CELL PENETRATING PEPTIDES; CHEMICAL MODIFICATION AND FORMULATION DEVELOPMENT

Kariem Ezzat
Cell penetrating peptides; chemical modification and formulation development

Licentiate thesis

Kariem Ezzat
To my family
List of publications

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


Additional publications


Abstract

Cell penetrating peptides (CPPs) have been extensively studied and exploited as drug delivery vectors for a wide variety of therapeutic cargos. However, several issues remain to be addressed regarding the enhancement of their efficiency and stability. In addition, to be available for patients, CPP-based therapeutics have to be formulated into suitable pharmaceutical forms that can be readily manufactured, transported, stored and conveniently used.

In this thesis, three chemically modified CPPs are developed having superior delivery properties for several nucleic acid-based therapeutic cargoes including: plasmids, small interfering RNA (siRNA) and splice switching oligonucleotides (SSOs), in different in-vitro and in-vivo models. In Paper I, we show that an N-terminally stearic acid-modified version of transportan-10 (TP10) can form stable nanoparticles with plasmids that efficiently transfect different cell types and can mediate efficient gene delivery in-vivo when administrated intramuscularly (i.m.) or intradermally (i.d.). In paper II, stearyl-TP10 is further modified with pH titratable trifluoromethylquinoline moieties to facilitate endosomal release. The new peptide, denoted PepFect 6 (PF6), elicited robust RNAi responses when complexed with siRNA in several cell models and promoted strong RNAi responses in different organs following systemic delivery in mice without any associated toxicity. In paper III, a new peptide with ornithine modification, PF14, is shown to efficiently deliver SSOs in different cell models including HeLa pLuc705 and mdx mouse myotubes; a cell culture model of Duchenne’s muscular dystrophy (DMD). Additionally, we have developed a method for incorporating this delivery system into solid formulation that could be suitable for several therapeutic applications. Solid dispersion technique is utilized and the formed solid formulations are as active as the freshly prepared nanoparticles in solution even when stored at elevated temperatures for several weeks.

Taken together, these results demonstrate that certain chemical modifications could drastically enhance the activity and stability of CPPs in-vitro and in-vivo. Moreover, we show that CPP-based therapeutics could be formulated into convenient and manufacturable dosage forms.
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<tr>
<td>CPP</td>
<td>Cell-penetrating peptides</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CR</td>
<td>Charge ratio</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne’s muscular dystrophy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HPRT1</td>
<td>Hypoxanthine-guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>i.d</td>
<td>Intradermal</td>
</tr>
<tr>
<td>i.m</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization – Time of flight</td>
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<tr>
<td>MBHA</td>
<td>p-methylbenzylhydralamine</td>
</tr>
<tr>
<td>MR</td>
<td>Molar ratio</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>ON</td>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>QN</td>
<td>N-(2-aminoethyl)-N-methyl-N’-[7-(trifluoromethyl)-quinolin-4-yl]ethane-1,2-diamine</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SSOs</td>
<td>Splice-switching oligonucleotides</td>
</tr>
<tr>
<td>SSPS</td>
<td>Solid-phase peptide synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilane</td>
</tr>
</tbody>
</table>
1. Introduction

Despite the tremendous success of basic biomedical research, new-drug output from pharmaceutical companies has been constant over the last decades and mostly based on small molecules (1). This gap between discoveries and therapeutics has recently led to intense interest in translational research; to transform biomedical discoveries into commercializable drug products (2). One of the major biomedical milestones in the last decade was the complete sequencing of the human genome (3), which has significantly deepened our knowledge about the genetic causes of diseases. This led to the emergence of several methods to interfere with disease pathophysiology on the molecular genetics level, a field that can collectively be called “gene therapy”. Gene therapy approaches can be roughly divided into 3 types according to their therapeutic effect (figure 1):

- Restoration of lost gene function by gene delivery via viral vectors or plasmids.
- Silencing of disease causing genes by antisense, antogene or RNAi (RNA interference) approaches.
- Modification of gene function by interfering with the splicing machinery via splice-switching oligonucleotides (SSOs)

However, all three approaches require the delivery of extremely large and charged nucleic-acid based molecules to their intracellular targets across the plasma membrane, which is inherently impermeable to such molecules. Difficulties in delivering such new therapeutic molecules to their target organs, tissues and subcellular compartments led to the development of several drug delivery technologies in recent years. In this thesis, the main gene therapy approaches will be briefly illustrated, and the ability of newly developed chemically modified CPPs to efficiently deliver plasmids, siRNA and SSOs will be highlighted.
1.1. Gene delivery

Loss of functional genes is the cause of several heritable diseases and cancers. Thus, the delivery of functional genes that could restore normal phenotype has been the holy grail of gene therapy for decades. Viral vectors have been utilized for gene delivery, however, promising results have been tempered by the potential for insertional mutagenesis that might lead to severe leukaemogenic side effects as has been reported recently (4, 5, 6). Also, humoral immunity directed against the viral vector particle is generally observed (7). Among the other safer alternatives for gene delivery is the delivery of plasmids carrying the desired gene via non-viral vectors. When the plasmid enters the cell, it is transcribed and translated by the cellular machinery without the need of genome integration. Cationic liposomes have been routinely used as delivery vectors for plasmids; however, toxicity remains a significant problem, according to several in-vivo findings (8).

1.2. Silencing of disease causing genes

Silencing of disease-causing genes can come in different ways including:

1.2.1. Antisense

There are two possible mechanisms for an antisense effect using antisense oligonucleotides (ONs). When the double-stranded DNA or genes situated in the nucleus are targeted, the approach is called the antigene strategy (9). The method that relies on targeting of the mRNA is called the antisense strategy. Antisense activity can be achieved either by blocking the binding sites for the 40S ribosomal subunit and for other translation initiation signals or by the formation of a double-stranded DNA/RNA complex that renders the RNA susceptible to RNase H digestion (9) (figure 1). Natural DNA and RNA have been used for antisense approaches together with several chemically modified analogues that offer better annealing with RNA targets and possess enhanced serum stability. Examples of chemically modified ONs include: phosphothiate DNA, 2’-O-methyl RNA (2’-OMe), locked nucleic acid (LNA), peptide nucleic acid (PNA) and phosphorodiamidate morpholino oligo (PMO)(10).
1.2.2. RNAi

RNAi is a fundamental pathway in eukaryotic cells, where long pieces of double stranded RNA are cleaved by an enzyme called dicer into shorter fragments called small interfering RNA (siRNAs) that can cleave complementary mRNA sequences by the help of the RISC complex and argonaute 2 (figure 1) (11). The proof-of-principle study in 2001 demonstrating that synthetic small interfering RNA (siRNA) could achieve sequence-specific gene knockdown in mammalian cells marked the birth of siRNA therapeutics (12). What makes the siRNA approach more appealing is that it cleaves target mRNA in a catalytic manner, thus, lower doses are required to achieve gene knockdown compared to the conventional antisense approaches. That is why intensive research has been carried out in the last decade to develop delivery vectors for siRNA therapeutics (11).

1.3. Splice-switching therapeutics

Modification of gene function can be achieved by interfering with the splicing machinery; an approach termed splice-switching (13). Recent studies using high-throughput sequencing indicate that 95–100% of human pre-mRNAs have alternative splice forms (14). Mutations that affect alternative pre-mRNA splicing have been linked to a variety of cancers and genetic diseases, and splice-switching oligonucleotides (SSOs) can be used to silence mutations that cause aberrant splicing, thus restoring correct splicing and function of the defective gene (figure 1) (13, 15). SSOs are antisense ONs ranging from 15 to 25 bases in length that do not activate RNase H, which would destroy the pre-mRNA target before it could be spliced (13, 15). One example of genetic diseases amenable for SSO therapy that will be addressed in this thesis is Duchenne’s muscular dystrophy (DMD). DMD is a neuromuscular genetic disorder that affects 1 in 3500 young boys worldwide (16). It is caused mainly by nonsense or frame-shift mutations in the dystrophin gene. SSOs are used to induce targeted ‘exon skipping’ and to correct the reading frame of mutated dystrophin pre-mRNA such that shorter, partially-functional dystrophin forms are produced (17). SSOs targeting exon 51 are currently in human clinical trials in various parts of Europe to treat DMD (18, 19). However, translating the promising results of SSOs into products re-
quires optimization of many parameters ranging from enhancement of cellular uptake and biodistribution to pharmaceutical formulation and long term stability.

Figure 1. Different gene therapy approaches. a. Viral delivery and genome integration. b. Plasmid delivery. c. Antisense steric block of translation. d. Antisense DNA/RNA hybrid and RNase H degradation. e. Antigene. f. Splice-switching ONs. g. siRNA.
1.4. Cell-penetrating peptides (CPPs)

CPPs are polybasic and/or amphipathic peptides, usually less than 30 amino acids in length, that possess the ability to penetrate cells (Cell Penetrating Peptides) or transduce (Protein Transduction Domains) over cellular plasma membranes directly in a receptor independent manner (20, 21). CPPs have attracted much interest in recent years as promising vectors for the delivery of a wide variety of therapeutics ranging from small molecules up to nanoparticles.

1.4.1. History

Many peptides and proteins have desirable therapeutic effects, but being large and often charged molecules, they have always been thought incapable of bypassing the plasma membrane. This view was challenged in the year 1988, when two groups independently published results in the same issue of CELL showing that both the recombinant and the chemically synthesized 86 amino acids long Tat protein were found to be rapidly taken up by cells in tissue culture (22, 23). Few years later, the 60 amino acid homeodomain of the Antennapedia protein in Drosophila was also shown to penetrate cells (24). A very important advancement in the field came by showing that the cell penetration capability is imparted by relatively short peptide sequences. The 16-mer peptide derived from the third helix of the homeodomain of Antennapedia termed penetratin (25), the 11-mer peptide derived from Tat protein (26), the 27-mer chimeric peptide termed transportan (27) and even simple polyarginines (R8) (28) were all shown to traverse the plasma membrane. These discoveries marked the birth of the field of CPPs. Since then, many CPPs have been discovered and studied as potential drug delivery vehicles, some of which are presented in Table 1.
Table 1. Selection of CPPs and their sequences\textsuperscript{a}

<table>
<thead>
<tr>
<th>CPP</th>
<th>Sequence</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penetratin</td>
<td>RQIKIWFQNRRMKWKK\textsuperscript{b}</td>
<td>(25)</td>
</tr>
<tr>
<td>Tat (48-60)</td>
<td>GRKKRRQRRRPQ</td>
<td>(26)</td>
</tr>
<tr>
<td>pVEC</td>
<td>LLIILRRIRKQAHASK-NH\textsubscript{2}</td>
<td>(29)</td>
</tr>
<tr>
<td>bPrPp</td>
<td>MVKSIGSWILVLFVAMWSDVGLCKKRPKP-NH\textsubscript{2}</td>
<td>(30)</td>
</tr>
<tr>
<td>Transportan</td>
<td>GWTLNSAGYLLGKINLKALAALAKKIL-NH\textsubscript{2}</td>
<td>(27)</td>
</tr>
<tr>
<td>TP10</td>
<td>AGYLLGKINLKALAALAKKIL-NH\textsubscript{2}</td>
<td>(31)</td>
</tr>
<tr>
<td>MAP</td>
<td>KLALKLALKAALKLA-NH\textsubscript{2}</td>
<td>(32)</td>
</tr>
<tr>
<td>Poly Arg</td>
<td>(RRR)\textsubscript{n}</td>
<td>(28)</td>
</tr>
<tr>
<td>Pep-1</td>
<td>KETWWETWTEWSQPKKKRKV\textsuperscript{d}</td>
<td>(33)</td>
</tr>
<tr>
<td>MPG</td>
<td>GALFLGWLGAAGSTMGAPKKKRKV\textsuperscript{d}</td>
<td>(34)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Peptides are C-terminal free acids unless stated otherwise. \textsuperscript{b} Originally with a free acid C-terminally but later shown also to have CPP properties when amidated. \textsuperscript{c} \textit{n} equals 2-4. \textsuperscript{d} C-terminal cysteamide group.

1.4.2. Uptake mechanism

The detailed structure activity relationship and the actual mechanism of cellular uptake for CPPs are not yet fully understood. Two main pathways have been debated to be solely responsible for CPP receptor-independent uptake; the direct translocation pathway and the endocytic pathway. There are also debated sub-models within each pathway. For the direct translocation, three different models have been proposed; the carpet model, the pore formation model and the inverted micelle-mediated model (35, 36). Also, for the endocytic pathway, three models have been proposed; classical clathrin mediated endocy-
tosis, caveolae-mediated endocytosis and macropinocytosis (37).

Recently, an increasing number of studies are emphasizing the role of endocytosis in their uptake (37, 38, 39, 40, 41, 42). However, it has become evident that different factors and experimental conditions affect the mechanism of uptake of CPPs and their cargos. Different CPPs, concentrations, incubation times and volumes, cell type, cargo type, cargo-coupling methodology, read-out assay, extent of toxicity and extent of degradation are all factors that affect the uptake mechanism. Moreover, endocytic entrapment has to be followed by physical membrane translocation if the peptide is to reach the cytoplasm (43). So, both the interpretation of results and the design of new CPPs should be projected on to the detailed background including the chemistry used, the type of cargo, the experimental conditions and the read-out assays.

1.4.3. CPPs as drug delivery vehicles

CPPs have attracted much interest as a promising alternative for intracellular delivery of therapeutic cargos, especially for proteins and nucleic acids. Most of the existing methodologies are either safe but not effective enough or very effective but suffer from safety concerns that limit their use. CPPs appear to combine both efficacy and safety being very potent for intracellular delivery of macromolecules and meanwhile easily biodegradable. Two main methods have been utilized to attach the CPP to its cargo, either via a covalent linkage or through the formation of a non-covalent complex.

1.4.3.1. Covalent linkage to cargo

CPPs have been linked to various cargos via covalent linkers. Small molecules like antineoplastic agents and antibiotics have been coupled to CPPs to enhance biodistribution and cellular uptake. For example, SynB peptide was utilized for the delivery of benzylpenicillin (B-Pc) to the brain and it was found that the brain uptake of coupled B-Pc was increased eight-fold on average compared to free B-Pc (44). An example of successful vectorization of chemostatics comes from our group where two different CPPs, YTA2 and YTA4, were utilized for the delivery of methotrexate (MTX) into MTX- resis-
tant breast cancer cells (45). CPPs have also been utilized to deliver a wide variety of proteins and peptides which are either coexpressed with CPPs or ligated with a disulphide bridge. For cancer treatment, a CPP derived from the fibroblast growth factor was conjugated to an anti-Akt single chain Fv antibody and administrated in vivo with subsequent reduction in tumor volume and neovascularization (46). Another interesting example is using undeca-arginine (R11) expressed recombinantly with the four transcription factors, Oct4, Klf4, Sox2, and c-Myc, to generate induced pluripotent stem cells (iPS cells) (47). This set-up was almost 10 times more efficient as compared to other approaches in generating iPS colonies without the risk of chromosomal integration associated with viral vectors. For peptide delivery, several groups have used both Tat and penetratin (Table 1) to convey peptides derived from the tumor suppressor p53, or peptides that modulate p53 activity, in an attempt to reduce tumor growth and induce apoptosis (48, 49).

For gene therapy approaches, covalently coupling CPPs, which are positively charged, to negatively nucleic acids has been not very easy to achieve. That is why in most gene therapy approaches using the covalent coupling strategy, neutral PNA or PMO were used instead of normal nucleic acids. Also, they can be directly attached to the CPP utilizing the solid phase peptide synthesis chemistry. Successful delivery of antisense ONs in vivo using CPPs was for the first time demonstrated with an antisense PNA complementary to human galanin receptor 1 (GalR1) mRNA coupled to transportan or penetratin that specifically down-regulated these receptors in rat brains (50). A number of endogenous proteins including dystrophin (51, 52), CD45, and the interleukin-2 (IL-2) receptor (53), have been targeted with PMOs using CPPs as well. However, the limitation of using only PNA or PMO has led the development of the other strategy of cargo linking to CPPs, which is the non-covalent complexation.

1.4.3.2. Non-covalent complexation with cargo

Some CPPs have been successfully exploited for plasmid and ON delivery using a non-covalently complexation strategy (54). Having net positive charge, such CPPs have been shown to form nano-sized particles with negatively charged nucleic acids, which are efficiently internalized by cells presumably via an endocytosis-dependant
mechanism (54, 54, 55, 56). This strategy has drastically expanded the range of therapeutic cargos that can be delivered via CPPs as it avoids laborious chemical conjugation and has almost no limitation on the size of the cargo. Moreover, it was found that lower concentrations of ONs are generally required to achieve a biological response utilizing this strategy (55, 57). Thus, it has been recently extensively utilized by our group and others for delivery of plasmids, siRNAs and SSOs (20).

1.4.4. Chemical modification

Several chemical modifications have been introduced to CPPs to enhance their membrane interaction, improve the nanoparticle formation capability or to facilitate endosomal escape and release of cargo after internalization (55). C-terminal cysteamide modification was shown to be crucial for CPP-mediated siRNA delivery using the MPG and CADY peptides by increasing membrane association and stabilizing particle formation by the formation of peptide dimers (58, 59, 60, 61). Also, addition of hydrophobic moieties to CPPs has been shown to be an efficient mean to increase the bioavailability of CPPs and associated cargo. Cholesteryl modification of some polyarginines was shown to enhance their activity for local delivery of siRNA in-vivo (62). Stearylation was also proven to be a successful method to introduce hydrophobicity and enhance delivery of CPPs in the case of plasmids (63). One major limitation of the non-covalent complexation strategy is that the formed nanoparticles are mainly internalized via endocytosis, and consequently much of the cargo is commonly retained in endosomes without reaching their target. Several approaches have been devised to enhance the endosomal escape of CPPs and their cargos out of the endosomes to their target subcellular compartments. One example is the development of a histidine-containing endosomolytic α-helical penetratin analogue, EB1, which was able to form complexes with siRNA and promote endosomal escape (64). Other methods utilized fusogenic peptides, like HA2-peptide, conjugated to CPPs in order to facilitate release from endosomes (65). One major conclusion of the papers discussed in this thesis is that stearylation does not only enhance nanoparticle formation and stability, but also enhances the endosomal escape.
1.5. Pharmaceutical formulation

Pharmaceutical formulation is the process of transformation of the active drug substance into a final medicinal product, which is an indispensable process before the drug could reach the market and be used by patients. It has a great effect on enhancing the efficiency and stability of active pharmaceutical ingredients. Additionally, specific formulations have to be tailored for different therapeutic approaches. Among the different pharmaceutical forms present, solid formulations remain the most widely used. It is the form used in tablets, capsules, powders for inhalation and even powders for injection. This is because they are easy to handle and very stable upon storage and transportation. That is why in this thesis a method was developed to incorporate CPP nanoparticles in a solid form that could be used in various therapeutic approaches.
2. Aims of the study

This thesis is primarily focusing on studying the effect of certain chemical modifications on the activity of TP10 and its analogues for the delivery of plasmids, siRNA and SSOs utilizing a non-covalent complexation strategy. Furthermore, it describes a method for formulating such drug delivery systems into a solid dosage form that could be suitable for several therapeutic applications.

**Paper I**, studies the activity of stearic acid-modified TP10 to deliver plasmid DNA to various cell types *in-vitro* and *in-vivo* upon *i.m* (intramuscular) and *i.d* (intradermal) injection.

**Paper II**, studies the activity of a new chemically modified CPP named PF6, where endosomolytic trifluoromethylquinoline moieties are added to enhance endosomal escape, to deliver siRNA in various cell types and when administered systemically *in-vivo*.

**Paper III**: In this paper, ornithine, a non-standard amino acid, is used to chemically modify a TP-10 analogue together with N-terminal stearylation. The new peptide named PF14 is tested in induction of splice-switching activity in several models and a method for developing solid formulations utilizing PF14/SSO nanoparticles is studied.
3. Methodological considerations

The methods used in this thesis are described in details in the contributing papers. This part will briefly discuss the main methods utilized with some theoretical aspects. The selections below are valid for all papers when nothing else is stated.

3.1. Solid phase peptide synthesis

All peptides in this thesis were synthesized by solid phase peptide synthesis (SPPS), which was pioneered by Bruce Merrifield in 1963 (66). SPPS is based on anchoring the growing peptide chain onto an insoluble solid matrix followed by adding an N-terminally protected amino acid then deprotection and adding a new amino acid in repeated cycles. This method enabled the synthesis of not only peptide chains but also other polymers including DNA and RNA revolutionizing entire fields of science.

All peptides used in this thesis were synthesized utilizing methylbenzyl hydrazine (MBHA) as an anchoring resin to produce C-terminal amidated peptides, and fmoc protection chemistry was used. For PF6, four novel trifluoromethylquinoline-based derivatives (QN) were introduced via a succinylated lysine tree. Peptides were thereafter cleaved from the resin using 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS) and 2.5% H₂O. Following cleavage and extraction, peptides were subsequently freeze-dried. Crude peptides products were purified using semi-preparative reversed-phase high performance liquid chromatography (HPLC) and analyzed using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer. The main peptides used in this thesis are presented in Table 2.
Table 2: The main peptides used in this thesis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Stearyl-TP10</td>
<td>Stearyl-AGYLLGKINLKAALAKKIL-NH₂</td>
</tr>
<tr>
<td>PF6</td>
<td><img src="image.png" alt="Four trifluoromethyquinolone based derivatives via a succinylated lysine tree" /></td>
</tr>
<tr>
<td>PF14</td>
<td>Stearyl-AGYLLGKLOOLAAAAALOOLL-NH₂</td>
</tr>
</tbody>
</table>

3.2. Cell cultures

The first propagation of a human cell line *in-vitro* was the HeLa cell line obtained from a cervical cancer patient named Henrietta Lacks and propagated by George O. Gey in the 1950’s. Since then, *in-vitro* cell culture models are serving as indispensible tools in assessing the activity of newly developed therapeutics before testing them on animal and human subjects. Two types of cell cultures exist, primary cultures that are obtained directly from the animal and can be kept for a limited period of time, and permanent cultures, which are usually of cancerous origin, and can be propagated indefinitely. More than 10 cell lines were used in this thesis including primary and permanent cell lines. All cell lines were grown at 37°C, 5% CO₂ in humidified environment with the appropriate culture media supplemented with nutrients and antibiotics.
3.3. Plasmid delivery (Paper I)

Non-covalent complexation strategy between the peptide and plasmids or ONs was exclusively utilized in this thesis. Different groups, including ourselves, have previously shown that CPPs can form nanoparticles with negatively charged ONs (including siRNAs) and plasmids with a diameter of 100–300 nm (67, 68). These nanoparticles are suggested to be taken up by the cells via an endocytosis-dependent process. In case of plasmids, pGL3 or pEGFP-C1 plasmid (4.7 kb), expressing luciferase or EGFP respectively were used. Plasmids were mixed with CPPs at different peptide:plasmid charge ratios (CRs), which were calculated theoretically, taking into account the positive charges of the peptide and negative charges of the plasmid. Complexes were formed for 1 h at room temperature then added to the cells. By measuring luciferase expression, the efficiency of delivery for different peptides and CRs can be assessed for different cell-lines.

3.4. siRNA delivery (Paper II)

In this paper, the nanoparticles were formed by mixing the peptide and siRNA in water at different molar ratios (MRs). Reporter cell-lines stably expressing luciferase were transfected with PF6 complexed with luciferase siRNA, and the decrease in luciferase expression was used to quantify the transfection efficiency. In addition, we investigated the targeting of an endogenous gene, hypoxanthine phosphoribosyl transferase 1 (HPRT1), in several other cell-lines by measuring mRNA levels using qPCR (real time quantitative polymerase chain reaction). HPRT1 was chosen due to the long cellular half-life of the protein (around 48 h) and thereby minimal impact on the vitality of the transfected cell and limited off-target effects of the specific HPRT1 siRNA sequence (69). In order to accurately determine the degree of down-regulation, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard for quantification.
3.5. SSOs delivery (Paper III)

Two cell lines were utilized in this study, Hela pLuc 705 and Mdx mouse myotubes. The HeLa pLuc705 cell-line is stably transfected with a luciferase-encoding gene interrupted by a mutated β-globin intron 2. This mutation causes aberrant splicing of luciferase pre-mRNA resulting in the synthesis of non-functional luciferase (70). Masking the aberrant splice site with SSOs redirects splicing towards the correct mRNA and consequently restores luciferase activity. Mdx mouse myotubes were obtained from confluent H2K mdx cells, which is a myogenic cell-line derived from transgenic mice carrying a point mutation in exon 23 of the dystrophin gene. Thus this cell-line serves as the leading cell-model system for development of drugs and drug delivery systems for treatment of DMD. Masking the point mutation in exon 23 with SSOs leads to the production of a shorter, partially-functional dystrophin mRNA that can be quantified with reverse transcriptase PCR (RT-PCR). SSOs were non-covalently mixed with PF14 at different MRs in water. Splice-switching efficiency was calculated as the percentage of the corrected band (exon skipping %) to the sum of corrected and aberrant bands.

3.6. Toxicity

To exclude that the activity of the peptides in-vitro is associated with toxicity, WST-1 assay was used. This assay measures cell viability as a function of mitochondrial metabolic activity. Wst-1 measures the activity of the mitochondrial dehydrogenases to convert tetrazolium salts to formazan and cell viability and proliferation is directly correlated to the amount of formazan dye formed, which is quantified spectrophotometrically.

3.7. Dynamic light scattering (DLS) and zeta-poteontial

To investigate the physicochemical characteristics of the nanoparticles formed upon complexation of the peptides and plasmids or ONs, DLS and zeta-potential measurements were carried out. The nanoparticles in solution after complexation can be regarded as a colloidal
dispersion system. Upon shining laser light on this system, the Brownian motion of the dispersed particles causes fluctuations in light-scattering intensity as a function of time, which can be correlated to the size of the scattering particles (71, 72). Each charged particle in a solution containing ions is surrounded by an electrical double layer of ions and counterions (figure 2) (73). The first layer of strongly bound counterions is commonly called Stern layer. Since it is strongly attached, this layer moves together with the particle in the medium. The second layer contains ions and counterions and is less strongly bound to the suspended particle, thus called “diffusion layer”. This layer is in direct contact with the bulk of the suspending liquid, and in contrast to the stern layer, ions of this layer do not move with the particle while moving in the medium. The potential difference across the double layer, i.e. between the edge of the Stern layer and the edge of the diffusion layer (bulk of suspending liquid) is called Zeta potential (figure 2) (72, 73).

**Figure. 2.** Different layers forming the zeta potential of the particle.
It is determined by electrophoresis of the sample and measuring the velocity of the particles using laser Doppler velocimetry (73). Zeta potential is highly sensitive to the type and amount of ions in the suspension and its pH.

3.8. Solid dispersion

In paper III, to test the feasibility of formulating PF14/SSO nanoparticles into solid formulation, solid dispersion technique was utilized. Although initially invented to increase the solubility of poorly water-soluble drugs by dispersing them over hydrophilic solid matrices (74), it is not used for the same purpose in the present work. This is because PF14/SSO nanoparticles are already water-soluble. However, the technique was adopted to enable us obtain uniform distribution of the nanocomplexes over water-soluble excipients by solvent evaporation. The suspension of nanoparticles was first mixed with different excipient solutions at different concentrations. For solvent evaporation (in this case water), we chose speed drying under elevated temperature (55 -60 °C) and reduced pressure. Despite being relatively stressful drying method, it is more relevant to the widely used, cost-effective, heat-based pharmaceutical drying techniques.

3.9. Animal Experiments

_In-vivo_ animal experiments were carried out in paper I and paper II. All animal experiments were approved by The Swedish Local Board for Laboratory Animals, and were designed to minimize the suffering and pain of the animals.

In paper I, stearyl-TP10/plasmid nanoparticles were injected _i.d._ or intramuscularly _i.m._ into _M. tibialis anterior_. Gene expression was assessed by imaging of the reporter gene (firefly luciferase) expression. In paper II, in luciferase experiments, mice were first injected by high dose of a luciferase expressing plasmid via hydrodynamic injection to stably express luciferase gene in the liver. After 3 weeks, the animals were systemically injected (tail vein injection) with PF6/luc-siRNA nanoparticles, and the downregulation was quantified by imag-
ing of luciferase expression in the liver. In HPRT1 experiments, animals were systemically injected with PF6/HPRT1-siRNA nanoparticles and downregulation of the HPRT1 mRNA was measured in different organs by qPCR. In addition, blood samples were collected in heparinized tubes to assess acute toxicity by measuring the levels of liver transaminases (ALT/AST), creatinine, white blood cells and C-reactive protein.
4. Results and discussion

The three papers in this thesis cover three newly developed chemically modified CPPs used for the delivery of different nucleic-acid based therapeutics in-vitro and in-vivo.

4.1. Delivery of plasmids via stearyl-TP10 in-vitro and in-vivo (Paper I)

Here, we demonstrate that stearylation of the TP10 peptide has a significant impact on plasmid delivery in different cell-lines including Chinese hamster ovary (CHO), Human Embryonic Kidney (HEK293), human glioblastoma (U87), human osteosarcoma (U2OS) and the hard-to-transfect primary mouse embryonal fibroblasts (MEF). Stearyl-TP10 (Table 2) transfection efficiency was in parity with the commercial lipid-based transfection reagent Lipofectamine-2000™, with less toxicity. In addition, stearyl-TP10 was able to transf ect entire cell population in a ubiquitous manner. However, the transfection efficiency was dependent on the charge ratio (CR) between the peptide and the plasmid. DLS studies showed that the nanoparticles are in the range of 120-150 nm. Compared to the unstearylated version, stearyl-TP10 resulted in around 4 log increases in luciferase expression levels as compared to plasmid-treated cells. Clearly, stearic acid renders the peptide more hydrophobic and presumably hydrophobicity plays an important role in particle formation, as it enables more pronounced plasmid DNA condensation and the formation of small, stable particles. This protects plasmid DNA, making it more stable against the degrading capacity of serum enzymes. The drastic increase in activity of stearyl-TP10 compared to TP10 could also be a result of increased endosomal escape. To corroborate this, the uptake levels of fluoresceinyl-labelled plasmid complexed with TP10 and stearyl-TP10 were assessed in serum free conditions, as TP10 nanoparticles are very la-
bile in the presence of serum. Minor differences were observed in the levels of uptake between the two peptides, which indicated that the higher activity of stearyl–TP10 might be due to better endosomal escape.

These stearyl-TP10/plasmid nanoparticles facilitated efficient gene delivery in muscle and skin, after i.m. or i.d. injections, respectively. In both cases, luciferase activity was increased around 1 log as compared to the background levels and these effects were shown to be dose-dependent. We also confirmed that the gene delivery did not trigger any immune response in-vivo and that these treatments were not associated with any systemic toxicity. Interestingly, these effects were only seen with the nanoparticles formed at CR1, while at other CRs, no effect on luciferase activity was seen. The critical dependency on certain CR possibly emanates from the avidity of stearyl-TP10 towards the plasmid and, therefore, the stability of these nanoparticles. Probably, at higher CRs release of the plasmid from the complexes is perturbed and the affinity of stearyl-TP10 towards it is too great for in-vivo applicability. Therefore, the plasmid cannot escape from the nanoparticle complex and, consequently, does not reach the cytoplasm throughout the tissue with higher efficacy than naked plasmid. These differences between the avidity at different CRs were confirmed by other assays in the paper.

4.2. Delivery of siRNA via PF6 in-vitro and in-vivo (Paper II)

siRNA has attracted a lot of attention in recent years as it was regarded as the ideal therapeutic molecule having the properties of specificity, potency, tolerability, and universality, except for being impermeable through the cell membrane due to its size and charge (75). In this paper, we tried to further enhance the endosomal escape properties of stearyl-TP10 and utilize it for siRNA delivery in-vitro and in-vivo. We hypothesized that incorporation of chloroquine (CQ) analogues into CPP backbone would mimic the pH buffering and osmotic endosome swelling effects of CQ (76). Hence, we introduced four novel trifluoromethylquinoline-based derivatives (QN) via a succinylated lysine tree into the stearyl-Tp10 sequence to improve endosomal release of siRNA (Table 2). The mean diameter of PF6/siRNA nano-
particles, formed at various molar ratios (MRs) was below 200 nm. The endosomolytic design rendered PF6 highly efficient in promoting siRNA-mediated RNAi in various cells, targeting either reporter genes (EGFP and luciferase), or endogenous genes (HPRT1 and Oct-4). In contrast to lipofection, PF6 promoted siRNA-mediated gene silencing in entire cell populations and, thus, dose-dependently exceeded the activity of the commercial lipofection reagents in practically all tested cells. Most importantly, the activity was well preserved in serum. Furthermore, while primary cells and suspension cells remain relatively refractory to most transfection strategies, PF6 efficiently promoted siRNA-mediated, dose-dependent gene silencing in different refractory cells such as Huvec, Jurkat, C17.2 neuronal stem cells and embryonic stem cells with IC₅₀ values ranging from 10–50 nM. These results collectively show that PF6 has the potential to transfect different cell types without affecting the phenotype of cells, which opens new possibilities for large-scale RNAi screens in disease relevant primary cells.

After confirming the potency of PF6 in cell culture, we next assessed the suitability of the vector for systemic delivery of siRNA in mice. Using the HPRT1 house-keeping gene as a target, we observed pronounced knockdown in liver, kidney and lung, following systemic delivery of PF6/HPRT1-siRNA, possibly reflecting their high blood supply. The majority of previous other successful RNAi reports are indeed based on results achieved from the liver, whereas efficient gene knockdown in other organs requires much more complicated strategies and multicomponent delivery vectors (77, 78). Interestingly, we observed over 60% gene silencing also in kidneys and lung, by using PF6 as a single-component vehicle without additional targeting motifs and without associated toxicity.

Based on the encouraging results of strong RNAi response in liver we utilized another animal model to analyze gene silencing in liver only. In this model, mice with luciferase expressed in the liver were treated with PF6/luc-siRNA nanoparticles. By using doses up to 1 mg/kg, substantial luciferase knockdown was observed, which lasted for more than a week. Hydrodynamic injection is frequently considered the golden standard technique for achieving siRNA-mediated RNAi responses in liver (79). Intriguingly, RNAi responses with PF6/siRNA in liver were at least in line with that standard technique. These results are comparable to recently published results on the use
of lipid nanoparticles (LNPs) for siRNA, targeting luciferase in liver of mice (80). Using LNPs with optimized composition (right amount of cationic liposomes, polyethylene glycol and cholesterol), the authors reported on significant RNAi responses for 10 days using doses of 3 mg/kg siRNA. The strong RNAi responses observed in liver could relate to the negative zeta-potential observed for PF6/siRNA in serum media since it has been previously reported that various lipid-based systems with negative surface charge efficiently target hepatic cells in-vivo (81, 82).

4.3. Delivery of SSOs via PF14 in-vitro and solid formulation development (Paper III)

In this paper we present a new chemically modified CPP, PF14. Starting from stearyl-TP10, we utilized ornithines as the main source of positive charges instead of lysines. The design of this peptide was based on earlier reports showing that poly-L-ornithine demonstrated superior transfection efficiency (up to x 10-fold) compared to equivalent poly-L-lysine-based systems (83). The superior efficiency of poly-L-ornithines was related to the higher affinity for DNA and the ability to make more stable complexes at lower charge ratios (83). Furthermore, we hypothesized that ornithine, being a nonstandard amino acid, would be less prone to serum proteases, and thus could retain the activity in serum conditions.

PF14 was tested in HeLa 705 and mdx mouse myotubes cell-lines utilizing different MRs. Robust splice-switching was observed in both cell lines in a dose dependent manner. This was confirmed on the protein and mRNA level by measuring the amount of functional luciferase enzyme produced in the HeLa 705 cell-line and by measuring the intensity of corrected mRNA bands via RT-PCR in both cell lines respectively. The activity was not significantly affected by serum conditions and at certain MRs in HeLa 705 cell-line PF14 significantly exceeded the activity of Lipofectamine2000® without associated toxicity. In addition, PF14 elicited a fast onset of its splice-switching activity as early as 8 hours and was able to transfect entire cell population without being very sensitive to increasing cell densities. Co-localization with endocytosis markers demonstrated that endocytosis is responsible for the uptake of PF14/SSO nanoparticles and this was
Further corroborated by the enhanced splice-switching activity upon addition of CQ.

Finally, we wanted to take our delivery system a step further by developing a suitable formulation for administration. Although CPPs have been extensively exploited in recent years for the delivery of various therapeutics (56), to our knowledge, formulation of CPPs into different pharmaceutical forms has never been thoroughly studied. Therefore, to expand the range of therapeutic applications of CPP-based therapeutics, there is a need for studies exploring the possibility of incorporating CPPs in different pharmaceutical forms; especially the solid form which is widely used in several pharmaceuticals. We applied the solid dispersion technique by mixing the nanoparticles in suspension with excipient solutions then drying at relatively high temperatures (55-60 °C) under vacuum. Mannitol, lactose and PEG 6000 were used as excipients at different concentrations. It was clear that different excipients and concentrations thereof had a huge impact on the activity of the formulation, with lactose at a concentration of 3.33% demonstrating splice-switching activity nearly identical to the freshly prepared nanoparticles in solution. On the contrary, Lipofectamine2000® almost lost its entire activity upon application of this drying and dispersion procedure. In order to assess that the presence of intact nanoparticles after the formulation procedure, DLS studies were performed comparing the freshly prepared nanoparticles with the solid formulations. We found that the particles size and particle-size distribution is highly affected by the type of excipient used and the formulation which mediated the highest splice-switching activity, also had DLS profile most similar to the freshly prepared nanocomplexes. Upon measuring the zeta potential of the nanoparticles, we found that they are negatively charged either freshly prepared or in formulation similar to the results obtained for PF6/siRNA nanoparticles.

Next, we subjected the best performing formulation (lactose at 3.33%) to stressful stability testing conditions at elevated temperatures for 2 months. Storage at high temperatures increases the rate of degradation reactions that could take years to occur (84). PF14 formulations demonstrated an excellent stability profile under such conditions where no statistically significant loss in transfection efficiencies at any time-point except for the 60 °C-8-weeks point where the transfection efficiency decreased to 70% of the initial value. Compared to lyophilized lipoplexes (85, 86), PF14 formulations demonstrated an excel-
lent stability profile without further additives or special storage conditions.
5. Conclusions

This thesis described three new chemically modified CPPs that were used to deliver nucleic-acid based molecules in various gene therapy approaches. We clearly demonstrate that these peptides form stable nanoparticles with the negatively charged cargos and are efficiently internalized in several cell-lines \textit{in-vitro} and \textit{in-vivo}. The nanoparticles were also active in solid formulation that can be used for different routes of drug administration.

**Paper I:** Introduces a new CPP, stearyl-TP10, for delivery of plasmids \textit{in-vitro} in various cell-lines and \textit{in-vivo} after \textit{i.m} and \textit{i.d} injection.

**Paper II:** Introduces a new CPP, PF6, for delivery of siRNA \textit{in-vitro} and after systemic administration \textit{in-vivo}.

**Paper III:** Describes a new CPP, PF14, for delivery of SSOs in different cell models including mdx mouse cell-line, an \textit{in-vitro} model for Duchenne’s muscular dystrophy. A method to formulate PF14/SSO nanoparticles into a solid form was also developed.
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7. References


