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ABSTRACT

Gene expression can be modified in a wide variety of ways, out of which the gene delivery via plasmids (pDNA) is considered as the most basic. In addition to the gene expression, post-transcriptional silencing through the RNA interference may provide a gene alteration method with high specificity and efficiency. Small interfering RNAs (siRNA) are oligonucleotides (ONs) that occur naturally in the cells as a part of a silencing mechanism and introducing an extracellular siRNA into the cells could be used to achieve a therapeutic effect. Besides gene expression and silencing, it is possible to modulate pre-mRNA splicing. For this, splice-correction antisense ONs (SCOs) could be used. Unfortunately, the chemical and the physical properties of the nucleic acid (NA) molecules narrow the use of NAs and a delivery method or a vector is needed to enable them to cross the membranes of the cells and reach their full potential. Cell-penetrating peptides (CPPs) are one type of vectors with high potential for the delivery of NAs. CPPs are short sequences consisting of approximately 5–30 amino acids (aa-s) with the ability to cross the membranes of the cells and carry along the associated cargoes.

In this thesis the role of modifications in the CPP sequence to the complex formation is investigated. Complexes are formed between the CPPs belonging to the NickFect or PepFect family and the NA cargo. The physico-chemical properties, internalization into the cells and intracellular trafficking of the formed CPP/cargo complexes are examined to understand the properties a CPP or a complex needs to achieve high biological effect from the delivered cargo. As the characteristics of the CPP/cargo complexes rely on the CPP and also the cargo it has to deliver, different types of NA are used: pDNA, siRNA and SCO. All the processes related to complex formation and interactions are determined in cell free and in *in vitro* conditions.

Taken together, our results demonstrate that although being all stearyl-transportan10 modifications, the NA delivery mechanisms and capabilities differ between the tested CPPs. In addition, the delivery process of different types of NAs poses diverging challenges to the delivery vector.

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IV Pärnaste, L.; Arukuusk, P.; Langel, K.; Tenson, T.; Langel, Ü. (2016) Rules behind siRNA delivery by peptides. *Submitted*

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

Paper I: took part in performing of the experiments

Paper II: took part in performing of the experiments

Paper III: designed the experiments, performed most of the experiments, analyzed the data and wrote the article

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LIST OF ABBREVIATIONS

A	amino acid alanine, Ala
A20	mouse B lymphoma cells
aa	amino acid
CHO	Chinese hamster ovary cells
CME	clathrin-mediated endocytosis
CPP	cell-penetrating peptide
CQ	chloroquine
CR	charge ratio
DLS	dynamic light scattering
EE	early endosome
EGFP	enhanced green fluorescent protein
FACS	fluorescence assisted cell sorting
Fmoc	9-fluorenylmethyloxycarbonyl
G	amino acid glycine, Gly
HEK	human embryonic kidney cells
HeLa	cervical cancer cell line
I	amino acid isoleucine, Ile
iTC	isothermal titration calorimetry
Jurkat	human T lymphocyte cells
K	amino acid lysine, Lys
kbp	kilo base pair
L	amino acid leucine, Leu
LAMP	lysosome-associated membrane protein
LDH	lactate dehydrogenase
LE	late endosome
LF2000	Lipofectamine 2000
Luc	luciferase
Lys	lysosome
MEF	mouse embryonic fibroblasts
MQ	ultrapure water
MR	molar ratio
N	amino acid asparagine, Asn
NA	nucleic acids
NF	NickFect
O	amino acid ornithine, Orn
ON	oligonucleotide
pDNA	plasmid DNA
PEG	polyethylenglycol
PF	PepFect
PLGA	poly(lactic-co-glycolic acid)
QMCF	stable episomal expression system
R	amino acid arginine, Arg

RNAi	RNA interference
RP-HPLC	reverse-phase high-performance liquid chromatography
SCARA	class A scavenger receptor
SCO	splice-correcting oligonucleotide
siRNA	small interfering RNA
SPPS	solid-phase peptide synthesis
T	amino acid threonine, Thr
t-Boc	tert-butyloxycarbonyl
TEM	transmission electron microscopy
TP10	transportan 10
U2OS	human osteosarcoma cells
U87	human glioblastoma-astrocytoma cells
Y	amino acid tyrosine, Tyr

INTRODUCTION

Gene therapy and genetic engineering have opened new approaches in the alteration of genes, with the aim of curing diseases or introducing new traits to the cells. Different nucleic acid molecules, including plasmids, SCOs, siRNAs, etc. could be used for it. Still, because of the high charge density, large size and lack of intrinsic internalization route to gain access to the cells, NAs have not yet reached their full therapeutic potential. The development of an efficient, biocompatible and non-toxic delivery method is the basis for broader use of the NAs.

Viral vectors are considered the most efficient delivery method this far, but several concerns related to their safety have led to the development of non-viral methods that are broadly divided into chemical and physical methods. These methods include the use of microinjections, lipid-based delivery vectors, cationic delivery vectors and CPPs. CPPs are short sequences consisting of approximately 5-30 aa-s and have the unique ability to gain access into the cell interior and carry into the cells the cargo attached to them. Although using the CPPs have some limitations, understanding the interactions between the CPP and the cargo, the structure and interactions with the cell of the formed nanoparticles, as well as intracellular trafficking of the latter give the basis upon which further development of the CPP-based carriers may proceed.

Plasmid DNA is a large circular DNA molecule from which a desired gene can be expressed when delivered to the cell nucleus. The size and the abundance of negative charges require condensation of the plasmid molecule by the delivery vector, before it can be efficiently protected from nucleases and delivered into the cell. They are one of the most commonly used NAs in biotechnology because of their versatility and ease of production. Small interfering RNA molecules are small, double stranded NAs with terminal overhangs susceptible to enzymatic degradation. Splice-correction ONs (SCOs) are antisense ONs that can be used to modulate splicing patterns. They are single-stranded and easily degraded by enzymes. Because of intrinsic difference of siRNA, SCO and pDNA, the properties expected from the carrier diverge. In order to develop the most effective transporters for the specific purposes, it is essential to understand the CPP-cargo interactions, complex formation and stability in addition to internalization and intracellular trafficking of the CPPs with different cargoes.

This thesis is dedicated to the investigation of the CPP-based delivery vectors for the complex formation with the NAs and their transport into the cells. The CPPs used in this thesis enable the use of the non-covalent strategy for CPP/NA nanoparticle formation and are thoroughly studied for complex stability to the premature dissociation and delivery of CPP/cargo complexes *in vitro*.

1. REVIEW OF LITERATURE

1.1. Gene therapy and genetic modification by nucleic acids

Gene therapy is a method that uses NA molecules as a drug in order to alleviate diseased state, improve the life quality of the patient or cure a disease permanently [1]. Genetic modification is the transfer of genetic material to introduce novel or improved organisms and also produce a gene product naturally not occurring in that cell [2]. Virtually all cells contain genes making them a potential target for gene alteration, if needed. There are various NAs that can be used for gene therapy. Based on their mechanism of action, they can be broadly divided into gene inhibitors, gene replacers or gene vaccines [3]. In addition to these, splicing patterns can be manipulated with SCOs. Genetic alteration of somatic cells could be achieved by manipulating the cells residing within the individual´s body (*in vivo*) or manipulating the cells obtained from the individual (*ex vivo*) and subsequently returning it to the host [4]. For both approaches the NA has to reach the target cell, be taken up by the cell and present bioactivity after reaching the intracellular target. For this, it has to overcome several barriers depending on the administration route and the intracellular target site for the NA. One of the setbacks of using NAs as drugs is their low stability to degradation, low membrane permeability, high charge density and the lack of efficient cellular uptake mechanism [5]. The success of gene therapy is largely dependent on the development of a vector or a transport vehicle that can selectively and efficiently deliver a NA to the target cells with minimal toxicity. In addition to increasing delivery efficacy, improving the genetic cargoes could result in a need for lower doses of the NA.

1.1.1. Gene delivery

Plasmids are circular DNA molecules, residing separately from the genomic DNA and are naturally found in the bacterial cells. They are a common kind of NA molecule to transfer genetic information into the cells, including eukaryotic cells, for gene therapy or for the production of a protein or RNA. The delivery of pDNA is one of the most basic techniques of molecular biology and a technological basis for *in vitro* and *in vivo* gene therapy. Only a few plasmids per cell are needed to have sufficient gene expression [6]. Gene transfer via pDNA has the potential to treat hereditary disorders and diseases that are caused by a combination of environmental factors and genetic predisposition [7].

Plasmids are generally considered non-toxic. They have low integration rate into the host´s genome, unless specifically engineered to do so. They exhibit a low specificity to a cell/tissue type, but by using distinct promoters and enhancers their specificity can be increased. Mainly a non-covalent vector-pDNA complexation strategy is used, because the circular pDNA lacks a site where a covalent linker could be added. Compared to other NAs, plasmids are several

times bigger, ranging from less than 1 kbp (minicircles) to tens of kbp-s. They carry high density of negative charges in the backbone per plasmid. The size and the charges make the condensation of the plasmid into a small nanoparticle and at least a partial neutralization of the negative charges one of the crucial steps in the delivery through the negatively charged phospholipid bilayers. The pDNA has to be delivered to the cell nucleus or into the cytosol of diving cells, because the expression of a gene from the pDNA uses host´s cell transcription and translation mechanisms. In the cytosol, the free pDNA may be recognized as a foreign substance and taken up by lysosomes (Lys) as a part of autophagy [8], a process possible and important in many physiological processes [9]. When still bound to a carrier after reaching the cytosol, the routing to lysosomes can be overcome or at least, postponed.

1.1.2. Splice correction by SCOs

Post-transcriptional modifications such as splicing are precicely regulated fundamental processes in the cells for generating an mRNA that can be translated into a protein. The splicing of the pre-mRNA requires the recognition of the splice site following the assembly of the spliceosome. Spliceosome is a multicomponent splicing complex, which catalyzes the cut-and-paste reactions that cleave the introns and re-joinin the exons. Alternative splicing of the pre-mRNA contributes to the functional diversity of the human genome. Abnormalities in the splicing may cause or contribute to the development or maintenance of numerous diseases [10]. The use of antisense ONs to modify expression patterns of the alternatively spliced mRNAs is an approach for potentially controlling such diseases.

SCOs are single stranded antisense NAs with the lenght of 15–20 nucleotides. They are able to hybridize with specific complementary sequences in the pre-mRNA and they can be applied to promote exon skipping in the spliceosome. Their use is limited due to their fast degradation by nucleases in the extracellular and intracellular environment.

1.1.3. RNA interference

RNA interference (RNAi) is a common process in the cells with the aim of inhibiting the expression of a gene. One of the advantages of using RNAi is the high target specificity facilitated by the base pairing between the target mRNA and the therapeutic ON [11]. The therapeutic ONs for inducing RNAi include antisense ONs, short hairpin RNAs, siRNAs, single-stranded RNAs and aptamers. The potential for RNAi therapeutics is very broad and is currently limited by the lack of an efficient delivery technology [12].

Synthetic siRNAs are small, double stranded NA molecules with terminal overhangs and a number of solvent-exposed negative charges. They can be

attached to the delivery vectors covalently or non-covalently. The low biological effect *in vivo* of the covalently linked siRNA has led to more frequent use of the non-covalent strategy [13, 14]. Still, linking via electrostatic interactions may result in the instability of the nanocomplexes and premature release of the cargo. The terminal overhangs of linear ONs are more susceptible for enzymatic degradation than circular pDNA. The small size and stiffness of siRNA molecules may help them to retain their initial structure. Therefore siRNAs do not necessarily require condensation to form a nanoparticle, as opposed to large pDNA molecules [15]. As an advantage compared to pDNA, inducing RNAi by siRNAs needs only the delivery of intact siRNAs into the cytosol.

The transport vector for siRNA must be able to deliver intact cargo from the site of application to the cell surface, across the plasma membrane to the cytoplasm, and then to the target RNA. The final achieved biological effect relies on the delivery method, the used cell line, the target mRNA concentration and also siRNA sequence [16].

1.2. Delivery of NAs

Owning to reduced bioavailability *in vivo*, many promising drug candidates, including NAs, are not developed to their full potential. Thus, the need for the effective methods that enable the delivery of a therapeutic compound across the plasma membrane is urgent. A wide range of viral, chemical and physical delivery methods (Figure 1) may be used as platforms for the delivery of NAs. The delivery method or transport vector should be able to carry the NA molecule to the target site, and also exhibit low toxicity and immunogenicity [17] and at the same time it has to maintain cargo activity, stability, be biocompatible, easy to produce, cost efficient, non-infectious and not causing adverse effects [3].

1.2.1. Viral vectors

Gene delivery systems are broadly divided into two groups: viral and non-viral vectors. Viral vectors harness viruses' innate internalization mechanisms with minimized cytotoxicity. Adenoviral, adenovirus associated viruses (AAV), retroviral and lentiviral derived vectors are the most commonly used types of vectors [18-20] and the newer generation viral vectors have been introduced to overcome the problems involving immunogenicity [21]. Several synthetic viruses featuring engineered components that are natural and synthetic [22] have been developed to obtain more controlled and predictable devices. Still, viruses' innate properties may lead to the unwanted side-effects and to an increased risk of adverse effects caused by the vector.

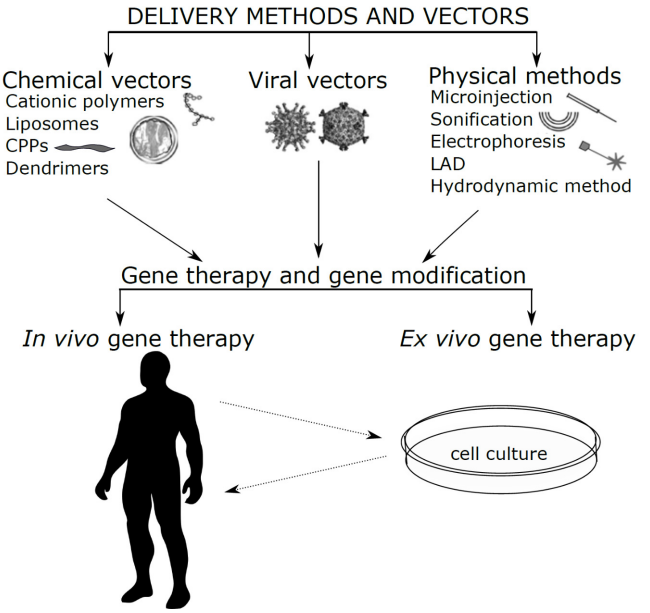


Figure 1. Figurative representation of gene therapy and gene modification approaches and the methods that can be used for this application. CPPs – cell-penetrating peptides, LAD – laser assisted delivery.

1.2.2. Non-viral delivery: physical and chemical methods

The non-viral approaches consist of (modified) nucleic acids alone [23] or in conjunction with a carrier. Non-viral methods can be divided into physical methods and chemical vectors. They are considered less toxic and non-immunogenic [24]. At the same time their use is limited due to their low gene transfer efficiency and in some cases *in vivo* instability and transient gene expression [25, 26]. The limitations of a non-viral delivery method may include the premature release of the cargo, degradation by the enzymes in the extracellular matrix and in the cells. The main limiting step is the internalization into the cells following entrapment in the endosomal compartments that leads to cargo's degradation in the Lys.

Physical methods involve using a physical force such as electric field or physical contact with cell membrane in order to enable the internalization of NAs into the cell. Electroporation is commonly used for cultured cells, but results in high losses of cell viability. Microinjection refers to using a small needle to directly inject the NA material into the cell or the cell nucleus. This method is efficient, but time consuming and needs high accuracy and competence. Physical methods include also the use of gene guns, hydrodynamic injections, etc. The main limitation for the use of physical methods is the low applicability for systemic delivery *in vivo*.

Chemical methods include the use of a chemical compound or a transporting molecule, which facilitates the delivery of NAs across the membranes of the cell. These vectors may be robust or finely tuned transporters and enhanced vectors may feature engineered components that are natural or synthetic [22]. The advantages of chemical vectors, such as cationic lipids and cationic polymers, include the ease of production and the ability to deliver higher molecular weight NAs [26]. The main disadvantage of chemical vectors is the low transfection efficiency [27]. This may be caused by the negatively charged NA cargo that has to be delivered across membranes also carrying a negative charge [28]. Therefore the carrier must be able to shield the negative charges in the NA backbone and at the same time maintain the ability to deliver the cargo into the cell.

1.3. Cell-penetrating peptides

One of the promising non-viral delivery vectors are peptide sequences consisting of ~5-30 aa-s that are able to internalize into the cells. They are called CPPs, protein-transduction domains, membrane translocating peptides or Trojan peptides. First proteins that were able to cross membranes were discovered in 1980s [29]. Firstly the protein-transduction domain of the trans-activator of transcription protein, Tat, was described by a group studying the human immunodeficiency virus (HIV) [30, 31]. Secondly, penetratin, a CPP derived from the drosophila homeobox protein (antennapedia, Antp) was described shortly after [32, 33].

Since then, hundreds of different CPPs have been developed with a common denominator that they can gain entry into the cells and can be used to facilitate the entry of proteins, small molecules, synthetic drugs, NAs, etc. linked to them [34] into various cells [35], usually with limited cell specificity. The CPPs are broadly classified into cationic or amphipathic CPPs. First discovered CPPs were highly cationic [36], but later several peptides that were with lower net charge and higher hydrophobicity were discovered [37]. Although many naturally derived sequences were identified, a number of chimeric (transportan), synthetically designed (polyarginines) [38, 39] or modified peptides (PepFect) have also been generated to improve the cellular uptake and to provide higher cell/tissue specificity. Another way to classify CPPs is based on their origin and dividing them into natural protein derived, chimeric and synthetic sequences.

The ability of the CPPs to deliver large cargoes to the intracellular environment without decreasing the functionality of the cargo provides an opportunity to transport biologically active molecules. CPPs provide a platform for the development of medical treatments using molecules that were considered improbable for therapeutic application. The cargo can be attached to the CPPs covalently or by using non-covalent complexation strategy [40, 41]. In covalent bonding the number of cargo molecules per peptide can be controlled resulting

in a defined structure [42, 43]. Non-covalent strategy is more versatile, easier to perform and driven mainly on electrostatic interactions between the CPP and the cargo. Cationic CPPs can pack NAs into nanoparticles, protect the NAs from degradation [44], and mediate the cargos internalization into the cells, the release from endosomes and the transport to the target organelles.

1.3.1. Transportan, PepFect, NickFect

Transportan is a chimeric CPP, consisting of 27 aa-s. Its N-terminal sequence is derived from the neuropeptide galanin and C-terminal sequence is derived from a peptide toxin from wasp venom - mastoparan. These two sequences are linked via an aa lysine (K) [45]. Transportan 10 (TP10) is a shortened version of transportan. In the TP10 the six first N-terminal aa-s of a peptide are removed, resulting in a less toxic CPP that harnesses the endocytotic pathways for gaining entry to the cells [46, 47]. N-terminally stearylated TP10 was named PepFect3 (PF3) and has been used to deliver ONs and pDNA [48]. The modifications of PF3 have led to the development of the new efficient CPPs, PepFects (PF) and NickFects (NF). PepFect6 (PF6) was further enhanced by covalent attachment of trifluoromethylquinoline-based moieties (CQ) via a lysine tree in the K7 position [49]. PepFect14 (PF14) was designed by replacing the K and isoleucines (I) by ornithines (O) and leucines (L) [50]. In NickFect1 I8 was replaced with threonine and tyrosine (Y) with phosphorylated analog [51].

1.4. Characterization of CPPs and CPP/cargo complexes

Over the past decades, numerous types of nanoparticles have been developed for medical and biotechnological applications. However, only a few are actually available on the market. One reason for this is the lack of understanding of the CPP/cargo complexes and their behavior upon contact with biological fluids and/or cell membranes. The complexes formed with the CPPs can be assessed on the basis of complex formation efficiency and the biological activity gained from the cargo. More importantly, the ability of the CPPs to protect the cargo from degradation and the possibility of CPP-cell interactions should be considered. For characterization of the CPP/cargo complexes various methods have been used, including intercalation of fluorescent dyes into pre-formed complexes, transmission electron microscopy (TEM), dynamic light scattering (DLS), etc. All these methods have some advantages, but also disadvantages. Therefore, several methods should be used simultaneously in order to determine the parameters a CPP should have in order to work as an efficient delivery vector. Easiest and the most efficient way to assess the CPPs and the complexes formed between CPPs and the cargos is to use cell free and *in vitro* assays, where experimental conditions can be controlled more than in complex systems, such as mouse models.

1.4.1. The net charge and structure of the CPPs

CPPs vary in the molecular weight, size and structure. The synthesis can be continued from the aa-s side chain [52] generating a branched or cyclic CPP instead of the linear peptide. The molecular weight varies from approximately thousand Da for short peptides to several kDa for longer, modified peptides. The secondary structure of a CPP, including random coils, α-helices or β-sheets, facilitates the interactions between the CPP and the cell membrane [53,54]. For some CPPs, the secondary structure is already formed in the free peptide. In others, they are induced when the peptide is interacting with the cargo or with a membrane. Although the most efficient CPPs have some similar features, no clear rules regarding the secondary structure can be proposed [55-57].

The charge distribution in the peptide sequence may play a role in the complex formation and the interactions with the cell membranes for some CPPs. As a different example, the aa residues introduced into the original sequence of Tat peptide failed to influence cellular uptake, indicating that the cationic charge alone is needed for translocation [58]. The structural properties of the CPP as well as the outer layer of a CPP/cargo complex can facilitate the interaction with membrane components. For Tat peptide uptake, the role of cell membrane glycosaminoglycans has been shown [58]. For negatively charged particles formed from PF14 and ONs, the involvement of SCARA receptors has been shown [59]. Since no clear consensus has been reached which membrane elements are involved [60, 61], several have been proposed. For example, in addition to glycosaminoglycans and SCARAs, cationic peptides are shown to interact with heparan sulfate proteoglycans [62].

1.4.2. The size, surface charge and dissociation of the complexes

The plasma membrane of the cell plays a crucial role in the selective permeability, intracellular osmotic balance maintenance, compartmentalization, and cellular uptake [63]. Small molecules such as aa-s, ions, etc. are able to permeate cells through carriers and channels on the membrane. Macromolecules, such as proteins, DNA, RNA have to rely on other modes of entry. Nanoparticles are identified as having dimensions ≤ 100 nm, but larger sized particles may be needed to contain sufficient amount of the drug [64]. The transport of the smallest cargo to even large 120 kDa can be mediated by CPPs [65] both *in vitro* and *in vivo*. Depending on the CPP concentration and the size of the cargo, CPP/NA nanoparticle size may vary. Nanoparticles may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials [66], but even small differences in the size may influence the actual distribution and thus bioavailability of the complex [67-69]. However, not all particles with the same size behave similarly; therefore composition of the particle is important as well.

The nanoparticle surface charge may also affect the fate of the complexes in the cells. Membrane potential was shown to have a huge role in the internalization of arginine (R) rich CPPs [70-72]. Because of the surface charge, CPP/cargo complexes may interact with the serum components, leading to a change in stability and/or lead to the formulation of aggregates. One way to modify the surface of the complexes and to improve the half-life of the peptide is the addition of polyethylenglycol (PEG) to the peptide sequence. Surface functionalization of gold nanoparticles with PEG has been used and resulted in efficient internalization and localization in the nuclear region [73]. Nanocomplexes consisting of the CPPs, poly(lactic-co-glycolic acid) (PLGA) and cargo have been used to improve doxorubicin delivery or drug delivery to the brain [74,75]. At the same time, masking the charges or modifying the nanoparticles may not improve the delivery as the CPP charge plays a role in the interactions with the cell surface components, such as receptors [76].

CPPs are a target for degradation by proteases. This degradation is an advantage, but also a disadvantage for the peptide-based delivery systems. The advantage comes from the ability of the cells to degrade the delivery agent, so no permanent damage or toxic effects remain. The disadvantage of being a target for proteases is that it may lead to the premature degradation of the CPP and the dissociation of the complexes. Several strategies have been used to increase the stability of the peptides, including the use on aa-s generally not included in the protein sequences, so they would not be as easily recognized by the enzymes [50]. At the same time the dissociation of the complexes at the target site is needed to release the cargo and gain desired biological effect. The CPPs that pack NAs into nanocomplexes without dissociation in the presence of serum show promise for use in non-covalent complexation strategy [77].

1.5. Internalization and trafficking of the cargo and the complexes

The mechanisms by which CPPs promote uptake of the associated molecules are influenced by the physico-chemical properties of the CPPs, the molecular cargo, and the variety of cells and tissues being treated. Two general models of CPP uptake have been proposed: energy-independent direct translocation across the plasma membrane to the cytosolic compartment [78, 79] and endocytosis [80-82]. Several internalization pathways, including caveolar, clathrin-mediated endocytosis and macropinocytosis, have been demonstrated for different CPPs [83, 84]. Results from studies conducted thus far suggest that the mechanism of uptake is highly dependent on the type of cargo, making it difficult to predict which penetrating peptide should be selected in each case [85-87]. In the case of endocytosis, the fate of the CPP-cargo complexes depends on their ability to escape the endosomal compartments before being delivered to the Lys for degradation. Endocytotic mechanism of cellular delivery can limit the effectiveness of the CPP-based deliveries by decreasing the amount of cargo that is active and

able to reach the target. Strategies for increasing endosomolysis, thereby making more active cargo available, involve the use of pH-responsive elements, which are active in the acidic environment of the endosomes without disrupting the membranes of other organelles [49, 88]. Examples include the addition of a short peptide derivative of the influenza virus protein hemagglutinin or the use of a synthetically designed peptide, GALA [89].

1.5.1. Endocytosis

The mechanisms of cellular uptake of the CPPs and the CPP/cargo complexes have been discussed extensively in several reviews [37, 85, 90]. In addition, CPPsite [91], an online database of CPPs, describes the almost 2000 known CPPs and provides information about uptake mechanisms for each peptide. Still, the exact internalization mechanism is not known and the probable reason behind these inconsistencies may lay in the diversity of the CPPs, used concentration, properties of the cargo (size, charge), temperature, proteins in the media, interactions with cell membrane and the state of the cells [92, 93]. In addition, the same CPP can harness more than one pathway, if needed [80].

The internalization of particles occurs after interactions with the cell membrane [94] and the two most likely pathways involve the direct penetration via the formation of an inverted micelle, membrane thinning, carpet-like model or of pore formation [32,95-97] or use of the endocytotic pathways. When attached to a cargo, the CPP/cargo complexes use mainly endocytosis to gain access to the cell interior. Endocytosis is a universal process occurring in the cells with the aim of gaining material from the cell exterior into the cell [98]. In addition to this, endocytotic pathways enable the sorting and transport of different products into the cell. Endocytosis is classically divided into different pathways, involving phagocytosis and pinocytosis. Pinocytosis is in addition divided into macropinocytosis, caveolin- and clathrin dependent endocytosis and caveolin and clathrin independent endocytosis [99] (Figure 2). Receptors in the receptor-mediated endocytosis assure the selectivity and cytosolic adaptor proteins assure the right sorting of endocytosed material.

Clathrin-coated pits and caveolae are two of the most recognizable features of the plasma membrane of the mammalian cells [100]. The importance of clathrin-mediated endocytotic (CME) pathway in uptake, trafficking, recycling, and degradation of the internalized compartments has been extensively studied [101] and clathrin controls several physiological functions, including synaptic vesicle internalization in neurons [102]. The CME involves the budding of clathrin coated plasma membrane inner layer to internalize receptor bound material. Following budding, the vesicle is uncoated and delivered to early endosomes (EE). Caveola/lipid raft-mediated, clathrin-independent endocytosis has emerged as another crucial trafficking pathway [103]. Caveolae and lipid rafts (membrane domains enriched in cholesterol and glycosphingolipids) participate in many cellular events, including cholesterol trafficking and inter-

nalization of certain toxins, viruses, GPI-anchored (glycosylphosphatidylinositol-anchored) proteins, and many other cargos [104, 105]. Caveolae are flask-shaped invaginations in the plasma membrane that co-localize with cholesterol and sphingolipid-rich membrane domains containing signaling molecules and membrane transporters. Caveolin is the driving dimeric protein behind the caveolae formation. After binding to the cholesterol it causes the inner surface membrane invaginations. Macropinocytosis is regarded as a nonspecific internalization route, because it is generally not mediated by receptors. Macropinocytosis involves the ruffling of the membrane and formation of lamellapodia. These structures are cell membrane protrusions and after collapsing into macropinosomes engulf large amount of the extracellular material. Following internalization, the formed vesicles are routed to transport vesicles – endosomes.

1.5.2. Endosomal trafficking of internalized particles

Endosomes are endocytotic vesicles and they are a part of a trafficking system in the cell. This system is responsible for the delivery of endocytosed material to the right cellular compartments. Each subtype of vesicles is characterized by distinct proteins and the transport is coordinated by Rab-GTPases [106]. For caveolin and clathrin dependent endocytosis, the vesicles are routed to EEs, a checkpoint and initial sorting depot [107]. From there the endocytosed material is re-routed to the cell membrane (exocytosis) or to the late endosomes (LE). Sorting initiated in the EEs predetermines the course for internalized material, prioritizing it for recycling, exocytosis, degradation in Lys or delivery to trans-Golgi network. During the maturation of an endosome (Figure 2) a set of vacuolar ATPases in the vesicle membrane change their activity and pH drops from 7.4 to pH 5 in mature Lys. In the EEs, the pH is slightly acidic compared to the extracellular environment and EEs can be recognized by the presence of Rab5 proteins in the membrane. From EEs the material is transferred to the LEs or the EEs are matured into LEs by the change of membrane proteins and the decrease of pH. LEs contain hydrolytically active lysosomal hydrolases and the first steps of degradation start in LEs. The low pH and activation of degradation enzymes by the pH enables the final lysis of content in the Lys [108]. The extended stay of nanoparticles in the endosomal compartments may lead to their degradation [94] and therefore the endosomal escape is needed in order to reach the target site intactly. Entrapment in endosomes is one of the major limitations for a non-viral delivery vector mediated transport [109]. After endosomal escape the nanoparticle has to reach the target in the cytosol or organelle and release the cargo. This is needed for the cargo to take its intended action.

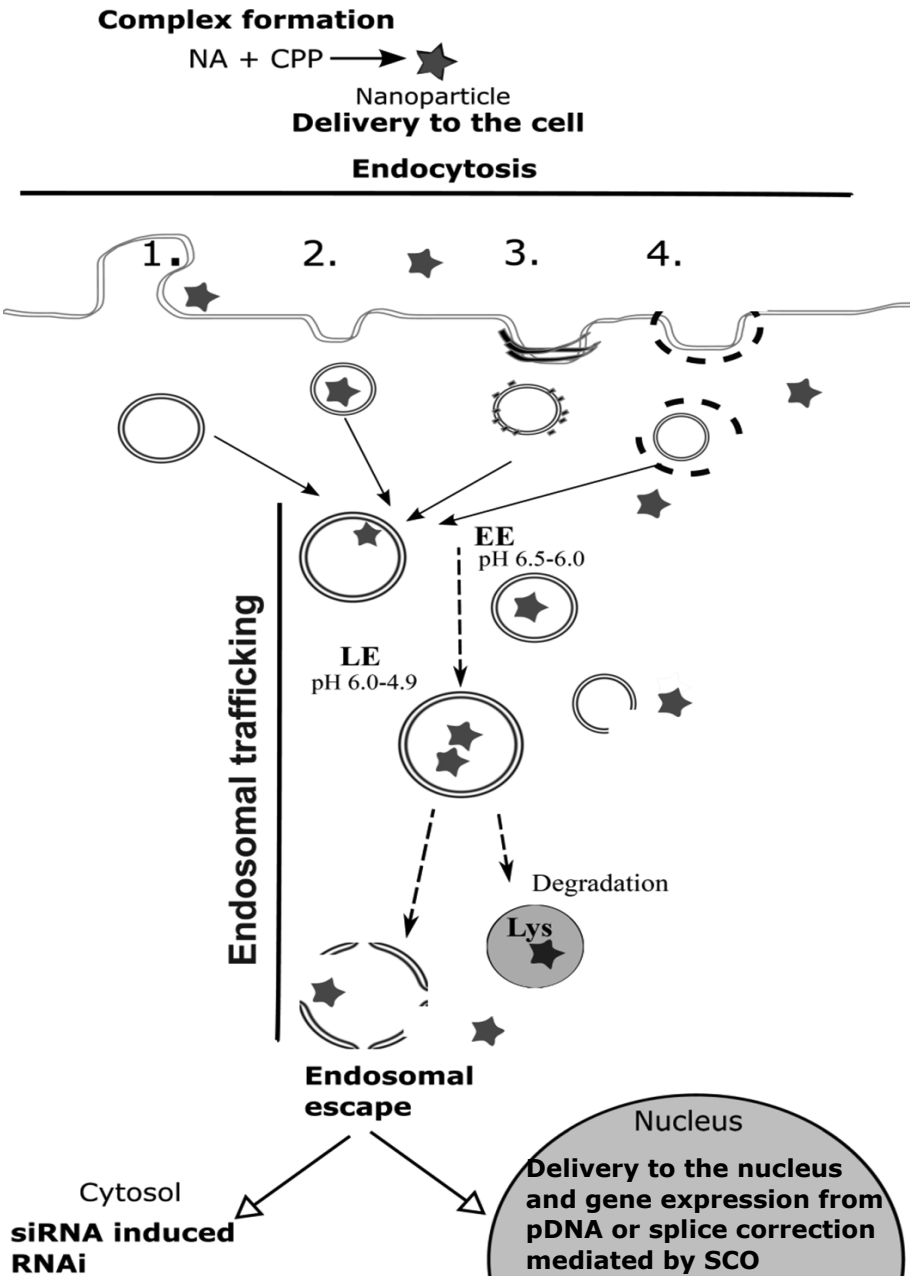


Figure 2. Schematic illustration of the main endocytotic pathways and intracellular routing of endocytosed nanoparticles with siRNA and pDNA. Endocytic pathways: 1. Macropinocytosis, 2. Clathrin- and caveolin-independent endocytosis, 3. Caveolin-mediated endocytosis, 4. Clathrin-dependent endocytosis. Endosomal vesicles: EE – early endosomes. LE – late endosomes, Lys – lysosomes. RNAi – RNA interference, NA – nucleic acid, CPP – cell-penetrating peptide, SCO – splice correction oligo-nucleotide, pDNA – plasmid DNA.

2. AIMS OF THE STUDY

The ability of CPPs to cross membranes and facilitate the uptake of various cargoes attached to the CPP has been widely recognized. Still, there are several limitations that restrict the use of the CPPs for the delivery of cargo in *in vivo* applicatios. The final biological effect gained from the delivered cargo relies on several aspects, including the interactions between the CPP and the cargo, the efficiency of nanoparticle formation, nanoparticle stability, and the interactions of the CPPs or the complexes with the cell membrane and intracellular trafficking of the internalized CPP/cargo particles. The *in vitro* testing of the CPPs and the characterization of the CPP/cargo particles provide data for their application *in vitro* and basis which further *in vivo* steps rely on.

More precisely, the aims of the study were:

Paper I: to evaluate the impact of two different modifications in the CPP sequence to the CPP/NA complex formation and the delivery of NAs. Test the applicability of the most efficient analogue for the delivery of pDNA in different cell lines and for protein production technology.

Paper II: to characterize the internalization and the intracellular trafficking of pDNA mediated by delivery vectors NF1 and NF51. Assess the role of the modifications in the peptide sequence on the internalization efficiency, internalization route and intracellular trafficking.

Paper III: to validate two methods that can be used for the tracking of the CPP/pDNA complexes after internalization and their endosomal release. Assess the influence of the modifications in the NF1 and NF51 on the intracellular trafficking of the cargo and endosomal release of the complexes.

Paper IV: to determine the binding properties of NFs and PFs to siRNAs, the CPP/siRNA complex structure and their stability to enzymatic degradation. Determine the physical and chemical properties of the formed CPP/siRNA complexes in order to obtain a model of complex formation between the most efficient tested CPP and siRNA.

3. METHODOLOGICAL CONCIDERATIONS

The methods used in this study are described comprehensively in the respective papers and only brief comments on the chosen methods are given here.

The *in vitro* models provide an opportunity to study the cellular response and the CPP/cargo properties in a closed and simplified system. Such models provide preliminary information about the complexes and provide alternative means for drug and chemical testing up to some levels. Advantages associated with the *in vitro* methods are time efficiency and cost effectiveness.

3.1. Peptides

In the current thesis the CPPs derived from TP10 were used (Table 1). Previous studies have shown an improved transfection efficiency of the CPPs when a N-terminal stearic acid was added. All tested peptides are considered as further modifications of PepFect3 (stearyl-TP10, PF3) and were synthesized by using a solid-phase peptide synthesis strategy (SPPS). In SPPS the first aa of the peptide is linked to a solid polymer, e.g. resin, and the synthesis is carried out in a stepwise manner. The advantage of using the SPPS is that it does not need purification between the aa coupling and deprotection. This synthesis strategy offers a versatile method to introduce modifications into the peptide sequence at any given step of the synthesis and without drastically changing the protocol. Prerequestite for this is the use of an aa with the suitable protection groups. Two main strategies regarding the protection of side chain amino groups are used in the SPPS. First uses 9-fluorenylmethyloxycarbonyl (Fmoc) and second uses tert-butyloxycarbonyl (t-Boc). The main difference arises from the needed conditions for the removal of Fmoc and t-Boc. Fmoc is removed by a strong base and t-Boc can be removed by a strong acid. The side chain groups of aa-s are protected with protection groups that are removed during the final peptide cleavage from the solid support, not during the synthesis. After cleavage from the resin and the final deprotection of aa side chain groups, the peptides were purified with reverse-phase high-performance liquid chromatography (RP-HPLC) and the correct product-containing fractions were identified by MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight) mass spectrometry.

In paper I the peptide sequence of stearyl-TP10 was modified by introducing O7 instead of K7 in the sequence and/or continuing the synthesis from the aa side chain, instead of α-amino group. These modifications were used to increase the serum stability of the CPP and also to create a flexible kinked structure in the peptide.

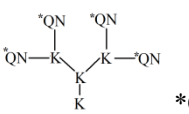
Papers II and III describe the CPP/pDNA complex formation, stability and intracellular trafficking of formed nanoparticles. Paper II focuses on thorough characterization of NF/pDNA complexes, their internalization and intracellular delivery. Paper III describes two methods that can be used to determine the endosomal transport and the release of complexes from the endosomes and how

the endosomal localization differs between the pDNA delivered by these CPPs. NF1 and NF51 are new CPPs that were previously used for the delivery of different NA cargoes [51,paper I]. From each set of designed CPPs, these two were the most efficient. We used NF1 and NF51 to deliver pDNA into HeLa cells and to determine the properties and cellular events involved with their high efficiency.

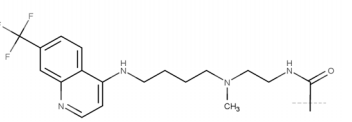
In paper IV the binding of CPPs to siRNA and complex formation were investigated. Five analogues of TP10 were compared (PF3, PF6, PF14, NF51, NF57) on the basis of complex formation, stability and ability to deliver siRNA into the cells and mediate downregulation of a target gene. These CPPs were chosen because of their previously shown efficiency in delivering siRNA.

Table 1. CPPs used in this thesis.

Name	Sequence	Paper	Reference
Transportan10, TP10	AGYLLGKINLKALAALAKKIL-NH_2	IV	[47]
PepFect3, PF3	Stearyl-AGYLLGKINLKALAALAKKIL-NH_2	IV	[48]
PepFect6, PF6	Stearyl-AGYLLGK*INLKALAALAKKIL-NH_2	IV	[49]
PepFect14, PF14	Stearyl-AGYLLGKLLOOLAAAALOOLL-NH_2	IV	[50]
NickFect1, NF1	Streayl-AGY*LLGKTNLKALAALAKKIL-NH_2	I, II, III	[51]
NickFect51, NF51	(Stearyl-AGYLLG)δOINLKALAALAKKIL-NH_2	I, II, III, IV	Paper I
NickFect53, NF53	(Stearyl-AGYLLG)εKINLKALAALAKKIL-NH_2	I	Paper I
NickFect61, NF61	Stearyl-AGYLLGOINLKALAALAKKIL-NH_2	I	Paper I
NickFect57, NF57	(Stearyl-AGYLLG)δOINLKALAALAKAIL-NH_2	IV	Paper IV



K* *QN



Y* Tyrosine with phosphoryl group

3.2. Non-covalent complex formation and nanoparticle characterization

There are two main strategies used to associate the cargo with the CPP. These are covalent conjugation and non-covalent complex formation. The non-covalent strategy was first utilized by G. Divita´s group [110] and relies on electrostatic interactions between the positively charged aa-s in the peptide sequence and the negatively charged phosphate backbone of the nucleic acid molecule. The first step of nanoparticle formation is the interaction of a CPP with a cargo. In order to have a stable complex a positively charged tripeptide may not be enough [111]. Hydrophobic and hydrophilic interactions and/or interactions with other aa-s in the sequence may be needed to stabilize the complex. The N-terminal fatty acid and positively charged aa-s in the C-terminal part enable to create two distinct regions in the same peptide sequence. Both hydrophobic and hydrophilic faces of amphiphilic peptides are likely involved in the interactions with RNAs [112]. Different methods have been used to describe CPP and cargo complexes and their formation. In this thesis gel electrophoresis, isothermal calorimetry, florescent dye intercalation assay and ethidium bromide quenching assay were used to characterize the complex formation between CPPs and nucleic acid cargoes.

3.2.1. Isothermal calorimetry

Stability of the formed nanoparticle determines the CPP/cargo complex dissociation at the target site. Isothermal calorimetry (iTC) is a highly accurate and sensitive method for simultaneous determination of K_A [M^{-1}], stoichiometry and thermodynamic parameters (ΔG, ΔH, and ΔS) in a simplified experimental set-up compared to *in vivo* experiments. The formation of siRNA and CPP complex is an exothermic process resulting in the release of heat. A cell containing a constant volume of siRNA is titrated with a series of additions of CPP molecules. When binding reaches saturation, fewer complexes are generated, therefore less heat is released. Although high picomolar Kd values have been reported using iTC, measurements in the 10^{-8} to 10^{-9} M^{-1} range are more common. Although iTC can work at lower sample concentrations, any concentrations lower than high nM is not sufficient. If a high nM sample concentration is chosen with a low picomolar Kd binding system, the titration curve is too steep, giving rise to a significant error in Kd. This limitation of iTC could be important, because complex formation is dependent on CPP and cargo concentrations, therefore results obtained by standard complex formation protocol and concentrations could be more relevant.

In paper IV iTC was used to determine the binding affinities of siRNA and CPP at biologically relevant pH-s and with CPPs differing in net charge, size and modifications.

3.2.2. Model calculations

Model calculations are based on the sequence of the CPP. The advantage of using modeling instead of experiment is that it improves the understanding of the system and permits exploration of alternatives without the need of synthesizing and going through a set of experiments for each peptide. The calculations are based on physical and chemical properties of each link in the peptide sequence. One of the ways to use mathematical models is to determine the net charge of the peptide at various biologically relevant pH-s. When exposed to the media and entering the cell, the particle is exposed to a pH drop in endosomes and increase after release from endosomes to the cytosol. This pH change may affect the stability of the complex. The mathematical calculation of net charge enables to assess the change of charge of the CPP during the delivery when exposed to intracellular and extracellular pH. For each cargo, there should be an optimal number of positive charges in the peptide sequence that interact with it to form the nanoparticle. Hydrophobicity, more specifically the regions in the peptide that are hydrophilic and hydrophobic and how they are located in the higher structures can also be estimated by mathematical modeling. Experimental setup for determining these parameters are time consuming and the conditions may alter the result.

In paper I, II and III the charge ratio (CR) was used to express the ratio of CPP over pDNA in complex formation solution. Charge ratio is based on the theoretical number of negative charges in the plasmid backbone, positively charged aa-s in the peptide sequence and their ratio to each other. The net charge of peptides and conversion from CR to molar ratio is shown in table 2.

In paper I and IV the molar ratios were used to express the excess of peptide over siRNA or SCO. This is based on the amount of moles of CPP and cargo. Molar ratios are widely used to express CPP/siRNA nanocomplex formation ratios, but can be expressed in charge ratios also (Table 2).

Table 2. The molar ratio to charge ratio conversion table for CPPs used in this thesis

CPP	Charge ratio CPP:cargo¤				Molar ratio CPP:siRNA/ CPP:SCO*						Net charge˅					
	1:1	2:1	3:1	4:1	6:1	10:1	15:1	20:1	30:1	40:1	pH 5	pH 5.5	pH 6	pH 6.5	pH 7	pH 7.5
TP10	2600:1	5200:1	7800:1	10400:1	0.5/1	1.0/2.0	1.5/3.0	1.9/3.8	2.6/5.2	3.8/7.6	+4.5	+4.2	+4	+4	+4	+4
PF3	2600:1	5200:1	7800:1	10400:1	0.5/1	1.0/2.0	1.5/3.0	1.9/3.8	2.6/5.2	3.8/7.6	+4.2	+4.1	+4	+4	+4	+4
PF6[#]	1040:1	2080:1	3120:1	4160:1	1.5/3	2.4/4.8	3.6/7.2	4.8/9.6	7.3/14.6	9.6/19.2	+11.7	+11.2	+10.8	+10.2	+9.1	+7.7
PF14	2100:1	4200:1	6300:1	8400:1	0.7/1.4	1.2/2.4	1.8/3.6	2.4/4.8	3.6/7.2	4.8/9.6	+5.2	+5.1	+5	+5	+5	+5
NF51	2600:1	5200:1	7800:1	10400:1	0.6/1.2	1.1/2.2	1.6/3.2	2.1/4.2	2.9/5.8	4.2/8.4	+5.7	+5.2	+4.8	+4.4	+4.2	+4
NF63	2600:1	5200:1	7800:1	10400:1	0.6/1.2	1.1/2.2	1.6/3.2	2.1/4.2	2.9/5.8	4.2/8.4	+5.7	+5.2	+4.8	+4.4	+4.2	+4
NF61	2600:1	5200:1	7800:1	10400:1	0.6/1.2	1.1/2.2	1.6/3.2	2.1/4.2	2.9/5.8	4.2/8.4	+5.7	+5.2	+4.8	+4.4	+4.2	+4
NF57	2600:1	5200:1	7800:1	10400:1	0.6/1.2	1.1/2.2	1.6/3.2	2.1/4.2	2.9/5.8	4.2/8.4	+5.7	+5.2	+4.8	+4.4	+4.2	+4
NF1	3400:1	6800:1	10200:1	13600:1	0.4/0.8	0.6/1.2	0.9/1.8	1.2/2.4	1.8/3.6	2.4/8.4	+3.2	+3	+2.8	+2.6	+2.3	+2.1

[#]Calculated for net charge +10, including positive charges from chloroquine moieties
[¤]Calculated for average number of positively charged aa-s in the sequence
*Calculated for net charge at pH 6.5.
˅ Charge per peptide calculated from ChemAxon MarvinSketch

3.2.3. Gel electrophoresis

Gel electrophoresis enables to assess the complex formation between CPPs and cargoes. The mobility in gel during electrophoresis depends on the molecules charge, size and shape. In nucleic acid bound to the CPPs the negative charges are neutralized by positive charges of the peptide. In addition to that, the movement of CPP/cargo complex is limited in the agarose gel network. The complex formation is assessed by the detection of nucleic acid band after electrophoresis. In case of pDNA, more than one band for one plasmid can be detected, because the plasmid can be in circular or supercoiled form and depending on that move in the gel faster or slower. In the case of siRNA, there is usually only one detectable band.

Gel electrophoresis on pre-formed complexes is a common method widely used for screening the CPP/cargo complex formation (used in paper II, III, IV). In paper IV gel electrophoresis was used to assess the complex formation of CPP/siRNA complexes at different pH conditions. For this, complexes were formed in different buffers, incubated at room temperature, mixed with loading dye and transferred to gel teeth.

3.2.4. Fluorescent dye intercalation assay for nanoparticle characterization

Fluorescent dyes that specifically bind to the nucleic acids can be used to assess the accessible cargo in the complexes and also the complex formation and the release of the cargo. Ethidium bromide is commonly used for that purpose, but today several other dyes have been developed. PicoGreen is a fluorescent dye that maintains its fluorescence intensity even after longer periods of excitation and can be used for prolonged measurements. It intercalates into the minor groove [113] of double stranded nucleic acid when NA is accessible.

In paper II and IV fluorescent NA binding dyes were used to assess the complex resistance to heparin displacement. The interaction between the CPP and the cargo has to be optimal in order to maintain stability during the delivery and also to enable the release of cargo at the target site. Heparin sodium salt is a molecule with high negative charge density and is able to replace the nucleic acid cargo in the loosely packed complex. The critical concentration at which 50% of nucleic acid is replaced in the complex enables to compare the stability of complexes. After complexes were formed, heparin solution at different concentrations was added and complexes incubated further. After incubation, a dye was added to detect the plasmid that had been replaced by heparin and therefore available for dye binding.

During delivery the complexes have to be stable to enzymatic degradation. The complexes consist of nucleic acid and peptides. Nucleases and proteases are two types of enzymes responsible for degrading protein/peptide and NA molecules in the cells and tissues. Proteinase K and RNase A were used to to

assess the complex resistance to enzymatic degradation and to pinpoint the component in the complex that causes the low stability.

In paper IV fluorescent dye intercalation was used both to assess the complex formation, and the resistances enzymatic degradation. For complex formation a fluorescent dye was added to the complex solution after incubation at room temperature and the percent of accessible siRNA was calculated based on the fluorescence intensities. For enzymatic degradation assay a fluorescent dye was added to pre-formed complexes to determine the initial percentage of accessible siRNA. Thereafter the enzymes Proteinase K or RNase A were added and incubated, following the measurement of changes in fluorescence intensities over the period of 24 h.

3.2.5. Dynamic light scattering and zeta potential

Dynamic light scattering (DLS) is a spectroscopic method that enables to determine the hydrodynamic mean size of formed complexes in solution. Nanoparticles in solution may be regarded as a part of colloidal dispersion system, where molecules follow the random Brownian motion, which causes the laser light to be scattered at different intensities. The time-dependent fluctuations are directly related to the rate of diffusion of the molecule through the solvent, which is related to the hydrodynamic diameter. The zeta potential correlates with the surface charge of the nanoparticles in a solution. Particle size and zeta potential are important parameters of a nanoparticle and may influence the internalization, stability and biodistribution of the complexes.

In paper I and II DLS was used to determine the size and zeta potential of complexes in MQ water and also in the presence of media and serum. In paper III DLS was used to determine whether the complexes formed with Cy-5 labeled pDNA differ significantly in the nanoparticle size.

3.3. Cell cultures

Experiments on cell cultures are alternative and also a crucial step before *in vivo* testing. Cell cultures are routinely used for preliminary screening of potential drug molecules/chemicals to test their efficacy. NA transfection with NFs and PFs was tested in several cell lines (Table 3), out of which CHO, HeLa and HEK 293 cell lines are widely used in cell biology research and biotechnology. They are easy to handle and are commonly used in drug delivery studies. MEF, Jurkat and A20 cells are considered hard-to transfect cells and were used to test if it is possible to efficiently deliver pDNA with NF51 into these cells. U87 cells are cancer cells and were used to compare the potential of NFs and PFs as siRNA delivery vechicles. U2OS and U87 were used to test NF51 ability to deliver pDNA into cancer cells.

Table 3. Cell lines used in this thesis

Cell line	Cell type	Paper
CHO (CHO-K1; EGFP-CHO; CHOEBNALT85)	chinese hamster ovary cells	I
MEF	mouse embryonic fibroblasts	I
HeLa (HeLa; HeLa pLuc705)	human cervical carcinoma cells	I, II, III
HEK 293	human embryonic kidney cells	I, III
U2OS (U2OS; U2OSSEBNALTD3)	human osteosarcoma cells	I
U87 (U87; U87-luc2)	human glioblastoma-astrocytoma cells	I, IV
Jurkat	human T lymphocyte cells	I
A20	mouse B lymphoma cells	I

3.4. Transfection experiments

The plasmid delivery efficiency of CPPs was determined by using a reporter system based on luciferase (luc) encoding pGL3 plasmid. This luciferin-luciferase system is sensitive, reproducible and easy to measure.

Plasmids are large molecules compared to other cargoes delivered by CPPs. The size varies from 4 kb for minicircles to tens of thousands base pairs for double reporter plasmids. In paper I, II and III the efficiency of CPPs was evaluated by transfecting cells with pGL3 (4.2 kbp or 5.2 kbp) reporter plasmid. Plasmid that is delivered to the nucleus can use the transcription and translation systems of the cell and the luc is expressed. To the cell lysate luciferase substrate luciferin is added and luminescence is measured. The measured luminescence correlates with the amount of luc expressed in the cell.

In paper I pQMCF-CMV-EGPF plasmid was delivered with NF51 into U2OSSEBNALTD3 and CHOEBNALT85 cells and number of transfected cells was measured with FACS (fluorescence assisted cell sorter) 24 h and 7 days post transfection, by detecting the EGPF. QMCF tehnology enables the expression of biologically active substances by generating expression cell banks within one week and production cell line for stable protein production in 6–8 months.

For gene downregulation in paper I and IV the cells that stably express luc or EGFP were used and siRNA targeting that gene product (mRNA) was delivered to the cytosol. SiRNA strand, when bound to its target, induces RNAi and the degradation of the target product, therefore no protein can be produced from this mRNA. In paper IV U87 cells encoding luc2 gene and in paper I CHO cells expressing EGFP gene gene were treated with CPP/siRNA. The efficiency of downregulation was measured by the percent of remaining luc2 or EGFP activity.

In paper I HeLa pLuc705 cells expressing non-functional luc gene were treated with CPP/SCO complexes delivering the SCO to modulate non-functional luc gene splicing. The efficiency of splice correction was expressed by the fold increase of luc gene activity after transfection.

As negative and positive controls cells treated with MQ water and the commercial reagent RNAiMAX or LF2000 were used.

3.5. Cellular uptake and intracellular trafficking of nanoparticles

There are several methods that are used to track the internalization and trafficking of nanoparticles. Most commonly different chemical inhibitors are used to block the pathways mediated by receptors. Chemical inhibitors directly bind to the receptor or disrupt their synthesis or presentation of these receptors on the cell membrane. The energy dependent endocytosis can be blocked by low temperature or by depletion of the cellular pool of ATP. This enables to differentiate energy dependent endocytosis from energy independent pathways i.e. direct translocation.

3.5.1. Membrane interactions – lactate dehydrogenase leakage

Lactate dehydrogenase (LDH) is an enzyme present in all living cells and catalyzes the reversible transformation of pyruvate to lactate under anaerobic conditions, coupled with the oxidation of NADH to NAD^+. Each normal tissue has a distinct LDH activity pattern, depending on their functions and levels of LDH increase in response decreased cell integrity in tissue injury. In cancer cells the LDH A levels are elevated and have been linked to tumor growth, maintenance and invasion [113]. The LDH A up regulation increases the efficiency of anaerobic glycolysis in tumor cells and reduces their dependency on oxygen. The release of LDH is commonly measured in order to test the cytotoxicity of compounds.

In paper IV U87 cells were used. U87 cell line has elevated levels of LDH and therefore an interaction between the CPP and the cell following a pore formation and release of LDH can be detected more easily than with non-cancerous cell lines where LDH levels are commonly lower.

3.5.2. Inhibition and downregulation of scavenger receptors

Scavenger receptors are cell-surface receptors that bind with low specificity anionic ligands. Scavenger receptors are involved in the uptake of negatively charged particles and the involvement of SCARA receptors has been shown for PF14/cargo complexes interactions with cells [59].

In paper II the role of SCARA in CPP/pDNA particle uptake was assessed by pre-treating cells with specific inhibitory ligands for SCARA: polyiosinic acid, dextran sulfate and fucoidan. Following the transfection of pDNA with CPPs, the effect was assessed by comparing the luciferase expression level in the cells treated with inhibitors and cells pre-treated with inhibitors. For SCARA downregulation SCARA3 and SCARA5 specific siRNAs were used. The cells were transfected with commercially available liposome based transfection reagent RNAiMAX 24 h prior of adding CPP/pDNA complexes. Expression from plasmid was measured 24 h after treating with CPP/siRNA.

3.5.3. Inhibition of endocytosis pathways

In eukaryotic cells, endocytosis is an important cellular process to internalize various cargo molecules, to regulate signal transduction pathways and is the main way for a cell to internalize solutions and particles in the solution. A number of endocytic pathways have been demonstrated to be involved in the uptake of DNA complexes [115, 116] and this is highly dependent on the cell type [117], the nature of the gene carrier [118] and the particle size [119]. This variability stresses the need to study the internalization of each of the different type of CPPs as gene carriers in the appropriate cell line model. CPP/cargo complexes are shown to use different endocytotic pathways by applying pharmacologic and molecular manipulations to selectively disrupt distinct endocytic pathways. In paper II chloropromazine, cytochalasine D, nystatin and chloroquine were used as pharmaceutical compounds to block certain pathways. Regarding their specificity, an increasing volume of evidence suggests that these inhibitors are not higly specific. Another drawback for using inhibitors is that nanocomplexes may use several internalization pathways simultaneously and blocking one pathway may cause the increased harnessing of another. In addition to this, off-target effects may occur due to the inhibitor. Therefore the results obtained by using inhibitors should be interpreted carefully.

Chloropromazine is a cationic amphiphilic drug which is deemed to inhibit clathrin-coated pit formation by a reversible translocation of clathrin and its adapter proteins from the plasma membrane to intracellular vesicles. As a side effect, it may insert into plasma membrane and alter the membrane fluidity. In addition it may inhibit phospholipase C and by that alter macropinocytosis and actin dynamics. Cytochalasin D is a drug that prevents actin polymerization and leads to the disassembly of the existing actin, inhibiting membrane ruffling and micropinocytosis. In addition, it may also affect the clathrin- or caveolin-mediated pathways. Nystatin is a polyene antifungal drug which binds to sterols, flattens caveolae and dissassembles the coat of clathrin-coated pits [120, 121].

Chloroquine (CQ) is an endosomolytic molecule and in its unprotonated form is able to cross membranes and accumulate in the acidic organelles of the cell. CQ is able to buffer the acidification of acidic organelles and its accumulation in the same organelle may cause its rupture. Addition of CQ enables to

assess the endosomal release capability of complexes from the acidic organelles such as Lys and LEs. If there was a significant increase in the final biological effect gained form delivered cargo, most probably the entrapment of complexes in LE and Lys may be one of the main limiting factors for the CPP. CQ was used in paper I, II and III to assess the endosomal release of complexes, and also in paper III as one component in a three component (CQ, Bafilomycin A, Nocodazol) assay designed to elucidate the CPP´s mechanism of action in endocytotic pathway. Bafilomycin A is an inhibitor of vacuolar ATPases and inhibits the acidification of endosomes. In HeLa cells, it is also able to block the maturation of endosomes from EE to LE. To assess the role of endocytosis and acidification Nocodazol was also used in comparison to Bafilomycin A and CQ. Nocodazol is able to block the actin filament synthesis in the cells, cause disassembly of microtubules and therefore block the endocytosis, without blocking the acidification of vesicles.

3.5.4. Separation of endosomes

Endosomes are membrane bound organelles in the cells, responsible for transporting endocytosed material and trafficking of the molecules produced in the cell. There have been several methods that enable the tracking of the CPP/cargo complexes, but most of them require labeling of the cargo or the CPP. The labels may affect the pathway and physico-chemical properties of the complexes; therefore a multi-step centrifugation offers an alternative to labeling and enables to assess the amount of cargo in certain organelles. Caused by different sediment velocities of organelles found in the cell, by choosing right density gradients and centrifugation forces, the organelles can be separated from each other. The drawbacks of using centrifugation methods, is the low reproducibility of experiments caused by biological variety and multi-step preparation, high time consumption and loss of material during the purification steps. Using self-forming gradient solutions, such as Percoll, enables to increase reproducibility and also decrease the hands-on time for the experiments. Still, centrifugation is used for the separation of cell populations from tissues, acquiring organells from the cells and separating and concentrating different types of organelles in a gradient. Determination of the complex location and change in quantity over time helps to understand the events during the delivery and contribute in CPP development or choosing the suitable application for the CPP.

In paper II the differential centrifugation and fractionation on Percoll was used to assess the amount of pDNA in early endosomes and Lys for HeLa cells transfected with NF/pDNA. In paper III HeLa and HEK293 cells were used in comparison. Centrifugation and fractionation of transfected cells was used to determine the localization and quantity of pDNA after CPP-mediated delivery in endosomal vesicles without the need of labeling either.

3.5.5. Single particle tracking

Single particle tracking (SPT) is a powerful tool to study the dynamics of intricate systems such as the living cell. The single molecule techniques over the past two decades have changed the field of cell biology by providing a more quantitative picture of the complex and highly dynamic organization of living systems. Amongst these techniques, single particle tracking (SPT) is an approach to study a variety of dynamic processes. SPT locates each particle and measures its individual dynamics and exploits the fact that the location of an isolated particle can be measured to a high accuracy. SPT provides access to single molecule behavior in the natural context of living cells, thereby allowing a complete statistical characterization of the system under study.

There have been several applications for SPT in the analysis of cell surface, viral infection of cells and processes in gene transcription. The advantage of SPT is the straightforward approach and imaging of live cells, whereas the cargo or organelles have to be fluorescently marked. To arrive at such single-particle trajectories, particles first need to be located within the images and, secondly, these locations have to be connected to trajectories.

In paper IV Cy5-pDNA was used and CPP without a label to form CPP/Cy5-pDNA complexes. Cells were pre-treated with BacMam reagent to have fluorescently labelled organelles (EE-Rab5; Lysosomes – LAMP1). Cells were treated with complexes only for a short period (15 min) to have optimal number for downstream SPT analysis. The co-localization events of labeled pDNA and EE or Lys were measured to elucidate the intracellular trafficking of CPP/pDNA complexes.

4. RESULTS AND DISCUSSION

In the papers included in this thesis, the aim was to investigate the selected CPPs on the basis of NA delivery efficiency and to link this to the CPP/cargo complex formation efficacy and the physicochemical properties of the complexes. Although derived from similar backgrounds, the modifications in the peptide sequences have led to differences in complex stabilities, internalization pathways and also to intracellular fate.

In paper I the novel stearyl-TP10 analogues belonging to the NF family were introduced for NA delivery. In paper II and III the NF/pDNA complexes were characterized, their internalization pathway determined and intracellular trafficking described. In addition, in paper III two methods of tracking the intacellular trafficking of CPP/pDNA were compared. In paper IV, the complex formation between NFs or PFs and siRNA was thoroughly described and a model of complex formation proposed. In this chapter, the results obtained from each paper will be summarized and discussed.

4.1. NickFect51 for the delivery of NAs and application in protein production (Paper I)

Modification of the CPP sequence is a way to improve their stability, chemical or physical properties. The sequence of stearyl-TP10 was modified to increase the CPP-mediated NA transfection efficiency. For this, two different modifications were tested. Firstly, to improve the serum stability of the CPP, O was introduced into the sequence. The use of O instead of K should increase the stability of the peptide [50], as O is naturally not included in the protein sequences and is less likely to be recognized by the proteases. The second modification was introduced to increase the flexibility and peptide bond stability [52] of the aa7. Both modifications were introduced separately, resulting in NF61 and NF53, or in combination, resulting in the CPP NF51. These CPPs were able to form stable non-covalent complexes with pDNA, siRNA and SCO, with the size between 60–140 nm in MQ water and 75–177 nm when serum was present. Introduction of either modification resulted in a CPP with greater efficacy than stearyl-TP10, but the use of both modifications in the same sequence resulted in a CPP with the highest efficacy.

As NF51 was the most efficient CPP out of three tested peptides the delivery with NF51 was investigated more thoroughly. It was tested for the delivery of pDNA in several cell lines, including hard to transfect cells and also in serum containing conditions. Even when serum was present, NF51 was able to mediate pDNA delivery into cells resulting in a high level of reporter gene expression from the delivered cargo. In addition to this, NF51 was able to deliver not only pDNA, but also siRNA and SCO resulting in efficient gene downregulation or reporter gene splice correction. The limited increase in

expression after addition of CQ and also TEM analysis revealed that NF51 has lysosomotropic properties and when associated with a cargo it is able to mediate endosomal escape after internalization. NF51 was tested for the applicability in QMCF protein production system as the delivery vector and was able to transfect the whole cell population and was more efficient even at lower DNA concentrations than commercially available reagents.

Out of designed CPPs, NF51 was the most efficient delivery vector for NAs and is applicable for the use in different cell lines.

4.2. NickFect1 and NickFect51 differ in uptake mechanism and intracellular trafficking (Paper II)

CPPs have been used to deliver variety of cargoes across the cell membranes, resulting in a high biological effect from the delivered cargo. However, the mechanism by which this effect is mediated is still vague. Understanding the circumstances behind the delivery enables to overcome the limitations of CPP-based transport vectors.

Modifications in the CPP stearyl-TP10 were introduced to the peptide sequence to increase the plasmid carrying efficacy of CPPs. NF1 and NF51 (a CPP designed and tested in paper I) were compared based on their complex formation, uptake mechanism and also intracellular trafficking of the formed complexes. In NF1, an extra negative charge was introduced by Y substitution phosphorylated tyrosine. Modifications in the NF1 and NF51 sequence influenced the nanoparticle formation, internalization route and intracellular trafficking of the nanoparticles, but for both CPPs the final biological effect gained from delivered cargo was similarly high. The transfection efficiency and previous work showed that NF1 and NF51 were the most efficient CPPs in the line designed with similar modifications [51, paper I]. In paper II we aimed to shed light on the mechanisms behind the efficiency. Both CPPs were able to form complexes with pDNA using the non-covalent strategy. In water NF1 and NF51 mixed with pDNA form nanoparticles with the size of <100 nm and addition of serum resulted in the increase of size and a negative surface charge. However, the serum did not significantly decrease the stability of the complexes. The negative surface charge of the complexes in the presence of serum components may play a role in the internalization of complexes. Although the negatively charged nanoparticle surface may repulse with the negative charges of the plasma membrane, scavenger receptors with low specificity may mediate the uptake of negatively charged particles. A role in the uptake of PF14/NA complexes has been shown for SCARA3 and SCARA5 previously [50, 122]. For NF1/pDNA and NF51/pDNA the use of scavenger receptor inhibitors and downregulation of SCARA 3 and 5 resulted in decreased biological effect. This indicates that SCARAs are involved in the uptake of the formed complexes.

The ability of CPPs to form a stable complex with the pDNA was assessed by adding heparin sodium salt to the pre-formed complexes. This revealed that

NF51/pDNA complexes were more stable to the replacement of pDNA by heparin, indicating that the formed complexes were more resistant to decomplexation compared to NF1/pDNA complexes. Similar dissociation tendencies were seen for NF1/pDNA also when observing the behavior of the complexes in the cells. For NF1, the fluorescence signals from peptide and pDNA were less co-localized in the same compartments than for NF51/pDNA. In addition, NF51/pDNA harnessed macropinocytosis as the main entry route and according to the endosomal compartment separation NF51/pDNA complexes stayed in less acidic endosomal compartments for extended period, compared to NF1/pDNA. NF51/pDNA complexes used different internalization mechanism than NF1/pDNA, as revealed by TEM (transmission electron microscopy) study. While NF51/pDNA formed large aggregates on the cell surface associated with macropinocytosis by membrane ruffling, NF1/pDNA complexes interacted with membrane as small clusters and were detected in the proximity of membrane pits and small ruffles. This indicates that NF1/pDNA may harness distinct endocytotic pathways simultaneously.

After endocytosis, the CPP/pDNA complexes are confined in the endocytotic vesicles and if the CPP is not able to disrupt the endosomal membranes, the complexes are routed for degradation to Lys. The escaping ability was assessed by the use of the lysosomotropic agent, chloroquine (CQ), during transfection. If CQ had effect on the increase of biological effect, then complexes were not co-localized with the CQ and not entrapped in the Lys. The addition of CQ did not significantly increase the biological effect gained for NF51/pDNA complexes, indicating that they are mainly located in EEs or escaped before reaching Lys. In the case of NF1/pDNA, the pDNA is located in LEs and Lys. Similar difference was detected when analyzing endosomal compartments separated form transfected cells.

Although having largely similar sequences, the modifications introduced in each peptide led to different mechanisms of action when delivering pDNA into the cell. NF51/pDNA was able to internalize cells rapidly and remain in EEs for longer periods, giving more time for complexes to escape from the endosomes and reach cytosol. However, the stability of complexes reduces the unpacking of the cargo and decreases the amount of free pDNA from which gene could be expressed. NF1/pDNA complexes were more prone to disscoiate prematurely, leading the pDNA to the Lys for degradation. At the same time the proportion of complexes that is able to escape endosomes and reach cytosol is more easily dissociated and pDNA is free for following gene expression. For both peptides different limiting factors were proposed, but they both led to high gene expression in *in vitro* conditions and the understanding of CPP-mediated delivery gives basis for further development of the vectors.

4.3. Intracellular trafficking of NF1 and NF51 complexes with pDNA (Paper III)

The intracellular trafficking and more importantly, the endosomal release of CPP/cargo complexes are processes not thoroughly studied for nanocomplexes. Several methods have been developed over the years, but all of them have some disadvantages. In paper III we aimed to find a suitable method to track the intracellular trafficking of the CPP/pDNA nanocomplexes and reveal the similarities and differences behind NF1 and NF51 as the transporters of pDNA.

Cells internalize CPP/cargo complexes predominantly via a pathway of endocytosis, which leads the entrapment of complexes in endosomal vesicles. To have a biological effect, the cargo has to be released from these vesicles and transported to the target site. For plasmid, the target organelle is the cell nucleus; therefore the CPP may have to mediate the transport of pDNA through the nucleus envelopes in addition to the plasma membrane and endosomes. Another strategy is for delivery into the nucleus is the CPP/pDNA complex release to the cytosol and during mitosis the pDNA can be incorporated to the cell nucleus. Before all these steps, the CPP/cargo complex has to escape endosomes; otherwise they are degraded in the LEs and Lys.

NickFects are able to deliver pDNA into the cells in large quantities. After 4 h almost 80% of pDNA was located in the cell and cellular compartments. To determine which endosomal mechanism NFs use, inhibitors were used to disrupt the endosome maturation, formation or acidification. For NF51/pDNA complexes, the inhibition of endosome acidification led to a decrease in the reporter gene expression, indicating that NF51 needed the change in acidity in order to gain high levels of gene expression from delivered plasmid. The role of acidification may also be for the inducement of dissociation of more stable NF51/pDNA complexes. For NF1, as it was located in the acidic Lys, the inhibition of acidification may have a different role, as it increases the time for the complexes to escape so pDNA is still intact and not degraded by Lys enzymes. Analysis of cellular contents after separation of endosomal compartments revealed that the complexes formed with NF51 and NF1 were located differently and the fraction of NF51/pDNA in endosomes decreased in time more than with NF1/pDNA, indicating that NF51/pDNA complexes were more capable to escape from the endosomes. At the same time, the proportion of complexes in Lys was higher with NF1/pDNA than with NF51/pDNA, showing that complexes formed with NF1 were more prone for degradation. The functionality of the pDNA in separated endosomes was checked by transfecting new cells, and it showed that the pDNA detected from EEs had the highest biological effect gained from pDNA. To confirm the results obtained with centrifugation method, single particle tracking (SPT) and co-localization of labelled vesicles and labelled pDNA was used. Firstly, the complex formation ability or size did not significantly increase when using labeled Cy5-pDNA, as confirmed with the gel electrophoresis and DLS. SPT revealed that there was a difference in internalization and localization of the complexes formed with NF1 and NF51.

For NF1/Cy5–pDNA a considerable amount of pDNA was co-located with LEs whereas NF51/Cy5–pDNA co-located mainly with EEs. In the case of complexes formed with NF1, the percentage of co-localization events with LEs increased more rapidly than with EEs whereas the percentage in EEs reached a plateau at higher point (70 min post-treatment) and remained fairly stable. For NF1/Cy5–pDNA, a higher amount of co-localization events with Lys was detected already at 15 min post-treatment in comparison to NF51/Cy5–pDNA.

Both NF51 and NF1 mediated efficient delivery of pDNA to the cell and enable expression from the delivered cargo, but the route of action differed. Endosomal transport and acidification of endosomal compartments play a role in delivery of pDNA. The final expression gained from the pDNA remained high, although NF1/pDNA complexes reach LEs and Lys faster and in larger proportion. For NF1/pDNA complexes the degradation in Lys in one of the hindering factors. NF51 mediated delivery enabled the pDNA to stay in the EEs for longer time period, giving higher opportunity for endosomal escape and bypassing the degradation in Lys. Both CPPs were efficient for pDNA delivery into different cell lines, although the characteristics of each should be taken into account when harnessing them for macromolecule delivery. Understanding the distinction between CPPs and relating them to differences in physico-chemical properties helps to implement modified CPPs with higher specificity for macromolecule delivery. In addition, SPT and centrifugation methods have proven to be valuable for understanding intracellular events leading to the biological effect from the cargo.

4.4. The binding of CPP and siRNA, complex formation and stability of formed complexes leads to biological activity of delivered siRNA (Paper IV)

Delivery of bioactive cargoes holds great potential, but the lack of efficient delivery methods for the NAs curbs their use. The first step towards delivery is the formation of CPP/cargo complexes and it relies on the physical and chemical properties of the CPP and also the cargo. In order to deliver bioactive cargoes into the cells efficiently, the CPP has to form stable nanoparticles that at the same time are able to release the cargo at the right target site. The complex formation and uptake mechanism of CPP/pDNA has been studied extensively. The siRNAs propose different challenges for the carrier and the complex formation. The siRNA complex formation cannot be based only on the previous knowledge of plasmid DNA behavior.

In the paper IV the previously developed CPPs belonging to the PepFect and NickFect family were included in addition to TP10 and NickFect57 to compare the effect of modifications in the sequence, net charge, complex formation, stability and their correlations with the biological effect from the delivered siRNA. PF6 had the highest charge density compared to other tested

CPPs and due to the chloroquine moieties has a pH dependent change of net charge. The high number of charges enabled PF6 to form complexes with siRNA at lower molar ratios and results in stable nanoparticles. The complex formation was investigated using gel electrophoresis, fluorescent dye intercalation assay and also iTC. All methods indicated that the mechanism of PF6 differs when compared to other CPPs. In addition, the CQ moiety increases the stability of PF6 to proteolytic enzymes.

The formation of complex itself did not ensure efficient delivery. NF51, NF57 and PF3 had a similar net charge and N-terminal stearyl modification and did not differ significantly in the binding to siRNA according to gel electrophoresis and PG assay results. Although PF3 formed complexes with siRNA, only low levels of gene knockdown were achieved. This could be linked to premature dissociation of complexes and insufficient destabilization of endosomal membranes. PF14 needed less peptide to form complexes with siRNA, but the complexes were unstable to enzymatic degradation and siRNA was released. The resistance of complexes to degradation and avoiding premature dissociation of siRNA is fundamental for CPP-mediated cargo delivery; however, this is usually compromised by the CPP or cargo cleavage before reaching the target. Intracellular uptake and protection of siRNA from degradation may be the leading limitations, because siRNA is prone to degradation by nucleases. To test the stability of the complexes heparin displacement assay and enzymatic degradation were used. Complexes formed with PF6/siRNA proved the most stable to enzymatic degradation and also to heparin displacement. PF6 is able to pack siRNA in a manner that it is not accessible to nucleases, therefore protecting it from premature degradation.

The presence of free peptide may be one of the forces behind the internalization and also endosomal escape. During intracellular trafficking in the endosomes, the pH is decreased and CPP net charge increases. This increase leads to reorganization of CPP/siRNA complexes and increase of free peptide fraction capable of interacting with membranes. Based on the results we proposed a model of nanoparticle formation with PF6 and siRNA, where the CPPs interacting directly with siRNA for the core of the particle and additional CPP is needed to form a shell that is able to interact with receptors and membranes. In addition, as the number of CPPs in the core changes when pH decreases or increases and leads to a change in CPP/siRNA complex stability, the siRNA is released from the acidic compartments to the cytosol.

5. CONCLUSIONS

This thesis is dedicated to the investigation of CPP-based delivery vectors for complex formation with NAs and their delivery into the cells. The CPPs used in this thesis enable non-covalent nanoparticle formation and are thoroughly studied for complex formation, stability and to the premature dissociation of the CPP/cargo complexes for the *in vitro* delivery. In addition to comparison between the CPPs, two intrinsically different cargoes were used to elucidate the challenges of the delivery that either poses. They key findings are listed below.

Paper I

- The replacing of K7 with an O7 and the continuation of synthesis through the δ-amino group of O7 side chain resulted in a highly efficient CPP, NF51, for the delivery of NAs. Introduction of either modification alone into stearyl-TP10 increased the efficacy of the CPP.
- NF51, NF53 and NF61 were able to form nanoparticles with pDNA, siRNA and SCO and mediate their transport into the cells and subsequent gene expression, gene downregulation or non-functional reporter gene splice correction.
- NF51 was an efficient pDNA delivery vector for tested adherent, suspension and primary cells.
- NF51 could be applied as a transfection reagent for QMCF technology.

Paper II

- Modifications introduced into the stearyl-TP10 sequence, such as a kinked structure or the addition of a negative charge, lead to the significantly different CPPs NF1 and NF51. Both peptides were able to exhibit high delivery efficiency and high biological effect from the delivered pDNA.
- Either CPP has a different challenge to overcome in order to mediate delivery.
- Scavenger receptors are involved in the internalization of the NF/pDNA complexes.
- NF1/pDNA complexes use several endocytosis pathways simultaneously. NF51/pDNA complexes harness macropinocytosis as the main entry pathway.
- The premature dissociation of the NF1/pDNA complexes is the biggest challenge for NF1 mediated delivery. Internalizaed complexes are routed to Lys for degradation. The endosomal escape of the NF1/pDNA complexes is limited, compared to the NF51/pDNA complexes.
- Plasmid DNA is forming more stable complexes with NF51 than with NF1. NF51 promotes the stay of the complexes in the EEs. Although endosomal escape is efficient, the released complexes are not dissociated as easily, therefore gene expression from pDNA is hindered.

Paper III

- Separation of endosomes from transfected cells enables to determine the location of the delivered pDNA without labeling the CPP or the cargo. Single particle tracking and analysis of co-localization of Cy5-pDNA with endosomal compartments is a fast and precise method to pinpoint the localization of pDNA and also the events of endosomal release. Both methods can be used to determine the trafficking and endosomal release of the CPP/NA complexes.
- Intracellular trafficking of the NF51/pDNA and the NF1/pDNA complexes differs, arising from the properties of the complexes. NF51/pDNA is located in the EEs for longer and the transport efficiency depends both on endosomal transport and the acidification of the endosomes. NF1/pDNA is rapidly routed to the LEs and Lys, but the proportion that is released to the cytosol is able to dissociate and release pDNA for subsequent gene expression.

Paper IV

- The CPPs NF51 and PF14 that are suitable for pDNA transfection are not as efficient for siRNA delivery.
- Electrostatic interaction driven by the net charge of the peptide and negatively charged NA backbone is the main force that promotes the complex formation, but hydrophobicity and peptide secondary structure of NFs and PFs affect the complex stability and the final biological effect from the delivered cargo.
- PF6 has the highest net charge and stability to degradation by proteases. The pH sensitive moiety leads to an increase in the net charge when pH decreases and causes a rearrangement of the formed complex. High net charge accompanied with resistance to degradation enables the formation of stable CPP/siRNA nanoparticles and is the pre-requisite for efficient siRNA delivery into cells.
- We propose that CPP and siRNA are arranged in a particle, forming a core and a shell. These structures dynamically reorganize depending on the environmental pH changes.

SUMMARY IN ESTONIAN

Rakku tungivate peptiidide ja nukleiinhapetega moodustatud nanokompleksidega seotud protsessid: kuidas, miks, mis ja kuhu

Nukleiinhapped omavad rakus olulist rolli nii pärilikkuse säilitamisel, geenide ekspressioonil kui ka geenide regulatsioonil. Nukleiinhappemolekule ja nende modifitseeritud analooge võiks potentsiaalselt kasutada ka geeniteraapiaks või uute geenide viimiseks rakkudesse, kus neid tavaliselt ei leidu. Nukleiinhapete kasutamise eeliseks on nende bioloogiline sobivus, kuid nukleiinhapete terapeutilist ja biotehnoloogilist kasutust piirab nende limiteeritud sisenemine rakkudesse ja kudedesse. Lisaks sellele lagundatakse vabad nukleiinhappemolekulid nii rakuvälises kui ka – siseses keskkonnas kiirelt. Sellest tulenevalt on nukleiinhapete laialdasemaks kasutamiseks vajalik efektiivse ning ohutu transportmeetodi rakendamine.

Viirusvektorite ja füüsikaliste meetodite kõrval on nukleiinhapete transpordiks kasutatud ka keemilisi vektoreid. Üheks suure potentsiaaliga vektorite tüübiks on rakku tungivad peptiidid. Neid iseloomustab võime siseneda rakkudesse ning sinna kaasa transportida ka endaga seotud lastmolekule. Rakku sisenevatele peptiididele on võimalik lasti siduda kovalentselt ja mittekovalentselt. Mittekovalentsel seondumisel on oluliseks peptiidis leiduvatel aminohapetel positiivselt laetud kõrvalrühmade olemasolu ning interaktsioon nukleiinhapete fosfaatselgroo negatiivsete laengutega. Lisaks sellele on hüdrofoobse rasvhappejäägi saba lisamine võimaldanud suurendada moodustunud komplekside stabiilsust ning interaktsioone membraanidega.

Nukleiinhappemolekulid erinevad nii suuruselt, kujult, kui ka struktuurilt, mistõttu on ühe, universaalse, transporteri leidmine keerukas. Plasmiidid on suured rõngasmolekulid, mille mittekovalentsel seondumisel on oluliseks vektori poolt indutseeritud plasmiidi kokku pakkimine nanopartikliks. Väiksemad oligonukleotiidi ja RNA molekulid, vajavad nukleaasidele kättesaadavate osade tõttu kaitset lagundamise eest.

Antud doktoritöö eesmärgiks on selgitada rakku sisenevate peptiidide ja nukleiinhapete vahel mittekovalentselt moodustunud komplekside füüsikalisi ning keemilisi omadusi, stabiilsust ning ka sisenemismehhanisme ja kompleksidega seotud protsesse rakus. Selleks võrreldi rakku sisenevaid peptiide, mis kuuluvad kimäärse peptiidi transportaan modifikatsioonide hulka ning mille puhul on eelnevalt näidatud võimet transportida rakku nukleiinhappeid. Lastmolekulidena kasutati peamiselt plasmiidset DNA-d ja siRNA-sid.

Esimeses artiklis testitakse kahe erineva modifikatsiooni mõju rakku sisseneva peptiidi transfektsiooniefektiivsusele. Lastmolekulidena kasutatakse nii pDNA-s, siRNA-t kui ka vaheltlõikust (*splicing*) korrigeerivaid oligonukleotiide. Plasmiidi transfektsiooni efektiivsust NF51-ga testitakse lisaks tavapäraselt kasutatavatele HeLa ja CHO rakuliinidele ka teistes rakuliinides, mida

loetakse raskemini transfekteeritavateks. Lisaks testitakse NF51-e kasutust QMCF tootmistehnoloogias transporterina.

Teises artiklis kirjeldatakse rakku sisenevate peptiidide NF1 ja NF51 komplekside stabiilsust, sisenemisteed rakku ning liikumist rakus ning kuidas on nendesse peptiidide järjestustesse viidud modifikatsioonid mõjutanud nende omadusi transporterina. Mõlemad peptiidid suudavad plasmiidi viia rakkudesse ning tagada kõrge ekspressioonitase plasmiidilt, kuid see, kuidas kumbki vektor selleni jõuab, on erinev. Kummagi peptiidi puhul ilmnevad kitsaskohad nende transpordiviisil kas komplekside ebastabiilsuses, kiires degradeerimises lüsosoomides või kompleksides, mis ei suuda piisavalt efektiivselt plasmiidi kompleksist sihtkohas vabastada.

Kolmandas artiklis võrreldakse kahte meetodit, mis võimaldavad täpsemalt jälgida sündmusi, mis järgnevad komplekside sisenemisele rakku. Kummalgi meetodil on eelised ja puudused, kuid mõlema puhul avaldub erinevus NF1 ja NF51 vahendatud plasmiidi transpordis, mis toetavad esimeses töös kirjeldatud sündmusi.

Neljandas artiklis kasutatakse lastmolekulina sünteetilisi siRNA molekule, sest need võimaldavad spetsiifilisemat geeni mõjutamist ning on oluliseks RNA interferentsi tekitamiseks rakus terapeutilisel või biotehnoloogilisel eesmärgil. Töö käigus selgub, et transporterid, mis suudavad kõige efektiivsemalt transportida plasmiidset DNAd, ei ole kõige sobivamad siRNA transpordiks. Lisaks sellele, tuginedes tulemustele, pakutakse mudel, kuidas toimub siRNA ja peptiidi vaheline interaktsioon ning komplekside moodustumine.

Antud töös on püütud selgitada peptiidide vahendatud nukleiinhapete transpordi eripärasid, liikumist rakus ning komplekside moodustumist, millele saaks tugineda peptiidide edasine disain.

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