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TOWARDS SMART ANTISENSE AND ANTIGENE THERAPEUTICS – DESIGN & SYNTHESIS OF NOVEL OLIGONUCLEOTIDE BASED BIOCONJUGATES.

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Towards smart antisense and antigene therapeutics – design & synthesis of novel based bioconjugates. THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family…

ABSTRACT

The use of nucleic acid technology has been of great importance in life science research. In several clinical trials patients with genetic disease are treated with help of innovations based on oligonucleotide therapy. The use of it is unfortunately limited in efficacy due to e.g., variable stability of oligonucleotides in biological fluids and in particular poor delivery to the site of action. Numerous modifications, introduced to functionalize oligonucleotides improve their properties and help to overcome the limitation connected to their uptake.

With this thesis, I present our development of novel bioconjugates and technologies for functionalization of oligonucleotides. In chapter 2 (paper I), the development of four different synthetic m3G-Cap (nuclear localization signals) constructs is described. We provide a protocol that allows the conjugation of these for derivatives to oligonucleotide using "click" reaction. As a result, novel bioconjugates equipped with constructs containing the m3G-Cap were synthesized for the investigation of nuclear delivery.

Chapter 3 (paper II) describes the synthesis of two biotinylated linkers that can be conjugated to both peptides and oligonucleotides for labeling purposes. The multi-step synthesis of the linker is followed by conjugation to oligonucleotides that was carried out on solid support as well as to a peptide in solution.

In chapter 4 (paper III), the research presented in paper I and II is combined to allow the visualization of m3G-Cap action. The biotin linkers were also conjugated with similar Cap constructs containing modifications of the triphosphate bridge. All bioconjugates were evaluated in cell assays for uptake and splice-switching and the results show that the minimal m3G-Cap-biotin construct should contain a trinucleotide in order to act as a nuclear transport signal.

Chapter 5 gives an insight into unpublished data on the development of a linker that enable multiple functionalization of oligonucleotides (ONs) with several different biologically active entities. The synthesis of the linker, together with the first oligonucleotide conjugates prepared using the protocol is reported.

In chapter 6 (paper IV), we describe the successful preparation of four different Zorro-LNA constructs with help of "click" chemistry. Their ability for DSI (double strand invasion) was tested and they proved to be able to invade into supercoiled DNA, providing a useful screening strategy when optimizing Zorro constructs directed against new anti-gene targets.

LIST OF SCIENTIFIC PAPERS

I. Malgorzata Honcharenko, Joanna Romanowska, Margarita Alvira, **Martina Jezowska**, Mikael Kjellgren, C. I. Edvard Smith and Roger Strömberg; Capping of oligonucleotides with "clickable" m_3G-CAPs.
RSC Advances, 2012, 2, 12949

II. **Martina Jezowska**, Joanna Romanowska, Burcu Bestas, Ulf Tedebark, Malgorzata Honcharenko;
Synthesis of biotin linkers with the activated triple bond donor [*p*-(*N*-propynoylamino)toluic acid] (PATA) or efficient biotinylation of peptides and oligonucleotides.
Molecules, 2012, 17, 14174

III. Malgorzata Honcharenko[§], Burcu Bestas[§], **Martina Jezowska**[§], Błażej A. Wojtczak, Pedro Moreno, Joanna Romanowska, Susanna M. Bächle, Edward Darzynkiewicz, Jacek Jemielity, C. I. Edvard Smith, Roger Strömberg;
Synthetic m_3G-CAP attachment necessitates a minimum trinucleotide cinstituent to be recognised as a Nuclear Import Signal.
Manuscript

IV. Olof I. Gissberg[§], **Martina Jezowska**[§], Eman M. Zaghloul, N.I. Bungsua, Roger Strömberg, C. I. Edvard Smith, Karin E. Lundin, Malgorzata Honcharenko;
Fast and Efficient Synthesis of Zorro-LNA Type 3'-5'-5'-3' Oligonucleotide Conjugates *via* parallel in-situ stepwise conjugation.
Org. Biomol. Chem., 2016,14, 3584

[§] Equal contribution.

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LIST OF ABBREVIATIONS

AON	antisense oligonucleotide
CPP	cell penetrating peptide
CuI	copper (I) iodide
DBCO	dibenzocyclooctyl
DCA	dichloroacetic acid
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DMTr	dimethoxy trityl
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DSI	double-strand invasion
Et_3N	triethyl amine
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyl hexafluorophosphate
HPLC	high performance liquid chromatography
LBL	long biotin linker
LNA	locked nucleic acid
MeCN	acetonitrile
MeOH	methanol
MFL	multiple functionalization linker
miRNA	microRNA
MMTr	monomethoxy trityl
MOE	methoxyl ethyl
mRNA	messenger RNA
MS	mass spectrometry
MV	microwave irradiation
NLS	nuclear localization signal
NMM	*N*-methylmorpholine

NPC	nuclear pore complex
ON	oligonucleotide
PAMBA	4-((2-(prop-2-yn-1-yloxy)acetamido)methyl)benzoic acid
PATA	*p*-(*N*-propynoylamino)toluic acid
PEGBL	PEG biotin linker
PMO	phosphorodiamidate morpholino oligomer
pre-mRNA	precursor mRNA
PS	phosphorothioate
PTD	protein transduction domain
PvCl	pivaloyl chloride
Py	pyridine
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNase	ribonuclease
Rt	room temperature
SBL	short biotin linker
siRNA	small interfering RNA
tBuOH	*tert*-butanol
TFO	triplex forming oligonucleotide
TIPS	triisopropylsilyl
TMS	trimethylsilyl

1 INTRODUCTION

1.1 OLIGONUCLEOTIDE BASED THERAPEUTICS

Biologically active nucleic acid based compounds are generating a strong interest in modern drug research. When designed accordingly, their unique features allow targeting specific sites responsible for many different diseases, including metabolic, infectious and genetic diseases. With their help we could individually tailor the treatment for each patient and thus maximize the benefit and minimize the harms of each medical therapy[1].

Oligonucleotides (ONs) are one example of such molecules. Therapeutics based on them are designed to hybridize targeted RNA or DNA and to regulate their function[2]. The type of the ON target molecule allows to classify them in to two groups: antisense oligonucleotides (AONs) that interact and form duplex with mRNA, microRNA or pre-mRNA and antigene oligonucleotides which enter the cell nucleus in order to form triplexes with double-stranded DNA (dsDNA).

1.1.1 Antisense therapy

In 1978 Zamecnik and Stephenson reported that a short oligonucleotide inhibits viral replication in cell culture[3]. They used single-stranded DNA-based oligonucleotides and called them antisense oligonucleotides for the first time. The field of antisense technology has rapidly developed with the invention of solid-phase synthesis methodology for preparation of oligonucleotides[4]. When the method became automatized libraries of compounds could be produced and evaluated in shorter time. Currently, AONs are undergoing several clinical trials for treatment of e.g., muscular dystrophy, asthma, diabetes, cardiovascular diseases, rheumatoid arthritis, Crohn's disease, HIV or cancer[5-7].

AONs are short stretches of DNA or RNA, usually 8 – 50 nucleotides, which use Watson-Crick base pairing to bind a complementary target RNA[2, 8]. Their structures are commonly altered, as unmodified oligonucleotides are very unstable and easily degraded in biological fluids[9]. It is important to mention, that these modifications can also improve the target affinity of AONs and their uptake by cells which are common challenges for their therapeutic use[10, 11].

Nucleic acid analogues can be classified into three generations (Figure 1):

- First generation – where the phosphate linkage is changed,
- Second generation – contain modification on 2'- position of the sugar moiety,
- Third generation – more diverse modifications of sugar rings and/or phosphodiester backbones.

Modifications of the phosphodiester backbone was some of the first made to nucleic acids, where one of the non-bridging phosphate oxygens was replaced. The most widely used are phosphorothioates (PS) which not only increase biological stability of the ON (half-life extended up to 10h[12-14]) but when used as deoxyribooligonucleotides they also activate RNase

H cleavage of the target RNA (see below). In addition, the PS-ONs are reported to bind to plasma proteins, which increases their circulation time and therefore prevents them to be cleared from the organism easily. The main disadvantage of this modification is the poor specificity of the mRNA binding which could lead to problems with toxicity[15].

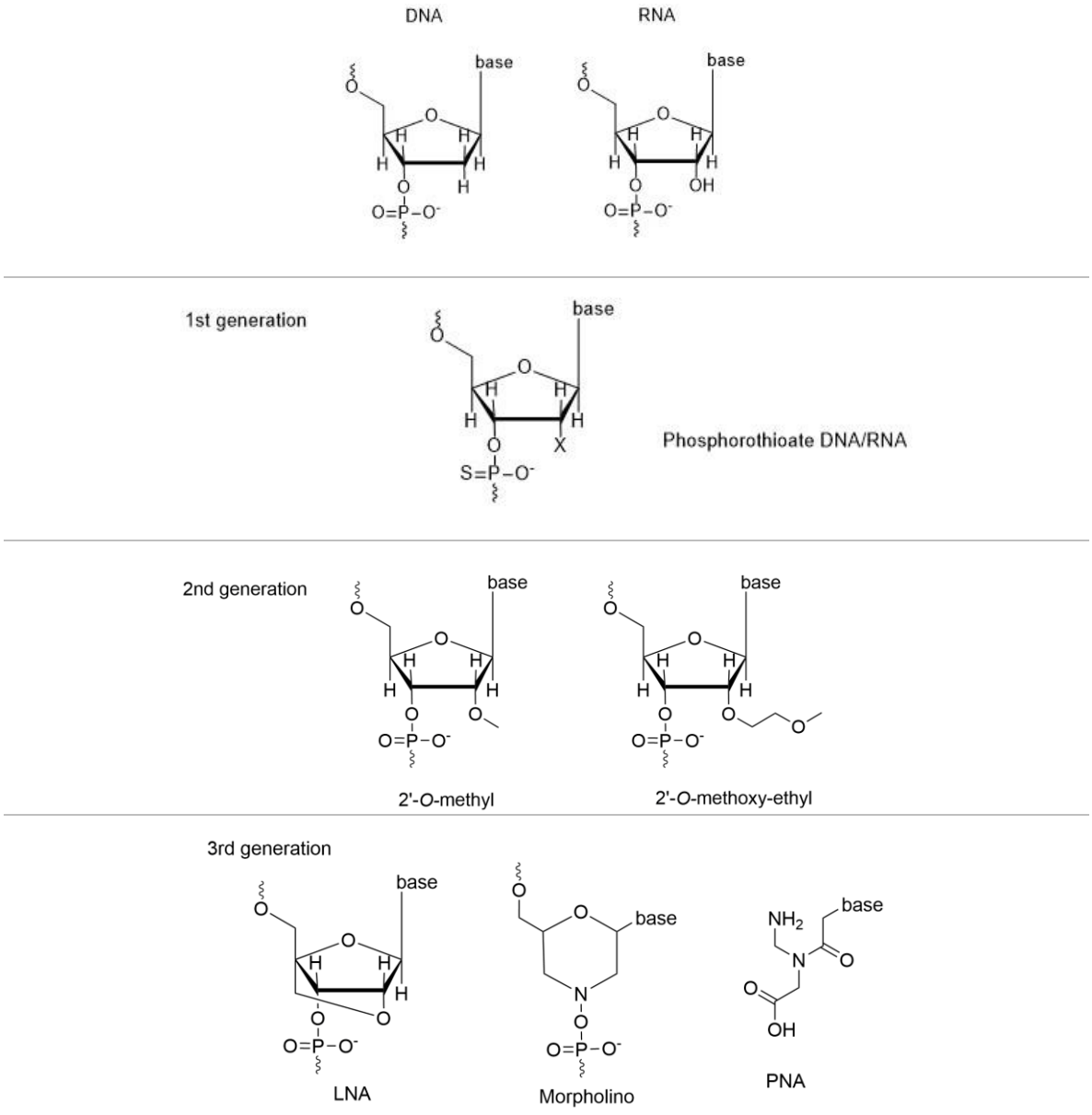


Figure 1: Basic nucleic acids and examples their modifications used in AONs.

The new generation of nucleic acid analogues brought a major improvement compared to the first. It has been shown that introducing modifications at the 2'-*O*-position of the sugar may increase the binding affinity and reduce toxicity. The most important second generation ONs are 2'-*O*-methyl[16] and 2'-*O*-metoxyethyl (MOE) RNAs. Their main drawback is the inability to activate the RNase H mechanism[17], which lowers the efficiency of the treatment. This problem has been solved by introduction of a "gapmer" concept[18], a construct where 2'-*O*-AlkylONs flank the central, RNase H activating part, DNA-PS ("gap").

The need for overcoming the limitations of the first and second generation AONs has driven researchers to prepare analogues with more extensive modifications including conformational restrictions as well as complete replacement of the ribose with different moieties. These third generation AONs include among others morpholino phosphoramidites (PMO)[19], locked nucleic acids (LNA)[20] and peptide nucleic acids (PNA)[21]. Although all of them demonstrate high affinity to the targeted RNA and are stable to nuclease degradation their duplexes with RNA are not efficiently recognized by RNase H.

LNA is a particularly interesting analogue achieving very high target affinity[22] thanks to its locked structure (bridging methylene carbon between 2' and 4' positions). This modification forces the nucleotide to be in north conformation and as a result the ON will resemble natural RNA in A-form[23, 24]. Other advantages of LNA are enhanced serum stability[25], slower renal clearance[26] and low toxicity[26, 27]. Similarly to 2'-*O*-AONs, LNA is a perfect tool for preparation of gapmers[28-30]. Its potential is also used in antigene therapy, discussed in chapter 1.1.2.

Antisense Oligonucleotide Mechanisms

There are several ways in which oligonucleotides influence processes in the cell[31]. Some examples are presented in the Figure 2. The first approach (Figure 2a) represents the steric block of messenger RNA (mRNA) by an AON, *via* Watson-Crick base pairing[3]. This action is strengthened by modifications of ONs which enhance their binding to the mRNA target.

Figure 2b illustrates the AON which after binding to mRNA recruits RNase H, thereby causing degradation of the mRNA. This type of action can be carried out by gapmers that consist of a DNA central part and outer modified AON "wings" allowing for more efficient RNA anchoring. A major advantage of a turnover mechanism compared to the steric block is that the dose of oligonucleotide used can be lower[31].

Another mechanism for mRNA cleavage is achieved *via* RNA interference (Figure 2c). Here, the siRNA (small interfering RNA) employs the RNA-induced silencing complex (RISC) in order to down-regulate the target mRNA[32].

MicroRNAs (miRNAs) presented in Figure 2d are small regulatory RNAs that at the end of biogenesis end up as single stranded RNA molecules on the RISC complex, which activates RNA interference and therefore degrades mRNA, in a similar mechanism as for siRNAs. If a complimentary oligonucleotide analogue binds strongly to the active strand of the miRNA, it cannot be loaded onto the RISC complex and regulation of gene expression is prevented. It has been reported that miRNA targeting ONs, even in submicromolar concentrations, rapidly enter cells and interfere with miRNAs[33, 34].

The RISC is also blocked in the next mechanism (Figure 2e). Here, the miRNA binding site on the mRNA is masked by an oligonucleotide analogue which prevents the mRNA degradation[35].

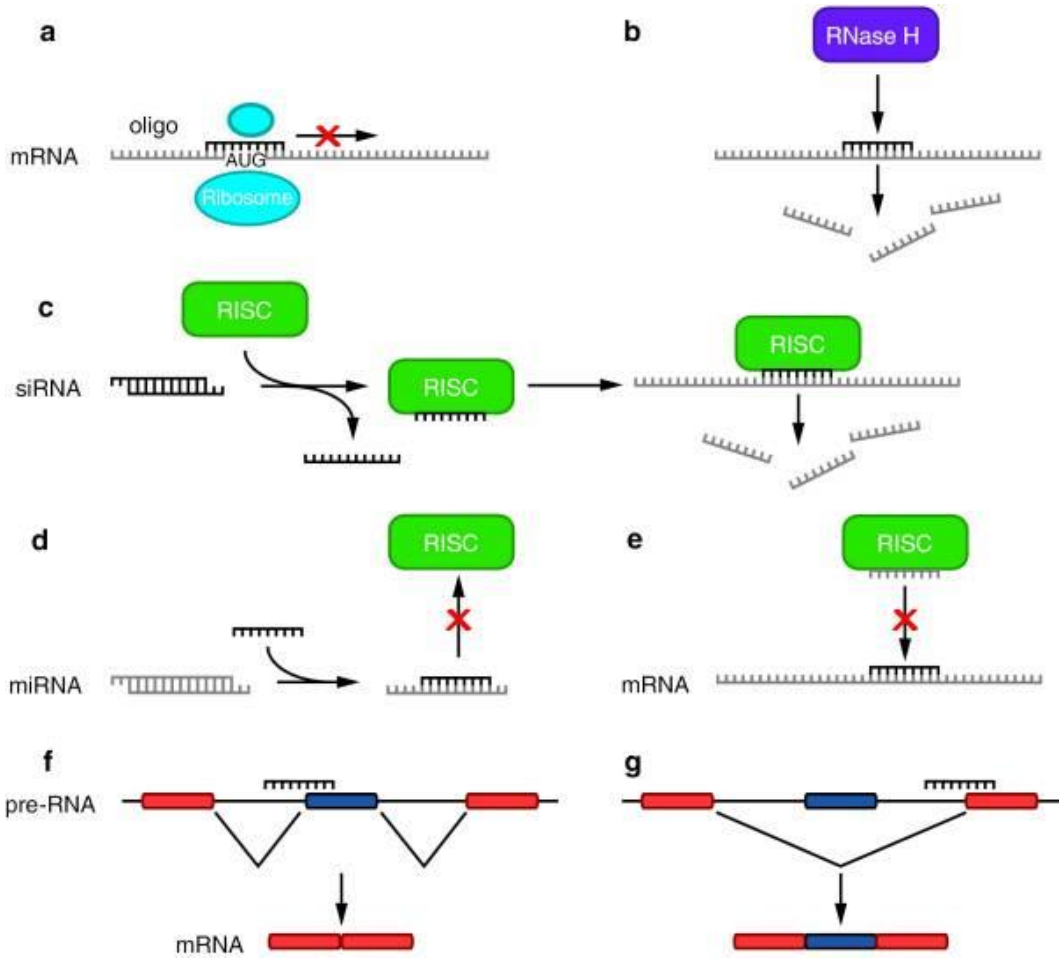


Figure 2: Schematic representation of how synthetic oligonucleotides influence processes in cells: a) steric block, b) recruitment of RNase H, c) RNA interference, d) binding to mRNA and blocking RISC, e) masking of miRNA binding site, e) exon skipping, f) exon inclusion. Reprinted by permission from Macmillan Publishers Ltd: Molecular therapy. Nucleic acids, Reference: 31, copyright 2012.

The two last Figures 2f and 2g represent a separate group of ON therapy mechanisms, taking place in the cell nucleus and targeting pre-mRNA. The splicing can be manipulated in different ways e.g., *via* steric hindrance, forcing splicing to an alternative route, or by employing the trans-splicing factors that can recognize the splice site, thus enhancing splicing. Here only two types of splicing are illustrated. The first is exon exclusion, also known as exon skipping, where an unnecessary exon is excluded, allowing restoration of the reading frame and generation of a partially functional protein. Exon inclusion, on the other hand, the splice-switching oligonucleotides are used as blocking agents to redirect the splice back to the correct splice sites.

1.1.2 Antigene therapy

DNA containing genetic information is found in the cell nucleus. It is responsible for development, reproduction and function of an organism and is therefore also a target for oligonucleotide therapeutics. The strategy where sequence specific ON is targeting dsDNA is called anti-gene therapy.

The mechanism of action, carried out by a synthetic ON, consists of a few steps. Most common is that the target cell first engulfs the ON by endocytosis. After endosomal escape into the

cytoplasm, it can also diffuse into the nucleus where it can bind to the target dsDNA and e.g., prevent transcription and formation of mRNA. When mRNA is less available the protein production is lower which should result in a lower concentration of protein and then lower activity.

Antigene oligonucleotide therapeutics can be divided in different strategies. Some are built through triple helix formation (triplexes) with dsDNA (triplex forming oligonucleotides - TFO) or invade the duplex DNA, binding one of the strands with Watson-Crick interactions and the other using both Hoogsteen[36] base pairing, thereby clamping the DNA.

Triplex Forming Oligonucleotides (TFOs)

The triple helix is formed, when the TFO attaches to the dsDNA *via* Hoogsteen base pairing, specific orientations of hydrogen bond. These can be in parallel or in antiparallel orientation. In parallel mode, when the TFO and a polypurine strand (**R**) are both in the 5' → 3'direction,

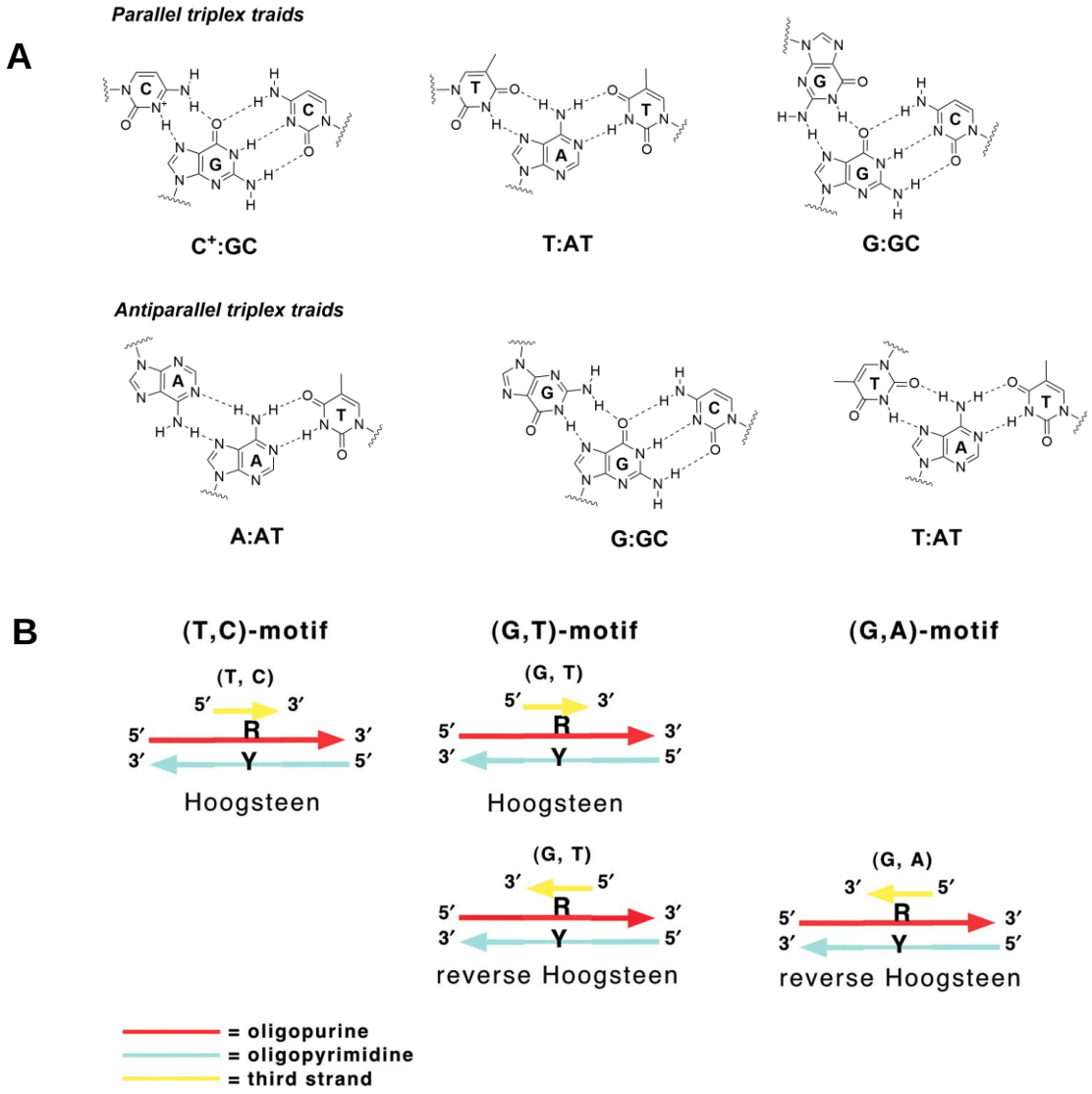


Figure 3: **A**: Parallel and antiparallel triplex compleses; **B**: Orientation of the three possible triplex motifs. Maria Duca, Pierre Vekhoff, Kahina Oussedik, Ludovic Halby, Paola B. Arimondo The triple helix: 50 years later, the outcome Nucl. Acids Res. (2008) 36 (16): 5123-5138, by permission of Oxford University Press.

the TFO can bind *via* TC or GT motif (Figure **3**). The triplexes formed in the TC motifs are: T:A*T and C:G*C+, where C+ stands for protonated cytosine. The protonation is a requirement for stable CGC triplets and makes the triplex dependent on slightly acidic conditions[37]. The parallel GT motifs are: C:G*G and T:A*T. The antiparallel orientation is obtained when the TFO is in the 3'→ 5' direction and a polypurine strand in the 5'→ 3' direction. Here the TFO binds also *via* GT- motif and a GA-motif, only this time the triplets are formed in the reverse-Hoogsteen orientation. In the GT- motif the TFO forms C:G*G and T:A*T triplets and in the GA- motif C:G*G and T:A*A triplets.

Double Strand Invasion (DSI)

When oligonucleotide therapeutics bind to one of the DNA strands *via* Watson-Crick interaction, the other strand is being displaced and as a result the DNA is opening up. Such a mechanism is recognized as double strand invasion. PNA and LNA are very important TFO modifications as they are suggested to use different pathways to invade dsDNA. Among these are duplex invasion and triplex invasion, where PNAs and LNAs bind homopurine sequences. A more complicated mechanism is double duplex invasion, using pseudo complimentary PNA and invader-LNA to bind overlapping sequences in both strands at the same time without self interaction[38, 39]. Another group of invading oligonucleotides are clamp ONs (bisLNA and bisPNA) that bind to dsDNA through a combination of Watson-Crick and Hoogsteen base pairing. Finally there are Zorro-LNAs which target two adjacent sites in both strands of DNA and due to their Z-shape structure the self-interaction is prevented[40, 41]. More about Zorro-LNA will be discussed in Chapter 6.

	Antisense therapy	**Anti-gene therapy**
Target	mRNA	genomic DNA
Target availability	Easier to achieve	Harder to achieve - dsDNA highly packed in chromatin
Target sequence	Hundreds to thousand copies available for AONs	Available once or twice in the genome
Mechanism of action	Most efficient when RNase H or RISC involved	Only blocks the transcription

Table 1: Comparison of antisense and anti-gene therapy.

1.2 BIOCONJUGATE PREPARATION - LINKING TECHNOLOGY FOR OLIGONUCLEOTIDES

As mentioned, unmodified oligonucleotides are not stable in biological fluids and their delivery to cells is poor because of their polyanionic nature. Chemical modifications have delivered

some solutions to these problems but major improvement can be obtained with introduction of conjugation[42-45]. ON-conjugate synthesis is achieved by attaching entities (molecule with particular properties) to ONs using covalent bonds, both by chemical and enzymatic reactions. The properties of such oligonucleotide conjugates are depending on the type of moiety attached[42] and can either improve properties that the ON already possess or give it completely new ones.

Conjugation to ONs can take place on either the 5'- or 3' terminus but also internally on the phosphodiester backbone, sugar or base. However, the 5' and 3' positions are easily accessible and therefore more straightforward for synthesis.

While designing the synthesis and the structure of entities a few things need to be taken into consideration. Among those are: chemical compatibility of the ON with the moiety, accessibility of conjugation sites, linker length and type of covalent linkage. The most important thing one must bear in mind is to make sure that the attachment of an entity does not weaken or hinder the affinity of the ON for the target site.

Numerous synthesis methods for preparation of oligonucleotide based bioconjugates can be categorized in many ways. One of them groups the approaches according to the medium used for their preparation: solid- or solution-phase[44, 45].

Solid-Phase Conjugate Synthesis

In this synthetic methodology the oligonucleotide is bound to a support, e.g., CPG (controlled-pore glass), and the conjugation can be performed with one or multiple steps and excess of reagents can be washed away before release from support. This makes purification less complicated and the procedure can often be automated on oligonucleotide synthesizer. If the entity is prepared in the form of either a phosphoramidite or an H-phosphonate, it could be conjugated to the ON in the course of the synthesis. This approach is commonly used with lipophilic molecules, fluorescent labels, dyes or PEGs which are commercially available as "ready-to-use" building blocks[46].

Another methodology used, mainly for conjugation of peptides or carbohydrates is called in-line or stepwise solid-phase synthesis. Here, the product is obtained either by synthesizing the ON first, on the solid-support, followed by elongation with the moiety or vice versa[46]. It is possible to synthesize a branched linker or take advantage of commercially available ones. Some limitations of this method includes the choice of protecting groups for the moiety, which needs to be chemically compatible with the preparation and deprotection conditions used in the course of the synthesis. It is still a great option for creating large libraries of products, in a short time, that can be scanned for therapeutic properties.

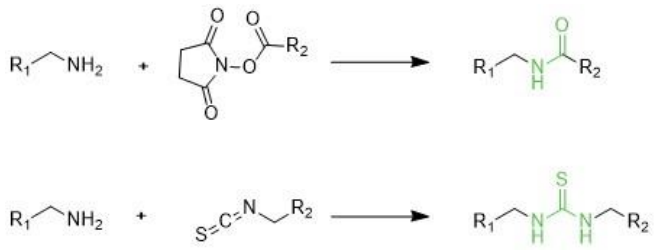
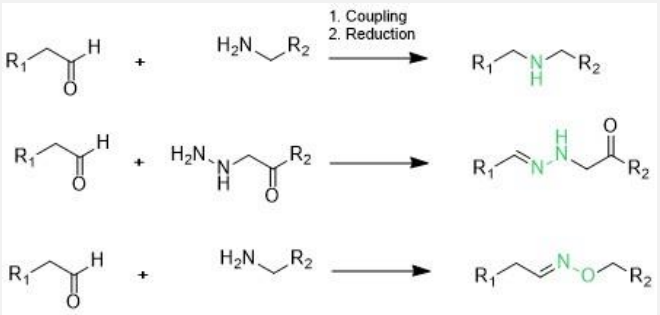
On-support fragment conjugation is quite similar to the stepwise solid-phase synthesis. The ON is still synthesized on solid support but the fragment conjugated to it is prepared separately and added when the moiety is fully prepared. This so called "fragment" is equipped with a functional group, like an amine or carboxylic acid that reacts with a preattached linker on the

ON to create the covalent linkage. For amide formation, coupling reagents typically used are reagents commonly used in peptide synthesis, such as HBTU/HOBt or HBTU/NMM[47-49]. Interestingly, copper catalyzed 1,3-dipolar cycloaddition reactions are also utilized for fragment conjugations[50] and microwave assisted conditions has been used to speed up the process[51].

Solution-Phase Conjugate Synthesis

When oligonucleotides and entities are conjugated in solution they are prepared and purified separately. They are then both equipped with mutually reactive groups that can result in formation of a covalent linkage. Compared to the solid-phase approach, it is an advantage that the chemistry used in the preparation of the two fragments does not need to be compatible with each other since it is done separately. Fully deprotected oligonucleotides are typically only soluble in solutions with high water content, so one should, however, make sure that the moiety is soluble under these conditions.

Examples of different solution-phase conjugations are grouped in the Table 2 below together with their most important characteristics.

Amide and Thiourea Linkages 	• Formed *via* reaction of NH group (readily attached to oligonuclotides) with activated carboxylic acid or isothiocyanate groups in basic conditions
Imine, Hydrazone and Oxyme Linkages 	• Nucleophilic addition on carbonyl group (present on ON) achieved *via* reaction of aldehyde with amino, hydrazine and aminooxy group respectivly[44, 45, 52], in mild acidic conditions • Imine linkageformation is reversable but can be reduced to secondary amine • Zatsepin et al. reported an approch where promoiety is equipped with an aldehyde group and the ON contains amino, hydrazine or aminooxy group[53]

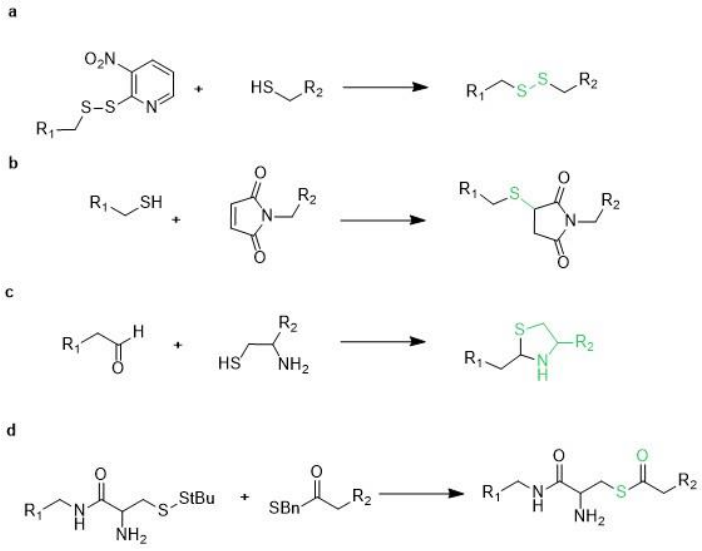
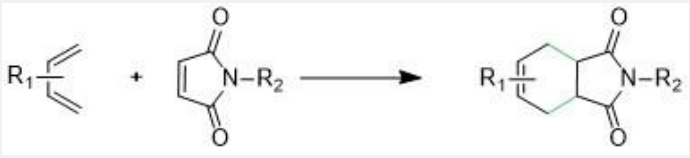
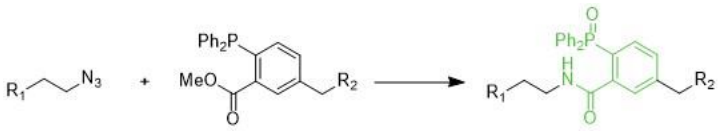
Disulfide, Thioether, Thiazolidine, Native Ligation 	• Reaction of thiol groups with others[46]: *a)* *Disulfide* – oxidative coupling of two thiol groups (one usually activated) *b)* *Thioether* – nucleophilic addition of a thiol to an electron-deficient carbon (halogeno-acetyl, acrylic, vinylic or maleimide group) *c)* *Thiazolidine* – reaction beween an aldehyde and a 1,2-aminothiol group *d)* *Native Ligation* - used for conjugation of two unprotected peptides one with aC-termnal thioester and the other with an N-terminal cysteine
Diels-Alder [4+2] Cycloaddition 	• Reaction between a diene with an electron-donating group and a dienophile carrying electron-withdrawing group; the diene group is incorporated on the ON as it is stable during solid-phase preparation of the ON[54]
Staudinger Ligation 	• Azide group reacting with phosphine at rt and in aqueous conditions[55]

Table 2: Examples of linker technology for preparation of bioconjugates in solution phase[44].

1,3-Dipolar Cycloaddition ("Click"-Chemistry)

"Click"- chemistry is a linking method that can be used both in solution and on solid support but since it is broadly used in this thesis the characteristics are discussed separately.

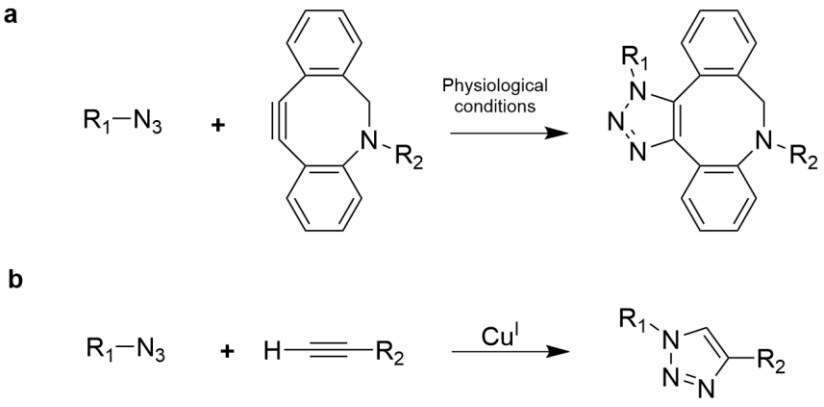


Figure 4: Copper free and copper-catalyzed "click" chemistry.

The efficiency of joining of an alkyne and an azide under mild conditions, with Cu (I) catalyst, was first reported by Sharpless and his co-workers in 2002, who also coined the expression "click" chemistry, and the reaction since then revolutionized the chemistry world[56]. This easy-to perform reaction is characterized by high chemoselectivity, which means that the two reagents involved in the process are only reacting with each other. The reaction can be accomplished in water giving a robustly ligated biomolecule in high yields. "Click"- reaction is also very versatile so both functional groups involved in the conjugation can often be placed on either molecule. Another advantage of the method is the reagents used. Their stability usually makes them resistant to hydrolysis over broad range of pH. Additionally, they are also biorthogonal which means they are often inert to groups like amines and hydroxyls[57].

1,3-dipolar cycloaddition for formation of triazoles from azides and alkynes can be classified into two broad categories (Figure **4**): copper free and copper-catalyzed reactions. In order to obtain reasonable rates in copper free chemistry the alkyne has to be strain-activates, i.e., the alkyne should be the part of a ring structure. This is exemplified by the reaction between dibenzocyclooctyne derivative (DBCO) and an azide group (Figure **4a**). An advantage of this method is biocompatibility and that it is suitable for live cells and even animal studies. Copper-catalyzed chemistry is the more common approach where the azide is conjugated with an alkyne in presence of a metal catalyst: copper (I)[56] (Figure **4b**). This method is very efficient, broadly used and has the advantage that it is regiospecific, i.e., only one isomer of the triazole product is formed. Since remains of copper reagent can cause biotoxicity special care must be

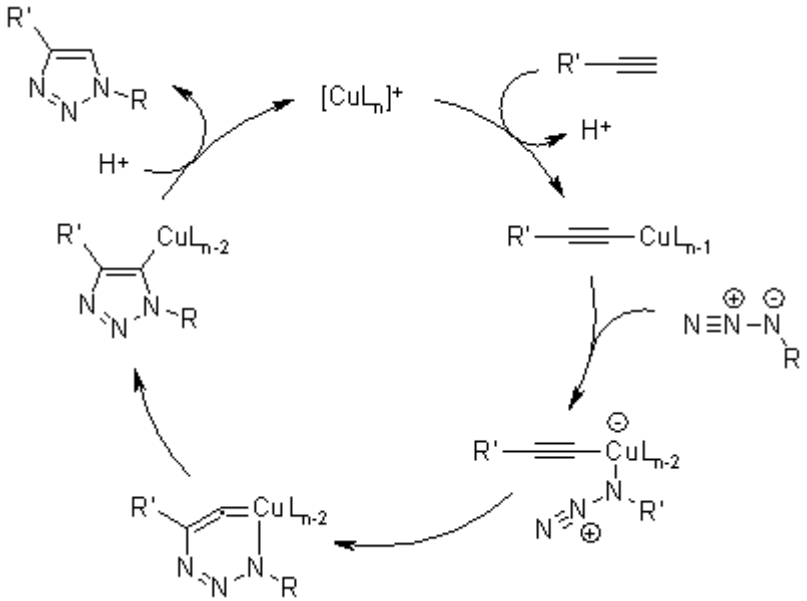


Figure 5: Proposed mechanism for copper-catalyzed cycloaddition. Reprinted with permission from J. Am. Chem. Soc., 2005, 127 (1), pp 210–216, Copper (I)-Catalyzed Synthesis of Azoles. DFT Study Predicts Unprecedented Reactivity and Intermediates Himo, F.; Lovell, T.; Hilgraf, R.; Rostovtsev, Vsevolod V.; Noodleman, L. ; Sharpless, K. B.; Fokin, V. . Copyright 2005 American Chemical Society.

taken to remove these if the product is to be used in biological experiments. In case of conjugates prepared on solid support this limitation can be overcome by washing with EDTA solution prior the cleavage of the conjugate from support. This way the copper amount can be reduced to the maximum limit for drinking water[58]. If RP-HPLC purification of the product is

done, EDTA can be added prior to the run to ensure complete removal of the copper ions (the Cu EDTA complex elutes more or less with the void volume of the column).

1.3 ENTITIES IMPROVING BIOCONJUGATE PROPERTIES

Over the years, scientists have developed many types of compounds which were conjugated to oligonucleotides to overcome different biological barriers, and to improve their therapeutic and diagnostic properties. This chapter presents a brief overview of the available options.

Fluorescent Labels

Being able to observe the action of oligonucleotide during their action is an important feature when studying novel bioconjugates. Fluorescent labels conjugated to oligonucleotides allow us to have such insight. They can be incorporated either on the 5'- or 3'- terminus as well as on the sugar or the nucleobase. Both solution- and solid-phase are appropriate mediums to carry out the conjugation and modified solid supports containing a fluorescent label are nowadays commercially available. These biomolecules cover very broad absorbance and emission wavelengths, ranging from UV-VIS to near infrared[59].

Fluorescent oligonucleotides play an important role in laboratory research, for example by helping to understand the mechanisms of RNA silencing by RNA interference. They are also used in clinics for DNA-based diagnostics such as Fluorescent In Situ Hybridization (FISH)[60]. They are also very useful tool for the Human Genome Mapping Project (HGMP)[46].

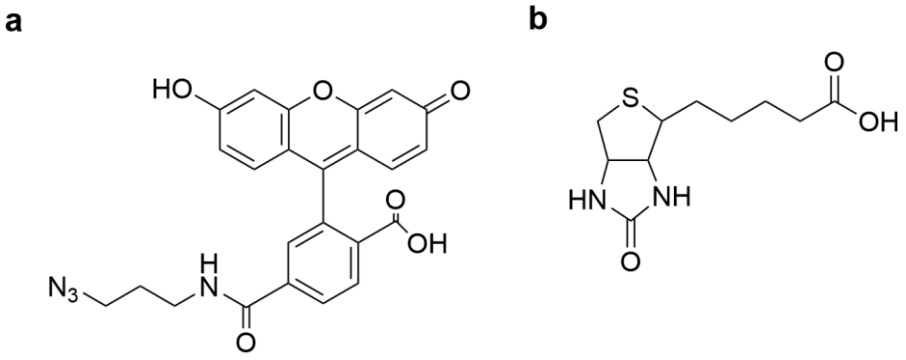


Figure 6: a) fluorescent linker with "clickable" handle, b) biotin.

As an example Figure **6** represents two routes to obtaining conjugated fluorescent entities. The first one is a fluorescent molecule equipped with an azide handle making it possible to conjugate *via* "click" reaction. The other one is biotin, which is not fluorescent on its own but possess exclusive affinity to fluorescent streptavidin/avidin proteins. The complex they create is a basis for many important biotechnological applications including studies of cellular transport[49].

Lipophilic-Oligonucleotide Conjugates

The cell membrane is a barrier that cells use to selectively regulate their environment. The phospholipid bilayers and proteins, which they are mainly made of, decide what is or is not allowed to enter. This largely hydrophobic border is very hard to cross for polyanionic

oligonucleotides and therefore the demand for lipophilic or amphiphilic transporters, supporting cellular uptake, is very high. The improvement can be achieved in two ways: by reducing the hydrophilic character of ON and/or by using the lipoprotein-mediated endocytosis pathway[61, 62].

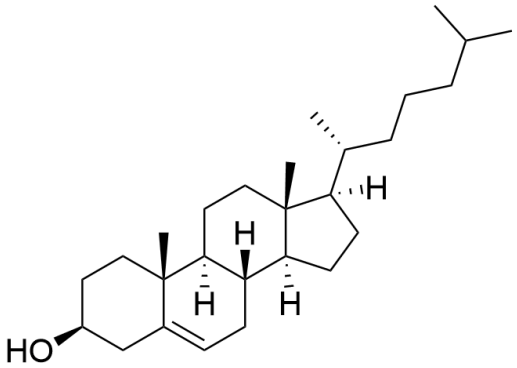


Figure 7: Example of a lipophilic entity, cholesterol, used in oligonucleotide conjugates to improve cellular uptake.

Figure 7 shows one example of an uptake-enhancing, lipophilic molecule. The first report on siRNA silencing of an endogenous gene *in vivo* under physiological conditions was describing the use of a cholesterol conjugate[63].

Polymer-Oligonucleotide Conjugates

Similarly to small lipophilic molecules, polymers are also used for oligonucleotide delivery[64, 65]. Their greatest advantage is the ability to reduce toxicity and immunogenicity. The most popular polymer in pharmaceutical applications is PEG (polyethylene glycol), which is known to prolong circulation time by lowering renal filtration rates[66, 67]. It is also important to mention that using polymers can offer a presence of multiple potential attachment sites[46].

Carbohydrate-Oligonucleotide Conjugates

Carbohydrates are a group of linkers for receptor-specific targeting. They can interact with proteins covering the cell surfaces which are able to recognize and internalize glycoprotein moieties through endocytosis. By conjugating specific sugars to ONs we can improve cell- and tissue-specific delivery[68, 69].

This group of bioconjugates has been reported to have potential in cancer treatment. An example here is hyaluronic acid, a linear polysaccharide, which is the main ligand for a transmembrane glycoprotein CD44 that is overexpressed in many cancers[70]. Hyaluronic acid conjugates of cancer drugs have been shown to exhibit increased uptake of ONs to cancer cells[71]

Peptide-Oligonucleotide Conjugates

An interesting group of entities improving oligonucleotide properties are highly cationic peptides that are able to cross cellular membranes[72]. These are known as cell-penetrating peptides (CPPs) or peptide transduction domains (PTDs)[73, 74] and typically of 5-40 amino acids (natural or non-natural) length. Many studies reported of cargo variety that CPPs were able to

transport various cargos, among these are: peptides[75], proteins[76], plasmid DNA[77], liposomes[78], nanoparticles[79] and oligonucleotides[80].

The association of ONs to a CPP can be achieved in different ways. If the oligonucleotide is neutrally charged both entities have to be covalently bound. In case of an ON carrying negative charges the molecules can also be connected *via* non-covalent interaction. Here, the peptide and ON are mixed in solution to form a complex that is further used. Overall, the uncharged oligonucleotides seem to give better results in conjugates with CPPs. The exact mechanism of CPPs uptake is not completely known. However, it is suggested that the peptides interact with anionic molecules like syndecans, present on the cell surface, which help them and their cargo to be internalize into the endosome[81]. No surprise that CPPs delivering the cargo to different organs including those usually hard to reach like the brain and the heart, have been recently reported[82-84]. Homing peptides are very promising group of delivery vehicles. They are carefully designed to target a specific cells or tissues[85].

Nuclear Localization Signals (NLS)

The conjugates mentioned above focus on entering the cells through the cytoplasm andtargeting of specific tissues or cells, which is a complex problem to tackle. However, sometimes the therapeutic oligonucleotide must be delivered even further – to the cell nucleus. This task is not easy and if we picture the nucleus as a commander center of the cell, we can easily imagine that its defense system will be very high. The membrane protecting the nucleus is very selective and its pore size restricts the molecules diffusing independently to be no bigger than 40KDa. That leaves proteins, ribosomal subunits and larger nucleotides (e.g., plasmids, certain RNA etc.) oligonucleotide therapeutics outside and in need of a carrier that will enable the active transport. These carriers are known as Nuclear Localization Signals (NLS). ONs can enter *via* diffusion but additional active transport should increase nuclear concentration.

The first and best characterized nuclear targeting signals are the classical nuclear localization peptides also referred to as sequences that contain one (monopartite) or two (bipartite) clusters of basic amino acids. The most studied example of a monopartite NLS peptide is described by Kalderon et al. and derived from the Simian Virus 40 (SV40) large T antigen which contains a cluster of 4-5 consecutive positively charged amino-acids[86, 87] (sequence: PKKKRKV). Bipartite NLSs contain two interdependent, basic clusters that are built in the way that a smaller upstream cluster, containing just two lysine or arginine residues, is separated by a mutation-tolerant linker from a downstream cluster that resembles a monopartite NLS[88]. The great example of this NLS group is nucleoplasmin where the two clusters required for nuclear targeting, are separated by 10-12 amino acids residues (sequence: **KR**PAATKKAGQA**KKKK**LDK)[89]. For the transport to happen two transport adapters are required: 1) importin-α that contains the NLS binding site and 2) importin-β, responsible for the docking of the importin-substrate complex to the cytoplasmic filaments of the nuclear pore complex (NPC) and the translocation through the pore[88]. Once the cargo-importin complex is inside the nucleus, RanGTP binds to importin-β leading to the release of cargo and dissociation of the complex.

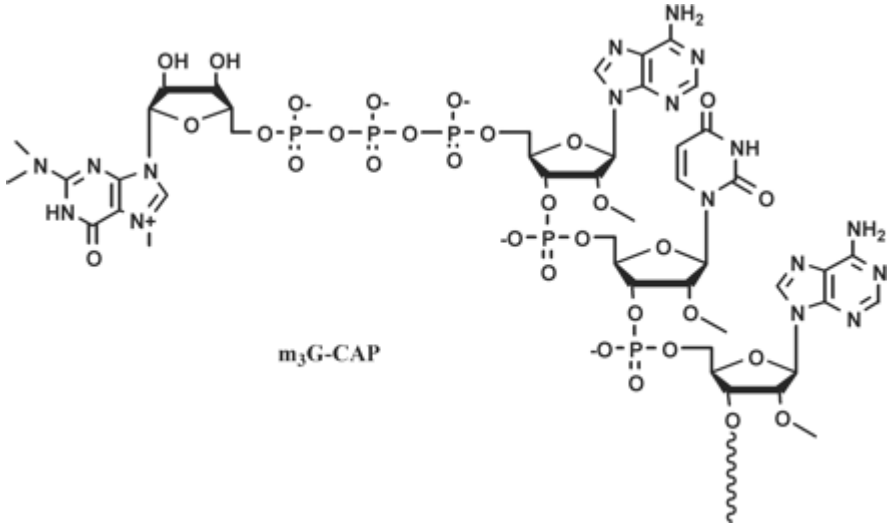


Figure 8: Structure of the 2,2,7-trimethylguanosine cap. Pedro M. D. Moreno, Malgorzata Wenska, Karin E. Lundin, Örjan, Wrange, Roger Strömberg, C. I. Edvard Smith, A synthetic snRNA m3GCAP enhances nuclear delivery of exogenous proteins and nucleic acids, Nucleic Acids Res. 2009, 37(6):1925-35, by permission of Oxford University Press.

The 2,2,7-trimethylguanosine cap (m_3G-Cap), is a different type of NLS. It is naturally found in uridine rich, small nuclear ribonucleoproteins (U snRNPs), involved in pre-mRNA splicing[90]. The RNA part of the snRNPs consists mainly of U1, U2, U5 and U4/U6, out of which only U6 is not transcribed by RNA polymerase II and capped by an m^7G-cap. The m_7G-cap is recognized by the cap binding complex (CBC) and transported to the cytoplasm where the snRNA associates with the survival of motor neurons (SMN) complex. When the complex accumulates the maturation events are promoted which ultimately leads to recruitment of methyltransferase Tgs1 that converts the m^7G-cap to the m_3G-cap[91, 92]. Subsequently, the m_3G-Cap is recognized by an adapter protein Snurportin 1 which then binds to importin-β for nuclear relocalization, where the final maturation of the snRNPs occurs[93, 94].

Moreno et al. has reported that use of the m_3G-Cap as a synthetic RNA 5'-end-NLS signal increases the nuclear transport of oligonucleotides as well as of a larger cargo protein[95]. In his study the m_3G-Cap was used as an adaptor for the nuclear transport of a biotin-streptavidin oligonucleotide complex, proving that 2,2,7-trimethylguanosine cap can promote nuclear transport. The splice-switching was also reported to be enhanced by additional active nuclear transport.

2 MODIFYING m_3G-CAPS FOR UNIVERSAL ATTACHMENT TO BIOLOGICAL CARGOS (PAPER I)

Delivering the oligonucleotide to the nucleus is of high importance, particularly with diseases like Duchenne muscular dystrophy (DMD), which have their origin in wrongly spliced pre-mRNA. It is therefore essential that we learn as much as we can about the possible nuclear localization signals.

The 2,2,7-trimethylguanosine cap, mentioned in the previous chapter, seems to be a most promising tool for promotion of the active transport of oligonucleotides from the cytoplasm to the nucleus. For preparation of oligonucleotide based bioconjugates, one could use existing capping methods where 5'-phosphonate is reacted with the activated m_3G-pyrophosphate[95, 96]. Unfortunately, the same approach cannot readily be used in case of other cargos like proteins or PNAs. Also, introducing modifications on the cap itself may cause difficulties for the conjugation.

A more universal system for attachment of caps was necessary. One that would allow us to efficiently prepare the conjugates time and subsequently evaluate under various conditions to find out more about m_3G-Cap properties and structural requirements for transport. We decided to equip the m_3G-Cap analogues with an azide handle to take advantage of the benefits that copper (I) catalyzed cycloaddition can bring. "Click" reactions, described in chapter 1.2, can be carried out in aqueous conditions which are essential for solubility of the triphosphate.

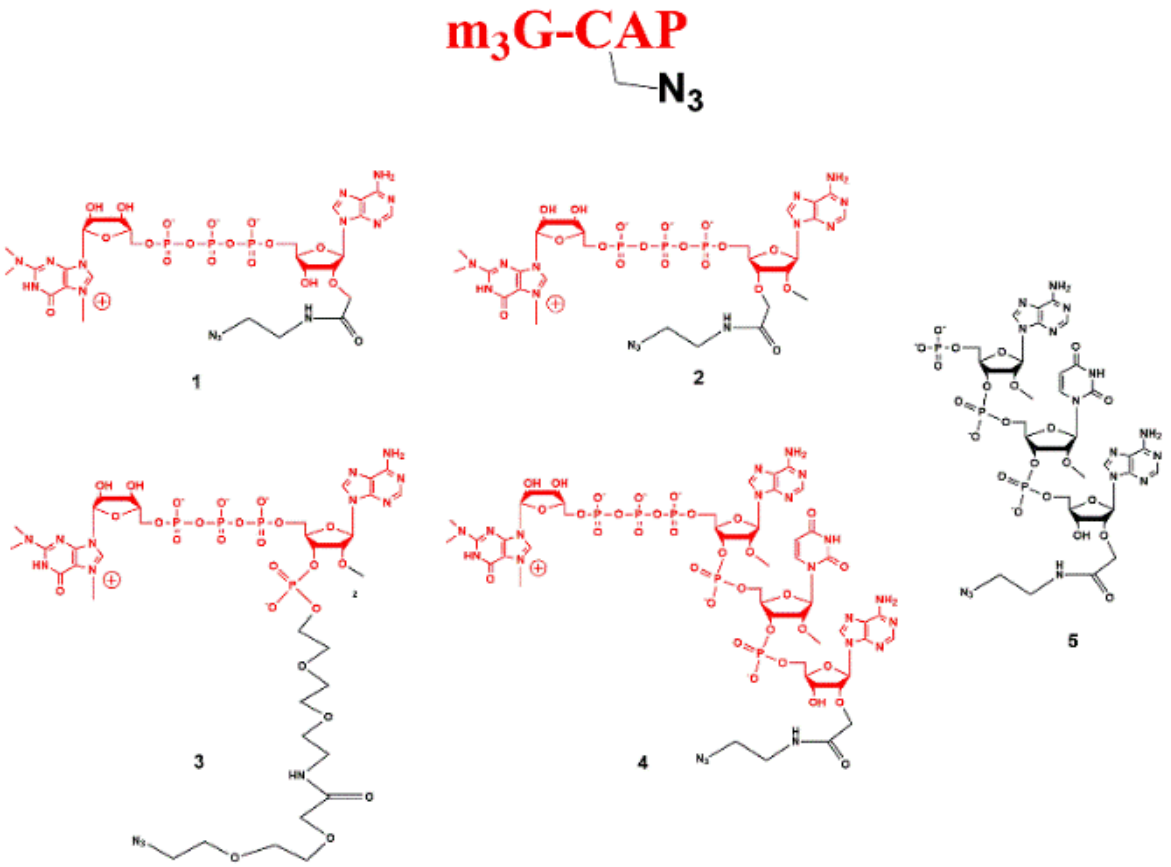


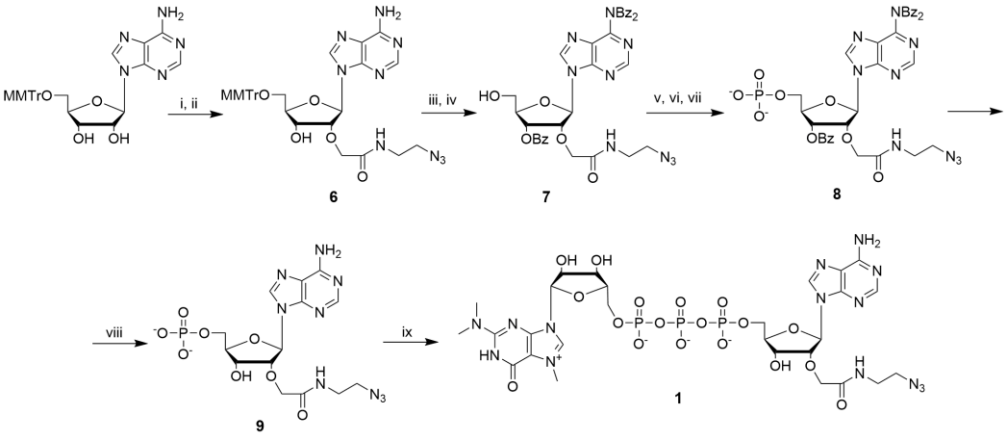
Figure 9: Structures of m_3G-Cap analogues (1-4) and the 5'- phosphorylated AUA trimer (5). Reproduced from Ref. 46 with permission from The Royal Society of Chemistry.

Furthermore, the process is efficient in mild conditions, no protecting groups are needed and the azides and alkynes are themselves inert to biological molecules[97]. It could also be speculated that an additional, artificial linker may affect the binding affinity towards Snurportin 1. Finally, [3+2] cycloaddition is a convenient form of synthesis as it can be easily scaled up for future animal experiments or clinical trials.

We have synthesized four different, ready-to-use m_3G-Cap constructs, each equipped with a functional azide group (Figure 9). The first one (**1**) is the most simplified version, where 2,2,7-trimethylguanosine is connected to adenosine *via* triphosphate bridge. The azide linker is attached to the 2'-position of the adenosine. Structure **2** is prepared with the thought that the 2'-*O*-methyl group can possibly promote the binding to Snurportin 1 and therefore the "clickable" azide group is conjugated at the 3'-position. In this way the cap analogue resembles the native one slightly more. The construct **3**, also based on 2'-*O*-methylated adenosine but in addition it has a 3'-phosphate as a part of a longer linker attachment. Ultimately, the closest to the natural one, m_3G-Cap constructs **4** was synthesized. Here, 2,2,7-trimethylguanosine is connected *via* triphosphate bridge to an AUA sequence, where the 5'-end A and U are 2'*O*-methylated and the azide linker is conjugated to the second A nucleoside at the 2'-*O*-position. We also prepared a control molecule – a 5'phosphorylated AUA trimer (Figure 9, structure 5), which can also be attached to various cargos.

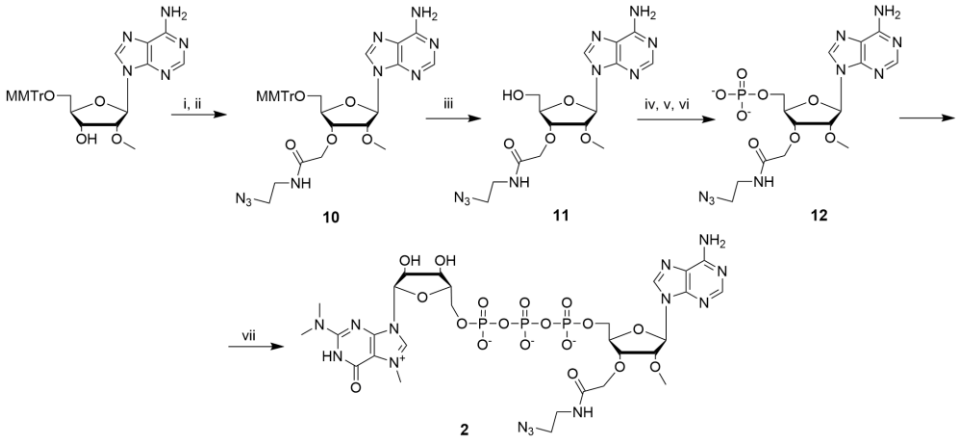
2.1 SYNTHESIS OF "CLICKABLE" m_3G-CAPS

Each of the constructs were prepared by several synthesis steps. Construct **1** was based on 5'-monomethoxytritylated (MMTr) adenosine that was first alkylated at 2'-positionand then subsequently reacted with 2-azidoethylamine to give compound **6**[98] (Scheme 1). Derivative **7** was achieved by base protection with benzoyl group and detritylation. This building block was also later used for the synthesis of m_3G-Cap construct **4**. Next, compound **7** was phosphorylated at the 5'-position (**8**) and the benzoyl group was removed with methanolic ammonia. In the final step compound **9** was reacted with m_3G 5'pyrophosphorylimidazolide to give product **1**.



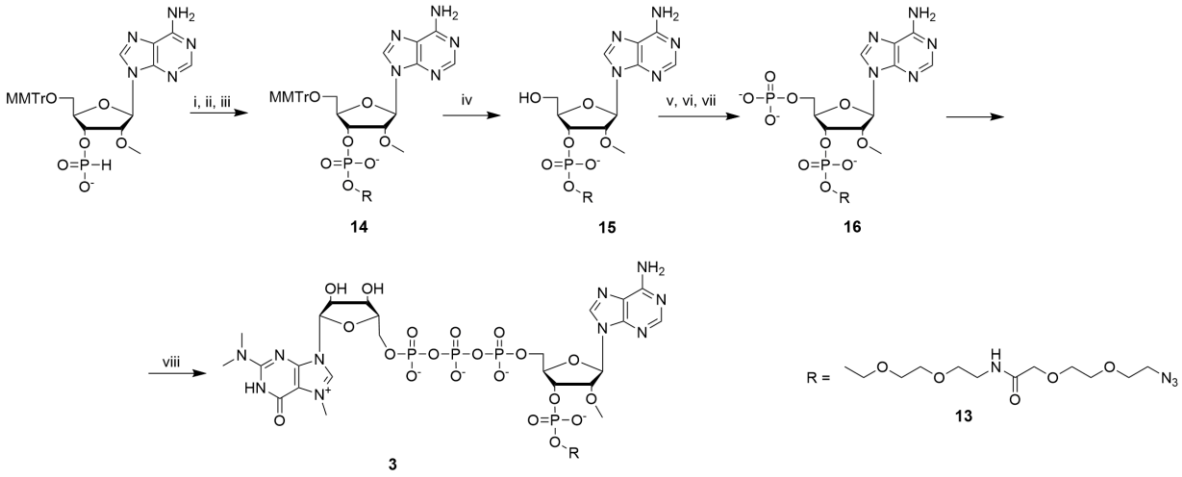
Scheme 1: Synthesis of m3G-Cap construct **1**. i: THF, *t*-BuOK, 20 min, allyl bromoacetate r.t., 6 h, r.t.; ii: dry MeOH, 2-azidoethylamine, 24 h, r.t. iii: benzoyl chloride, pyridine, overnight, r.t.; iv: 80% acetic acid, r.t., 2 h; v: (9H-fluoren-9-yl)methyl H-phosphonate, DCM:Py 9:1, PvCl, r.t., 30 min; vi: I_2, water, r.t., 10 min; vii: MeCN:Et_3N 2:1, overnight, r.t.; viii: MeOH/NH_3 sat., r.t. 50 h; ix: m_3GppIm, $MnCl_2$, N-methylmorpholine x HCl pH 7, 30 °C, 7 days; Adapted from Ref. 46 with permission from The Royal Society of Chemistry.

Scheme 2 illustrates preparation of m_3G-Cap structure **2** which starts with alkylation and aminolysis of 5'-*O*-MMTr 2'-*O*-methylated adenosine to give **10**. After the MMTr group was removed compound **11** was 5'-phosphorylated to give **12** and then m_3G-Capped, in the same matter as **1**, giving compound **2**.



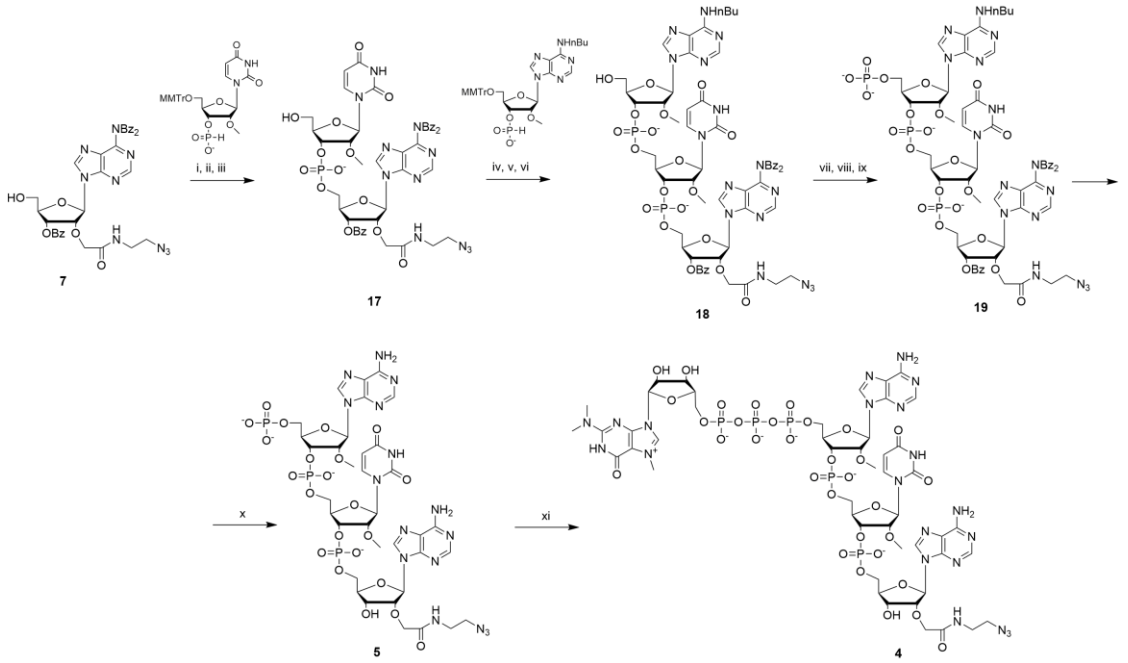
Scheme 2: Synthesis of m3G-Cap construct **2**. i: THF, *t*-BuOK, 20 min, allyl bromoacetate r.t., 6 h, r.t.; ii: dry MeOH, 2-azidoethylamine, 24 h, r.t. iii: 80% acetic acid, r.t., 2 h; iv: (9H-fluoren-9-yl)methyl H-phosphonate, DCM:Py 9:1, PvCl, r.t., 30 min; v: I_2, , water, r.t., 10 min; vi: MeCN:Et_3N 2:1, overnight, vii: m_3GppIm, $MnCl_2$, N-methylmorpholine x HCl pH 7, 30 °C, 7 days; Reproduced from Ref. 46 with permission from The Royal Society of Chemistry.

Synthesis of analogue **3** is preceded by preparation of azidoalcohol **13**, which was then coupled to 5'-*O*-MMTr-2'-*O*-methyladenosine 3'-H-phosphonate and oxidized to give compound **14** (Scheme 3). **14** is then consecutively detritylated to **15**, 5'-phosphorylated to **16** and capped resulting in product **3**.



Scheme 3: Synthesis of m_3G-Cap construct **3**. i: DCM : Py 9 : 1, PvCl, 5 min; ii: compound 13, 20 min; iii: I_2, water, r.t., 5 min; iv: 80% acetic acid, r.t., overnight; v: (9H-fluoren-9-yl)methyl H-phosphonate, DCM : Py 9 : 1, PvCl, r.t., 40 min; vi: I_2, water, r.t., 10 min; vii: MeCN : Et_3N 2 : 1, overnight, r.t.; viii: m_3GppIm, $MnCl_2$, NMM x HCl pH 7, 30 °C, 7 days; Reproduced from Ref. 46 with permission from The Royal Society of Chemistry.

The most complex m_3G-Cap structure **4** required initial preparation of the AUA trinucleotide for which we used H-phosphonate chemistry[99-101]. This means that compound **7** was fist couples to the previously prepared[102, 103], 5'-*O*-MMTr 3'-H-phosphonate of 2'-*O*-methyluridine and then *N'*-butyryl-5'-*O*-MMTr-2'-*O*-methyl adenosine 3'-H-phosphonate. Next, the trinucleotide **18** was 5'-phosphorylated to give **19** and deprotected with ammonia to "clickable" control substance **5**, which after capping resulted in m_3G-Cap structure **4**. A more detailed description of different constructs synthesis is presented in Paper I.



Scheme 4: Synthesis of m_3G-Cap construct **4** and control **5**. i: DCM:Py 9:1, PvCl, 10 min; ii: I_2, water, r.t., 5 min; iii: 80% acetic acid, r.t., overnight; iv: DCM:Py 9:1, PvCl, 30min; v: I_2, water, r.t., 10 min; vi: 80% acetic acid, r.t., overnight; vii: (9H-fluoren-9-yl)methyl H-phosphonate, DCM:Py 9:1, PvCl, r.t., 40 min; viii: I_2, water, r.t., 10 min; ix: MeCN:Et_3N 2:1, overnight, r.t.; x: NH_3 aq. sat., r.t., overnight; xi: m_3GppIm, $MnCl_2$, NMM x HCl pH 7, 30 °C, 7 days; Reproduced from Ref. 46 with permission from The Royal Society of Chemistry.

2.2 FUNCTIONALIZATION OF OLIGONUCLEOTIDE FOR CONJUGATION OF "CLICKABLE" CAPS

At this point our cap analogues could be conjugated to various molecules by use of "click chemistry". We were, primarily interested in conjugation to splice-switching oligonucleotides. The particular sequence has been used for splice correction in the HeLa-Luc 705 reporter cell line, containing a luciferase reporter construct, which has β-globulin 705 intron inserted into the luciferase ORF[104].

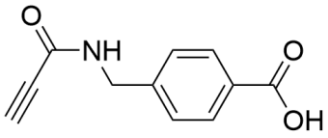


Figure 11: Triple bond donor PATA.

To allow the "click" reaction we needed to equip the ONs with the correct compatible functional group. From our previous studies, involving [3+2] cycloaddition, we learned that low concentration of reacting biomolecules requires a more active triple bond functionality to achieve sufficient conversion[58]. Therefore use of the (*N*-propynoylamino)-*p*-toluic acid (PATA) linker that possesses an electron-withdrawing carbonyl group adjacent to the triple bond would give a substantial advantage when conjugated to the ON (Figure 10).

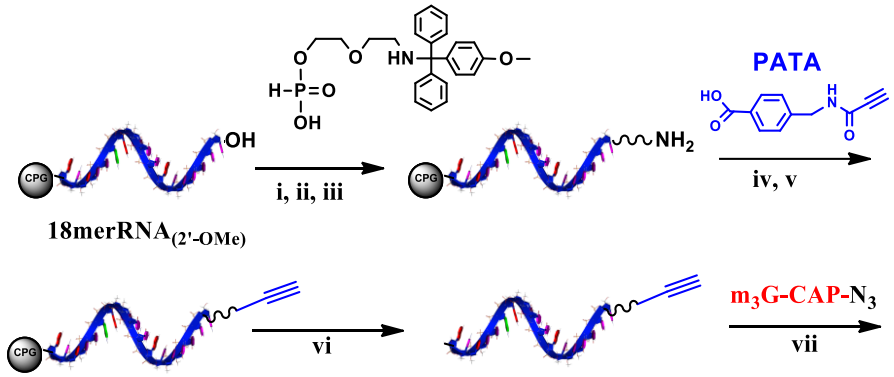


Figure 10: Schematic representation of ON-m$_3$G-Cap bioconjugates. **i**: 2-(*N*-(4-monomethoxytrityl)aminoethoxy)ethyl H-phosphonate, Py, PvCl, 20 min, r.t.; **ii**: I2, water, Py; **iii**: 2% DCA, DCM, 2 min; **iv**: pre-activation of (*N*-Propynoylamino)-*p*-toluic acid (PATA), HBTU, NMM, DMF, 0.5 h, r.t., **v**: addition of activated PATA to solid-supported ON, 2 h; **vi**: MeOH/NH$_3$ sat., 18 h, r.t., **vii**: "click": CuI, DIPEA, overnight, water/DMSO 9/1; Reproduced from Ref. 46 with permission from The Royal Society of Chemistry.

Figure 11 schematically presents the stepwise synthesis of ONs conjugated to m$_3$G-Cap structures. Functionalization of oligonucleotide with an alkyne linker, was performed on solid support, in a two-step procedure. First commercially available 2'-*O*-methyl-modified ON was 5'-detritylated and then the 5'-aminolinker H-phosphonate[58] was attached. Next, the 5'-amino linker was detritylated to enable the conjugation with pre-activated PATA (activation with HBTU and NMM in DMF for 30 min). Finally, the ON with the linked triple bond donor is deprotected and cleaved from support using methanolic ammonia at r.t., overnight.

Theoretically, the "click" itself could also be carried out as a subsequent step on the solid support, before ammonia treatment but since, we, at the time, were not sure about the stability of the caps under these basic conditions we decided to perform this reaction in solution. For our conjugation we used crude alkyne ON, as PATA was observed to undergo partial degradation in the triethylammonium acetate buffer used for the HPLC purification. It is important to mention that reaction between functionalized cap and modified ON is carried out with an excess of cap. This is because we wanted the ON substrate to be more or less fully consumed, which also simplifies purification, since the product has a retention time close to the substrate oligonucleotide.

2.3 CONCLUSIONS AND FUTURE PROSPECTS

As a result of this study we prepared four different "clickable" m_3G-Cap constructs and we have demonstrated that they can be "clicked" to modified oligonucleotides (Figure 12). These bioconjugates are being evaluated in biological assays but at the time of writing these are yet to be completed.

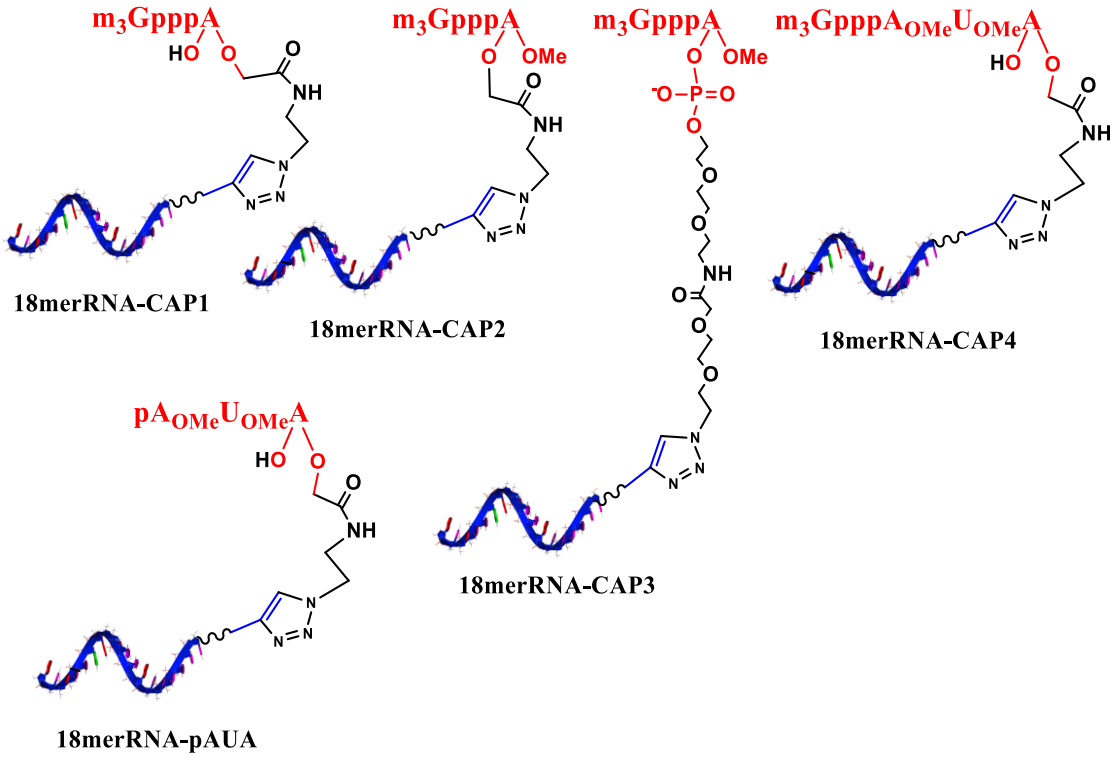


Figure 12: 18merON-m_3G-Cap bioconjugates. Reproduced from Ref. 46 with permission from The Royal Society of Chemistry.

As predicted introduction of modifications to the triphosphate bridge m_3G-Caps significantly improves the stability against enzymatic degradation[105]. The successful preparation of the cap structures inspired also the collaboration with a scientific group from Poland which resulted in preparation of "clickable" m_3G-Caps that contain modifications in the triphosphate bridge[106]. To further explore the structural requirements for effective nuclear delivery the m_3G-Caps were also conjugated to biotin linkers (described in Chapter 3) and evaluated for nuclear uptake of a streptavidin cargo that cannot enter into the nucleus by diffusion (Chapter 4).

3 UNIVERSAL LINKER FOR BIOTINYLATION OF BIOLOGICAL TARGETS (PAPER II)

Biotin plays an important role in modern biotechnology, which is mainly due to its exclusive affinity for fluorescent streptavidin and avidin. The unique complex they create is an excellent tool for studies of cellular transport or visualization of nuclear import[95, 107]. The affinity of the biotin–streptavidin interaction is extremely strong, with a dissociation constant (*Kd*) of 10^{-15} M[108-110]. Biotin fits inside a 'pocket' (Figure 13) of the streptavidin protein, creating an enveloping effect which supplements the binding interaction.

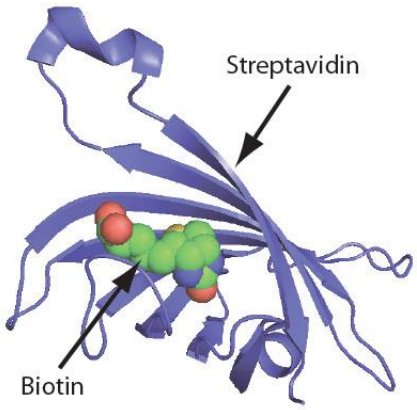


Figure 13: Biotin in streptavidin pocket. From: Weber, P.C.,Ohlendorf, D.H.,Wendoloski, J.J.,Salemme, F.R., Structural origins of high-affinity biotin binding to streptavidin.1989. Science 243 (85-88) . Reprinted with permission from AAAS.

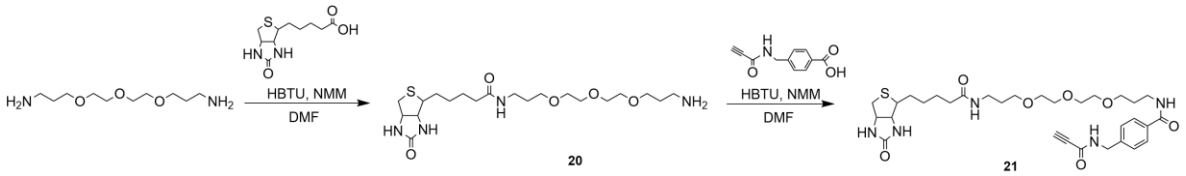
High interest in biotin lead to development of various derivatives that are currently available on the market. Biotin can be attached both to the 5'- and 3'- terminus of ONs as well as internally through a modified thymidine residue (Biotin-dT). Researchers can choose between biotin phosphoramidites, constructs containing two functional biotin groups that help to increase biotin–streptavidin binding affinity or biotin azides, which allow "click" conjugation to alkyne equipped oligonucleotides.

In the approach presented here I am taking advantage of the benefits that "click" chemistry offers, especially since reported methods seemed to be more laborious[111] or less universal[112]. An advantage is also that linkers prepared in this study are equipped with an activated alkyne functional group, achieved by conjugation of the formerly mentioned PATA moiety. The length of the linker was varied in order to test the minimum spacer requirement for the relatively deep streptavidin pocket, especially when larger molecules like oligonucleotides are attached to the biotin.

3.1 SYNTHESIS OF BIOTIN LINKERS

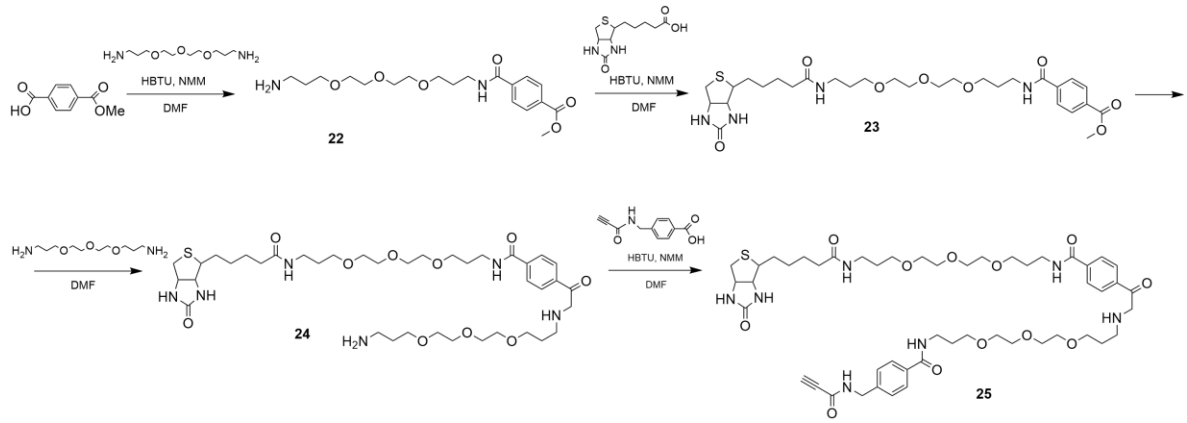
Synthesis of the shorter biotin linker (**21**, SBL) starts with conjugation of pre-activated commercial biotin (activation: HBTU, NMM in DMF, 30 min) with 4,7,3-trioxa-1,13-tridecanediamine. The crude product **20** was purified with column chromatography on silica. It is important to mention that both product and substrate are not UV active, which made isolation somewhat more cumbersome and MS analysis of eluting fractions was used to evaluate the presence of product. After PATA was pre-activated (30 min, in DMF with HBTU

and NMM) **21** was added and reacted for 2 h. The need for high purity of the final linker led to the use of RP-HPLC (C-18 column and triethylammonium acetate buffers, pH 6.5) for the purification.



Scheme 5: Synthesis of short biotin linker (SBL) carrying the activated triple bond donor PATA.

During purification it seemed as if trimethylamine present in the buffer formed an adduct with PATA ($M^+ + Et_3NH$ = 732.98, 10–15%), possibly due to Michael addition[113]. To decrease its formation, the heating generally used during HPLC purifications was avoided and buffers were changed to just water and acetonitrile. This led to complete absence of the adduct.



Scheme 6: Synthesis of long biotin linker (LBL) carrying the activated triple bond donor PATA.

With the second linker the spacer was designed to be not only longer but also to contain a UV-active chromophore which would facilitate the detection of the conjugation products during TLC analysis. Therefore, in the first step of the synthesis monomethyl terephthalate was activated (in the same manner as biotin in case of SBL) and conjugated with commercial 4,7,3-trioxa-1,13-tridecanediamine. After completion of the reaction the product **22** was purified using silica gel column chromatography and reacted with activated biotin (as described for synthesis of compound **21**). Since the product of that reaction (**23**) is a methyl ester, aminolysis with 4,7,3-trioxa-1,13-tridecanediamine was possible. Compound **23** was simply dissolved with the excess of amine and left to react overnight at 44 °C. This led to the expected non-symmetrical product **24** with one free amine which after conjugation with the triple bond donor PATA gave final product – long biotin linker (LBL, **25**). The crude product was pre-purified by silica gel column chromatography but to obtain a high purity of the final product we used RP-HPLC in a similar manner as for the short linker.

3.2 BIOTINYLATION OF OLIGONUCLEOTIDES AND PEPTIDE ON SOLID SUPPORT AND IN SOLUTION

After successful preparation of the linkers, the study of successful "click" reaction in solution and on solid support was initiated. For this purpose conjugation was performed with the biotin

linkers with two oligonucleotides on solid support and with a peptide in solution. The choice of the biologically active substrates was (Table 3):

- commercially obtained 18mer 2'-*O*-methylated ON (the same used in experiments presented in Chapter 2),
- commecially synthesized 14mer, also 2'-*O*-methylated additionally containing two LNA-A building blocks for improvement of hybridization of the oligonucleotide
- a C-myc derivied peptide suggested to have cell penetrating properties and modified with an azido group.

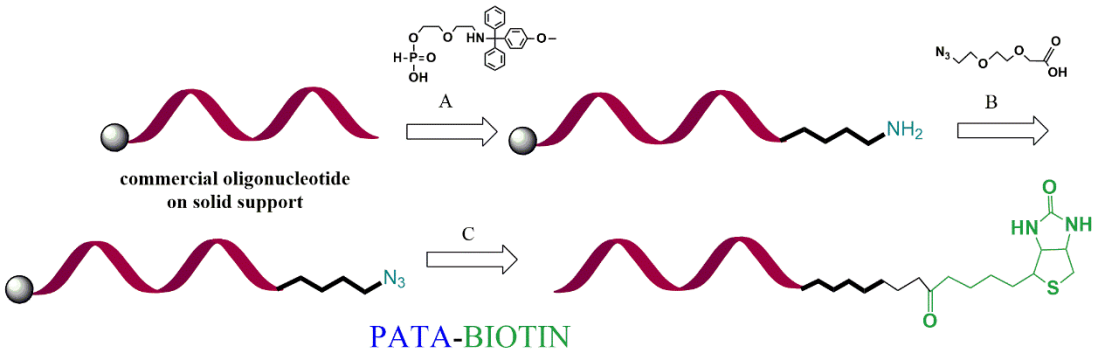


Figure 14: Schematic representation of biotinylation procedure for oligonucleotides on solid support. **A**: Deprotection of 5'-*O*-DMTr followed by reaction with 2-(*N*-(4-monomethoxytrityl)aminoethoxy)ethyl H-phosphonate, Py, PvCl, 20 min, r.t.; I_2, water, Py; 2% DCA, DCM, 2 min; **B**: pre-activation of azido acid, HBTU, NMM, DMF, 0.5 h, r.t., and addition of activated azido acid to solid-supported ON, 2 h; **C**: "click": CuI, DIPEA, overnight, water/DMSO 9/1;

All precursors of these substrates were purchased still attached to the solid support and the process of functionalizing them into "clickable" molecules is presented in Figure 14 and 15 respectively.

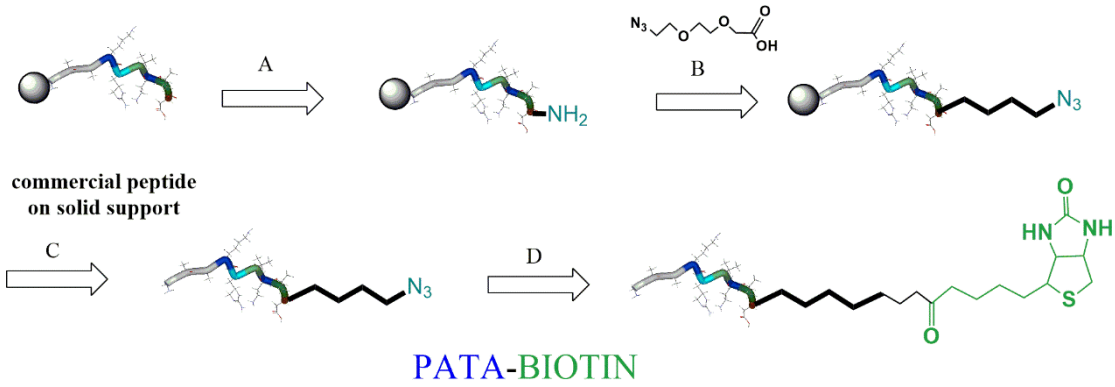


Figure 15: Schematic representation of biotinylation procedure for peptides. **A**: Deprotection of *N*-terminal Fmoc protecting group (20% piperidine in DMF); **B**: reaction with 2-(2-azidoethoxy)ethoxyacetic acid (pre-activated with HBTU and NMM in DMF, 0.5 h, r.t.), DMF, 2 h, r.t.; **C**: 90% TFA, 4% TIS, 4% H2O, 2% 3,5-dioxo-1,8-octanedithiol, 4 h, r.t.; **D**: "click": CuI, DIPEA, water/DMSO 9/1, overnight.

Biotinylation of ONs relied on stepwise conjugation starting with attachment of an aminolinker to the 5'position of the ON. Next, the MMTr group was removed from the linker under mild acidic conditions and the unprotected amino group was conjugated with pre-activated 2-(2-azidoethoxy)ethoxyacetic acid. Finally, CuI catalyzed Hüisgen dipolar [3+2]-cycloadditions with biotin linkers containing the triple bond donor were carried out. This was followed by ammonia treatment to remove the complete bioconjugates from support. The high degree of

conversion, as evidenced by HPLC analysis of the desired crude products, is presented in Figure **16**.

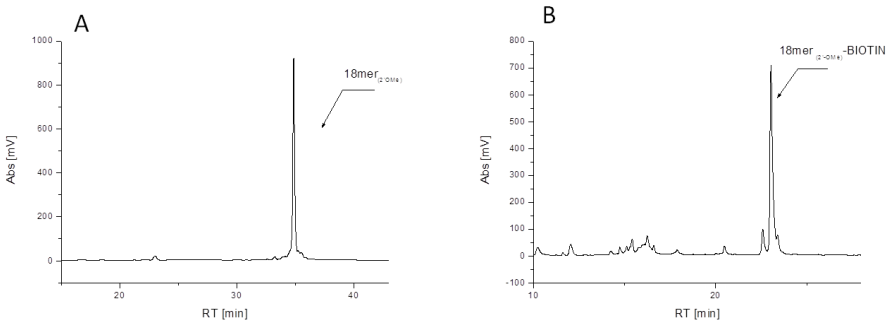


Figure 16: HPLC chromatograms of crude products: A) crude 18mer (2'-OMe) ON, B) crude reaction mixture of the synthesis of 18mer (2'-OMe)-BIOTIN conjugate.

As mentioned, conjugation of biotin linkers to a peptide was accomplished in solution after the peptide substrate was modified with an azido group on solid support[48, 58]. The procedure of converting commercially purchased molecules into "clickable" ones is quite straightforward. It requires the solid supported peptide to be first deprotected at the N-terminus and then conjugated with pre-activated 2-(2-azidoethoxy)ethoxyacetic acid. The cleavage from support was performed using standard conditions with trifluoroacetic acid (TFA) cocktail and purified with HPLC. This is in many cases desired but it is likely that the crude material can also be used directly. The peptide was then readily converted into a biotin conjugate using copper-catalyzed cycloaddition.

Substrate	Conditions	Biotin Linker	Product MS calc/found
18merRNA$_{(2'\text{-OMe})}$ oligonucleotide 5'-CCUCUUACCUCAGUUACA-3'	Solid-Phase	SBL	6796/6798
14merRNA$_{(2'\text{-OMe/LNA})}$ oligonucleotide 5'-AAAUGU***A***ACUG***A***GG-3'	Solid-Phase	LBL	6031/6032
Azido- (C-myc) N_3-L-GAAKRVKLD	Solution Phase	LBL	2111/2112

Table 3: Summary of substrates and products used for preparation of biotin linkers. SBL- short biotin linker, LBL- long biotin linker, ***A***- LNA-A building block.

3.3 CONCLUSION AND FUTURE PROSPECTS

New tools for visualization of transport are always in demand, especially since they can expand the scope of possible applications. Having more options to choose from when labeling biologically active molecules may only be to our benefit. Many biotin tags are already available on the market. However, linkers prepared in this project give alternatives for molecules with properties that restrict them to carry only azide functional groups. Arming biotin with a new

activated click tag provides an additional universal method for scanning potential therapeutics and development of novel versatile technologies.

An example of successful application of the synthesized linkers is presented in the following chapter where labeling of bioactive macromolecules (m_3G-Cap derivatives) allow us to learn more about their properties.

4 INVESTIGATING STRUCTURAL REQUIREMENTS FOR m_3G-CAP PROMOTED NUCLEAR DELIVERY (PAPER III)

In the previous studies complementary building blocks were prepared. These can be conjugated together in order to assess biological function. Biotin linkers are the excellent tools for labeling and observing the transport of a meaningful cargo. The m_3G-Cap derivatives, on the other hand, are promising devices for improved nuclear delivery and addition of a fluorescent label enables visualization of the action. Putting these two pieces of the puzzle together creates a useful bioconjugate for evaluation of minimum structural requirements for retained nuclear transport ability *in vitro*. This information can help us to improve therapeutic effects at lower doses by achieving higher nuclear concentrations *via* active transport.

In addition to the m_3G-Cap analogues presented in chapter 2, clickable caps prepared in collaboration[106], that contain modifications of the triphosphate bridge were evaluated. This kind of alteration is done to improve the resistance of the caps to specific and non–specific enzymatic degradation, like e.g., by NUDIX pyrophosphatase that have recently been reported as a decapping agent for modified RNA[114]. The Dcp2 enzyme targeting primarily monomethylguanosine (m^7G) capped RNA, was also shown to hydrolyze m_3G-capped snRNA, as a part of a quality control mechanism[114, 115]. Several modifications have been demonstrated to protect capped oligonucleotides against enzymatic degradation[106, 116-122]. Examples of these are β-phosphorothioate, α,β- and β,γ-methylenebisphosphonate and α,β- and β,γ-imidodiphosphate groups into the triphosphate bridge. These and other modifications have displayed significant improvements of cellular half-life of exogenously delivered capped-constructs[105, 123]. For instance, the methylene group introduced between α and β phosphonates

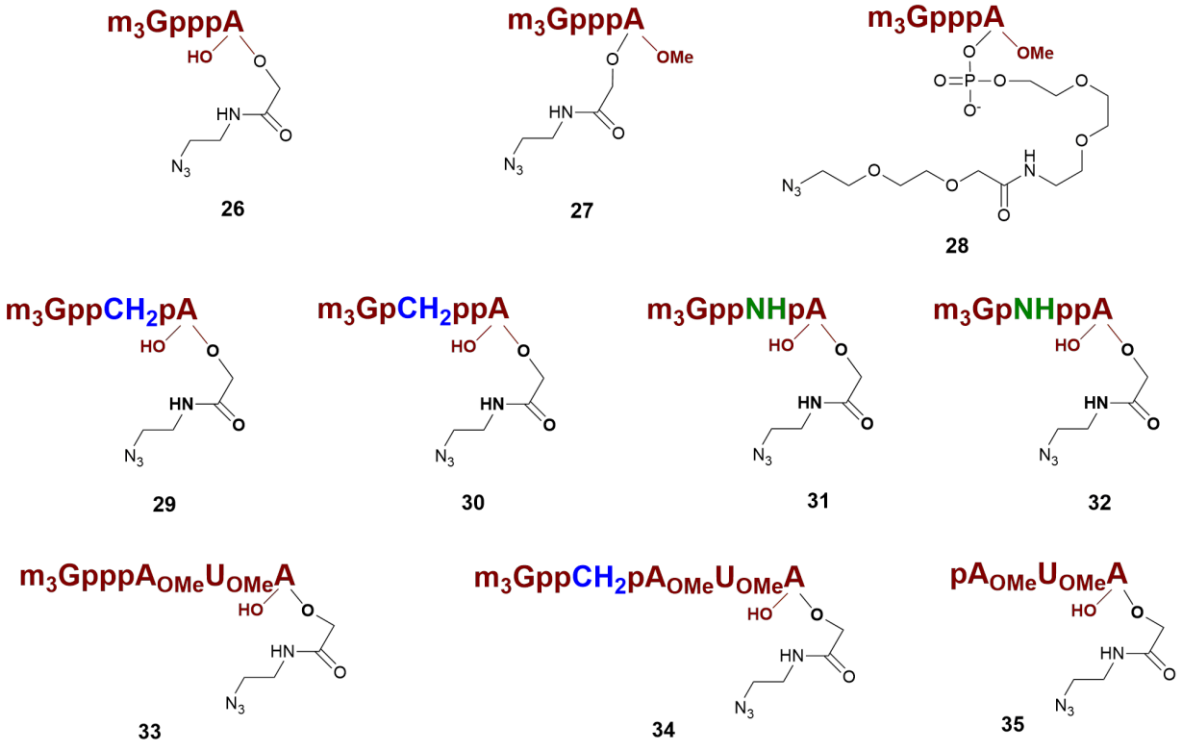


Figure 17: Structures of m_3G-Cap analogues.

in the m_3G-Cap analogue, offers a significant increase of the half-life in 10% serum as well as substantial protection against decapping in the cytosol. Methylene modification is one of the modifications introduced in the triphosphate bridge that was tested below. Another one is the introduction of an imidodiphosphate into the bridge (Figure 17).

The group of biotin tags used for this study has also been expanded by addition of a much longer linker compared to the ones prepared in Paper II. PEG biotin linker (PEGBL) was synthesized by functionalization of commercially purchased Biotin-PEG(23)-NH_2 with pre-activated PATA, giving a "clickable" terminal alkyne group (Figure 18).

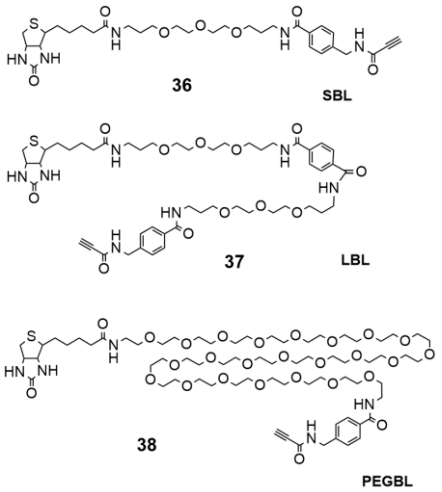


Figure 18: Structures of biotin linkers used in the study.

To prepare the m_3G-Cap–biotin constructs copper-catalyzed cycloaddition was carried out overnight in a solution of tert-butanol/water (1:1). When reaction completion was confirmed by HPLC and MS the crude products were purified by RP-HPLC using a linear gradient of TEAA buffers.

4.1 BIOLOGICAL EVALUATION

The novel bioconjugates were evaluated in cell assays after incubating them with Streptavidin –Alexa 488 (STV-488), which is an excellent tool for studies of active nuclear delivery primarily due to its size. On its own the molecular weight of STV is approximately 56 KDa, and after creating a complex with our biotinylated product the mass rises up to 60-70 KDa, which is clearly above the threshold for passive diffusion through the nuclear pores. The newly prepared complexes were than transfected into U2OS cells by PULS in protein transfection agent and after six hours the cells were analyzed by fluorescent microscopy.

Figure 19 depicts the extensive variety of biotinylated cap structures ranging from the adenosine based ones with different linker length and modifications on the triphosphate bridge (**39**-**46**) to more complex molecules with an AUA sequence (**47**-**49**). Additionally, the non-capped controls are also presented (Figure 20, **50**-**51**). However, only cells treated with compound **47**, prepared from cap derivative 33 and LBL (**37**), displayed a signal from the nucleus in the form of speckles (Figure 22). This would suggest that having the natural

trinucleotide AUA after the triphosphate bridge is the necessary minimum requirement for nuclear transport.

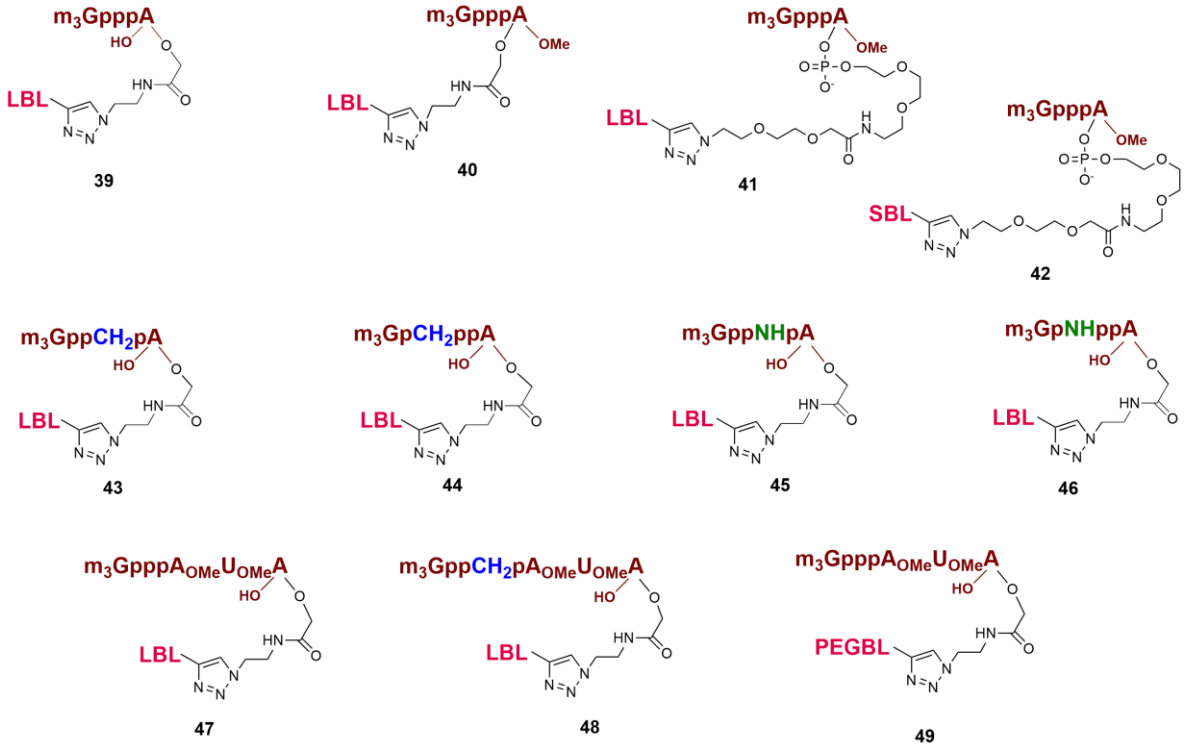


Figure 19: Biotinylated cap derivatives.

Due to this not completely unexpected result it is no surprise that the shorter modified caps also did not promote nuclear uptake. Modified caps raised high expectations after demonstrating improved resistance to enzymatic degradation as well as good binding to snurportin[105, 118]. Most

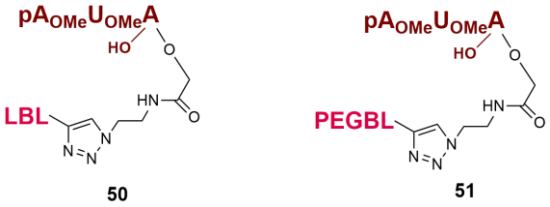
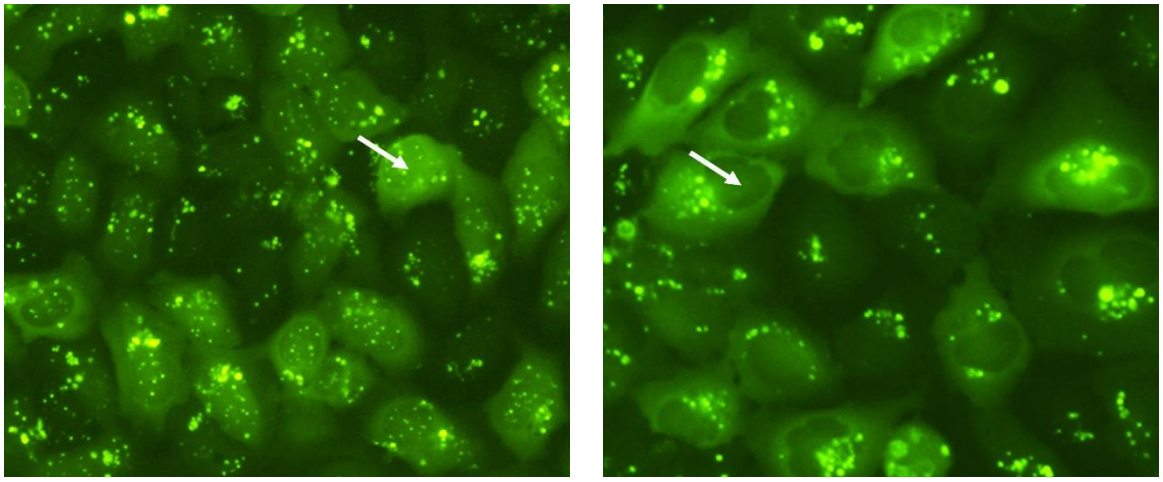


Figure 20: Biotinylated cap derivative controls.

modified caps tested here are apparently too short, since at least an additional trinucleotides is required for nuclear transport. It is then more of a surprise that the methylenemodified cap **48**, which fulfills the trinucleotides requirement, did not reveal any nuclear localization. As even the short caps can bind efficiently to snurportin[105] but still fail to promote nuclear delivery, it is evident that binding to this protein is not the only parameter that matters and it is possible that interaction of the capped cargo-snurportin complex with importin β is quite sensitive to the additional oligonucleotide stretch as well as to structural changes imposed by modifications.

Another interesting observation is that the long biotin linker PEGBL (**38**) did not improve the properties of the bioconjugate. Construct **49** only showed some minor activity. It is possible that its length reduced the availability of the m_3G-cap due to steric hindrance and decrease the efficiency of nuclear transport.



Bioconjugate 47 **Control 50**

Figure 22: Zoomed image of cap-biotin conjugate **47** (Figure 19) and cap-biotin conjugate **50** (Figure 20, control) treated cells. Nuclear region of the cells were marked with a white arrow. Nuclear transport is evaluated by the Alexa-488 signal from the nucleus.

Further on, following the results obtained with fluorescent microscopy we wanted to confirm if the signal obtained with bioconjugate **47** indeed originates from the cell nucleus. For this purpose we ran confocal microscopy experiments, comparing biotin-m_3G-Cap **47** with control **50**. The result is presented in Figure 21 where on the left the confocal microscopy picture is shown and on the right the processed image with the defined nuclear volume and the signal coming from STV-Alexa488 within that volume. The STV complex with m_3G-capped ON conjugate **47** displays a clear signal from the nucleus while the STV complex with control **50** does not. Further analyses indicated that in **47**-SVT some aggregates seem to form within the nucleus near the edge of the volume implying that there could be some interference with the nuclear transport and thus room for further improvement.

Bioconjugate 47

Control 50

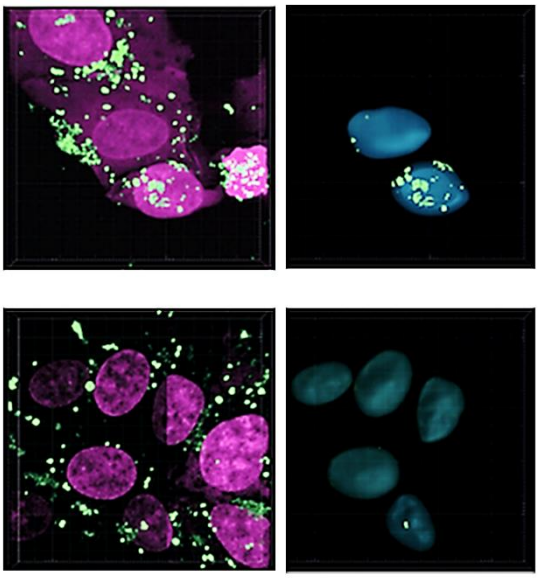


Figure 21: Confocal microcopy analysis of the cells treated with complexes constitutive of STV-Alexa488- and m_3G-Cap – biotin conjugates (**47** and **50**). Left panel shows the confocal image whereas the right panel shows the defined nuclear volume and the STV-Alexa488.

4.2 CONCLUSIONS AND FUTURE PROSPECTS

Preparation of the novel biotin-m_3G-Cap structures not only assured us that the methodology is useful for the creation of bioconjugates, but probably more importantly allowed us to study and understand some of the aspects that need to be considered when using these nuclear localization signals. With the result obtained we know now that the minimum requirement for active nuclear transport is equipping the m_3G-Cap construct with at least an additional trinucleotide. However, we are aware that this story does not finish here and that additional experiments need to be carried out and different construct need to be prepared to fully comprehend the mechanism. We suspect that additional nucleotides added after the trinucleotide will be even more efficient in promoting the nuclear uptake of the cargo. After optimizing the ideal length of the oligonucleotide attached to the cap we should come back to the idea of modifications on the triphosphate bridge and test if their presence can improve the overall process.

Another interesting idea to investigate is attachment of most promising m_3G-Cap derivative **47** to various alternative cargos and observe how well these are transported. For that purpose a multiple linker, as discussed in Chapter 5 could allow the attachment of an m_3G-Cap derivative and a fluorescent dye to the same molecule, would be handy.

When it comes to future prospects for m_3G-Cap derivatives, strictly from a synthesis point of view, there are few aspects that might be worth considering. The idea of automation of cap attachment would be of great benefit as preparing caps on solid support could lead to straight assembly during oligonucleotide synthesis. The optimized methodology would give us a chance to prepare more constructs in a shorter time, which in turn helps in scanning of more potential conjugates resulting in learning more about this NLS.

Furthermore, speeding up the overall synthesis would be very advantageous, not least to allow the preparation of constructs in shorter time. To try this, one could use microwave irradiation in the “click” reaction leading to cap constructs, of course under conditions where caps are not being degraded. It is likely that successful development in this direction will significantly shorten preparation of “clickable” conjugates.

5 MULTIPLE FUNCTIONALIZATION LINKER (MFL) ENABLING ATTACHMENT OF SEVERAL DIFFERENT ENTITIES TO ONS (UNPUBLISHED RESULTS)

The possibilities and combinations for preparation of ON therapeutics seem to be endless, however the question of how to deliver the oligonucleotide to the site of action still does not have a clear answer. We seem to have almost all tools in our hands, like the therapeutic ON, entities that will deliver it into the tissue/cell and/or nucleus as well as potential signals for tissue specificity but to put all these functionalities together is a most demanding task. This is where the idea of a universal multiple functionalization linker (MFL) emerged.

One thing that seemed clear while designing the linker was to equip it with a functional group that would allow the use of "click" conjugation of desired entities. This methodology has already proven its efficiency several times before. Additionally, not only we have previously synthesized "clickable" derivatives for this purpose as well as in house methods to prepare azides and triple bond donors, but there are also many ready-to-click biologically active molecules that are commercially available. With that in mind, literature was browsed to learn what modifications have been studied to introduce the azido and alkyne functional group to oligonucleotides. A large variety can be found, however most of them focus on alteration of the nucleotide itself, which can later serve as building blocks during oligonucleotides synthesis[124, 125]. As presented in Figure 23 different positions are used to introduce the required functional groups. Among them we can distinguish introduction of triple bond or azide at the 2', 3'and 5'–positions of the nucleoside[51, 126, 127]. Modifications are also often introduced at the nucleobase[128-131]. A different approach is preparation of scaffolds based either on amino acids/cyclic peptides[132, 133] or carbohydrates derivatives[134].

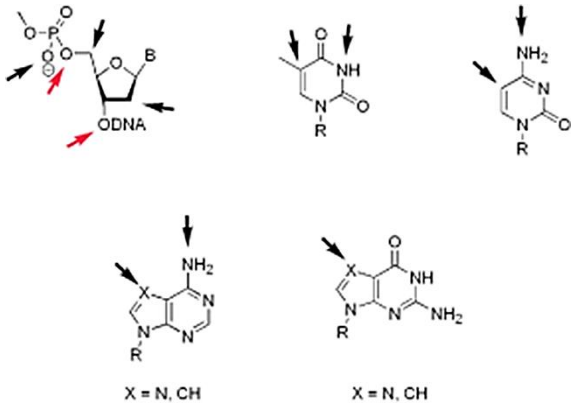


Figure 23: Overview of different positions used for the attachment of azides or alkynes. Red arrows - attachment point for terminal azide and alkyne residues only. B - nucleobase. From Gramlich P. M., Wirges C. T., Manetto A., Carell T. Postsynthetic DNA modification through the copper-catalyzed azide-alkyne cycloaddition reaction.; Angew Chem Int Ed Engl. 2008;47(44):8350-8, provided by John Wiley and Sons.

Two of the articles, in particular drew my attention. Bouillon et al. have presented a method where solid-supported DNA was functionalized with three H-phosphonate monoester units[51]. These where then converted to alkyne moieties and clicked with galactosyl azide using microwave irradiation (MW) to shorten the reaction time[135]. The method is suitable for multi-labeling of oligonucleotides with the same tag for example to enhance the visualization or

uptake (when several units are attached). A substantial advantage here is the use of MW that improved not only the reaction time but also the overall yield.

A most interesting approach was also presented by Carell and his group[130, 136]. They attached alkynyl side chains to the 5-position of pyrimidines or to the 7-position of 7-deazapurines to enable versatile labeling of DNA. As the oligonucleotides are synthesized the modified nucleotides are introduced and some of them carry a protecting groups like trimethylsilyl (TMS) or tri-*iso*-propylsilyl (TIPS) on the alkyne. After the synthesis is completed the non-protected triple bonds can be clicked directly on support followed by removal of TMS group with mild acidic conditions or during cleavage from support. Once the second label is clicked on the oligonucleotide the TIPS group is displaced in solution to allow the third cycloaddition reaction. Although, this methodology has some limitations[137, 138], it is a substantial breakthrough and DNA constructs with three different entities were prepared with yields of about 50%. A year after Valverde et al. screened a range of commonly used alkyne protecting silyl groups to check their compatibility with iterative copper catalyzed 1,3-cycloaddition. Their results suggest that TIPS/triethylsilyl (TES) or *tert*-butyldiphenylsilyl (DPS)/TES protecting schemes are effective for multiple successive "click" reactions[133].

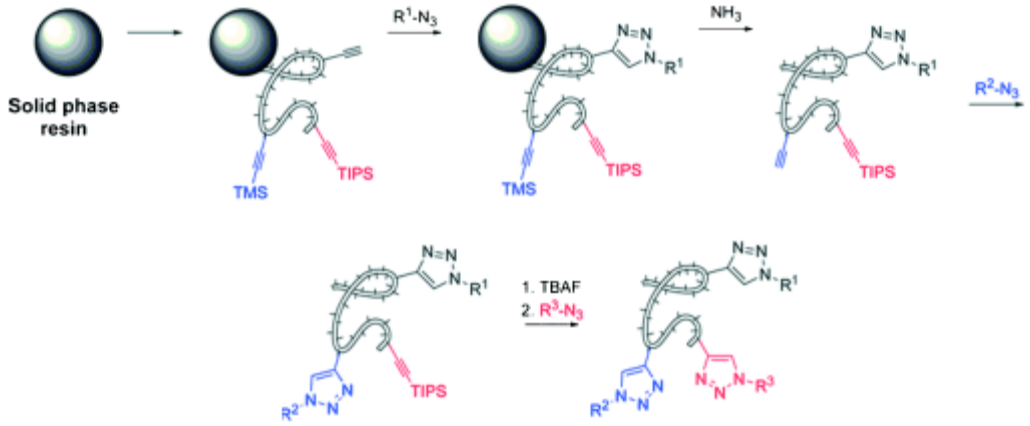


Figure 24: Sequential modification of DNA proposed by Carell et al. . From Gramlich P. M., Wirges C. T., Manetto A., Carell T. Postsynthetic DNA modification through the copper-catalyzed azide-alkyne cycloaddition reaction.; Angew Chem Int Ed Engl. 2008;47(44):8350-8, provided by John Wiley and Sons.

These two examples are efficient for the attachment of multiple entities to an oligonucleotide and they both have one important feature in common – their "clickable" functional groups or masked precursors are incorporated in the oligonucleotide during ON synthesis on soild support. The only obvious drawback is that incorporation into bases as well as internally into the oligonucleotide sequence may hamper the function of the ON.

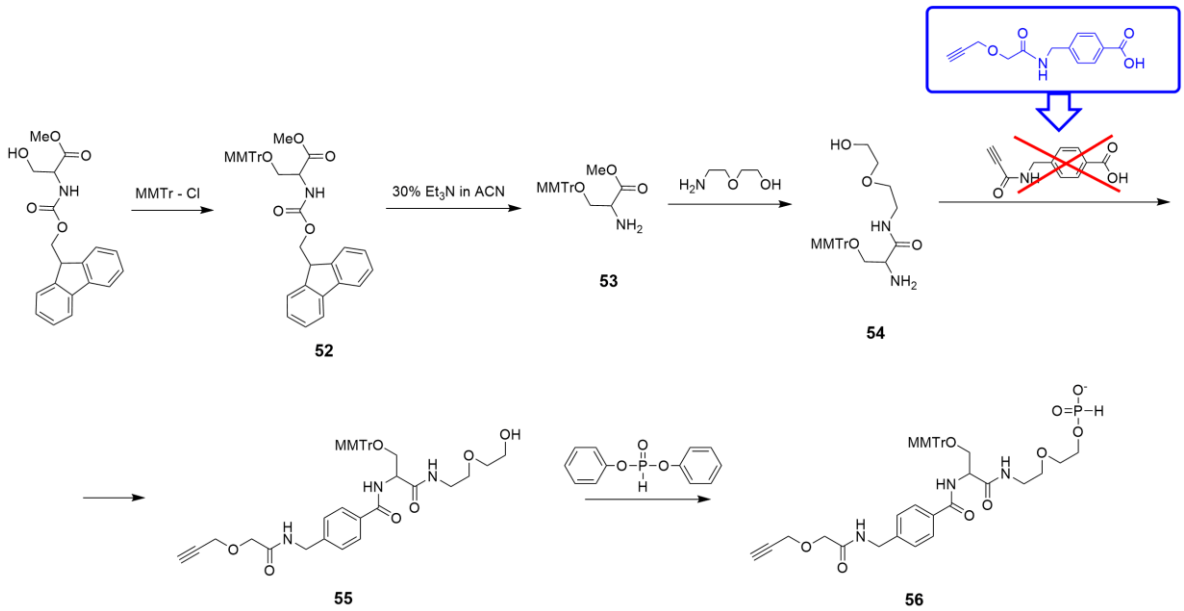
The modification that we wanted to prepare is based on a slightly different approach. Our design was an independent molecule which would be conjugated at the 3' or 5'-terminus to avoid interference with the ON sequence of therapeutic significance.

5.1 DESIGN AND SYNTHESIS OF A MULTIPLE FUNCTIONALIZATION LINKER (MFL)

While planning the structure of our multiple functionalization linker (MFL) we set some goals that should be fulfilled: 1) the linker should possess a clickable functional group to give an

attachment point for efficient conjugation of biologically active entities, 2) straight forward attachment to 5' oligonucleotide position, preferably *via* H-phosphonate or phosphoramidite chemistry and 3) an additional functionality where, if desired, another linker unit can be attached. As we also wanted to carry out the entire process of multiple conjugation on an oligonucleotide that is attached to solid support, it was important that the linker's chemistry is compatible with that.

As starting point for synthesis of the linker Fmoc (fluorenylmethyloxycarbonyl) serine methyl ester was chosen. The remaining free OH group was protected with MMTr and the crude product **52** was purified by flash column chromatography. The Fmoc group was then removed to give **53** and aminolysis of the methylester was performed with 2-(2-aminoethoxy)ethanol, in absence of solvent and at 45 ºC. After purification, **54** was reacted with pre-activated triple bond donor PATA resulting in a product with the correct mass. Although, the phosphonylation reaction with salicyl chlorophosphite was also successful, the column purified final product gave a mixture due side reactions of the added PATA group, most likely related to what we observed before with this activated triple bond.

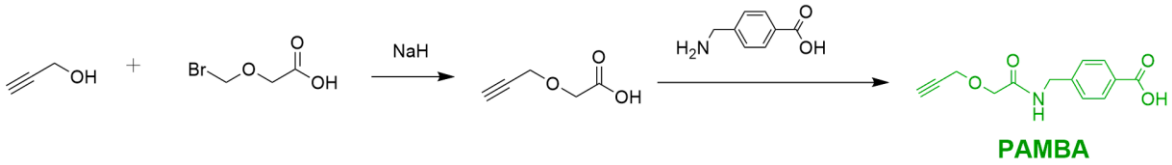


Scheme 7: Synthesis of multiple functionalization linker (MFL) using PAMBA.

p-(*N*-propynoylamino)toluic acid (PATA) was our linker of choice when preparing conjugates requiring triple bond donor. Thanks to its unique structure it allows the "click" reaction to be efficient also at low concentration, where standard triple bonds gave virtually no results. It has a handle for attachment to oligonucleotides carrying aminolinkers and is UV visible, which is convenient upon preparation and purification. The synthesis is also very straightforward. In just one step reacting propynoic acid and aminomethylbenzoic acid we obtain the final product PATA in high purity. An additional advantage is that the starting materials are inexpensive, which means that the cost for the reagent is quite low. The linker can be made in a relatively large scale (grams, rt and column chromatography) and is stable upon storage[58]. Unfortunately, the reactivity of PATA has also caused some problems. In many cases when base has been present we have observed side reaction, which is most probably due to Michael addition to the

conjugated triple bond. These problems were observed on several occasions, e.g., during HPLC purification with use of TEAA buffers or deprotection with aqueous ammonia at 50 °C. The moment this became an obstacle for the synthesis of an MFL we decided to change the structure of PATA, preserving it best features but avoiding the possibility of Michael addition.

The PAMBA linker (4-((2-(prop-2-yn-1-yloxy)acetamido)methyl)benzoic acid) is very similar to PATA but the triple bond is not conjugated to the carbonyl function. The triple bond is somewhat less activated. However, a recent comparison of reaction rates for different alkynes in "click" cycloadditions suggested that electron withdrawal by the oxygen, connected to the propynyl group, should give sufficient reactivity. Before the new molecule was used in the



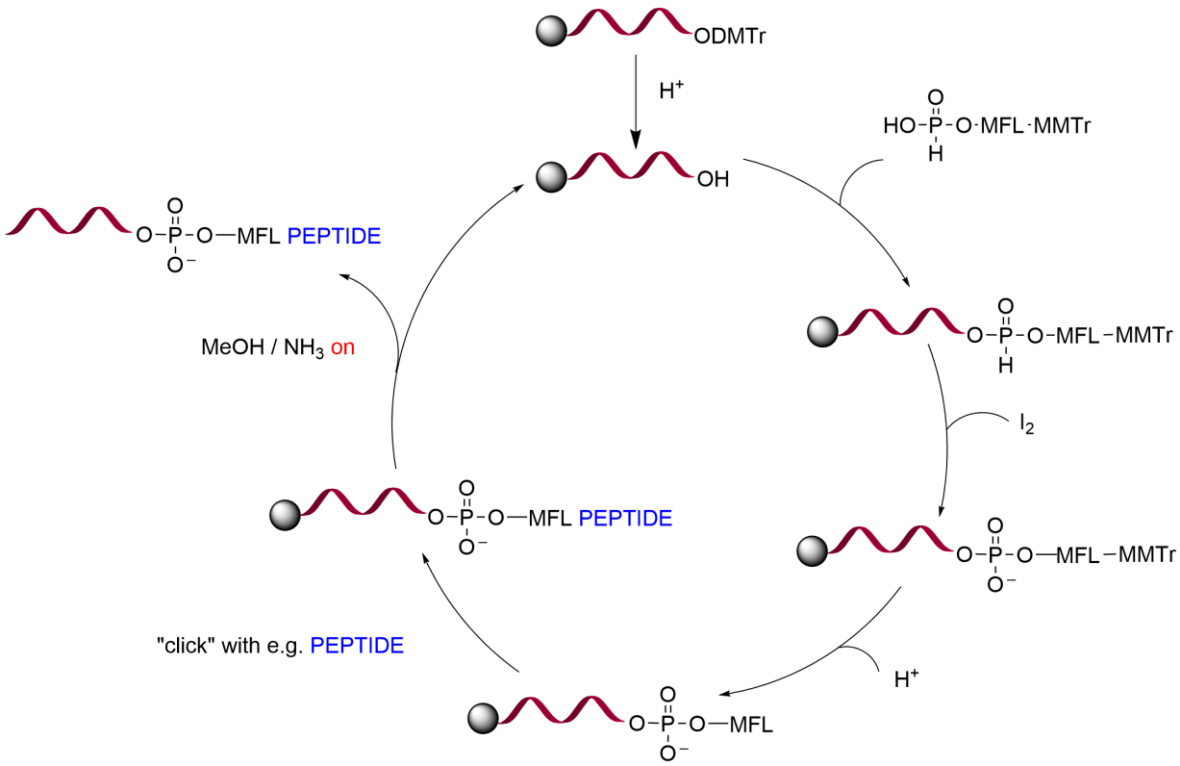
Scheme 8: Synthesis of PAMBA linker.

synthesis of MFL, it was tested under two different conditions: 1) it ability to perform the click reaction, which was successful and 2) its stability in basic conditions, which also gave a positive result. Since both results were positive we proceeded and coupled with compound **54**. After **55** was purified it was reacted with diphenyl phosphite in dry pyridine to give the multiple functionalization linker **56** in 63% yield (Scheme 7).

5.2 CONJUGATION OF LINKER AND "CLICK" REACTION ON SOLID-SUPPORTED OLIGONUCLEOTIDE

Schematic representation of the linker attachment to oligonucleotide and the "click" reaction on support are illustrated in Scheme 9. In a way it is almost a cyclic procedure. The cycle, attachment of the linker unit, "click" reaction, deprotection can be performed multiple times and equip the oligonucleotide with multiple different entities.

The general procedure for synthesis began with placing the solid-supported ON in Eppendorf tube and washing it extensively with methanol (MeOH), acetonitrile (MeCN) and dichloromethane (DCM). To deprotect the ON from the DMTr group, 3,5% DCA solution in DCM was used. The support was then washed repeatedly with DCM and then a mixture of MeCN-pyridine (3:1), to prepare it for the introduction of the MFL. Next, the linker in anhydrous pyridine was added to support followed by 1.5 eq pivaloyl chloride (PvCl) dissolved in anhydrous MeCN (30 mM solution) and the reaction mixture was shaken for 5 min. After this time, the coupling solution was removed and the support was washed with MeCN-pyridine (3:1) to ensure removal of remaining reagents. In the following step the solid-supported crude product was oxidized with iodine in pyridine/water for 15 min. The support was then washed extensively with pyridine/water (9:1), MeCN and DCM. Next the MMTr group, protecting the hydroxyl group of the multiple linker was removed with 3.5% DCA solution in DCM. The support was subsequently washed repeatedly with DCM and MeCN, to prepare it for the "click" reaction. The desired azido-modified molecule (e.g. peptide or fluorescent label) was dissolved in a 1:1 mixture of *t*BuOH:H_2O and added to the support followed by *N*,*N*-



Scheme 9: Preparation of oligonucleotide bioconjugates with versatile biologically active entities using multiple functionalization linker – reaction scheme.

diisopropylethylamine (DIPEA) and copper iodide (CuI) and the reaction was left shaking for 24 h. Next day, the conjugation solution was removed and the support washed with 1:1 tBuOH:H_2O, EDTA solution, MeCN and DCM. Then, depending on the desired product, the final is either cleaved from the support with methanolic ammonia or subjected to another round of the conjugation steps, which first includes addition of another MFL unit.

5.3 MULTI-SUBSTITUTED OLIGONUCLEOTIDE CONJUGATES – SUMMARY OF SUCCESSFULLY PREPARED CONSTRUCTS

Before the first oligonucleotide construct was prepared several optimizations of the linker attachment step were done. To improve the solubility of the compounds the reaction was carried out in MeCN-pyridine (3:1). Initially both MFL and PvCl were dissolved in this mixture but partially due to solubility and partially due to the stability of PvCl this was changed. The MFL was dissolved in one part of pyridine and added to the resin, followed by PvCl in three parts of MeCN. This gave a "cleaner" crude product as analyzed by HPLC and led to an increase in overall yield. In addition, the amount of PvCl used also had an influence on the efficiency of the coupling. The initial use of 5 eq (to ensure dry conditions and complete conversion in a short time) resulted in additional by-products and therefore somewhat suppressed the yield of the desired oligonucleotide conjugate. Reducing the PvCl to 3 eq gave a slight improvement and use of 1.5 eq clearly generated less side products and more desired product.

With these improvements introduced the first multiple-entities-oligonucleotide construct was prepared. Initially, the short non-charged PLG peptide in *N*-acetylated form (this tripeptide also known as MIF was reported to be a blood-brain barrier penetrating peptide[139]) was used as a

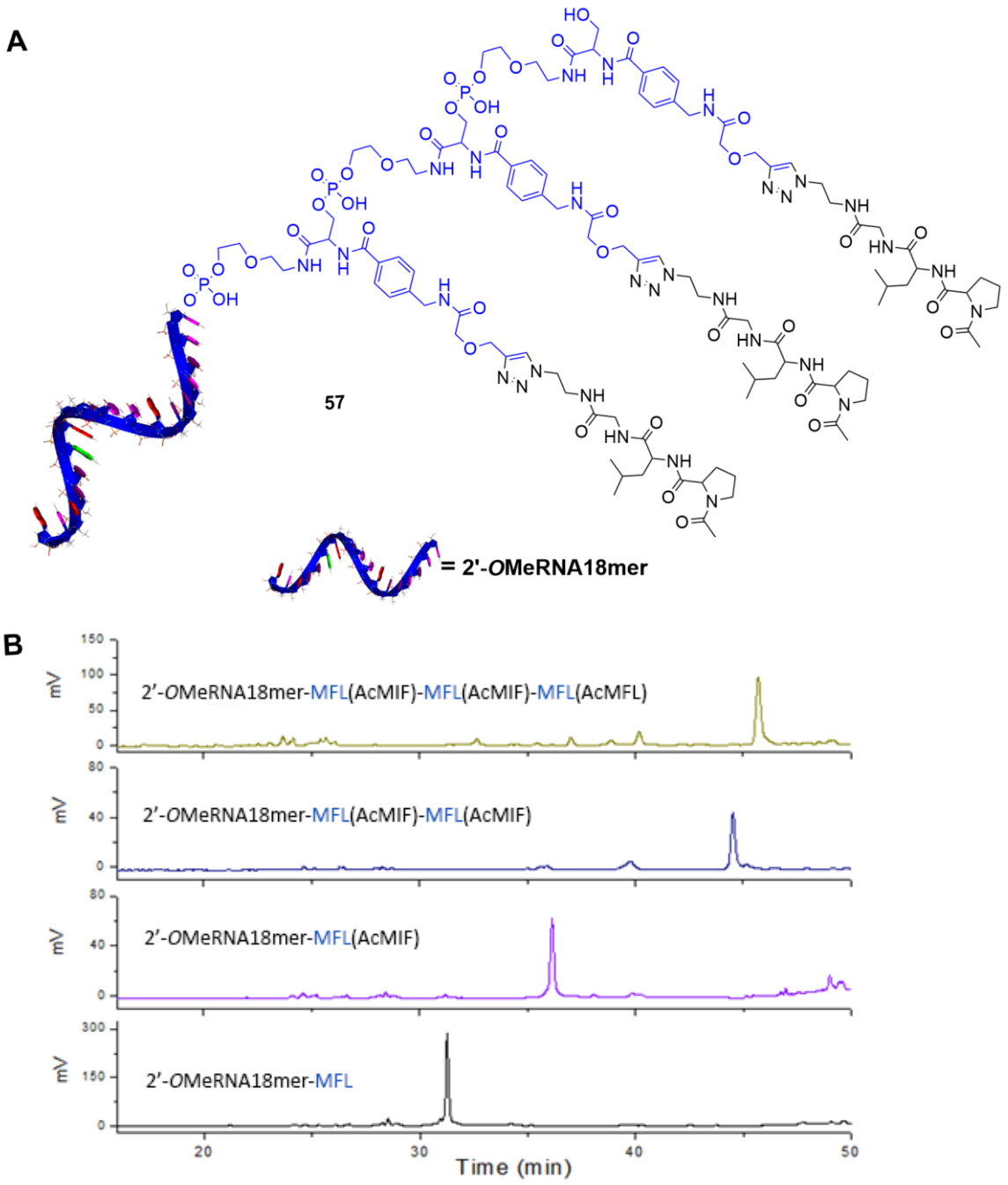


Figure 25: **A**: Structure of product **57** the 2'-OMeRNA18mer equipped with three multiple linkers with AcMIF peptides attached to each linker, **B**: HPLC chromatograms of crude products after different steps towards preparation of product **57**, from bottom to top: ON with only linker attached, ON with one linker with AcMIF attached, ON with two linkers each with an AcMIF attached, ON with three linkers each with AcMIF attached.

model for later reactions. The preparation of the clickable form (*N*-acetylProLeuGlyNHCH$_2$CH$_2$N$_3$ or AcMIF-N$_3$) is achieved in a single coupling of the acetylated peptide with azidoethylamine[58]. The idea behind preparation of a first model conjugate was to observe how the process is develops for each addition and if there are stages that still require some optimization. The first attempt was not as clear due to poor purity of the commercially obtained 18mer 2'-*O*Me oligonucleotide. However, when the solid-supported oligonucleotide was synthesized in our lab (the same sequence as the purchased one) the results were really good. The three units of linkers were attached one by one and each conjugation

step was followed by "click" reaction with AcMIF-N_3 according to the above described general procedure. Figure 25A shows the structure of the final trisconjugated compound (**57**).The mono and bisconjugated ONs were also prepared in a similar fashion and the overall syntheses were remarkably clean as evidenced by HPLC profiles of crude materials shown in Figure 25B. The correct structure of each construct was confirmed by mass spectrometry and it is also clearly

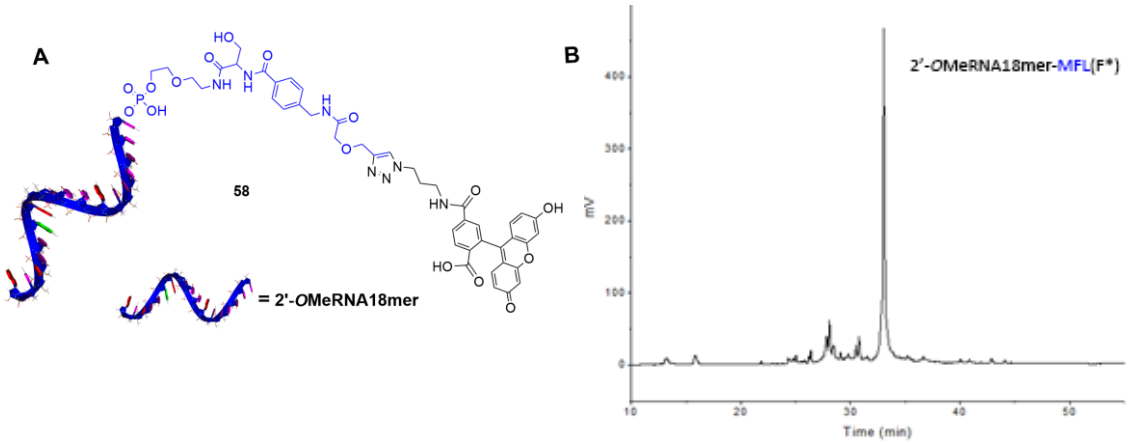


Figure 26: **A**: Structure of product **58** 2'-*O*MeRNA18mer equipped with multiple functionalization linker and fluorescent label, **B**: HPLC chromatogram of product **58**.

visible how the time of retention (t_R) increases for each additional unit.

After confirming that the multiple functionalization linker can fulfil its function the next test was if addition of other entities with different properties and solubility can cause some problems in the developed procedure. Two different constructs based on the same ON were synthesized: 1) with a fluorescein derivative that can serve as a labeling tag (Figure 26) and 2) a 14mer tumor cell-targeting CPP (target: osteosarcoma- U2OS cell line)[140, 141] (Figure 27). As both results were positive and the correct mass of products was confirmed it was encouraging to prepare oligonucleotide bioconjugates with two or three different biologically relevant entities.

Several attempts were done in order to achieve the diverse conjugations which gave knowledge on how to design the synthesis of desired compounds. The conclusions and considerations drawn from these attempts are described later in this chapter but for the moment let's focus on the constructs that were successfully synthesized. First an ON conjugate containing a peptide, a sugar and a fluorescent label (product **60**) was prepared. MFL was coupled to the 18mer 2'-*O*-methyloligoribonucleotide, followed by 1,3-dipolar cycloaddition with the *N*-acetylated peptide MIF azide. Next, another linker unit was attached and "clicked" with an acetylated mannopyranoside equipped with azide handle. Finally, after the third MFL unit is attached the bulky fluorescein azide derivative is "clicked" to result in ON triple conjugate **60** after deprotection.

The second triple conjugate (**61**) was prepared in similar manner. Apart from the AcMIF peptide and sugar derivative it is also equipped with a muscle-homing peptide ASSLNIA (purchased still on resin and equipped with an azide handle by microwave assisted coupling of 2-(2-azidoethoxy)ethoxyacetic acid to the *N*-terminus). This peptide has been shown to display a 9- to 20-fold increase in affinity to muscle cells[142, 143] and is therefore a potential targeting

signal for therapeutics focused on diseases of the heart and skeletal muscles such as muscular dystrophies.

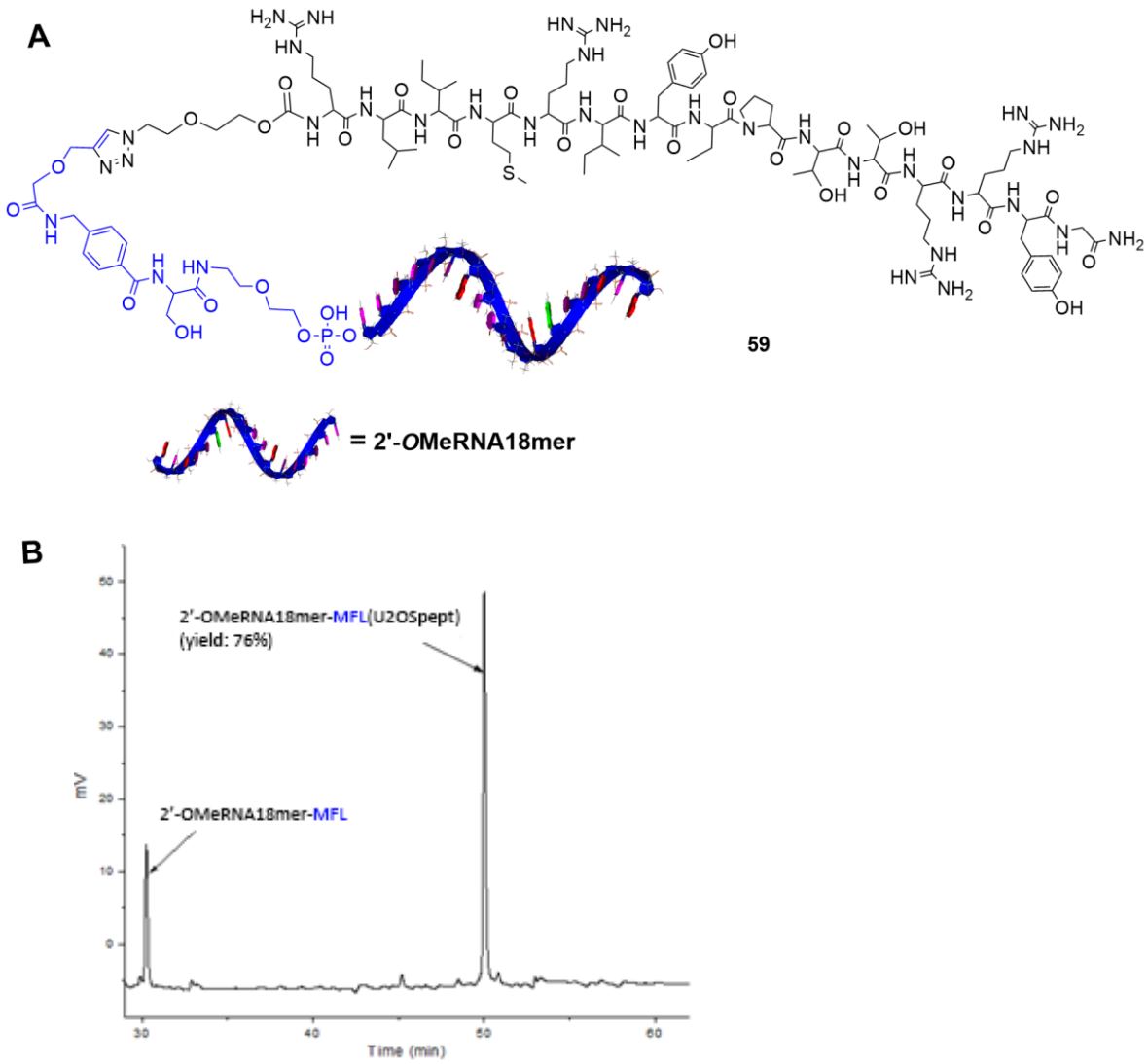


Figure 27: **A**: Structure of product **59** 2'-*O*MeRNA18mer equipped with MFL and a peptide targeting U2OS cell lines, **B**: HPLC chromatogram of crude product **59**.

Figure 30 presents the third oligonucleotide multiconjugate **62**, prepared with help of the multiple functionalization linker. The difference here is that an m_3G-Cap construct is attached in the last step of the synthesis. In this particular case the ON-construct was cleaved off support after the third linker unit was added and the "click" reaction with the Cap was carried out in solution. Product **62** is particularly interesting, since to my knowledge, it is the first presented multiple signal where three different classes of biomolecules (peptide, sugar, oligonucleotide with triphosphate bridge/Cap) were clicked on the same ON scaffold. This conjugate opens the door to the study of various combinations of signals with joint action that have not been tested yet.

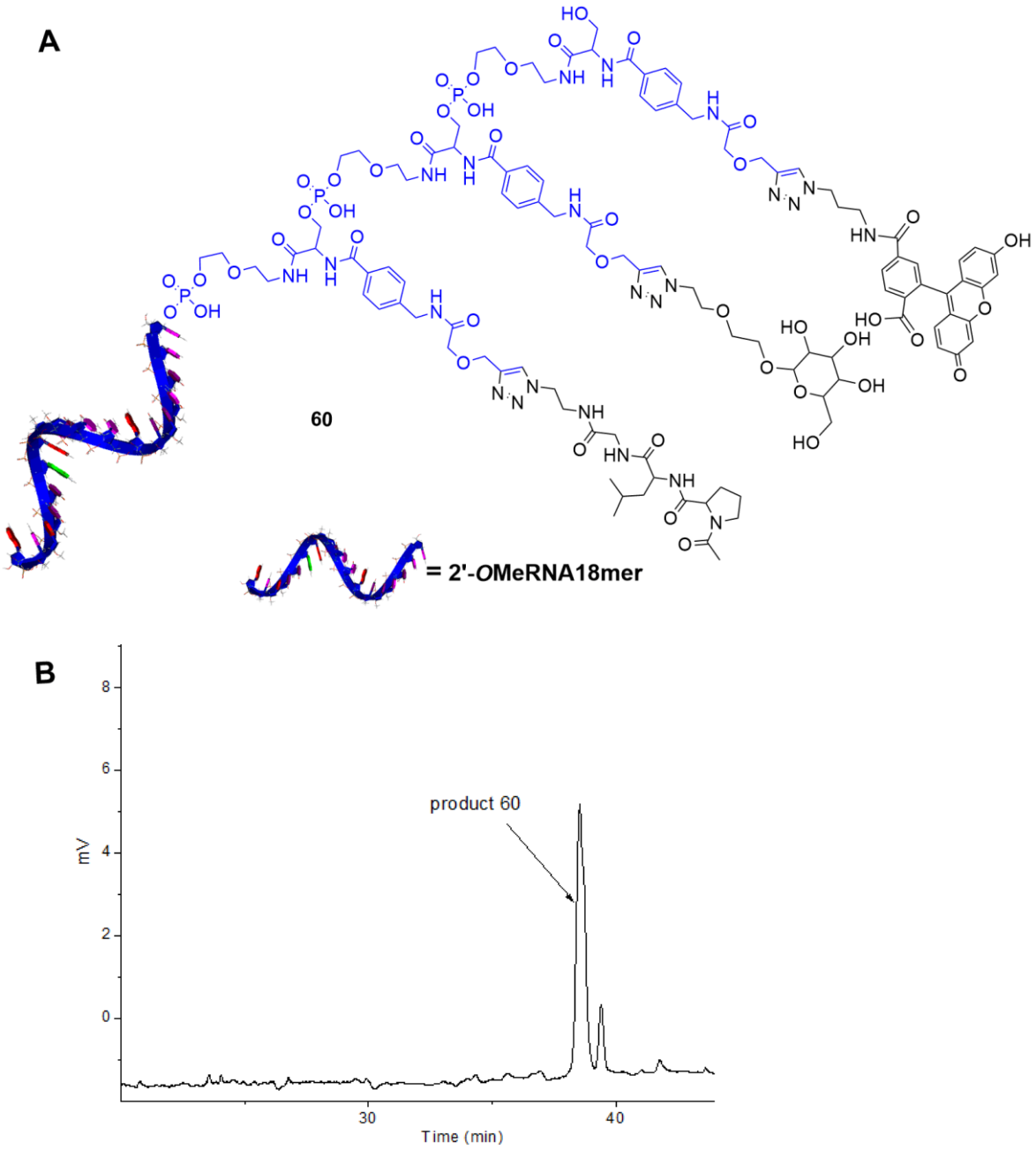


Figure 28: **A**: Structure of product **60** 2'-*O*MeRNA18mer equipped with three multiple functionalization linkers and an AcMIF peptide, a mannopyranoside and a fluorescent label respectively, **B**: HPLC chromatogram of purified product **60** (mass of the additional peak corresponds to the mass of the product).

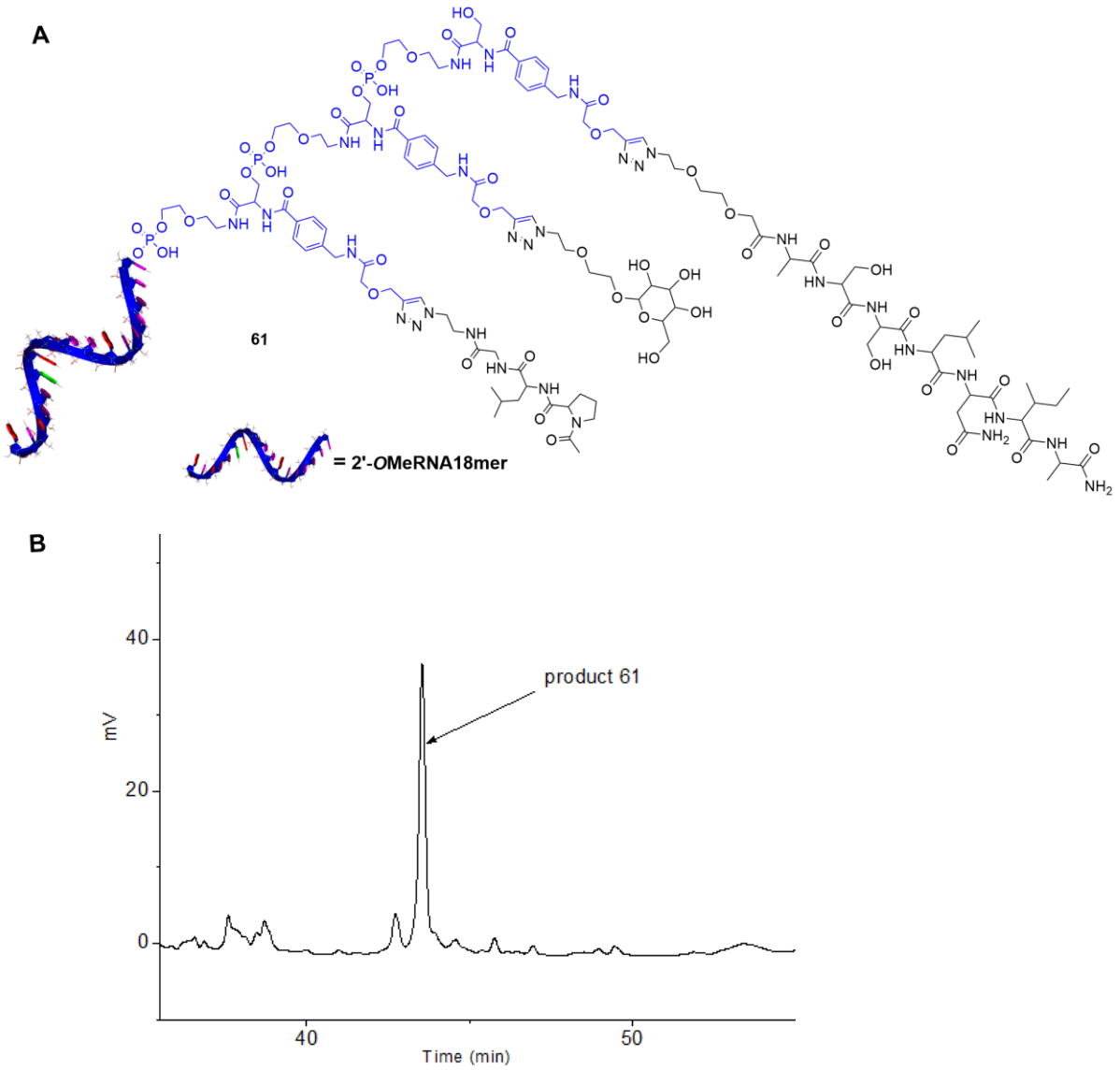


Figure 29: **A**: Structure of product **61** 2'-*O*MeRNA18mer equipped with three multiple functionalization linkers and an AcMIF peptide, a mannopyranoside and an ASSLNIA peptide respectively, **B**: HPLC chromatogram of purified product **61**.

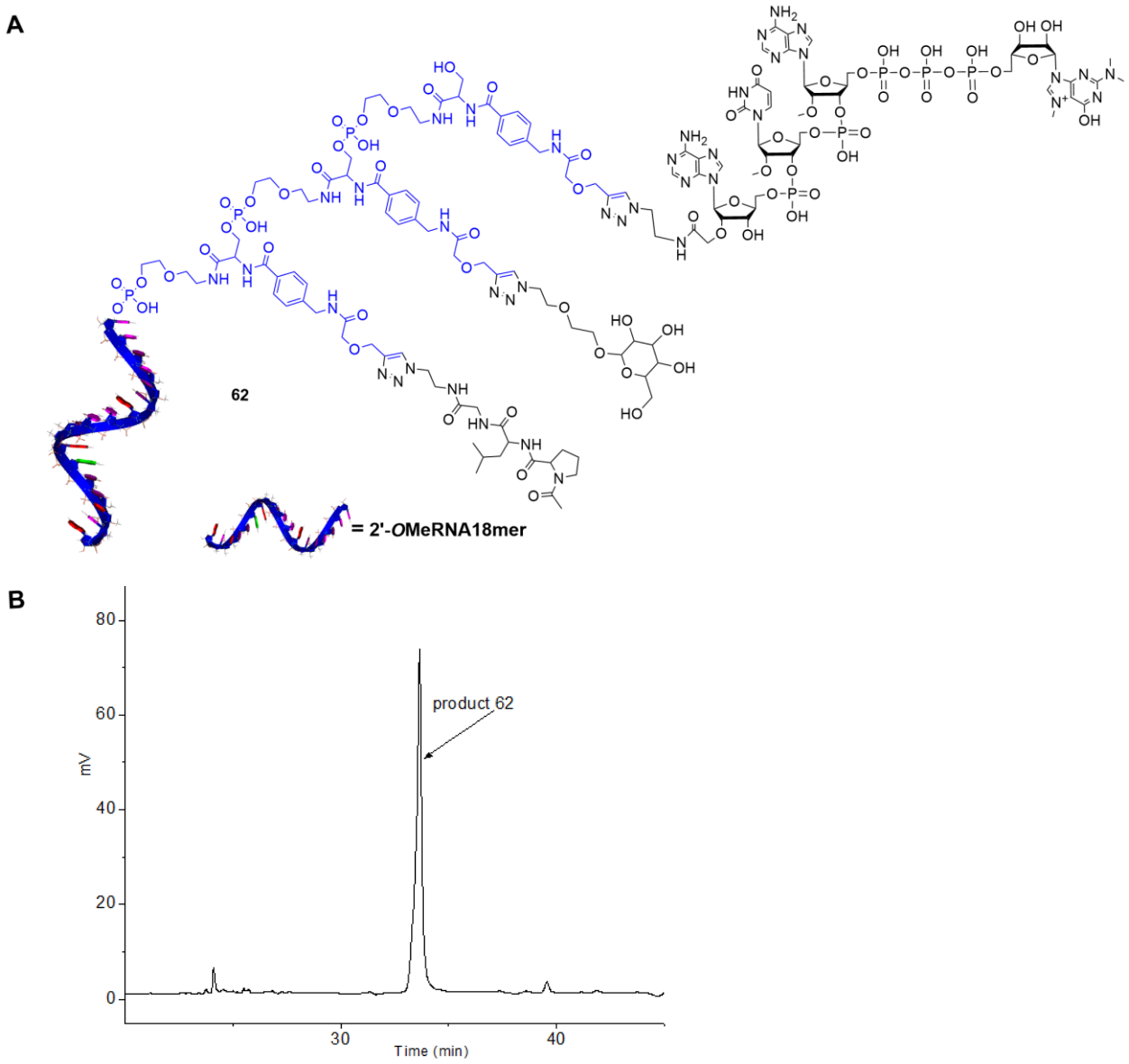


Figure 30: **A**: Structure of product **62** 2'-*O*MeRNA18mer equipped with three multiple functionalization linkers and an AcMIF peptide, a mannopyranoside and an m_3G-Cap respectively, **B**: HPLC chromatogram of purified conjugate **62**.

5.4 CONSIDERATIONS AND CONCLUSIONS ON HOW TO DESIGN THE OLIGONUCLEOTIDE BIOCONJUGATES USING MULTIPLE FUNCTIONALIZATION LINKER

The oligonucleotide bioconjugates presented in the previous chapter are the result of several experiments which helped in understanding how to benefit most from the multiple functionalization linker. In this part of my thesis the part of the study that was not successful but still gives a valuable insight into the possibilities and limitations of the methodology, is presented.

Preparation of an oligonucleotide construct possessing both the previously presented fluorescent label and the U2OS-targeting CPP failed, despite the observations made during the course of the synthesis that indicated successful attachment of the MFL. Repeating the experiment and testing the reaction progress both after the first "click" and after the synthesis was completed, revealed full conversion of substrate after the "first" cycle but analysis of the

crude final product gave quite inconclusive results. We realized that we had not thought about the fact that we actually "clicked" on a compound containing a free hydroxyl and the lack of success could be assigned to that. This hydroxyl group, present on the fluorescent label, is an attractive site for the next MFL unit added right after. Clearly the clickable entity used, should not contain a free hydroxyl unless it is the last entity added.

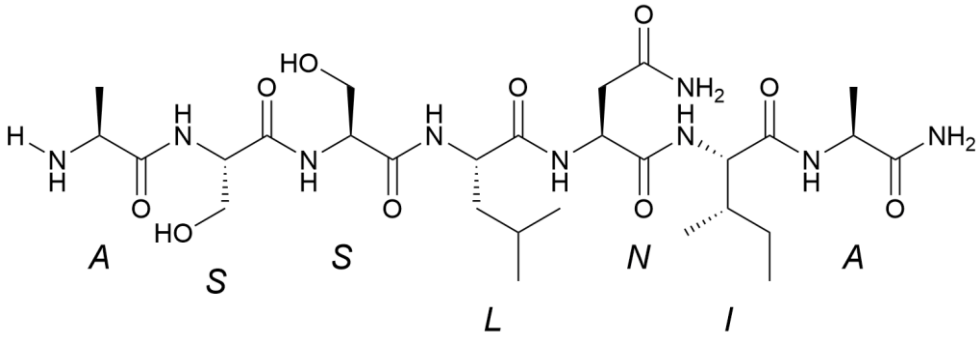


Figure 31: ASSLNIA peptide possessing two free hydroxyl groups.

Trying to improve the procedure experiments were performed with use of the ASSLNIA peptide, which possesses two free hydroxyl groups (Figure 31). After attaching the first MFL unit followed by "clicking" the peptide, an additional step with capping of the peptide was performed by adding a 4:1 solution of pyridine:acetic anhydride to the resin and stirring for 30 min. This should acetylate the OH groups and prevent their reaction with the next MFL. After extensive washing another linker unit was coupled, oxidized and the MMTr group was deprotected before the whole construct was cleaved off support. Unfortunately, the HPLC analysis presented in Figure 32 clearly shows that the capping step was not successful and after checking the MS of individual peaks not only product but also product with one and two additional MFLs were detected.

Conclusions and Future Prospects

The observations made while working with ASSLNIA peptide were somewhat disappointing. However, there are several possibilities to get past this hurdle and it is not unlikely that the conditions for capping can be optimized. Being able to carry this out as one of the consecutive steps in solid-phase synthesis would make the methodology more versatile and elegant, not least considering the option of automating the method in the future. Another alternative would be to acetylate the free hydroxyl groups before the peptide or any other entity is "clicked" to the solid-supported oligo-construct. This is quite possible but requires additional handling.

Use of fully protected peptides would of course remove any limits of peptide sequence for the above procedure as they can also be prepared in different ways. One example is by use of a 2-chlorotrityl resin. Peptides can be cleaved off this resin within an hour with hexafluoroisopropanol (HFIP)-DCM (1:4) with all protecting groups intact[144]. Addition of a "clickable" azide handle would definitely be compatible and many interesting peptides prepared with this methodology could be later used with the multiple functionalization linker.

However, care must be taken in choice of protection to ensure that its removal leaves the oligonucleotide intact and is also compatible with the ammonia treatment used for cleaving the ON from support.

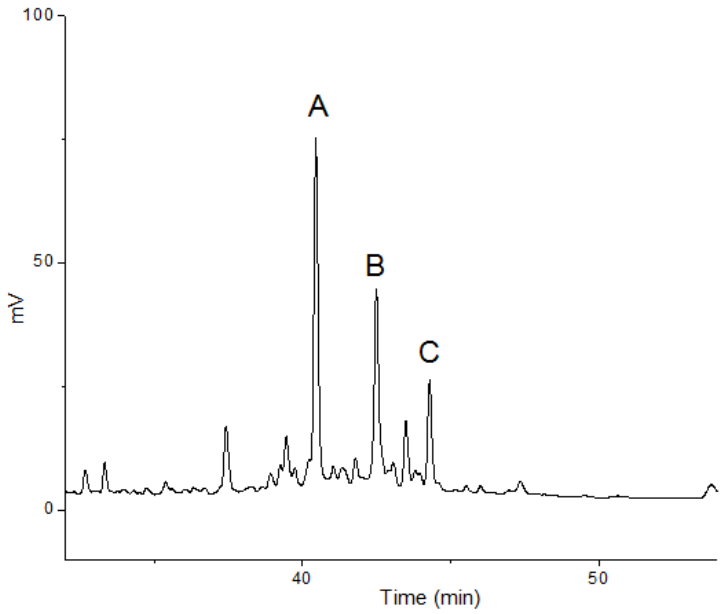


Figure 32: HPLC chromatogram showing the result of the experiment where free hydroxyl groups of ASSLNIA peptide were to be capped to prevent the multiple linker attacking these sites; A – product: 18mer MFL(ASSLNIA)MFL, B – product with one additional multiple functionalization linker, C – product with two additional multiple functionalization linkers.

Another worthwhile effort would be to shorten the time of the conjugate synthesis. The answer here could be the use of microwave irradiation for the 1,3-dipolar cycloaddition reaction, as proposed by Bouillon el al[51]. His suggestion of performing the reaction at 60ºC for 20 min is very tempting considering that in the present study 24h for each "click" step was used to make sure that the reaction is complete. Even if this could be shortened somewhat it would not be close to the potential time gain one could get by use of microwave conditions. If the entities used for preparation of oligonucleotide-based bioconjugate are not harmed by MV it could mean reduction of the time for complete synthesis with days.

Investigating further possibilities of MFL use is highly interesting. It is designed to enable the combined effect of many useful entities, which were invented and investigated before but never observed working together. Such prepared constructs have a strong potential to deliver the oligonucleotide therapeutics to the site of action. The constructs that have already been prepared and other similar ones should now undergo biological evaluation. The methodology could even be automated and used for scanning of various combinations of biologically active entities. A substantial advantage of the MFL is its versatility which means that it could be used for diverse targets and in many different ways. One idea could also be preparation of Zorro-LNA constructs, described in Chapter 6, where they could serve as a non-nucleotide linker.

6 "CLICK" ZORRO-LNA AS A TOOL FOR SCANNING THE DOUBLE-STRAND INVASION (DSI) REQUIREMENTS (PAPER IV)

Silencing of specific genes by targeting double strand DNA with oligonucleotides is one of the methods with potential for therapeutics. The task for an anti-gene agent is still not easy as it must cross both the plasma membrane of the cells as well as the nuclear membrane and subsequently find its way through chromatin to find its target[41]. A high affinity of binding, achieved *via* Watson-Crick base-pairing, and possibly additional interactions, is also of great importance together with sequence specificity towards the target DNA. Locked nucleic acids (LNAs) have often been used in these strategies as they not only possess the above mentioned properties but also can invade super-coiled dsDNA under physiological pH and salt conditions[22].

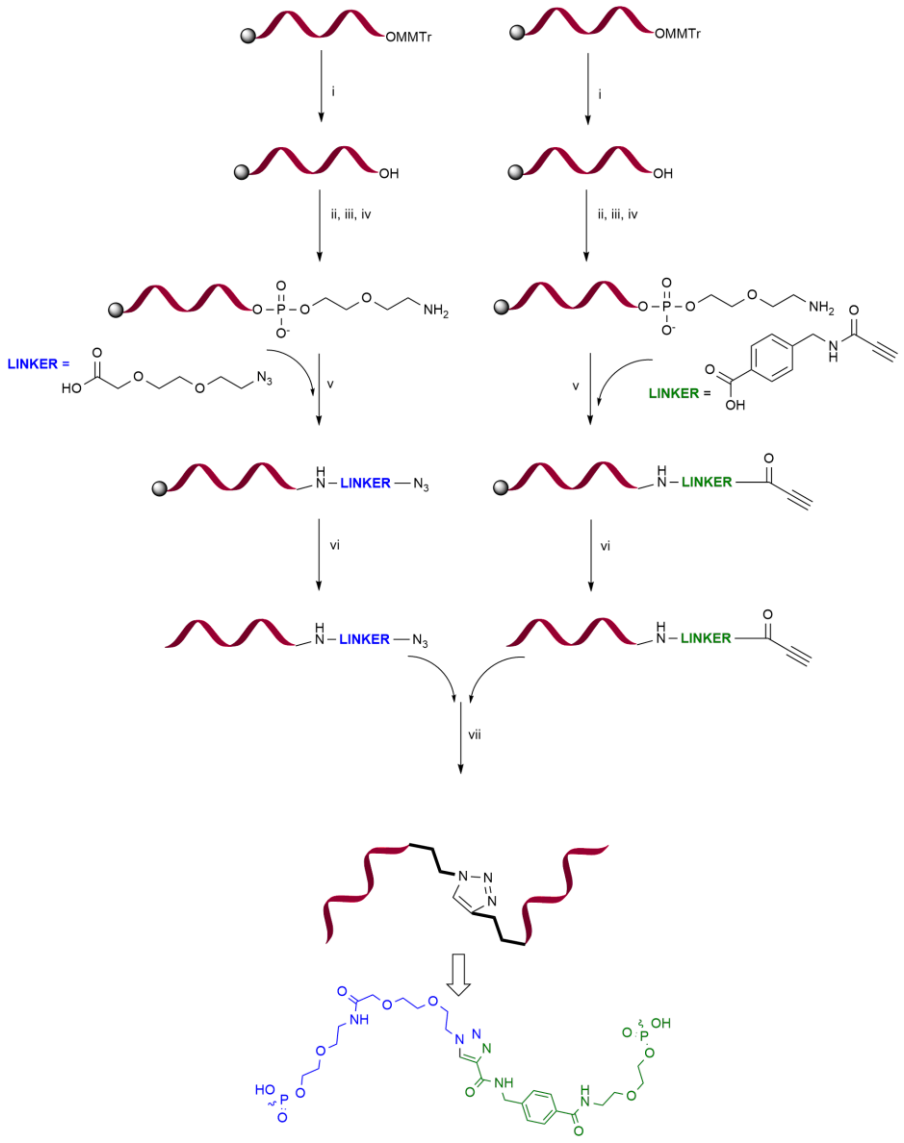
Zorro-LNA is a class of anti-gene oligonucleotides known for almost a decade. It is suggested that due to its Z shape it has the potential to specifically bind to neighboring sites on opposite strands of a duplex DNA[40]. Zorro-LNAs are typically prepared by using LNA/DNA mixmer ONs (Zorro "arms") connected to each other *via* nucleotides or a non-nucleotide based linker. The group of my co-supervisor, Professor Edvard Smith, has been working with different Zorro constructs, developing and testing various options for assembling the two "arms" mainly in order to improve double strand invasion (DSI) but also to ease the synthesis of the required constructs. Originally, the Zorro-LNA consisted of two LNA/DNA mixmers attached to each other through a 7-nucleotide, complimentary linker region. However, despite the success in interaction with the DNA, the potential intra-molecular binding between bases in the linker region and bases in the arms was a troublesome limitation. To overcome this problem in the next design the Zorro was prepared in the form of a single stranded–ON, where the two arms were covalently connected. The complex, referred to as "single-stranded Zorro" (ssZorro) was smaller in size which should be an advantage as small LNA-based constructs have been reported as more efficient[29, 145]. The ssZorros were even better in strand invading the DNA than the first generation Zorros that consisted of two strands that hybridized. However, their synthesis was laborious and required both 3'- and 5'-phosphoramidites of unmodified and LNA building blocks. Reversed amidites for DNA are commercially available but the LNA counterparts have to be synthesized in house prior to synthesis of the constructs, which is particularly laborous[146].

6.1 PREPARATION OF "CLICKABLE ARMS" AND "CLICK" ZORRO-LNA

A simplified and universal method for preparation of Zorro-LNA would be highly advantageous and would to allow preparation of the arms in the form of "building blocks". Such an approach would make it easier to screen various Zorro- constructs for: 1) the best DNA/LNA sequence combination and 2) different lengths of the conjugated arms.

Scheme 11: Schematic representation of Click-Zorro preparation.

With the experience in hand an obvious choice was to utilize Cu (I) [3+2] catalyzed cycloaddition to prepare "Click-Zorro-LNAs" where the arms are first equipped with an alkyne and azide functionality, respectively, and then linked by formation of a triazole (Scheme 11)[147].



Scheme 10: Schematic representation of parallel stepwise in situ synthesis of "clickable oligonucleotide arms" followed by their conjugation. i: 2% DCA, DCM, 2 min; ii: 2-(*N*-(4-monomethoxytrityl)aminoethoxy)ethyl H-phosphonate, Py, PvCl, 20 min, r.t.; iii: I_2, water, Py; iv: 2% DCA, DCM, 2 min; v: pre-activation of azido acid or (N-propynoylamino)-*p*-toluic acid (PATA) with HBTU, NMM in DMF, 0.5 h, r.t., then addition of their activated form to solid-supported ON, 2 h; vi: MeOH/NH_3 sat., 18 h, r.t., vii: CuI, DIPEA, overnight, water/*t*BuOH (dipolar cycloaddition [3 + 2] of ON-PATA with ON-N_3). Reproduced from Ref. 147 with permission from The Royal Society of Chemistry.

The functionalization of the "clickable arms" was done on solid support using the commercially obtained oligonucleotide mixmers, which were first 5'-detritylated (Scheme 10). Next, the 5′-

amino modifier: 2-(*N*-(4-monomethoxytrityl)aminoethoxy)ethyl H-phosphonate, which has previously been proven to give high yields, was coupled to the ONs with the help of PvCl[100]. After oxidation of the intermediate H-phosphonate, the MMTr group on the aminolinker is removed to prepare the solid-supported ON for conjugation with the pre-activated triple bond donor PATA and the azido-acid (2-(*N*-(4-monomethoxytrityl)aminoethoxy)ethyl H-phosphonate respectively. Finally, the two modified mixmers are cleaved off support with methanolic ammonia at r.t overnight. The "click" reaction could theoretically be performed with one ON on solid support, but to avoid potential complication, due to the polarity of deprotected ONs, we decided to carry out the "click" reaction in solution. The purification of

Name	Sequence of Zorro arm	Crude intermediate
D1	CCCtCCtcTTtcTTCa	D1 azido
D4	TgCcCCtCCtcTTTcTTCa	D4 azido
U1	GgCACccATgCgcTgA	U1 PATA
U3	cAacAaGcaCggCctC	U3 PATA
D3	TtgCcAgaCtcTgCc	D3 azido
D2	CccCagCcaCccTctG	D2 azido
U2	GgaAacCtcCctAaG	U2 PATA

Table 4: Nucleotide sequence of Zorro arms and the modifications added for preparation of "clickable" intermediates. Azido = 2-(2-(2-azidoethoxy)ethoxy)acetate acid, PATA = *(*N-*propvnylamino)*-p-*toluic acid. LNA monomers in uppercase, DNA in lower case.* Reproduced from Ref. 147 with permission from The Royal Society of Chemistry.

Entry	Name	Overlap*	Sequence
1	Z2BS-1	1	3′-AgTcgCgTAccCACgG click CCCtCCtcTTtcTTCa-3′
2	Z2BS-4	4	3′-AgTcgCgTAccCACgG click TGCcCCtCCtcTTtcTTCa-3′
3	Zi5-2	2	3′-GaAtcCctCcaAagG click CccCagCcaCccTctG-3′
4	Zi5-3	3	3′-CtcCggCacGaAcaAc click TtgCcAgaCtcTgCc-3′
5	Z-2ON	1	3′-AgTcgCgTAccCACgGTCtAaCt-5′ 5′-AGATtGACCCtCCtcTTtcTTCa3′
6	Z-2HEG	1	3′-AgTcgCgTAccCACgG-HEG-HEG-CCCtCCtcTTtcTTCa-3′

Table 5: Zorro constructs used in the study: Entry 1-4 "Click-Zorro-LNA", entry 5, 6 Zorro-LNA controls. * bases overlapping the two arms when the construct is bound to its dsDNA target. Reproduced from Ref. 147 with permission from The Royal Society of Chemistry.

each arm is neither desired (degradation of PATA mentioned in previous chapters) nor required as reacting equal amounts of both clickable oligonucleotides gave effective conjugation. For

the same reason the methodology can also be considered as time- and cost-efficient as only the final Zorro-construct is purified with HPLC. The sequence and modifications introduced to the individual arms are presented in Table 4 and the structures of "Click-ZorroLNAs" synthesized are shown in Table 5 (Entry 1-4).

6.2 INVESTIGATION OF "CLICK" ZORRO-LNA DOUBLE-STRAND INVASION

The general procedure for analyzing the double-strand invasion involves incubating the target plasmids with potential candidate constructs at 37°C and collecting samples at specific time points. The strand invading capacity of each sample is then determined using S1 nuclease, an enzyme capable of cleaving ssDNA, as used in previous Zorro-LNA studies[41].

Sequence overlap influence on the DSI

As the influence of the base overlap of Zorro-LNA arms was not thoroughly investigated before we decided to first incubate unlinked D1+U1 (sequences corresponding to Z2BS-1) and D4+U1 (sequences corresponding to Z2BS-4) together with plasmid pN252BS for 8, 24, 48 and 72h at 37°C and intracellular physiological salt and pH conditions. The results of the study, obtained at 1μM concentration are presented in Figure 33. They show clearly that the extended overlap does not hamper the strand invasion as the readings are about the same for the unlinked D4+U1 as the control 2-ON Zorro (Z-2ON was reported previously). Additionally, each individual arm was also tested separately under the same conditions which led to an interesting outcome: the ON D4 was quite efficiently invading on its own giving a result comparable to the D1+U1, whereas the individual arms D1 and U1 were not productive.

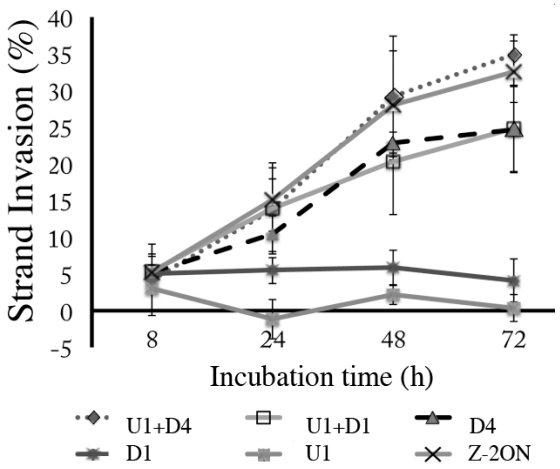


Figure 33: Extent of DSI achieved by unlinked Zorro-arms, alone, in combination and in comparison with the old Z-2ON as a control. Reproduced from Ref. 147 with permission from The Royal Society of Chemistry.

"Click-Zorro-LNA" influence on the DSI

To evaluate the double strand invasion of the "click-Zorro-LNA" we incubated constructs Z2SB-1 and Z2SB-4 together with the plasmid and compared with the previously reported Z-2HEG and Z-2ON as controls. Both tested Zorros have invaded DNA at both 1 μM and 3 μM

concentrations. However, they were clearly less efficient then the Z-2HEG construct and slightly worse than with the Z-2ON. After 72 h the Z-2HEG gives 75% DSI at 3 μM, while the "Click"-Zorros give about 40–45% (Figure 34A). It is, however, important to notice that the

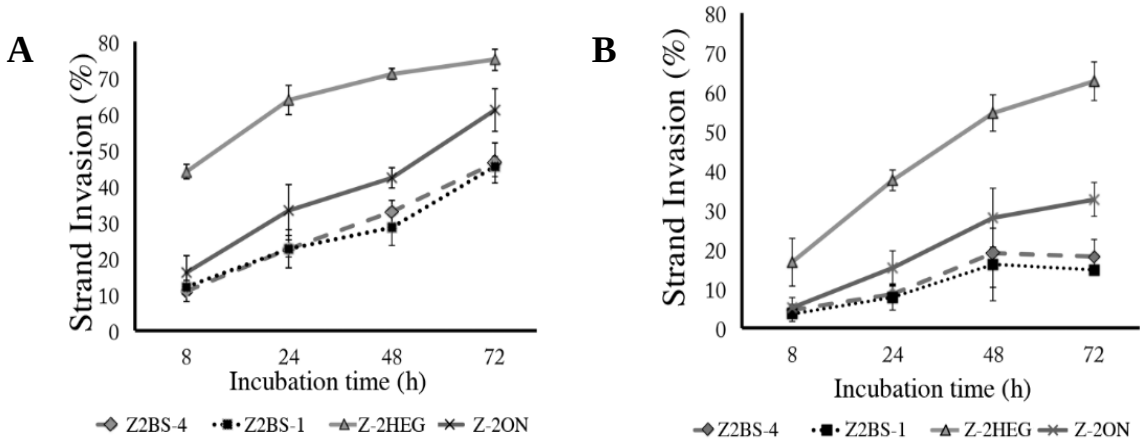


Figure 34: "Click-Zorro-LNA" in comparison with Z-2ON and the best of the earlier synthesized single stranded Zorros, Z-2HEG: A – at 3 μM concentration, B – at 1 μM concentration. Reproduced from Ref. 147 with permission from The Royal Society of Chemistry.

invasion for the "Click"-Zorros is steadily increasing with time. At lower concentration the differences are more visible (Figure 34B).

It can be concluded that the flexibility of the HEG linker could be of importance for the invasion process. However, having the advantage of the fast and convenient synthesis that "click-Zorro-LNA" offers is an interesting option for preparation of compound libraries that can be scanned for their activity towards various targets. We suggest that the two approaches can be combined for development of new anti-gene ONs, which would mean that when a promising construct is found a construct with similar length and hydrophobicity could be prepared using reverse amidites and HEG linkers.

Additionally, the new PAMBA linker presented in the previous chapter could replace the PATA to give some additional flexibility to the Zorro structure. The multiple linker could also be an attractive tool for "clicking" the two arms together.

Tandem Zorro-LNAs

In previous Zorro studies only a single construct was targeting two neighboring sites. This situation is not quite realistic if we look at a genomes existing in nature that rarely possess suitable repeat sequences. For this reason we decided to synthesize two different "click-Zorro-LNAs", Zi5-2 and Zi5-3 (with 2 and 3 bases overlap between the arms respectively) which were supposed to bind side by side in the genome. Before incubation the corresponding sites were inserted into the pN25 plasmid replacing the old sequence.

Comparing the results to the previous reports where two identical sites were targeted by a single constructs, the DSI was reduced when two different Zorros were used to bind adjacent sites. The invasion extend did, however, increase steadily with time (Figure 35).

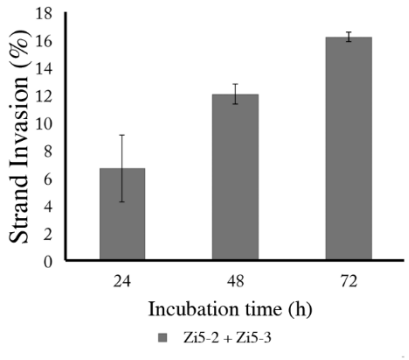


Figure 35: The extent of DSI achieved with a combination of the two different ZorroZi5-2 and Zi5-3 against the new target at 2 μM concentration. Reproduced from Ref. 147 with permission from The Royal Society of Chemistry.

7 ACKNOWLEDGEMENTS

I could never have written this thesis on my own, so I would like to express my sincerest gratitude to the following people, who have directly or indirectly contributed:

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