Stockholms universitet

Gene therapy tools: oligonucleotides and peptides

Ph.D. thesis in Neurochemistry with Molecular Neurobiology

Stockholm University, Sweden 2016

Jonas Eriksson

Cover: Jonas Eriksson

ISBN 978-91-7649-460-8

Printed in Sweden by Holmbergs, Malmö 2016

Distributor: Department of Neurochemistry

To my late grandmother Inga

An expert is a person who has found out by his own painful experience all the mistakes that one can make in a very narrow field.

Niels Bohr

Abstract

Genetic mutations can cause a wide range of diseases, e.g. cancer. Gene therapy has the potential to alleviate or even cure these diseases. One of the many gene therapies developed so far is RNA-cleaving deoxyribozymes, short DNA oligonucleotides that specifically bind to and cleave RNA. Since the development of these synthetic catalytic oligonucleotides, the main way of determining their cleavage kinetics has been through the use of a laborious and error prone gel assay to quantify substrate and product at different time-points. We have developed two new methods for this purpose. The first one includes a fluorescent intercalating dye, PicoGreen, which has an increased fluorescence upon binding double-stranded oligonucleotides; during the course of the reaction the fluorescence intensity will decrease as the RNA is cleaved and dissociates from the deoxyribozyme. A second method was developed based on the common denominator of all nucleases, each cleavage event exposes a single phosphate of the oligonucleotide phosphate backbone; the exposed phosphate can simultaneously be released by a phosphatase and directly quantified by a fluorescent phosphate sensor. This method allows for multiple turnover kinetics of diverse types of nucleases, including deoxyribozymes and protein nucleases.

The main challenge of gene therapy is often the delivery into the cell. To bypass cellular defenses researchers have used a vast number of methods; one of these are cell-penetrating peptides which can be either covalently coupled to or non-covalently complexed with a cargo to deliver it into a cell. To further evolve cell-penetrating peptides and understand how they work we developed an assay to be able to quickly screen different conditions in a high-throughput manner. A luciferase up- and downregulation experiment was used together with a reduction of the experimental time by 1 day, upscaling from 24- to 96-well plates and the cost was reduced by 95% compared to commercially available assays. In the last paper we evaluated if cell-penetrating peptides could be used to improve the uptake of an LNA oligonucleotide mimic of GRN163L, a telomerase-inhibiting oligonucleotide. The combination of cell-penetrating peptides and our mimic oligonucleotide lead to an IC_{50} more than 20 times lower than that of GRN163L.

List of publications

This thesis is based on the following four publications, from here on referred to as **Paper I**, **II**, **III** and **IV** respectively.

I. **Eriksson J**, Helmfors H, Langel Ü. A high-throughput kinetic assay for RNA-cleaving deoxyribozymes. PLoS ONE. 2015;10(8):e0135984.

II. **Eriksson J**, Langel Ü. Quantitative microplate assay for real-time nuclease kinetics. PLoS ONE. 2016;11(4):e0154099.

III. Helmfors H, **Eriksson J**, Langel Ü. Optimized luciferase assay for cell-penetrating peptide-mediated delivery of short oligonucleotides. Anal Biochem. 2015;484:136–42.

IV. Muñoz-Alarcón A, **Eriksson J**, Langel Ü. Novel efficient cell-penetrating, peptide-mediated strategy for enhancing telomerase inhibitor oligonucleotides. Nucleic Acid Ther. 2015;25(6):306–10.

Additional publications

V. Lindberg S, Regberg J, **Eriksson J**, Helmfors H, Muñoz-Alarcón A, Srimanee A, et al. A convergent uptake route for peptide- and polymer-based nucleotide delivery systems. J Control Release. 2015;206:58–66.

VI. Arukuusk P, Pärnaste L, Margus H, **Eriksson NKJ**, Vasconcelos L, Padari K, et al. Differential endosomal pathways for radically modified peptide vectors. Bioconjug Chem. 2013;24(10):1721–32.

VII. Regberg J*, **Eriksson J***, Langel Ü. Cell-penetrating peptides: from cell cultures to *in vivo* applications. Front Biosci. 2013;E5(2):509–16.

VIII. Baumgarten T, Schlegel S, Wagner S, Löw Klepsch M, **Eriksson J**, Bonde I, Herrgard M, Nørholm M, Slotboom DJ, de Gier J-W. Promoting the evolution of E. coli BL21(DE3) towards enhanced membrane protein production. Submitted to Nature Communications.

* Both authors contributed equally to this work.

Paper I and II of this thesis have previously been presented in my licentiate thesis. ISBN 978-91-7649-323-6.

Contents

Abbreviations

2'OMe	2'-O-methyl RNA
7-MEG	7-Methylguanosine
CPP	Cell-penetrating peptide
CRISPR	Clustered regulatory interspaced short palindromic repeats
DMD	Duchenne muscular dystrophy
DNAzyme	Deoxyribozyme
dNMP	Deoxyribonucleoside-5'-monophosphate
DSB	Double-stranded break
eGFP	Enhanced green fluorescent protein
EtBr	Ethidium bromide
ExoIII	*E. coli* exonuclease III
Fmoc	9-Fluorenylmethyloxycarbonyl
HDR	Homology-directed repair
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA
k_{cat}	Turnover number
K_M	Michaelis constant
k_{off}	Dissociation rate constant
k_{on}	Association rate constant
LNA	Locked nucleic acid
M^{2+}	Divalent metal ion
MDCC	7-Diethylamino-3-[N-(2-maleimidoethyl)carbamoyl]coumarin
NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
ON	Oligonucleotide
PBP	Phosphate-binding protein
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PepFect	PF

P_i	Inorganic phosphate
PNPase	Purine nucleoside phosphorylase
PolyI	Polyinosinic acid
PS	Phosphorothioate
RISC	RNA-induced silencing complex
RNAi	RNA interference
SCARA	Class A scavenger receptors
SCO	Splice-correcting oligonucleotide
SELEX	Systematic evolution of ligands by exponential enrichment
sgRNA	Single-guide RNA
siRNA	Small interfering RNA
SNALP	Stable nucleic acid lipid particles
SPPS	Solid-phase peptide synthesis
T4PNK	T4 polynucleotide kinase
TALE	Transcription activator-like effector
tcDNA	Tricyclo-DNA
TFO	Triplex-forming oligonucleotide
TNA	Threose nucleic acid
TRAP	Telomerase repeat amplification protocol
v_0	Initial velocity
V_{max}	Maximal velocity

1. Introduction

For millennia mankind has used different compounds to treat diseases. Over the last few centuries the molecular mechanisms of many diseases have been determined and therapeutics have been developed from this knowledge. The identification of DNA as the carrier of genetic material and the role of RNA has led to a whole new branch of knowledge and treatments. Treatment of genetic disorders through gene addition, deletion, modification or regulation is called gene therapy.

Based on previous findings of DNA transfer to *Pneumococcus,* gene therapies for mammalian cells were conceptualized in the 1960s and early 70s with the introduction of exogenous DNA into mammalian cells through co-incubation. These experiments exhibited extreme inefficiency and the experiments were not reproducible; regardless, this showed a possibility of stable gene transfer to mammalian cells and researchers hypothesized there must be a better way to introduce the exogenous DNA into the cells.

The definition of gene therapy further expanded to include not only gene addition but also gene regulation, either by removal, down- or upregulation. Not only endogenous genes are included but also exogenous genes such as those introduced by viruses. Many gene therapy agents have been developed, from recombinant viruses to short synthetic oligonucleotides (ON) to engineered enzymes. Only a few of these have reached approval to be marketed as medicines. The great potential of gene therapy to directly treat disease-causing genes pushes the scientific community to further develop new and improved therapeutic ONs. However the problem of therapeutic ONs is many times not the therapeutic mechanism but rather to deliver the ONs to the correct cells with high efficiency. To do so a cell-penetration strategy has to be incorporated in the treatment; for example viral vectors, lipid nanoparticles or cell-penetrating peptides (CPP) can be used for this purpose.

1.1. Antisense oligonucleotides

The first ON drug to get FDA approval was fomivirsen in 1998, an antisense drug which blocks the translation of cytomegalovirus to treat retinitis[1]. Since then several ON drugs have been approved. An antisense ON binds a target RNA through Watson-Crick or Hoogsteen base pairing[2-4]. This interaction, the formation of a duplex or triplex, inhibits mRNA translation either through initiation of RNase H-degradation (only in the case of duplexes) or sterically hindering translation.

The RNase H-mediated degradation is sensitive to the chemical composition of the antisense ON; some ON chemistries such as 2'-O-methyl RNA (2'OMe) can't initiate RNase H degradation as the enzyme recognizes heteroduplexes of RNA-DNA based on the helical structure and 2'OMe doesn't form the same helical structure with RNA as DNA would[5]. It is therefore common to keep the core nucleotides as deoxynucleotides or other chemistries that permit RNase H degradation, so called gapmers. To protect the core nucleotides from degradation by exonucleases, nuclease-resistant nucleotides are added up- and downstream, commonly referred to as 'flanking nucleotides', of the core nucleotides. An alternative protection strategy is circularization of the antisense ON, to 'hide' the 5' and 3' ends from exonucleases. This strategy was used to create ONs which form highly stable and selective triplexes with target mRNA through both Watson-Crick and Hoogsteen base pairing[2].

1.1.1. Inhibition of telomerase

The end caps of chromosomes, telomeres, shorten with every cell division because the replication machinery can't copy the end of the chromosome completely[6]. Ultimately the telomeres become 'critically short' meaning the cells can't divide anymore and they become senescent[7,8]. The number of times a cell can divide before entering the senescent state is called the Hayflick limit. This mechanism is thought to prevent cells from becoming immortal and thus be one step closer to becoming cancerous. However, there is an enzyme, telomerase, which can elongate the telomeres[9]. Human telomerase is constituted by a catalytic protein subunit (human telomerase reverse transcriptase; hTERT) and a guide-and-template-RNA (human telomerase RNA; hTR)[10]. To elongate, the telomere hTERT is guided by hTR to the 3' flanking end of a telomere where hTERT adds nucleotides to the 3' end that are complementary to hTR, 6 nucleotides at a time, before relocating

to the newly formed end where this process is repeated. The formed long single stranded telomere 3' overhang is then turned into a double strand by primase and polymerase α[11]. Telomerase is expressed in more than 90% of all cancers and is needed to allow the cancer to grow indefinitely[12].

A number of attempts have been done to target either hTERT or hTR, the most notable example is using a cell-penetrating antisense ON called GRN163L or imetelstat developed by the Geron Corporation[13,14]. GRN163L is a 13 nt long N3'→P5' *thio*-phosphoramidate ON which specifically binds hTR to inhibit telomerase activity. GRN163L also has a palmitoyl moiety at the 5' end, increasing the cellular uptake of the antisense ON. GRN163L is currently in phase II clinical trials for the treatment of several types of cancer.

1.1.2. Splice-correction

Post-transcriptional modifications are common in eukaryotic cells; one of these modifications is pre-mRNA splicing, the excision of introns and fusing of flanking exon sequences by the spliceosome, a large riboprotein complex located in the nucleus. In 1980 it was discovered that the same pre-mRNA can yield different mRNAs; a phenomenon called alternative splicing[15]. It is estimated that more than 95% of human pre-mRNAs which contain more than one exon can undergo alternative splicing to form multiple mRNAs[16]. Genetic mutations may cause aberrant splicing, ultimately forming mRNAs containing parts of intact introns or where exons have been removed. This event can be countered by masking these aberrant splice sites by ON therapy.

A splice-correcting ON (SCO) binds and masks the splice site through Watson-Crick base pairing, causing the splicing machinery to bypass this site and continue to the next splice-site. This therapy can rescue mutations causing aberrant splicing without actually modifying the gene. The SCO is 15-25 nucleotides long and composed of nucleotides which don't activate RNase H, as this would cause the degradation of the pre-mRNA. SCOs can also be used to skip whole exons to form truncated proteins; which, among others, is the strategy used to treat Duchenne muscular dystrophy (DMD), a disease caused mainly by nonsense or frame-shift mutations in the dystrophin gene[17]. The SCO used for DMD causes the spliceosome to 'skip' an exon, effectively excising it to form a shorter functional form of dystrophin.

1.2. Ribozymes

The idea of RNA being able to catalytically speed up biological reactions was conceived in the 1960's by scientists such as Francis Crick and Leslie Orgel among others[18,19]. The theory was that for the first Darwinian creature to exist the genetic material would have to be copied in order to be passed on. However, in a very primitive system the need for both RNA and protein, and all the systems needed for translation, from the start of life would be too complex; thus the idea of RNA having both the ability to pass on information and to catalyze biological reactions was proposed.

It took about 20 years before the first catalytic RNA (ribozyme) was found; the pre-mRNA of the 26S ribosomal RNA in *Tetrahymena thermophila* was shown to self-splice, a reaction causing the intron to autoexcise and autocyclize without the help of any protein enzyme[20]. Quickly following this discovery the following year Sidney Altmans group proved the RNA component of RNase P to be the sole catalyst of the riboprotein complex, the protein part showing no catalytic activity[21].

Even though there has been proof of protein-free RNA catalysis, the prevalence in nature seem limited as these have only been found in a few viral-like sources[22]. All the naturally found RNA catalysts, except for rRNA in the ribosome, catalyze the scission of RNA, either in *cis* (e.g. hammerhead ribozyme) or *trans* (e.g. RNase P). Self-splicing ribozymes also catalyze ligation of the surrounding exons. Since the discovery of naturally occurring ribozymes a wide range of synthetic ribozymes have been developed with the ability to catalyze different biological reactions; expanding the catalytic diversity further than those of natural ribozymes. Ribozymes promote catalysis using ribosyl hydroxyl groups, metal ions, nucleobases and small molecule cofactors[23].

1.3. DNAzymes

Since RNA had been proven to have catalytic activities and there being a high similarity between RNA and DNA, Breaker and Joyce hypothesized that DNA, much like RNA, could be used for enzymatic catalysis even though no naturally occurring examples have been found[24]. Through a modified systematic evolution of ligands by exponential enrichment (SELEX) protocol (figure 3) they isolated several sequences with the ability of intramolecular cleavage of a ribonucleotide; these sequences were used as inspiration for the design

of an RNA-cleaving deoxyribozyme (DNAzyme) with the capability of multiple turnover intermolecular cleavage of a ribonucleotide-containing substrate strand. This publication was followed by the selection of the, to date, most studied DNAzymes, '8-17' and '10-23' (numbers depict the selection cycle followed by the number of the sequence within the cycle; figure 1), which were selected to cleave a target RNA in a buffer simulating intracellular conditions[25].

Since these discoveries, a number of DNAzymes have been designed and selected for several catalytic activities such as RNA or DNA ligation[26-28], DNA phosphorylation[29], Diels-Alder reaction[30] or nucleopeptide linkage formation[31] among others. Multiple uses for these different DNAzymes have been found, either as sensors for intracellular sodium ions[32] or specific RNAs[33], signal amplifiers[34], or DNAzyme walkers/biomimetic nanomotors[35].

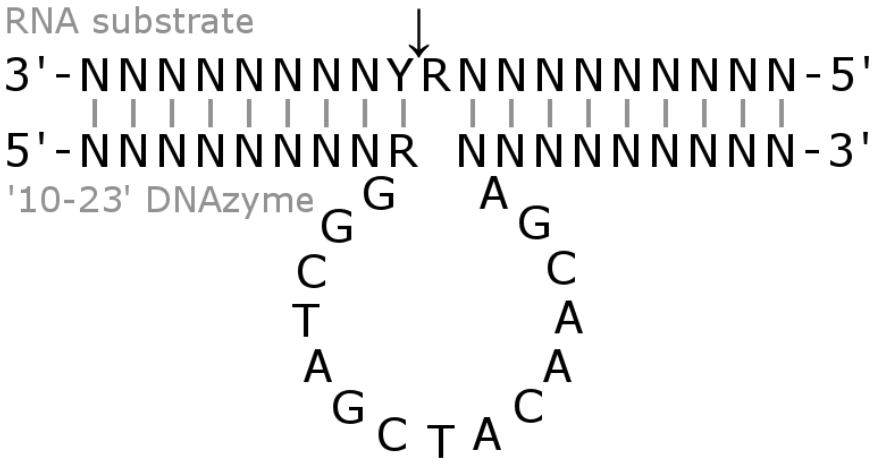

Figure 1. '10-23' DNAzyme bound to RNA substrate. Catalytic loop of DNAzyme is shown as the non-complementary bulge. Cleavage site in RNA is indicated by the arrow and the surrounding nucleotides are a purine (R) and a pyrimidine (Y). The length of binding arms vary and can contain any type of nucleotide (N) depending on the target RNA sequence.

1.3.1. DNAzymes as RNAi agents

The use of 10-23 DNAzymes as knockdown agents in cells has been criticized as the intracellular concentration of free Mg^{2+} has been estimated to be between 0.05 and 2 mM[36-38] while the 10-23 DNAzyme, and others, have been selected under conditions with 10 mM Mg^{2+} or

more; however, the use of 10-23 DNAzymes for RNA interference (RNAi) has been proven several times, both *in vitro*[39-43] and *in vivo*[44,45].

Another problem of using DNAzymes for RNAi is the susceptibility of natural nucleotides to nucleases present extra- and intracellularly. Many strategies have been used to circumvent this problem; introduction of nuclease-resistant nucleotides[46,47] (the most common strategy), addition of nuclease-resistant hairpin sequences to the 5' and 3' ends[41], coupling the DNAzyme to gold nanoparticles[43], circularization to 'hide' the 5' and 3' ends from exonucleases[48], among others. A fascinating version of the introduction of nuclease-resistant nucleotides to DNAzymes is the exchange of all nucleotides by L-DNA, the enantiomer of naturally occurring D-DNA, to form so called Spiegelzymes® which avoid all nucleases[49]; interestingly the exchange to D-DNA neither inhibits the complementary base pairing with L-RNA nor the cleavage activity of the 10-23 catalytic loop.

During DNAzyme SELEX (covered below) the target substrate is an intramolecular stretch of ribonucleotides which is far from similar to the final intended substrate (full length mRNA if used for RNAi), therefore the use of these DNAzymes for the mRNA has to be evaluated as mRNAs are highly structured, only about 10% of putative cleavage sites are accessible to DNAzymes[50,51], and the DNAzyme has to be able to work for multiple turnovers. To screen for accessible sites of the mRNA, different techniques have been used: individually testing DNAzyme cleavage at each putative cleavage site of the mRNA[50]; multiplex cleavage of target mRNA with several DNAzymes and determining which sites have been cleaved by primer extension[52]; using peptide nucleic acid antisense ONs which bind the flanking sequences of the DNAzyme target site can 'open' up the structured mRNA to allow DNAzyme binding and cleavage[53]; also, introduction of modified nucleotides such as 2'OMe or locked nucleic acids (LNA) which increase the T_m can allow the DNAzyme to invade the target strand even if the mRNA is structured[54].

The great selectivity of DNAzymes has been shown to be useful for specifically cleaving single-nucleotide polymorphisms[53,55] and disease-causing mRNA fusions[52]. Control of the catalytic activity of the 10-23 DNAzyme has been done by introducing photolabile groups into the DNAzyme molecule to gain an 'on/off switch' or increase/decrease the cleavage activity by shining UV light on the molecule[56,57]. Alternative ways of introducing DNAzymes to specific cells have been explored and Sugiyama et al. introduced a gene, which transcribes an RNA template for a DNAzyme, by lentiviral transfer to cells; these cells would then be

challenged with wild-type HIV-1 and the RNA template which was previously introduced would serve as a substrate for HIV-1 reverse transcriptase, effectively producing DNAzymes by reverse transcription specifically in the HIV-1 infected cells[42].

1.3.2. '10-23' catalytic loop

Catalytic loops of DNAzymes differ greatly, both in length and nucleotide composition. The 10-23 DNAzyme catalytic loop was thought to be highly intolerant to changes[58]; this was later shown to not hold true for many of the nucleotides of the catalytic loop where the nucleotides close to the border were highly conserved while nucleotides 7-12 could be replaced without high loss of catalytic activity[59]. The thymidine at position 8 allows for the most changes, exchange for another natural nucleotide only decreases the activity up to ~25% while a deletion of this nucleotide retained or slightly decreased the activity[60,61]. Dividing the DNAzyme into two ONs is possible if the division is made next to the thymidine at position 8 or adenine at position 12 causing only a 50-70% decrease in activity[56].

The nucleotide least susceptible to exchange or modification is guanosine at position 14, which upon either exchange of the nucleotide for another, or removal of either functional group of the guanine group cause almost complete loss of function. Nucleotides 1-6 and 13-14 of the catalytic loop are implicated to be directly involved in the catalytic function of the 10-23 DNAzyme. These data show that the catalytic loop is flexible both in structure and to changes. Furthermore 2'OMe modifications have been introduced into the catalytic core to increase resistance to endonucleolytic degradation without significant changes of the catalytic activity[62]. Nucleotides with modified bases, containing imidazole, ammonium and guanidinium groups, have been added to other DNAzyme catalytic loops to remove the need for divalent metal ions (M^{2+})[63].

The first crystal structure of a DNAzyme, RNA-ligating 9DB1, in active conformation was published in 2016[65]. Unlike ribozymes which have a 2'-hydroxyl group to use during catalysis, the DNAzymes have a larger degree of flexibility allowing them to explore a wider range of conformations, making up for the lack of a 2'-hydroxyl group. Attempts have been made to crystallize 10-23 DNAzyme, however the attained crystal structures showed conformations which are not the active form of the 10-23 DNAzyme[66].

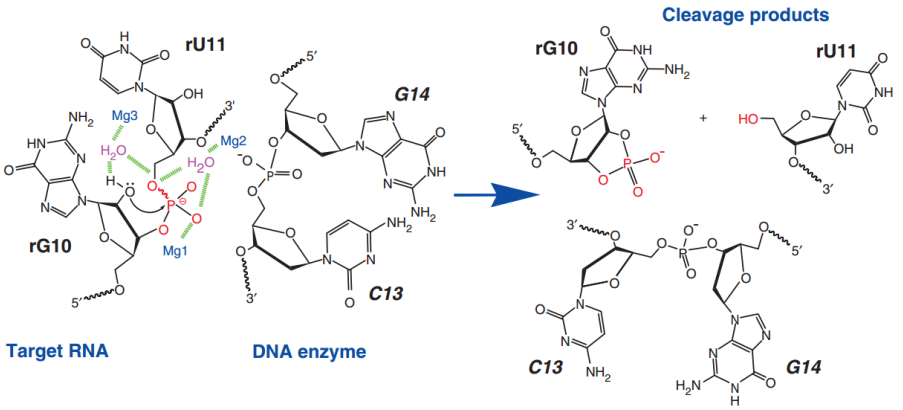

Figure 2. Structure of the catalytic site based on simulations. Suggesting three Mg^{2+} are involved in the catalytic mechanism of 10-23 DNAzyme and the direct involvement of C13 and G14[64].

Simulations of the 10-23 DNAzyme folding comparing different mutants and metal ion conditions suggests an electrostatic pocket is formed close to the scissile phosphate by the catalytic loop and the RNA substrate backbone[64]. The nucleotides suggested to be part of the electrostatic pocket is G1-G7 and C13-G14 which is in coherence with the findings of Zaborowska et al[59]. The electrostatic pocket is thought to attract, hold and direct M^{2+} toward the scissile bond. Mutations of the catalytic core is thought to cause changes in the folding of the catalytic core and in turn the electrostatic pocket, changing its ability to coordinate the substrate and M^{2+}. The two, or possibly three, Mg^{2+} in the electrostatic pocket are thought to stabilize the transition state during 2'-oxyanion attack and stabilizing the leaving 5'-OH product (figure 2). Furthermore the similar structure of the duplexes formed between the binding arms of the DNAzyme and the substrate for both active and inactive DNAzyme while the electrostatic pocket differ suggest the catalytic loop is solely responsible for the catalytic activity of the DNAzyme. The formation of a 2'-oxyanion next to the scissile phosphate has been postulated to be generated by metal-hydroxide assisted deprotonation or by the coordination of M^{2+} to act as a Lewis acid at the 2'-OH, enhancing its acidity. The activated 2'-oxyanion performs an S_N2 attack on the scissile phosphate. Similarities to some ribozymes such as hammerhead ribozymes could give clues to the 10-23 DNAzyme catalytic mechanism[23].

1.4. Systematic evolution of ligands by exponential enrichment

SELEX is a process to select ONs with a specific property, such as catalytic activity or binding affinity, from a library of random ONs[67-69]. Several rounds of selection are made with varying conditions; varying the buffer composition, reaction time, temperature or doing negative selection by removing a vital component. After each round the selected ONs are amplified, usually by polymerase chain reaction (PCR), to increase the number of molecules in the library. After selection steps sequencing can be performed either by cloning the library into a plasmid and transforming bacteria to select single colonies to sequence single sequences by general sequencing methods such as Sanger sequencing or, for a deeper sequence coverage, by Next-Generation Sequencing[24,70,71]. SELEX was originally created to select DNA ONs which specifically bind to yeast GCN4 protein from a library of ONs[67]. From the repeated selection cycles the researchers could identify a number of different sequences and from these determine the sequence specificity of GCN4 DNA-binding. Since then many different ONs with various catalytic activities and binding affinities have been selected by modified SELEX protocols.

In short, application of SELEX to selection of RNA-cleaving DNAzymes has been done by coupling the DNAzyme library to a biotin-labeled ON containing at least one ribonucleotide (figure 3). The resulting ON would be bound to a streptavidin bead and mixed with reaction buffer. Any sequences capable of *cis*-cleaving a phosphate next to a ribonucleotide is subsequently eluted from the beads while sequences without this ability are kept bound to the beads. The selected sequences would be amplified by PCR and the biotin-labeled ON would be coupled again to the DNAzyme library for further selection, usually in more stringent conditions such as lower concentration of M^{2+} or shorter reaction time.

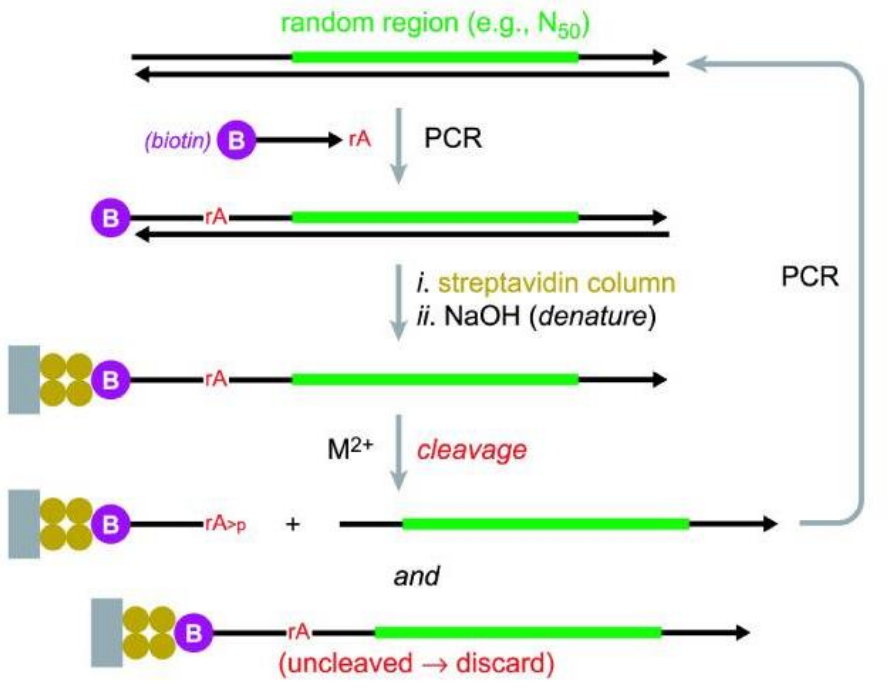

Figure 3. DNAzyme systematic evolution of ligands by exponential enrichment. Adapted from Silverman, 2005[72].

1.5. Oligonucleotide aptamers

Aptamers are a class of ONs which can bind specifically to a wide range of molecules. Aptamers are similar to antibodies but with the added benefits of the possibility to be produced completely in a test tube by chemical synthesis, better storage capability and elicit low to no immunogenicity[73]. As previously described, SELEX is the main method used to develop aptamers. However, when using SELEX the choice of nucleotides is limited as not all types of modified nucleotides can be incorporated by polymerases. This lowers the possibility to select nuclease-resistant aptamers. However, lately there have been advances in using modified nucleotides directly in the SELEX process[74].

The main choice in making nuclease-resistant aptamers has been to introduce phosphorothioate (PS) linkages during or after SELEX; however, changing nucleotides after SELEX can often result in loss of affinity of the aptamer. Another strategy to create nuclease-resistant aptamers is to select aptamers against a mirror-image of the target molecule and after SELEX invert the stereochemistry of the nucleotides,

using L-nucleotides, forming Spiegelmers®, instead of the naturally occurring D-nucleotides; this allows the aptamer to bind to the target molecule with the same affinity as the SELEX-selected molecule binds the mirror-image[75,76]. In 2004 the first aptamer received FDA-approval, Macugen, for treating age-related macular degeneration administered by intravitreal injection[77].

1.6. Nucleotide modifications

There are many endogenous exo- and endonucleases expressed in humans, which usually are the targets for gene therapy. These nucleases are both found in cells and in extracellular environments such as the blood; endonucleases tend to be less prevalent extracellularly, however this varies greatly between species[76]. The nucleases found in blood degrade natural RNA- or DNA-containing ONs quickly[62,78]; however, this degradation can be reduced by introduction of modified nucleotides.

There are five classes of modifications which can be done to ONs: modification of the phosphate backbone, 2' modifications, bridged or cyclic NAs, modification of the NA base, or replacing the phosphate and/or ribose backbone (examples of these can be seen in figure 4). PS is the most commonly used nucleotide modification, replacing one of the non-bridging oxygens in the phosphate backbone with a sulfur. The PS modification renders the phosphodiester bond more stable to nuclease attack. However, PS modification can often cause innate immune-responses and hepatotoxicity[79]. 2'OMe is a naturally occurring modification which can be found in small RNAs such as tRNAs, introduced as post-transcriptional modifications[80]; 2'OMe and also 2'-fluoro modifications are commonly introduced to chemically synthesized ONs, both 2'-fluoro and 2'OMe modifications increase oligonucleotide resistance to nuclease degradation, mainly due to the lack of a 2' hydroxyl group, and increases the T_m for duplex formation. 'Bridging' or 'locking' a nucleic acid signifies connecting a methylene (for LNA, others can be used) bridge between the 2' and 4' positions of the ribose ring, this 'locks' the ribose conformation in N-type, which is adapted in A-form RNA duplexes, increasing the affinity of the ON for RNA and increasing nuclease resistance. Base modifications tend to have modest effect on nuclease resistance, however these modifications are generally employed to decrease the innate immune response to the ONs; base modifications are also used, as mentioned previously, in probing the importance of functional groups in catalytic ONs. The last class of modifications, replacing the ribose backbone, is of rising interest as more

research is put into them, one example is threose nucleic acid (TNA), where the ribose has been exchanged to a threose and the phosphate backbone is connected 2'-3'. TNAs have been shown to be essentially completely resistant to nuclease degradation[81]; also, the possibility to perform SELEX of TNA ONs using a polymerase and a reverse transcriptase has been developed[82]. Expansion of the sugar backbone to incorporate bi- or tricyclic sugars is tested in several labs, the most prominent modification being the tricyclo-DNA (tcDNA) which spontaneously form nanocomplexes by itself and is taken up by cells similarly to CPPs[83]; tcDNAs have met great success through splice-correction in a mouse model of DMD, restoring most tissues of the mice to a healthier state[17]. Modified nucleotides have further been used to select for catalytic 'XNAzymes'[84,85].

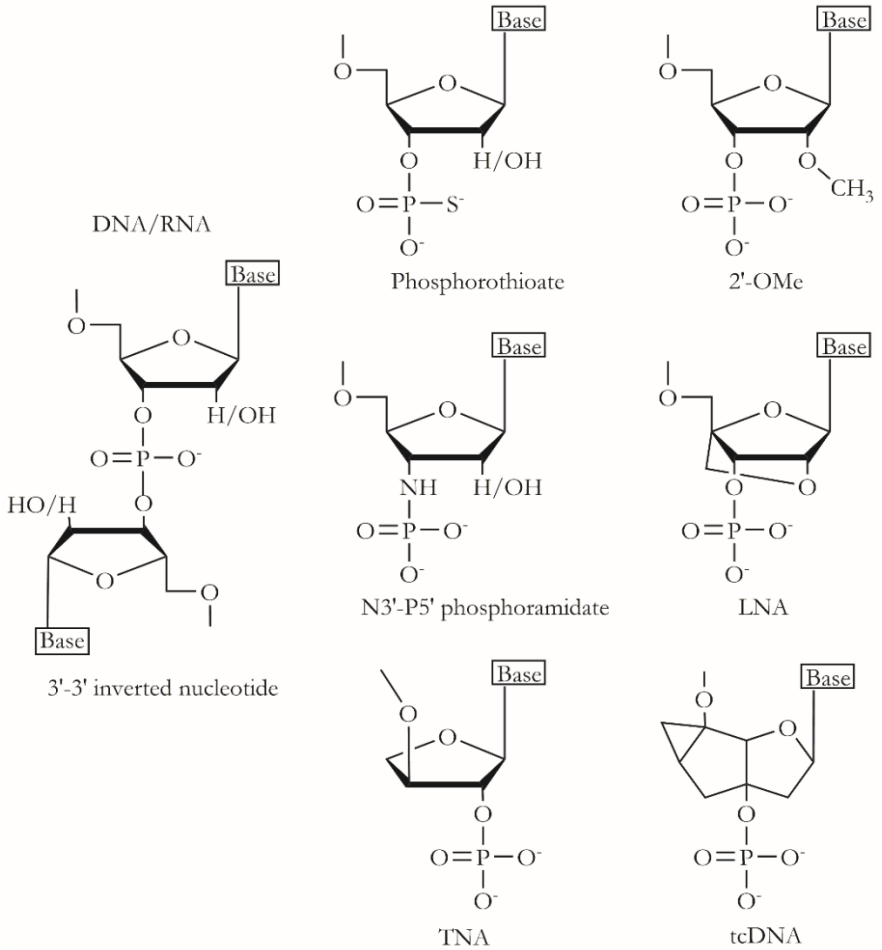

Figure 4. Examples of nucleotide modifications.

1.7. RNA-induced silencing complex

In 1998 *Fire et al.* discovered that injecting nematodes with dsRNA produced substantially more knockdown of a target gene than injecting either the antisense or sense strand alone[86]. They showed that only a few molecules of dsRNA per cell could elicit a strong knockdown effect over a long time indicating either an amplification or catalytic process to be involved, which was proven to be the latter. The interference pathway that was discovered was that of the so called RNA-induced silencing complex (RISC), a term which was coined in a later publication[87]. Transcribed or exogenously added dsRNA can be digested by an enzyme named Dicer into short, 21-23 nt, dsRNAs called small interfering RNA (siRNA)[88]; these are further unwound by, and one strand is loaded into, RISC. The loaded RISC then searches for and binds to mRNAs with reverse complementary sequence of the guide strand. The bound mRNA is cleaved by an enzyme, Argonaute 2 (Ago2), incorporated in RISC[89]. This enzymatic cleavage is what confers the high efficiency and multiple turnover capability of the siRNA-mediated knockdown. Loaded RISC can also cause steric hindrance of mRNA translation[90].

1.8. Transient gene expression

Plasmids are circular extrachromosomal genetic material and are most commonly found in bacteria. Plasmids can be used for transient or stable transfection to deliver large genetic material, express proteins and transcribe small hairpin RNA among other applications. For plasmids to elicit their function in cells they need to reach the cell nucleus, a process which has proven to be highly inefficient in cells as the nuclear envelope is basically impermeable to molecules, which in this case would have to pass through the nuclear pore complex. During mitosis the nuclear envelope is disassembled and permeation is increased, allowing plasmids to enter the nucleus to a greater extent in dividing cells compared to non-dividing cells[91-93]. Transfection of plasmids raise several safety risks such as the possibility of illegitimate DNA integration, integration through homology-dependent recombination, prokaryotic sequences (e.g. origin of replication, antibiotic resistance gene) being present or the plasmid could induce an immune response in the host organism[94,95]. To minimize the size and remove prokaryotic sequences from plasmids minicircles were created. By adding specific recombination sites and a recombinase to the plasmid, excision and recircularization of the expression cassette can be achieved, ultimately degrading the prokaryotic

part of the plasmid allowing purification of the minicircle. Minicircles further elicit activation of the exogenous silencing machinery to a lesser extent than plasmids allowing for extended functional time of expression[96].

Another approach to transient gene expression is the transfection of *in vitro* transcribed mRNA. This vector has several advantages over plasmids; mRNA allows for more control over expression levels, removes the risk of insertion into the host genome, no introduction of prokaryotic sequences, and mRNA doesn't need to enter the nucleus. As mRNA is translated in the cytoplasm the need for the gene therapy vector to enter the nucleus is abrogated, allowing for high expression even in quiescent and post-mitotic cells; which is difficult for plasmids as the nuclear import is greatly diminished[97]. A novel variant of an mRNA translation vector was published in 2015; the mRNA was circularized and included a kozak sequence, allowing translation inititation of the circular mRNA through a rolling-circle mechanism creating concatameric proteins in human cells[98].

1.9. Genome engineering

The idea of inserting genes into the chromosomes of a living cell has existed for a long time and was the initial definition of gene therapy. Integrating a functional gene in a cell to replace or even repair a dysfunctional gene has been the main interest in the genome engineering field of gene therapy. The major difficulty of the methods developed for this field is controlling the site of integration.

Sambrook et al. made the discovery that simian virus 40 integrates its genome into chromosomes of the infected cell[99]; ever since, different types of viruses have been discovered to integrate their genome in a similar fashion and have subsequently been engineered to integrate genes of interest. Especially retro- and lentiviruses have been used for this purpose. However, viral integration is mainly unspecific or has slight preference for certain regions of the genome. This seemingly random integration can cause disease to develop, mainly cancer, through insertional mutagenesis.

The need for targeting of integration was initially met by zinc-finger nucleases, a fusion of zinc-finger domains which can specifically bind target dsDNA and FokI endonuclease to induce double-strand breaks (DSB), and transcription activator-like effector (TALE) nucleases, fusions between DNA-binding domains from TALE proteins and FokI

endonuclease[100]. Both of these techniques induce double-stranded breaks which cause the cell to initiate either of two repair mechanisms, nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ can cause insertions or deletions at the DSB site while HDR can insert a gene of interest with homology around the DSB site. These two fusion proteins are difficult to design and thus expensive.

Recently a new method has been developed from the discovery that many prokaryotes use clustered regulatory interspaced short palindromic repeats (CRISPR) together with a Cas nuclease as a form of acquired immunity[101]. The current state of this technique utilizes a Cas nuclease, usually Cas9, and a single-guide RNA to specifically bind a dsDNA region and cleave it[102,103]. The design of this single-guide RNA (sgRNA) is simple and the system can be multiplexed to use several different sgRNAs in the same cell to target several locations in the genome simultaneously. Introducing these into the cells can be made in different ways such as viral transduction, incorporating them in plasmids or even *in vitro* translating Cas and transcribing the sgRNA before delivering them into the cell.

1.10. Transcriptional activation and repression

Strategies for transcriptional activation have been developed, mainly focusing on attracting transcription factors to the vicinity of a promoter. Early systems used triplex-forming ONs (TFO), relying on their ability to bind the dsDNA of plasmids and chromosomes by Hoogsteen or reverse-Hoogsteen base pairing with a polypurine tract. The TFO would be connected to a hairpin which allowed the binding of endogenous transcription factors for subsequent transcriptional activation of target gene in treated cells[104]. Another recent method based on a TALE system has been developed to activate transcription. By coupling a TALE protein to the TET1 hydroxylase catalytic domain transcription could be activated by demethylation of specific critically methylated promoters. With the excellent targeting and multiplexing capability of Cas9 researchers have been able to activate transcription of several genes simultaneously. A nuclease-deficient Cas9 was fused to three transcription factors inducing an upregulation of RNA expression of up to 20000 times for a specific gene[105]. This construct was used to differentiate human induced pluripotent stem cells to neuronal cells without the use of permanent cell modifications such as lentiviruses. By using Cas9 fused to the transcription factor VP64 three independent groups could activate latent HIV-1 in cell culture models, addressing a

possible future path of curing HIV-1 infection[106]. Furthermore, repression of transcription can be induced by catalytically dead Cas9; utilizing this variant of Cas9 and guide RNAs researchers could block transcriptional initiation or elongation simply by steric hindrance by Cas9 binding to a gene or promoter[107].

1.11. Enzyme kinetics

Since the discovery of enzymes there has been a need to elucidate reaction mechanisms and rate constants of enzymatic reactions; this has been done by various enzyme kinetic assays. Simple enzymatic reactions can be described by Michaelis-Menten kinetics[108]. This model for enzyme kinetics describes an enzyme binding to a substrate, by rate constants defining association (k_{on}) and dissociation (k_{off}), and the catalytic activity of the enzyme upon the substrate and release of product, defined as the turnover number (k_{cat}) as seen in figure 5. An enzymatic reaction, which can be described by the Michaelis-Menten kinetics, can be divided into three states: pre-steady state, steady state (multiple turnover, $E < S$) and single turnover ($E \geq S$) as seen in figure 6. The pre-steady state is defined as the time between the reaction initiation until the enzymes get saturated with substrate; after which the concentration of enzyme-substrate complex becomes constant ($d[ES]/dt \approx 0$). The pre-steady state of the reaction is generally very fast.

Figure 5. A simple enzymatic reaction where free enzyme (E) and free substrate (S) is in equilibrium with enzyme-substrate complex (ES), with two rate constants (k_{on} and k_{off}) describing the association and dissociation rates of the equilibrium. The turnover number (k_{cat}) is the rate which product is formed and released from the ES.

Steady-state occurs as long as [ES] is constant. During this time-frame the formation and release of product is constant ($d[P]/dt$). As the product formed may influence further enzymatic catalysis, through product inhibition, the assumption of constant [ES] and product formation is not always true. Therefore, the steady-state enzyme reaction

rate, v, is measured before these effects may take place, avoiding rather than preventing them[108]. Plotting the reaction rate against [S] in a Michaelis-Menten saturation curve, where the maximal reaction rate is V_{max} and the [S] at half V_{max} is defined as K_M, the Michaelis constant. V_{max} is equal to the product of initial enzyme concentration $[E]_0$ and k_{cat}; as V_{max} is the maximal reaction rate when enzyme is saturated with substrate. K_M can be viewed as a measure of enzyme-to-substrate affinity since it is defined as: $K_M = (k_{off} + k_{cat}) / k_{on}$. Using these constants, a specificity constant ($= k_{cat} / K_M$) can be calculated and used as a measure for the enzyme efficiency, the conversion of substrate to product in a multiple turnover fashion, and for easy comparison between different enzymes.

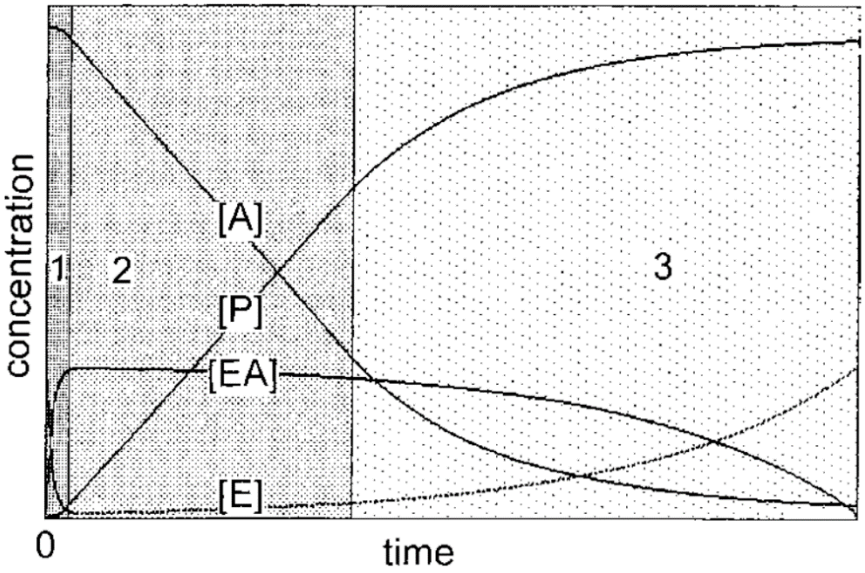

Figure 6. Progress curves of components of a Michaelis-Menten reaction[109]. Substrate [A] and product [P] concentrations decreasing and increasing, respectively, over time. 'Steady state' (2) is defined as the time when enzyme-substrate complex concentration [EA] is approximately constant. 'Pre-steady state' (1) is the period prior to steady state. The time after steady state is 'post-steady state' (3) where the rate of product formation is declining.

1.11.1. Nuclease kinetics assays

Since the discovery of nucleases there have been many published assays for nuclease kinetics. The simplest methods involve periodically retrieving samples from nuclease reactions for further substrate/product

separation and quantification in agarose or polyacrylamide gels, a laborious and inaccurate method[25,110]. Many other assays demand modified substrates, for example labeling with fluorescent molecules creating a non-natural substrate that might skew reaction kinetics by interfering with binding and or catalysis[111-113]. Some assays are incapable of detecting multiple cleavage events of the same substrate, e.g. when the substrate has been cleaved once the following cleavage events are not detected by the assay[114].

Development of kinetic assays for RNA-cleaving DNAzymes has been scarce. The standard of the field is still to periodically retrieve samples from nuclease reactions to separate and quantify either the substrate or product in gels. One of the few assays developed is an assay employing an extrinsic fluorescent intercalating dye, ethidium bromide (EtBr), for single turnover DNAzyme kinetics; however it has several drawbacks, including being limited to single turnover, the inability to measure highly structured DNAzymes as well as the inability to use full length mRNA as substrate[52]. In paper I, a further development of this method is presented which is able to measure highly structured DNAzymes and use full length mRNA as substrate[115]. A completely novel assay to determine nuclease, either protein- or nucleotide-based, kinetics is presented in paper II[116]. The assay is based on the common denominator among nucleases, they expose an ON backbone phosphate for each cleavage event (figure 7); the exposed phosphate can be released by a phosphatase and quantified in real-time by a fluorescent phosphate sensor, which is described below.

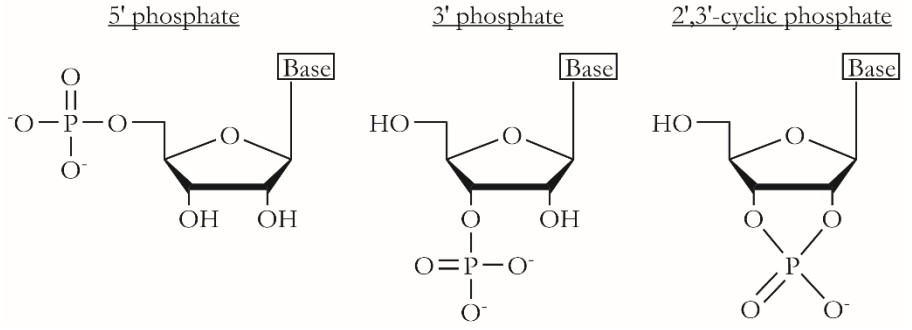

Figure 7. The different forms of exposed phosphates after nuclease cleavage.

1.11.2. Phosphate-binding protein

A sensor for inorganic phosphate (P_i) was developed in 1994 by Brune et al. using the *E. coli* phosphate-binding protein (PBP) with an A197C mutation and coupling an environment-sensitive fluorophore to the cysteine[117]. PBP is composed of two domains which are connected by a hinge; the two domains together form a binding cleft for P_i. Binding of P_i causes a structural change, bringing the two domains closer together[118]. PBP is produced and transported to the periplasm in response to low levels of nutrients. In the periplasm PBP scavenges P_i as a first step to transport it into the bacteria. The binding of P_i is quick, tight and specific.

Introduction of the A197C mutation to PBP with subsequent covalent coupling of the environment-sensitive fluorophore 7-diethylamino-3-[N-(2-maleimidoethyl)carbamoyl]coumarin (MDCC) forms a biosensor for P_i (MDCC-PBP, commercial name Phosphate Sensor)[117]. As PBP undergoes a large structural change upon binding of P_i, the environment-sensitive MDCC responds to the induced structural change, where the two domains move closer to each other, and increases its fluorescence 6- to 8-fold. This sensor was originally used to determine kinetics of an ATPase; other enzymes have been used together with the sensor, such as a protein phosphatase[119] or measuring pyrophosphate release kinetics of a DNA polymerase by adding pyrophosphatase for a coupled enzyme kinetic assay[120].

1.12. The gene therapy delivery problem

As cells rarely take up exogenous ONs without degrading them the whole idea of gene therapy comes to a stop without the help of a transfection agent; either by direct modification of ONs to increase uptake or using any of the plethora of transfection agents evolved by nature (e.g. viruses) or synthetic (i.e. lipids or peptides). Barriers which need to be overcome include extracellular sequestration by the liver and kidneys, degradation of ONs by extra- and intracellular nucleases, passing the plasma membrane, endosomal escape if endocytosis is the mode of uptake, entry into the nucleus (not needed for all gene therapy strategies) and delivery to specific cells through targeting.

1.12.1. Viral vectors

In 1968 *Rogers* and *Pfuderer* demonstrated the first proof-of-concept gene transfer mediated by a virus[121]. They introduced a polyadenylate stretch to the 3' end of tobacco mosaic virus RNA. Once the viral RNA was taken up by plant cells the RNA would translate the viral RNA into protein fused to a lysine stretch because of the polyA stretch. Many viral gene transduction experiments and clinical trials followed causing a boom in the gene therapy field. Until the tragic death of a patient suffering from partial deficiency of ornithine decarbamylase which was treated with a very high dose of adenovirus causing a massive immune response; he died 4 days later from multiorgan failure[122]. Later, other clinical trials have had problems with insertional mutagenesis causing leukemia in treated patients[123,124]. The largest fears of viral-mediated gene therapy today are the risks of insertional mutagenesis and immunogenicity.

China was the first (2003) country to approve a gene therapy product based on a virus; the adenoviral vector Gendicine™ is used to introduce human p53 cDNA to treat head- and neck squamous cell carcinoma. Two years following the approval of Gendicine™ another virus-based gene therapy was approved; Oncorine™ which is a conditionally replicative adenovirus capable of replication and cell lysis only in p53-deficient cells, a condition often met in cancerous cells. The first gene therapy product in Europe, Glybera®, was approved in 2012. Glybera® is an adeno-associated viral vector targeted to muscle tissue to express human lipoprotein lipase in patients with severe lipoprotein lipase deficiency. The cost of Glybera is high, it has a recommended retail price of €53000 per vial, each patient needing an average of 21 vials bringing the total cost of treatment to €1.1M[125].

The three main viral vector systems are integrase-defective lentiviral vectors, adeno-associated viral vectors and adenoviral vectors[126]. These have different advantages and disadvantages, mainly genetic instability with integrase-defective lentiviral vectors while adeno-associated and adenoviral vectors may be sequestered by pre-existing neutralizing antibodies as most of the human population have already been exposed to these viruses. There is also a risk of insertional mutagenesis involved in viral transduction. The limited size of some viral genomes can only allow for insertion of relatively small genetic material (4.5-5 kb in adeno-associated viral vectors[126,127]). There is also the risk of immune response toward the viral vector itself and the production efficiency of viruses is low.

1.12.2. Lipid vehicles

Liposomes and lipid nanoparticles have been studied for a long time and in many different compositions. They've been used both for ON and conventional drug delivery. The fundamental idea being lipids enclosing an aqueous (liposome), lipid (nanoemulsion) or solid (lipid nanoparticle) core. The lipids can be modified to contain different types of lipid tails and head groups, changing the particle characteristics such as charge, size and drug release[128]. The lipids can further be decorated with various molecules such as targeting ligands or polyethylene glycol to increase circulation time[129]. Later, so called stable nucleic acid lipid particles (SNALPs) have been developed to deliver ONs. SNALPs contain both cationic and fusogenic lipids, for cellular uptake and endosomal escape respectively. SNALPs also contain cholesterol to stabilize the lipid bilayer of the particle. SNALPs have been used to efficiently deliver many ONs both *in vitro* and *in vivo*; for example, a single dose of SNALP with siRNA was used to knockdown ApoB in cynomolgus monkeys, inducing a reduction in ApoB levels for 11 days[130].

1.12.3. Non-lipid vehicles

Two of the most used non-lipid transfection reagents are polyethyleneimine (PEI) and poly-L-lysine. The use of poly-L-lysine as a transfection reagent for ON delivery has not seen much success; with low transfection efficiencies and showing some cytotoxicity *in vitro* this reagent is rarely used. PEI on the other hand has seen a wide range of use owing to its high transfection efficiency and low cytotoxicity.

The transfection efficiency is speculated to be due to the so called "proton sponge effect", a supposed event occurring in endo- or lysosomes where a molecule with protonatable residues, at neutral pH, buffers the pH and in turn inducing osmotic swelling and eventually rupturing the endo- or lysosome[131,132]. This theory has since never been proven and is questioned[133]. Recent findings suggest a cooperative action between PEI-pDNA complexes and free PEI chains; the addition of extra PEI to fully PEI-complexed pDNA (N:P = 3) increased total uptake and speed of uptake[134]. The buffering capacity, or proton sponge effect, of PEI has been shown to delay the maturation of endosomes and the fusion of endosomes to lysosomes; much like lysosomotropic agents (e.g. ammonium chloride or chloroquine) or bafilomycin A_1, a vacuolar H^+-ATPase inhibitor, preventing endosomal acidification[135,136].

Other non-lipid vehicles which are not based on polymers have been created such as covalent coupling of cargo to metal nanoparticles or even cell-penetrating RNA aptamers. DNAzymes coupled by disulfide bridges to gold nanoparticles was used to knock down tumor necrosis factor-α in a myocardial infarction mouse model to reduce irreversible damage caused to the heart post-myocardial infarction[137]. The development of cell-penetrating RNA aptamers was done by SELEX with the use of nuclease-resistant nucleotides; a library of RNA ONs were applied to cells in culture and extracellular RNA was degraded by strong nucleases while internalized RNAs were purified and reamplified for further rounds of SELEX[138]. CPPs are another kind of non-lipid transfection vehicles; CPPs are covered below. The wide range of molecules which can be used to initiate transfection shows both how versatile and complex the field is.

1.13. Cell-penetrating peptides

In 1988 the first reports of a protein, and truncations thereof, being capable of passing the cellular membrane were reported[139,140]. The protein was trans-activator of transcription (tat) from HIV-1. Later the minimal truncation needed for the transduction capability was determined for tat and also for the protein Antennapedia from *Drosophila* and these short peptides were called 'cell-penetrating peptides'[141,142].

There have been many modifications of CPPs while the most important one being the addition of an N-terminal lipid which could increase transfection efficiency 100-fold for most CPPs[143]. Throughout the following two decades a plethora of new CPPs would be reported; protein-derived, designed and chimeric (a mix of protein-derived and/or designed) CPPs. To the protein-derived category belong such peptides as penetratin and Tat(48-60), while synthetic CPPs which have been designed by man contain peptides such as polyarginine and CADY[144,145]. The last category, chimeric CPPs, contain the peptide families PepFects and NickFects which have been developed by our lab. They are based on two naturally occurring peptides, galanin and the wasp venom peptide mastoparan[146]. Through a series of modifications the PepFects have gone from being modified with lipids[147] (stearic acid; PepFect3) to including covalently linked endosomolytic chloroquine analogs to branch structures[148] (PepFect6) and replacing lysines with ornithines and rearranging the hydrophobic residues[149] (PepFect14). These designs were logically followed up with PepFect15, incorporating all three

modifications for further improvement of the peptides cell-penetrating effect[150].

CPPs have been used as fusions together with proteins to transfect active proteins into cells and even live animals[151,152]. Covalent coupling of cargo ONs to CPPs have been a popular strategy however a much simpler setup is to mix CPPs with ONs to form non-covalent complexes, a strategy first used in 2007[153].

1.13.1. Mechanism of uptake

Early experiments on CPP uptake indicated a direct penetration mechanism. Cells would take up fluorescently labeled CPPs both at 37°C and 4°C, which should prevent all endocytotic activity. In some cases this would later be disproven as researchers realized that the fixation of cells in these experiments led to disruption of the cell membrane, allowing any peptide to pass into the cells[154]. Following this, new experimental setups were introduced to evaluate the uptake of CPPs which would also show that most, arguably all[155,156], CPPs at low concentration were endocytosed and not taken up by direct penetration (figure 8). The common way of determining which endocytotic pathway is used is to pretreat the cells with inhibitors of these pathways[157]. Mostly there is no one specific pathway utilized by a specific CPP but rather several pathways used simultaneously.

Our group recently found a class of receptors which might be part of the endocytosis of CPP nanoparticles[158]; class A scavenger receptors (SCARA) is a widely expressed family of receptor and highly expressed in macrophages. The SCARAs are associated with the 'scavenging' or 'cleaning' of the blood stream for exogenous particles. Inhibiting the action of SCARAs by application of either of three inhibitors (Fucoidan, dextran sulfate or polyinosinic acid; PolyI), but not chemically similar reagents without inhibitory effect (galactose, chondroitin sulfate or polycytosinic acid), prevented cultured cells from being transfected by CPP and polymer nanoparticles[156,158]. Targeting peptides have been fused to CPPs to target specific cells or enhance transcytosis (figure 8)[159].

Nanoparticles used for *in vivo* delivery tend to be sequestered in the liver and kidneys. These are the major organs for cleaning the blood and it is only logical that this should occur. The particles which end up in the liver don't uniformly go to every cell of the organ but are rather mostly taken up by the Kupffer cells, macrophages of the liver[160]. The Kupffer cells are rich in SCARAs to help with their role as blood stream cleaners.

It has previously been reported that the sequestration effect of the Kupffer cells upon adenoviruses can be abrogated by pretreating the animal with polyI; the pretreated animals would then be treated with adenovirus which would be taken up by hepatocytes instead through targeting to the coxsackievirus-adenovirus receptor[161].

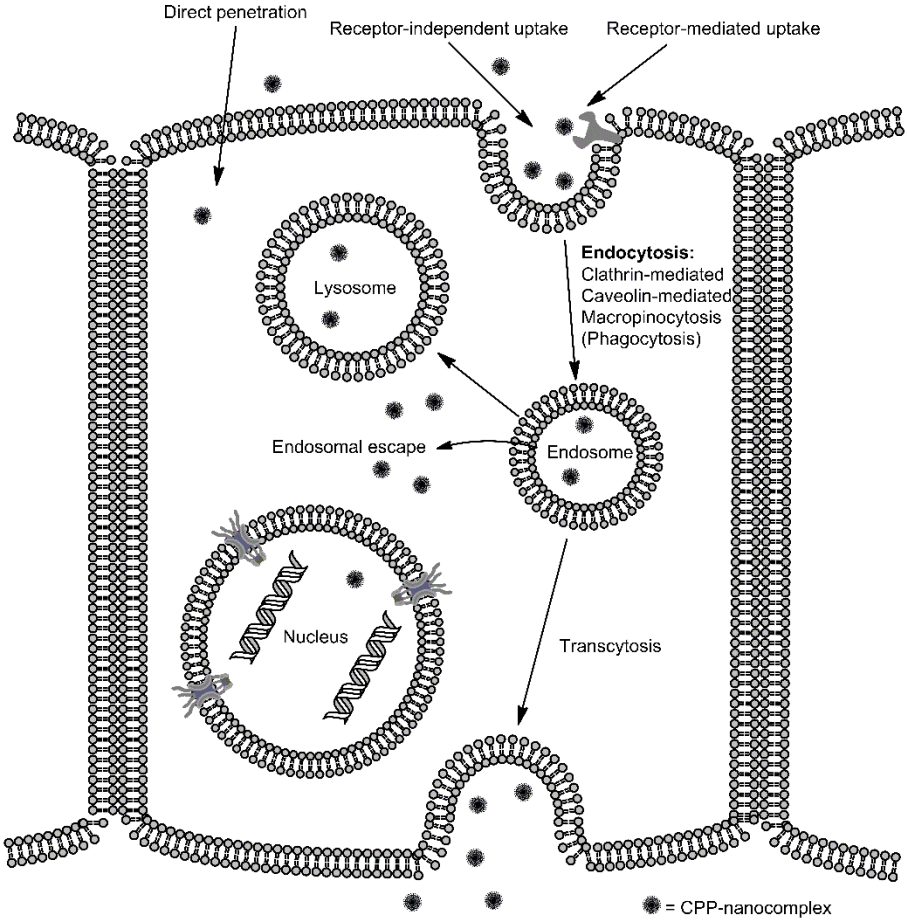

Figure 8. Representation of the uptake and transcytosis of CPP-nanocomplexes[162].

1.13.2. Scavenger receptors

Even though scavenger receptors are mainly associated with macrophages they are found in a wide range of tissues and cell types. There are 8 different classes of membrane-bound and soluble scavenger receptors, each class containing several proteins. The main purpose of scavenger receptors is thought to be the removal (scavenging) of foreign

particles and waste molecules in the organism by endocytosis and subsequent degradation. In a previous publication from our group the SCARA 3 and 5 were detected in the HeLa cell line used for determining transfection efficiency[158]; knocking down these receptors by siRNA prior to pGL3 transfection with PF14 showed a significant decrease in transfection efficiency (~50% less) and pretreatment with agonists of the scavenger receptors could remove the transfection capability completely. This discovery was followed up with testing the uptake of other CPPs and polymeric transfection reagents by scavenger receptors[156]; this publication showed scavenger receptors constitute some or all of the endocytosis of CPP, alone or complexed with ONs, and polymeric transfection reagents with ONs. These results indicate scavenger receptors may be a common denominator for transfection reagents. This is however far from proven as direct interaction between the receptor(s) and nanocomplexes are yet to be confirmed, the receptors may only play part in the endocytosis and the use of such general agonists might not only target the scavenger receptors.

1.14. High-throughput assay development

The introduction of microplates containing a range of wells up to 1536 and microplate readers allowed the scientific community to greatly upscale the number of compounds in each screening experiment. However, with the rising number of samples and screening of libraries a way to evaluate assay robustness and the detection of "hits" was needed. Using Z- and Z'-factors we can both score 'hits' during screening and also evaluate the assay itself[163]. To determine the robustness of an assay a Z'-factor can be calculated as in equation 1.

$$(1) \qquad Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} + \mu_{c-}|}$$

σ is the standard deviation and μ is the mean of the positive (c+) and negative (c-) controls respectively. A Z'-factor value of 1 is an ideal assay were standard deviations are close to zero and/or the difference between means are going toward infinity. Z'-factors above 0.5 are considered excellent assays. $0 < Z' < 0.5$ is considered a marginal assay while Z'-factors below 0 are essentially impossible to use as the signals of negative and positive controls overlap.

2. Aims of the studies

Since the general method of measuring DNAzyme kinetics is a laborious and discontinuous gel assay, the thesis was aimed at developing novel assays which are measured in real-time and less laborious. Another aim was to see if we could combine CPPs with different ONs for gene therapy. We also saw a need in streamlining the screening capabilities with CPP:ON transfection.

Paper I

We wanted to address the drawbacks of the already published EtBr assay for single turnover DNAzyme kinetics and we reasoned that a dye with higher specificity for dsDNA and RNA:DNA heteroduplexes, and a higher signal strength could possibly replace EtBr and allow for the use of structured DNAzymes and also full length mRNA substrates.

Paper II

The common denominator of nucleases prompted us to explore if we could utilize the exposed phosphate after nuclease cleavage to quantify, preferably in real-time, and calculate the nuclease kinetics. A method to remove background P_i from reaction components was also sought after.

Paper III

There was a need to upscale and simplify the CPP transfection methods used for screening purposes. To accommodate the increased use of reagent for screening in 96-well plates a homemade luciferin reagent needed to be produced.

Paper IV

As the addition of a lipid moiety to GRN163 to increase the uptake of the ON would also decrease the binding affinity we wanted to see if we instead could use CPPs to deliver a non-lipidated ON mimic of GRN163 so as not to lose any binding affinity. The reduction of cell proliferation and induction of senescence in HeLa cells was the final aim.

3. Methodological considerations

The methods used are described in detail in each paper. This chapter contains a discussion about choices done within the methods and an overview of the methods.

3.1. Oligonucleotides

The DNAzymes that were chosen for papers I and II were DzSJ and Dz451; two DNAzymes differing in binding arm length (7 vs. 13 nt per arm), ability to form secondary structure and target cleavage site composition (AU vs. GU)[25,115]. As previously discussed different mutations of the '10-23' catalytic loop can either decrease or abrogate the DNAzyme cleavage. To construct inactive DNAzyme controls we introduced a mutation at the sixth nucleotide of the '10-23' catalytic core, changing a guanosine to a cytidine. Changing G6 of the catalytic core to any of the other three natural nucleotides decreased cleavage activity to close to zero[50].

As substrates we used short synthesized RNA ONs; for DzSJ the same substrate was used as the one used for the EtBr assay while the substrate for Dz451 had 4 nt overhangs on each side which was a relic from previous pilot experiments with different binding arm lengths (up to 17 nt in each arm). To also investigate the applicability of the PicoGreen assay for full length mRNA substrates, we amplified the enhanced green fluorescent protein (eGFP) gene from pEGFP-C1 (Clontech) using two primers (Table 1) and upon doing so a T7 promoter sequence is incorporated upstream of the gene. The mRNA could then be *in vitro* transcribed in high yields using T7 Transcriptaid kit (Life Technologies).

The main substrate of *Escherichia coli* exonuclease III (ExoIII) is dsDNA with blunt or 5' overhang ends; therefore we chose to anneal two 20 nt long synthetic DNA ONs allowing for a theoretical maximum of 19 cleavage events per substrate molecule. An EcoRI cleavage site (GAATTC) was incorporated for the possibility to include EcoRI in the

experiments; however, this was not included due to problems with removing background P_i.

Table 1. Oligonucleotides used in the papers of this thesis

	Sequence (5'-3')	Paper #
DNAzymes		
DzSJ	GCA CCC AGG CTA GCT ACA ACG ACT CTC TC	**I & II**
I-DzSJ	GCA CCC AGG CTA **C**CT ACA ACG ACT CTC TC	**I & II**
Dz451	GCC ATG ATA TAG AGG CTA GCT ACA ACG AGT TGT GGC TGT TG	**I**
I-Dz451	GCC ATG ATA TAG AGG CTA **C**CT ACA ACG AGT TGT GGC TGT TG	**I**
DNAzyme substrates		
17 nt DzSJ substrate	*GGA GAG AGA | UGG GUG CG*	**I & II**
35 nt Dz451 substrate	*ACU ACA ACA GCC ACA ACG | UCU AUA UCA UGG CCG AC*	**I**
ExoIII substrates		
Sense strand	GGT GTT GGA ATT CGC CTT AG	**II**
Antisense strand	CTA AGG CGA ATT CCA ACA CC	**II**
PCR primers		
Forward eGFP	TAA TAC GAC TCA CTA TAG GG ATG GTG AGC AAG GGC GAG	**I**
Reverse eGFP	TTA CTT GTA CAG CTC GTC CA	**I**
SCO		
SCO705	c*c*u*c*u*u*a*c*c*u*c*a*g*u*u*a*c*a	**III**
siRNA		
Firefly luciferase GL3?	*ACGCCAAAAACAUAAAGAAAG*TT	**III**
hTR-binding ONs		
ON1	T*A*G*G*G*T*T*A*G*A*C*A*a	**IV**
ON3 (sense control)	A*T*C*C*C*A*A*T*C*T*G*T*t	**IV**

Underlined letters denote '10–23' catalytic loop of DNAzymes; Bold letters are inactivating G-C mutations in catalytic loop; Italics are ribonucleotides; | represent the cleavage site; * denotes a PS linkage; blue letters are LNA; lower case letters represent 2'OMe.

For Paper III a splice-correction assay and a reporter gene knockdown assay was used to optimize transfection. The splice-correction assay uses HeLa cells stably transfected with a plasmid encoding luciferase which has been modified to include a mutated intron. This mutation forms an aberrant splice site causing the luciferase expressed to be non-functional. By transfecting a SCO, this aberrant splice site can be bypassed and a functional version of the protein can be expressed, which can now be measured by the luciferase activity. We chose to use the same ON that has been used in previous publications[164]; an 18 nt long RNA ON modified at each nucleotide with 2'OMe and at each phosphate backbone with PS.

We chose to make a mimic of GRN163 using LNA nucleotides and PS linkers. A 2'OMe was included at the 3' end due to patent issues. The decision to not include a lipid moiety in the ON was based on two observations: previous research has indicated a possibility for LNA ONs to be readily taken up by cells without the use of transfection reagents, indicating there being no need for the lipid moiety to enhance cell permeation[165]; also, the publication on GRN163L showed that introduction of the palmitoyl moiety increased the IC50 more than 5 times in a cell-free assay[13], indicating the palmitoyl might inhibit the interaction of the ON with hTR.

3.1.1. DNAzyme cleavage

For general DNAzyme cleavage assays different ratios of DNAzyme:substrate has been employed. For multiple turnover ratios of 1:10 and up has been used to make sure each enzyme can cleave multiple substrates if it is able to. When measuring single turnover kinetics enzyme:substrate ratios of 10:1 or even higher ratios of enzyme has been used. However, Ferrari and Perrachi[52] used a DNAzyme:substrate ratio of 1:1 to emulate a single turnover reaction without introducing unnecessary amounts of background fluorescence from surplus DNAzyme. We also used this ratio when developing the PicoGreen assay in Paper I. This strategy has seemingly worked well as cleavage kinetics of DzSJ with a short synthetic substrate (Table 1) showed similar kinetics (0.179 min^{-1}) when measured using the PicoGreen assay as previously published values (0.13 – 0.15) min^{-1}) and compared to generic gel assay (0.1653 min^{-1}) and an adaptation of the published EtBr assay (0.1738 min^{-1})[52,115]. The validity of the assay was further proven by the similar single turnover cleavage kinetics when using Dz451 together with a short synthetic RNA or full length eGFP mRNA as substrate

compared to generic gel assay. The use of the previously published EtBr assay to measure Dz451 kinetics could not be done; as previously stated the EtBr assay can't be used with highly structured DNAzymes or substrates as the difference in fluorescence over time would be difficult to measure.

To prevent RNA degradation during electrophoresis either polyacrylamide gels with 8M urea (for short RNA substrates) or agarose gels with 1% (v/v) household bleach (for full length mRNA) was used. The use of urea in polyacrylamide gels has long been standard for electrophoresis of single-stranded ONs because of its efficient denaturation of both proteins (e.g. RNases) and ONs. For agarose gels the equivalent to urea has been to use MOPS buffer (light sensitive and needs to be filtered) and formaldehyde (toxic); instead we added household bleach to TBE agarose solution. The bleach effectively denatures both RNases and RNA and reduces cost by about 7-fold[166]. Using this method we could quickly set up gels for analysis of DNAzyme cleavage of full length eGFP mRNA[115].

3.1.2. Nucleic acid dyes

There is a seemingly endless variety in nucleic acid dyes, which generally are fluorescent and this increases upon binding of the ONs. Some dyes are specific for either ss- or dsNAs, RNA or DNA; like PicoGreen which has detection limits for dsDNA as low as a few picograms[167,168]. The previously published EtBr assay by Ferrari and Perrachi is illustrated in figure 9; this assay however has several drawbacks such as the inability of using structured DNAzymes or substrates (such as full length mRNA)[52]. To address this drawback we tested several dyes, SYTO 61, PO-PRO 1, DRAQ5, Hoechst 33258, DAPI, Propidium iodide, GelRed and PicoGreen; the latter turned out to have a much higher specificity for dsDNA and heteroduplexes compared to EtBr at the same time as giving a strong signal[169]. PicoGreen together with RNA yields low signals compared to dsDNA or heteroduplexes allowing full length mRNAs to be used without giving a too high background signal[167].

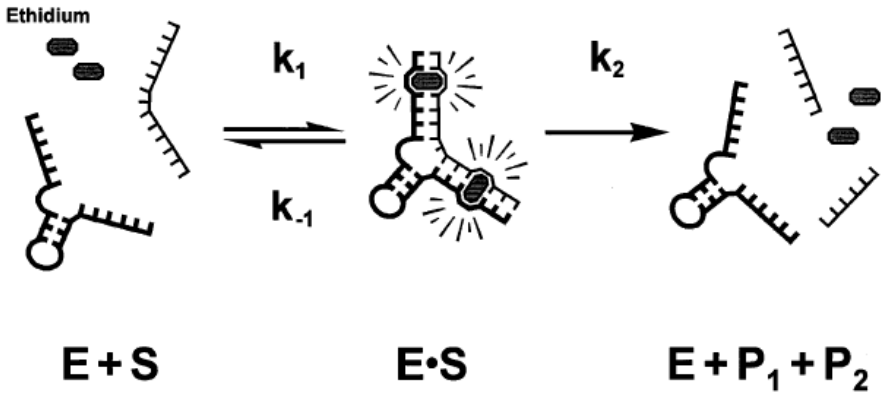

Figure 9. Schematic representation of the ethidium assay[52]. DNAzyme (E) binds RNA substrate (S) and EtBr intercalates the double-stranded regions of the enzyme-substrate complex. After cleavage the DNAzyme, products and ethidium dissociate, causing a fluorescence decrease.

3.2. Nucleic acid structure prediction

Over the years several structure prediction softwares have been developed. The basic idea is to find the structure with the lowest energy level to determine the most probable and stable structure which the ON will form. For this, each plausible folding configuration is computed and these are ordered according to their predicted energy level. These prediction softwares are far from perfect and they are estimated to predict the correct base pairing in 50-70% of cases[170]. The use of mFold in Paper I gives us an indication of the DNAzyme structures, DzSJ being almost completely unstructured while Dz451 most likely form a lot of intramolecular base pairs. This indication is further supported by the large difference in fluorescence increase by intercalating dyes such as EtBr or PicoGreen seen with these DNAzymes.

3.3. Phosphate sensor assay

The basic idea of the phosphate sensor assay was to use the common denominator among nucleases, they leave a single exposed phosphate for each cleavage event; meaning if this exposed phosphate could be released and quantified, a direct correlation between P_i formation could be drawn to nuclease cleavage kinetics. This could be done by using phosphatases to release the exposed phosphate as P_i and subsequently quantifying it in real-time using MDCC-PBP. Removal of background P_i

is the main problem of this assay as it is a very common contaminant. The most common way to solve this, and the method we used, is the addition of purine nucleoside phosphorylase (PNPase) and 7-methylguanosine (7-MEG) to buffers and reactants, causing the phosphate to be bound to the ribose[171]. This could however have a deleterious effect on the reaction by removing P_i released during the reaction. Two different methods were tested to remove background P_i without addition of enzyme. Using a copper-loaded Dowex M4195 chelating resin or treating reagents with lanthanum carbonate[172,173]. Removing P_i from water and buffers by these methods proved to be highly efficient, however when applying them to enzyme-containing solutions the enzymes were either removed or inactivated.

3.3.1. Phosphatases

To release P_i from deoxyribonucleoside-5'-monophosphates (dNMP) products released by ExoIII in the phosphate sensor assay we used FastAP alkaline phosphatase. Both pro- and eukaryotes express alkaline phosphatase, a nonspecific phosphomonoesterase[174]. The exposed phosphate after RNA cleavage by the 10-23 DNAzyme is a 2',3'-cyclic phosphate and alkaline phosphatase is incapable of releasing it as P_i[175]. To remove the 2',3'-cyclic phosphate we used T4 polynucleotide kinase (T4PNK), a multi-purpose enzyme which can act as both a kinase and phosphatase. The ability to remove 2',3'-cyclic phosphates is naturally used by the T4 bacteriophage to thwart innate immune responses of the host bacteria which cleaves host cell tRNAs to inhibit viral protein synthesis; the tRNA cleavage creates a 2',3'-cyclic phosphate on the tRNA, preventing the tRNA to be used for translation of viral genes, however the 2',3'-cyclic phosphate can be removed by T4PNK to evade the immune response[176].

3.3.2. Exonuclease III

To show the diversity of the phosphate sensor assay we included a protein nuclease, ExoIII; a 31 kDa monomeric enzyme isolated from *E. coli*. Four different activities have been demonstrated for ExoIII[110]; 3'-5' exonuclease activity upon dsDNA were dNMPs are cleaved from the 3' end of nicks, recessed ends, blunt ends and 3'-overhangs of less than 4 bases in dsDNA; exonucleolytic ribonuclease H activity for the RNA strand in RNA:DNA heteroduplexes; 3' phosphatase activity; the fourth activity is thought to be the main activity inside the cell, apurinic/apyrimidinic endonuclease activity, used for reparation of

apurinic/apyrimidinic sites. A single active site is thought to be used for all four activities.

3.4. Microplate readers

Throughout this thesis two different microplate readers have been used; a GloMax 96 microplate luminometer (Promega) for the luciferase assays in paper III, and a Flexstation II (Molecular Devices) for following the DNAzyme assays developed in paper I and II in real-time. Using the Flexstation II we could add reagents through the built-in microfluidics so that there would be no void between the reaction initiation and start of the measurement. An important note when setting up experiments for these microplate readers is that the GloMax reads wells row-by-row while the Flexstation II measures column-by-column. To improve and speed up the assays of paper I, II and III the use of a plate reader which reads all wells of the whole plate simultaneously would be a good choice.

3.5. Solid-phase peptide synthesis

Bruce Merrifield introduced the solid-phase peptide synthesis (SPPS) method in 1963[177]. The basic idea of SPPS is to use a resin, large enough to not pass through a filter, and couple amino acids to it. The amino acids are coupled one-by-one starting from the C-terminus. The method we used, and the most common, to produce our peptides is the use of amino acids protected N-terminally by 9-fluorenylmethyloxycarbonyl (Fmoc). The carboxylic acid of each amino acid is reacted with the previous amino acids amine group to form a peptide bond; the Fmoc group can then be removed for subsequent addition of the next amino acid. After each step byproducts and reagents can easily be washed of as the solid resin support allows the growing peptide chains to be easily retained in the reaction vessel. To produce PF15 a succinylated lysine tree was introduced on the side chain of K7 and to the tree four trifluoromethylquinoline-based derivatives were introduced. Peptides were cleaved from the solid support using 95% trifluroacetic acid, 2.5% triisopropylsilane and 2.5% H_2O. The crude peptide was further freeze-dried and purified by semi-preparative reverse-phase high performance liquid chromatography and the mass of the purified product was analyzed by using a matrix-assisted laser desorption/ionization-time of flight mass spectrometer.

3.6. Dynamic light scattering and zeta-potential

Dynamic light scattering is a method to determine the size or particles in solution. Our research group has used this to determine the size of CPP:ON complexes in several publications; one important realization was made, which often is not thought of in most research papers, that the measurement of particles in a biologically relevant media is of more use than to measure them in water as this can change the size of the particle significantly[158]. Together with the size we can also attain a polydispersity index, which tells us the distribution of particle sizes. Furthermore, applying an electric field to the particles will cause them to move to either electrode depending on the overall charge of the particle; the parameter which can be defined through this experiment is the zeta-potential. A particle in solution will attract a layer of ions around it, the Stern layer, and outside of that another diffuse layer of ions and counterions; the charge difference between these layers is the zeta-potential.

3.7. Cells

All cells were passaged in 75 cm^2 flasks at 37°C, 5% CO_2, in DMEM with Glutamax supplemented with 0.1 mM non-essential amino acids, 10% fetal bovine serum, 200 U/ml penicillin and 200 μg/ml streptomycin. The cells used in this thesis were U-87 MG-luc2 and HeLa pLuc 705 for use as luciferase knockdown and upregulation targets in paper III and HeLa cells in paper IV as they grow fast and express functional telomerase. CPP:ON complexes were mixed in either ultrapure Milli-Q water or a phosphate buffer (pH 7.4) at $1/10^{th}$ of the final culture volume.

3.7.1. Splice-correction and siRNA assays

The previously published method to induce luciferase expression by ON transfection is based on a stably transfected plasmid containing the luciferase gene which has been interrupted by a mutated human β-globin intron 2; the mutation forms a cryptic splice-site in the intron and causes aberrant splicing of the luciferase pre-mRNA, ultimately forming a nonfunctional protein[164]. To promote correct splicing of the intron an 18 nt long ON, ON705, can mask the aberrant splice-site allowing the spliceosome to excise the whole intron and form full length luciferase mRNA to be translated to functional luciferase. For the ON to gain

access to the nucleus of the cell a transfection reagent has to be used as the ON alone is not taken up. This method can then be used to evaluate the ON transfection efficiency of different transfection reagents, such as CPPs, by quantifying the relative amount of functional luciferase in transfected cells by the use of a luciferin reagent. Another way to evaluate transfection efficiency was to use siRNA targeted toward luciferase stably expressed by a cell line (U-87 MG-luc2), showing the versatility of our assay; downregulating instead of upregulating luciferase was equally effective in terms of coefficients of variance and Z'-factors.

3.8. TRAPeze RT telomerase detection

The basis for the TRAPeze real-time telomerase detection assay is the telomerase repeat amplification protocol (TRAP)[12,178]; which allows telomerase to add telomeric repeats to the 3' end of an ON which is then followed by PCR amplification of the single-stranded product; the products are then run on gels to be quantified by imaging. The introduction of a self-quenching fluorescent hairpin primer allows for real-time fluorescent quantification of telomerase activity as the hairpin primer unfolds during PCR amplification and becomes fluorescent.

4. Results and discussion

Paper I

The introduction of the EtBr-assay for single turnover DNAzyme kinetics allowed for much faster and simpler kinetic determinations over the conventional gel assay[52]. Several drawbacks reduce the usefulness of this assay; inability of multiple turnovers, structured DNAzymes and full length mRNA substrates. To address this we decided to try other extrinsic fluorophores which could replace EtBr. More favorable characteristics such as specificity for dsNAs and higher fluorescence increase (>1000 fold) upon binding were found in PicoGreen, a cyanine dye with excitation and emission maximum at 502 and 530 nm, respectively. Using this fluorophore the assay could be used together with DNAzymes, irrespective of the degree of secondary structure as judged by folding of the two DNAzymes using Mfold[179], and full length mRNA substrates. As controls, we used G6C mutated DNAzymes, causing the catalytic effect to drop to close to zero[59]. The inactive DNAzymes did not show any cleavage as measured by the assay. Using either short RNA substrates or full length mRNA together with PicoGreen showed true kinetic constants as they were similar (less than 8% difference) to the ones achieved through the conventional gel assay. Being able to measure DNAzyme kinetics upon cleaving full length mRNA in an easy way allows for more relevant kinetic studies as the short synthetic RNA strands are often substitutes for the full length mRNA, disregarding the secondary structure of flanking sequences. When using EtBr in the same setup we confirmed the inability of this fluorophore to be used with structured DNAzymes. The inability to measure multiple turnover remains as this assay follows the [ES], while during multiple turnover or steady-state $d[ES] / dt \approx 0$.

Paper II

With the definition of the common denominator among nucleases, the exposure of a single NA backbone phosphate for each cleavage event, we set out to see if we could use this phenomenon to quantify and determine the kinetics of different types of nucleases. The basic idea is to couple the nuclease cleavage with a phosphatase, specific for the type of

phosphate formed after cleavage, to release the exposed phosphate as P_i which would subsequently be quantified. To quantify the released P_i we chose MDCC-PBP or Phosphate Sensor which can bind P_i quickly and tightly for highly specific and sensitive real-time quantification as seen in figure 10. MDCC-PBP was only slightly affected by the reaction components and standard curves for each buffer composition was made. In a microplate setup several wells could be measured simultaneously to increase the throughput.

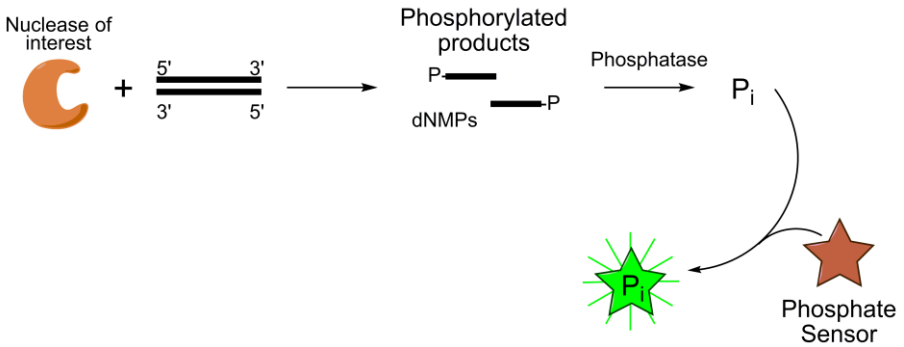

Figure 10. General schematic of the phosphate exposure assay.

We successfully determined kinetics of two very different nucleases, a '10-23' DNAzyme and ExoIII, showing the versatility of the assay. From this we could determine that neither protein- nor NA-based nucleases, exo- or endonucleases, 5' phosphorylated or 2',3'-cyclic phosphorylated products obstructed the capability of the assay to measure nuclease kinetics. As the phosphorylation of the products formed by these two nucleases are different (5' vs. 2',3'-cyclic) we needed to use two different phosphatases, FastAP to release 5' phosphate and T4PNK to release 2',3'-cyclic phosphate as this is not cleavable by any alkaline phosphatase. The kinetics determined by this method were multiple turnover and could be fit to Michaelis-Menten equations to attain k_{cat} and K_M values. This was only possible for ExoIII as the K_M of the DNAzyme used is slightly below the limit of detection for the assay (0.9 nM vs. ~2 nM).

Removing the background P_i from reaction components is important for this assay as it will interfere with measuring the release P_i. The contaminating P_i is often in high concentration, both in solutions and on lab plastics. We tried three methods to remove the background P_i; using a copper-loaded Dowex M4195 chelating resin[173] to specifically bind the P_i or using lanthanum carbonate to precipitate P_i[172] proved to both be

highly efficient methods in removing P_i, however when applied to enzyme-containing solutions the enzymes would either be removed from the solution or inhibited. Using PNPase and 7-MEG pretreatment proved to be a good method to remove P_i as PNPase would bind the P_i to the ribose of 7-MEG resulting in the formation of a nucleobase and ribose-1-phosphate[171]. The addition of PNPase to enzymes had to be kept at a minimal concentration as residual PNPase in the final assay could affect the readout negatively by removing P_i released by the phosphatases. The optimal concentration was determined by measuring the speed of P_i removal by PNPase in real-time using MDCC-PBP.

Paper III

To upscale from 24-well plates which we had used in previous publications to 96-well plates was not as straight forward as we had expected. The direct scaling between the two plate sizes produced highly variable results; the scale ratios of 24- and 96-well plates are 5 times in volume but 6 times in bottom surface area. The variability was determined to be due to rapid signal decrease of the commercial luciferin reagent (20% in 3 min), over-seeding of cells due to improper scaling, inconsistent lysis with conventional lysis buffer, and too long time between cell-seeding and the experimental end-point also causing column and row effects. To address each of these sources of variability a home-made luciferin reagent was composed (CoA, DTT, luciferin and ATP), the optimal number of cells was determined for seeding, the lysis buffer was abandoned in favor of a freeze-thaw cycle, and reverse transfection was used instead of forward transfection to save a whole day of experimental time and at the same time decreasing the variance and column and row effects.

When optimizing the luciferin reagent the following was determined for each component: CoA is the main effector in maximum initial signal and also has some effect on signal decrease; DTT affects signal decrease over time the most and is the most expensive component; an approximate equimolar balance of ATP and luciferin is needed to get the maximum possible signal. With this information in mind a compromise between price per plate, signal longevity and signal strength; we settled for a composition showing less than 20% decrease during the initial 20 minutes but which still gave a high signal and contained as little DTT as possible without significantly affecting signal strength and longevity. The composition which was used for the high-throughput assay development decreased the cost per plate from €90 to €3. The optimal cell seeding number was determined for forward transfection and was later increased

for reverse transfection to account for the 1 day decrease of cell growth. Variance from lysis was decreased by skipping the lysis buffer and instead removing the media by putting the plate upside down on a paper tissue to remove most of the media and centrifuging the plate upside down at low speed; this was followed by a single freeze-thaw cycle at -80°C.

With the optimized assay conditions we could determine the applicability of CPP transfection using both SCO and siRNA to up- and downregulate luciferase expression, respectively. The CV% of PF14:SCO was 7% and for PF14:siRNA 6% while Z' scores were 0.71 and 0.73 respectively indicating both to be excellent assays.

Paper IV

The 'end-replication problem' and free radicals cause the ends of chromosomes, the telomeres, to shorten with each cell division[7,180] . Once the telomeres reach a critical length the cells undergo replicative senescence and cell crisis, often leading to apoptosis. The telomeres can be elongated by telomerase, an endogenous enzyme normally not active in somatic cells[9,12]. 90% of all cancers express telomerase while at the same time having short telomeres, indicating an intervention to knockdown or inhibit telomerase could be a treatment for cancer[12]. As most other cells don't express telomerase the risk of side-effects is low.

One of the gene therapies which have been developed to inhibit telomerase activity is GRN163L, a 13 nt long ON with a palmitoyl moiety[13]. By binding to hTR, the RNA-component of telomerase, GRN163L can inhibit the activity of telomerase at an IC_{50} of 150 nM. It has been shown that without the palmitoyl moiety, which allows the ON to be taken up by cells, the ON can inhibit telomerase at lower concentrations (IC_{50} 1.4 nM vs. 7.8 nM) in a cell-free assay; this prompted us to explore the use of CPPs with an hTR-binding ON without a lipid moiety.

Using GRN163L as a model we designed an LNA mixmer to target hTR and decrease the activity of telomerase. This LNA mixmer named ON1 was taken up by HeLa cells, determined by the use of a nonradioactive real-time PCR-based TRAP assay, without the use of a transfection reagent or a covalently coupled lipid moiety, such as the palmitoyl of GRN163L. By the use of transfection reagents such as Lipofectamine 2000 and PF15 the inhibitory effect of ON1 could be significantly advanced compared to naked delivery. By dose-response experiments and curve fitting an IC_{50} of approximately 8.4 nM was

determined, which is more than 20 times less than the published value for GRN163L in HeLa cells[181]. Particle sizes were measured by dynamic light scattering as previous publications of noncovalent complexation between ONs and PFs have proven these to form nanocomplexes. Nanocomplexes of approximately 400 nm with a polydispersity index of 0.4 indicating the population of nanocomplexes to be polydisperse. The zeta-potential of -27.1 mV indicates high stability of the nanocomplexes. The role of scavenger receptors in CPP:ON nanocomplex uptake has previously been shown by our group and is here also proven for the delivery of PF15:ON1 nanocomplexes to HeLa cells by inhibiting the uptake with scavenger receptor agonists.

5. Conclusions

Paper I

After testing a range of extrinsic fluorophores, PicoGreen was found to be more suited for real-time kinetics of single turnover DNAzyme cleavage of RNA targets than EtBr; the switch of extrinsic fluorophore allows for the use of highly structured DNAzymes and substrates as well as full length mRNA targets with this method. The use of a Flexstation II with an integrated fluidics module allowed for high-throughput measurements of DNAzyme kinetics.

Paper II

This paper is based on a novel idea and method to measure kinetic activity of nucleases in real-time. Highly diverse nucleases can be used with this method; ON- and protein-based nucleases, exo- and endonucleases, and nucleases can be paired with different phosphatases to allow for measurement irrespective of the form of the exposed phosphate. Using MDCC-PBP to quantify P_i allowed us to measure nuclease kinetics in real-time in this phosphatase-coupled assay.

Paper III

The proposed method is an optimization of previous transfection setups to be used for screening for the most optimal transfection conditions. The optimization allows the procedure to be completed in 1 day less than previously, significantly cheaper due to the in house mixing of luciferin and the upscaling to 96-well plates. Using this method one can quickly screen different compositions and ratios in the buildup of nanocomplexes. Calculated Z'-scores, both with siRNA and SCOs, determine this method to be graded as an 'excellent' method; meaning it has low variation and a high separation of positive and negative controls.

Paper IV

Using a novel CPP to mediate ON transfection instead of a lipid moiety covalently coupled to the ON a significantly decreased IC_{50} could be achieved. Compared to Imetelstat which is currently in clinical trials

the CPP-mediated transfection of the LNA mixmer was more than 20-times more efficient in inhibiting telomerase activity. This is attributed to the use of a separate transfection reagent from the ON and at the same time allowing the ON to be free from the lipid moiety to freely exert its binding to hTR. Our results indicate that CPPs could be used to significantly enhance the efficiency of small ONs inhibiting telomerase.

In conclusion, this thesis describes two novel assays for DNAzyme and nuclease kinetics which will simplify the determination of kinetic constants. Utilizing the common denominator among nucleases provided an excellent assay in paper II for nuclease kinetics, allowing for real-time multiple turnover kinetics in 96-well plates. Furthermore, we have streamlined and upscaled the process of elucidating CPP mechanisms through the assay in paper III. The efficiency enhancing effect of combining CPPs and ONs was shown by combining PF15 with an imetelstat-mimic, effectively lowering the IC_{50} 20-fold.

6. Populärvetenskaplig sammanfattning

Under årtusenden har människan använt olika substanser för att behandla sjukdomar. Det är dock under det senaste seklet och speciellt de senaste decennierna som de underliggande mekanismerna för många sjukdomar har blivit upptäckta. Med en ökad förståelse för sjukdomsmekanismer har även behandlingar kunnat utvecklas. En viktig upptäckt var att DNA bär på det genetiska materialet; DNA kopieras till RNA som i sin tur kodar för skapandet av ett protein. Eftersom DNA är grunden, eller huvudritningen, i den här processen så kan mutationer och förändringar i DNAt ge upphov till många olika sorters sjukdomar. De tidigaste idéerna om genterapi var att lägga till en gen för att ersätta en icke-funktionell. Genterapi har sedan expanderats till att även inkludera avlägsnandet, modifiering eller reglering av gener. Detta kan göras på flera sätt, t.ex. korta oligonukleotider (RNA- eller DNA-strängar) kan användas för att nedreglera en gen, s.k. antisense. I de första två artiklarna har vi använt oss utav RNA-klyvande DNA-enzymer (DNAzymes) som är korta DNA-oligonukleotider som kan binda till specifika RNA-strängar och klippa isär dem, på så sätt hindra RNAt att agera ritning till skapandet av ett protein. Utvecklingen av de första DNAzymes skedde i mitten av 90-talet och sedan dess har man använt en primitiv metod som är både tidskrävande och problematisk för att bestämma hur snabbt DNAzymes klyver RNA-strängarna. Vi har därför utvecklat två olika metoder för att mäta klyvningshastigheten hos DNAzymes, den senare av de två metoderna fungerar även för andra enzymer som klyver RNA eller DNA. Den första metoden går ut på att man inkluderar ett fluorescerande färgämne som lyser starkare då det är bundet till dubbelsträngat DNA eller en blandning av RNA och DNA, under reaktionens gång kommer mängden av RNA och DNA som är bundet till varandra att minska och på så sätt kan vi följa reaktionen då ljuset blir svagare med tiden. Den andra metoden baseras på den minsta gemensamma nämnaren hos alla enzymer som klyver DNA eller RNA, de exponerar alltid en fosfatgrupp i DNA- eller RNA-molekylens ryggrad. Den exponerade fosfatgruppen kan sedan klippas loss och då kvantifieras med hjälp av ett speciellt protein som binder fosfat, när det händer så blir en fluorofor bundet till proteinet mer ljusstarkt.

Det stora problemet med genterapi är ofta inte om, eller hur väl, genterapin i sig fungerar utan att få in genterapin i cellen. Eukaryota celler har ett yttre membran och ett inre membran som innehåller cellens genom, DNAt. Cellen har många försvarsmekanismer för att skydda sig från främmande partiklar och därför är det svårt att få in molekyler i celler. Man upptäckte dock under 60-talet att virus kunde överföra genetiskt material till människoceller, sedan dess har många alternativ utvecklas för att föra in genetiskt material i celler. Ett alternativ är att använda cellpenetrerande peptider (CPP) som är uppbyggda av aminosyror. CPPer har utvecklats länge i vår forskningsgrupp och de har sett många förändringar genom åren. För att ytterligare förbättra våra CPPer och förstå hur de fungerar behövde vi utveckla en snabb metod för att undersöka många förhållanden samtidigt. Med detta mål utformade vi en metod i artikel III där vi kan mäta 96 förhållanden på en gång (jämfört med 24 tidigare som dessutom tog 1 hel dags arbete längre), samtidigt som vi sparar 95% av kostnaderna.

En genterapi som är i kliniska studier just nu är inhiberingen av telomeras, ett enzym som förlänger ändarna på kromosomerna (som innehåller cellens genom). Kromosomerna blir kortare varje gång en cell delar på sig och kan liknas vid cellens klocka för hur länge den kan leva, då ändarna av kromosomerna har nått en kritisk längd så slutar cellen att dela på sig och begår ofta självmord. 85% av alla cancerceller men nästan inga 'normala' celler producerar telomeras, detta gör att de kan leva och dela sig oändligt antal gånger. Men om man med genterapi hindrar telomeras från att fungera så dör cancercellerna så småningom. Vi tillverkade därför i artikel IV en liknande antisense-oligonukleotid som används i de kliniska studierna och resonerade att om vi använder våra CPPer så kan vi få en mycket effektivare genterapi. Effekten vi fick var en ökning av effektiviteten med över 20 gånger jämfört med den genterapi som är i kliniska studier.

7. Acknowledgements

Two countries, three apartments, three computers, five years, six grants, seven publications, fourteen kilograms later I have finally finished my thesis. I have faced a lot of challenges and difficulties during my time as a PhD student. However, I also had a lot of fun. Here is a brief mentioning of the people who helped me through this time.

As expected, the person who has had the biggest influence on my studies has been my supervisor, Professor Ülo Langel. I have to once again thank you for letting me and the other PhD students in your lab have the liberty of presenting an idea to you, and financing it even if it was crazy. You have taught me not to get ahead of myself in my research. You were right; I do want to be awarded the Nobel Prize one day.

In the future I hope to have as good colleagues as I have had during my time at the department of Neurochemistry. This last time has been very intense and my biggest help during this time has been Jakob; without you this thesis would be worse, a lot worse, also thanks again for all the great food. Henrik "Uncasual Friday" Helmfors, thank you for the great dinners and fun times. Next comes the person who would probably win an Olympic gold medal if talking was an Olympic sport, Andrés, it has been a pleasure and sometimes nuisance to work with you; also, thanks for the times we played role-playing games. Staffan, I'm glad I made someone feel better by coming in to the office late at times. To the four guys above, thanks for all the time in the gym. Thanks to Carmine for all the time spent together bouldering. Ying, don't ever stop being such a fun and friendly person. Thanks to Daniel "the Dude" for the great discussions both on science and philosophy, and also laughing at "Flat-Earthers" with me. Tõnis, you're a funny guy; try to refrain from your heritage and not destroy your liver. Kristin, I'm happy to see you having a great life with your family. I'm also envious of your house, but not its location. I want to thank Kariem for all the nice words and support in the beginning of my PhD studies. Jens H, you were my best student and we had a great time together. You're going to do great with your PhD. To all of the above, thank you so much for being my friends

and coworkers, you have all made a big impact on me. Also big thanks to everyone at the department for making my time there great.

I lived in Estonia for five months because I was invited to work on a project idea of mine involving HIV-1 in Andres Merits' lab. Thank you, Andres, for this time and your guidance.

I would like to express my very great appreciation to my mother, father, Kenta, Sofie, Marie and Camilla, who have supported and believed in me throughout my studies. The same goes for my friends, especially Robert, Robin, Jens B, Daniel, Tina, and Patrik.

For those of you, whom I have not mentioned, thank you too!

Finally I would like to say that words can't express how much you mean to me Maria. You're always there for me, with never-failing sympathy and encouragement. I'm so excited and looking forward to see what our future holds for us. You complete me.

8. References

1. Azad R, Driver V, Tanaka K, Crooke R, Anderson K. Antiviral activity of a phosphorothioate oligonucleotide complementary to human cytomegalovirus RNA when used in combination with antiviral nucleoside analogs. Antivir Res. 1995;28(2):101.
2. Kool ET. Circular oligonucleotides: new concepts in oligonucleotide design. Annu Rev Biophys Biomol Struct. 1996;25(1):1–28.
3. Watson JD, Crick FHC. Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. Nature. 1953;171(4356):737–8.
4. Hoogsteen K. The crystal and molecular structure of a hydrogen-bonded complex between 1-methylthymine and 9-methyladenine. Acta Crystallogr. 1963;16(9):907.
5. Deleavey GF, Damha MJ. Designing chemically modified oligonucleotides for targeted gene silencing. Chem Biol. 2012;19(8):937–54.
6. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;345(6274):458.
7. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Experimental Cell Research. 1961;25(3):585.
8. Hayflick L. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res. 1965;37(3):614.
9. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 1985;43(2 Pt 1):405–13.
10. Greider CW, Blackburn EH. The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell. 1987;51(6):887–98.
11. Ray S, Karamysheva Z, Wang L, Shippen DE, Price CM. Interactions between telomerase and primase physically link the

telomere and chromosome replication machinery. Mol Cell Biol. 2002;22(16):5859–68.

12. Kim N, Piatyszek M, Prowse K, Harley C, West M, Ho P, et al. Specific association of human telomerase activity with immortal cells and cancer. Science. 1994;266(5193):2011-15.

13. Herbert B-S, Gellert GC, Hochreiter A, Pongracz K, Wright WE, Zielinska D, et al. Lipid modification of GRN163, an N3'→P5' *thio*-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition. Oncogene. 2005;24(33):5262–8.

14. Asai A, Oshima Y, Yamamoto Y, Uochi T-A, Kusaka H, Akinaga S, et al. A novel telomerase template antagonist (GRN163) as a potential anticancer agent. Cancer Res. 2003;63(14):3931–9.

15. Alt F. Synthesis of secreted and membrane-bound immunoglobulin mu heavy chains is directed by mRNAs that differ at their 3' ends. Cell. 1980;20(2):293-301.

16. Nilsen TW, Graveley BR. Expansion of the eukaryotic proteome by alternative splicing. Nature. 2010;463(7280):457–63.

17. Goyenvalle A, Griffith G, Babbs A, Andaloussi El S, Ezzat K, Avril A, et al. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. Nat Med. 2015;21(3):270–5.

18. Crick FHC. The origin of the genetic code. J Mol Biol. 1968;38:367–79.

19. Orgel LE. Evolution of the genetic apparatus. J Mol Biol. 1968;38(3):381.

20. Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, Cech TR. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. Cell. 1982;31(1):147–57.

21. Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell. 1983;35(3 Pt 2):849–57.

22. Serganov A, Patel DJ. Ribozymes, riboswitches and beyond: regulation of gene expression without proteins. Nat Rev Genet. 2007;8(10):776–90.

23. Strobel SA, Cochrane JC. RNA catalysis: ribozymes, ribosomes, and riboswitches. Curr Opin Chem Biol. 2007;11(6):636–43.

24. Breaker RR, Joyce GF. A DNA enzyme that cleaves RNA. Chem Biol. 1994;1(4):223–9.

25. Santoro SW, Joyce GF. A general purpose RNA-cleaving DNA enzyme. Proc Natl Acad Sci USA. 1997;94(9):4262–6.

26. Coppins RL, Silverman SK. A DNA enzyme that mimics the first step of RNA splicing. Nat Struct Mol Biol. 2004;11(3):270–4.

27. Hoadley KA, Purtha WE, Wolf AC, Flynn-Charlebois A, Silverman SK. Zn^{2+}-dependent deoxyribozymes that form natural and unnatural RNA linkages. Biochemistry. 2005;44(25):9217–31.

28. Sreedhara A, Li Y, Breaker RR. Ligating DNA with DNA. J Am Chem Soc. March 18, 2004;126(11):3454–60.

29. Wang W, Billen LP, Li Y. Sequence diversity, metal specificity, and catalytic proficiency of metal-dependent phosphorylating DNA enzymes. Chem Biol. 2002;9(4):507–17.

30. Pradeepkumar PI, Höbartner C, Baum DA, Silverman SK. DNA-catalyzed formation of nucleopeptide linkages. Angew Chem Int Ed Engl. 2008;47(9):1753–7.

31. Chandra M, Silverman SK. DNA and RNA Can Be Equally Efficient Catalysts for Carbon−Carbon Bond Formation. J Am Chem Soc. 2008;130(10):2936.

32. Torabi S-F, Wu P, McGhee CE, Chen L, Hwang K, Zheng N, et al. In vitro selection of a sodium-specific DNAzyme and its application in intracellular sensing. Proc Natl Acad Sci USA. 2015;112(19):5903–8.

33. Zhao Y, Zhou L, Tang Z. Cleavage-based signal amplification of RNA. Nat Commun. 2013;4:1493.

34. Wen Y, Xu Y, Mao X, Wei Y, Song H, Chen N, et al. DNAzyme-based rolling-circle amplification DNA machine for ultrasensitive analysis of microRNA in Drosophila larva. Anal Chem. 2012;84(18):7664–9.

35. Tian Y, He Y, Chen Y, Yin P, Mao C. A DNAzyme that walks processively and autonomously along a one-dimensional track. Angew Chem Int Ed Engl. 2005;44(28):4355–8.

36. Grubbs RD. Intracellular magnesium and magnesium buffering. BioMetals. 2002;(15):251–9.

37. Romani A, Scarpa A. Regulation of cell magnesium. Archives of Biochemistry and Biophysics. 1992;298(1):1.

38. Jahnen-Dechent W, Ketteler M. Magnesium basics. Clin Kidney J. 2012;5(Suppl 1):i3–i14.

39. Warashina M, Kuwabara T, Nakamatsu Y, Taira K. Extremely high and specific activity of DNA enzymes in cells with a Philadelphia chromosome. Chem Biol. 1999;6(4):237.

40. Yuan B-F, Xue Y, Luo M, Hao Y-H, Tan Z. Two DNAzymes targeting the telomerase mRNA with large difference in Mg^{2+} concentration for maximal catalytic activity. Int J Biochem Cell Biol. 2007;39(6):1119–29.

41. Abdelgany A, Wood M, Beeson D. Hairpin DNAzymes: a new tool for efficient cellular gene silencing. J Gene Med. 2007;9(8):727–38.

42. Sugiyama R, Hayafune M, Habu Y, Yamamoto N, Takaku H. HIV-1 RT-dependent DNAzyme expression inhibits HIV-1 replication without the emergence of escape viruses. Nucleic Acids Res. 2010;39(2):589–98.

43. Yehl K, Joshi JP, Greene BL, Dyer RB, Nahta R, Salaita K. Catalytic deoxyribozyme-modified nanoparticles for RNAi-independent gene regulation. ACS Nano. 2012;6(10):9150–7.

44. Iversen PO, Nicolaysen G. DNA enzyme targeting TNF-α mRNA improves hemodynamic performance in rats with postinfarction heart failure. Am J Physiol Heart Circ Physiol. 2001:281(5):H2211-7.

45. Hurtado A, Podinin H, Oudega M, Grimpe B. Deoxyribozyme-mediated knockdown of xylosyltransferase-1 mRNA promotes axon growth in the adult rat spinal cord. Brain. 2008;131(Pt 10):2596–605.

46. Vester B, Hansen LH, Lundberg LB, Babu BR, Sørensen MD, Wengel J, et al. Locked nucleoside analogues expand the potential of DNAzymes to cleave structured RNA targets. BMC Mol Biol. 2006;7:19.

47. Abdelgany A, Uddin MK, Wood M, Taira K, Beeson D. Design of efficient DNAzymes against muscle AChR alpha-subunit cRNA in vitro and in HEK 293 cells. J RNAi Gene Silencing. 2005;1(2):88–96.

48. Seifert G, Taube T, Paal K, Einsiedel von HG, Wellmann S, Henze G, et al. Brief communication: stability and catalytic activity of novel circular DNAzymes. Nucleos Nucleot Nucl. 2006;25(7):785–93.

49. Wyszko E, Mueller F, Gabryelska M, Bondzio A, Popenda M, Barciszewski J, et al. Spiegelzymes® mirror-image hammerhead ribozymes and mirror-image DNAzymes, an alternative to siRNAs and microRNAs to cleave mRNAs *in vivo*? PLoS ONE. 2014;9(1):e86673.

50. Kurreck J, Bieber B, Jahnel R, Erdmann VA. Comparative study of DNA enzymes and ribozymes against the same full-length messenger RNA of the vanilloid receptor subtype I. J Biol Chem. 2001;277(9):7099–107.

51. Cairns MJ, Hopkins TM, Witherington C, Wang L, Sun LQ. Target site selection for an RNA-cleaving catalytic DNA. Nat Biotechnol. 1999;17(5):480–6.

52. Ferrari D, Peracchi A. A continuous kinetic assay for RNA-cleaving deoxyribozymes, exploiting ethidium bromide as an extrinsic fluorescent probe. Nucleic Acids Res. 2002;30(20):e112.

53. Kim JE, Yoon S, Mok H, Jung W, Kim D-E. Site-specific cleavage of mutant ABL mRNA by DNAzyme is facilitated by peptide nucleic acid binding to RNA substrate. FEBS Lett. 2012;586(21):3865–9.

54. Schubert S, Fürste JP, Werk D, Grunert H-P, Zeichhardt H, Erdmann VA, et al. Gaining target access for deoxyribozymes. J Mol Biol. 2004;339(2):355–63.

55. Fluiter K, Frieden M, Vreijling J, Koch T, Baas F. Evaluation of LNA-modified DNAzymes targeting a single nucleotide polymorphism in the large subunit of RNA polymerase II. Oligonucleotides. 2006;15(4):246–54.

56. Ruble BK, Richards JL, Cheung-Lau JC, Dmochowski IJ. Mismatch Discrimination and Efficient Photomodulation with Split 10-23 DNAzymes. Inorganica Chim Acta. 2012;380:386–91.

57. Xu Q, Cao A, Zhang L, Zhang C. Rapid and Label-Free Monitoring of Exonuclease III-Assisted Target Recycling Amplification. Anal Chem. 2012;84(24):10845-51.

58. Joyce GF. RNA cleavage by the 10-23 DNA enzyme. Meth Enzymol. 2001;341:503–17.

59. Zaborowska Z, Furste JP, Erdmann VA, Kurreck J. Sequence Requirements in the Catalytic Core of the "10-23" DNA Enzyme. J Biol Chem. 2002;277(43):40617–22.

60. Zaborowska Z, Schubert S, Kurreck J, Erdmann VA. Deletion analysis in the catalytic region of the 10-23 DNA enzyme. FEBS Lett. 2005;579(2):554–8.

61. Richards JL, Seward GK, Wang Y-H, Dmochowski IJ. Turning the 10-23 DNAzyme on and off with light. Chembiochem. 2010;11(3):320–4.

62. Schubert S, Gül DC, Grunert H-P, Zeichhardt H, Erdmann VA, Kurreck J. RNA cleaving "10-23" DNAzymes with enhanced stability and activity. Nucleic Acids Res. 2003;31(20):5982–92.

63. Hollenstein M, Hipolito CJ, Lam CH, Perrin DM. Toward the combinatorial selection of chemically modified DNAzyme RNase A mimics active against all-RNA substrates. ACS Comb Sci. 2013;15(4):174–82.

64. Plashkevych O, Chattopadhyaya J. Structure of 10-23 DNAzyme in Complex with the Target RNA in silico-A Progress Report on the Mechanism of RNA Cleavage by DNA Enzyme. In: Zhang L, Xi Z, Chattopadhyaya J, editors. Medicinal Chemistry of Nucleic Acids. John Wiley & Sons, Inc., Hoboken, NJ, USA; 2011. p. 272-91.

65. Ponce-Salvatierra A, Wawrzyniak-Turek K, Steuerwald U, Höbartner C, Pena V. Crystal structure of a DNA catalyst. Nature. 2016;529(7585):231–4.

66. Nowakowski J, Shim PJ, Prasad GS, Stout CD, Joyce GF. Crystal structure of an 82-nucleotide RNA-DNA complex formed by the 10-23 DNA enzyme. Nat Struct Biol. 1999;6(2):151–6.

67. Oliphant AR, Brandl CJ, Struhl K. Defining the sequence specificity of DNA-binding proteins by selecting binding sites from random-sequence oligonucleotides: analysis of yeast GCN4 protein. Mol Cell Biol. 1989;9(7):2944-9.

68. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science. 1990;249(4968):505-10.

69. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. Nature. 1990;346(6287):818-22.

70. Schütze T, Wilhelm B, Greiner N, Braun H, Peter F, Mörl M, et al. Probing the SELEX process with next-generation sequencing. PLoS ONE. 2011;6(12):e29604.

71. Beier R, Boschke E, Labudde D. New strategies for evaluation and analysis of SELEX experiments. Biomed Res Int. 2014:849743.

72. Silverman SK. In vitro selection, characterization, and application of deoxyribozymes that cleave RNA. Nucleic Acids Res. 2005;33(19):6151–63.

73. Keefe AD, Pai S, Ellington A. Aptamers as therapeutics. Nat Rev Drug Discov. 2010;9(7):537–50.

74. Burmeister PE, Lewis SD, Silva RF, Preiss JR, Horwitz LR, Pendergrast PS, et al. Direct in vitro selection of a 2'-O-methyl aptamer to VEGF. Chem Biol. 2005;12(1):25.

75. Vater A, Klussmann S. Turning mirror-image oligonucleotides into drugs: the evolution of Spiegelmer® therapeutics. Drug Discov Today. 2014;20(1):147–55.

76. Williams KP, Liu XH, Schumacher TN, Lin HY, Ausiello DA, Kim PS, et al. Bioactive and nuclease-resistant L-DNA ligand of vasopressin. Proc Natl Acad Sci USA. 1997;94(21):11285–90.

77. Gragoudas ES, Adamis AP, Cunningham ET, Feinsod M, Guyer DR. Pegaptanib Sodium in the Treatment of Neovascular Age-Related Macular Degeneration. N Engl J Med. 2004;351:2805-16.

78. Dass CR, Saravolac EG, Li Y, Sun L-Q. Cellular uptake, distribution, and stability of 10-23 deoxyribozymes. Antisense Nucleic Acid Drug Dev. 2002;12(5):289–99.

79. Levin AA. A review of issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. BBA-Gene Struct Expr. 1999;1489(1):69-84.

80. Kiss T. Small nucleolar RNA-guided post-transcriptional modification of cellular RNAs. The EMBO Journal. 2001;20(14):3617-22.

81. Culbertson MC, Temburnikar KW, Sau SP, Liao J-Y, Bala S, Chaput JC. Evaluating TNA stability under simulated physiological conditions. Bioorg Med Chem Lett. 2016;26(10):2418–21.

82. Yu H, Zhang S, Dunn MR, Chaput JC. An efficient and faithful in vitro replication system for threose nucleic acid. J Am Chem Soc. 2013;135(9):3583–91.

83. Ezzat K, Aoki Y, Koo T, McClorey G, Benner L, Coenen-Stass A, et al. Self-Assembly into Nanoparticles Is Essential for Receptor Mediated Uptake of Therapeutic Antisense Oligonucleotides. Nano Lett. 2015;15(7):4364–73.

84. Taylor AI, Holliger P. Directed evolution of artificial enzymes (XNAzymes) from diverse repertoires of synthetic genetic polymers. Nat Protoc. 2015;10(10):1625–42.

85. Taylor AI, Pinheiro VB, Smola MJ, Morgunov AS, Peak-Chew S, Cozens C, et al. Catalysts from synthetic genetic polymers. Nature. 2014;518(7539):427–30.

86. Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. 1998;391(6669):806-11.

87. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature. 2000;404(6775):293–6.

88. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409:363–6.

89. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. 2004;15(2):185–97.

90. Iwakawa H-O, Tomari Y. Molecular insights into microRNA-mediated translational repression in plants. Mol Cell. 2013;52(4):591–601.

91. Lechardeur D, Verkman AS, Lukacs GL. Intracellular routing of plasmid DNA during non-viral gene transfer. Adv Drug Deliv Rev. 2005;57(5):755–67.

92. Dean DA, Strong DD, Zimmer WE. Nuclear entry of nonviral vectors. Gene Ther. 2005;12(11):881–90.

93. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and Molecular Barriers to Gene Transfer by a Cationic Lipid. J Biol Chem. 1995;270(32):18997-9007.

94. Bessis N, GarciaCozar FJ, Boissier M-C. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. Gene Ther. 2004;11 Suppl 1:S10–7.

95. Würtele H, Little KCE, Chartrand P. Illegitimate DNA integration in mammalian cells. Gene Ther. 2003;10(21):1791–9.

96. Chen ZY, He CY, Ehrhardt A, Kay MA. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. Molecular Therapy. 2003;8(3):495.

97. Bettinger T, Carlisle RC, Read ML, Ogris M, Seymour LW. Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. Nucleic Acids Res. 2001;29(18):3882–91.

98. Abe N, Matsumoto K, Nishihara M, Nakano Y, Shibata A, Maruyama H, et al. Rolling Circle Translation of Circular RNA in Living Human Cells. Sci Rep. 2015;5:16435.

99. Sambrook J, Westphal H, Srinivasan PR, Dulbecco R. The Integrated State of Viral DNA in SV40-Transformed Cells. Proc Natl Acad Sci USA. 1968;60(4):1288-95.

100. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 2013;31(7):397–405.

101. Mojica FJM, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol. 2005;60(2):174–82.

102. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, et al. RNA-guided human genome engineering via Cas9. Science. 2013;339(6121):823–6.

103. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science. 2013;339(6121):819.

104. Ghosh MK, Katyal A, Chandra R, Brahmachari V. Targeted activation of transcription in vivo through hairpin-triplex forming oligonucleotide in Saccharomyces cerevisiae. Mol Cell Biochem. 2005;278(1-2):147-55.

105. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, Iyer EPR, et al. Highly efficient Cas9-mediated transcriptional programming. Nature Methods. 2015;12(4):326–8.

106. Saayman SM, Lazar DC, Scott TA, Hart JR, Takahashi M, Burnett JC, et al. Potent and Targeted Activation of Latent HIV-1 Using the CRISPR/dCas9 Activator Complex. Mol Ther. 2016;24(3):488-98.

107. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 2013;152(5):1173–83.

108. Michaelis L, Menten ML. Die kinetik der invertinwirkung. Biochem z. 1913;49:333–69.

109. Bisswanger H. Enzyme Kinetics: Section 2.1-2.5. Enzyme Kinetics: Principles and Methods. 2008. p. 59–124.

110. Hoheisel JD. On the Activities of Escherichia coli Exonuclease III. Anal Biochem. 1993;209(2):238-46.

111. Lee J, Min D-H. A simple fluorometric assay for DNA exonuclease activity based on graphene oxide. Analyst. 2012;137(9):2024–6.

112. Trubetskoy VS, Hagstrom JE, Budker VG. Self-Quenched Covalent Fluorescent Dye-Nucleic Acid Conjugates as Polymeric Substrates for Enzymatic Nuclease Assays. Anal Biochem. 2002;300(1):22-6.

113. Xu Q, Cao A, Zhang L-F, Zhang C-Y. Rapid and Label-Free Monitoring of Exonuclease III-Assisted Target Recycling Amplification. Anal Chem. 2012;84(24):10845-51.

114. Li JJ, Geyer R, Tan W. Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA. Nucleic Acids Res. 2000;28(11):E52.

115. Eriksson J, Helmfors H, Langel Ü. A high-throughput kinetic assay for RNA-cleaving deoxyribozymes. PLoS ONE. 2015;10(8):e0135984.

116. Eriksson J, Langel Ü. Quantitative microplate assay for real-time nuclease kinetics. PLoS ONE. 2016;11(4):e0154099.

117. Brune M, Hunter JL, Corrie JE, Webb MR. Direct, real-time measurement of rapid inorganic phosphate release using a novel

fluorescent probe and its application to actomyosin subfragment 1 ATPase. Biochemistry. 1994;33(27):8262–71.

118. Brune M, Hunter JL, Howell SA, Martin SR, Hazlett TL, Corrie JE, et al. Mechanism of inorganic phosphate interaction with phosphate binding protein from Escherichia coli. Biochemistry. 1998;37(29):10370–80.

119. Vlad F, Rubio S, Rodrigues A, Sirichandra C, Belin C, Robert N, et al. Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. Plant Cell. 2009;21(10):3170–84.

120. Beckman JW, Wang Q, Guengerich FP. Kinetic analysis of correct nucleotide insertion by a Y-family DNA polymerase reveals conformational changes both prior to and following phosphodiester bond formation as detected by tryptophan fluorescence. J Biol Chem. 2008;283(52):36711-23.

121. Rogers S, Pfuderer P. Use of viruses as carriers of added genetic information. Nature. 1968;219(5155):749-51.

122. Stolberg SG. The biotech death of Jesse Gelsinger. N Y Times Mag. 1999:136-40, 149-50.

123. Fehse B, Roeder I. Insertional mutagenesis and clonal dominance: biological and statistical considerations. Gene Ther. 2007;15(2):143–53.

124. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000;288(5466):669–72.

125. Ylä-Herttuala S. Glybera's second act: the curtain rises on the high cost of therapy. Mol Ther. 2015;23(2):217–8.

126. Chen X, Gonçalves MAFV. Engineered viruses as genome editing devices. Mol Ther. 2015;24(3):447–57.

127. Flotte TR. Gene therapy progress and prospects: recombinant adeno-associated virus (rAAV) vectors. Gene Ther. 2004;11(10):805–10.

128. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, et al. Liposome: classification, preparation, and applications. Nanoscale Res Lett. 2013;8(1):102.

129. Deshpande PP, Biswas S, Torchilin VP. Current trends in the use of liposomes for tumor targeting. Nanomedicine. 2013;8(9):1509–28.

130. Zimmermann TS, Lee ACH, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. RNAi-mediated gene silencing in non-human primates. Nature. 2006;441(7089):111–4.

131. Behr J-P. The proton sponge: a trick to enter cells the viruses did not exploit. 1997;51:34–6.

132. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA. 1995;92(16):7297.

133. Benjaminsen RV, Mattebjerg MA, Henriksen JR, Moghimi SM, Andresen TL. The possible "proton sponge " effect of polyethylenimine (PEI) does not include change in lysosomal pH. Mol Ther. 2012;21(1):149–57.

134. Yue Y, Jin F, Deng R, Cai J, Chen Y, Kung H-F, et al. Revisit complexation between DNA and polyethylenimine - Effect of uncomplexed chains free in the solution mixture on gene transfection. J Control Release. 2010;155(1):67–76.

135. Johnson LS, Dunn KW, Pytowski B, McGraw TE. Endosome acidification and receptor trafficking: bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. Mol Biol Cell. 1993;4(12):1251-66.

136. Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacol Rev. 2006;58(1):32–45.

137. Somasuntharam I, Yehl K, Carroll SL, Maxwell JT, Martinez MD, Che P-L, et al. Knockdown of TNF-α by DNAzyme gold nanoparticles as an anti-inflammatory therapy for myocardial infarction. Biomaterials. 2015;83:12–22.

138. Magalhães MLB, Byrom M, Yan A, Kelly L, Li N, Furtado R, et al. A general RNA motif for cellular transfection. Mol Ther. 2012;20(3):616–24.

139. Green M, Loewenstein PM. Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell. 1988;55(6):1179-88.

140. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. Cell. 1988;55(6):1189-93.

141. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, et al. Tat-mediated delivery of heterologous proteins into cells. Proc Natl Acad Sci USA. 1994;91(2):664–8.

142. Derossi D, Joliot AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodornain translocates through biological membranes. J Biol Chem. 1994;269(14):10444–50.

143. Futaki S, Ohashi W, Suzuki T, Niwa M, Tanaka S, Ueda K, et al. Stearylated arginine-rich peptides: a new class of transfection systems. Bioconjug Chem. 2001;12(6):1005–11.

144. Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, et al. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J Biol Chem. 2000;276(8):5836–40.

145. Crombez L, Aldrian-Herrada G, Konate K, Nguyen QN, McMaster GK, Brasseur R, et al. A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. Mol Ther. 2008;17(1):95–103.

146. Pooga M, Hällbrink M, Zorko M, Langel Ü. Cell penetration by transportan. FASEB J. 1998;12(1):67–77.

147. Mäe M, Andaloussi El S, Lundin P, Oskolkov N, Johansson HJ, Guterstam P, et al. A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy. J Control Release. 2008;134(3):221–7.

148. Andaloussi SEL, Lehto T, Mäger I, Rosenthal-Aizman K, Oprea II, Simonson OE, et al. Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically *in vivo*. Nucleic Acids Res. 2011;39(9):3972–87.

149. Ezzat K, Andaloussi SEL, Zaghloul EM, Lehto T, Lindberg S, Moreno PMD, et al. PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. Nucleic Acids Res. 2011;39(12):5284–98.

150. Lindberg S, Muñoz-Alarcón A, Helmfors H, Mosqueira D, Gyllborg D, Tudoran O, et al. PepFect15, a novel endosomolytic cell-penetrating peptide for oligonucleotide delivery via scavenger receptors. Int J Pharm. 2012;441(1-2):242–7.

151. Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, et al. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Med. 1998;4(12):1449–52.

152. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. *In vivo* protein transduction: delivery of a biologically active protein into the mouse. Science. 1999;285(5433):1569–72.

153. Crombez L, Divita G. A non-covalent peptide-based strategy for siRNA delivery. Methods Mol Biol. 2010;683:349–60.

154. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, et al. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. J Biol Chem. 2002;278(1):585–90.

155. Rydström A, Deshayes S, Konate K, Crombez L, Padari K, Boukhaddaoui H, et al. Direct translocation as major cellular uptake for CADY self-assembling peptide-based nanoparticles. PLoS ONE. 2011;6(10):e25924.

156. Lindberg S, Regberg J, Eriksson J, Helmfors H, Muñoz-Alarcón A, Srimanee A, et al. A convergent uptake route for peptide- and polymer-based nucleotide delivery systems. J Controll Release. 2015;206:58–66.

157. Madani F, Lindberg S, Langel Ü, Futaki S, Gräslund A. Mechanisms of cellular uptake of cell-penetrating peptides. J Biophys. 2011;2011:414729.

158. Ezzat K, Helmfors H, Tudoran O, Juks C, Lindberg S, Padari K, et al. Scavenger receptor-mediated uptake of cell-penetrating peptide nanocomplexes with oligonucleotides. FASEB J. 2011;26(3):1172–80.

159. Srimanee A, Regberg J, Hällbrink M, Kurrikoff K, Veiman K-L, Vajragupta O, et al. Peptide-Based Delivery of Oligonucleotides Across Blood–Brain Barrier Model. Int J Pept Res Ther. 2013;20(2):169-78.

160. Greaves DR, Gordon S. The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. J Lipid Res. 2008;50 Suppl:S282–6.

161. Haisma HJ, Kamps JAAM, Kamps GK, Plantinga JA, Rots MG, Bellu AR. Polyinosinic acid enhances delivery of adenovirus

vectors in vivo by preventing sequestration in liver macrophages. J Gen Virol. 2008;89(Pt 5):1097–105.

162. Regberg J, Eriksson J, Langel Ü. Cell-penetrating peptides: from cell cultures to in vivo applications. Frontiers in Bioscience. 2013;E5(2):509–16.

163. Zhang JH, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen. 1999;4(2):67.

164. Kang SH, Cho MJ, Kole R. Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. Biochemistry. 1998;37(18):6235–9.

165. Moschos SA, Frick M, Taylor B, Turnpenny P, Graves H, Spink KG, et al. Uptake, efficacy, and systemic distribution of naked, inhaled short interfering RNA (siRNA) and locked nucleic acid (LNA) antisense. Mol Ther. 2011;19(12):2163–8.

166. Aranda PS, LaJoie DM, Jorcyk CL. Bleach gel: A simple agarose gel for analyzing RNA quality. 2012;33(2):366-9.

167. Singer VL, Jones LJ, Yue ST, Haugland RP. Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. Anal Biochem. 1997;249(2):228–38.

168. Rengarajan K, Cristol SM, Mehta M, Nickerson JM. Quantifying DNA concentrations using fluorometry: a comparison of fluorophores. Mol Vis. 2002;8:416–21.

169. Cosa G, Focsaneanu KS, McLean JR, McNamee JP, Scaiano JC. Photophysical properties of fluorescent DNA-dyes bound to single- and double-stranded DNA in aqueous buffered solution. Photochem Photobiol. 2001;73(6):585–99.

170. Eddy SR. How do RNA folding algorithms work? Nat Biotechnol. 2004;22(11):1457–8.

171. Nixon AE, Hunter JL, Bonifacio G, Eccleston JF, Webb MR. Purine nucleoside phosphorylase: its use in a spectroscopic assay for inorganic phosphate and for removing inorganic phosphate with the aid of phosphodeoxyribomutase. Anal Biochem. 1998;265(2):299-307.

172. Pennick M, Poole L, Dennis K, Smyth M. Lanthanum carbonate reduces urine phosphorus excretion: evidence of high-capacity phosphate binding. Renal Failure. 2012;34(3):263–70.

173. Zhao D, Sengupta AK. Ultimate removal of phosphate from wastewater using a new class of polymeric ion exchangers. Water Research. 1998;32(5):1613-25.

174. Simopoulos TT, Jencks WP. Alkaline phosphatase is an almost perfect enzyme. Biochemistry. 1994;33(34):10375–80.

175. Morse DP, Bass BL. Detection of inosine in messenger RNA by inosine-specific cleavage. Biochemistry. 1997;36(28):8429–34.

176. Das U, Shuman S. Mechanism of RNA 2',3'-cyclic phosphate end healing by T4 polynucleotide kinase–phosphatase. Nucleic Acids Res. 2013;41(1):355–65.

177. Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc. 1963;85(14):2149-54.

178. Kim NW, Wu F. Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). Nucleic Acids Res. 1997;25(13):2595–7.

179. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003;31(13):3406–15.

180. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, et al. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. The EMBO Journal. 1992;11(5):1921–9.

181. Ludlow AT, Robin JD, Sayed M, Litterst CM, Shelton DN, Shay JW, et al. Quantitative telomerase enzyme activity determination using droplet digital PCR with single cell resolution. Nucleic Acids Res. 2014;42(13):e104.