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PIRET ARUKUUSK

NickFects – novel cell-penetrating peptides. Design and uptake mechanism

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NickFects – novel cell-penetrating peptides. Design and uptake mechanism

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Supervisor: Prof. Ülo Langel
Institute of Technology, Faculty of Science and Technology,
University of Tartu,
Tartu, Estonia; and
Department of Neurochemistry,
Stockholm University,
Stockholm, Sweden

Opponent: Prof. Ines Neundorf
Institute of Biochemistry, Department of Chemistry,
University of Cologne,
Cologne, Germany

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ABSTRACT

Nucleic acids and their analogues are highly potential candidates to be utilized for the treatment of various devastating diseases. The clinical potential of these biomolecules remains restricted so far because of their poor stability in the presence of serum and low uptake into the cells resulting from the high molecular weight, negative charge and hydrophilic nature of the nucleic acids. Therefore, the development of macromolecule-based drugs is dependent on the progress and improvement of carrier molecules that can facilitate their transfection and protect the cargo from degradation. CPPs are relatively short peptides, 5–30 amino acids in length, with the ability to gain access to the cell interior via energy-dependent and/or independent mechanisms, and facilitate intracellular delivery of associated cargo molecules to intracellular targets.

This thesis focuses on the design and characterization of a new family of CPPs, named NickFect. The peptides are designed to deliver various types of bio-active cargos, including plasmid DNA, splice-correcting oligonucleotides and small interfering RNAs, using non-covalent nanoparticle formation approach. In order to enhance the nanoparticle formation activity, uptake efficacy and endosomolytic properties, we insert different modifications or make amino acid substitutions to the backbone of the parental peptide, stearyl-TP10. For instance, addition of phosphoryl-group yielded NF1 and NF2, highly efficient peptide-based transfection reagents for the intracellular delivery of splice-correcting oligonucleotides. Another radical modification, insertion of a kink, resulted in NF51 that proved to deliver nucleic acids to the targets both in the nucleus and cytoplasm. Additionally, we demonstrate the applicability of NF51 in protein production system. Furthermore, we unravel the uptake mechanism of two NickFect family members, NF1 and NF51, related to the biological activity of transfected plasmid DNA.

Taken together, our results demonstrate that the performed chemical modifications in NickFects enhanced the activity of these peptides for delivering nucleic acids. Studies of the uptake mechanism gave us valuable information on how to enhance the bioavailability of different genetic materials non-covalently linked to NickFects for further *in vivo* applications and for designing more efficient carrier vectors and achieving bioavailability of the macromolecule-based drugs. Conclusively, NickFects have remarkable potential for the delivery of nucleic acids both *in vitro* and *in vivo*.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I Oskolkov, N., **Arukuusk, P.,** Copolovici, D. M., Lindberg, S., Margus, H., Padari, K., Pooga, M., and Langel, Ü. (2011) NickFects, phosphorylated derivatives of Transportan 10 for cellular delivery of oligonucleotides. *Int J Pept Res Ther17*, 147–157.

II **Arukuusk, P.,** Pärnaste, L., Oskolkov, N., Copolovici, D. M., Margus, H., Padari, K., Möll, K., Maslovskaja, J., Tegova, R., Kivi, G., Tover, A., Pooga, M., Ustav, M., and Langel, Ü. (2013) New generation of efficient peptide-based vectors, NickFects, for the delivery of nucleic acids. *Biochim Biophys Acta 1828*, 1365–73.

III **Arukuusk, P.,** Pärnaste, L., Margus, H., Eriksson, J., Vasconcelos, L., Padari, K., Pooga, M., and Langel, Ü. (2013) Differential endosomal pathways for radically modified peptide vectors. *Bioconjug Chem*. In press

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

Paper I: synthesized the peptides, performed most of the experiments, participated in data analysis and in the writing of the paper;

Paper II: synthesized the peptides, performed transfection and nanoparticles characterization experiments, analysed most of the data, wrote the paper as the corresponding author;

Paper III: synthesized the peptides, performed many of the experiments, analysed most of the data, wrote the paper as the corresponding author.

ADDITIONAL PUBLICATIONS

Langel, K., Lindberg, S., Copolovici, D. M., **Arukuusk, P.**, Sillard, R., and Langel, Ü., (2010) Novel fatty acid modifications of transportan 10., *Int J Pept Res Ther*, 16, 247–255.

Suhorutšenko, J., Oskolkov, N., **Arukuusk, P.**, Kurrikoff, K., Eriste, E., Copolovici, D. M., and Langel, Ü. (2011) Cell-penetrating peptides, PepFects, show no evidence of toxicity and immunogenicity in vitro and in vivo. *Bioconjug Chem* 22, 2255–62.

Veiman, K. L., Mäger, I., Ezzat, K., Margus, H., Lehto, T., Langel, K., Kurrikoff, K., **Arukuusk, P.,** Suhorutšenko, J., Padari, K., Pooga, M., Lehto, T., and Langel, Ü. (2012) PepFect14 peptide vector for efficient gene delivery in cell cultures. *Mol Pharm* 10, 199–210.

Rytkönen, J., **Arukuusk, P.**, Xu, W., Kurrikoff, K., Langel, Ü., Lehto, V.P., Närvanen, A. (2013) Mesoporus silicon nanoparticles functionalized with cell penetrating peptides: a delivery platform for oligonucleotide drugs. *Mol Pharm*. Submitted

Patent application

Langel, Ü., **Arukuusk, P.,** Oskolkov, N., Copolovici, D. M. (2011) A system for cargo delivery into the cells. PCT/EP2012/053036

ABBREVIATIONS

CME	clathrin-mediated endocytosis
CPP	cell-penetrating peptide
CR	charge ratio
CQ	chloroquine
DIEA	diisopropylamine
DLS	dynamic light scattering
EBV	Epstein-Barr virus
EBNA	Epstein-Barr nuclear antigen
EE	early endosome
EGFP	enhanced green fluorescent protein
EtBr	ethidium bromide
FACS	fluorescence-activated cell sorter
FM	serum-containing medium
Fmoc	9-fluorenylmethoxycarbonyl
GAG	glycosaminoglycan
GPCR	G protein coupled receptor
LAMP	lysosome-associated membrane protein
LDL	low density lipoprotein
LE	late endosome
LF2000	Lipofectamine 2000
Luc	luciferase
MBHA	methylbenzylhydrylamine
MEND	multifunctional envelope-type nanodevice
MR	molar ratio
NLS	nuclear localization signal
NF	NickFect
ON	oligonucleotide
PCR	polymerase chain reaction
pDNA	plasmid DNA
PE	phosphatidylethanolamine
PF	PepFect
SCARA	class A scavenger receptor
SCO	splice-correcting oligonucleotide
SFM	serum-free medium
siRNA	small interfering RNA
SPPS	solid-phase peptide synthesis
t-Boc	tert-butyloxycarbonyl
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
TP10	transportan 10

INTRODUCTION

During last decades, a crucial need for new drugs with increased specificity and lower side effects than currently used low-molecular-mass drugs for the treatment of devastating diseases such as cancers or genetic diseases has emerged. Through rational design based on molecular, cellular and structural data, the macromolecules with protein modulatory functions can be developed to treat different disorders. One of the main obstacles in drug delivery today, is the reaching of a drug molecule to the intracellular targets. Therefore, there is a great need for efficient and non-toxic delivery vehicles that are able to cross the cell membrane and carry macromolecule-based drugs with them. Cell-penetrating peptides (CPPs) is a class of non-viral delivery vectors that has been used for the intracellular delivery of various bioactive cargos. CPPs have been successfully used for the transfection of plasmid DNA, splice-correcting oligonucleotides and small interfering RNAs, both *in vitro* and *in vivo*.

The focus of this thesis is to design novel efficient cell-penetrating peptides applicable for the intracellular delivery of nucleic acids, using non-covalent co-incubation strategy. This study presents several novel peptides that are all analogues of the well-studied cell-penetrating peptide TP10 and evaluates their potential to transfect different nucleic acid-based cargos. Special focus is paid to the properties of formed CPP-cargo nanoparticles, their uptake mechanism and intracellular trafficking, and how these are related to biological activity of the transfected bioactive cargo.

1. LITERATURE OVERVIEW

1.1. Macromolecules for gene therapy

The development and production of new therapeutic macromolecules that can overcome limitations of small-molecule drugs such as poor specificity and rapid elimination from the blood stream, has dramatically increased during the past decade (*1, 2*). Different gene therapy approaches have gained significant focus lately because of their potential to treat severe and hard-to-cure diseases, like inherited genetic diseases, viral infections and cancer (*3, 4*). In gene therapy genetic material is delivered into target cells to replace a gene that is missing, mutated or poorly expressed (*5*). Second approach in gene therapy, is to silence disease-causing genes expression and production of proteins using RNA interference machinery (*5*). Besides that, gene function can be modified by interfering with splicing machinery using splice-correcting oligonucleotides (*6*).

Physico-chemical properties of nucleic acids, e.g. high molecular weight and anionic backbone, make them impermeable for the plasma membrane. The inability of nucleic acid-based therapeutics to reach their designated cellular and intracellular target sites is one of the main obstacles for their application (*7, 8*). Hence the success or failure of gene therapy critically relies on gene delivery and the development of macromolecule-based drugs is dependent on the progress in the design of carrier molecules (*9*).

Delivery vehicles can be broadly divided into two classes – viral and non-viral vectors. Viral vectors can efficiently transfect therapeutic into genome of the cells, but safety concerns, such as immune response and mutations caused by randomly integrated viral genomes, have limited their applications in clinics (*10*). Although much progress has been accomplished in developing viral vectors without serious side effects and the safety profile of them has improved, they still have their limitations (*11, 12*). Importantly, viral vectors are restricted by cargo-carrying capacity and are not compatible with the transient delivery of synthetic antisense-based oligonucleotides (ONs), including splice-correcting ONs (SCOs) and small interfering RNAs (siRNAs) (*1*). This has increased interest towards non-viral delivery methods, to find safer alternatives for gene delivery. Non-viral vectors are usually based on different cationic entities, like different lipids, synthetic polymers or peptides (*13*). Among these, cationic lipids and synthetic polymers, known as lipoplexes and polyplexes, respectively, have been studied most extensively.

A novel approach for the intracellular delivery of various bioactive molecules is using peptides with membrane permeation activity. The peptides, named cell-penetrating peptides (CPPs) or peptide transduction domains (PTDs) have been used to transport nucleic acids into cells both *in vitro* and *in vivo* (*14*).

1.2. Cell-penetrating peptides

1.2.1. Background

Protein transduction into cells was first described in 1988 in parallel by Frankel and Pabo (*15*), Green and Lowenstein (*16*), who discovered that the transactivator of transcription (TAT) protein of Human Immunodeficiency Virus (HIV) can enter tissue-cultured cells and promote viral gene expression. In 1991 it was reported by the group of Alain Prochiantz that homeodomain of Antennapedia was also capable to enter nerve cells and regulate neural morphogenesis (*17*). These findings were followed in 1997 by Vives et al., who identified a minimal sequence that enabled cell entry, $Tat_{48–60}$ (*18*) and in 1994 Derossi et al., who determined that only 16-mer peptide, known as penetratin or pAntp, was sufficient for intracellular delivery (*19*). These findings marked the birth of the field of CPPs. Over the past 20 years, a rapidly growing number of CPPs has been identified or designed through structure-activity relationship studies and today the number of reported CPPs exceeds 100.

Table 1. Selection of CPPs

CPP	**Sequence**	**Origin**	**Ref.**
Tat $_{(48–60)}$	GRKKRRQRRRPPQ	Protein derived	(*18*)
Penetratin	RQIKIWFQNRRMKWKK-NH_2	Protein derived	(*19*)
pVEC	LLIILRRRIRKQAHAHSK-NH_2	Protein derived	(*20*)
Transportan	GWTLNSAGYLLGKINLKALAA LAKKIL-NH_2	Chimeric Galanin/Mastoparan	(*21*)
TP10	AGYLLGKINLKALAALAKKIL-NH_2	Chimeric Truncated analog of transportan	(*22*)
MPG	GALFLGWLGAAGSTMGAPKK KRKV-cya	Chimeric HIV-gp41/SV40-antigen	(*23*)
Pep-1	KETWWETWWTEWSQPKKKR KV-cya	Chimeric HIV-reverse transcriptase/ SV40-antigen	(*24*)
MAP	KLALKLALKALKAALKLA-NH_2	Synthetic	(*25*)
Polyarginine	(R)n ; 6 <n<12	Synthetic	(*26, 27*)
CADY	GLWRALWRLLRSLWRLLWRA -cya	Synthetic	(*28*)

cya, C – terminal cysteamide.

Despite the fact that peptides from diverse origin and with various physico-chemical properties have cell-penetrating properties, they possess some common features. CPPs are relatively short peptides, 5–30 amino acids in length, with the ability to gain access to the cell interior via energy-dependent and/or independent mechanisms, and facilitate intracellular delivery of attached bioactive cargo (*29*). Mostly CPPs are positively charged peptides, but transduction properties have been demonstrated for several anionic and hydrophobic peptides as well (*30, 31*). While CPPs are hard to define, the common denominator of all the CPP is that they are capable of mediating the delivery of various macromolecules across the cellular membranes, including peptides, proteins, plasmid DNA (pDNA), antisense ONs (asONs), splice-correcting oligonucleotides (SCOs), short interfering RNAs (siRNAs), anti-microRNAs (antiMiRs), contrast agents, drugs and various nanoparticles both *in vitro* and *in vivo* (*7, 14, 32*). A selection of most broadly used CPPs are presented in Table 1.

1.2.2. Classification

According to their origin, three main classes of CPPs can be distinguished: peptides derived from proteins, chimeric peptides that are formed by the fusion of two natural sequences, and synthetic peptides, which are rationally designed sequences usually based on structure-activity studies. CPPs can also be classified according to their physico-chemical properties, e.g. amphipathicity or hydrophobicity. According to Miletti et al., CPPs can be divided into three major classes: cationic, amphipathic and hydrophobic (*5, 33*).

Cationic CPPs, also referred to as non-amphipathic CPPs, contain a stretch of positive charges that is essential for their uptake. Importantly, they do not have specific 3D-strctural requirements for transduction (*33*). The most prominent members of this group are Tat peptide, derived from HIV type I transcription factor Tat, and synthetic peptide polyarginine. Studies on arginine-based peptides have shown that the minimal sequence for cellular uptake is octaarginine (R_8), and increasing the number of arginines up to 12 enhances the uptake level. On the other hand, longer oligomers tend to display toxicity (*34*). Likewise, it has been shown that at least eight positive charges are needed for efficient uptake of several other cationic CPPs (*27*). The main membrane interaction and binding comes from the electrostatic forces between the positively charged peptides and the negatively charged membrane constituents. The secondary structure of the peptide has no direct impact on the uptake (*35*).

Nuclear localization sequences (NLSs) can also be classified as cationic CPPs. NLSs are short peptides, based on lysine-, arginine- or proline-rich motifs, that can be transported to the nucleus through nuclear pore complex. The cell transduction efficacy of most NLSs is not on a high level, but they can be covalently linked to hydrophobic peptide sequences, resulting in amphipathic CPPs with improved cell-penetrating properties (*33*).

Amphipathic CPPs that consist of hydrophilic and hydrophobic parts, and contain mainly Lys residues as net positive charge sources, can be subdivided into primary and secondary amphipathic peptides. Primary amphipathic CPPs have distinct hydrophobic and hydrophilic domains separately located in the sequence, e.g. hydrophobic C-terminus and hydrophilic N-terminus. Usually, these peptides have more than 20 amino acid long sequence to, at least theoretically, span the hydrophobic core of the membrane bilayer (*35*).

Transportan (TP) and its truncated version TP10 that contains several hydrophobic and hydrophilic blocks in their sequences, belong to this class. Transportan is a chimeric peptide, consisting N-terminal fragment of neuropeptide galanin and wasp venom peptide mastoparan that are linked via a Lys-residue (*21*). The other primary amphipathic CPP, fully derived from natural proteins is pVEC, consisting of 13 cytosolic and 5 transmembrane residues from vascular endothelial cadherin. Several peptides that belong to this group are chimeric and obtained by covalently attaching a hydrophobic domain and NLS sequence for efficient targeting. For example, MPG and Pep-1 peptides are both based on the SV40 NLS sequence. The hydrophobic domain of MPG was derived from the fusion sequence of HIV glycoprotein 41, while that of Pep-1 contains tryptophan-rich cluster, which has high affinity for membranes. In both MPG and Pep-1 peptides, the hydrophobic domain and NLS domain are separated by a linker sequence (WSQP) (*23, 24*).

Secondary amphipathic CPPs display their amphiphilic properties upon the interaction with cell surface molecules and subsequent changes in the secondary structure of the peptide. These CPPs obtain α-helical or β-sheet secondary structure, where polar residues are pointed to one face and the nonpolar residues to the opposite side of the structure. The amphiphilicity of a peptide can be imaged as looking through a helical wheel. Although most amphipathic peptides are also cationic, the membrane translocation is mainly a consequence of amphiphilicity and not electrostatic interactions. For example displacement of lysines with other polar residues in synthetic MAP peptide, resulted in new anionic or neutral peptides with membrane transduction properties (*33*). Penetratin, 16 amino acid long peptide from the third helix of the Drosophila Antennapedia, and synthetic CADY, both have secondary amphipathicity. The minimal length of the peptide that is required for the uptake is unclear. Although, studies with MAP suggest that minimum four helix turns are essential for cell penetration, also shorter amphipathic CPPs have been reported. However, the helicity of the secondary structure is highly important. These CPPs need to be at least partially helical near the membrane interface to expose the hydrophobic face to the membrane and hydrophilic face to the solvent (*33*).

Lately, novel hydrophobic CPPs, with low net charge and no amphiphatic arrangement have been reported. These peptides either contain only polar residues or have low net charge, or have chemical modifications based on hydrophobic chains that are essential for their uptake. Cationic or anionic pentapeptides, e.g. PMLKE, VPALR (*36*), stapled peptides and pepducins can

be classified to this group. In stapled peptides hydrocarbon staple increases peptide helicity by rigidifying the peptide structure, thus contributing to cellular uptake (*33*). Pepducins, N-terminally lipidated peptides derived from a G protein coupled receptor (GPCR) intracellular loop, translocate over the membrane and bind to the cytosolic region of transmembrane proteins. Contrary to ordinary CPPs, pepducins remain anchored to the cell membrane and target the GPCR /G protein interface. Pepducins for over 15 different GPCRs have been successfully produced, several of which have shown activity in preclinical *in vivo* models (*37*).

1.3. Physico-chemical properties of CPPs

CPPs form a diverse group of peptides according to their physico- chemical characteristics. The typical properties of CPPs are positive charge and amphiphaticity. Furthermore, a recent study that evaluated 186 peptides, demonstrated the importance of the shape, structure complexity and the 3D-pattern of the constituting atoms influence on the uptake capacity of peptides (*38*).

1.3.1. Charge

A feature shared among CPPs is a high degree of positive charges, due to the content of arginine and lysine residues. At physiological pH, both arginine (pKa 12) and lysine (pKa 10.5) are protonated and interact with negatively charged phosphate and sulphate groups of the extracellular matrix. Still, the impact of positively charged residues differs and arginine residues are more effective in terms of internalization than lysines (*30*). The efficient internalization of arginine-rich CPPs has been shown to depend on the high basic guanidine moiety of arginine. The guanidinium groups can form divalent hydrogen bonds with negatively charged phosphate, sulfate and carboxylate groups on the cell surface. This enhances the affinity of arginine-rich peptides for cell surfaces and neutralization of these positive charges by counter anions is suggested to be beneficial for translocation through the membranes (*39*). It has even been proposed that backbone of the CPP is not essential for translocation and serves as a scaffold to expose guanidinum groups (*40*). On the other hand, ammonium group in lysine can form only one hydrogen bond, and the replacement of arginine with lysine residues that have the same net charge, significantly reduces the peptide uptake (*41*).

It is worth noting that apart from being essential for membrane transduction, positive charges are valuable for non-covalent binding of negatively charged cargos, e.g. nucleic acids.

1.3.2. Hydrophobicity

Hyrophobicity is a common feature that is shared by the majority of CPPs.It has the impact on complex formation with the cargo and solubility of the complex in aqueous solutions, which is valuable property for drug development (*41*). Furthermore, hydrophobic residues in the sequence of CPPs play a major role in the interaction with the plasma membrane bilayer and are thought to enhance the translocation of the peptide across the membrane. For instance, in pVEC sequence the substitution of hydrophobic -N-terminal amino acids to alanine, significantly decreased the cellular uptake (*42*). On the other hand, attachment of small hydrophobic molecule biotin to Tat peptide, enhanced cellular uptake (*43*). Besides that, a study where the content of hydrophobic Leu and hydrophilic Lys residues in amphipathic Hel peptides were varied, showed that the peptide with the highest portion of hydrophobic amino acids was the most efficient for delivering DNA. It also validated the importance of hydrophobic region for the formation of aggregates that were supposed to support cellular uptake and protect DNA. Moreover, hydrophobic amino acids stabilized the α-helical structure (*43*).

In addition to hydrophobicity, the role of aromatic functional groups is even more sophisticated. Aromatic residues, that are highly represented in membrane surface proteins, especially tyrosine and tryptophan, have favorable free energy for insertion into the membrane bilayer (*30*). In antimicrobial peptides, tryptophan residues were reported to destabilize the cell membranes (*44*), and in the case of arginine-rich peptides tryptophan residues played crucial role in the uptake (*45, 46*). For example, Jafari et al. developed novel carrier vector C6MI for siRNA delivery, by replacing three leucine residues in synthetic secondary amphipathic C6 peptide with tryptophan residues. This resulted in improved helicity, solubility in aqueous solutions and uptake efficacy (*41*). Besides that, Rydberg et al. reported that additional tryptophan residues to oligoarginine sequence enhanced the uptake efficacy while positioned in the middle part of the sequence or distributed evenly along the backbone (*46*).

Introduction of hydrophobic fatty acid moiety to the N-terminus of the CPP has been shown to improve the transfection properties of the peptide. This strategy has been utilized by several groups. Already in 2001, Futaki et al., reported significant improvement in gene delivery due to stearylation of octaarginine (R8) (*47*). Later, Wang et al. designed stearylated nuclear localization signal (NLS) modified R8, which increased luc gene expression *in vitro* (*48*). Similarly, cholesterol moiety incorporation to polyarginines enhanced the activity for siRNA delivery *in vivo* (*49*).

In 2009, Mäe et al. showed that stearylated TP10, also named PepFect3 (PF3), efficiently vectorized SCO and these complexes facilitated splice correction at low SCO concentrations (*50*). Furthermore, PF3 proved to be efficient vector for pDNA not only *in vitro*, but also *in vivo* (*51*). Stearylation of TP10 backbone has paved the way to the development of PepFect family of peptides in our group. PF6, contains chloroquine derivative trifluoromethyl-

quinoline (*52*), in PF14 lysines were replaced with ornithine residues (*53*) and in PF15 both the above mentioned modifications have been utilized (*54*). NickFects, a novel family of CPPs that is the main subject of the current thesis, is also designed on the backbone of stearyl-TP10 (*55, 56*).

1.3.3. Secondary structure

The secondary structure of CPPs has mainly been studied with model membranes. Still, the impact of the secondary structure of the peptide is not clear and its correlation to transduction properties is controversial. For example, in contact with model membranes penetratin obtains α-helical secondary structure. However, it has been shown that this α-helical structure is not important for the membrane interaction and internalization route (*30*). Relaying on structural and biophysical studies of CADY, MPG and PEP complexes with various cargos, Deshayes et al. pointed out the importance of carrier structural polymorphism in triggering cellular uptake (*57*). Comparative analysis of 10 well-known CPPs also confirmed the importance of structural plasticity, which determines their ability to interact with phospholipid membranes and route of internalization (*58*). The importance of structural polymorphism correlates with the fact that the peptides have to interact with different lipid environments, and therefore to adapt needed conformation (*30*).

1.4. Peptide modifications to promote endosomal escape

CPPs utilize mainly endocytic pathways to gain access to the cells and this leads to the entrapment of CPP-cargo complexes in endosomal vesicles. The endosomal escape of the therapeutic macromolecules is critical for obtaining the desired biological effects. Without reaching their target in cytosol or in nucleus they cannot exert their function and prolonged stay in the endosomes increases the risk of degradation by the lysosomal enzymes (*59*). This has led to intense investigations to overcome this limitation and a myriad of strategies have been developed, e.g. pore formation in the endosomal membrane, pH-buffering effect of protonatable groups and fusion into lipids bilayer of endosomes (*60*).

In *in vitro* settings, endosomal entrapment can be overcome with the use of lysosomotropic agents, e.g. chloroquine, ammonium chloride and methylamine, which accumulate in endosomal vesicles in unprotonated form. In acidic environment they become protonated, unable to escape from endosomes and so cause destabilization of endosomal membranes (*61*). Although enhancement of biological response in co-treatment with chloroquine has been reported repeatedly (*62–64*), the strategy is not suitable for *in vivo* applications due to the toxicity of chloroquine. On the other hand, this fact clearly indicates that escape from endocytotic organelles to the cytosol is a limiting step for efficient transfection.

Bacteria and viruses are pathogens that use different mechanisms to facilitate their infection into the host cells and simulation of these functions can be used in bioactive macromolecule delivery systems. Small peptide domains from viral proteins have been identified that are responsible for endosomal escape. For example, the second subunit of haemagglytinin (HA) protein of influenza virus, HA2, has shown fusogenic activity. At low pH, the protonation of glutamic acid and aspartic acid residues changes conformation of the peptide. This facilitates fusogenic activity and subsequent insertion of the peptide into the endosomal membrane, resulting in membrane destabilization. In order to promote endosomal release, in several studies HA2 has been used together with CPPs (*65, 66*). In addition, glutamic acid enriched analogue of HA2 peptide, INF7, with the ability to induce more potent pH-sensitive membrane perturbations, has been used with cationic liposomes and polymers (*67*). Furthermore, the design of novel peptides with endosomes destabilizing properties (KALA, GALA), was based on HA2 sequence. Similarly to virus-derived peptides, the 30 amino acid long synthetic GALA peptide contains repeated sequence of Glu-Ala-Leu-Ala, to mimic viral fusion protein segments. The GALA peptide converts its structure from random coil to helical at pH 5 and this leads to the destabilization of the membranes (*68*). GALA peptide has been utilized as a coating to liposomes and in multifunctional envelope-type nanodevice (MEND) (*67*). Fusion strategy is also utilized by several liposome formulations that contain a specific phospholipid, phosphatidylethanolamine (PE). At low pH, PE changes its structure from lamellar to inverted micelles, which allows fusion of liposomal and endosomal membranes and results in destabilization of endosomes (*69*).

Histidine-rich peptides have been used in several studies for endosomal escape purposes (*70–73*). The imidazole ring of histidine has a pKa ~6.5. Therefore, it can be protonated into positive charge in the acidic environment, while it remains lightly negatively charged at normal physiological conditions. Protonation of imidazole groups in acidic endosomal compartments leads to osmotic swelling, followed by rupture of endosomes and release of the cargo (*74*). Relaying on that property, different pH-sensitive peptides with cell-penetrating and membrane-lytic properties have been developed. Histidine residues have been inserted into various CPPs, e.g. MPG, penetratin, R9 andTat (*73, 75, 76*).

In addition, photosensitizers and light sources for their excitation have been used to enhance the diffusion of molecules from endosomes into the cytosol (*77*). However, this strategy may not be practical for therapeutic applications because the phophosensitizer may exhibit a distribution differing from the CPP/siRNA complex *in vivo* (*78*). Besides that, photoinduced endosomal escape has been used, where fluorescent dyes were covalently linked to CPPs (Tat, penetratin, polyarginine) and cytosolic diffusion was observed after photo-stimulation (*79*).

Furthermore, hydrophobic modifications of CPPs, e.g. stearylation, has been shown to enhance endosomal escape (*80, 81*). For instance, stearylation of TP10 resulted in higher luciferase expression level, while the uptake of CPP-cargo complexes was similar to the uptake TP10, suggesting endosomal release of the complexes (*51*). Thereafter, a successive endosomolytic modification was accomplished in stearyl-TP10, where trifluoromethyl- quinoline derivative was added to the backbone. The novel TP10 analog, PF6 proved to be highly effective delivery vector for siRNA both *in vitro* and *in vivo* (*52*).

1.5. CPPs for the delivery of nucleic acids

Gene therapy approaches can be roughly classified according to their pharmacological effect: 1) restoration of lost gene function by gene delivery; 2) silencing disease-causing genes by RNA interference (RNAi) approaches; 3) modification of gene function by interfering with the splicing machinery. All these approaches are based on nucleic acids, e.g. pDNA, short siRNA, SCO. In order to reveal its biological activity, nucleic acids have to reach their target site inside the cells, either in the nucleus or in the cytoplasm.

The ability to transport cargoes over the cellular plasma membrane makes CPPs promising vehicles for macromolecules-based drugs. Numerous groups have reported efficient intracellular delivery of nucleic acids, either covalently or non-covalently linked to CPPs. Since the initial publication of non-covalent complex for the delivery of ONs by the group of Giles Divita (*23*), this strategy has been utilized with several well-known CPPs. Furthermore, a large number of novel CPPs have been developed specifically with complex formation and transfection properties. All these studies have uncovered that certain chemical modifications in the CPPs help to enhance efficient complex formation, cellular uptake and further endosomal escape. The most often exploited modifications are C-terminal cysteamidation (*23, 28, 82, 83*) or introduction of fatty acid residue to the N-terminus (*47, 50, 83*), and incorporation of endosome disruptive moieties to the backbone of the peptide (*52, 73*). Short overview of CPP-mediated ON cellular delivery is presented in Table 2.

Tabel 2. Examples of CPP-mediated nucleic acids intracellular delivery

CPP	Modification	Cargo	Biological effect	Reference
MPG	Cysteamidation	pDNA	Luciferace expression	(*84*)
Stearyl-Arg8	Stearylation	pDNA, siRNA	Luciferace expression; GFP and MAP2b knock-down	(*27, 85*)
Stearyl-$(RxR)_4$	Stearylation	pDNA, SCO	Luciferace and EGFP expression, Hela pLuc 705 model- luciferase expression	(*86*)
Stearyl-TP10	Stearylation	pDNA, SCO	Luciferace expression, Hela pLuc 705 model- luciferase expression	(*50, 51*)
Stearyl-NLS-Arg8	Stearylation, NLS	pDNA	Luciferace expression	(*87*)
C-5H-Tat-5H-C	His, Cys	pDNA	Luciferace expression	(*73*)
PF14	Stearylation, Orn	SCO, siRNA	Hela pLuc 705 model-luciferase expression, HPRT1 mRNA knock-down	(*81, 88*)
PF6	Stearylation, QN	siRNA	Luc, EGFP, HPRT1, Oct-4, GAPDH knock-down	(*52*)
CADY	Cysteamidation	siRNA	GAPDH knock-down	(*28*)
Chol-Arg9	Cholesterylation	siRNA	VEGF knock-down	(*49*)
MPG-8	Cholesterylation	siRNA	Cyc B1 knock-down	(*83*)
NF1	Stearylation, phosphorylation	SCO, pDNA	Hela pLuc 705 model-luciferase expression; luciferace expression	(*56, 89*)
NF51	Stearylation, Orn, branched	pDNA, SCO, siRNA	Luciferace and EGFP expression; Hela pLuc 705 model-luciferase expression; EGFP knock-down	(*55, 89*)

EGFP, enhanced green fluorescent protein; stearyl, stearic acid moiety; GFP, green fluorescent protein; MAP2b, microtubule-associated protein; NLS, nuclear localization signal; His, histidine residues; Cys, cysteine residues; Orn, ornithine residues; HPRT1, hypoxanthine phosphoribosyl transferase; QN, trifluoromethylquinoline derivative; Luc, luciferase; Oct-4, octamer-binding transcription factor4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; chol, cholesteryl moiety; VEGF, vascular endothelial growth factor; Cyc B1, cyclin B1 protein

1.6. Attachment of cargo to CPPs

For the intracellular delivery, CPPs can be linked to ON either covalently or non-covalently. Covalent conjugation results in a chemical compound, where the molecule of CPP is conjugated via covalent bond to the molecule of bioactive cargo. The well defined conjugate can be easily verified by mass-spectrometry analysis and the structure-activity relationships are straightforward to interpret (*10, 90*). Still, covalent conjugation is often complicated and laborious chemical synthesis where high concentrations of compounds are needed. Furthermore, it is not suitable for large nucleic acids delivery, e.g. pDNA. For the design of CPP-cargo conjugates several critical factors have to be considered. Effective dose ratio between the CPP and cargo, steric hindrance, suitable linker, conjugation site in the backbone of CPP, are just some of them (*10*).

On the other hand, non-covalent strategy, which is applicable only for negatively charged ONs, is easy to perform, cost-efficient and low concentrations of compounds can be utilized. The main drawback of non-covalent strategy is the difficulty in generating and characterizing homogenous nanoparticles.

1.7. Characterization of the nanoparticles

The stable complex formation is an important prerequisite for the therapeutic application of nucleic acids using non-covalent complexation strategy with carrier vectors. The formed complex has to meet certain criteria to master all the obstacles before reaching the target. First, the complex has to overcome extracellular barriers: it has to avoid particle clearance mechanisms, to target specific cells or tissues and to protect nucleic acid from enzymatic degradation by nucleases in blood circulation. Thereafter, the complex has to master the cellular barriers, i.e. cellular uptake, endosomal escape, nuclear entry and nucleic acid release (*91, 92*).

Negative charges in the backbone of nucleic acids allow their electrostatic interaction with cationic polymers or peptides, to form nanoparticles. Due to condensing, DNA is able to assemble into nanostructures, which are smaller in size (30–100 nm) than naked DNA (up to 1000 nm). Furthermore, condensing interactions are not only of electrostatic nature, as hydrophobic interactions also take place between the DNA and the delivery vector (*93*).

The size of the nanoparticles plays an important role regarding *in vivo* and *in vitro* applications and can influence the mechanisms and specificity of uptake by different organs and cells and also passive tumor targeting (*92*). Receptor-mediated endocytosis has been shown to be a powerful method for specific delivery of nucleic acids to certain cell types or tissues. Due to the size of endocytotic vesicles, nanoparticles should be small enough to be taken up by this mechanism (*94*). Ideally, they should be less than 200 nm in diameter to

ensure optimal endocytotic uptake and diffusion through tissue *in vivo* by enhanced permeation and retention effect (*95*).

Nanoparticle size can be influenced by many factors, including medium composition. For instance, investigations of DNA/PEI nanoparticles showed that they formed large aggregates in physiological salt concentrations. But the presence of fetal bovine serum in the culture medium hampered the growth of the particles, plausibly due to the adsorption of serum proteins on the particle surface and so preventing particle-to-particle interaction (*96*). Similar results were obtained with CPP-siRNA nanoparticles. The presence of physiological salt concentration resulted in more loosely packed and bigger particles, probably due to the formation of salt bridges between the particles. In contrast, the addition of serum yielded smaller complexes (*97*). Interestingly, the small DNA/PEI complexes were taken up rapidly in human erythromyeloid leukemia cells, while big aggregated complexes mainly stuck on the cell surface. Nevertheless, large aggregates showed higher luciferase gene-transfer level compared to small nanoparticles. The lower transfection level of small particles could result from reduced cell association and endosomolytic activity (*96*). This data shows vividly the importance of environment for the complex formation and transfection. However, in *in vivo* applications, especially intravenous administration, the nanoparticles should be small and preferably uncharged to prevent aggregation, plasma interaction and to prolong circulation in the blood stream (*96*).

Besides the size, the stability of the nanoparticles is also crucial for the successful delivery. During circulation in blood or tissue's infiltration, nanoparticles interact with serum proteins and encounter polyanions, e.g. heparan sulfates on the cell surface. These interactions may result in premature cargo release from the nanoparticle (*97*). On the other hand, after reaching the target, cargo should be released from the complex for subsequent functions. The interaction between the cargo and the delivery agent has to be balanced in order to provide stability as well as to enable dissociation of the nucleic acid to exert its activity (*91, 98*). Various strategies have been applied for nanoparticles stabilization. For example crosslinking with disulphide- and iminothiolane-crosslinks or inserting hydrophobic domains in polyplexes or stearylation of CPPs (*92*). Besides stearylation, in paper II and III NickFect/pDNA nanoparticles stability was altered by the replacement of lysine in the backbone of TP10 with ornithine. This replacement was based on earlier reports, showing that polyornithine formed more stable particles with pDNA and demonstrated superior transfection efficacy than polylysin (*99, 100*).

The surface charge, characterized by zeta potential, determines the mode of interaction of the nanoparticle with the cell surface. Zeta potential is greatly affected by the dispersion medium, especially by the pH and ion concentration. For that reason, it is highly important to measure zeta potential of nanoparticles in relevant medium. The positive surface charge is commonly believed to allow interaction with polyanionic glycosaminoglycans on the cell surface (*97*).

Recently, it was shown that CPP-ON nanoparticles obtain negative surface charge in the presence of serum proteins and their uptake is mediated by scavenger receptors (*101*). Acquisition of negative zeta potential in serum-containing medium was also measured for arginine-rich peptides, although the negative surface charge was weaker compared to TP10 analogues. Furthermore, a linear correlation between the zeta potential under the serum conditions and the sensitivity to serum was observed. The complexes with the most negative potential were also the most stable in the presence of serum. It is still unclear whether the high negative surface charge protects the nanoparticles from the interaction with serum components, or alternatively, induces the formation of a protective layer of serum proteins that protects the nanoparticles from degradation (*97*).

1.8. Cell surface binding of CPP-cargo complexes

Initial step of internalization requires the interaction of the CPP-cargo complex with the plasma membrane. From the structural point of view, CPPs share a high content of basic amino acids and are positively charged at physiological pH. This property confers them the ability to bind electrostatically membrane constituents such as anionic lipids and glycosaminoglycans (GAGs) (*35*). Several reports confirm the affinity of CPPs towards negatively charged lipids that may assist the anchoring of the peptide to the membrane surface and might afford a passive translocation of the peptide across the membrane (*35, 102*). On the other hand, CPPs bind with higher affinity to the proteoglycans than anionic lipids, the content of which in membranes is about 10% and that are mainly localized in the cytosolic leaflet (*35*). As a consequence the direct peptide interaction with lipids is quite rear.

Acidic polysaccharides, GAGs, which are expressed ubiquitously on the cell surface, provide high charge density and due to the far-extending side-chains exhibit supreme potential for electrostatic interactions. CPPs bind GAGs with high affinity that increases as a function of sulphate content and chain length of GAGs (*35*). Increasing number of work validate the importance of arginine-rich peptides interaction with membrane associated proteoglycans, including heparan or chondroitin sulphate proteoglycans (*103–105*). The ability of arginine to form bidentate hydrogen bonds with sulphate, phosphate and carboxylate groups is likely important not only for binding, but also for membrane translocation (*104*). Several studies suggest that cell surface heparan sulphate chains of proteoglycans are essential for Tat binding, leading to Rac1 activation, which triggers membrane reorganization and internalization by macropinocytosis (*103, 106*). On the other hand, glycans have been shown to form a pool of negative charge that Tat binds on the cell surface, but this binding is independent of Tat-mediated transduction by macropinocytosis (*107*). More evidence is required to conclude that these proteoglycans are actual receptors that can induce macropinocytosis, as proteoglycans can often couple with other receptors and activate them (*108*).

Recently, Ezzat et al. showed that PF14-SCO complexes obtained negative surface charge in bio-relevant conditions, which excludes their possibility to interact with negatively-charged cell surface constituents due to electrostatic repelling. This finding lead to a hypothesis that particular receptors may mediate negatively charged nanoparticles interactions with cell surface. Further experiments proved that a group of cell surface receptors, scavenger receptors were involved in the uptake of PF14-SCO complexes (*101*). Later, the impact of scavenger receptors was also shown in the uptake of PF14-pDNA nanoparticles (*109*).

Lately, Futaki and co-workers reported that chemokine receptor (CXCR4) stimulates the uptake of arginine 12 mer peptide (R12) (*108*). CXCR4 is also a co-receptor for HIV-1 infection. Binding R12 to CXCR4 stimulates actin organization and macropinocytosis. Interestingly, R8 and Tat, other typical arginine-rich CPPs that are also reported to use macropinocytosis, did not significantly activate CXCR4-mediated uptake pathway. Higher internalization efficacy of R12 and higher valency of guanidine groups, resulting in greater interaction with cell surface, was suggested as an explanation (*108*).

1.9. Scavenger receptors

Scavenger receptors (SR) were discovered in 1979 by M. Brown and J. Goldstein, who found that acetyl-low density lipoprotein (acLDL) can accumulate in macrophages in atherosclerotic plaques of patients lacking LDL receptors (*110*). Scavenger receptors were named after their role of scavenging modified forms of LDL. SRs comprise a structurally diverse group of soluble and membrane bound proteins, divided into 8 classes (A-H). Despite the lack of sequence similarity or identity, SRs retain the capacity to bind modified LDL particles and other polyanionic ligands (*111*). The range of ligands that SRs bind is extremely diverse and includes microbial structures as well as an array of endogenous molecules (*111*). Following ligand binding, SR can mediate ligand internalization and/or activate signalling cascades leading to diverse cellular functions including lipid peroxidation, apoptosis, endothelial cell dysfunction, monocyte attachment and differentiation leading to foam cell formation (*112*).

Besides the function in LDL metabolism, SRs are pattern recognition receptors that play an important role in innate immunity by the identification and endocytosis of wide variety of pathogen-associate molecules. SRs are highly expressed in macrophages and involved in several immune responses (*113*).

1.9.1. SCARAs

Class A scavenger receptors (SCARAs) are characterized by the presence of collagen-like domain and to date 5 different subtypes have been identified: SR-A I/II/III (SCARA1), MARCO (SCARA2), CSR1 and CSR2 (SCARA3), SRCLI and SRCLII (SCARA4) and SCARA5 (*111, 114–116*). SCARAs have similar domain structure. Being trimeric type II transmembrane glycoproteins, they consist of cytoplasmic tail, transmembrane domain, spacer region and collagenous domain. Still, SCARA2 and SCARA4 do not contain α-helical coiled-coil domain (*111*). All characterized SCARA ligands are polyanionic (e.g. dextran sulfate, polyinosinic acid), although many polyanions with similar chemical structure (e.g. chondroitin sulphate, polycytidylic acid) fail to bind SCARAs (*117*).

SCARA1 and SCARA2 are largely expressed on macrophages. Additionally, SCARA1 isoforms can be detected on endothelial and smooth muscle tissues and SCARA2 on splenic dendritic cells (*114*). The main functions of SCARA1 and 2 are binding to modified LDL, and bacteria. SCARA1 functions in ligands uptake and degradation rather than intracellular signalling. SCARA2 may act as cell-surface sensor (*115*). SCARA3, named after cellular stress response (CSR), was detected in normal fibroblasts by the property of scavenging oxidative molecules or harmful products of oxidation (*118*). SCARA4 is scavenger receptor with C-type lecithin (SRCL) and has at least 2 isoforms. This membrane-expressed receptor is found on the surface of endothelial cells but not on the surface of macrophages and may be involved in innate immune response against fungal infections (*112, 119*). SCARA5 is expressed on plasma membrane of selected epithelial cells, especially in testis, trachea, lung and bladder, but not in macrophages. This expression pattern has prompted researchers to postulate a role of this receptor in epithelial homeostasis. SCARA5 binds ferritin, which is followed by endocytosis into an acidic compartment and incorporation of iron into the cell, supporting cell survival. Whether ferritin binds to other scavenger receptors or uniquely to SCARA5, is still a pending issue. SCARA5 has been reported to act as a tumor suppressor. Its lower-expression decreases tumor growth and inhibits metastasis (*116, 120*). Differently from original macrophage, scavenger receptors from class A (SR-A and MARCO) that were determined by investigating acLDL binding, SCARA4 and 5 do not bind acLDL. The ability of SCARA3 to bind acLDL has to be elucidated (*114*).

SCARAs that are expressed in a broad variety of cells have been shown to participate in the uptake of nucleic acids and different anionic nanoparticles. They are involved in the uptake of single-stranded RNA, like poly I and poly G (*121*), as well as synthetic double-stranded RNA, such as poly IC (*114*). Lately, it was shown that SCARAs are responsible for the detection of virus infections. They bind extracellular, viral dsRNA and mediate its entry into the cell, where it is delivered to other intracellular sensors that activate antiviral responses (*114*). SRs mediate cellular uptake of oligonucleotide-functionalized gold nanoparticles (*122*), polymer-functionalized iron oxide nanoparticles (*123*),

silver and polystyrene nanoparticles (*124, 125*). The ability of SCARAs to bind and mediate the uptake of CPP-nucleic acid nanoparticles was first reported by K. Ezzat (*101*) and is particularly important for this thesis.

SR-ligand complexes can undergo receptor-mediated endocytosis, trafficking trough the endosome-lysosome system leading to degradation or accumulation of ligand. It is likely that after endocytosis, many SRs are recycled back to the plasma membrane, where they mediate further ligand binding, clearance or accumulation (*112*). Different mechanisms of endocytosis have been postulated for SR and several publications demonstrate that multiple pathways are active simultaneously (*123, 124, 126, 127*).

1.10. Endocytosis

Cell membranes display a tremendous complexity of lipids and proteins designed to perform the functions cell require. The outer leaflet of the plasma membrane is the surface through which each cell communicates with the environment. In order to appropriately respond or affect its environment, cell membrane composition must be tightly regulated by the cell. Endocytotic mechanisms control the lipid and protein composition of the plasma membrane, thereby controlling cells/ environment interaction (*128*). During endocytosis, the extracellular material is engulfed by the invagination of the plasma membrane to form a small vesicle. Each vesicular trafficking event involves membrane budding formation of spherical vesicles from flat membrane areas, typically followed by its regulated transport (*129*). Endocytosis can be classified into two broad categories: phagocytosis that only occurs in certain cell types (e.g. macrophages) and pinocytosis that occurs in all cells (*130*). Therefore, endocytosis and pinocytosis are often considered synonymous. At least four morphologically distinct endocytotic pathways have been characterized: clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, macropinocytosis, and clathrin/caveolae-independent endocytosis. They differ in the composition of the coat, in the size of the detached vesicles, and in the fate of internalized particles (*131*). The main endocytotic pathways and intracellular routing of endocytosed material are presented on Figure 1.

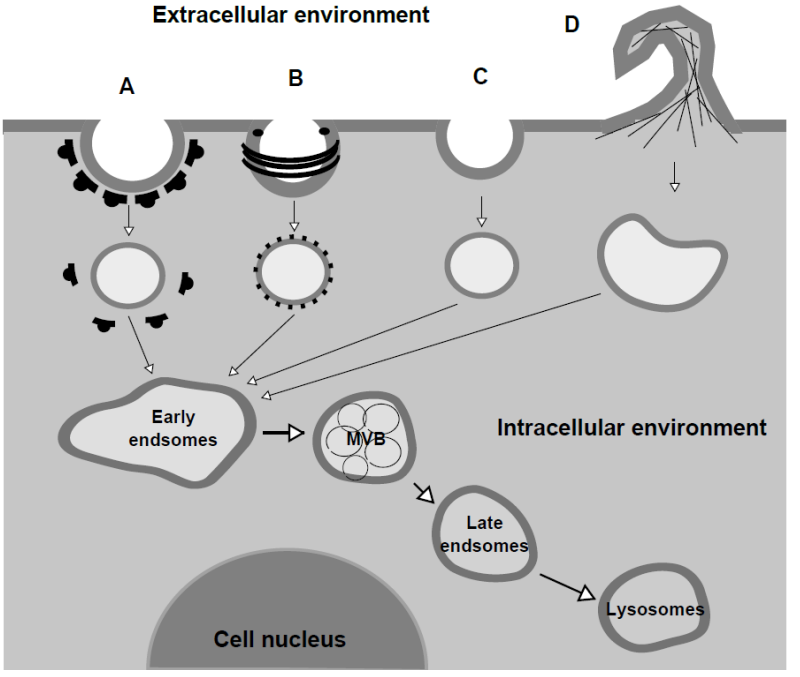

Figure 1. Schematic illustration of the main enocytotic pathways and intracellular routing of endocytosed material. A – clathrin-dependent, B – caveolin-mediated, C – clathrin- and caveolin- independent endocytosis, D–macropinocytosis. Endosomal trafficking routes are indicated by arrows.

1.10.1. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME), the major and best-characterized endocytotic pathway, occurs constitutively in all mammalian cells and carries out the continuous uptake of essential nutrients, antigens, growth factors, and pathogens (*132*). Low-density proteins with their receptors, transferrin and hormones use CME (*133*). In CME, a wide variety of transmembrane receptors and their ligands are packed with the use of cargo adaptors into clathrin coated vesicles (CCVs) (*128*). The binding of a ligand to a specific cell-surface receptor results in clustering of the receptor-ligand complex in coated pits of plasma membrane, which are formed by the assembly of cytosolic coat proteins, mainly clathrin. The coated pits invaginate and pinch of the plasma membrane, aided by dynamin, to form intracellular CCVs, usually 100–150 nm in diameter (*131*). Clathrin coat then depolymerizes, resulting in early endosomes (EE), which join with each other to form late endosomes (LE) that fuse with lysosomes. Molecules entering by this pathway experience a drop in pH from neutral to pH 5.9 in EE, with further reduction to pH 5 in lysosomes (*131*).

Clathrin itself binds neither membranes nor cargo, but specific adaptor proteins (e.g. AP-2 complex) recruit it to the membrane. However, clathrin is absolutely required for the budding and in the absence of clathrin plasma membrane remains flat and endocytosis does not take place (*129*). The GTPase dynamin is required for CME and the self-assembly of dynamin functions to constrict the neck of invaginated coated pits (*131*). By many accounts also cholesterol is important for CME, although this pathway is less sensitive to cholesterol depletion than most coat-independent budding pathways (*134*).

1.10.2. Caveolae-mediated endocytosis

Caveolae are 60–80 nm in size, hydrophobic membrane microdomains that are rich in cholesterol and glycosphingolipids (*135*). They reside only in the strictly ordered membrane microdomains called lipid rafts (*136*). Lipid rafts are dynamic nanoscale assemblies enriched in sphingolipid, cholesterol, and glycosylphosphatidylinositol-anchored proteins (*137*). Caveolae are flask-shaped invaginations of the plasma membrane characterized by their association with a family of cholesterol-binding proteins, called caveolins. There are three mammalian caveolin proteins: caveolin1 and 2 that are found widely in almost all cells, lacking only in neurons and leukocytes, and caveolin 3 that is muscle specific (*128*). Caveolins are pentahelical proteins. Two of the helices insert deeply into the membrane, while the other three helices are amphipathic and are thought to wedge themselves into the interfacial region of the membrane (*138*). Caveolae contain a quantified number of caveolin molecules (~ 144), which suggests the formation of highly organized coat (*139*). They are highly stable and are internalized slowly (*131*). Cholesterol is required for caveolar uptake and drugs that bind cholesterol pertube internalization through caveolae (*140*). Caveolae also depend on the actin cytoskeleton, and drugs that cause the depolymerisation of the actin cytoskeleton (e.g. cytochalasin D) can inhibit caveolae uptake.

Caveolae are present in many cell types and are especially abundant in endothelial cells (*130*). They are involved in several cellular processes, including cholesterol homeostasis, glycosphingolipid transport, endocytosis/ transcytosis of certain viruses, bacteria and toxins (*131*). It is generally belived that caveolar uptake does not lead to lysosomal degradation of internalized material (*135, 141*). Therefore, this pathway may be advantageous in terms of DNA delivery and has shown to be involved in Tat-mediated DNA uptake (*141*). From the gene delivery point of view the main drawback is that caveolae are slowly internalized and their fluid volume is small. Still, this is a promising gene delivery strategy, if the internalization can be increased, for example through the use of specific receptors for caveolae (*131*).

1.10.3. Macropinocytosis

Macropinocytosis describes a form of larger-scale internalization that frequently involves protrusions from the plasma membrane that subsequently fuse with each other or with the plasma membrane, resulting in the uptake of extracellular components (*128*). In this actin-driven evagination large endocytotic vesicles of irregular size and shape are formed (*142*). Macropinocytosis usually accompanies cell surface ruffing that is induced in many cell types upon stimulation of growth factors or other signals (*130*). A ruffle is formed by a linear band of outward-directed actin polymerization near the plasma membrane that lengthens into a planar extension of cell surface. After stimulation by mitogenic factor, the ruffles enlarge and finally close into macropinosomes (*143*). Macropinosomes have no coat nor specific receptors and their size varies, up to 5 μm (*131*). The directed actin polymerization is stimulated by small GTPases, e.g. Rac1, that regulate the advancement of protrusion and the formation of pinocytic cap (*144*). Macropinocytosis is cholesterol dependent, which is required for anchoring the activated Rac1 to the sites of premature macropinosome (*145*).

The unique feature that distinguishes macropinocytosis from the other endocytotic pathways is the variation of the ultimate fate of the macropinosome in different cell types. In macrophages, they move towards the center of the cell, shrink due to loss of water, become acidified and fuse to lysosomal compartments (*146*). On the other hand, in human A-431 and NIH-3T3 cells they remain relatively isolated from the rest of endosomal system, interact only with other macropinosomes and finally fuse back with the plasma membrane (*143, 147*). Although the pH of macropinosomes decreases, they do not fuse into lysosomes in this case (*131*). Furthermore, macropinosomes are thought to be inherently leaky compared to other types of endosomal vesicles (*66, 148, 149*). Originating from that, macropinocytosis provides several advantageous aspects for drug delivery: high volume uptake of macromolecules, the avoidance of lysosomal degradation and relatively leaky nature of macropinosomes, which enables endosomal escape (*131*).

1.10.4. Clathrin- and caveolin-independent endocytosis

In addition to CME, caveolin-mediated endocytosis and macropinocytosis, other, so-called clathrin- and caveolin-independent endocytosis pathways exist in mammalian cells that have been less studied. In non-clathrin and non-caveolar endocytosis the formed vesicles do not possess a coat and have been shown to display a rather diverse array of carrier morphology, i.e. small uncoated vesicles, ring-shaped structures or large tubular carries (*150, 151*). These pathways can be further subdivided into dynamin-dependent and independent routes. Similarly to caveolin-dependent endocytosis, lipid rafts are required for these internalization routes. For example internalization of interleukin-2 receptors into lymphocytes is suggested to use clathrin-inde-

pendent endocytosis (*152*). These pathways are also required for high and sustained synaptic activity, and for rapid endocytosis by neuroendocrine cells (*130, 153*).

1.11. Uptake of CPP-cargo complexes

In 1990s, most CPPs were presumed to enter cells by directly crossing the cellular membrane in energy- and temperature-independent manner (*19*). However, in 2003 Lundberg et al. showed that the experimental basis for this proposition was redistribution artefact caused by fixation before microscopy (*154*). This led to a rush of studies re-examining how CPPs enter the cells.

In general, CPPs can convey their cargo into the cells by two principally different mechanisms- endocytosis and direct penetration. The direct penetration mechanism constitutes the interaction of positively charged CPP with the negatively charged components of the membrane, causing transient or stable destabilization of the membrane associated with folding of the peptide on the lipid membrane (*155*). Direct penetration is most probable at high peptide concentrations (*156–158*). Still, it is not the prevailing mechanism for the majority of CPP-cargo complexes. Nevertheless, this pathway cannot be excluded, as it is occasionally reported for certain CPPs and cargos. For example it was recently proved that CADY peptide complexes with siRNA utilize direct penetration (*159*).

Internalization via some pathways of endocytosis has been shown for most CPP-cargo complexes. To pinpoint the certain internalization pathway is a difficult task, as several endocytotic routes can be utilized in parallel (*160*) and down-regulation of specific path may lead to the up-regulation of some alternative pathways. For Tat and other arginine-rich peptides-nucleic acids complexes, macropinocytosis accompanied by actin reorganization, has been shown to be the main endocytosis pathway (*161*). However, in the case of Tat, also caveolae-mediated endocytosis has been reported (*162*). TP10-protein complexes use simultaneously at least 4 pathways: macropinocytosis, CME, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis (*163*). PF6/ON complexes are mainly taken up by clathrin-coated pits (*32*).

It is important to emphasize that the uptake mechanism of CPP-cargo complex depends in addition to the properties of the CPP on numerous other parameters and conditions. Utilized concentrations and properties of the cargo, variations in membrane composition, cell type and experimental conditions, are just some of them.

1.12. Intracellular trafficking and endosomal release

After budding from the plasma membrane, the CPP-cargo complexes are confined in endocytotic vesicles. Despite the existence of numerous internalization routes, early endosomes (EE) serve as a focal point of the endocytic pathways. Sorting events initiated at this compartment determine the subsequent fate of the internalized material, destining them either for recycling to the plasma membrane, for degradation in lysosomes or for delivery to the trans-Golgi network (*164*). The quick sorting is accomplished through the formation of distinct microdomains within EEs. Besides the primary function, sorting, EEs are responsible for the initial dissociation of receptor-ligand complexes. Although the pH in EEs is only slightly acidic (pH 6.3–6.8), it is still sufficient to result in the dissociation of ligands from receptors. For instance transferrin or low-density lipoprotein receptor slated for recycling from EEs, whereas low-density lipoprotein and the ligand-bound epidermal growth factor receptor are transported to late endosomes/lysosomes for degradation (*164*). The multivesicular parts of EEs lead to the formation of globular multivesicular bodies (MVBs). Precisely, MVBs evolve from the early endosomal compartment by forming intraluminal vesicles from the limiting membrane of the compartment (*129*). Although, endocytotic organelles can be devided into early and late endosomes (LEs), these types of endosomes do not exist as distinct stable organelles, but instead undergo a maturation process by changing their repertoire of membrane proteins. For example losing the Rab5, prevalent in EEs, and gaining the Rab7 in LEs (*165*).

The transfer of material from EEs to LEs involves the dissociation of vacuolar elements from EE network and their subsequent migration on microtubules to the perinuclear cytoplasm where they fuse to LEs (*166*). Late endosomes are enriched in lgp/LAMP-s, Rab7, Rab9. They also contain hydrolytically active lysosomal hydrolases, and are therefore sometimes referred as prelysosomal compartments (*166*). Lysosomes are acidic organelles (pH 4.5–5) that contain a high number of soluble lysosomal hydrolases with each having a specific substrate (*167*). The relationship between LEs and lysosomes is dynamic and not easily defined. Still lysosomes can be distinguished from LEs as the lysosomes have higher density in Percoll. Although in transmission electron microscopy or fluorescence microscopy EEs and lysosomes are often labeled simply on morphological appearance, intracellular position and morphology are often very different in each cell type, making this kind of assumption unhelpful. It is important to identify subcellular compartments using specific markers, e.g. Rab5 for EE and Rab7 for LE/lysosomes (*168*).

To avoid degradation of the cargo by enzymes in lysosomes or recycling of it back to the cell surface, the release from endosomes is essential for the efficient transfection. Compared to cell membrane translocation, the concentration of CPP-cargo complexes in the endosomal lumen extensively elevates the local concentration of the peptide per unit area of the membrane. At high

concentration, the peptide accumulates and clusters on the membrane, thus interfering with the regularity of its packing and inducing its subsequent leakage. Such destabilization and pore formation has been shown for TP10 (*169*) and arginine-rich peptides (*170*). The liberation of CPP-cargo complexes from endosomes thereby occurs via destabilized and leaky endosomes, as seen in the electron microscopy images (*171*). Still, the exact amount of escaped complex is complicated to define. In paper III fractioning of endosomal compartments with Percoll gradient was used to study the amount of complexes in early endosomes and late endosomes/lysosomes.

Lysosomotropic agent chloroquine (CQ) has been used to promote the escape of complexes from endosomal compartments. CQ accumulates in endosomal vesicles, buffers the pH and prevents the fusion with lysosomes. Furthermore, CQ destabilizes endosomal membranes, converts them leakier and penetrable for complexes (*13*). Albeit improved transfection in the presence of CQ has been reported frequently (*50, 86, 172, 173*) , this compound is toxic at higher concentrations and not suitable for *in vivo* applications (*66*).

Therefore, modification of carrier vectors in order to improve their endosomolytic properties is highly important and this is discussed in more details in chapter 1.4.

2. AIMS OF THE STUDY

The main target of this thesis was to design novel efficient peptide-based vectors that can be utilized for the intracellular delivery of nucleic acids via non-covalent nanoparticle formation strategy. The impact of different chemical modifications in the backbone of the parental peptide was correlated to the transfection efficacy of nucleic acids and biological activity.

Paper I: To study if the insertion of phosphoryl-group in the backbone of stearyl-TP10 improves the delivery and endosomolytic properties of the peptide to be used for SCO intracellular delivery.

Paper II: To evaluate the impact of two novel modifications, insertion of a kink and substitution of L-lysine with L-ornithine. To study the potential of the novel peptides for the delivery of pDNA, SCO and siRNA. Moreover, to evaluate the applicability of the novel peptide NF51 in protein production technology.

Paper III: To investigate two novel cell-penetrating peptides NF1 and NF51, with superior delivery properties according to paper I and paper II. To study their complex formation activity, uptake mechanism, intracellular trafficking and determine the impact of different internalization steps on the biological activity in gene delivery for successive development of these delivery vectors and their further *in vivo* applications.

3. METHODOLOGICAL CONSIDERATIONS

3.1. Design of peptides

In the current thesis a novel family of peptides, named NickFects, was designed. Previous studies of our group have proved that addition of stearyl-group to the N-terminus of TP10 significantly improved the transfection efficacy and endosomal release of CPP/ON complexes (*50, 51*). Hence, we designed all the NFs as modifications of stearylated-TP10.

Paper I describes two new CPPs, NF1 and NF2. With the design of these CPPs, we aimed to enhance interactions of the peptide with intracellular and endosomal membranes. Phospholipids are the main component of plasma membrane. Moreover, most liposome formulations that are utilized as transport vectors for nucleic acids, contain phospholipid PE. Relaying on that, we added phosphoryl-group to the backbone of stearyl-TP10. Earlier study about cationic α-helical peptides, including mastoparan, indicates that hydrophobicity balance of the peptide affects its complex formation with DNA (*174*). To change the hydrophobicity of NickFects, we replaced Ile8 in the TP10 sequence with more hydrophilic amino acid, threonine. With these modifications, we reduced the net positive charge and increased hydrophilicity of the peptide, and obtained pH-dependent vectors NF1, NF2 (*56*).

In paper II, we modified Lys7, which is the linker between neuropeptide galanin motif and mastoparan residues in stearyl-TP10 sequence. The first modification was inspired by poly-L-ornithine's higher ability to condense DNA and this lead to superior transfection efficacy compared to corresponding poly-L-lysine-based systems (*99*). Moreover, non-coded amino acid ornithine has shown higher resistance to serum proteases and should increase the stability of the peptide. Furthermore, recently a novel CPP named PF14, was developed in our group, where lysines were replaced with ornithines. PF14 proved to be highly efficient transport vector for nucleic acids (*81*). In order to improve the serum stability of the peptide and enhance its transfection efficacy for pDNA, we replaced Lys7 with ornithine in NF53.

The second modification was based on an earlier study comparing α- and ε-lysine systems. Polylysine, where Lys residues were linked via side chain ε-amino group, had more flexible backbone and the ε-peptide bond was more resistant to protease degradation. ε-polylysine condensed DNA into smaller particles than ordinary α-polylysines and these particles exposed lower cytotoxicity and higher transfection efficacy *in vivo* (*175*). Relaying on that, we utilized side chain ε-amino group of Lys7 for subsequent synthesis and gained NF61. In the third novel peptide, NF51, we implemented these above-mentioned modifications simultaneously. We replaced Lys7 with ornithine and continued synthesis by coupling Gly to the δ-amino group of ornithine (*55*).

3.2. Peptide synthesis

All peptides were synthesized by solid-phase peptide synthesis (SPPS), a strategy introduced by Bruce Merrifield in 1963 (*176*). The SPPS is based on anchoring the growing peptide chain onto an insoluble solid polymer and amino acids are attached in a step-wise manner without purification between coupling and deprotection cycles. Two alternative strategies according to temporary protecting group on α-amino group of amino acid prevail in SPPS: tert-butyloxycarbonyl (t-Boc) and 9-fluorenylmethoxycarbonyl (Fmoc) strategy. The difference between these two strategies is that t-Boc group can be removed by strong acid, while Fmoc group is removed by strong base. This enables to combine these two chemistries when orthogonal deprotection of specific side chain is necessary, e.g. in NF51 synthesis orthogonal deprotection strategy was utilized. The side chain amino groups of amino acids are protected with permanent protective groups that are removed during final cleavage of the peptide from the solid support.

Throughout this thesis, peptides were synthesized on an automated peptide synthesizer or manually using Fmoc chemistry (*177*) by assembly of protected amino acids to Rink-amide methylbenzylhydrylamine (MBHA) resin to obtain C-terminally amidated peptides. The final cleavage was performed with trifluoroacetic acid (TFA), 2.5% triisopropylsilane and 2.5% water. Peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) and analyzed using matrix-assisted laser desorption-ionization/time of flight (MALDI-TOF) mass spectrometry. The peptides used in this thesis are presented in Table 3.

Table 3. CPP sequences used in the thesis.

CPP	Sequence	Ref.
Stearyl-TP10, PF3	stearyl-AGYLLGKINLKALAALAKKIL-NH_2	(*55, 56*)
p-Pepfect3, p-PF3	stearyl-AGY(PO_3)LLGKINLKALAALAKKIL-NH_2	(*56*)
NickFect1, NF1	stearyl-AGY(PO_3)LLGKTNLKALAALAKKIL-NH_2	(*56, 89*)
NickFect2, NF2	stearyl-AGYLLGKT(PO_3)NLKALAALAKKIL-NH_2	(*56*)
NickFect3, NF3	stearyl-AGY(PO_3)LLGKT(PO_3)NLKALAALAKKIL-NH_2	(*56*)
NickFect11, NF11	stearyl-AGYLLGKTNLKALAALAKKIL-NH_2	(*56*)
NickFect 51, NF51	(stearyl-AGYLLG)δ-OINLKALAALAKKIL-NH_2	(*55, 89*)
NickFect 53, NF53	(stearyl-AGYLLG)ε-KINLKALAALAKKIL-NH_2	(*55*)
NickFect 61, NF61	stearyl-AGYLLGOINLKALAALAKKIL-NH_2	(*55*)

Stearyl, stearic acid moiety; PO_3, phosphoryl group

3.3. Non-covalent nanoparticle formation

In general, two main strategies can be utilized to associate CPPs to different cargos: non-covalent complex formation or covalent conjugation, where the cargo is linked to the CPP via covalent bond. The non-covalent strategy that relies on complex formation between the CPP and cargo due to electrostatic and hydrophobic interactions was first demonstrated by the group of G.Divita for linking pDNA (*23*). Thereafter non-covalent complex formation strategy has been successfully employed for different ON delivery (*24, 50, 178*).

In the current thesis nanoparticles of CPPs and ON-based cargo, i.e. pDNA, SCO, siRNA, were formed by mixing CPP with cargo molecules and co-incubating for 45 min at the room temperature for spontaneous complex formation.

To characterize the amount of CPP over cargo the charge ratio (CR) for pDNA and the molar ratio (MR) for siRNA and SCO were used. CR was calculated theoretically, taking into account positive charges of the peptide and negative charges of the plasmid while MR is a ratio between the amount of moles of CPP and cargo.

3.4. Nanoparticle characterization

The physico-chemical properties of the nanoparticles significantly affect pharmacokinetics and biological activity of the cargo. A variety of methods that characterize the size, surface charge and stability of the nanoparticles were utilized in the current thesis.

3.4.1. Dynamic light scattering and zeta potential

Dynamic light scattering (DLS) is a spectroscopic technique mainly used to determine the particle size and polydispersity in solution. Nanoparticles in solution can be regarded as a colloidal dispersion system where molecules undergo random movement called Brownian motion. The Brownian motion of particles in suspension causes laser light to be scattered at different intensities. In DLS the time-dependent fluctuations in the scattered light are measured by a fast photon counter. The fluctuations are directly related to the rate of diffusion of the molecule through the solvent, which is related to the hydrodynamic diameter of the nanoparticles (*179*).

Charged particles, e.g. peptides, suspended in a solution attract ions of opposite charge to their surface. The liquid layer surrounding the particle exists in two parts: an inner region, called the Stern layer, where the ions are strongly bound and an outer, diffuse, region where ions are less firmly attached to the particles. Within the diffuse layer, there is a notional boundary inside which the ions and particles form a stable entity. The potential that exists at this boundary is known as the zeta potential. This potential is calculated by the electrophoretic

mobility of the particles in solution, measuring intensity fluctuation of scattered light (*180*). Zeta potential correlates the surface charge of the particle in a current medium (*181*). That is why it is highly important to carry zeta potential measurements in a biorelevant medium.

Throughout the current thesis CPP-ON nanoparticles hydrodynamic diameter, polydispersity index that characterizes the homogeneity of nanoparticles and zeta potential were measured using a Zetasizer Nano ZS apparatus. Measurements were conducted in water, in serum-free and serum-containing media. In Paper III, an automated in-place titration device of the apparatus was used to mimic the physiological conditions in endosomal compartments and evaluate the stability of the nanoparticles in acidic environment.

3.4.2. Heparin displacement assay

The interaction between the delivery agent and the cargo in nanoparticle should be balanced to provide stability and protection of the cargo from degradation, and at the same time to allow the dissociation of the nucleic acid to exert its activity when reached the target (*91*). Heparin sodium salt is an anionic molecule that has higher binding affinity to the delivery vector than nucleic acid, and the concentration of heparin needed to dissociate CPP-nucleic acid complex characterizes the stability of the nanoparticles. In paper III, the heparin displacement assay was utilized. The complex stability was assessed by electrophoresis or by fluorimeter, capturing the amount of released pDNA.

3.5. Cell cultures

In the current thesis, several cell lines have been used. Most of them are commonly used in CPP field and enable comparisons of previously published data.

HeLa cells are an immortalized cell line derived from cervical cancer cells taken from Henrietta Lacks in 1951. Since then this rapidly growing and robust cell line has been used in many research areas. In paper III, HeLa cells and in paper I and II, HeLa pLuc 705 cells were utilized. HeLa pLuc 705 cells are stably transfected with luciferase-encoding gene interrupted by a mutated intron 2 derived from β-globin gene that carries a cryptic splicing site resulting in aberrant and non-functional luciferase. Masking the cryptic splice site with SCO enables the redirection of splicing and production of functional luciferase protein. Thus using these cells vectors ability to transfect SCO can be evaluated by luciferase activity.

In paper II, regular adherent cell lines e.g. Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK293) cells and cancer cells, namely human osteosarcoma (U2OS) and human glioblastoma (U87) cells were used to screen

the potential delivery vehicles. CHO-K1 cells are subclones from parental cell line initiated from a biopsy of an ovary of adult Chinese hamster. The HEK293 cell line was generated by adenoviral transformation of normal human embryonic kidney cells. U87 cells are human glioblastoma-astrocytoma cells with epithelial morphology, obtained from a stage IV cancer patient and U2OS cell line was derived from the bone tissue of a fifteen-year-old human female suffering from osteosarcoma. To evaluate siRNA-mediated gene silencing we used CHO cells stably expressing EGFP.

In paper II, we also utilized more refractory primary mouse embryonic fibroblasts (MEFs) and lymphocyte suspension cells Jurkat and A20. Pluripotent MEF cells are often used as "feeder cells" in human embryonic stem cells research. The Jurkat cell line was established in the late 1970s from the peripheral blood of a 14-year old boy with T cell leukemia. Jurkat is an IL-2 producing T lymphocyte cell line, commonly used to study T cell signaling. The A20 is immortalized B-cell lymphoma cell line derived from a spontaneous reticulum cell neoplasm found in mouse.

Additionally, in paper II, we employed U2OSEBNALTD3 and CHOEBNALT85 cell lines to study the applicability of NF51 for protein production. U2OSEBNALTD3 and CHOEBNALT85 are stable cell lines derived from U2OS and CHO-S, respectively. These cells stably express mouse polyomavirus large T antigen and Epstein-Barr virus (EBV) EBNA-1 protein for stable replication and maintenance of QMCF plasmids containing polyomavirus minimal origin and FR element with EBNA-1 binding sites.

3.6. Transfection experiments

The efficacy of the designed CPPs to deliver nucleic acids was examined by using various reporter systems, such as luciferase- or EGFP-encoding plasmids, EGFP-silencing RNA or pLUC 705 SCO.

3.6.1. pDNA delivery

To evaluate pDNA transfection efficacy we utilized luciferase-encoding pGL3 plasmid. Luciferase is an enzyme that catalyzes the conversion of luciferin into oxyluciferin that produces bioluminescence, which can be measured by luminometer. To be transcribed, the plasmid has to reach the nucleus. Assessment of pGL3 plasmid delivery efficacy is a widely used positive read-out method and it was used in papers II and III.

In paper II, EGFP-encoding plasmid was used to assess the percentage of transfected cells using fluorescence-activated cell sorting (FACS). Enhanced green fluorescent protein (EGFP) is ideal for subcellular labelling and visualization. FACS analysis is a laser-based biophysical technique that is employed in cell counting, cell sorting, biomarker detection and protein

engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. With FACS we counted the number of EGFP-transfected cells in comparison to the whole population of viable cells.

3.6.2. SCO delivery

In paper I and II, we utilized HeLa pLuc 705 cell line-based functional assay developed by R. Kole et al. in 1998 (*182*), which is a widely exploited positive read-out method to quantify ON delivery into the nucleus by various delivery vectors. Masking the aberrant spice site with SCO redirects splicing towards the correct mRNA and restores luciferase activity that can be quantified by luminescence.

3.6.3. siRNA delivery

In paper II we used EGFP-CHO reporter cell line to evaluate the efficacy of new vectors to deliver siRNA. The cell populations stably expressing EGFP and cell populations where EGFP was downregulated by siRNA/CPP nanoparticles were measured by FACS.

3.7. Toxicity studies

The ability of CPPs to translocate through cell membranes can be accompanied by toxic effects resulting from membrane perturbation especially at higher peptide concentrations. CPP concentrations exceeding a toxic threshold, can cause irreversible damage to the cells and cytotoxicity of novel CPPs has to be carefully evaluated (*10*).

To exclude that the activity of the peptides is associated with toxicity MTS assay was utilized in paper I and CytoTox-Glo assay in paper II. MTS assay is a tetrazolium salt-based colorimetric method. The amount of metabolizing cells is detected by the activity of intracellular enzyme mitochondrial dehydrogenase that reduces tetrazolium to formazan dye having adsorbance maximum at 490–500 nm. CytoTox-Glo assay is based on distinct protease activity associated with cytotoxicity and signal of luminogenic peptide substrate (alanyl-alanylphenylalanyl- aminoluciferin substrate) added to the cells indicates the number of cells that have lost membrane integrity.

3.8. Methods to study cellular uptake and intracellular trafficing

3.8.1. Inhibition and downregulation of scavenger receptors

Scavenger receptors are cell-surface receptors that bind with low specificity to polyanionic ligands (*183*). To assess if scavenger receptors type A are involved in the uptake of NF/pDNA nanocomplexes we tested the effect of well-known specific inhibitory ligands for SCARA (*113, 183*) polyinosinic acid (poly I), fucoidan and dextran sulfate on the pDNA transfection activity mediated by NickFects. Structurally similar compounds poly C, galactose and chrondroitin sulfate that lack affinity to SCARA were utilized as negative controls. HeLa cells were treated for 30 min with inhibitory compounds prior to addition of pre-formed NF/pDNA nanocomplexes and bioluminescence was measured 24 h after transfection.

To downregulate scavenger receptors expressed in HeLa cells, SCARA3 and SCARA5 siRNAs were used in mixture and scrambled siRNA was used as a negative control. The cells were transfected with siRNA using Lipofectamine RNAiMAX and NF/pDNA nanocomplexes were added 24 h post-transfection with siRNAs. Bioluminescence of luciferase was measured 24 h post-transfection with NF/pDNA nanocomplexes.

3.8.2. Inhibition of endocytosis pathways

Endocytotic pathway is an energy-dependent mechanism and therefore it can be inhibited by lowering the temperature or by the use of metabolic inhibitors to deplete the ATP pool (*184*). An extensively used method to pinpoint the particular endocytotic pathways is blocking a certain internalization route with pharmaceutical compounds, endocytosis inhibitors. Chloropromazine is a cationic amphipathic drug that causes the dissociation of clathrin lattice and therefore inhibits clathrin-mediated endocytosis (*185*). The main drawbacks using chloropromazine are possible insertion into plasma membrane and alteration of the membrane fluidity. Besides that it can inhibit phospholipase C, important for actin dynamics and macropinocytosis, which can give rise to misinterpretations (*186*). Nystatin that forms large aggregates upon binding to cholesterol has been used to inhibit lipid raft caveolae-mediated endocytosis (*186*). Caused changes in caveolar shape and inhibition of lipid raft ligands does not affect CME and macropinocytosis. Cytochalasin D that causes the polymerization of the actin cytoskeleton and disassembly of the existing actin, inhibits membrane ruffling and macropinocytosis. However, affecting actin cytoskeleton may partly influence CME and caveolae-mediated endocytosis (*187*). Chloroquine (CQ) is a lysosomotropic compound that can cross the membrane of lysosomes in the neutral unprotonated form. As a weak base CQ adsorbs protons and inhibits acidification in endosomal compartments and endosome/lysosome fusion. While in protonated form CQ, not capable to cross

the membrane, accumulates in endosomes, induces their swelling and rupture (*13, 61*). Co-transfection with CQ helps to release the entrapped cargo from endosomal compartments and to enhance transfection efficacy (*61*).

In paper III we used low temperature, chloropromazine to inhibit CME, cytochalasine D known to suppress macropinocytosis and nystatin for caveolin-associated process. In papers I, II and III, lysosomotropic agent CQ was used to evaluate the amount of entrapped CPP/ON nanoparticles in endosomal compartments. Gene delivery efficacy was measured and normalized to the control experiment performed in the absence of inhibitory compounds.

3.8.3. Confocal and transmission electron microscopy

Confocal laser scanning microscopy enables live-cell imaging of fluorophore labeled CPPs and provides valuable information about their intracellular localization. In paper III we produced double-labeled complexes, where the peptide and plasmid DNA were tagged with different fluorochromes and tracked the uptake in real-time using confocal laser scanning microscopy.

The advantage of transmission electron microscopy (TEM) compared to confocal microscopy is high resolution and magnification, although only fixed cells can be used. In papers I and III, TEM was utilized to obtain ultrastructural analysis of the uptake and intracellular trafficking of NF/ON nanoparticles. SCO or pDNA were labeled with nanogold clusters and thereafter complexed with respective peptide. After the treatment, the cells were fixed, sectioned and examined with transmission electron microscope.

3.8.4. Separation of endosomal compartments

Microscopy methods enable to visualize the location of nanoparticles, but it is complicated to obtain quantifiable data. In order to measure the amount of nanocomplexes in early and late endosomal/lysosomal compartments, we separated and purified endosomal compartments of transfected cells using Percoll-based ultracentrifugation method in paper III.

Density gradient medium, silica colloid Percoll has often been used to separate different cell populations from tissues and organelles of cells (*188–190*). According to our knowledge, Percoll has not been used to separate endosomal fractions earlier. We fragmented the separated endosomes and verified early endosomes and late endosomes/lysosomes containing fractions with Western blot analysis using anti-Rab5 and anti-LAMP2 antibodies. Quantification of DNA content in these fractions using PCR provided information about CPP/pDNA complexes in endosomal compartments at certain time points.

3.9. QMCF technology

QMCF technology is a stable episomal expression system that uses mammalian cells and appropriate plasmids for expression of biologically active substances, e.g. proteins, recombinant antibodies, virus-like particles. Used plasmids contain mouse polyomavirus (Py) DNA replication origin and EBV EBNA-1 protein binding site to ensure the stable propagation of plasmids in the QMCF cells. Utilized cells are derived from CHO and U2OS cell lines and support stable maintenance and partitioning of QMCF plasmids by combination of two proteins EBV EBNA-1 and Py Large-T antigen. QMCF technology enables to generate expression cell banks within one week and stable protein production cell line in 6–8 months.

In paper II, NF51 was tested for the applicability as a transfection reagent in QMCF technology.

4. RESULTS AND DISCUSSION

Papers I and II in this thesis present the design and transfection efficacy studies of NickFects (NFs), the new family of cell-penetrating peptides. All members of NickFect family are based on the structure of stearylated TP10 peptide.

The earlier studies by group show that stearylation of TP10 peptide significantly improves the transfection of nucleic acids using non-covalent nanoparticle formation strategy (*50*). Stearyl-TP10, also known as PF3, was able to deliver SCO and pDNA into a large variety of cell lines, including hard-to-transfect mouse embryonic fibroblasts. Furthermore, it also showed potential as gene delivery vehicle for *in vivo* applications (*51*). Still, the efficacy of stearyl-TP10 in the presence of serum remained lower than that of the widely used transfection reagent LF2000. Besides that, co-transfection experiments with lysosomotropic agent CQ suggested partial endosomal entrapment of PF3/ON complexes yielding reduced biological activity. On that basis, we designed novel cell-penetrating peptides, named NickFects.

In paper III we perform a detailed analysis of the physico-chemical characteristics and intracellular trafficking of nanoparticles of two NFs with plasmid DNA.

4.1. NF1 and NF2 for the delivery of SCO (paper I)

The current study presents NickFect1 (NF1) and NickFect2 (NF2), novel phosphorylated analogues of stearyl-TP10.

In order to enhance the interactions of the peptide with the intracellular membrane and induce endosomal release, we incorporated the phosphoryl-group to the backbone of the original peptide. This modification was inspired by the fact that phospholipids are the main components of plasma membrane and that most liposomes formulations for nucleic acids transport contain phospholipid PE (*69*). Moreover, we replaced Ile to Thr at the 8th position of TP10 sequence. With the above-mentioned modifications we reduced the net positive charge and increased hydrophilicity of the peptide, obtaining pH-dependent vectors NF1, NF2. Furthermore, our study demonstrated that exclusive addition of phosphoryl-group only or Ile/Thr substitution in the backbone, resulted in p-PF3 or NF11 respectively that showed less efficacy *in vitro* than NF1 and NF2. On the other hand, NF3 peptide, which contains two phosphoryl-groups, formed less stable complexes with SCO probably due to repulsion and steric hindrance.

The SCO delivery efficacy of NFs was benchmarked to the transfection efficacy of the parental peptide stearyl-TP10 and the widely used commercial reagent LF2000 in Hela pLuc 705 cells. NF1 and NF2 showed significant improvement in splice correction compared to stearyl-TP10. Importantly, they surpassed the activity of LF2000 both in serum-free and serum-containing

medium. Furthermore, co-transfection of NF/SCO complexes with CQ demonstrated that the improvement in transfection efficacy was achieved due to the escape of the complexes from endosomal compartments.

In order to characterize the structure of NF/ON nanoparticles and to gain more insight into their internalization process, we performed ultrastructural analysis using TEM. Both NF1 and NF2 packed ON into dense and regular spherical particles. These particles were efficiently engulfed by cells, retained their dense packing inside endosomal vesicles, were able to translocate to cytosol where they dissociated into smaller assemblies. Moreover, delivered ONs were often detected close to the nuclear membrane and some of them in the nucleus. Unlike NickFects, stearyl-TP10 formed heterogenous linear rope-like assemblies with ON that associated with the cell surface and induced invaginations. A large number of the nanoparticles were entrapped in endosomal compartments and translocation to the cell nucleus was detected very rarely. Furthermore, stearyl-TP10/ON rope-like nanoparticles that were able to release from endosomes remained in place rather than dissociated into smaller particles. It is reasonable to speculate that NFs form less stable particles with ONs probably due to implemented negatively charged phosphoryl-groups in the backbone of the peptides. Our findings once more emphasize the importance of the shape and properties of CPP/ON nanoparticles.

DLS analysis showed that NF1 and NF2 condense SCO into nanoparticles with 300–500 nm size that, opposite to our expectations, had negative surface charge in reduced medium, i.e. in Opti-Mem. Subsequent papers from our group confirmed the importance of this finding and shed light to the intracellular internalization mechanisms of negatively charged nanoparticles (*101*).

The interaction of CPP with the cell membrane may also cause toxicity, which should be considered during the selection of novel potent CPP candidates (*10*). Albeit, NFs were able to deliver ONs with higher efficacy than LF2000, the high transfection efficacy was not associated with toxicity. On the other hand, LF2000 significantly reduced cell viability at concentrations suggested by the manufacturer (*191*).

Taken together, NF1 and NF2 are very promising vectors for ON delivery. As NF1 had a higher transfection efficacy in the presence of serum, it is plausible to suggest this peptide for further *in vivo* applications.

4.2. NF51 for the delivery of pDNA, siRNA, SCO and for protein production (paper II)

In paper II, we improved the complex formation activity, stability in the presence of serum and endosomolytic properties of stearyl-TP10. We modified the Lys7, which in rational design served as a linker between neuropeptide galanin motif and mastoparan residues in TP10 peptide sequence.

In order to enhance the transfection efficacy and serum stability of the NF/ON complex, the Lys7 was replaced with Orn, resulting in NF53. This modification was based on poly-L-ornithine´s higher ability to condense DNA compared to corresponding poly-L-lysine-based systems (*99*) and higher resistance of ornithine to serum proteases (*81*). The rationale behind the second modification was the higher transfection efficacy of ε-polylysine compared to α-polylysine. Authors of this earlier study suggest that the linkage of Lys residues via side chain ε-amino group created a more flexible backbone of the peptide. Nonetheless, the ε-peptide bond was more resistance to protease degradation (*175*). Relaying on that, we used side chain ε-amino group of Lys7 for subsequent synthesis to gain NF61. Thereafter, we implemented these above-mentioned modifications simultaneously. In NF51 we replaced Lys7 with ornithine and continued synthesis by coupling Gly to the δ-amino group of ornithine.

All the novel peptides, NF51, NF53 and NF61, condensed pDNA, SCO and siRNA into stable nanoparticles with less than 170 nm size in serum-containing medium, indicating that they should meet the size criteria for *in vivo* applications (*192*). Interestingly, all these nanoparticles had negative zeta potential in the presence of serum.

Our results evidenced that both introduced modifications were well-justified as all the novel peptides, NF51, NF53 and NF61 surpassed the efficacy of stearyl-TP10 in gene delivery efficacy. Remarkably high transfection level was achieved with NF51 that mediated pDNA delivery in all tested cell lines, including hard-to-transfect cells, such as mouse embryonic fibroblasts, and T and B lymphocytes. Moreover, in several cell lines gene expression obtained with NF51/pDNA nanoparticles exceeded the level achieved with LF2000. Furthermore, NF51 was able to fulfill an important prerequisite for efficient delivery vector, to transfect the whole cell population. Importantly, this criterion was not feasible for LF2000. Next we showed that besides gene transduction, NF51 is also applicable for RNA delivery. NF51 induced higher splice correction than LF2000 even in the presence of serum. Moreover, NF51 facilitated better siRNA delivery than commercial reagent LF RNAi MAX, resulting in target gene knockdown at low siRNA (25–50 nM) concentrations. Taken together, these results suggest that NF51 efficiently targets intracellular machineries both in the nucleus and in cytoplasm. Furthermore, NF51 was not toxic even in refractory cells, e.g. MEF and Jurkat.

Ultrastructural analysis of NF51/SCO trafficking by TEM and NF51/pDNA co-transfection experiments with CQ both evidenced the lysosomotropic properties of NF51.

Encouraged by these findings, we took our delivery system a step further by testing the applicability of NF51 in protein production system named QMCF. This system uses mammalian cells and appropriate plasmids for the expression of biologically active substances (i.e. recombinant proteins, recombinant antibodies, virus-like particles) and for the generation of cell-based assays for

screening of active compounds for drug development. The main advantage of QMCF technology is the possibility to generate the expression cell banks within 2–3 weeks after transfection. Transfection reagent for this system has to transfect the whole cell population without causing toxic effects to delicate suspension and adherent modified cells. Most chemical reagents that are on market today do not fulfil these requirements and so electroporation is commonly utilized to provide the most efficient transfection. NF51 effectively facilitated pDNA intracellular delivery and 7 days after single treatment almost whole cells population was transfected with the gene of interest. Our data corroborated the applicability of NF51 in QMCF technology as a safe and effective alternative to electroporation.

We may conclude that the insertion of a kink in the backbone of stearyl-TP10 has a significant impact on the complex formation and endosomolytic properties of the peptide. It has been suggested that the structural polymorphism and structural plasticity of the delivery vector determine the ability to interact with phospholipid membranes (*57, 58*). Furthermore, the introduction of flexible kink in amphipathic antimicrobial peptide Maximin allowed the adjustment of two helical segments according to local environment and caused changes in the properties of α-helical peptide (*193, 194*). It is plausible to speculate that the flexible-kink between galanin and mastoparan segments in NF51 sequence is highly important for complex formation and delivery properties.

Conclusively, NF51 is a highly efficient and versatile transfection agent for the intracellular delivery of nucleic acids using non-covalent strategy that has great potential in gene therapy and in protein production.

4.3. Uptake mechanism of NF1 and NF51 complexes with pDNA (paper III)

In the current work we characterized how radical modifications in stearyl-TP10 peptide, such as introducing a kink in the structure of NF51 or including extra negative charge by phospho-tyrosine substitution in NF1 influenced the nanoparticle formation, uptake mechanism and intracellular trafficking with regard to the biological activity of transfection.

In water, NF1 and NF51 formed nanoparticles of similar 60 nm size with plasmid DNA. For both peptides the nanoparticles obtained negative surface charge and remained stable in the presence of serum, although the size of the particles gradually increased, probably due to coating of them with serum proteins. However, NF51/pDNA nanoparticles were more resistant towards the heparin-induced decomplexation compared to NF1/pDNA nanoparticles. These differences in decomplexation were corraborated by confocal microscopy studies with nanoparticles from fluorescein-labeled CPP and rhodamine-labeled DNA. For NF1 fluorescence in both channels was separated to a larger degree

than for NF51 substantiating a higher degree of dissociation. Furthermore, for NF51, the maintenance of colocalization inside endosomal structures suggests trafficking into endosomal compartments that do not undergo acidification.

Recently, it was demonstrated that stearyl-TP10 analogue PF14 condenses SCO into nanoparticles that obtain negative surface charge in biorelevant conditions, e.g. the presence of serum proteins and/or isotonic NaCl concentrations. The negative charge of the particles suggested that they should repel from the cell surface, not interact as expected. Search for plausible cell surface receptors led to scavenger receptors that bind with low specificity polyanionic ligands and negatively charged particles. The authors demonstrated that scavenger receptors from type A, more precisely SCARA5 and SCARA3, mediate the uptake of PF14/SCO nanoparticles (*101*). Later, the involvement of these receptors was also shown in PF14-mediated gene delivery (*109*).

In order to elucidate whether negatively charged NF/ON nanoparticles also exploit scavenger receptors to mediate their intracellular uptake, we used well-known scavenger receptor ligands for blocking the receptor-binding sites or down regulated SCARA3 and SCARA5 receptors with specific siRNAs. Our data corroborated the high engagement of SCARA3 and SCARA5 receptors both in NF1- and NF51-mediated gene transduction. Furthermore, knocking down the two above-mentioned receptor subtypes reduced gene transfection by NF1 and NF51 to the level of 15% and 10% respectively from ordinary uptake, while in the case of PF14/SCO the subsequent down regulation resulted in a decrease up to 45%.

Transmembrane scavenger receptors, which bind various anionic ligands, have been previously shown to utilize different endocytotic pathways. Several studies demonstrate that multiple pathways are active simultaneously (*123, 126, 127*). Furthermore, it was recently reported that negatively charged silver nanoparticles in murine macrophage cells induced gradual clustering of the nanoparticles during the uptake and this clustering determines the internalization pathway. For instance, small nanoparticle clusters are taken up via clathrin-dependent but actin-independent pathway, whereas, the large clusters are engulfed in by actin-dependent pathway (*124*). Similarly, in our TEM study we discovered that NF51/pDNA complexes formed large conglomerates on the cell surface, which associated with membrane ruffles or localized in their close proximity, referring to macropinocytosis as the prevalent path of cell entry. On the other hand, NF1/pDNA complexes associated with the plasma membrane as smaller clusters, being simultaneously detected in close proximity to small membrane pits and membrane ruffles. These data was supported by the experiments with endocytosis inhibitors, suggesting macropinocytosis as the prevailing path of uptake for NF51/pDNA complexes. At the same time NF1/pDNA complexes were shown to utilize different endocytic pathways in parallel.

After budding from plasma membrane, endocytosed NF/pDNA nanoparticles are confined in endosomal compartments and the subsequent endo-

somal release is highly important to avoid their degradation in lysosomes. Co-transfection with CQ suggested that NF51/pDNA complexes can disrupt endosomal membranes releasing from the vesicles without the support of lysosomotropic agent, while large amount of NF1/pDNA complexes were entrapped in endosomal compartments.

In order to quantitatively characterize the amount of NF/pDNA nanoparticles in different endosomal-compartments, we followed the kinetics of trafficking through early and late endosomes using cell fractionation and a detection of recovered DNA by PCR. The obtained data confirmed lysosomotropic properties of NF51. Lower amount of NF51/pDNA nanoparticles compared to NF1/pDNA complexes reached LE/lysosomal compartments, although a totally large amount of NF51/pDNA nanoparticles was detected in EEs. To our knowledge, this approach has never been used to study CPPs endosomal release earlier. Consecutive studies utilizing Percoll gradient provides us quantitative information about CPP/ON nanoparticles intracellular trafficking.

Taken together, we suggest that NF1 and NF51 complexes are passing two different bottlenecks for efficient gene expression. High quantity of NF51/pDNA complexes rapidly enters the cells, being subsequently released from endosomes. Yet, after reaching cytosol densely condensed particles hamper pDNA unpacking and biological activity. On the other hand, the uptake of NF1/pDNA nanoparticles is slower and a large amount of them are entrapped in endosomal compartments. However, these particles are more loosely packed and pDNA is released while reached the target. As a result of these two limiting processes the gene transfection mediated by NF1 and NF51 is on the same level of efficacy. These findings enable further development of these vectors in order to achieve higher bioavailability of macromolecule-based drugs and higher potency in *in vivo* applications.

5. CONCLUSIONS

This thesis describes a new family of cell-penetrating peptides – NickFects that are applicable for the intracellular delivery of various nucleic acids using non-covalent strategy. The key findings of this thesis are listed below.

Paper I

- Insertion of phosphoryl group and substitution of Ile8 with Thr in stearyl-TP10 sequence resulted in highly efficient and non-toxic vectors NF1 and NF2 for intracellular delivery of splice-correcting oligonucleotides.
- NF1 and NF2 form stable nanoparticles with SCOs with negative surface charge in Opti-Mem environment.
- NF/ON nanoparticles are able to release from endosomal compartments and reach the nucleus, as confirmed by TEM analysis and gene expression.
- NF1- and NF2-mediated SCO transfection efficacy surpasses activity of LF2000 both in serum-free and serum-containing media.

Paper II

- Substitution of Lys7 with ornithine and continuing the synthesis through using the δ-amino group of ornithine side-chain resulted in highly active and non-toxic cell-penetrating peptide NF51, for efficient nucleic acids intracellular delivery.
- NF51 condenses pDNA, SCO and siRNA into stable nanoparticles less than 170 nm in size bearing negative surface charge in serum-containing medium.
- NF51 enables highly efficient gene expression, splice correction and gene silencing. The achieved transfection levels exceeded frequently the commercial reagents LF2000 and LF RNAi MAX. NF51 is capable of delivering pDNA into a large variety of cell lines, including refractory suspension and primary cells, e.g. MEF, Jurkat and A20 cells.
- Applied modifications in NF51 enhance the endosomolytic properties of the peptide.
- NF51 as a transfection agent is applicable in QMCF technology for the expression and production of recombinant proteins in hard-to-transfect suspension cell lines.

Paper III

- Dissimilar modifications in stearylated TP10, such as inserting a kink into the backbone or including extra negative charge by adding phosphoryl-group resulted in NF51 and NF1 respectively. Both peptides form similar low nanometer sized and negatively charged nanoparticles with pDNA exhibiting equally high gene transfection efficacy.
- Different stages at the transfection define the biological activity of NF51- and NF1-delivered pDNA. For NF51 the cargo release from the complex is

crucial for transfection efficacy, whereas for NF1 escape of the complexes from endosomal compartments is the main bottleneck in gene transfection

- Scavenger receptors SCARA3 and SCARA5 are highly engaged in the uptake of negatively charged NF1/pDNA and NF51/pDNA nanoparticles.
- NF51/pDNA nanoparticles are internalized by HeLa cells prevalently via macropinocytosis. NF1/pDNA complexes utilize several internalization routes simultaneously, i.e. CME, caveolae-mediated endocytosis and macropinocytosis.
- NF51 has superior lysosomotropic properties compared to NF1
- NF51 and NF1 are equally efficient delivery vectors for pDNA delivery exploiting different internalization mechanisms, which enables their utilization in different applications.

SUMMARY IN ESTONIAN

NickFecktid – uued rakku sisenevad peptiidid. Disain ja sisenemismehanism

Vajadus uute ja spetsiifilisemate ravimite järgi, kui hetkel kasutatavad madalmolekulaarsed ravimid, kasvab pidevalt. Nukleiinhapped ja nende analoogid omavad väga suurt potentsiaali paljude raskete haiguste raviks. Oma füsikokeemilistest omadustest tingituna ei ole nukleiinhapete molekulid võimelised iseseisvalt rakku sisenema ja vajavad terapeutilise toimekohani jõudmiseks transportvektorite abi. Viirustel põhinevad vektorid on väga efektiivsed, kuid nende kasutamisel võivad ilmneda mitmed kõrvaltoimed nagu immunoloogiline reakstioon või transporditava DNA insertsioon valesse genoomi piirkonda. Sellest tulenevalt keskendutakse üha enam mitteviiruslike vektorite arendamisele.

Rakku sisenevad peptiidid (RSPd) on üks grupp mitteviiruslikke vektoreid, mida on laialdaselt kasutatud nukleiinhapete rakku viimiseks nii *in vitro* kui ka *in vivo* tingimustes. RSPd on lühikesed, 5–30 aminohappe jäägist koosnevad katioonsed ja/või amfipaatsed peptiidid, mis on võimelised sisenema rakku ja transportima sinna ka endaga seotud lastmolekuli. Lastmolekuli sidumiseks on peamiselt kaks moodust: kovalentne, kus lastmolekul seotakse RSPga kovalentse sideme kaudu ja mitte-kovalentne, kus kasutatakse elektrostaatilisi ja hüdrofoobseid interaktsioone. Antud töös kasutati mitte-kovalentset sidumise strateegiat, kus lastmolekulidest ja RSPdest moodustati nanopartiklid. Kuigi mitte-kovalentne sidumisstrateegia on palju lihtsam ja odavam kui kovalentne strateegia, on selle peamiseks puuduseks moodustunud nanopartiklite iseloomustamise keerukus. Seetõttu pöörati antud töös erilist tähelepanu RSP-nukleiinhapete komplekside omadustele. Moodustunud kompleksid sisenevad rakku enamasti endotsütoosi teel, millest tulenevalton suur tõenäosus jääda kinni endotsütootilistesse vesiikulitesse. See omakorda vähendab oluliselt lastmolekulide bioloogilist aktiivsust, sest lastmolekulide sihtmärgid asuvad kas rakutuumas või tsütoplasmas. RSPde kui transportvektorite kasutamisel on väga oluline tagada RSP/lastmolekuli komplekside vabanemine endotsütootilistest vesiikulitest ning lastmolekuli jõudmine oma sihtmärgini rakus.

Käesoleva töö peamiseks eesmärgiks oli disainida uued efektiivsed peptiidsed vektorid, NickFectid (NF), erinevate nukleiinhapete transpordiks rakku. Uute vektorite disainil lähtuti stearüleeritud TP10 peptiidist. Disaini eesmärgiks oli parandada peptiidse vektori võimet moodustada komplekse lastmolekuliga, suurendada komplekside sisenemist rakkudesse ja vabanemist endostütootilistest vesiikulitest.

Esimeses töös kirjeldame NF1 ja NF2. Disanil aluseks võetud peptiidi järjestusse lisame fosforüülrühma ja hüdrofoobsuse muutmiseks asendame isoleutsiin treoniiniga. Nimetatud modifikatsioonid parandavad olulisest RSP võimet transportida rakkudesse splaissingut-korrigeerivaid oligonukleotiide.

Teises töös modifitseerime TP10 koostisosi, mastoparaani ja galaniini fraktsioone siduvat ühenduslüli ning disainime „kõvera“ peptiid NF51. Saadud peptiid transpordib rakkudesse nii plasmiidset DNAd, splaissingut-korrigeerivaid oligonukleotiide kui ka väikseid interfeeruvaid RNAsid. Lisaks näitame, et NF51 on kasutatav transfetsiooni reagendina valkude tootmises, antud juhul kasutades QMCF tehnoloogiat.

Kolmandas töös uurime kahe erineva RSP, NF1 ja NF51 omadusi. Näitame, et kuigi käesolevad peptiidid omavad erinevat keemilist struktuuri on nende võime transportida rakku plasmiidset DNAd sarnane. Võrdleme nende peptiidide ja plasmiidse DNA komplekside omadusi, interaktsiooni rakupinnaga, rakku sisenemiseks kasutatavaid endotsütoosi radasid ning rakusisest liikumist.

Kokkuvõttes on antud töös disainitud peptiidid, NickFectid, on vähe toksilised, sobivad nukleiinhapete transpordiks ja omavad head perspektiivi nii geeniteraapias kui valkude tootmises.

REFERENCES

(1) Kole, R., Krainer, A. R., and Altman, S. (2012) RNA therapeutics: beyond RNA interference and antisense oligonucleotides. Nat Rev Drug Discov 11, 125–40.

(2) Shin, M. C., Zhang, J., Min, K. A., Lee, K., Byun, Y., David, A. E., He, H., and Yang, V. C. (2013) Cell-penetrating peptides: Achievements and challenges in application for cancer treatment. J Biomed Mater Res A.

(3) Heitz, F., Morris, M. C., and Divita, G. (2009) Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br J Pharmacol 157, 195–206.

(4) Jang, Y. L., Yun, U. J., Lee, M. S., Kim, M. G., Son, S., Lee, K., Chae, S. Y., Lim, D. W., Kim, H. T., Kim, S. H., and Jeong, J. H. (2012) Cell-penetrating peptide mimicking polymer-based combined delivery of paclitaxel and siRNA for enhanced tumor growth suppression. Int J Pharm 434, 488–93.

(5) Alhakamy, N. A., Nigatu, A. S., Berkland, C. J., and Ramsey, J. D. (2013) Non-covalently associated cell-penetrating peptides for gene delivery applications. Ther Deliv 4, 741–57.

(6) Sazani, P., and Kole, R. (2003) Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. J Clin Invest 112, 481–6.

(7) Koren, E., and Torchilin, V. P. (2012) Cell-penetrating peptides: breaking through to the other side. Trends Mol Med 18, 385–93.

(8) Margus, H., Padari, K., and Pooga, M. (2012) Cell-penetrating peptides as versatile vehicles for oligonucleotide delivery. Mol Ther 20, 525–33.

(9) Sibley, C. R., Seow, Y., and Wood, M. J. (2010) Novel RNA-based strategies for therapeutic gene silencing. Mol Ther 18, 466–76.

(10) Lee, S. H., Castagner, B., and Leroux, J. C. (2013) Is there a future for cell-penetrating peptides in oligonucleotide delivery? Eur J Pharm Biopharm 85, 5–11.

(11) Herzog, R. W., Cao, O., and Srivastava, A. (2010) Two decades of clinical gene therapy – success is finally mounting. Discov Med 9, 105–11.

(12) Kay, M. A. (2011) State-of-the-art gene-based therapies: the road ahead. Nat Rev Genet 12, 316–28.

(13) Pack, D. W., Hoffman, A. S., Pun, S., and Stayton, P. S. (2005) Design and development of polymers for gene delivery. Nat Rev Drug Discov 4, 581–93.

(14) Brasseur, R., and Divita, G. (2010) Happy birthday cell penetrating peptides: already 20 years. Biochim Biophys Acta 1798, 2177–81.

(15) Frankel, A. D., and Pabo, C. O. (1988) Cellular uptake of the tat protein from human immunodeficiency virus. Cell 55, 1189–93.

(16) Green, M., and Loewenstein, P. M. (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell 55, 1179–88.

(17) Joliot, A. H., Triller, A., Volovitch, M., Pernelle, C., and Prochiantz, A. (1991) alpha-2,8-Polysialic acid is the neuronal surface receptor of antennapedia homeobox peptide. New Biol 3, 1121–34.

(18) Vives, E., Brodin, P., and Lebleu, B. (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 272, 16010–7.

(19) Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 269, 10444–50.
(20) Elmquist, A., Lindgren, M., Bartfai, T., and Langel, Ü. (2001) VE-cadherin-derived cell-penetrating peptide, pVEC, with carrier functions. Exp Cell Res 269, 237–44.
(21) Pooga, M., Hällbrink, M., Zorko, M., and Langel, Ü. (1998) Cell penetration by transportan. FASEB J 12, 67–77.
(22) Soomets, U., Lindgren, M., Gallet, X., Hällbrink, M., Elmquist, A., Balaspiri, L., Zorko, M., Pooga, M., Brasseur, R., and Langel, Ü. (2000) Deletion analogues of transportan. Biochim Biophys Acta 1467, 165–76.
(23) Morris, M. C., Vidal, P., Chaloin, L., Heitz, F., and Divita, G. (1997) A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. Nucleic Acids Res 25, 2730–6.
(24) Morris, M. C., Depollier, J., Mery, J., Heitz, F., and Divita, G. (2001) A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol 19, 1173–6.
(25) Oehlke, J., Scheller, A., Wiesner, B., Krause, E., Beyermann, M., Klauschenz, E., Melzig, M., and Bienert, M. (1998) Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically. Biochim Biophys Acta 1414, 127–39.
(26) Mitchell, D. J., Kim, D. T., Steinman, L., Fathman, C. G., and Rothbard, J. B. (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res 56, 318–25.
(27) Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J Biol Chem 276, 5836–40.
(28) Crombez, L., Aldrian-Herrada, G., Konate, K., Nguyen, Q. N., McMaster, G. K., Brasseur, R., Heitz, F., and Divita, G. (2009) A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. Mol Ther 17, 95–103.
(29) Lindgren, M., and Langel, Ü. (2011) Classes and prediction of cell-penetrating peptides. Methods Mol Biol 683, 3–19.
(30) Bechara, C., and Sagan, S. (2013) Cell-penetrating peptides: 20 years later, where do we stand? FEBS Lett 587, 1693–702.
(31) Yamada, T., Das Gupta, T. K., and Beattie, C. W. (2013) p28, an Anionic Cell-Penetrating Peptide, Increases the Activity of Wild Type and Mutated p53 without Altering Its Conformation. Mol Pharm.
(32) Hassane, F. S., Abes, R., El Andaloussi, S., Lehto, T., Sillard, R., Langel, Ü., and Lebleu, B. (2011) Insights into the cellular trafficking of splice redirecting oligonucleotides complexed with chemically modified cell-penetrating peptides. J Control Release 153, 163–72.
(33) Milletti, F. (2012) Cell-penetrating peptides: classes, origin, and current landscape. Drug Discov Today 17, 850–60.
(34) Tünnemann, G., Ter-Avetisyan, G., Martin, R. M., Stockl, M., Herrmann, A., and Cardoso, M. C. (2008) Live-cell analysis of cell penetration ability and toxicity of oligo-arginines. J Pept Sci 14, 469–76.

(35) Ziegler, A. (2008) Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans. Adv Drug Deliv Rev 60, 580–97.
(36) Gomez, J. A., Chen, J., Ngo, J., Hajkova, D., Yeh, I. J., Gama, V., Miyagi, M., and Matsuyama, S. (2010) Cell-Penetrating Penta-Peptides (CPP5s): Measurement of Cell Entry and Protein-Transduction Activity. Pharmaceuticals (Basel) 3, 3594–3613.
(37) Kubo, S., Ishiki, T., Doe, I., Sekiguchi, F., Nishikawa, H., Kawai, K., Matsui, H., and Kawabata, A. (2006) Distinct activity of peptide mimetic intracellular ligands (pepducins) for proteinase-activated receptor-1 in multiple cells/tissues. Ann N Y Acad Sci 1091, 445–59.
(38) Stalmans, S., Wynendaele, E., Bracke, N., Gevaert, B., D'Hondt, M., Peremans, K., Burvenich, C., and De Spiegeleer, B. (2013) Chemical-functional diversity in cell-penetrating peptides. PLoS One 8, e71752.
(39) Nakase, I., Tanaka, G., and Futaki, S. (2013) Cell-penetrating peptides (CPPs) as a vector for the delivery of siRNAs into cells. Mol Biosyst 9, 855–61.
(40) Wender, P. A., Rothbard, J. B., Jessop, T. C., Kreider, E. L., and Wylie, B. L. (2002) Oligocarbamate molecular transporters: design, synthesis, and biological evaluation of a new class of transporters for drug delivery. J Am Chem Soc 124, 13382–3.
(41) Jafari, M., Karunaratne, D. N., Sweeting, C. M., and Chen, P. (2013) Modification of a Designed Amphipathic Cell-Penetrating Peptide and Its Effect on Solubility, Secondary Structure, and Uptake Efficiency. Biochemistry.
(42) Elmquist, A., Hansen, M., and Langel, Ü. (2006) Structure-activity relationship study of the cell-penetrating peptide pVEC. Biochim Biophys Acta 1758, 721–9.
(43) Pujals, S., Fernandez-Carneado, J., Lopez-Iglesias, C., Kogan, M. J., and Giralt, E. (2006) Mechanistic aspects of CPP-mediated intracellular drug delivery: relevance of CPP self-assembly. Biochim Biophys Acta 1758, 264–79.
(44) Chan, D. I., Prenner, E. J., and Vogel, H. J. (2006) Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. Biochim Biophys Acta 1758, 1184–202.
(45) Walrant, A., Correia, I., Jiao, C. Y., Lequin, O., Bent, E. H., Goasdoue, N., Lacombe, C., Chassaing, G., Sagan, S., and Alves, I. D. (2011) Different membrane behaviour and cellular uptake of three basic arginine-rich peptides. Biochim Biophys Acta 1808, 382–93.
(46) Rydberg, H. A., Matson, M., Amand, H. L., Esbjörner, E. K., and Norden, B. (2012) Effects of tryptophan content and backbone spacing on the uptake efficiency of cell-penetrating peptides. Biochemistry 51, 5531–9.
(47) Futaki, S., Ohashi, W., Suzuki, T., Niwa, M., Tanaka, S., Ueda, K., Harashima, H., and Sugiura, Y. (2001) Stearylated arginine-rich peptides: a new class of transfection systems. Bioconjug Chem 12, 1005–11.
(48) Wang, H. Y., Chen, J. X., Sun, Y. X., Deng, J. Z., Li, C., Zhang, X. Z., and Zhuo, R. X. (2010) Construction of cell penetrating peptide vectors with N-terminal stearylated nuclear localization signal for targeted delivery of DNA into the cell nuclei. J Control Release 155, 26–33.
(49) Kim, W. J., Christensen, L. V., Jo, S., Yockman, J. W., Jeong, J. H., Kim, Y. H., and Kim, S. W. (2006) Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. Mol Ther 14, 343–50.

(50) Mäe, M., El Andaloussi, S., Lundin, P., Oskolkov, N., Johansson, H. J., Guterstam, P., and Langel, Ü. (2009) A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy. J Control Release 134, 221–227.
(51) Lehto, T., Simonson, O. E., Mäger, I., Ezzat, K., Sork, H., Copolovici, D. M., Viola, J. R., Zaghloul, E. M., Lundin, P., Moreno, P. M., Mäe, M., Oskolkov, N., Suhorutšenko, J., Smith, C. I., and El Andaloussi, S. E. (2011) A peptide-based vector for efficient gene transfer in vitro and in vivo. Mol Ther 19, 1457–67.
(52) El Andaloussi, S., Lehto, T., Mäger, I., Rosenthal-Aizman, K., Oprea, II, Simonson, O. E., Sork, H., Ezzat, K., Copolovici, D. M., Kurrikoff, K., Viola, J. R., Zaghloul, E. M., Sillard, R., Johansson, H. J., Said Hassane, F., Guterstam, P., Suhorutsenko, J., Moreno, P. M., Oskolkov, N., Halldin, J., Tedebark, U., Metspalu, A., Lebleu, B., Lehtio, J., Smith, C. I., and Langel, Ü. (2011) Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. Nucleic Acids Res 39, 3972–3987.
(53) Ezzat, K., El Andaloussi, S., Zaghloul, E. M., Lehto, T., Lindberg, S., Moreno, P. M., Viola, J. R., Magdy, T., Abdo, R., Guterstam, P., Sillard, R., Hammond, S. M., Wood, M. J., Arzumanov, A. A., Gait, M. J., Smith, C. I., Hällbrink, M., and Langel, Ü. (2011) PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. Nucleic Acids Res 39, 5284–98.
(54) Lindberg, S., Munoz-Alarcon, A., Helmfors, H., Mosqueira, D., Gyllborg, D., Tudoran, O., and Langel, Ü. (2012) PepFect15, a novel endosomolytic cell-penetrating peptide for oligonucleotide delivery via scavenger receptors. Int J Pharm 441, 242–7.
(55) Arukuusk, P., Pärnaste, L., Oskolkov, N., Copolovici, D. M., Margus, H., Padari, K., Möll, K., Maslovskaja, J., Tegova, R., Kivi, G., Tover, A., Pooga, M., Ustav, M., and Langel, Ü. (2013) New generation of efficient peptide-based vectors, NickFects, for the delivery of nucleic acids. Biochim Biophys Acta 1828, 1365–73.
(56) Oskolkov, N., Arukuusk, P., Copolovici, D. M., Lindberg, S., Margus, H., Padari, K., Pooga, M., and Langel, Ü. (2011) NickFects, phosphorylated derivatives of Transportan 10 for cellular delivery of oligonucleotides. Int J Pept Res Ther 17, 147–157.
(57) Deshayes, S., Konate, K., Aldrian, G., Crombez, L., Heitz, F., and Divita, G. (2010) Structural polymorphism of non-covalent peptide-based delivery systems: highway to cellular uptake. Biochim Biophys Acta 1798, 2304–14.
(58) Eiriksdottir, E., Konate, K., Langel, U., Divita, G., and Deshayes, S. (2010) Secondary structure of cell-penetrating peptides controls membrane interaction and insertion. Biochim Biophys Acta 1798, 1119–28.
(59) Lundin, P., Johansson, H., Guterstam, P., Holm, T., Hansen, M., Langel, Ü., and El Andaloussi, S. (2008) Distinct uptake routes of cell-penetrating peptide conjugates. Bioconjug Chem 19, 2535–42.
(60) Varkouhi, A. K., Scholte, M., Storm, G., and Haisma, H. J. (2011) Endosomal escape pathways for delivery of biologicals. J Control Release 151, 220–8.
(61) Erbacher, P., Roche, A. C., Monsigny, M., and Midoux, P. (1996) Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/ lactosylated polylysine complexes. Exp Cell Res 225, 186–94.

(62) Wolf, Y., Pritz, S., Abes, S., Bienert, M., Lebleu, B., and Oehlke, J. (2006) Structural requirements for cellular uptake and antisense activity of peptide nucleic acids conjugated with various peptides. Biochemistry 45, 14944–54.
(63) Shiraishi, T., Pankratova, S., and Nielsen, P. E. (2005) Calcium ions effectively enhance the effect of antisense peptide nucleic acids conjugated to cationic tat and oligoarginine peptides. Chem Biol 12, 923–9.
(64) Veldhoen, S., Laufer, S. D., Trampe, A., and Restle, T. (2006) Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect. Nucleic Acids Res 34, 6561–73.
(65) Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. J Biol Chem 269, 12918–24.
(66) Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med 10, 310–5.
(67) Nakase, I., Kobayashi, S., and Futaki, S. (2010) Endosome-disruptive peptides for improving cytosolic delivery of bioactive macromolecules. Biopolymers 94, 763–70.
(68) Li, W., Nicol, F., and Szoka, F. C., Jr. (2004) GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. Adv Drug Deliv Rev 56, 967–85.
(69) Fattal, E., Couvreur, P., and Dubernet, C. (2004) "Smart" delivery of antisense oligonucleotides by anionic pH-sensitive liposomes. Adv Drug Deliv Rev 56, 931–46.
(70) Kichler, A., Leborgne, C., Danos, O., and Bechinger, B. (2007) Characterization of the gene transfer process mediated by histidine-rich peptides. J Mol Med (Berl) 85, 191–201.
(71) Hatefi, A., Megeed, Z., and Ghandehari, H. (2006) Recombinant polymer-protein fusion: a promising approach towards efficient and targeted gene delivery. J Gene Med 8, 468–76.
(72) Zhang, Q., Tang, J., Fu, L., Ran, R., Liu, Y., Yuan, M., and He, Q. (2013) A pH-responsive alpha-helical cell penetrating peptide-mediated liposomal delivery system. Biomaterials 34, 7980–93.
(73) Lo, S. L., and Wang, S. (2008) An endosomolytic Tat peptide produced by incorporation of histidine and cysteine residues as a nonviral vector for DNA transfection. Biomaterials 29, 2408–14.
(74) Yoshida, T., Lai, T. C., Kwon, G. S., and Sako, K. (2013) pH- and ion-sensitive polymers for drug delivery. Expert Opin Drug Deliv.
(75) Liu, B. R., Lin, M. D., Chiang, H. J., and Lee, H. J. (2012) Arginine-rich cell-penetrating peptides deliver gene into living human cells. Gene 505, 37–45.
(76) Hart, S. L. (1999) Integrin-mediated vectors for gene transfer and therapy. Curr Opin Mol Ther 1, 197–203.
(77) Oliveira, S., Fretz, M. M., Hogset, A., Storm, G., and Schiffelers, R. M. (2007) Photochemical internalization enhances silencing of epidermal growth factor receptor through improved endosomal escape of siRNA. Biochim Biophys Acta 1768, 1211–7.
(78) Endoh, T., and Ohtsuki, T. (2009) Cellular siRNA delivery using cell-penetrating peptides modified for endosomal escape. Adv Drug Deliv Rev 61, 704–9.

(79) Matsushita, M., Noguchi, H., Lu, Y. F., Tomizawa, K., Michiue, H., Li, S. T., Hirose, K., Bonner-Weir, S., and Matsui, H. (2004) Photo-acceleration of protein release from endosome in the protein transduction system. FEBS Lett 572, 221–6.
(80) Khalil, I. A., Futaki, S., Niwa, M., Baba, Y., Kaji, N., Kamiya, H., and Harashima, H. (2004) Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. Gene Ther 11, 636–44.
(81) Ezzat, K., El Andaloussi, S., Zaghloul, E. M., Lehto, T., Lindberg, S., Moreno, P. M., Viola, J. R., Magdy, T., Abdo, R., Guterstam, P., Sillard, R., Hammond, S. M., Wood, M. J., Arzumanov, A. A., Gait, M. J., Smith, C. I., Hällbrink, M., and Langel, Ü. (2011) PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. Nucleic Acids Res.
(82) Simeoni, F., Morris, M. C., Heitz, F., and Divita, G. (2003) Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. Nucleic Acids Res 31, 2717–24.
(83) Crombez, L., Morris, M. C., Dufort, S., Aldrian-Herrada, G., Nguyen, Q., Mc Master, G., Coll, J. L., Heitz, F., and Divita, G. (2009) Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. Nucleic Acids Res 37, 4559–69.
(84) Morris, M. C., Chaloin, L., Mery, J., Heitz, F., and Divita, G. (1999) A novel potent strategy for gene delivery using a single peptide vector as a carrier. Nucleic Acids Res 27, 3510–7.
(85) Tonges, L., Lingor, P., Egle, R., Dietz, G. P., Fahr, A., and Bahr, M. (2006) Stearylated octaarginine and artificial virus-like particles for transfection of siRNA into primary rat neurons. RNA 12, 1431–8.
(86) Lehto, T., Abes, R., Oskolkov, N., Suhorutsenko, J., Copolovici, D. M., Mäger, I., Viola, J. R., Simonson, O. E., Ezzat, K., Guterstam, P., Eriste, E., Smith, C. I., Lebleu, B., Samir El, A., and Langel, Ü. (2010) Delivery of nucleic acids with a stearylated (RxR)4 peptide using a non-covalent co-incubation strategy. J Control Release 141, 42–51.
(87) Wang, H.-Y., Chen, J.-X., Sun, Y.-X., Deng, J.-Z., Li, C., Zhang, X.-Z., and Zhuo, R.-X. (2010) Construction of cell penetrating peptide vectors with N-terminal stearylated nuclear localization signal for targeted delivery of DNA into the cell nuclei. J Control Release In Press, Corrected Proof.
(88) Ezzat, K., Zaghloul, E. M., El Andaloussi, S., Lehto, T., El-Sayed, R., Magdy, T., Smith, C. I., and Langel, Ü. (2013) Solid formulation of cell-penetrating peptide nanocomplexes with siRNA and their stability in simulated gastric conditions. J Control Release 162, 1–8.
(89) Arukuusk, P., Pärnaste, L., Margus, H., Eriksson, J., Vasconcelos, L., Padari, K., Pooga, M., and Langel, Ü. (2013) Differential endosomal pathways for radically modified peptide vectors. Bioconjug Chem.
(90) Lehto, T., Kurrikoff, K., and Langel, Ü. (2012) Cell-penetrating peptides for the delivery of nucleic acids. Expert Opin Drug Deliv 9, 823–36.
(91) Viola, J. R., El-Andaloussi, S., Oprea, II, and Smith, C. I. (2010) Non-viral nanovectors for gene delivery: factors that govern successful therapeutics. Expert Opin Drug Deliv 7, 721–35.

(92) Scholz, C., and Wagner, E. (2011) Therapeutic plasmid DNA versus siRNA delivery: common and different tasks for synthetic carriers. J Control Release 161, 554–65.
(93) Patel, M. M., and Anchordoquy, T. J. (2005) Contribution of hydrophobicity to thermodynamics of ligand-DNA binding and DNA collapse. Biophys J 88, 2089–103.
(94) Ogris, M., Steinlein, P., Carotta, S., Brunner, S., and Wagner, E. (2001) DNA/ polyethylenimine transfection particles: influence of ligands, polymer size, and PEGylation on internalization and gene expression. AAPS PharmSci 3, E21.
(95) Maeda, H. (2010) Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. Bioconjug Chem 21, 797–802.
(96) Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., and Wagner, E. (1998) The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. Gene Ther 5, 1425–33.
(97) van Asbeck, A. H., Beyerle, A., McNeill, H., Bovee-Geurts, P. H., Lindberg, S., Verdurmen, W. P., Hallbrink, M., Langel, U., Heidenreich, O., and Brock, R. (2013) Molecular parameters of siRNA – cell penetrating peptide nanocomplexes for efficient cellular delivery. ACS Nano 7, 3797–807.
(98) Kwok, A., and Hart, S. L. (2010) Comparative structural and functional studies of nanoparticle formulations for DNA and siRNA delivery. Nanomedicine 7, 210–9.
(99) Ramsay, E., and Gumbleton, M. (2002) Polylysine and polyornithine gene transfer complexes: a study of complex stability and cellular uptake as a basis for their differential in-vitro transfection efficiency. J Drug Target 10, 1–9.
(100) Ramsay, E., Hadgraft, J., Birchall, J., and Gumbleton, M. (2000) Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection in-vitro. Int J Pharm 210, 97–107.
(101) Ezzat, K., Helmfors, H., Tudoran, O., Juks, C., Lindberg, S., Padari, K., El Andaloussi, S., Pooga, M., and Langel, Ü. (2012) Scavenger receptor-mediated uptake of cell-penetrating peptide nanocomplexes with oligonucleotides. Faseb J 26, 1172–80.
(102) Cahill, K. (2009) Molecular electroporation and the transduction of oligo-arginines. Phys Biol 7, 16001.
(103) Nakase, I., Akita, H., Kogure, K., Gräslund, A., Langel, Ü., Harashima, H., and Futaki, S. (2011) Efficient intracellular delivery of nucleic acid pharmaceuticals using cell-penetrating peptides. Acc Chem Res 45, 1132–9.
(104) Gump, J. M., and Dowdy, S. F. (2007) TAT transduction: the molecular mechanism and therapeutic prospects. Trends Mol Med 13, 443–8.
(105) Nakase, I., Niwa, M., Takeuchi, T., Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S., Ueda, K., Simpson, J. C., Jones, A. T., Sugiura, Y., and Futaki, S. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. Mol Ther 10, 1011–22.
(106) Imamura, J., Suzuki, Y., Gonda, K., Roy, C. N., Gatanaga, H., Ohuchi, N., and Higuchi, H. (2011) Single particle tracking confirms that multivalent Tat protein transduction domain-induced heparan sulfate proteoglycan cross-linkage activates Rac1 for internalization. J Biol Chem 286, 10581–92.

(107) Gump, J. M., June, R. K., and Dowdy, S. F. (2010) Revised role of glycosaminoglycans in TAT protein transduction domain-mediated cellular transduction. J Biol Chem 285, 1500–7.
(108) Tanaka, G., Nakase, I., Fukuda, Y., Masuda, R., Oishi, S., Shimura, K., Kawaguchi, Y., Takatani-Nakase, T., Langel, Ü., Gräslund, A., Okawa, K., Matsuoka, M., Fujii, N., Hatanaka, Y., and Futaki, S. (2012) CXCR4 stimulates macropinocytosis: implications for cellular uptake of arginine-rich cell-penetrating peptides and HIV. Chem Biol 19, 1437–46.
(109) Veiman, K. L., Mäger, I., Ezzat, K., Margus, H., Lehto, T., Langel, K., Kurrikoff, K., Arukuusk, P., Suhorutsenko, J., Padari, K., Pooga, M., Lehto, T., and Langel, Ü. (2012) PepFect14 peptide vector for efficient gene delivery in cell cultures. Mol Pharm 10, 199–210.
(110) Brown, M. S., Goldstein, J. L., Krieger, M., Ho, Y. K., and Anderson, R. G. (1979) Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. J Cell Biol 82, 597–613.
(111) Plüddemann, A., Neyen, C., and Gordon, S. (2007) Macrophage scavenger receptors and host-derived ligands. Methods 43, 207–17.
(112) Stephen, S. L., Freestone, K., Dunn, S., Twigg, M. W., Homer-Vanniasinkam, S., Walker, J. H., Wheatcroft, S. B., and Ponnambalam, S. (2010) Scavenger receptors and their potential as therapeutic targets in the treatment of cardiovascular disease. Int J Hypertens 2010, 646929.
(113) Peiser, L., and Gordon, S. (2001) The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. Microbes Infect 3, 149–59.
(114) DeWitte-Orr, S. J., Collins, S. E., Bauer, C. M., Bowdish, D. M., and Mossman, K. L. (2010) An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. PLoS Pathog 6, e1000829.
(115) Murphy, J. E., Tedbury, P. R., Homer-Vanniasinkam, S., Walker, J. H., and Ponnambalam, S. (2005) Biochemistry and cell biology of mammalian scavenger receptors. Atherosclerosis 182, 1–15.
(116) Jiang, Y., Oliver, P., Davies, K. E., and Platt, N. (2006) Identification and characterization of murine SCARA5, a novel class A scavenger receptor that is expressed by populations of epithelial cells. J Biol Chem 281, 11834–45.
(117) Platt, N., and Gordon, S. (2001) Is the class A macrophage scavenger receptor (SR-A) multifunctional? – The mouse's tale. J Clin Invest 108, 649–54.
(118) Han, H. J., Tokino, T., and Nakamura, Y. (1998) CSR, a scavenger receptor-like protein with a protective role against cellular damage causedby UV irradiation and oxidative stress. Hum Mol Genet 7, 1039–46.
(119) Bowdish, D. M., and Gordon, S. (2009) Conserved domains of the class A scavenger receptors: evolution and function. Immunol Rev 227, 19–31.
(120) Martinez, V. G., Moestrup, S. K., Holmskov, U., Mollenhauer, J., and Lozano, F. (2011) The conserved scavenger receptor cysteine-rich superfamily in therapy and diagnosis. Pharmacol Rev 63, 967–1000.
(121) Pearson, A. M., Rich, A., and Krieger, M. (1993) Polynucleotide binding to macrophage scavenger receptors depends on the formation of base-quartet-stabilized four-stranded helices. J Biol Chem 268, 3546–54.

(122) Patel, P. C., Giljohann, D. A., Daniel, W. L., Zheng, D., Prigodich, A. E., and Mirkin, C. A. (2010) Scavenger receptors mediate cellular uptake of polyvalent oligonucleotide-functionalized gold nanoparticles. Bioconjug Chem 21, 2250–6.
(123) Lunov, O., Zablotskii, V., Syrovets, T., Rocker, C., Tron, K., Nienhaus, G. U., and Simmet, T. (2010) Modeling receptor-mediated endocytosis of polymer-functionalized iron oxide nanoparticles by human macrophages. Biomaterials 32, 547–55.
(124) Wang, H., Wu, L., and Reinhard, B. M. (2012) Scavenger receptor mediated endocytosis of silver nanoparticles into J774A.1 macrophages is heterogeneous. ACS Nano 6, 7122–32.
(125) Kanno, S., Furuyama, A., and Hirano, S. (2007) A murine scavenger receptor MARCO recognizes polystyrene nanoparticles. Toxicol Sci 97, 398–406.
(126) Zhang, L. W., Yang, J., Barron, A. R., and Monteiro-Riviere, N. A. (2009) Endocytic mechanisms and toxicity of a functionalized fullerene in human cells. Toxicol Lett 191, 149–57.
(127) Zhang, L. W., and Monteiro-Riviere, N. A. (2009) Mechanisms of quantum dot nanoparticle cellular uptake. Toxicol Sci 110, 138–55.
(128) Doherty, G. J., and McMahon, H. T. (2009) Mechanisms of endocytosis. Annu Rev Biochem 78, 857–902.
(129) Hurley, J. H., Boura, E., Carlson, L. A., and Rozycki, B. (2010) Membrane budding. Cell 143, 875–87.
(130) Conner, S. D., and Schmid, S. L. (2003) Regulated portals of entry into the cell. Nature 422, 37–44.
(131) Khalil, I. A., Kogure, K., Akita, H., and Harashima, H. (2006) Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. Pharmacol Rev 58, 32–45.
(132) Takei, K., and Haucke, V. (2001) Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol 11, 385–91.
(133) Ehrlich, M., Boll, W., Van Oijen, A., Hariharan, R., Chandran, K., Nibert, M. L., and Kirchhausen, T. (2004) Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell 118, 591–605.
(134) Sandvig, K., Torgersen, M. L., Raa, H. A., and van Deurs, B. (2008) Clathrin-independent endocytosis: from nonexisting to an extreme degree of complexity. Histochem Cell Biol 129, 267–76.
(135) Harris, J., Werling, D., Hope, J. C., Taylor, G., and Howard, C. J. (2002) Caveolae and caveolin in immune cells: distribution and functions. Trends Immunol 23, 158–64.
(136) Tagawa, A., Mezzacasa, A., Hayer, A., Longatti, A., Pelkmans, L., and Helenius, A. (2005) Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. J Cell Biol 170, 769–79.
(137) Lingwood, D., and Simons, K. (2010) Lipid rafts as a membrane-organizing principle. Science 327, 46–50.
(138) Parton, R. G., Hanzal-Bayer, M., and Hancock, J. F. (2006) Biogenesis of caveolae: a structural model for caveolin-induced domain formation. J Cell Sci 119, 787–96.
(139) Pelkmans, L., and Zerial, M. (2005) Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. Nature 436, 128–33.
(140) Lamaze, C., and Schmid, S. L. (1995) The emergence of clathrin-independent pinocytic pathways. Curr Opin Cell Biol 7, 573–80.

(141) Ferrari, A., Pellegrini, V., Arcangeli, C., Fittipaldi, A., Giacca, M., and Beltram, F. (2003) Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. Mol Ther 8, 284–94.
(142) Amyere, M., Mettlen, M., Van Der Smissen, P., Platek, A., Payrastre, B., Veithen, A., and Courtoy, P. J. (2002) Origin, originality, functions, subversions and molecular signalling of macropinocytosis. Int J Med Microbiol 291, 487–94.
(143) Swanson, J. A., and Watts, C. (1995) Macropinocytosis. Trends Cell Biol 5, 424–8.
(144) Hoppe, A. D., and Swanson, J. A. (2004) Cdc42, Rac1, and Rac2 display distinct patterns of activation during phagocytosis. Mol Biol Cell 15, 3509–19.
(145) Grimmer, S., van Deurs, B., and Sandvig, K. (2002) Membrane ruffling and macropinocytosis in A431 cells require cholesterol. J Cell Sci 115, 2953–62.
(146) Meier, O., and Greber, U. F. (2003) Adenovirus endocytosis. J Gene Med 5, 451–62.
(147) Kerr, M. C., and Teasdale, R. D. (2009) Defining macropinocytosis. Traffic 10, 364–71.
(148) Meier, O., Boucke, K., Hammer, S. V., Keller, S., Stidwill, R. P., Hemmi, S., and Greber, U. F. (2002) Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. J Cell Biol 158, 1119–31.
(149) Norbury, C. C., Hewlett, L. J., Prescott, A. R., Shastri, N., and Watts, C. (1995) Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. Immunity 3, 783–91.
(150) Kirkham, M., Fujita, A., Chadda, R., Nixon, S. J., Kurzchalia, T. V., Sharma, D. K., Pagano, R. E., Hancock, J. F., Mayor, S., and Parton, R. G. (2005) Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. J Cell Biol 168, 465–76.
(151) Romer, W., Berland, L., Chambon, V., Gaus, K., Windschiegl, B., Tenza, D., Aly, M. R., Fraisier, V., Florent, J. C., Perrais, D., Lamaze, C., Raposo, G., Steinem, C., Sens, P., Bassereau, P., and Johannes, L. (2007) Shiga toxin induces tubular membrane invaginations for its uptake into cells. Nature 450, 670–5.
(152) Subtil, A., Hemar, A., and Dautry-Varsat, A. (1994) Rapid endocytosis of interleukin 2 receptors when clathrin-coated pit endocytosis is inhibited. J Cell Sci 107 (Pt 12), 3461–8.
(153) Cardenas, A. M., and Marengo, F. D. (2010) Rapid endocytosis and vesicle recycling in neuroendocrine cells. Cell Mol Neurobiol 30, 1365–70.
(154) Lundberg, M., Wikstrom, S., and Johansson, M. (2003) Cell surface adherence and endocytosis of protein transduction domains. Mol Ther 8, 143–50.
(155) McNeil, P. L., and Steinhardt, R. A. (2003) Plasma membrane disruption: repair, prevention, adaptation. Annu Rev Cell Dev Biol 19, 697–731.
(156) Duchardt, F., Fotin-Mleczek, M., Schwarz, H., Fischer, R., and Brock, R. (2007) A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. Traffic 8, 848–66.
(157) Kosuge, M., Takeuchi, T., Nakase, I., Jones, A. T., and Futaki, S. (2008) Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. Bioconjug Chem 19, 656–64.
(158) Alves, I. D., Jiao, C. Y., Aubry, S., Aussedat, B., Burlina, F., Chassaing, G., and Sagan, S. (2010) Cell biology meets biophysics to unveil the different

mechanisms of penetratin internalization in cells. Biochim Biophys Acta 1798, 2231–9.
(159) Rydström, A., Deshayes, S., Konate, K., Crombez, L., Padari, K., Boukhaddaoui, H., Aldrian, G., Pooga, M., and Divita, G. (2011) Direct translocation as major cellular uptake for CADY self-assembling peptide-based nanoparticles. PLoS One 6, e25924.
(160) Räägel, H., Säälik, P., and Pooga, M. (2010) Peptide-mediated protein delivery-which pathways are penetrable? Biochim Biophys Acta 1798, 2240–8.
(161) Nakase, I., Akita, H., Kogure, K., Gräslund, A., Langel, Ü., Harashima, H., and Futaki, S. (2012) Efficient intracellular delivery of nucleic acid pharmaceuticals using cell-penetrating peptides. Acc Chem Res 45, 1132–9.
(162) Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F., and Giacca, M. (2003) Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins. J Biol Chem 278, 34141–9.
(163) Säälik, P., Padari, K., Niinep, A., Lorents, A., Hansen, M., Jokitalo, E., Langel, Ü., and Pooga, M. (2009) Protein delivery with transportans is mediated by caveolae rather than flotillin-dependent pathways. Bioconjug Chem 20, 877–87.
(164) Jovic, M., Sharma, M., Rahajeng, J., and Caplan, S. (2010) The early endosome: a busy sorting station for proteins at the crossroads. Histol Histopathol 25, 99–112.
(165) Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005) Rab conversion as a mechanism of progression from early to late endosomes. Cell 122, 735–49.
(166) Mellman, I. (1996) Endocytosis and molecular sorting. Annu Rev Cell Dev Biol 12, 575–625.
(167) Saftig, P., and Klumperman, J. (2009) Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nat Rev Mol Cell Biol 10, 623–35.
(168) Duncan, R., and Richardson, S. C. (2012) Endocytosis and intracellular trafficking as gateways for nanomedicine delivery: opportunities and challenges. Mol Pharm 9, 2380–402.
(169) Hällbrink, M., Floren, A., Elmquist, A., Pooga, M., Bartfai, T., and Langel, Ü. (2001) Cargo delivery kinetics of cell-penetrating peptides. Biochim Biophys Acta 1515, 101–9.
(170) Herce, H. D., Garcia, A. E., Litt, J., Kane, R. S., Martin, P., Enrique, N., Rebolledo, A., and Milesi, V. (2009) Arginine-rich peptides destabilize the plasma membrane, consistent with a pore formation translocation mechanism of cell-penetrating peptides. Biophys J 97, 1917–25.
(171) Padari, K., Koppel, K., Lorents, A., Hällbrink, M., Mano, M., Pedroso de Lima, M. C., and Pooga, M. (2010) S4(13)-PV cell-penetrating peptide forms nanoparticle-like structures to gain entry into cells. Bioconjug Chem 21, 774–83.
(172) Abes, R., Arzumanov, A., Moulton, H., Abes, S., Ivanova, G., Gait, M. J., Iversen, P., and Lebleu, B. (2008) Arginine-rich cell penetrating peptides: design, structure-activity, and applications to alter pre-mRNA splicing by steric-block oligonucleotides. J Pept Sci 14, 455–60.
(173) El-Andaloussi, S., Johansson, H. J., Lundberg, P., and Langel, Ü. (2006) Induction of splice correction by cell-penetrating peptide nucleic acids. J Gene Med 8, 1262–73.
(174) Niidome, T., Anzai, S., Sonoda, J., Tokunaga, Y., Nakahara, M., Hatakeyama, T., and Aoyagi, H. (1999) Effect of amino acid substitution in amphiphilic alpha-

helical peptides on peptide-phospholipid membrane interaction. J Pept Sci 5, 298–305.
(175) Huang, D., Korolev, N., Eom, K. D., Tam, J. P., and Nordenskiold, L. (2008) Design and biophysical characterization of novel polycationic epsilon-peptides for DNA compaction and delivery. Biomacromolecules 9, 321–30.
(176) Merrifield, R. B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. Journal of the American Chemical Society 85, 2149–2154.
(177) Fields, G. B., and Noble, R. L. (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int J Pept Protein Res 35, 161–214.
(178) Kilk, K., El-Andaloussi, S., Järver, P., Meikas, A., Valkna, A., Bartfai, T., Kogerman, P., Metsis, M., and Langel, Ü. (2005) Evaluation of transportan 10 in PEI mediated plasmid delivery assay. J Control Release 103, 511–23.
(179) Philo, J. S. (2006) Is any measurement method optimal for all aggregate sizes and types? Aaps J 8, E564–71.
(180) Domingues, M. M., Santiago, P. S., Castanho, M. A., and Santos, N. C. (2008) What can light scattering spectroscopy do for membrane-active peptide studies? J Pept Sci 14, 394–400.
(181) Borm, P. J., Robbins, D., Haubold, S., Kuhlbusch, T., Fissan, H., Donaldson, K., Schins, R., Stone, V., Kreyling, W., Lademann, J., Krutmann, J., Warheit, D., and Oberdorster, E. (2006) The potential risks of nanomaterials: a review carried out for ECETOC. Part Fibre Toxicol 3, 11.
(182) Kang, S. H., Cho, M. J., and Kole, R. (1998) Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. Biochemistry 37, 6235–9.
(183) Platt, N., and Gordon, S. (1998) Scavenger receptors: diverse activities and promiscuous binding of polyanionic ligands. Chem Biol 5, R193–203.
(184) Saraste, J., Palade, G. E., and Farquhar, M. G. (1986) Temperature-sensitive steps in the transport of secretory proteins through the Golgi complex in exocrine pancreatic cells. Proc Natl Acad Sci U S A 83, 6425–9.
(185) Wang, L. H., Rothberg, K. G., and Anderson, R. G. (1993) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J Cell Biol 123, 1107–17.
(186) Ivanov, A. I. (2008) Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? Methods Mol Biol 440, 15–33.
(187) Parton, R. G., Joggerst, B., and Simons, K. (1994) Regulated internalization of caveolae. J Cell Biol 127, 1199–215.
(188) Campanella, M., Sciorati, C., Tarozzo, G., and Beltramo, M. (2002) Flow cytometric analysis of inflammatory cells in ischemic rat brain. Stroke 33, 586–92.
(189) Jouret, F., Courtoy, P. J., and Devuyst, O. (2011) Segmental and subcellular distribution of CFTR in the kidney. Methods Mol Biol 741, 285–99.
(190) Gao, J., Xia, L., Lu, M., Zhang, B., Chen, Y., Xu, R., and Wang, L. (2012) TM7SF1 (GPR137B): a novel lysosome integral membrane protein. Mol Biol Rep 39, 8883–9.
(191) Dass, C. R. (2004) Lipoplex-mediated delivery of nucleic acids: factors affecting in vivo transfection. J Mol Med (Berl) 82, 579–91.
(192) Petros, R. A., and DeSimone, J. M. (2010) Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discov 9, 615–27.

(193) Heinzmann, R., Grage, S. L., Schalck, C., Bürck, J., Banoczi, Z., Toke, O., and Ulrich, A. S. (2011) A kinked antimicrobial peptide from Bombina maxima. II. Behavior in phospholipid bilayers. Eur Biophys J 40, 463–70.
(194) Toke, O., Banoczi, Z., Kiraly, P., Heinzmann, R., Bürck, J., Ulrich, A. S., and Hudecz, F. (2011) A kinked antimicrobial peptide from Bombina maxima. I. Three-dimensional structure determined by NMR in membrane-mimicking environments. Eur Biophys J 40, 447–62.

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Siirad tänud mu perele ja sõpradele toetuse ja mõistmise eest.

PUBLICATIONS

CURRICULUM VITAE

Name: Piret Arukuusk
Date of birth: March 8, 1964
Citizenship: Estonian
Contact: Institute of Technology, University of Tartu
Nooruse 1, 50411, Tartu, Estonia
E-mail: piret.arukuusk@gmail.com, piret.arukuusk@ut.ee

Education and employment:

1971–1982	Tartu Miina Härma Gymnasium
1982–1987	University of Tartu, Faculty of Physics and Chemistry, Bioorganic chemistry
1986–1989	University of Tartu, specialist
1995–2003	AS Estiko Plastar, technologist, sales manager, sales director
2004–2005	AS Valga Lihatööstus, sales and marketing director
2005–2009	OÜ Cafemo, chairman
2009–	University of Tartu, Institute of Technology, specialist

Special courses:

2013	Visiting PhD student in Stockholm University, Department of Neurochemistry

List of publications:

1. Langel, K., Lindberg, S., Copolovici, D. M., **Arukuusk, P.**, Sillard, R., and Langel, Ü., (2010) Novel fatty acid modifications of transportan 10., *Int J Pept Res Ther*, 16, 247–255.
2. Oskolkov, N., **Arukuusk, P.,** Copolovici, D. M., Lindberg, S., Margus, H., Padari, K., Pooga, M., and Langel, Ü. (2011) NickFects, phosphorylated derivatives of Transportan 10 for cellular delivery of oligonucleotides. *Int J Pept Res Ther17*, 147–157.
3. Suhorutšenko, J., Oskolkov, N., **Arukuusk, P.**, Kurrikoff, K., Eriste, E., Copolovici, D. M., and Langel, Ü. (2011) Cell-penetrating peptides, PepFects, show no evidence of toxicity and immunogenicity in vitro and in vivo. *Bioconjug Chem* 22, 2255–62.
4. Veiman, K. L., Mäger, I., Ezzat, K., Margus, H., Lehto, T., Langel, K., Kurrikoff, K., **Arukuusk, P.,** Suhorutšenko, J., Padari, K., Pooga, M., Lehto, T., and Langel, Ü. (2012) PepFect14 peptide vector for efficient gene delivery in cell cultures. *Mol Pharm* 10, 199–210.
5. **Arukuusk, P.,** Pärnaste, L., Oskolkov, N., Copolovici, D. M., Margus, H., Padari, K., Möll, K., Maslovskaja, J., Tegova, R., Kivi, G., Tover, A., Pooga, M., Ustav, M., and Langel, Ü. (2013) New generation of efficient peptide-based vectors, NickFects, for the delivery of nucleic acids. *Biochim Biophys Acta 1828*, 1365–73.

6. **Arukuusk, P.,** Pärnaste, L., Margus, H., Eriksson, J., Vasconcelos, L., Padari, K., Pooga, M., and Langel, Ü. (2013) Differential endosomal pathways for radically modified peptide vectors. *Bioconjug Chem*. In press
7. Rytkönen, J., **Arukuusk, P.**, Xu, W., Kurrikoff, K., Langel, Ü., Lehto, V.P., Närvanen, A. (2013) Mesoporus silicon nanoparticles functionalized with cell penetrating peptides: a delivery platform for oligonucleotide drugs. *Mol Pharm*. Submitted

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ELULOOKIRJELDUS

Nimi: Piret Arukuusk
Sünniaeg: 08.03.1964
Kodakondsus: Eesti
Kontaktandmed: Tehnoloogiainstituut, Loodus- ja tehnoloogiateaduskond, Tartu Ülikool Nooruse 1, 50411 Tartu, Eesti
E-post: piret.arukuusk@gmail.com, piret.arukuusk@ut.ee

Haridus ja teenistuskäik:

1971–1982	Tartu Miina Härma Gümnaasium
1982–1987	Tartu Ülikool, keemia teaduskond
1986–1989	Tartu Ülikool, stažöör
1995–2003	AS Estiko Plastar, tehnoloog, müügijuht, müügidirektor
2004–2005	AS Valga Lihatööstus, müügi- ja turundusdirektor
2005–2009	OÜ Cafemo, juhataja
2009–	Tartu Ülikool, Tehnoloogiainstituut, spetsialist

Erialane enesetäiendus:

2013	Vahetus-doktorant Stockholmi Ülikoolis, Neurokeemia Instituudis

Teaduspublikatsioonid:

1. Langel, K., Lindberg, S., Copolovici, D. M., **Arukuusk, P.**, Sillard, R., and Langel, Ü., (2010) Novel fatty acid modifications of transportan 10., *Int J Pept Res Ther*, 16, 247–255.
2. Oskolkov, N., **Arukuusk, P.,** Copolovici, D. M., Lindberg, S., Margus, H., Padari, K., Pooga, M., and Langel, Ü. (2011) NickFects, phosphorylated derivatives of Transportan 10 for cellular delivery of oligonucleotides. *Int J Pept Res Ther17*, 147–157.
3. Suhorutšenko, J., Oskolkov, N., **Arukuusk, P.**, Kurrikoff, K., Eriste, E., Copolovici, D. M., and Langel, Ü. (2011) Cell-penetrating peptides, PepFects, show no evidence of toxicity and immunogenicity in vitro and in vivo. *Bioconjug Chem* 22, 2255–62.
4. Veiman, K. L., Mäger, I., Ezzat, K., Margus, H., Lehto, T., Langel, K., Kurrikoff, K., **Arukuusk, P.,** Suhorutšenko, J., Padari, K., Pooga, M., Lehto, T., and Langel, Ü. (2012) PepFect14 peptide vector for efficient gene delivery in cell cultures. *Mol Pharm* 10, 199–210.
5. **Arukuusk, P.,** Pärnaste, L., Oskolkov, N., Copolovici, D. M., Margus, H., Padari, K., Möll, K., Maslovskaja, J., Tegova, R., Kivi, G., Tover, A., Pooga, M., Ustav, M., and Langel, Ü. (2013) New generation of efficient peptide-based vectors, NickFects, for the delivery of nucleic acids. *Biochim Biophys Acta 1828*, 1365–73.

6. **Arukuusk, P.,** Pärnaste, L., Margus, H., Eriksson, J., Vasconcelos, L., Padari, K., Pooga, M., and Langel, Ü. (2013) Differential endosomal pathways for radically modified peptide vectors. *Bioconjug Chem*. In press
7. Rytkönen, J., **Arukuusk, P.**, Xu, W., Kurrikoff, K., Langel, Ü., Lehto, V.P., Närvanen, A. (2013) Mesoporus silicon nanoparticles functionalized with cell penetrating peptides: a delivery platform for oligonucleotide drugs. *Mol Pharm*. Submitted

Patendi taotlus:

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