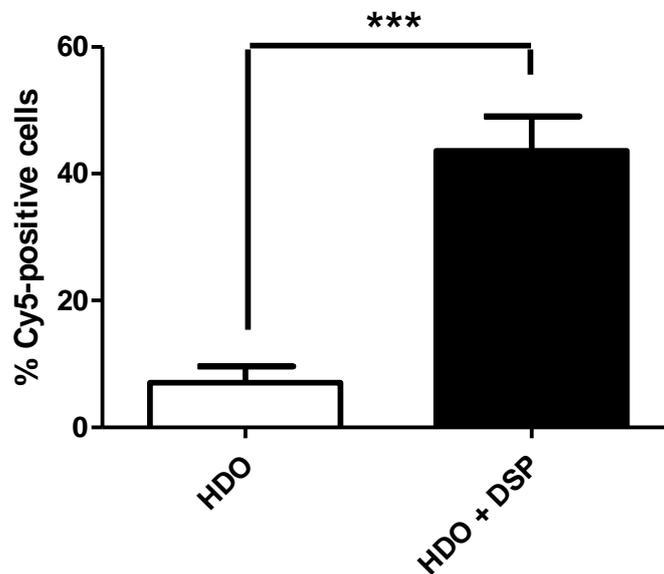


Figure 11: Flow cytometric assay of polyplex uptake at 1 hour after transfection; summary of three individual measurements for each formulation ($p < 0.0005$). White: HD-O polyplexes (w/w 1/1). Black: HD-O polyplexes (w/w 1/1) + 0.05/1 DSP.

3.1.5 Knockdown evaluation on mRNA level

Target gene silencing can be proven on protein level, as shown with the luciferase reporter gene assay, or by direct measurement of the levels of targeted mRNA. Successful siRNA mediated gene silencing leads to decreased mRNA levels and in consequence to a reduced amount of protein. In this experiment the gene encoding for AHA1, activator of 90 kDa heat shock protein ATPase [130], was chosen for evaluation of mRNA knockdown with quantitative real time PCR. AHA1 plays no critical role in cell survival, which makes it an ideal target for unbiased mRNA knockdown experiments. In order to determine successful silencing of this endogenous gene, the level of AHA1 mRNA after treatment with AHA1-siRNA containing polyplexes was verified. As an internal standard the stably expressed house keeper gene ACTB was chosen (Figure 12).

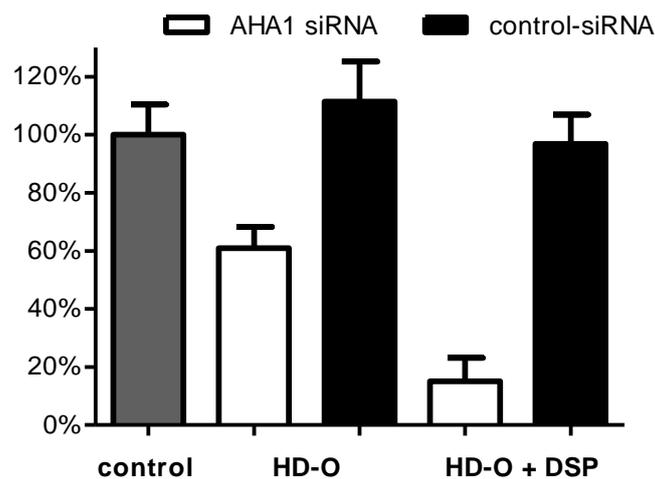


Figure 12: AHA1 mRNA levels in Neuro2A-eGFPLuc cells 48 hours after treatment with HD-O polyplexes (w/w 1/1) +/- 0.05/1 DSP. Gray: buffer control, white: AHA1 siRNA, black: control siRNA. qPCR experiments were performed by Daniel Edinger.

Significant differences in AHA1 expression between cells treated with HD-O/AHA1-siRNA polyplexes with or without lateral stabilization were detected. The nonstabilized particles achieved a moderate knockdown of 39%, whereas treatment with DSP crosslinked polyplexes resulted in 85% AHA1 gene knockdown. Application of control siRNA did not affect AHA1 expression for both formulations.

3.2 Solid phase derived, precise oligomers for siRNA delivery

Polymers represent an interesting class of delivery vehicles for nucleic acids because they can be tailored exactly to the individual requirements of the payload and functionalities like targeting moieties or shielding agents can be incorporated. However, the synthesis of such polymers with the help of solution chemistry yields polydisperse products due to incomplete coupling reactions and high amounts of impurities along with the desired product. Purification of the polymers, especially of those with additional functional groups, is difficult, time consuming and often only possible to a certain extent. A promising solution to this problem is a solid phase supported synthesis method for the design of monodisperse, sequence defined polymers for nucleic acid delivery [127, 131].

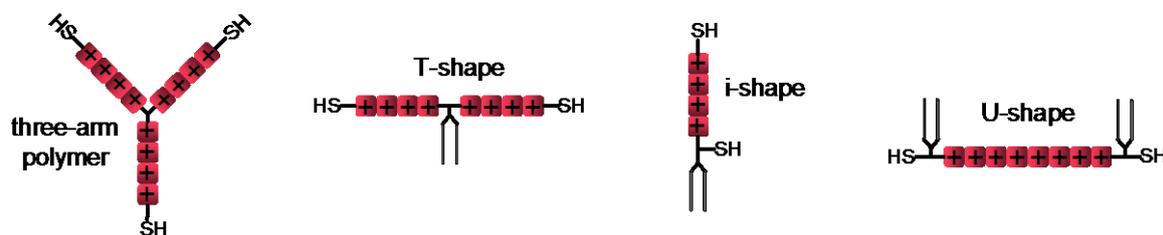
Employing this synthesis strategy, our group assembled several hundred oligomeric structures, with the aim of finding suitable siRNA delivery agents and rendering clear and reliable structure-activity relationships between polymer design and delivery efficacy.

3.2.1 Design of sequence defined oligomers based on oligo (ethane amino) amides

The new solid phase derived oligomers were synthesized from a set of building blocks, see Scheme 2. New artificial oligoamino acids (AA) form the backbone of the oligomers: Ptp, Stp, Gtp and Gtt. They contain tetraethylenepentamine (tp) or triethylentetramine (tt), coupled to succinic, glutaric or phthalic acid. In the past, polymers like polyethylenimine have shown successful nucleic acid delivery [70, 132-134]. The essential subunit of these structures, the 1,2-diaminoethane motif, enables the binding of nucleic acid and facilitates endosomal escape through interaction with the vesicle membrane and the proton sponge effect [32, 67]. Because of these interesting features, this motif was also incorporated into the new building blocks. For the introduction of lytic domains, the incorporation of fatty acids with increasing chain length and varying degrees of saturation at predefined positions within the oligomers was examined. Additionally, selected natural amino acids were used: lysine served as a branching point during polymer synthesis, cysteines were utilized for the

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Scheme 3: Sketch of the oligomer shapes: three-armed structure with a lysine as branching point; T-shape with two fatty acids incorporated in the middle of the polymer backbone; i-shape with two fatty acids on one side of the backbone; U-shape with two fatty acids at each end of the polymer backbone. “+” in red represents a cationic oligoamino acid building block (Ptp, Stp, Gtp, Gtt).



The other three structures all contain fatty acids at defined positions of the oligomer backbone: the T-shape has two vicinal fatty acids located in the middle of the oligomer, the i-shape has the two fatty acids attached to one side of the oligomer and the U-shape has two fatty acids linked to each end of the oligomer chain. Various lipid moieties were screened, studying the effect of different locations of the lipid components within the oligomer and the hydrophilic/lipophilic balance, optimal for stable and effective siRNA nanoparticles. Optionally, cysteines were incorporated at each end of the fatty acid containing oligomers. A list of sequences of all discussed oligomers can be found in Table 2 (see below).

Table 2: List of tested oligomers.

polymer ID	topology	polymer sequence
229	i-shape	C-Stp-Stp-Stp-C-K-LinA2
510	i-shape	C-Ptp-Ptp-Ptp-C-K-LinA2
226	i-shape	C-Gtp-Gtp-Gtp-C-K-LinA2
228	i-shape	C-Gtt-Gtt-Gtt-C-K-LinA2
230	i-shape	C-Gtp-Gtt-Stp-C-K-LinA2
386	three-arm	(C-Stp-Stp-Stp)2]K-Stp-Stp-Stp-C
515	three-arm	(C-Ptp-Ptp-Ptp)2]K-Ptp-Ptp-Ptp-C
514	three-arm	(C-Gtp-Gtp-Gtp)2]K-Gtp-Gtp-Gtp-C
513	three-arm	(C-Gtt-Gtt-Gtt)2]K-Gtt-Gtt-Gtt-C
413	T-shape	C-Stp-Stp-K(K)-Stp-Stp-C
461	T-shape	C-Stp-Stp-K(K-ButA2)-Stp-Stp-C
68	T-shape	C-Stp-Stp-K(K-CapA2)Stp-Stp-C
48	T-shape	C-Stp-Stp-K(K-MyrA2)Stp-Stp-C
462	T-shape	C-Stp-Stp-K(K-SteA2)Stp-Stp-C
49	T-shape	C-Stp-Stp-K(K-OleA2)Stp-Stp-C
463	T-shape	C-Stp-Stp-K(K-LinA2)Stp-Stp-C
51	i-shape	C-Stp-Stp-Stp-C-K
45	i-shape	C-Stp-Stp-Stp-C-K-MyrA2
46	i-shape	C-Stp-Stp-Stp-C-K-OleA2
197	three-arm	(C-Stp)2]K-Stp-C
199	three-arm	(C-Stp-Stp)2]K-Stp-Stp-C
277	three-arm	(C-Stp-Stp-Stp-Stp)2]K-Stp-Stp-Stp-Stp-C
387	three-arm	(A-Stp-Stp-Stp)2]K-Stp-Stp-Stp-A
76	T-shape	C-Stp-(OleA)2K]K-Stp-C
80	T-shape	C-Stp-Stp-Stp-(OleA)2K]K-Stp-Stp-Stp-C
84	T-shape	C-Stp-Stp-Stp-Stp-(OleA)2K]K-Stp-Stp-Stp-Stp-C
281	U-shape	C-(LinA)2K]K-Stp-(LinA)2K]K-C
280	U-shape	C-(LinA)2K]K-Stp-Stp-(LinA)2K]K-C
278	U-shape	C-(LinA)2K]K-Stp-Stp-Stp-(LinA)2K]K-C
352	U-shape	C-(LinA)2K]K-Stp-Stp-Stp-Stp-(LinA)2K]K-C
389	U-shape	A-(LinA)2K]K-Stp-(LinA)2K]K-A
390	U-shape	A-(LinA)2K]K-Stp-Stp-(LinA)2K]K-A
279	U-shape	A-(LinA)2K]K-Stp-Stp-Stp-(LinA)2K]K-A
388	U-shape	A-(LinA)2K]K-Stp-Stp-Stp-Stp-(LinA)2K]K-A
379	i-shape	A-Gtp-Gtt-Stp-A-K-LinA2
470	i-shape	C-Stp-Stp-Stp-Stp-C-K-OleA2
488	U-shape	K-(Stp-Stp-Stp-Stp-K-(K-OleA2)-C)2
471	i-shape	C-Stp-Stp-Stp-Stp-C-K-LinA2
489	U-shape	K-(Stp-Stp-Stp-Stp-K-(K-LinA2)-C)2

3.2.2 Influence of different cationic building blocks on gene silencing efficiency

The new artificial amino acids, Ptp, Stp, Gtp, and Gtt were screened for their ability to bind siRNA, form stable polyplexes and mediate gene silencing (Figure 13). Two model oligomer topologies were chosen for testing efficiencies of the different incorporated artificial amino acids (aa): on the one hand, an i-shape lipopolymeric structure, based on three aa units and two fatty acids attached to a lysine at one side of the oligomer backbone, $(\text{LinA})_2\text{K-C-aa-aa-aa-C}$ (Figure 13 left). On the other hand, a branched three-armed structure free of lipidic residues, consisting of three arms, each built up of three aa units containing a terminal cysteine, $(\text{C-aa-aa-aa})_2\text{K-aa-aa-aa-C}$, (Figure 13 right).

Physicochemical tests show the ability of the i-shape oligomers to bind (Figure 13A) and compact (Figure 13B) siRNA. In the gel shift (Figure 13A left) most of the siRNA is retained in the gel pockets for the Stp, Ptp and Gtp building blocks even at a low nitrogen/phosphate (N/P) ratio of 3. Interestingly, the EtBr assay (Figure 13B left) revealed that the compaction ability is higher for the Stp and Gtp blocks than for Ptp or Gtt, but dye exclusion is much lower than for the high molecular weight linear PEI control polymer. Considering both assays, the binding/compaction ability of the building blocks in i-shape oligomers decreases in the following order: Stp > Gtp > Ptp > Gtt. The mixture of building blocks (Stp-Gtt-Gtp) in one oligomer had no beneficial effects.

In vitro gene silencing (Figure 13C left) was determined using Neuro2A cells, stably expressing eGFPLuc. Treatment with eGFP siRNA polyplexes (but not control siRNA polyplexes) showed effective gene silencing for all tp –based amino acids (Stp, Ptp, Gtp). Some reduced knockdown activity was observed for the building block mixture, and only moderate knockdown for the oligomer with the Gtt building block (Figure 13C left). Luciferase activity was determined for cells treated either with GFP siRNA (directed against the eGFPLuc fusion protein) or with control siRNA and always compared (in relative %) with the luciferase activity (100%) of buffer treated control cells (Figure 13C left). Remarkably, none of the presented structures caused significant toxicity, indicated by the absence of unspecific knockdown signal in the control siRNA treated cells.

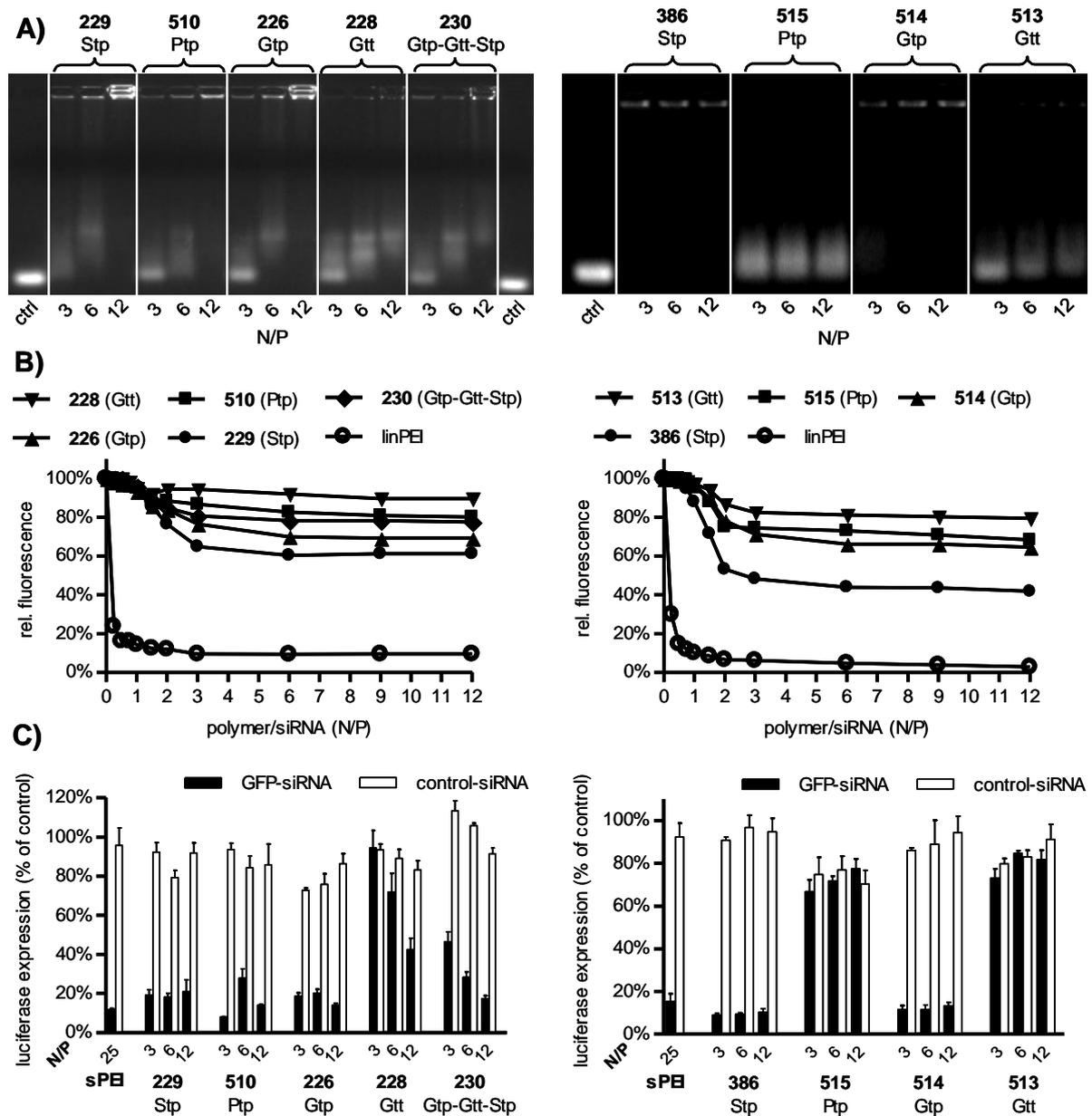


Figure 13: Left: i-shape oligomers with two cysteines, two terminal LinA, and varying building blocks, right: three-armed oligomers with a terminal cysteine at the end of each arm. A) siRNA binding assay at different N/P ratios. B) Ethidium bromide exclusion assay at increasing N/P ratios. Control polymer: linear PEI (22 kDa). C) Gene silencing ability of polyplexes in Neuro2A-eGFP/Luc cells with eGFP-targeted siRNA or control siRNA was tested (at different nitrogen/phosphate ratios, N/P). Positive control: succinylated PEI (sPEI) [73]. Black: GFP-siRNA, white: control-siRNA.

The ethidium bromide assay revealed a big difference between the control polymer linear polyethylenimine (linPEI) and the i-shape formulations. linPEI is able to form very compact particles, leading to a drastic decrease in fluorescent signal in this

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assay. In contrast the i-shapes seem to form stable particles with less compact structure, as the fluorescent signal from EtBr / siRNA interaction only decreases to a maximum of 60% when oligomer is added.

Table 3: Size and Zeta potential of i-shape oligomer (LinA)2K-C-aa-aa-aa-C (N/P 12) with different building blocks.

Building block	Size [nm]	Zeta potential [mV]
Stp	182 ± 7	27,7 ± 0,2
Ptp	144 ± 2	25,6 ± 0,7
Gtp	18 ± 1	21,4 ± 1,2
Gtt	314 ± 20	26,2 ± 0,4
Gtp-Gtt-Stp	172 ± 1	26,1 ± 0,6

However, the i-shape lipopolymers effectively bind siRNA (Figure 13A left), form polyplexes of approximately 200 nm or smaller with a zeta potential above 20 mV at N/P 12 (Table 3) and mediate efficient reporter gene knockdown in serum containing medium (Figure 13C left), whereas linear PEI only achieves moderate knockdown, including unspecific knockdown due to significant toxicity at low N/P ratios (Figure 14).

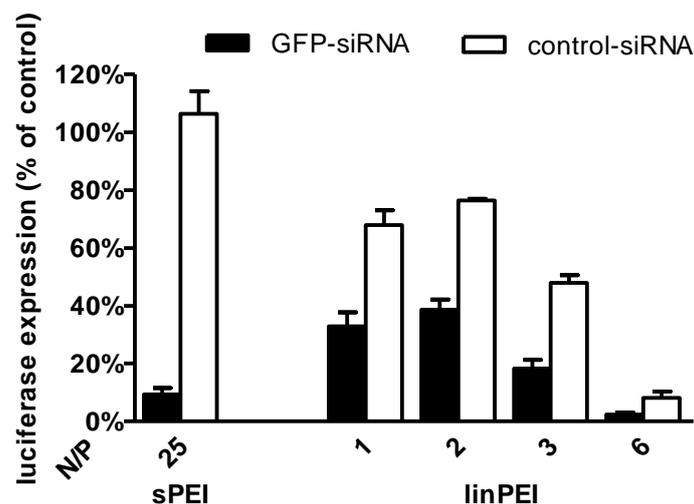


Figure 14: Gene silencing ability of linear PEI (22 kDa) in Neuro2A-eGFPLuc cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

Apparently, apart from electrostatic compaction of the nucleic acid, other stabilizing mechanisms exist in assembly with i-shape lipopolymers, such as disulfide formation via cysteine crosslinking and stabilizing effects through the lipid modification (see below).

For the three-arm structure (Figure 13 right), similar results were obtained: the Stp building block, followed by Gtp showed best physicochemical properties in agarose gel shift and ethidium bromide assay. Ptp and Gtt based oligomers did not bind siRNA to a sufficient extent (Figure 13A right). Consistently, only the three-arm oligomers with Stp or Gtp facilitate siRNA mediated gene silencing (Figure 13C right).

3.2.3 Lipid modification at selected positions of the oligomers

The incorporation of lipid moieties into cationic polymers can improve particle stability and also can introduce pH sensitive amphiphilicity, leading to enhanced membrane disruption at an endosomal pH of 5 to 6 and thereby enhance endosomal escape. We systematically tested the effect of various incorporated fatty acids differing in hydrocarbon chain length (from C4 to C18) and saturation. As a model oligomer topology, a T-shape structure with four Stp units and two terminal stabilizing cysteines was chosen. Two vicinal fatty acids were incorporated in the middle of the oligomer backbone by attachment to both alpha- and epsilon amino groups of a grafted lysine. Preliminary siRNA knockdown tests had shown that it was necessary to incorporate two fatty acids in close proximity in order to achieve effective gene silencing. The gene silencing assay (Figure 15A) revealed that out of the six tested fatty acids, the two unsaturated fatty acids with 18 carbon atoms, oleic acid and linolic acid, were the most effective lipid moieties. The incorporation of myristic acid also resulted in knockdown activity but also significant cytotoxicity. Similar results were obtained for i-shape structures (Figure 16) emphasizing oleic acid and linolic acid as most attractive hydrophobic oligomer modifications. The corresponding saturated C18 modification, stearylolation, showed only moderate silencing activity.

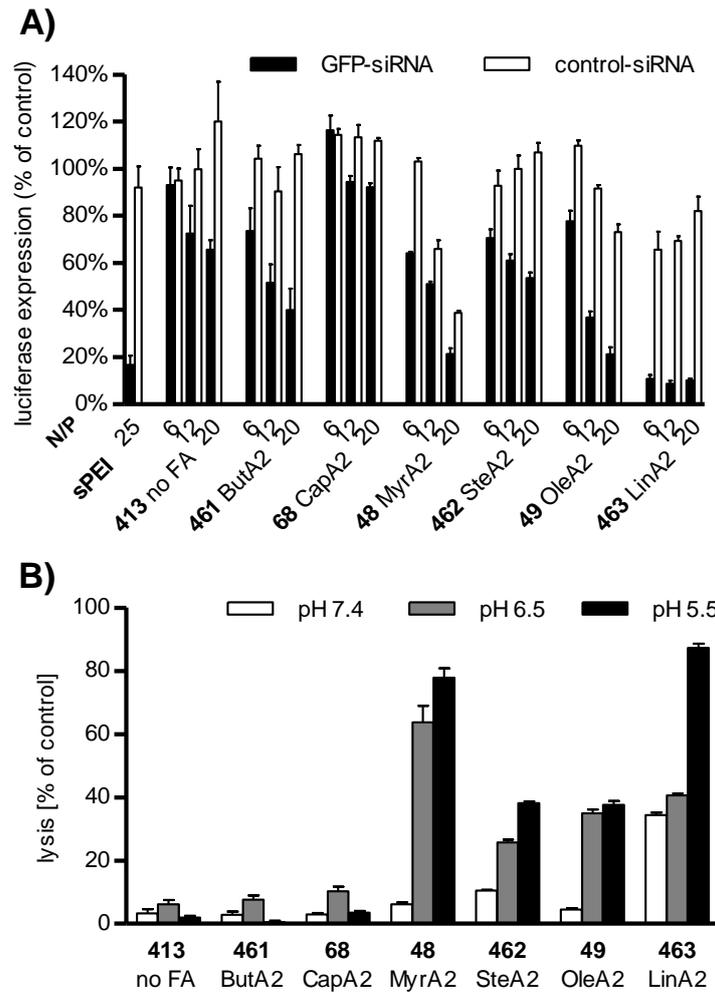


Figure 15: A) Gene silencing ability of T-shape oligomer with different lipid moieties in Neuro2A-eGFP-Luc cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA. B) Erythrocyte leakage assay of plain oligomer (2.5 μ M) at different pH values (white: pH 7.4, grey: pH 6.5, black: pH 5.5); leakage assay was performed by Christina Troiber.

The erythrocyte leakage assay (Figure 15B) demonstrates the lytic effect of oligomer on fresh mouse erythrocytes at different pH values. Structures with \geq C14 modifications (myristic, stearic, oleic or linolic acid) showed pH specific behaviour with no or moderate lytic activity at physiological pH 7.4 and high hemolysis at endosomal pH 5.5, respectively. The myristic acid modification results in a potent lysis activity already at pH 6.5 which correlates with the observed cytotoxicity of this oligomer.

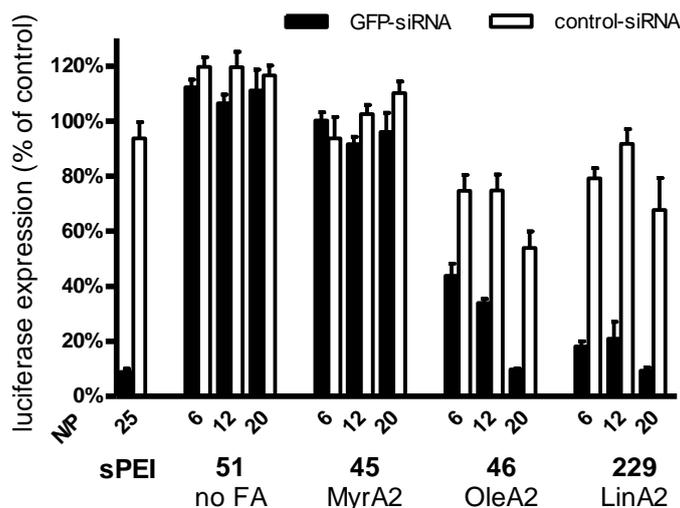


Figure 16: Influence of different lipid moieties on the gene silencing ability of an i-shape oligomer, (FatA)2K-C-Stp-Stp-Stp-C, in Neuro2A-eGFPLuc cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

3.2.4 Effect of chain length variation of the oligomers

The effect of the chain length of the oligomers on transfection efficiency is another interesting aspect which has to be considered in polymer design. In this library different shapes were tested with increasing numbers of Stp building blocks, from one to four units in each arm (Figure 17). Three-arm and T-shape structures with only one Stp unit in each arm are ineffective in siRNA delivery, whereas the very lipophilic U-shape oligomer with one Stp unit per molecule, with or without cysteines, shows activity. All three shapes enabled gene silencing when two or more Stp units per arm were assembled. Surprisingly, a further increase above two Stp units per arm did not enhance transfection efficiency but also did not increase toxicity of the oligomers.

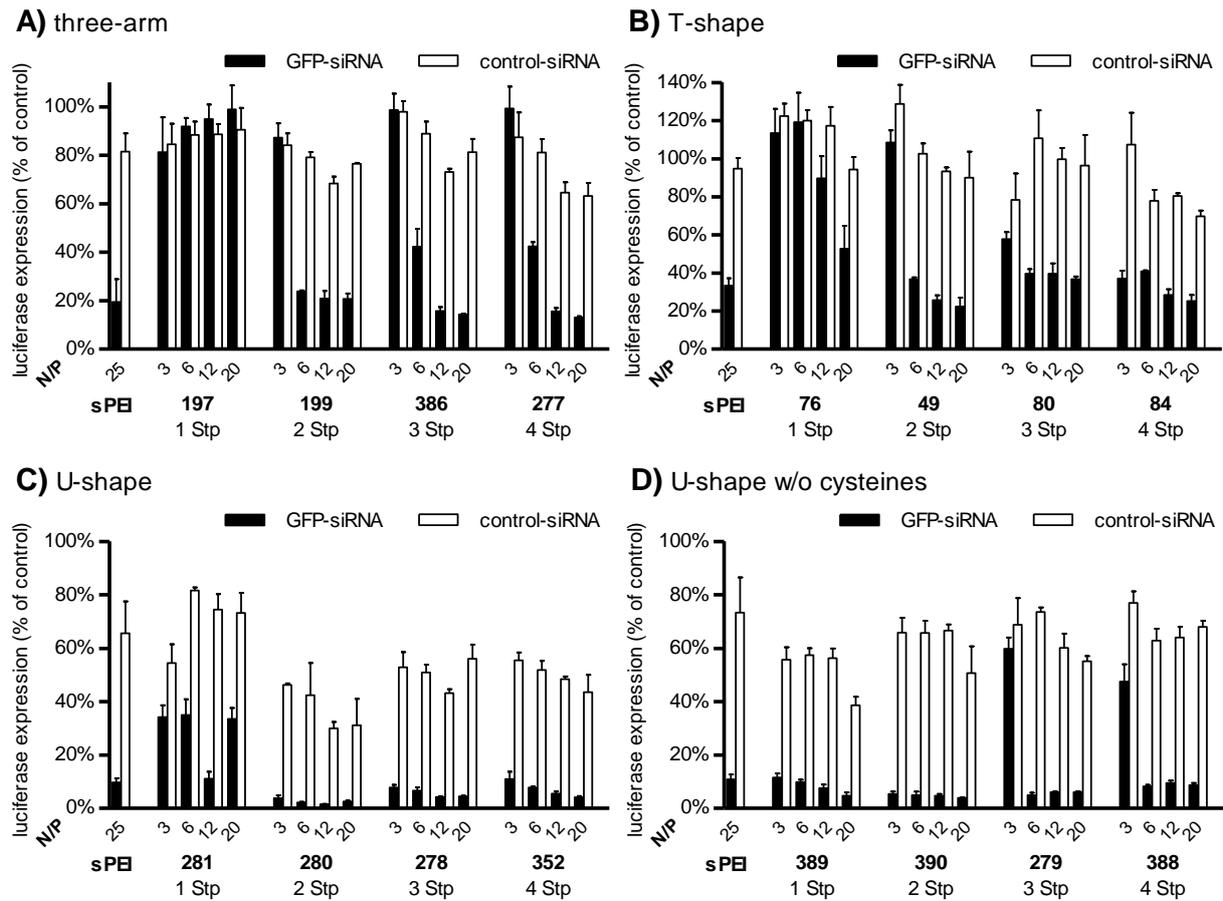


Figure 17: Gene silencing ability of oligomers with increasing chain length (1 to 4 Stp units in each arm) in Neuro2A-eGFP/Luc cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). A) Three-armed oligomers with terminal cysteines B) T-shape oligomers with two oleic acids and terminal cysteines C) U-shape oligomers with four linolic acids and terminal cysteines D) U-shape oligomers with four linolic acids without terminal cysteines. Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

3.2.5 Disulfide stabilization of polyplexes

Due to the far fewer negative charges of siRNA compared to pDNA, electrostatic interaction between nucleic acid and the positively charged polymer in the polyplexes does not provide sufficient stability of the particles. Apart from additional hydrophobic modification of polymers, which also improves polyplex stability, the introduction of cysteines for disulfide crosslinking is a versatile way to increase particle stability. Especially for *in vivo* applications, particle stability might be essential for successful delivery. As an example, the disulfide formation during polyplex incubation time of the

three-armed oligomer **386** with a terminal cysteine in each arm was monitored (Figure 18).

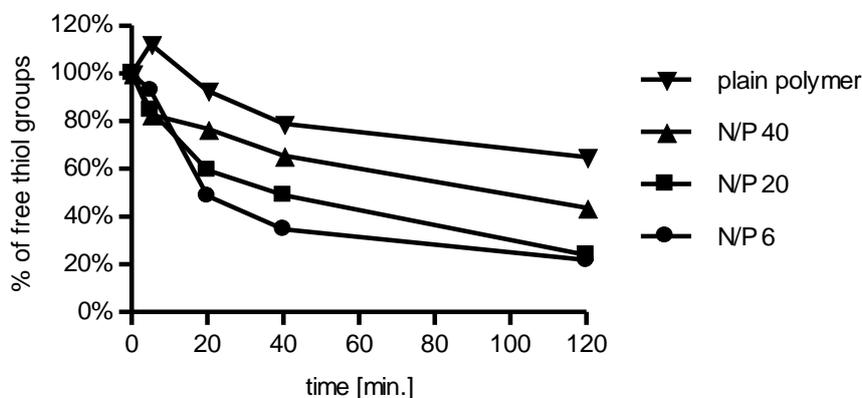


Figure 18: Thiol oxidation kinetics of three-armed oligomer **386**, (C-Stp-Stp-Stp)₂K-Stp-Stp-Stp-C, during polyplex formation. The oligomer was incubated without siRNA (at a concentration corresponding to N/P 20/1) or with siRNA at different N/P ratios (siRNA concentrations as in cell culture: 370 nM).

After 40 minutes of polyplex formation time approximately 60% of all free thiols were oxidized at an N/P ratio of 6. The oxidation rate decreased with rising N/P ratios, reaching the lowest rate for plain oligomer. Presumably the presence of siRNA accelerates disulfide formation because the nucleic acid acts as a template, bringing the positively charged oligomer molecules together into close distance [135].

The necessity of cysteines in three-arms was demonstrated by comparison with their mutated alanine analogs (Figure 19); the exchange of cysteines by alanines prevents stabilization of the polyplexes by disulfide crosslinking and results in loss of gene silencing (Figure 19A). The gel shift assay reveals that in contrast to **386**, the alanine mutant **387** is not able to bind siRNA even at high N/P ratios (Figure 19B), making cellular delivery of nucleic acid impossible.

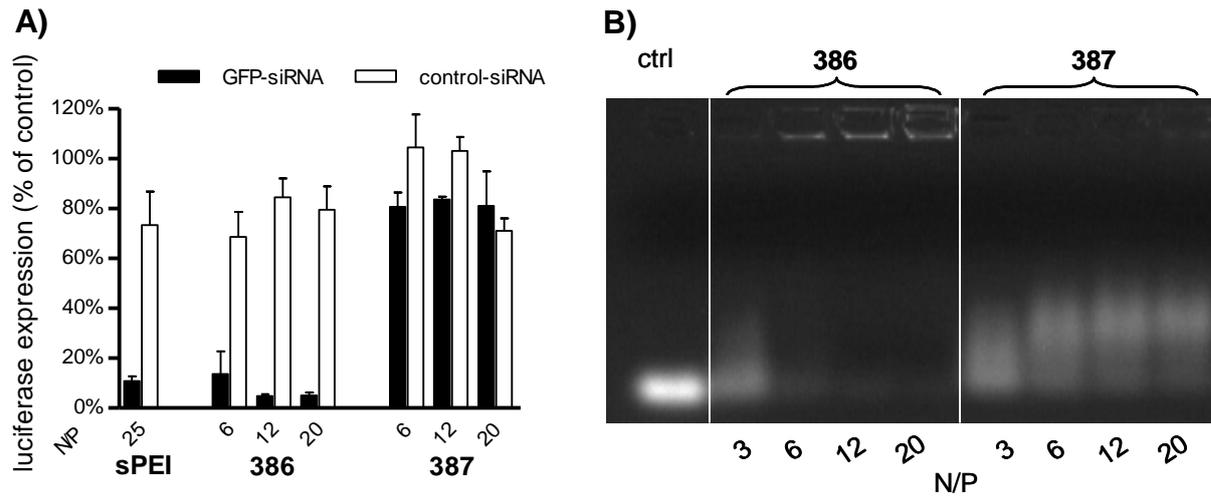


Figure 19: A) Gene silencing efficiency of three-armed structure **386** and its alanine analog **387** in Neuro2A-eGFPLuc cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA. B) Agarose gel shift at different N/P ratios. Ctrl: free siRNA.

Similar requirements are demonstrated for the i-shape topology (Figure 20A); the cysteine containing i-shape oligomer **230**, C-Gtp-Gtt-Stp-C-K-LinA₂, showed efficient gene silencing, whereas the alanine analog **379** lost activity.

Differently from the three-armed structures, the fatty acid containing i-shape oligomer is able to bind siRNA independent of cysteines (Figure 20B). An explanation for the the absence of gene silencing activity of the alanine mutant is given by Figure 20C and D: the cellular uptake study shows that far less labeled siRNA reaches the cytosol when complexed with the cysteine free version of the oligomer than with polymer **230**. Size measurements indicate that only the i-shape with cysteines leads to the formation of nanoparticles with homogenous size distribution of around 100 nm. The polyplex size of alanine analog polyplexes cannot be determined by Dynamic Light Scattering due to inhomogeneous and incomplete particle formation.

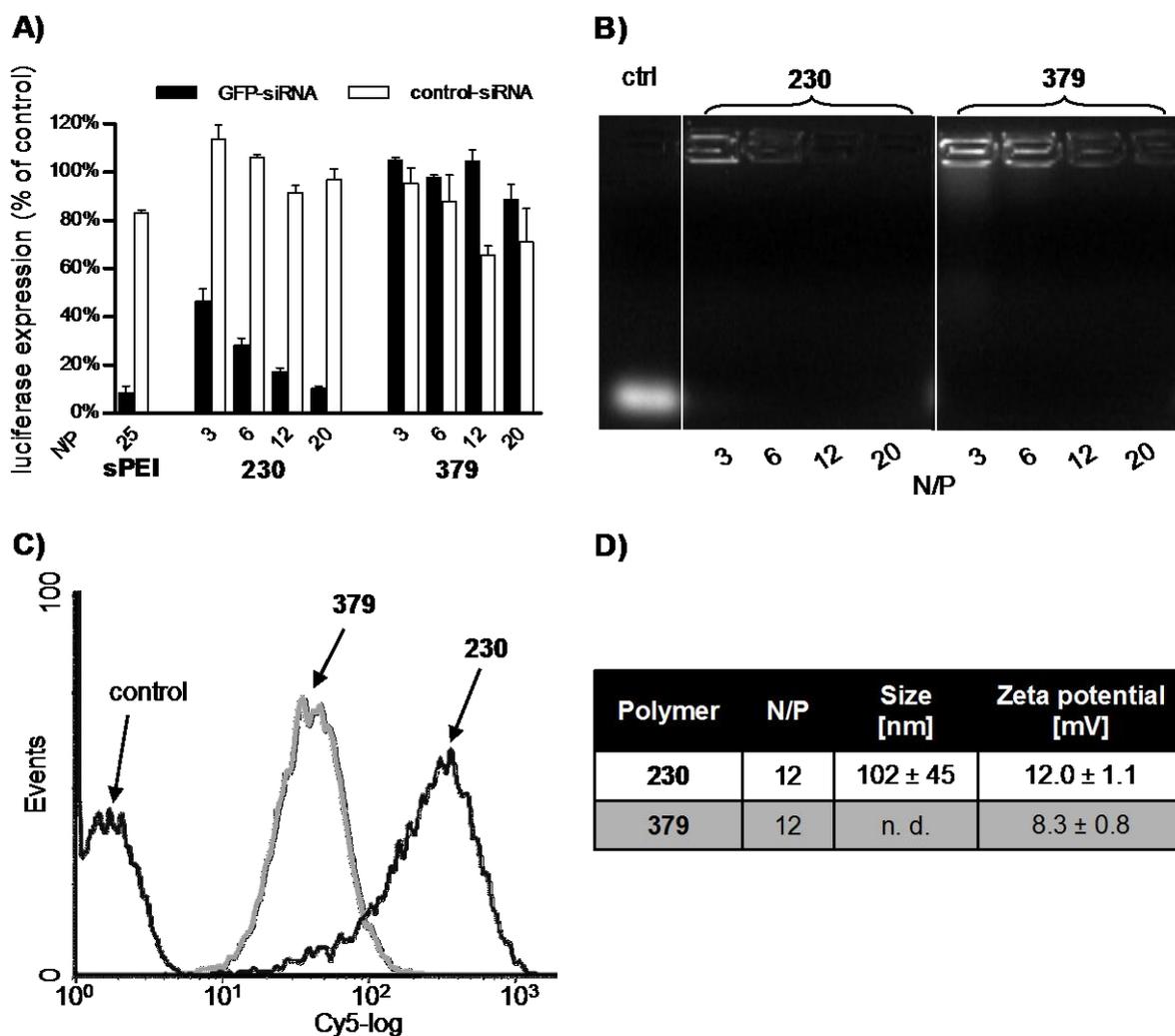


Figure 20: A) Gene silencing efficiency of i-shape structure **230** and its alanine analog **379** in Neuro2A-eGFP_{Luc} cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA. B) Agarose gel shift at different N/P ratios. Ctrl: free siRNA. C) Cellular internalization study using flow cytometry. Cellular uptake of Cy5 labeled siRNA, complexed with i-shape **230** and its alanine analog **379**, was determined via flow cytometry analysis 4 hours after transfection. D) Size and Zeta potential of siRNA polyplexes with i-shape oligomer **230** and its alanine analog **379**, respectively, at an N/P ratio of 12; n.d. = not detectable.

Another way of testing the necessity of cysteines is the chemical blockade of the thiols (Figure 21A). For this reason T-shape **49** was reacted with N-ethylmaleimide (NEM), irreversibly blocking the thiol groups by thioether formation. The NEM-blocked version of the oligomer completely lost gene silencing efficiency (Figure 21B).

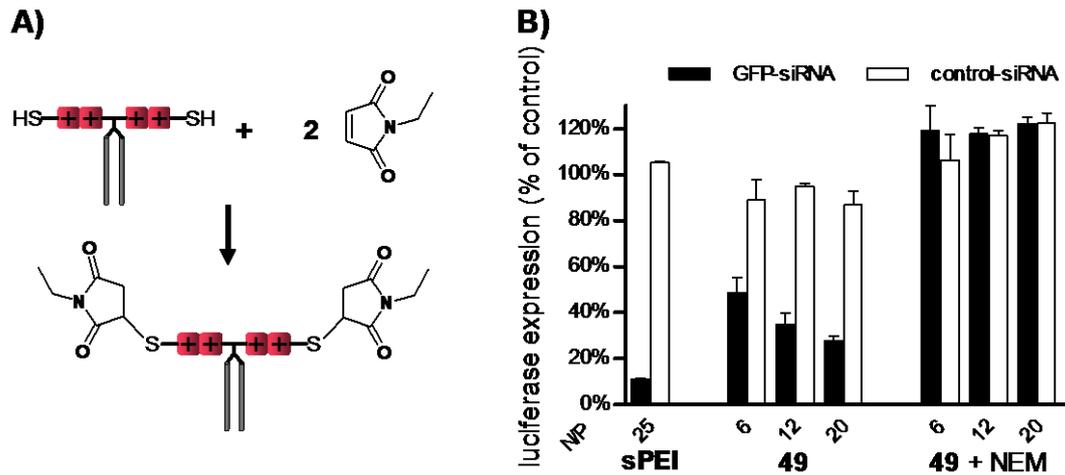


Figure 21: A) Reaction scheme NEM blockade of T-shape **49**, C-Stp-Stp-K(K-OleA2)Stp-Stp-C. B) Gene silencing ability of T-shape oligomer **49** w/o or with NEM blockade in Neuro2A-eGFPLuc cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

In contrast, for U-shape oligomers gene silencing efficiency does not depend on disulfide formation in the polyplexes (Figure 22A). This can be explained by the strongly lipophilic character of these oligomers, which carry four fatty acids per molecule.

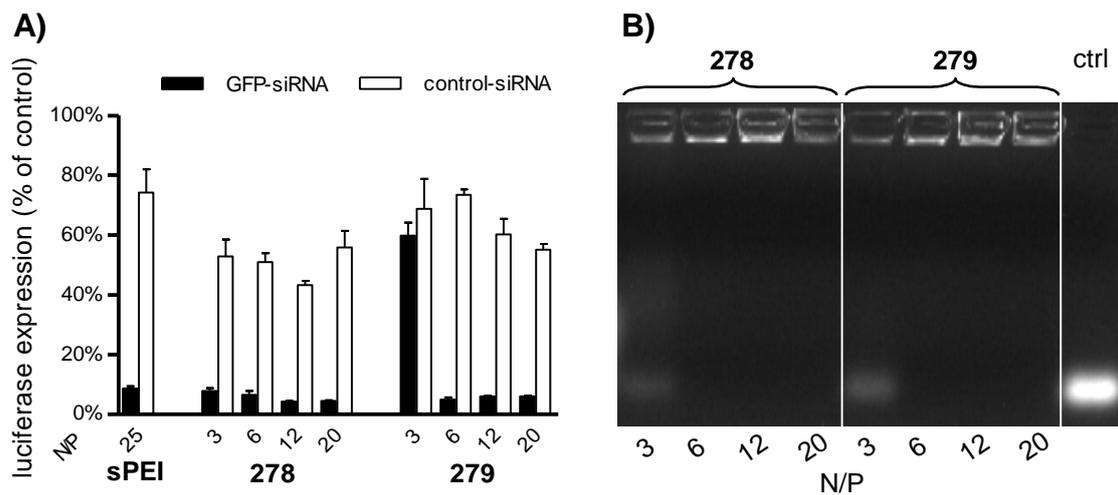


Figure 22: A) Gene silencing efficiency of U-shape **278** and its alanine analog **279** in Neuro2A-eGFPLuc cells with eGFP-targeted siRNA or control siRNA (at different N/P ratios). Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA. B) Agarose gel shift at different N/P ratios. Ctrl: free siRNA.

These hydrophobic modifications enable sufficient particle stabilization for siRNA delivery. The gel shift experiment (Figure 22B) illustrates the very efficient nucleic acid binding of both versions of the oligomer and Table 4 shows the formation of nanoparticles in both cases, both essential requirements for successful siRNA transfection.

Table 4: Size and Zeta potential of siRNA polyplexes with U-shape oligomer **278** and its alanine analog **279**, respectively, at an N/P ratio of 12.

Polymer	N/P	Size [nm]	Zeta potential [mV]
278	12	248 ± 26	25.2 ± 1.2
279	12	128 ± 1	17.1 ± 0.3

3.2.6 Impact of the various oligomer topologies

Molecular topology of the oligomers seems to play a minor role in delivery efficiency compared to the effect of incorporating different building blocks, lipid moieties or cysteines.

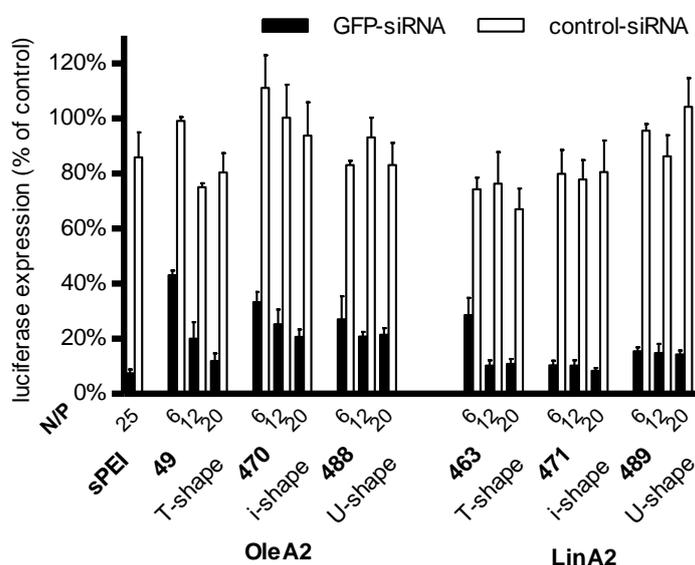


Figure 23: Comparison of gene silencing ability of T-shape, i-shape and U-shape oligomers with the same building blocks and hydrophilic/lipophilic structure in Neuro2A-eGFPLuc cells with eGFP-targeted siRNA or control siRNA 200 nM, at different N/P ratios. T-shapes: C-Stp-Stp-(FatA)2K]K-Stp-Stp-C, i-shapes: (FatA)2K-C-Stp-Stp-Stp-Stp-C, U-shapes: C-(FatA)2K]K-Stp-Stp-Stp-Stp-K-Stp-Stp-

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Stp-Stp-(FatA)2K]K-C. Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

These findings were obtained by comparing the transfection efficiency of i-shape and T-shape oligomers with four Stp units and identical lipid moieties with U-shape oligomers with eight Stp units and the same lipid moieties, where no significant difference in transfection efficiency was detectable (Figure 23 and 24). Eight Stp units in the U-shape oligomers were necessary for this comparison in order to keep the hydrophilic/lipophilic balance the same as with the i- and T-shapes (two fatty acids per oligomer), as the U-shapes contain four fatty acids per oligomer.

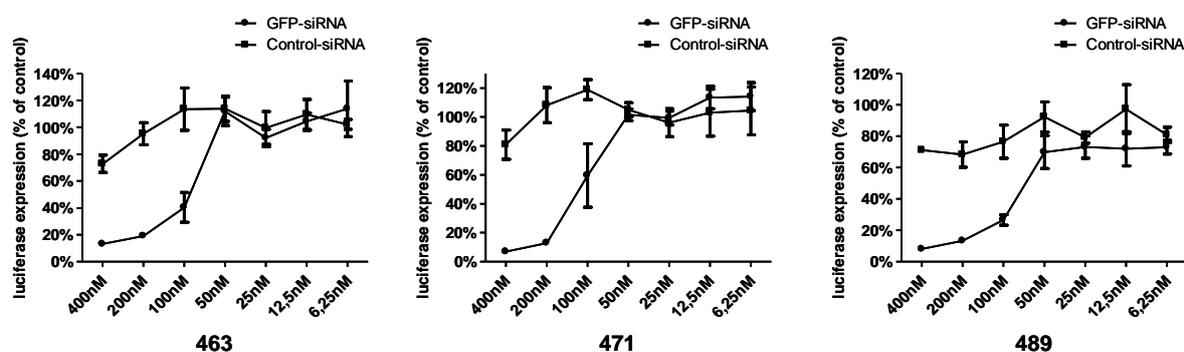


Figure 24: Gene silencing ability of T-shape **463**, C-Stp2-K-(K-LinA2)-Stp2-C, i-shape **471**, C-Stp4-C-K-LinA2 and U-shape **489**, C-(LinA)2K]K-Stp-Stp-Stp-Stp-K-Stp-Stp-Stp-Stp-(LinA)2K]K-C, in Neuro2A-eGFPLuc cells with eGFP-targeted siRNA or control siRNA at decreasing siRNA concentrations.

3.2.7 Cellular uptake of fluorescently labeled polyplexes

The actual delivery and uptake of polyplexes into cells can be proven by fluorescently labeling of oligomer, siRNA or both. In order to prove delivery capability of the novel structures, i-shape **229**, C-Stp₃-C-K-LinA₂, which had already shown efficient reporter gene silencing in Neuro2A-eGFPLuc cells, was labeled with the fluorescent marker carboxyfluorescein and formulated with siRNA that was labeled with Cy5 dye. Microscopic pictures at according wavelengths of the fluorescent markers were taken 24 hours after transfection (Figure 25).

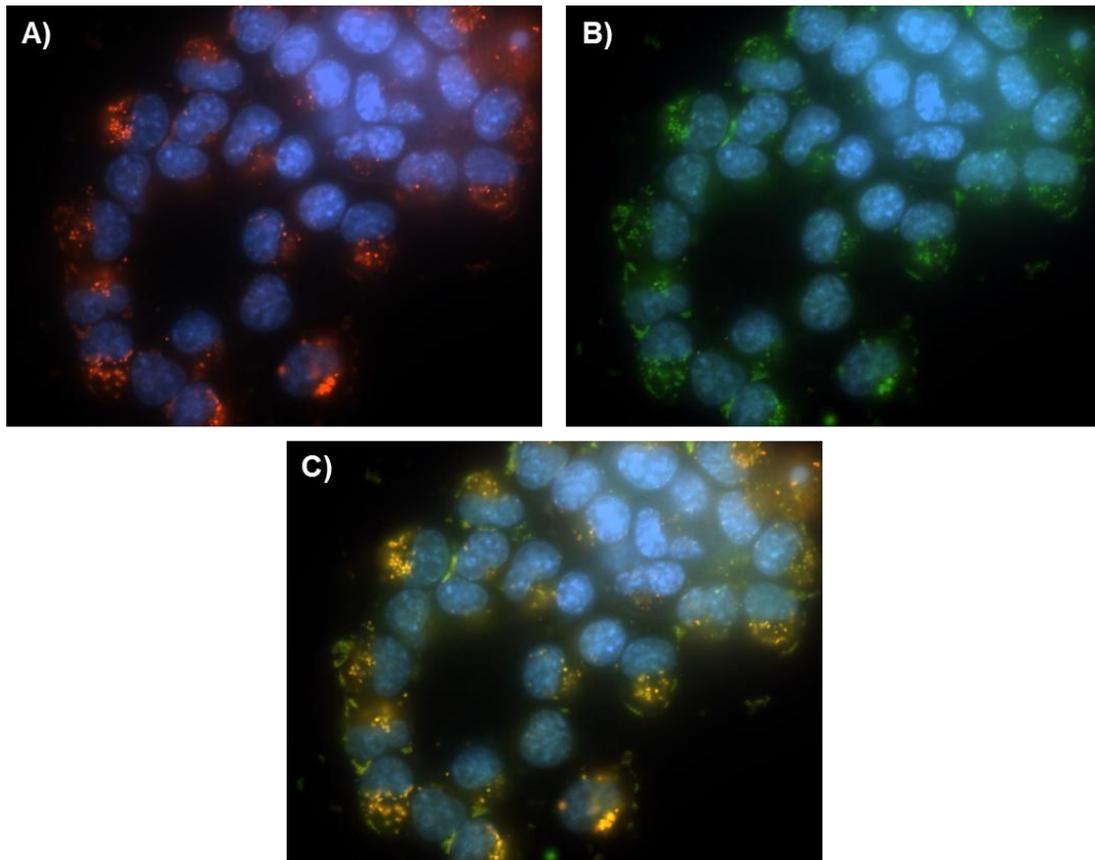


Figure 25: Delivery of fluorescently labeled polyplexes of **229** (N/P 12) in Neuro2A cells 24 hours after transfection. Blue: cell nuclei stained with Hoechst 33342. A) Red: Cy5-labeled siRNA. B) Green: Carboxyfluorescein-labeled **229**. C) Overlay picture of siRNA and oligomer.

Figure 25A and B clearly show that both siRNA and oligomer have accumulated in the cytosol of Neuro2A cells. The overlay picture of both channels shows that most of the siRNA is still sticking to oligomer. The most likely reason for this is that only the spots with concentrated siRNA and oligomer are visible in the presented microscopic picture, in this case probably endo-/lysosomes, and free siRNA in the cytosol is not detectable in this setting because it is too diluted. The results of the previously mentioned luciferase reporter gene assay show that siRNA was set free into the cytosol, leading to specific gene silencing.

3.2.8 Delivery efficacy with decreasing siRNA dosages

So far, the screening of the oligomer library was performed with 500 ng siRNA / well (370 nM). Three promising oligomers were also tested with decreasing siRNA concentrations (Figure 26).

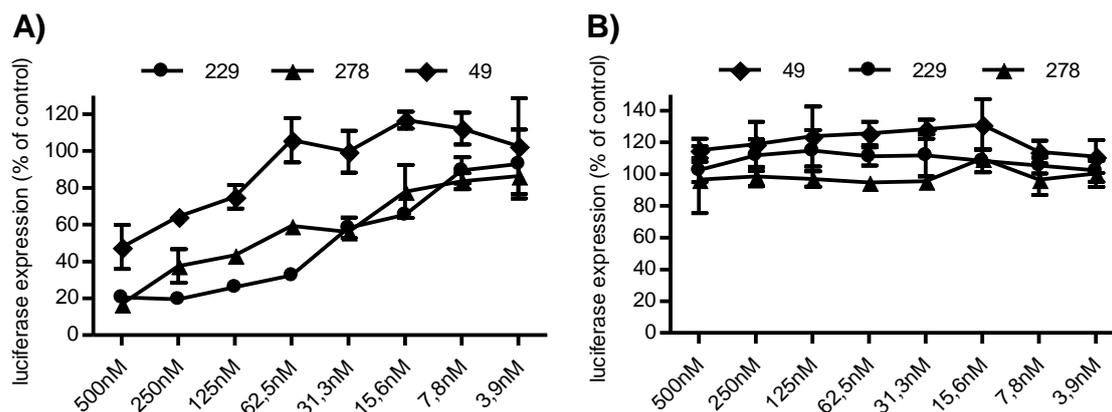


Figure 26: Dose-dependent gene silencing ability of selected transfected oligomers at constant oligomer concentrations. A) Gene silencing ability of i-shape **229**, C-Stp₃-C-K-LinA₂ (3.5μg/well), U-shape **278**, C-K-(K-(LinA)₂)-Stp₃-K-(K-(LinA)₂)-C (4.7μg/well), and T-shape **49**, C-Stp₂-K-(K-OleA₂)-Stp₂-C (3.1μg/well), in Neuro2A-eGFP_{Luc} cells with eGFP-targeted siRNA at decreasing siRNA concentrations. B) Analogous experiment using control siRNA.

For example, i-shape oligomer **229** and U-shape oligomer **278** achieved a knockdown of approximately 50% with an siRNA concentration of 30 nM without unspecific effects on the cells.

3.2.9 Gene silencing efficiency on different cell lines

All synthesized oligomers were originally screened on the murine neuroblastoma cell line N2A, stably transfected with the eGFP_{Luc} reporter gene, for their gene silencing efficiency. In figure 27, the efficiency of the oligomers was examined on a broader scale. One oligomer of each topology class, which had successfully delivered siRNA in N2A cells, was tested on a variety of other cell lines (human prostate carcinoma DU-145, human hepatoma HUH-7, human hepatoma HepG2 and human cervix carcinoma KB cells).

Especially the lipopolymers i-shape **229** and U-shape **278** showed high transfection efficiency on a broad range of cell lines, occasionally with some unspecific gene silencing at high N/P ratios. Treatment of HUH-7 hepatoma cells with these oligomers resulted in activation of cell metabolism, indicated by the induction of luciferase expression in the control-siRNA group far above that of buffer treated control cells. Despite this induction, a clear difference in reporter gene expression

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between GFP-siRNA treated and control-siRNA treated cells could be observed, showing successful siRNA delivery.

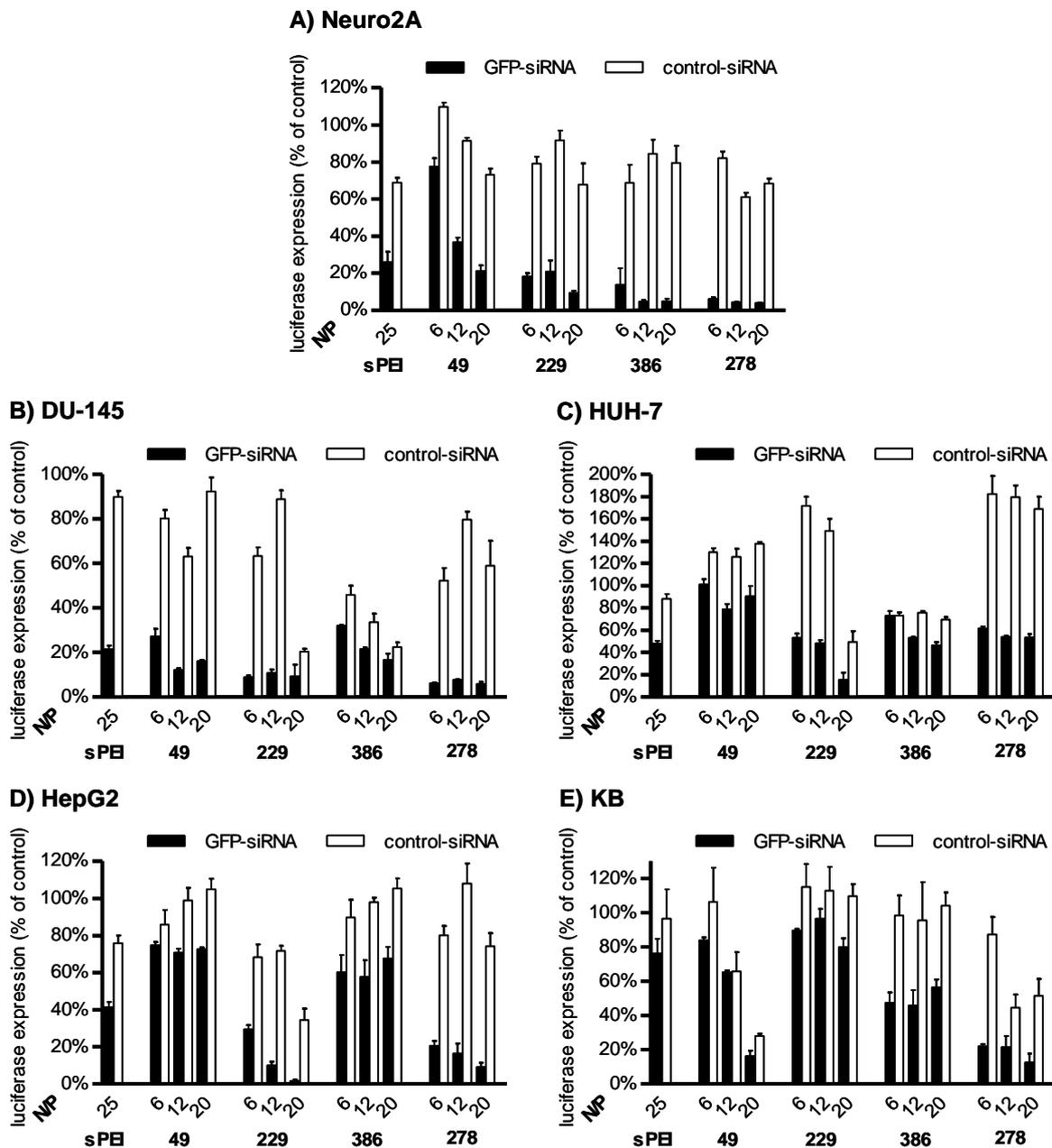


Figure 27: Gene silencing ability of selected oligomers of the four major molecular shapes in various cell lines, all stably transfected with the eGFPLuc fusion protein. A) Neuro2A murine neuroblastoma cells. B) DU-145 human prostate carcinoma cells. C) HUH-7 human hepatoma cells. D) HepG2 human hepatoma cells. E) KB human cervix carcinoma cells. Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA. Oligomer sequences: **49** C-Stp-Stp-K(K-OleA2)Stp-Stp-C, **229** (LinA)2K-C-Stp-Stp-Stp-C, **386** (C-Stp-Stp-Stp)2]K-Stp-Stp-Stp-C, **278** C-(LinA)2K]K-Stp-Stp-Stp-(LinA)2K]K-C.

T-shape **49** was especially successful in prostate carcinoma DU-145 cells, whereas the three-armed **386** only achieved moderate specific gene silencing in other cell lines apart from N2A. These findings show that from the synthesized catalog of oligomers, there is an appropriate delivery agent available for each tested cell type, but there is no universal carrier suitable for all cell lines. In every individual case, the most effective structure has to be detected experimentally.

3.2.10 Cytotoxicity of plain oligomer

Selected oligomers, which were effective in siRNA delivery, were tested for their cytotoxic impact on Neuro2A cells. Therefore, the cells were treated with serial dilutions of i-shape **230**, three-arm **386** or U-shape **278**. The effect on cell viability was compared to that of buffer treated control cells. The well characterized polymer linear PEI was chosen for comparison of the new carriers with established structures (Figure 28).

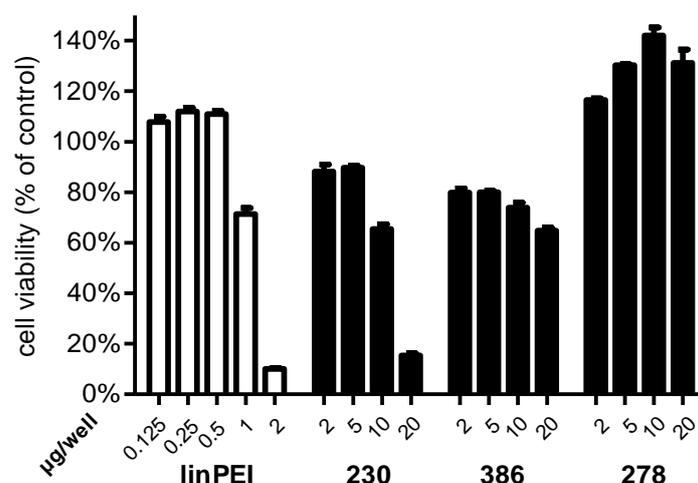


Figure 28: Toxicity of oligomers on Neuro2A-eGFPLuc cells 48 hours after oligomer addition in comparison to linear polyethylenimine (linPEI, 22 kDa).

Results show that linPEI leads to cell death at concentrations of 2 µg/well, whereas i-shape **230** has the same toxic effect only at a tenfold higher dose. The three-arm **386** only shows very moderate toxicity even at the highest tested dose of 20 µg oligomer / well and U-shape **278** does not show any toxic effect at all tested concentrations.

Taken together the results demonstrate a dramatic decrease of cytotoxicity compared to the widely used standard polymer linear PEI.

3.2.11 Polyplex serum stability

Stability of polyplexes in the blood stream is essential for successful systemic delivery of siRNA *in vivo*. Interactions with blood components, especially electrolytes or proteins like the negatively charged albumine, can lead to dissociation or aggregation of administered polyplexes. In order to determine the stability, an agarose gel shift assay was performed with the polyplexes of selected oligomers. Prior to the loading of the gel, the formed polyplexes were incubated with increasing concentrations of fetal bovine serum, thereby imitating *in vivo* conditions (Figure 29).

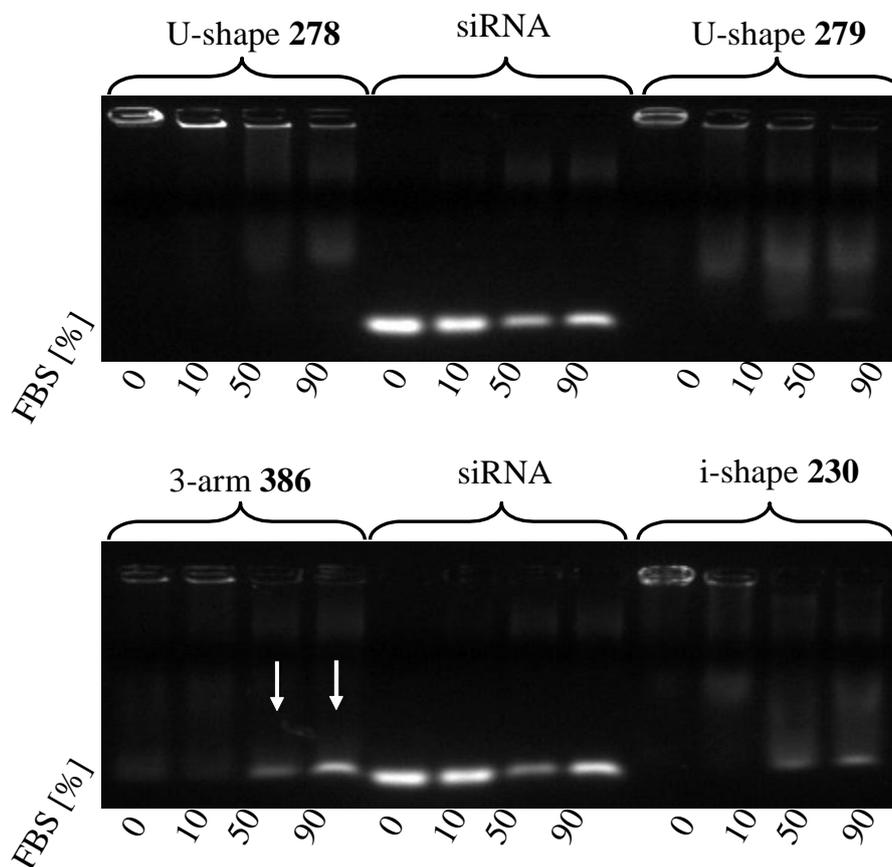


Figure 29: siRNA polyplex stability in serum determined by agarose gel shift assay at N/P 12 and indicated FBS serum concentrations. Serum was added to the polyplexes after particle formation and the samples were incubated for 15 minutes at ambient temperature. As in all experiments, chemically stabilized siRNA was used.

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The polyplexes of lipophilic U-shape oligomers **278** and **279** in particular showed sustained stability under these conditions. Some moderate siRNA release could be observed for all oligomers at the highest possible FBS concentration in this assay (90%), especially for three-armed oligomer **386**.

3.3 Functionalized and targeted siRNA delivery systems

3.3.1 Folate-PEG-siRNA conjugate for receptor mediated siRNA uptake

3.3.1.1 Design of folate receptor targeted siRNA

Receptor targeted delivery of polyplexes by incorporation of targeting ligands into the carrier system enables specific uptake and accumulation of the nanoparticles in the desired tissue. In this case, the targeting ligand folic acid was not linked to a carrier but directly to the payload, the siRNA (Scheme 4).

Scheme 4: Folate-PEG-siRNA conjugate. The targeting ligand is linked to the siRNA via PEG spacer (24 ethylene glycol units) and a reducible disulfide bond.



Incorporation of the longest available defined PEG spacer between nucleic acid and targeting ligand, consisting of 24 ethylene glycol units, should ensure the presentation of the targeting ligand on the surface of the resulting polyplexes and at the same time reduce the surface charge of the particles for reduced unspecific interactions with blood components or normal tissue in case of systemic application. The PEGylated targeting ligand was synthesized by solid phase peptide synthesis and the siRNA conjugate was purified to maintain a high degree of purity (synthesis by C. Dohmen, see [136]). This way, reliable conclusions about structure-activity relationships can be drawn from the combination of the siRNA conjugate with the monodisperse carriers like the three-armed oligomer **386**.

3.3.1.2 Determination of folate receptors on KB cells

For clear examination of the folate-PEG-siRNA conjugate, a cell line is required which expresses the targeted receptor, in this case the human folic acid receptor (FOLR-1). The KB cell line, established by HeLa cell contamination, has been reported to highly express this specific receptor [137, 138]. For confirmation of the

receptor status, cells were examined *via* antibody staining with anti-human FOLR-1 antibody (Figure 30).

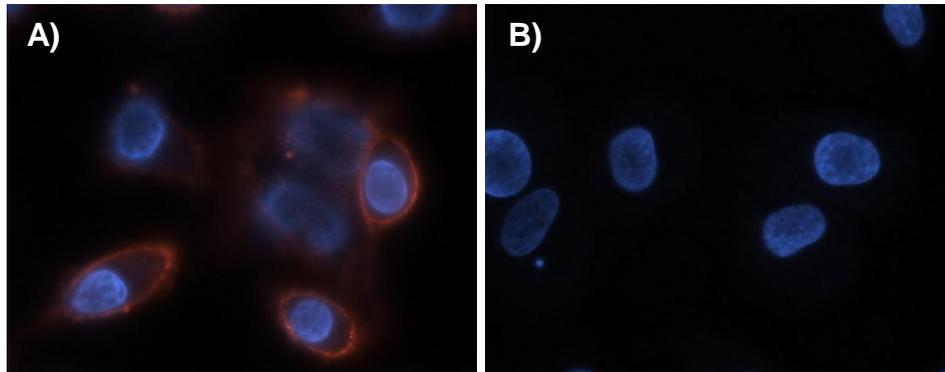


Figure 30: Folate receptor staining with human FOLR-1 antibody conjugated to Allophycocyanin (red). A) Human cervix carcinoma KB cells. B) Human hepatoma HUH-7 cells as control. Blue: cell nuclei stained with Hoechst 33342 dye.

The microscopic images after antibody staining show that the KB cells express FOLR-1 on the cell surface, whereas no signal is observable on the surface of the control cell line HUH-7. Thus, the purchased KB cell line is suitable for investigation of folic acid receptor targeted siRNA delivery.

3.3.1.3 Uptake of plain FA-PEG-siRNA into folate receptor expressing cells

After confirmation of the high folic acid receptor expression on the surface of KB cells, the folate-PEG-siRNA conjugate (FA-PEG-siRNA) was applied to the cells and receptor mediated uptake of the conjugate was monitored. After 16 hours of incubation, the microscopic images showed that an immense amount of fluorescently labeled conjugate had been taken up into KB cells, but addition of lysotracker green revealed that all of the siRNA was trapped in endo-/lysosomes, where it is degraded and cannot take action by incorporation into the cytosolic silencing complex of the RNAi machinery (Figure 31A and B). Control experiments proved that the uptake took place in a receptor mediated fashion, as internalization in KB cells could be completely blocked by preincubation with an excess of free folate or treatment with untargeted PEG-siRNA (Figure 31C and D).

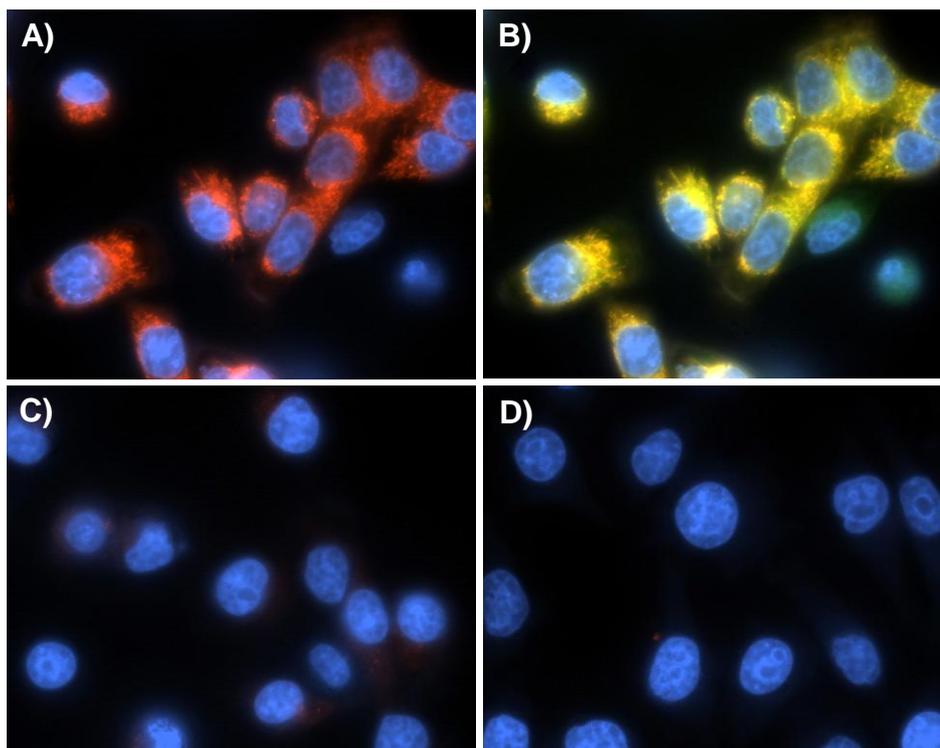


Figure 31: Cellular uptake of FA-PEG-siRNA in human cervix carcinoma KB cells (siRNA concentration: 370 nM). A) FA-PEG-siRNA treated KB cells. B) Overlay of A) with additional channel of lysotracker[®] green for staining of endo-/lysosomes (overlay of red and green channel causes yellow color). C) FA-PEG-siRNA treated KB cells after preincubation with an excess of folate. D) PEG-siRNA treated KB cells. Blue: Hoechst 33342, red: Cy5 labeled siRNA, green: lysotracker[®] green.

These findings were underlined by flow cytometry experiments (Figure 32). Again, a strong uptake into KB cells was detected for the FA-PEG-siRNA conjugate, whereas competition with an excess of free folic acid or treatment with untargeted PEG-siRNA only showed a minor shift in fluorescence intensity compared to buffer treated control. Additionally, a control experiment with Neuro2A cells with low folic acid receptor expression was performed, in which only marginal uptake of the FA-PEG-siRNA conjugate was detectable, supporting the receptor specificity of internalization. The internalization studies showed that the ligation of folic acid to siRNA *via* PEG spacer led to efficient receptor mediated uptake of the conjugate into folic acid receptor expressing cells. However, the entrapment in endosomes and the small size of these molecules, which would cause rapid clearance from blood circulation *in vivo*, make formulation with a carrier system, e.g. polymers with endosomal escape functionalities, indispensable for successful cytosolic delivery of the nucleic acid and effective gene silencing.

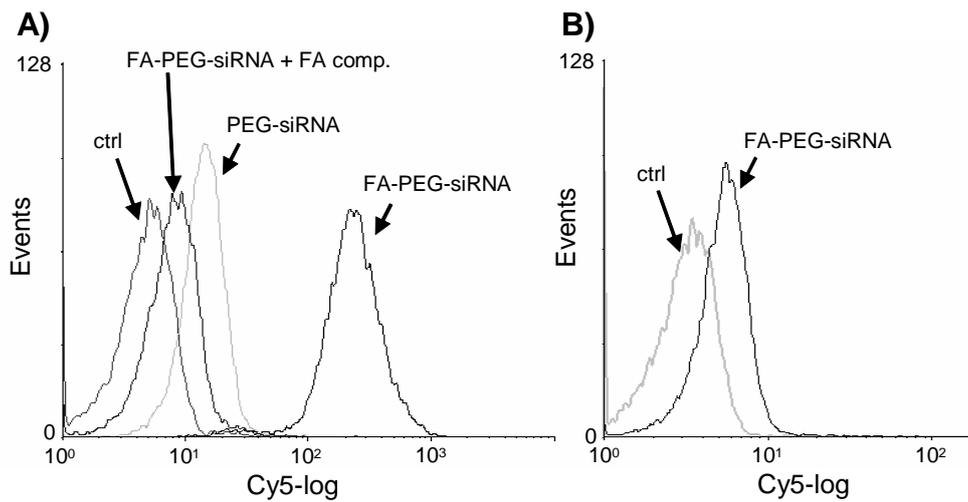


Figure 32: Cellular internalization study using flow cytometry. Cellular uptake of Cy5 labeled FA-PEG-siRNA was determined via flow cytometry analysis (“+ FA comp.” = preincubation of cells with an excess of folate). A) KB cells. B) Folic acid receptor negative Neuro2A cells.

3.3.1.4 Proof of functionality of folate receptor targeted siRNA in a non-targeted setting

Functionality of siRNA is often affected by modification of the double strand due to insufficient cleavage of the double helix before integration into the silencing complex or imperfect incorporation of the antisense strand into the RISC.

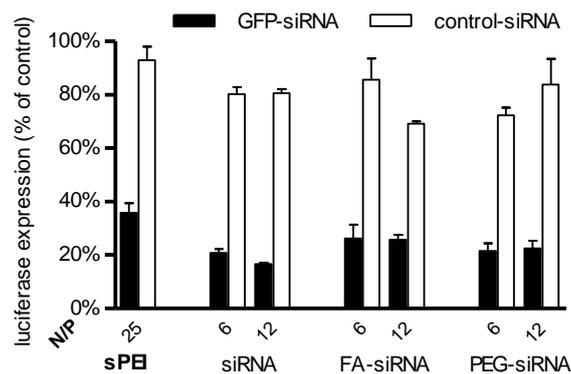


Figure 33: Gene silencing ability of modified (FA-PEG-siRNA and PEG-siRNA) versus unmodified siRNA complexed with oligomer **230**, C-Gtp-Gtt-Stp-C-K-LinA2, in Neuro2A-eGFPLuc cells (N/P 12 and 20). No medium change was performed after transfection with an incubation time of 48 hours. Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

Although modification with PEG and targeting ligand took place at the 5' end of the sense strand, functionality of the modified siRNA was confirmed by comparing the silencing efficiency of the FA-PEG-siRNA conjugate to PEG-siRNA and unmodified siRNA in a non-targeted reporter gene assay. For this purpose, the three different siRNA batches were individually complexed with the lipophilic oligomer **230** and Neuro2A-eGFPLuc cells were transfected with the resulting polyplexes for 48 hours (Figure 33). Both PEG-siRNA and FA-PEG-siRNA showed equal silencing ability to unmodified siRNA, affirming that the strand modification had no impact on the functionality of the siRNA inside the cell.

3.3.1.5 Formulation of folate receptor targeted siRNA with three-armed oligomer 386

The lipophilic oligomer **230** successfully delivered the targeted siRNA conjugate into the cytosol of cells in a non-targeted transfection setting. For folate receptor targeted delivery this oligomer proved inadequate because of its lipophilic tails, leading to micellar structures of the resulting polyplexes and thus masking the targeting ligand of the siRNA conjugate. In consequence, the masking of the targeting ligand led to undesired unspecific cellular uptake (data not shown).

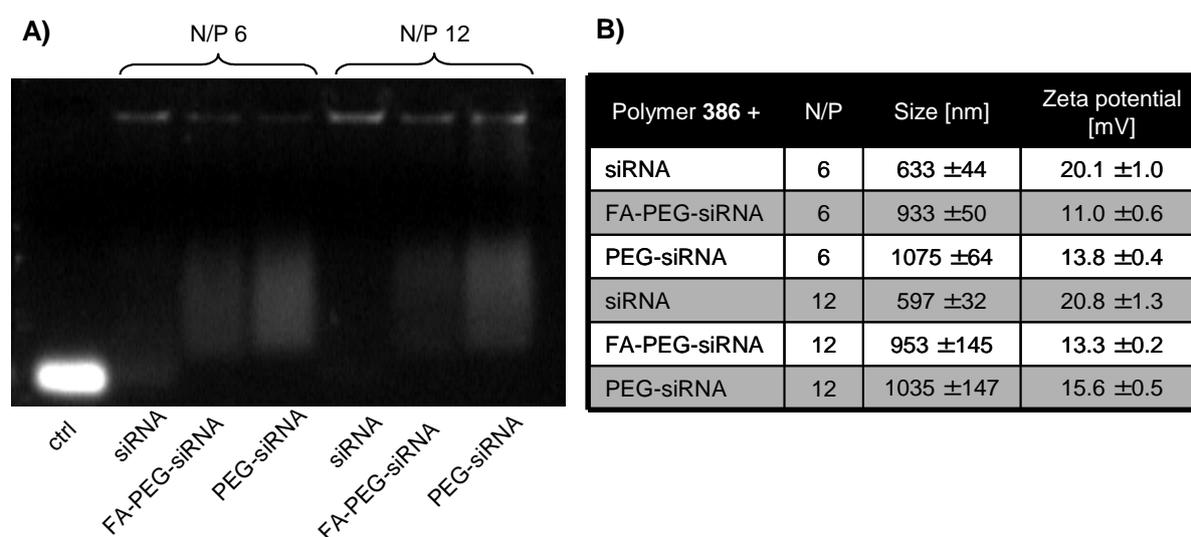


Figure 34: A) Gel shift assay of modified and unmodified siRNA, complexed with three-armed polymer **386** (N/P 6 and 12). Ctrl: free siRNA. B) Size and Zeta potential of the corresponding polyplexes.

Three-armed **386**, another carrier from the previously mentioned oligomer library, possesses more favourable characteristics. The strongly hydrophilic structure, free of lipid moieties, bears a terminal cysteine in each arm for crosslinking during polyplex formation and gene silencing ability of this oligomer has already been shown in Neuro2A-eGFPLuc cells in a non-targeted fashion (Figure 19A). Thus, FA-PEG-siRNA was complexed with **386** and siRNA binding ability, particle size as well as Zeta potential was determined for the formed polyplexes.

The gel electrophoresis assay showed stable particle formation for the oligomer with the targeted siRNA conjugate (Figure 34A). Particle size of the polyplexes with PEGylated siRNA increased, but the decrease of the Zeta potential for the PEGylated particles in comparison to polyplexes with unmodified siRNA confirmed that the PEG moiety with the targeting ligand attached is present on the surface of the polyplexes (Figure 34B).

Receptor mediated uptake of the targeted polyplexes was determined *via* flow cytometry (Figure 35).

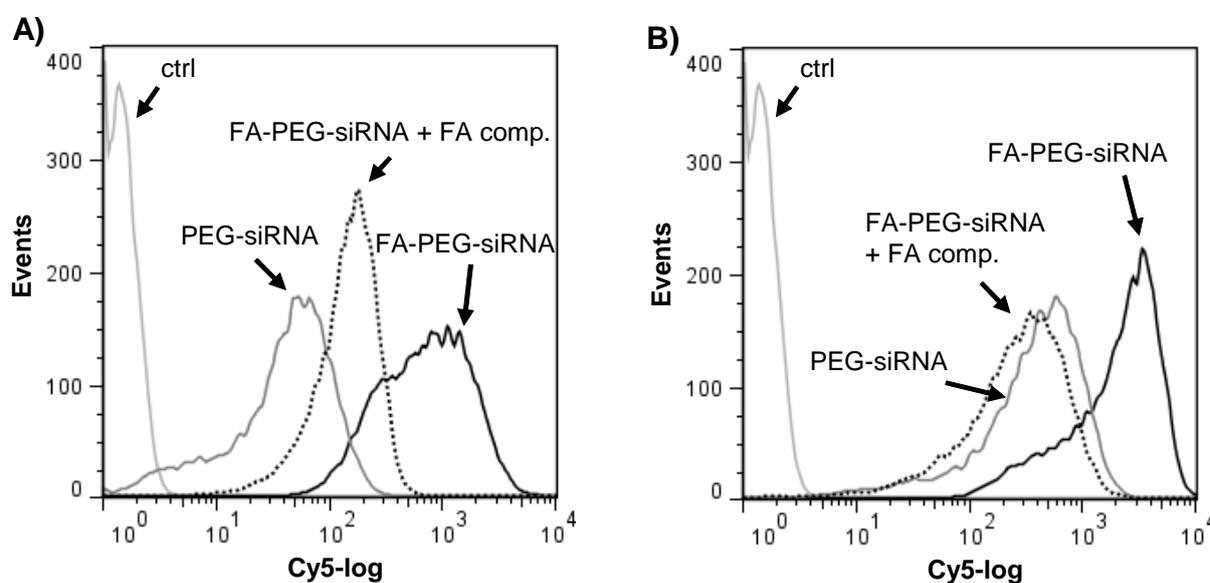


Figure 35: Flow cytometric analysis of cellular uptake of polyplexes formed with Cy5 labeled siRNA conjugates and oligomer **386**. Uptake of FA-PEG-siRNA polyplexes was tested with or w/o preincubation of cells with an excess of folate. A) N/P 6. B) N/P 12.

A more than tenfold increased cellular uptake was detected for polyplexes containing the FA-PEG-siRNA conjugate compared to particles with PEG-siRNA. In addition, the

reduced cellular internalization of folic acid receptor targeted polyplexes in the presence of excess folic acid confirmed the receptor specific endocytosis pathway. Some unspecific internalization in this assay, shown by an uptake of untargeted PEG-siRNA polyplexes, can be explained by the incomplete shielding of the polyplexes by PEGylation of the siRNA. This was indicated by a reduced but still positive Zeta potential of the PEGylated particles. Hence, ionic interactions between the positively charged polyplex with the negatively charged cell surface are not completely abrogated, leading to minor unspecific internalization.

After having increased the size of the FA-PEG-siRNA, the major limitation of this conjugate, which should be solved by formulation with a carrier system, was examined: the entrapment in endosomes. For this reason different mixtures of FA-PEG-siRNA with plain siRNA were complexed with **386** and tested in a luciferase reporter gene assay (Figure 36).

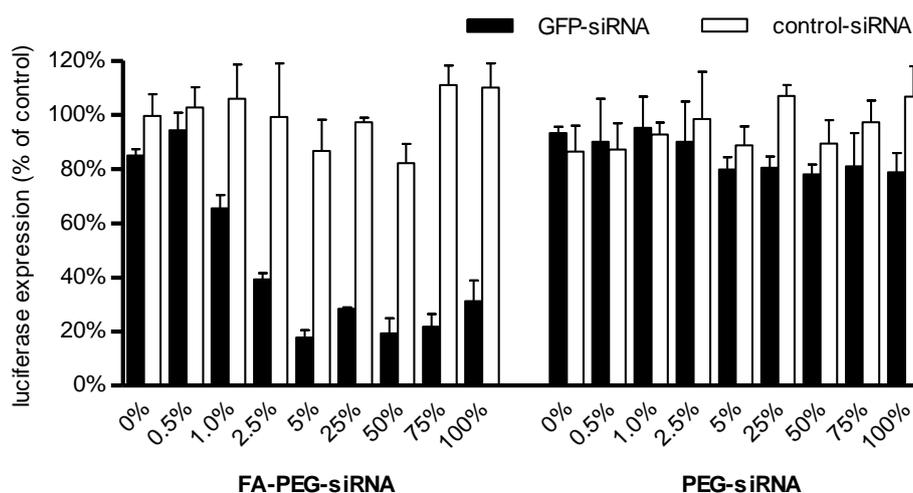


Figure 36: Gene silencing activity of polyplexes with different mixtures of targeted and nontargeted siRNA (% of targeted siRNA in total siRNA), complexed with three-armed polymer **386** (N/P of 6) in folate receptor positive, human cervix carcinoma KB-eGFPLuc cells. Medium change after 30 minutes. Final siRNA concentration: 200 nM. Black: GFP-siRNA, white: control-siRNA.

Polyplexes containing only 2.5% targeted siRNA of total siRNA and higher were able to successfully mediate gene silencing in a receptor targeted fashion. Proof for the receptor specific manner is provided by the lack of gene silencing efficiency for PEG-siRNA polyplexes in all ratios. As RNAi takes place in the cellular cytosol, this assay

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gives evidence for successful endosomal escape of the targeted siRNA. Remarkably, the complexes have no impact on the viability of the treated cells, indicated by the absence of knockdown in the control-siRNA treated cells.

The reporter gene assay in figure 36 was performed with a final siRNA concentration of 200 nM. Because high potency is an important factor for successful and repeated application of new entities, the gene silencing ability of the presented system was tested with decreasing siRNA dosages at a fixed level of oligomer concentration (Figure 37).

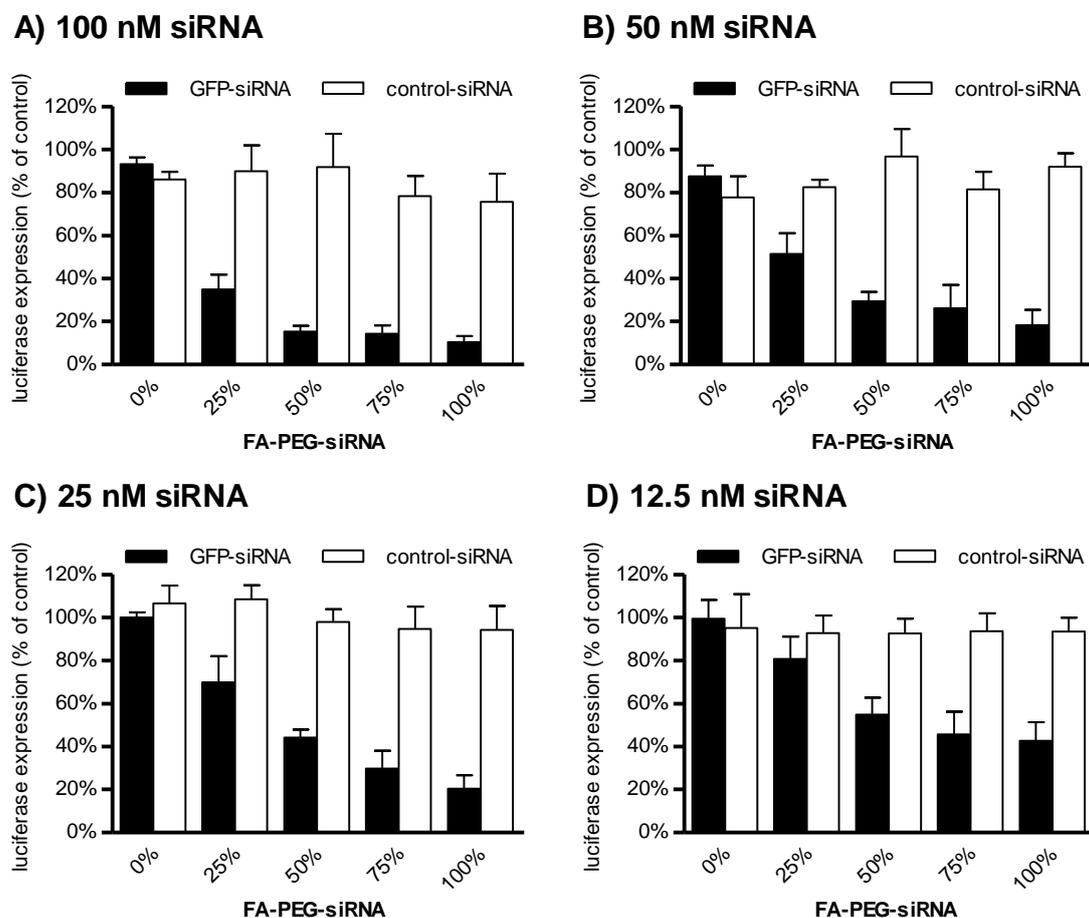


Figure 37: Gene silencing efficiency of targeted polyplexes with decreasing siRNA dosages in KB-eGFP-Luc cells. Polyplexes were formed using constant amounts of polymer **386** (0.53 μ g) and (in the highest concentration) 135 ng siRNA (final concentration 100 nM) or stepwise reduced siRNA amounts (final concentrations 50 nM, 25 nM, or 12.5 nM). For each siRNA concentration, indicated mixtures of targeted and non-targeted siRNA were applied (label of x-axis refers to the % of targeted siRNA of total siRNA). Medium change after 30 minutes.

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As demonstrated, high transfection efficiency was conserved down to siRNA concentrations of 25 nM. At these low doses, a higher amount of targeted siRNA was necessary to achieve equal levels of gene silencing as with higher siRNA concentrations. Decrease of efficiency was observed at 12.5 nM siRNA.

3.3.2 Influenza peptide – siRNA conjugate (INF-siRNA)

3.3.2.1 Design of the influenza peptide – siRNA conjugate

The lytic peptide INF7, an acidic modified version of the 23 amino-terminal amino acid sequence of the influenza virus hemagglutinin subunit HA-2, has proven pH specific lytic activity by switching to an alpha-helical conformation at endosomal pH [139]. Incorporation of components with pH specific membrane activity into polyplexes facilitates the rupture of endosomes after acidification and thereby improves transfection efficiency of the polyplexes after endocytosis. The overall negative charge of INF7 makes it an ideal candidate to be directly linked to siRNA, as this modification does not reduce the binding between cationic polymer and nucleic acid by electrostatic interaction (Scheme 5).

Scheme 5: INF-siRNA. The lytic peptide INF7 is linked to siRNA by a reducible disulfide bond.



As attachment site, again the 5' end of the sense strand was chosen for minimizing a potential impact on the silencing efficiency of the siRNA. The linkage between peptide and nucleic acid contains a reducible disulfide bond, which can be cleaved in the reductive environment of the cytosol. The conjugate was purified, resulting in a defined structure (synthesis by C. Dohmen, see Diss. Dohmen 2012).

3.3.2.2 Formulation of INF-siRNA with sequence defined oligomers for enhanced endosomal release and efficient gene silencing

For some oligomers of the discussed library, effective siRNA delivery could be detected but gene silencing efficiency of the polyplexes was insufficient. Reason for this might be the missing endosomal escape ability of these particular oligomers. The microscopic images in figure 38 show an example where this is the case. Oligomer **76**, C-Stp-(OleA)₂K]K-Stp-C, forms stable particles with regular siRNA, which are readily internalized into cells (Figure 38A) but remain stuck in endosomes (Figure 38B), where they face degradation in the endo-/lysosomal process.

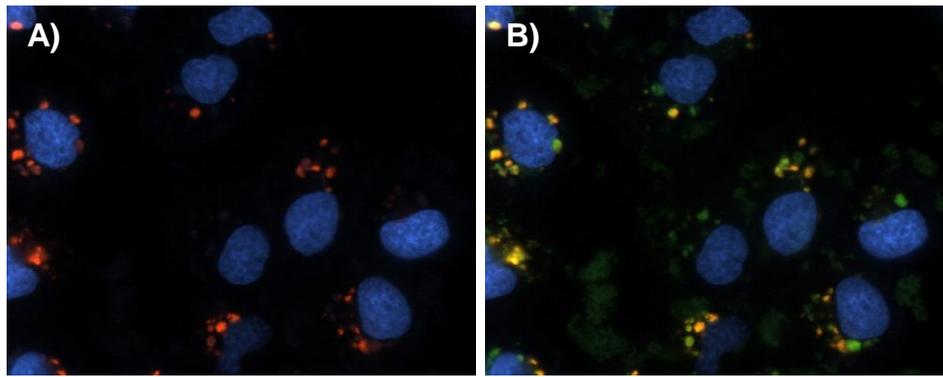


Figure 38: Cellular uptake of fluorescently labeled siRNA complexed with oligomer **76** in HUH7 cells. A) Cy5 labeled siRNA in endosomes. B) overlay of siRNA channel with lysotracker green channel. Blue: cell nuclei (Hoechst 33342), red: siRNA; green: Lysotracker green, yellow: fusion of red and green.

This carrier system presents the perfect test object for the activity of INF-siRNA. The membrane disruptive character of INF-siRNA at pH 5 should facilitate endosomal release of the internalized polyplexes and enable siRNA mediated gene knockdown. Efficiency of the combination of oligomer **76** and INF-siRNA is presented in Figure 39.

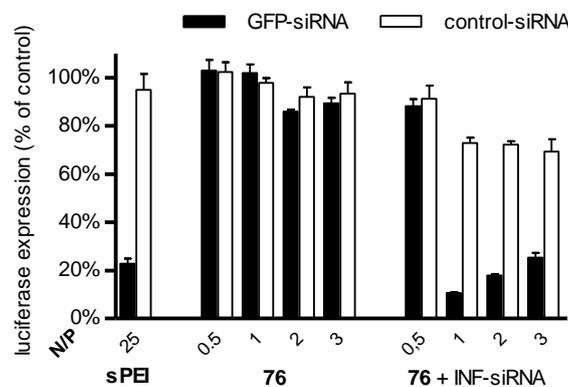


Figure 39: Gene silencing efficiency of oligomer **76** complexed with regular siRNA or INF-siRNA in Neuro2A-eGFPLuc cells at different N/P ratios. Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

Compared to regular siRNA, the influenza peptide modified siRNA achieves potent silencing of the luciferase signal at low N/P ratios of oligomer **76**, with moderate impact on cell viability, demonstrated by the minor decrease of protein expression in

the control-siRNA treated cells. This experiment shows the lytic potential of the INF-siRNA conjugate at endosomal pH and its ability to grant gene silencing ability to otherwise unfeasible carriers.

Another example for successful restoration of transfection efficiency is shown in figure 40.

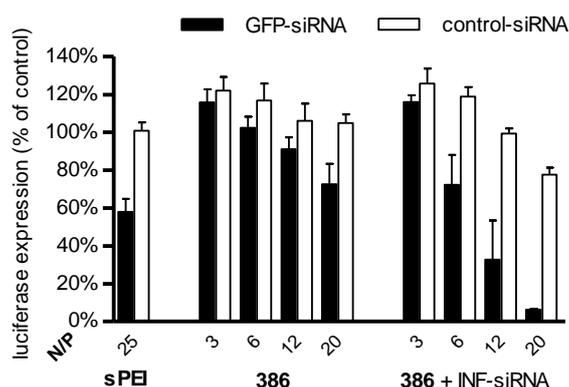


Figure 40: Gene silencing efficiency of three-armed oligomer **386** complexed with regular siRNA or INF-siRNA in KB-eGFPLuc cells at different N/P ratios. Positive control: succinylated PEI (sPEI). Black: GFP-siRNA, white: control-siRNA.

Three-armed oligomer **386**, which demonstrated high gene silencing in murine neuroblastoma Neuro2A-eGFPLuc cells, only had a very moderate effect in the human cervix carcinoma KB cell line (Figure 40). With the help of INF-siRNA, luciferase expression was reduced below 20% at low cytotoxicity levels. Obviously, the polyplexes lacked endosomal escape activity in KB cells, possibly because of variations in endosomal acidification in the different cell lines, reducing endosomal swelling through the proton sponge effect of the oligomer by its buffering capacity.

3.3.2.3 Comparison of INF-siRNA conjugates coupled by disulfide or maleimido linkage

In the described INF-siRNA conjugate the linkage between nucleic acid and influenza peptide contains a disulfide bond for cleavage in the reductive environment of the cytosol after endosomal escape, so the siRNA retains its full functionality. The necessity of this reversible linkage was explored by comparison of the conjugate with

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its non-degradable counterpart INF-Mal-siRNA, where the two components are coupled *via* a maleimide linker.

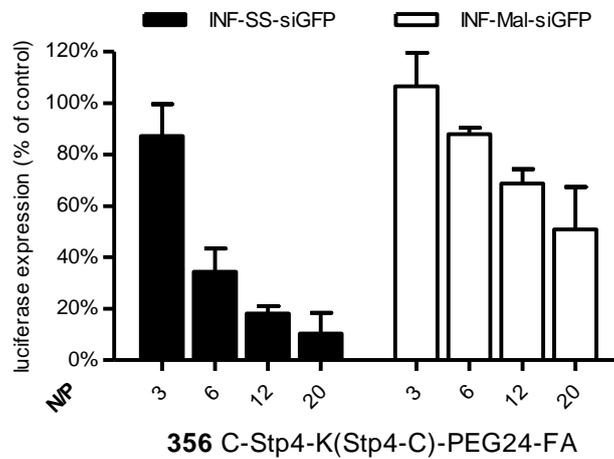


Figure 41: Gene silencing efficiency of oligomer **356** complexed with INF-SS-siGFP or INF-Mal-siGFP in KB-eGFPLuc cells at different N/P ratios. Medium change after 30 minutes.

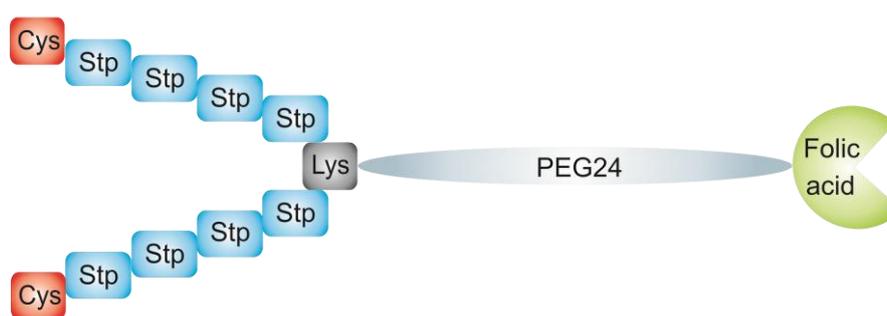
When complexed with oligomer **356** (see below), a clear reduction of knockdown efficiency is observable for the non-cleavable conjugate (Figure 41), demonstrating the necessity of the reducible disulfide linkage within the construct. Although the lytic peptide is coupled to the 5' end of the sense strand, the modification seems to hinder the incorporation of the antisense strand into the RNAi machinery.

3.3.3 Multifunctional, folate receptor targeted carrier system for receptor mediated uptake of lytic INF-siRNA

3.3.3.1 Design of the folate receptor targeted, pegylated oligomer

The previous work has described functionalization of siRNA either with targeting ligand or lytic peptide for improved delivery capabilities of siRNA carriers, based on oligo (ethane amino) amides. A combination of both functionalities should have synergistic effects on transfection efficiency of polyplexes. In addition, shielding, e.g. by incorporation of polyethylene glycol (PEG) into the system, can prevent interaction with blood components and thereby prolong circulation half life of nanoparticles in the blood stream. A simple mixture of both siRNA conjugates, in combination with upper mentioned oligomers, did not result in enhanced, targeted gene silencing (data not shown). The present study combines the targeting and shielding function of the new oligomer **356**, also synthesized by solid phase peptide synthesis, with the lytic activity of the characterized INF-siRNA conjugate (Scheme 6; synthesis by C. Dohmen, see Diss. Dohmen 2012).

Scheme 6: Functionalized oligomer **356** with eight cationic Stp units as oligomer backbone, two terminal cysteines (Cys), a PEG spacer consisting of 24 ethylene oxide monomers inserted in the middle of the backbone by coupling to lysine (Lys) and the targeting ligand folic acid at the end of the PEG chain.



The oligomer backbone consists of eight Stp building blocks with a terminal cysteine at each end of the chain for crosslinking during polyplex formation. In the middle of the backbone, a PEG chain, consisting of 24 ethylene glycol units (PEG24), is incorporated, with folic acid attached to its distal end for targeting of the human folic acid receptor (FOLR-1). The construct combines siRNA binding with shielding and

targeting functionalities. For efficient endosomal escape, the lytic activity of INF-siRNA was exploited, as previous studies with the Stp-oligomer **386** showed that polyplexes with unmodified siRNA are not able to mediate efficient gene silencing in folic acid receptor positive KB cells, due to insufficient endosomal escape (Figure 36).

3.3.3.2 Cellular association of targeted carrier system on folate receptor positive cells

The first step in targeted siRNA delivery is the successful binding of the polyplexes to the targeted receptor, in this case the folic acid receptor. Cellular association of polyplexes, consisting of fluorescently labeled INF-siRNA, complexed with the new construct **356**, C-Stp4-K(Stp4-C)-PEG24-FA, was determined by flow cytometry. Cells were incubated with the polyplexes for 30 minutes on ice in order to minimize cellular uptake. Receptor specific attachment to the cell surface was examined in control samples by competition of receptor binding by preincubation of the cells with an excess of free folic acid (Figure 42).

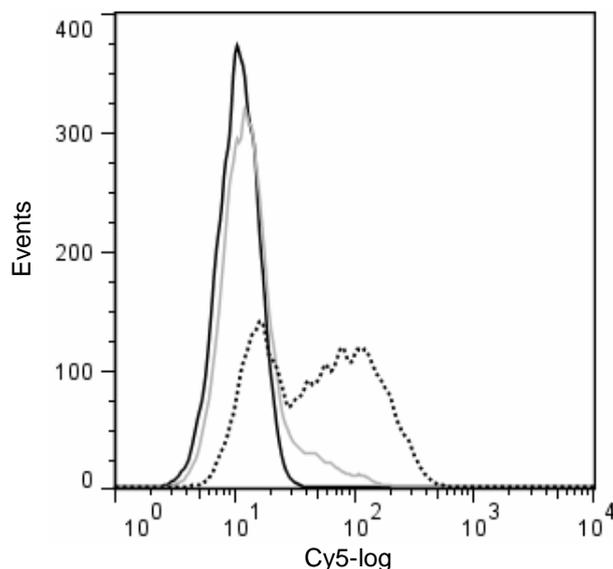


Figure 42: Cellular association assay of fluorescently labeled (Cy5)-INF-siRNA (200 nM) complexed with **356**, C-Stp4-K(Stp4-C)-PEG24-FA, on KB cells. Incubation was performed on ice and medium was exchanged after 30 minutes. Black: HBG control. Gray: **356** polyplexes on KB cells, preincubated with an excess of folate on ice for 30 minutes. Dashed: **356** polyplexes on KB cells.

Results showed that the polyplexes could only bind to the cell surface when the folic acid receptors were not blocked by an excess of free folate, demonstrating the receptor specific binding to KB cells.

3.3.3.3 Functional groups in the folate targeted carrier system for receptor mediated siRNA delivery and gene silencing

The construct **356**, C-Stp4-K(Stp4-C)-PEG24-FA, carries functional groups for crosslinking, shielding and folic acid receptor specificity. For the determination of the necessity of the incorporated functional groups for successful siRNA delivery, two mutated versions of the construct were synthesized by solid phase peptide synthesis, depleting either the cysteines or the targeting ligand. This way, clear structure-activity relationships of the carrier system can be determined. In structure **420**, S-Stp4-K(Stp4-S)-PEG24-FA, the two cysteines were replaced by serines, hindering thiol crosslinking during polyplex formation. Sequence **188**, A-PEG24-K-(Stp4-C)₂, lacks the targeting ligand folic acid, which makes receptor specific uptake impossible. The extent of cellular uptake of siRNA mediated by the serine and alanine analog of **356** was monitored by flow cytometry and confocal microscope (Figure 43 and 44).

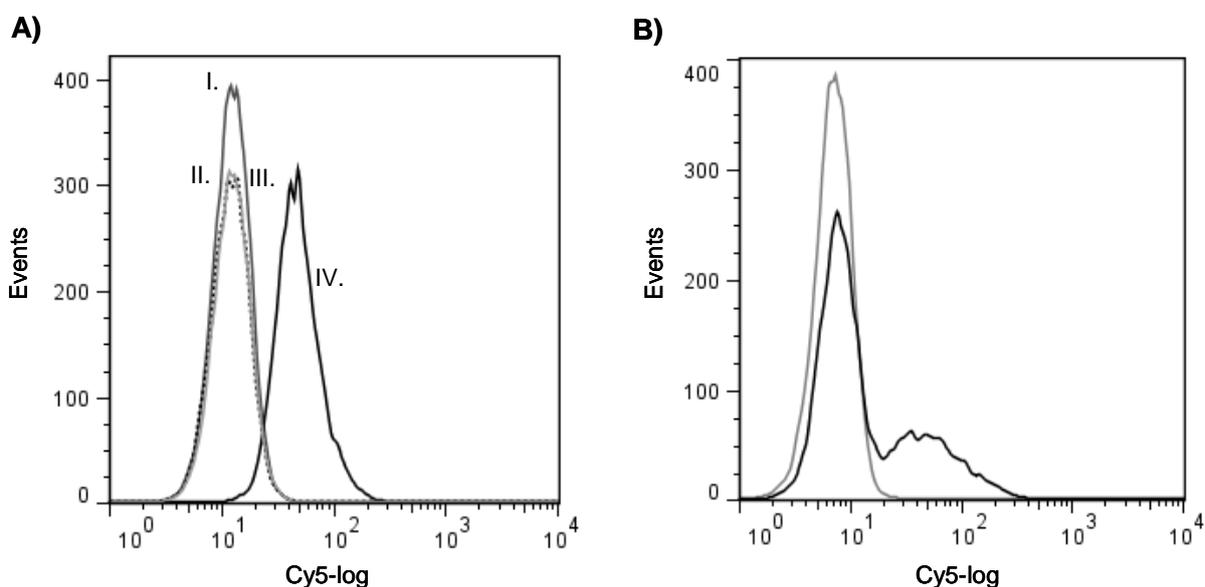


Figure 43: Cellular internalization assay of fluorescently labeled (Cy5)-INF-siRNA (200 nM) complexed with **356**, C-Stp4-K(Stp4-C)-PEG24-FA, and its mutated forms. A) KB cells. I. (dark gray): HBG control. II. (light gray): **420**, S-Stp4-K(Stp4-S)-PEG24-FA. III. (dashed): **188**, A-PEG24-K-(Stp4-C)₂. IV. (black): **356**. B) Neuro2A cells. Gray: HBG control. Black: **356**.

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The FACS experiments revealed that both analogs were not able to deliver the labeled siRNA into KB cells, emphasizing the importance of cysteines and targeting ligand within the construct. In a control experiment the cellular uptake of **356** polyplexes was determined in Neuro2A cells with low folic acid receptor expression (Figure 43B). The diminished rate of uptake further proved the receptor based internalization mechanism.

Identical results were obtained with the confocal microscopic images (Figure 44). Out of the three tested constructs, again only **356** was able to transfect KB cells with labeled INF-siRNA. The low folic acid receptor expressing control cell line, in this case HUH-7, which was chosen because of better adhesion characteristics than N2A cells, only showed marginal transfection with INF-siRNA, complexed with **356**.

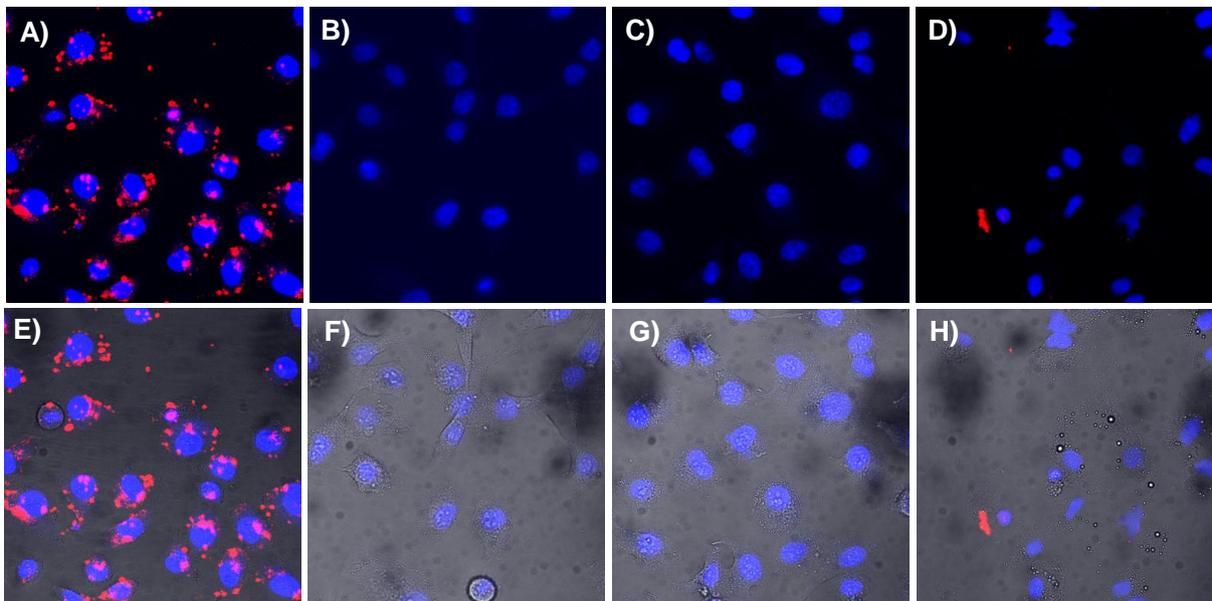


Figure 44: Cellular uptake of fluorescently labeled (Cy5)-INF-siRNA (200 nM) complexed with **356**, C-Stp4-K(Stp4-C)-PEG24-FA, and its mutated forms (N/P 16) using a confocal scanning fluorescence microscope. Final siRNA-concentration per well: 200 nM; transfection time: 30 minutes. Upper panel with two channels: Hoechst 33342 channel blue for staining of nuclei, Cy5 channel red; bottom panel with three channels: transmission light, Hoechst 33342 channel blue, Cy5 channel red. A) – C) KB cells; D) Folic acid receptor negative HUH-7 cells. A) **356**. B) **420** (serine analog). C) **188** (alanine analog). D) **356**.

So far, successful cellular attachment and internalization of the targeted particles was demonstrated. Shielding of the resulting positively charged polyplexes was achieved

by the PEG chain: the Zeta potential of the particles was shown to be around zero (see Diss. Dohmen 2012).

For the knockdown of a specific gene target by siRNA, one last hurdle has to be overcome inside the cells, the endosomal escape. Complexation of regular GFP-siRNA with **356** only mediated marginal gene silencing in the luciferase reporter gene assay (Figure 45).

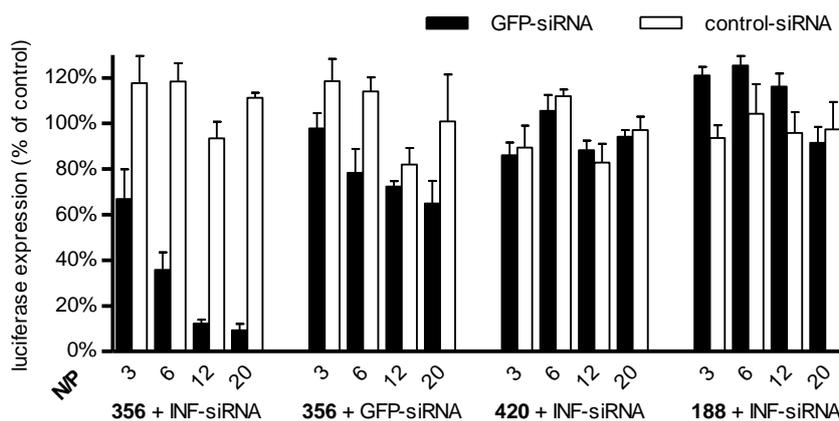


Figure 45: Gene silencing activity of INF-siRNA or regular siRNA (GFP-siRNA) complexed with **356**, C-Stp4-K(Stp4-C)-PEG24-FA, and INF-siRNA complexed with the mutated analogs **420** S-Stp4-K(Stp4-S)-PEG24-FA and **188**, A-PEG24-K-(Stp4-C)₂, in KB-eGFPLuc cells. Medium change after 30 minutes.

However, combination of the targeted, shielded and crosslinked construct with membrane disruptive INF-siRNA, directed against the eGFPLuc protein, led to strong silencing of luciferase signal in KB-eGFPLuc cells. Transfection with this carrier system was well tolerated by the cells, indicated by the unchanged protein expression in the control-siRNA treated samples. The absence of knockdown efficiency for the mutated analogs of **356**, even with INF-siRNA, confirmed the findings of the previous cellular internalization studies.

For the proof of specificity of receptor mediated uptake, control studies were also performed for the luciferase reporter gene assay (Figure 46). The effect of blocking the folic acid receptors on KB cells with an excess of free folate on transfection efficiency of **356** polyplexes and their gene silencing ability in Neuro2A cells with low folic acid receptor status was determined.

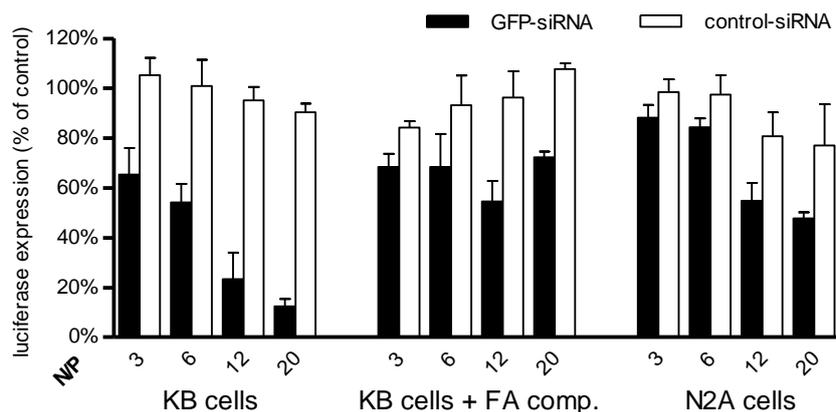


Figure 46: Gene silencing activity of INF-siRNA, complexed with **356**, C-Stp4-K(Stp4-C)-PEG24-FA in KB-eGFPLuc cells, in KB-eGFPLuc cells which had been preincubated with an excess of folic acid (+ FA comp.) and in Neuro2A-eGFPLuc cells with low folic acid receptor level. Medium change after 30 minutes.

In both cases, luciferase expression was diminished only to a minor extent, indicating that particle uptake was insufficient because of the blocking or absence of folate receptors on the cell surface.

3.3.3.4 Influence of variations of PEG chain length on gene silencing efficiency of the multifunctional, folate receptor targeted carrier system

First *in vivo* trials with the new construct revealed that the small size of the polyplexes leads to rapid clearance from blood circulation through the kidneys (see Diss. Dohmen 2012). Therefore, an increase of size was crucial for future *in vivo* applications. This was accomplished by elongation of the PEG chain in the construct by coupling two or three PEG24 instead of just one (synthesis by C. Dohmen). Before further *in vivo* experiments could be conducted, gene silencing ability of such strongly shielded particles had to be determined, as a large extent of shielding can hamper endosomal escape of polyplexes, the so called PEG dilemma [140, 141]. Figure 47 shows that the elongation of the PEG chain has no impact on gene silencing efficiency of the construct, as both modifications show identical efficiency like the original carrier system **356**.

Results

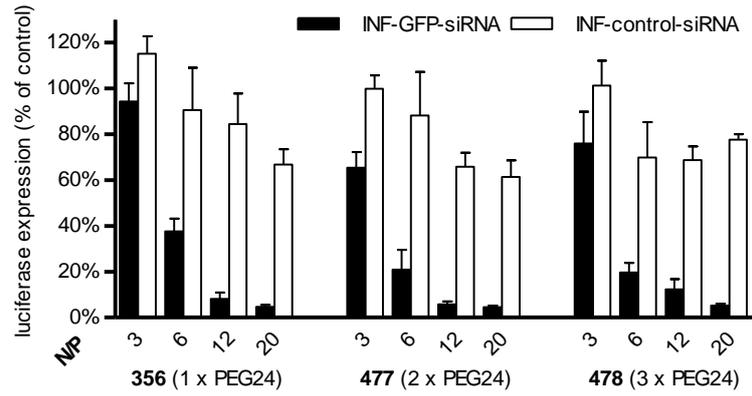


Figure 47: Influence of variation of PEG chain length on gene silencing efficiency in KB-eGFPLuc cells. **356:** C-Stp4-K(Stp4-C)-PEG24-FA, **477:** C-Stp4-K(Stp4-C)-(PEG24)₂-FA, **478:** C-Stp4-K(Stp4-C)-(PEG24)₃-FA. Medium change after 30 minutes.

4 DISCUSSION

4.1 Evaluation of lateral stabilization of siRNA polyplexes via DSP crosslinking

Cross linking strategies have been reported to strongly enhance stability and efficiency in a broad range of nanoparticles [142-145]. In this work, the amine reactive, homobifunctional NHS-ester DSP was utilized for the *in situ* surface cross linking of siRNA polyplexes, formed with the biodegradable, pseudodendritic polymer HD-O. Successful reaction of crosslinking agent with the surface amines of the polyplexes could be shown by a decrease of the positive Zeta potential after the modification step (Table 1). A similar study had previously applied surface crosslinking for polymeric siRNA delivery but with a different linker, which is only reactive at alkaline pH, far higher molar ratios between crosslinker and polymer, and with a non degradable PAMAM dendrimer [146].

Optimization of the HD-O/siRNA ratio, as well as tuning of the linker/polymer ratio proved essential for the formation of stable, nano-sized particles. Nevertheless, the surface cross linking with DSP presents a simple and reproducible method for increasing the lateral stability of siRNA polyplexes. This was demonstrated in the successful delivery of siRNA directed against the luciferase reporter gene, which led to effective luciferase silencing in two different cell lines, stably expressing the eGFP-luciferase fusion protein (Figure 8). Control experiments with non-coding siRNA, as well as measurement of the cell viability after polyplex treatment, confirmed the good tolerability of the stabilized particles *in vitro*.

Actual intracellular delivery of siRNA by the DSP-stabilized polyplexes was proven by flow cytometry (Figure 10 and 11). Internalization of plain HD-O polyplexes was only detectable to a minor extent. This is another hint that the presented polyplexes are on the edge of surviving in serum and electrolyte containing medium but need just some improvement in stability for actually doing so.

Replacement of the Luc-siRNA in the polyplexes by siRNA directed against the endogenous target AHA1 resulted in strong, specific silencing of AHA1 mRNA, again only for the DSP stabilized polyplexes (Figure 12). Even though a moderate

reduction of AHA1 mRNA was detected in samples treated with nonstabilized polyplexes, the analogous luciferase siRNA delivery experiments (measuring the actual end product of gene expression, the protein) indicate that the level is insufficient for the silencing. The current work presents encouraging data for future applications of the DSP linker strategy for stabilizing siRNA polyplexes, while preserving the biodegradability of the applied polymers. This stabilization strategy might be optimized for a future successful *in vivo* application of such siRNA polyplexes.

4.2 Evaluation of solid phase derived, sequence-defined siRNA carriers based on oligo (ethane amino) amides

For a systematic optimization of polymeric carriers for siRNA delivery, we chose a strategy based on solid phase supported synthesis which allows the generation of precise sequence-defined oligomer structures [122, 126, 127]. In this study a small library of 39 solid phase derived oligomers assembled into four different topologies using four different novel oligo (ethane amino) building blocks, six different fatty acids and/or cysteines, were examined for their ability to form siRNA polyplexes and mediate gene silencing.

Initially, four different artificial oligoamino acids building blocks (Stp, Gtp, Ptp, Gtt; see [126] and Scheme 2) were screened in two oligomer topologies (i-shapes, three-arms). These building blocks contain the same proton-sponge 1,2-diaminoethane motif as present in polyethylenimine and analogous efficient polymeric nucleic acid carriers [39, 74, 147, 148]. siRNA binding and gene silencing studies (Figure 13) revealed that only the three tetraethylenpentamine (tp) containing building blocks, but not the triethylentetramine (tt) containing building block (Gtt), have promising properties as oligomer backbone units. Stp, succinamidyl-tetraethylenpentamine, displayed the greatest potential amongst the new artificial amino acids. The glutaric acid-containing Gtp and phthalic acid-containing Ptp building blocks displayed weaker siRNA binding ability than Stp but still have effective gene silencing efficiency when incorporated into the i-shape topology. In contrast, Gtt with only two protonable

nitrogens per repeating unit results in oligomers with lowest siRNA binding ability and thus appears not as useful for assembly of siRNA carriers.

Within the investigated classes of oligomers, polyplex stability appears as an important parameter. Stability appears as even more critical for siRNA as compared with plasmid DNA polyplexes [127, 129]. Therefore functional modifications were incorporated into oligomers to stabilize polyplexes: cysteines, which after siRNA complexation provide covalent stabilizing crosslinks within the formed nanoparticle, and/or lipophilic α,ϵ -diacyl-modified lysines which provide nanoparticle stabilization by non-covalent hydrophobic interactions (see below).

Systematic variation of the chain length of Stp units in carriers with three-arm, T-shape and U-shape topology (Figure 17) revealed that small, hydrophilic oligomers with only one Stp unit per arm are incapable of mediating gene silencing, despite the incorporation of stabilizing cysteines. Only for the lipophilic, poorly water-soluble U-shape oligomer already one Stp unit was sufficient for siRNA delivery. More general, the minimum required and also sufficient repeat number was two Stp subunits per arm. Further elongation did not significantly enhance gene silencing activity *in vitro*.

For the water-soluble oligomers with three-arm, i-shape and T-shape topology, cysteines were essential to achieve siRNA transfection activity. Through disulfide crosslinks, the small oligomers form larger structures during the polyplex formation process, but biodegradability should be maintained by cytosolic reduction. Monitoring of the disulfide formation process during polyplex formation with a three-armed structure revealed that the crosslinking reaction is accelerated in the presence of siRNA, which most probably acts as a template in the process (Figure 18). The requirement of disulfide formation was demonstrated either by blocking the cysteine mercapto groups with NEM (Figure 21) or by lack of activity when cysteine was replaced by alanine (Figures 19 and 20). For the alanine mutants of the three-armed structures a poor siRNA binding ability could be shown, resulting in absence of gene silencing efficiency (Figure 19), whereas the fatty acid containing i-shape alanine mutant effectively retained siRNA in the gel shift assay but also lacked gene silencing ability. Reason for this is most likely the insufficient particle formation, underlined by the reduced cellular uptake compared to their cysteine containing counterparts (Figure 20). The more lipophilic U-shape oligomers, carrying diacyl groups at both ends of the polyamine oligomer backbone, do not need cysteines for gene silencing

activity (Figure 22). Here the twofold hydrophobic domains lead to a strong hydrophobic stabilization of siRNA polyplexes, making disulfides dispensable for efficient siRNA binding and formation of nanosized polyplexes.

The type of lipidic diacyl moieties incorporated into the oligomers were analyzed in detail, screening saturated fatty acids from C4 to C18 and two unsaturated C18 fatty acids. Incorporation of the two unsaturated C18 fatty acids, oleic and linolic acid, resulted in pH specific membrane lysis behavior and most effective gene silencing, both in the case of T-shapes (Figure 15) and i-shapes (Figure 16). This positive effect of lipidic moieties is consistent with previous reports with polymerizable surfactants and lipopolymers [40, 147].

Within the evaluated lipid-modified Stp oligomers, the different topologies (i-shape, T-shape, U-shape) proved to play only a minor role in the gene silencing efficiency of the oligomers when compared at a defined fixed ratio (2/4 or 4/8) of hydrophobic fatty acid / hydrophilic Stp building blocks (Figure 23 and 24).

Proof for successful delivery of siRNA into cells with the help of the novel oligomers was provided by flow cytometry (Figure 20) and microscopic imaging experiments (Figure 25) with fluorescently labeled polyplexes, ruling out possible unspecific effects in the reporter gene assays. The microscopic pictures showed that signals from labeled oligomer and labeled siRNA could be overlaid, confirming that intact polyplexes were internalized into the cells.

Efficacy of polyplexes at low siRNA dosages is especially important with regard to *in vivo* applications, as low doses are favorable for avoiding side effects and for repeated treatment options. For this reason three lipid modified oligomers with different topologies were tested for gene silencing efficiency at decreasing siRNA dosages. The oligomers showed activity even at low siRNA concentrations, highlighting the potential of the new structures (Figure 26).

Structure-activity relationships of the new siRNA carriers were explored in the murine neuroblastoma cell line N2A. For a broad application, efficient delivery of nucleic acids in various different cell types is required. Especially the oligomers with lipidic moieties demonstrated efficient silencing ability in a broad range of human cancer cell lines (Figure 27).

Established polymers for nucleic acid delivery, e.g. PEI [23, 149] or PAMAM [150-152], often show significant signs of cytotoxicity at high concentrations or after

repeated application. Although good tolerability of the new carriers, based on oligo (ethane amino) amides, has already been shown in the luciferase reporter gene assays, where complexation with control-siRNA had no effect on luciferase protein expression, dose escalation studies were performed with selected oligomers for the evaluation of their cytotoxic potential. The data showed a tenfold reduced or even lower cytotoxicity for the oligomers compared to linear PEI (Figure 28), opening up the possibility of repeated *in vivo* application without causing severe side effects.

The harsh environment in the bloodstream with electrolytes, proteins and mechanic stress often destroys the majority of systemically applied polyplexes. However extracellular stability is essential for the cytosolic delivery of siRNA at the target site. A serum stability assay (Figure 29), which is supposed to simulate extracellular *in vivo* conditions, demonstrated high stability for most tested structures, especially for the lipid modified oligomers, making them interesting candidates for *in vivo* application.

In conclusion, the application of solid phase peptide synthesis on oligomer assembly enables clear insights into structure-activity relationships between the shape, sequence and functional modifications of oligomers and their efficiency in siRNA delivery. With the help of this strategy, several new promising polymer classes were identified for siRNA delivery. The new strategy also enables further optimization through precise, site-specific functionalization of the carrier systems, such as introduction of PEG shielding and receptor targeting domains.

4.3 Evaluation of functionalized and targeted siRNA delivery systems

4.3.1 Folate conjugated siRNA for receptor mediated siRNA uptake

The described new class of oligomers has shown to efficiently bind siRNA, form stable nanoparticles and transfect cells. For successful systemic application of these siRNA carriers, functionalization of the resulting polyplexes is essential for specific uptake into the desired tissue. The folate-PEG-siRNA conjugate proved a suitable way for introducing a targeting ligand into the particles, enabling receptor specific cellular internalization. The plain siRNA conjugate was readily taken up into folic acid

receptor positive cells by receptor mediated endocytosis (Figure 31 and 32). However, reporter gene silencing could not be achieved by the plain construct due to insufficient endosomal escape (Figure 31). Similar findings have already been reported in literature [153]. Functionality of the siRNA was fully maintained after modification of the double strand, which was demonstrated by its delivery with a lipophilic transfection agent in a non-targeted setting (Figure 33). Entrapment and degradation of the construct in the endolysosomal compartment was circumvented by formulation of the FA-PEG-siRNA conjugate with three-armed oligomer **386**. The hydrophilic PEG spacer between targeting ligand and nucleic acid within the siRNA construct reduced the surface charge of the polyplexes, confirming the presentation of the ligand on the polyplex surface, which is essential for receptor mediated internalization (Figure 34). Furthermore, the decrease of the Zeta potential reduces unspecific interactions between polyplex and cell surface, increasing the selectivity of cellular uptake. Receptor specific internalization of the stable particles was demonstrated by comparing the cellular uptake rate of functionalized FA-PEG-siRNA polyplexes with polyplexes of folic acid depleted PEG-siRNA, which showed a substantially reduced internalization rate (Figure 35). Additional proof was obtained by receptor competition experiments with an excess of free folic acid, in which the internalization rate of polyplexes with the construct were diminished. The folic acid receptor targeted polyplexes mediated strong gene silencing in FOLR-1 positive KB cells, confirming the endosomal release of siRNA, as this is a requirement for the activity of siRNA within the cytosolic RNAi machinery (Figure 36). Three-armed oligomer **386** contains 1,2-diaminoethane units, similar to polyethylenimine, which are responsible for the proton sponge effect in endosomes, leading to endosomal swelling and in consequence endosomal rupture. A positive concomitant effect of the formulation of the siRNA construct with **386** is the increased size of the complexes (Figure 34). After systemic application, plain FA-PEG-siRNA with a hydrodynamic size < 10 nm would rapidly be cleared from blood circulation by the kidneys, hindering receptor mediated uptake into the desired tissue, e.g. distant tumor tissue. The concept of linking targeting ligands directly to siRNA for receptor specific cellular uptake has previously been described [153-155], but the systems lacked structural precision due to the chosen synthesis route or insufficient purification. The present work combines a high degree of purity and structural definition for both components,

siRNA conjugate and oligomer, yielding reliable and reproducible data for clear structure-activity relationships of the delivery system. The high efficiency of this targeted siRNA system, even at low siRNA dosages, shows its potential for future *in vivo* applications.

4.3.2 Influenza peptide – siRNA conjugate

Incorporation of membrane active moieties like fatty acids [95, 147] or lytic peptides [119, 156, 157] into siRNA carriers has proven to facilitate endosomal release and enhance gene silencing efficiency of the internalized particles. Instead of attaching this functionality to the carrier, with possible complications during synthesis due to multiple modification steps of the carrier, our group attached the lytic peptide INF7 [139] directly to activated siRNA in a straightforward procedure by disulfide linkage and subsequent purification by HPLC (see Diss. Dohmen 2012). The resulting membrane active INF-siRNA conjugate enables the use of carriers like oligomer **76**, which cannot promote sufficient endosomal release, for efficient gene silencing. Lytic activity of INF7 and functionality of the coupled siRNA were not affected by the hybridization (see Diss. Dohmen 2012), enabling the use of the construct in polyplexes with full efficiency of both components. The membrane disruptive character of INF-siRNA at endosomal pH of 5 releases polyplexes into the cytosol, which would otherwise face endo-/lysosomal degradation (Figure 38 and 39). After cleavage of the reducible disulfide bond between the modified influenza peptide and siRNA, caused by elevated glutathione levels in the cytosol, the antisense strand of the nucleic acid can be incorporated into the silencing complex and take action without impairment from the modification. Necessity of the reducible disulfide bond was demonstrated by the reduced gene silencing activity of an INF-siRNA analog with stable maleimido linkage (Figure 41).

Transfection efficiency of a polycationic carrier can vary in different cell lines, amongst others because of variations in endosomal acidification, impacting the protonation degree of the polycationic carrier and endosomal swelling, causing differences in endosomal escape. When the buffering capacity and membrane interaction of the carrier in a specific cell line is not sufficient for effective endosomal rupture, the combination with INF-siRNA can boost transfection efficiency, like shown with three-armed oligomer **386** in KB cells (Figure 40). Without lytic component, **386**

polyplexes are completely dependent on the proton sponge effect and interaction of the oligomer with the endosomal membrane, as it contains no additional functional groups like lipid modification for enhanced membrane destabilization. For this type of carrier, the functionalization of siRNA with INF7 is a useful endorsement, yielding efficient siRNA delivery systems.

As endosomal escape is a major bottleneck in polymeric siRNA delivery, the INF-siRNA conjugate presents an effective agent for overcoming this critical hurdle in the delivery process. With regard to shielded polyplexes, e.g. by PEGylation, this becomes even more important, as shielding reduces the interaction between polycationic carriers and the cell membrane, which is favorable in the extracellular environment for a prolonged circulation half life in the blood stream but disadvantageous after cellular internalization. Within endosomes, strong interaction between cationic carrier and endosomal membrane is required for destabilization of the membrane. This “PEG dilemma” can be solved by the use of additional membrane active agents like the INF-siRNA conjugate.

4.3.3 Folate receptor targeted, defined carrier for receptor mediated uptake of lytic INF-siRNA

For efficient systemic delivery of siRNA to a specific tissue a polycationic carrier system has to be functionalized and bioresponsive in order to adapt to the changing environment and requirements in the course of the delivery process. Several examples of bioresponsive systems have been reported for siRNA delivery [31, 95, 146, 158] but the various introduced functional domains increased the polydispersity of these constructs even beyond the original poor definition of the cationic carrier. The discussed sequence defined, folic acid receptor targeted, PEGylated, polycationic carrier **356**, in combination with lytic INF-siRNA, proved to be a promising approach, maintaining the sequence defined character of the carrier platform even after functionalization. This system addresses several critical bottlenecks in siRNA delivery. Strong siRNA binding and stable polyplex formation is provided by the oligomer backbone with 8 Stp building blocks and a terminal cysteines at the end for crosslinking during polyplex formation. PEGylation of the

cationic backbone masks the positive surface charge of the polyplexes, reducing unspecific interactions of the polyplexes with blood components and prolonging circulation half life (see Diss. Dohmen 2012). The targeting moiety folic acid at the distal end of the PEG chain ensured receptor specific attachment and uptake into folic acid receptor positive cells (Figure 43 and 44). The construct is assembled by solid phase peptide synthesis, using defined building blocks. This ensures the reliability and reproducibility of the results and at the same time allows the assembly of mutated sequences of the construct, depleting one functional group at a time. This way, exact structure-activity relationship studies can be performed. Two different analogs of **356** were synthesized in parallel to the original construct, one without targeting ligand, the other one lacking cysteines for crosslinking. Complexes of the analogs with siRNA were not internalized and did not mediate gene silencing, highlighting the necessity of these functional groups for the intracellular delivery of siRNA (Figure 43, 44 and 45). Oligomer **356** however also showed very moderate gene silencing ability with regular siRNA. Obviously, the structure of the oligomer plus PEGylation of the construct does not support endosomal escape of the polyplexes [140, 141]. Only the combination with INF-siRNA facilitated efficient endosomal escape, once the polyplexes had been internalized in endosomes, leading to strong reporter gene knockdown (Figure 45). Unspecific gene silencing was ruled out by receptor competition experiments and the absence of gene silencing ability in Neuro2A-eGFPLuc cells with low folic acid receptors status. Encouraged by the high gene silencing efficiency and good biocompatibility of the construct in cell culture, systemic *in vivo* delivery experiments were conducted in KB tumor bearing mice, which revealed that the polyplexes were rapidly cleared from the blood circulation before reaching tumor tissue, due to their small size. Short circulation time dramatically reduces the therapeutic potential of such targeted systems. Therefore the size of the particles was increased by elongation of the PEG chain with one or two additional PEG24 molecules (see Diss. Dohmen 2012). PEG24 was chosen because it is the longest available defined PEG chain, maintaining the monomolecular shape of the construct. Gene silencing experiments demonstrated that the higher degree of PEGylation did not affect knockdown efficiency of the polyplexes (Figure 47), opening up the possibility for further systemic delivery experiments.

5 SUMMARY

Nucleic acids like siRNA embody a next step in biological treatment options, following proteins and peptides. For resounding success of these new entities, appropriate delivery agents are essential for accumulation of the payload inside target cells. Polymers represent an interesting class of carriers for achieving this goal. In this thesis, improved as well as novel delivery systems, based on polycationic carriers, were discovered and evaluated for efficient delivery of siRNA.

The reported pseudodendritic, biodegradable polymer HD-O, consisting of oligoethylenimine building blocks, had previously demonstrated effective gene delivery of pDNA but lacked gene silencing efficiency when complexed with siRNA in the present study. Reason for this was insufficient stability of the resulting polyplexes. *In situ* lateral stabilization of the resulting polyplexes by the amine reactive crosslinker DSP improved stability of the complexes, which led to greatly improved delivery efficiency and reporter gene knockdown. Biodegradability of the polymer after linker stabilization is maintained by disulfide bonds in the crosslinking agent. This study underlines the importance of stability of siRNA nanoparticles for delivery efficiency as one of the major bottlenecks in the delivery process. One of the drawbacks of this and other reported polycationic carriers is the polydispersity of the polymers, making batch comparisons and reproducibility difficult. Therefore the developed concept of crosslinking for increased stability of siRNA polyplexes was transferred to a novel class of sequence defined oligomers, incorporating additional functional groups for enhanced siRNA delivery.

By exploiting solid-phase supported peptide synthesis, sequence defined, monodisperse oligo (ethane amino) amides were assembled, using different novel non-peptidic building blocks, and various molecular shapes were tested for structure-activity relationships in siRNA delivery. Efficient reporter gene knockdown was obtained in a variety of cell lines using either branched three-armed structures, or lipid-modified structures with i-shape, T-shape, U-shape configuration. For the majority of structures, apart from U-shapes, the presence of cysteines was strictly required for lateral polyplex stabilization and silencing activity, like previously also found for the HD-O polyplexes. Although all four tested building blocks contain the

ethylenediamine proton sponge motif, only oligomers assembled with the tetraethylenepentamine based amino acids (Stp, Gtp, Ptp) but not with the triethylenetetramine based amino acid (Gtt) were able to mediate efficient gene silencing. For the lipopolymeric structures, out of the tested saturated (from C4 to C18) and unsaturated (C18) fatty acid moieties, two proximate oleic acids or linolic acids provided the oligomers with the best gene silencing activity and also pH specific lytic activity at pH 5.5, presumably facilitating endosomal escape of the polyplexes. Evaluation of oligomer chain length revealed a minimal number of at least two oligo (ethane amino) building blocks per oligomer arm as necessary for the vast majority of structures, but only marginal changes were found with higher numbers. Molecular topology of the oligomers played a subordinate role in delivery efficiency compared to the effect of incorporating different building blocks and functional groups. The high efficiency of these defined delivery systems even at low siRNA dosages, in combination with low cytotoxicity, makes them promising candidates for *in vivo* applications. Proven serum stability of the polyplexes strengthens their potential as systemic siRNA delivery agents.

Changing requirements during the delivery process of siRNA request the functionalization of polyplexes, allowing adaptation to the different biological surroundings. The first important step, selective uptake into target cells, can be achieved by incorporation of targeting ligands, e.g. folic acid, targeting the folic acid receptor. The FA-PEG-siRNA conjugate, in combination with a three-armed oligomer from the novel oligomer series, showed selective uptake and efficient gene silencing in folic acid receptor positive cells. The PEG spacer of the conjugate reduced the overall positive surface charge of polyplexes, thereby minimizing unspecific interactions with blood components or cell surfaces.

Another major barrier in siRNA delivery is effective escape from the endosomal compartment. Coupling the membrane active peptide INF7 directly to siRNA via degradable disulfide linkage boosted endosomal escape ability of otherwise ineffective oligomeric systems, facilitating siRNA mediated reporter gene silencing *in vitro*.

Ideally, a siRNA carrier system should comprise functionalities for all essential steps during the delivery process: efficient binding of siRNA into stable polyplexes, shielding of the polyplex during circulation, a targeting moiety for receptor specific

cellular uptake and finally effective release of the payload from the endosomal compartment. An approach to realize this kind of multifunctional system is the folic acid receptor targeted, PEG shielded carrier **356**, assembled by solid phase supported synthesis, in combination with INF-siRNA. The Stp-based polycationic oligomer with two cysteines for crosslinking during polyplex formation mediated receptor specific cellular uptake of INF-siRNA, resulting in strong gene silencing. High purity of the entities and sequence defined structures allowed to study the impact of every single functional domain on delivery efficiency. Based on the applied synthesis strategy and the findings of this study, further rational design of novel potent carriers can be achieved by introducing new cationic building blocks, elongation of the polymeric backbone or screening of new functional domains.

6 APPENDIX

6.1 Abbreviations

A	alanine
ACTB	beta-actine (gene name)
AHA1	activator of 90 kDa heat shock protein ATPase isoform 1
Boc	tert-butyloxycarbonyl
brPEI	branched polyethylenimine (25 kDa)
ButA	butyric acid
C	cysteine
CapA	caprylic acid
DAPI	4,6-Diamidin-2-phenylindol
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPE	dioleoyl-phosphatidylethanolamine
DOTAP	dioleoyl-trimethylammoniumpropane
DSP	dithiobis (succinimidylpropionate)
DSPC	distearoyl-glycero-3-phosphocholine
dsRNA	double stranded ribonucleic acid
DSS	disuccinimidyl suberate
EDTA	ethylenediaminetetraacetate
eGFP	enhanced green fluorescent protein
EtBr	ethidium bromide
FA	folic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
Fmoc	fluorenylmethyloxycarbonyl chloride
FOLR-1	human folic acid receptor 1
HBG	HEPES-buffered glucose
HD	hexanediol-diacrylate

HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethansulfonic acid)
HPLC	high pressure liquid chromatography
K	lysine
LinA	linolic acid
linPEI	linear PEI (22 kDa)
Luc	luciferase
miRNA	micro RNA
mRNA	messenger RNA
MTT	dimethylthiazolyldiphenyl-tetrazolium bromide
MW	molecular weight
MyrA	myristic acid
NEM	N-ethyl maleimide
N/P-ratio	molar ratio of nitrogen to phosphate (conjugate to nucleic acid)
OEI	oligoethylenimine
OEI 800	OEI (800 Da)
OleA	oleic acid
PAMAM	polyamidoamines
PBS	phosphate-buffered saline
pDNA	plasmid DNA
PEI	polyethylenimine
PEG	polyethylene glycol
PEG24	polyethylene glycol with 24 ethylene glycol units
PLL	poly-L-lysine
poly-IC	poly-inosine-cytosine
RISC	RNA-induced silencing complex
RLU	relative light units
RNA	ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
siRNA	small interfering RNA
SPDP	succinimidyl 3-(2-pyridyldithio) propionate
sPEI	succinylated branched PEI (25 kDa)

Appendix

SPPS	solid phase peptide synthesis
SteA	stearic acid
Tf	transferrin
TNBS	trinitrobenzenesulfonic acid
w/w ratio	weight to weight ratio (conjugate to nucleic acid)

6.2 Publications

6.2.1 Original papers

Russ V, Fröhlich T, Li Y, Halama A, Ogris M, Wagner E (2010) *Improved in vivo gene transfer into tumor tissue by stabilization of pseudodendritic oligoethylenimine-based polyplexes*. J Gene Med 2010 Feb; 12 (2); 180-93.

Schaffert D, Troiber C, Salcher EE, Fröhlich T, Martin I, Badgujar N, Dohmen C, Edinger D, Kläger R, Maiwald G, Farkasova K, Seeber S, Jahn-Hofmann K, Hadwiger P, Wagner E (2011) *Solid-Phase Synthesis of Sequence-Defined T-, i-, and U-Shape Polymers for pDNA and siRNA Delivery*. Angew Chem Int Ed Engl. 2011 Sep 12; 50 (38): 8986-9.

Dohmen C, Fröhlich T, Lächelt U, Röhl I, Vornlocher HP, Hadwiger P, Wagner E (2012) *Defined Folate-PEG-siRNA Conjugates for Receptor Specific Gene Silencing*. Mol. Ther. – Nucleic Acids. 1e7

Fröhlich T, Edinger D, Kläger R, Troiber C, Salcher E, Badgujar N, Martin I, Schaffert D, Cengizeroglu A, Hadwiger P, Vornlocher HP, Wagner E (2012) *Structure-activity relationships of siRNA carriers based on sequence-defined oligo (ethane amino) amides*. J Control Rel. In press

Dohmen C, Edinger D, Fröhlich T, Schreiner L, Lächelt U, Troiber C, Rädler J, Hadwiger P, Vornlocher HP, Wagner E *Nano sized multifunctional polyplexes for receptor mediated siRNA delivery*. In revision

Fröhlich T, Edinger D, Russ V, Wagner E *Stabilization of polyplexes via lateral crosslinking for efficient siRNA delivery*. Submitted

Salcher E, Kos P, Fröhlich T, Badgujar N, Scheible M, Wagner E *Sequence-defined Four-arm Oligo(ethanamino)amides for pDNA and siRNA Delivery: Impact of Building Blocks on Efficacy*. In revision

6.2.2 Reviews

Fröhlich T, Wagner E (2010) *Peptide- and polymer-based delivery of therapeutic RNA*. *Soft Matter*, 2010, 6 (2), 226-234.

6.2.3 Abstracts

Maier K, Fröhlich T, Russ V, Li Y, Halama A, Ogris M, Schaffert D, Schlossbauer A, Bein T, Wagner E: *Biocompatible Nanosystems for Nucleic Acid & Protein Delivery*. Third Annual Symposium on Nanobiotechnology: New Directions in NanoHealth, 2009, California NanoSystems Institute, UCLA, Los Angeles, USA (poster presentation).

Dohmen C, Fröhlich T, Foralosso R, Wagner E: *pH-Labile Degradable Pseudodendritic Polycations for In Vitro and In Vivo DNA Delivery into Cancer Cells*. 17th Annual Meeting German Society for Gene Therapy (DG-GT e.V.), October 7–9, 2010, LMU, Munich, Germany (poster presentation).

Fröhlich T, Phillip A, Russ V, Edinger D, Wagner E: *Biodegradable Pseudodendritic Oligoamines for siRNA Delivery*. 17th Annual Meeting German Society for Gene Therapy (DG-GT e.V.), October 7–9, 2010, LMU, Munich, Germany (poster presentation).

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