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ABSTRACT

Gene therapy has advanced greatly in the last few decades, offering treatment to diseases that are caused by genetic abnormalities. However, the nucleic acids used for this therapy can't reach their intracellular targets due to their physio-chemical properties, requiring delivery vectors to cross the cellular barriers. Unfortunately, the development of safe, affordable, and efficient gene delivery vectors remains the primary obstacle for the wider translation of gene therapy into clinics. Although viral gene therapy is abundantly used in clinical trials, there are still safety and financial concerns associated with the viral vectors, necessitating further research into non-viral alternatives.

Cell penetrating peptides (CPPs) are one of such non-viral vectors that can be used for the delivery of nucleic acids into cells. CPPs are short peptides that can form non-covalent nanoparticles with nucleic acids and transport them across cell membranes. However, the main problems of CPPs are their non-specific uptake, low *in vivo* gene delivery efficiency, and acute toxicity.

The work in this thesis focuses on modifications of the CPPs belonging to the PepFect family to address the aforementioned problems. To reduce non-specific uptake in cancer therapy, the surface of CPP/nucleic acid complexes was modified to reduce interactions with non-targeted cells, but increase accumulation in the diseased tissue. Additionally, the capability of these newly designed tumor specific CPPs to induce a therapeutic effect was investigated. The acute toxic effects of the CPPs were addressed by modifying both the CPP/nucleic acid complex formulation strategies, and the net charge and hydrophobicity of the CPP. Our results demonstrate that safety, tumor specificity, and thereby gene delivery efficiency of the CPPs can be improved by utilizing these modifications.

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The following publications form the basis of this dissertation and are referred to in the text using Roman numerals:

I Veiman, K.-L., **Künnapuu, K.**, Lehto, T., Kiisholts, K., Pärn, K., Langel, Ü., and Kurrikoff, K. (2015). PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo. J. Controlled Release 209, 238–247.

II **Künnapuu, K.**, Veiman, K.-L., Porosk, L., Rammul, E., Kiisholts, K., Langel, Ü. and Kurrikoff, K. (2018). Tumor Gene Therapy by Systemic Delivery of Plasmid DNA with Cell Penetrating Peptides. FASEB Bio-Advances 00, 1–10. In press

III Kurrikoff, K., Veiman, K.-L., **Künnapuu, K.**, Peets, E.M., Lehto, T., Pärnaste, L., Arukuusk, P. and Langel, Ü. (2017). Effective in vivo gene delivery with reduced toxicity, achieved by charge and fatty acid -modified cell penetrating peptide. Scientific Reports 7 (1), 17056−17056.

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The author's contribution to each article is as follows:

I: Designed and performed many of the experiments, synthesized peptides, analyzed data, and participated in the writing of the manuscript.

II: Designed and performed most of the experiments, synthesized all of the peptides, analyzed most of the data, participaed in the writing of the manuscript as a corresponding author.

III: Designed and synthesized some of the peptides, performed some of the experiments, and analyzed some of the data.

Other publications:

IV Cerrato, C.P., **Künnapuu, K.**, and Langel, Ü. (2016) Cell penetrating peptides for intracellular organelle targeting. Expert Opin. Drug Deliv.

ABBREVIATIONS

CPP	Cell penetrating peptide
CR	Charge ratio
DLS	Dynamic light scattering
i.v.	Intravenous
i.p.	Intraperitoneal
MALDI-TOF MS	Matrix assisted laser desorption/ionization time of flight mass spectrometry
MMP	Matrix metalloproteinase
NA	Nucleic acid
N/P	The ratio of the amine groups of cationic polymers to the phosphate groups of nucleic acids
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PF	PepFect
PF14	PepFect14
pshVEGF	Plasmid expressing short hairpin RNA against vascular endothelial growth factor
RFU	Relative fluorescence unit
RLU	Relative light unit
RP-HPLC	Reverse phase high performance liquid chromatography
siRNA	Small interfering RNA

INTRODUCTION

The last few decades have seen important advances in the field of gene therapy, which has great potential to offer curative treatment to disorders caused by genetic abnormalities by modifying the patient's genome or correcting abnormal gene expression. For the treatment to be effective, the therapeutic nucleic acids that are large negatively charged macromolecules, must get to their site of action – the cytosol or nucleus inside cells. To reach those sites, numerous biological barriers need to be overcome, which these molecules can cross only with the help of gene delivery vectors. Although viral vectors have exhibited great efficiency, they have several critical drawbacks like immunogenicity and expensive production. This has prompted extensive investigation into non-viral gene therapy, where synthetic molecules that can form nanoparticles with nucleic acids and deliver them into cells are used as delivery vectors, and although they are safer than their viral counterparts, they still need improvement in gene delivery efficiency.

Cell penetrating peptides (CPPs) are a class of non-viral delivery vectors. They are short, usually cationic or amphipathic peptides that can transport bioactive cargo, including nucleic acids into the cells. However, CPPs tend to exhibit nonspecific distribution in the organism after administration, which can cause toxicity to healthy tissues and reduce available therapeutic material for the diseased cells. For example, in tumor gene therapy, the therapeutic material is often cytotoxic to cells, and nonspecific distribution of the drug can create severe side effects. This means that CPPs need further modification to increase their specificity. Also, acute toxic effects have been observed with some CPPs, necessitating optimization of nanoparticle formulation and peptide design strategies.

The work presented in this thesis was aimed at addressing both the lack of specificity and acute toxicity of the CPPs. First, CPPs were modified to increase tumor specificity, and increase tumor accumulation to achieve more efficient tumor therapy. Secondly, nanoparticle formulation strategies and peptide net charge were modified to reduce acute toxicity of the particles. The investigations presented in this study provide efficient strategies for reducing both off-target and acute toxicity of CPPs.

1. LITERATURE OVERVIEW

1.1. Gene therapy

Gene therapy involves the therapeutic delivery of nucleic acids into a patient's cells with the aim of treating or preventing disease. In contrast to conventional drugs that often treat symptoms, gene therapy has the potential to treat the disease at its genetic roots by replacing or counteracting a defective gene in affected cells, and thereby offering curative treatment (1, 2). For gene therapy to work, the therapeutic gene must be delivered into the affected cells with efficient and safe delivery vehicles (3, 4). Gene therapy can be carried out *ex vivo*, where gene transfer is undertaken outside the body, or *in vivo*, where the drug is administered directly into the patient.

Hematopoietic stem cell (HSC) gene therapy is a preferred target for *ex vivo* gene therapy, and the modified HSCs can be used to treat conditions where mature hematopoietic lineages don't function correctly (1). This approach has been tested for treating severe inherited diseases of the immune system, such as severe combined immunodeficiency (SCID) and Wiskott-Aldrich syndrome (5–8), diseases of the blood (β-thalassemia) (9), and neurodegenerative storage diseases (adrenoleukodystrophy) (10). For *in vivo* gene therapy, the liver has been the preferred target for a long time, mainly due to its high accessibility to the administered particles. Targeting the liver could provide a strategy for treating metabolic diseases and plasma protein deficiencies, and a lot of testing has gone into the treatment of severe hemophilia B (11).

Targeted gene editing with the development of artificial DNA endonucleases has further boosted the development of gene therapy, since they improve the ability to make precise changes in the genome by introducing DNA double-strand breaks at specific loci, which are then repaired by the body's endogenous DNA repair machinery (12). For example, targeted gene editing is achievable through clustered regularly interspaced short palindromic repeat (CRISPR)/ Cas9 RNA based nucleases, but it needs further investigation of off target activity (13).

There is a wide range of methods that allow to restore activity of defective genes, and to block harmful genes. These methods are all based on nucleic acids or their synthetic analogues. The delivery of plasmid DNA and mRNA can boost the expression of specific genes or proteins, short interfering RNA (siRNA) and antisense oligonucleotides (ASO) can specifically downregulate gene expression by binding to mRNA that leads to enzyme-dependent degradation of the targeted mRNA (14). Splice correcting oligonucleotides that are single stranded antisense oligonucleotides that can correct splicing mutations (15).

Plasmid DNA (pDNA) is a circular double stranded DNA molecule. Compared to RNA-based vectors, pDNA is easier to produce and store, because DNA is quite stable while RNA is more easily degradable (16). Plasmid DNA is also much bigger than siRNA, and shows more electrostatic interactions with

polycations, increasing stability of the complexes. Plasmid DNA is versatile. It can be used for gain of gene function via replacing a missing gene, and plasmids can also be used to deliver genes which express nucleic acids that knock down gene expression, leading to loss of gene function. The last approach helps to overcome some of the hurdles associated with RNA delivery, namely, the sensitivity of RNA to degradation, and the short duration of the therapeutic effect, since siRNA molecules are destroyed along with their target during RNA interference. This means that expressing a construct from a plasmid allows for greater duration of the transgene effect (17). However, one big advantage of delivering siRNA is that its place of action is in the cytosol, while pDNA needs further transport to the nucleus, increasing the difficulty of plasmid delivery (18).

1.2. Gene therapy for cancer treatment

Since cancer is a disease characterized by genetic abnormalities, gene therapy is a prospective way to treat this disease. Strategies of gene-based cancer therapy include blocking the expression of an oncogene, expressing a gene to induce apoptosis, enhance chemotherapeutic drug sensitization, or enhance the immunogenicity of the tumor (19, 20). For example, the TRAIL ligand is expressed in several tumor cells to induce apoptosis (21), and expressing interleukin-24 sensitizes tumor cells to toll-like receptor 3 mediated apoptosis (22, 23). The most extensively studied tumor suppressor gene is p53, and the expression of wild type p53 with a recombinant adenovirus for the treatment of head and neck squamous cell cancer was among the first commercialized gene therapies (24). Anti-apoptotic genes can be silenced with siRNA or microRNA (miRNA) mediated silencing (25). MicroRNA can also be used as replacement therapy by replacing miRNAs that are downregulated in tumors. For example, miR34 inhibits cell growth and its expression is frequently reduced in many cancer types, and replacing miRNA-34 can lead to normal regulation of cell death. This approach is in clinical trials for liver cancer (26).

Tumor angiogenesis is one of the drivers of tumor development, and inhibiting angiogenesis can be an effective way to impede tumor growth as part of combinatorial treatment (27). Angiogenesis is also an accessible target when the drugs are administered by particles with specificity toward tumor endothelial cells – inhibition of endothelial cell proliferation and migration in the tumor blood vessels by cytokines with anti-endothelial activity, like interleukin-23 and IFNα, can damage numerous tumor cells from limited supply of oxygen, nutrients and growth factors (28). Interleukin-12, which is also a pro-inflammatory cytokine with immunoregulatory functions, has also been suggested to exert anti angiogenic effects (29, 30). Vascular endothelial growth factor (VEGF) is critical for angiogenesis. Once the new blood vessels are formed, VEGF inhibits apoptosis of the vasculature (31). In pre-clinical models, VEGF-targeted therapy had minimal effect on tumor burden when administered alone, but as a combi-

nation therapy with paclitaxel, the tumor burden was decreased (32, 33). Bevacizumab, an angiogenesis inhibitor, was approved by the FDA in 2018 for patients with epithelial ovarian, fallopian tube, or peritoneal cancer when used in combination with paclitaxel and carboplatin (34).

Immunotherapy is also gaining popularity as a tumor therapy strategy. Cytokine based therapy can enhance anti-tumor immune response; IL-2, IL-12, IFNα, IL-4 and IFNγ have so far been investigated for this purpose. IL-12 is widely studied (35), and IL-12 plasmid lipopolymer complexes have been shown to be safe and well tolerated in treating recurring ovarian cancer (37). AAV vector based IL-12 therapy is also under clinical trials for persistent prostate cancer therapy (37). T cells are popular for *ex vivo* cancer immunotherapy by gene therapy. The underlying mechanism for the anti-cancer effect of this therapy lies in the *ex vivo* activation and amplification of tumor specific cytotoxic T cells that lead to an adaptive immune response against the tumor upon administration (38). In this approach, exogenous T cell receptors (TCRs) are added to T cells, which should ideally target tumor specific neoantigens that originate uniquely from random mutations in the tumors of the patient, meaning that this therapy must be highly personalized (1). Recently, synthetic chimeric antigen receptors (CARs) have been introduced to T cells that combine the binding specificity of an antibody against cancer associated surface markers with domains from the TCR (39). For example, CARs have been directed against B cell surface molecule CD19 in B cell malignancies that exhibit normal to high levels of CD19 expression (40, 41). Currently there is very high interest in studying this approach (42), and two CAR-T cell therapies are already authorized by the European Medicines Agency for the treatment of B-cell acute lymphoblastic leukemia, large B-cell lymphoma and non-Hodgin lymphoma (43, 44).

1.3. Gene delivery

Although therapeutic nucleic acids show great therapeutic potential, they can't overcome cellular and tissue barriers to get into cells due to their large size and high negative charge. This means that efficient and safe delivery systems are the fundamental basis for gene therapy. Viruses are naturally highly effective vectors for gene delivery, and thus they have been modified to carry therapeutic genetic material into cells for gene therapy purposes. However, viruses are also naturally immunogenic, and viral gene therapy exhibits other unwanted side effects like insertional mutagenicity (45). Viral gene therapy is also very expensive. The first commercially approved therapy, Glybera, for the treatment of familial dyslipidemia (a disease with abnormal amounts of lipid in the blood) which uses adeno-associated viral vectors cost 1 million euros per treatment, which can challenge reimbursement and insurance policies (46). Its marketing authorization was not renewed because of lack of interest in the drug (47). Luxturna, an adeno-associated virus vector-based gene therapy for the treatment of retinal

dystrophy that was approved in 2018, costs around $420 000 per eye (48, 49). The high price reflects cost of preclinical development, manufacturing and distribution of the medicine. As an alternative to viral vectors, non-viral vectors are being developed which are less immunogenic, don't cause genotoxicity, and that are easier and more affordable to produce.

1.4. Non-viral vectors

Non-viral vectors are synthetic molecules, usually lipids, peptides, and polymers. They can pack nucleic acids into nano-sized complexes by electrostatic interactions, and unlike viral vectors, their insert size is unrestricted. Non-viral vectors have lower immunogenicity, high biocompatibility, and better potential for scale-up manufacturing. Polymeric carriers can also deliver different types of therapeutic nucleic acids, including antisense oligonucleotides, SCOs, siRNA, miRNA, plasmid DNA, and mRNA (50–52). However, central concerns of nonviral vector development are still small gene transfer efficiency, and short duration of transgene expression compared to viral vector mediated gene therapy.

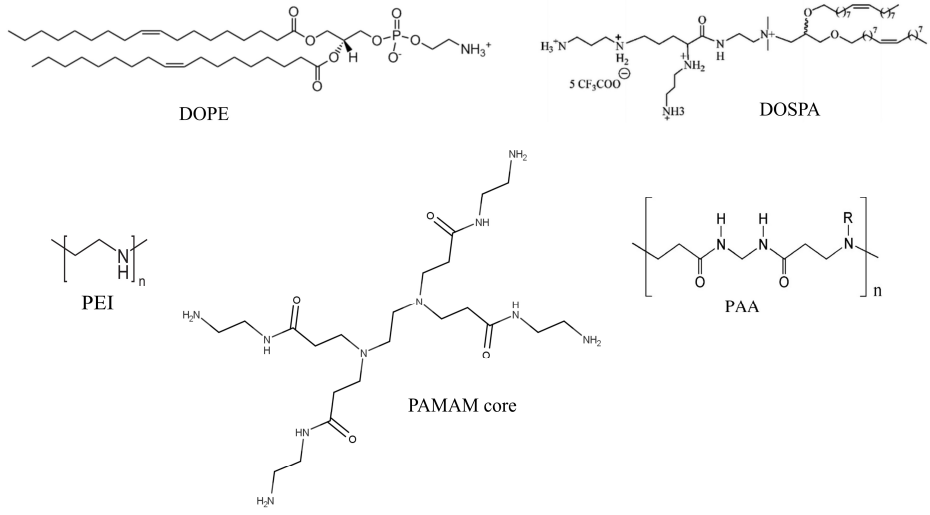


Figure 1. The molecular structures of some of the most common non-viral vectors.

Liposomes are usually formed by self-assembly of lipid molecules that contain both a hydrophilic head, and hydrophobic tail groups, forming a bilayer in aqueous solution that forms a vesicle (53). Their hydrocarbon tails are usually between 8–18 carbons in length, and it has been shown that asymmetric lipid mixtures with both shorter and longer carbon chains produce higher transfection efficiencies than symmetric formulations (54). Widely used cationic lipids

include N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and cholesterol (DC-Chol). A neutral lipid, dioleoylphosphatidyl-ethanolamine (DOPE) is often used with cationic lipids because of its membrane destabilizing effects at low pH, aiding in endolysosomal escape (55). The mixture of ({2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate} (DOSPA), which is a derivative of DOTMA, and DOPE at a 3 : 1 ratio is commercially available under the name Lipofectamine (53).

Polyethyleneimine (PEI) has been the most investigated cationic polymeric carrier for plasmid DNA delivery, it has robust gene delivery efficiency in both *in vitro* and *in vivo*. Every third atom of PEI is a protonatable amino nitrogen giving PEI a high cationic charge density. It also has good buffering capability at a wide range of pHs. The main obstacle for the use of PEI has been the heterogeneity and polydispersity of the polymer; the chemical composition of the polymer is ill-defined, which is not favorable from a clinical perspective. It's not specific in terms of further chemical functionalization, the targeting ligand numbers are only statistical, and modifications are done at undefined conjugation sites on the polymer. Also, defined modifications at specific sites are difficult to achieve, and PEI is toxic (52).

Another type of polymer used for gene delivery are dendrimers. They have highly branched and well defined structure, and they are monodisperse. Polyamidoamine (PAMAM) dendrimers have a high density of amines which are only partially protonated at a physiological pH. The secondary and tertiary amines provide buffer capacity and can act as the proton sponge for endosomal escape (56, 57). The high surface charge density of PAMAM is associated with cytotoxicity, and partial PEGylation and acetylation have been used to reduce the cytotoxic effects (58). Fluorinated dendrimers have also been synthesized and they have shown lower toxicity and improved transfection efficacy in several cell lines (59).

Polyamidoamines are also used in linear form. The structure of their backbone differs from dendritic polyamidoamines by the sequence of amine and amide groups. In the dendritic form, each amide group is followed by an amine, but the linear backbone has two amide groups followed by one or two amine groups (60). Linear amphoteric polyamidoamines (PAAs) have high transfection efficiency and low toxicity, but the synthesis products have high polydispersity (61). Sequence defined PAAs on the other hand have precisely defined products, and they have shown less toxicity than PEI (62). Sequence defined PAAs have low molecular weight and are more biocompatible, but the particles they form with DNA are less stable. Lipid modifications have been used to increase its stability (63).

1.5. Cell penetrating peptides

Cell penetrating peptides (CPPs) are another class of non-viral drug delivery vectors. They usually consist of 30 or less amino acids, and are generally cationic or amphipathic (64). Their most remarkable property is their capability to deliver bioactive cargo, including nucleic acids, through the cell membrane (65). One of the advantages of CPPs over other polymer vectors is their defined structure, which is obtained through solid phase peptide synthesis (52).

The first observations about proteins capable of crossing cell membranes were noted about 30 years ago, when the transcription trans-activating protein (Tat) from HIV-1 was discovered to be capable of entering cells, and even the cell nucleus (66, 67). The same property was found in the homeodomain of Antennapedia, a protein from the fruit fly (*Drosophila melanogaster*) (68), and VP22, a herpes virus protein (69). The minimal sequences necessary for the translocation of the proteins were identified several years later (70, 71). Thus the field of CPPs was born, and since then the number of CPPs discovered has been rapidly increasing. CPPs are diverse in terms of their amino acid composition, polarity, and charge. Although most CPPs are cationic, cell penetration ability has also been demonstrated for negatively charged peptides (72, 73). Based on their physiochemical properties, CPPs can be divided into three classes: cationic, amphipathic, and hydrophobic (74).

Table 1. Sequences of common CPPs from different classes.

CPP	Sequence	Class	Ref.
Tat(48–60)	RKKRRQRRR	Cationic	(71)
Penetratin	RQIKIWFQNRRMKWKK	Cationic	(70)
Polyarginine	R_n-NH_2	Cationic	(75)
pVec	LLIILRRRIRKQAHAHSK-NH_2	Amphipathic	(76)
Pep-1	KETWWETWWTEWSQPKKKRKV-Cya	Amphipathic	(77)
P28	LSTAADMQGVVTDGMASGLDKDYLKPDD	Anionic	(73)
TP2	PLIYLRLLRGQF-NH_2	Hydrophobic	(78)

Cya – cysteamide

Cationic (non-amphipathic) CPPs contain clusters of positively charged residues, which is essential for their cell uptake. Their membrane binding primarily arises from electrostatic interactions between the cationic peptide and negatively charged membrane components – they bind more strongly to anionic membrane lipids than neutral parts of the membrane (74, 79). Studies on arginine-based CPPs have shown that increasing the number of positive charges enhances cellular uptake, but increases toxicity, with the optimal number of arginines being around 8 (80).

Amphipathic CPPs contain both hydrophilic and hydrophobic domains, and are further divided into primary and secondary amphipathic peptides (74).

Primary amphipathic CPPs contain sequential hydrophobic and cationic domains in their primary structure. They bind strongly to both neutral and anionic lipid membranes (79). Secondary amphipathic CPPs display amphipathicity only through secondary structure, where cationic residues are grouped in distinct faces of the molecule, and they show greater affinity to anionic than neutral membranes (79). In some cases their cell transduction ability is retained even when substituting a cationic amino acid with another polar residue, given that the amphipathicity is conserved (81).

Hydrophobic CPPs contain only non-polar residues, have a low net charge, or have a hydrophobic domain that is crucial for uptake. Unlike most cationic and amphipathic CPPs, their transduction ability isn't affected by sequence scrambling (74). Some hydrophobic CPPs can reportedly translocate directly across membranes into cytosol, which eliminates the risk of endosomal entrapment (82).

CPPs can be attached, either covalently or non-covalently, to bioactive molecules that inherently have limited uptake and little to no bioavailability. They have been used to deliver various high-molecular-weight molecules like proteins, antibodies, and DNA into cells for both imaging and therapeutic purposes (83). These conjugates can enter cells at sufficient levels to achieve a biological effect, and conjugating these molecules to CPPs also help to reduce their dose, weakening dose-dependent side effects (84, 85).

CPPs can additionally be used for enhancing the intracellular delivery of other nanocarriers, like liposomes, improving their interaction with cells and reducing their cytotoxicity (84, 86, 87). They can also increase cell entry of inorganic particles like silica, iron, gold, and silver nanoparticles (84). For example, TAT modified nanosilver displays antitumor activity in cancer cells, while unmodified nanosilver lacks an efficient cellular uptake mechanism (88).

CPPs can be used for the delivery of nucleic acids. When this strategy was first developed, nucleic acids were conjugated to CPPs covalently, PNA antisense of galanin receptor 1 to transportan for example, which was able to suppress the gene expression in rats (89). Nucleic acids can also be non-covalently conjugated to CPPs with a positive net charge, where they are condensed into particles through electrostatic interactions that can effectively cross the cell membrane (90–92).

The lack of cell specificity continues to be a drawback of CPPs. They have also been found to have toxic effects (93, 94). The complexes of CPPs and their cargo accumulate in sites that are reached earliest, like the lung, that are often not the desired target, and which also causes the loss of material into untargeted tissues (84, 95). A possible solution is to add targeting moieties to the CPP, or adding stimuli sensitive modifications that activate the CPP in a certain environment only present in the targeted tissue (96). CPPs can be functionalized with antibodies or their fragments (97–100), tumor targeting moieties (101, 102), BBB penetrating moieties (103, 104), etc.

1.5.1. The cell entry of CPPs

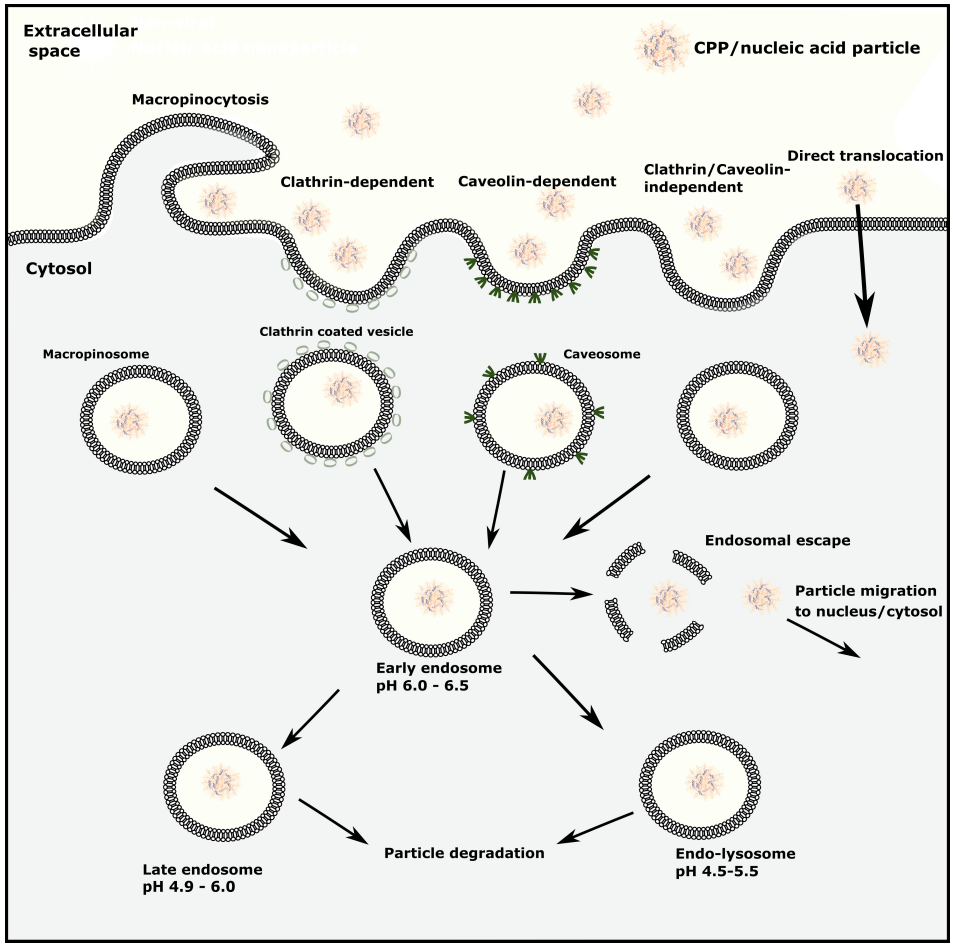


Figure 2. Scheme of the main endocytosis pathways of endocytosed nanoparticles.

Most CPPs bind to cell membranes through amphipathic or electrostatic interactions (105). CPPs and CPP-cargo conjugates can enter cells through different pathways of pinocytosis, like macropinocytosis (106), clathrin-mediated endocytosis, clathrin/caveolae-independent endocytosis (107), caveolae/lipid raft mediated endocytosis (108), or through direct penetration (109). Which pathway is utilized, is influenced by properties, like length and charge distribution of the specific CPP, the properties of the cargo molecule, and cell type (110). During endocytosis particles are engulfed by the cell, and the CPP-cargo nanoparticles end up inside endocytic vesicles. They must escape into the cytosol, or they will be degraded (107). Various strategies have been developed to overcome endosomal entrapment, which will be discussed in future chapters.

1.5.2. Transportan and its analogs

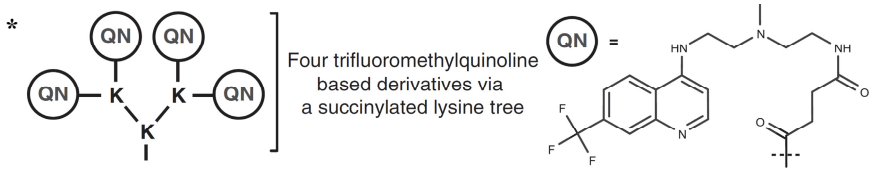
The work presented in this dissertation is based on the development of CPPs that are all analogues of transportan. Transportan is a CPP consisting of 27 amino acids that's a combination of the neuropeptide galanin in the N-terminal part, and mastoparan, a peptide from wasp venom, in the C terminal end. These two moieties are connected by a lysine residue (111). It has higher transfection efficiency than penetratin, and the C terminal alpha helix is important for transfection (112). However, transportan exhibited toxic effects that arose from its inhibitory effect on GTPase activity and its recognition by galanin receptors. To remedy this, an analog of transportan, TP10, was designed by truncating its N terminus. This didn't affect cellular uptake, but showed less toxicity compared to transportan (113). Futaki et al. had showed that stearylation of a CPP increased its transfection efficiency (114), and so the effect of stearylation was also evaluated for TP10. The modified TP10 was 30 times more effective than un-stearylated TP10 in a splice correction assay (115).

TP10 has been modified in several ways, primarily to increase its gene delivery efficiency. PF6 was created by introducing a proton-accepting moiety, four trifluoromethylquinoline based derivatives to stearyl-TP10 via a succinylated lysine tree. This new CPP, PF6, mediated efficient gene knockdown by siRNA delivery *in vivo* and *in vitro* (116). In another stearyl-TP10 modification approach, PF14 was created by utilizing ornithines and leucines instead of lysines and isoleucines, which turned out to be more efficient. This design was based on the finding that poly-L-ornithine demonstrated superior transfection efficiency compared to equivalent poly-L-lysine, because of its higher affinity to DNA (117, 118).

Another line of stearyl-TP10 analogs was created by replacing Ile8 with Thr to increase hydrophilicity, and the phosphoryl group of Tyr3 or Thr8 was added to create a pH dependent vector. These analogs were named NickFects, and NF1 with the phosphorylated Tyr3 was the most efficient of the analogs (119). In another NickFect analogue, Lys7 was replaced with ornithine, and its δ-NH_2 group was used for continuing peptide synthesis to create NF51 (120). Further modification of NF51 by increasing secondary amphipathicity and reducing net charge of NF51 lead to the design of NF55 which resulted in two fold increase transfection efficiency over NF51, and increased efficiency *in vivo* (121). Both NF55 and PF14 are both efficient pDNA vectors for *in vivo* gene delivery.

Table 2. Peptide sequences of transportan and its analogs.

Name	Sequence	Ref.
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	(111)
TP10	AGYLLGKINLKALAALAKKIL-NH_2	(113)
Stearyl-TP10	Stearyl-AGYLLGKINLKALAALAKKIL-NH_2	(115)
PF6	Stearyl-AGYLLGK*INLKALAALAKKIL-NH_2	(116)
PF14	Stearyl- AGYLLGKLLOOLAAAALOOLL-NH_2	(117)
NF1	Stearyl-AGY(PO_3)LLGKTNLKALAALAKKIL-NH_2	(119)
NF51	(Stearyl-AGYLLG)δ-OINLKALAALAKKIL-NH_2	(120)
NF55	(Stearyl-AGYLLG) δ-OINLKALAALAKAIL-NH2	(121)



1.6. Biological barriers for non-viral gene delivery

Nanoparticle formulations face several biological obstacles that limit their bio-availability. When nanoparticles are injected into the body, they come into contact with different barriers that affect how many of the particles actually reach the target site. Some of the main barriers nanoparticles face are degradation, opsonization, nonspecific distribution, and endosomal entrapment (122, 123). Upon systemic administration, the vector/nucleic acid nanoparticles come into contact with a plethora of different blood components, cells, proteins, enzymes, etc. The nucleic acid vectors have to protect the genetic material from degradation by nucleases, and have to resist degradation themselves (124).

Living organisms have different mechanisms to defend themselves against foreign objects in the bloodstream. One of the defense mechanisms involves opsonization – the adsorption of different types of plasma proteins also called opsonins (immunoglobulins, blood serum proteins etc.) on the surface of particles (125, 126). These opsonized particles are recognized by phagocytic cells like macrophages and monocytes which leads to the clearance of nanoparticles from the organism within minutes and thus therapeutic material is lost (123). Surface properties of the particles influence opsonization. Negatively and positively charged particles are associated with rapid removal from the blood compared with neutral particles. There is also positive correlation between the size of nanoparticles and internalization by macrophages (127).

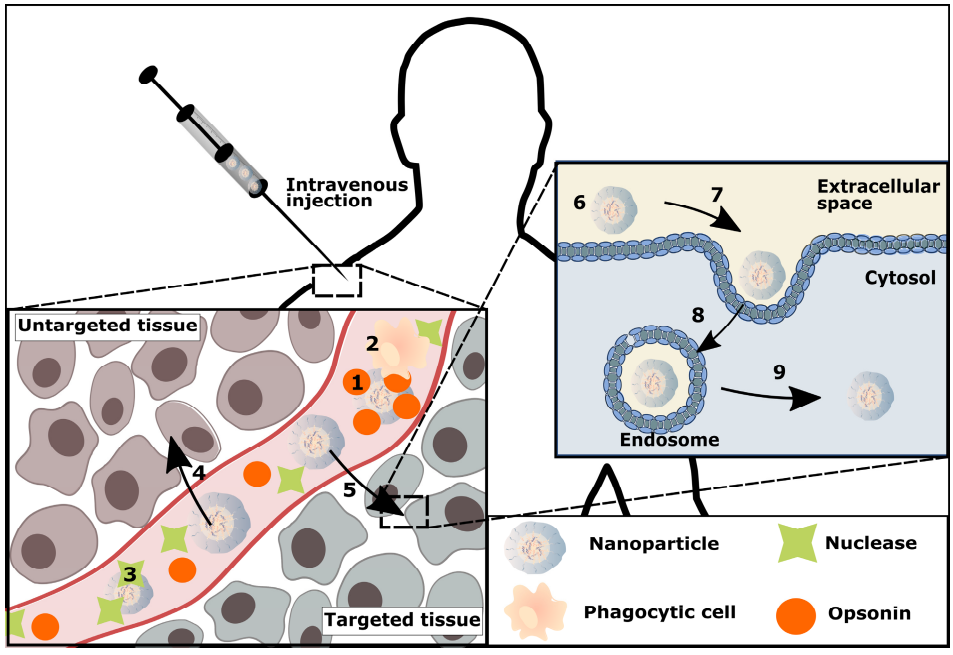


Figure 3. Barriers to nanoparticle delivery for gene therapy. Upon intravenous injection, the nanoparticles face opsonization (1) which can lead to clearance of the nanoparticles by phagocytic cells (2); and degradation of the genetic material in the nanoparticles by nucleases (3). After that a problem is distribution of particles into non-targeted tissues (4). When reaching the target tissue (5), the nanoparticles need to interact with the cell membrane (6) and be endocytosed (7) where they'll end up in endocytic vesicles (8). To reach the target sites of nucleic acids, the particles need to escape from endosomes (9).

Once the particle is able to evade immediate clearance, its next challenge is to reach the target tissue. Nonspecific distribution of the injected constructs leads to loss of material into non-targeted tissues and toxicity. A key factor influencing biodistribution and therapeutic efficacy is nanoparticle size. Large particles (> 200 nm) accumulate in liver and spleen, while small nanoparticles (< 5nm) are filtered out by the kidneys. Nanoparticles with size around 100 nm generally have long-lasting circulation times (128). Another factors influencing biodistribution are the deformability and charge of particles. Rigid particles are cleared more quickly while deformable, "softer" nanoparticles have longer circulation times and reduced accumulation in the spleen (129). Deformable particles can also travel through small capillaries, like those found in the lung for example (130). Although nanoparticles with a positively charged surface usually have a high cell uptake rate, they also exhibit high nonspecific internalization (131). Usually the particles escape the circulation only at the sites where the blood vessels have open fenestrations (like in the liver endothelium) or where they're perturbed, like by inflammatory processes or by tumors (129). Lung and liver uptake are associated with adverse effects – lung endothelium

activation and liver damage. The lung cell transfection could also be linked to the formation of micro-thrombi, because transfection decreases when animals are pretreated with anti-aggregant molecules. The endothelial surface can become strongly pro-coagulant upon injection of the complexes (132).

If the particles have successfully reached the target tissue, they also need to be able to enter the cells. Nano- and microscale constructs need to trigger endocytosis, a process where substances are internalized by the cell, to get inside cells and release their cargo. The material is engulfed into membrane invaginations that form membrane-bound vesicles called endosomes (125). Endosomes mature or fuse with lysosomes which become acidic, which in turn activates hydrolytic enzymes. The site of action for nucleic acids is usually the cytosol (siRNA, mRNA), or the nucleus (pDNA). If the nanoparticles can't escape the endosomes before the endosome maturation or its fusion with lysosomes, they will be degraded by the enzymes and won't reach their target (133).

If all these barriers are not addressed, nanoparticle based drugs can not realize their clinical potential. Fortunately, several strategies exist to overcome these obstacles.

1.7. Modifying the nanoparticles to overcome the barriers

17.1. Charge and size considerations

The fate of the nanoparticle and its interactions with biological systems is largely determined by its charge. Neutral and zwitterionic particles exhibit lower clearance after intravenous (i.v.) administration, and particles with a small positive or negative charge have minimal self-self, and self-non-self interactions. The higher the surface charge, the more the macrophage scavenging is increased, and the particles are rapidly cleared after i.v. injection, and are unable to cross the peritoneal barrier after intraperitoneal injection (123). However, higher surface charge also leads to more efficient interactions with cells, and positively charged particles show greater endocytosis in cells whereas negative particles do not (134). Also, negatively charged particles tend to be taken up by macrophages more than positively charged particles after opsonization. Control of the surface charge can minimize undesired loss of the particles and determine particle transfection efficiency.

Cationic particles favor uptake by endothelial cells (135). For example, cationic liposome/pDNA complexes are attached to and internalized into endothelial cells of blood vessels, extravascular leukocytes and macrophages. Most of the complexes are taken up by the endothelial cells of lung capillaries and macrophages in the liver and spleen (136). Systemic administration of high doses of PEI-DNA complexes mostly transfect the lung, which has the highest level of transgene activity after intravenous delivery. However, around half of the injected complexes appear to be taken up by the liver, but the level of transgene expression in the liver is low. This is probably cause by the phagocytosis by Kupffer cells, where the complexes are rapidly degraded (137).

Currently it is thought that the nanoparticle diameter should fall between 10–100 nm for *in vivo* use, although the upper limit is not as well defined. The lower limit is the threshold for elimination by the kidneys (138). Generally, nanoparticles with an average diameter around 100 nm show prolonged circulation, and they can escape filtration by the liver and spleen. With nanoparticle diameter over 150 nm, more nanoparticles are entrapped in the liver, spleen, and large particles tend to accumulate in the capillaries of the lungs (128).

1.7.2. Reducing rapid clearance

Opsonization and the resulting uptake of the particles by phagocytic cells is one of the mechanisms of rapid clearance of nanoparticles. Several strategies have been investigated to minimize opsonization, conjugating polyethylene glycol (PEG) to the surface of the nanoparticles for example (139). PEG provides steric hindrance via water shell formation which prevents protein binding to the nanoparticle, which has also been shown to prolong circulation time (140). However, although PEG delays immune response, it can also hinder uptake of the nanoparticles by cells. This is why active shielding has also been used, where self-peptides like CD47 can be attached to the surface of nanoparticles. CD47 is a marker of self that hinders phagocytosis, and CD47 decorated nanoparticles exhibit a delayed clearance by macrophages (141). Also, biomimetic particles obtained by surface modification with leukocytic membranes have been used to reduce opsonization. Leukocytes have all the functions required for drug delivery: they can evade the immune system, cross biological barriers in the body and localize at target tissues, so particles coated with purified membranes from white blood cells could delay clearance by phagocytic immune cells, and transport cargo across endothelium (142).

1.7.3. Increasing endosomal escape

Various strategies, like fusogenic peptides, and pH sensitive particles are being used to address endosomal entrapment (143). In these cases, the low pH of endosomes can be exploited, where the particles can go through pH-dependent conformational change, and the new conformation can fuse with the membrane to create pores in the endocytic vesicle, releasing its content into the cytosol (144). Another strategy is to use cationic lipids that enhance endosomal escape by interacting with the endosomal membrane and creating pores. The lipids that facilitate endosomal escape can be attached to CPPs and other polymers and they can thus improve the ability of other drug delivery vectors to enter the cytosol (145). Polymers that have a buffering capacity can induce osmotic swelling and the subsequent burst of the endocytic vesicle, which is called the proton sponge effect (146). For example, about 20% of the amino groups of PEI are protonated at physiological pH; the rest act as a proton sponge, being increasingly more protonated as the pH in the endosome decreases (51, 147,

148). The chloroquine analogs in the CPP PF6 are another example of vector modifications that increase endosomal escape by taking advantage of the proton sponge effect (116). Endosomal escape can also be enhanced by adding histidine residues (149, 150), or fusogenic peptides with a high amount of basic amino acids or amphipathic sequences (151).

1.8. Tumor targeting nanoparticles

When attempting tumor therapy, both the tumor and the mononuclear phagocytic system compete for the nanoparticles upon systemic administration. On average, 0.7% of the injected nanoparticles actually reach the tumor (122). Tumor gene delivery efficiency has been shown to increase when the particles are smaller (< 100 nm), have neutral zeta potential (from –10mV to +10 mV), or have active targeting moieties. This shows that the optimal nanoparticle modifications can be critical for overcoming barriers to cancer therapy.

An ideal anti-tumor therapeutic is stable in the bloodstream, and internalizes only into tumor cells leaving healthy tissues unharmed. CPPs generally need improvement in both categories, but fortunately, many strategies have been devised to address this problem. PEGylation is one of the possible strategies to increase the circulation time of nanoparticles, and additionally it has been found that nanoparticles that have longer circulating times are able to accumulate in tumors via the enhanced permeability and retention (EPR) effect that results in leaky tumor vessels. This is possible due to the neovasculature of tumors having a discontinuous basement membrane (152). A drawback of this approach is that PEG inhibits interactions of gene carriers with cell surfaces, greatly reducing cellular uptake. PEG has also been found to reduce endosomal escape capabilities of nanoparticles. These drawbacks reduce the capability of the nucleic acid to reach the cytosol and nucleus, lowering the therapeutic efficiency (153). Although the longer drug circulation times increase the likelihood of the drugs being extravasated into the tumor through the EPR effect, this also means that the drug can enter normal tissues as well, even though at a slower rate. The effect of the EPR effect is modest – providing less than 2-fold increase in tumor delivery compared to the levels in normal organs (154).

The EPR varies substantially between tumor types and patients (155) and simply relying on the EPR effect might not be enough to achieve efficient tumor specific gene delivery. Classic targeting approaches are passive and active targeting, with the first mainly exploiting the environmental factors of the tumor, such as a lack of draining lymphatic vessels, upregulated levels of certain enzymes, local pH value, etc., and the latter usually depends on specific markers at tumor sites which are targeted by specific moieties (156, 157). In many cases, the CPP activity is masked to avoid unspecific uptake in the healthy tissues, and it is fully exposed after reaching the target site, allowing for efficient internalization (158). A selection of these targeting strategies is discussed in more detail in the following chapters.

1.8.1. Matrix metalloproteinase sensitive particles

In this approach, the upregulated levels of matrix metalloproteinases (MMPs) are exploited. MMPs are overexpressed in many types of tumors; an upregulation of MMP-2 and MMP-9 levels is necessary for the degradation of basement membrane components, which allows the tumors to grow, and metastasize. They also regulate angiogenesis, and their activity correlates with tumor stage (159–161). In 2004, Jiang et al. introduced an activatable CPP, where the polyarginine conjugated to a fluorophore was attached to a polyanionic peptide moiety over a MMP-sensitive linker, forming a hairpin structure. In this form, the construct couldn't enter cells since the positive charges of the arginines were masked, and the low concentration of MMPs in the bloodstream wasn't enough for CPP activation. However, in an environment where MMP was present at higher concentrations, the linker was cleaved, and the polyarginine was unmasked. Since MMP is overexpressed in tumor sites, this leads to tumor-specific activation and uptake of the CPP (162).

1.8.2. pH sensitive particles

The extracellular pH in normal tissues and blood is 7.4, and the intracellular pH is slightly more acidic, pH 7.2. However, this is reversed in most tumors, where the extracellular pH is lower than the pH of healthy tissues, ranging from 5.7 to 7.8, depending on the tumor type (163, 164). This pH imbalance is caused by the high rate of glycolysis in cancer cells. Also, the acidic environment weakens the extracellular matrix which benefits the tumor (165). This presents an opportunity to use pH sensitive shielding: the cationic CPP can be masked with a polymer that is negatively charged under neutral conditions, and becomes neutral in acidic conditions. This approach was introduced by Sethuraman et al., who attached polysulfonamide to TAT (166). Polysulfonamide is negatively charged at pH 7.4, but becomes neutral below pH 7.0 – the extracellular tumor pH – leading to tumor-sensitive activation. Jin et al. used an approach where the primary amines of the lysine residues in TAT were amidized to succinyl amides, which inhibits TAT's interactions with non-targeted cells. The amide groups are hydrolyzed at acidic pH, generating corresponding amines that are positively charged (167).

1.8.3. Hypoxia-sensitive particles

Another tumor specific microenvironmental condition is hypoxia – regions in the solid tumors that have considerably lower oxygen concentration than normal tissue, because of the rapid growth of the tumor that outgrows its blood supply. A hypoxia-induced size-shrinkable nanoparticle that increases the penetration of drugs and nucleic acids into tumors was designed, where a hypoxia-sensitive azobenzene (AZO) was utilized as a linker between positively charged poly-

amidoamine (PAMAM) loaded with doxorubicin, and PEG2000. When entering the tumor site and being subjected to the hypoxic environment, the PEG groups were detached upon the breaking of the hypoxia-responsive linker AZO, exposing the PAMAM (168).

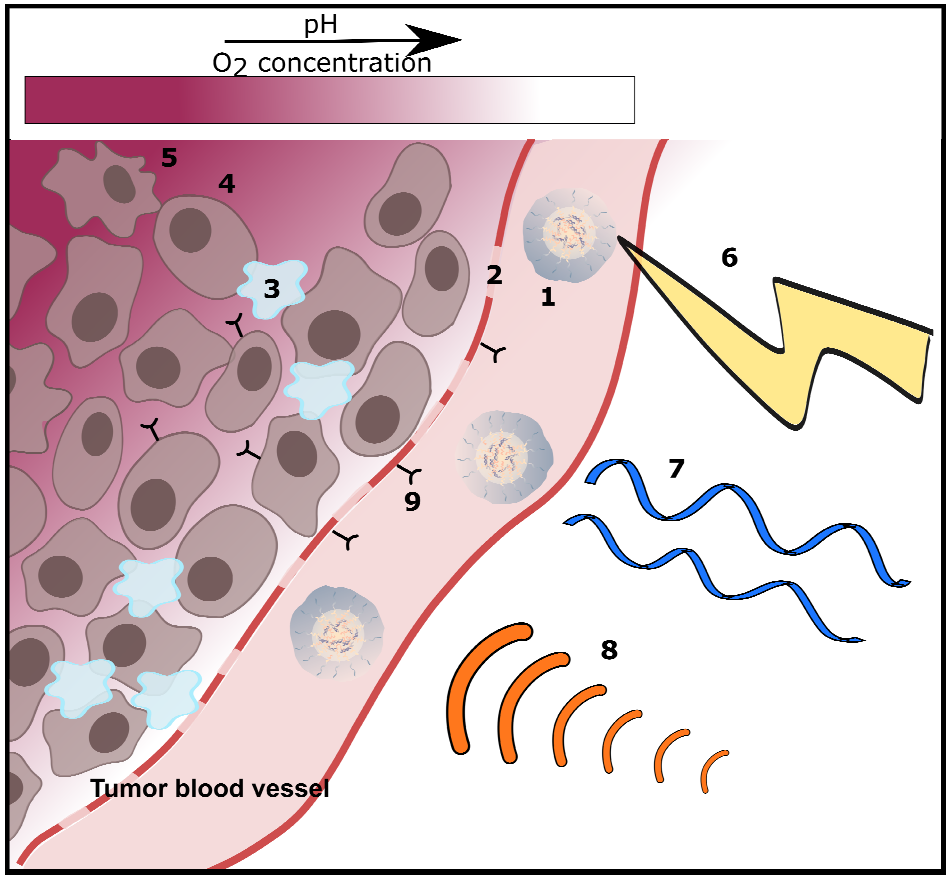


Figure 4. Different tumor specific properties and external stimuli that can be exploited for tumor targeting. 1 – nanoparticle; 2 – leaky blood vessel; 3 – tumor-specific enzymes; 4 – tumor area with lower pH; 5 – tumor area with lower oxygen concentration; 6 – external nanoparticle activation with light; 7 – external nanoparticle activation with ultrasound; 8 – external nanoparticle activation with heat; 9 – tumor specific cell or vascular surface markers.

1.8.4. External stimuli sensitive constructs

With a localized pathology like solid tumors, external stimuli such as heat, ultrasound, magnetic field, and light can be used to activate the drug delivery vehicles after they have passively diffused to the target tissue.

1.8.4.1. Temperature

A CPP-doxorubicin conjugate was used in a macromolecular carrier that is made of an elastin-like polypeptide (ELP). ELP has a phase transition occurring between 39 °C and 42 °C. Since this temperature range is above normal body temperature, systemic activation is prevented. Upon inducing hyperthermia in the desired site, the CPP-doxorubicin conjugate is released, and taken up by the cells in the heated tissue. ELP is soluble at body temperature, where it increases the plasma half-life of the particle, but it becomes insoluble and accumulates in locally heated regions (169, 170). The most common thermosensitive lipid is dipalmitoylphosphatidylcholine (DPPC). It has a gel-to-liquid phase transition at 41°C. Liposomes containing DPPC release more than 80% of their load within 30 minutes after the temperature is increased form 37 °C to 41 °C, which results in a high drug accumulation in the target tissue (171). Temperature sensitive constructs have also been made of carbon nanotubes. Upon injecting and heating with radio-frequency waves, the nanotubes increase in temperature to a point that leads to the death of cancer cells (172). Carbon nanotubes could also be decorated with CPPs to improve their cellular uptake.

1.8.4.2. Ultrasound

Sonication – application of ultrasound – can also be used to deliver drugs to target tissues. The process reversibly increases micro-permeation of the membrane, which can result in a more potent delivery of the drug molecules into the area of sonication. Constructs that are sable in the blood stream, but are exclusively sensitive to ultrasound can be designed (173).

1.8.4.3. Light

Light-sensitive tumor targeting includes photodynamic therapy where a photo-sensitizing agent (chlorins, phthalocyanines, porphycenes etc.) that is nontoxic, is activated with light, resulting in the generation of radical oxygen species that destroy the tumor cells (174). The photosensitizing agents are hydrophobic, re-quiring stabilization with micelles or liposomes. A short angiogenic endothelial cell targeting peptide APRPG was conjugated to PEGylated liposomes containing a photosensitizer, which lead to light sensitive tumor cell-specific targeting (175). Also, light sensitive lipids can be used that go through photo-triggered structural changes, leading to either leakage or collapse of the particles (176).

1.8.5. Active targeting

Active targeting ligands on drug carriers can further increase their accumulation at the target site, and allow delivery to specific cells. Monoclonal antibodies (mAB) are highly specific binders, targeting cell surface antigens. For example, nucleosome-sensitive antibody mAb 2C5 has been used for additional tumor cell targeting in pH sensitive TAT decorated PEGylated liposomes (172).

However, one of the limitations of using mAbs for targeting is the large size of the antibody molecule, which makes it difficult to reach into the interior of the large tumor mass where blood supply is inadequate. Also, antibodies tend to be taken up by the reticuloendothelial system (178).

Tumor targeting peptides are short peptide sequences that show high specificity and affinity to targeted cells, they're easier to prepare than antibodies, and thus make useful alternatives. They can also be chemically modified without adversely affecting their targeting ability (179). For example, they can be attached to other polymers, and cyclized. Cyclization puts the peptide under a constrained conformation that increases the interaction between the targeting peptide and its receptor (180, 181). Tumor specific peptides usually target either cell surface receptors, intracellular receptors, or the extracellular matrix that are often overexpressed in tumors. Some of the receptors targeted include aminopeptidase N, mucin1, urokinase plasminogen activator receptor, transferrin receptors, vascular endothelial growth factor receptors, etc. Intracellular receptors important in cancer signaling include cyclin A, and BCR/ABL (179). CREKA is one of the peptides that targets the extracellular matrix, and binds to the fibronectin-fibrin complex which is indicative of transition of normal epithelial cells to epithelial-mesenchymal that have stronger ability to migrate and invade than normal cells (182). The CAGALCY peptide targets the brain microvasculature (183).

The first tumor targeting peptide discovered, was RGD which binds the $\alpha_v\beta_3$ integrins (184). The $\alpha_v\beta_x$ integrin family are receptors for various extracellular matrix proteins (179), and several peptides containing the RGD motif have been used since (185–188). The LyP-1 peptide primarily accumulates in myeloid cells/macrophages in tumors after intravenous injection. The macrophages are abundant in the hypoxic areas of the tumors, meaning that Lyp-1 also reaches poorly vascularized areas (189, 190).

Similar tumor penetrating properties to Lyp-1 were discovered in the iRGD peptide (191). The iRGD is cyclic and it contains the integrin binding motif RGD, but its effect differs from the RGD peptide. The iRGD sequence spreads much deeper into extravascular tumor tissue, and it binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins expressed on tumor cells. Upon integrin binding, the CendR motif is activated through a protease cleavage event. The CendR motif then binds to neuropilin-1 or 2, activating an endocytic transport pathway – the CendR pathway (192), which is a form of receptor-initiated macropinocytosis. This pathway is also primed by nutrient depravation (193). The endocytic vesicles that form are large, around 200 nm, and so they take up a lot of fluid around the cell – potentially including drug molecules around the cells, meaning that the iRGD peptide can be co-administered with the drug and they don't necessarily have to be conjugated to each other (194). Lyp-1 also contains a CendR motif, and uses the CendR pathway (195).

Tumor targeting peptides provide tumor targeting, but can't often penetrate the cell membrane, or deliver large drug molecules like nucleic acids. In this regard, CPPs and tumor-targeting peptides could work together synergistically.

2. AIMS OF THE STUDY

CPPs have shown potential as gene delivery vectors, but they are still in need of improvement in terms of toxicity and tissue specificity. The main aim of this research was to develop CPPs with improved tumor targeting and reduced toxicity.

The specific aims of the study are described below:

- Paper I: The aim was to create tumor-sensitive CPPs for cancer gene therapy. To achieve this, polyethylene glycol (PEG) was conjugated to the CPP PF14 over a tumor sensitive linker.
- Paper II: Testing the capability of tumor-sensitive CPPs designed in Paper I to induce tumor growth reduction when complexed with therapeutic pDNA. An additional aim was to explore effects of active tumor targeting on the previously designed tumor-sensitive CPPs.
- Paper III: The aim was to explore alternative methods to PEG for reducing CPP toxicity. For this purpose, modifying nanoparticle formulation strategies, and changing CPP charge density and hydrophobicity were explored.

3. METHODOLOGICAL CONSIDERATIONS

3.1. Peptide design

Table 3. Peptides used in the thesis.

Name	Sequence	Paper
PF14	Stearyl-AGYLLGKLLOOLAAAALOOLL-NH_2	I, II, III
PF141	Stearyl-AGYLLGKLLOOLAAAALOOLL-PEG_{600}-NH_2	I
PF142	Stearyl-AGYLLGKLLOOLAAAALOOLL-PEG_{1000}-NH_2	I
PF143	Stearyl-AGYLLGKLLOOLAAAALOOLL-PEG_{2000}-NH_2	I
PF144	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG_{600}-NH_2	I, II
PF147	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-LALGPG-PEG_{600}-NH_2	I, II
PF145	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG_{1000}-NH_2	I, II
PF148	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-LALGPG-PEG_{1000}-NH_2	I, II
PF146	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG_{2000}-NH_2	I
PF149	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-LALGPG-PEG_{2000}-NH_2	I
iRGD	C*RGDKGPDC*-NH_2	II
PF144-iRGD	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG_{600}-C*RGDKGPDC*-NH_2	II
PF145-iRGD	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG_{1000}-C*RGDKGPDC*-NH_2	II
PF148-iRGD	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-LALGPG-PEG_{1000}-C*RGDKGPDC*-NH_2	II
PF6	Stearyl-AGYLLGK(a)INLKALAALAKKILL-NH_2	III
PF14-O	Stearyl-AGYLLGKLLOOLA**OO**ALOOLL-NH_2	III
PF14-E	Stearyl-AGYLLGKLL**E**OLAAAALOOLL-NH_2	III
C0-PF14	NH_3-AGYLLGKLLOOLAAAALOOLL-NH_2	III
C10-PF14	Decanyl-AGYLLGKLLOOLAAAALOOLL-NH_2	III
C22-PF14	Behenyl-AGYLLGKLLOOLAAAALOOLL-NH_2	III
C22-PF14-O	Behenyl-AGYLLGKLLOOLA**OO**ALOOLL-NH_2	III

O – Ornithine;
X – Aminohexanoic acid;
* – Disulphide bond between marked cysteines;
(a)Four trifluoromethylquinoline moieties attached to succinylated lysine tree.

All the CPPs used in this thesis are presented in Table 3. The CPPs designed in this study are all based on PepFect14 (PF14), which is a transportan analog that mediates efficient gene delivery *in vitro* and *in vivo* (117). However, PF14 has shown acute toxicity in animal models, and accumulates mostly in the lungs, so we studied different strategies in CPP design to increase their accumulation in the tumors for tumor therapy, and reduce the toxic effects by combining modifications in peptide sequence and complex formulation strategies.

In paper I our goal was to modify PF14 to decrease its accumulation in the lungs and liver, and increase accumulation in tumors. To achieve this, PEG moieties with different sizes (PEG600, PEG1000, PEG2000) were conjugated to the C terminal end of PF14 (creating PF141, PF142, PF143). To make the PEG removable in a tumor sensitive manner, MMP-2 sensitive linkers (PLGLAG) were introduced between the CPP and PEG, with aminohexanoic acid (X) linker between the cleavage site and PF14, to increase accessibility for the enzyme (PF144, PF145, PF146). For the uncleavable control CPP, the cleavage site was scrambled (LALGPG) (PF147, PF148, PF149).

For paper II, the iRGD moiety was introduced to the C terminal end of the previously designed MMP-2 sensitive PF14 analogues PF144 and PF145, to study the effect of additional active tumor targeting on the MMP-sensitive particles.

In paper III our goal was to study the effect of changing the net charge and hydrophobicity of PF14 on its transfection efficiency, with the overall goal of reducing CPP dose, and reducing the surface charge of the particles.

3.2. Peptide synthesis

The peptides used in this study were synthesized according to standard protocols for Fmoc (fluorenylmethyloxycarbonyl) solid phase peptide synthesis (SPPS) introduced by Bruce Merrifield in 1963 (196). The principle of SPPS is the stepwise synthesis of the peptide by adding amino acids with protected α-amino groups to the growing peptide chain that is anchored to an insoluble solid resin. Fmoc chemistry has an orthogonal protection strategy: the α-amino groups are protected by Fmoc which is removed in basic conditions (piperidine), while removal of the side chain protection groups and the peptide from the solid phase occurs under acidic conditions (95% TFA). An alternative to Fmoc-SPPS is t-Boc (tertbutyloxylcarbonyl) strategy, where all the protection groups are removed under acidic conditions. The α-amino group is protected by t-Boc, which is removed in milder acidic conditions (50% TFA), but the removal of peptide from resin requires a strong acid such as HF. Although using the Boc strategy gives better coupling results and less side reactions, the acid lability of both the temporary and permanent protecting groups can lead to the side chain deprotection during repeated TFA treatment. Additionally, HF is highly toxic and corrosive, requiring expensive equipment, and more fragile

peptides don't often survive the harsh HF treatment. This is why the Fmoc-strategy was used for the synthesis of the peptides in this work.

The deletion analogues of the synthesized peptide, and other impurities were purified using reverse-phase high-performance liquid chromatography (RP-HPLC), where the analytes are separated based on their hydrophobicity: the more hydrophobic a molecule is, the bigger its affinity to the stationary phase and its retention in the column. TFA was used in the mobile phase as a pH stabilizer, and an ion pair reagent, which interacts with the basic residues in the peptide, helping to increase retention of the charged analytes in the column and thereby increase selectivity. TFA is also volatile, allowing easier recovery of peptides (197). The molecular weight of the cleaved and purified peptides was analyzed by matrix-assisted laser desorption-ionization/time-of-flight (MALDI-TOF) mass spectrometry. MALDI is one of the best ionization techniques for analyzing peptides, because it is capable of ionizing large molecules with minimal fragmentation, making the obtained mass spectrum easier to analyze. Electrospray ionization (ESI) can also be used to ionize peptides for mass spectrometry, but it fragments the peptides. The TOF mass analyzer is often used together with MALDI because of its broad mass range.

3.3. Non-covalent CPP/nucleic acid complex formation

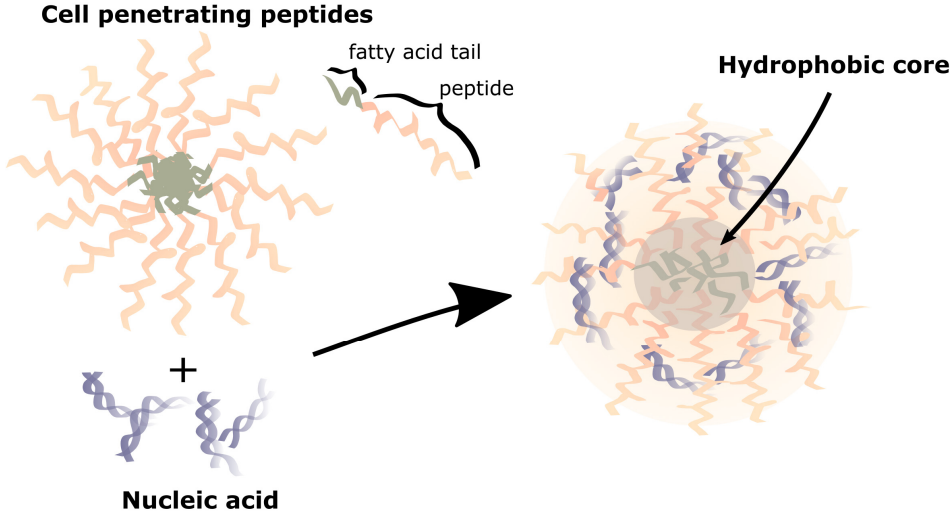


Figure 5. Schematic representation of hypothetical non-covalent complex formation. Since the CPPs are initially already in an aqueous environment, they form micelles. The CPP/nucleic acid complex hypothetically has a hydrophobic core.

After the peptides are synthesized, the next step before further experiments is to form complexes between the peptide and nucleic acids. In this study we used the non-covalent complex formation strategy between the CPP and plasmid

DNA (pDNA) which is widely used in non-viral gene delivery. With this method, the negatively charged DNA molecules are condensed by positively charged peptides by electrostatic interactions, although hydrophobic forces between non polar amino acid residues and the alkyl moieties also play a role in stabilizing the complexes. We used the charge ratio (CR) to characterize the amount of CPP over the plasmid DNA. CR corresponds to a widely used term N/P ratio, which represents the ratio of the amine groups of cationic polymers to the phosphate groups of DNA (198). CR is calculated theoretically, it takes into account the positive charges of the CPP (N), the negative charges of the DNA (P), and shows the amount of positive charges of the CPP to one negative charge of the DNA (N/P). For example, CR1 means that theoretically, there are as many positive charges arising from the CPP than there are negative charges arising from DNA. Usually we use an excess of CPP, and CR2 means that there are twice as many positive charges from the CPP than negative charges from the DNA.

3.3.1. Formulation of complexes for *in vitro* studies

The CPP/pDNA nanoparticles for cell culture experiments were prepared by mixing plasmid DNA with the CPP in MQ water followed by a 40 minute incubation at room temperature. As the transfection efficiency of the CPP/pDNA complexes is highly dependent on the CR, we used CR3 for cell culture transfection in this work based on previous studies.

When doing experiments with the matrix metalloproteinase (MMP) sensitive CPPs, the complexes had to be activated with the recombinant MMP enzyme before transfection to study the effect of MMP cleavage on transfection efficiency. For this, the complexes were prepared as described above, followed by adding the recombinant MMP-2 enzyme to the mixture, and incubating at 37 °C for 40 minutes before the transfection experiment.

3.3.2. Formulation of complexes for *in vivo* studies

The complex formulations for animal studies were optimized for systemic intravenous injections by tail vein. The first part of complex formation for *in vivo* experiments is similar to complex formation for in vivo studies: CPP and pDNA are mixed in MQ, and incubated for 40 minutes. The plasmid dose normally used is 20 µg (1 mg/kg) of pDNA that is mixed with CPPs at CR4. In this study we also tried additional formulations: 20 µg plasmid and 50 µg (2.5 mg/kg) plasmid mixed with CPPs at CR2. After the 40 minute incubation, the complex solution was mixed with glucose to achieve the final glucose concentration of 5% so the injection solution would be isotonic. This mixture was injected immediately via tail vein.

When forming complexes with PEGylated CPPs, the PEGylated CPPs were mixed with the non-PEGylated PF14 at different ratios. This is characterized by the PEGylation rate, which shows how big part of the total peptide content is made of the PEGylated CPP. For example, PEGylation rate of 50% means that there are equal molar amounts of the PEGylated CPP and PF14. These were prepared by first mixing the pDNA with the PEGylated component to ensure their presence in the complexes, and 5 minutes later adding PF14. This was followed by the 40 minute incubation and addition of glucose as described previously.

When doing *in vivo* experiments to study the effect of iRGD co-administration, the CPP/pDNA complexes were formed as described above, and the iRGD peptide was added after the complexes had formed after incubation.

3.4. CPP/pDNA nanoparticle characterization

3.4.1. Dynamic light scattering

The size of the CPP/pDNA nanoparticles is an important parameter, determining the fate of the complexes upon administration, toxicity and biodistribution, so studying this property is important. The diameter of the particles can be measured by dynamic light scattering (DLS) that measures the light fluctuations caused by Brownian motion of the particles, and calculates the size of the particles based on this information. DLS measures the hydrodynamic size of the particles which is the effective diameter of the solvated particle in a solution. For this study, the Zetasizer Nano ZS apparatus was used. Complexes for these studies were formulated as described above.

The Zetasizer Nano ZS apparatus also enables us to measure the zeta potential of the particle, which correlates with the surface charge – another important parameter of nanoparticles that influences biodistribution, toxicity and efficacy. When a particle is in a solution, it attracts other ions onto its surface. This creates two layers around the particle: the layer, where ions are adsorbed to the particle, and the diffuse layer where ions are loosely associated with the particles. Zeta potential shows the difference in potentials between the mobile fluid and adsorbed layer around the particle, and it is an indicator of the stability of the colloidal solution. When zeta potential is between 0 and ±5, the particles coagulate rapidly. For these measurements, complexes were again formulated as described above, and diluted 3x with MQ before pipetting into cuvettes.

3.4.2. Resistance of complexes to the presence of serum

The adsorption of serum proteins to nanoparticles and degradation of DNA by nucleases present in the bloodstream influence the efficiency of the therapy. This means the stability of CPP/pDNA complexes in the presence of serum components is an important parameter of the particles. They have to be stable in the bloodstream among serum components in order to have hope of reaching their target site and not be immediately degraded after injection. In this study we assessed this property by incubating the CPP/pDNA complexes in 50% FBS solution for 4 hours at 37 °C, followed by *in vitro* transfection (described in more detail in the chapter '*in vitro* transfection'). Since the serum concentration in the blood can be around 30–50%, depending on various conditions, incubating the nanoparticles at 50% serum concentration at 37 °C mimics some of the conditions the nanoparticles face in the bloodstream, and we can assess how stable the particles are in this environment. The following cell transfection experiment shows us how much the transfection efficiency is reduced upon prolonged contact with serum proteins. The more stable the complexes are, the less their transfection efficiency is influenced by serum incubation.

3.4.3. Heparin Displacement

The heparin displacement assay helps us judge the strength of interactions between CPP and pDNA in the nanoparticle. CPP/pDNA complexes must be strong enough to be stable in the bloodstream and protect DNA from degradation by nucleases, but at the same time the complex must be able to dissociate in the cytosol, so the nucleic acid could exert its effect. Heparin sulfate is an anionic molecule with a high negative charge density that has higher affinity to CPPs than DNA does, and the heparin concentration necessary for dissociating the complexes shows the stability of the particles – the bigger the heparin concentration needed, the stronger the interactions between CPP and pDNA. The nanoparticles were incubated at heparin at different concentrations for 30 minutes at 37 °C, followed by detection of the pDNA that had been displaced from the complexes by heparin, by adding the fluorescent dye PicoGreen® to the mixture that emits fluorescence when it intercalates with accessible DNA. The bigger the measured fluorescence signal is, the bigger is the amount of complexes that had dissociated.

3.4.4. Resistance against enzymatic degradation

Resistance to enzymatic degradation is critical for the use of nanoparticles in *in vivo* systems. The bigger resistance to proteinase treatment can indicate the longer half-life of the particles in the blood stream that has a protease-rich environment. To assess this resistance, CPP/pDNA complexes, formulated as

described above, were mixed with PicoGreen®. Proteinase K was then added to the complexes, and the resulting change in fluorescence emission resulting from CPP degradation by the protease was measured over a period of time.

3.5. Cell culture and tumor induction

The use of 2D cell cultures is a simple way to assess the biological activity of CPPs. In this study, multiple cell lines were used. In papers I and II, tumor cell lines U87-MG (human glioblastoma) and Neuro2a (murine neuroblastoma) were used to assess transfection efficiencies of the CPP/pDNA complexes on tumor cells *in vitro*, and in paper III, the peptides were tested on the CHO (Chinese hamster ovary) cell line, which is a commonly used cell line in biotechnology.

For primary *in vivo* studies for assessing reporter gene induction by CPP/pDNA complexes, tumors were induced with Neuro2a cells, since they form well vascularized tumors that also express the MMP-2 enzyme. We have observed that these tumors tend to disappear spontaneously in some animals when inducing the tumors in Balb/c mice who are immunocompetent. This is why other tumor models need to be used when studying the effects of the therapeutics on tumor growth. So for this purpose, tumors were induced with HT1080 (human fibrosarcoma) and 4T1 (murine mammary gland tumor) cells. Since HT1080 is a human cell line, athymic Nude-Foxn1nu mice had to be used as hosts since they have weakened immune systems, which prevent the organism from destroying the foreign cells. The tumors were implanted into the right flank of the mice. Since 4T1 and Neuro2a cells are murine cell lines, BALB/c mice with intact immune systems could be used. Neuro2a tumors were induced in the right flank of the mouse, and 4T1 were implanted into the 4^{th} mammary fat pad of female mice.

3.6. *In vitro* transfection

The efficiency of the CPP/pDNA complexes was evaluated by using a luciferase reporter system, and the p-CMV-Luc2 plasmid was used for expressing firefly luciferase. This reporter system is highly sensitive and specific, allowing us to detect even small changes in transfection levels. It also reflects the biological activity of the transgene, unlike fluorescence assays that measure the physical presence of the fluorescently labelled complexes in cells. The complexes were prepared as described above, and added to cells. The cells were lysed 24 hours after adding the complexes, which is the optimal time point for analyzing luciferase expression from the transfected plasmid. When the cells are lysed, the expressed luciferase is released to the lysate, and we can measure its amount with the luciferase assay. This assay contains luciferin, which is oxidized in a reaction catalyzed by luciferase. During this reaction, light is emitted,

and it can be measured by a luminometer. The amount of light emitted correlates to the concentration of luciferase in the solution and thus the transfection efficiency for luciferase encoding plasmid delivery.

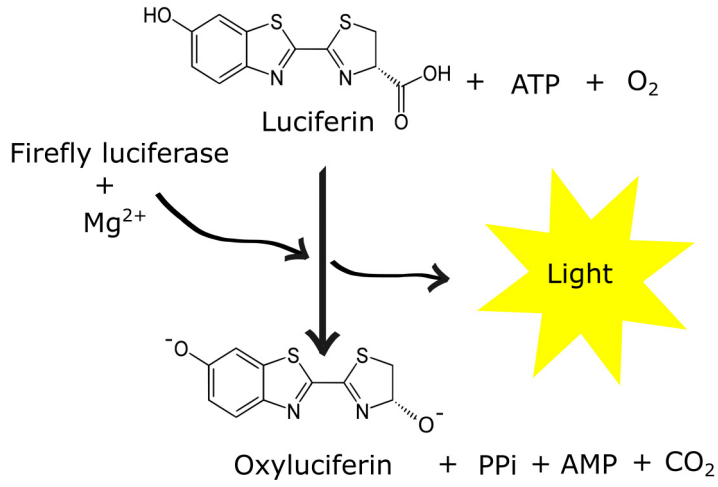


Figure 6. Luciferase-luciferin reaction.

3.7. Reporter gene delivery assessment *in vivo*

The luciferase reporter system was also used to evaluate gene delivery efficiency *in vivo*. Luciferase is an excellent reporter for *in vivo* studies, because the gene induction levels are lower than in cell culture, and high sensitivity is needed. However, the assay only gives us information about the general tissue that is transfected, not about the specific cell types that express the signal. The plasmid used was p-CMV-Luc2 which encodes the firefly luciferase. The complexes were prepared as described above in the in vivo complex formulation protocol, and injected immediately via tail vein in mice. The mice were sacrificed 24 hours after the injection as this is the time point where the luciferase expression from the plasmid has reached maximum levels. The tissues were harvested, homogenized, and the cells lysed before luciferase measurement to release the intracellular luciferase. The luciferase assay containing luciferin was also used in these experiments.

3.8. Tumor reduction measurements

To assess the effect of the anti-tumor therapeutic on tumor growth, one of the simplest way is to monitor the size of the tumor over time by measuring its size. In this study we could simply use calipers to measure the size since both tumor

models had visible subcutaneous tumors, but with models where the tumor isn't visible, other strategies like various imaging methods must be used. With the HT1080 model, the animals were assigned into 6 groups of 10 according to their tumor size when the tumors first became visible. This allowed us to create similar starting conditions for each treatment group. However, since we knew from previous experiments that the 4T1 model is very aggressive and grows fast, we had to assign mice into groups and start treatment before the tumors became visible – if we waited until that point, the tumors would've reached the cutoff size before the end of the treatment. All animals received 3 injections of the CPP/pshVEGF complexes or pshVEGF in 5% glucose to maximize the therapeutic plasmid concentration and the time of transgene expression in the tumors. The tumors were measured three times a week, and the experiment was terminated and mice euthanized at the first signs of lethargy.

4. RESULTS AND DISCUSSION

4.1. Tumor sensitive CPP PF144 is able to deliver reporter gene encoding plasmid DNA specifically into tumors (Paper I)

Tumor gene therapy vectors should ideally only enter tumor cells, and leave healthy tissues unharmed. The CPP PF14 (117, 199) has previously been shown to be able to induce transgene expression *in vivo*, and it rapidly transfects the liver and lung. If we used PF14 as a vector in tumor gene therapy, and the tumor was located outside of those tissues, PF14's inherent transfection profile would lead to off-target toxicity and loss of material. This means that PF14 has to be modified to decrease its interactions with healthy cells, and increase accumulation in tumors. We hypothesized that if we inactivated PF14 in a reversible, tumor sensitive manner, we would increase gene delivery efficiency in tumors, and reduce transgene expression in healthy tissues, leading to a safer and more efficient vector for cancer therapy. To achieve this, two modifications were utilized: PEGylation, and matrix metalloproteinase (MMP) sensitivity.

We explored polyethylene glycol (PEG) as the shield to inactivate the cell penetrating ability of PF14. PEGylation has been shown to reduce unwanted interactions with blood components, which increases the circulation time and the probability of the particle reaching its target tissue (200). However, the steric hindrance of PEG chains can interfere with the release of drugs from nanoparticles, and they also block nanoparticle uptake in target cells (201). PEGylation would thus allow us to increase plasma half-life of PF14 while also hindering its interactions with non-targeted cells.

Firstly, to study the effect of PEGylation on gene delivery efficiency, different sized PEG chains were conjugated to the C-terminal part of PF14 (PEG600, PEG1000, and PEG2000; corresponding CPPs PF141, PF142, PF143; sequences are presented in Table 3). The use of PEG molecules with different sizes would allow us to assess which of them would be most efficient for transfection with PF14. Also, the C-terminal end was chosen for modification since PEG is hydrophilic, and introducing it near the stearyl moiety would disturb the hypothetical micelle-like structures where the stearic acid chains form the hydrophobic core.

The results showed that PEGylated PF14 analogues were able to form complexes. The size of the complexes of PEGylated PF14 and pDNA was around 100–120 nm, and increasing the content of PEGylated CPPs in the complexes (increase in PEGylation rate), the zeta potential of the particles decreased. Since high surface charge has been linked to toxicity, reduction in zeta potential was a welcome result.

In cell culture experiments we found that 100% PEGylation rate showed no transfection activity regardless of the size of the PEG moiety. When decreasing PEGylation rate, the transfection activity was restored. This confirmed that PEGylation could be used to prevent the interactions of the complexes with cells.

We also investigated the effect of PEG on serum stability, circulation times and biodistribution of the complexes. First, the stability of the PEGylated complexes in the presence of DNase and serum, which are both encountered by the particles in the bloodstream, was studied. The PEGylated peptides were slightly less resistant to degradation than PF14, showing that PEG can protect PF14/pDNA complexes from serum components and enzymes. Complex stability in blood was tested by collecting blood from mice after intravenous injection of the complexes. The effect of PEGylation was most strikingly seen 30 minutes after injection, when the concentration of PEGylated complexes in the blood was around 3 times higher than that of PF14, showing that PEGylation reduced the rate of degradation, potentially increasing bioavailability. The tissues were collected from the animals 24 hours after the injection of complexes, and biodistribution was assessed by homogenizing and analyzing the tissues. Interestingly, the bioaccumulation patterns of complexes containing fluorescently labelled pDNA were similar regardless of PEG content of the complexes.

Continuing with *in vivo* experiments, we observed that already a 10% presence of a PEGylated PF14 with PEG1000 or PEG2000 in the complexes increased gene expression in tumors, and when increasing the PEGylation rate to 50%, the transgene expression in the tumor was increased for all PEGylated complexes regardless of PEG size. This suggests passive accumulation of the complexes in tumors through the EPR effect (202). In all cases, increasing the amount of PEGylated peptide in the complex decreased gene expression levels in the lungs, while gene expression in the liver was low with all PEGylation rates tested. A reduction in gene expression levels in the lungs was a welcome result, since unmodified PF14 shows high transfection levels in the lung which reduces the amount of therapeutic material available for the tumor, and correlates with acute toxic effects.

These findings supported our initial goal which was to confirm that PEG is able to shield the activity of PF14 and increase bioavailability. Next we wondered if we could increase nanoparticle accumulation in tumors and protect healthy tissues by keeping the PEGylation rate high, while making the PEG moiety removable in a tumor-sensitive manner by introducing a tumor specific matrix metalloproteinase-cleavable linker between PF14 and PEG.

MMP-2 activatable CPPs were first presented by Jiang et al. (162), who masked the activity of polyarginine with a construct consisting of anionic amino acids and PEG, which were linked to polyarginine over an MMP-2 cleavable peptide sequence PLGLAG. This allowed them to deliver the fluorescently labelled polyarginine selectively into tumors. While their approach was meant mainly for diagnostic purposes and for delivery of fluorescent dyes, we decided to apply this strategy for tumor sensitive gene delivery. So we investigated whether adding the MMP-2 sensitive linker PLGLAG between PF14 and the PEG moiety would further increase gene delivery into tumors, since theoretically the transfection efficiency of nanoparticles should increase when PEG is removed. The MMP-2 sensitive sequence was added to all the previously tested

PF14 analogues, and the new peptides were named PF144 (PEG600), PF145 (PEG1000), and PF146 (PEG2000) (Table 3).

First, we wanted to see whether MMP-2-sensitive restoration of transfection ability was taking place by incubating the CPP/pDNA complexes with recombinant MMP-2. We transfected cells with the PEGylated complexes with and without MMP-2 incubation, and saw an increase in transfection efficiency after the MMP-2 treatment. The transfection efficiency of the cleavable CPPs was restored to levels comparable to PF14/pDNA complexes, which confirmed that we are able to fully restore PF14 activity. The CPPs with scrambled cleavage sites (LALGPG) didn't regain their activity after MMP-2 incubation, confirming that the increase in transfection efficiency of cleavable peptides arose specifically from linker cleavage by MMP-2. The MMP-2 dependent activation was present even at lower PEGylation rates.

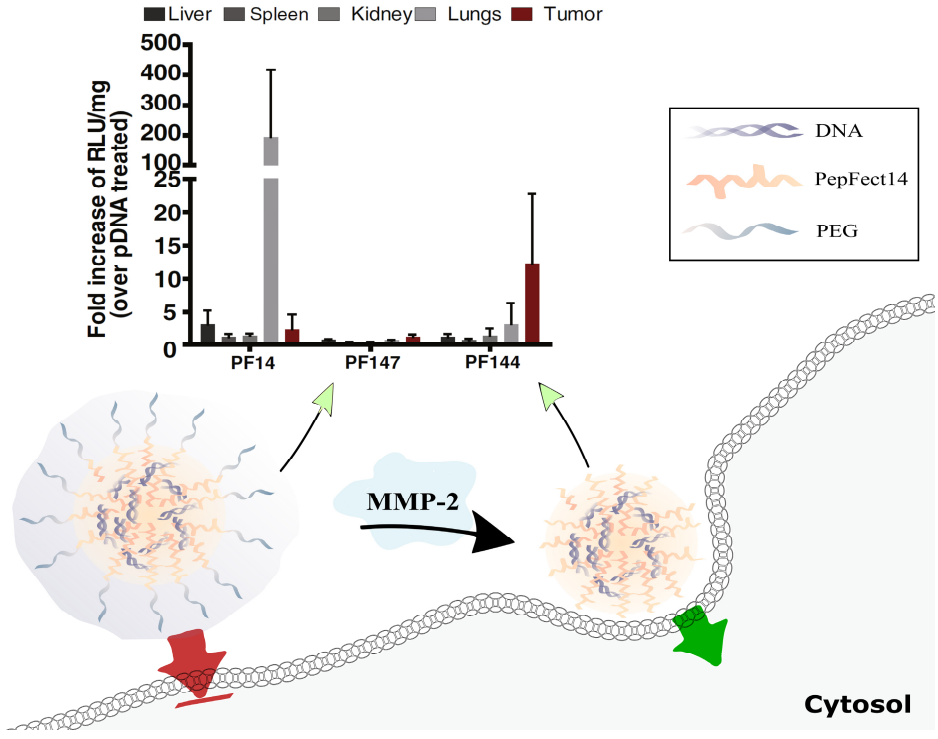


Figure 7. Activation of MMP-2 activatable nanoparticles.

The MMP-2 sensitive complexes would ideally be 100% PEGylated to achieve minimal interaction with non-targeted tissues. Unfortunately 100% PEGylated constructs failed to show uptake in any tissue in tumor bearing mice, indicating that the endogenous levels of MMP-2 are not high enough to be able to reach their target site in heavily PEGylated complexes. We adjusted the PEGylation rate to find the optimal ratio where gene expression in normal tissues would be

minimal, but prevalent in tumors at the same time. This was fortunately easy to optimize since it only required using varying amounts of PF14 and the PEGylated analogue. We found that at 50% PEGylation rate, reporter gene expression was lowered in non-targeted organs like lung, and increased in the tumors with PF144. At 70% PEGylation rate, luciferase expression was almost exclusively observed in tumor tissue, while it remained on basal level in other tissues. PF145 and PF146 showed similar tendencies, but with lower luciferase expression levels in tumors. Control peptides with the uncleavable linker showed no tumor accumulation. As seen from the experiments, the transfection profile of the complexes is highly dependent on the amount of PEG in the complex. The importance of fine-tuning the PEGylation rate has also been demonstrated by other groups (203).

These results confirm that the activatable CPPs were working as intended: achieving the tumor selective gene expression is probably mediated by the sufficient masking of CPP activity via PEGylation, which is followed by tumor sensitive removal of the PEG by MMP-2 near the tumor tissue, resulting in gene delivery to tumor cells.

4.2. Tumor sensitive CPP PF144 complexed with an anti-VEGF plasmid can slow tumor growth and it's superior to PF145-iRGD (Paper II)

Now that the tumor selectivity of MMP activatable CPPs was confirmed, it was time to test whether the MMP-2 sensitive complexes could induce an anti-tumor effect when complexed with a therapeutic plasmid. Although reporter gene expression in tumors was evident, it doesn't indicate whether the number of cells transfected is high enough for a therapeutic nucleic acid to be able to affect the tumor growth at all. Luciferase assay is very sensitive, allowing the detection of even small signals. Our second goal was to see if we could further increase transgene expression levels in tumors without losing tumor specificity, by adding an active tumor targeting ligand to the construct.

PF144 from the previous study was chosen for the tumor treatment study, since it could induce the highest gene expression levels in tumors out of the PEGylated analogues. For this experiment we also needed pDNA that could affect tumor growth, and we chose anti-VEGF pDNA (pshVEGF – plasmid expressing short hairpin RNA for VEGF knockdown) for this purpose. Using a plasmid that expresses short hairpin RNA against VEGF has a chance to induce a longer lasting effect than delivering anti-VEGF siRNA, although it faces an additional barrier by having to migrate to the cell nucleus. When the plasmid is expressed, a short hairpin RNA is generated after transcription, which is an RNA molecule with a tight hairpin turn in its secondary structure. This RNA can be used to silence target gene expression, VEGF expression in our case, via RNA interference. Its other advantage over siRNA, which can also silence target gene expression via RNA interference, is its lower degradation rate (204).

VEGF (vascular endothelial growth factor) expression is up-regulated in cancer, it stimulates the growth of new blood vessels, and knocking down VEGF expression leads to the suppression of angiogenesis, which hinders tumor growth (31). The downregulation of VEGF has previously been reported to lead to tumor growth suppression, but in most cases it has been administered as co-therapy (205–207). Since our goal was to evaluate the effect of CPP/pDNA nanoparticles alone, adding a co-therapeutic would have complicated the picture, which is why we didn't add a co-therapeutic. VEGF knockdown by plasmid expression was verified prior the tumor therapy experiments in cell culture, where clear knockdown of VEGF expression was seen, so we knew the pshVEGF plasmid was working as intended.

The ability of PF144/pshVEGF plasmid complexes to induce tumor growth reduction was evaluated with two tumor models, HT1080 and 4T1, which both express MMP-2 and $\alpha_v\beta_3$ integrins (208–211). Only the nanoparticles containing activatable PF144 were able to inhibit tumor growth rate in both tumor models, showing that PF144 mediates gene delivery into tumors is at a high enough level to induce a biological effect (in this case, reduction of tumor growth rate), and so it is a promising vector for tumor gene therapy

Our second goal was to evaluate how additional tumor targeting would affect the MMP-sensitive CPPs. Since MMP-2 has been shown to co-localize with $\alpha_v\beta_3$ integrins on tumor endothelium (212, 213), the tumor homing $\alpha_v\beta_3$ integrin specific peptide iRGD (191) was chosen as the targeting moiety. We hypothesized that the nanoparticles are concentrated around cells through binding to the integrins, which leads to more efficient linker cleavage since MMP concentration is higher near cell surfaces. This idea is illustrated on Figure 8.

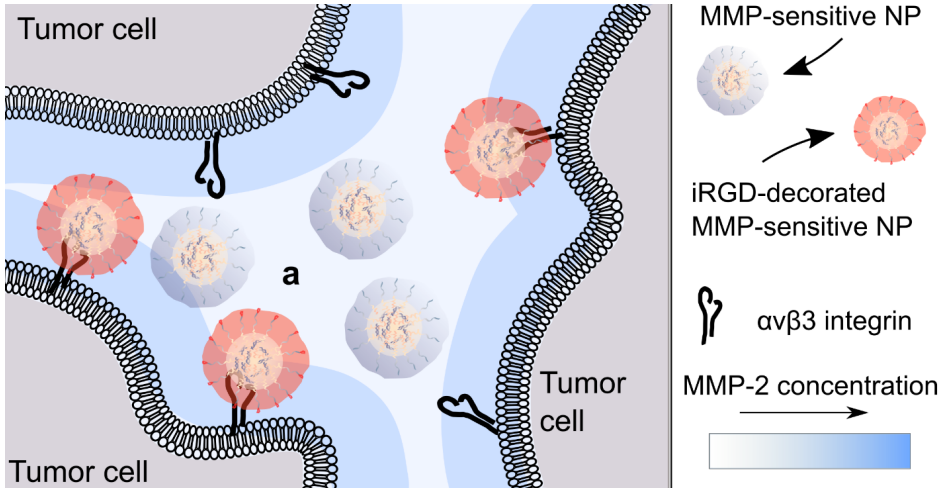


Figure 8. Theoretical advantage of iRGD decorated peptides. The MMP-2 cleavage should be more efficient after iRGD anchors the complex near cell surfaces where MMP-2 concentration is higher.

The iRGD moiety needs to be located on the surface of the particle to be effective so it could come into contact with the $\alpha_v\beta_3$ integrins. This is why we conjugated the iRGD moiety to the chain terminus of the PEG moieties of activatable CPPs (sequences in Table 3). If the iRGD were attached somewhere else in the PF14 sequence, the PEG chains could easily mask it. After iRGD conjugation to the MMP-activatable peptides, an increase in zeta potential was observed, suggesting that the surface charge of the nanoparticles had probably changed thanks to the charges of the iRGD peptide.

Since PF144 was the most efficient out of the CPPs in Paper I, we initially conjugated iRGD to PF144, creating PF144-iRGD. To test how the conjugation of iRGD influences the transfection ability of the CPP, transfection experiments in cell culture were carried out. We saw that although the transfection ability of the CPP was restored upon incubation with MMP-2, conjugating iRGD to PF144 had increased its baseline transfection ability in the PEGylated form that hadn't been activated with MMP-2. The PEGylated construct should have minimal transfection efficiency to reduce off target gene delivery. This rise in activity of the non-activated peptide would mean a decrease in specificity, since the complexes could more actively interact with non-targeted tissue. Indeed, in Neuro2a tumor bearing animals, complexes containing PF144-iRGD induced gene expression in liver, spleen and lung, and this also reflected in reduced accumulation of the particles in tumors compared to PF144, likely because of material loss in other tissues.

This was not an ideal situation, so next we conjugated iRGD to PF145 that has a longer PEG chain, creating PF145-iRGD. We hypothesized that the longer PEG chain should be more effective in counteracting the activity of iRGD. The transfection results showed that although complexes containing PF145-iRGD also exhibited higher baseline activity, it was still lower than that of PF144-iRGD. This was also reflected in *in vivo* studies, where PF145-iRGD containing complexes retained their tumor specificity, and had an increase in tumor transgene expression. These results showed that we had achieved our goal of increasing tumor gene delivery efficiency by introducing an active targeting moiety to the CPP.

The iRGD peptide has also been demonstrated to work as a co-delivery agent, where it facilitates the cellular uptake of drug molecules it's not covalently attached to (194). We tried this approach *in vivo* with PF144 and PF145 to see how it performs when compared to the CPP-iRGD conjugates. The co-administration of iRGD with activatable PepFects resulted in a higher luciferase induction in the spleen with both peptides, and the tumor specificity was reduced, which also diminished transgene levels in the tumors, possibly due to loss of material into other tissues. Transgene expression in spleen and other tissues has been observed before with iRGD co-delivery (214, 215), and it has been hypothesized that $\alpha_v\beta_3$ expressing macrophages can increase the clearance of the nanoparticles from the circulation (216), thus reducing therapeutic material available for tumors. We concluded that the PepFect peptides are better suited to be conjugated to iRGD as opposed to co-administration.

Next we performed anti-tumor therapy in HT1080 and 4T1 tumor models with the PF145-iRGD complexes and anti-VEGF plasmid DNA, which we had also used with PF144. Theoretically, complexes containing PF145-iRGD should perform as well as PF144, as luciferase experiments would suggest. However, to our surprise, PF145-iRGD performed worse, with no tumor growth reduction observed in either tumor models.

Puzzled by this outcome, we sought answers from serum stability of the CPPs. When drugs are administered intravenously, they come into contact with many blood components, and need to be stable in that environment. The stability of the nanoparticles in the presence of serum was assessed by evaluating the effect of 50% FBS on the transfection ability of the complexes, and the strength of the complexes in the presence of varying concentrations of heparin, which binds to CPPs more strongly than DNA. Serum incubation decreased the transfection efficiencies of the CPPs that contained PEG, and the effect was more notable with increasing size of the average PEG moiety – PF145-iRGD with the longest average PEG chain was the most sensitive, while PF14 with no PEG moiety the least sensitive to the presence of serum. Also, PF145-iRGD was more strongly influenced by serum treatment than PF145, suggesting that the iRGD moiety also contributes to serum susceptibility. Similar trends were also seen with the heparin displacement assay, where the strength of the CPP/pDNA complexes was evaluated by how easily heparin could displace pDNA in the nanoparticles. Again, PF14 with no PEG or iRGD formed the most condense complexes with pDNA, while PF144 with the shortest PEG chain and no iRGD moiety was the second best out of the CPPs tested. This indicates that CPPs with longer and more polydisperse PEG chains tend to form less condensed complexes which are more accessible to binding of serum components, like β globulin, that has been shown to adsorb to PEG (217). This also provides answers to the unexpected tumor treatment result – PF144 containing complexes perform better than PF145-iRGD because they are more stable in the bloodstream which hypothetically increases their effective dose.

To conclude, the activatable CPP created in Paper I can efficiently reduce tumor growth rate in anti-VEGF therapy. Since the nucleic acid used with the CPP is not restricted to anti-VEGF plasmid, other targets could also be tested in the future.

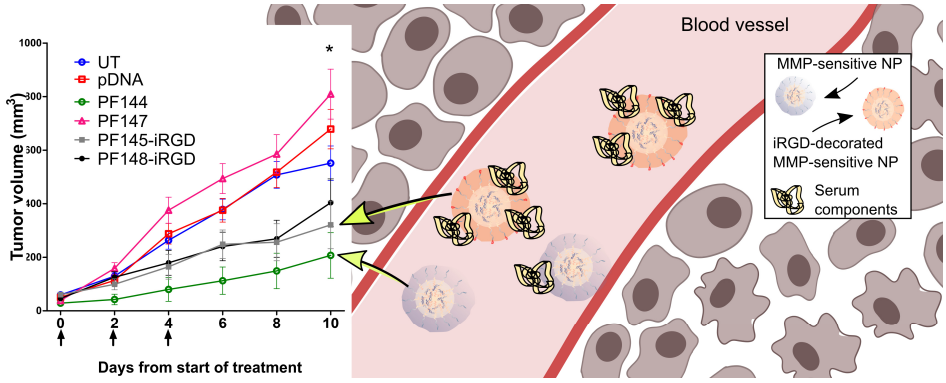


Figure 9. Hypothetical fate of MMP-2 activatable, and iRGD-decorated CPPs upon intravenous administration *in vivo* and the resulting effect on tumor treatment. Yellow arrows point to the corresponding treatment group.

4.3. Nanoparticle formulation optimization reduces side effects (Paper III)

Since we had observed occasional acute toxic effects upon injecting mice with PF14/pDNA complexes during previous studies, we sought to find ways to reduce toxicity of the particles in this research. It is known that the surface charge of the particles affects their binding to blood plasma proteins – opsonization – which leads to the elimination of the particles from the organism, and activation of the immune system (218). Since PF14 has a high cationic zeta potential at CR4, we hypothesized that the toxic side effects might be lessened by decreasing the surface charge. Although PEGylation is commonly used for reducing surface charge of particles, it also reduces favorable contacts of CPPs with cells, as we also saw in Paper I, and without an additional mechanism for eliminating the PEG, transfection efficiency is also reduced.

First, we studied the effect of modifying the charge ratio (CR) on the surface charge of the particles. We titrated the CR of PF14/pDNA complexes, and saw that a decrease in charge ratio was accompanied by a reduction in the zeta potential, meaning that the surface charge could be easily reduced by just lowering the charge ratio, and thus reducing the amount of unbound CPPs. We were then interested in how the CR affects transfection efficiency and toxicity of the particles. In general, transfection efficiency was only influenced by pDNA dose, regardless of the CR. However, toxic effects were clearly dependent the amount of excess CPP, which increases with the CR. The correlation between uncomplexed polymer and toxicity has also been proposed by other groups (219). We could increase the pDNA dose up to 2 µg *in vitro* at CR2, without observing toxicity, while at CR6, where the amount of free CPPs is bigger, we could only use up to 0.125 µg plasmid. Lowering the CR also lead to a reduction in opsonization. This suggests that lowering the charge ratio reduces

toxicity and thus allows the usage of higher plasmid DNA doses, resulting in increased efficiency *in vitro*.

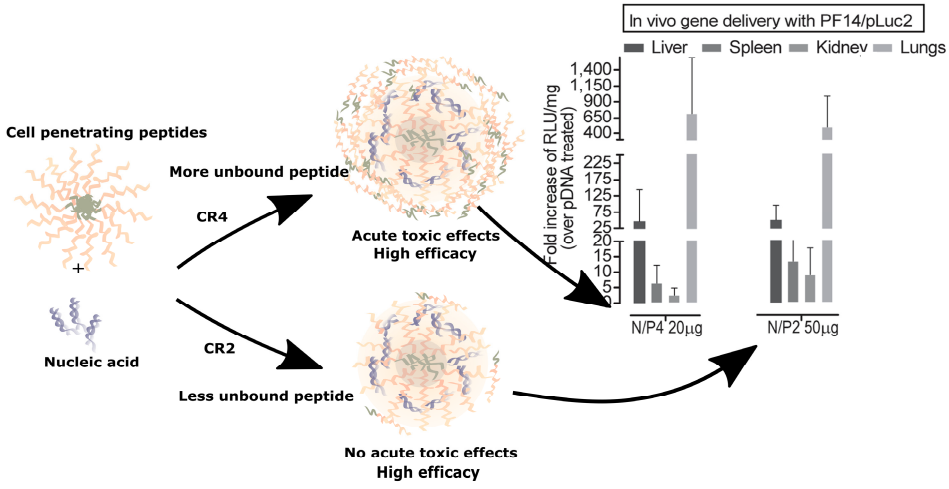


Figure 10. Effect on charge ratio and plasmid dose on acute toxicity and *in vivo* gene delivery.

To see whether this strategy would also work *in vivo*, we lowered the charge ratio from CR4 that is used typically, to CR2, where we observed zero lethality. Relying on cell transfection studies, we also increased the pDNA dose, and with 50 µg pDNA dose at CR2, we saw a similar gene induction profile as with CR4 with 20 µg pDNA dose, but without the lethality, mirroring the previously obtained *in vitro* results. Lungs are an important organ from the perspective of bioactivity and side effects, so we evaluated how the nanoparticles accumulate in the lungs after intravenous administration of complexes containing fluorescently labeled pDNA. When the animals were injected with the CPP/pDNA complexes at CR4, a large amount of fluorescence was observed in lungs, but at CR2, the signal in the lungs was significantly lower which is possibly the reason complexes at CR2 are also less toxic. The physical accumulation of the nanoparticles, as seen with CR4, could be one of the reasons behind its lethality.

Another component of nanoparticles that can cause toxic effects is the dose of the CPP. Thus we hypothesized that if we can decrease the amount of the CPP in the nanoparticles, we could also reduce adverse effects. Since we formed complexes between PF14 and pDNA according to the CR, we could reduce the amount of peptide by increasing its net charge – the charge ratio would stay the same, but the dose of CPPs would be reduced. We first designed a PF14 analogue, PF14-O, where two alanines were substituted with ornithines (Table 3) to increase the net charge by 2 (+7 total). We also made a control peptide PF14-E, where one ornithine was substituted with glutamic acid, resulting in a –2 change in net charge (+3 total). Interestingly, the size and zeta potential of the new analogues did not significantly differ from PF14. When

doing cell culture experiments, we compared these new CPPs to other delivery vectors with a high net charge, PF6 (111) (+10), and PEI, a commonly used cationic polymer for gene transfection that exhibits very high charge density (137). According to *in vitro* results, the highest transfection was mediated by vectors with highest net charge, PF14-O and PF6, followed by PF14, jetPEI, and lastly, PF14-E, which correlates with previous knowledge about the effects of higher positive charge on transfection efficiency (128).

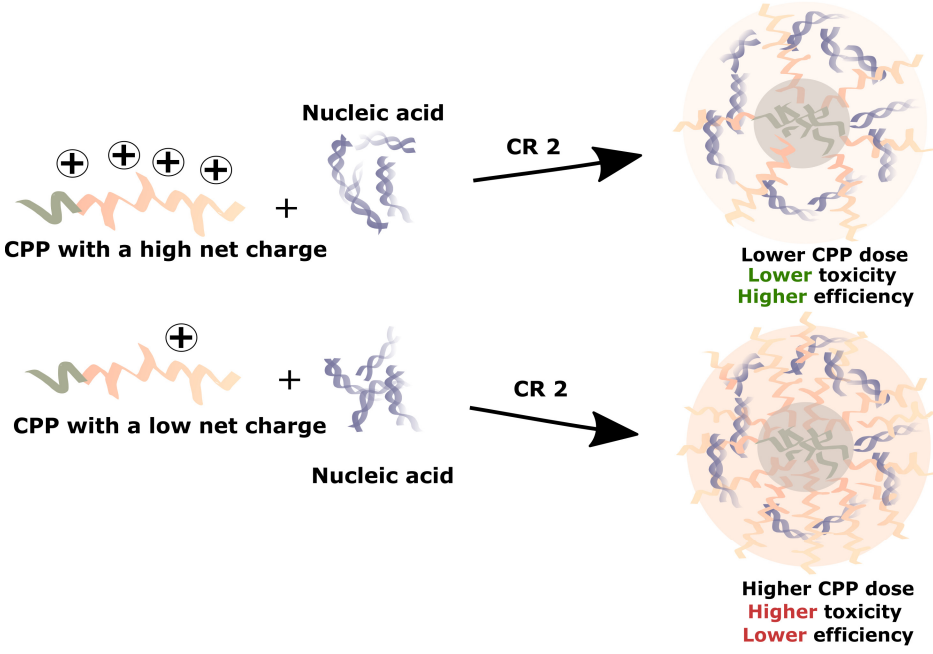


Figure 11. Effect of CPP net charge on peptide toxicity and gene delivery efficiency.

We also studied the effect of hydrophobicity on the size and surface charge of the nanoparticles. For this, the N terminal fatty acid of PF14 was either eliminated (C0-PF14), shortened (C10-PF14) or increased (C22-PF14). All the sequences are presented in Table 3. The DLS results showed that the longer the fatty acid chain length, the bigger the zeta potential of the particle, and thereby the surface charge. When performing *in vitro* tests, it was observed that an increase in fatty acid chain length also increased the transfection efficiency of the CPP, but also toxic effects were observed with CPPs with longer fatty acid chains, which probably comes from their higher surface charge.

For gene delivery to be effective, the nanoparticle must be protected against degradation. We assessed the susceptibility of the CPP component of the complexes to proteinases by incubating the complexes with proteinase K, which can degrade peptides and has broad substrate specificity. This experiment demonstrated that modifications in electrostatic and hydrophobic properties of the CPPs affect complex stability – decreasing the charge makes complexes

more susceptible to proteinase treatment, while increasing the charge has no effect on stability. Additionally, the longer the N-terminal carbon chain length, the more stable the complex are against proteinase treatment.

After gaining valuable information from stability experiments, we carried on with gene induction experiments *in vivo*. PF14 and its analogues with a higher charge (PF14-O) and longer fatty acid chain (C22-PF14) were most efficient out of the tested PF14 analogues, inducing transgene expression in lungs, liver, and spleen. Also, to take into account what we had learned earlier with CR studies, we assessed the *in vivo* performance of the PF14 analogues at CR2 with a higher amount of pDNA. Interestingly, both PF14 analogues PF14-O and C22-PF14 showed less gene expression in lung, but more in liver when compared to the same CPPs at CR4. Again, zero lethality was observed with the new PF14 analogues at CR2.

So far we had seen that PF14 analogues with highest net charge (PF14-O) and longest fatty acid chain (C22-PF14) were the most efficient, and C22-PF14 additionally had high stability to proteinase treatment, so we decided to combine these CPPs and synthesized a peptide with a long fatty acid chain and +7 net charge (C22-PF14-O, Table 3). Interestingly, this new peptide exhibited even higher stability to proteinase treatment than any of the parent peptides, and when administering this CPP to mice, we saw an even further increase in gene induction in the liver than with the parent peptides, but again, no acute toxic effects at CR2. Taking into account that the toxicity and lung accumulation are possibly linked, the decrease of gene induction in the lungs could be one of the mechanisms behind reduced toxicity.

To conclude, we found several efficient, yet simple ways of reducing CPP toxicity: lowering the surface charge by reducing charge ratio and increasing hydrophobicity, and decreasing peptide dose by increasing its net charge. These modifications can lead the way in the design of new safer CPPs.

5. CONCLUSIONS

The main aim of this paper was to create tumor sensitive CPPs for cancer treatment applications, and study ways to increase overall CPP toxicity while preserving efficiency.

The key findings from all the papers are described below.

Paper I:

- PEG is able to shield PF14 transfection ability.
- Adding a tumor sensitive linker between PF14 and PEG allows for MMP-2 sensitive PEG removal and resulting CPP activation.
- The activatable CPPs induced gene expression mostly in tumors, while the gene expression remained at baseline levels in other analyzed tissues.

Paper II:

- The delivery of MMP-2 activatable CPP PF144 complexed with anti-angiogenic plasmid DNA is able to induce tumor growth.
- Conjugating iRGD to MMP-2 activatable PepFects requires a PEG with at least 1000 Da average size to retain tumor specificity.
- Longer PEG and the addition of iRGD make PepFect nanoparticles more susceptible to blood serum components.
- PF144 is superior to PF145-iRGD in tumor reduction experiments.

Paper III:

- Simple change from charge ratio 4 to charge ratio 2 reduces nanoparticle surface charge and acute toxic effects of PF14.
- CPP/pDNA nanoparticle surface charge is influenced rather by the hydrophobic component of the CPP than the number of charges in the CPP.
- Increasing both the charge and fatty acid chain length of PF14 increase resistance to proteinase digestion and enhances liver transfection.

SUMMARY IN ESTONIAN

Rakku siseneva peptiidi PepFect14 modifitseerimine kasvajaspetsiifilisuse suurendamise ja toksilisuse vähendamise eesmärgil

Viimastel aastakümnetel on geeniteraapia alal toimunud olulised edasiminekud, mis on teinud sellest ühe potentsiaalseima ravimeetodi, mis võimaldab ravida haigusi geenitasandil, kas vigast geeni asendades või selle ekspressiooni maha surudes. Efektiivseks raviks peavad terapeutilised nukleiinhapped, mis on suured negatiivselt laetud makromolekulid, rakus jõudma oma toimekohta, milleks on näiteks tsütosool või rakutuum. Õigete kudedeni jõudmiseks peavad need molekulid läbima mitmeid bioloogilisi takistusi, mida nad saavad efektiivselt teha ainult transportvektorite abiga.

Kuigi viirusvektorid on väga efektiivsed, on neil kliinilistes katsetes avaldunud mitmeid olulisi puudusi, nagu immunogeensus ning tootmise kõrge hind. Need puudujäägid on ajendanud uuringuid mitteviirusliku geeniteraapia vallas, kus transportvektoriteks on sünteetilised molekulid, mis suudavad moodustada nukleiinhapetega nanoosakesi ning need seejärel rakkudesse toimetada. Kuigi sünteetilised transportvektorid on ohutumad kui viirusvektorid, vajavad nad siiski täiendavat arendamist efektiivsuse parandamiseks.

Üks klass mitteviiruslikke transportvektoreid on rakku sisenevad peptiidid (RSP). Nad on lühikesed, tavaliselt katioonsed või amfipaatsed peptiidid, mis suudavad rakkudesse transportida bioaktiivseid lastmolekule. RSPde probleemiks on siiani manustamisjärgne mittespetsiifiline jaotumine organismis, mis suurendab toksilisust mittehaigetele kudedele ning vähendab kättesaadava terapeutilise materjali hulka haigusliks koes. Näiteks vähi kemoteraapia korral on terapeutikum tavaliselt rakkudele tsütotoksiline ning mittespetsiifline ravimi jaotumine võib põhjustada patsiendile tõsiseid kõrvalnähtusid. Selle vältimiseks vajavad RSPd, nagu ka teiste klasside vektorid, edasist arendust suurendamaks nende koespetsiifilisust. Lisaks teistele transportvektoritele on ka RSPde puhul märgatud probleeme toksilisusega. Toksilisuse vähendamiseks on vajalik optimiseerida nanoosakese valmistamise ning peptiidi disaini strateegiaid.

Käesoleva doktoritöö eesmärgiks oli olemasolevate RSPde modifitseerimine, et suurendada nende spetsiifilisust kasvajate suhtes, vähendada mittespetsiifilist osakeste kogunemist kopsudesse ning uurida, kuidas RSP/nukleiinhappe nanoosakeste moodustamise optimeerimine mõjub nende efektiivsusele ning toksilisusele.

Töö esimeseks eesmärgiks oli modifitseerida rakku sisenevat peptiidi PepFect14 nii, et suureneks selle kasvajaspetsiifilisus. Nimelt PF14 indutseerib transgeeni ekspressiooni väga tugevalt kopsus ja maksas ning kui seda kasutada vektorina vähivastases geeniteraapias, koguneks suur osa materjalist just nendesse organitesse ja suureneks ka teraapia toksilisus. Et seda vältida, konjugeeriti PF14 C-terminaalsesse otsa polüetüleenglükooli (PEG) molekul, mis kirjanduse

andmetel vähendab partikli kontakte tervete kudedega ning suurendab osakeste akumulatsiooni kasvajas. PEGüleeritud PF14 suurendab nanoosakeste võimet indutseerida geeniekspressiooni tuumoris ning vähendab seda kopsus ning maksas. On teada, et PEG konjugeerimine RSPga suudab maskeerida selle transfektsioonipotentsiaali. Parandamaks transfektsiooniefektiivst kasvajakoes, lisati PEG molekuli ja PF14 vahele lisalüli, mis on substraadiks spetsiifiliselt vähikoes üleekspresseeritud ensüümile. Kui selline PEGüleeritud peptiidi sisaldav RSP/nukleiinhappe kompleks satub vähikoesse, lõikab ensüüm oma substraati, mille tulemusena PEG molekul eemaldub ning vabanenud RSP on suuteline efektiivselt ümbritsevaid rakke, milleks on eelistatult kasvajarakud, transfekteerima. Nende PF14 modifikatsioonidega saavutasime oma eesmärgi, luues PF14 analoogi, mis suudab indutseerida kasvajaspetsiifilist geeniekspressiooni.

Edasi jätkati tööd varem loodud kasvajaspetsiifiliste RSPdega. Eelmises töös uuriti vaid reportergeeni ekspressiooni, kuid see ei näita, kuidas antud osakesed töötavad terapeutilise plasmiidiga. Seega testiti järgnevalt kuidas vähispetsiifiline PF14, PF144, komplekseeritud VEGF maha suruva plasmiidiga, mõjub tuumori kasvule. VEGFi (vaskulaarse endoteeli kasvufaktori) ekspressioon on kasvajates ülesreguleeritud, see stimuleerib uute veresoonte kasvu ning VEGF ekspressiooni mahasurumine surub alla uute verestoonte teket, mis omakorda takistab kasvaja arengut. Kasvajavastase toime hindamiseks süstiti tuumoreid kandvatesse hiirtesse PF144/shVEGF komplekse ning mõõdeti ajas tuumorite suurust. Tuumorite kasvukiirus aeglustus – PF144 suudab transgeeni kasvajarakkudesse transportida piisaval tasemel, et saavutada bioloogiline efekt. Teiseks eesmärgiks oli näha, kas kasvajat sihtiva peptiidi iRGD lisamine eelnevas töös loodud kasvajaspetsiifilistele RSPdele suurendab nende transfektsiooniefektiivsust kasvajas veelgi. Selleks konjugeeriti iRGD PEGüleeritud RSPde PEG ahela otsa. Leiti, et reportergeeni ekspressioon modifitseeritud peptiide kasutades oli palju kõrgem kui modifitseerimata peptide sisaldavates RSP/nukleiinhappe kompleksides. Uus iRGD-ga konjgeeritud RSP siiski ei suutnud mõjutada kasvaja kasvukiirust samal määral mis PF144, tõenäoliselt kuna see on palju tundlikum lagundamisele seerumikomponentide poolt.

Kolmandas töös keskendusime samuti PF14 toksilisuse ning kopsutransfektsiooni vähendamisele, kuid seekord ilma PEGi kasutamata. Nimelt, nagu ka eelnevates töödes näha, inhibeerib PEG nanoosakeste kontakti rakkudega, pikendades küll biosaadavust, kuid tehes osakesed vähem kättesaadavaks ka kudedele, kuhu tahetakse geeni transportida. Selle saavutamiseks keskenduti peptiid-nukleiinhappe komplekside erinevate omaduste muutuse mõjule toksilisusele ning kopsutransfektsioonile. Esiteks optimeeriti PF14/nukleiinhappe omavahelist suhet ning leiti, et väiksema laengusuhte puhul, kus suurem osa peptiidist on nukleiinhappega seotud, on osakesed vähem toksilised. Selle põhjuseks võib olla vaba peptiidifraktsiooni, mis ei ole nukleiinhappega seotud, vähenemine. Teiseks optimeeriti peptiidi kogulaengut ning rasvhappejäägi pikkust ning sünteesiti vastavad PF14 analoogid. Selle käigus selgus, et mida positiivsem on peptiidi kogulaeng kompleksis, seda suurem on selle transfektsiooniefektiivsus. Lisaks ilmnes, et pikem rasvhappejääk peptiidi küljes suudab seotud

nukleiinhapet efektiivsemalt kaitsta lagundavate ensüümide eest. Kombineerides peptiidi kogulaengu suurendamist, rasvhappejäägi pikendamist ning RSP/ nukleiinhappe suhte vähendamist, suudeti viia alla toksilisust põhjustav kõrge kopsutransfektsiooni tase. Need modifikatsioonid on heaks juhiseks uute ja turvalisemate RSPde disainil.

Kokkuvõtteks, rakku sisenevad peptiidid suudavad efektiivselt nukleiinhappeid *in vivo* erinevatesse kudedesse transportida ning kasutades spetsiifilisi modifikatsioone, nagu PEGüleerimine, laenguarvu ning RSP/nukleiinhappe suhte muutmine, on võimalik parandada RSP koespetsiifilisust ning toksilisust.

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