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Petra Prohaska

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Abstract

Only twenty years ago RNA interference with double stranded RNA was observed in Caenorhabditis elegans and opened the door for a new promising method in disease therapy, though with some limitations. In the meantime, many of those limitations such as off-target effects, unintended immune response and instability against serum nucleases have been overcome through research and development of modifications within the double stranded RNA and conjugates with polymers and lipids. Still, obstacles like rapid elimination through the kidney and the reticuloendothelial system and retention in the endosome, which leads to inactivation and degradation through lysosomes, remain.

This diploma thesis will discuss the effect of a previously developed 4-arm spacer loaded equimolarly with four distinct siRNAs. Those are directed against the production of different proto-oncogenes which are all overexpressed in the human breast cancer cell line HeLa.

After the successful synthesis of the oligonucleotides and their complementary DNA-overhangs for self-assembly to the 4-arm complex the sequences were verified via denaturing polyacrylamide gel electrophoresis. Through a 2.5% agarose gel the successful assembly of the synthesized siRNA with the 4-arm scaffold was determined. The construct was stable in serum-supplemented buffer over 48 hours. Then the efficacy of the delivery system was tested in cell experiments with HeLa cells through recording the inhibition of the respective proto-oncogenes via quantitative real-time reverse transcriptase PCR. The evaluation showed a correlation of the effect with the applied concentrations of siRNA and lipofectamine, a cationic transfection agent. The potential synergistic effect on tumor inhibition, which was assumed because of the attack on multiple targets in the arising of cancer, was overshadowed by interactions around RISC, the core complex in the RNA interference machinery. This competition was observed especially in higher concentrations. The 4-arm scaffold resulted in equivalent gene silencing in comparison to standard siRNA mixtures. In contrast, a luciferase assay showed more potent RNA interference of the 4-arm complex with four molecules of siRNA than of uncomplexed siRNA.

The gathered data indicates that exact conditions of lipofectamine-mediated transfection are essential for successful and potent silencing of the self-assembled

constructs and play an important role for toxicity and competitive inhibition. The oligonucleotide scaffold provides many benefits such as higher stability against nucleases and the equimolar delivery of multiple or combinative therapeutic agents.

Zusammenfassung

Vor nur 20 Jahren wurde RNA Interferenz mit doppelsträngiger RNA in Caenorhabditis elegans beobachtet und öffnete das Tor für eine neue vielversprechende Methode in der Krankheitstherapie, wenn auch mit einigen Limitationen. Inzwischen wurden viele dieser Limitationen wie off-target Effekte, unerwünschte Immunantworten und Instabilität gegenüber Serumnukleasen durch Erforschung und Entwicklung von Modifikationen an der doppelsträngigen RNA und Konjugate mit Polymeren und Lipiden überwunden. Dennoch bleiben Hindernisse wie die rasche Elimination durch Niere und retikuloendotheliales System und die Retention im Endosom, die zu Inaktivierung und Abbau durch Lysosomen führt, erhalten.

In dieser Diplomarbeit wird die Wirkung eines zuvor entwickelten 4-Arm Spacers, der äquimolar mit vier verschiedenen siRNAs beladen ist, untersucht. Diese siRNAs richten sich gegen die Produktion unterschiedlicher Proto-Onkogene, welche alle in der humanen Brustkrebszelllinie HeLa überexprimiert vorkommen.

Nach erfolgreicher Synthese der Stränge und ihrer komplementären DNA-Überhange zur Bindung an den 4-arm Komplex wurden die Sequenzen mittels denaturierender Polyacrylamid-Gelelektrophorese überprüft. In einem 2.5%igen Agrosegel wurde die erfolgreiche Assemblierung der synthetisierten siRNA mit dem 4-Arm Gerüst festgestellt. Das Konstrukt war über 48 Stunden in serumhältigen Puffer stabil. Anschließend wurde die Effektivität des Transportsystems in Zellversuchen an HeLa Zellen getestet indem die Inhibition der entsprechenden Proto-Onkogene via quantitativer real-time reverse Transkriptase PCR erfasst wurde. Die Auswertungen zeigten eine Abhängigkeit von der applizierten Konzentration an siRNA und Lipofectamin, einem kationischem Transfektionsreagenz. Ein potentieller synergistischer Effekt auf die Tumorinhibition, der durch den Angriff unterschiedlicher Ziele in der Krebsentstehung vermutet wurde, wurde durch Interaktionen rund um RISC, den Kernkomplex der RNA Interferenz Maschinerie, überschattet. Diese Konkurrenz wurde vor allem in höheren Konzentrationen beobachtet. Das 4-Arm Gerüst erzielte die gleichen Silencing-Aktivitäten im Vergleich zu den einzelnen siRNAs. Im Luciferase Assay wurde dagegen eine potentere RNA Interferenz des mit vier

Molekülen siRNA beladenen 4-arm Komplexes gegenüber einzelner siRNA ersichtlich.

Die erfassten Daten zeigen, dass exakte Bedingungen der Lipofectamin-Transfektion essentiell für erfolgreiches und potentes Silencing des selbst-assemblierten Konstrukts sind und eine ausschlaggebende Rolle in Bezug auf Toxizität und kompetitive Inhibition spielen. Das Oligonukleotid-Gerüst bietet viele Vorteile in der RNA Interferenz wie erhöhte Stabilität gegenüber Nukleasen und dem gezielten äquimolaren Transport von multiplen oder kombinativen therapeutischen Agentien.

1 Introduction

1.1. RNA interference

RNA interference (RNAi), the mechanism of double-stranded RNA (dsRNA) to manipulate gene expression[1], was first observed in the nematode worm Caenorhabditis elegans in 1998[2]. While purified single stranded antisense and sense RNAs only showed marginal gene silencing, dsRNA was not only more potent but interference even persisted into the next generation. This heritable occurrence does not apply to Drosophila and mammalian cells, but the core mechanism of RNAi remains the same.[3]

After endocytosis of dsRNA the enzyme Dicer of the RNase III ribonuclease family cleaves the dsRNA under ATP consumption into sequences of about 22 nucleotides.[4,5] Then the formed double stranded short interfering RNA (siRNA) is loaded onto RNA-induced silencing complex (RISC) which is initially still inactive.[1] Only after the protein Argonaute 2 (Ago2) dissociates the double stranded RNA under ATP consumption into single stranded RNA, RISC is activated.[1,6] The strand interacting with RISC is called guide or antisense strand while the other one is termed passenger or sense strand.[7] Through Watson-Crick base-pairing with its complementary target mRNA and Ago-nuclease activity gene silencing is induced (Figure 1).[3,7]

The gene silencing machinery does not only apply to nematode worms, flies and mammals, but also to plants, moths, fish and the fungus Neurospora[3,5] which indicates genesis in early RNA-development.[8]

1.1.1. Types of RNAi

There are three major types of RNAi therapeutics: miRNA, shRNA and siRNA.[9] Though RISC is their core in the gene silencing mechanism, they differ in efficiency and specificity.[1]

The natural non-coding single stranded micro-RNA (miRNA), which is transcribed and spliced into its functional equivalent over several steps, does not bind perfectly with its target mRNA. Because of the size of 70 nucleotides of the precursor hairpin RNA, it can be transformed into more than one siRNA, and the imperfect complementarity can easily lead to potential interaction with many target mRNAs[10].

Concurrent silencing of several genes can boost functional outcomes, but this feature can also enhance the risk of off-target effects.

Short hairpin RNA (shRNA) consists of a single RNA strand with self-complementary passages, which align to a small loop.[11] To be cleaved into siRNA, shRNA needs to be processed by Dicer.[12] Even though plasmid-generated shRNA is generally more potent in gene knockdown and has a longer effect than the use of siRNA, it has more obstacles to overcome like the passage into the nucleus of the cell to be transcribed into its effective counterpart.[13,14]

With a length of 19-30 nucleotides short interfering RNA (siRNA) has the advantage of a low molecular weight which facilitates the delivery into the cell and enables modifications for stabilization.[1] Further less off-target effects are to be expected because of its perfect complementary fit to its target mRNA.

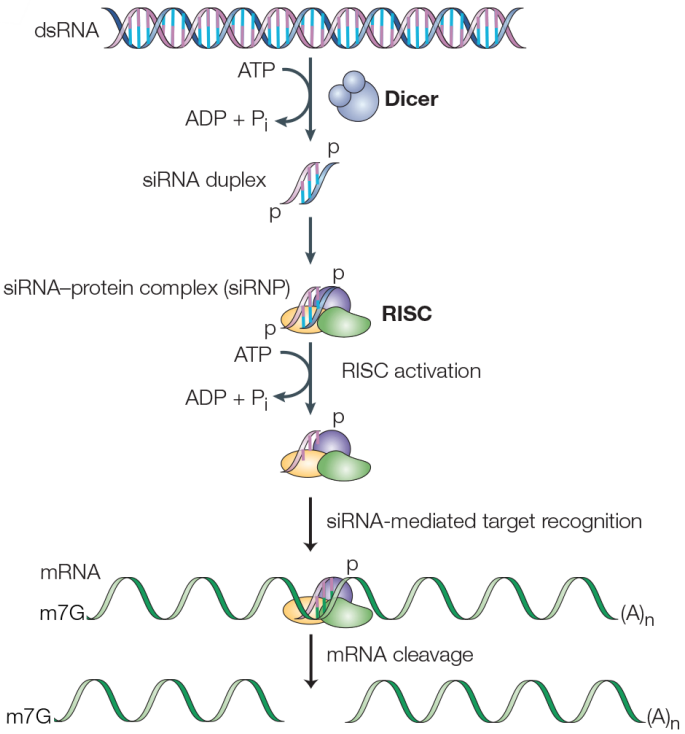

Figure 1 pictures the process of RNA interference: The double stranded siRNA is cut by the Dicer into duplexes of ~22 nucleotides. Those are loaded onto RISC where Ago2 cleaves the strands to activate the complex. Through Watson-Crick base-pairing the guide strand targets its complementary mRNA which is cleaved and thus made ineffective for transcription. (Adapted by permission from [6], © 2003 Nature Publishing Group)

1.2. Barriers and carriers

One of the most challenging hurdles of siRNA therapeutics is their delivery into the cytoplasm of the target cells.[1,15] The transport of RNA into the cell is nothing else than the imitation of viruses and bacteriophages which also use DNA or RNA to modulate expression for their purposes. As an answer the organism put many obstacles in the path to prevent the intrusion of external DNA and RNA. On the one hand many of those obstacles can be avoided through choosing more efficient application routes like local delivery to target organs such as the eye or the nose[1] and intravenous injection[16] but to unfold the full potential of RNAi we need to overcome those barriers.

Extracellular defense mechanisms of the organism against foreign DNA and RNA include degradation through nucleases, phagocytosis, elimination through the reticuloendothelial system (RES) and glomerular filtration in the kidney and extravasation, though the last one can be exploited with suitable sizes of nanoparticles to accumulate siRNA into tumor cells.[16,17]

The degradation through serum nucleases can be reduced through chemical modifications of the backbone and with the help of nanocarriers.[1,7] To avoid the activation of phagocytes lipophilicity, stability, size and the electrostatic charge of the carrier have to be taken into account.[16] While enhanced lipophilicity and stability reduce the detection by the scavenger cells of the immune system, size and charge should be kept small, ideally below 200 nm. On the other hand, particle size below about 10 nm induces increased elimination in the kidney.

The passage through the cell membrane is also hampered by the negative charge, large molecular weight and hydrophilicity of siRNA[1]. Intracellular obstacles are digestion in lysosomes after endocytosis[16] and unintended off-target effects as well as immune responses through Toll-like receptors (TLR) and protein kinase R (PKR)[17], though TLR-mediated immune response can be abrogated by insertion of 2'-O-methyl uridine or guanosine into the passenger strand of siRNA.[1,7] (Figure 2)

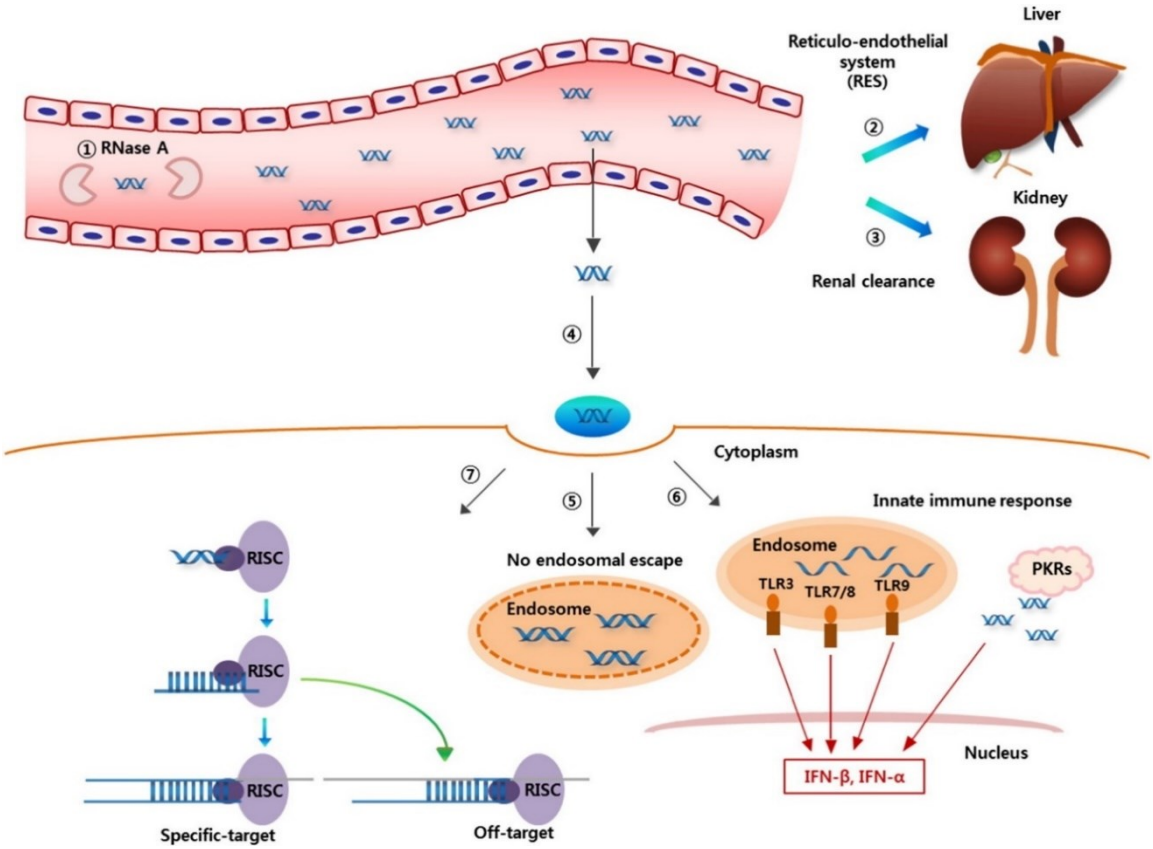

Figure 2 shows the extra- and intracellular barriers of RNAi delivery: ① symbolizes the degradation through serum nucleases in the blood system. The enhanced elimination through RES ② and glomerular filtration ③ depict further obstacles. ④ indicates the poor transportation through cell membranes. In the cell entrapment in endosomes ⑤, which leads to digestion in lysosomes on the one hand and on the other hand to unwanted immune responses through TLR and PKR ⑥, and off-target effects caused by interaction with non-target mRNA ⑦ represent additional hurdles. (Reprinted from [17], © 2016, with permission from Elsevier)

Now that we captured the obstacles of RNAi delivery, what are possible solutions to overcome them? For one thing the siRNA itself can be modified, another method is the conjugation with receptor ligands or packaging into nanoparticles.[17]

In siRNA, modifications in the backbone like phosphorothioate-groups and the ribose such as 2'-O-methylation and locked nucleic acid (LNA) promote stability and reduce immune response, though too many of each modification respectively can induce toxic or decreased RNAi effects.[1,7,17] Next to the already described major types of RNAi further structures of siRNA have been developed to enhance potency and stability or to reduce off-target effects like dumbbell shaped RNA, a variation of shRNA with one more loop, asymmetric RNA duplexes and small internally segmented interfering RNA.[17]

Possible conjugation compounds for siRNA are cholesterol and α-tocopherol which induce lipophilicity and thereby enhance stability and uptake into organs such as

liver and jejunum.[15,17] Fusion with polymers like polyethylene glycol (PEG) or polyethyleneimine (PEI) additionally avoid immune response and toxicity through reducing the electrostatic charge[1,17,18] while folate and lactose can induce targeted delivery through receptor binding, likewise aptamers bind to their molecular targets.[15,17]

Even though many systems for siRNA delivery have been developed some obstacles remain to be mastered especially in systemic delivery.[19]

1.3. Therapy with RNAi

After the discovery of RNAi in 1998[2], the first treatment in clinical trial through siRNA was in patients with age-related macular degeneration (AMD) in 2004[1,7]. In the meantime several companies like Alnylam Pharmaceuticals (USA), Santaris Pharma (Denmark) and InteRNA Technologies (Netherlands) have been established which focus on the development of RNAi therapeutics.[20]

Due to the various hurdles of RNAi delivery many therapeutics enrolled in clinical trials are indicated for local and topical use[20] or because of the entrapment in RES for organs with high accumulation such as the liver[1]. Patisiran, also known as ALN-TTR02, is a lipid nanoparticle formulation of an siRNA targeted at the gene transthyretin in hepatocytes and has recently completed clinical trials and is currently reviewed for approval in Europe and the USA.[19,20] Some further examples of RNAi drugs in clinical trial are as follows: ALN-RSV01 as treatment of respiratory syncytial virus infection, PF-04523655 against AMD and diabetic macular edema, ApoB SNALP (apolipoprotein B stable nucleic acid lipid particles) a siRNA targeting LDL cholesterol in patients with hypercholesterolemia, Excellair treating inflammatory disorders such as asthma, ALN-VSP as treatment for liver cancer and many more.[1,7,15,20]

Mipomersen is the first siRNAs for systemic administration, approved in 2013 in the US for management of homozygous familial hypercholesterinemia[21], but was declined in Europe because the company wanted to apply it to a wider patient range which raised the question of safety.[15] The next RNAi and first miRNA-targeted therapeutic in human clinical trials is miravirsen targeting miR122 in patients with hepatitis C virus infection.[15,20,22]

Even though not all clinical trials were successful, for example the trial of Bevasiranib was stopped because of its unsatisfying performance[20], the research of RNAi and improvements in its delivery open new possibilities in the treatment of various diseases.

1.4. DNA/RNA nanoparticles

While delivery systems such as polymers and lipids with their rather heterogeneous characteristics make the prediction of pharmacodynamics and pharmacokinetics of their attached drugs difficult, DNA/RNA nanoparticles overcome this obstacle and show further advantages such as the possibility to produce any clearly defined shape and size, the transportation of multiple siRNAs and potentially less immunogen reactivity.[17]

The correct assembly of those complexes based on base-pairing is only dependent on certain temperatures above about 45°C and a slow nucleation process, which keeps the production simple.[23,24] Several structures have been built with this method to transport multiple siRNAs for example packaging RNA (pRNA), the RNA nanoring and tetrahedron oligonucleotide nanoparticle (ONP).[17] (Figure 3)

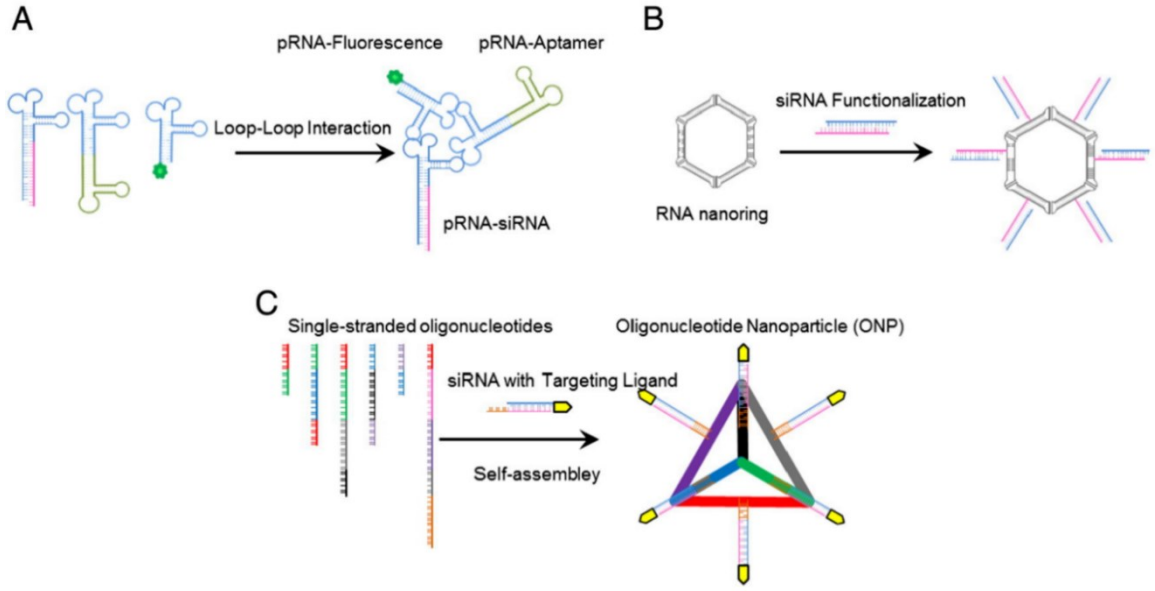

*Figure 3 depicts examples of DNA/RNA nanocarriers as delivery agents for siRNA and their assembly: pRNA (**A**), the RNA nanoring (**B**) and ONP (**C**). (Adapted from [17], © 2016, with permission from Elsevier)*

pRNA has been developed out of a bacteriophage and contains a dsRNA helical domain and an interlocking domain with two loops.[25] Those loops can align complementarily to build structures of more than one pRNA, to transport several siRNAs or other functional compounds at once.[25,26]

The RNA nanoring is a complex of 6 loop sequence-modified RNA modules which self-assemble into a hexamer ring onto which 6 siRNAs can be attached.[27,28]

ONP is an assembly of 6 DNA strands with overhangs attached to each edge so that siRNA with complementary overhangs can hybridize onto the structure.[29] The

size of this complex avoids renal clearance and enhances the accumulation on the tumor site. Not only does ONP show successful gene silencing without cationic carriers, also a longer blood circulation could be detected in vivo.

1.5. sticky siRNA

Another method to stabilize siRNA against nucleases is to polymerize themselves. siRNA with overhangs of 5 to 8 deoxy-adenosine or deoxy-thymidine rich 3‘-overhangs are called sticky siRNA (ssiRNA).[18] Through synthesis of complementary overhangs sticky siRNA can form “gene-like” double strands of RNA which induce better stability (Figure 4). The study also showed that this feature protects the RNA from the degradation through nucleases and induces in combination with the transfection agent polyethyleneimine (PEI) more potent silencing in vitro. Further no immunogen responses were detected and in vivo application in the mouse also determined the higher stability and more potent gene silencing of ssiRNA next to siRNA.

Non-complementary ssiRNA too pictured enhanced RNAi, but still complementary overhangs induce more potent silencing.[30] Another interesting result was that the potency of gene silencing is dependent on length, nature and flexibility of the overhangs in combination with the fifth generation triethanolamine core poly-amidoamine (TEA-core PAMAM) dendrimer G5.

Further studies demonstrate successful in vitro and in vivo gene silencing in prostate cancer and melanoma indicating the potential lying within sticky siRNA.[31,32]

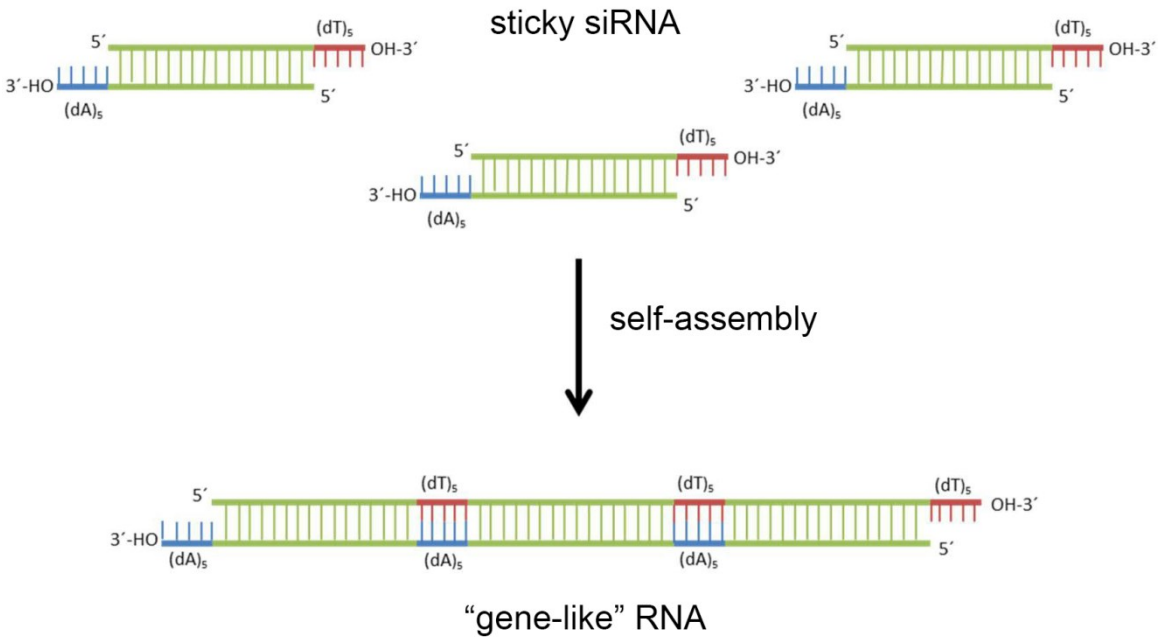

Figure 4 depicts the self-assembly of sticky siRNA into “gene-like” RNA. (Adapted from [33])

For this thesis four different sticky siRNAs with DNA-overhangs were synthesized and attached to a 4-arm spacer out of DNA silencing genes coding for the following proteins: aurora kinase (AURK), GRP78, β-catenin (CTNNB) and Myc2 (MYC). All of them are overexpressed proto-oncogenes in HeLa, the immortal human breast cancer cell line.[34–37]

1.5.1. Aurora kinase

Aurora kinases play an important role during mitosis and have been identified as proto-oncogenes due to their induction of polyploid cells with multiple centromeres in many types of cancer. Though they appear to induce instability in chromosomes when overexpressed, other factors are needed to develop carcinogenesis.[34,38]
In mammals three types have so far been identified: Aurora kinases-A, -B and -C which are activated through autophosphorylation and phosphorylate further substrates, which are important in mitosis.[34,38] Their name originates from the resemblance of Aurora kinase-A to the northern lights, aurora, in screening of Drosophila mutants with defects at the mitotic spindle apparatus because of its location in the spindle poles.[39,40] There Aurora kinase-A supports the assembly and stability of the spindle and further plays a role in the maturation of duplicated centromeres.[34,38] One of its substrates is the tumor suppressor p53 which is inactivated and degraded in cells overexpressed with Aurora kinase-A.
Aurora kinases-B and -C have similar functions though Aurora kinase-C is under normal expression only detectable in testis[38], whereas in cancer cells like HeLa it is observed outside of testis.[34] Those kinases are a part of the chromosome passenger protein family and assist in the correct localization of kinetochores during mitosis and face an important role during cytokinesis.[41]

1.5.2. GRP78

GRP78 is the acronym for glucose-related protein 78 and got its name from its enhanced appearance in cells with glucose shortage and its weight of 78 kDa.[42] Other names for this protein are binding immunoglobulin protein (BiP) and heat shock 70 kDa protein 5 (HSPA5), the last synonym indicating its family relationship with the heat shock proteins. As its related proteins GRP78 is a chaperone which folds proteins, induces degradation of misfolded ones and plays a role in transportation.[36] Its activation is caused by unfolded proteins which escape the overworked endoplasmic reticulum to reduce their physiologic stress.[43] This stress is non-transient in tumors because of their uncontrolled growth, leading to overexpression of GRP78. On the one hand GRP78 can be used as indicator for tumors, but also as a target in cancer therapy because of its malfunctions due to overexpression. In high concentrations GRP78 triggers resistance to apoptosis and its increased appearance on the surface of cells can even lead to enhanced proliferation and metastasis.[44]

1.5.3. β-catenin

In the cell β-catenin has two tasks: one is structural, the other refers to signaling.[45] Its name originates from the Latin word catena, meaning chain, due to its binding to E-cadherin with which it indirectly modulates the cytoskeleton of the cell.[46] Free β-catenin is either phosphorylated for degradation or it forms a complex with APC, Adenomatous polyposis coli (a tumor suppressor).[45] For the signaling function its degradation is stopped by Wnt-1 and it translocates into the nucleus where it connects to transcription factors to induce the synthesis of Wnt-1 and β-catenin. Wnt-1 further plays a role in tissue homeostasis, cell renewal and regeneration. In case of cancer, the balance between the structural and signaling function of β-catenin is disturbed and high cytoplasm concentrations are found.[35] Those inhibit regulators like NF-κB, which is important for inducing apoptosis through Fas, and p53. Furthermore, β-catenin can form a complex with T-cell factor and enhance Wnt-1 signaling which causes resistance to apoptosis inducing chemotherapeutical drugs.[47]

1.5.4. Myc2

The family of MYC received its name from its association with myelocytomatosis in early development.[48] The gene is able to express two different proteins through different upstream promotors located before the start codon: Myc1 and Myc2.[48,49] Though many functions of MYC are not jet fully unraveled, we know that it is involved in ribosome biogenesis, cell growth and cell proliferation through transcriptional and translational mechanisms.[37] Myc2 seems to additionally be important in the stress response of cells.[48] Because of its important role and therefore being a perfect point of attack in the cell cycle, the expression of MYC is regulated by many factors such as growth factors, availability of nutrition and checkpoints like p53.[37] The loss of those controls lead to overexpression and MYC can even become independent to growth factors. As a result, the emerging malfunctions include amplification of gene expression, angiogenesis, metastasis and cell migration.

2 Objective

As a previously developed 4-arm spacer showed promising results concerning stability against nucleases and luciferase gene silencing[33], the next step to test its potency had to be taken. This thesis discusses the efficacy of the 4-arm scaffold hybridized with four different siRNAs for concurrent silencing of four targets. In consideration of many factors triggering the initial development of cancer and its further growth, the possibility of synergistic effects with different targets enhancing the gene silencing had to be researched. The synthesized siRNAs were chosen to be effective in HeLa cells even though different pathways in cancer development were addressed and DNA-overhangs were added for the attachment to the 4-arm spacer.

For synthesis RNA-phosphoramidites were selected because of their high gains and low synthesis of unwanted symmetric dimers while for the DNA-overhangs, which were chosen to respectively attach to the complementary strands in the 4-arm spacer, derivatives with 2'-OH-methylated ribose were added to reduce the immune response and increase stability.

For transfection and endosomal escape, the cationic transfection agent Lipofectamine RNAiMAX was used. Further a comparison of different concentrations of lipofectamine was made to evaluate the effectiveness on the one hand and on the other hand to allow a better comparability with the positive control of double stranded siRNA due to the differences in molecular weight and negative charge. Also, various concentrations of the oligonucleotide complex were arranged to capture the most potent gene silencing activity and verify concentration-dependent silencing.

For verification of successful hybridization, stability and transfection into the cell denaturing polyacrylamide gel electrophoresis, stability test on a 2.5% agarose gel and a Luciferase assay with normalization to the protein concentration by a Bradford assay were performed.

3 Materials and methods

3.1. Synthesis of siRNA and its purification

3.1.1. Synthesis of RNA-tiles and its DNA strands

All strands were synthesized by a POLYGEN DNA-Synthesizer from PolyGen GmbH (Langen, Germany) with the oligo synthesis manager from POLYGEN as software. The RNA and DNA building blocks and all reagents used for the oligonucleotide syntheses were from Sigma Aldrich/Merck (SAFC, Proligo). The resins applied were CPG rU, rC, rG and rA. For the synthesis of RNA DMT-2'O-TBDMS RNA-phosphoramidites were used, for the partially 2'-methoxylated DNA-synthesis DMT-2'O-Me-rAdenosine and -rGuanosine phosphoramidites and DMT-dCytidine and -dThymidine phosphoramidites were utilized.

For synthesis phosphoramidite method, where the strands are stepwise build from 3' to 5', was conducted under the absence of air with the help of argon. The reagents for the four synthesis steps were the following: For detritylation, the removal of DMT protection groups from 5'-OH, TCA Deblock was supplemented. For the coupling of the nucleic acids ETT activator was used. For the capping, which is the acetylation of all free 5'-OH to prevent the subsequent attachment of nucleotides (false sequences), CAP A and CAP B were mixed directly in the synthesis column. And for the last step, the oxidation of the phosphite to a phosphate, the Oxidizer 0.02 M was supplemented.

3.1.1.1. Synthesis of siRNA

First the RNA building blocks were removed from the -20°C refrigerator and the cleaned and dried glass vials from the heating oven. To warm them up to room temperature under exclusion of moisture, both were put in a desiccator. Before applying the RNA phosphoramidites into the POLYGEN Synthesizer, 300 mg of each were dissolved in 4.5 ml water-free acetonitrile (diluent).

The detritylation was monitored and the 5'-DMT of the full-length strand was cleaved for the antisense strands, while it was retained for the sense strands for further synthesis of DNA strands.

The sequences used were specific for aurora kinase (AURK), 78 kDa glucose regulated protein (GRP78), also known as heat shock 70 kDa protein 5 (HSPA5) and binding immunoglobulin protein (BiP), β-catenin (CTNNB) and Myc2 (MYC), all of them are proto-oncogenes. (Table 1)

3.1.1.2. Synthesis of DNA

DNA building blocks were prepared equally as those of RNA and synthesized on top of the sense strands of siRNA to fit onto the 4-arm DNA complex that had been developed previously[33]. The sequences are noted in Table 1.

Code	Sequence
Aurora Kinase-SS	5'-AAAAAAAAAAAAAAAGUAAAGGAAAGUUUGGUAA-3'
GRP78-SS	5'-GTATATGCTCGAGTGGAAAUGAGUUGGAAAGCUA-3'
β-Catenin-SS	5'-CAATTAATGGTGTCAAGCUGAUAUUGAUGGACAG-3'
Myc2-SS	5'-CCTGTGTCTGTTGTGGAGGAUAUCUGGAAGAAAU-3'
Aurora Kinase-AS	5'-UUACCAAACUUUCCUUUAC-3'
GRP78-AS	5'-UAGCUUUCCAACUCAUUUC-3'
β-Catenin-AS	5'-CUGUCCAUCAAUAUCAGCU-3'
Myc2-AS	5'-AUUUCUUCCAGAUAUCCUC-3'

Table 1: sequences of sense (blue) and antisense (red) strands of the siRNA with DNA-overhang (green) for hybridization with the 4-arm complex.

3.1.2. Purification of DNA-RNA-hybrids and siRNA antisense strands

To cleave the synthesized sequences from the CPG and deprotect them, the resins with the synthesized oligonucleotides were put into vials, 1 ml AMA (ammonium hydroxide/methylamine 1:1) were added and the mixtures were heated at 55°C for 10 minutes in the tightly closed vials. For 5 minutes the samples were cooled down in the fridge before being transferred (without the resin) into Eppendorf tubes. Then the resins were rinsed two times with 100 µl RNase-free water to increase the yield. In a SpeedVac (Alpha 2-4 LSC from Christ GmBH, Osterode am Harz, Germany) the solvents were evaporated until the samples were dry. Subsequently 50 µl DMSO were added to the pellet and if necessary for complete solution, the oligonucleotides were heated to 65°C for a few minutes in a thermomixer from Eppendorf (Hamburg, Germany). Under a fume hood, 50 µl TEA-3HF (triethanolamine-trihydrofluoride) were added to remove the protection groups from the 2'-OH of the ribose and the mixtures were heated for 2.5 hours at 65°C on a thermomixer. Two µl 3 N sodium acetate and 1 ml isopropyl alcohol were added after a short cooling period and the samples were vortexed after each application. For ensuring complete precipitation, the oligonucleotides were stored at -70°C overnight.

The next day all samples were centrifuged at 4°C at 12500 rotations per minute for 10 minutes, the supernatants were removed and collected separately. Samples were washed 3 times with 75% ethanol and centrifuged after each washing step. Finally, the air-dried pellets were dissolved in 30 µl RNase-free water and their concentrations were measured on a Nanodrop® Spectrophotometer ND-1000 from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

3.2. Gel electrophoresis

3.2.1. Denaturing polyacrylamide gel electrophoresis (den. PAGE)

To verify the successful synthesis and deprotection of the oligonucleotides and to monitor their purity, a denaturing polyacrylamide gel electrophoresis was performed.

3.2.1.1. Preparing the gel

To produce two gels, 6.8 g urea were dissolved in 7.5 ml 40% acrylamide stock solution (acrylamide/bisacrylamide 29:1) and 1.5 ml 10x TBE-buffer (TRIS-borate-EDTA-buffer) through shaking and if necessary short heating. After eventual cooling down of the solution 75 µl APS and 7.5 µl TEMED were added. The gel apparatus was filled swiftly and a comb was added for formation of pockets. After at least one hour of polymerization, the forerun was started at 150 V for 30 minutes in a tank filled with 1x TBE as buffer.

3.2.1.2. Preparing the samples and gel electrophoresis

During the forerun the synthesized oligonucleotides were prepared for analysis. Five µl of each 100 µM sample (comply with 0.5 nmol) were mixed with 5 µl formamide buffer (95 % formamide in water) and heated for 3 minutes at 95°C.
First 5 µl of 6x DNA Loading Dye (a mixture of bromophenol blue and xylene cyanol from Thermo Scientific), which was used for monitoring the electrophoresis run, were applied into the two outer gel chambers, then 10 µl of each oligonucleotide solution were applied into the other chambers. The gel was run for 1.5 hours at 150 V until the two dyes reached about two thirds of the running surface.
Then the gel was stained with methylene blue (0.02 % in water) for 15 to 20 minutes and washed repeatedly with water until the bands were clearly visible and the background destained.
The denaturing gel was scanned with GS-170 Calibrated Imaging Densitometer from Bio-Rad (Hercules, California, USA) and processed with Quantity One software.

3.2.2. 2.5 % native agarose gel

The 2.5% native agarose gel was performed on the one hand to verify the success of the hybridization of the 4-arm-complex and the sense and antisense strand of siRNA and on the other hand to evaluate the stability of siRNA and the 4-arm-complex with its siRNA.

Because of the teratogenicity of ethidium bromide, it is important to use nitrile gloves and work very cautiously when preparing and evaluating this gel.

3.2.2.1. Preparing the gel

Agarose (1.5 g) were dissolved in 60 ml 1x TBE through swaying and heating in the microwave until the solution boiled up shortly. Further shaking was done while cooling the solution down to around 50 °C. Then 6 µl ethidium bromide (1:10 000 from 10 mg/ml) were added and the solution swayed until it was dispersed and filled into the horizontal gel chamber. The comb was attached immediately.

3.2.2.2. Preparing the samples and gel electrophoresis

During the polymerization of the gel (about 1 hour), the samples were prepared for application. They were diluted to 10 µM with 1x PBS buffer to a 30 µl solution and first pipetted to hybridize to the 4-arm construct and sense with antisense strands of siRNA. After heating at 90°C for 10 minutes and slowly cooling down to 4°C, the following samples for application were hybridized at 80°C for 10 minutes and slowly cooled down to 4°C: 4-arm and hybridized siRNA, 4-arm and sense strands.

Three µl 6x DNA Loading Dye were added to the prepared samples. The agarose gel was placed into its apparatus and filled with ice cooled 1x TBE. After removing the comb, the samples were pipetted into the gel chambers and the electrophoresis was run at 90 V. When the 5 µl GeneRuler™ 50bp DNA Ladder standard from Thermo Scientific (Waltham, Massachusetts, USA) reached half of the running surface (approximately 1 h), the gel electrophoresis was stopped and the agarose gel scanned with ChemiDoc™ XRS from Bio-Rad (Hercules, California, USA).

3.3. Hybridization of siRNA

After each hybridization step the samples were slowly cooled down to 4°C in a thermomixer comfort from Eppendorf (Hamburg, Germany).

For the assembly of the siRNA sense strands and the 4-arm complex first the siRNA itself and the 4-arm tiles itself had to be hybridized separately. This was done at 90°C for 10 minutes. The hybridization of siRNAs with the 4-arm complex was done at only 80°C for 10 minutes to prevent the dissociation of the already hybridized parts. Figure 5 pictures the self-assembly of the scaffold.

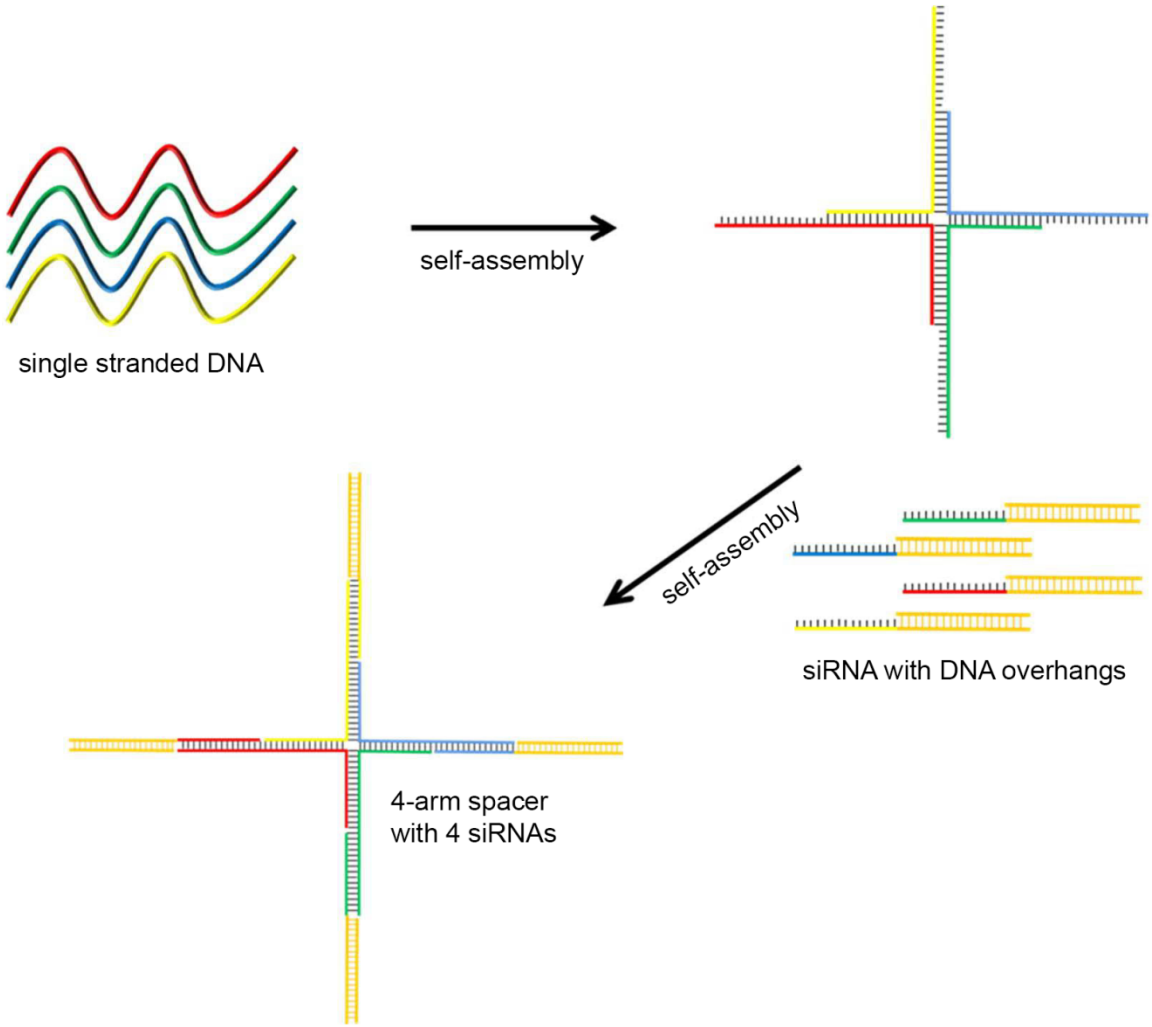

Figure 5: schematic representation of the self-assembly of 4-arm spacer and siRNAs during hybridization (adapted from [33])

3.4. Stability test

The stability test was conducted on a thermomixer comfort from Eppendorf (Hamburg, Germany) to check the stability of the hybridized oligonucleotide scaffold.

First 10 µl of each siRNA sense and antisense strand (10 µM) were mixed and hybridized. Then 20 µl of the solution and 20 µl of the beforehand prepared 4-arm complex[33] (10 µM) were mixed, this time at 80°C. Sequences of the 4-arm spacer are shown in Table 2.

Oligo1: overhang= linker to siRNA AURK 5'-TTTTTTTTTTTTTTTCACGATAAGATTCGGGTCGCCGCTATCAAC-3'
Oligo5: overhang= linker to siRNA GRP78 5'-CACTCGAGCATATACGTTGATAGCGGCGACCCCTATTTACAGAGG-3'
Oligo3: overhang= linker to siRNA CTNNB 5'-TGACACCATTAATTGCCTCTGTAAATAGGGGCTGAAAAGGACTGC-3'
Oligo6: overhang= linker to siRNA MYC 5'-CACAACAGACACAGGGCAGTCCTTTTCAGCCCGAATCTTATCGTG-3'

*Table 2: The sequences of the self-assembling 4-arm complex parts[33]: overhangs for hybridization with siRNA (**black**) and its complementary parts (each complementary part in the same colors). The overhangs of each strand were complementary to the ones of siRNA and assembled as follows: Oligo1 with AURK, Oligo5 with GRP78, Oligo3 with CTNNB and Oligo6 with MYC.*

Six µl of the hybridized siRNA solution and 6 µl of the 4 arm-complex were separately mixed with 10 µl DMEM GlutaMAX® Medium containing 10% fetal bovine serum from Gibco®, Life Technologies.

At five specific points in time samples were collected: after 0 hours, 1 hour, 3 hours, 24 hours and 48 hours of incubation at 37°C. The samples were heated at 90°C for 2 minutes after incubation to stop further enzymatic cleavage. Then 2 µl 6x DNA-Loading Dye were added before storing the samples at -70°C until all had finalized the incubation period. The samples and, as a standard, 3 µl GeneRuler™ 50bp DNA Ladder from Thermo Scientific (Waltham, Massachusetts, USA) were applied onto a 2.5% agarose gel and run in 1x TBE for about 50 minutes at 90 V. The agarose gel was scanned in a ChemiDoc™ XRS from Bio-Rad (Hercules, California, USA) and processed with Quantity One software.

3.5. Cell experiments

The cell line used for all cell experiments was HeLa. The cells were incubated in a Celculture® CO_2 INCUBATOR from Esco (Singapore) and a Laminar Biosafe 7-130-2 from Ehret (Emmendingen, Germany) served as workplace.
For cultivation, DMEM medium, FBS and TrypLE™ Express from Gibco®/Life Technologies/Thermo Fisher Scientific were used.
T-test for two samples assuming unequal variances was performed to evaluate statistical significance, where the treated cells were juxtaposed with untreated cells.

3.5.1. Splitting cells

During propagation, the cells in the culture flask were split before reaching confluency. Therefore, PBS and DMEM (containing Penicillin/Streptomycin and 10% FBS) were preheated in a water bath to 37°C. First the medium in the cell culture flask was aspirated and the flask cautiously flushed with 10 ml 1x PBS which were then also aspirated. To detach the cells from the surface they were incubated with 1 ml TrypLE™ Express (stable trypsin replacement enzyme) for 3 minutes. After that about 10 ml of DMEM were added to disperse the cells through clashing them onto the flasks surface.
A new flask was filled with about 8 ml DMEM (+PenStrep+10% FBS) and 2 ml of the cell suspension were added, dispersed and after labeling stored in the incubator at 37°C and 5% CO_2.

3.5.2. Transfection trials

The transfection trials were done to evaluate the effect of the samples on HeLa cells and to estimate whether the application of siRNA with a 4-arm-complex is more effective than the siRNAs alone.

3.5.2.1. Preparation of siRNA

Each siRNA was prepared in a separate tube. Five µl of the sense and antisense strands were added and hybridized at 90°C. The siRNA samples were diluted from 5 µM to 1 µM (10 µl sample with 40 µl 1x PBS) and further diluted to receive different concentrations in each trial. The process to reach the final concentrations is further described in the results part.

As transfection reagent 50 µl Lipofectamine®RNAiMAX from Invitrogen by Thermo Fisher Scientific were added in different dilutions to the siRNAs. The diluting solution was Opti-MEM® Reduced Serum Medium from Gibco®.

3.5.2.2. Seeding cells

Other than when splitting cells, DMEM + 10% FBS without PenStrep was used to avoid toxic effects in conjunction with the transfection reagent.

After splitting a proportion of the cells, 10 µl were transferred into a counting chamber to determine the dilution. The target was 75000 cells in 400 µl to put into each well of a 24 well plate. For diluting DMEM GlutaMAX™-I by Gibco® (Carlsbad, USA) life technologies plus 10% fetal bovine serum (without PenStrep) was added.

After applying 100 µl of transfection mix into each well 400 µl of the cell suspension were added.

3.5.2.3. Extraction of RNA

For the extraction of RNA, the Thermo Scientific GeneJET RNA Purification Kit (K0731, K0732) was used according to the manufacturer's instructions. All steps were performed under the hood and all centrifuging was done at 12000 x g in Centrifuge 5810R from Eppendorf (Hamburg, Germany).

3.5.2.3.1. Washing

First 7.7 ml lysis buffer were supplemented with 156 µl β-mercaptoethanol for each 24 well plate. Then the growth medium was removed from the cells and they were rinsed with 1x DPBS without calcium chloride and without magnesium chloride (Gibco®) to remove residual medium. After removing and discarding PBS, 300 µl of the lysis buffer supplement with β-mercaptoethanol and 180 µl ethanol were added and the 24 well plate was shaken for about 5 minutes on a shaker.

The lysates were transferred into appropriately labeled GeneJet RNA Purification Columns inserted in a collection tube. The tubes were centrifuged for 1 minute after which the collection tube was discarded. The column was placed into a new 2 ml collection tube with 700 µl Wash Buffer 1 and again centrifuged for 1 minute. After discarding the flow through, the column was centrifuged two times with Wash Buffer 2. The first time 600 µl were used and centrifuged for 1 minute, the second time 250 µl were added and centrifuged for 2 minutes, then discarded. To make sure all Wash buffer was removed the columns were emptied, re-spinned and put into a new collection tube.

3.5.2.3.2. Extraction

Fifty µl nuclease-free water were pipetted into the center of the purification column, which was subsequently centrifuged for 2 minutes and re-spinned until no water was left in the column. Then the eluted RNA was transferred into sterile 1.5 ml RNase-free tubes.

3.5.2.4. Synthesis of cDNA

The concentrations of RNA were measured with a NanoDrop® Spectrophotometer ND-1000 from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The necessary volumes containing 500 ng of RNA were calculated, transferred to a fresh tube and filled up with RNase-free water to 11 µl.

The synthesis of cDNA was prepared in a DNA/RNA UV-cleaner box UVT-S-AR from Biosan (Riga, Latvia) with RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific.

First the 8 well strips were labeled and the samples were cooled on ice. A master mix was made in a sufficient volume for each sample plus 20%: 4 µl 5X Reaction Buffer, 1 µl RiboLock RNase Inhibitor (20 U/µl), 2 µl 10 mM dNTP Mix and 1 µl RevertAid M-MuLV RT (200 U/µl) per well were mixed and stored on ice while the samples were prepared.

One µl random hexamer primer were put into the bottom of each tube, followed by the appropriate amount of water to reach 500 ng of sample in 11 µl. Next the aliquoted samples were added. Then 8 µl of master mix were added. The master mix for RT- control was put directly into the tube without RevertAid M-MuLV RT (200 U/µl) but RNase free water instead.

Samples were mixed and centrifuged and the synthesis was run in an Eppendorf Mastercycler gradient device with the following temperature settings: 5 minutes at 25°C, 60 minutes at 42°C and 10 minutes at 70°C to inactivate RT enzyme as indicated in the manual. For quantitative real-time reverse transcriptase PCR, the cDNA products were diluted 1:10 (20 µl samples with 180 µl RNase free water) and two of the untreated pools were mixed together.

3.5.2.5. Quantitative real-time reverse transcription PCR (qRT-PCR)

qPCR preparation was also performed in the DNA/RNA UV-cleaner box UVT-S-AR from Biosan (Riga, Latvia) and 96 well plates were used. Again, all samples were cooled on ice.

First a master mix was prepared: 13 µl nuclease free water and 4 µl 5x Solis EVA Green Mix per well were mixed with an excess of 20%. One µl primer and 2 µl template cDNA were pipetted into each well. After that 17 µl of the prepared master mix were added. Then a sealing foil was placed on top and the plate was shaken cautiously and centrifuged.

Each plate was prepared just before the quantitative real-time RT-PCR was started. The cycle program was selected according to the manufacturer's instructions and run on a Light Cycler® 480 from Roche (Rotkreuz, Schweiz). For 10 minutes the samples were heated at 95°C, then up to 50 cycles were repeated with the following temperatures: 15 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C. After that a melting curve was initiated.

As a reference gene, all samples were analyzed for RNA polymerase II.

The received CP scores were calculated with REST 2009 software to compute the relative expression according to the 2^ΔΔCt method.

3.5.3. Fluorescence microscopy

The efficiency of the lipofectamine transfection was monitored by fluorescence microscopy. Therefore, a fluorescently tagged dA18 oligonucleotide and the 4-arm-spacer were diluted to 5 µM with 1x PBS and hybridized for 5 minutes at 50°C.
First the solutions of the 4-arm-spacer/dA18 mixture and lipofectamine were prepared to apply 50 µl onto the cells of each well. The 2.5 µM 4-arm/dA18 were diluted with Opti-MEM to give final concentrations of 10 nM and 1 nM. Old and freshly opened batches of lipofectamine were separately diluted to give concentrations of 6 µl/pmol and 0.6 µl/pmol of the samples of 10 nM and 1 nM, respectively. As a control, 50 µl Opti-MEM were added instead of 50 µl LF-sample mixture onto a separate cell batch.

3.5.3.1. Seeding cells

A part of the cells was split and the remaining ones were put into a falcon tube, mixed and 10 µl of the suspension were transferred into a counting chamber to estimate the dilution. The target was 75000 cells in 250 µl to put into each well of a 48 well plate, which was reached through dilution with DMEM GlutaMAX™-I (Gibco®, Carlsbad, USA) containing 10% fetal bovine serum (without PenStrep). After applying 250 µl of cell suspension into each well 50 µl of the prepared solutions were added.
The seeded HeLa cells were incubated at 37°C and 5% CO_2 and examined after 1-1.5 hours, 3.5 hours and 24 hours in the fluorescence microscope EVOS FL from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

3.5.4. Luciferase and Bradford assay

To investigate whether the 4-arm-complex is functionally internalized into the cells, a Luciferase assay was performed. For the reverse transfection HeLa PGK-EGFPLuc with modified Oligos MS were seeded.

3.5.4.1. Seeding the cells

After splitting a part of the cells, the remaining ones were put into a falcon tube and mixed. Ten µl were put into a counting chamber to estimate the dilution. This time the target was 25000 cells in 90 µl to put into each well of a 96 well plate of Greiner Bio One (Kremsmünster, Austria). Again, DMEM GlutaMAX^{TM}-I (Gibco®, Carlsbad, USA) containing 10% fetal bovine serum (without PenStrep) was used for dilution. Then the samples were diluted to add 4 pmol, 1 pmol and 0.1 pmol to the cells, respectively, and lipofectamine was prepared for an amount of 0.3 µl/pmol siRNA. The dilutions of siRNA and lipofectamine were mixed, vortexed, and left at room temperature for 5 minutes.

Ten µl of each sample or Opti-MEM for the untreated cells were added into each of the 96 wells, followed by 90 µl cell suspension. The cells were incubated at 37°C and 5% CO_2 for 48 hours.

3.5.4.2. Cell lysis

After the incubation the medium was aspirated and 90 µl 1x PBS were added to each well with a multichannel pipette before examining the cells under Nikon TMS-F inverted microscope from Nikon (Chiyoda, Tokyo, Japan).

Passive lysis buffer (468 µl, 5x) from Promega (Mannheim, Germany) and 1872 µl water were mixed and 30 µl of this dilution were added into each well separately after the aspiration of 1x PBS and the plate was shaken for 30 minutes.

3.5.4.3. Preparation of Bradford assay

Meanwhile another 96 well plate from Greiner was prepared for a Bradford assay: 18 µl water were put into each well. 0, 1, 2, 3, 4, 5 and 10 µl BSA (bovine serum albumin) were applied into the outer wells for the calculation of the calibration curve and filled up with aqua purificata to 20 µl.

When the cell lysis was completed, 2 µl of the samples were added and pipetted up and down to ensure a good dispersion.

3.5.4.4. Evaluation of Luciferase and Bradford assay

The substrate for luciferin prepared by the laboratory group of Manfred Ogris, consisting of 3.0 mg/ml luciferin in 20 mM glycyl glycine, 1 mM $MgCl_2$, 1 mM EDTA, 3.3 mM DTT, 0.5 mM ATP and 0.3 mM coenzyme A was inserted into channel B of the plate reader. The 96-well plate without cover was inserted into the infinite® M200Pro from Tecan (Grödig, Austria) and 100 µl reagent were added and luminescence was measured with the Tecan-Platereader. The operating program was Tecan-i-control.

After Luciferase assay was nearly finished 180 µl Bradford reagent from Bio-Rad were added into the prepared 96 well plate and a cover was put on top. Then the plate was inserted into the infinite® M200 Pro and the absorbance measured at 595 nm.

The results were saved in a Microsoft Excel table and visualized into column charts. The Bradford assay gave a calibrating curve of the proteins to compensate cell amounts and to normalize the measured data. Further the luciferase outcomes were relativized to the untreated control cells.

4 Results and Discussion

4.1. Synthesis of siRNA and DNA-tiles

The sequences of 4-arm spacer and DNA-overhangs of siRNA were designed to not interfere with the human genome and induce off-target effects. The DNA-overhangs of siRNAs were respectively complementary to the ones of the scaffold to avoid false assembly and linked as described in materials and methods part.
The synthesis of the oligonucleotides was overall satisfactory and, after they were purified as descripted in materials and methods, they were quality controlled by denaturing polyacrylamide gel electrophoresis to ensure no shortened sequences were generated.

4.1.1. denaturing PAGE

To verify purity of the synthesized oligonucleotides denaturing polyacrylamide gel electrophoresis was done as per description in materials and methods. To this end, 0.5 nmol of each siRNA strand were applied into the gel chambers. After the run the gel was scanned on GS-170 Calibrated Imaging Densitometer and processed with the Quantity One software.

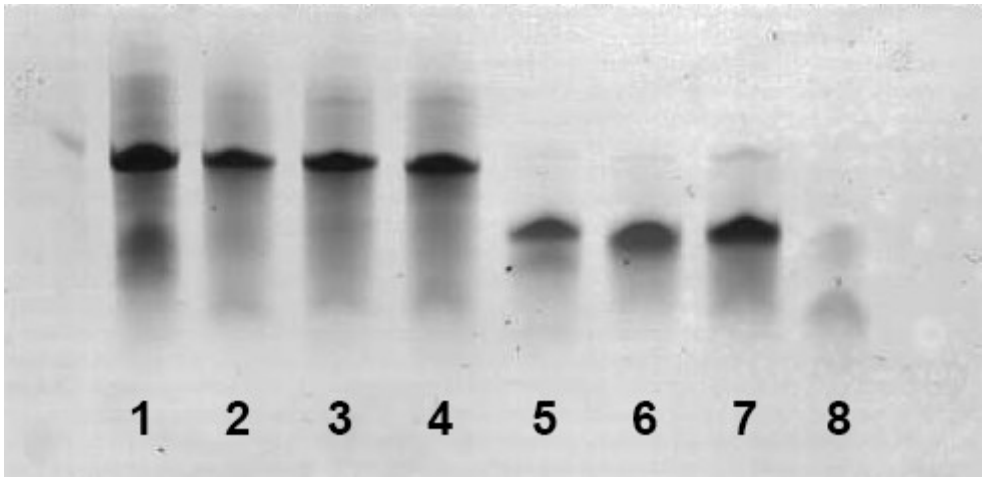

*Figure 6: denaturing PAGE of the sense and antisense strands of the synthesized siRNAs. On the left side are the sense strands with DNA-overhang: AURK (**1**), GRP78 (**2**), CTNNB (**3**) and Myc2 (**4**). On the right the four antisense strands: AURK (**5**), GRP78 (**6**), CTNNB (**7**) and Myc2 (**8**).*

In Figure 6 the bands of the synthesized siRNA of both sense and antisense strands are depicted. The 34mer sense strands with their overhangs of DNA (for hybridization with the 4-arm construct) have a higher molecular weight and because of this did not migrate as far as the antisense strands, which are composed of 19 nucleic acids.

The sense strands are all very prominent while the antisense strands show more variation in intensity. This could be due to variations in applied amounts on the gel, caused by errors in concentration determination or an irregularity in application on the gel. Further you can notice lighter bands under the trails of some strands like AURK which indicate that during synthesis a few wrong sequences were built.
The antisense strand of Myc2 is only slightly visible which could lead to the assumption of poor yields during the synthesis or the purification and partial degradation by nucleases, but in following transfection trials gene silencing with Myc2 was similar as with the other strands. Thus, it seemed more likely that a problem occurred during gel analysis.
In total, the denaturing polyacrylamide gel electrophoresis shows the successful synthesis of all sense and antisense strands while only minimal and acceptable amounts of secondary sequences were included. Further the differences of the molecular weight underline the complete synthesis of DNA-overhangs on top of the sense strands.

In later transfection trials a positive control of siRNAs that were synthesized and used earlier[50], was utilized and before testing them on cells their purity after storage at -70°C for more than one year was verified via denaturing PAGE as depicted in Figure 7.

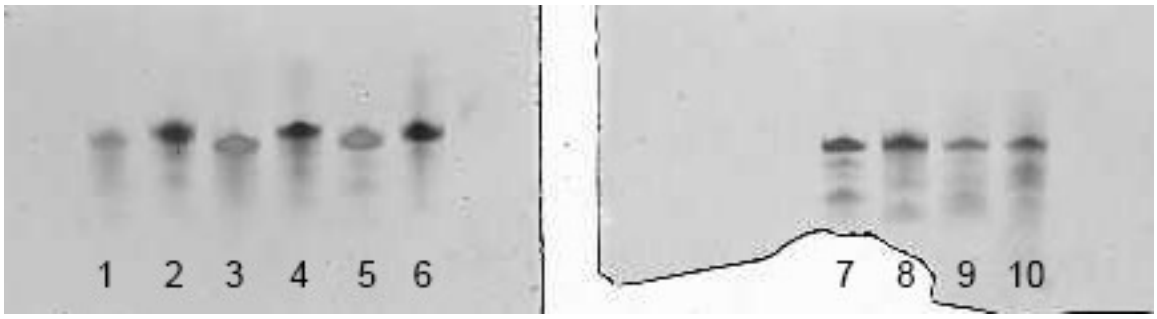

*Figure 7 pictures the den. PAGE of additional siRNAs. The prominent bands of aurora kinase antisense (**1**) and sense (**2**) strands, HSP27 antisense (**3**) and sense (**4**) strands, GRP78 antisense (**5**) and sense (**6**) strands, MYC antisense (**7**) and sense (**8**) strands and CTNNB antisense (**9**) and sense (**10**) strands show that the siRNAs remained stable since their synthesis. Lighter bands under the prominent ones reveal that some degradation seems to occur, but adequate stability for use in silencing experiments is maintained.*

The sequences are depicted in Table 3. HSP27 is another siRNA against the heat shock protein family and was inspected because of assumed instability of GRP78. Denaturing PAGE and a transfection trial to monitor the silencing effect depicted stability and similar efficacy of both. Therefore, GRP78 was used as more immediate control.

Code	sequences
GRP78	5‘-UAGCUUUCCAACUCAUUUC-dTdT-3’
HSP27	5‘-AUCUCCACCACGCCAUCCU-dTdT-3’
β-Catenin	5‘-CUGUCCAUCAAUAUCAGCU-dTdT-3’
MYC	5‘-UUCCUCAUCUUCUUGUUCC-dTdT-3’
AURK	5‘-UAACUCUCUUCGAAUGACA-dTdT-3’

Table 3 pictures the sequences of siRNA antisense strands used as positive control synthesized in earlier studies[50]*.*

4.1.2. 2.5% agarose gel

To verify the successful self-assembly of the siRNAs and the 4-arm spacer after hybridization, a native agarose gel was prepared and 15 µl of each sample consisting of the respective hybridized strands were added into the chambers. The scan was done and analyzed with ChemiDoc XRS.

At first a 2% agarose gel was made, but due to its blur the percentage of agarose was increased to 2.5% to provide a tighter molecular filter and receive more focused bands of the nucleic assemblies of around 200 bp.

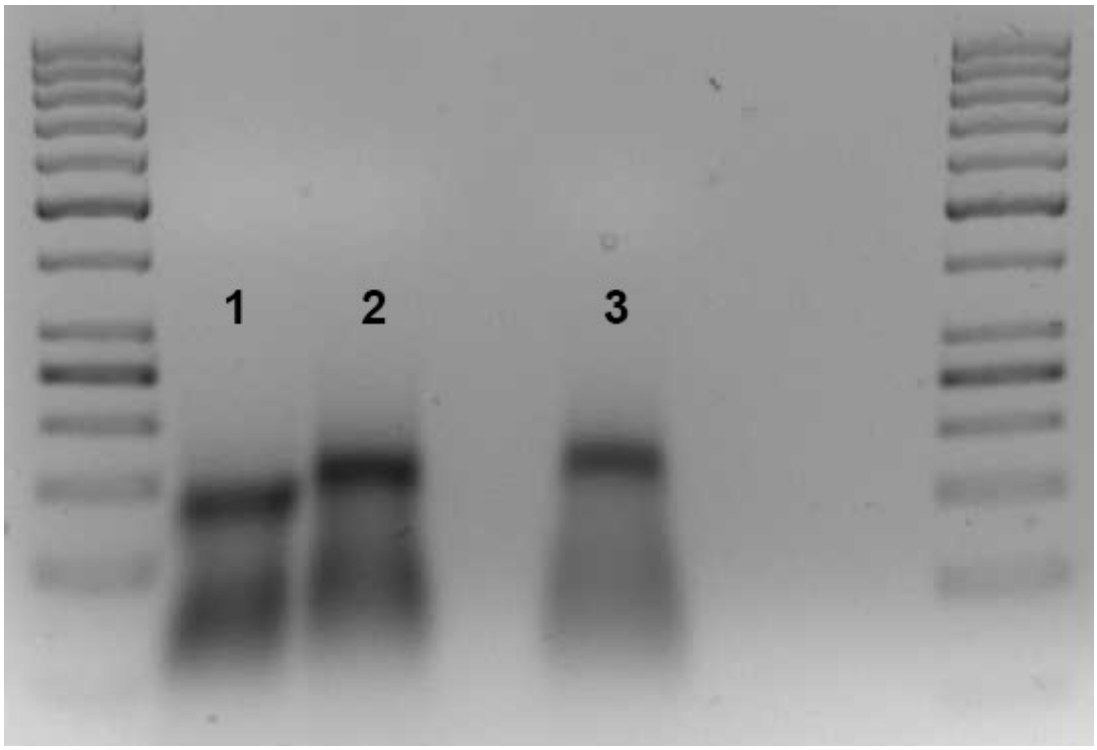

*Figure 8: 2.5% native agarose gel with the hybridized 4-arm (**1**), 4-arm spacer hybridized only with sense siRNA (**2**) and 4-arm spacer with double stranded siRNA (**3**). The GeneRuler 50bp DNA Ladder on both sides serves as standard.*

Again, a molecular weight difference is visible in Figure 8, this time of the hybridized 4-arm spacer which has the fewest nucleic bases and wanders the farthest, the hybridized 4-arm construct only with sense siRNA and the 4-arm hybridized with sense and antisense strands of siRNA. Between the last two a small difference in walking distance is visible, and thus a stepwise increase of the apparent molecular weight of assemblies with increasing numbers of base pairs. The GeneRuler 50bp DNA Ladder on both sides serves as indicator for the sizing of oligonucleotides and the hybridized structures were located around the 150 bp band even though the complete construct should be at 200 bp. Importantly, these three-dimensional assemblies have different analytical properties, smaller size, and show different

migration in gels than standard, linear double stranded nucleic acids. Thus, a straight-forward estimation of the size in bp by comparison to the marker bands is not possible. However, the apparent size of the constructs is slightly below linear nucleic acids with the same number of base pairs, which corresponds well to the expected migration behavior.

The strong bands prove a successful assembly of both the 4-arm spacer alone and with its siRNA attachments through hybridization, and a certain percentage of uncomplexed oligonucleotides (either because of slight variations in amounts or an equilibrium of association and dissociation) is indicated by the slight tailing below.

4.2. Stability test

The stability test was done to ensure the stability of the siRNA hybridized with the 4-arm construct against nucleases in comparison to the siRNA alone. To this end, the samples were incubated in 10% serum in cell culture medium at 37°C for the indicated times in Figure 9.

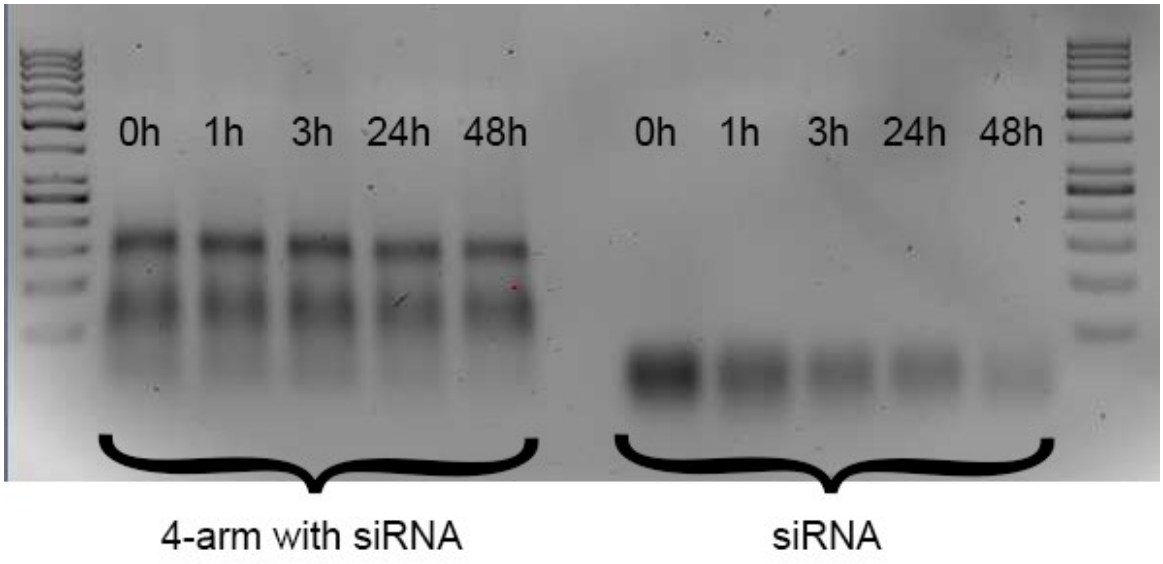

Figure 9 shows the 2.5% agarose gel for stability testing. On the left side, 4-arm with hybridized siRNA at incubation times 0, 1, 3, 24 and 48 hours, on the right side, double stranded siRNAs with the same incubation times. Once more, on both outer lanes GeneRuler 50bp DNA Ladder as standard. Since the siRNAs have less than 50 bp they are located below the first mark of standard while the bands of 4-arm complex are again at 150-200 bp.

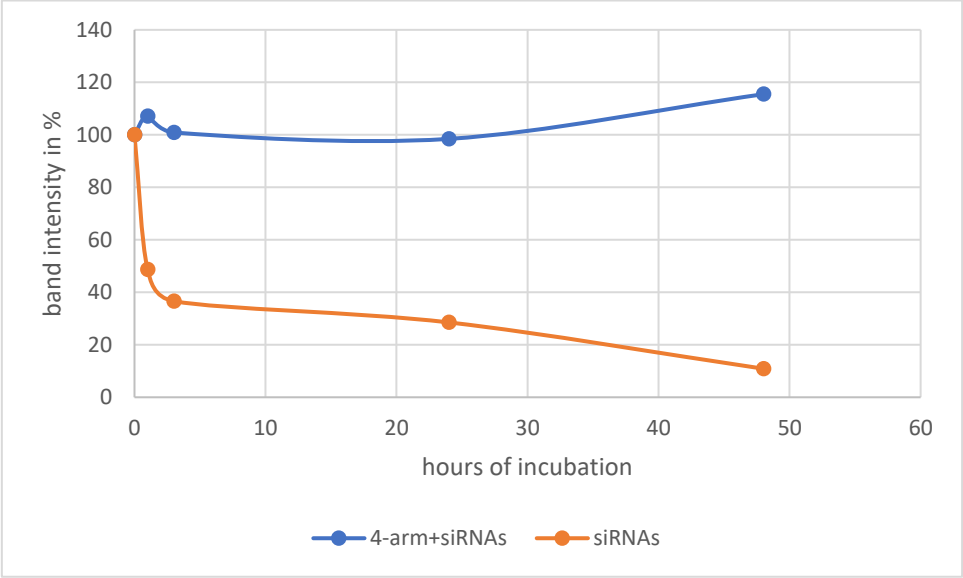

Figure 10 shows the percentage of band intensity of the stability test calculated by densitometric quantification of individual bands. 4-arm spacer with siRNA (blue) is more stable in serum-containing medium in comparison to siRNAs without spacer (orange).

The stronger stability of the 4-arm complex is clearly visible at all incubation times and even after 48 hours of exposure, strong bands resulted. There are slight differences visible in the intensity as shown in Figure 10, which can be caused by inaccuracies during the application of the samples and detection of the ethidium bromide signal.

While the 4-arm complex stays stable over 48 hours, siRNA starts to degrade after 1 hour. The degradation continues until only 10% of siRNA are detectable after 48 hours. To determine whether the DNA-overhangs induce a higher vulnerability of siRNA against serum nucleases a comparison between those and siRNA without DNA-overhang should be made. Besides to evaluate the maximal stability of the 4-arm spacer a longer incubation time could be set, or stricter conditions could be applied.

This shows that the complexation of siRNAs with the 4-arm scaffold stables them and can serve as a carrier to transport siRNA into the cells with less vulnerability against nucleases. This is likely explained the 4-arm spacer being a difficult to reach target for serum nucleases because of its unnatural junction and secondary structure.[33]

4.3. Transfection trials

The cells were prepared as per description in materials and methods. The siRNA samples and lipofectamine were prepared differently in each transfection experiment because of the varied concentrations in each trial. For each data point (dilution of each individual siRNA) 3 biological replicates were analyzed.

4.3.1. Equal volume of lipofectamine

4.3.1.1. Preparation of the samples

First the 4-arm spacer and siRNA had to be combined. Therefore, 5 µl of each siRNA (10 µM, sense and antisense strand) were mixed for hybridization. Those were separately attached to the 4-arm spacer, which means there were 4 samples of the complex with only one site of the 4-arm construct occupied by each respective siRNA. For an occupation of all sites of the 4-arm, a previously prepared stock mixture of all 4 hybridized siRNAs (5 µM), which was also used for the stability test, was added to the 4-arm spacer who had a concentration of 10 µM. The same solution was also used for a comparison of efficacy between siRNAs and 4-arm construct samples.

For optimized transfection, lipofectamine was added to each sample after dilution with Opti-MEM to their final concentrations which are shown in Table 4. For the control either Opti-MEM only or an Opti-MEM-lipofectamine mixture were applied.

Code	concentration per siRNA	concentration all siRNAs	µl of lipofectamine
siRNA	0.3125 nM	1.25 nM	0.375
4-arm + all siRNAs	0.15625 nM	0.625 nM	0.375
4-arm +one siRNA	0.625 nM	0.625 nM	0.375

Table 4 shows the final concentrations of the samples and volume of lipofectamine in the wells of the first transfection trial. Each sample had three biological replicates.

After the samples, the cells were prepared. Then 100 µl of all samples were transferred into the wells of a 48-well plate and 400 µl of the freshly dispersed cell suspension were added. The plate was incubated for 48 hours at 37°C and 5% carbon dioxide.

4.3.1.2. Analysis

After the seeding of the cells and their incubation, the cells were lysed and the RNA was extracted. Then the RNA was translated into cDNA for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). The obtained relative quantification values were calculated with REST 2009 software according to 2^ΔΔCt method. (Figure 11)

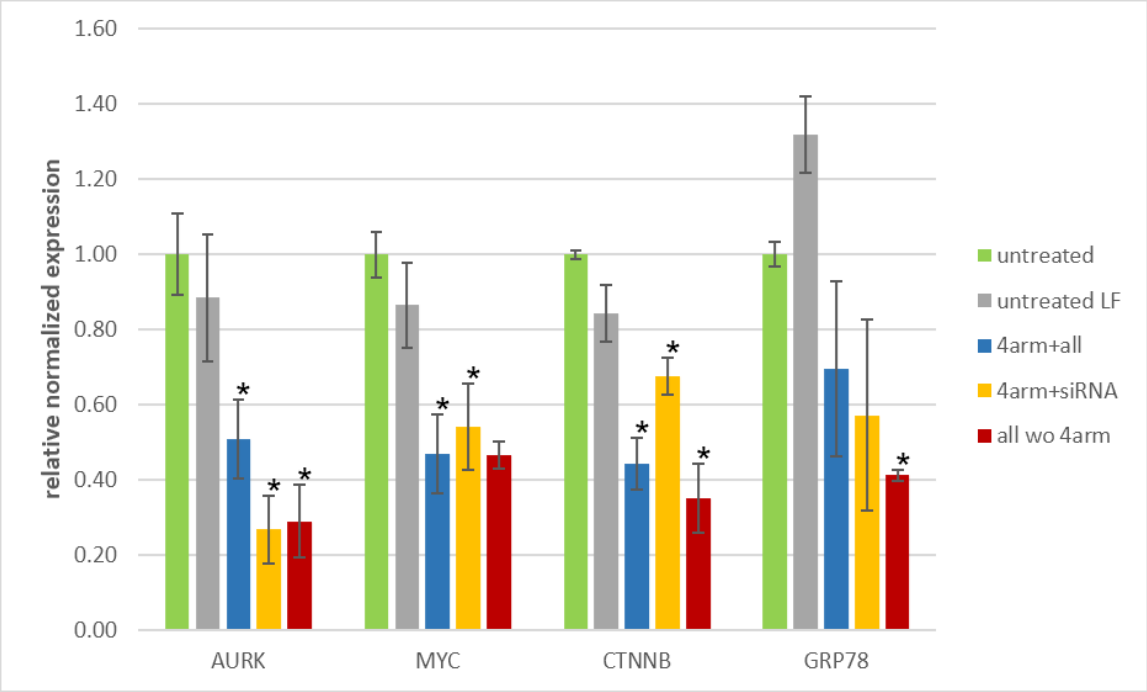

*Figure 11 shows the qRT-PCR relative quantification calculated with REST 2009 software according to 2^ΔΔCt method of the transfection trial with equal volume of lipofectamine. The controls were **untreated** (only Opti-MEM added) and **untreated LF** (an Opti-MEM-lipofectamine mixture added). Onto the treated cells was either the 4-arm spacer with only one siRNA (**4arm+siRNA**) or the 4-arm spacer with all siRNAs (**4arm+all**) or a mixture of all siRNAs (**all wo 4arm**) applied. Significant values are marked with * (p<0.05).*
The controls show both no significant gene silencing while all other samples are effective. siRNAs alone have in most cases a better result as the 4-arm complexes caused by the overall higher concentration of siRNAs and the higher ratio of lipofectamine in relation to the nucleic acids. Comparing the two 4-arm constructs it becomes obvious, that there seems to be competitive behavior when all siRNAs are attached resulting in differences in their effectiveness.

In general, significant mRNA degradation is caused by all siRNAs. The 4-arm complex seems to have less effect than siRNAs alone which could be due to the high negative charge and size which causes a less effective transportation into the cells. It should be noted that in this experiment, the same volume of lipofectamine was used for all samples. The spacer nucleic acids contribute to the overall nucleic acid size and charge, and thus, lower transfection efficiency is expected. A higher dose of lipofectamine could improve the transfection efficacy and lead to better

scores. Then again, the concentrations referring siRNA were not equal. The 4-arm spacer with all silencing genes carried only half the amount of siRNA without spacer and still we see in MYC and β-catenin silencing gene nearly the same effect. This indicates a better stability of the construct with the DNA carrier.

Also, it is observable in aurora kinase and GRP78 genes that the inhibition of the complex with only one siRNA was more successful than with all four while in MYC and β-catenin the opposite is happening. There can be various reasons for this. The different siRNAs may be unequally loaded into RISC and if we follow this thought further competitive behavior around RISC can too, lead in the case of the 4-arm spacer combined with four different siRNAs to unequal silencing. In this trail of thought other questions come to mind: can RISC reach saturation and are there more limiting factors?

Already studies have shown that competitive behavior around RISC are observable in mammalian cells and there is a factor that can limit RNA interference, though it is not jet known for sure which factor or if there are more than one who are accountable for this occurance.[51–54] For shRNA and miRNA a limiting factor is exportin-5 which is not required for siRNA gene silencing.[51,55] Still, siRNA can compete against those.[51] Saturation seems to occur at around 50 to 150 ng applied[51] but in our trials much less amount was transfected into the cells. The study further showed, that non-saturating amounts of about 25 ng can still induce competition. Not only different forms of siRNA such as microRNA and shRNA[51], but also inactive siRNA can compete with active siRNA[53].

4.3.2. Influence of lipofectamine volume on gene silencing

The goal of this transfection trial was to show whether a different volume of lipofectamine shows a better transfection through higher gene silencing activity.

4.3.2.1. Preparation of the samples

The stock solution of the 4-arm spacer hybridized with all siRNAs or with only one siRNA attached as described earlier were used here once again. Other than that, solutions of single siRNAs were freshly prepared: in separate tubes 2 µl of 10 µM of the sense and antisense strands of the AURK, Myc2, CTNNB and GRP78 siRNAs were added up with 1x PBS to 40 µl and hybridized, respectively. The now 5 µM solutions were further diluted to reach a final concentration of 1.25 nM in the wells with 100 µl applied. This time different lipofectamine concentrations were prepared as depicted in Table 5.

Code	concentration per siRNA	concentration all siRNAs	µl of lipofectamine
siRNA LF1	1.25 nM	1.25 nM	3.875
siRNA LF2	1.25 nM	1.25 nM	0.375
4-arm+all LF1	0.15625 nM	0.625 nM	3.875
4-arm+all LF2	0.15625 nM	0.625 nM	0.375
4-arm+siRNA LF1	0.625 nM	0.625 nM	3.875
4-arm+siRNA LF2	0.625 nM	0.625 nM	0.375

Table 5: the final concentrations of siRNA for the transfection trial with variable lipofectamine volumes. Samples were applied in triplicates into two 48-well plates.

When all samples were ready, the cells were prepared. One hundred µl of the transfection mixtures were added into the wells before 400 µl of the cell suspension were mixed into them. For the negative control 100 µl of Opti-MEM were applied.
After 24 hours of incubation the cells were monitored in the microscope. While the untreated cells were confluent lacking any non-viable cells or cell debris, the ones medicated with lipofectamine were not fully confluent with some dead floating cells visible. Also, with higher volumes of transfection agent, lower cell numbers were observable and in marginal wells more cells were dead.
After 48 hours the cells were harvested and prepared for qRT-PCR.

4.3.2.2. Analysis

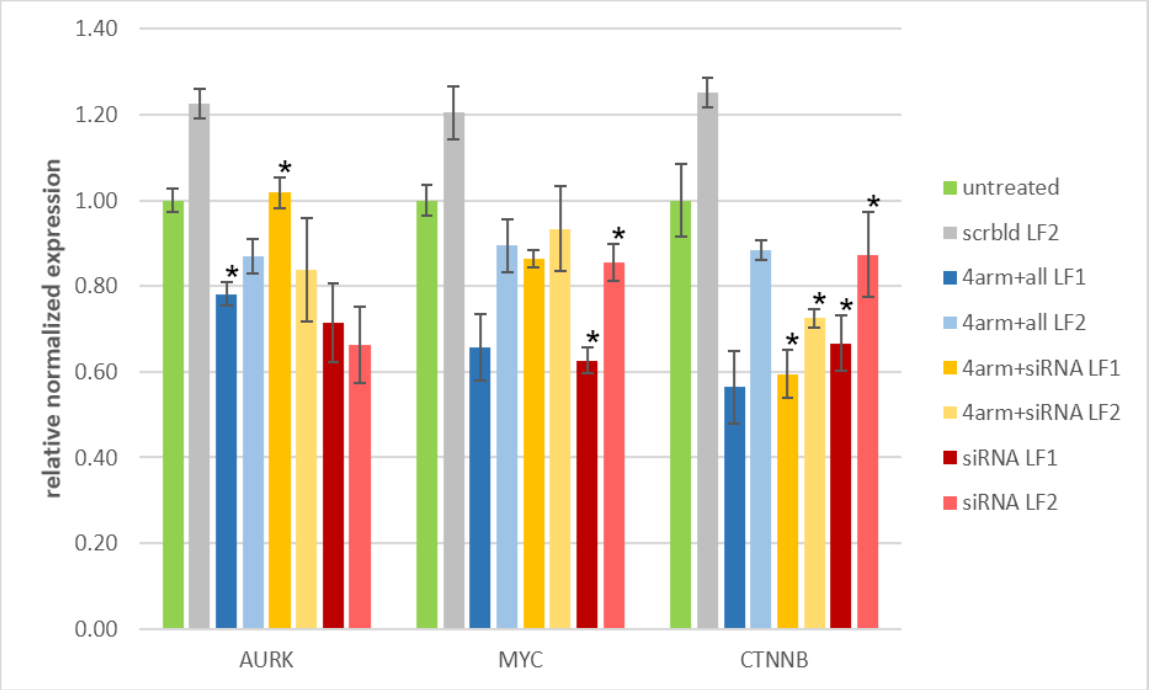

*Figure 12 displays the outcome of the influence of lipofectamine volume on gene silencing. All samples but out of scrambled RNA were applied with two different concentrations of lipofectamine (**LF1** 3.875 µl, **LF2** 0.375 µl). Significant values are marked with * ($p<0.05$). While untreated cells (**untreated**) and cells treated with inactive siRNA (**scrbld**) show no silencing of any gene, in most other samples an effect is visible. The 4-arm with all siRNAs attached (**4arm+all**) shows higher effect with higher volumes of lipofectamine than siRNA alone (**siRNA**) and 4-arm with only one siRNA attached (**4arm+siRNA**). Only in the samples treated with aurora kinase silencing siRNA the opposite is visible for siRNA and 4arm+siRNA, but the differences are not statistically significant.*

As Figure 12 shows, the 4-arm spacer is more effective with a higher concentration of lipofectamine. The same was observed for siRNA alone. In general, the silencing efficacies were very similar between constructs with one and all four siRNAs. Since the same lipofectamine volumes were applied, a lower transfection efficiency of the full assembly can be assumed. However, the 4-arm spacer with a single siRNA failed to induce pronounced silencing of AURK und MYC.

The effect of the 4-arm is compared to siRNA the same or better as we can observe in the cells treated with β-catenin with the same amount of lipofectamine added.

This shows that through the compensation of the negative charge the complex but also siRNA can more easily pass into the cell. To present a better comparison between siRNA of lower weight and less negative charge and the 4-arm spacer in future transfection trials four times more transfection agent was applied to the spacer, resulting in nearly equal ratios of lipoplexing agents to oligonucleotides relative to their length.

Another adaption happened because the siRNAs alone seemed to have less effect than in the first transfection trial which could be caused by their instability: after checking stability in denaturing PAGE and silencing in separate transfection trials, siRNAs with validated silencing activities and without DNA-overhangs[50] were used as positive control.

Another reason for varying silencing outcomes could be changes of the cells with increasing number of passages or due to slightly different culturing or seeding conditions. Thus, to guarantee better reproducibility the cells were first prepared and then samples were mixed to adapt the exposure time with lipofectamine.

4.3.3. Evaluation of optimal siRNA concentration for silencing

In this trial different concentrations of siRNA were applied to prove concentration dependent effectiveness. Aurora kinase silencing siRNA served as positive control and for comparison.

4.3.3.1. Preparation of the samples

After preparing the cells as per description in materials and methods, they were stored in the incubator during the preparation of the samples.

In this transfection trial different concentrations of aurora kinase inhibiting siRNA was applied as double stranded siRNA and as 4-arm spacer with all four siRNAs (against AURK, GRP78, CTNNB and MYC) onto the cells, and silencing of AURK was determined. This time double stranded siRNA lacking overhangs were used as positive control, kindly supplied by Volker Baumann.

As preparation the samples of 1 µM were diluted with Opti-MEM to reach the concentrations shown in Table 6.

	1	2	3	4	5	6
A	untreated	untreated	untreated	AURK 2.5nM	AURK 2.5nM	AURK 2.5nM
B	AURK 5nM	AURK 5nM	AURK 5nM	AURK 7.5nM	AURK 7.5nM	AURK 7.5nM
C	AURK 10nM	AURK 10nM	AURK 10nM	4arm+all 2.5nM	4arm+all 2.5nM	4arm+all 2.5nM
D	4arm+all 5nM	4arm+all 5nM	4arm+all 5nM	4arm+all 7.5nM	4arm+all 7.5nM	4arm+all 7.5nM

Table 6 pictures the application plan for the evaluation of optimal siRNA concentrations for silencing. For each sample three wells were prepared. ***AURK*** *is the double stranded siRNA against aurora kinase while* ***4arm+all*** *indicates the 4-arm spacer hybridized with all four different siRNAs. Next to the sample the concentration is noted. In the wells marked as untreated 100µl Opti-MEM were added instead of the siRNA-lipofectamine mixture.*

For aurora kinase silencing siRNA, the highest destined concentration was 10 nM in 500 µl, so 5 pmol had to be applied within 100 µl. Therefore, 40 µl of 1 µM sample were diluted to 400 µl. For the double stranded siRNA without spacer 0.1 µl lipofectamine per pmol siRNA were added.

The 4-arm complex was prepared to a final concentration of 7.5 nM in 500 µl which means 3.75 pmol in 100 µl had to be applied into the wells. Here 30 µl of 1 µM 4-arm complex were diluted to 400 µl. For better transfection and comparison four times as much lipofectamine were added which means in numbers: 15 µl lipofectamine were diluted to 500 µl and of this solution 400 µl were added to the prepared mixture of the 4-arm spacer. Thus, lipofectamine ratios relative to siRNA were equal, but a neutralizing effect of the spacer oligonucleotides was not taken into account.

After the addition of the transfection agent lipofectamine the solutions were incubated for 10 minutes at room temperature to guarantee the formation of a complex of these two agents.

These solutions were further diluted. Aurora kinase inhibiting siRNA reached final concentrations of 10 nM, 7.5 nM, 5 nM and 2.5 nM, while the 4-arm construct reached 7.5 nM, 5 nM and 2.5 nM concentrations in the wells. Quantities of siRNA and volume of lipofectamine are noted in Table 7.

Code	quantity per siRNA	quantity of all siRNAs	µl of lipofectamine
AURK 2.5nM	1.25 pmol	1.25 pmol	0.125
AURK 5nM	2.5 pmol	2.5 pmol	0.25
AURK 7.5nM	3.75 pmol	3.75 pmol	0.375
AURK 10nM	5 pmol	5 pmol	0.5
4arm+all 2.5nM	1.25 pmol	5 pmol	0.5
4arm+all 5nM	2.5 pmol	10 pmol	1
4arm+all 7.5nM	3.75 pmol	15 pmol	1.5

Table 7 shows the material quantities of the samples of the evaluation of optimal siRNA concentrations for silencing with the applicated volume of lipofectamine. The 4-arm spacer with all siRNAs was applicated with four times the lipofectamine to compensate the higher negative charge and size next to the siRNA alone.

Of these prepared solutions 100 µl were pipetted into each well and 400 µl of the freshly dispersed cells were added. As negative control 100 µl Opti-MEM were applied. This time the cells were harvested after for 30 hours.

4.3.3.2. Analysis

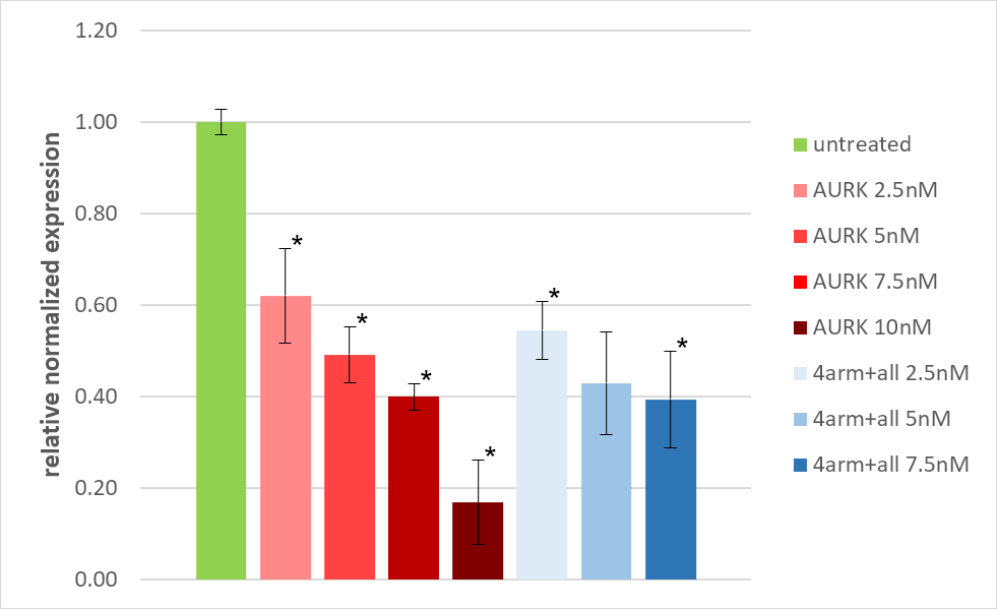

*Figure 13 displays the results of the evaluation of optimal siRNA concentrations for silencing. Significant values are marked with * ($p<0.05$).*
*All samples resulted in concentration-dependent silencing effect on aurora kinase. The 4-arm spacer (**4arm+all**) shows minimal, but non-significant superior results compared to the positive control of aurora kinase silencing siRNA (**AURK**). This indicates that the four times higher volume of lipofectamine applied to the 4-arm spacer to compensate its size and high negative charge induces equal transfection.*

As Figure 13 indicates, in comparison to the untreated cells gene silencing is significant in all applied samples and it is dependent on the applied concentration of siRNA in both, the double stranded siRNA alone and siRNA hybridized with the 4-arm spacer. The 4-arm construct shows the same outcomes as the aurora kinase siRNA alone, which can have different reasons. On the one hand the scaffold has a better stability against nucleases in comparison to siRNA which should lead to better effects. But on the other hand the interaction of more than just one siRNA can induce decreased silencing effects due to competition and, as mentioned in the first transfection trial, studies have already proven that combined application of different siRNAs show reduced efficacy.[51–54] This leads to the assumption that the construct with all siRNAs shows less gene silencing for each gene alone than only one siRNA. To evaluate whether the competition within the 4-arm spacer induces significant poorer outcomes in comparison to a spacer with only one siRNA further research should be done.

In general, this trial proves that the effect of gene silencing is dependent not only on the volume of transfection agent applied, as discussed before, but also on the concentration of the siRNA itself.

4.3.4. Confirmation of silencing by all four siRNAs

After the promising outcome of the evaluation of optimal siRNA concentration for silencing which indicated that higher concentrations of siRNA lead to more potent gene silencing, a higher concentration of 10 nM 4-arm spacer was applied in comparison to a concentration of 5 nM. This time all siRNAs were respectively applied as positive controls.

4.3.4.1. Preparation of the samples

While the prepared cells were stored in the incubator, the samples were arranged. In this transfection trial the silencing effects of all four siRNAs attached to the 4-arm spacer or alone were compared to untreated cells and cells treated with ineffective RNA (scrbld). Of the 4-arm complex with all siRNAs two different dilutions were applied while the siRNAs without spacer all had only 10 nM concentration in the wells, except aurora kinase siRNA which had the same concentrations as the 4-arm assembly (5 and 10 nM). Table 8 displays the application into the 48-well plates.

	1	2	3	4	5	6
A	untreated	untreated	untreated	HSPA5 10nM	HSPA5 10nM	HSPA5 10nM
B	CTNNB 10nM	CTNNB 10nM	CTNNB 10nM	MYC3 10nM	MYC3 10nM	MYC3 10nM
C	4arm+all 5nM	4arm+all 5nM	4arm+all 5nM	4arm+all 10nM	4arm+all 10nM	4arm+all 10nM
D	AURK 5nM	AURK 5nM	AURK 5nM	AURK 10nM	AURK 10nM	AURK 10nM

	1	2	3	4	5	6
A	scrbld 10nM	scrbld 10nM	scrbld 10nM	untreated		
B	untreated	untreated	untreated	untreated		
C						
D						

*Table 8: Two 48-well plates were prepared to fit all samples. Aurora kinase silencing siRNA (**AURK**) and 4-arm with all siRNAs attached (**4arm+all**) were applied in concentrations of 5 and 10 nM, respectively, while all other positive controls (**HSPA5** for GRP78, **CTNNB** for β-catenin, **MYC3** for MYC2 in the 4-arm spacer, respectively) were applied in a concentration of 10 nM only. The cells treated with ineffective siRNA (**scrbld**) as well had a concentration of 10 nM.*

First the transfection agent was prepared: 15 µl lipofectamine were diluted to 1500 µl for the siRNA samples and 16 µl were diluted to 400 µl for the 4-arm spacer. Then of each 1 µM siRNA, except AURK, and the ineffective scrambled RNA 20 µl were put into separate tubes and Opti-MEM was added to a volume of 200 µl.

For the 4-arm with all siRNAs and AURK 30 µl of 1 µM were filled up to 300 µl with Opti-MEM to provide enough excess for further dilution.

After that the equivalent volume of lipofectamine were added to all samples which means to all 200 µl dilutions 200 µl transfection agent were added and to the 300 µl dilutions 300 µl were added. Thus, for each siRNA 0.1 µl lipofectamine per pmol siRNA were used. Then the samples were set aside for 10 minutes of reaction time. Out of the 10 nM solutions of AURK and 4-arm spacer dilutions of 5 nM were prepared. Applied quantities of siRNA and volume of lipofectamine are shown in Table 9.

Code	quantity per siRNA	quantity of all siRNAs	µl of lipofectamine
HSPA5 10 nM	5 pmol	5 pmol	0.5
CTNNB 10 nM	5 pmol	5 pmol	0.5
MYC3 10 nM	5 pmol	5 pmol	0.5
AURK 5 nM	2.5 pmol	2.5 pmol	0.25
AURK 10 nM	5 pmol	5 pmol	0.5
scrbld 10 nM	5 pmol	5 pmol	0.5
4arm+all 5 nM	2.5 pmol	10 pmol	1
4arm+all 10 nM	5 pmol	20 pmol	2

Table 9 shows the material quantities applied into each well compared in each sample with the applied volume of transfection agent.

As in the previous transfection trials first 100 µl samples and on top of that 400 µl cell suspension were pipetted into the 48-well plates after which they were incubated for 30 hours at 37°C and 5% carbon dioxide.

After RNA extraction, cDNA synthesis and qRT-PCR the results were transformed into a column chart.

4.3.4.2. Analysis

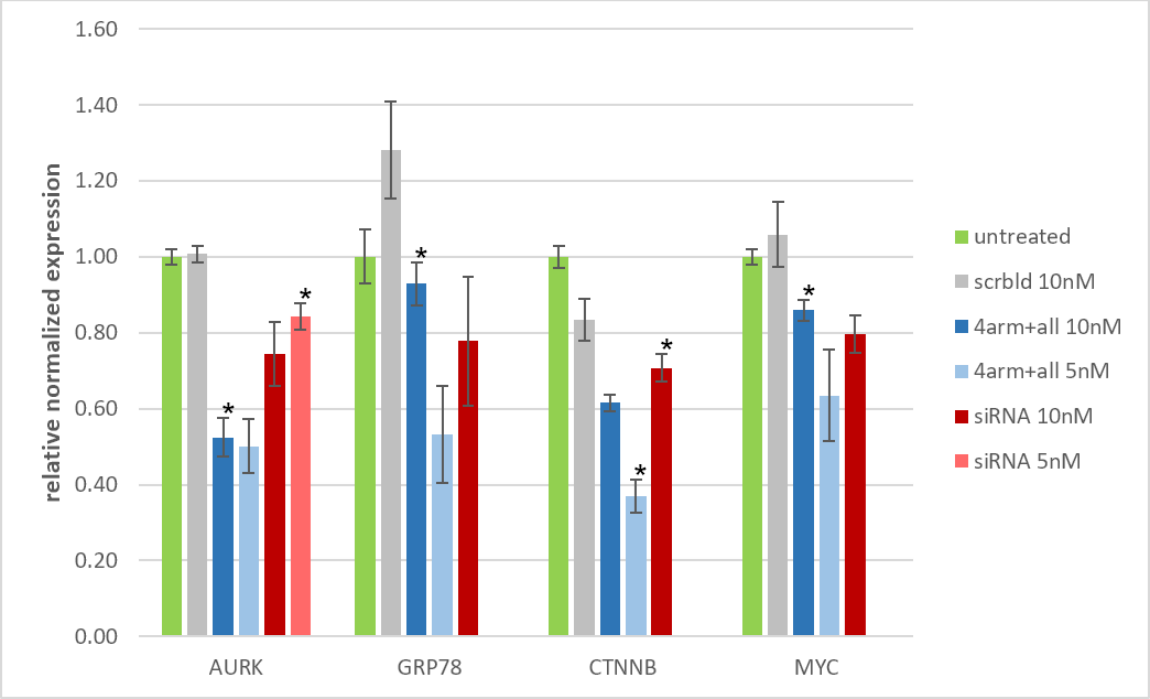

*Figure 14: The ineffective scrambled siRNA (**scrbld**) shows in all cases no significant silencing while the siRNAs all silence their target gene. In all cases the 4-arm complex shows better results with 5 nM concentration than with 10 nM. The aurora kinase silencing siRNA the opposite is visible, the effect is higher with a higher concentration.*
*Significant values are marked with * ($p<0.05$).*

As pictured in Figure 14 the 5 nM 4-arm complex shows next to the 10 nM siRNAs alone a better silencing effect which can be pinpointed to the better stability against serum nucleases. This time the difference is more prominent but on the other hand the 10 nM 4-arm spacer shows lower effects for most genes.

After the evaluation of optimal siRNA concentration for silencing, a concentration of 10 nM for the 4-arm spacer with its siRNAs was applied with the expectation of even better gene silencing effects but those were not fulfilled. This can have various reasons: either the competitive behavior of siRNAs intensifies with its concentration which could be interpreted into the better silencing of aurora kinase next to the other genes or a higher level of toxicity caused by high concentrations of lipofectamine.

Studies with cell penetrating agents have shown the safety of Lipofectamine RNAiMAX in low concentrations[56] but with other transfection agents such as Lipofectamine 2000 toxic effects were visible in high concentrations[57]. In vitro, toxicity is dependent on several parameters, such as incubation time and cell number. The difference within this and the prior experiment, in which 5 and 7.5 nM

of the complete assembly had nearly identical effects, may be the higher concentration, or a lightly lower cell number, both of which contribute to elevated toxicity.

With siRNAs there can also be possible toxicities through immunogenicity.[7] Here several modifications within the backbone for example a 2'-O-methyl-group[58] or modifications in the sense strand[7] can reduce off-target effects such as the immune reaction and induce a safer use.

4.4. Fluorescence microscopy

The aim of the fluorescence microscopy study was to ensure adequate transfection efficiency.

The fluorescence microscope sends out certain wavelengths to stimulate the dye to fluoresce. As described in the materials and methods part the 4-arm was hybridized with a fluorescently tagged dA18 oligonucleotide and two different concentrations were mixed. Equally for lipofectamine, an old and a freshly opened batch were compared and two different concentrations were prepared. The final concentrations of the 4-arm spacer were 10 nM and 1 nM and lipofectamine was diluted to give concentrations of 6 µl/pmol siRNA and 0.6 µl/pmol siRNA, respectively.

Again, HeLa cells were used, and images were taken after 1 hour, 3.5 hours and 24 hours incubation.

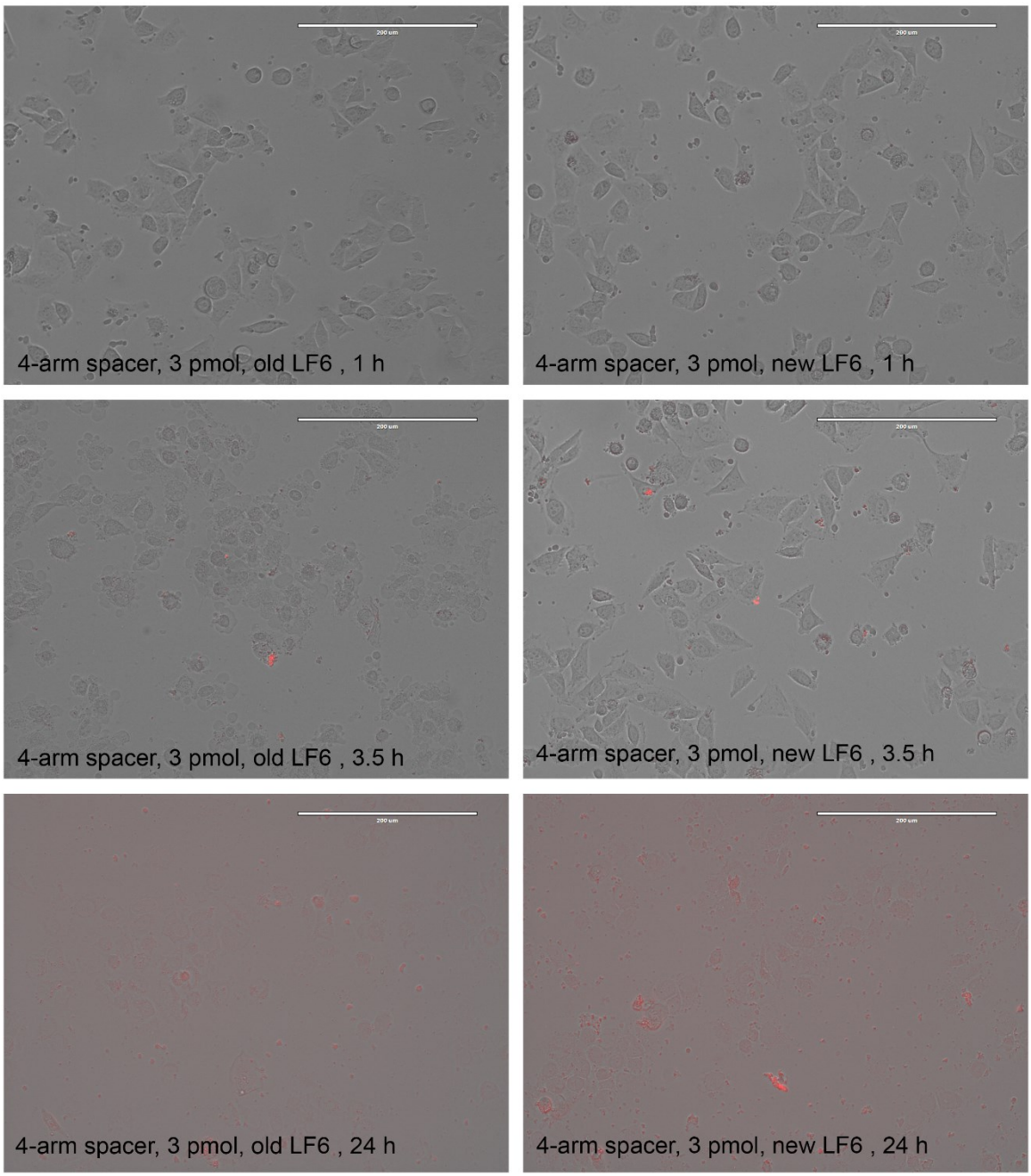

*Figure 15: On the left side: the 4-arm spacer with a concentration of 10 nM and 6 µl/pmol of the old batch lipofectamine (**old LF6**) at different observation times is depicted. On the right side is the 4-arm with the same concentration of 10 nM transfected with the freshly opened lipofectamine with 6 µl/pmol (**new LF6**) concentration at different times.*

As we can see in Figure 15 after one hour of incubation the fluorescence is not yet clearly visible. After 3.5 hours transfection seems to be visible through the fluorescence of dA18 in some of the cells which indicates a successful transfection. After 24 hours we see a number of dead cells fluoresce in the wells.

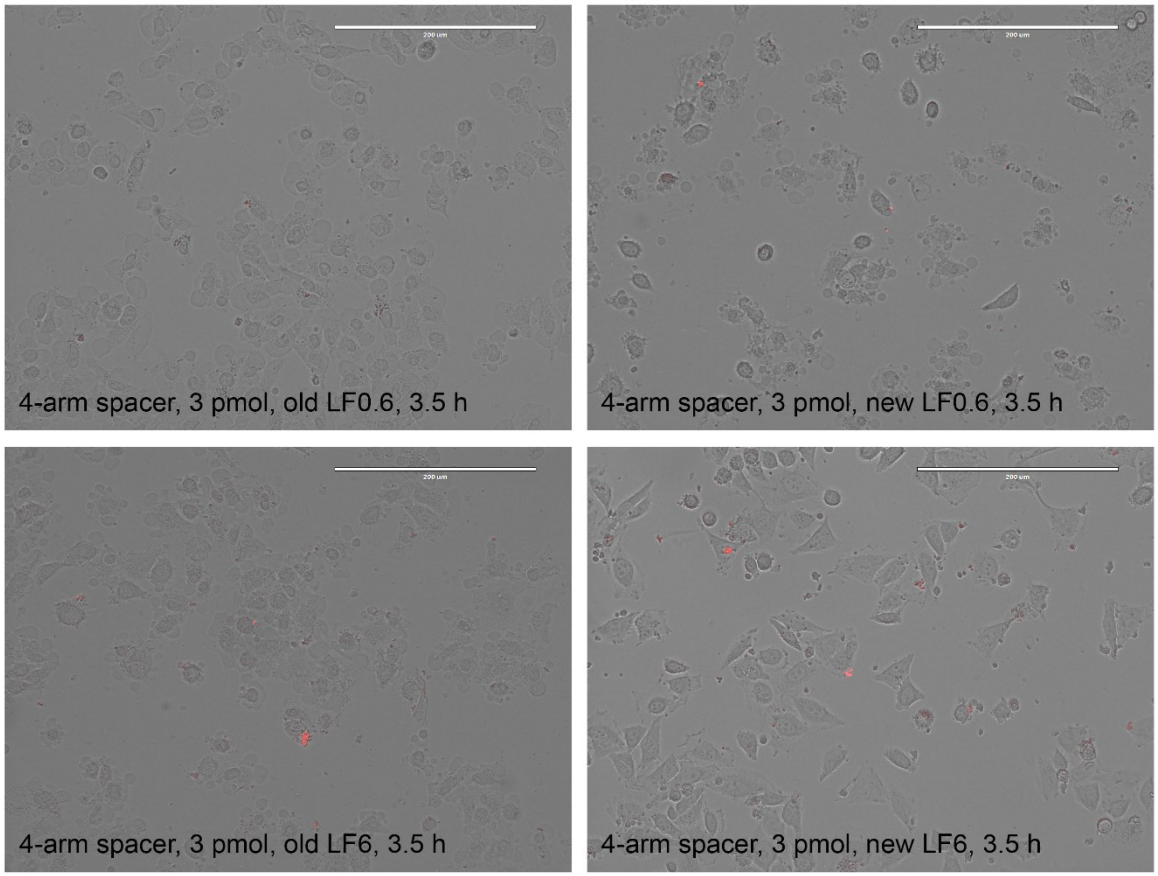

*Figure 16: here are the different concentrations of lipofectamine pictured after the same incubation time. On the top left the old lipofectamine batch at 0.6 µl/pmol (**old LF0.6**) and on the bottom with 6 µl/pmol (**old LF6**), respectively with a concentration of 10 nM 4-arm spacer. On the top right the fresh opened lipofectamine of 0.6 µl/pmol concentration (**new LF0.6**) while on the bottom right its concentration was 6 µl/pmol (**new LF6**) again with the 4-arm spacer at a concentration of 10 nM, respectively.*

Figure 16 shows that higher concentrations of the transfection agent lipofectamine induce slightly better cell penetration of 4-arm spacer visible through the fluorescence of dA18 and low concentrations show lower fluorescence. Also, in the low lipofectamine concentration of the old batch no fluorescence is visible while in the freshly opened batch occasional cases are apparent.

As shown in Figure 15, the HeLa cells suffer from toxicity after 24 hours of exposure to 10 nM 4-arm spacer with 6 µl/pmol lipofectamine. To examine whether this occurrence is dependent on the concentrations of 4-arm spacer and lipofectamine or also visible in untreated cells a comparison of those two is shown in Figure 17. A clearly more confluent cell growth is visible in the untreated well.

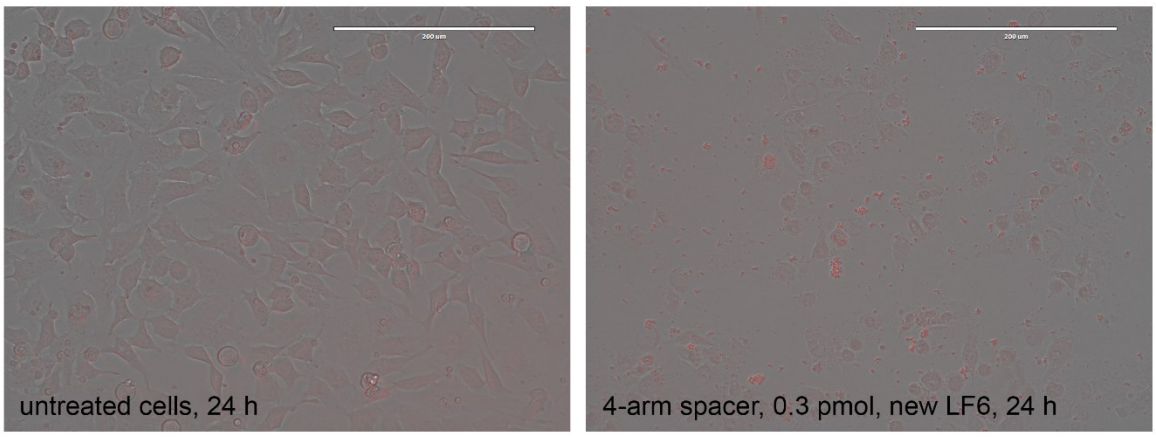

Figure 17 presents a comparison of untreated cells and cells handled with 4-arm spacer of 1 nM and 6 µl/pmol fresh lipofectamine after 24 hours.

The fluorescence microscopy displays after 1 hour of incubation in all samples, irrelevant if respectively 10 nM or 1 nM 4-arm spacer with 6 µl/pmol or 0.6 µl/pmol lipofectamine, only minor intracellular fluorescence. Also, no difference is visible in old and freshly opened batch of transfection agent as depicted in Figure 15 which indicates unfinished transfection.

After 3.5 hours of incubation a moderate cell growth and intracellular fluorescence are visible. In Figure 16 both old and fresh transfection agent show at a concentration of 6 µl/pmol same levels of fluorescence through 10nM 4-arm spacer with dA18 while with a lower concentration of 0.6 µl/pmol lipofectamine and 3 pmol 4-arm complex differences appear in the fluorescence: in the old batch of transfection agent there is none visible whereas the freshly opened batch shows some.

At 24 hours of incubation most of the fluorescence comes from dead cells as Figure 15 shows. To exclude that all cells are affected in Figure 17 the untreated cells and cells at low concentrations of 4-arm spacer are shown. While the untreated wells show confluent growth, all treated cells have less cell growth. This could be due to the potential toxicity of lipofectamine. As mentioned before, studies show that Lipofectamine RNAiMAX, which was used in this thesis, is safe in low concentrations[56] while high concentrations can picture a risk of apoptosis, especially if applied on sparsely seeded cells[57].

Because of this finding in future transfection trials the incubation time was set to 30 hours instead of 48 hours.

4.5. Luciferase and Bradford assay

The goal of the luciferase assay was to find out whether the luciferase gene of the genetically modified HeLa cells would be degraded through transfection of the 4-arm construct with a luciferase siRNA and the siRNA alone and to compare the potency of the two agents.

The luciferase siRNA for the 4-arm spacer was provided by Mathias Stadlbauer with the sequences noted in Table 10.[59] The sense strands were synthesized with the four different DNA-overhangs to be able to attach to the spacer.

Code	sequences
Luc AS	5'-UCGAAGUACUCAGCGUAAGdTdT-3'
Luc Sense	5'-CUUACGCUGAGUACUUCGA-(PS)-dT-(PS)-dT-3'
DNA-overhang oligo 1	5'-AAAAAAAAAAAAAAA-3'
DNA-overhang oligo 5	5'-GTATATGCTCGAGTG-3'
DNA-overhang oligo 3	5'-CAATTAATGGTGTCA-3'
DNA-overhang oligo 6	5'-CCTGTGTCTGTTGTG-3'

Table 10 shows the sequences of the transfected luciferase siRNA and the DNA-overhangs synthesized onto the 3'-ends of sense strands of Compact Luc Sense. The antisense strand had one phosphorothioate (PS) in 3' and the blue nucleotides of the sense strand were methylated at the 2'-OH of ribose.[59]

The positive control was Luc siRNA while the cells for negative control were incubated with 10 µl of Opti-MEM. The positive control and the 4-arm spacer were diluted to different amounts of 4, 1 and 0.1 pmol as shown in Table 11. As transfection agent 0.3 µl lipofectamine per pmol siRNA were added.

	1	2	3	4	5	6	7	8	9	10	11	12
A	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*
B	*u*	u	4 pmol	4 pmol	4 pmol	u	4 pmol	4 pmol	4 pmol	u	u	*u*
C	*u*	u	4 pmol	4 pmol	4 pmol	u	4 pmol	4 pmol	4 pmol	u	u	*u*
D	*u*	u	1 pmol	1 pmol	1 pmol	u	1 pmol	1 pmol	1 pmol	u	u	*u*
E	*u*	u	1 pmol	1 pmol	1 pmol	u	1 pmol	1 pmol	1 pmol	u	u	*u*
F	*u*	u	0.1 pmol	0.1 pmol	0.1 pmol	u	0.1 pmol	0.1 pmol	0.1 pmol	u	u	*u*
G	*u*	u	0.1 pmol	0.1 pmol	0.1 pmol	u	0.1 pmol	0.1 pmol	0.1 pmol	u	u	*u*
H	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*	*u*

*Table 11 shows the transfection plan for Luciferase and Bradford assay. The untreated wells are marked with **u**, the 4-arm spacer with four luciferase siRNAs was pipetted with the indicated amount of substance into the blue-colored wells while the luciferase siRNA GSK was added into the pink-colored wells. For the Luciferase assay only the thick boarded area was measured. For the Bradford assay the samples were pipetted into a similar plan of a 96-well plate, but the two outer columns were used for the calibrating curve with BSA. Therefore, the whole plate was measured with all the wells included. The samples were diluted to 4 pmol, 1 pmol and 0.1 pmol to add into each well, respectively.*

All values were calculated in relation to untreated cells with the help of Bradford assay to compensate the number of cells in each well.

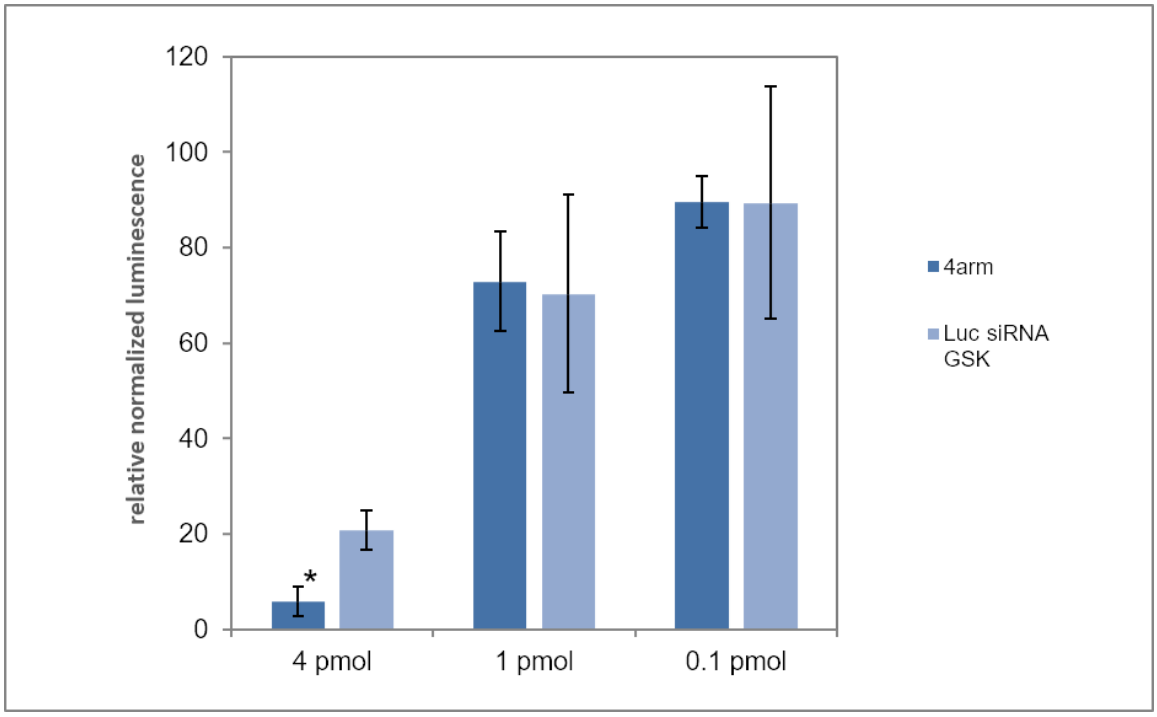

*Figure 18 shows the outcome of the Luciferase assay normalized with the Bradford assay. On the x-axis the three different concentrations applied onto the cells are noted. The dark blue column on the left is the result of the 4-arm spacer hybridized with luciferase siRNA (sequences see Table 10), on the right the lighter blue results of the positive control (free siRNA). Significant differences between the 4-arm spacer and free siRNA at the same concentrations are marked with * ($p<0.05$).*

In Figure 18 the differences in potency of the 4-arm spacer with luciferase siRNA and the positive control Luc siRNA are presented. At an amount of 0.1 pmol substance there are no variations between the cells treated with respectively both samples to be seen. Also, the luminescence reduction is only minor, indicating poor effectiveness at this concentration. The samples of 1 pmol also show minor gene silencing of the luciferase gene in HeLa. Only at an amount of 4 pmol of substance in 100 µl which equals a concentration of 40 nM, substantial gene silencing was detected due to the RNA interference of the relevant gene. Here the 4-arm spacer shows a significantly better efficacy, which is due to its better stability against nucleases as depicted in the stability test.

The volume of lipofectamine added was the same for both samples. Due to the higher negative charge and size of the 4-arm spacer compared to siRNA alone a higher dose of transfection agent could induce even better outcomes for comparison.

Taken together, the silencing effects of scaffolded siRNAs were generally equal to that of the free siRNA oligonucleotides. The experiments have shown that precise transfection conditions, including lipofectamine:nucleic acid ratio, and siRNA concentration play an essential role for successful and optimal silencing next to cell type and incubation conditions. As the carrier scaffold is larger than the siRNAs alone, an adequate increase of lipofectamine is necessary to avoid poorer transfection. The differences between potencies of four individual siRNAs when transfected in complexed or free form may be caused by intermolecular competition to RISC, or by varying transfection efficacies of a mixture of different siRNA sequences, meaning several of the four siRNAs could preferentially interact with the transfection reagent. On the other hand, complexation to the 4-arm spacer assures in principle equimolar transfection of each oligonucleotide, however the biochemical analysis shows some dissociated components next to the fully assembled construct. Furthermore, the varying sequences of the DNA-overhangs may have different stabilities which could lead to more or less release of siRNA in the cell. The 4-arm spacer and similar RNA-based nanoscaffolds are promising concepts for further development for basic scientific and therapeutic applications. Opportunities for further optimization include the incorporation of delivery systems for self-delivering constructs and the generation of more complex and higher stable assemblies. For in vivo use, the safe application and the absence of off-target and immunogenic effects caused by the scaffolding nucleic acids need to be established.

5 Conclusion

Since delivery of siRNA into the cell and the prevention of its degradation remain one of the most significant obstacles for RNAi therapeutics many modifications and carriers have been developed in the past years to overcome those hurdles. In a previous study[33] a 4-arm spacer out of DNA was synthesized which showed promising results concerning the enhanced stability and gene silencing in Luciferase assay. As a step to the next level this thesis concentrated on the effect of the 4-arm construct attached to four siRNAs which target different genes, respectively.

After the successful synthesis of each antisense and sense strand of siRNA with suitable DNA-overhangs for the attachment to the nanocarrier, the construct was hybridized. The 2.5% agarose gel and stability test showed promising outcomes regarding the self-assembly and stability. In the densitometric quantification of the stability test, the synthesized double stranded siRNAs demonstrated a degradation of about 50% in only one hour of incubation indicating a disadvantage of the 15mer DNA-overhangs next to sticky siRNA of 5mer to 8mer overhangs shown in different studies[18] where improved stability was observed. Further in the first few transfection trials a diminished RNAi was noted which could be due to the defrosting process before each use inducing further degradation. To guarantee reproducibility previously synthesized and used siRNAs[50] were appropriated in later trials. The same degradation process seems to be visible in the 4-arm scaffold with only one siRNA attached and was therefore excluded from later trials.

Careful evaluation of gene silencing was undertaken with optimization of various parameters. With the same amount of lipofectamine the same impact on silencing was detected for free and scaffolded siRNA even though a less effective transfection is to be expected due to the higher negative charge of the scaffold. Furthermore, higher lipofectamine concentrations induce enhanced RNAi but are also able to induce toxicity as seen in fluorescence microscopy experiments.

Another important factor concerning RNAi is the applied concentration of siRNA. In the transfection trials it is apparent that exposure to increased concentrations of siRNA induces less expression of targeted genes. Within the 4-arm spacer the possibility of interactions between the different siRNAs became visible, especially in higher concentrations. By performing a Luciferase assay with Bradford

calibration curve to compensate for different cell counts, the effective transport of the 4-arm spacer into the cytosol was ensured. As noted, studies[51–54] have shown that competitive behavior around RISC is observable in mammalian cells. Another study[60] pictured that the simultaneous RNAi of two proteins is possible. So, to evade the occurrence of competition a concentration with the lowest interaction potential and the highest gene silencing effect has to be found or siRNAs better suited for combination have to be evaluated.

Though the results of this thesis were not as predicted with a uniformly higher gene silencing impact assumed through enhanced stability, the oligonucleotide scaffold remains a potential carrier for siRNA, inducing better stability and the advantage of transporting multiple siRNAs of equimolar concentrations for combinational therapy and therefore the potential of synergistic effects in tumor inhibition by targeting various mRNAs.

List of abbreviations

Ago2	Argonaute 2
AMA	Ammonium hydroxide/Methylamine 1:1
APS	10% ammonium persulfate solution
AURK	aurora kinase targeting siRNA
CPG	controlled pore glass
CTNNB	β-catenin targeting siRNA
den. PAGE	denaturing polyacrylamide gel electrophoresis
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DMT	Dimethoxyltrityl
DNA	deoxyribose nucleic acid
DPBS = PBS	Dulbecco's Phosphate Buffered Saline
dsRNA	double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
ETT	5-Ethylthio-1*H*-tetazol
FBS	fetal bovine serum
GRP78	GRP78 targeting siRNA
LF	lipofectamine = Lipofectamine RNAiMAX
miRNA	micro RNA
MYC = Myc2	MYC targeting siRNA
ONP	oligonucleotide nanoparticle
PenStrep	Penicillin/Streptomycin
PKR	protein kinase R
pRNA	packaging RNA
qRT-PCR	quantitative real-time reverse transcriptase PCR
RES	reticuloendothelial system
REST	Relative Expression Software Tool
RISC	RNA-induced silencing complex
RNA	ribose nucleic acid
RNAi	RNA interference
shRNA	short hairpin RNA
siRNA	short interfering RNA
ssiRNA	sticky siRNA
TBE-buffer	TRIS-Borate-EDTA-buffer
TCA	Trichloroacetic acid
TEA-3HF	Triethanolamine-trihydrofluoride
TEMED	N,N,N',N'-Tetramethylethylenediamine
TLR	Toll-like receptor
TRIS	Tris(hydroxymethyl)-aminomethane

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