Cell-penetrating peptides, novel synthetic nucleic acids, and regulation of gene function

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Reconnaissance for designing functional conjugates

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Doubt is not a pleasant condition, but certainty is an absurd one

-*Voltaire*

List of publications

This thesis is based on the following publications, referred to in the text as paper I and paper II:

I. EL Andaloussi S, Guterstam P, Langel Ü
Assessing the delivery efficacy and internalization route of cell-penetrating peptides
Nat. Prot. (2007) 2(8): 2043-2047

II. Guterstam P, Lindgren M, Johansson H, Tedebark U, Wengel J, EL Andaloussi S, Langel Ü
Splice switching efficiency and specificity for oligonucleotides with locked nucleic acid monomers
Biochem J. 2008, in press

Abstract

Our genome operates by sending instructions, conveyed by mRNA, for the manufacture of proteins from chromosomal DNA in the nucleus of the cell to the protein synthesizing machinery in the cytoplasm. Alternative splicing is a natural process in which a single gene can encode multiple related proteins. During RNA splicing, introns are selectively removed resulting in alternatively spliced gene products. Alternatively spliced protein products can have very different biological effects, such that one protein isoform is disease-related while another isoform is desirable. Splice switching opens the door to new drug targets, and antisense oligonucleotides (asONs), designed to switch splicing, are effective drug candidates. Cellular uptake of oligonucleotides (ONs) is poor, therefore utilization of cell-penetrating peptides (CPPs), well recognized for intracellular cargo delivery, is a promising approach to overcome this essential issue. Most CPPs are internalized by endocytosis, although the mechanisms involved remain controversial.

Here, evaluation of CPP-mediated ON delivery over cellular membranes has been performed. A protocol that allows for convenient assessment of CPP-mediated cellular uptake and characterization of corresponding internalization routes is established. The protocol is based on both fluorometric uptake measurements and a functional splice-switching assay, which in itself is based on biological activity of conveyed ONs. Additionally, splice switching ONs (SSOs) have been optimized for high efficiency and specificity. Data suggest that SSO activity is improved for chimeric phosphorothioate SSOs containing locked nucleic acid (LNA) monomers. It is striking that the LNA monomers in such chimeric constructs give rise to low mismatch discrimination of target pre-mRNA, which highlight the necessity to optimize sequences to minimize risk for off-target effects.

The results are important for up-coming work aimed at developing compounds consisting of peptides and novel synthetic nucleic acids, making these entities winning allies in the competition to develop therapeutics regulating protein expression patterns.

Contents

Abbreviations

2OMe RNA	2’-O-Methyl RNA
AEC	Anion exchange chromatography
asON	Antisense oligonucleotide
BMD	Becker muscular dystrophy
CFTR	Cystic fibrosis transmembrane conductance regulator
CME	Clathrin-mediated endocytosis
CPP	Cell-penetrating peptide
DCC	Dicyclohexylcarbodiimide
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
HATU	2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HIV	Human immunodeficiency virus
HOBt	Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
LNA	Locked nucleic acid
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MBHA	4-Methylbenzhydrylamine
mRNA	Messenger RNA
Npys	3-Nitro-2-pyridinesulfenyl
ON	Oligonucleotide
PEI	Polyethyleneimine
PEN	Penetratin, also named pAntp
PI3K	Phosphatidylinositol 3-kinase
PNA	Peptide nucleic acid
PO	Phosphodiester
PS	Phosphorothioate
RISC	RNA-induced silencing complex
RLU	Relative luminescence units
RNA	Ribonucleic acid
RNAi	RNA interference

RT-PCR	Reverse transcriptase polymerase chain reaction
siRNA	Short interfering RNA
SPPS	Solid phase peptide synthesis
SSO	Splice switching oligonucleotide
TAT	CPP derived from the trans-activator of transcription protein
t-Boc	*tert*-butyloxycarbonyl
TBTU	O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TP	Transportan
TP10	Transportan 10

1. Introduction

Regulation of gene function means alteration of protein expression from a specific gene. Cellular protein synthesis can be manipulated to achieve desired protein expression patterns by utilizing antisense oligonucleotides (asON) which bind complementary to cellular target RNA and interfere with its further processing[1].

A great number of diseases arise from aberrant protein expression and specific, artificially mediated, regulation of gene function is therefore of great interest from a therapeutic point of view. Several synthetic nucleic acids, with improved properties for targeting of biologically important RNAs, have been developed lately[2] but vectors for oligonucleotide (ON) delivery is still a topic causing aggravation.

Cell-penetrating peptides (CPPs) have gained increasing attention since the initial discovery in 1994[3] due to their remarkable ability to penetrate cells and convey cargo, such as ONs. Understanding the mechanisms for CPP-mediated delivery and ON-induced regulation of protein expression may prove to be a prerequisite for therapeutic applications of synthetic nucleic acids.

1.1 Oligonucleotide-mediated regulation of gene function

The mediator of hereditary qualities, DNA, was structurally described by Watson and Crick in 1953[4]. As a result of extensive cell biological research explaining the proteomic background to diseases and the full sequencing of the human genome[5], development of many novel therapeutics are based on regulation of protein expression. To compensate aberrant protein synthesis, ON-mediated regulation of gene function is about to transform the development of novel drugs. Regulating asONs bind to target RNA and influence cellular processes, such as translation and pre-mRNA splicing. Another antisense mechanism interfering with gene function, which will not be discussed here, is the antigene strategy acting at DNA level as triplex forming asONs[6]. Alterna-

tive splicing of pre-mRNA is the cellular target process for the asONs, utilized in this thesis.

1.1.1 Antisense oligonucleotides and alternative splicing

The antisense mechanism is a process in which a foreign asON hybridizes to target RNA and interfere with its further processing. One mechanism of action for RNA targeting asONs is digestion of target mRNA-mediated by RNAse H[2]. Other mechanisms are steric hindrance of mRNA to be translated by the ribosome or steric influence on spliceosomal processing of target pre-mRNA. Another member in the family of gene modulating mechanisms is RNA interference (RNAi)[7,8], in which exogenous short double-stranded RNA fragments (siRNA) are bound to proteins in the cytosolic RNA-induced silencing complex (RISC) generating cleavage of target mRNA[9]. In this thesis, steric block asONs that interfere with spliceosome processing are utilized as reporters for cellular delivery and for evaluation of splice switching asONs (SSOs) with novel synthetic nucleic acids monomers.

Post transcriptional modifications, including alternative splicing (i.e. removal of introns and fusion of exons), are fundamental for generating mRNAs that can be translated into proteins. In contrary to constitutive splicing where the immature pre-mRNA transcript always is processed in the same manner, generating only one type of mRNA, alternative splicing produces various mRNAs with different sequences, and concomitantly, different protein isoforms

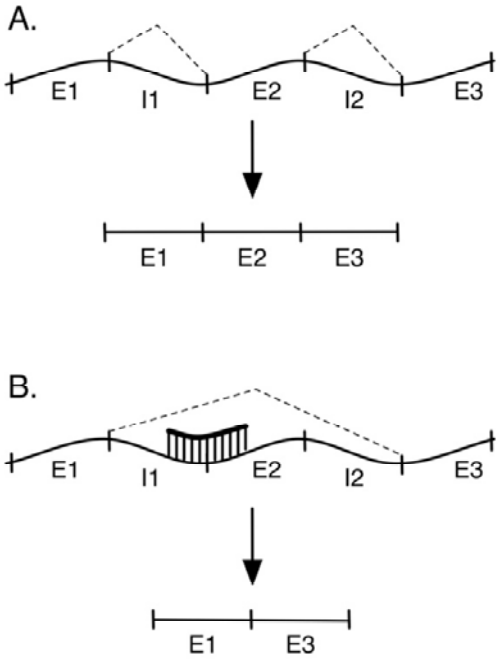


Figure 1.1: *Exon skipping induced by splice switching oligonucleotides (SSOs).* ***A****. Normal splicing of the pre-mRNA.* ***B****. An SSO interferes with the splicing machinery and give rise to another mRNA and thereby another protein isoform.*

with potentially different functions. Considering that an average gene encodes pre-mRNAs with eight different exons and that approximately 70% of all genes undergo alternative splicing, this is most likely the major source of protein diversity present in human cells[10]. Pre-mRNA splicing is an essential, precisely regulated process that occurs in the nucleolus of cells. Splicing requires exon recognition, followed by accurate cleavage and rejoining of exons, which are determined by invariant GT and AG intronic nucleotides at the 5′ and 3′ intron-exon junctions, respectively. Components of the basal splicing machinery recognize and bind to splice site sequences and promote assembly of multicomponent splicing complex known as the spliceosome that catalyzes the cut-and-paste reactions that remove introns and join exons[11]. Hence, introduction of an SSO to the cell can direct splicing to the desired protein isoforms (Figure 1.1).

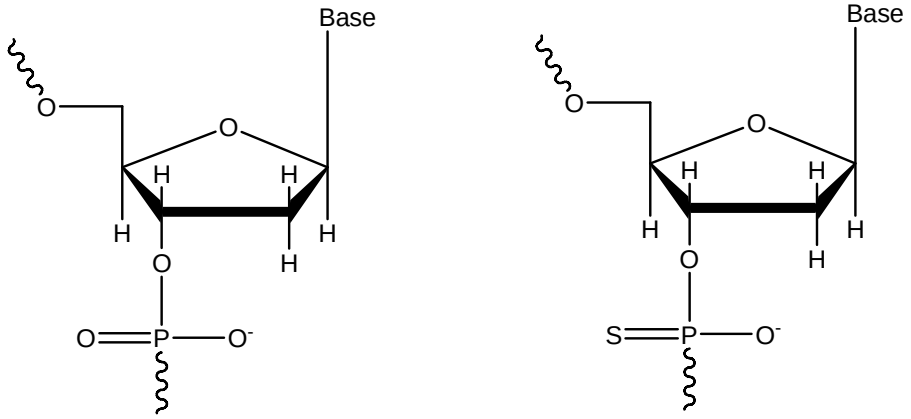
1.1.2 Alternative splicing and diseases

Several diseases including β-thalassemia, cystic fibrosis, muscular dystrophies, cancers, and several neurological disorders, are associated with alteration in alternative splicing, caused by mutations affecting the splicing process[12-14]. It is estimated that 20-30% of all disease causing mutations affect pre-mRNA splicing. *Cis*-acting mutations (i.e. mutations within the pre-mRNA to be spliced) either disrupts existing splice sites, generating intron inclusions or exon exclusions, or produce novel splice sites[13]. *Trans*-acting splicing mutations can affect the function of the basal splicing machinery or factors that regulate alternative splicing, resulting in changed preferences for choice of splice sites[15]. From here on, only *cis*-acting mutations are discussed.

One of the first splicing mutations described was found in β-thalassemia patients, where mutations in intron 2 of β-globin pre-mRNA create an aberrant 5′splice site, concomitantly activating a cryptic 3′splice site which leads to an intron inclusion, and therefore, expression of non-functional protein[16]. Same type of mutations has been identified in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, resulting in aberrant splicing and development of cystic fibrosis[17]. Duchenne muscular dystrophy (DMD), characterized by progressive degenerative myopathy, and its milder allelic disorder, Becker muscular dystrophy (BMD), are both caused by mutations in the dystrophin gene[18]. These are only mere examples of diseases caused by alterations in alternative splicing, for reviews see[14,19].

1.1.3 Synthetic nucleic acids

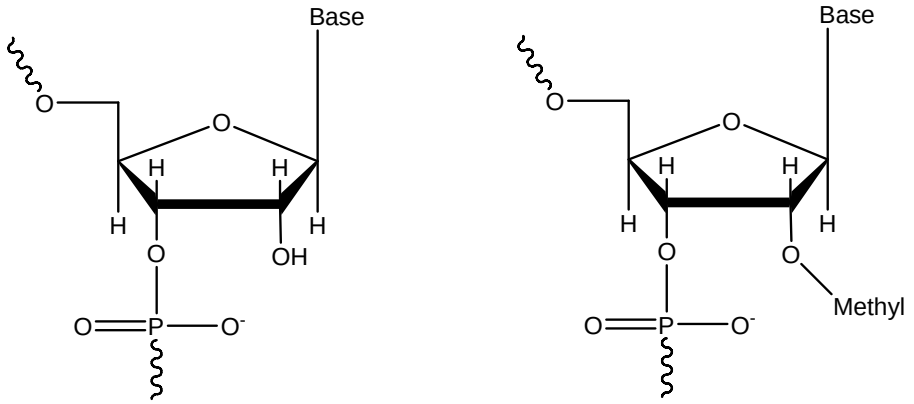
The major issue with ON-based therapeutics is cellular delivery, provided that gene regulating synthetic nucleic acids already are stable in serum, hybridize effectively to target RNA, and are non-toxic. Additionally, asONs for inhibition of protein expression should preferably recruit the RNA cleaving enzyme RNAse H while ONs for splice switching should not recruit RNAse H[2]. The first generation of synthetic asONs, represented by phosphorothioate (PS) DNA (Figure 1.2), has a modification on the phosphodiester (PO) linkage, recruit RNAse H and has high serum stability[20,21].



Phosphodiester Phosphorothioate

Figure 1.2: Phosphate backbone modification of phosphorothioate, DNA. Oxygen is replaced by sulfur in the first generation synthetic nucleic acids as compared to natural phosphodiester backbone.

The second generation of synthetic nucleic acids has 2'-O modifications, such as 2'-O-Methyl RNA (2OMe RNA) (Figure 1.3), with increased melting temperature when hybridized to RNA. Modification of the ribose sugar ring implies that the second generation does not recruit RNAse H[22], and they are therefore suitable as SSOs.



RNA 2'-O-Methyl RNA

Figure 1.3: Ribose modifications characterize the second generation synthetic nucleic acids, here exemplified by 2'-O-Methyl RNA. The 2'-O modifications increase binding affinity and abolish RNAse H recruitment.

The third generation asONs are characterized by further modifications of the riboses and / or the backbone. Synthetic nucleic acids with constrained ribose ring forming bicyclic nucleic acids, such as locked nucleic acid (LNA) have been developed (Figure 1.4). LNA has considerably higher affinity for target RNA than asONs from the second generation, confirmed by increased melting temperature for LNA strands hybridized to RNA[23]. Another type of third generation synthetic nucleic acid is peptide nucleic acid (PNA), which is achiral and has uncharged backbone. PNA comprise N-(2-aminoethyl) glycine units where nucleobases are attached to central amines with methylene carbonyl linkers[24] (Figure 1.4). Oligomers of PNA are less water soluble than most other synthetic nucleic acids but solubility can be improved by addition of positively charged lysine residues to the sequence[25,26].

Since, second and third generation synthetic nucleic acids do not recruit RNAse H their utility in gene silencing is limited. To avoid this drawback, gapmers with an internal stretch of 7-10 PS DNA monomers flanked by e.g. LNA monomers, are designed to achieve RNAse H mediated cleavage of target mRNA[27]. Gapmers have lowered affinity to target RNA as compared with corresponding mixmers, which have an even sequence distribution of different synthetic nucleic acids[28]. Hence, mixmers containing, e.g. PS DNA and LNA monomers, do not recruit RNAse H, and have advantageous affinity for target RNA. These are desirable characteristics for SSOs.

Due to intensive research in the field of novel synthetic nucleic acids, and as a result the introduction of third generation

asONs, issues with serum stability and target affinity are already solved to a great extent while delivery issues remain unsolved.

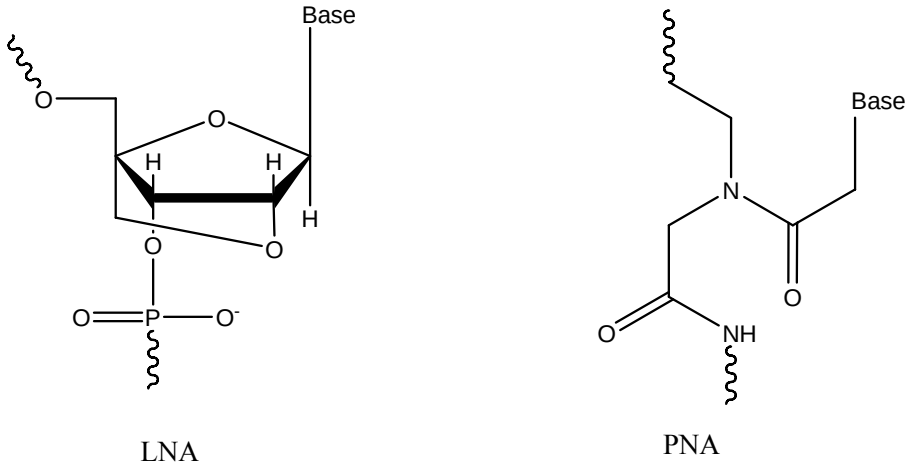


Figure 1.4: Third generation synthetic nucleic acids have significant ribose modifications as for the bicyclic LNA or complete replacement of the ribose phosphate backbone as in uncharged PNA.

1.2 Delivery of oligonucleotides over cell membrane

The main obstacle with the use of asONs, regulating gene function, is their low bioavailability. Most delivery vectors available to date are far from optimal and they have mainly been formulated and optimized for the delivery of gene expressing plasmids. For example, several viral vectors have been developed and utilized in gene therapy with promising results. Despite being very efficient, the viral vectors potentially suffer from several detrimental effects such as acute immune responses, immunogenicity, and viral recombination. Therefore, methods of non-viral gene delivery have been explored using various physical or chemical approaches. Physical approaches include needle injection, electroporation, gene gun, ultrasound, and hydrodynamic delivery, with the common denominator being employment of physical force to permeate the cell membrane, facilitating intracellular gene transfer. These approaches have been utilized both *in vitro* and *in vivo* with varying success, as reviewed by Gao 2007[29]. The main impediments with these methods are practical issues *in vivo* and cytotoxicity associated with plasma membrane perturbation.

The most frequently utilized strategy in nonviral gene delivery is, by far, formulation of DNA into condensed particles using

cationic lipids or cationic polymers[29]. These particles are subsequently taken up by cells via endocytosis into vesicles, from which a small fraction of DNA is released into the cytoplasm.

1.2.1 Non-viral oligonucleotide delivery vectors

Cationic lipids are increasingly used since Felgner and co-workers' initial discovery, in 1987, that a double-chain monovalent quaternary ammonium lipid, referred to as lipofectin, could efficiently bind to and convey DNA into cultured cells[30]. Mixing of cationic lipids and ONs creates small nuclease protected particles, liposomes, that allows cellular uptake and alleviate the release from endosomal structures[31]. Although cationic lipids have been successfully exploited *in vivo*, most of these vectors are not suited for *in vivo* use, as a result of their sensitivity for serum proteins. A dramatic change in surface charge and size occur when cationic lipids in complex with ONs are exposed to overwhelming amounts of negatively charged proteins that are abundant in the blood and extracellular matrices[29].

Apart from liposomes, cationic polymers represent the other large group of carriers that have been applied widely for ON-delivery. These linear or branched conformation polymers range from DNA condensing polylysine[32] to the most extensively used polyethyleneimine (PEI)[33] One major drawback using PEI as a transfection reagent is its non-biodegradable nature, raising toxicity concerns[34]. Cellular uptake through receptor-mediated endocytosis in absence of any ON condensing agent is another approach in which cholesterol or non-toxic polyethers are linked to the ON[35]. Recently, potent cell-specific systemic delivery vectors have been introduced which might alleviate potentially adverse side effects stemming from unwanted delivery to non-targeted cells and decreasing doses required to attain a biological response *in vivo*[36].

Aptamers are one group of targeting ligands with high specificity that have been used for delivery of siRNAs to prostate cancer cells both *in vitro*[37] and *in vivo*[36]. Aptamers are ON sequences that have been engineered through repeated rounds of selection to have affinity for a specific molecular target[38]. Since aptamers are ONs themselves it is easy to covalently fuse asONs and aptamer through continuous synthesis. For the purpose of siRNA delivery, significant effort has also been invested in optimizing liposomal delivery vectors. Such optimized liposomes are based on cationic lipids forming a bilayer that encapsulate the ONs (Figure 1.5). The liposomes can be

functionalized with stabilizing ties, such as thylene glycol or targeting ligands[39]. Despite some progress, pharmacokinetic properties like delivery efficiency and tissue targeting have to be further improved[39].

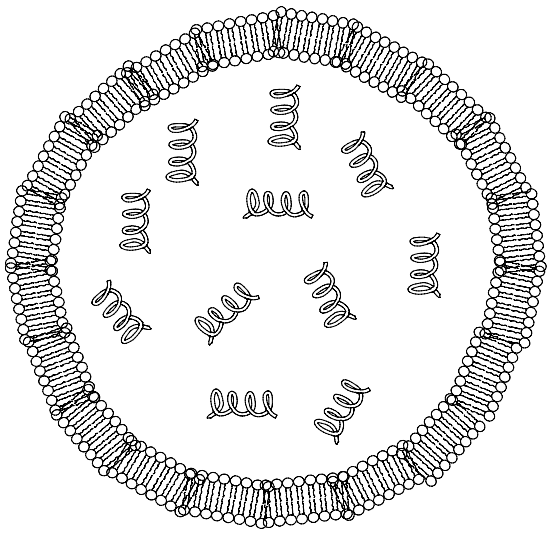


Figure 1.5: Liposomal delivery vector carrying anionic ONs. The liposome can be functionalized, e.g. with ligands.

Even though the mentioned vectors only are a limited selection of delivery vehicles, it is essential to find more efficient and non-toxic vectors for transport of ONs. Delivery efficacy, targeting, and toxicity are the main concerns for ON delivery vectors.

1.3 Cell-penetrating peptides

Cell-penetrating peptides (CPPs) were first identified while investigating proteins involved in infection of mammalian cells by HIV-virus. The trans-activator of transcription protein (TAT) was found to penetrate cells, thereby being able of bystander effects[40,41]. A positively charged peptide of 9 amino acids was later found to be the minimum sequence required for translocation, hence a CPP[42]. Before the TAT derived CPP was discovered, another naturally derived CPP was identified, namely penetratin (PEN), from the antennapedia homeoprotein in *Drosophila*[3]. Furthermore, promising peptides like transportan (TP) have been synthesized[43], later a deletion analogue named transportan 10 (TP10)[44] was introduced, but more simple designs like polyarginines have also been found potent[45].

As the name indicates, CPPs were originally thought to directly penetrate the plasma membrane and gain access to the cytoplasm. This was later found to be a experimental artifact and CPPs

were revised to employ endocytotic pathways[46,47]. Nevertheless, this topic is still under debate and a few CPPs are still considered to be capable of entering cells via non-endocytic pathways[48,49]. CPPs are alternatives to cationic polymers, liposomal or viral delivery vectors promoting cellular uptake of asONs regulating gene function.

There are two strategies for CPP-mediated ON delivery, namely covalent attachment of cargo to CPP or a strategy based on non-covalent interactions, mainly electrostatic interactions, between anionic ON and cationic peptide. In paper I, both strategies are employed in an attempt to characterize potency and endocytic pathways for CPPs. It is important to be aware of uptake mechanism for CPP-mediated ON delivery to understand the opportunities and limitations a particular CPP conjugate possesses.

For non-covalent strategies it is in most cases a prerequisite to co-add agents that enhance endosomal release, e.g. chloroquine[50] , together with the ON and CPP complex. The quantities of these agents needed are at magnitudes that could be toxic *in vivo*, leading to destabilization of cellular membranes, and this is not suitable for novel therapeutics. Consequently, it is preferable with CPPs that are covalently conjugated to its cargo. However, non-covalent complexes of ON and CPP that are well-characterized and can be applied to cells without co-addition of endosomolytic agents may have a future as therapeutic compounds[51-53].

1.4 Internalization routes for cell-penetrating peptides

The use of CPPs as delivery vectors are nowadays considered as a functional and effective method for ONs, *in vitro*, while the underlying mechanism for cellular uptake is a controversial matter. It is generally concluded that endocytosis is involved at low, e.g. non-toxic, treatment concentrations[54]. Endocytosis is a complex and ambiguous process involving several pathways[55]. The main pathways can be roughly summarized in macropinocytosis, endocytosis dependent on the coat proteins clathrin or caveolin, and pathways independent of clathrin and caveolin. Dynamin is a protein involved when membrane invaginations are budding off from the plasma membrane to form independent vesicles which later, to various extents, end up in early endosomes[56] (Figure 1.6).

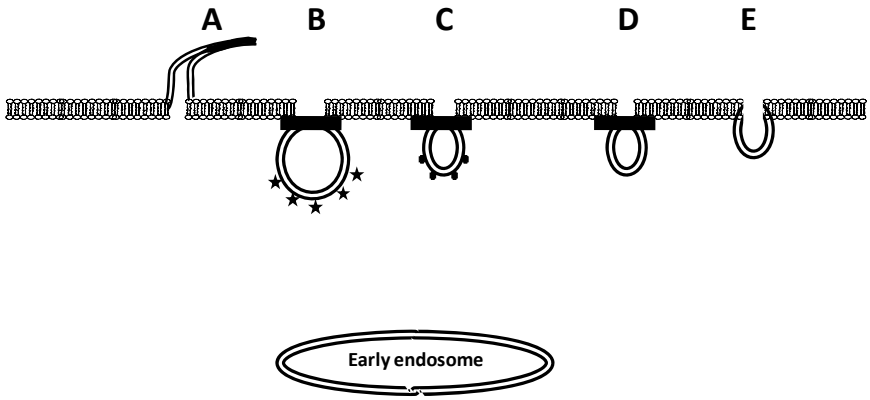


Figure 1.6: Scheme over pathways for endocytotis from cell exterior (upper part) to cell interior (lower part). Macropinocytososis is dependent on actin filaments in the plasma membrane (A). Clathrin-mediated endocytosis (B), and caveolin-dependent endocytosis (C) are dependent on dynamin for vesicle excision. Clathrin- and caveolin-independent pathways can either be dependent (D) or independent (E) of dynamin. Clathrin is represented by stars, caveolin by dots, dynamin by solid line.

To assess CPP uptake mechanisms experiments are performed under conditions where one or several pathways are inhibited. The most widely used strategy has been to treat cells at +4 ^{o}C where all energy dependent processes, including endocytosis, are slowed down so potentially energy independent pathways can be detected[52] Initial interaction between CPP and cell is often mediated by anionic heparane sulfates on the cell surface[57]. By pre-treating cells with the enzyme heparinase, these structures are cleaved from the cell surface, and the importance of CPP interaction with heparane sulfates for cellular uptake can be elucidated. Uptake pathways can also be specifically manipulated by treating cells with endocytosis inhibitors (Table 1.1) prior to peptide exposure[58,59] This approach has been greatly exploited in attempts to demarcate endocytotic pathways for different CPPs. The main setback with endocytosis inhibitors is specificity of the agents as no inhibitor can completely shut down a specific pathway[58]

2. Aims of the study

The main objectives with this thesis have been to develop methods for evaluation of CPP-mediated ON delivery over cellular membranes and to develop methods for optimizing SSOs to achieve high efficiency and specificity utilizing novel synthetic nucleic acids. The main goals of the respective publications are presented below.

Paper I: Describe a protocol that allows for convenient assessment of internalization routes for CPP-mediated cellular ON uptake.

Paper II: Establish a protocol for convenient ON synthesis and purification. Evaluate SSOs containing novel synthetic nucleic acids for splice switching efficiency and specificity, and optimization of the most potent SSO sequences.

3. Methodological considerations

Experimental procedures are extensively covered in paper I, this part will briefly discuss these methods with some theoretical aspects. For paper II, this part will add some information about the methods used. The sections below are valid for both papers when nothing else stated.

3.1 Oligonucleotide synthesis and purification

The solid-phase ON synthesis, and subsequent purification are extensively described in the supplementary data to paper II. Synthesis of PS DNA and 2OMe RNA (Table 3.1) were performed using ÄKTA^{TM} oligopilot^{TM} plus 10 synthesizer (Figure 3.1). The synthesis scale was 15 µmol scale utilizing disposable Oligosynt^{TM} 15 columns, specially packed with polystyrene based solid support, Primer Support 200^{TM}. The solid support was functionalized for synthesis of ON sequences with 2OMe RNA monomers in the 3'- end.

A previously described protocol[60] for purification was optimized and crude ONs were purified by anion exchange chromatography (AEC). The buffer system was based on NaCl gradient implying that purified ON had to be desalted. The final product was freeze dried and thereafter diluted to 100 µM stock solutions and kept at -20 ^{o}C until use.

Figure 3.1: Oligonucleotide synthesis was performed with an ÄKTA oligopilot synthesizer.

3.2 Synthesis of peptides, PNA, and conjugates thereof

All peptides and PNAs were synthesized by solid phase peptide synthesis (SPPS), a strategy introduced by Bruce Merrifield in 1963[61]. Both peptides and PNAs were assembled by *t*-Boc chemistry using a 4-methylbenzhydrylamine (MBHA) resin to generate amidated C-terminus. The loading of resin used for peptide synthesis was approximately 1 mmol/g while the loading was lowered tenfold for PNA synthesis due to the bulky nature of PNA monomers. Amino acids were coupled as hydroxybenzotriazole (HOBt) esters using a mixture of HOBt and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) activators. Alternatively, amino acids were coupled with HOBt and dicyclohexylcarbodiimide (DCC) using an Applied Biosystems 433A peptide synthesizer. PNA monomers were coupled on a similar synthesizer with 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU). Anhydrous hydrogen fluoride was used for cleavage from the resin. To avoid side-reactions of carbocations formed during cleavage, *p*-cresol, and if the peptide or PNA contained sulfur, *p*-thiocresol, was added to the cleavage mixture to act as scavengers. Following cleavage, peptides, and PNAs were filtrated to remove the resin and then freeze dried. Crude products were finally purified using preparative reverse-phase high performance liquid chromatography and analyzed using matrix-assisted laser desorption/ionization-time of flight (MALDI – TOF) mass spectrometer. Conjugation of PNA to CPP was made by generating a disulfide bridge between the two moieties. The advantage with disulfide linker is its cleavage in the reducing environment inside

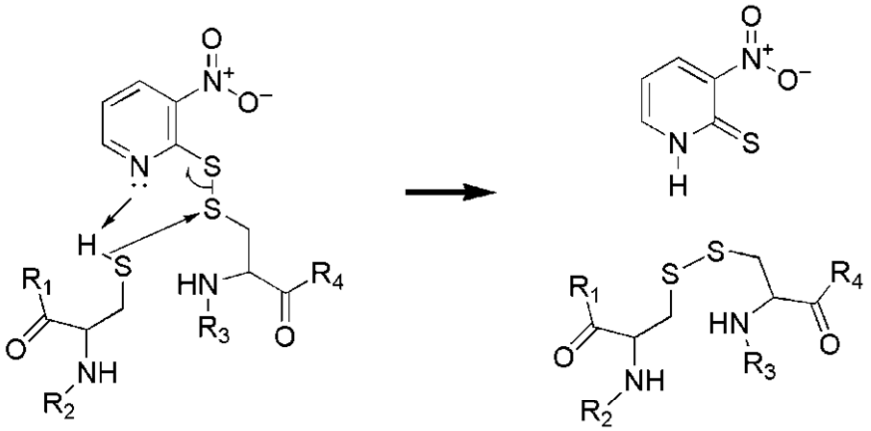


Figure 3.2: Formation of disulfide bridge using Npys.

the cell releasing PNA from CPP [62]. Peptides used for conjugation were synthesized with a 3-nitro-2-pyridinesulfenyl (Npys) derivative of cysteine, which rapidly reacts with thiols to form disulfides (Figure 3.2). The peptide with Npys was mixed with PNA holding a cysteine residue (Table 3.1) in 20% acetonitrile/water containing 0,1% TFA, incubated over night, purified by HPLC, and thereafter characterized by MALDI-TOF.

Table 3.1: Oligonucleotide and peptide sequences. Control ONs have internal inverted stretches which give rise to mismatches to target pre-mRNA. Both peptide and PNA have amidated C-terminus. Peptides, except TP and TP10, have N-terminal cysteine with Npys if utilized for conjugation to PNA. TP and TP10 has the Cys(Npys) attached orthogonally on the lysine in position 13 and 7, respectively. For experiments in paper I, the PNA is fluorescein labeled orthogonally at the C-terminal lysine.

Name	Sequence	#Mismatches	
		Total	LNA
2OMe	5'- CCU CUU ACC UCA GUU ACA	0	0
2OMeinv	5'- CCU CUU ACA CUC GUU ACA	4	0
LNA2-2OMe	5'- CcU cUt AcC tCa GtU aCa	0	0
LNA2-DNA	5'- CcU cUt AcC tCa GtU aCa	0	0
DNA	5'- CCT CTT ACC TCA GTT ACA	0	0
L-DNA	5'- CCT CTT ACC TCA GTT ACA	0	0
LNA1	5'- cCU cUU aCC UcA GUt ACa	0	0
LNAinv1	5'- cCU cUU aCA CtC GUt ACa	4	1
LNAinv2	5'- cCU cUU aGA CtC CUt ACa	6	1
LNAinv3	5'- cCU aCU cCA UtC GUt ACa	6	3
LNAinv4	5'- cCU cUU aGA CtC CUt CAa	8	1
LNAtr1	5'- cCU cUU aCC UcA GUt	0	0
LNAtrinv1	5'- cCU cUU aCA CtC GUt	4	1
LNAtr2	5'- cUU aCC UcA GUt	0	0
LNAtrinv2	5'- cUU aCA CtC GUt	4	1
LNAtr3	5'- cUU aCC UcA	0	0
LNAtrinv3	5'- cUU aCA CtC	4	1
LNAscr	5'-tCA gAU tCC AtC ACc UUc	14	6
PNA	**C KK** CCT CTT ACC TCA GTT ACA **KK**-NH_2	0	0
PNAinv	**C KK** CCT CTT ACA CTC GTT ACA **KK**-NH_2	4	0
M918	**MVT VLF RRL RIR RAS GPP RVR V**-NH_2		
TAT	**YGR KKR RQR RR**-NH_2		
TP	**GWT LNS AGY LLG KIN LKA LAA LAK KIL**-NH_2		
TP10	**AGY LLG KIN LKA LAA LAK KIL**-NH_2		
PEN	**RQI KIW FQN RRM KWK K**-NH_2		

LNA ONs are PS LNA/2'-OMe RNA mixmers where the LNA monomers are indicated as small letters. PS 2'-OMe RNA positions are capitals. For DNA, capitals indicate PS DNA positions, and for L-DNA capitals indicate L-DNA phosphorodiester positions (spiegelmer). Inverted stretches are underlined and bold letters represent amino acids. Observe for LNAinv3, the stretch of nine inverted monomers give rise to only six mismatches.

3.3 Splice switching reporter assay

To facilitate evaluation of SSOs, Kole and co-workers have constructed a splicing reporter system[63] with a plasmid carrying a luciferase-coding sequence that is interrupted by an insertion of intron 2 from β-globin pre-mRNA, carrying an aberrant splice site that activates a cryptic splice site. Unless this aberrant splice site is masked by an SSO, the pre-mRNA of luciferase will give rise to expression of non-functional luciferase (Figure 3.3). By using HeLa pLuc 705 cells which are stably transfected with this plasmid, it is possible to evaluate various SSOs by measuring induced luciferase activity and thereby generate a positive read-out[64]. Luciferase activity in lysate from HeLa pLuc 705 cells has been an indicator for successful cellular delivery of SSOs (paper I) and as a tool to verify splice switching efficiency and specificity for various synthetic nucleic acids (paper II).

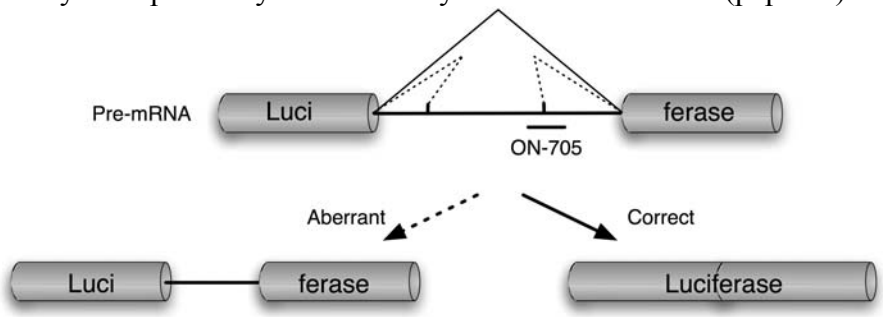


Figure 3.3: Reporter system for splice switching based on a plasmid carrying a luciferase-coding sequence with insertion of intron 2 from β-globin pre-mRNA containing an aberrant splice site that activates a cryptic splice site. Unless the aberrant splice site is masked by a splice switching oligonucleotide non-functional luciferase is expressed.

3.4 Analysis of splice switching by RT-PCR

Quantification of activity from functional luciferase, as carried out here and also in most published work to date, is a sensitive and convenient assay, which allows comparison of several conjugates quickly in terms of efficiency or specificity. However, such data are expressed in relative light units (RLU) and do not allow direct determination of the extent to which aberrant splicing has been corrected[65]. RT-PCR products from the aberrantly and correctly spliced luciferase pre-mRNA can be separated easily by gel electrophoresis, thus allowing evaluation of the extent of splice switching. RT-PCR measurements in combination with luciferase luminescence measurements is an excellent

procedure to ascertain splice switching activity. Details for RT-PCR analysis is carefully described in paper II.

3.5 Cell culture

Throughout all cell experiments in this thesis HeLa cells stably transfected with p705 have been used[63]. This cell type derives from human immortalized cervical cancer cells, initially taken from a patient named Henrietta Lacks. HeLa cells are widely used as they are robust, proliferate rapidly, and are relatively easy to transfect.

3.6 Toxicity measurements

The wst-1 proliferation assay assesses long-term viability after treatment of cells to address the toxic profile. Knowledge about toxic profile is important for *in vivo* experiments, and to exclude uptake artifacts, arising from damaged cells, in the splice switching reporter assay. The metabolic activity, and thereby the viability can be measured by the activity of the mitochondrial dehydrogenases. Several viability assays based on tetrazolium salts are available, and in the wst-1 assay, used in this thesis, the wst-1 salt is cleaved by the succinate-tetrazolium reductase system in viable cells generating formazan (Figure 3.4)[66]. The formazan dye is quantified spectrophotometrically at 420 nm, and the absorbance is directly proportional to mitochondrial dehydrogenase activity, and thereby to the amount of viable cells.

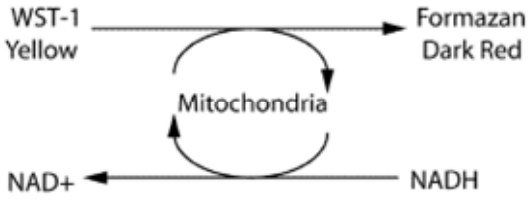


Figure 3.4: *Proliferation assay. The wst-1 salt is cleaved by mitochondrial succinate-tetrazolium reductase forming the soluble formazan dye, which is detected by absorbance measurements as an indicator of viable cells.*

3.7 Quantitative uptake measurement by fluorometry

One commonly used method to quantify CPP uptake is employment of fluorophore labeling which allows fast screening of various CPPs and conjugates[67]. In paper I, this method is used to quantify the uptake of

disulfide coupled conjugates, with CPP and fluorescein-labeled PNA. The main drawback of the method is the issue of distinguishing between internalized fluorophores, and those that are retained in cell surface structures. One way to reduce this artifact is to trypsinize and wash cells before they are lysed. Another issue is the impact that the fluorophore has on the cell-penetrating properties of the conjugate. The conjugates that actually are internalized inside cells may also be trapped in endosomal vesicles and in that way not bioavailable[68,69]. However, quantitative fluorometry can give valuable information when results are interpreted with respect to the limitations of the method, and conclusions should only be drawn after confirmation of results using other uptake assays.

4. Results and discussion

The two reports in this thesis cover different aspects of regulating gene function by novel synthetic nucleic acids and CPPs. In paper I, a method to address CPP-mediated delivery efficiency and internalization route for uncharged PNA and anionic 2OMe RNA ONs is developed. In paper II the focus changed from CPPs to optimization of bioactive SSOs exploiting the opportunities that has arisen, as the third generation of synthetic nucleic acids is introduced.

4.1 Delivery efficacy and internalization route for CPPs (paper I)

This is a protocol based on splice switching in combination with regular quantitative uptake by fluorometry to assess CPP activity, published with the intention to unify divergent experimental settings used by different research groups. The protocol presented here is optimized, not only to assess the relative delivery efficacy of different CPPs, but also to delineate the uptake mechanism of CPPs. Described in the protocol is the use of CPP and PNA conjugated by disulfide bridge and non-covalent mixtures of CPP and anionic 2OMe RNA. To dissect CPP uptake mechanisms consistently and systematically, a number of endocytosis inhibitors are tested. Experiments performed with several different endocytosis inhibitors give a relatively reliable characterization of employed pathways for CPPs when summarizing results (Table 4.1).

The most potent CPP conveying PNA was M918 followed by TP and TP10. Since most studies on CPPs have employed TAT and PEN, we decided to further investigate the role of endocytosis in uptake of PEN-S-S-PNA conjugates. TAT-S-S-PNA was omitted in this study due to very low levels of splice switching activity. Inhibitors of macropinocytosis significantly decreased the splicing efficiency for PEN, and pre-treatment with heparinase reveal that PEN-S-S-PNA is dependent on initial interaction with heparane sul-

fates for subsequent internalization, which is in line with previous results from our group[70].

Non-covalent strategies for CPP and 2OMe RNA are described to avoid cumbersome conjugate preparations when screening for novel CPPs or investigating CPP uptake mechanisms. We show, using PEN, that the non-covalent complexes are able to promote splice-correction, however, not in absence of a lysosomotrophic agent. Modification of CPP and SSO to allow for easy assembly generating robust complexes that can penetrate into cells, and accomplish intended biological activity, e.g, splice-switching, will be a topic for future investigation.

Table 4.1: Overview of treatments employed in paper I to elucidate uptake mechanism for CCPs. Cellular uptake is affected when pre-treating cells with inhibitors. Summarizing results from various treatments and uptake measurements gives indication of endocytic pathway for the CPP utilized.

Treatment	Effect	Uptake ↑	Uptake unchanged	Uptake ↓
+4 °C	Inhibit all endocytic pathways		Endocytosis independent uptake	Endocytosis
Cytochalasin D	Inhibit actin elongation and thereby macropinocytosis	not Macropinocytosis	not Macropinocytosis	Macropinocytosis
Heparinase III	Cleave heparan sulfates from the cell surface	HS inhibit uptake	HS independent uptake	HS dependent uptake
Wortmannin	Inhibitor of a kinase (PI3K) that is believed to affect both macropinocytosis and CME.		Clathrin independent but dynamin dependent uptake	Clathrin-mediated uptake or macropinocytosis
Chloroquine	Buffering endosomes, delaying formation lysosomes and thereby promoting endosomal escape	Endocytosis	Endocytosis independent uptake	

4.2 Splice switching efficiency and specificity for oligonucleotides with LNA (paper II)

Paper II is an investigation of target specificity for SSOs, applicable for everyone working with LNA. Splice switching efficiency and specificity for SSOs based on LNA and PNA has been evaluated together with 2OMe RNA, ordinary PS DNA, and enantiomeric DNA, also called spiegelmers or L-DNA. The intention with these experiments was to compare a selection of synthetic nucleic acids, and conclude which ON that is most suitable as SSO. The focus is merely on the ONs in this paper, and therefore, a commercially available transfection agent (Lipofectamine 2000) was used to convey ONs into cells. Chimeric PS SSOs with 2OMe RNA and LNA were the most powerful. It was confirmed by RT-PCR that such chimeric PS SSO with 33% LNA monomers induced splice switch for more than one third of the pre-mRNA at 100 nM treatments. While measurements of luciferase activity only gives a relative appreciation of splice switching activity, use of RT-PCR allows the evaluation of completeness of splice switching by comparison of the amounts of uncorrected and corrected mRNA. The achievement of a fair proportion of correction at low treatment concentration is a key issue in the development of SSOs as potential therapeutics[65].

It was established that the LNA control sequence, carrying four mismatches to target pre-mRNA, generated similar splice-switching activity as the correct sequence. The LNA monomers are very potent in raising the melting temperature for complexes with target RNA[23]. Accordingly, non-cautious introduction of LNA monomers when designing SSO sequences seems to result in implications for target specificity that obviously must be avoided. It was observed that LNA sequences did not have target specificity even when lowering treatment concentration. Preliminary results from experiments performed after publication of paper II reveal that shortening of sequence length from 18 to 12 monomers gives specificity for the LNA sequences (Table 4.2) without severely compromising efficiency (data not shown). Shortening SSO sequences will enable higher doses, in molar, when performing *in vivo* experiments since such experiments are limited by amount of active substance, in milligram, per dose. Future experiments will therefore, most likely, be based on optimized LNA sequences.

Table 4.2: Sequences of truncated SSOs and luciferase activity in RLU after treatment with 100 nM SSO. Splice switching activities for sequences with mismatches are related to respective correct sequence to give an estimation of specificity. None of the 9mers induced any splice switching activity as compared to untreated cells. Experiments are performed in triplicate (n=2).

Name	Sequence	Mismatches	Length	LNA monomers	Activity* (%)
2OMe	5'- CCU CUU ACC UCA GUU ACA	0	18	0	**28**
2OMeinv	5'- CCU CUU ACA CUC GUU ACA	4			
LNA1	5'- cCU cUU aCC UcA GUt ACa	0	18	6	**99**
LNAinv1	5'- cCU cUU aCA CtC GUt ACa	4			
LNAtr1	5'- cCU cUU aCC UcA GUt	0	15	5	**74**
LNAtrinv1	5'- cCU cUU aCA CtC GUt	4			
LNAtr2	5'- cUU aCC UcA GUt	0	12	4	**17**
LNAtrinv2	5'- cUU aCA CtC GUt	4			
LNAtr3	5'- cUU aCC UcA	0	9	3	**No activity detected**
LNAtrinv3	5'- cUU aCA CtC	4			

*Percentage of induced luciferase activity for control sequences with mismatches compared to activity for correct sequences.

5. Conclusions

The essential findings in the two papers that constitute the framework of this thesis are described below.

Paper I: The most potent CPP to convey ONs turned out to be M918, followed by TP and TP10. We delineated the uptake mechanism for various CPPs and concluded that PEN is taken up via macropinocytosis when conjugated to PNA. The described method to assess uptake mechanism is easy and reliable, as it is based on both traditional fluorometric uptake measurements and the functional splice-switching assay which in itself is based on true biological activity induced by the cargo.

Paper II: Splice switching activity increases when introducing LNA monomers to PS 2OMe RNA SSOs, and activity is further increased with higher proportion of LNA. The splice switching efficiency for PS LNA mixmers complemented with 2OMe RNA or DNA monomers is similar. However, LNA monomers give rise to low mismatch discrimination to target pre-mRNA.

This thesis has increased the understanding of uptake mechanisms for CPPs, and revealed the impact of introducing monomers of the novel synthetic nucleic acid, LNA, into SSOs. The results are important for the up-coming work aimed at making CPPs and novel synthetic nucleic acids winning allies in the competition to develop therapeutics, regulating protein expression patterns.

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7. References

1. Green, P.J., Pines, O. & Inouye, M. The role of antisense RNA in gene regulation. *Annu Rev Biochem* **55**, 569-97 (1986).
2. Kurreck, J. Antisense technologies. Improvement through novel chemical modifications. *Eur J Biochem* **270**, 1628-44 (2003).
3. Derossi, D., Joliot, A.H., Chassaing, G. & Prochiantz, A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269**, 10444-50 (1994).
4. Watson, J.D. & Crick, F.H. Genetical implications of the structure of deoxyribonucleic acid. *Nature* **171**, 964-7 (1953).
5. Venter, J.C. et al. The sequence of the human genome. *Science* **291**, 1304-51 (2001).
6. Besch, R., Giovannangeli, C. & Degitz, K. Triplex-forming oligonucleotides - sequence-specific DNA ligands as tools for gene inhibition and for modulation of DNA-associated functions. *Curr Drug Targets* **5**, 691-703 (2004).
7. Elbashir, S.M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15**, 188-200 (2001).
8. Fire, A. et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-11 (1998).
9. Peters, L. & Meister, G. Argonaute proteins: mediators of RNA silencing. *Mol Cell* **26**, 611-23 (2007).
10. Lander, E.S. et al. Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921 (2001).
11. Matlin, A.J., Clark, F. & Smith, C.W. Understanding alternative splicing: towards a cellular code. *Nat Rev Mol Cell Biol* **6**, 386-98 (2005).
12. Black, D.L. Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem* **72**, 291-336 (2003).
13. Faustino, N.A. & Cooper, T.A. Pre-mRNA splicing and human disease. *Genes Dev* **17**, 419-37 (2003).
14. Pajares, M.J. et al. Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol* **8**, 349-57 (2007).
15. Mercatante, D.R., Bortner, C.D., Cidlowski, J.A. & Kole, R. Modification of alternative splicing of Bcl-x pre-mRNA in prostate and breast cancer cells. analysis of apoptosis and cell death. *J Biol Chem* **276**, 16411-7 (2001).

16. Busslinger, M., Moschonas, N. & Flavell, R.A. Beta + thalassemia: aberrant splicing results from a single point mutation in an intron. *Cell* **27**, 289-98 (1981).
17. Rowntree, R.K. & Harris, A. The phenotypic consequences of CFTR mutations. *Ann Hum Genet* **67**, 471-85 (2003).
18. Koenig, M. et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *Am J Hum Genet* **45**, 498-506 (1989).
19. Garcia-Blanco, M.A., Baraniak, A.P. & Lasda, E.L. Alternative splicing in disease and therapy. *Nat Biotechnol* **22**, 535-46 (2004).
20. Campbell, J.M., Bacon, T.A. & Wickstrom, E. Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. *J Biochem Biophys Methods* **20**, 259-67 (1990).
21. Spitzer, S. & Eckstein, F. Inhibition of deoxyribonucleases by phosphorothioate groups in oligodeoxyribonucleotides. *Nucleic Acids Res* **16**, 11691-704 (1988).
22. Monia, B.P. et al. Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* **268**, 14514-22 (1993).
23. Kumar, R. et al. The first analogues of LNA (locked nucleic acids): phosphorothioate-LNA and 2'-thio-LNA. *Bioorg Med Chem Lett* **8**, 2219-22 (1998).
24. Nielsen, P.E., Egholm, M., Berg, R.H. & Buchardt, O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **254**, 1497-500 (1991).
25. EL Andaloussi, S., Johansson, H.J., Lundberg, P. & Langel, Ü. Induction of splice correction by cell-penetrating peptide nucleic acids. *J Gene Med* **8**, 1262-73 (2006).
26. Fabani, M.M. & Gait, M.J. miR-122 targeting with LNA/2'-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates. *Rna* **14**, 336-46 (2008).
27. Jepsen, J.S. & Wengel, J. LNA-antisense rivals siRNA for gene silencing. *Curr Opin Drug Discov Devel* **7**, 188-94 (2004).
28. Petersen, M. & Wengel, J. LNA: a versatile tool for therapeutics and genomics. *Trends Biotechnol* **21**, 74-81 (2003).
29. Gao, X., Kim, K.S. & Liu, D. Nonviral gene delivery: what we know and what is next. *Aaps J* **9**, E92-104 (2007).
30. Felgner, P.L. et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* **84**, 7413-7 (1987).
31. Xu, Y. & Szoka, F.C., Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35**, 5616-23 (1996).
32. Laemmli, U.K. Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine. *Proc Natl Acad Sci U S A* **72**, 4288-92 (1975).

33. Boussif, O. et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **92**, 7297-301 (1995).
34. Kunath, K. et al. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J Control Release* **89**, 113-25 (2003).
35. Soutschek, J. et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173-8 (2004).
36. McNamara, J.O., 2nd et al. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotechnol* **24**, 1005-15 (2006).
37. Chu, T.C., Twu, K.Y., Ellington, A.D. & Levy, M. Aptamer mediated siRNA delivery. *Nucleic Acids Res* **34**, e73 (2006).
38. Ellington, A.D. & Szostak, J.W. In vitro selection of RNA molecules that bind specific ligands. *Nature* **346**, 818-22 (1990).
39. de Fougerolles, A., Vornlocher, H.P., Maraganore, J. & Lieberman, J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov* **6**, 443-53 (2007).
40. Frankel, A.D. & Pabo, C.O. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55**, 1189-93 (1988).
41. Green, M. & Loewenstein, P.M. Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. *Cell* **55**, 1179-88 (1988).
42. Vives, E., Brodin, P. & Lebleu, B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* **272**, 16010-7 (1997).
43. Pooga, M., Hällbrink, M., Zorko, M. & Langel, Ü. Cell penetration by transportan. *Faseb J* **12**, 67-77 (1998).
44. Soomets, U. et al. Deletion analogues of transportan. *Biochim Biophys Acta* **1467**, 165-76 (2000).
45. Futaki, S. et al. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* **276**, 5836-40 (2001).
46. Lundberg, M., Wikström, S. & Johansson, M. Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther* **8**, 143-50 (2003).
47. Richard, J.P. et al. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* **278**, 585-90 (2003).
48. Deshayes, S. et al. On the mechanism of non-endosomial peptide-mediated cellular delivery of nucleic acids. *Biochim Biophys Acta* **1667**, 141-7 (2004).
49. Deshayes, S. et al. Insight into the mechanism of internalization of the cell-penetrating carrier peptide Pep-1 through conformational analysis. *Biochemistry* **43**, 1449-57 (2004).

50. Shiraishi, T. & Nielsen, P.E. Enhanced delivery of cell-penetrating peptide-peptide nucleic acid conjugates by endosomal disruption. *Nat Protoc* **1**, 633-6 (2006).
51. Crombez, L. et al. A non-covalent peptide-based strategy for siRNA delivery. *Biochem Soc Trans* **35**, 44-6 (2007).
52. Langel, Ü. *Cell-penetrating peptides: Processes and Applications*, (CRC Press, Boca Raton, FL, 2002).
53. Lundberg, P., EL Andaloussi, S., Sütlü, T., Johansson, H. & Langel, Ü. Delivery of short interfering RNA using endosomolytic cell-penetrating peptides. *Faseb J* **21**, 2664-71 (2007).
54. Duchardt, F., Fotin-Mleczek, M., Schwarz, H., Fischer, R. & Brock, R. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* **8**, 848-66 (2007).
55. Jones, A.T. Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. *J Cell Mol Med* **11**, 670-84 (2007).
56. Mayor, S. & Pagano, R.E. Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol* **8**, 603-12 (2007).
57. Horonchik, L. et al. Heparan sulfate is a cellular receptor for purified infectious prions. *J Biol Chem* **280**, 17062-7 (2005).
58. Sieczkarski, S.B. & Whittaker, G.R. Dissecting virus entry via endocytosis. *J Gen Virol* **83**, 1535-45 (2002).
59. Wadia, J.S., Stan, R.V. & Dowdy, S.F. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* **10**, 310-5 (2004).
60. Shanagar, J. Purification of a synthetic oligonucleotide by anion exchange chromatography: method optimisation and scale-up. *J Biochem Biophys Methods* **64**, 216-25 (2005).
61. Merrifield, R.B. Solid Phase Peptide Synthesis. 1. The synthesis of a tetrapeptide. *J Am Chem Soc* **85**, 2149-2154 (1963).
62. Hallbrink, M. et al. Cargo delivery kinetics of cell-penetrating peptides. *Biochim Biophys Acta* **1515**, 101-9 (2001).
63. Kang, S.H., Cho, M.J. & Kole, R. Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. *Biochemistry* **37**, 6235-9 (1998).
64. El-Andaloussi, S., Johansson, H.J., Lundberg, P. & Langel, Ü. Induction of splice correction by cell-penetrating peptide nucleic acids. *J Gene Med* **8**, 1262-73 (2006).
65. Abes, S. et al. Efficient splicing correction by PNA conjugation to an R6-Penetratin delivery peptide. *Nucleic Acids Res* **35**, 4495-502 (2007).
66. Slater, T.F., Sawyer, B. & Straeuli, U. Studies On Succinate-Tetrazolium Reductase Systems. Iii. Points Of Coupling Of Four Different Tetrazolium Salts. *Biochim Biophys Acta* **77**, 383-93 (1963).

67. Holm, T. et al. Studying the uptake of cell-penetrating peptides. *Nat Protoc* **1**, 1001-5 (2006).
68. EL Andaloussi, S., Järver, P., Johansson, H.J. & Langel, Ü. Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study. *Biochem J* **407**, 285-92 (2007).
69. Fischer, R., Waizenegger, T., Kohler, K. & Brock, R. A quantitative validation of fluorophore-labelled cell-permeable peptide conjugates: fluorophore and cargo dependence of import. *Biochim Biophys Acta* **1564**, 365-74 (2002).
70. EL Andaloussi, S., Johansson, H.J., Holm, T. & Langel, Ü. A novel cell-penetrating peptide, M918, for efficient delivery of proteins and peptide nucleic acids. *Mol Ther* **15**, 1820-6 (2007).

Paper I

and

PDF proof of Paper II

with supplementary material