Targeted siRNA Delivery Using a Lipo-Oligoaminoamide Nanocore with an Influenza Peptide and Transferrin Shell

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Developing RNA-interference-based therapeutic approaches with efficient and targeted cytosolic delivery of small interfering RNA (siRNA) is remaining a critical challenge since two decades. Herein, a multifunctional transferrin receptor (TfR)-targeted siRNA delivery system (Tf&INF7) is designed based on siRNA complexes formed with the cationic lipo-oligoamino amide 454, sequentially surface-modified with polyethylene glycol-linked transferrin (Tf) for receptor targeting and the endosomolytic peptide INF7 for efficient cytosolic release of the siRNA. Effective Tf&INF7 polyplex internalization and target gene silencing are demonstrated for the TfR overexpressing tumor cell lines (K562, D145, and N2a). Treatment with antitumoral EG5 siRNA results in a block of tumor cell growth and triggered apoptosis. Tf-modified polyplexes are far more effective than the corresponding albumin- (Alb) or nonmodified 454 polyplexes. Competition experiments with excess of Tf demonstrate TfR target specificity. As alternative to the ligand Tf, an antimurine TfR antibody is incorporated into the polyplexes for specific targeting and gene silencing in the murine N2a cell line. In vivo distribution studies not only demonstrate an enhanced tumor residence of siRNA in N2a tumor-bearing mice with the Tf&INF7 as compared to the 454 polyplex group but also a reduced siRNA nanoparticle stability limiting the in vivo performance.

1. Introduction

Nucleic acid therapy, the utilization of nucleic acid to express or silence genes for disease treatment, offers novel opportunities for cancer therapy.[1] Recently small interfering RNA (siRNA) was introduced for oncotherapy through RNA interference (RNAi) inducing sequence specific target messenger RNA degradation in the cytoplasm.[2] Successful antitumoral siRNA delivery vehicles need to acquire the following capabilities: protection of siRNA against degradation by nucleases during in vivo circulation; particle surface shielding to reduce interaction with serum proteins; passive crossing of the leaky tumor vasculature to accumulate in tumors via the enhanced permeability and retention effect,[3] enhanced cellular uptake into tumor cells based on specific targeting ligands; and efficient cytosolic release.[4] Varieties of siRNA carriers were developed in recent years, such as cationic polymers, liposomes, dendrimers, inorganic nanoparticles, and hybrid systems.[5] Despite encouraging progresses, successful targeted and efficient delivery of therapeutic siRNA is still full of challenges.

Targeting natural cellular receptors with the corresponding ligand-containing nanoparticles has been found as an encouraging strategy for delivery of nucleic acid materials both in vitro and in vivo.[6] Transferrin (Tf), an iron transporting protein, is efficiently transported into cells through transferrin receptor (TfR)-mediated endocytosis.[7] Since TfR is overexpressed in most proliferating cells and especially tumor cells, it has been used as a target in drug, peptide, protein, toxins, and gene delivery.[8] Encouragingly, Tf-containing nucleic acid formulations have already reached the stage of clinical testing in human.[9] Davis and co-workers developed a TfR-targeted siRNA delivery system based on cyclodextrin-oligocations complexed with adamantyl-polyethylene glycol (PEG)-Tf.[10] This siRNA nanoparticle formulation already was evaluated in a phase I clinical trial, where systemic nanoparticle delivery resulted in gene silencing in the tumor of the treated patient.[9b]

In order to achieve efficient gene silencing, sufficient cytosolic release of siRNA is essential for effective formation of the RNA-induced silencing complex. However, investigations of lipid- and polymer-based nanoparticles revealed a very low efficiency of 1%–2% of nucleic acid escaping from endosomes into the cytosol, which only happens during a particular, limited time window.[11] Hence, endosomal escape after endocytosis is a key rate-limiting step in siRNA delivery.
Several strategies had been developed to enhance siRNA release, including the use of cell penetrating peptides or lipid components and other means. The synthetic peptide INF7, a glutamic acid-enriched analogue of the influenza hemagglutinin membrane protein HA2, exhibits ability to specifically disrupt membrane bilayers at endosomal pH around 5 to 6, therefore it appears to be ideally for enhancing endosomal escape. In fact, incorporation of synthetic influenza peptides like INF7 in a covalent or noncovalent manner strongly increased transfection efficiency of pDNA polyplexes. Direct attachment to siRNA also improved activity of corresponding siRNA polyplexes.

To combine the option of multifunctional polymer design with the requirement of precise chemical synthesis, our group has developed sequence-defined cationic oligoamino amides generated via solid-phase assisted synthesis using the artificial amino acid succinoyl-tetraethylene pentamine (Stp) as building block in properly protected form. Like the well-established transfection carrier polyethylenimine, the sequence-defined oligomers contain repeats of the chemical 1,2-diaminoethane motif, which has capacity for electrostatic nucleic acid binding and enhancing endosomal escape through the proton sponge effect. Encouraging siRNA delivery activity was achieved with the T-shaped lipo-oligomer containing two lipophilic oleic acids combined in a T-shaped topology with two cationic arms, each containing two Stp units, three tyrosines, and a terminal cysteine. Introduction of hydrophobic fatty acids and aromatic tyrosines as well as the capability of disulfide cross-linking had been found important for the stability of formed siRNA polyplexes.

In the present study, a TfR-targeted siRNA delivery system was assembled based on oligomer siRNA complexes. These sized core nanoparticles were surface-modified with Tf via a PEG linker, followed by linkage of INF7 peptides through disulfide bonding. Efficient eGFP (enhanced green fluorescent protein)-luciferase marker gene silencing and antitumoral EG5 gene silencing in vitro was dependent on both TfR interaction and the presence of the endosomolytic INF7 peptide for cytosolic siRNA release. In vivo pilot studies revealed delivery into tumors, the extent of which however was limited by suboptimum in vivo stability of siRNA polyplexes.

2. Results

2.1. Generation of Tf-PEG and INF7 Modified siRNA Polyplexes

Targeted siRNA polyplexes were assembled as displayed in Figure 1A. At first, siRNA was complexed with the sequence-defined cationic oligomer. As can be seen from agarose gel shift assay (Figure 1B), particle size by dynamic laser-light scattering (DLS) and zeta potential measurements (Figure 1C), the complexation resulted in formation of stable nanoparticles of around 107 nm size and a highly positive zeta potential of +23 mV, consistent with our previous published work. The hydrophobic oleic acid and tyrosine residues of contributed

| Samples | Size (Intensity nm) | PDI | Zeta Potential (mV) |
|---------|---------------------|-----|---------------------|
| 454     | 106.9 ± 3.8         | 0.14 ± 0.01  | 23.4 ± 0.4          |
| 5% Tf   | 144.3 ± 1.1         | 0.12 ± 0.01  | -0.3 ± 0.04         |
| Tf & INF7 | 243.9 ± 5.7       | 0.19 ± 0.02  | -2.9 ± 0.3          |

Figure 1. A) Schematic illustration of Tf and INF7-modified siRNA polyplexes. A, K, Y, and C in lipo-oligomer 454 are the corresponding (L) α-amino acids in one-letter code. Both Tf-PEG-OPSS and INF7-TNB contain reactive groups able to react with cysteine thiol groups of oligomer 454. The schematic presentation of Tf-PEG and INF7 attached to the polyplex via covalent disulfide bonds does not imply any accurate topology, but reflects the much larger size of the PEG-linked 80 kDa Tf protein as compared to the much smaller INF7 peptide. B) Agarose gel shift of siRNA polyplexes. C) Size by DLS and zeta potential of siRNA polyplexes.
to the stability of the siRNA polyplexes.\textsuperscript{[17a,19]} Furthermore, the two terminal cysteines in the oligomer structure enhanced the stability of siRNA polyplexes by partial disulfide cross-linkage, while residual free cysteine thiois persisted on the polyplex surfaces, available for further chemical modification. In the second assembly step, polyplexes were modified with the rather large (80 kDa) and negatively charged Tf protein via a 5 kDa PEG-ortho-pyridyl-2-disulfide (OPSS) linker through a disulfide bond exchange with the polyplex (see Figure 1A). In the final third step, the INF7 peptide containing a C-terminal activated cysteine TNB derivative was coupled by disulfide bond formation. The successful modification of polyplexes was accompanied by distinct changes in the biophysical properties (see Figure 1C). The zeta potential of siRNA polyplexes decreased from +23 mV to neutral (Tf&INF7) upon the attachment of 5 mol% Tf and 10 mol% negatively charged INF7 peptide. Meanwhile, the particle size increased from 107 to 144 nm upon linkage with Tf-PEG5K-OPSS, and to ~240 nm after additional incorporation of the INF7 peptide, but remains in dimensions well suitable for biological application. DLS particle count rate remained unchanged, indicating that similar nanoparticle numbers maintained in solution (Table S1, Supporting Information). Transmission electron microscopy (TEM) images (Figure S1, Supporting Information) show that the size and morphology of individual polyplexes remains unaltered (around 50–75 nm nanospheres) suggesting that the larger DLS size of TF&INF7 are caused by a tendency of polyplexes to cluster into multimeric structures. Stable Tf and Tf&INF7 postmodified particles were also obtained with a siRNA concentration of 200 µg mL\textsuperscript{−1}; notably both 5% Tf and Tf&INF7 particles are well maintained even after six cycles of freeze and thawing (Table S2, Supporting Information). Direct modification of 454 polyplexes with 10 mol% INF7 at N/P 6 without Tf coating decreased size uniformity and even induced agglomeration.

2.2. GFP Gene Silencing

GFP gene silencing was investigated using two TfR overexpressing cell lines, the human K562-eGFP-Luc erythroleukemia suspension cell line (Figure 2A), which has been widely used for TfR targeting, meanwhile is difficult to transfact according to previous studies,\textsuperscript{[23]} and the adherent murine neuroblastoma cell line N2a-eGFP-Luc (Figure 2B). GFP siRNA transfection with 454 core polyplexes was compared with multifunctional TfR-targeted polyplexes (Tf or Tf&INF7) and control protein (albumin)-coated polyplexes (Alb or Alb&INF7). The corresponding control siRNA polyplexes did not reduce luciferase reporter gene expression. Noncoated 454 and Tf-coated siGFP polyplexes displayed gene silencing in the N2a reporter cell line but not in the K562 reporter cell line. Combining targeting ligand Tf and endosomolytic peptide (TF&INF7) further enhanced reporter gene silencing. In contrast, Alb or Alb&INF7 polyplexes had a decreased gene silencing activity as compared to plain 454 polyplexes (Figure 2). The combined TF&INF7 coating triggered efficient gene silencing also in the K562 eGFP-Luc reporter cell line (Figure 2A). This could be verified also by flow cytometric measurement of intracellular GFP fluorescence intensity (Figure S3, Supporting Information). Attachment of INF7 to 454 polyplexes without Tf was less effective than the combined coating.

In a proof-of-concept study, new 454 polyplexes were generated, with the Tf protein ligand being replaced by the monoclonal IgG1 antibody 8D3 (TfRab) directed against the murine TfR. Encouragingly, beneficial GFP gene silencing was detected with TfRab polyplexes containing INF7 peptide comparing to plain 454 polyplexes in TfR overexpressing murine N2a-eGFP-Luc cells (Figure S4, Supporting Information).

2.3. Cellular Internalization of siRNA in Modified Carriers

To verify the connection between Tf-mediated uptake and increased gene silencing, cellular internalization was investigated. Improved cellular uptake of Tf&INF7 polyplexes in K562 cells was observed by laser scanning confocal microscopy (Figure 3A). In contrast, coating with Alb&INF7 blocked the internalization of Alb&INF7 polyplexes (Figure 3B). Interestingly, the blockade of TfR with excess of free competitive Tf reduced the cellular internalization of Tf&INF7 (Figure 3C). Similarly, superior cellular uptake of the TfR-targeted polyplexes compared to control polyplexes was observed in the N2a-eGFP-Luc cell line (Figure 3D).
internalization of Tf-modified polyplexes was demonstrated in the TfR overexpressing prostate carcinoma cell line DU145 (receptor detection in Figure S2, Supporting Information) in comparison to 454 or Alb-modified polyplexes (Figure 3D,E). Again, the blockade of TfR with excessive Tf reduced the cellular internalization of Tf&INF7 polyplexes (Figure S5, Supporting Information), indicating a TfR-mediated endocytotic uptake.

2.4. Antitumoral Gene Silencing of EG5

Next steps toward therapeutic delivery were made using siRNA for silencing the EG5 gene. EG5 is a key protein required during mitosis. Silencing of EG5 blocks centrosome separation, induces cell cycle arrest in G2 stage, finally resulting in apoptosis. As EG5 gene silencing is closely related with antitumoral effects, we monitored the effect of EG5 siRNA polyplexes in DU145 cells (Figure 4A) and K562 cells (Figure 4B) by changes in the metabolic activity as measured by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. One hour incubations with unmodified siEG5 454 polyplexes were compared with Tf-PEG or Alb-PEG coated polyplexes. Polyplexes of control siRNA (siCon) served as negative controls. Neither standard 1 h (Figure 4) nor extended 24 h (Figure S6, Supporting Information) incubations with siCon polyplexes resulted in significant reduction of metabolic cell
activity, ruling out the possibility of RNAi independent effects. Shielding of siRNA polyplexes with the large proteins Tf or Alb reduced the antitumoral effect of siEG5 as compared to plain cationic polyplexes formed with 454 at N/P ratio of six in the Tf overexpressing cell line DU145 (Figure 4A). For K562 cells (Figure 4B), the Tf-coated but not the Alb-coated siEG5 polyplexes retained their antitumoral activity. As the protein coating of 454 polyplexes was hypothesized to strongly reduce endosomal escape properties after endocytosis, the endosomolytic agent chloroquine was included (Figure 4A). This treatment did not alter metabolic activity of cells treated with siCon control polyplexes or siEG5 polyplexes with plain 454 (without targeting) or ineffective Alb-coated polyplexes. However, it strongly enhanced the antitumoral activity of TF-PEG shielded polyplexes. Consistently, the incorporation of endosomolytic INF7 peptide into the Tf polyplexes resulted in the highest potency in DU145 (Figure 4A), K562 (Figure 4B) and N2a cells (Figure S7, Supporting Information). In contrast, Alb-PEG 454 polyplexes displaying reduced cell entry (Figure 3) did not benefit from INF7 peptide incorporation.
In addition to the metabolic activity assay, the effect of EG5 gene silencing was monitored with an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay performed by flow cytometry (Figure 4C–H). Annexin V has a high affinity for membrane phosphatidylserine (PS), thus FITC-labeled Annexin V can be used for the detection of outer membrane translocated PS in apoptotic cells. Apoptosis of cells treated with polyplexes containing control siRNA or EG5 siRNA was detected at 24 h (C–E) or 48 h (F–H) after transfection. At 24 h after treatment, the majority of the cells treated with control siRNA (Figure S8A, Supporting Information) or with Alb&INF siEG5 polyplexes were found as healthy cells in the FITC−/PI− Q4 zone. In the Tf&INF7 siEG5 group, the percentage of viable cells was reduced to 54%, with 22% of cells found in early apoptosis (FITC+/PI− Q3), and 24% of cells already in the FITC+/PI+ Q2 zone, presenting late apoptosis or necrosis. At 48 h after transfection, control siRNA-treated cells were again found as healthy cells (Figure S8B, Supporting Information). In Tf&INF7 siEG5 group, less than 8% of cells were in a normal viable state, 35% of cells were found in early apoptosis, and 57% of cells in late apoptosis or necrosis. In sum, both metabolic activity (MTT assay) and apoptosis (Annexin V-FITC/PI assay) demonstrated that Tf&INF7 siEG5 polyplexes are promising antitumoral nanoparticles. The observed antitumoral effect of EG5 gene silencing was reduced by TfR blockade using excessive Tf (Figure S9, Supporting Information), indicating the biological function of the targeting ligand Tf for cellular internalization of polyplexes via TfR-mediated endocytosis.

2.5. Cell Cycle Analysis of K562 Cells Treated with EG5 siRNA Polyplexes

EG5 knockdown blocks mitotic separation of daughter chromosomes, thus resulting in a G2/M stage arrest. Cell cycle analyses of polyplex-treated K562 cells at 24 or 48 h (Figure 5A or 5B) displayed no alterations for the groups transfected with control siRNA. Only in the Tf&INF7 siEG5 group, at 24 h after treatment a strong increase of cells in the G2 stage (more than 70% of cells) was observed, accompanied with a small abnormal sub-G1 peak. In contrast, no obvious changes were detectable in the Alb&INF siEG5 polyplex group. At 48 h, the G2 peak in the Tf&INF7-treated group was significantly reduced, meanwhile the sub-G1 peak had largely increased, indicating that more cells undergo apoptosis resulting in subcellular fragment. EG5 silencing at protein expression levels as determined by Western blot (Figure 5C) was found to be downregulated after transfection with Tf&INF7 454 siEG5 far more effectively than with INF7 454 siEG5 (some effect) or Alb&INF7 454 siEG5 (no effect). An especially strong downregulation was observed at 48 h. Due to EG5 gene knockdown, frequent mitotic aster formation was observed in cells at 24 h after transfection with Tf&INF7 siEG5 (Figure 5D) but not with Alb&INF7 siEG5-treated K562 cells (Figure S10, Supporting Information).

2.6. In Vivo Distribution

Polyplexes (50 µg siRNA (labeled with Cy7 to 50%) per mouse complexed at N/P 6 with 454 with and without further surface
modifications) did not show any sign of acute toxicity in mice. Near infrared (NIR) fluorescence bioimaging was performed to determine the distribution of polyplexes after tail vein injection. As shown in Figure 6, siRNA polyplexes spread throughout the whole body along with the blood circulation immediately after injection. NIR fluorescence indicated that siRNA largely accumulated in liver and lung both for the 454 and the Tf&INF7 group (Figure 6A,B). In addition, an early decay of fluorescence was observed, although slower than that with free siRNA (Figure S11, Supporting Information), indicating insufficient stability and in vivo disassembly of the siRNA polyplexes. Nevertheless, a notable tumor residence of Tf&INF7 but not plain 454 polyplex-delivered siRNA was detected after injection. Although no tumor NIR fluorescence signal was retained at 8 h after injection, quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Figure 6C,D) demonstrated a more than twofold enhanced AHA1 siRNA dose in tumor in the Tf&INF7 group as compared to the 454 group. Interestingly, this enhancement was not present in a Tf without INF7 group (Figure 6D), suggesting a Tf receptor-independent cause. Possibly the slightly larger size of Tf&INF7 (244 nm) as compared to 454 polyplexes (107 nm) may contribute to this type of passive entrapment.

3. Discussion

Gene silencing by small interfering RNA is a promising therapeutic option for nucleic acid medicines. To exploit its full potential, special modifications and/or carriers are required for successful intracellular delivery both in vitro and in vivo. While therapeutic siRNA delivery targeted to the liver is close to reach the medical market in patients, systemic tumor-targeted delivery is still in its infancy. Protease-antibody fusion proteins were designed as siRNA delivery vehicle, achieving gene silencing in targeted tumor cells but not in normal tissue. The serum protein transferrin (Tf) has been widely used as targeting ligand for the delivery of nucleic acids into
Tf may act not only as a targeting ligand but also as a shielding domain to reduce nonspecific binding. More than 80% targeted gene silencing and reduced tumor growth were reported with transferrin-shielded oligoethyleneimine derivative (OEI-HD) polyplexes containing therapeutic siRNA Ran in mice bearing N2a tumor. In another approach, a well-defined TIR-targeted siRNA delivery system was assembled by surface modification of cyclodextrin-oligocation siRNA complexes with adamantyl-PEG-Tf utilizing an elegant host–guest interaction. Intensive evaluation in pharmacological models was followed by the first phase I human clinical trial of systemic receptor-targeted siRNA delivery, successfully demonstrating gene silencing in tumors.

Our group recently designed a novel kind of siRNA carrier based on sequence-defined lipo-oligoamino amides, which are able to complex siRNA into nanoparticles of around 100 nm through electrostatic interactions between negatively charged siRNA and positively charged Stp building blocks, further stabilized by hydrophobic interactions from oleic acid dimers and tyrosine trimer residues, and covalent bonds formed between terminal cysteines, leaving additional surface free cysteines for functional modification. The precision of sequence-defined solid-phase-assisted synthesis enables the design of multifunctional carriers containing PEG shielding domains and small chemical or peptidic targeting ligands. However, as Tf is a 80 kDa large protein, direct chemical linkage with the novel class of rather small sequence-defined oligomers resulted in the loss of nucleic acid binding ability of such oligomer conjugates. The present work describes an alternative route for the successful preparation of TIR-targeted polyplexes. Through a surface modification of already formed siRNA lipo-oligomer complexes with Tf-PEG, not only the compaction of siRNA in the inner lipo-oligomer core is maintained but also surface shielding with Tf via a flexible PEG5K spacer is obtained. Superior cellular internalization of Tf-modified polyplexes into TIR overexpressing cells was detected by flow cytometry and also laser scanning confocal microscopy. Additionally, Tf blockade experiments verified TIR-mediated endocytosis as responsible for increased uptake of Tf-polyplexes, contributing to the observed enhanced gene silencing (Figure 3C and Figures S5 and S9, Supporting Information). On the contrary, surface modification of polyplexes with albumin decreased the uptake of polyplexes, probably because of loss of electrostatic attraction between cationic polyplexes and the slightly negatively charged cell membrane. Encouragingly, superior GFP gene silencing was also achieved with anti-murine TIR antibody-modified polyplexes (Figure S4, Supporting Information), indicating the procedure as applicable for modification with other large ligands.

Successful cytosolic release is indispensable for an efficient siRNA delivery system, because endolysosomal enzymes degrade the majority of internalized cargos and siRNA has its site of action in the cytosol. Although Tf-modified polyplexes display increased cellular internalization, this results in predominant entrapment in endolysosomes. Beneficial gene silencing activity was only detected in the presence of the endosomal release agent chloroquine (Figure 4). The Stp building blocks in lipo-oligomer exhibit endolysosomal buffer capacity and together with the membrane activity of incorporated oleic acids may trigger endosomal escape. However, this endosomal escape capability is greatly reduced by the shielding of polyplexes with 80 kDa Tf and 5 kDa PEG. Herein, INF7 peptide with endolysosomal membrane lysis ability had to be introduced into the siRNA polyplexes. Contributed by the endosomolytic activity of INF7, a significant increase of Tf&INF7 hemolytic activity was detected comparing with Tf polyplexes (Figure S14, Supporting Information). Notably, Tf&INF7 polyplexes showed significantly increased gene silencing in DU145 cells (Figure 4A) through the INF7 assisted cytosolic cargo release, comparable to Tf polyplexes with chloroquine. Both luciferase assay and GFP fluorescence detection demonstrated increased GFP gene silencing of Tf&INF7 containing siRNA in K562-GFP-Luc cells (Figure 2A and Figure S3, Supporting Information). Moreover, application of antitumoral EG5 siRNA polyplexes resulted in cell growth arrest in the G2/M stage, with the phenotype of mitotic aster formation well detectable by fluorescence microscopy. EG5 protein expression was reduced at 24 h after transfection with Tf&INF7 containing EG5 siRNA (Figures 5C), manifesting EG5 gene silencing in K562 cells. Interestingly, Alb-modified polyplexes were still inefficient in siRNA silencing even after linkage with INF7. Altogether the data suggest that both cellular internalization of polyplexes and their cytosolic release are essential steps for successful siRNA delivery. Efficient gene silencing with GFP or EG5 siRNA was achieved only by the combined effect of targeting ligand Tf and the endosomolytic peptide INF7.

First in vivo pilot studies revealed some advantages and disadvantages of the current siRNA polyplex system. Consistent with previous polyplex distribution studies, no signs of toxicity were observed, and the polyplexes largely accumulated in liver (Figure 6). The Tf&INF7 polyplexes, displaying a neutral zeta-potential caused by INF7 incorporation and shielding with Tf and PEG5K, experienced a reduced lung retention as compared to the positively charged polyplexes (Figure 6C). Encouragingly, both NIR fluorescence and qPCR studies verified that Tf&INF7 polyplexes are able to deliver siRNA into N2a tumors in mice. However, this accumulation was strongly hampered by insufficient in vivo polyplex stability and low circulation time. Interestingly, these stability problems were not predictable from ex vivo studies (Figure S15, Supporting Information). Encouraging stability ex vivo with 90% serum was detected with DLS as shown in Figure S15A,B (Supporting Information). Both polyplexes show an only slightly increased particle size, which remained the same for 18 h. Both and Tf polyplexes showed well siRNA compaction even after 24 h incubation with serum as shown in Figure S15C (Supporting Information). A related sharp discrepancy between ex vivo serum and in vivo blood circulation stability of RNA polyplexes was most recently also reported by Kataoka and co-workers, suggesting anionic macromolecules such as with high charge densities on cell surfaces as responsible factors for the difference. The low molecular weight of sequence-defined oligomers such as lipo-oligoamine, which is far smaller than classical transfection polymers, on the one hand contributes to the favorable biocompatibility but apparently on the other hand limits the siRNA polyplex stability. Disulfide cross-linkage and hydrophobic oleic acids and tyrosines stabilized polyplexes.
in vitro studies; however, these stabilization motifs are probably not enough for remaining their integrity in the more challenging in vivo environment, inducing polyplexes dissociation during circulation or in tumor before cellular uptake. Future research needs to aim at improved extracellular in vivo stability of this chemically precise class of siRNA carriers, such as by extension of the polycationic domain[31] or additional hydrophobic stabilization,[29,32] further cross-linkage of polyplexes before or after postmodification,[13] design of novel sequenced defined oligomers forming more stable siRNA complexes,[34] or sticky ended siRNA,[35] which make siRNA more DNA-like to stabilize polyplexes by enhanced electrostatic interaction with the cationic oligomers.

4. Conclusion

In summary, a TIR-targeted siRNA delivery system was developed based on the T-shaped lipo-oligomer 454 complexed with siRNA molecules into core nanoparticles, which were subsequently decorated with PEG-linked Tf as an 80 kDa large serum protein targeting ligand for surface shielding and cellular uptake, and INF7 as small pH-triggered peptide for endolysosomal escape. With such TIR-INF7-modified 454 siRNA polyplexes, TIR-dependent delivery and effective target gene silencing was demonstrated in several cell systems. Use of antitumoral siRNA directed against the EG5 gene resulted in potent tumor cell killing. In vivo distribution studies showed enhanced siRNA homing to the tumor for TIR-INF7 polyplexes. Further oligoamino amide core modifications will be required to improve the in vivo stability of polyplexes suitable for systemically targeted siRNA therapy. The presented postcoupling strategy for protein and peptide attachment to polyplexes opens the perspective also for alternative targeting proteins such as antibodies, as exemplified with anti-TIR 8D3, and other endosomolytic agents.

5. Experimental Section

Materials: Cell culture media Roswell Park Memorial Institute (RPMI)-1640, Dulbecco’s modified Eagle’s medium (DMEM), bovine serum albumin (Alb), and human transferrin (Tf) were obtained from Sigma-Aldrich (Munich, Germany). Anti-murine TfR antibody 8D3 (TfRab) was provided by Sanofi (Frankfurt, Germany). Antibiotics and antimycotics (penicillin, streptomycin, and 100 µg mL\(^{-1}\) of amphotericin B) were from Sigma-Aldrich (Munich, Germany). Fetal bovine serum (FBS) were purchased from Invitrogen (Karlsruhe, Germany). INF7 peptide (Glu-Ala-Glu-Thr-Ser) was from Polymere GmbH (Tuebingen, Germany). peQGOLD TriFast is from PEQLAB Biotechnology GmbH (Erlangen, Germany). INF7-TfRab: NHS-PEG5K-OPSS and contained TfRab 8D3 with 2.3 molar ratio point with branching at the α,ε-amino group of lysine was synthesized by solid-phase-assisted synthesis as previous reported.[19] Syntheses of OPSS-PEG5K-Conjugated Tf, Alb, and Anti-Murine TfR Antibody 8D3: Human transferrin (8.4 mg mL\(^{-1}\)) was dissolved in PBS pH 7.4 buffer (20 × 10\(^{-3}\) M HEPES, 150 × 10\(^{-3}\) M NaCl). To couple Tf with PEG5K, NHS-PEG5K-OPSS (50 mg mL\(^{-1}\)) in dimethyl sulfoxide (DMSO) was added into Tf solution with molar ratio Tf : PEG5K-OPSS 1:2, the mixture reacted for 3 h by shaking at 25 °C. Free NHS-PEG5K-OPSS was removed through ultrafiltration with 10 kDa pore size. The modification degree of Tf with PEG5K was measured by absorbance of the 2-pyridinethione chromophore at 343 nm with UV–vis after treated with 1,4-dithioothreitol (DTT). The Tf-PEG5KOPSS conjugate contained Tf with 1.2 molar ratio PEG5K-OPSS. Alb-PEG5K-OPSS was synthesized with the same procedure, with 0.8 molar ratio PEG5K-OPSS per albumin. Rat monoclonal anti-murine TfR antibody 8D3 TfRab-PEG5K-OPSS was synthesized analogously with react molar ratio 1:5 of TfRab: NHS-PEG5K-OPSS and contained TfRab 8D3 with 2.3 molar ratio PEG5K-OPSS.

Polyplex Preparation: 500 ng siRNA (siControl, siGFP, or siEG5) and oligomer 454 at N/P 6 were separately diluted with 20 × 10\(^{-3}\) M HEPES buffer with 5% glucose pH 7.4 (HBG buffer) to final volume 10 µL. The two solutions were mixed by pipetting up and down (about 15 times), further incubated for 45 min at room temperature. Tf-PEG5KOPSS or Alb-PEG5KOPSS with different molar ratio to oligomer was added into the mixture with gently shaking 300 rpm on Thermomixer comfort (Eppendorf, Hamburg, Germany) for 15 min at room temperature. Finally, for polyplex modification with INF7, INF7-TNB (10 mol% activated INF7 through reacting with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)) peptide was added into the solution and incubated for 15 min with gently shaking at room temperature. Agarose Gel-Shift Assay: A 2.5% agarose gel was prepared with GelRed (Biotium, Inc., Hayward, CA, USA) for siRNA detection. Polyplexes were prepared with 500 ng siRNA in 20 µL then mixed with loading buffer. Electrophoresis was performed at 80 V for 40 min.

Particle Size and Zeta Measurement: Polyplexes were prepared as described above with a final siRNA concentration of 25 µg mL\(^{-1}\) in HBG buffer (unless indicated differently). Sizes and zeta potentials were measured using a Zetasizer Nano ZS (Malvern Instrument, Herrenberg, Germany), at 25 °C.

Transmission Electron Microscopy: Polyplexes were negatively stained with 1% phosphotungstic acid pH 7.0, then characterized by TEM with a JEM-1011 (JEOL) operating at 80 kV.

Cell Culture: Human chronic myelogenous leukemia K562 cells, K562-eGFP-Luc cells stably transfected with an eGFP-Luc fusion gene, and human prostate carcinoma DU145 cells were cultured in RPMI 1640 medium. Mouse neuroblastoma N2a cells and N2a-eGFP-Luc cells, stably transfected with the eGFP-Luc fusion gene, were grown in DMEM medium at 37 °C in 5% CO\(_2\). All media were supplied with 10% FBS, 100 U mL\(^{-1}\) penicillin, and 100 µg mL\(^{-1}\) streptomycin.

eGFP-Luc Gene Silencing Measured with a Luciferase Assay: eGFP gene silencing was performed in 96 well plates with 1 × 10\(^{4}\) K562-eGFP-Luc suspension cells or 5 × 10\(^{3}\) N2a-eGFP-Luc cells per well with 500 ng control siRNA or GFP siRNA polyplexes. The transfection medium was replaced with fresh medium after 1 h incubation with polyplexes for K562-eGFP-Luc cells and 4 h for N2a-eGFP-Luc cells. Cells were cultured for 48 h before determination of luciferase activity. Luciferase activity of cell lysates was measured using a Centro LB 960 plate reader luminometer (Berthold Technologies, Bad Wildbad, Germany) and a luciferin-LAR (1 µM glycylglycine, 100 × 10\(^{-3}\) M MgCl\(_2\), 500 × 10\(^{-3}\) M ethylenediaminetetraacetic acid (EDTA), DTT, adenosine 5′-triphosphate (ATP), coenzyme A) buffer solution. The relative light units were measured.
presented as percentage of the luciferase gene expression obtained with buffer-treated control cells.

**Cytotoxicity Mediated by EGS Gene Silencing (MTT Assay):** K562 suspension cells were seeded in 96 well plates, 1 × 10^4 cells per well 2–3 h before transfection. DU145 cells or N2a cells (5 × 10^4 per well) were seeded in 96 well plate at 24 h before transfection, with the medium exchanged for fresh serum-containing RPMI 1640 right before transfection. K562 or DU145 cells were incubated with control siRNA or EGS siRNA polyplexes (0.5 µg siRNA per well) for 1 h, in case of N2a cells for 4 h, then medium was exchanged by fresh one. When indicated, 100 × 10^{-6} M chloroquine was added and cells were further incubated for 4 h, followed by another change of medium. Cells were further incubated for 48 h. For Tf competition experiments, cells were incubated with free iron saturated Tf (5 mg mL^{-1}) on ice for 30 min to block TfR before polyplexes added. To measure metabolic activity, 10 µL MTT (5 mg mL^{-1}) was added into each well and incubated for 2 h at 37 °C until formazane formed. Finally formazane (solved by DMSO) absorbance was measured at 590 nm (with 630 nm as reference wavelength) using micro plate reader (Spectrafluor Plus, Tecxan Austria Gmbh).

**Cellular Internalization of siRNA Polyplexes:** K562 suspension cells were seeded in 24 well plates with 1.2 × 10^6 cells per well 2–3 h before the experiment. DU145 cells were seeded with 5 × 10^4 cells per well at 24 h before, and fresh media were provided before the experiment. Polyplexes containing 1.35 µg siRNA (including 20% Cy5-labeled siRNA) per well were added into each well. After 45 min incubation at 37 °C in 5% CO_2, cells were washed with phosphate-buffered saline (PBS) for three times to remove free polyplexes, then incubated with 100 µL heparin to remove polyplexes nonspecifically associated to the cell surface. After washing with PBS, cells were collected and resuspended in PBS buffer with 10% FBS. For Tf competition experiment, cells were incubated with free Tf (5 mg mL^{-1}) for 30 min on ice to block TfR before polyplexes added. Internalization of the polyplexes was measured by flow cytometry with CyanTM ADP (Dako, Hamburg, Germany) through excitation at 635 nm and detection of emission at 665 nm. Dead cells were detected by 4′,6-diamidino-2-phenylindole (DAPI) fluorescence and removed by gating in order to analyze polyplex uptake into living cells. Data were analyzed by Flowjo 7.6.5 flow cytometric analysis software. Polyplexes were incubated with K562 cells for 2 h, and Hoechst dye was used as nucleus staining probe. Cellular internalization was observed by laser scanning confocal microscopy (Leica TCS SP8, Germany).

**Measurement of Apoptosis with an Annexin V-FITC/PI Assay:** K562 cells were seeded in 12 well plates with 8 × 10^4 per well and transfected with polyplexes (prepared the same as polyplex preparation part with 1.5 µg siRNA per well) containing control or EGS siRNA. Cells were collected by centrifuge at 24 or 48 h after transfection, then apoptosis was detected with an Annexin V-FITC/PI assay (BioVision) by Cyan ADP flow cytometry. Data were analyzed by Flowjo 7.6.5 flow cytometric analysis software.

**Cell Cycle Analysis:** K562 cells transfected with control siRNA or EGS siRNA (the same concentration as in the apoptosis experiment) were collected by centrifugation at 24 or 48 h after transfection and washed with PBS, then incubated with 100 µL propidium iodide staining solution (0.1% sodium citrate, 0.1% Triton X-100, 50 µg mL^{-1} propidium iodide in Millipore water) for 3 h on ice in dark. Cells were centrifuged after adding 1 mL PBS buffer and resuspended in 500 µL PBS and measured with the CyanTM ADP flow cytometer. Data were analyzed by Flowjo 7.6.5 flow cytometric analysis software.

**Fluorescence Microscopy for Mitotic Aster Formation:** K562 cells transfected with control siRNA or EGS siRNA (the same concentration as in the apoptosis experiment) were collected by centrifugation and washed with PBS. For fixation, cells were incubated with 4% paraformaldehyde for 30 min at room temperature. After washed by PBS, nuclei were stained by DAPI for 15 min at room temperature. Cell samples were analyzed by fluorescence microscopy.

**In Vivo Experiments:** Nude mice (Rij; NMRI-nu (nu/nu), 6 weeks old) were purchased from Janvier, France and housed in a 12 h day and night cycle. N2a cells (5 × 10^6) were injected subcutaneously into the left flank. Bioluminescence experiments were performed about 10 d after tumor cell injection with three mice per group. Polyplexes, prepared as previously described containing 50 µg AHA1 siRNA (50% Cy7 labeled) at N/P 6 in 250 µL HBGC buffer, were injected into the tail vein. NIR fluorescence bioimaging was performed at 0 min, 30 min, 1 h, and 8 h after injection with a charge coupled device (CCD) camera using IVIS Lumina system with Living Image software 3.2 (Caliper Life Sciences, Hopkinton, MA, USA) for the subsequent analysis. Animals were sacrificed 8 h after the treatment. Organs (including lung, liver, kidney, and spleen) and tumors were collected for further investigation by qRT-PCR. All animal experiments were performed according to the guidelines of German law for animal protection.

**In Vivo siRNA Accumulation Analyzed with Real-Time qRT-PCR:** Total RNA was isolated from organs and tumors of different groups using peqGOLD TriFast method (PEQLAB, Germany), and cDNA synthesis was performed using 1 µg of total RNA with qScript microRNA cDNA synthesis kit (Quanta BioScience, USA). qRT-PCR was implemented with PerfeCTa SYBR Green SuperMix (Quanta BioScience, USA) on a LightCycler 480 system (Roche, Germany) using mir-191 as housekeeper. Primers used are shown as follows: mir-191 forward: GCCCAACCGGCAATCCCACAAAG, AHA1 forward: GAGACTATCTCCACACTC (Sigma-Aldrich, Germany). ΔCt is the difference between Ct (threshold cycle) values of the measured RNA of interest and the housekeeper RNA, meaning a normalization to the housekeeper. Thus, 2^-ΔΔCt represents the relative quantity of the target RNA. It assumes an exponential growth and a doubling of product in each PCR cycle.

**Hemolysis Assay:** After washed with PBS, human red blood cells (obtained from LMU Clinics—Campus Grosshadern, Munich, Germany) were diluted to 5 × 10^8 erythrocytes per mL with PBS pH 7.4, 6.5, or 5.5. INF7 peptide or polyplexes 75 µL in PBS with indicated pH was added to a V-bottom 96-well plate (NU NC, Denmark). Wells containing 1% Triton X-100 in PBS were positive controls for 100% lysis. PBS-treated wells with indicated pH were used as negative controls. Erythrocyte suspension previously prepared 75 µL was added to each well, resulting in a final concentration of INF7 0.5, 1, or 3 × 10^{-6} M, and 454 oligomer 2 × 10^{-6} M for modified polyplexes. The plates were incubated under constant shaking for 1 h at 37 °C. After centrifugation, hemoglobin release in 80 µL supernatant was measured at 405 nm with a microplate reader (Tecan Spectrafluor Plus, Tescan, Switzerland). Relative hemolysis activity was defined as hemolysis activity (%) = (A_{0,05} (samples) / A_{0,05} (PBS)) × (A_{0,05} (Triton X) / A_{0,05} (PBS)) × 100 and presented as the mean ± SD (n = 3).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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