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CARMEN TALI

Scavenger receptors as a target for nucleic acid delivery with peptide vectors

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UNIVERSITY OF TARTU
Press

Department of Developmental Biology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Dissertation is accepted for commencement of the degree of Doctor of Philosophy in Cell Biology on March 6, 2017 by the Council of the Institute of Molecular and Cell Biology, University of Tartu.

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Commencement: Room No. 105, 23B Riia Street, Tartu, Estonia, at 10:15 on April 21, 2017.

The publication of this dissertation is granted by the Institute of Molecular and Cell Biology, University of Tartu.

ISSN 1024-6479
ISBN 978-9949-77-374-9 (print)
ISBN 978-9949-77-375-6 (pdf)

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University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

I. 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(3): 1172–1180

II. **Juks, C.,** Padari, K., Margus, H., Kriiska, A., Etverk I, Arukuusk, P., Koppel, K., Ezzat, K., Langel, Ü., Pooga, M. (2015) The role of endocytosis in the uptake and intracellular trafficking of PepFect14-nucleic acid nanocomplexes via class A scavenger receptors. *Biochim. Biophys. Acta* 1848(12): 3205–3216.

III. Margus, H., **Juks, C.,** Pooga, M. (2015) Unraveling the mechanisms of peptide-mediated delivery of nucleic acids using electron microscopy. *Methods Mol. Biol.* 1324: 149–62.

IV. **Juks, C.,** Lorents A., S., Arukuusk P., Langel Ü., and Pooga M. (2017). Cell penetrating peptides recruit type A scavenger receptors to the plasma membrane for cellular delivery of nucleic acids. *FASEB J* 31(3): 975–988.

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My contribution to the articles referred to in this study is following:

Paper I	Designed and performed confocal and electron microscopy experiments, participated in revision of the manuscript.
Paper II	Conceived and designed the study, performed all experiments except luciferase activity measurement and colocalization studies with ER and Golgi apparatus markers, analyzed the data and wrote the manuscript.
Paper III	Participated in writing of the manuscript.
Paper IV	Conceived and designed the study, performed all experiments except Western blot analysis and experiments with LAMP-2 and ionomycine, analyzed the data and wrote the manuscript.

ABBREVIATIONS

AP2	adaptor protein 2
APO	apolipoprotein
cA-SR	class A scavenger receptor
CCP	clathrin-coated pit
CCV	clathrin-coated vesicle
CLIC	clathrin-independent carrier
CLSM	confocal laser scanning microscopy
CME	clathrin-mediated endocytosis
CPP	cell penetrating peptide
CSR	cellular stress-response protein
CT	cholera toxin
DMD	Duchenne muscular dystrophy
DOPE	1, 2-Dioleoyl-sn-glycero-3-phosphoethanolamine
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EE	early endosome
EED	endosomal escape domain
EGF	epidermal growth factor
ER	endoplasmic reticulum
GAPDH	glyceralderhyde-3-phosphate dehydrogenase
GEEC	GPI-anchored protein-enriched early endocytic compartment
GPI	glycosylphosphatidylinositol
HA	hemagglutinin
HCC	human hepatocellular carcinoma
HIV	human immunodeficiency virus
HPRT1	hypoxanthine phosphoribosyltransferase 1
IL-2	interleukin-2 receptor
LAMP	lysosome associated protein
LDL	low-density lipoprotein
LE	late endosome
LF2000	Lipofectamine 2000
LNP	lipid-nanoparticle
MAP	model amphipathic peptide
MARCO	macrophage receptor with collagenous structure
MR	molar ratio
MVB	multivesicular body
NA	nucleic acid
NF	NickFect
ON	oligonucleotide
PAS	penetration accelerating sequence
pDNA	plasmid DNA
PEG	polyethylenglycole
PEI	polyethylenimine

PF	PepFect
PFA	paraformaldehyde
$PI(4,5)P_2$	phosphatidylinositol (4, 5) bisphosphate
PI3K	phosphatidylinositide 3-kinase
PLL	poly-L-lysine
PLO	poly-L-ornithine
PMO	phosphorodiamidate morpholino oligonucleotide
PNA	peptide nucleic acid
Poly-C	polycytidylic acid
Poly-I	polyinosinic acid
PS-2`-OMe	phosphorothioate 2` O methyl
RISC	RNA induced silencing complex
RNAi	RNA interference
RVG	rabies virus glycoprotein
SCO	splice-correcting oligonucleotide
SMA	spinal muscular atrophy
SNA	spherical nucleic acid
SR	scavenger receptor
SR-A3	class A scavenger receptor 5
SR-A5	class A scavenger receptor 3
SR-B1	class B scavenger receptor 1
SV40	simian virus 40
Tat	transactivator protein
TEM	transmission electron microscopy
TGN	trans-Golgi network
TLR	Toll-like receptor
TP	transportan

INTRODUCTION

Nucleic acids (NAs) and synthetic oligonucleotides (ONs) hold great promise in medical perspective to treat variety of genetic or acquired diseases due to their high affinity and specificity towards the target. However, the plasma membrane of the cells poses an impermeable barrier that prevents the large and highly negatively charged molecules to pass into cells. Since the vast majority of NA and ON targets locate inside the cells, their access to the cell interior is inevitable perquisite for exhibiting their functionality. To overcome the issue, various viral- and non-viral delivery vector systems have been designed and introduced. One class of such promising non-viral vectors is cell penetrating peptides (CPP), which according to the name are able to efficiently cross the barrier of the plasma membrane. Most importantly, CPPs have the ability to transduce other molecules into cells, when coupled with either covalently or non-covalently.

Currently, CPPs have been harnessed in highly diverse applications, however, their wider utilization *in vivo* and for biomedical purposes has been limited due to the incomplete knowledge of their functioning. Quite recently it was believed that due to the positive charge, CPPs form with negatively charged ONs complexes carrying positive charge that associate with cell surface and deliver the payload into the cells by taking advantage of the distinct endocytic pathways. Contrary to expectation, it was recently discovered that the PepFect family peptide PepFect14 (PF14) forms negatively charged complexes with splice-switching oligonucleotides (SSO). Such negatively charged complexes should instead of association repel from the plasma membrane, however, these complexes are highly active in correcting the splicing within cells. This surprising finding suggests that the uptake of such complexes might be receptor mediated, even though it has been speculated that CPPs function independently of receptors.

The main objective of the current thesis was to unravel the cellular uptake mechanism of PF14 complexes with splice-correcting oligonucleotide (SCO), and in more detail we focused on the role and regulation of class A scavenger receptors (cA-SRs). We demonstrate the contribution of two members of cA-SRs- SR-A3 and SR-A5 in the cellular uptake of the nanocomplexes. More precisely, we show that the respective receptors are not present on the plasma membrane of HeLa pLuc705 cells constantly, but are recruited there in response to the exposure of cells to the nanocomplexes with the help of calcium and phosphatidylinositol 3-kinase (PI3K) dependent process. In addition, we also aim to determine the uptake mechanisms that nanocomplexes use for entering the cells, and analyze their subsequent intracellular fate. We demonstrate that binding of nanocomplexes to respective receptors induces the uptake by cells mainly via macropinocytosis and caveolae-dependent endocytosis, and after internalization the nanocomplexes are trafficked into rather non-acidic vesicles where nanocompelexes can induce their escape to the cytosol.

1. LITERATURE OVERVIEW

1.1 Nucleic acid based gene therapy approaches

Majority of NA- and ON-based therapies act through antisense mechanism to block the gene expression or to restore its normal function. Antisense approaches can be divided into RNA interference, antisense therapy and splice-switching approach.

RNA interference (RNAi) was discovered in 1998 in the nematode *C. elegans* (Fire and others 1998), and to date, it has turned to the most powerful post-transcriptional gene regulation mechanism. RNAi response is initiated in response to long double-stranded RNA (dsRNA) that is cut into small (21–25 nucleotides) interfering RNA (siRNA) sequences by cytosolic RNase III-like enzyme Dicer (Bernstein and others 2001; Kole and others 2012), and subsequently, siRNA is incorporated into RNA induced silencing complex (RISC) (Sontheimer 2005). Within the RISC complex, siRNA strands are separated, the antisense strand remains associated with RISC, and the sense strand is cleaved and released. Within the RISC complex, the antisense strand recognizes and binds in a sequence-specific manner to the target messenger RNA (mRNA), and the endonuclease Argonaut 2 guides the degradation of target mRNA (Kole and others 2012).

Antisense therapy is based on short (15–20 nucleotides) single stranded antisense oligonucleotides that bind to target mRNA via Watson-Crick hybridization and modulate the target mRNA translation in sequence specific manner. Antisense effect can be achieved either by inducing enzymatic cleavage of the target mRNA by RNase H, or by inhibition of translation by steric blockage of the ribosome (Kurreck 2004; Kole and others 2012).

Splice-switching approach is a method to correct mutations caused by aberrant splicing, or to redirect splicing (Bauman and others 2009). Majority of human genes undergo alternative splicing, a post-transcriptional regulation mechanism by which different forms of mRNAs are produced from one gene, and results in production of structurally and functionally different protein variants (Siva and others 2014). However, alterations in splicing may lead to various genetic disorders, like Duchenne muscular dystrophy (DMD) or cystic fibrosis. DMD is a severe genetic neuromuscular disorder caused by mutations in dystrophin gene open-reading frame, and by loss of functional dystrophin protein (Wood 2010). Targeting of splice-switching oligonucleotides (SCO) complementary to the mutation site, gives an opportunity to correct the reading-frame and production of semi-functional dystrophin via exon-skipping mechanism (Lu and others 2003).

mRNA-based therapeutics comprise a new class of drugs to introduce genetic material into cells (Sahin and others 2014). Once the mRNA is reached to the cell cytoplasm, it will be immediately translated by cellular translation machinery into functional protein. Basically, there are two options to introduce the mRNA into cells- the first approach is to deliver the mRNA into patients through *ex vivo* transfection, or secondly via direct delivery using various transfection methods (Sahin and others 2014).

1.2 Delivery methods for therapeutic molecules

Delivery of ONs into target cells or tissues is extremely challenging. The large size and hydrophilic nature impedes ONs to transverse across the protective cell plasma membrane. Therefore an efficient and safe delivery vectors are required. A suitable delivery vector must overcome several of extra- and intracellular barriers, enable to condense ONs into stable complex to avoid the degradation, provide the access to the target cell/tissue, facilitate an efficient endosomal release, and allow cargo dissociation from the vector to ensure the delivery of the gene of interest to its target site in the cytoplasm or nucleus.

Delivery vectors can be divided into two major classes: viral vectors and non-viral synthetic vectors. Viral vectors have been exploited in numerous gene delivery applications due to their natural property to invade host cells and carry their genetic material from cell to cell. In drug delivery purposes, the viral genome is replaced with therapeutic gene/drug in order to avoid viral replication and pathogenicity. To date, the vast majority of ongoing clinical trials are based on viral vectors (Ginn and others 2013). Although, viral vectors have high transfection efficiency, still their wider usage is impeded mainly because of safety concerns associated with their immunogenicity, and insertional mutagenesis and tumorigenesis (Donsante and others 2001; Thomas and others 2003; Russell 2007).

The aforementioned issues related to viral vectors have prompted to seek for less toxic non-viral vectors. Non-viral gene delivery vectors are mainly based on synthetic cationic lipids and polymers, or peptides. Due to the cationic charge, lipids and polymers bind ONs via electrostatic interactions, and condense them into nanosized particles, lipoplexes and polyplexes, respectively.

Cationic lipids have been extensively exploited in plenty of applications since their first utilization as gene delivery vector for plasmid DNA (pDNA) in 1987 (Felgner and others 1987). To date, hundreds of lipids have been developed, all of which have common structural features: a positively charged head group, one or two hydrophobic tails, and a linker between the polar and hydrophobic regions (Lv and others 2006; Mintzer and Simanek 2009). In addition to cationic lipids, some helper lipids, like cholesterol or 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), are incorporated into lipoplex formulation to improve their transfection efficiencies and promote endosomal escape (Zuhorn and others 2005). Among the **cationic polymers**, polyethylenimine (PEI) is one of the most widely used polymers (Boussif and others 1995; Pack and others 2005; Mintzer and Simanek 2009). PEI is synthetic polymer with high cationic charge density and protonable amino groups (Werth and others 2006). Due to the polymeric nature, PEI as well as other polymers, can be synthesized with different lengths, geometries and molecular weights, e.g. with branched or linear structure (Godbey and others 1999; Elouahabi and Ruysschaert 2005). Other widely used polymers are polyornithines (PLO), polyamidoamidine dendrimers and chitosan (Pack and others 2005; Mintzer and Simanek 2009).

Cationic polyplexes and lipoplexes are mainly taken up via endocytic pathways initiated by electrostatic interactions between the positively charged polyplexes/lipoplexes and negatively charged cell surface components (Mislick and Baldeschwieler 1996; Mounkes and others 1998; Wiethoff and others 2001). However, the induction of endosomal escape and release of cargo from endosomes is fundamentally different (Elouahabi and Ruysschaert 2005). Endosomal release of lipoplexes is mainly driven by hydrophobic interactions assisted by fusogenic helper lipids, e.g. DOPE (Zuhorn and others 2005). Endosomal escape of cationic polymers, e.g. PEI and dendrimers, is believed to rely on their intrinsic endosomolytic activity (Patil and others 2009). Due to the high amount of tertiary amine groups, cationic polymers can act as "proton sponges" to raise the endosomal pH by promoting influx of ions and water, rupture of endosomal membrane and release of sequestered material into the cytosol. However, recent experimental data suggests that the "proton sponge" effect is not the dominant mechanism of how PEI induces the endosomal leakage (Benjaminsen and others 2013).

Still, the polyplexes and lipoplexes suffer from relatively poor transfection efficiency, especially *in vivo,* caused by their highly charged nature and due to the adsorption of serum proteins (Li and Szoka 2007; Gao and others 2007). On the other hand, polyplexes and lipoplexes are easily modified in order to improve their targeting capabilities or endosomal escape. For example, addition of targeting ligand, e.g. ligands of nutrient receptors for folate or transferrin, improves their uptake into cancer cells that often express high amount of folate or transferrin receptors (Biswal and others 2010; Kircheis and others 2001). In order to improve the endosomal escape, incorporation of fusogenic peptides such as KALA or GALA into lipoplex/polyplex have been described (Kakudo and others 2004). To overcome their poor serum stability, covering the surface of the nanoparticles with polyethylene glycol (PEG) reduce interactions with serum components and prolong the circulation time (Petersen and others 2002; Huang and others 2010a).

1.3 Cell penetrating peptides

Cell penetrating peptides (CPP) compromise another class of non-viral delivery vectors with membrane penetrating properties. The research field of CPPs was established nearly 30 years ago when two independent groups discovered the membrane translocation property of human deficiency virus 1 (HIV-1) trans-activator protein TAT (Frankel and Pabo 1988; Green and Loewenstein 1988). A couple of years later, in 1991, a 60 amino acid region of the *Drosophila* antennapedia homeobox protein was demonstrated to be capable of penetrating nervous cells (Joliot and others 1991), and few years later, the same group identified that only a 16 amino acid long sequence from the third helix of the full-length protein is sufficient for membrane penetration, and was named as penetratin (Derossi and others 1994). Subsequently, the minimal sequence

responsible for the uptake of TAT-protein was determined and named as Tat peptide (Vives and others 1997). Soon after the discovery of "natural" CPPs, the synthetic model amphipathic peptide MAP (Oehlke and others 1998), the first chimerical CPP transportan (TP) (Pooga and others 1998), and fully synthetic sequences composed of arginines (Mitchell and others 2000) with cell penetrating ability were designed. Ever since, the family of CPPs has been increasingly expanded and up to date hundreds of CPPs with different chemistries and properties have been discovered or designed. Due to their great sequence variety and different physio-chemical properties, it is difficult to define the uniform concept for all CPPs, however they still share some common features: they are relatively short sequences, usually 5–40 amino acids long, with the capacity to promote the intracellular delivery of bioactive cargos by means of different mechanism, mainly via endocytosis (Langel 2015).

1.3.1 Classification of CPPs

CPPs can be divided by their physical-chemical properties into three classes: amphipathic, hydrophobic and cationic (Milletti 2012; Pooga and Langel 2015).

Amphipathicity corresponds to the partitioning of hydrophilic and hydrophobic parts into opposite side of the molecule (Hristova and others 1999), and such property on the one hand favors the interactions with negatively charged membrane components via electrostatic interactions, and the hydrophobic domain on the other hand is responsible for insertion into lipid membrane. Amphipathic CPPs, in turn are split into two, the primary and secondary amphipathic CPPs. **Primary amphipathic CPPs** comprises hydrophobic and hydrophilic domains in their primary structure, often after folding to α-helices (Song and others 2011). Experiments with model membranes have revealed that the primary amphipathic CPPs bind equally efficiently to both anionic and neutral lipids, suggesting that hydrophobic interactions with membranes dominate over the electrostatic interactions (Magzoub and others 2001). CPPs belonging to this class are usually composed of more than 20 amino acids, however, only a few of them are reported to be basic. The long enough sequence is thought to allow the peptides to submerge into lipid bilayer, and therefore potentially destabilize the membranes (Ziegler 2008). A prototypical primary amphipathic CPP is chimeric TP consisting of 27 amino acids, of which the first 12 amino acids are derived from neuropeptide galanin, and linked via lysine residues to 14 amino acid long mastoparan peptide from wasp venom (Pooga and others 1998). Studies on model membrane systems have demonstrated that the amphipathic α-helix of the peptide is provided by C-terminal mastoparan (Barany-Wallje and others 2004). In order to diminish the inhibitory effect of TP to GTPase activity, a shorter TP analogue TP10 was introduced (Soomets and others 2000). TP10 lacks 6 amino acids in the N-terminal part of TP, however, deletion of these amino acids did not affect the uptake of the peptide (Soomets and others 2000). Introduction of further chemical modifications to the TP10 backbone lead to the

development of PepFect (PF) and NickFect (NF) family CPPs (El Andaloussi and others 2011; Ezzat and others 2011; Oskolkov and others 2011). Other well-known primary amphipathic CPPs are MPG (Morris and others 1997) and Pep-1 (Morris and others 2001) (Table 1).

In **secondary amphipathic CPPs**, the hydrophobic and hydrophilic domains are not segregated in their primary structure, however, after binding to membranes, peptides undergo a conformational change to adopt either α-helical (Lamaziere and others 2007; Crombez and others 2009) or β-strand (Magzoub and others 2001) structure, which allows positioning of hydrophobic amino acids on the one side and cationic parts on the opposite side. Secondary amphipathic CPPs are generally shorter than primary amphipathic CPPs, and unlike primary amphipathic CPPs, secondary amphipathic CPPs bind preferentially with membranes containing high amount of anionic lipids, however, affinity to neutral lipids has been reported to be rather low (Ziegler 2008; Binder and Lindblom 2003). Penetratin (Derossi and others 1994), CADY (Crombez and others 2009) and MAP (Oehlke and others 1998) are examples of secondary amphipathic CPPs (Table 1).

Cationic CPPs, also referred as non-amphipathic CPPs, contain high number of positively charged amino acids, most often arginines or lysines. In analogy to secondary amphipathic CPPs, cationic CPPs associate only with membranes that contain high amount of anionic lipids (Ziegler 2008). However, due to the highly cationic nature, the association with membranes is predominantly forced by electrostatic interactions with negatively charged plasma membrane components. Nevertheless, not all cationic amino acids exhibit similar binding and internalization efficiency; arginines are considered to be by far more effective at entering the cells than other basic amino acids, for example lysine, histidin or ornithine (Mitchell and others 2000). The phenomenon of arginine relies on the guanidinum headgroups that form stable hydrogen bonds after interactions with anionic membrane components (Mitchell and others 2000). Interestingly, the rate of cellular entry of cationic CPPs is also dependent on the number of arginine residues in the sequence. Peptide sequences with more than 6 arginine residues possessed significantly higher uptake rate than shorter ones, however, the optimal length for delivery purposes have been proposed to possess 8–9 arginine residues. The longer sequences lead to toxic side effects and lower uptake rates (Mitchell and others 2000; Futaki and others 2001; Suzuki and others 2002). In addition to polyarginines, also Tat peptide (Vives and others 1997) and M918 (El-Andaloussi and others 2007) belong to this group (Table 1).

Hydrophobic CPPs comprise the smallest number of CPPs. They are predominantly composed of nonpolar amino acids with no or few basic amino acids (Milletti 2012). Hydrophobic CPPs are for example anionic and cationic pentapeptides (Gomez and others 2010), and stapled peptides (Bernal and others 2007; Brown and others 2013; Milletti 2012).

Table 1. Selection of the most often used CPPs and their sequences.

CPP	Sequence	Reference
Primary amphipathic CPPs		
Transportan (TP)	GWTLNSAGYLLGKINLKALAALAKKIL-NH2	Pooga and others 1998
TP10	AGYLLGKINLKALAALAKKIL-NH2	Soomets and others 2000
MPG	GALFLGWLGAAGSTMGAPKKKRKV-cysteamide	Morris and others 1997
PF14	Stearyl-AGYLLGKLLOOLAAAALOOLL-NH2	Ezzat and others 2011
Secondary amphipathic CPPs		
Penetratin	RQIKIWFQNRRMKWKK-NH2	Derossi and others 1994
MAP	KLALKLALKALKAALKLA-NH2	Oehlke and others 1998
CADY	GLWRALWRLLRSLWRLLWRA-cysteamide	Crombez and others 2009
Cationic CPPs		
Oligoarginines	(R)n	Mitchell and others 2000
Tat	GRKKRRQRRRPPQ-NH2	Vives and others 1997

1.3.2 Coupling of cargo molecules with CPPs

In principal, there are two main strategies to attach cargo molecules to CPPs: a covalent linkage, or a non-covalent complex formation strategy. The covalent linkage is based on chemical crosslinking, e.g. disulfide bridges, to couple CPPs with cargo molecules (Pooga and others 1998; Turner and others 2005). The disulfide bridge in general, is cleavable in the reducing environment of the cytosol, and thus allows the dissociation of the cargo from delivery vector. Chemical linkage results in conjugates with well-defined structural characteristics, however, this strategy is not beneficial for large and charged molecules like pDNA, SCO and siRNA. Covalent strategy has been mainly exploited for non-charged or neutral cargo molecules, like peptide nucleic acids (PNA) and phosphorodiamidate morpholino oligonucleotides (PMO). As the covalent strategy relies on chemical linkage, it may have also an impact on cargo functionality.

The non-covalent strategy was introduced by the group of Divita and Heitz in 1997, and this approach relies on electrostatic and hydrophobic interactions associating highly positively charged CPPs and negatively charged nucleic acids (Morris and others 1997). Unlike covalent-linkage, non-covalent strategy is less laborious and time-consuming as complexes are formed by simple mixing. However, the formed nanoparticles might be heterogeneous in terms of size and shape, therefore their exact characterization is complicated.

1.4 CPP mediated oligonucleotide delivery

CPPs have extensively been utilized for delivery of different of ONs, including PNAs, PMOs, siRNAs, SCOs, and pDNAs, both via covalent or non-covalent strategy in multiple of applications.

The first successful CPP-based ON delivery study was introduced in 1997 by a group of G. Divita (Morris and others 1997). The authors demonstrated that the chimeric peptide MPG efficiently condensed short single- or double-stranded ONs into nanoparticles that entered the cells mainly via non-endocytic pathway (Morris and others 1997). Soon after this, the very first successful CPP-based *in vivo* experiment was conducted (Pooga and others 1998). In this study, PNA complementary to human galanin receptor 1 mRNA was covalently coupled with transportan and penetratin, and such conjugates were readily able to down-regulate galanin receptors in rat brain (Pooga and others 1998). Thereafter, numerous of CPP-based *in vivo* ON delivery approaches have been reported. The first *in vivo* experiment with non-covalent nanocomplexes was demonstrated in 2007 by using PNA analogue, negatively charged HypNA-pPNA targeting the cyclin B1, and Pep-3 peptide (Morris and others 2007). HypNA-pPNA-Pep3 showed high activity in cell culture, unfortunately, these nanocomplexes were found to be rather inefficient in *in vivo* models. After intravenous administration, only 20% of reduction in tumor growth was observed, suggesting that the stability of the complexes in the presence of serum is severely affected. To solve this issue, nanoparticles were modified with PEG molecules, and such modification substantially improved the stability and delivery of complexes, yielding in 90% of reduction of tumor growth (Morris and others 2007).

Majority of the splice-switching applications *in vitro* are based on using HeLa pLuc705 reporter cells introduced by Prof. R. Kole (Kang and others 1998). These cells carry a luciferase gene interrupted by mutated β-globin intron 2 that leads to production of nonfunctional protein. However, administration of SCOs complementary to this site, masks the mutation, redirects the splicing machinery and leads to production of a functional protein. One of the first most potent SCO delivery vector was (RxR)4, an oligoarginine analogue in which arginine residues are linked with 6-aminohexanoic acid linker (Abes and others 2006). Covalent conjugates of (RxR)4 and PMO or PNA against mutated site have been shown to induce efficient splice redirection and production of

functional protein in HeLa pLuc705 cells, as well as in the mdx DMD mice model (Said Hassane and others 2010; Fletcher and others 2006; Ivanova and others 2008); however, such complexes were frequently found to be sequestered into endosomes. Further, modifying the (RxR)4 with stearyl moiety substantially increased the splice-correction activity of SCOs compared to unmodified RxR4 in both, cell culture and in DMD models *in vivo*, probably due to the positive effect of fatty acid moiety on endosomal escape. Although, the efficiency of stearylated and un-modified (RxR)4 to deliver PMO was quite comparable, still in case of stearylated-(RxR)4, 10-times lower PMO concentration was required (Lehto and others 2010). Recently, new chemically modified stearyl-TP10, and its analogues PepFects were designed for ON delivery (El Andaloussi and others 2011; Hassane and others 2011; Ezzat and others 2011). PepFect14 (PF14) is stearylated TP10 analogue, in which isoleucines are substituted with leucines, and ornithines are introduced instead of lysines as a source of positive charges (Ezzat and others 2011). The design of PF14 was based on earlier reports where poly-L-ornithines were found to possess superior transfection efficiency compared to poly-L-lysine based systems, mainly due to the higher affinity for DNA and formation of more stable complexes (Ramsay and Gumbleton 2002). Indeed, PF14-SCO nanoparticles exceeded the transfection efficiency of stearyl-TP10 in HeLa pLuc705 cells, and exhibited high splice-correction activity even in mdx mouse myotubes, a cell culture model of DMD (Ezzat and others 2011). Importantly, PF14 exceeded the delivery efficiency of commercially used transfection reagents Lipofectamine 2000 (LF2000), RNAiMAX™ and jetPEI™ (Ezzat and others 2011). During the recent years, a great progress has made in the field of SCO delivery in terms of DMD therapy. The PNA/PMO internalization peptide (Pip), designed on basis of (RxR)4 and R6-Penetratin, has been confirmed to be the most intriguing delivery vector for ONs in numerous of therapeutic applications of DMD (Yin and others 2011; Betts and others 2012) and spinal muscular atrophy (SMA) (Hammond and others 2016). The most prominent peptides of this group are Pip5 and Pip6, and conjugated with PMO, are capable of restoring dystrophin expression at high level in nearly all muscle types, including cardiac muscles (Yin and others 2011; Betts and others 2012 and 2015), as well as restoration of survival moto neuron protein in SMA disease models (Hammond and others 2016).

The first successful CPP-mediated siRNA delivery was achieved with MPG peptide (Simeoni and others 2003). The noncovalent complexes of MPG and siRNA against luciferase yielded in about 80% of reduction in luciferase production, in both HeLa and Cos-7 cells. In 2007, it was reported that the fusion peptide of R9 and rabies virus glycoprotein (RVG) is able to transport siRNA across the blood-brain-barrier (Kumar and others 2007). Upon intravenous injection of RVG-R9-siRNA complexes against GFP into mice, the complexes were readily detectable in the brain where a 50% reduction in GFP expression was observed, concurrently no significant uptake was seen in the liver or spleen (Kumar and others 2007). Soon after this, a new potent siRNA

delivery vector, CADY was introduced (Crombez and others 2009). CADY is a secondary amphipathic CPP with C-terminal cysteamide modification to improve the interactions with membranes and to stabilize formed complexes (Morris and others 2001; Simeoni and others 2003). Indeed, CADY formed stable complexes with siRNA that in high efficiency mediated the down-regulation of glyceralderhyde-3-phosphate dehydrogenase (GAPDH) in suspension and primary cells. Importantly, the uptake of CADY-siRNA nanocomplexes was independent of endocytosis (Crombez and others 2009; Rydström and others 2011); however, very recent experimental data revealed that CADY-siRNA nanoparticles enter the cells mainly via receptor-mediated endocytosis (Lindberg and others 2015). At the same time, a group of S. Dowdy introduced an interesting strategy for siRNA delivery that relies on double-stranded RNA binding domain (DRBD) and Tat peptide (Eguchi and others 2009). Tat-DRBD-siRNA against GFP or GAPDH was shown to induce efficient gene knock-down in primary cell-lines without notable toxic side effects. Tat-DRBD also exhibited efficient RNAi response against luciferase upon intranasal administration *in vivo* (Eguchi and others 2009). To improve the endosomal escape of siRNA, a group of Prof. Ü. Langel developed another stearyl-TP10 based peptide, PF6, which has an extra modification, an endosomolytic trifluoro-methylquinoline moiety (El Andaloussi and others 2011). PF6-siRNA nano-complexes targeting the hypoxanthine phosphoribosyltransferase 1 (HPRT1) or GAPDH exceeded the transfection efficiency of stearyl-TP10-siRNA and yielded in high RNAi response in even hard-to-transfect cells, including primary mouse embryonal fibroblasts, human umbilical vein endothelial cells, mouse embryonic stem cells and Jurkat cells. Moreover, systemic administration of PF16-siRNA nanocomplexes against HPRT1 *in vivo*, yielded in high RNAi responses in variety of organs, but more than 60% of knock-down was achieved in liver, lung and kidney, notably, without any acute toxicity for mice. Additionally, PF6-siRNA nanoparticles at the dose of 1mg/kg siRNA substantially silenced the luciferase expression in mice liver, and the effect lasted more than a week (El Andaloussi and others 2011). Recently, it was introduced that the SPACE peptide is able to penetrate across the defensive skin barrier (Hsu and Mitragotri 2011). The SPACE peptide was discovered through *in vitro* phage display assay, and it shows excellent penetration through upper *stratum corneum* as well as into deeper dermis. Covalently coupled SPACE-siRNA conjugates induced high RNAi response in cell culture and in *in vivo* mice models to silence GAPDH and IL-2 (Hsu and Mitragotri 2011).

1.5 Uptake mechanisms of CPP-cargo complexes

In general there exist two main mechanisms to cross the protective lipid bilayer of the plasma membrane: endocytosis, and direct penetration across the plasma membrane. However, to date there is no consensus at which extent either of these entry mechanisms contributes to the uptake of CPPs. Direct penetration

across the plasma membrane is considered to occur at relatively high peptide concentration (Duchardt and others 2007; Kosuge and others 2008), and in the presence of small hydrophobic cargos, such as fluorescent dyes (e.g AlexaFluor488) (Tünnemann and others 2006; Hirose and others 2012). However, when CPPs are coupled with high molecular weight cargos, including ONs, the switch from direct penetration to endocytosis occurs (Tünnemann and others 2006). Nevertheless, direct penetration cannot be exclusively eliminated, as it has been described to be a prevalent entry route for amphipathic CPPs such as MPG, CADY, and Pep-1 even with large cargo molecules (Deshayes and others 2006; Konate and others 2010; Rydström and others 2011). Still, the majority of studies, mainly based on pharmacological inhibitors or RNAi, highlight the role of endocytosis as primary mechanism for the uptake of CPP-cargo complexes (Richard and others 2003 and 2005; Khalil and others 2004; Eguchi and others 2009; Säälik and others 2009; Al Soraj and others 2012). Cell entry via endocytosis includes clathrin-mediated endocytosis, caveolin-dependent endocytosis, macropinocytosis, and clathrin- and caveolin independent pathways (Figure 1), and frequently, CPPs may utilize different mechanisms simultaneously (Duchardt and others 2007; Säälik and others 2009; Arukuusk and others 2013; Juks and others 2015), making the understanding of action of CPPs more complicated.

Whether it is endocytosis or direct penetration, the internalization of CPP-cargo complexes is initiated by binding to cells. Due to the cationic nature of CPPs, it is believed that association with cells is triggered by electrostatic interactions with negatively charged cell surface components, e.g. glygosaminglycans or phospholipids (Nakase and others 2007; Lundin and others 2008; Ziegler and Seelig 2011). Contrarily, group of S. Dowdy demonstrated that the entry of Tat-Cre fusion protein via macropinocytosis is not triggered through interactions with cell surface heparan sulfates or sialic acids, since the internalization of the fusion-protein was not affected in cells lacking glycans. Instead, the binding and uptake was found to be dependent on cell surface proteins (Gump and others 2010). Analogously, Ezzat et al discovered that PF14 nanocomplexes with SCOs obtain negative surface charge in transfection medium (Ezzat and others 2011), and thus, binding of such complexes to cell proteoglycans on the cell surface is not favorable, and requires specific receptor. Recently, we demonstrated that such complexes are recognized by class A scavenger receptors (cA-SR), which efficiently promote their entry into cells by means of different endocytic mechanisms (Ezzat and others 2012; Juks and others 2015). Now it is clear that cA-SR dependent endocytosis is more general mechanism among PF and NF family peptides in complex with ONs (Arukuusk and others 2013; Veiman and others 2013; Lindberg and others 2013 and 2015). However, harnessing of cA-SR for internalization into cells is not only restricted to this type of peptides, but also CADY-siRNA nanoparticles, earlier believed to enter cells independently of endocytosis, engage cA-SRs to gain access into cells (Lindberg and others 2015). As the majority of CPP-ON complexes acquire negative zeta-potential in transfection media (van Asbeck

and others 2013), therefore it is feasible to speculate that scavenger receptors with promiscuous binding of anionic molecules (Pearson and others 1993), might contribute to the uptake of other CPP-ON complexes as well. Moreover, the transfection of commercial transfection regent's PEI and PLO relies on cA-SR dependent endocytosis (Lindberg and others 2015). Scavenger receptors will be discussed in more detail below.

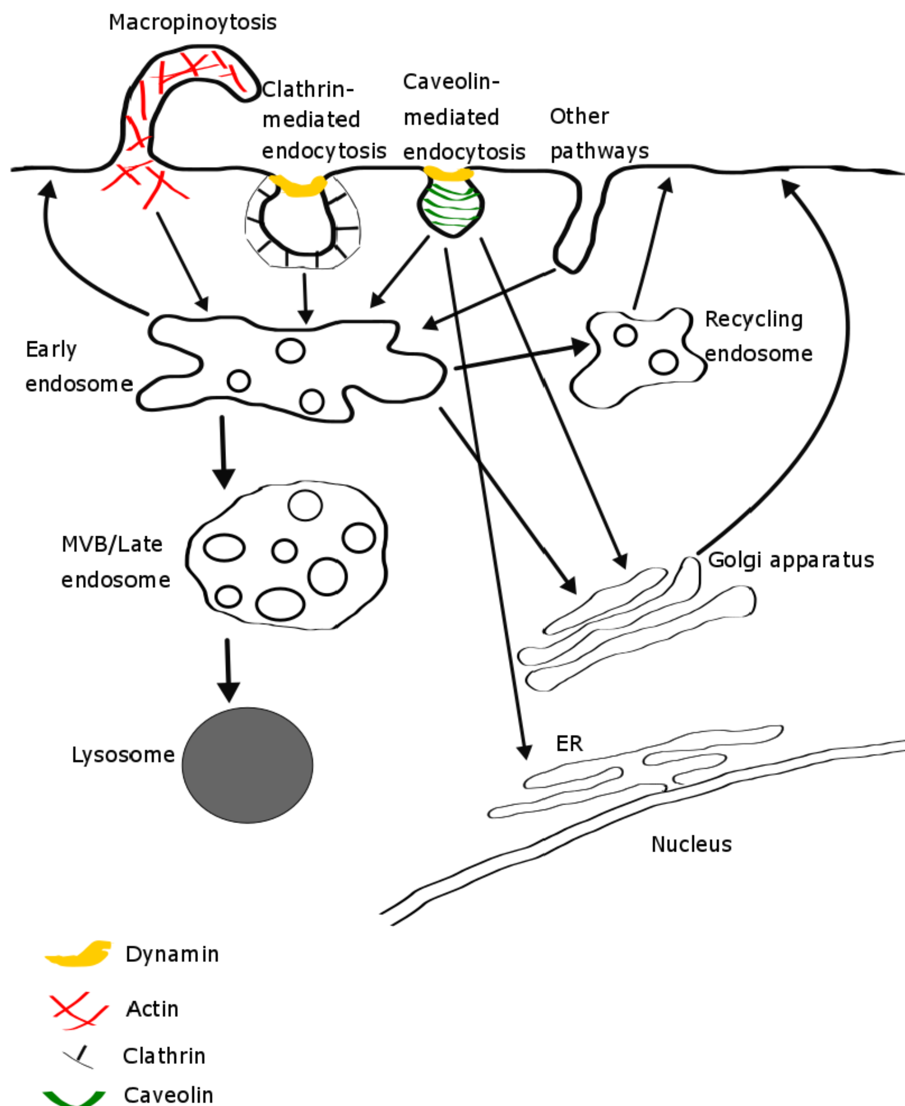

Figure 1. Schematic representation of endosomal entry routes and intracellular trafficking pathways for CPP-cargo complexes. MVB- multivesicular body, ER- endoplasmic reticulum.

1.5.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is by far the most extensively studied endocytic entry route that is constitutively active in all mammalian cells. Through the CME cells internalize nutrients, regulate the number of cell surface proteins, and participate in receptor endocytosis (Schmid 1997; Kirchhausen 2000). Moreover, CME also contribute to generation of transport vesicles derived from trans-Golgi network (TGN) (Griffiths and others 1988). Clathrin coated vesicles (CCV) are relatively small, 100–150 nm in diameter, and characterized by the presence of coat formed by three-legged protein (triskelion) clathrin (Kirchhausen 2000). However, clathrin itself is unable to directly bind the membrane, and requires a coordinated assembly of adaptor and accessory proteins (Kaksonen and others 2005; McPherson 2010). It has been speculated that at least more than fifty different proteins contribute to CME and formation of clathrin coated pit (CCP). The assembly of CCP is thought to be initiated in phosphatidylinositol (4,5) bisphosphate (PI(4,5)P_2) rich regions of the inner leaflet on the plasma membrane (Antonescu and others 2010), where PI(4,5)P_2 is specifically required for docking the main CME regulatory protein adaptor protein 2 (AP2) to the membrane (Abe and others 2008). Abe and colleagues demonstrated that after depletion of PI(4,5)P_2 from the plasma membrane, AP2 showed diffusive distribution, and the internalization of transferrin receptor was reduced. However, in control cell, AP2 was found to be clustered to the distinct plasma membrane regions that also contained tranferrin receptors, suggesting that PI(4,5)P_2 is the main regulator of AP2 (Abe and others 2008). AP2 can bind with both, PI(4,5)P_2 and specific motifs in the cytoplasmic domain of the receptors, and thus participate in cargo selection to the nascent site of the pit. Subsequently, clathrin is recruited to the membrane via AP2, followed by its polymerization and conversion of the flat plasma membrane into highly curved CCP (Ungewickell and Hinrichsen 2007). The budding of CCP depends on small cytoplasmic GTPase dynamin that polymerizes around the nascent vesicle neck and assist the membrane scission and budding of the vesicle (Stowell and others 1999). Subsequently, upon release into cytosol and before fusion with endosomal compartments, clathrin coat is quickly dissociated by ATPase heat shock cognate 70 and its cofactor auxilin (Kirchhausen 2000).

Transferrin is prototypical cargo that enters the cells via CME. Its binding to transferrin receptor induces rapid entry within few minutes (Ciechanover and others 1983), followed by targeting to classical endo-lysosomal pathway, along which vesicles undergo progressive maturation with gradual drop in pH of vesicles from early- to late endosomes, and finally, the pathway terminates in lysosomes where transferrin is degraded.

Engagement of CME for internalization have been demonstrated for Tat-PNA complexes (Richard and others 2005), for conjugates of TP or TP10 with PNA (Lundin and others 2008), and for nanocomplexes of PF6 and SCO (Hassane and others 2011).

1.5.2 Caveolin-dependent endocytosis

Caveolae are small, 50–100 nm in diameter, bulb-shaped invaginations of the plasma membrane, where major constituent is a cholesterol-binding protein caveolin (Cav) (Pelkmans and others 2001; Cheng and Nichols 2016). There are three mammalian caveolins, Cav-1, Cav-2 and Cav-3. Cav-1 and Cav-2 are expressed in non-muscle cells (Scherer and others 1997), whereas expression of Cav-3 is restricted to muscle cells (Tang and others 1996), however, only Cav-1 and Cav-3 share the capacity to induce caveolae (Hayer and others 2010). Each of the caveola is estimated to contain 140–150 copies of caveolin proteins (Pelkmans and Zerial 2005). The caveolae rise from distinct plasma membrane regions, called rafts, that are rich in cholesterol, sphingolipids and glyco-phosphatidylinositol (GPI) linked proteins (Pelkmans and others 2001; Harris and others 2002). In addition to caveolin, caveolae are also enriched in cavin family proteins (Briand and others 2011), recruited to stabilize caveolin oligomers (Liu and Pilch 2008), and to regulate the dynamics and budding of caveolae (Hill and others 2008). High cholesterol content during caveolae formation is essential, since its depletion has yielded in dissociation of cavin proteins from the membrane and subsequent flattening of the invaginations (Hill and others 2008). In analogy to CME, dynamin is recruited for fission of caveolae from the plasma membrane (Henley and others 1998).

Caveolae are present in most of eukaryotic cells, but are considered to be essentially abundant in endothelial cell, adipocytes and muscle cells (Chidlow and Sessa 2010). Caveolae have been implicated in numerous cellular processes, including cholesterol- and lipid metabolism, calcium signaling, endocytosis and transcytosis (Parton and del Pozo 2013; Cheng and Nichols 2016). Since the raft domains contain enormous amount of signaling molecules (Okamoto and others 1998; Shaul and Anderson 1998), caveolae also contribute to numerous signal transduction pathways. Caveolae are also responsible for uptake of some pathogens and their associated molecules, such as cholera toxin (CT) and simian virus SV40 (Pelkmans and others 2001).

Unlike CCPs that are rapidly internalized into cells, caveolae are considered to be long-lived structures, which can be associated with the plasma membrane for long time periods without being internalized (Kirkham and others 2005; Tagawa and others 2005; Pelkmans and Zerial 2005; Hayer and others 2010). However, upon stimulation, e.g. simian virus SV40, caveolae are pinched-off from the membrane and enter the cells. The early reports have proposed that budding of caveolae leads to the formation of distinct vesicular structure, the caveosomes that lack classical endosomal markers and possess neutral pH (Pelkmans and others 2001). However, subsequent studies have disproved the existence of such vesicles, and claimed that caveolae still fuse with early endosomes, and follow the trafficking through the endo-lysosomal system (Pelkmans and others 2004; Hayer and others 2010). Still, several experiments have revealed that the uptake via caveolae is not degradative, mainly because of the slow trafficking/maturation of vesicles aroused from caveolae (Harris and

others 2002; Fittipaldi and others 2003; Tagawa and others 2005; Kirkham and others 2005). Entry through caveolae may also address the material directly to Golgi apparatus or ER (Pelkmans and others 2001; Le and Nabi 2003).

Cholesterol depletion by methyl-β-cyclodextrin has revealed that caveolin-dependent endocytosis is the main entry pathway for Tat-protein construct (Fittipaldi and others 2003). Knock-down of caveolin-1 with siRNA significantly reduced the uptake of TP- and TP10-protein conjugates as well (Säälik and others 2009). Also, the uptake of NFs and PFs complexed with ONs occurs partially via caveolae-dependent manner (Arukuusk and others 2013; Veiman and others 2013; Juks and others 2015). Recently, by using a nystatin, an inhibitor of caveolin-dependent endocytosis, or RNAi to deplete Cav-1, we demonstrated that the uptake of PF14-SCO complexes was reduced to 30 and 40% level, respectively (Juks and others 2015), suggesting that PF14-SCO complexes are partially internalized via caveolin-dependent pathway.

1.5.3 Macropinocytosis

Macropinocytosis is highly regulated form of endocytosis, that leads to internalization of extracellular fluids and membrane bound bulk cargo (Lim and Gleeson 2011). Macropinocytosis is initiated by extensive actin rearrangement that results in formation of membrane ruffles, which subsequently collapse back to the plasma membrane and fuse with it to form large macropinosomes (Swanson and Watts 1995). Unlike to caveolae and clathrin-coated vesicles, macropinosomes lack an apparent coat structure, and are considerably larger in their size ranging from 0.2–5 μM (Swanson and Watts 1995). Macropinocytosis occur in many cell types, but is constitutively active only in dendritic cells to capture antigens (West and others 2000), however, in many other cell types it is considered to be a transient response to external stimulation, most commonly a growth-factor stimulated process (Falcone and others 2006; Commisso and others 2013; Kamphorst and others 2015). Binding of growth-factors to respective receptor-tyrosine kinase, cause activation of multiple signaling events initiated by activation of small GTPases like Rac1 and Cdc42. Rac1 and CdC42 contribute to excessive actin-cytoskeleton reorganization, formation of plasma membrane ruffles and eventually macropinosome formation (West and others 2000; Garrett and others 2000). Rac1 and CdC42, in turn activate and recruit kinases such as p21-activated kinase 1 (Pak1) and phosphatidylinositol-3-kinase (PI3K) to the plasma membrane ruffling areas, that are involved in regulation of actin-cytoskeleton dynamics (Dharmawardhane and others 2000), and macropinosome closure (Araki and others 1996), respectively. In addition, the reorganization of actin cytoskeleton and subsequent membrane ruffling is highly sensitive to cholesterol and $PI(4,5)P_2$ depletion from the membrane (Grimmer and others 2002; Kwik and others 2003). Unlike CME and caveolae, fission of macropinosomes from the plasma membrane is independent of dynamin activity; instead, it recruits C-terminal-binding protein-1/brefeldinA-

ADP ribosylated substrate that associates around the neck of forming macropinosome (Liberali and others 2008).

Once the macropinosomes are formed and entered the cells, they are believed to undergo classical endo-lysosomal pathway from early endosomes towards the lysosomes (Racoosin and Swanson 1993). However, in human epidermoid A431 cells, a somewhat different trafficking has been described (Hewlett and others 1994). In these cells, after epidermal growth factor (EGF) stimulation, macropinosomes remained as a distinct population of vesicles that failed to fuse with conventional endosomes, and did not mature into late endosomes/lysosomes (Hewlett and others 1994). Instead, macropinosomes were directed to recycling pathway.

Macropinocytosis has been pointed out to be the major entry route for arginine-rich CPPs and their complexes with ONs (Nakase and others 2004; Al Soraj and others 2012). Nakase et al demonstrated that binding of TAT and R8 with plasma membrane heparan sulfates can induce Rac1-dependent actin cytoskeleton remodeling and induction of macropinocytosis (Nakase and others 2004). Macropinocytosis also contributes to the biological activity of PF14-SCO and NF-pDNA nanocomplexes (Juks and others 2015; Arukuusk and others 2013).

1.5.4 Clathrin- and caveolin-independent uptake routes

Inhibition of CME or caveolin-dependent endocytosis does not arrest the endocytosis from the plasma membrane, and along with the three major pathways described above, there are also alternative pathways that are distinct from clathrin- and caveolin coated vesicles, e.g. CLIC (clathrin independent carrier)/ GEEC (GPI-anchored protein-enriched early endocytic compartment), flotillin- and Arf6-dependent entry routes (Sandvig and others 2011; Mayor and others 2014). Similarly to caveolae, all these alternative pathways are associated with lipid-raft domains and are sensitive to cholesterol depletion (Lamaze and others 2001; Howes and others 2010). These pathways are further divided by their recruitment of dynamin into dynamin-dependent or -independent routes (Mayor and Pagano 2007).

For example, the entry of interleukin-2 receptor (IL-2) and EGF at high concentration have been proposed to occur via dynamin-dependent but clathrin-independent way (Lamaze and others 2001; Sigismund and others 2005). Both, the IL-2 and EGF accumulate in raft domains before being internalized via uncoated vesicles.

The most studied dynamin-independent pathway is CLIC/GEEC pathway, which is involved in the uptake of GPI-anchored proteins, extracellular fluid, bacteria and toxins (for example CT and SV40) (Kirkham and others 2005; Howes and others 2010). Internalization of GPI-anchored proteins induces formation of tubular invaginations, CLICs, from the plasma membrane that further mature into early endosomal compartments GEECs (Sabharanjak and others 2002). CLIC/GEEC pathway recruits small GTPases Cdc42 and GTPase

regulator associated with focal adhesion kinase 1 (GRAF1) to regulate the actin polymerization, and to give rise to tube-shaped membrane invaginations, respectively (Lundmark and others 2008). Another pathway for GPI-anchored proteins is dependent on raft-associated proteins flotillins/reggie, but do not require dynamin (Glebov and others 2006). There are two types of flotillins, flotillin-1 and flotillin-2, and their co-assembly on the plasma membrane lipid-raft domain induces plasma membrane invaginations in similar shape and size range as caveolae, but lack the caveolin proteins (Glebov and others 2006; Frick and others 2007).

1.5.5 Endosomal trafficking

After being endocytosed, the internalized material is subsequently directed to endosomal network, a complex vesicular system which main function is to receive and sort the internalized material to the final destination. Basically, two main intracellular trafficking pathways exist; a recycling pathway to direct internalized material back to the plasma membrane, and endo-lysosomal pathway for degradation. There are four major endocytic compartments: early endosomes (EE), recycling endosomes, late endosomes (LE) and lysosomes (Figure 1), which undergo multiple of fission and fusion steps in order to sort cargo to appropriate destination. The key regulators in endosomal membrane trafficking are the small GTP-binding Rab family proteins that can exist in active GTP-bound state or inactive GDP-bound state (Zerial and McBride 2001; Lee and others 2009). At active state, Rab-proteins are localized to vesicle membrane, and in association with specific effector proteins guide endosomal maturation. For example, Rab5 is present on EEs (Gorvel and others 1991), Rab4 and Rab11 are main markers of recycling endosomes (Sonnichsen and others 2000), and Rab7 and Rab9 for LEs (Rink and others 2005).

No matter along which pathway the materials enter the cells; they are firstly converged into early endosomes (EEs) that act as initial cargo-sorting stations. EEs are complex structures that consist of tubular domains and large intraluminal vesicles (~400 nm in diameter) (Gruenberg 2001; Jovic and others 2010), both of which give rise to vesicles with discrete function. The material, that is destined to recycle back to the plasma membrane, is sorted into tubular region of EEs, and is subsequently targeted to the cell surface directly or through recycling endosomes (Figure 1). On the other hand, the material that is destined for degradation accumulates in vesicle part of EEs (Sonnichsen and others 2000). Importantly, different regions of EEs are also characterized by different Rab regulatory proteins. Rab5 that is considered to be the key regulatory factor of EE fusion (Gorvel and others 1991), is mainly localized to vesicular part of EEs; however the tubular part of EEs lack Rab5, but it is rich in Rab4 (Sonnichsen and others 2000). In addition, the lumen of early endosomes is mildly acidic with pH~6, which is an appropriate environment for dissociation of many receptor-ligand complexes.

The cargo entrapped into intraluminal vesicles of EEs follows further trafficking towards lysosomes. During endosomal maturation from EEs to LEs, the composition of the endosomal membrane changes and Rab5 is substituted with Rab7 (Rink and others 2005; Vonderheit and Helenius 2005). In comparison with EEs, the environment of LEs is more acidic (pH~5), and LEs are generally bigger with round or oval shape. LEs also contain numeral intraluminal vesicles, and therefore are often called as multivesicular bodies (MVB) (Miller and others 1986) (Figure 1). The pH of endosomes during their maturation is controlled by the concentration of endosomal membrane bound proton pump ATPases (Forgac 2007). Finally, the internalized material terminates in more acidic lysosomes (pH 4.5–5), where it is being degraded by lysosomal hydrolases.

1.5.6 Endosomal release of CPP-cargo complexes

CPPs are promising tools to deliver ONs into cells, however, being mainly internalized via endocytosis, the main limiting obstacle in CPP-mediated ON delivery is their entrapment into endosomal vesicle; however, to be functionally active, cargo needs to escape into cytosol or reach the nucleus. The exact mechanism, how CPP-cargo complexes promote the endosomal escape, and at which state and extent it occurs, remains to unknown yet, mainly because of lack of suitable methods to follow this explicitly.

Recently, a couple of groundbreaking studies conducted by two separate groups provided new insights into endosomal release of siRNA-lipid nanoparticles (LNP) (Gilleron and others 2013; Wittrup and others 2015). Using a combination of confocal laser scanning and electron microscopy, Gilleron et al revealed that the endosomal escape of LNPs occurs from maturing endosomes with characteristics of both early and late endosomes, and carrying respective markers. Moreover, only a very small fraction of siRNA was found to be released from endosomes, accounting only 1–2% of the total amount of internalized siRNA (Gilleron and others 2013). Similar conclusions were also made by Wittrup and his colleagues (Wittrup and others 2015). Furthermore, the authors did not detect endosomal release from late endosomes or lysosome, showing that endosomal escape occurs during a very narrow time window. Moreover, only few siRNA molecules per single endosome reached the cytosol, suggesting that endosomal escape is not achieved through entire endosome burst, but rather as a consequence of slight permeabilization of the limiting membrane of endosome (Wittrup and others 2015). Whether the CPP-ON complexes also act in a similar way, needs further studies.

To increase the rate of endosomal escape of CPP-cargo complexes, several approaches have been introduced. One possible strategy is to increase the hydrophobicity of CPPs by attachment of fatty acids to CPP sequence, and thereby promoting their interactions with membranes. Futaki and coworkers showed that attachment of N-terminal stearyl moiety to R8 increased its

efficiency to transfect plasmid DNA into cells by almost 2 orders of magnitude compared to unmodified R8 (Futaki and others 2001). Later, stearylated-R8 was also used to improve siRNA delivery (Tönges and others 2006). In addition, modifying the N-terminus of TP10 with stearyl moiety showed positive effect on SCO delivery and yielded in transfection efficiency comparable to the commercial reagent LF2000 without toxicity (Mäe and others 2009). Likewise, addition of hydrophobic amino acid sequences composed of tryptophan or phenylalanine, have shown to increase the transfection efficiency and endosomal release of CPP-cargo complexes (Takayama and others 2009; Rydberg and others 2012; Lönn and others 2016). For example, attachment of hydrophobic penetration accelerating sequence (PAS) to R8 enhanced its internalization as well as widespread cytosolic distribution (Takayama and others 2009). The hydrophobic PAS sequence inserts into the lipid membrane of endosomes, leads to membrane destabilization and thereby enhances the endosomal escape of sequestered material into the cytoplasm. Recently, Lönn et al introduced a tryptophane rich synthetic endosomal escape domain (EED) to improve the endosomal escape of Tat peptide (Lönn and others 2016). Conjugation of EED to GFPβ11-TAT significantly improved the cytoplasmic delivery compared to the parent peptide without the modification.

Another strategy to overcome endosomal entrapment is attachment of fusogenic peptides to CPP sequences. One of such peptides is derived from the influenza virus hemagglutinin protein HA2 that at low pH undergoes conformational change and adopts hydrophobic helical structure that inserts into lipid membrane, and promotes fusion of viral membrane with endosomal membrane (Han and others 2001). Attachment of fusogenic peptide HA2 to TAT peptide drastically improved its transduction properties (Wadia and others 2004). Increasing the endosomolytic properties of CPPs with incorporation of histidine residues in the CPP sequence is a third strategy to induce endosomal escape via “proton sponge effect”. The imidazole group of histidine has a pK_a of ~6.0, which allows absorbing protons in the acidic environment of the endosome, leads to osmotic swelling, membrane disruption and eventually endosomal escape (Lo and Wang 2008). Incorporation of histidine residues to arginine-rich CPPs such as Tat and R9 significantly improved their efficiency to deliver plasmid DNA into cells (Lo and Wang 2008; Liu and others 2013). Similarly, replacement of certain amino acids in penetratin sequence by histidine yielded in endosomolytic α-helical EB1 peptide that exhibited superior siRNA delivery efficiency over the parent peptide penetratin (Lundberg and others 2007).

Alternative approach for provoking the endosomal escape is to apply endosomolytic agents, e.g chloroquine. Chloroquine is weak base that accumulates in acidic endosomes or lysosomes, and depending on its concentration, it can interfere with the acidification of endosomes by preventing accumulation of protons into endosomes (at low concentrations), or induce influx of ions and water, and subsequent rapture of endosomes (at high concentrations) (Ciftci and Levy 2001). Wadia *et al* demonstrated that Tat-Cre fusion protein internalization into nucleus was significantly improved in the presence of chloroquine

(Wadia and others 2004). Analogously, stearyl-TP10 mediated SCO delivery and splice-correction activity was drastically enhanced when chloroquine was included into transfection medium (Mäe and others 2009). Despite of its positive effect on endosomal release, chloroquine is toxic to cells, and its application in gene therapy or clinical trials is questionable. Recently, a chloroquine analogue, trifluoromethylquinoline incorporation into steary-TP10 sequence resulted in a new peptide PF6 (El Andaloussi and others 2011). PF6 drastically improved the siRNA delivery and its functionality compared to its parent peptide stearyl-TP10, moreover, PF6 did not exhibit any significant cytotoxic effects (El Andaloussi and others 2011). In addition, PF15, an analogue of PF14 with trifluoromethylquinoline, exceeds the SCO induced splice-correction efficiency mediated by PF14 by almost two-fold (Lindberg and others 2013).

1.6 Scavenger receptors

Scavenger receptors (SR) are transmembrane proteins that were discovered by Brown and Goldstein in 1979, who originally defined SRs by their ability to bind and endocytose modified low density lipoproteins (LDL) (e.g. acetylated and oxidized LDLs), but not native LDL molecules (Goldstein and others 1979). Therefore the early studies on SRs were mainly concentrated on their role in lipid metabolism and progression of atherosclerosis. However, since their first discovery nearly four decades ago, the family of SRs has expanded and now it is clear that SRs comprise a large supergroup of receptors with a range of physiological and pathological functions. The receptors of this family are divided into 10 classes, from A-J, based on their structural characteristics (Figure 2) (Prabhudas and others 2014; Zani and others 2015). Interestingly, comprising structurally very diverse family with barely no or little structural homology between the classes, they all share similar function to bind polyanionic ligands, including acetylated or oxidized LDLs, high density lipoproteins, apolipoproteins, lipopolysaccharides, polyribonucleotides, apoptotic cells, double stranded (ds) RNA and -DNA, and synthetic nanoparticles (Pearson and others 1993; Kanno and others 2007; Patel and others 2010; Lunov and others 2011; Yu and others 2012). Exhibiting such an unusually broad ligand binding specificity, it is not surprising that SRs participate in multiple cellular processes, including endocytosis and phagocytosis (Thomas and others 2000; Peiser and others 2002) cellular adhesion (Cholewa and others 2010), antigen presentation (Abraham and others 1995 and 1997; Nicoletti and others 1999). SRs also participate in activation of diverse cellular signaling events (Jozefowski and Kobzik 2004; Baranova and others 2008; Huang and others 2010b; Zani and others 2015), as well as contribute to the progression of Alzheimer diseases (El Khoury and others 1996; Husemann and others 2002). SRs belong to the class of pattern recognition receptors (PPR), and contribute to innate immune responses by recognizing intact bacteria and viruses, or pathogen-associated molecules (Hampton and others 1991; Dunne and others

1994), which is why they are so abundantly expressed in macrophages and other myeloid cells (Hughes and others 1995). However, SRs are not restricted to these types of cells, but are also found on numerous other cell types, including epithelial and endothelial cells, fibroblasts and smooth muscle cells (Plüddemann and others 2007; Prabhudas and others 2014). Recently it was speculated that due to their wide ligand binding repertoire, SRs do not function alone but rather belong to heteromultimeric signaling complexes together with Toll-like receptors (TLR), integrins and tetraspanins (Canton and others 2013). In line of this, members of SRs have been shown to cooperate with TLR2, TLR3, TLR4 and TLR9 (Peiser and others 2002; DeWitte-Orr and others 2010; Yu and others 2012). Furthermore, TLRs and SRs often exhibit overlapping ligand-binding specificity.

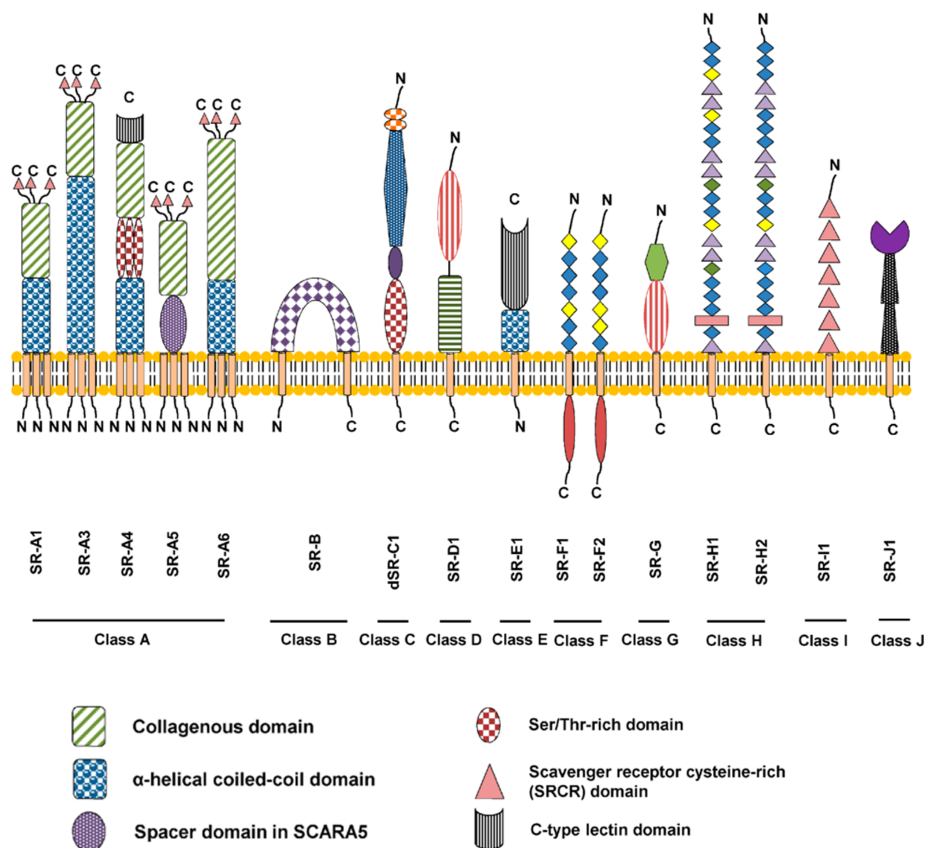

Figure 2. Illustrative figure of scavenger receptors (adopted from Zani and others 2015). SRs constitute a large family of receptors, which according to the structural domains are divided into 10 classes, from A-J. The specific domains of class A receptors are indicated in the figure.

1.6.1 Class A scavenger receptors

Class A scavenger receptors (cA-SR or SCARA) belong to a group of type two transmembrane glycoproteins with heterotrimeric structure (Kodama and others 1990). To date, five members of this class have been identified: SR-A1, and its splice variants SR-A II and SR-A III; MARCO; SR-A3; SR-A4 and SR-A5 (Figure 2), where all monomers have a short cytoplasmic tail, single transmembrane domain, spacer region, and long extracellular domain (Plüddemann and others 2007). A unique structural feature among the members of cA-SR is the collagenous domain in extracellular domain of receptors (Figure 2). Additionally, SR-A1, MARCO and SR-A5 have a cysteine-rich domain in the extracellular part (Plüddemann and others 2007). Nevertheless, being under investigation for years, only a few aspects about the multi-domain organization are known. By far, the best studied cA-SR receptors are SR-A1 and MARCO, and it has been proposed that positively charged regions in the extracellular domain are responsible for ligand binding. MARCO binds ligands mainly via the cysteine-rich domain (Ojala and others 2007); however, recognition of acetylated and oxidized LDLs by SR-A1 is believed to be assisted rather by collagenous domain (Doi and others 1993). The cytoplasmic domain of cA-SR receptors regulates their membrane localization and internalization (Kosswig and others 2003; Chen and others 2006).

SR-A1 and MARCO are generally expressed in macrophages and other myeloid cells (Hughes and others 1995) with main function to endocytose modified LDLs, but also bacteria and other pathogen associated proteins, and thus provide innate defense against pathogens (Elomaa and others 1995; Limmon and others 2008; Zhu and others 2011). SR-A1 has also been implicated in lipid metabolism by its ability to bind apolipoproteins (Gao and others 2003). In addition to macrophages, SR-A1 receptors are also present on endothelial and smooth-muscle cells (Pitas 1990; Mietus-Snyder and others 2000). SR-A3, also known as cellular stress-response protein (CSR), is upregulated under oxidative stress conditions (UV irradiation), and thus protects cells against oxidative stress by scavenging reactive oxygen species (Han and others 1998). Unlike to SR-A1 and MARCO, SR-A3 was found to localize in the cell cytoplasm of HeLa cells near the endoplasmic reticulum (ER)/Golgi complex (Han and others 1998). SR-A4, a scavenger receptor with C-type lectin, is mainly expressed on endothelial cells, and contributes to innate immunity responses by recognizing microbes (Nakamura and others 2001). Importantly, SR-A4 binds only with oxidized LDLs, not acetylated LDLs (Ohtani and others 2001). SR-A5 is mainly expressed on fibroblasts and epithelial cells, and its expression was found to be particularly high in testis, lung, bladder, and small intestine (Jiang and others 2006; Li and others 2009; DeWitte-Orr and others 2010). In analogy to SR-A3, SR-A5 localizes to the regions near the ER and TGN in CHO cells (Jiang and others 2006). SR-A5 has the capacity to bind bacteria, viral dsRNA, but not modified LDLs (Jiang and others 2006; DeWitte-Orr and others 2010). SR-A5 also functions as a receptor

of serum ferritin, and thus participates in transferrin-independent iron uptake (Li and others 2009). Recent studies have identified the possible role of SR-A3 and SR-A5 to act as tumor-suppressor genes. Low SR-A5 levels in human hepatocellular carcinoma (HCC) have been linked with the progression of the disease (Huang and others 2010b). In contrary, elevating the SR-A5 levels in HCC cells *in vitro* or *in vivo* mice model significantly suppressed the colony formation of the cells and tumor growth in mice. The molecular mechanism by which SR-A5 inhibit tumorigenesis was found to rely on its ability to bind focal adhesion kinase, and to suppress its phosphorylation (Huang and others 2010b). Therapeutic potential of SR-A5 was also confirmed in glioma and lung cancer models *in vivo* (Yan and others 2012). Over-expression of SR-A3 in mice with induced prostate cancer has resulted in decreased metastasis and inhibition of tumor growth (Yu and others 2006; Zhu and others 2009). In contrary, high SR-A3 levels in ovarian cancer are related with the progression of cancer (Bock and others 2012).

The ability of cA-SR receptors to bind NAs made them an exceptionally important in regard of this thesis. Members of cA-SRs have been shown to bind polyribonucleotides (Pearson and others 1993), unmethylated CpG oligonucleotides (Jozefowski and others 2006), dsDNA (DeWitte-Orr and others 2010), dsRNA (Limmon and others 2008; Dieudonne and others 2012), synthetic dsRNA (Nellimarla and others 2015), polyvalent oligonucleotide-functionalized gold nanoparticles (Patel and others 2010), and spherical nucleic acid nanoparticles (SNA) (Choi and others 2013). Importantly, we demonstrated the involvement of two members of cA-SR, SR-A3 and SR-A5, in the uptake of PF14-SCO nanocomplexes (Ezzat and others 2012). Pre-treatment of HeLa pLuc705 cells with cA-SR competitive ligands poly-I and dextran sulfate, or simultaneous down-regulation of SR-A3 and SR-A5 with siRNAs, substantially decreased the binding and internalization as well as the biological effect of PF14-SCO nanoparticles (Ezzat and others 2012). Furthermore, using transmission electron microscopy (TEM), we demonstrated that both cA-SRs can directly bind PF14-SCO nanocomplexes on the plasma membrane, and the colocalization was retained even after internalization inside the vesicles (Juks and others 2015).

SRs are known to bind anionic ligands, however, the negative charge is not the only feature necessary for binding to cA-SRs. Prof. M. Krieger and his workgroup showed that cA-SRs bind and internalize with high efficiency negatively charged polyribonucleotides poly-I and poly-G. However, negatively charged but structurally distinct molecules poly-A and poly-C are not recognized by cA-SRs (Pearson and others 1993). It was found that poly-I and poly-G aggregate under physiological conditions and acquire a four-stranded helical structure. Poly-C and poly-A, in contrary, cannot assemble into such structures, suggesting that only aggregated forms of polyribonucleotides can bind cA-SRs (Pearson and others 1993). The requirement for distinct ligand structure was recently demonstrated by Choi and coworkers when studying the uptake of negatively charged SNAs (Choi and others 2013). The authors demonstrated

that SNA with three-dimensional architecture are efficiently recognized and endocytosed by cA-SRs, however, their linear counterparts did not show any affinity to cA-SRs (Choi and others 2013), demonstrating that in addition to negative charge the structure of the ligand is also a decisive feature to be recognized by cA-SRs.

The mechanism by which SRs gain access into cells relies mainly on endocytosis, and all the major endocytic pathways have been implicated in their uptake, i.e clathrin-mediated endocytosis (Chen and others 2006; Lunov and others 2011), caveolin-mediated endocytosis (Zhu and others 2011; Choi and others 2013), and macropinocytosis (Yao and others 2009). Following the internalization, receptor-ligand complex is thought to be dissociated under low-pH of endosomes, and the ligands are destined for degradation. Receptors are probably targeted to the plasma membrane through TGN to aid in further ligand bindings (Murphy and others 2005).

2. AIMS OF THE STUDY

The aim of the current thesis was to examine the cellular uptake and intracellular trafficking of negatively charged PF14-SCO nanocomplexes with emphasis to class A scavenger receptors.

Specific aims:

1. To study the potential role of scavenger receptors in the uptake of PF14-SCO nanocomplexes, by utilizing pharmacological inhibitors and RNAi in combination with microscopic approaches (Paper I, II and III).
2. In order to provide more insight into role of cA-SRs, to determine whether the PF14-SCO nanocomplexes can directly interact with respective cA-SRs receptors on the plasma membrane (Paper II and III).
3. To elucidate the particular endocytosis mechanism(s) responsible for the uptake and functionality of PF14-SCO nanocomplexes, as well as to examine the intracellular trafficking of the nanocomplexes (Paper II and III).
4. Since SR-A3 and SR-A5 are not present on the cell surface of control cells, to unravel the mechanisms that trigger the recruitment of SR-A3 and SR-A5 from the cell cytoplasm to the plasma membrane (Paper IV).

3. METHODOLOGICAL CONSIDERATION

The methods used in the current thesis are described in detail in the respective papers included in the current thesis, and the following section only provides a brief overview of selected methods.

3.1 Cell lines

Throughout the current thesis, the majority of the experiments were conducted on human cervical cancer cells, HeLa pLuc705 cell line, a kind gift from Prof. R. Kole (Kang and others 1998). HeLa pLuc705 cells are stably transfected with luciferase encoding plasmid that contains a mutated β-globin intron. The mutation causes aberrant splicing of luciferase mRNA and loss of functional luciferase. However, targeting of splice-correcting oligonucleotides (SCOs) complementary to the mutated region redirects the splicing-machinery and restores the production of the protein (Figure 3) (Kang and others 1998). HeLa pLuc705 cell line is a commonly used *in vitro* model to evaluate the delivery efficiency of SCOs by CPPs in terms of splice-correction. In order to place our results in the broader context and to enable comparison with previously published results, the respective cell line was used in current study. Additionally, the human T lymphocyte cell line, Jurkat cells were included into Paper IV as control for recruitment of receptors to the plasma membrane.

All the cell-lines were grown at 37°C in humidified atmosphere containing 5% CO_2 with the appropriate culture media supplemented with 10% fetal bovine serum, and 200 U/mL penicillin and 200 μg/mL streptomycin.

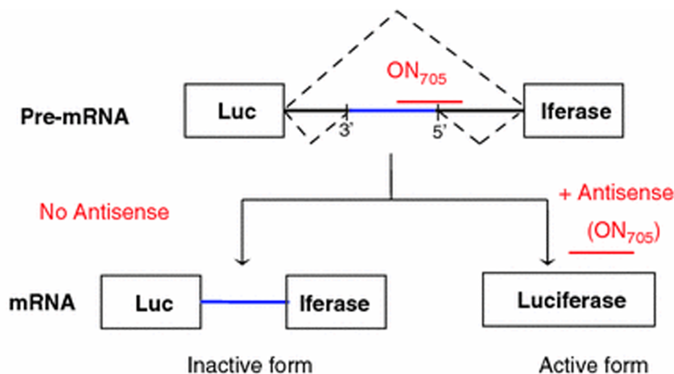

Figure 3. Schematic illustration of splice-correction assay (adopted from Said Hassane and others 2010). Luciferase reporter gene is interrupted by insertion of human β-globine intron (blue) that carries point mutation at nucleotide position 705. This mutation causes abberant splicing and loss of functional protein. Nuclear delivery of specific oligonucleotide, ON_{705}, aigainst the mutated region redirects splicing-machinery and restores production of functional luciferase protein.

3.2 Used CPPs and oligonucleotides

The current thesis aimed to elucidate the mechanisms of one of the effective SCO delivery vector, PepFect14 in more detail. The design of PF14 is based on stearyl-TP10 in which the lysines are replaced by ornithines and isoleucines are substituted with leucines. The idea of such modifications was based on earlier reports where the poly-L-ornithines possessed superior transfection efficiency over the poly-L-lysine based systems in terms of better nucleic acid binding and stable nanoparticle formation (Ramsay and Gumbleton 2002). Furthermore, as ornithine is a nonstandard amino acid, thus it would be less prone to degradation by serum proteases, and therefore would also improve the stability of the nanocomplexes in the presence of serum.

Additionally, in Paper IV also other amphipathic CPPs TP10, PF6, CADY and pepnetratin, and cationic R9 (Table 2) were included to the study to assess their potency to recruit SR-A3 and SR-A5 to the plasma membrane.

The phosphorothioate 2` OMe (PS-2`-OMe, 5′-CCU CUU ACC UCA GUU ACA-3′) SCO was used as a nucleic acid cargo molecule whose effect can be quantified in HeLa pLuc705 cells. Depending on the assay, the 5′ end of the oligonucleotide was labeled either with Cy5 or nanogold tag (d=1.4 nm), or was left unmodified.

Table 2. Examined CPPs and their sequences.

CPP	Sequence	Reference
PF14	Stearyl-AGYLLGKLLOOLAAAALOOLL-NH2	Ezzat and others 2011
TP10	AGYLLGKINLKALAALAKKIL- NH2	Soomets and others 2000
PF6	Stearyl-AGYLLGK*INLKALAALAKKIL-NH2	El Andaloussi and others 2011
CADY	GLWRALWRLLRSLWRLLWRA-cysteamide	Crombez and others 2009
Penetratin	RQIKIWFQNRRMKWKK- NH2	Derossi and others 1994
R9	RRRRRRRRR	Mitchell and others 2000

* Four molecules of chloroquine analogue coupled at this position via lysine tree.

3.3 Formation of non-covalent nanocomplexes

There are two main strategies to couple ONs with delivery vectors: a covalent conjugation and non-covalent complexation. The non-covalent strategy is suitable for negatively charged ONs, and therefore throughout the studies presented

in the current thesis, we used the non-covalent association strategy that relies on electrostatic and hydrophobic interactions of positively charged CPPs and negatively charged ONs to spontaneously form nanocomplexes.

The noncovalent complexes were formed in a similar manner throughout the study. Briefly, PF14 and PS-2`- OMe SCOs were co-incubated in MilliQ water at molar ratio (MR) 5 to achieve 1 μM and 200 nM final concentrations, respectively. Complexes were formed at room temperature for 30–60 min, and subsequently, nanocomplexes were directly added to the cells grown in the presence or absence of serum and incubated for appropriate time periods.

3.4 Methods to study the involvement of cA-SRs in the cellular uptake of PF14-SCO nanocomplexes

3.4.1 Pharmacological inhibition of SR-A3 and SR-A5, and RNAi

Scavenger receptors comprise a large supergroup of receptors with the ability to bind poly-anionic ligands (Pearson and others 1993). In order to assess whether the cA-SRs participate in the uptake of negatively charged PF14-SCO nanocomplexes, the effect of cA-SRs specific inhibitory ligands polyinosinic acid (poly-I), dextran sulfate and fucoidan was examined on the cellular uptake and functionality of the nanocomplexes. The respective controls, polycytidylic acid (poly-C), chondroitin sulfate and galactose are structurally similar to inhibitory ligands, although with no affinity against cA-SRs. The effect of the inhibitors on the association with cells and cellular uptake, or splice-correction activity and luciferase expression was examined with TEM or luminometer, respectively.

Since the aforementioned inhibitors are not exclusively selective, we also used RNAi to down-regulate SR-A3 and SR-A5. siRNAs for respective receptors were used as a mixture at 25 nM concentration of each, and were transfected into cells using Lipofectamine RNAiMAX. 24 or 48 h after the transfection with siRNAs, unlabeled or Cy5 labeled PF14-SCO nancomplexes were added to the cells and the enzymatic activity of luciferase, or cellular uptake of the nanocomplexes was examined with luminometer, or FACS or fluorescence plate reader, respectively.

3.4.2 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) is quick and informative approach to examine the colocalization of the molecules of interest, and follow the trafficking and cellular localization of the nanoparticles in relation to cellular structures by using fluorescently labeled probes in both live and fixed cells. Therefore, in the current thesis CLSM was exploited for immunofluorescence experiments with specific antibodies in fixed cells, or to follow the trafficking of the nanocomplexes in relation to intracellular organelles in live cells. For immunofluorescence experiments, after treatment of cells with nanocomplexes

or SCOs for appropriate time, the cells were fixed with 4% paraformaldehyde (PFA) fixative in PBS and permeabilized with Triton X-100 to allow the antibodies to reach their intracellular antigen. Thereafter, the cells were treated with respective primary and secondary antibody carrying fluorescent dyes. Finally, to preserve the morphology of the cells, cells were mounted with Fluoromount G.

In order to retain the integrity of the plasma membrane and to visualize receptors only on the plasma membrane, cells were fixed with non-permeabilizing fixative by using 3% PFA in 0.1 M phosphate buffer (pH 7.4). The intactness of the plasma membrane after fixation was controlled either by DNA-stain DAPI or lysosomal protein LAMP-2 staining. If the cells were intact, DAPI could not pass the plasma membrane and stain the nucleus, and the integral LAMP-2 was not targeted to the plasma membrane.

The CLSM images were captured with Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Japan).

3.4.3 Transmission electron microscopy (TEM)

Although, CLSM is powerful and informative method for following the cellular localization and trafficking of the fluorescent molecules, the method still has some limitations. The resolution of optical microscopy does not allow to study the cellular structures and interactions of molecules in detailed manner. Moreover, fluorescent dyes are susceptible for photobleaching that might decrease the signal. The high magnification and resolution provided by TEM can solve the issues related with CLSM, and therefore in order to provide more detailed data about the interactions with cells, i.e. cellular localization and interactions with receptors, this technique was used throughout the current study. In the current thesis, we utilized two different TEM methodologies- “classical” TEM and immuno-TEM. For classical TEM analysis, we used nanocomplexes of PF14 with nanogold-labeled (d=1.4 nm) SCO. After the transfection of the cells with the nanocomplexes for appropriate time, cells were fixed with 2.5% glutaraldehyde. Due to the small size of the gold-tag (1.4 nm) it is not readily detectable by TEM, and thus the gold tag was enlarged by silver deposition for better visualization. After the silver enhancement, specimens were post-fixed with 1% OsO_4 and dehydrated with ethanol series. Finally, specimens were flat-embedded in epoxy resin and ultrathin sections from the resin were cut. Images were captured at 100 kV accelerating voltage on JEM-100S (JEOL, Japan) TEM.

To confirm the results obtained with confocal microscopy and in order to examine the interactions of SR-A3/SR-A5 and PF14-SCO nanocomplexes more precisely, we used the immuno-TEM and labeled the receptors and nanocomplexes with colloidal gold tags with different sizes. Such labeling allowed us to simultaneously detect the nanocomplexes and receptors, map their cellular distribution, and ascertain whether the nanocomplexes can associate with respective receptors directly, i.e. whether they reside in close proximity. Briefly, SCOs labeled with 10 nm colloidal gold were complexed with PF14, and the

receptors were labeled with the respective primary antibodies and secondary antibodies labeled with 6 nm colloidal gold particle. As 6 and 10 nm labels can be easily distinguished under TEM, no silver enhancement is required. In order to preserve the ultrastructure of the cells and the antigenicity of the antibodies, instead of classical fixation for TEM with 2.5% glutaraldehyde, we used milder PLP (paraformaldehyde-lysine-periodate) fixative and permeabilization with 0.01% saponin. Images were captured at 120 kV accelerating voltage on FEI Tecnai G2 Spirit (FEI, The Netherlands) TEM.

3.5 Methods to study the cellular uptake mechanism and intracellular trafficking of PF14-SCO nanocomplexes

3.5.1 Cellular uptake experiments

The most common methods to study the cellular uptake of nanocomplexes are the inhibition of respective pathways by pharmacological inhibition of endocytosis, or RNAi. In the current thesis we used the both methods. Among the pharmacological inhibitors, nystatin was used to inhibit the caveolin-dependent endocytosis, amiloride or EIPA to interfere with macropinocytosis and chlorpromazine to inhibit CME. The effect of the endocytosis inhibitors on the splice-correction activity of the nanocomplexes was examined by measuring the luciferase expression with luminometer. The effect of down-regulation of cavoeolin-1 with specific siRNA on the cellular uptake of Cy5 labeled PF14-SCO nanocomplexes was examined by using fluorescence plate reader.

In order to better visualize the induction of macropinocytosis and caveolin-dependent endocytosis, we analyzed the colocalization of Cy5-labeled PF14-SCO nanocomplexes with fluorescently tagged 70 kDa dextran in live cells; or staining caveolin-1 in fixed cells by specific antibody, respectively. All the images were captured with CLSM and quantified with FluoView1000 program (Olympus).

3.5.2 Intracellular trafficking of PF14-SCO nanocomplexes

In order to unravel the intracellular trafficking and destination of PF14-SCO nanocomplexes after internalization, we mapped the localization of nanocomplexes in relation to acidic/degradative vesicles, Golgi network and endoplasmic reticulum (ER) by fluorescence microscopy.

Trafficking of nanocomplexes or SCOs along the endo-lysosomal pathway was examined with pH sensitive probe LysoSensor DND. The fluorescence intensity of the probe rises gradually as the pH of the vesicle drops, and thus the change in fluorescence intensity allows us to estimate the acidity of the vesicles containing PF14-SCO nanocomplexes. Additionally, to assess whether the nanocomplexes are targeted to lysosomes, final destinations in endo-lysosomal

pathway, immunofluorescence experiment against lysosomal membrane protein LAMP-2 was conducted.

In order to determine whether the PF14-SCO nanocomplexes enter the recycling pathway for being exocytosed, we mapped their localization in relation to trans-Golgi network with a TGN46 specific antibody or fluorescently labeled ceramide in fixed and live cells, respectively. Localization of complexes to ER was mapped with fluorescently labeled ER marker Blue-White DPX.

4. RESULTS AND DISCUSSION

4.1 Involvement of scavenger receptors in the cellular uptake of PF14-SCO nanocomplexes (Paper I, III)

It has been expected that due to the cationic nature of the CPPs and its molar excess over ONs in nanoparticle formulation, the CPP-ON nanoparticles possess positive charge that enables interactions with negatively charged plasma membrane components and subsequent entry into cells in analogy with "naked" CPPs. Nevertheless, recently it was demonstrated that PF14-SCO nanocomplexes have negative zeta potential (Ezzat and others 2011). The anionic nature of the nanocomplexes should repel them from the plasma membrane, however, PF14-SCO nanocomplexes still exhibit high splice-correcting activity. This finding hints that the internalization of PF14-SCO might be assisted by some receptors. Therefore, in Paper I we aimed to elucidate the possible receptor/binding partners that facilitate the internalization of PF14-SCO nanocomplexes.

At first we aimed to examine the changes in zeta-potential in more detail in different environments. We found that in MilliQ water, where the nanoparticles are initially formed, the zeta-potential was highly dependent on the molar ratio of the nanocomplexes. At MR3 nanocomplexes were negatively charged, but at MR5 or MR10, nanocomplexes acquired nearly neutral or positive charge, respectively. However, in the presence of serum or in NaCl solution, nanoparticles were negatively charged at all MRs tested in this paper, suggesting that the negative zeta potential is not barely determined by negatively charged serum components, but rather by the salts and pH of the environment.

Further, we shifted our focus on the elucidation of the possible receptors that might contribute to the uptake of PF14-SCO nanocomplexes. One of the probable candidates were class A scavenger receptors with their ability to bind polyanionic ligands (Pearson and others 1993). Importantly, cA-SRs also exhibit promiscuous binding of variety of nucleic acids (Pearson and others 1993; DeWitte-Orr and others 2010; Patel and others 2010). First, we conducted a pilot experiment with well-known cA-SR inhibitory ligands poly-I, dextran sulfate and fucoidan, which binding to receptors should interfere with the association of other possible ligands. Poly-C, chondroitin sulfate and galactose were used as respective negative controls. These molecules are chemically related with inhibitory ligands, however, have no affinity for cA-SRs. Pre-incubation of HeLa pLuc705 cells with inhibitory ligands substantially decreased the splice-correction activity of PF14-SCO in a concentration-dependent manner. Poly-I and fucoidan at 25 µg/mL and 5 µg/mL concentration, respectively, almost completely blocked the activity of PF14-SCO nanocomplexes. The most potent inhibitory ligand was dextran sulfate, yielded in more than 80% of reduction in luciferase expression already as low as at 0.5 µg/mL concentrations, and at 5 µg/mL concentration the activity of PF14-SCO was completely abolished. Conversely, respective negative control compounds did not affect the uptake and functionality of the nanaocomplexes. Furthermore, to confirm the effect of the

cA-SR inhibitors on the cellular association and uptake of nanocomplexes, we used TEM. In control cells, PF14-SCO complexes were abundantly associated with cells and already after 1 h, nanocomplexes had entered the cells where they located mainly in endosomal vesicles. However, in concordance with splice-correction assay, pre-treatment of cells with dextran-sulfate almost completely blocked the association and internalization of the complexes to cells, whereas the pre-treatment with chondroitin sulfate did not affect the ability of complexes to bind and internalize to cells. Collectively, these results demonstrate that when cA-SRs are not available for ligand binding at the cell surface, i.e. the sites for ligand docking on the receptor are already occupied; association of PF14-SCO nanoparticles with cells and subsequent entry into cells are abolished.

4.1.1 SR-A3 and SR-A5 are the binding partners for PF14-SCO nanoparticles

After verifying that the cA-SR inhibitory ligands compete with and block the binding of nanocomplexes to cA-SRs, our next aim was to identify whether the HeLa pLuc705 cells express the cA-SRs at all, and if they do so, which type of cA-SRs participate in the uptake of PF14-SCO nanocomplexes. Using the real-time PCR, we found that only two members of cA-SRs, SR-A3 and SR-A5, are expressed in HeLa pLuc705 cells. The participation of the respective receptors in functionality of nanocomplexes was further verified by RNAi experiment. We found that simultaneous knock-down of SR-A3 and SR-A5 substantially decreased the splice-correcting activity of the complexes, yielding in slightly more than 50% of reduction in luciferase expression. However, the pharmacological cA-SR inhibitors yielded in substantially higher inhibition rates. Such moderate effect of cA-SR downregulation to cell entry of ONs has also been observed in earlier studies (Patel and others 2010; Saleh and others 2006), and might suggest that multiple receptors with overlapping functions participate in uptake of nanocomplexes. We speculate that the residual activity of nanocomplexes after RNAi might be due to rapid regeneration of the receptors pool after down-regulation, or other scavenger or pattern recognition receptors might participate in the uptake. Recently, it was found that the uptake of PF14 analogue, PF32, into brain endothelial cells and across the blood-brain barrier model besides to SR-A3 and SR-A5, is also dependent on SR-B1 receptors (Srimanee and others 2016). The possible involvement of SRs form other classes during the uptake of PF14-SCO needs still to be further tested.

In brief, the results from inhibition assay and RNAi strongly support the involvement of SR-A3 and SR-A5 in the uptake of PF14-SCO nanocomplexes.

4.1.2 PF14-SCO nanocomplexes colocalize with SR-A3 and SR-A5

In order to assess whether the nanoparticles can associate with respective receptors, we analyzed the colocalization of SR-A3/-A5 and PF14-SCO nanocomplexes by immunofluorescence assay. Here, the Cy5 labeled SCOs were complexed with PF14, and SR-A3 and SR-A5 were visualized with SCARA3 and SCARA5 antibodies and respective fluorescently-labeled secondary antibodies. After 1 h of incubation, the nanocomplexes showed prevalent colocalization with SR-A3 and SR-A5 on the plasma membrane, however, the colocalization was found to be more prominent under 4ºC, a condition that avoids the endocytosis. Moreover, the colocalization was retained even inside the cells in vesicles, corroborating the fact that SR-A3 and SR-A5 participate in the uptake of the nanocomplexes. Still, the overlap was not complete and the SR-A´s signal appeared only at the edges of the nanoparticles that might be due to the large size of the complexes and localization of receptors in one site.

Surprisingly, the naked SCO at high concentration (1 µM), but not at nanomolar range (200 nM), showed also a substantial colocalization with both receptors. Moreover, the naked SCO at as high as 5 µM concentration induced more than 3-fold increase in luciferase expression compared to untreated cells. However, the naked SCO is still by far less efficient in switching splicing than in complexes with PF14 that induced more that 120-fold increase in luciferase activity compared to untreated cells. As the binding with cA-SRs is also dependent on the structure of the ligand, it is likely that at higher concentrations SCOs starts to form aggregates (Ezzat and others 2015), which is an essential structural feature for binding to cA-SRs (Pearson and others 1993).

Still the question remains, what gives the overall negative charge of the nanocomplexes? The nanoparticles obtained negative zeta-potential also in the absence of serum, thus, the negative charge is not a result of adsorption of anionic serum proteins, but must be determined by other factors. Previously, it has been shown that the binding of oligonucleotide-functionalized gold nanoparticles (DNA-Au-NP) to cA-SRs is highly dependent on the density of DNA exposed on the surface of nanoparticles (Patel and others 2010). During the examination of the effect of SR inhibitory ligands on nanoparticles with different amount of DNA molecules, the authors revealed that reducing the density of DNA on nanoparticles reduced the inhibitory effect as well as the uptake of the nanocomplexes. They suggested that the dense monolayer of DNA around the nanoparticle mimics the helical structure of cA-SR inhibitory ligands, i.e. poly-I (Pearson and others 1993), and thus facilitates association with scavenger receptors (Patel and others 2010). Similarly, the dense oligonucleotide shell of spherical nucleic acids (SNAs) contributes to the uptake via cA-SRs (Choi and others 2013). According to published papers and our results, it is tempting to speculate that during the nanoparticle formulation, SCOs are partially exposed on the surface of the nanoparticles that gives the overall negative charge and facilitates recognition by SR-A3 and SR-A5 receptors.

4.2 Cell entry mechanism and intracellular trafficking of PF14-SCO nanocomplexes (Paper II, III)

In paper II, we focused on the uptake mechanisms and intracellular trafficking of PF14-SCO nanocomplexes. There is no unique or universal mechanism for cellular uptake of CPPs, and frequently more than one internalization pathway is exploited simultaneously. Moreover, SRs can induce their internalization via all common endocytic pathways. In order to elucidate the exact uptake mechanism and subsequent cellular destination for PF14-SCO nanocomplexes, we used a combination of confocal- and electron microscopy techniques to map their localization in relation to cellular structures.

4.2.1 Binding to SR-A3 and SR-A5 induces uptake via macropinocytosis and caveolin-dependent endocytosis

In Paper I, using TEM, we demonstrated that after internalization the PF14-SCO nanocomplexes reside mainly in vesicular structures, suggesting that the internalization of the nanocomplexes occurs mainly via endocytosis, however, we did not elucidate the exact mechanism. The most common methods to determine the endocytic pathway(s) along which the material is internalized into cells relies mainly on different pharmacological inhibitors of endocytosis, or RNAi to knock-down specific proteins involved in respective pathways. Since SRs can induce their entry into cells via all endocytic pathways described so far, i.e. CME, caveolin-dependent endocytosis and macropinocytosis, and in order to determine which one(s) contribute the activity of PF14-SCO nancomplexes, we first utilized a set of pharmacological inhibitors. We ascertained that the inhibition of macropinocytosis with EIPA reduced the splice-correction activity of nanocomplexes by about 80%, and nystatin, an inhibitor of caveolin-dependent endocytosis, yielded in 30% drop of luciferase activity. However, inhibition of CME, the main entry route for receptor-mediated endocytosis, did not affect the activity of the nanocomplexes at all. To further confirm the minor role of caveolin-dependent entry route, we knocked down caveolin-1 and assessed its effect on the uptake of PF14-SCO nanocomplexes. In concordance with inhibitory assay, down-regulation of caveolin-1 substantially reduced the uptake of the nanocomplexes of PF14 and Cy5 labeled SCOs, and yielded in 40% of reduction compared to untreated cells, or cells treated with non-targeted siRNA. Since the inhibition of endocytic routes with pharmacological inhibitors is not very specific, and in order to further validate the results, colocalization of PF14-SCO nanoparticles with 70 kDa dextran and caveolin-1, markers for macropinocytosis and caveolae respectively, was examined. The nanocomplexes showed substantial colocalization with both markers at the plasma membrane as well as inside the cells in vesicular structures, corroborating the involvement of macropinocytosis and caveolin-dependent endocytosis in their uptake.

Macropinocytosis and caveolin-dependent endocytosis are also prevalent uptake routes for PF14-pDNA nanocomplexes (Veiman and others 2013), as well as for other TP10 analogue, NickFects with pDNA (Arukuusk and others 2013).

Collectively, our results demonstrate that at least two cell entry mechanisms, macropinocytosis and caveolin-mediated endocytosis, are active in parallel during the uptake of PF14-SCO.

4.2.2 PF14-SCO nanocomplexes do not enter the recycling pathway

After being internalized, endocytosed material is being subsequently sorted and directed to its final destination mainly via two major intracellular pathways: endosomal recycling pathway that directs the cargo back to the plasma membrane, or endo-lysosomal pathway to target the material to lysosomes for degradation. The exact internalization pathway might also influence the final destination of the payload. For example, CT and SV40 that are internalized via caveolae are directly trafficked to the Golgi apparatus and ER (Pelkmans and others 2001; Le and Nabi 2003). An interesting finding was recently published by Sahay et al, who showed that after internalization via macropinocytosis siRNA-LNP nanoparticles are directly targeted to late endosomes where siRNA is dissociated from the LNP, and is subsequently recycled back to the plasma membrane (Sahay and others 2013). Moreover, after internalization, cA-SRs reach the TGN, where they are packed into secretory vesicles and are trafficked to the plasma membrane (Mori and others 1994). In order to assess the role of recycling pathway, the localization of Cy5 labeled SCO and PF14 nanocomplexes were mapped in relation to Golgi apparatus either with antibody-staining against TGN46 (trans-Golgi network protein), or fluorescently labeled ceramide in time dependent manner. Here, we found that only a negligible fraction of internalized nanocomplexes colocalized with TGN46 or ceramide after 30 min, and in time after 1 h, the number was even decreased, and only about 1% of the total internalized complexes showed colocalization with Golgi markers. Thus, PF14-SCO nanocomplexes avoid targeting to Golgi apparatus and recycling pathway after internalization, and other pathways might contribute to the cellular trafficking of the nanocomplexes. Additionally, we also did not observe any colocalization of nanocomplexes with ER marker.

4.2.3. PF14-SCO nanocomplexes avoid targeting to lysosomes

The other major intracellular route is the endo-lysosomal pathway that targets the endocytosed material through gradually acidified vesicles, and it finally terminates in lysosomes where the payload is degraded. The acidity of the intracellular vesicles can be monitored with pH sensitive probe LysoSensor. LysoSensor enters the cells via endocytosis and its fluorescence intensity depends on the pH of the vesicles, i.e. the more acidic vesicles emit brighter fluorescence

signal than vesicles with neutral or slightly acidic pH. Trafficking of the Cy5 labeled PF14-SCO nanocomplexes through endo-lysosomal pathway was monitored in time and the results were compared with the naked SCO. After 1 h, majority of the internalized complexes were mainly located in cell periphery in rather non-acidic vesicles that lacked LysoSensor signal. However, further in time, the complexes-containing vesicles had increased in size and moved deeper into the cells, but still the majority of them did not colocalize with LysoSensor, and only very rare colocalization with LysoSensor-positive vesicles was observed after 4 h. Moreover, no significant colocalization was detected for lysosomal protein LAMP-1 and PF14-SCO nanocomplexes over 4 h examination time, suggesting that PF14-SCO avoid targeting to acidic lysosomes, and they are mainly retained in non-acidic or slightly acidic early/late endosomes, at least during the time window examined in the current thesis. This result is also in good concordance with the results obtained with pharmacological inhibitors of endocytosis. The endocytic vesicles derived from caveolae are considered to give rise to intracellular vesicles with rather neutral pH due to the slower maturation rate; and thus, this might provide an opportunity to retain the functionality of the SCO and promote the endosomal escape before reaching the highly acidic lysosomes. These results are also well in line with earlier results from our laboratory. Räägel et al showed that the internalization of CPP-protein complexes, specifically TP-avidin constructs are partially sequestered into non- or slightly acidic vesicles (Räägel and others 2009). Here, the authors also suggested that the vesicles with slightly acidic interior were probably derived from caveolae-dependent pathway, as the caveolin-dependent entry route is the main uptake mechanism for TP-protein complexes (Säälik and others 2009). In analogy with this and our current results, also spherical nucleic acids (SNA) that enter the cells via cA-SR and caveolin-dependent manner (Choi and others 2013) are not targeted to acidic lysosomes, but rather reside in slightly acidic early/late endosomes, even over 24 h study-time (Wu and others 2014).

However, unlike to nanocomplexes, naked SCOs at 1 μM concentration were trafficked to acidic vesicles already within 2 h, as confirmed by LysoSensor and LAMP-1 staining. Further in time, even higher proportion of the SCOs were sequestered into acidic vesicles, and after 4 h the majority of internalized SCOs showed intense colocalization with LAMP-1.

In order to pinpoint the time frame after which the nanoparticles start to escape from endososomes, we mapped their cellular localization in time by using TEM. We found out that already after 30 min of incubation, some of the nanocomplexes had entered the cells and located in endosomal vesicles, most commonly in MVBs. Further in time, the number of the nanocomplexes that had entered the cells was even increased, and starting from 1 h we could also detect vesicles with discontinuous membranes and escape of the nanocomplexes to the cytosol. However, over 4 h of incubation time only few gold-labeled SCOs had reached the nucleus where they can redirect splicing. In order to estimate the efficiency of endosomal escape, we counted the number of total internalized nanocomplexes and the fraction that reached the cytosol. We found

that the vast majority of the nanocomplexes remain entrapped in endosomes, and the cytosolic fraction accounts only for 8% of the total internalized nanocomplexes, corroborating data from earlier reports and suggesting that only tiny amount of nanocomplexes could reach the cytosol, and the endosomal escape is still the major hurdle in ON-based therapies (Gilleron and others 2013; Sahay and others 2013; Wittrup and others 2015). Even though, in Paper I we delineated that naked SCO at as high as 5 μM concentration is able to promote splice-switching and restoration of luciferase expression, still the small amount of SCO that reached the cell interior remained entrapped in endosomes and no endosomal escape or nuclear localization was detected by TEM after 4 h.

Collectively, these results suggest that PF14 helps to interfere with the acidification of the endosomes and promotes endosomal escape of the SCO.

4.2.4 SR-A3 and SR-A5 are recruited to the plasma membrane after exposure to PF14-SCO nanocomplexes

In parallel with the examination of the uptake and intracellular trafficking of PF14-SCO nanocomplexes, we also aimed to analyze their interactions with receptors more precisely, i.e. we aimed to find out whether the nanocomplexes can directly bind with SR-A3 and SR-A5. Although, in Paper I we already demonstrated the colocalization between the receptors and nanocomplexes by confocal microscopy, however, the resolution provided by fluorescence microscopy is by far too low to provide detailed information about their actual interactions. Therefore, to solve the issue, we used high resolution TEM to examine the interactions in more detail. For the analysis we used two gold labels with different sizes: the receptors were labeled with respective primary antibodies and secondary antibodies that carried 6 nm colloidal gold tag, and nanocomplexes with 10 nm gold tag. After 1 h of incubation, we found that both, SR-A3 and SR-A5 reside in close proximity to nanocomplexes on the plasma membrane as well as after internalization in endosomal vesicles, suggesting that nanocomplexes can directly interact with respective receptors. Moreover, we could detect only 2–3 gold-labeled receptors near the complexes. This result is in good concordance with confocal microscopy results from Paper I where we detected only partial overlap of nanocomplexes with receptors, suggesting that only a few receptors are required for efficient association and internalization of the PF14-SCO nanocomplexes.

To our surprise, we discovered that SR-A3 and SR-A5 are not exposed on the plasma membrane of uninduced control cells, but are recruited there after exposure to PF14-SCO nanocomplexes. Still, their intracellular concentration in control cells is rather high, where receptors are mainly found in small vesicles near the plasma membrane, or more centrally in the cell cytoplasm. This phenomenon was also confirmed by confocal microscopy. Two of the earlier reports have shown that in addition to the temporal presence on the plasma membrane, SR-A3 and SR-A5 are mostly localized inside the cells near the ER-

Golgi complex region in HeLa and CHO cells, respectively (Han and others 1998; Jiang and others 2006). Many other reports have also demonstrated that in the presence of ligands, cA-SRs are overexpressed (Mietus-Snyder and others 2000; Fukuhara-Takaki and others 2005). However, these papers do not shed light to their initial localization, i.e. whether the receptors were constantly present on the plasma membrane and the presence of ligands just recruits more of them there, or receptors are absent from the plasma membrane and are recruited there after ligand induction. In the present study we showed for the first time that PF14-SCO nanocomplexes induce the translocation of SR-A3 and SR-A5 from their cytoplasmic localization to the plasma membrane of HeLa pLuc705 cells to assist in binding and internalization of the nanocomplexes.

4.3 Mechanisms behind cell surface recruitment of SR-A3 and SR-A5 (Paper IV)

In Paper IV, we aimed to further delineate the mechanisms behind the cell surface recruitment of SR-A3 and SR-A5. The majority of the papers published in the field of SRs are mainly concentrated on their ability to bind and internalize different ligands, or the consequences of their down or up-regulation. Still, only few studies have addressed the mechanisms that regulate the cellular localization of SR-As. In this paper we concentrated on the role of calcium and PI3K, well-known regulators of vesicular trafficking and exocytosis, in the cell surface recruitment of SR-A3 and SR-A5.

4.3.1 SR-A3 and SR-A5 are recruited to the plasma membrane by CPPs

Recently, an interesting discovery was made by Lindberg and co-workers, who demonstrated that when the binding sites of SR-A3 and SR-A5 are blocked with inhibitory ligands, the uptake of fluorescently labeled PF14 is diminished (Lindberg and others 2015), suggesting that PF14 on its own can interact with these receptors. To our surprise, we found that PF14 alone is also able to trigger the translocation of receptors from the cytoplasm to the cell surface, contradicting the fact that SRs can bind only anionic ligands. Moreover, similar phenomenon was also observed with other amphipathic CPPs like TP10, PF6, CADY and penetratin, as well as with cationic CPP R9. Our workgroup has previously demonstrated that during the cellular uptake of amphipathic CPPs such as MAP and TP, the plasma membrane is temporarily reorganized at the level that enables the influx of calcium ions into cells which in turn activate the membrane repair responses (Lorents and others 2012). Since TP10, the parent peptide of PF14, and PF14 itself are membrane-active peptides, we investigated, whether the mobilization of SR-A3 and SR-A5 from their intracellular locations to the cell surface is caused by the temporal breaches of the lipid bilayer induced by PF14. Moreover, recently we showed that not all the CPP molecules

are associated with ONs into nanoparticles (Arukuusk and others 2013), but there is also a fraction of free peptide that might concentrate on the plasma membrane and cause its permeabilization. In order to assess the effect of PF14 to the integrity of the plasma membrane, we examined whether PF14 is able to induce the plasma membrane repair response. Plasma membrane repair response is activated by influx of calcium ions through the damaged site of the plasma membrane that increases the cytosolic calcium concentration, and subsequently induces the exocytosis of intracellular vesicles, and usually the membranes of lysosomes are used for the replacement of damaged regions of the plasma membrane (Jaiswal and others 2002). Thus, detection of the lysosomal protein LAMP-2 on the plasma membrane allowed us to estimate the integrity of the plasma membrane. As a result, we confirmed that PF14 did not interfere with the integrity of the plasma membrane at the level to induce influx of calcium ions and subsequent recruitment of LAMP-2 to the plasma membrane, even at as high as 4 μM peptide concentration. Collectively, this excludes the possibility that SR-A3 and SR-A5 are mobilized to the plasma membrane as a result of the damaged membrane, but rather is more indirectly regulated process.

4.3.2 Extra- and intracellular calcium ions are essential for both, the cell surface recruitment of SR-A3 and SR-A5 and internalization of PF14-SCO nanoparticles

Still, the calcium ions are ubiquitous intracellular signaling molecules that have central role in a multiple signal transduction pathways that control a variety of cellular processes, among which the regulation of intracellular membrane trafficking is the most relevant regarding this study. Calcium concentration is kept relatively low in intracellular environment, however, its effect on multiple of processes reveals when its concentration increases. The most well-studied calcium-dependent membrane fusion process is regulated exocytosis that occurs in neuronal cells in response to high intracellular calcium concentration to target the receptors to the plasma membrane, or to secrete hormones and neurotransmitters into extracellular space (Burgoyne and Morgan 1998). However, calcium-dependent exocytosis also occurs in non-secreting cells (Jaiswal and others 2002; Coorssen and others 1996; Burgoyne and Clague 2003). For example, calmodulin, a calcium-binding protein, is required for transferrin receptor recycling (Apodaca and others 1994; Hunziker 1994), suggesting the role of calcium to trigger the trafficking of the receptors between the cell interior and the plasma membrane. Since calcium plays an essential role in intracellular membrane trafficking, we hypothesized that the calcium ions could also trigger the trafficking of SR-A3 and SR-A5 and their insertion into the plasma membrane. The requirement for calcium ions for the cell surface recruitment of receptors and biological activity of the nanocomplexes was analyzed by depletion of calcium ions from the extra- or intracellular environment with calcium chelators EGTA or BAPTA-AM, respectively. Indeed, pre-treatment of

HeLa pLuc705 cells with EGTA or BAPAT-AM before the addition of PF14-SCO nanocomplexes substantially impeded the trafficking of receptors form intracellular pool to the plasma membrane. This effect was even higher under conditions where extra- and intracellular calcium ions were depleted simultaneously. Depletion of calcium not only reduced the number of cell surface receptors, but also decreased the number of nanoparticles interacting with the cells, as well as reduced the luciferase activity of PF14-SCO nanocomplexes. EGTA and BAPTA-AM treatment reduced the luciferase activity by about 60% and 30%, respectively. These results demonstrate that calcium ions are critical regulators that control the cellular localization of SR-A3 and SR-A5. Still, elevation of intracellular calcium concentration with ionophore on its own was not sufficient to trigger the translocation of SR-A3 and SR-A5 to the plasma membrane, suggesting that rather quick and local increase in calcium concentration triggers the mobilization of receptors to the plasma membrane. It is also possible that calcium ions are not the sole players in this process, and probably other regulators are necessary as well.

4.3.3 Recruitment of SR-A3 and SR-A5 to cell surface is dependent on PI3K and actin cytoskeleton

Phosphatidylinositol-3 kinase (PI3K) activity is required for regulation of various cellular processes, including endocytosis, intracellular vesicle trafficking, and exocytosis (Corvera 2001; Lindmo and Stenmark 2006). Numerous studies have demonstrated that PI3K activity is crucial for cell surface localization of various transmembrane receptors, for example GLUT-4 and transferrin receptors (Yang and others 1996; Spiro and others 1996; van Dam and others 2002). Moreover, the activity of PI3K is necessary for SR-A1 localization to the cell surface during macrophage adhesion, as demonstrated by using PI3K specific inhibitors wortmannin and LY294002 treatment (Nikolic and others 2007; Cholewa and others 2010). Furthermore recruitment of class B scavenger receptor SR-B1 from cytoplasmic pool to the plasma membrane in adipocytes and hepatocytes requires active PI3K (Tondu and others 2005; Shetty and others 2006), suggesting that PI3K activity is crucial for targeting of different SRs to the plasma membrane. Inspired by these results, we hypothesized that the activity of PI3K is also essential for regulation of SR-A3 and SR-A5 levels on cell surface. Treatment of cells with wortmannin or LY294002 significantly decreased the cell surface recruitment of respective receptors as well as reduced the splice-correction activity of the nanocomplexes, yielding in about 20% and 30% reduction of luciferase activity, respectively. These results demonstrate that activation of PI3K is also required for the regulation of SR-A3 and SR-A5 receptors localization. Moreover, PI3K activity in cells can be controlled by calcium ions (Danciu and others 2003; Liu and others 2007) that we also discovered to be crucial for the trafficking of SR-A3 and SR-A5 from cytoplasm to cell surface. PI3K is also an important regulator of actin cytoskeleton

and one of the key regulators in macropinocytosis (Clague and others 1995; Araki and others 1996), the latter of which is the major internalization mechanism for PF14-SCO nanocomplexes.

In addition to calcium ions and PI3K activity, we delineated that the efficient translocation of SR-A3 and SR-A5 to the plasma membrane relies on intact actin cytoskeleton- disruption of the actin cytoskeleton with Lantruculin B or Cytochalasin D exerted a negative effect on the cell surface recruitment of the receptors. However, microtubules did not seem to be important, since their destabilization did not decrease the number of SR-A3 and SR-A5 on the plasma membrane. Collectively, our results demonstrate that the recruitment of SR-A3 and SR-A5 to the cell surface is highly regulated process that requires calcium ions, active PI3K and intact actin cytoskeleton.

4.3.4 Localization of SR-A3 and SR-A5 to cell surface, but not internalization is dependent on serum proteins

To our surprise we also found that in the presence of serum higher amount of the SR-A3 and SR-A5 receptors are targeted to the plasma membrane after nanoparticle treatment compared to the serum-free conditions. Negatively charged molecules are abundant in serum and could be possible ligands for SRs, and thus, this might help to explain why in the presence of serum more of SR-A3 and SR-A5 are found on the plasma membrane. However, with or without the serum, in Paper I we showed that PF14-SCO nanoparticles possess a negative net charge, -12.1 ± 1.41 in serum-containing media, and -14.62 ± 1.49 in the absence of serum (Ezzat and others 2012), suggesting that the negative charge is not determined by anionic serum components. Besides, nanoparticles in serum-free media produced slightly higher splice-correction effect than in serum containing media. However, the size of the nanocomplexes differs greatly depending on the used media. In the absence of serum, nanoparticles are much bigger than in serum-containing media (623.16 ± 79.69 in the absence of serum and 363.8 ± 52 in the presence of serum as measured by dynamic light scattering), suggesting that adsorption of serum proteins onto nanoparticles prevents the formation of agglomerates and thus, might help to pack the PF14-SCO nanocomplexes into smaller particles and mask their surface. Thus, one might speculate that the bigger particles are more prone to interfere with the integrity of cellular membranes, and thereby promote their internalization/endosomal escape more easily. Additionally, we also determined that independently of the presence or absence of serum, the nanocomplexes follow the same cell-entry routes: macropinocytosis and caveolae-mediated endocytosis. Although, less receptors were targeted to the plasma membrane by PF14-SCO nanocomplexes under serum-free conditions, knock-down of SR-A3 and SR-A5 by siRNA suppressed the uptake of the nanocomplexes in both media. Still, in the presence of serum the down-regulation of both receptors lead to stronger reduction in cellular uptake of nanocomplexes than in serum-free media.

One important factor that affects the biological fate of the nanoparticles is the protein corona. Upon administration of the nanocomplexes to biorelevant media, proteins of body fluid start to quickly adsorb onto the nanoparticles by forming a protein corona that strongly influences the interactions with cell, organs and immune system (Monopoli and others 2012; Kelly and others 2015). Generally, it is believed that protein corona hinders the binding of the nano-complexes to specific target receptors (Mirshafiee and others 2013; Salvati and others 2013), but there are also some contradictive claims (Caracciolo and others 2013; Mortimer and others 2014; Fleischer and Payne 2014; Pozzi and others 2015; Palchetti and others 2016). For example, apolipoproteins (APO) such as APOA1 and -A2, APOB, APOC2/3, APOH, vitamin K dependent protein, integrin beta3, Ig heavy chain V–III regions are considered to promote the association of different nanoparticles with cells (Palchetti and others 2016; Pozzi and others 2014 and 2015). APOs are the main components of lipoproteins such as LDLs and high density lipoproteins, and thus are recognizable by several lipoprotein receptors on the cell surface (Pozzi and others 2014). Additionally, APOC3 can promote also interactions with class B scavenger receptors (Pozzi and others 2015). Inter-alpha trypsin inhibitor is a hyaluronan binding protein, a major constituent of the extracellular matrix that might promote interactions with cells, and thereby also facilitate the interaction of nanoparticles with cell surface receptors and their internalization into cells (Pozzi and others 2015). As already mentioned, in addition to the negative charge, ligand binding to cA-SRs also requires a specific structure: a four stranded helix. The growing number of papers in the field of protein corona has pointed to the fact that proteins might undergo a conformational change during adsorption of serum components onto nanoparticles, and thereby expose new binding motifs for specific receptors. For example, albumin is the major serum protein that upon adsorption to nanoparticles can undergo conformational changes and thereby be recognizable by SRs (Yan and others 2013; Mortimer and others 2014; Fleischer and Payne 2014). Moreover, considering that SRs may function as a signalosome complex with other receptors, it is possible that adsorbed proteins promote interactions with other possible receptors to promote interactions with cell surface and endocytosis of the nanoparticles. Indeed, cells plasma membrane contains a myriad of different receptors, e.g. folate and transferrin receptors, integrins and multiple of growth factor receptors, therefore, it has been recently hypothesized that numerous epitopes on different protein corona proteins could target multiple receptors simultaneously (Palchetti and others 2016). Thus, it is highly essential to map the exact composition of the protein corona of nanoparticles to be able to predict their interactions with cells.

CONCLUSIONS

Cell penetrating peptides hold great potential as vehicles for oligonucleotide delivery in the fields of biotechnology and biomedicine. However, their application is limited by incomplete understating of their action mechanisms, *i.e.* how CPP-ON nanoparticles internalize, where they are sorted in cells after the internalization, and whether and how efficiently they can promote endosomal escape in order to ensure the functionality of the payload.

The main goal of the current research was to examine the cellular uptake mechanisms of one of the most effective delivery vectors for ONs, PepFect14 in complex with SCO, with special focus on the possible involvement of class A scavenger receptors. By using a combination of biochemical assays with confocal and transmission electron microscopy, we specifically aimed to delineate the role of cA-SRs in the cellular uptake of PF14-SCO nanocomplexes, and to elucidate the endocytic pathways and intracellular destiny of the nanocomplexes after they bind to the receptors. The knowledge obtained from the current research expands the understanding of how CPPs function, as well as provides new insights into the action SR-A3 and SR-A5, a field that is currently poorly explored.

The main results of the current thesis are:

- Negatively charged PF14-SCO nanocomplexes are recognized and internalized by class A scavenger receptors SR-A3 and SR-A5. (Paper I and Paper II)
- SR-A3 and SR-A5 are not constantly present on the plasma membrane of HeLa pLuc705 cells, but are recruited there only after treatment with nanocomplexes. Moreover, in addition to nanocomplexes, PF14 itself can promote the cell surface mobilization of the receptors. Although at lower efficiency, similar phenomenon is also characteristic for other amphipathic CPPs, like TP10, PF6 and CADY, and penetratin, and even for cationic R9. (Paper II and Paper IV)
- The cell surface recruitment of the receptors is highly regulated process that in addition to nanocomplexes is also dependent on intra- and extracellular calcium ions, PI3K activity, and actin cytoskeleton. (Paper IV)
- The number of the receptors on the plasma membrane is also controlled by serum proteins. However, the number of the receptors is not directly correlated with the efficiency to induce the internalization and functionality of PF14-SCO complexes, since the uptake and splice-correction activity of the nanocomplexes in serum-containing and serum-free culture media appeared to be rather equal. (Paper IV)
- Association of PF14-SCO nanocomplexes with SR-A3 and SR-A5 induces their internalization by cells mainly via macropinocytosis and caveolin-dependent endocytosis. (Paper II)
- After internalization, PF14-SCO nanocomplexes avoid targeting to low-acidic vesicles, but instead are rather located in non- or slightly acidic

vesicles. In contrary, the naked SCO is quickly targeted to lysosomes for degradation. (Paper II)

- Only a small portion of PF14-SCO nanocomplexes are able to escape from endosomes to the cytosol, indicating that the major limitation in cellular delivery of oligonucleotides by CPPs in the form of nanocomplexes is their entrapment into endosomes. (Paper II)

SUMMARY IN ESTONIA

Püüdurretseptorid kui peptiididega nukleiinhapete rakku transportimise sihtmärgid

Oligonukleotiididel (ON) põhinevad geeniregulatsiooni muutmise mehhanismid on püüdnud üha enam tähelepanu biotehnoloogias ja biomeditsiinis nii kaasasündinud kui omandatud haiguste raviks. Paraku suure molekulmassi ning negatiivse laengu tõttu ei ole sellised molekulid võimelised iseseisvalt läbima raku plasmamembraani. Kuna enamik ON sihtmärkidest asuvad rakus sees tsütoplasmas või raku tuumas, on vaja kasutada transportvektorit, mis üheltpoolt peab kaitsma ON lagundamise eest ning teisalt tagama selle jõudmise toimekohani. Ühed potentsiaalseimad transportvektorid on rakku sisenevad peptiidid (RSP). RSPd on lühikesed, kuni 40 aminohappe pikkused ning enamasti positiivset laengut kandvad peptiidid, mis on võimelised läbima raku membraane ning rakkudesse suunama erineva suuruse ja omadusega bioaktiivseid molekule, kasutades selleks kovalentset või mitte-kovalentset strateegiat. Vaatamata sellele, et RSP-sid kui transportvektoreid on aktiivselt uuritud üle 20 aastat, on nende laiem rakendamine konkreetse ravimina piiratud peamiselt puudulike ning tihti vastukäivate teadmistega nende toimimise mehhanismide kohta. Varem arvati, et RSPd moodustavad ON-ga positiivse laenguga komplekse, mis interakteerudes rakupinna negatiivselt laetud komponentidega indutseerivad rakkudesse sisenemise kasutades selleks peamiselt erinevaid endotsütootilisi mehhanisme. Hiljuti aga tuvastati, et vastupidiselt eeldatule moodustuvad teise põlvkonna RSP PepFect14 (PF14) ja splaissingut korrigeeriva oligonukleotiidi (SKO) vahel hoopis negatiivse laenuga nanokompleksid (Ezzat and others 2011), mistõttu selliste komplekside seondumine rakkudega peaks olema väheefektiivne. Vaatamata sellele endotsüteeritake PF14-SKO kompleksid rakkude poolt väga efektiivselt ning nad omavad kõrget bioloogilist aktiivsust. Praeguseks on teada, et enamik RSP-ON komplekse omavad negatiivset laengut bioloogilises keskkonnas (van Asbeck and others 2013; Arukuusk and others 2013; Veiman and others 2013; Lindberg and others 2015). Kuna otsene interaktsioon raku negatiivselt laetud struktuuridega on ebasoodne, vajavad sellised kompleksid rakkudesse sisenemiseks tõenäoliselt retseptorit. Ühed potentsiaalsed retseptori-kandidaadid on püüdurretseptorid (i. k. *scavenger receptors*), mille omaduseks on siduda ja endotsüteerida negatiivse laenguga ligande.

Käesolev uurimustöö eesmärgiks oli uurida püüdurretseptorite toimimismehhanisme PF14 ja SKO komplekside sisenemisel rakkudesse, sealhulgas keskendusime komplekside endotsütoosi mehhanismide selgitamisele ning nende rakusisesele suunamisele ja paiknemisele. Samuti analüüsisime komplekside võimet indutseerida endosoomidest vabanemist, mis on äärmiselt oluline ON funktsionaalsuse tagamiseks.

Esimeses artiklis me tuvastasime, et PF14-SKO kompleksid kasutavad rakkudesse sisenemiseks klass A püüdurretseptoreid SR-A3 ja SR-A5. Rakkude töötlemine klass A püüdurretseptoreid inhibeerivate ligandide polüinosiinhappe,

dekstraan sulfaadi ja fukoidaaniga, või kasutades RNA interferentsi, et maha suruda SR-A3 ja SR-A5 ekspressioon, blokeeris nanopartiklite seondumise plasmamembraaniga, nende sisenemise rakkudesse ja SKO funktsionaalse aktiivsuse. Lisaks me kasutasime immunofluorestsents analüüsi ning tuvastasime, et vastavad retseptorid kolokaliseeruvad kompleksidega nii rakkude pinnal kui rakus sees vesiikulites, kinnitades et PF14-SKO kompleksid sisenevad rakkudesse SR-A3 ja SR-A5 retseptorite vahendusel.

Teises artiklis suunasime oma fookuse konkreetsete endotsütoosi mehhanismide ja rakusisese paiknemise selgitamiseks. Selleks, et uurida, milline endotsütoosirada indutseeritakse pärast komplekside seondumist retseptoritele, me kasutasime farmakoloogilisi endotsütoosi inhibiitoreid: amiloriidi, nüstatiini ja kloorpromasiini, mis inhibeerivad vastavalt makropinotsütoosi, kaveoliin-sõltuva endotsütoosi ning klatriin-sõltuva endotsütoosi rajad. Me tuvastasime, et interaktsioon rakupinna retseptoritega indutseerib PF14-SKO komplekside sisenemise rakkudesse peamiselt makropinotsütoosi ja kaveoliin-sõltuva endotsütoosi teel. Seevastu klatriin-sõltuva raja inhibeerimine komplekside võimet indutseerida splaissingu korrektsiooni ei mõjutanud ning seega ei ole see rada oluline komplekside sisenemisel ja funktsionaalsuse avaldumisel. Pärast sisenemist rakku, suunatakse endotsüteeritud materjal mööda erinevaid radasid erinevatesse rakusisestesse organellidesse, mis määravad ära endotsüteeritud materjali edasise saatuse. Rakkudele on iseloomulikud kaks peamist rada: esiteks ringlusrada, mille kaudu endotsüteeritud materjal suunatakse tagasi raku plasmamembraanile. Teiseks peamiseks rajaks on endo-lüsosomaalne rada, mööda mida endotsüteeritav materjal suunatakse happelistesse lüsosoomidesse, kus toimub selle lõplik lagundamine. Rakusisene saatus võib olla määratud ka kindla endotsütoosiraja poolt. Näiteks on teada, et sisenemine rakkudesse läbi kaveoliin-sõltuva raja võib suunata endotsüteeritava materjali otse Golgi kompleksi või endoplasmaatilisse retiikulumi (ER). Antud komplekside rakusisese suunamise uurimine näitas, et PF14-SKO komplekse ei suunata trans-Golgi võrgustiku sekretoorsetesse vesiikulitesse ning ei seega ei saadeta neid rakust välja. Samuti ei täheldanud me komplekside paiknemist ERi. Me tuvastasime, et PF14-SKO komplekse ei suunata ka klassikalisse endo-lüsosomaalsesse ratta, vaid enamik rakku sisenenud PF14-SKO kompleksidest paiknesid pigem neutraalse pH-ga vesiikulites ning ainult väike hulk PF14-SKO komplekse suunati happelistesse lüsosoomidesse, isegi pärast 4-tunnist inkubatsiooni. Vastupidiselt kompleksidele, vaba SKO suunati juba 2-tunni jooksul happelisesse vesiikulitesse, viidates sellele, et PF14 takistab endosoomide hapustumist ja küpsemist lüsosoomideks. Saadud tulemused on hästi kooskõlas endotsütoosi inhibiitoritega läbi viidud katsetega, kuna arvatakse, et kaveoliin-sõltuv rada suunab endotsüteeritud materjali nõrgalt happelistesse vesiikulitesse, kust osaliselt indutseeritakse endosoomidest vabanemist ning paiknemist tsütoplasmasse. Siiski, valdav osa rakku sisenenud PF14-SKO kompleksidest jääb endosoomidesse kinni ning vaid umbes 8% nendest jõudis raku tsütoplasmasse, mis viitab sellele, et endosoomidesse kinnijäämine on peamine limiteeriv faktor ON-de rakendamisel rakus toimuvate protsesside mõjutamiseks ja biomeditsiinis.

Esimeses artiklis me tuvastasime retseptorite ja komplekside koospaiknemise rakkude pinnal ja sees fluorestsents mikroskoopia abil, kuid selleks et uurida nende interaktisoone detailsemalt, kaasasime ka elektronmikroskoopilise analüüsi antud uurimustöösse. Märgistades kompleksid ja retseptorid erineva suurusega kullamärgisega, me näitasime, et kompleksid paiknevad retseptorite vahetus läheduses, mistõttu võib arvata, et nende interaktsioon on otsene. Sealjuures me tuvastasime, et efektiivseks sisenemiseks rakku on vajalik vaid väheste retseptorite osalus. Üllatavalt me tuvastasime, et antud retseptorid ei ole rakkude pinnale eksponeeritud pidevalt, vaid need suunatakse plasmamembraanile komplekside toimel nende lisamisel rakkude kasvulahusesse. Samas, vastavate retseptorite hulk raku tsütoplasmas oli märkimisväärne.

Töö kolmandas artiklis me keskendusime detailsemalt mehhanismidele, mis võiksid osaleda retseptorite pinnale toomisel. Toetudes juba avaldatud tulemustele, keskendusime kaltsiumi ioonide ning fosfoinositool-3-kinaasi (PI3K) rollile selles protsessis. Mõlemad eelnimetatud on vajalikud erinevate rakuliste protsesside reguleerimises, sealhulgas mitmete retseptorite regulatsioonis. Me tuvastasime, et nii rakuvälise kui -sisese kaltsiumi eemaldamine vastavalt EGTA või BAPTA-AM-ga pärsib retseptorite translokatsiooni rakkude pinnale, kus neid võrreldes inhibeerimata kontroll-rakkudega oli oluliselt vähem. Lisaks me tuvastasime, et nii rakuväline kui -sisene kaltsium on olulised ka komplekside toime avaldumiseks, kuna kaltsiumi eemaldamine inhibeeris oluliselt SKO bioloogilist aktiivsust. Sarnased tulemused saime ka PI3K inhibeerimisel wortmannini või LY294002-ga, mis vähendasid oluliselt raku pinnale suunatud retseptorite hulka kui ka splaissingu korrektsiooni taset. Saadud tulemused näitavad, et SR-A3 ja SR-A5 ümberpaiknemine raku tsütoplasmast rakkude pinnale on kõrgelt reguleeritud protsess, mis lisaks nanokompleksidele vajab ka kaltsiumioone ja PI3K aktiivsust.

Erinevate nanopartiklite kasutamisel biomeditsiinis on hakatud üha enam uurima ka nende interaktsioone bioloogilise keskkonnaga, mis võib oluliselt mõjutada nanopartiklite eluiga ning jõudmist sihtmärgini. Seetõttu me uurisime, kas retseptorite translokatsioon rakkude välismembraanile võib sõltuda rakkude kasvulahuse koostisest, st. kas seerumiga ja seerumvabas söötmes on see protsess sarnane. Me tuvastasime, et seerumvabas söötmes suunatakse SR-A3 ja SR-A5 retseptoreid rakkude pinnale väga vähesel määral. Sellest järeldub, et SR-A3 ja SR-A5 translokatsioonil rakkude pinnale on lisaks RSP-ON kompleksidele ning kaltsiumioonidele ja PI3K aktiivsusele vajalikud ka rakusöötmes oleva vereseerumi komponendid. Samas me leidsime, et retseptorite koguarv raku pinnal ei ole otseses seoses komplekside rakkudesse sisenemise ja splaissingu korrektsiooni efektiivsusega, kuna nii seerumvabas kui ka seerumit sisaldavas söötmes oli SKO indutseeritud splaissingu korrektsiooni tase üsna sarnane.

Töösse on kaasatud ka metodoloogiline artikkel oligonukleotiidide märgistamisest rakutranspordi uurimiseks ultrastruktuuri tasemel.

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ACKNOWLEDGEMENTS

First and foremost, I want to express my deepest gratitude to my supervisor, Margus Pooga for introducing me the world of CPPs, sharing his knowledge and providing encouragement during my studies. I am also very thankful to Kärt Padari for her guidance during my BSc and MSc studies, and for introducing me the secrets of electron microscopy.

I am also thankful to my colleagues from the Institute of Technology, especially Prof. Ülo Langel for welcoming me to his group and for introducing me the peptide synthesis.

Additionally, I want to express my gratitude towards the present and former labmates from the department of Developmental Biology, especially Janely, Helerin, and Annely for invaluable help in everyday lab work and for being such a great colleagues. It has been a pleasure to work with you.

I am also thankful to Viljar Jaks for proofreading my thesis, and for his helpful comments and suggestions on the thesis.

Finally, I would like to thank all of my closest friends who have inspired and motivated me. Eva, and Maia, Tuuli, Regina, Kertu, Madli and Holar from "Ruuni gang" – this gratitude goes to you!

Above all, I am grateful to my family, especially my mother and brother for all the support and encouragement. My special thanks go to my dear husband Karmo for always being there for me and appreciating my achievements.

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Publikatsioonid:

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(3): 1172–1180

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