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Cell-penetrating peptide mechanism studies: from peptides to cargo delivery

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ABSTRACT

Cell-penetrating peptides (CPPs) are short amino acid sequences, consisting of up to 30 amino acids, capable of delivering bioactive cargos inside cells in an efficient and non-toxic fashion. Several cellular internalization pathways have been proposed for these molecules and the majority of studies conclude that CPPs utilize several uptake routes simultaneously with some favored more than others. The reports vary as internalization depends on the type and concentration of the CPP, the nature of the cargo, the specific cell membrane composition of the studied cell line, the intracellular target and other experimental conditions. In the present thesis, uptake mechanisms of several common CPPs are characterized by studying their cytosolic uptake kinetics using fluorescent and bioluminescent cargos. In addition, transfection mechanisms of non-covalent CPP-oligonucleotide nanocomplexes are investigated by assessing the effects of hydrophobic CPP modifications and complex formation on their efficacy.

We utilized kinetic assays to determine the simultaneous involvement of several uptake pathways in CPP internalization. The chosen peptides displayed very different and concentration dependent uptake kinetic profiles that were strongly affected by endocytosis inhibitors. Both of the studies support the simultaneous involvement of several endocytotic pathways in their cellular uptake. To study the hydrophobic effects on transportan 10 mediated splice-correcting antisense oligonucleotide delivery, we conjugated different fatty acids to the peptide and measured how these modifications affect the hydrodynamic size and transfection efficacy of CPP-oligonucleotide complexes. We determined the optimal hydrophobic CPP modification and nanocomplex size for maximal splice correction efficiency. In order to elucidate the role of complex formation in CPP-mediated siRNA transfections, we utilized isothermal calorimetry in conjunction with dynamic light scattering studies. The results revealed that although the complexes are with the same size at acidic and physiological pH, the amount of CPPs in the nanocomplexes varies, indicating that the dynamic equilibrium between the peptides and the cargo is pH-dependent.

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LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications, which will be referred to by the corresponding Roman numerals in the text:

I. **Langel, K**., Lindberg, S., Copolovici, D.M., Arukuusk, P., Sillard, R., Langel, Ü. (2010) Novel Fatty Acid Modifications of Transportan 10. *International Journal of Peptide Research and Therapeutics, Volume 16, Number 4*, 247–255.

II. Mäger, I., Eiríksdóttir, E., **Langel, K**., EL Andaloussi, S., Langel, Ü. (2010). Assessing the uptake kinetics and internalization mechanisms of cell-penetrating peptides using a quenched fluorescence assay. *Biochimica et Biophysica Acta-Biomembranes, 1798(3)*, 338–343.

III. Mäger, I., **Langel, K***., Lehto, T., Eiríksdóttir, E., Langel, Ü. (2012). The role of endocytosis on the uptake kinetics of luciferin-conjugated cell-penetrating peptides. *Biochimica et Biophysica Acta-Biomembranes, 1818(3)*, 502–511.

IV. **Langel, K**., Arukuusk, P., Tenson, T., Langel, Ü. (2014). Characterization of CPP:siRNA nanocomplexes by Isothermal Titration Calorimetry. S*ubmitted*.

* Shared first author

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My personal contribution to the articles referred to in this thesis is as follows:

I. planned and performed most of the experiments and data analysis, wrote the paper as corresponding author;
II. performed the synthesis of the peptides, participated in writing of the paper;
III. performed most of the experiments, analyzed the data and participated in writing of the paper;
IV. planned and performed most of the experiments and all data analysis, wrote the paper as corresponding author

ADDITIONAL PUBLICATIONS

Järver, P., **Langel, K**., EL Andaloussi, S., and Langel, Ü. (2007) Applications of cell-penetrating peptides in regulation of gene expression. *Biochem. Soc.Transactions, 25(4)*, 770–774.

Veiman, K-L., Mäger, I., Ezzat, K., Margus, H., Lehto, T., **Langel, K**., Kurrikoff, K., Arukuusk, P., Suhorutsenko, J., Padari, K., Pooga, M., Lehto, T., Langel, Ü. (2013) PepFect14 peptide vector for efficient gene delivery in cell cultures. *Mol Pharm, 10(1)*, 199–210.

Saar, I., Lahe, J., **Langel, K**., Runesson, J., Karlsson, K., Järv, J., Rytkönen, J., Närvänen, A., Kurrikoff, K., Langel, Ü. (2013) Novel systemically active galanin receptor 2 ligands in depression-like behaviour. *Journal of Neurochemistry. 127(1)*, 114–123.

Copolovici, D.M., **Langel, K**., Eriste, E., and Langel, Ü. (2014) Cell-Penetrating Peptides: Design, Synthesis and Applications. *ACS Nano. 8(3)*, 1972–94.

ABBREVIATIONS

Abz	2-Aminobenzoic acid
C/LR	Caveolae/lipid raft dependent endocytosis
CME	Clathrin-mediated endocytosis
CPP	Cell-penetrating peptide
Cpz	Chlorpromazine
CQ	Chloroquine
CvME	Caveolae-mediated endocytosis
CyD	Cytochalasin D
DLS	Dynamic light scattering
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EPR	Enhanced permeability and retention
Fmoc	9-Fluorenylmethyloxycarbonyl
HSPGs	Heparan sulphate proteoglycans
ITC	Isothermal titration calorimetry
LDH	Lactate dehydrogenase
MP	Macropinocytosis
MPS	Mononuclear phagocytic system
MQ	MilliQ water
MR	Molar ratio
NF51	NickFect51
Nys	Nystatin
ON	Oligonucleotide
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PF6	PepFect6
PI3K	Phosphatidylinositol 3-kinase
PMO	Phosphorodiamidate morpholino oligonucleotide
PNA	Peptide nucleic acid
SCO	Splice-correcting antisense oligonucleotide
siRNA	Small interfering RNA
SPPS	Solid-phase peptide synthesis
t-Boc	*tert*-Butoxycarbonyl
TP10	Transportan 10

INTRODUCTION

In order to successfully utilize the vast number of available potential drugs in clinical settings, they need to reach their required target. More often than not, this requires an efficient and safe delivery vector. Cell-penetrating peptides (CPPs) are a class of non-viral delivery vectors that meet these criteria.

As of today, more than one hundred CPPs are available with varying physicochemical properties and internalization mechanisms. In order to sift out the most effective delivery vectors, their exact uptake mechanisms, kinetics and cargo delivery properties need to be determined.

Kinetic uptake studies coupled with endocytic pathway studies enable to determine the involvement and extent of each uptake route in CPP internalization in a more transparent fashion compared to single-endpoint methods. We therefore assessed the cytoplasmic uptake kinetics and mechanisms of several common CPPs using a quenched fluorescence assay and a semi-biological bioluminescence assay.

Physicochemical studies of CPP-cargo complexes can reveal the uptake mechanisms of these dynamic non-covalent conjugates that can be used in the further development of more potent delivery vectors. We assessed the role of CPP hydrophobicity in the delivery of splice-correcting antisense oligonucleotides (SCOs). In addition, we characterized the interactions between CPPs and siRNA to shed light on their transfection mechanism.

The results presented in this thesis exemplify the advantage of kinetic assays as well as the importance of studying the physicochemical properties of non-covalent CPP-oligonucleotide nanocomplexes in understanding CPP and CPP-cargo uptake mechanisms. This thesis brings out crucial aspects that have to be accounted for when designing novel delivery vectors for biotechnological and clinical applications.

1. LITERATURE OVERVIEW

Advances in biochemistry and molecular biology over the years, together with the human genome sequencing project, have increased our understanding of the genetics of numerous diseases and has led to the identification of many disease-causing genes. In order to treat these diseases by inhibiting, modulating or introducing new genes to the affected cells, appropriate drugs need to be transported into their point of action. Due to their physicochemical properties, many drugs are unable to enter cells and require delivery vectors which are efficient, safe, and resistant to degradation. Generally, there are two types of vehicles, viral and non-viral. The most studied non-viral delivery vectors are liposome-, polymer- and micelle- based systems, and more recently, cell-penetrating peptides (CPPs) have gained interest as well.

This thesis concentrates on the cellular internalization mechanisms and CPP uptake kinetics characterization of CPP and CPP-cargo conjugates.

1.1 Cell-penetrating peptides (CPPs)

CPPs are short peptide sequences consisting of up to 30 amino acids that are able to cross the cellular membrane and transport bioactive cargos into cells in a non-toxic and efficient fashion. CPPs generally are positively charged and amphipathic, possessing both hydrophilic and lipophilic properties, enabling them to interact both with cargos and the cell membrane.

1.1.1 CPP origins

The inception of the CPP field is closely related to the finding that full length HIV-1 transcription transactivation (Tat) protein [1] and Drosophilia Antennapedia homeodomain [2] were shown to be able to translocate to the interior of cells and accumulate in the cell nucleus. This led to the discovery of protein transduction domains (PTDs), launching the field of CPPs. The first CPPs penetratin [3] and Tat [4] were exclusively derived from naturally occurring transport proteins. However, later many novel CPPs with chimeric nature, such as MPG [5], Pep-1 [6] and TP10 [7] or of completely designed amino acid sequences, such as polyarginines [8, 9], YTA-2 and YTA-4 [10], MAP [11] and CADY [12], have been proposed.

1.1.2 CPP classification

CPPs can be classified according to their origin or chemical properties, such as their charge, hydrophobicity or amphipathicity. Classification by origin, as exemplified in the previous chapter, focuses more on CPP evolution and is therefore less informative. In order to study CPP cellular internalization mechanisms, the peptides need to be categorized based on their structural and chemical properties. As a result, CPPs can be divided into cationic, hydrophobic and amphipathic peptides (Table 1).

Table 1. Examples of common CPP by classification.

CPPs	Origin	Sequence	Ref.
Cationic			
R_9	Designed	RRRRRRRRR	[8]
RxR	Designed	RxRRxRRxRRxR	[13]
Tat	HIV-1 transactivator protein	GRKKRRQRRRPPQ	[4]
Hydrophobic			
SG3	Plasmid selection	RLSGMNEVLSFRWL	[14]
Pep-7	Phage display	SDLWEMMMVSLACQY	[15]
KF	Kaposi fibroblast growth factor	AAVALLPAVLLALLAP	[16]
Primary amphipathic			
TP10	Galanin and Mastoparan	AGYLLGKINLKALAALAKKIL	[7]
Secondary amphipathic			
Penetratin	Antennapedia homeodomain	RQIKIWFQNRRMKWK	[3]
MAP	Designed	KLALKLALKALKAALKLA	[11]
CADY	Chimeric peptide PPTG1	GLWRALWRLLRSLWRLLWRA	[12]
pVEC	Murine VE-cadherin	LLIILRRRIRKQAHAHSK	[17]
EB1	Chimeric	LIRLWSHLIHIWFQNRRLKWKKK	[18]
Proline-rich			
SAP	Designed	VRLPPPVRLPPPVRLPPP	[19]

x-aminohexanoic acid

Typical cationic CPPs, R_9 [8], Tat [4] and $(RxR)_4$ [13, 20] all possess a high positive net charge and no acidic amino acid residues. Although the number of arginine residues can vary, studies suggest that at least six positive charges are required to induce efficient CPP uptake [21]. These peptides interact with anionic cell membrane constituents such as glycosaminoglycans and rely less on interactions with membrane lipids [22, 23]. Arginine-rich peptides were initially considered as the "Trojan horse" delivery vectors that enter cells without eliciting any adverse cellular responses [24]. Nevertheless, later studies have shown that these CPPs can induce a wide range of side-effects, including reduced membrane integrity and cell viability [25].

CPPs containing only apolar residues can be classified as hydrophobic and so far only a few have been discovered, such as the signal sequence from Kaposi fibroblast growth factor [26] and SG3, a CPP identified by a plasmid display-based selection platform [14]. However, hydrophobic amino acid residues are crucial in case of many amphipathic and other long chimeric CPPs.

One of the most informative methods of CPP classification is based on their physicochemical properties. Peptides, such as TP10 [27], contain both polar and non-polar regions and are categorized as primary amphipathic CPPs. The hydrophobic character of these CPPs has been shown to be responsible for their cellular internalization mechanism as indicated by their affinity to both neutral and anionic lipid membranes [22, 28, 29]. However, the interaction between the

cell membrane and these CPPs is strongly dependent on the membrane potential and composition [30], as primary amphipathic peptides can induce concentration dependent leakage in model membranes and often lyse bacteria without affecting eukaryotic cells [31].

Secondary amphipathic CPPs have a highly hydrophobic region on one face of the peptide while the other face may be cationic, anionic or polar. Common examples include penetratin [3], MAP [11], CADY [12] and pVEC [17]. These peptides maintain a random coil structure in solution but adapt an α-helical or in some cases β-sheet structure once in contact with cell membrane components, such as glycosaminoglycans and lipids. Secondary amphipathic CPPs interact weakly with neutrally charged membranes and bind to cell membranes which contain at least 20% anionic lipids [22, 32], with the possible exception of MAP, which causes membrane leakage at high concentrations [33, 34].

Another class of amphipathic CPPs is proline-rich peptides, which contain a proline or pyrrolidine template and exhibit self-assembly properties [19].

1.1.3 CPPs in cargo delivery

Cargos can be conjugated to CPPs covalently or complexed non-covalently. Generally, in order to attain the desired biological effect, once internalized, the cargo needs to be released. To achieve this in case of covalent CPP-cargo conjugates, biodegradable linkers have to be used. One of the most commonly used strategies is conjugation through a disulfide linker [35–38], as it remains sufficiently stable in the extracellular media and is cleaved once reaching reducing intracellular compartments, particularly the cytosol [39, 40]. In addition, disulfide bond cleavage in endosomes and lysosomes is very inefficient as endocytotic pathways are oxidizing [40]. However, evidence suggests that in some cases disulfide cleavage can also occur on the cell surface [36] and therefore it is important to observe the free thiol contents in the extracellular environment when utilizing disulfide linkers. Examples of successful covalent CPP-cargo conjugates include peptides [41], proteins [42], chemotherapeutics [43] and oligonucleotides (ONs) [35, 44]. Because the negatively charged oligonucleotides can mask the positive CPP charges and render the conjugate ineffective [45, 46], in covalent conjugation strategies, uncharged ON analogues such as peptide nucleic acids (PNAs) and phosphorodiamidate morpholino oligonucleotides (PMOs) have to be utilized as cargos [47, 48].

Non-covalent CPP conjugation strategy can be used virtually for any negatively charged molecule and it has been widely applied for short splice-correcting antisense oligonucleotides (SCOs), small interfering RNAs (siRNAs) and large plasmid DNA (pDNA). The benefits of non-covalent complexation are convenient sample preparation, easily variable CPP-to-cargo ratios and that the intrinsic activities of both CPPs and cargo are preserved. These nanocomplexes can also be easily modified with additional targeting and/or shielding moieties. However, there are limitations associated with the use of non-covalent complexes *in vivo*. The CPP-cargo nanocomplexes have to be resistant to degradation

and clearance and reach the target site in sufficient doses. Therefore, clinical applications might be limited to local administration [49].

1.1.4 Evolution of CPPs

In order to utilize CPPs as effective cellular delivery vectors they have to associate with cargo molecules optimally, so that the complexes are intact in the extracellular medium and dissociate once taken up by endosomes and enable the release of the cargo into cells. CPPs also have to be resistant to degradation by serum enzymes but at the same time remain non-toxic. The systemic toxicity of certain CPPs could be reduced by improving their cell or tissue specificity, thereby decreasing required effective concentration [50].

Covalent conjugation strategies have been used more frequently for cargo delivery as most peptides are not able to condense cargo into nanocomplexes and/or subsequently release it from endosomes. In case of non-covalent conjugates, mainly three types of interactions occur between positively charged CPPs and negatively charged oligonucleotides – electrostatic, hydrophobic and hydrogen bonding. In order to develop efficient delivery vectors, all of these properties have to be optimized. For example, stearoylation of octaarginine increases its plasmid transfection efficiency by approximately 100-times [51].

However, the major issue for novel CPP delivery vectors remains endosomal escape, as most studies indicate endocytosis as the dominant internalization route for non-covalent CPP complexes. A common method for overcoming endosomal entrapment is co-incubation of cargo complexes with the endosomolytic agent chloroquine [52]. Recently, this rationale was used for the development of a highly potent CPP, PepFect6 (PF6), by incorporating chloroquine analogues in its chemical structure [53]. PF6 was additionally modified with a fatty acid which could contribute to endosomal membrane disruption [54, 55]. Hydrophobic modifications could also increase the CPPs resistance to proteases and peptide stability can be further increased by using D-form or unnatural amino acids [56, 57]. Some CPPs, such as KALA [58] and EB1 [18] can also display inherent endosomolytic properties as their conformation adapts to changes in pH as they are transported to acidic endosomal compartments.

In order to enhance the clinical applicability of CPPs, they should be cell- or tissue type-specific. This can be achieved by adding targeting moieties such as certain amino acid sequences to CPPs. For example, rabies virus glycoprotein-derived peptide is capable of crossing the blood-brain-barrier [59] and CendR peptides internalize into cells and bind to the tumor cell-specific receptor neuropilin-1 [60]. Numerous other targeting ligands have been tested over the years with some showing great promise [61].

1.1.4.1 Hydrophobic modifications

As mentioned in the previous chapter, the first successful hydrophobic modification in the CPP field was stearoylated octaarginine. The improved transfection efficiency can be a result of increased CPP-oligonucleotide binding as described in case of stearoylated cationic polyaspartamide derivatives that displayed more effective siRNA condensation and siRNA mediated gene knockdown than their non-stearoylated parent peptides [62]. In addition, hydrophobic moieties might be exposed on the surface of the formed nanoparticles and thereby contribute to the absorption of the complexes on the cell membranes and the subsequent disruption of endosomal membranes. Hydrophobic fatty acid chains could also increase resistance against serum degradation [63].

Fatty acid modifications have improved the cargo delivery properties of numerous CPPs. For example, stearoylation highly increased the SCO transfection efficiency of $(RxR)_4$ and TP10 peptides [13, 64], however several reports indicate that the optimal length of the fatty acid modification can differ between CPPs. Therefore numerous different fatty acids have been tested in conjunction with this strategy. For Alexa-labelled octaarginine, a hexanoyl modification showed the highest efficiency of cellular uptake of the studied variants [65] and in case of an anti-proliferative somatostatin analog, pamitoylation significantly increased its activity [63]. Also, addition of capric acid to human calcitonin derived CPPs resulted in efficient cytosolic delivery of siRNA. The developed vectors were used for knockdown of the G protein-coupled human neuropeptide YY_1 receptor [66]. Another fatty acid, laurylic acid, combined with the use of D-amino acids, was shown to increase the cellular uptake of oligoarginines. These CPPs efficiently internalized to the cells and exerted low cellular toxicity, however, if lacking the lipid chain, peptides were degraded and transported out of the cells [67]. The lauroyl modification was chosen based on the screening of several fatty acid modifications stressing the need for an optimal fatty acid length [68]. Recently a series of highly potent stearoylated CPPs, NickFects and PepFects, were developed that are able to deliver various nucleic acids inside cells [53, 69–71].

In paper I, the effect of twelve different hydrophobic modifications on TP10 mediated splice-correcting antisense oligonucleotide delivery was investigated.

1.2 CPP internalization mechanisms

Ever since the introduction of the first CPPs, penetratin and Tat, more than 100 CPPs have been developed [61]. These peptides can possess very different physicochemical properties and have therefore shown to utilize various cellular internalization mechanisms (Figure 1).

Studying the mode of action of all types of CPPs involves registration of cellular entry after inhibiting certain cellular entry pathways. Most studies concentrate on affecting endocytic pathways by either chemical inhibitors, knocking down selected genes responsible for certain endocytotic routes, or by simply

lowering the temperature to where active transport processes are stopped. From these types of studies it has been concluded that generally CPPs are taken up by several endocytotic pathways simultaneously with one path favored more than others in case of certain CPPs. Endocytosis is mainly triggered by interactions between the peptide and cell membrane constituents, such as negatively charged glycosaminoglycans and phospholipids. In addition, especially when attached to larger cargos, CPPs can utilize certain receptors in their uptake, such as class A scavenger receptors [70, 72]. As chemical modifications have to be introduced to CPPs in order to study their uptake, interpretations should be made in the context of the CPP and the cargo together.

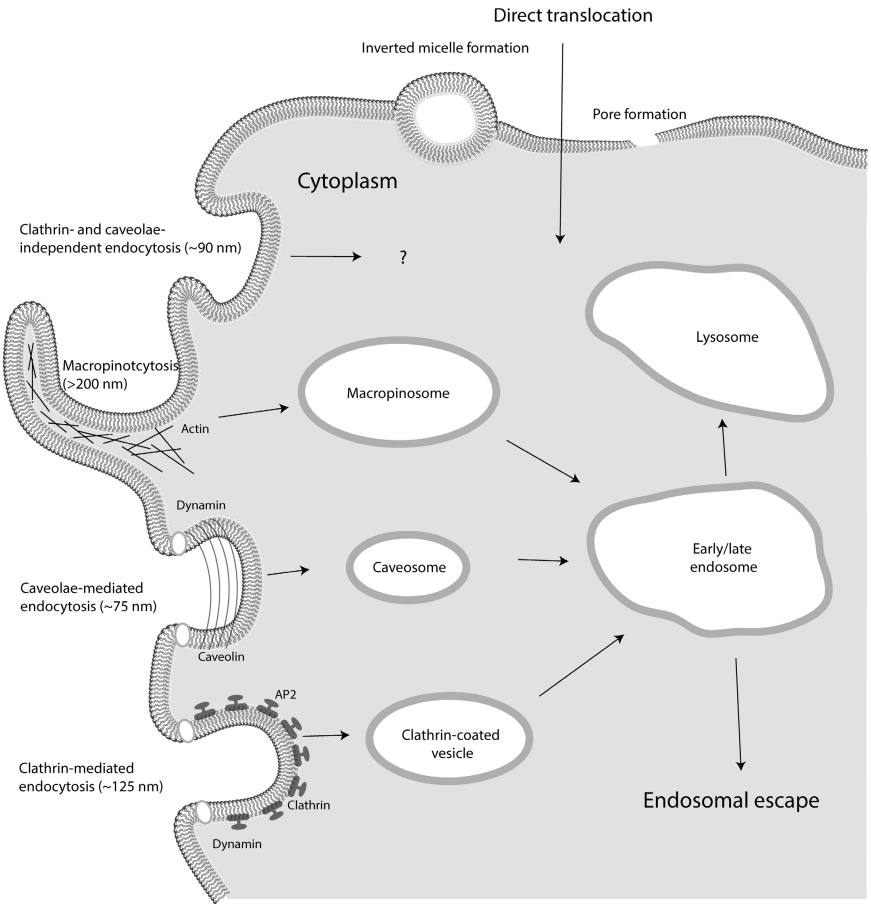

Figure 1. Cellular uptake mechanisms utilized by CPPs. Dynamin dependent clathrin- and caveolae-mediated endocytosis as well as dynamin independent macropinocytosis and clathrin- and caveolae independent endocytosis pathways are all involved in CPP uptake. The resulting vesicles are of different diameter in case of each pathway. Initial CPP uptake is followed by intracellular trafficking into a variety of endomembrane vesicular compartments including early/late endosomes and lysosomes. Endosomal escape of CPPs can occur at any point during this process. In addition, some CPPs destabilize the cellular membrane and are taken up by direct translocation, i.e. through inverted micelle- or pore formation.

1.2.1 Endocytosis

Endocytic mechanisms can affect the lipid and protein composition of the plasma membrane, thereby regulating the transport of bioactive molecules into cells [73]. Classically, cellular uptake pathways are divided into endocytic pathways and non-endocytic pathways. Endocytosis is commonly said to consist of four types of pathways: phagocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), macropinocytosis (MP) and clathrin- and caveolin independent endocytosis [74].

Phagocytosis has generally not been associated with CPPs as it occurs only in macrophages, monocytes, neutrophils, and dendritic cells [75] whereas CME, CvME, and MP occur in almost all kinds of cells including the aforementioned [74, 76]. The phagocytic pathway is mediated by cup-like membrane extensions, usually larger than 1 μm, to internalize large particles such as bacteria or dead cells.

1.2.1.1 Clathrin-mediated endocytosis (CME)

Clathrin-mediated endocytosis is the most-well studied endocytic pathway. It is receptor-dependent and requires clathrin proteins and the GTPase dynamin [77]. Its first step consists of coat nucleation mediated by the internalized molecules and phosphatidylinositol(4,5)-bisphosphate, $PI(4,5)P_2$. During this process, cargo molecules are clustered and concentrated within the emerging clathrin coated pit with a size about 100–150 nm [78, 79]. As the clathrin lattice formation continues, the pit becomes deeply invaginated, until vesicle fission occurs, driven by the GTPase dynamin [77]. The next step in the CME pathway is transport of endocytosed vesicles from the vicinity of plasma membrane towards late endosomes and ultimately to lysosomes [80]. During endosome maturation from early endosomes to lysosomes, intraluminal pH is gradually being lowered by proton pumps. Once reaching lysosomes, endosomal cargo is enzymatically degraded [81]. Thus, therapeutic cargos must escape from endosomes to retain their biological activity and avoid degradation.

Clathrin consists of three clathrin heavy chains which are tightly bound to clathrin light chains [82]. During clathrin coat assembly the terminal domain of clathrin serves as a second protein-protein interaction hub that clusters AP-2 and accessory proteins into a clathrin-centered interaction network [79]. To inhibit CME in the current thesis, hypertonic sucrose was utilized which is known to dismantle these clathrin structures. As sucrose modifies the actin cytoskeleton and can therefore induce cytotoxicity, a more specific CME inhibitor, chlorpromazine (Cpz) was used additionally. Cpz depletes the clathrin and AP2 adaptor protein complex from the plasma membrane to the endosomal membranes.

1.2.1.2 Caveolae-mediated endocytosis (CvME)

CvME, also termed as lipid raft-mediated endocytosis, begins with the formation of a wedge-shaped structure by a scaffolding membrane protein, caveolin. CvME is a cholesterol, dynamin-dependent and receptor-mediated pathway [83] in the sense that caveolins, the structural proteins of caveolae, are a family of cholesterol-binding proteins. In addition, the fission of the caveolae from membranes is mediated by dynamin, which locates in the neck of caveolae and then generates the cytosolic caveolar vesicle, caveosome, with a diameter in the range of 50–100 nm [74]. Examples of receptors located in caveolae that can mediate CvME include insulin receptors [84] and activated epidermal growth factor receptors [85]. The intracellular fate of caveosomes, most likely, does not suffer in a drop in pH, and therefore the cargos can be directly transported to the Golgi and/or endoplasmic reticulum, avoiding normal lysosomal degradation [86].

In order to inhibit the CvME pathway, nystatin was used, which can bind to cholesterol in plasma membranes and alter membrane functions, including caveolae formation, thus inhibiting lipid raft engulfment.

1.2.1.3 Macropinocytosis (MP)

Macropinocytosis is a type of dynamin-independent [87] fluid-phase endocytosis responsible for engulfing relatively large amounts of non-specific substances [88]. MP is a signal dependent process that normally occurs in macrophages and cancer cells via the formation of actin-driven membrane protrusions which collapse onto and fuse with the plasma membrane [74]. During this process, Ras-related proteins in brain (Rab) are essential for the vesicle fission from the cell membrane [89]. Distinct from clathrin-coated vesicles and caveosomes, macropinosomes have no apparent coat structures and are heterogeneous in size, generally considered larger than 200 nm in diameter [90]. The exact intracellular fate of macropinosomes is unclear; it seems to be cell type dependent. In some cases, for example EGF-treated A431 cells, macropinosomes do not seem to mature beyond the early endosomal stage [91], but in other cases late macropinosomes begin to acquire Rab7 and lysosomal glycoprotein A (lgp-A), which are lysosome markers [92, 93].

Macropinosome processing requires extensive actin remodeling that incorporates several acting binding proteins such as Rho-family GTPases Rac1 and Cdc42 [94]. Therefore cytochalasin D (CyD), which blocks actin polymerization, was used to inhibit macropinocytosis. In addition, macropinosome closure requires PI3K and this process can be inhibited by wortmannin which was used in this thesis as well.

1.2.1.4 Clathrin- and caveolin-independent endocytosis

Clathrin- and caveolin independent endocytosis pathways are less studied as the formed vesicles do not possess a coat and display diverse morphology, such as uncoated vesicles, ring shaped structures and large tubular carriers [95, 96]. Clathrin- and caveolin independent endocytosis is further divided into dynamin-dependent and independent routes and requires, similarly to caveolae-dependent endocytosis, lipid-rich structures for internalization. This type of endocytosis has been shown to internalize extracellular fluid, the B subunit of cholera toxin, sphingolipids, glycosylphosphatidylinositol-linked proteins, as well as receptors for interleukin-2, growth hormone, autocrine motility factor, endothelin, and many other molecules [97]. Clathrin- and caveolin-independent endocytosis is reliant on specific cell substituents and morphologies [73].

1.2.2 Cell membrane interaction partners

Endocytosis of CPPs is triggered by an interaction between the peptides and the cell membrane. There are several potential anionic cell membrane binding partners that are utilized by CPPs through electrostatic interactions and hydrogen bonding, such as carboxylates, phosphates and sulfates. These functional groups are present in cell membrane proteoglycans, phospholipids and fatty acids.

Proteoglycans (PG) are a heterogeneous group of proteins containing long, linear, polysulphated and negatively charged glycosaminoglycan (GAG) polysaccharides. The most prevalent GAGs in PGs are chondroitin sulphate (CS), dermatan sulphate (DS) and heparan sulphate (HS) [38]. These membrane constituents act as anionic acceptors for extracellular positively charged CPPs, ultimately leading to peptide uptake at multiple sites on the cell surface.

Studies using cell lines which do not produce HS or CS revealed that the internalization of nonaarginine was impaired [98]. Adding exogenous heparin to cells also produced the same effect in case of Tat(46–56) and Antp(43–58) [99], hCT(9–32)-br and Sweet Arrow Peptide (SAP) [100]. Calorimetric studies have revealed a strong affinity between nonaarginine and HS at binding stoichiometries close to charge neutralization [101]. In case of polyarginines, in addition to electrostatic interactions the guanidinium group of arginine is able to form hydrogen bonds with sulphate and carboxylate groups present in heparan sulphate proteoglycans (HSPGs) [102–104], which greatly enhances their uptake potential. Also a specific peptide motif, consisting of basic amino acids flanked by hydrophobic residues, has been found to interact with HSPGs [105, 106]. The interaction between CPPs and these negatively charged cell membrane constituents has been shown to induce aggregation and ligand clustering in such way that the CPPs are concentrated at the cell surface for subsequent internalization [104, 107]. Recently it has been shown that treating cells with octaarginine resulted in enhanced clustering of syndecan-4, a type I HSPG, on plasma membranes that led to protein kinase Cα activation and initiation of

macropinocytosis [108]. CPP aggregation however, does not always guarantee cellular uptake [109].

After the initial interaction at the cell surface, CPPs can be taken up by the cell as the CPP-HSPG complex is enzymatically cleaved and CPP passes through the plasma bilayer to the cytoplasm directly or by endocytosis [110]. However, once the CPP-HSPG construct is in endosomes, the CPP can remain trapped, as the affinity of the interaction between the CPP and HSPGs diminishes due to cleavage of HS from the core protein by heparanases, causing a decrease in its anionic charge [111].

The utilization of such unspecific electrostatic interactions suggest that CPPs are simply taking advantage of the cells natural membrane recycling mechanisms as ca. 2–5% of a cell-membrane is recycled every minute [112, 113]. More precisely, the CPP may also simply attach to the cell surface and exploit inherent cell-membrane turnover to be internalized [38, 114].

1.2.3 Direct translocation

Direct translocation involves destabilization of the cellular membrane in an energy- and temperature-independent manner [115]. Several mechanisms have been proposed which rely on the interactions between positively charged CPPs and negatively charged phospholipids in the plasma membrane, such as inverted micelle formation, adaptive translocation and pore-formation.

Inverted micelle formation refers to an invagination in the cell membrane as a result of electrostatic interactions and the subsequent interaction of hydrophobic residues with the membrane core [116]. Reorganization of the neighboring lipids [117] leads to the formation an inverted micelle that encapsulates the CPPs in its interior and membrane disruption releases the peptide into the intracellular side. This model was first proposed for the translocation of penetratin [118] and since then numerous other studies have described membrane deformations, such as tabulation and formation of multivesicular structures when CPPs interact with model and cellular membranes [119, 120].

Adaptive translocation is associated with arginine-rich CPPs and involves the guanidinium groups of arginines that form bidentate hydrogen bonds with the phospholipid headgroups on the cell membrane. This can mask the peptide charge, attenuating its polarity and enabling its adaptive diffusion into and across the membrane [21].

The pore formation model is based on the formation of transient toroidal pores that can enable the passive diffusion of CPPs across the plasma membrane and was first observer for Tat and arginine-rich peptides [121, 122]. These CPPs accumulate in the outer leaflet and position between the phosphate and the carbon chains of the lipids causing a thinning of the bilayer. The subsequent attraction between the side chains of arginine and lysine and the phospholipid headgroups of the distal layer leads to the formation of a transient pore. Similar observations have also been made with other CPPs, such as TP10 [123–125], Pbeta and Pep-1 [126]. In addition, pore formation has been

associated with membrane repair response, induced by calcium influx into the cells, where internal vesicles will fuse together and serve as a patch to reseal the membrane [34, 127, 128].

Although most CPPs primarily utilize endocytosis in their cellular uptake, direct translocation certainly plays a part, especially in case of naked peptides. MPG [12], Tat [129], Pep [130], R_9 [121] and CADY [127] have all been associated with endocytosis-independent uptake routes and, based on microscopy observations as well as flow cytometry experiments, there seems to be a certain concentration threshold above which direct translocation of cationic CPPs is favored [131, 132]. Therefore a better description of these proposed mechanisms of internalization is required.

1.2.4 Uptake mechanisms of CPP-cargo complexes

The uptake mechanisms of CPP-cargo complexes, similarly to naked CPP uptake, depend on a myriad of factors such as the composition of the cell membrane, cell type, experimental conditions, CPP concentration and of course, the nature of the cargo [133–135]. The first step in CPP-cargo uptake involves interactions with membrane-associated proteoglycans. However, CPP-oligonucleotide complexes form substantially larger particles than naked CPPs and the hydrodynamic size of the formed nanocomplexes is one of the factors that may determine their subsequent endosomal uptake route. As macropinosomes are the largest endosomal vesicles, macropinocytosis is most often associated with CPP-cargo complex internalization [136]. Therefore, endosomal escape of these complexes is crucial as was discussed in previous chapters.

Whereas certain CPPs can be taken up by both endocytosis and direct translocation [134], CPP-cargo nanoparticles mainly utilize endocytosis. One exception is CADY-siRNA nanocomplexes that induce transient cell membrane permeabilization, which is rapidly restored by cell membrane fluidity. Therefore, the designers of this peptide propose that the major mechanism of cell entry for these complexes is direct translocation [127]. Endocytosis independent translocation was also observed in case of non-covalent siRNA-MPG8 and Pep-3 complexes [137]. In contrast, PF6-oligonucleotide complexes are mainly taken up by smaller clathrin-coated pits [138] and in case of CPP mediated protein delivery, similarly to naked CPPs, several endocytic pathways can be utilized simultaneously [139, 140].

Recently, scavenger receptors have been linked to the uptake mechanism of CPP-oligonucleotide complexes [72]. These nanocomplexes become negatively charged upon interacting with serum proteins that electrostatically bind to the positively charged CPPs [141]. Scavenger receptors recognize negatively charged particles and are involved in the uptake of PepFects [70, 72], NickFects [142] and octaarginine-modified lipid nanoparticles [143]. After binding to scavenger receptors, the nanocomplexes are taken up by receptor-mediated endocytosis or traffic through the endo-lysosomal system [144], and as previously mentioned, these processes can occur simultaneously.

Another proposed uptake mechanism of CPP-cargo complexes involves the formation of self-assembled CPPs around a CPP-cargo core, which interact with cell membrane proteoglycans, destabilizing the membrane and enabling subsequent uptake of the cargo [38, 136, 145]. In order to determine the exact mechanism however, the physicochemical properties of CPP-cargo nanoparticles need to be determined and their interactions with cell- and endosomal membranes have to be assessed.

1.3 CPP internalization kinetics

The uptake rate of CPPs is an important factor when developing CPPs for drug delivery as it gives more information on their uptake routes compared to simple end-point studies. As discussed, CPP mediated uptake is dependent on peptide concentration and more importantly the nature of the delivered cargo, therefore the origin of a measured readout is crucial for interpreting the experimental data correctly. For example, uptake studies utilizing fluorescent labels do not distinguish between endosomally trapped and released peptides which can lead to misinterpretations. Therefore, measuring CPP kinetics using a suitably designed assay serves as a valuable tool for assessing the effect of different cargos and peptide modifications on their uptake.

Examples of successful CPP kinetic studies include flow cytometry analysis utilizing fluorescein-tagged Tat, which indicate that its uptake kinetics is similar to the kinetics of endocytosis [146]. In Jurkat cells, radioisotope labelled Tat peptide also showed concentrative cell accumulation and rapid uptake kinetics [147]. Time course of the cellular uptake of rhodamine labelled Tat (48–60) and Drosophila Antennapedia (43–58) suggested that the arginine-rich basic peptides share a certain part of the internalization pathway [148]. This was clarified by internalization kinetics studies utilizing 7-nitrobenz-2-oxo-1,3-diazol-4-yl (NBD) labelled pAntp, which suggest that the peptide enters into cells by a mechanism that involves both energy-dependent and independent processes [149].

In order to emulate drug-conjugate delivery into a cell, drug release, and drug turnover by an intracellular target, releasable luciferin conjugates were developed that allow the quantification the real-time uptake of any delivery vector [150]. This assay was used to assess the *in* vivo skin uptake kinetics of octa-D-arginine [151]. By using an improved version of this assay, the cytosolic delivery kinetics measurements of eight common CPPs revealed that, based on those results, CPPs can be divided into fast and slow internalization groups [152]. The uptake of CPPs can be very rapid, with uptake half-times as low as 2–12 min [33, 147, 148], however in the majority of cases they remain between 20–60 min [33, 152, 153]. In the current thesis, CPP internalization mechanisms were studied by systematically inhibiting different endocytosis routes and measuring their cytosolic uptake kinetics utilizing either a quenched fluorescence assay (paper II) or a biological readout (paper III).

1.4 Characterization of CPP-cargo complexes

Covalently formed CPP-cargo conjugates can be described by their molecular mass, secondary structure and other similar characteristics. The structure of non-covalently formed conjugates however, is much more dynamic and therefore their physicochemical properties must be described. Minor changes in parameters such as nanocomplex size and surface charge can strongly influence their biological interactions, pharmacological activity and safety [154]. According to the FDA [155], the required physicochemical studies that have to be performed on new nanoparticle based drugs include:

- morphology of the delivery vector,
- amount of delivery vector,
- degradation products of the delivery vector,
- particle size and size distribution of the nanoparticle,
- light scattering index,
- net surface charge of the nanoparticle,
- amount of complexed and free drug,
- *in vitro* release.

In the following chapters, some of the most important parameters for CPP-oligonucleotide nanocomplexes will be discussed.

1.4.1 Characterizing CPP-cargo complexes by DLS

One common physicochemical method for studying non-covalently formed CPP-cargo conjugates is dynamic light scattering which measures the hydrodynamic size and charge of the formed nanocomplexes. The size of these particles can be crucial to their efficacy, especially in *in vivo* and clinical settings, as biodistribution and elimination from blood circulation strongly depends on particle size and surface properties.

Rapid renal clearance and urinary excretion has been demonstrated for small particles with a hydrodynamic diameter below 5 nm [156]. On the other hand, nanoparticles with a hydrodynamic diameter of more than 200 nm typically exhibit rapid opsonisation and subsequent uptake by the mononuclear phagocytic system (MPS) and will be sequestered by liver and spleen [157, 158]. Opsonisation is a process by which a foreign structure becomes covered with opsonin proteins, thereby allowing its recognition by the MPS [155]. The opsonisation of CPP nanoparticles can be reduced by modifying their surface by certain molecules such as polyethylene glycol (PEG), which shields their charge and decreases unspecific interactions with plasma proteins [159]. As mentioned previously, smaller particles, 50–100 nm, are filtered by caveolae [160, 161] and CPP nanocomplexes need to be released in order to exhibit their biological effect. Another mechanism associated with nanocomplex uptake *in vivo* is the

enhanced permeability and retention (EPR) effect which is responsible for the enhanced accumulation of macromolecules in solid tumors [162, 163]. The inflammation and lesions of the vascular bed allow the accumulation of macromolecules and particles of a size between 50 and 500 nm inside the interstitial space. The discovery of the EPR effect has supported the formulation development of nanoparticle systems for cancer therapy but in many cases the influence of this effect on systemic distribution has been overestimated. In most cases particles are cleared from the systemic circulation before the EPR effect can be utilized [155].

The net charge of the CPP-cargo nanocomplex, which can also be measured by DLS, determines its stability and circulation time, as it greatly influences its aggregation, opsonisation and clearance. However, it is also strongly influenced by environmental parameters, such as ionic strength, protein concentration and pH of the solute. A better indicator for the integrity of CPP nanocomplexes would be their pH-dependent zeta potential profile [155].

In conclusion, depending on the surface charge, a particle size between 100 and 300 nm is required for the optimization of circulation times and tumor accumulation. Particles in this size range also exhibited the most efficient transfection properties in *in vitro* conditions in papers I and IV.

1.4.2 Characterizing CPP-cargo complexes by ITC

Isothermal titration calorimetry (ITC) can be utilized to determine the thermodynamic parameters of CPP binding processes. Most studies focus on interactions between CPPs and their cell membrane counterparts [101, 104, 164–169] with only a few examples of CPP-cargo interaction studies.

In order to elucidate CPP-cargo binding, ITC must be coupled with other physicochemical characterization methods. For example, the interaction between HIV-1 Tat(47–57) and double-stranded DNA was investigated by ITC and static right-angle light scattering. The results revealed that the CPP condenses the DNA in an exothermic fashion and the affinity of the CPP for DNA is 1–2 orders of magnitude higher than for extracellular heparan sulfate [170]. Another study focusing on the interactions between dendrimers, consisting of Tat, a nuclear localization signal peptide, dendritic polylysine, and salmon sperm DNA showed that dendrimers are forming stable complexes with DNA. In addition, the binding affinity and thermodynamic parameters were found to increase as the number of positive charges on the dendrimer increased, indicating that ionic interactions were the major binding forces between the two molecules. Also, the binding affinity was stronger at pH 3.2 and precipitation of the complex was more prominent at pH 7.2 [171]. Isothermal titration calorimetric data from a study where interactions between poly(amidoamine) (PAMAM) dendrimers and siRNA were studied suggested a simple binding for one of the dendrimers, whereas a biphasic binding was evident for others, with an initial exothermic binding and a secondary endothermic formation of larger dendriplex aggregates, followed by agglomeration [172]. Another biophysical

characterization study of an amphipathic peptide that mediates the uptake of non-covalently bound siRNA into cells and its subsequent release into the cytosol revealed that even though the peptide enhanced the uptake of siRNA into cells, no direct interaction between siRNA and peptide was observed at neutral pH by isothermal titration calorimetry. By applying additional endosomal-escape assays and cell-fractionation techniques, authors concluded that the peptide-mediated siRNA delivery occurs through a pH-dependent and conformation-specific interaction with cellular membranes and not with the cargo [173].

The ITC studies on the binding processes between an amino-acid-pairing (AAP) peptide and siRNA revealed their interaction enthalpy and stoichiometry values. However, they were obtained based on a single-site independent model. In addition, the gel shift assay revealed that molar ratio 10:1 (peptide:siRNA) was required to form stable complexes, which further questions the use of the aforementioned model [174]. A recent study focusing on interactions between a retro-inverso dioleoylmelittin (riDOM) CPP and DNA as well as heparan sulfate revealed that riDOM binds tightly to DNA through an entropy-driven binding reaction with a microscopic binding constant and a stoichiometry of 12 riDOM per 10 DNA base pairs. The CPP interacts less strongly with heparan sulfate and the authors hypothesize that depending on the concentration of sulfated glycosaminoglycans on the cell surface this process could compete with DNA binding and inhibit riDOM induced transfection [175]. ITC was also utilized in paper IV to determine the CPP-siRNA binding properties of two efficient siRNA delivery vectors in different solution conditions.

2. AIMS OF THE STUDY

The objective of this thesis was to characterize the cellular internalization kinetics and mechanisms of CPPs and covalently or non-covalently formed CPP-cargo constructs. The aims included the assessment of the performance of kinetic assays compared to conventional single end-point studies in categorizing CPPs. The further goal also included assessing the importance of CPPs' hydrophobicity and complex formation properties in nucleic acid delivery efficiency. The precise goals of each paper are described below.

Paper I	To assess the role of hydrophobic CPP modifications in non-covalent CPP-oligonucleotide nanocomplex delivery.
Paper II	To determine the kinetic parameters of CPP cytosolic internalization utilizing a quenched fluorescence assay and to assess the effect of endocytosis inhibitors on this process in order to determine CPP uptake mechanisms.
Paper III	To determine the effect of endocytosis inhibitors on CPP cytosolic internalization kinetics profiles using a bioluminescence assay in order to assess CPP uptake mechanisms.
Paper IV	To study the physicochemical properties of CPP-siRNA binding processes in order to elucidate their cellular internalization mechanism.

3. METHODOLOGICAL CONSIDERATIONS

The methods and materials used in the current thesis are presented in detail in each publication. This chapter briefly describes the principles of the used protocols and why each particular method was chosen.

3.1 Peptide selection and design

In this thesis, the CPP transportan 10 (TP10) was modified with a variety of naturally occurring fatty acids in order to determine the impact of hydrophobicity on their ability to deliver splice-correcting antisense oligonucleotides. In addition to TP10, the uptake kinetic profiles and endocytic uptake routes of several other widely used CPPs (Table 2) were determined. Further, the cargo delivery mechanism of TP10 based CPPs NickFect51 and PepFect6 was assessed.

Table 2. CPPs used in this thesis.

CPP	Application	Paper	Ref.
TP10	Hydrophobic modifications, SCO delivery, uptake kinetics (quenched fluorescence, bioluminescence), ITC	I,II,III, IV	[7]
M918	Uptake kinetics (quenched fluorescence, bioluminescence)	II,III	[176]
pVEC	Uptake kinetics (quenched fluorescence, bioluminescence)	II,III	[17]
EB1	Uptake kinetics (bioluminescence)	III	[18]
MAP	Uptake kinetics (bioluminescence)	III	[11]
Penetratin	Uptake kinetics (bioluminescence)	III	[118]
Tat	Uptake kinetics (bioluminescence)	III	[4]
TP10(Cys)	Uptake kinetics (bioluminescence)	III	[7]
PF6	siRNA delivery, ITC, DLS	IV	[53]
NF51	siRNA delivery, ITC, DLS	IV	[69]

In paper I, as it had previously reported that a stearoyl modification greatly enhances the SCO delivery efficacy of TP10 [64], fatty acids of different lengths and structure were conjugated to the N^{α}-terminus of TP10 to evaluate the overall effect of hydrophobicity on the delivery efficacy of a splice-correcting 2′-OMe phosporothioate oligonucleotides.

In paper II the overall cargo delivery kinetics, with emphasis on cytoplasmic delivery, of three established CPPs, i.e. M918, TP10 and pVEC, were determined. The uptake kinetics were measured using a quenched fluorescence assay [33] with some modifications.

In paper III, the uptake mechanisms of eight established CPPs, including TP10, MAP, Tat, TP10(Cys), pVEC, M918, penetratin and EB1, were evaluated

using a semi-biological real-time uptake kinetics assay reported previously [150–152].

In paper IV, the siRNA delivery ability of cell-penetrating peptides, NickFect51 and Pepfect6, was assessed by siRNA transfection, DLS and ITC experiments. These peptides were chosen for their excellent siRNA delivery properties [53, 69].

3.2 Peptide synthesis

All peptides were synthesized by solid-phase peptide synthesis (SPPS) first described by Bruce Merrifield [177]. Briefly, the peptide is anchored to an insoluble support, a resin, which is stable to the chemical reactions carried out during the synthesis. The peptide is assembled in a step-wise manner by repeated cycles of formation of a peptide bond between the amino group and a N^{α}-protected amino acid, followed by deprotection of the temporary N^{α}-protecting group. At the end of the synthesis the assembled peptide is cleaved from the solid support. The functional groups of the amino acid side chains are protected with permanent protecting groups, which are also cleaved after completion of the synthesis. Presently, two permanent protective group strategies are being used, *tert*-butoxycarbonyl (Boc)/benzyl-based and the 9-fluorenylmethyloxycarbonyl (Fmoc)/*tert*-butyl-based strategy.

After cleavage, the peptides were purified by reverse-phase HPLC and the correct molecular weight was determined by matrix- assisted laser desorption/ ionization-time of flight mass spectrometry.

3.3 Covalent cargo conjugation

In this thesis, several CPPs were chemically modified. In paper I, different fatty acids that are present in most eukaryotic cell membranes, were added to the N-terminus of TP10. In paper II and III, cysteines were added to the peptide sequences in order to induce further modifications that enable the assessment of CPP internalization. Paper II utilized a quenched fluorescence assay and in paper III a covalently attached luciferin was used in order to determine cellular uptake of CPPs by measuring bioluminescence. In both papers, the cargo was conjugated to a CPP via a disulfide bond. This method was chosen because disulfide bonds are only reduced in the cytosol and therefore overestimation of the signal arising from extracellular or endosomally trapped conjugates is avoided [178]. It has been reported that the disulfide bridge is cleaved by glutathione, which represents the major component of the cellular redox system [40], and to some extent enzymes such as protein disulfide isomerase [179].

3.4 Non-covalent nanoparticle formation

In papers I and IV, a non-covalent conjugation strategy was used to assess the cellular uptake mechanisms of CPP-cargo complexes. Non-covalent nanoparticle formation in the CPP field was first described for pDNA delivery [180] and has been utilized for a wide range of negatively charged cargos [181]. Non-covalent complexes are formed by electrostatic and hydrophobic interactions and therefore do not require chemical modifications to the CPP or cargo. In the current thesis, CPPs were mixed with SCOs or siRNA for 1 h at different molar ratios (MRs) to determine the delivery efficacy of modified TP10 analogues in paper I and to assess PF6 and NF51 mediated siRNA downregulation efficacy in paper IV.

3.5 Nanoparticle characterization

In order to understand the delivery mechanism of non-covalent nanoparticles, several physicochemical parameters have to be described. In this thesis, the particle size and binding of CPP-cargo complexes in different solution conditions was studied.

3.5.1 Dynamic light scattering

DLS is a spectroscopic technique which measures the fluctuations in light-scattering intensity as a function of time over time scales from approximately 100 ns to 30 ms. Those fluctuations are due to the Brownian motion of the scattering particles. The time scale of the scattering fluctuations is directly related to the translational diffusion coefficient of the scattering particles in the solvent, which in turn is related to their hydrodynamic diameter [182].

The size and homogenicity of CPP-cargo complexes can determine their delivery efficacy as the nanoparticles have to obtain an optimal size in order to be internalized by cells. In *in vitro* experiments, the sizes can vary more as seen in paper I, but *in vivo* conditions require more condensed and smaller particles as different size nanocomplexes may target different organs and have different tissue distribution profiles [155].

3.5.2 Isothermal titration calorimetry

In short, ITC measures the heat absorbed or released from the interaction between a ligand and a receptor. ITC enables the determination of binding affinities (K_a), stoichiometry of the binding (n) and also the enthalpy (ΔH), entropy (ΔS) and Gibbs free energy changes (ΔG). ITC was used in paper IV to elucidate CPP-siRNA binding processes by measuring the heat output when positively charged CPPs interact with negatively charged siRNA.

3.6 Cell cultures

Three different cell lines were used in this thesis. Regular HeLa cells were used in paper II. HeLa cells are immortalized cervical cancer cells taken from Henrietta Lacks in 1951. These cells have shown high durability and versatility in research and have been used for applications such as drug discovery, study of viruses, cancer research and are also widely used in the field of CPPs. In paper I and III a modified HeLa cell line, HeLa pLuc 705 was used. This cell line is stably transfected with a luciferase-encoding gene interrupted by a mutated β-globin intron 2 [183]. This mutation introduces an aberrant pre-mRNA splicing site, and thereby leads to the synthesis of nonfunctional luciferase. However, binding of the complementary 2′-O-Me RNA (pLuc 705 SCO) to the aberrant splicing site restores the normal pre-mRNA splicing and functional luciferase is expressed as shown in paper I (Figure 2A).

In paper IV we evaluated siRNA mediated gene silencing in U87 cells stably expressing Luc2. The U-87 MG-Luc2 cell line is a luciferase expressing glioblastoma cell line stably transfected with firefly luciferase gene (Luc2 vector).

3.7 Transfection experiments

In papers I and IV, the nucleic acid delivery efficacy of various CPPs was estimated by using either pLuc 705 SCOs or Luc2-silencing siRNA, respectively. Additionally, a luciferase encoding plasmid was used in paper III to induce transient luciferase expression.

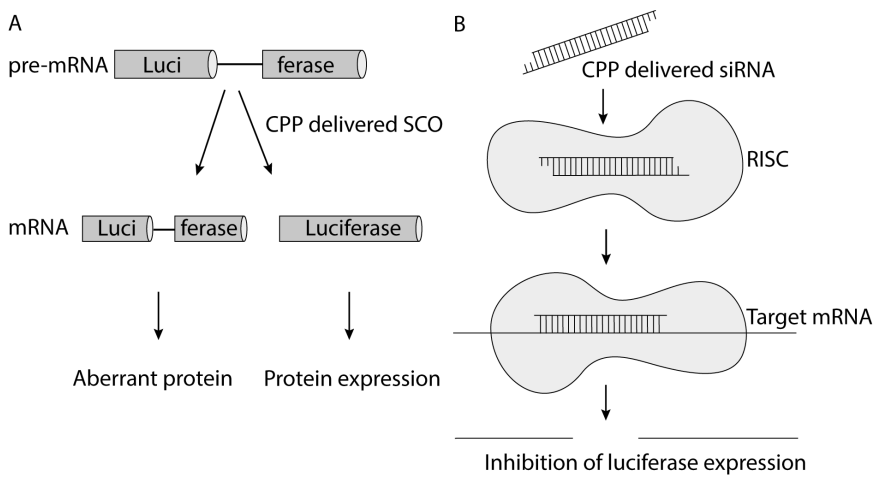

Figure 2. Transfection assays used in this thesis. (A) CPPs were used to deliver pLuc 705 antisense oligonucleotides which restore luciferase expression into cells. The luciferase-encoding gene is interrupted by an aberrant pre-mRNA splicing site which results in the synthesis of non-functional luciferase. By introducing a pLuc 705 SCO, luciferase expression is restored. (B) CPPs were used to deliver Luc2-silencing siRNA into cells stably expressing luciferase. Once reaching the cytosol, siRNA is incorporated into the RISC complex which selectively binds to Luc2 mRNA, degrades it and leads to the downregulation of protein expression.

3.7.1 SCO delivery

In paper I HeLa pLuc 705 cells were utilized to assess CPP mediated cellular delivery efficiency of splice-correcting antisense oligonucleotides (SCOs). This assay is widely used as a positive read-out method for quantifying SCO delivery by various delivery vectors.

3.7.2 siRNA delivery

In paper IV, U-87 MG-Luc2 reporter cell line was used to compare the delivery efficiency of two established CPPs. By downregulating luciferase expression, the decreased luciferase activity can be normalized to untreated cells and gene silencing efficacy can be measured (Figure 2B). In order to compare different CPPs, measurements were performed at different CPP to siRNA molar ratios.

3.8 Measuring CPP uptake kinetics

Traditionally, CPP uptake mechanisms are measured using static endpoint assays. Because different CPPs internalize into cells at different rates, certain aspects of their cell entry mechanism could therefore be overlooked. CPP uptake kinetics studies can serve as a valuable tool in describing their uptake mechanisms.

In the current thesis, two different methods were used in order to describe the cytosolic uptake of CPPs. In paper II, a quenched fluorescence assay was utilized, consisting of CPP and cargo. Both were modified with a cysteine residue, enabling covalent conjugation and a quencher-fluorophore pair was incorporated into the system. The cargo was carrying a fluorophore while a corresponding fluorescence quencher was attached to the CPP. Once reaching the cytosol, the disulfide bond would be cleaved leading to separation of the fluorophore and the quencher which in turn leads to an increase in fluorescence (Figure 3A). Therefore, this assay can be used to quantify CPP uptake kinetics in cells using a spectrofluorometer.

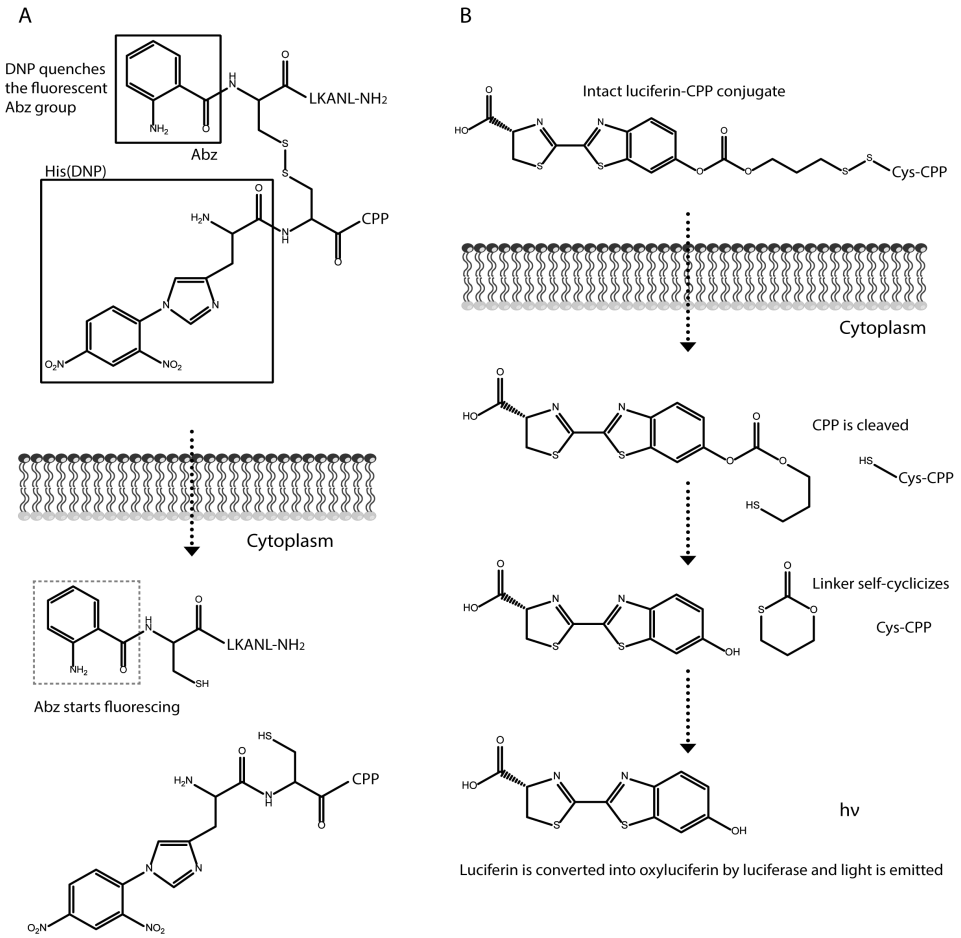

Figure 3. Uptake kinetics assays used in this thesis. (A) In the quenched fluorescence assay, once reaching the cytoplasm, the quencher (DNP) and fluorophore (Abz) are separated and fluorescence is produced. (B) In the bioluminescence assay, luciferin is conjugated to CPPs through a self-cyclicizing linker. Once reaching the cytoplasm, luciferin is released and converted to oxyluciferin by luciferase and light is emitted.

In paper III, this method was improved by incorporating luciferin as the cargo. A spacer was chemically attached to luciferin which could be covalently conjugated to a cysteine residue on CPPs via a disulfide bridge. The luciferin linker was designed so once reaching the cytoplasm, the disulfide bridge would be cleaved and the spacer self-cyclizes, allowing the release of free luciferin (Figure 3B). Next, the luciferin is oxidized by luciferase enzyme, emitting a photon of light which can be quantified at any time-point using a luminometer. This method has several advantages over the method used in paper II. Firstly, any complication arising from incomplete quenching are avoided. This assay is also semi-biological and can be used in adherent cells either stably or transiently transfected with a luciferase gene.

3.9 Uptake pathway studies utilizing endocytosis inhibitors

Cellular internalization mechanisms can determine the trafficking and ultimately, the intracellular fate of CPPs [184]. Therefore it is important to study which endocytic routes CPPs use for cellular internalization. In this thesis, several commonly used endocytosis inhibitors were utilized to elucidate the uptake mechanism of CPPs. However, none of the used inhibitors of different uptake pathways is absolutely specific. They either have side-effects resulting from affecting the actin cytoskeleton or interfering with alternative uptake pathways simultaneously. In paper II, chlorpromazine (10 μM), cytochalasin D (4 μM), wortmannin (50 nM) and sucrose (0.4 M) were used whereas in paper III chlorpromazine (10 μM), cytochalasin D (4 μM), nystatin (50 μM) and chloroquine (100 μM) were used (Table 3).

Table 3. Endocytosis inhibitors used in this thesis.

Inhibitor	Mechanism	Affected pathway		
		CME*	MP*	C/LR*
Chlorpromazine	Depletion of clathrin/AP2 from the cellular membrane to endosomal membranes	+++	+	-
Sucrose	Clathrin dispersion from the cellular membrane	+++	+	+
Wortmannin	Phosphoinositide metabolism (PI3K) inhibitor	+	+++	+
Cytochalasin D	Depolymerization of F-actin and disassembly of the actin cytoskeleton	+	+++	+
Nystatin	Interaction with cholesterol in the cellular membrane	-	-	+++
Chloroquine	Promotion of endosomal escape/slowing down overall endocytosis	+	+	+

* CME – clathrin mediated endocytosis; MP – macropinocytosis; C/LR – Caveolae/ lipid raft dependent endocytosis

Chlorpromazine and sucrose were used to inhibit clathrin mediated endocytosis (CME). This pathway is named after the clathrin protein, containing heavy and light chains, that forms clathrin coted pits. Sucrose disperses plasma membrane clathrin structures and thus inhibits CME. Chlorpromazine is a cationic amphipathic drug that triggers the depletion of clathrin and AP2 adaptor protein complex from the plasma membrane and leads to their artificial assembly on endosomal membranes [185]. However, both of these inhibitors also inhibit macropinocytosis. Furthermore, sucrose can influence the cortical actin cytoskeleton which can cause non-specific cytotoxicity which affects all major endocytic routes to some extent. Therefore, it was used only in paper II and together with chlorpromazine which discriminates the clathrin-mediated endo-

cytic pathway initially and is therefore more specific [185]. That said, chlorpromazine can also insert into the plasma membrane and change its fluidity. It also affects actin dynamics by inhibiting phospholipase C, which could influence macropinocytosis.

Cytochalasin D and wortmannin were used to inhibit macropinocytosis (MP). Cytochalasin D inhibits membrane ruffling and endocytosis by depolymerizing F-actin and disassembling the actin cytoskeleton [185]. Wortmannin inhibits phosphoinositide metabolism, which is needed for actin cytoskeleton reorganization. However, neither of these inhibitors is highly specific because depolymerizing F-actin and inhibition of phosphoinositide metabolism may also disrupt the other two endocytic pathways. For example, cytochalasin D is also used as the inhibitor for caveolae-mediated endocytosis [186].

Nystatin was used to inhibit the caveolae mediated endocytic pathway. Nystatin is an antifungal drug that forms large aggregates upon binding to cholesterol [185]. This changes the shape of the caveolae and inhibits the binding and subsequent internalization of the lipid-raft ligand. Nystatin is therefore quite specific in inhibiting the caveolae pathway as is does not affect CME or MP.

Chloroquine slows down the acidification of endosomal vesicles and accumulates in endosomes/lysosomes causing them to swell and disrupt by osmotic effects [187]. This leads to either (i) endosomal escape which increases uptake or (ii) slowing down the overall endocytosis pathway resulting in inhibiting the uptake [188]. Uptake of CPP-luciferin conjugates was measured in the presence of chloroquine in paper III in order to determine which of these effects prevail.

3.10 Toxicity studies

Several assays were used throughout this thesis to determine the possible cytotoxic effects of CPPs and CPP conjugates. In addition, in paper II the extracellular thiol concentrations were determined in order to assess whether the conjugates are cleaved before entering cells or as a result of an outflow of glutathione into the extracellular media. Cells were incubated with Ellmann's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) that reacts with free thiols, leading to the release of 3-thio-6-nitrobenzoate (TNB^-), which then ionizes into measurable yellow TNB^{2-}.

In paper I, the cytotoxicity of the designed CPPs was tested by WST-1, a cell viability assay that measures the metabolic activity of the mitochondria.

In paper III, the integrity of the cellular membrane after incubation with CPPs was determined by lactate dehydrogenase (LDH) assay. LDH assay is a rapid, fluorescent measure of the release of LDH from cells with a damaged membrane. LDH released into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into resorufin, the fluorescent end product of the assay. The amount of measured resorufin is proportional to the amount of released LDH and is therefore proportional to membrane leakage.

4. RESULTS AND DISCUSSION

In the four papers included in this thesis (three published articles and one submitted manuscript), selected CPPs are characterized in terms of their cellular uptake kinetics, internalization mechanisms and cargo delivery mechanisms. In papers II and III, kinetic assays were used in order to determine how fast and efficiently and, even more importantly, by which kinetic profile CPPs are being internalized. The readout of these assays was based on cytosolic delivery in order to assess their biological availability. In paper I, several hydrophobic modifications were conjugated to transportan 10 in order determine how CPP hydrophobicity affects its ability to deliver non-covalently attached oligonucleotides inside cells. In paper IV, the physicochemical properties of CPP-siRNA binding was assessed in order to elucidate their transfection mechanism. In this chapter, the results obtained from each paper will be summarized and discussed.

4.1 Hydrophobic modifications are crucial to transportan 10 oligonucleotide delivery efficacy (Paper I)

In paper I, the cell-penetrating peptide transportan 10 (TP10) was modified with a variety of hydrophobic molecules in order to determine their effect on non-covalently attached oligonucleotide delivery. Hydrophobic CPP modifications were first reported when stearoylation of arginine rich peptides was utilized to transport plasmid DNA inside cells [51]. Thereafter, stearoylated TP10 was used as a delivery vector for splice-correcting antisense oligonucleotides using a non-covalent co-incubation strategy. However, the optimal length of fatty acid may depend on the CPP in question [63]. For example, in case of Alexa-labelled octaarginine, hexanoic acid showed the highest improvement in the cellular uptake of CPP conjugates, whereas butanoic and decanoic acid were less efficient [65].

In order to determine the optimal hydrophobic modification for TP10, various fatty acids with different chain lengths, varying from 8 carbons to 24, were conjugated to the peptide and the conjugates' delivery efficacy was assessed using a splice correction assay (Figure 2A). In total, 12 delivery vector analogues were non-covalently complexed with 2´-OMe splice-correcting antisense oligonucleotides and the splice correction was measured at different peptide-to-oligonucleotide molar ratios in serum-free and serum-containing media using the HeLa pLuc 705 cell line. The results showed that heptadecanoyl modification had higher splice correction ability than stearoyl, although statistically it was not significant. However, the effect was obtained at a lower molar ratio, which indicates that the heptadecanoic acid modification could be more efficient in delivering SCOs. Furthermore, each vector requires a different molar ratio in order to achieve the highest splice correction. This suggests that

an optimal number of hydrophobic molecules or suitable overall hydrophobicity may be needed to induce the required conformation for most efficient nanoparticles. In serum-containing medium stearoyl-TP10 remained most potent but the overall splice correction efficiency of all conjugates was significantly reduced. The results showed that in order to maximize splice correction efficacy, the optimal hydrophobicity for TP10 can be achieved by conjugating it to a fatty acid containing at least 16 carbons in its tail. When the fatty acid chain length exceeds 22 carbons, splice correction efficiency starts to decrease again.

In order to determine the dependency of the uptake of these complexes on their size we used DLS to measure the hydrodynamic diameters of the formed nanoparticles. Several studies focusing on siRNA, plasmid DNA and also protein delivery have shown that the optimal complex size for achieving maximal therapeutic effect *in vitro* and also *in vivo* ranges from 70 nm to 300 nm [155]. No correlations between the hydrophobicity of the added modification and the resulting size of the CPP-SCO nanocomplexes were observed. The general range for all formed nanoparticles was from 100 nm to 250 nm when the complexes were formed in water and 500 nm to 1 μm when in Opti-MEM. A likely cause for bigger particles obtained in Opti-MEM could be due to aggregation by the components present in the reduced cellular growth media. This could also explain why splice correction was significantly reduced in serum-containing media as particles around 1 μm are proposed to be only taken up by macropinocytosis [90].

As the increase in length of the hydrocarbon chain can potentially result in increased toxicity [189], the safety of the peptide oligonucleotide complexes was determined using the Wst-1 assay. Although the arachidoyl and behenoyl modifications showed slight toxicity at higher concentrations, none of the complexes displayed statistically significant toxicity values at their most effective molar ratios. In contrast, Lipofectamine 2000, which showed the highest splice correction efficiency, was also the most toxic of the tested delivery vectors.

In conclusion of Paper I, the most efficient range of hydrophobicity for TP10 analogs for delivering oligonucleotides into cells was determined. In order to assess how the transfection efficacy of these particles is dependent on their size, the hydrodynamic diameter of the formed nanoparticles was correlated with the vectors' activity. The experiments revealed that in order to induce efficient SCO transfection, the particle size should remain between 100–200 nm. These findings were utilized in the development of highly efficient non-viral gene therapy delivery vectors, PepFects and NickFects.

4.2 Quenched fluorescence assay shows how endocytosis inhibitors affect the kinetics of CPP uptake (Paper II)

Most CPP-uptake mechanism studies employ single end-point measurements which can overlook crucial aspects in their mode of internalization. In Paper II, we assessed CPP uptake mechanisms by measuring their real-time uptake kinetics. In addition, the effect of endocytosis inhibitors on real-time uptake kinetics was quantified for the first time. In order to account for the potential endosomally entrapped CPP portion, a quenched fluorescence assay was employed, where only the signal arising from cytoplasmic delivery will be registered.

Measurement of Abz-Cys-LKANL-amide pentapeptide uptake kinetics mediated by selected CPPs was first described by Hällbrink et al. [33]. The assay was based on measuring the increase in fluorescence by using an energy transfer quenching method where the Abz molecule acts as a fluorophore and nitrotyrosine acts as a quencher. In Paper II the side chain protective group of His, DNP, was used as the quencher in order to increase synthesis yields (Figure 3A). The ability to deliver pentapeptide cargo into HeLa cells with and without the presence of endocytosis inhibitors of three different CPPs (M918, TP10 and pVEC) was assessed. The uptake kinetic curves of these conjugates were registered and the measured data points were fitted to one-phase exponential association curves according to the formula

$$Y = Y_{max} \cdot [1-\exp(-K \cdot x)] \qquad \text{(Eq.1)},$$

where Y is the uptake level (pmol of internalized peptide), Y_{max} is the maximal uptake level, K is the first order constant in s^{-1} and x is time in s.

The uptake rate constants and plateau values for each CPP depend on their concentration but in terms of total uptake, M918 is superior to the other two, leaving TP10 with the lowest maximal uptake level. It is notable that a similar relationship does not apply to their rate constants as the values for pVEC and TP10 are higher than for M918 at 1 μM conjugate concentration.

Endocytosis inhibitors affect both the first-order rate constant and the total uptake level of the tested CPP conjugates. For instance, endocytosis inhibitors have the strongest effect on M918-mediated uptake, whereas pVEC-mediated uptake is less affected. Overall, there are several pathways involved in the uptake of these conjugates that are affected by different inhibitors in various extents. In case of M918, all the inhibitors lowered the total uptake, whereas wortmannin and sucrose increased the rate constant. In case of TP10, the total uptake is lowered and rate constant increased by Cpz, wortmannin and sucrose while CyD does not affect the rate constant but slightly increases total uptake. For pVEC, only sucrose lowers the total uptake and increases its rate constant. Overall the data revealed that the main uptake routes of M918 and pVEC include both macropinocytosis and CME and in case of TP10, the predominant

cellular entry route is CME. These findings are in line with previously published reports [190–192].

In conclusion of Paper II, these data demonstrate the advantages of real-time CPP uptake kinetics assays, as a more thorough picture of their uptake mechanisms is obtained. The results show that CPP concentration affects the kinetics and the total uptake of different CPPs to a different extent. This is an important observation since the majority of CPP delivery studies are single end-point measurements, which may not take these specialties into account. The results obtained in this paper led to the development of a more complex CPP kinetic assay used in Paper III which focused on these aspects more in-depth.

4.3 Bioluminescence assay shows how endocytosis inhibitors affect CPP uptake kinetic profiles (Paper III)

In Paper III, a bioluminescence assay (Figure 3B) was used to measure the cytosolic uptake kinetic profiles of eight different CPPs in the presence of endocytosis inhibitors. Previous results showed that CPPs can be differentiated into slow and fast cellular internalization groups [152] which hinted the possibility of an endocytosis independent internalization route.

In order to determine which type of endocytosis is employed by each CPP; Cpz, a CME inhibitor; CyD, a MP inhibitor and Nys, a C/LR inhibitor (Table 3) were used. Although endocytosis inhibitors are not exclusively specific, the long term activation of compensatory transport pathways can be minimized [73, 185]. The inhibitors used in paper III were chosen to minimize cross-inhibition effects [185]. In addition to common endocytosis inhibitors, chloroquine (CQ) was used, which is used to inhibit the acidification rate of early endosomes and to promote release of endosomally entrapped material. In addition the decrease in acidification is accompanied by slower vesicle recycling which could slow down the overall rate of endocytosis [188, 193]. CQ was used to determine which of these mechanisms prevails in the first 2 hours of CPP uptake.

First we analyzed how the used endocytosis inhibitors affect the overall luciferin-CPP uptake at different incubation time points – 15, 30, 60 and 120 min. The overall cytosolic uptake of all the peptide conjugates, except luciferin-Tat, was affected most by CyD treatment, which indicates the involvement of macropinocytosis. Also, similarly to previous reports, the overall uptake inhibition level may depend on both the incubation time and CPP conjugate concentration [194, 195].

In addition to calculations performed at chosen time-points, the uptake rate kinetic curves of the studied CPP-luciferin conjugates were characterized. Results revealed that incubation with endocytosis inhibitors significantly changes their cellular internalization kinetics suggesting a clear involvement of CME and caveolae/lipid raft mediated endocytosis in their cytosolic entry. At higher conjugate concentrations, the involvement of caveolae/lipid raft mediated endocytosis in the slow uptake group peptides internalization was

more pronounced. Also, contrary to M918 and pVEC, CME was not involved in the uptake of penetratin and EB1 at lower concentrations.

In conclusion, paper III demonstrated that the prevailing uptake route in the cytosolic delivery of luciferin-CPP conjugates is macropinocytosis, even for the fast internalizing group of peptides (MAP, Tat, TP10). In accordance with literature, to a smaller extent, CME and caveolae/lipid-raft dependent endocytosis are involved as well. Furthermore, based on uptake kinetic data analysis, the involvement of different endocytosis sub-types may depend on luciferin-CPP concentration in case of certain CPPs but not in case of others. The involvement of these pathways in the cytosolic luciferin-CPP delivery was determined when comparing the overall total uptake in selected time points but they are more clearly revealed when analyzing the kinetic parameters and shapes of the uptake kinetics curves which reflects the importance of CPP uptake kinetic studies. The information about the intracellular fate of CPPs is valuable for designing novel peptide-based delivery vectors because the incorporated endocytosis route and uptake kinetics might define the fate of the internalized material and its subsequent intracellular trafficking [155].

4.4 Physicochemical characterization of non-covalently formed CPP-siRNA nanocomplexes elucidates their cellular uptake mechanism (Paper IV)

The end goal of CPPs is the delivery of bioactive cargos into cells. In order to achieve this, the uptake mechanism of CPP-cargo complexes needs to be elucidated. In paper IV, the non-covalent complex formation of two efficient siRNA transfection vectors, PF6 [53] and NF51 [69], was studied.

In order to determine siRNA transfection efficacy of these CPPs, different CPP to siRNA molar ratios (MRs) were used to form the complexes. Maximal siRNA-mediated target gene downregulation for each CPP was reached at MR 20, while PF6 showed 60% downregulation already at MR 3, whereas the same efficacy with NF51 was reached at MR 6. This indicated that although both CPPs are efficient in delivering siRNA at higher MRs, there could be differences in their mechanism of action, both in terms of particle formation and interaction with cell membranes.

To study the physicochemical properties of the PF nanocomplexes, their hydrodynamic particle size was measured at different MRs by DLS in milliQ water (MQ) and at physiological pH 7.5. Results revealed that in case of both CPPs, in water, at lower MRs the particles were 200–300 nm in size and reached 100 nm after MR 10, which indicates that the nanocomplexes became condensed once the required CPP concentration was reached. At pH 7.5 the same result was achieved at MR 20. This indicated that internalized PF6- and NF51-siRNA nanoparticles undergo pH-dependent structure changes. In addition, DLS measurements performed with naked CPPs revealed that PF6 and NF51 form larger particles at pH 7.5 than at pH 5.0. Also, naked PF6 became

more compact at higher concentrations whereas NF51 particles remained unchanged.

One of the most difficult steps in characterizing CPP internalization is their endosomal release. In light of the latter data, we sought to investigate more thoroughly whether the CPP-siRNA complexes are different at physiological and acidic pH, thereby mimicking extracellular and endosomal conditions. Based on the data gathered by DLS, ITC studies were performed where the released heat from the interaction between the CPPs and siRNA was measured. Results revealed that at both endosomal pH 5.0 and physiological pH 7.5, CPPs bind siRNA. The data correlated with DLS data as no heat was released after CPP MR 10 in MQ and at pH 5.0. In case of pH 7.5, the same affect was obtained after MR 20, indicating again that the formation of a stable nanocomplex requires higher CPP concentrations. These results also correlate with the siRNA transfection results as maximum mRNA downregulation was reached at MR 20 in case of both CPPs.

Based on these results, we propose that PF6- and NF51-siRNA nanocomplexes are dynamic systems composed of a layer of CPPs that encapsulate siRNA and a second layer of CPPs that are responsible for membrane interactions. Once reaching the acidic environment of endosomes, this second self-assembled CPP layer destabilizes due to increased charge repulsion within the layer caused by changes in pH and enables the escape and subsequent cytosolic localization of siRNA. According to this theory, CPP-cargo complexes may not be as different from naked CPPs as previously considered and CPP uptake studies could also give valuable insights into their internalization mechanisms.

5.CONCLUSIONS

This thesis characterizes the uptake mechanism of several commonly used CPPs. In addition, this thesis focuses on the cellular internalization mechanisms of non-covalent CPP-cargo nanocomplexes. The key findings of this thesis are listed below.

Paper I In this paper we aimed to determine the impact of different hydrophobic modifications on transportan 10 for splice-correcting antisense oligonucleotide delivery. We determined the optimal required CPP hydrophobicity and CPP-SCO nanocomplex size range for maximal splice correction efficacy.

Paper II In this paper we proved that studying the uptake kinetic data provides a more thorough picture of CPP internalization compared to end-point assays. Endocytosis inhibitors affected both the first-order rate constant and the total uptake level of the CPP uptake kinetic curves.

Paper III In this work, we showed that in case of certain CPPs the involvement of different endocytosis sub-types may depend on CPP concentration. In addition, we demonstrated that the main uptake route in the cytosolic delivery of CPP conjugates is macropinocytosis and to a smaller extent, CME and caveolae/lipid-raft dependent endocytosis are involved as well

Paper IV In this work we determined that CPP to siRNA molar ratio 20 is required for efficient siRNA transfection. In addition, physicochemical studies revealed that at physiological pH 7.5, MR 20 is required in order to form a condensed nanoparticle with 100 nm hydrodynamic range and only MR 10 is required at endosomal pH 5.0.

SUMMARY IN ESTONIAN

Rakku sisenevate peptiidide mehhanismide uurimine: peptiididest transpordini

Aastatepikkune molekulaar- ja biokeemia areng koos inimese genoomi sekveneerimisprojektiga on oluliselt suurendanud meie arusaama paljude haiguste mehhanismidest ning on aidanud tuvastada mitmeid haigustega seotud geene. Nende haiguste raviks võib kasutada geeniteraapiat, mis seisneb geeni ekspressiooni inhibeerimises, vigase geeni väljavahetamises või geeni transportimises soovitud rakkudesse. Kuna paljud ravimimolekulid ei ole ise võimelised rakkudesse sisenema, tuleb kasutada transportmolekule, mis jagunevad viirusel põhinevateks ja mitte-viiruslikeks. Viirustel põhinevad transporterid on efektiivsed, kuid omavad tihti kõrvaltoimeid. Enim uuritud mitte-viiruslikud ravimite transportvektorid põhinevad hüdrofoobsetel süsteemidel, nagu liposoomid, polümeerid ja mitsellid. Viimasel ajal on üha enam hakatud kasutama rakkudesse sisenevaid peptiide (RSP), mis on võimelised transportima rakkudesse efektiivselt ja ilma kõrvaltoimeteta erinevaid bioloogiliselt aktiivseid molekule, alates madalmolekulaarsetest ravimitest kuni kõrge molekulmassiga negatiivselt laetud nukleiinhapeteni.

Alates nende avastamisest 20 aastat tagasi on loodud üle saja erineva RSP, mis erinevad üksteisest füsikokeemiliste omaduste ja rakkudesse sisenemise mehhanismide poolest. Selleks, et sõeluda välja kõige efektiivsemad transportvektorid on vajalik välja selgitada nende täpne sisenemismehhanism, sisenemiskineetika ja teised molekulide transpordi efektiivsust määravad omadused.

RSP-de rakku transportimises osalevad mitmed erinevad mehhanismid, mille kasutuse ulatus oleneb paljudest erinevatest asjaoludest, sealhulgas RSP tüüp ja kontsentratioon, transporditava molekuli tüüp, uuritava raku membraani struktuur, rakusisene sihtmärk ning muud katsetingimused. Samuti on kindlaks tehtud, et mitu mehhanismi võivad olla aktiivsed samaaegselt.

Antud töös uuriti mitme laialdaselt kasutatava RSP rakkudesse sisenemise mehhanisme kasutades fluorestsentsil ja bioluminestsentsil põhinevaid meetodeid, mis võimaldavad määrata RSP-de tsütoplasmasse jõudmise kineetikat. Lisaks uuriti, kuidas mõjutavad hüdrofoobsed interaktsioonid ja nanoosakeste omadused RSP-de võimet transportida erinevaid nukleiinhappeid rakkudesse.

Koos kasutati nii RSP-de rakkudesse sisenemise kineetika määramist võimaldavaid meetodeid kui ka lähenemist, mille abil saab hinnata, millist endotsütoosi rada antud RSP rakku sisenemiseks vajab. Kahe meetodi koostöö tulemusel saab detailsemat informatsiooni RSP-de sisenemise mehhanismide kohta kui lihtsamate lõpppunkti mõõtmistega, kuna sama lõpptulemust omavad RSP-d võivad omada täiesti erinevaid kineetilisi profiile. Leidsime, et endotsütoosi radade inhibeerimine mõjutab nii üldist rakku sisenemise taset kui ka kineetilist profiili. Iga uuritud RSP puhul oli mõju ulatus erinev. Samuti selgus, et valitud peptiidide rakku sisenemise profiil võib olla väga erinev, sõltudes RSP kontsentratsioonist ja endotsütoosi inhibiitoritest. Mõlemad tulemused

näitavad, et nende peptiidide rakku sisenemisel on samaaegselt kasutuses mitu endotsütoosi rada.

RSP-de abil on võimalik suure molekulmassiga negatiivselt laetud nukleiinhappeid pakkida kindla suurusega nanoosakestesse. Kuna need kompleksid moodustuvad läbi mitte-kovalentsete sidemete, määrati ka nende füsikokeemilised omadused. Uuriti, kuidas mõjutavad erinevad hüdrofoobsed modifikatsioonid RSP transportan 10 võimet transportida rakkudesse splaissingut muutvaid oligonukleotiide. Määrati moodustunud osakeste suurus ning RSP hüdrofoobsuse vahemik, kus oligonukleotiidide transport on kõige efektiivsem.

Et teha kindlaks, kuidas moodustuvad RSP-siRNA nanokompleksid, määrati nende osakeste suurus ja moodustumisel eralduva soojuse hulk ning korreleeriti saadud tulemused siRNA transpordi efektiivsusega erinevatel RSP kontsentratsioonidel. Viies läbi mõõtmisi nii endosoomide happelise pH kui ka raku füsiloogilise pH juures, selgus, et komplekside suurus ei olene pH muutusest. Samas varieerub RSP hulk kompleksis umbes kaks korda, millest järeldasime, et RSP ja siRNA vahel on dünaamiline, pH-st sõltuv, tasakaal.

Antud doktoritöös esitatud tulemused toovad välja RSP-de rakku sisenemise mehhanismide uurimisel kasutatavate kineetiliste meetodite eelised ning mitte-kovalentselt moodustunud RSP-nukleiinhappe nanoosakeste füsikokeemiliste parameetrite olulisuse RSP-de aktiivsuse jaoks. Kokkuvõtvalt käsitletakse doktoritöös olulisi aspekte, mis on vajalikud uudsete transpordivektorite arendamiseks ja kasutuseks biotehnoloogilistes ning kliinilistes rakendustes.

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List of publications

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