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KRISTA FREIMANN

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Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

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ABSTRACT

Recent advances in genomics and genome editing strategies have created novel prospects for gene therapy. Advancements in genomic sequencing able us to regulate the expression of pathogenic phenotype-causing genes through the utilization of therapeutic nucleic acids. The main obstacle for gene therapy is the poor cellular uptake of such therapeutics. Therefore, the major challenge in creating effective gene-based therapies is the development of selective and safe delivery vectors. Currently, the most effective and abundantly utilized gene delivery vehicles in clinical trials are virus-derived vectors. Despite the encouraging results with the viral delivery systems, concern about the safety of these vectors remains. Therefore, increasingly more effort have been applied into the research of non-viral vehicles as a safer alternative to the viral counterparts.

Cell-penetrating peptides are short cationic and/or amphipathic peptides that upon the non-covalent complexation with nucleic acids can enhance significantly nucleic acid uptake into cells. The main problems that hinder CPP wider application in gene delivery are non-specific and low transfection efficacy in vivo and poor control over the size and size distribution of the non-covalently formed nanoparticles.

The aim of this research was to design more effective transfection reagents for the systemic in vivo gene delivery. We designed a novel peptide specifically for in vivo utilization by using previous knowledge about the physicochemical properties and internalization mechanism of different CPPs. We thereafter developed more efficient CPP and nucleic acid complex formulation strategy to prepare more uniformly sized particles to enhance gene delivery and decrease the risk of potential side-effects from aggregated particles. In addition, we tackled the problem of non-specificity of the peptide vector and incorporated magnetic particles into the CPP-nucleic acid complexes to enhance the efficacy and increase the targeted delivery into specific organs. In conclusion we developed an approach to produce non-conglomerating effective nanoparticles with uniform size for in vivo gene delivery.

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LIST OF ORIGINAL PUBLICATIONS

I. **Freimann K**, Arukuusk P, Kurrikoff K, Vasconcelos LDF, Veiman K-L, Uusna J, et al. Optimization of in vivo DNA delivery with NickFect peptide vectors. J Control Release Off J Control Release Soc. 2016 Nov 10; 241:135–43.

II. **Freimann K**, Arukuusk P, Kurrikoff K, Pärnaste L, Raid R, Piirsoo A, et al. Formulation of stable and homogenous cell penetrating peptide NF55 nanoparticles for efficient gene delivery in vivo. Mol Ther – Nucleic Acids. 2017 Oct 20

III. Dowaidar M, Abdelhamid HN, Hällbrink M, **Freimann K**, Kurrikoff K, Zou X, et al. Magnetic Nanoparticle Assisted Self-assembly of Cell Penetrating Peptides-Oligonucleotides Complexes for Gene Delivery. Sci Rep. 2017 Aug 22;7(1):9159.

Author's contribution to the articles referred in this thesis:

Paper I. Wrote the paper as a corresponding author, designed, and performed many of the experiments (synthesized and purified most peptides, screened bioactivity of nanoparticles in the cell culture and participated in in vivo experiments) and analyzed the data.

Paper II. Wrote the paper as a corresponding author, designed, and performed most of the experiments (synthesized and purified peptides, performed the cell culture experiments and participated in in vivo studies) and analyzed the data.

Paper III. Participated in in vivo studies.

ABBREVIATIONS

CPP-cell	– penetrating peptide
TP	– Transportan peptide
NF	– NickFect peptide
PF	– PepFect peptide
NA	– nucleic acid
ON	– oligonucleotide
pDNA	– plasmid DNA
DLS	– dynamic light spectroscopy
SCO	– splice-correcting oligonucleotide
PEI	– polyethylenimine
TEM	– transmission electron microscopy
PEG	– polyethylene glycol
siRNA	– small interfering RNA
EDS	– energy dispersive X-ray Spectroscopy
MALDI-TOF	– matrix assisted laser desorption ionization-time of flight mass spectrometry
CR	– charge ratio
MR	– molar ratio
MQ	– MilliQ water
pDMAEMA	– (poly(2-dimethylamino)ethyl methacrylate) dendrimer
CCC	– cryo-concentrated complex

INTRODUCTION

In recent years, important advances have been achieved in the use of genome editing approaches to precisely alter cellular genomes. Gene therapy can regulate practically any sequence in the genome and repair expression of pathogenic phenotype-causing genes by using therapeutic nucleic acids. Due to their size and anionic nature, exogenous nucleic acids need vectors to enhance their delivery into the diseased tissue. Although impressive accomplishments have been accomplished with virus-derived gene delivery vectors their utilization is not without risks. Increasingly more effort has been directed into the research of non-viral vectors. Although being safer alternatives to viral counterparts, they lack efficient transfection and often result in short-lived gene expression.

Cell-penetrating peptides are one family of non-viral delivery vectors. Cell-penetrating peptides (CPP) are short cationic and/or amphipathic peptides, which have been shown to significantly improve the delivery of various bio-molecules in vivo. These non-viral peptide delivery vehicles offer a safer alternative to convey therapeutic nucleic acids into cells. The transport of nucleic acids is usually facilitated through the non-covalent attachment to the CPP. Although non-covalent complex formation is a great and efficient method for formulating stable particles for transfection, these particles display very heterogeneous size distribution. The size of the particle, along with other physico-chemical properties (such as surface charge, morphology), influences the biodistribution of particles in vivo. Furthermore, large and aggregated particles can cause side-effects and increase toxicity of nanoparticles. Besides the heterogeneous size of formulated particles, the CPP efficient utilization is hindered by non-specific and low transfection in vivo. Therefore, all these major obstacles have to be overcome before the broader utilization of CPPs for in vivo gene delivery.

The aim of this thesis was to tackle these previously described main problems of non-viral gene delivery systems. A novel effective CPP for the systemic delivery of DNA was designed and an improved formulation approach to prepare stable and uniformly sized particles was developed. Incorporation of magnetic particles to increase the efficacy and specificity of the peptide vectors was harnessed.

1. LITERATURE OVERVIEW

1.1. Gene therapy

Recent advancements in genomics have able us to regulate the expression of almost any gene in the genome through the introduction of exogenous nucleic acids (NA) into cells. Therefore, gene therapy can be utilized to regulate the expression of the pathogenic phenotype-causing genes for therapeutic purposes. In addition to double-stranded DNA vectors which is often used as a therapeutic agent to replace defective genes in patients suffering from genetic disorders, small oligonucleotides can also be utilized to regulate the abnormal gene expression. These include small single stranded antisense oligonucleotides which can be used to correct aberrant splicing in many genetic disorders (such as Duchenne muscular dystrophy)(1). Additionally, double-stranded interfering RNAs (siRNAs) or more complicated system such as plasmid DNA vectors which encodes small hairpin RNAs which then are processed into siRNAs inside cells which can subsequently utilized for RNA interference to regulate the pathogenic gene expression at the post-transcriptional level(2).

The idea of gene therapy arose initially during the 1960s with the development of genetically marked cell lines(3–6) and with better understanding of mechanism of cell transformation by the papovaviruses (6–8). Although gene therapy offers a great promise, the main problem lies in the development of efficient gene-delivery vehicles which can deliver these therapeutic nucleic acids into the diseased tissue.

In 1990 the FDA approved the first clinical trial of gene therapy with attempt to treat adenosine deaminase deficiency, a monogenetic disease leading to severe immunodeficiency(9). One of the two patients exhibited a temporary response, but the successfulness of the gene therapy is debatable as she received simultaneously enzyme replacement therapy with polyethylene glycol adenine deaminase (9,10). After this initial gene therapy attempt further gene transfer trials were started for several diseases (10) until the 1999 when the patient died from multiple organ failure after immune system responded to high dose adenovirus administration(11). This unfortunate incident demonstrated the first time the need for safer gene delivery systems. The first real successful gene therapy was in 2000 when Cavazzana-Calvo et al was able to provide full correction of disease phenotype in human severe combined immunodeficiency (SCID)-X1 disease(12). Although the study experienced a major setback when 25% of the treated boys developed leukemia due to insertional mutagenesis, it led to the public discussion about the risks of gene therapy and the development of sensitive polymerase chain reaction techniques to detect viral integration sites, thereby considerably increasing the safety of integrating vectors(13).

Despite some success (shown in Table 1), many challenges remain to fully utilize the potential of gene therapy. The main obstacles are the persistent issues of safety and delivery. Currently utilized vectors for nucleic acid delivery can

be divided into viral and non-viral vectors. Although viral vectors are the most efficient and extensively used delivery vehicles in clinical trials they can exhibit serious side-effects. Therefore, a lot of energy has been applied for designing non-viral vectors for safer gene delivery. Despite the great progress made with delivery vectors the lack of efficient delivery of therapeutic nucleic acids still hindrance the practical utilization of gene therapy.

Table 1. Gene therapy products approved for marketing.

Product	The objective	Vector	Approval	Ref
Gendicine	Gene therapy-based product for the clinical use for the treatment of head- and neck squamous cell carcinoma	adenoviral vector, wherein the E1 gene is replaced with a human p53 cDNA	2003	(14)
Glybera	The European Medicines Agency approves the gene therapy Glybera for the treatment of severe lipoprotein lipase deficiency	an adeno-associated viral vector engineered to express lipoprotein lipase in the muscle tissue	2012	(15)
Strimvelis	the European Commission granted the first market approval for Strimvelis for the treatment of adenosine deaminase-deficient (ADA) severe combined immunodeficiency disease	autologous CD34+ enriched cell fraction that contains CD34+ cells transduced with retroviral vector that encodes for the human ADA cDNA sequence	2016	(16)
Luxturna	In 2018, Spark Therapeutics could market a product for an inherited retinal disease	AAV2 vector containing modified human RPE65 cDNA	2018	(17)

1.1.1. Gene therapy in cancer

Gene therapy helps to eliminate cancer by delivering nucleic acids to express pro-apoptotic proteins or substitute mutated genes, downregulate or silence onco-genic pathways, produce anti-cancer cytokines and activate the immune system against cancer(18). In combination with chemotherapy, gene therapy can be utilized by delivering therapeutic nucleic acid against a chemotherapeutic resistance pathway(18).

Targeting oncogenes is often used for cancer gene therapy. Mutations, amplifications, or chromosomal rearrangements in normal genes can give arise to oncogenes that are important for carcinogenesis. Among others, the three RAS genes are the most frequently mutated gene family in cancer and are found

in around 25% of human tumors(19). RAS genes belong to the group of eukaryotic genes that are necessary for cell proliferation and differentiation(19). Therefore, this oncogenic activity of RAS genes is considered as very potential molecular target for various cancer gene therapies.

Another group of oncogenes are transcription factors. Aberrant activation of transcription factors alters expression of multiple gene sets associated with tumor development and progression. Therefore, targeting transcription factor can be utilized to design therapeutics for cancer gene therapy. One of the potential molecular targets for the cancer therapy is the transcription factor p53 tumor suppressor gene that regulates the pathways of cell-cycle arrest, apoptosis, and DNA repair(18,20). p53 mutation occurs in over half of all human tumors and even with tumors that have wild-type p53, the pathways of p53-induced cell-cycle arrest and apoptosis are usually deficient(20,21).

As tumor angiogenesis is important for creation of new blood vessels and for tumor mass to grow larger than a few millimeters, other gene therapy strategy is to alter the function of angiogenic factors (22,23). This is achieved either by inhibition of production or function of pro-angiogenic cytokines or by delivering genes that encode inhibitors of angiogenesis to suppress tumor growth(24). There are various potential targets such as vascular endothelial growth factor(25), basic fibroblast growth factor, platelet-derived growth factor and others(23).

An efficient method for cancer therapy is to facilitate genetically-modified T-cells for antitumor effect. Adoptive cell therapy is personalized cancer therapy which use patient immune system for direct anticancer activity(26). Adoptive cell therapy can utilize either natural host tumor-reactive lymphocytes or host cells that have been genetically engineered with antitumor T cell receptors or chimeric antigen receptors(26). The first successful clinical application of adoptive cell gene therapy was reported in 2010 when treatment with cells expressing anti-CD19 chimeric antigen receptors in patient with refractory lymphoma resulted in cancer regression(27).

Up to date, cancer is by far the most common disease treated by gene therapy. It composes over 64% of all ongoing clinical gene therapy trials worldwide(28). The first gene therapy-based product was Gendicine for treatment of head- and neck squamous cell carcinoma(14). This was followed by Philippine in 2007 when Rexin-G was approved for marketing to treat solid tumors(29). Rexin-G, is a tumor-targeted retrovector encoding an N-terminal deletion mutant of the cyclin G1 gene with antineoplastic activity(29). Ten years later, in 2017 FDA approved genetically-modified autologous T-cell immunotherapy Kymriah for patients with acute lymphoblastic leukemia(30). Marketing of these gene therapy-based products demonstrates already the potential of gene therapy for future personalized, effective, and safer cancer therapy.

1.2. Gene delivery systems

The poor gene delivery is the limiting factor for most in vivo gene therapies. Carriers used for systemic in vivo gene delivery can be divided broadly into viral vectors and non-viral vectors, and both have its own set of benefits and limitations.

1.2.1. Viral vectors

Various viruses have been studied for development of new gene therapy carriers, such as adenovirus, lentivirus, herpes simplex virus, adeno-associated virus and baculovirus. Viruses have evolved various mechanisms for overcoming cellular barriers to efficiently transport their genomes inside cells and achieve very high gene expression. Thus, it is not surprising that viral vectors are most commonly utilized carriers in gene therapy trials. Over half (69%) of gene vectors in clinical trials are viral vectors(31). Actually, adenovirus was the first effective gene delivery vector and it is the most commonly used vector in worldwide in clinical trials, accounting for >20% of all vectors used in gene therapy trials(31). In addition to adenoviruses, adeno-associated virus vectors can infect a wide range of cell types and transfect both dividing and non-dividing cells and at the same time demonstrate only low immunogenic profile(32). Although viral carriers are very efficient at transfection, some of the major obstacles of using these vectors are reactions with the immune system, viral longevity and vector packaging capacity(31).

1.2.2. Chemical compounds for nucleic acid non-viral delivery

Compared to viral vectors non-viral gene delivery vehicles usually exhibit lower immunogenicity, higher packaging capacity and have the potential for scale-up manufacture. On the other hand, current non-viral vectors are not able to achieve as high gene transfection efficiency as viral counterparts.

The most commonly used materials in non-viral vectors are lipids (such as sphingolipids), cationic polymers (mainly polyethyleneimine(33), chitosan(34), pDMAEMA dendrimers(35)), peptides (poly-L-lysine(36), poly-arginine(37)), various inorganic components(38) (such as iron oxide, silica, gold) and combinations of them (shown in Figure 1).

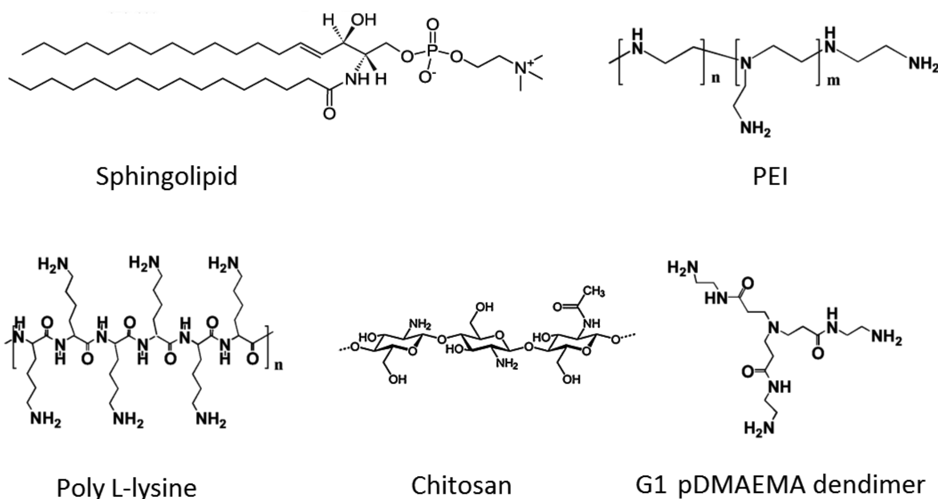

Figure 1. The most broadly used non-viral gene delivery vectors. These includes sphingolipids commonly used to prepare liposomes, polyethyleneimine (PEI), poly-L-lysine, chitosan and generation 1 pDMAEMA (poly(2-dimethylamino)ethyl methacrylate) dendrimer.

1.3. Cell-penetrating peptides

Cell-penetrating peptides (CPPs) are short peptides that can penetrate and deliver various macromolecules through tissue and cell membranes. CPPs efficiently improve the intracellular delivery of various macromolecules such as pDNA(39), oligonucleotides(40), siRNA(41), peptide nucleic acids(42) and proteins(43). CPPs consist of around 30 amino acids and can be broadly divided into three groups based on physical-chemical properties of peptides(44,45):

1. cationic peptides,
2. amphipathic peptides,
3. hydrophobic peptides.

Most CPPs are characterized as amphipathic and/or positively charged peptide at physiological pH. CPPs are known to utilize both energy-dependent endocytosis and energy–independent direct translocation internalization methods. The selection of internalization pathway depends on cell lines and their confluency, concentration of peptide, environment and delivered cargo(45). At standard concentrations of CPPs which are utilized to deliver nucleic acids in cell culture experiments, the main entrance mechanism is endocytosis(44). All different pathways of endocytosis and they co-occurrence have been observed with CPPs, including clathrin- and caveolae-mediated endocytosis, macropinocytosis and phagocytosis(44,46–48).

1.3.1. Origins of cell-penetrating peptides

The first indication that proteins can translocate through biological membranes was in 1988 when rapid uptake in cell culture of tat transcription-transactivating protein of HIV was observed(49,50). Thereafter, three years later the Drosophila antennapedia gene homeobox (pAntp) polypeptide was shown to internalize into nerve cells(51). One year later, the sequence of pAntp was shortened and peptide of 16 amino acids in length from the third helix of the homeodomain was discovered to enter the cells(52). After discovery of these protein-derived peptides hundreds of CPPs have been synthesized. Naturally occurring proteins have been altered yielding so called chimeric CPPs such as Transportan (53), MPG(54), Pep-1(55) and M918(56). In addition, completely synthetic peptides have been designed such as polyarginines(37), CADY(57) and MAP(58).

1.3.2. Transportan-derived peptides

Transportan (TP) is a 27 amino acid-long peptide containing 12 highly conserved amino acids from the amino terminus of the neuropeptide galanin connected via a lysine with wasp venom peptide toxin mastoparan in the carboxyl terminus(59) (shown in Figure 2). To find the exact region of the TP sequence that is essential for the membrane translocation property of the peptide various deletion analogues of TP have been investigated(60). From various synthesized TP analogues the peptide TP10 which lacks first six first amino acids of N-terminus of TP demonstrated efficient penetration through cell membrane. Furthermore, this deletion decreased binding affinity towards galanin receptors and abolished modulation of basal GTPase activity. This is essential for avoiding the potential side-effects of Transportan- derived CPPs. This deleted new analogue TP10 lead to designing of multiple TP-based CPPs named PepFects (PF) and NickFects (NF).

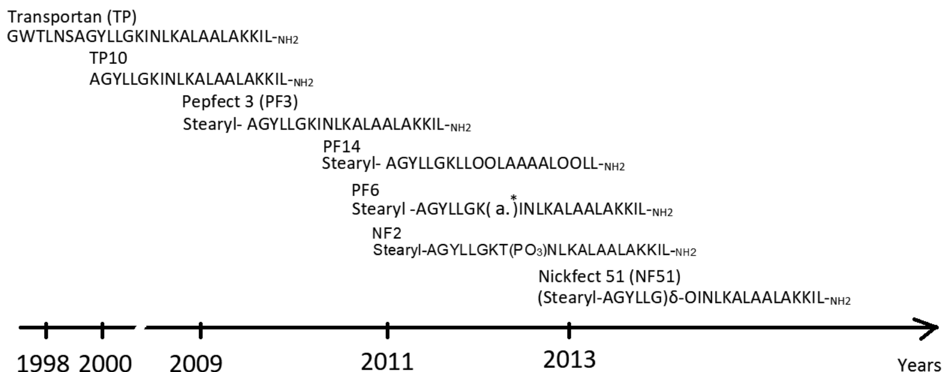

Figure 2. Design of Transportan and the main Transportan-derived peptides over the past years. a.* indicates the succinylated trifluoroquinoline based derivative.

1.3.3. PepFects

One of the first effective modifications of TP10 was stearylation of N-terminus of the peptide yielding PepFect3 (PF3) which reduced the toxicity and increases TP10-mediated splice correction approximately 30-fold compared to non-stearylated TP10(61). Stearylation strategy was applied as results of Futaki and colleagues showed significantly enhanced transfection efficacy of stearylated polyarginine(62).

Next, PF3 sequence was further modified by changing the sequence and by replacing lysines with ornithines to create leucine-zipper motif to enhance peptide binding with nucleic acid and increase the transfection(63,64). The ornithine introduction was inspired by Ramsay et al. results which demonstrated 10-fold higher transfection efficacy of polyornithine and stronger complex formation with pDNA compared to polylysine peptide(65). The yielded peptide PF14 is one of the most efficient peptide of our group and standardly used transfection regent for SCO, siRNA and pDNA delivery in vitro or in vivo.

Similarly to other CPPs TP-derived peptide utilize mainly endocytosis pathways to deliver cargo into cells. Therefore to enhance endosomal escape pH titratable trifluoromethylquinoline group was attached covalently to PF3 sequence(66). The created peptide PF6 formed stable complexes with siRNA and demonstrated high RNAi response in various cell cultures with different confluency and even in vivo.

Various other PF analogues have been synthesized and investigated to further enhance nucleic acid delivery, such as PF15(67) which contains combined modifications from PF14 and PF6 or PF14 analogues with different fatty acid acyl chain length (68).

1.3.4. NickFects

The first Nickfect (NF) peptides synthesized were stearylated TP10 analogues where isoleucine in the TP10 sequence was substituted with threonine and/or additionally phosphorylated at different sites in the sequence of the peptide(69). The yielded peptides NF1 and NF2 exhibited significantly greater splice correction in the cell culture compared to the parental TP10 analogue. Few years later next efficient transfection reagent NF51 with incorporated branched structure into the stearylated TP10 sequence backbone was designed(70). The physicochemical and internalization studies of these NF analogues complexes with nucleic acids(71) lead to the design of NF55 peptide for specifically in vivo systemic gene delivery(72).

Continuous studies are underway to further improve NF utilization in vivo such as incorporation of histidines to make the peptides pH-responsive and therefore improve their interaction with cell membrane and escape form endosomes through so called proton sponge effect(73).

1.4. Barriers for non-viral in vivo gene delivery

Despite a century of continues discovery and development, todays therapeutic formulations are not capable of delivering nanoparticles specifically at sites of interest after systemic administration(74). Furthermore, gene vectors confront multiple biological barriers during systemic gene delivery. Therefore, site-specific delivery of therapeutics will not be efficient unless design of nano-particles considers all of the biological barriers that delivery reagents encounter upon intravenous administration.

The biggest obstacles for in vivo systemic gene delivery today are (shown in Figure 3):

1. Serum lability of nanoparticles
2. Nonspecific distribution and inadequate accumulation of nanoparticles into the target organ and cells.
3. Internalization of nanoparticles into cells and trafficking into the nucleus.

1.4.1. Serum lability of nanoparticles

First, the size of nanoparticle dictates the bio-distribution and pharmacokinetics of the particle after the administration and it has been shown that smaller than 10 nm in diameter vectors are filtered out of circulation by the kidneys and particles over 200 nm by mononuclear phagocyte system(74,75). In general, nanoparticles ranging from 10 to 200 nm in diameter are considered ideal for avoiding the renal clearance or accumulation into the liver and spleen(74).

After intravenous administration, nanoparticles encounter a harsh environ-ment of blood which consist of sugars, salts, plasma proteins, and immune cells(76,77). As non-viral vectors are generally formed through assembly of the vector and nucleic acids via electrostatic interaction these interactions are weakened at this high salt concentrations causing complex to disassemble and expose nucleic acids(77). Exposed nucleic acids are thereafter very prone to fast degradation by serum nucleases(77,78). It has been even reported that the half-life of free pDNA can be as short as only about ten minutes in mouse whole blood(78). In addition, serum may also cause nanoparticle aggregation into large particles, which can lead to opsonization of the vector and thereby clearance by mononuclear phagocytic system. This aggregation of nanoparticles in serum can also lead to severe pulmonary embolism(79). The adsorption of plasma proteins onto the surface of nanoparticle depends on many factors such as the size, surface charge and chemistry and hydrophobicity of particle(74,80). Therefore, surface charge and functionalization of the particle significantly influence the circulation halftime of the nanoparticle. Highly cationic particles have been shown to be opsonized and subsequently cleared by the phagocytic cells at greater extent than negatively charged or neutral particles(74,81). Moreover, nanoparticles with different surface functionalization display diverse in vivo bio-distribution and circulation time and therefore surface of the particle

has been modified to prolong the half-life of the particle in blood. These alterations include incorporation of inert polymers (such as PEG (polyethylene glycol))(82) or covering the surface of the particle with various biomolecules (for example membrane glycoprotein CD47 peptide and extracted cell membrane) to biomimetic the particle and prevent protein adsorption(83,84). After serum proteins cover the nanoparticles and form so called protein corona the biological efficiency of the particles are decreased and usually only the liver and lungs are transfected non-specifically (77,85,86). Opsonization also hinders the active-targeting (such as using the targeting or shielding moieties) strategies and adhered protein corona masks targeting ligands which consequently results with reduction in specificity(74). This has been demonstrated by Salvati et al. with transferrin-functionalized nanoparticles that lost their targeting capabilities when a biomolecule corona adsorbed on the surface(87).

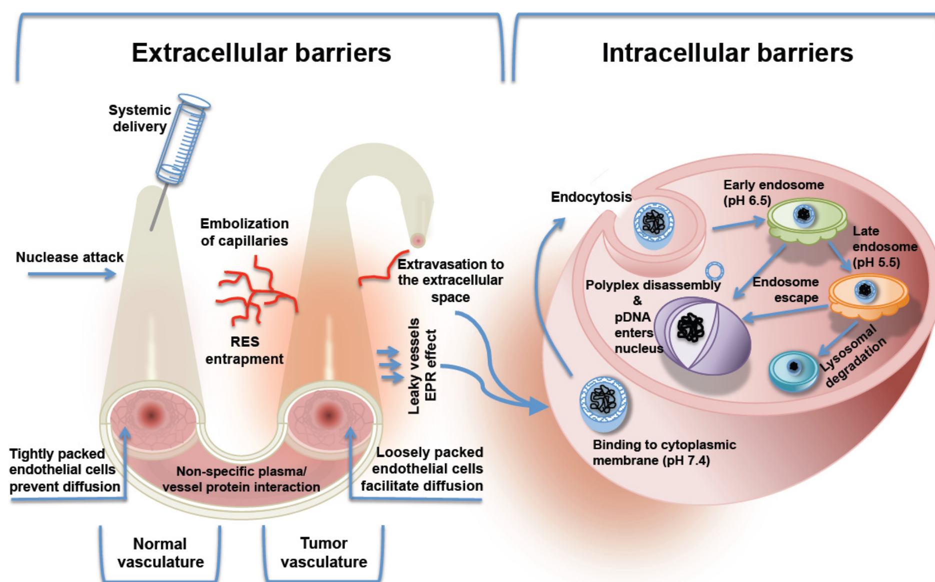

Figure 3. Graphical representation of the extra- and intracellular barriers faced by non-viral gene therapies following systemic delivery (from McCrudden and McCarthy et al. 2013 (88) Cancer Gene Therapy – Key Biological Concepts in the Design of Multifunctional Non-Viral Delivery Systems, Gene Therapy – Tools and Potential Applications, Dr. Francisco Martin (Ed.), InTech).

1.4.2. The nonspecific distribution and inadequate accumulation of nanoparticles into the target tissue

After reaching to the targeted organ or diseased tissue other extracellular barriers exist depending on the targeted tissue and/or the state of the disease. For example, to deliver nanoparticles into the central nervous system, the particles

must pass tight junctions between capillary endothelial cells in the blood brain barrier (BBB) capillaries(89,90). In addition, endothelial cells express high levels of active efflux transport proteins such as the P-glycoprotein pumps that quickly remove any foreign substance that bypasses the BBB (91). In contrast to tight junctions between endothelial cells in brain stable and long circulating nanoparticles are expected to passively accumulate in the tumor via extra-vasation through leaky vasculature. This kind of enhanced permeability of particles through fenestrated vasculature and endothelial dysfunction is known as enhanced permeability and retention (EPR) effect (92,93). This disorganized vasculature is very characteristic to tumors but it is not unique phenomena only to cancers as fenestrated vasculature has been described with infection(94) and with heart failures(95). Although description of the EPR effect by Maeda and Matsumura et al.(93) was the foundation of many macromolecular therapeutics designs it was soon discovered that elevated intratumoral pressure from vascular enhanced permeability and dysfunction of lymphatic vessels prevented the delivery of nanoparticles into distal regions in tumor tissue(96). The lack of ability of particles to reach into distal parts of the tumor greatly reduces the effectiveness of therapeutics(97).

1.4.3. Internalization of nanoparticles into cells and trafficking into the nucleus

After nanoparticles pass various extracellular barriers the intracellular barriers remain. The first obstacle is the internalization of particles through cell membrane. Depending on the properties of nanoparticles various internalization pathways are used and the fate of internalized vector depends largely on uptake mechanism(98). Nanoparticles are not able to diffuse through cell membrane due to their size, surface charge or other physicochemical properties and therefore require an active transport. Earlier studies indicated non-endocytotic internalization methods for many non-viral vectors. For example, studies with lipoplexes indicated the direct release of DNA into cytosol via fusion between the lipids and the plasma membrane(99) and with cell-penetrating peptides energy-independent direct penetration was first proposed as main internalization method(46). Although, it is now believed that endocytosis is the essential entrance mechanism for these vectors. Various non-viral vectors are known to enter into cells mainly via different endocytosis pathways(100–105). The predominantly utilized internalization pathway for nanoparticles is endocytosis via clathrin-coated vesicles(98). As the endocytosis is the main entrance pathway the biological efficacy of delivered cargo is greatly dependent whether particles can escape from intracellular vesicles and therefore avoid lysosomal degradation or recycling of the vector back to the cell surface. Various modifications to non-viral carriers have been made to improve the release from endosome, for example addition of pH-sensitive fusogenic lipids(106) and peptides(107). After release from endosomes the DNA must then dissociate from carrier in cytosol

or even close to the nuclear membrane for nuclear transfection. Even when vectors reach from endosomes to cytosol the carried nucleic acids will be then vulnerable to cytoplasmic nucleases. The half-life of pDNA in cytoplasm has been shown to be between 50–90 min(108). The import of nanoparticles into the nucleus occurs through nuclear pore complex and only molecules less than 40 kDa and 10 nm size in diameter are able to diffuse through pores(109). Since even the condensed pDNA is larger it causes poor transfection of non-dividing cells. The importance of nuclear transfection has been also demonstrated by Pollard and co-workers when they observed significantly increased reporter gene expression after microinjection to the nucleus compared to injection into cytosol(110).

1.5. CPP and nucleic acid nanoparticles

1.5.1. Vectorization of nucleic acids with CPPs for the transfection

There are mainly two ways to form CPP/nucleic acid complexes either covalently or non-covalently. In covalent coupling most often, a disulfide bond but also ester and peptide bonds are used to conjugate cargo onto peptide(111–113). This kind of linkage yields more stable and defined complexes. These stable and uniformly sized nanoparticles are more suitable for in vivo utilization as these particles exhibit longer circulation time and do not non-specifically accumulate into liver, lungs or renal tissue depending on their size(74). Covalent strategy is advantageous for delivering uncharged nucleic acids as peptide nucleic acid and phosphorodiamidate morpholino oligomer, but it is not suitable for the transporting of pDNA and siRNA(114). On the other hand, non-covalent formulation process is less expensive and only simple co-incubation of peptide and nucleic acid is needed. In non-covalent complex formation, the positive-charge bearing peptide is used to condense negatively charged nucleic acids into the particle mainly via electrostatic interaction (shown in Figure 4). This kind of nanoparticle assembly is widely used for transfecting cells with oligonucleotides, siRNA, splice-correcting oligonucleotides (SCO) and plasmid DNA with various poly-cationic molecules such as poly-lysine, chitosan, cationic lipids and polyethyleneimine(115). Although non-covalently formed complexes might not exhibit so defined homogenous size distribution as covalently linked counterparts. This heterogenous size distribution of non-covalent complexes could hinder their further utilization in vivo because of the potential side-effects from agglomerated particles.

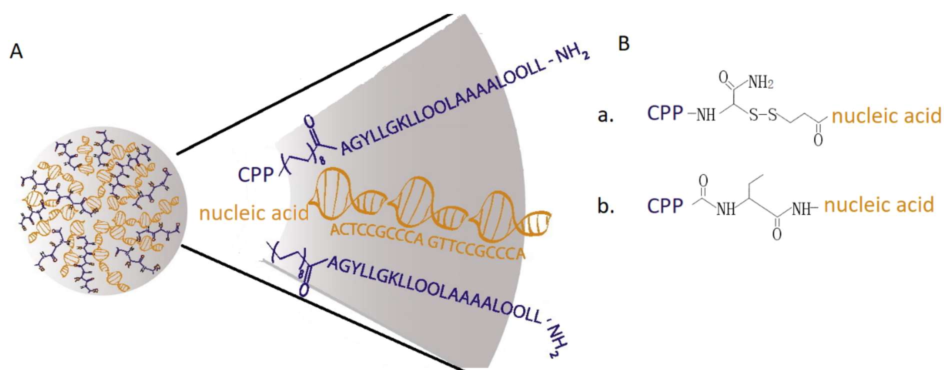

Figure 4. Graphical illustration of non-covalent complexes of CPP and nucleic acids (A) and covalent complexes (B), a. demonstrates the disulfide and b. amide bond between CPP and nucleic acid. Modified figure from Veiman et al. 2015(116).

The first efficient CPP mediated oligonucleotide transfection in vivo was achieved through covalent attachment of Galanin receptor 1 (GalR1) antisense peptide nucleic acid to Transportan or Penetratin CPP(117). After intrathecal administration of these CPP constructs GalR1 receptors were specifically down-regulated in rat brain. Thereafter many groups have covalently coupled phosphorodiamidate morpholino oligomers to various CPPs to correct the splicing of dystrophin mRNA in the dystrophinopathy mdx mouse model (118,119).

After the discovery of Divita group that CPPs can non-covalently form particles with negatively-charged nucleic acid, greater extent of transfection studies have utilized the electrostatic forces between peptide and nucleic acids to facilitate the delivery(54). In addition to electrostatic forces, hydrophobic interactions have been also shown to be participating in non-covalent complexation of particles(120,121). This non-covalent particle formulation method enables to avoid laborious chemical conjugation of construct and transfect larger nucleic acids into cells. Non-covalent formulation method has been widely used to delivery different nucleic acids such as pDNA(122), siRNAs(123) and oligonucleotides(114) into various organs in vivo.

1.5.2. Common modifications of CPPs to enhance the in vivo gene delivery

To conquer biological barriers and enhance in vivo transfection various modifications to CPP have been utilized to design more suitable vectors. Some of these modifications which have been introduced into the Transportan-derived CPPs include:

1. The incorporation of hydrophobic moieties and alteration of amphipathicity of the peptide to increment the interaction between cell membrane and CPP particles.
2. Attachment of inert polymers to enhance the circulation time of the peptide in blood
3. Addition of shielding moieties to the peptide to enhance the transfection specificity and increase the half-life of the peptide
4. Conjugation of the CPP to the targeting moiety to increase the cell or tissue specific transfection
5. Incorporation of magnetic iron oxide for more efficient targeting of CPP-nucleic acid particles

1.5.2.1. The incorporation of hydrophobic moieties and alteration of amphipathicity of the peptide to increment the interaction between cell membrane and CPP particles.

Incorporation of hydrophobic moieties and increment of the amphipathicity of the peptide have been utilized to enhance the transfection. The enhancement of hydrophobicity of the peptide has been shown to improve the CPP and nucleic acid (NA) complexation and transfection. Futaki et al. and Khalil et al. were first to demonstrate that incorporation of stearyl moieties to polyarginine condensed DNA into stable particles(62,124). These stearylated peptide-NA particles were able to adsorb on to the surface of the cell membrane due to the increased hydrophobic interactions between the particles and cell membrane and thereby improve the transfection efficacy. Thereafter, multiple studies have demonstrated the improved transfection after increment of CPP hydrophobicity(61,68,125,126). Another way to improve transfection efficacy of CPPs is to increase the helicity and amphipathicity of the peptide. This has been illustrated nicely with the first synthetic CPP MAP (Model Amphipathic Peptide) peptide. The alteration of MAP peptide sequence (to modify the amphipathicity of the MAP peptide) showed that the positive charge is not the only factor for the penetration and that helical amphipathicity was important for these peptides to cross the plasma membrane(127). Disruption of the helicity of the peptide significantly decreased the translocation of the peptide into cells. Thereafter, various studies have used tuning of the amphipathicity of the peptide to enhance cellular uptake efficiency of the peptide (128). In addition, Niidome et al. have found correlation between the helical character of peptides and DNA transfection(126). Moreover, Niidome et al. results indicated that peptides with continuous large hydrophobic region could form stable particles with the DNA and efficiently transfer the nucleic acid into cells.

1.5.2.2. Attachment of inert polymers to enhance the circulation time of the peptide

To tackle the stability problem of the CPP-nucleic acid particle in the blood attachment of inert polymers to CPP has been utilized to reduce the protein adsorption onto the surface of CPP-NA particle. The most commonly incorporated polymer to decrease opsonization and increase CPP-NA particles resistance against hydrolytic enzyme is hydrophilic PEG (polyethylene glycol). PEG can be used to cover the surface of the nanoparticle to enhance water solubility of the particle and to suppress non-specific interaction with blood components for a reduced blood clearance. First illustrations of the potential of surface pegylation of nanoparticles arose in early 1990s when different research groups reported that pegylation of liposomes enhanced blood circulation times of liposomes(129,130). Thereafter, Grief and colleagues showed the similar results with pegylated poly(lactic-co-glycolic acid) nanospheres(131). Although pegylation is a great strategy to increase half-life of particles it can inhibit binding with target molecules thereby reducing the efficacy of the delivery (known as PEG dilemma)(132). To overcome this problem various groups have utilized cleavable linkers which when cleaved release the sterically shielding PEG from nanoparticle. Several cleavage conditions can be applied to design reducible(133), pH-dependent(134–136) or protease-cleavable(137,138) linkers. Pegylated CPPs have shown to increase circulation time and tumor tissue transfection(116) and incorporated into formation of liposome-based nanoparticle to further enhance the drug delivery(139–141).

1.5.2.3. Conjugation of the CPP with shielding sequences to enhance the transfection specificity

Several shielding moieties have been conjugated to CPPs to increase the specificity of the transfection. Shielding anionic sequences (usually sequences containing Glu amino acids) have been conjugated to CPPs to create activatable CPP (aCPPs)(142). aCPP concept was first introduced by Tsien and colleagues(142) and they used shielding properties of Glutamate sequences to target specifically tumor tissue (shown in Figure 5). Tumor targeting with aCPP takes advantage of the diseased tissue microenvironment (such as acidity, reducing environment) to dissociate shielding moiety from CPP once the target tissue has been reached. Besides addition of shielding anionic sequences other masking strategies have been applied such as pH sensitive oligopeptides. Cationic CPP MAP fusion with pH sensitive His-Glu oligopeptide have been synthesized to mask cell-penetrating moiety at neutral pH(143). After exposure to mildly acidic pH this pH-sensitive masking moiety significantly enhanced the surface binding and internalization of the CPP specifically into the targeted cells.

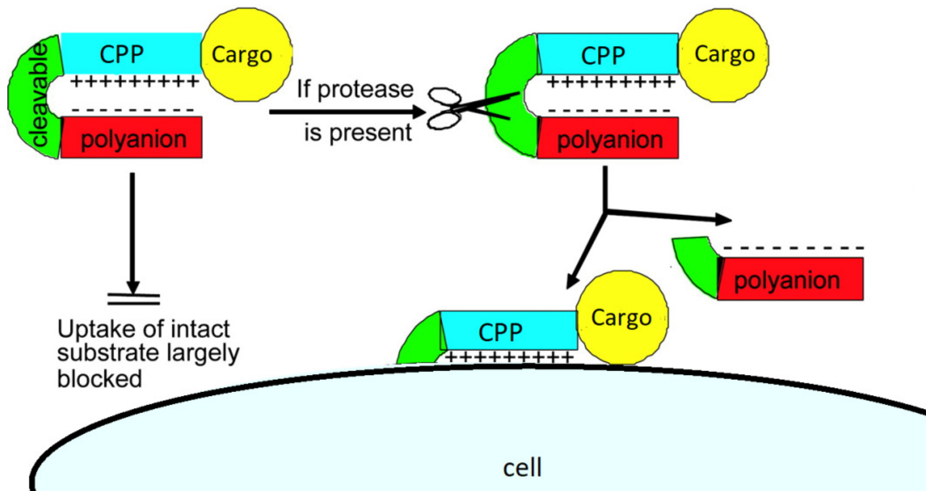

Figure 5. The specificity of the CPP towards specific tissue or cells can be enhanced through the incorporation of shielding moieties (modified figure from Jiang et al. 2004 (142), Copyright (2004) National Academy of Sciences, U.S.A.).

1.5.2.4. Conjugation of the CPP with targeting moieties to enhance the transfection specificity and efficacy

In addition to shielding strategy various targeting moieties are also used to enhance specificity of the transfection. Various antibodies(144,145), homing peptides(146,147) and targeting sequences of organells(148) have been utilized. One of the most broadly used targeting moieties with CPPs are so called homing sequences which take advance of target tissue distinct microenvironment and unique expression pattern of membrane receptors to target specific cells. For example, compared to the normal tissue environment, the tumor microenvironment displays stromal cell abnormalities, abnormal protein expression, acidosis, and hypoxia(149). These differences can be utilized for the selective delivery of therapeutics. With the advancement in the phage-displayed peptide library screening technique, various homing peptides have been identified that have tumor homing capacity(150). These peptides can specifically bind to various receptors that are uniquely expressed or over-expressed in tumor tissues. Compared with antibodies, homing peptides are much smaller in size and more efficient in tissue penetration and relatively easy to synthesize at much lower cost(149). Thus these homing peptides can be can be integrated into specifically tumor targeting formulation of particles(146,147,151,152). Despite broad utilization of homing peptides their in vivo application is still hindered mainly because of their short half-life and rapid renal removal from the circulation(153,154).

1.5.2.5. Incorporation of magnetic iron oxide for more efficient targeting of CPP particles

Although antibodies for receptors (as antigens) expressed in the target tissue have been used in targeting tumors their bulk size hinders their penetration into solid tumors(155). Also, reengineering of antibodies is usually needed to minimize possible immunogenic side-effects(155). In addition to antibodies, various small molecule ligands have been widely utilized for targeted delivery, but they lack efficient tumor targeting specificity required for in vivo gene therapy(149,156).

To enhance specific accumulation of nanoparticles into the target tissue one possibility is to use magnetic targeting. Magnetic targeting utilizes para-magnetic particles to guide their accumulation into the target tissue with local strong magnetic fields(157). These magnetic particles exhibit superparamagnetism properties which mean that on application of an external magnetic field, they become magnetized and when magnetic field is removed they no longer exhibit any residual magnetic interaction. These magnetic nanoparticles can facilitate the transport of biomolecules to their target site in the body under the influence of an applied magnet field(157). Magnetic nanoparticles have been used since 1970s when Freeman et al first introduced the concept of using magnetism in medical and biological sciences(158). In biomedical application, the most commonly used magnetic particles are iron oxides magnetite (Fe_2O_4) and maghemite (γ-Fe_2O_3) with a size ranging between 10 nm and 100 nm in diameter(157). Iron oxide particles are mostly used because of their relative ease of synthesis, biocompatibility and approval of Food and Drug Administration compare to other magnetic particles such as cobalt based particles (157,159).

The current research on iron oxide particles aims to use these particles as diagnostic agents in magnetic resonance imaging as well as for drug delivery vehicles(157). For biological utilization magnetic particles must be coated to enhance their colloidal stability, circulation time in blood, dispersity in aqueous solution and for achieving functional surfaces(157,159). Magnetic Fe_2O_4 particles have been coated and combined with various molecules such as lipids(160), ethyl cellulose(161), poly(D,L-lactide-co-glycolide)(162) and chitosan(163) to enhance accumulation of nanoparticles into target tissue.

2. AIMS OF THE STUDY

The main aim of this thesis was to design more efficient and safer formulations of CPP/nucleic acid particles for the systemic in vivo delivery of nucleic acids. For that the peptide vector complexes with nucleic acids were investigated to improve the formulation method of particles to enhance the gene delivery in vivo.

The specific goals of this study are as follows:

Paper I, the aim of this research was to rationally design an improved novel peptide for efficient in vivo gene delivery using knowledge from internalization studies of different CPPs.

Paper II, the aim of this study was to develop a new formulation approach to prepare uniformly sized nanoparticles for their further enhanced utilization in vivo.

Paper III, the aim of this research was to reduce the non-specific transfection of the peptide vector by incorporation of magnetic particles to enhance the peptide vector efficacy and specificity.

3. MATERIALS AND METHODS

3.1. Synthesis of peptides with solid phase peptide synthesis method

All synthesized peptides are described in Table 1. Peptides were synthesized in stepwise manner at 0.1 mmol scale on an automated peptide synthesizer using Fmoc (fluorenylmethyloxycarbonyl) solid-phase peptide synthesis strategy (SPPS) (164) with Rink-amide MBHA (methylbenzylhydrylamine) resin as solid phase to obtain C-terminally amidated peptides. Fmoc SPPS was preferred over Boc based SPPS because it abled us to use milder deprotection and final cleavage (TFA acid) and monitoring of the deprotection rate via UV detection compared to harsh conditions (HF acid) needed for Boc SPPS.

Table 2. Synthesized peptides.

Peptide	Sequence	Ref
NF1	Stearyl-AGY(PO_3)LLGKTNLKALAALAKKIL-$_{NH2}$	(69)
NF2	Stearyl-AGYLLGKT(PO_3)NLKALAALAKKIL-$_{NH2}$	(69)
NF16	Stearyl-AGELLGKTNLKALAALAKKIL-$_{NH2}$	(72)
NF17	Stearyl-AGYLLGKENLKALAALAKKIL-$_{NH2}$	(72)
NF18	(Stearyl-AGELLG)δ-OINLKALAALAKKIL-$_{NH2}$	(72)
NF19	(Stearyl-AGYLLG)δ-OENLKALAALAKKIL-$_{NH2}$	(72)
NF51	(Stearyl-AGYLLG)δ-OINLKALAALAKKIL-$_{NH2}$	(70)
NF54	(Stearyl-AGYLLG)δ-OINLKALAALAAKIL-$_{NH2}$	(72)
NF55	(Stearyl-AGYLLG)δ-OINLKALAALAKAIL-$_{NH2}$	(72)
NF551	(Stearyl-AGYLLG)δ-OINLKAK(C*)AALAKAIL-$_{NH2}$	(72)
NF552	(Stearyl-AGYLLG)δ-OINLKALAALAKAILC*-$_{NH2}$	(72)
PF14	Stearyl- AGYLLGKLLOOLAAAALOOLL-NH2	(64)
PF220	Stearyl- KWLKLWFLKLLKKFL-$_{NH2}$	(165)
PF221	Stearyl- FLKLLKKFLFLKLLKKFL-$_{NH2}$	(165)
PF222	Stearyl- WLRLLWKKWFLKL-$_{NH2}$	(165)
PF223	Stearyl- KWLKLWAGYLLGKINL-$_{NH2}$	(165)
PF224	Stearyl- WKKWINLKALINLKAL-$_{NH2}$	(165)

* PEG2000-Cys is conjugated via disulfide bond

In Fmoc solid-phase peptide synthesis, the peptide chain is created stepwise using amino acids protected either Fmoc or *t*-Boc (tert-butyloxycarbonyl) groups. In paper I for the synthesis of NF51, NF54 and NF55 *t*-Boc monomers Boc-l-Orn(Fmoc)-OH were used. For synthesis of phosphorylated peptides phosphothreonine Fmoc- Thr(PO(OBzl)OH)–OH (Fluka, Germany) and

phosphotyrosine Fmoc-Tyr(PO(OBzl)OH)–OH (Merck, Germany) monomers were used and the coupling was carried out manually. The final cleavage was performed using standard protocol. Peptides were purified by reversed-phase HPLC using C4 column (Phenomenex Jupiter C4, 5 µm, 300 A, 250 × 10 mm) using a gradient of 20–80% acetonitrile/water containing 0.1% TFA. The identity of peptides was analyzed by matrix-assisted laser desorption ionization time-of flight (MALDI-TOF) mass-spectrometry (The Voyager-DE™ PRO Bio-spectrometry™ System) in positive linear mode using α-cyano-4-hydroxycinnamic acid as matrix (Sigma–Aldrich).

3.2. Formulation strategies of nanoparticles applied in the thesis

In this thesis, we use non-covalent complex formation where we condense negatively charged nucleic acids with positive-charge bearing peptide mainly via electrostatic interaction. This kind of nanoparticle assembly is widely used for transfecting cells with oligonucleotides, siRNA, SCO and plasmid DNA with various poly-cationic molecules such as poly-lysine, chitosan, cationic lipids and polyethyleneimine.

To assemble peptide-pDNA complexes with different charge ratios (CR) the negative charges of DNA phosphate, the positive charges of the peptide amino groups and the negative charges of the phosphoryl group or amino acid side chain group of peptides were considered. This means that the peptide/nucleic acid charge ratio is the proportion of moles of positively charged amino acid groups in the CPP to those of phosphate ones in the nucleic acid. The CR corresponds to more extensively used nitrogen to phosphate ratio N/P.

3.2.1. Formulation 1 method for preparation of nanoparticles for in vitro studies

Nanoparticles are prepared by mixing pDNA with the peptide in MQ water. Thereafter nanoparticles are incubated for 30 min at room temperature to assemble complexes before starting transfection experiments. In this thesis the term formulation 1 is used to indicate nanoparticle formulation protocol for the cell culture experiments. As transfection efficacy is highly dependent on the used CRs, cell type, temperature, and other conditions of the experiment we have optimized this so called in vitro formulation method for the transfection of various mammalian cell lines in our previous studies. The optimal amount of plasmid for generally transfected mammalian cells is 0.5 µg per well of 24-well plate containing 50 000 seeded cells which have been allowed to grow at confluency of 70–80%. Standardly charge ratios between 1 to 5 is first used to screen and find the best formulation charge ratio to continue further with more extensive studies.

3.2.2. Formulation 2 method for preparation of nanoparticles for in vivo studies

In this thesis the term formulation 2 indicates the protocol for assembling particles for the systemic administration in vivo. In the previous studies we have optimized the formulation method of particles to find the charge ratios which are safe and effective for the plasmid delivery via intravenous administration. To achieve efficient systemic delivery of pDNA in vivo 10 times higher dose of nanoparticle is needed compared to formulation 1 described earlier. Standardly 20 µg of pDNA is mixed with peptide at charge ratio 2 to 4 to yield dose administered per one animal (1 mg/kg). Thereafter 30 min incubation at room temperature is used to formulate nanoparticles. After complexation glucose is added to achieve an isotonic injection solution (5%).

3.2.3. Filtration of complexes

For the removal of aggregated particles and to create more homogenous size distribution of nanoparticles, complexes were formulated as described above (formulation 2) and then filtered through 100 nm or 200 nm pore-sized polypropylene filters (Pall, Acrodisc, USA). The filter sizes were chosen based on the aim to prepare particles between 100 to 200 nm which are suitable for in vivo administration. To quantify the recovery of plasmid and peptide after filtration, PicoGreen (Quant-iT™ PicoGreen®, ThermoFisher Scientific) and the Lowry based protein detection method (DC protein determination kit, Bio-Rad Laboratories, Inc., USA) were used. For in vitro studies, we diluted complexes ten-fold, thereby achieving a concentration equal to formulation 1 which are standardly used protocol for the cell culture experiments in our lab.

3.2.4. Cryo-concentrated formulation of nanoparticles

To enable better control over properties of formulated nanoparticles and to further improve transfection efficacy we aimed to prepare particles with more uniform size. For that we developed a cryo-concentrated formulation protocol. To formulate the cryo-concentrated complexes (CCC), a combination of formulation 1 and 2 was used. 20 µg of plasmid was mixed with the peptide at CR4 according to the formulation 1 (final peptide concentration 40 µM in a total volume of 2 ml of MQ water). After 30 min of incubation, glucose was added according to the formulation 2 (to achieve an isotonic injection solution (5%)), and thereafter, the formulated complexes were lyophilized. Prior to use in transfection experiments, the complexes were reconstituted in appropriate volume of MQ water that represents a dose for 1 animal (1 mg/kg).

3.2.5. Iron oxide incorporation into PF/nucleic acid particles

In the paper III we selected PF14-derived peptides because the parent peptide PF14 and its complexes with pDNA, SCO and siRNA have been thoroughly studied both in vitro and in vivo. Utilized PF14 analogues were first designed and described by Dowaidar et al. with a statistical method based on regression data analysis quantitative structure–activity relationships (QSAR)(165). From this study the most efficient PF peptides for nucleic acid transfection were selected. Iron oxide nanoparticles were incorporated into PF/nucleic acid nanoparticles to increase the selectivity and efficiency of gene therapy.

Iron oxide/PF/pDNA particles were prepared by mixing a suspension of iron oxide particles with pDNA and thereafter PF peptide was added to reach charge ratio 5 between peptide and pDNA. Complexes were then allowed to assemble through incubation at room temperature for 2h.

Iron oxide/PF/SCO particles were formulated at PF and SCO molar ratio 10. Again, first nucleic acid (at final concentration 10 µM) was added to iron oxide solution and thereafter PF peptide was introduced. To assemble particles 2h incubation was used.

Iron oxide/PF/siRNA complexes were formulated at PF and siRNA molar ratio 20. First siRNA (at final concentration 25 µM or 50 µM) was mixed with iron oxide solution and thereafter PF peptide was added. Complexes were incubated 2h at room temperature.

3.3. Characterization of peptide nanoparticles

3.3.1. Computational methods

To investigate the impact of amino acid sequence alterations to the secondary structure of new peptide analogues computational analysis was used. Structures of individual peptides were built or adapted and minimized in implicit solvent using chemical simulation software Schrödinger Software Package version 2015. For the resulting structures, the molecular surfaces, molecular electrostatic potentials, and hydrophilic/hydrophobic maps were calculated with Maestro. Maestro is the graphical user interface for Schrödinger's computational programs. Primary sequences of standard amino acids were used to calculate the potential for aggregation using statistical mechanics in Tango. Tango, is an algorithm for identifying β-sheet aggregation propensity and can be used to predict which peptides and their mutants will aggregate(166).

3.3.2. Circular dichroism spectroscopy

Structural properties of peptide play a major role in the cellular delivery efficacy of peptide-based nanoparticles. In this thesis we used Circular dichroism (CD) spectroscopy to study the effect of alterations in peptide sequence on secondary structure of peptide. CD spectroscopy is wildly used method for analyzing the conformation of proteins and peptides in solution. CD detects wavelength-dependent differences in the absorption of right and left circularly polarized light by optically active molecules such as peptides. The CD spectrum of unordered peptides is described by a single band below 200 nm, at the same time alpha-helical structures usually present two negative bands at 208 and 222 nm along with one positive band at 192 nm; beta-sheet structures typically show a negative band at 217 nm and a positive band at 195 nm(167).

Estimation of α helix and β strand content of the peptides, from their circular dichroism spectra, was calculated using the K2D2 algorithm.

3.3.3. Dynamic light spectroscopy

As size and surface charge of the nanoparticles can determine the intracellular fate, toxicity, and bio-distribution of particles after administration it is essential to study these physicochemical properties. One of the most commonly used techniques for the analysis of nanoparticles and protein aggregates is dynamic light scattering (DLS)(168). DLS measures the rate at which the intensity of the scattered light fluctuates due to Brownian motion and correlates this to the size of the particles. The DLS obtains a hydrodynamic diameter based on the diffusion of the particles and the hydrodynamic diameter indicates the effective diameter of a particle in a liquid. The mean hydrodynamic mean diameter of the nanocomplexes was determined by dynamic light scattering studies using a Zetasizer Nano ZS apparatus (Malvern Instruments, United Kingdom). All results were based on three or four measurements from two independent samples. All data were converted to relative by intensity or volume plots from which the mean hydrodynamic diameters were derived.

3.3.4. Transmission Electron Microscopy

For the morphological characterization of complexes, a Transmission Electron Microscopy (TEM) analysis was performed. TEM is a characterization method for imaging nanoparticles to obtain quantitative measures of particle size (without the hydration layer), size distribution and morphology. TEM visualizes the transmission of a focused beam of electrons through a sample, forming an image similarly to a light microscope, but because electrons are used rather than light to visualize the sample, TEM imaging has significantly higher resolution compared to light microscope.

3.3.5. Energy-dispersive X-ray spectroscopy

Energy Dispersive X-ray Spectroscopy (EDS) was used in this thesis to map the elemental composition of nanoparticles. EDS is a chemical analysis method that can be used with the two major electron beam based techniques of Scanning Electron Microscopy and Transmission Electron Microscopy. EDS analysis can be utilized to determine the elemental composition of individual points, line scans or to map out the lateral distribution of elements from the imaged area.

3.3.6. Characterization of stability of particles

3.3.6.1. Gel retardation assay

The gel retardation assay is widely used method to study the peptide and nucleic acid interaction and nanoparticle formulation. In this assay electrophoresis is used to analyze the complex formation between positively charged peptide and negatively charged nucleic acids. The rationale behind this assay is that electrophoretic mobility of negatively charged DNA, complexed with positive CPPs, should decreases gradually on the agarose gel with increasing charge ratio. The decreased mobility shift of nucleic acids indicates stronger interaction with peptide and hence the more tightly packed complexes between nucleic acid and CPP.

3.3.6.2. Competitive displacement assay

Formulation of stable and compact nanoparticles that would not dissociate after the exposure to serum is essential for the efficient transfection. The high stability of particles is required to deliver nanoparticles into the cells in serum and thereafter intracellularly into the nucleus. At the same time DNA needs to be released inside the cell to be expressed. Hence, it is necessary to form nanoparticles that are neither too stable nor labile. We therefore investigated plasmid DNA condensation by peptides at different charge ratios and dissociation of the complexes by heparin competitive displacement assay. To study the dissociation of peptide and DNA particles many polyanions have been used to competitively displace DNA from complexes. The most extensively used polyanion is heparin sulfate. Heparin sulfate has high negative charge density and can displace nucleic acids from loosely packed nanoparticles. Heparin sulfate has previously been found to mediate DNA release from polyelectrolytes as well as cationic liposomes(169). The displacement of DNA in labile and loosely packed particles by heparin salt is grater compare to more tightly packed particles. In heparin displacement assay peptide/pDNA complexes were mixed with fluorescent nucleic acid stain PicoGreen reagent (Invitrogen) for quantitating double-stranded DNA. Thereafter heparin sulfate (Sigma-Aldrich) was added to the complex solution and then incubated and analyzed. In each experiment,

naked DNA stained with PicoGreen was used to normalize the PicoGreen fluorescence signal detected from the complexes. After this fluorescence was analyzed and the higher fluorescence signal was correlated with more loosely packed nanoparticles.

3.3.6.3. Resistance of complexes to enzymatic degradation

The resistance of particles to protease degradation is critical to their use in drug delivery in vivo. Resistance to protease digestion might indicate the longer half-life of nucleic acid and therefore greater bioactivity in protease-rich environment like in blood serum. The aim of this assay was to estimate how tightly are nanoparticles packed and how accessible is peptide in complex for protease degradation. Proteinase K treatment was carried out to evaluate the resistance of the pre-formed NF/DNA complexes to enzymatic degradation of CPP by proteinases. Proteinase K is proteolytic enzyme with broad specificity purified from the fungus Tritirachium album.(170)

3.3.7. Screening of bioactivity of particles

In this thesis the term bioactivity of peptide/nucleic acid particles indicates the ability of the formulated nanoparticles to induce reporter gene expression in transfected cells.

3.3.7.1. Cell culture

Utilization of different cell lines is essential for better understanding of uptake of peptide-based nanoparticles as internalization mechanism of particles is dependent on cell type. We used multiple adherent cell lines which are commonly used to study transfection efficacy. Cell types used in this thesis are described in Table 2.

In paper I, epidermal primary keratinocytes were additionally used to further investigate efficacy of new NF analogues in primary cell culture.

In paper III, HeLa pLuc 705 cell line was utilized to study the transfection efficiency of PF/SCO particles in the widely used assay for splice correction. HeLa pLuc 705 cells are extensively utilized as a functional assay system to study the vector efficacy to deliver antisense oligonucleotides into cells. HeLa pLuc 705 cells are HeLa cells stably transfected with recombinant plasmid (pLuc/705) carrying the luciferase gene interrupted by a mutated human β-globin intron 2 (IVS2-705)(171). This mutation causes aberrant splicing of luciferase pre-mRNA in HeLa pLuc/705 cells. After the delivery of antisense oligonucleotide and thereby the correction of splicing, the functional luciferase is produced, and luminescence signal can be measured to examine the efficacy of the transfection.

Table 3. Cells used in this thesis.

Cells	Cell type	Paper
HeLa	Epithelial cervix adenocarcinoma	I, III
HeLa pLuc 705	Epithelial cervix adenocarcinoma, stably transfected with recombinant plasmid (pLuc/705)	III
CHO	Epithelial-like ovary derived cells	I, II
U87-MG	Epithelial glioblastoma astrocytoma cell line	I, III
U87-MG-luc	Epithelial glioblastoma astrocytoma cell line, stably expressing luciferase	III
Neuro2A	Neuroblastoma neuroblast neuronal and amoeboid stem cells	I
HT-1080	Epithelial fibrosarcoma	I
Keratinocytes	Human epidermal primary keratinocytes	I

3.3.7.2. Transfection studies

In this thesis CPP transfection efficacy was evaluated using plasmid DNA, SCO and siRNAs. We utilized multiple reporter systems for the screening of the efficiency of the peptide to facilitate the nucleic acid transport into cells.

In all three papers, pDNA transfection studies were investigated using p-CMV-Luc2 plasmid which express firefly luciferase. This is broadly utilized reporter system which is sensitive, robust, and easy to measure. p-CMV-Luc2 is 10kb-sized reporter plasmid which codes firefly (Photinus pyralis) luciferase that has been optimized for monitoring transcriptional activity in eukaryotic cells. After pDNA transfection cell or tissue lysate is mixed with luciferin and thereafter expressed luciferase activity is measured. The quantified luminescence signal correlates with transfection efficacy.

In paper I, we also transfected cells with two additional plasmids, p-EGFP plasmid encoding GFP and p-7SK-shRRM2 plasmid. p-EGFP plasmid was used to estimate the proportion of transfected population of cells using FACS by measuring the fluorescence signal from transfection-positive cells. p-7SK-shRRM2 plasmid was used to study gene modification in biologically relevant context, outside reporter system. This plasmid encodes short hairpin RRM2 and we aimed to down-regulate ribonucleotide reductase subunit M2 (RRM2) gene. Down-regulation of RRM2 gene have been shown to significantly reduce the cell growth and adhesion. Therefore, we treated cells with NF/ p-7SK-shRRM2 nanoparticles and determined population of viable cells with flow cytometry from a scatter plot.

In paper III, we investigated CPP efficacy to deliver also splice-correcting oligonucleotides into cells. Treatment of the cells with a SCO targeted to the aberrant splice site restores luciferase expression and quantified luminescence

signal correlates with efficacy of SCO delivery. We used SCO to correct aberrant splicing of luciferase pre-mRNA in HeLa pLuc/705 cells.

In paper III, we also used stably expressing luciferase U87-MG-luc cell line to deliver siRNA that targets mRNA of luciferase and causes degradation of the luciferase mRNA that leads to a decrease in the signal of luminescence.

3.3.7.3. Toxicity evaluation of particles

The CytoTox-Glo™ Assay is a luminescent cytotoxicity assay that measures the relative number of dead cells in cell populations. The assay is based on detection of differential ubiquitous proteolytic activities associated with intact viable cells and cells that have lost membrane integrity (172). The method uses a luminogenic peptide substrate (alanyl-alanylphenylalanyl-aminoluciferin) which cannot cross intact membranes to measure protease activity which is released from cells that have lost membrane integrity. Therefore, luminescent signal can be used to calculate dead cells.

We also used WST-1 assay which uses tetrazolium salt WST-1 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt) which is enzymatically cleaved to formazan by cellular mitochondrial dehydrogenases in viable cells. The formazan dye produced from WST-1 by viable cells can be quantified by measuring the absorbance of the dye at 440 nm.

For in vivo toxicity evaluation of nanoparticles, we used frequently used liver function blood test. We analyzed two liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) for detecting any inflammation or damage to cells in the liver after administration of peptide-based nanoparticles.

4. RESULTS AND DISCUSSION

4.1. Design of the effective peptide vector for systemic in vivo transfection

Progress in genomic sequencing have given us opportunity to regulate almost any gene expression in transcriptome through exogenous therapeutic nucleic acids. The main drawback of that kind of gene manipulation is poor cellular uptake of nucleic acids, especially in systemic administration in vivo. Therefore, the main challenge in creating effective gene-based therapies is the development of selective and safe delivery vectors.

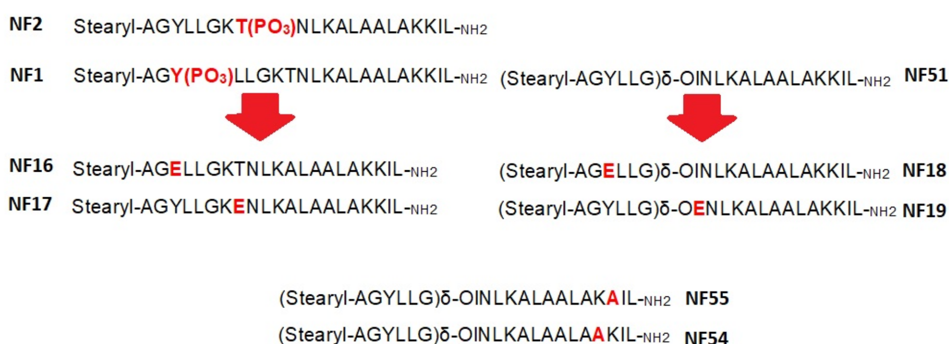

Figure 6. Illustration of the design of novel analogues of NFs.

In the Paper I, previous knowledge from internalization studies and physio-chemical properties of different analogues of NickFects (NF) was utilized to rationally design new NF peptides for in vivo use. We have previously designed two efficient NF analogues, both displaying similar efficacy despite having different structure and uptake mechanism(69,70). NF51 formulated stronger complexes with pDNA and delivered plasmid into cells via macropinocytosis while NF1 nanoparticles were less stable and entered cells through macropinocytosis and clathrin- or caveolae-mediated endocytosis(71). Although showing great transfection in cell culture, both peptides lacked in vivo delivery. We therefore hypothesized that we could combine modifications made to these peptides to create the vector more suitable for in vivo application. The graphic scheme of rational design of NF peptides can be seen in Figure 6.

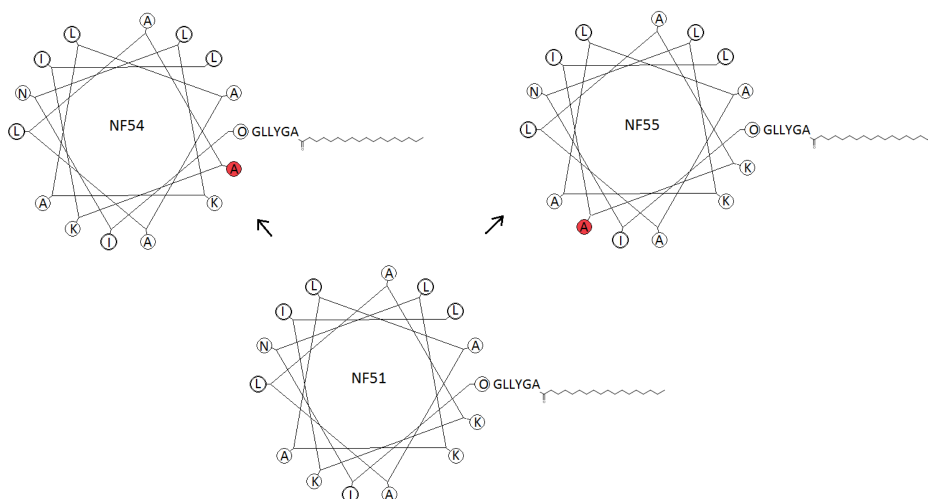

Figure 7. α-helical wheel projectors shown for NF51 and its analogues, indicating rationality of the location of amino acid replacement.

We first investigated if phosphorylation itself or the negative charge from phosphorylation is required for NF1 and NF2 transfection. As addition of a phosphoryl group has been shown to increase the transfection(173) we first studied the importance of phosphorylation and charge location within NF1 and NF2 sequence by synthesis of NF16 and NF17. In NF16 and NF17 phosphorylated amino acids were replaced with Glu which bears negative charge at physiological pH. After verifying that charge, not phosphorylation is essential for the transfection; similar alterations were introduced into NF51 sequence (NF18, NF19). These modifications in NF51 sequence however decreased transfection efficacy and uptake studies revealed that C-terminus modifications were less deleterious than alterations in the N-terminus sequence. Since insertion of Glu into N-terminus sequence decreased transfection, further modifications were made into C-terminus. After seeing no advantage by insertion of negative charge we continued to reduce the charge by replacing the positively charged lysines with alanines. These modifications were introduced again into C-terminus sequence and therefore two Lys at C-terminus in NF51 sequence, 18th and 19th, were replaced with Ala (α-helical wheel projectors of designed peptides are shown in Figure 7). Interestingly, only one analogue, NF55 showed improved transfection. Further CD spectroscopy studies revealed that this higher delivery efficacy might arise because of NF55 increased α-helical secondary structure compared to other NF peptides (in the Paper I, Figure 2). Indeed, the importance of helicity in TP10 and other CPPs for transfection has been shown previously by Song et al.(174) and Niidome et al. (126).

Next, after we had screened and selected the best peptide for gene delivery, we characterized NF55/pDNA particles and found that NF55 condenses pDNA into stable nanoparticles that are more resistant to protease degradation than

parental peptide NF51 nanoparticles (in the Paper I, Table 2). We propose that this can be because NF55 is more hydrophobic (in the Paper I, Table 2) than NF51 and hydrophobicity has been shown to increase peptide-DNA binding. The development of the more protease-resistant particles is essential, especially for the systemic in vivo administration as the half-life of the particle in blood stream greatly determines the transfection efficacy of the nanoparticle. Before continuing in vivo, we estimated the NF55 ability to escape from endosomes as entrapment of particles into endosomes has been shown to hinder the transfection. Endosomal escape studies revealed that in contrast with other NickFects, NF55 is not significantly hindered by endosomal entrapment (in the Paper I, Figure 3B).

After verifying that NF55 is highly efficient CPP in vitro we aimed to investigate its utilization in vivo (Figure 8). When delivering pDNA with NF55 systemically, we noticed like often reported with cationic particles, significantly high gene induction in lungs and liver. Surprisingly, we observed over 22-fold higher gene induction in cerebellum and 7-fold higher reporter gene expression in the rest of the brain tissue after NF55/pDNA treatment compared to naked pDNA treatment. Reporter gene induction was significantly higher in NF55 treatment compared to parental peptide NF51 and naked pDNA treatment. Furthermore, NF55/pDNA particles demonstrated comparable transfection with the best commercial in vivo transfection reagents in main organs (especially in lung tissue) and 5-fold greater bioactivity in cerebellum. This demonstrate the potential of NF55 to be an effective gene delivery vector and as a safer alternative to currently used transfection reagents which although very efficient, might cause side-effects.

After we have verified NF55 ability to transfect various tissues in vivo we aimed to deliver pDNA specifically into tumor and reduce lung and liver transfection. Because we detected high luminescence signal from cerebellum and brain after NF55 treatment we were interested to investigate it further and studied the transfection with intracranial glioblastoma model. Therefore, we compared NF55 and the parental-peptide NF51 efficacy to transfect intracranial tumor tissue. Interestingly, we detected 45-fold higher reporter gene expression in tumor-containing striatum compare to naked pDNA treated mice (in the Paper I, Figure 4B). Unfortunately, despite this high accumulation of NF55 particles into tumor tissue the similar gene expression was also observed in unaffected brain regions (in the Paper I, Figure 4B). Moreover, highest transfection was detected again in lungs and liver. This indicated the need to optimize the NF55 nanoparticles to specifically transfect distinct cell population or tissue.

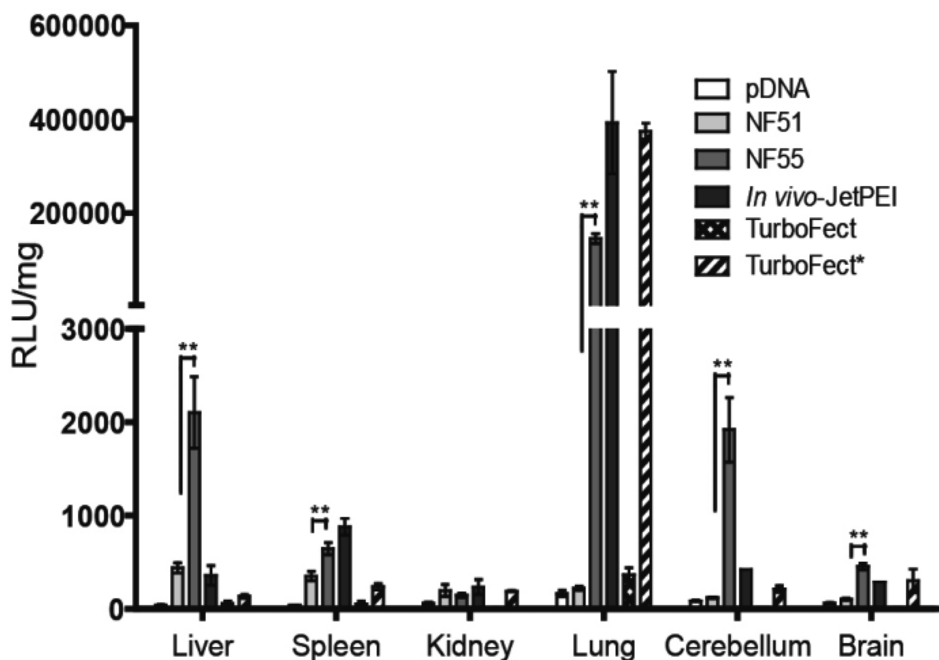

Figure 8. The novel analogue NF55 significantly enhances the transfection in balb/c mice at CR4. 20 μg pDNA was used to form nanoparticles with different delivery vectors except TurboFect termed with *, where 50 μg pDNA was used. The data is from at least three representative experiments performed in triplicate, presented as mean ± SEM, *P < 0.05; **P < 0.01 (Student t-test, two-tailed distribution, two-sample unequal variance.

Therefore, to increase the delivery of NF55/pDNA particles into the tumor tissue and to decrease the non-specific lung and liver accumulation we conjugated PEG polymer to NF55. As the helicity of the peptide is important for the transfection, the pegylation site in the peptide was selected and screened to maximize α-helicity of the NF55 (in the Paper I, Table S2). Various pegylated NF55 peptides were designed to utilize PEG shielding ability to decrease accumulation into lungs and liver and increase the transfection into tumor. To screen for the best formulation, we then moved away from intracranial model and continued studies using subcutaneous tumor. Two pegylated NF55 analogues NF551 and NF552 demonstrated the highest percentage of α-helix secondary structure and were chosen to formulate nanoparticles with different ratios with NF55. We aimed to optimize and find the optimal ratio of NF55 and the pegylated variant of NF55 (NF551 or NF552) which would form stable complexes with DNA and reduce the non-specific accumulation, but at the same time do not decrease the transfection into the tumor tissue. The latter phenomenon is known as PEG dilemma and it has been observed that incorporation of high concentration of PEG could significantly decrease the transfection. The most effective formulation was with 20% NF552/80% NF55/pDNA as it

increased tumor accumulation over three times and reduced the lung transfection over 38-fold compared to NF55/pDNA treatment alone. These results indicate that NF55/pDNA particles can be optimized for more efficient tumor accumulation through the incorporation of pegylated peptides.

Finally, we investigated the possibility to produce solid formulation of NF55/pDNA particles. This is essential as solid formulations are more stable during storage and transportation and remain the most extensively used pharmaceutical formulation currently utilized. The lyophilized pre-formed NF55/pDNA complexes did not conglomerated after lyophilization, and their size and shape were similar to that of non-lyophilized particles. Moreover, the storage at room temperature for seven days did not affect the bioactivity of these lyophilized particles. These results demonstrate that NF55/pDNA particles can be prepared and stored as a solid formulation without sacrificing its bioactivity.

Taken together, these results demonstrate that we have designed very efficient transfection reagent for in vivo utilization and illustrated the potential of NF55 to be modified for further targeting gene delivery.

4.2. Formulation of stable and homogenous nanoparticles for the efficient gene delivery in vivo

In clinical settings, it is necessary for pharmaceutical to possess strictly controlled and defined molecular composition and structural characteristics. It is essential as physicochemical parameters, including size and size distribution influence the biodistribution and toxicity of nanoparticles. Therefore, in the Paper II we continued to further investigate the physicochemical properties of the previously designed and formulated NF55/pDNA particles and to develop a new formulation approach to prepare NF55/DNA nanoparticles with more homogenous size distribution. The main aim was to prepare particles with more controlled size and size distribution for more safe and efficient systemic in vivo gene delivery. The graphic scheme of different formulation methods applied in the Paper II can be seen in Figure 9.

We have previously shown that our CPPs condense pDNA into approximately 100 nm sized stable homogenously distributed nanoparticles, but during characterization of NF55/pDNA particles, we discovered that in addition to major fraction of 100-nm sized particles a small fraction of these particles aggregated and formed larger conglomerates (Figure 10, formulation 1). This conglomeration was surprising and to investigate this further we used DLS to compare particles prepared using two formulation protocols which differ in used concentration of nanoparticles. We compared formulation 1 protocol which is our usual complex formulation method for transfecting the cell culture to formulation 2 method.

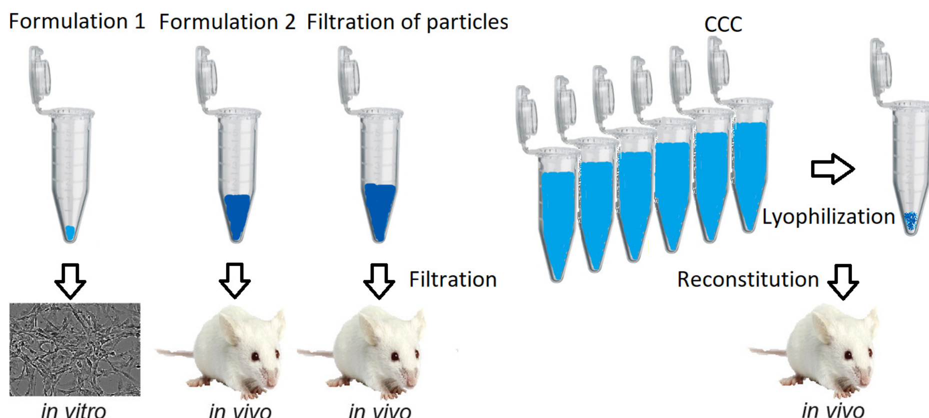

Figure 9. Graphic scheme of different formulation methods applied in the Paper II. This includes formulation 1 which is used in cell culture studies, formulation 2 which is prepared for the systemic in vivo gene delivery, formulation method of filtrated formulation 2, and the modified formulation strategy to produce cryo-concentrated complexes (CCC). The darker blue color indicates the higher concentration of particles.

In formulation 2 protocol we use ten times higher concentration of particles as higher concentration of particles is needed for the efficient systemic in vivo transfection (for example the concentration of peptide is 400 µM compare to 40 µM used in formulation 1). DLS measurements with formulation 1 revealed mainly one distinctive population of NF55/pDNA nanoparticles with an average size of 100 nm. On the other hand, when we repeated the measurements at higher concentration of particles we observed significantly more agglomerated particles (Figure 10, formulation 2). Particles formulated at higher concentration (formulation 2) displayed very heterogeneous size distribution and only 1/3 of population of nanoparticles were approximately in the desired size range of 100 nm for the in vivo application (Figure 10). This demonstrated the need for the development of more suitable formulation method of particles to prepare nanoparticles with controlled size and with more homogenous size distribution.

To reduce heterogeneous size distribution of NF55/pDNA particles we utilized filtration method (Figure 9). In our experiments both, DLS and TEM studies also confirmed that filtration successfully removed large conglomerates from formulation 2 and similarly to formulation 1, only one distinctive population of about 125-nm diameter nanoparticles were obtained. Moreover, filtration also lowered significantly polydispersity index compared to formulation 2. Despite successful elimination of aggregates bioactivity in cell culture was reduced over 1000-fold and transfection in vivo also diminished after nanoparticle filtration (Figure 12). These results demonstrate that although filtration of complexes yield homogenous size distribution of particles the transfection efficacy is significantly decreased.

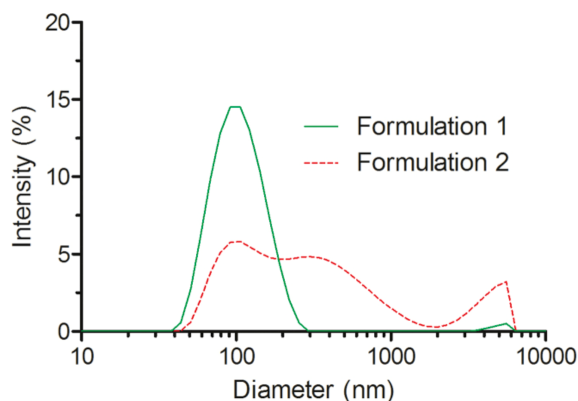

Figure 10. NF55/DNA particles formed at the higher concentration needed for the systemic in vivo gene delivery (formulation 2) tend to aggregate and form larger conglomerates compare to formulation 1 which is used in the cell culture studies. DLS measurements with formulation 1 (green line, peptide concentrations 40 μM) and formulation 2 (dashed red line, peptide concentrations 400 μM).

As filtration did not gave us the desired outcome we investigated alternative method to produce more uniformly sized particles. Therefore, we next utilized alternative strategy where we aimed to mimic the nanoparticle formulation 1 protocol which produces particles with more homogenous size distribution and adapt it for in vivo settings (Figure 9, CCC). We assembled the particles at the same concentration as we did for formulation 1, lyophilized the complexes and reconstituted the specimen in a smaller volume to prepare higher concentration of cryo-concentrated complex (CCC, peptide concentration 400 μM at the same peptide: DNA charge ratio 4) formulation required for administration in vivo. According to DLS, CCC particles revealed markedly higher size homogeneity than the formulation 2 and around 98% of particles were around the desired size range (around 100 nm size) and more suitable for in vivo administration (Figure 11). We thereafter analyzed morphology of CCC nanoparticle with TEM. After lyophilization, the CCC formulation sample contained mostly 50 nm-sized separate spherical particles (in the Paper II, Figure 5C). Both DLS and TEM results indicated that CCC formulation method can be utilized to produce particles with homogenous size distribution.

After we had verified that CCC formulation method can be applied to prepare nanoparticles with homogenous size distribution we then aimed to investigate the stability and bioactivity of CCC formulated particles. As the stability of nanoparticles in the blood stream is crucial for their utilization, we analyzed the resistance of the nanoparticles to protease digestion. CCC nanoparticles demonstrated slightly higher resistance to protease digestion compared to the standard formulation, thus indicating that CCC nanoparticles are more stable and tightly packed and therefore more suitable for the systemic in vivo delivery.

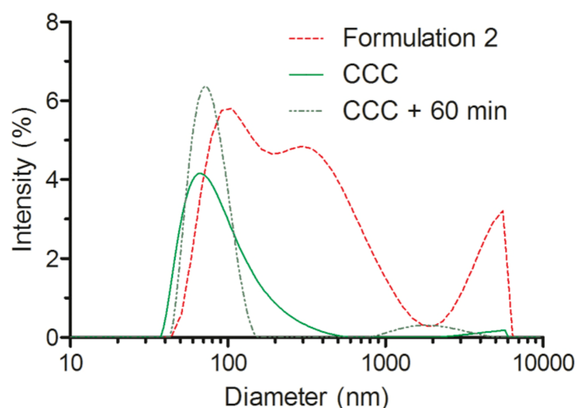

Figure 11. DLS analyzis of samples indicates that CCC formulation strategy produces non-conglomerating nanoparticles with homogenous size distribution compare to standard formulation 2 method. Particles obtained by complex formulation method 2 are represented with a dashed red line, and the cryo-concentration complex (CCC) formulation strategy is illustrated with a green line. The dotted darker green line shows the size distribution of CCC particles after incubation for 60 min.

Subsequently, confirming that CCC formulation yields more uniform and stable particles we continued with transfection studies. CCC particles exhibited similar delivery efficiency in cell culture as standard formulation 1. These results encouraged us to investigate the CCC bioactivity in vivo. Interestingly, CCC particles were twice as effective in transfecting lung tissue compared to standard formulation 2 treatments (Figure 12). The fact that the transfection efficacy increases after CCC formulation method offers a very promising strategy to generate efficient transfection reagent with uniform size distribution of particles. Moreover, these nanoparticles can be stored in lyophilized state (at least seven days as shown in the Paper I, Figure 5) at room temperature until needed and after convenient reconstitution in water these particles still maintain their homogenous size distribution (Figure 11) and high systemic in vivo gene delivery efficacy (Figure 12).

Because the filtered samples where we managed to produce smaller particles with more homogenous size distribution showed the reduced bioactivity, we hypothesized that maybe the larger conglomerates facilitate the transfection. There have been reports that sedimentation of larger aggregated nanoparticles can enhance the transfection in vitro(175). It has also been demonstrated that conglomerated large particles tend to accumulate within capillaries of the lung(74). These studies might help to explain why the filtered fraction of nano-particles that contains homogenous small nanoparticles exhibit reduced bioactivity in lungs. To investigate the importance of the size and the size distribution of the particle on transfection efficacy, we increased the conglomeration of the CCC particles by incubating these at room temperature for 60 min, instead of using immediately after the reconstitution of nanocomplexes. DLS

shows that incubation of CCC particles for 60 min induces positive skew to the size distribution, increasing the fraction with larger size (Figure 11). This incubation encouraged aggregation of particles up to 13% of overall particle population according to DLS. Increased conglomeration however decreased transfection of CCC particles back to the similar efficacy as was detected after treatment with the standard formulation 2 particles (Figure 12). This suggests that aggregation of particles alone cannot directly correlate with their efficacy in vivo.

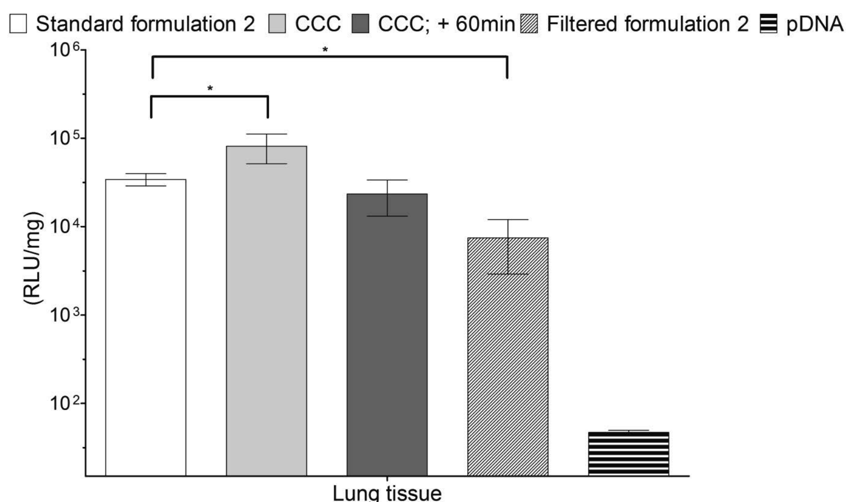

Figure 12. CCC formulation strategy significantly improved the systemic in vivo gene delivery whereas filtration of particles reduced the transfection. Transfection efficacy in vivo of standard formulation 2, CCC and filtered formulation 2 nanoparticles at CR4 and 20µg pDNA. The data is from at least three representative experiments performed in triplicate, presented as mean ± SEM, *P < 0.05 (Student t-test, two-tailed distribution, two-sample unequal variance.

Conclusively, in the Paper II we developed novel nanoparticle formulation using cryo-concentration that yields stable particles with homogenous size distribution and displays significantly higher lung transfection compared to the standard formulation.

4.3. Incorporation of iron oxide particles to improve the efficacy and specificity of peptide vectors

The important obstacle to overcome is the non-targeted delivery of nano-particles. This off-targeted uptake of nanoparticles could increase side effects and reduce the efficacy of treatment. Magnetic nanoparticles can be used to create selective targeting drug delivery systems(157). Recent studies have shown that

incorporation of magnetic nanoparticles can enhance cell or tissue specific uptake of nanoparticles in the presence of magnetic field(157,161,176). In the Paper III, we aimed to study whether these magnetic particles could be incorporated into our CPP- nucleic acid formulations. We incorporated iron oxide nanoparticles into CPP and nucleic acid particles to investigate the physicochemical properties of the formulated particles. The future goal of this study is to improve the efficacy and specificity of the CCP transfection through the incorporation of magnetic particles and utilization of magnetic field.

We used our previously designed effective PepFect14 peptide as the most thoroughly described peptide from our designed CPPs and its analogues to form nanoparticles with magnetic iron oxide and nucleic acid (NA).

First, we studied the ability of PF analogues to complex Fe_3O_4 and nucleic acids. Most PF analogues were able to form stable nanoparticles with Fe_3O_4 and nucleic acids as these formulations displayed zeta potential ≥30 mV or ≤ – 30 mV which is usually considered a good indicator for stable colloidal dispersions. In addition to describing stability of particles, surface charge is important for their cellular uptake. Nanoparticles with positive surface charge are typically taken up by the cells more efficiently compared to negatively charged counterparts(74). This can be mainly due to the greater interaction between nanoparticles and negatively charged plasma membrane. Most PF analogue formulations with Fe_3O_4 and nucleic acids indeed had the positive surface charge. Taken together these results demonstrate that iron oxide can be incorporated into our PF/NA formulations to produce stable nanoparticles which is essential for the efficient transfection.

Next, after the characterization of the size and surface charge of Fe_3O_4 /PF/NA particles we continued to investigate their transfection efficacy. First, we tested the transfection efficacy of Fe_3O_4 /PF/NA particles in the cell culture. We studied the ability of Fe_3O_4 /PF particles to enhance plasmid delivery and three PF analogues (PF221, PF222 and PF223) complexed with Fe3O4 demonstrated 2-100-fold higher plasmid transfection compared to standard PF/pDNA particles. Thereafter, we studied SCO delivery and the most effective one from the screened PF peptides was the parent peptide Fe_3O_4 /PF14 particles which increased splice correction 4-times compared to standard PF14/SCO complexes. Fe_3O_4 /PF14 /siRNA particles also demonstrated highest downregulation compared to other Fe_3O_4 /PF/siRNA formulations and standard PF14/siRNA particles. As there was no magnetic field involved with these transfection studies this increased efficacy might be caused because of the enhanced sedimentation of the particles after the addition of Fe_3O_4. We concluded that PF-nucleic acid formulation can be further complexed with Fe_3O_4 to significantly enhance the biological activity in the cell culture.

The internalization mechanism of CPP-nucleic acid particles is still debatable, but the likeliest entering pathway of particles is thought to be endocytosis, although energy-independent translocation has been described. The internalization mechanism of vectors modified with magnetic particles are thought to be remain the same. Huth et al.(177) and Sauer et al.(178) have studied the

internalization of magnetic particles coated with PEI polyplexes or lipoplexes, respectively. Both studies concluded that the addition of Fe_3O_4 did not change the internalization mechanism of these vectors and the coated magnetic particles still used mainly endocytosis to enter cells. We used therefore confocal microscopy to investigate the cellular uptake of our Fe_3O_4 /PF/NA particles. Indeed, we similarly concluded that the incorporation of Fe_3O_4 did not alter the cellular uptake compared to conventional PF/NA particles without the magnetic particles.

Finally, we decided to use PF14, which has been extensively described and utilized in vivo already, to form complexes with Fe_3O_4 for systemic delivery of pDNA. The highest reporter gene expression was detected in lungs and liver tissue (in the Paper III, Figure 8). Incorporation of Fe_3O_4 decreased the luminescence signal 5- and 20-fold in lung and liver respectively. Although incorporation of Fe_3O_4 into PF14/pDNA particles reduced the bioactivity in the analyzed tissues further study is necessary to assess usefulness of iron oxide for tissue specific targeting via magnetic field.

Taken together, we described Fe_3O_4 /PF/nucleic acid particles and showed the effectiveness of incorporation of iron oxide into standard PF/NA complexes to increase the transfection in cell culture. In vivo results demonstrate that Fe_3O_4 /PF14/pDNA bioactivity is not entirely diminished and application of magnetic field could be used to enhance particle accumulation and biological activity in specific tissue.

5. CONCLUSIONS

The main aim for this research was to design more efficient and safer formulations of CPP/nucleic acid particles for the systemic in vivo delivery of nucleic acids.

The key results can be concluded as described below.

Paper I

- ✓ Optimization of the secondary amphipathicity and charge of the NickFect peptide analogues improved the transfection efficiency.
- ✓ Novel peptide NF55 formed stable nanoparticles with DNA that were resistant to protease degradation.
- ✓ NF55 mediated DNA delivery in vivo with excellent gene induction efficiency that is comparable to the commercial transfection reagents currently available offering a safer alternative for the systemic in vivo gene delivery.
- ✓ NF55/DNA nanoparticles demonstrated increased tumor transfection in various mouse tumor models. Efficiency of NF55 to deliver DNA into tumor tissue increased even further after incorporation of PEG2000 polymer.
- ✓ Solid formulation of NF55/DNA particles displayed a good stability profile without additives or special storage conditions.

Paper II

- ✓ Filtration of the NF55/DNA nanoparticles successfully removed large aggregates and increased the homogeneity of the particle size distribution.
- ✓ Cryo-concentration formulation strategy of NF55/DNA particles produced non-conglomerating nanoparticles with uniform size, increased resistance to protease degradation and significantly improved bioactivity in vivo.

Paper III

- ✓ PF peptides were able to form stable complexes with nucleic acid and Fe_3O_4 particles.
- ✓ Incorporation of Fe_3O_4 particles significantly enhanced PF peptides ability to transfect nucleic acids in cell culture.
- ✓ Application of magnetic field might be used in future to increase the accumulation of formulated complexes with Fe3O4 into the specific tissue.

6. SUMMARY IN ESTONIAN

Genoomika ja sekveneerimismeetodite areng on täiendanud meie teadmisi geneetikast ning geneetilisetest haigustest. Geneetiliste haiguste tekkepõhjuste mõistmine võimaldab meil sünteesida erinevalt modifitseeritud seerum-stabiilseid terapeutilisi nukleiinhappeid, reguleerimaks spetsiifiliselt mingi konkreetse geeni avaldumist. Nii on võimalik rakendada geeniteraapiat ning taastada kahjustunud või puuduva geeni funktsioon või hoopis vaigistada ebanormaalselt üle-ekspresseeruv geen. Kahjuks takistab nende nukleiinhapete edukat kasutamist antud terapeutiliste molekulide laeng ja suurus, mis vähendab oluliselt nende biomolekulide jõudmist kahjustunud koe rakkudesse.

Nukleiinhapete rakkudesse sisenemise efektiivsuse tõstmiseks kasutatakse mitmesuguseid geenivektoreid, mida võib jagada viiruslikeks ja mitte-viiruslikeks vektorites. Kuigi viirusvektorid on hetkel kasutatavatest vektoritest ühed efektiivseimad ning üle 69% kliinilistes katsetes kasutatavatest geenivektoritest on just viirusvektorid, siis segab nende kasutamistveelgi edukamat rakendamist vektorite võimalik immunogeensus ja transfekteeritava nukleiinhape suuruse piirang. Mitte-viiruslikud vektorid on küll madalama transfektsiooni efektiivsusega, kuid nad on võrreldes viirusvektoritega tavaliselt vähem toksilisemad.

Üheks mitte-viiruslikuks transfektsioonivektoriks on rakku sisenevad peptiidid (RSP). RSP-d on tavaliselt kuni 30 aminohappe pikkused katioonsed ja/või amfipaatsed peptiidid. RSP saab siduda kovalentselt või mittekovalentselt mitmesuguste terapeutiliste ühenditega (nukleiinhapetega, valkudega, peptiididega jt molekulidega), et suurendada nende transporti rakkudesse nii in vitro, kui ka in vivo. Peamiseks probleemiks RSP kasutamisel on nende peptiidide vähene transfektsiooni efektiivsus in vivo. Lisaks on RSP tavaliselt vaja modifitseerida, et muuta neid selektiivseks konkreetse koe ja rakutüübi suhtes, vähendades seeläbi võimalike kõrvalmõjude tekke riski. Samuti on oluline kontrollida moodustatud RSP-nukleiinhappe osakeste füsiko-keemilisi omadusi, sest ka sellest sõltub osakeste biodistributsioon organismis. Nukleiinhapete transfektsiooniks kasutatakse enamasti mittekovalentset peptiidiga seondamist, mis on odavam ning lihtsam, kuid selle meetodiga moodustunud nanopartiklid on küll stabiilsed ent väga heterogeense suurusjaotusega. Selline erinev nanopartiklite suuruse distributsioon mõjutab nii rakukultuuri kui ka in vivo tulemusi. Erineva suurusega osakesed akumuleeruvad erinevatesse organitesse ja ekskreteeritakse organismist erineva kiirusega. Samuti sõltub osakeste suurusest kui hästi tuntakse partikkel ära retikuloendoteliaalsüsteemi poolt ning kui pikk on komplekside poolväärtusaeg seerumis. Lisaks suurendab selline suurusest tulenev mittespetsiifiline osakeste kuhjumine kõrvalnähtude teket. Seega on oluline leida õige formuleerimise tehnika, et kontrollida paremini osakeste suurusjaotust.

Antud doktoritöö eesmärk oli valmistada efektiivseks ja ohutuks süsteemseks in vivo manustamiseks mõeldud RSP -nukleiinhappe formulatsioon.

Esimeses töös disainiti NickFect peptiidide ja nukleiinhappega moodustunud komplekside füsiko-keemiliste omaduste ning sisenemismehhanismide põhjal uued NickFect peptiidi analoogid. Optimeerides nii peptiidi laengut kui ka amfipaatsust, sünteesiti NickFect55 peptiidi, mis moodustab nukleiinhappega proteaasitöötlusele vastupidavaid stabiilseid osakesi. See on oluline NF55 nanopartikli poolväärtusaja suurendamiseks seerumis. Lisaks suurendas NF55 peptiid oluliselt DNA transfektsiooni nii rakukultuuris, kui ka in vivo mitmes hiire vähkkasvaja mudelis.

Teises artiklis keskenduti NF55-DNA osakeste formulatsiooni modifitseerimisele, et komplekside kasutamine oleks ohutum ja efektiivsem. Selgus, et in vivo kasutamiseks mõeldud formulatsioonis on vaid 1/3 tekkinud osakeste diameeter alla 200 nm ning seega on umbes 30% osakestest ainult sobilik süsteemseks manustamiseks. Osakeste heterogeense suurusjaotuse vähendamiseks ja aglomeratsiooni vältimiseks filtreeriti või lüofiliseeriti osakesi väiksemas kontsentratsioonis (CCC-cryo-concentrated complex formulation). Mõlemad meetodid vähendasid märgatavalt partiklite aglomeerumist ning suurendasid väiksema läbimõõduga (<200 nm) osakeste osakaalu. Kui filtratsioon vähendas nanopartiklite bioloogilist aktiivsust, siis CCC formulatsiooni tehnika suurendas oluliselt moodustunud osakeste in vivo transfektsiooni ning stabiilsust proteaasi töötlusele.

Kolmandas töös lisati RSP ja nukleiinhappe kompleksidele magnetilisi rauaoksiidi osakesi suurendamaks transfektsiooni raku-ning koespetsiifilisust. Uuriti PepFect peptiidide võimet moodustada osakesi erinevate nukleiinhapete ja magnetiliste Fe_3O_4 partiklitega. Fe_3O_4 lisamine PF-nukleiinhapete kompleksidesse ei vähendanud osakeste stabiilsust ning suurendas oluliselt bioloogilist aktiivsust rakukultuuris. Samas näitasid loomkatsed, et rauaühendite lisamine vähendab transfektsiooni. Edasine uuring koos magnetvälja kasutamisega on vajalik, et selgitada kas magnetilisi osakesi saaks kasutada konkreetse organi transfektsiooniks.

Kokkuvõtvalt valmistati antud doktoritöös rakkusisenev peptiid ning formulatsiooni meetod, millega on võimalik moodustada stabiilseid homogeense suurusjaotusega osakesi efektiivseks in vivo transfektsiooniks.

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