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KADI-LIIS VEIMAN

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ABSTRACT

Gene therapy is widely considered to have great therapeutic potential for a wide variety of diseases that occur due to malfunctioning genes. To achieve therapeutic effects, genetic material needs to reach target organelles within cells, and thus must overcome complex intra and extracellular barriers. Because the physicochemical properties of nucleic acids, such as their high molecular weight and negative net charge, prohibit translocation over cell membranes, the successful application of gene therapy relies on the development of gene delivery vehicles.

Cell-penetrating peptides (CPPs) are one class of non-viral transport vectors that have been widely exploited to deliver nucleic acids into cells. CPPs can be up to 30 amino acids long, are typically cationic and/or amphipathic, and can facilitate both the condensation of large nucleic acid molecules such as plasmid DNA (pDNA) and their intracellular delivery.

The main purpose of the research presented in this dissertation was to develop an efficient CPP in cell culture that is applicable for systemic gene delivery *in vivo*, and has potential to treat diseases caused by aberrant gene expression, such as cancer. First, we characterized various aspects of peptide based gene delivery, such as potential gene induction efficacy and the uptake mechanisms in cell culture. Next we evaluated the potential for CPP-mediated pDNA delivery after systemic administration in mice and found that improvements were required, including the need to achieve tumor specific gene delivery. For that, we evaluated various strategies such as the conjugation of either targeting peptides or polyethyleneglycol (PEG) molecules to the CPPs. The latter strategy improved the biocompatibility of CPP/pDNA complexes and permitted us to shield the universal transfection property of CPPs, which could be further activated specifically in specific tumor tissues and induce gene expression. We also optimized the complex formulation to improve their gene delivery properties without PEGylation and characterized other CPP properties such as cationic charge density and fatty acid modification. We found both to be important aspects that govern CPP-mediated gene delivery not only in cell culture, but also *in vivo*. In conclusion, the potential of CPP-based gene delivery system could be further extended for gene therapy applications in relevant disease models.

TABLE OF CONTENTS

ABSTRACT 5
TABLE OF CONTENTS 6
LIST OF PUBLICATIONS 8
ABBREVIATIONS 10
INTRODUCTION 11
1. LITERATURE OVERVIEW 12
1.1 Gene therapy 12
1.1.1 Practical aspects about the design of gene expression vectors 13
1.1.2 Regulation of gene expression with nucleic acid-based technologies 14
1.2 Non-viral gene delivery platforms 16
1.2.1 Barriers for non-viral gene delivery 16
1.2.2 Common cationic gene delivery reagents for *in vivo* administration 18
1.2.3 Strategies for improvement for cancer gene delivery 22
1.3 Cell-penetrating peptides 25
1.3.1 Overview of history and classification 25
1.3.2 Uptake mechanism and endosomal release 26
1.3.3 CPP mediated nucleic acid delivery 28
1.3.4 Tuning specificity of CPPs 32
2. AIMS OF THE STUDY 33
3. METHODOLOGICAL CONSIDERATIONS 34
3.1 Peptide modification 34
3.2 Peptide synthesis 35
3.3 Complex formulation strategies 37
3.3.1 Non-covalent formulation strategy 37
3.3.2 Modification of PEGylation rate 37
3.4 Characterization of formed complexes 37
3.4.1 DNA binding and condensation 38
3.4.2 Complex size and surface charge 38
3.4.3 Heparin displacement assay 38
3.5 Cell cultures 38
3.6 Bioactivity of CPP/pDNA complexes in cell culture 39
3.6.1 Receptor dependent uptake and endosomal escape 39
3.6.2 Evaluation of gene delivery efficiency 40
3.6.3 Toxicity profile and induction of innate immunity 40
3.6.4 Gene delivery potential of CPP/pDNA complexes *in vitro* BBB model 41
3.7 Evaluation of CPP/pDNA complex interactions with blood components 41

3.7.1 Hemolysis 41
3.7.2 Serum incubation 41
3.7.3 Stability against enzymatic attack 42
3.8 *In vivo* bioactivity of systemically administered CPP/pDNA complexes 42
3.8.1 Induction of tumor models 42
3.8.2 Biodistribution 42
3.8.3 Pharmacokinetic profile 43
3.8.4 Gene expression induction in different tissues 43
4. RESULTS AND DISCUSSION 44
4.1 Characterization of PF14 mediated gene delivery in cell culture (Paper I) 44
4.2 CPP-based delivery of pDNA across the blood–brain barrier (Paper II) 45
4.3 PF14 gene delivery potential and its improvements for *in vivo* applications (Paper III) 47
4.4 Formulation characteristics governing PF14 gene delivery *in vivo* (Paper IV) 49
5. CONCLUSIONS 52
SUMMARY IN ESTONIAN 54
REFERENCES 57
ACKNOWLEDGEMENTS 68
PUBLICATIONS 69
CURRICULUM VITAE 131
ELULOOKIRJELDUS 133

LIST OF PUBLICATIONS

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

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

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

I. performed most of the experiments, and participated in both the data analysis and in the writing of the manuscript;
II. performed part of the gene delivery experiment, and participated in both the data analysis and in the writing of the manuscript;
III. designed and performed many of the experiments, analyzed most of the data and participated in the writing of the manuscript as a corresponding author;
IV. designed and performed many of the experiments, analyzed most of the data and participated in the writing of the manuscript as a corresponding author.

Other publications:

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VI. Kurrikoff, K., **Veiman, K.-L**., and Langel, Ü. (2015). CPP-Based Delivery System for *In Vivo* Gene Delivery. In Cell-Penetrating Peptides, Ü. Langel, ed. (New York, NY: Springer New York), pp. 339–347.

ABBREVIATIONS

ACPP	Activatable cell-penetrating peptide
ANG	Angiopep-2
BBB	Blood-brain barrier
CF	Cystic fibrosis
CMV	Cytomegalovirus
CPP	Cell-penetrating peptide
CQ	Chloroquine
CR	Charge ratio
DLS	Dynamic light scattering
DMD	Duchenne muscular dystrophy
EPR	Enhanced permeability and retention effect
EtBr	Ethidium bromide
FACS	Fluorescence-activated cell sorter
gHo	Glioma-homing peptide
HC50	Hemolytic concentration 50%
i.m	Intramuscular
i.p	Intraperitoneal
i.v	Intravenous
LF2000	Lipofectamine™ 2000
MMP	Matrix metalloproteinase
ON	Oligonucleotide
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PF14	PepFect14
PF6	PepFect6
PLL	Poly-L-Lysine
RBC	Red blood cells
RES	Reticuloendothelial system
RFU	Relative fluorescence unit
RISC	RNA-induced silencing complex
RLU	Relative light unit
RNAi	RNA interference
S.c	Subcutaneous
SCARA	Class A scavenger receptor
SCO	Splice-correcting oligonucleotide
shRNA	Short-hairpin RNA
siRNA	Small interfering RNA
TJ	Tight junctions
TP10	Transportan10
UT	Untreated

INTRODUCTION

Advances and improvements in genetics and biotechnology have provided a completely new understanding about how the human genome functions. An ever-expanding amount of data is becoming available to identify the causes underlying disorders caused by aberrant gene expression. In light of this progress, gene therapy has received renewed consideration as a potential treatment strategy for a very wide variety of diseases that occur due to malfunctioning genes, including cancer. Gene therapy applies strategies that modify the expression of an individual's genes or correct the expression of abnormal genes. To achieve these objectives, therapeutic nucleic acids must reach their targets within cells, and thus must overcome complex intra and extracellular barriers. Because the physicochemical properties of nucleic acids preclude their spontaneous translocation into cells, successfully applying gene therapy relies on the development of gene delivery vectors, the most challenging part of developing gene therapy-based applications.

After initial failures, the development of viral vectors proved to effectively deliver their cargo, however, their utilization is still associated with drawbacks, which creates potential for the use of non-viral gene delivery vectors. Numerous studies have demonstrated their efficacy to deliver genetic material into cells, however, the effectiveness of non-viral gene delivery vectors is often reduced when applied for *in vivo* gene delivery. While significant advances have been made in this field, and several cationic polymers and lipid-based materials have reached preclinical and clinical studies, not one single non-viral vector has been granted a marketing license after decades of studies.

Cell-penetrating peptides (CPPs) are a relatively new class of non-viral transport vector that has considerable potential for both drug delivery and the delivery of genetic material. They can both condense nucleic acids into nano-sized complexes and initiate their uptake into cells. CPPs share common traits with other non-viral vectors, including their property of universal transfection in cell cultures and difficulties using them *in vivo*. First, the work presented in this dissertation demonstrates the potential of CPPs to deliver genes in cell culture. Next, we present efforts to translate the potential of this non-viral gene delivery platform for the optimized gene delivery *in vivo*. The approaches presented herein could provide a new means to deliver therapeutic nucleic acids into relevant disease models.

1. LITERATURE OVERVIEW

1.1 Gene therapy

Advances and technological improvements have made it both possible and feasible to sequence human genome [1], [2]. These advances have also significantly deepened our understanding of the mechanisms that cause many genetic disorders. Taken together, recent developments have greatly expanded the potential use of gene therapy as a therapeutic intervention. Although therapeutic applications are now reaching clinical trials [3], gene therapy has not yet realized its potential, which may require a major breakthrough to achieve. Yet, in light of recent technological advances, gene therapy is considered to be in the midst of a renaissance [3], [4]. In recent years several gene therapy products have been authorized for marketing in different countries (Table 1) and expanding rate of clinical trials have emerged [5].

Gene therapy could be defined as a set of strategies that either modify the expression of an individual's genes or correct abnormal genes. Each of these strategies involve the administration of a specific DNA (or RNA) molecule [6]. In EU regulation, gene therapy has been placed under the guidelines for advanced therapy medicinal products (ATMPs), which refers to medicinal products for human use. Besides gene therapy products, this class of treatment also includes somatic cell therapy and tissue engineering products [7]. This clearly demonstrates that gene therapy is now considered to be a more realistic approach than it was twenty years ago, when the first clinical trials were conducted [8], [9]. Applications are not restricted to the delivery of genes into cells because the definition now includes constructing or manipulating cells or tissues whose genome has already been modified. This simultaneously opens up a new venue for applications, and a number of issues that have yet to be addressed.

Table 1. Gene therapy products approved for marketing

Gene therapy product	Approval	Disease	Delivery method	Delivered Gene	Reference
Gendicine	2003 China	Head and neck squamous cell carcinoma	Viral	TP53	[10]
Rexin-G	2007 Philippines	Solid tumors	Viral	Cytocidal cyclin G1 construct	[11], [12]
Neovasculgen	2011 Russia	Peripherial arterial disease	Intramuscular injection	VEGF	[13], [14]
Glybera	2012 European Union	Lipoprotein lipase deficiency	Viral	LPL	[15], [16]

One of the most important milestones for the development of gene therapy could be considered the approval to market Glybera® in Europe [15], [16]. This is a gene therapy product for the treatment of a metabolic disease, lipoprotein lipase deficiency (LPLD, incidence of ~1/500 000), where the gene that encodes lipoprotein lipase is administered via a viral vector after intramuscular (i.m.) injection. Its efficacy has been evaluated in three clinical trials where the long term expression of this protein has been achieved, together with therapeutic effects [17].

Cystic fibrosis (CF) is another disease which treatment has been often associated with gene therapy. It is a monogenic autosomal recessive disorder that affects about 70000 people worldwide and is caused by a mutated gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is a cAMP regulated anion channel expressed on the apical surface of epithelial cells that lining airways, pancreatic ducts, and other tissues [18]. The affected cells are located in an environment filled with mucous, which makes gene delivery extremely difficult and necessitates the need for repeated administration. Cationic lipid nanoparticles, which are a non-viral gene delivery vehicles, have been harnessed for CF gene therapy [19].

There are also several trials where genetic material has been delivered *ex vivo* by viral vectors into hematopoietic stem cells for the treatment of β-thalassemia [20], Wiskott-Aldrich syndrome [21] and X-linked severe combined immunodeficiency [22]. These examples rely on an improved viral gene delivery method, and highlight the importance of validating and studying other relevant targets to address the needs of emerging and advanced gene modulation technologies. Furthermore, it is important to investigate alternative delivery vectors that have the potential to lead gene therapy into completely new level.

1.1.1 Practical aspects about the design of gene expression vectors

To deliver therapeutic genes into cells it is common that the gene of interest is inserted into a plasmid expression vector (pDNA) of prokaryotic origin. These expression vectors contain the elements required to initiate gene expression, such as promoters and their enhancer sequences and an antibiotic resistance gene is commonly available for the production of expression molecules. Being of bacterial origin, pDNA contains unmethylated CpG sequences, which are recognized by the host's immune system, and more specifically, by Toll-like receptors, which, in turn, could lead to the development of an immune reaction [23], [24]. Although it is highly probable that these sequences are initially shielded by the gene delivery vector, once they have dissociated from the delivery vector, unmethylated CpG sequences will be silenced and eventually discarded [25]. To avoid this, gene expression vectors used for gene therapy should be designed carefully with reduced CpG content to avoid an immune response and enable sustained gene expression [26], [27], [28]. Furthermore, it

is possible to remove all of the bacterial sequences that are unnecessary to express the required genes via the minicircle DNA strategy. Advantages to this approach have been reported both *in vitro* and *in vivo* [29], [30], and although constructing these such expression vectors was initially considered to be laborious, technical improvements have made it possible to produce them in higher quantities [31].

Another aspect to consider is the choice of promoters. The most commonly used viral SV40 or CMV driven expression cassettes provide rapid gene expression [32], which can be beneficial to study the effect of the gene delivery reagent, however, for therapeutic applications, prolonged gene expression is more desirable to avoid repeated administrations. For that, the human elongation factor 1α (EF1 α) promoter alone or in combined with CMV-derived enhancer elements can be beneficial [27] .

1.1.2 Regulation of gene expression with nucleic acid-based technologies

Originally, gene therapy research targeted monogenic disorders but recent advances in biotechnology have opened very intriguing and potent possibilities to carry out gene therapy via short synthetic nucleic acids. RNA interference (RNAi) is a naturally occurring process that silences the expression of specific genes at the post-transcriptional level. The ability to silence genes using double stranded RNA (dsRNA), was first characterized in 1998 [33], followed by the delivery of a synthetic dsRNA molecule into mammalian cells that silenced a gene via RNAi using synthetic short interfering RNAs (siRNAs) [34]. This technology has since been widely used in a variety of applications and has even reached into clinical trials [35], [36].

RNAi technology relays on the delivery of siRNA molecules into the cell cytosol where the RNA-induced silencing complex (RISC) is available to mediate gene knockdown. siRNAs, the effector molecules of RISC, are 21–23 nucleotides long and commonly possess modifications [37], [38] to make them more stable against extra- and intracellular enzymatic influences and to some extent these modifications limit off-target effects [39]. Once siRNA is taken up by the RISC complex, passenger strand is excluded and the guide strand then binds to the target mRNA. Thereafter, the RNase of RISC cleaves the mRNA, which leads to rapid degradation of target RNA, followed by reduced gene expression.

Another approach to silence genes via RNAi is to express short hairpin RNA (shRNA) molecules inside cells [40], [41] using pDNA expression vectors. The silencing mechanism also relies on the RISC complex, however, the shRNA, expressed by the pDNA vector, must first be processed to 21–22 nucleotide long siRNAs by the Dicer molecule [42]. This technique uses the endogenous processing machinery to produce siRNA from shRNA encoding pDNA, and potentially allows for persistent gene silencing using lower copy numbers of

effector molecules thereby resulting in less off-target effects and greater safety [43].

RNAi is a very appealing technology for cancer treatment due to its ability to simultaneously silence several targets, however, the size of siRNAs limits their translocation over cellular membranes yet are small enough to be excreted by the kidneys if administered as a single agent. For RNAi to become an effective therapeutic intervention, these limitations must be met and a number of other issues addressed: (i) effective delivery, (ii) possible off-target effects, and (iii) pharmacokinetics and pharmacodynamics [43]. Non-targeted delivery of siRNA molecules leads to their accumulation in liver and possible uptake by hepatocytes. Because of this, several RNAi gene therapy applications have targeted the liver. To achieve selective delivery, incorporating siRNA into nanoparticles [44] or direct conjugation with targeting moieties can be beneficial. One example of binding a targeting moiety to siRNA is conjugating N-acetylgalactosamine (GalNAc) to siRNA, which mediates hepatocyte uptake via an asialoglycoprotein receptor. This approach leads to efficient and durable gene silencing at low and well tolerated doses [45], [46]. The siRNA-conjugates that have reached the clinical trials have been reviewed by Wittrup and Lieberman [47].

Another very potent approach for advanced gene therapy, or more specifically genome editing, is a technology termed the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9) system. First described and derived from the adaptive immune system of the bacterium *Streptococcus pyogenes* [48], its first application in mammalian cells was reported in 2013 [49] and is now very broadly used for genome editing in various organisms, including humans and other mammals [50]. It is based on the cut-and-paste approach that removes aberrant genetic information and replaces it with functional sequences. CRISPR/Cas ribonucleoprotein complex is composed of endonuclease Cas9, needed to bind and cleave target DNA, and two single-stranded RNA molecules – CRISPR RNA (crRNA), which binds to a complementary DNA sequence and trans-activating RNA (tracrRNA). Cas9 Induced breaks are repaired via two mechanisms. The most common is non homologous end-joining (NHEJ), which leads to the introduction of insertions and deletions at the break site. Although, due to its simplicity it is most commonly used but NHEJ is very error prone and alternative mechanisms are being developed. The more precise homology-directed repair (HDR) requires the delivery of an exogenous DNA repair template, together with components. This extra delivery step makes the HDR method much more difficult to apply, however, the benefits are very desirable and significant effort has been made to utilize HDR in practice [51],[52].

As mentioned above, to apply genome editing in practice, all of the required components must be delivered into the target cells, because the ribonucleoprotein complex involved is only natively expressed in bacteria. Mainly viral vectors are employed to deliver and express these components, however, the stable expression of Cas9 endonuclease has been shown to increase side-effects

[53] and because of that, non-viral and transient delivery systems may be more beneficial [52]. It has been reported that CRISPR/Cas enables the correction of genes such as CFTR that underlies CF [54] and a potential therapeutic treatment for Duchenne muscular dystrophy has been demonstrated [55]. The major advantage of the CRISPR/Cas system over RNAi is that once the genetic defect is repaired, the result is inheritable and, CRISPR/Cas can also be used to correct gene expression, while RNAi can only be used to mediate temporal gene silencing [56]. Due to its prokaryotic origin it is likely that the CRISPR/Cas approach will have less cross-reactions in eukaryotic cells and lead to a reduction in off-target effects. It is important to note that advances in the field of RNAi and the delivery of its components into cells has made a significant impact on applying CRISPR/Cas in practice and in the delivery and/or expression of its effector molecules. The lessons learned on how to increase the intracellular stability of short synthetic RNA molecules and reduce their side-effects have made it both easier and faster to apply advanced nucleic acid-based therapies [57]. So far, the major drawback for all gene therapy applications is still the method of delivery and advances in this field could lead to the blossoming of gene therapy into routine clinical practice.

1.2 Non-viral gene delivery platforms

As discussed above, several gene therapy strategies are available in the market or are under investigation in clinical trials. Viral vectors and advances made in this field have contributed significantly to the development of gene therapy, yet, these have not avoided the need for extensive optimization (particle modification, loading capacity). This, together with persistent safety concerns such as immunogenicity and insertional mutagenesis [58], have driven research towards efficient and safe non-viral delivery platforms. These platforms typically use cationic polymers or lipids that complex genetic material into particles via electrostatic and/or hydrophobic interactions. Many of these have already reached preclinical evaluation and several have reached into clinical trials. However, not one single non-viral gene therapy product has been granted a market license.

1.2.1 Barriers for non-viral gene delivery

The biggest challenge for non-viral gene therapy lies in *in vivo* gene delivery. The common limitations for most of these systems are [59] :

(i) relatively low efficacy of gene transfection into the target cells,
(ii) physicochemical instability of the vector/DNA complexes,
(iii) induced cytotoxicity.

To achieve a bioeffect, the therapeutic material must be sufficiently stable in the hostile environments within the organism and overcome various extracellular

and intracellular barriers before reaching its intracellular target in a functional state. To some extent, delivery vectors can protect the therapeutic genetic material with their ability to pack or condense nucleic acids. Administration of the therapeutic material is conventionally carried out using either oral or local administration, or intravenous injection (i.v.) [60]. Although local administration enables to avoid most of the the extracellular barriers, this method is not applicable for most genetic diseases, and thus systemic administration is preferred. Upon entering the blood stream, the therapeutic material is immediately affected by blood cells, degradative proteins, complement proteins, and reticulo-endothelial system (RES) recognition, all of which lead to rapid inactivation or even aggregation. This typically results in decreased gene delivery efficiencies, and, in the case of large aggregates, may trigger toxic reactions due to lung embolization.

Physicochemical parameters such as surface charge and size are important factors that determine the fate of delivery complexes. A net positive charge reduces the aggregation between particles in formulation solution through electrostatic repulsion, however, once in the bloodstream they rapidly associate with negatively charged serum albumins and red blood cells (RBCs), which possess a negative surface charge [61]. In addition, the particles can interact with vascular endothelial cells and engulfed via phagocytosis. Another aspect is the size of preformed complexes and the size they obtain once they have reached into bloodstream. Although large aggregates may induce toxic effects, particles that are too small are rapidly excreted from the body through glomeruli in the kidney [60], so it is important to optimize the size they attain in the bloodstream.

To reach targets in the brain, the therapeutic material must pass the blood-brain barrier (BBB), which consists of brain capillary endothelial cells that do not have fenestrations, have extensive tight junctions (TJs), and very sparse pinocytotic vesicular transport [62]. This physical barrier actively regulates the transport and access of molecules to brain cells, and only very low molecular weight molecules are able to diffuse through the TJs; All other nutrients cross this barrier via transporters [63] [64]. The BBB is a selective barrier that possess a significant challenge for the delivery of therapeutic agents into the brain. However, with our increasing knowledge about neurodegenerative diseases and glioblastomas, the impetus to target these issues is also growing and gene therapy could be a prospective treatment method. It has been observed that growing brain tumors induce TJs to open and provide a slight therapeutic window for the delivery of therapeutic molecules, however, no significant therapeutic effects have been accomplished by taking advantage of these open junctions and this delivery strategy allows molecules that can be toxic to neurons access to the brain, which may lead to permanent damage and severe effects [65]. In general, no delivery method should provoke permanent damage to either the BBB or the brain.

Once gene delivery complexes traverse the barriers present after systemic administration and reach the vicinity of their target cells, they must translocate

over the cellular membrane and overcome many intracellular barriers. Several strategies are used to bind delivery complexes to specific receptors or fuse them with cellular membranes. Usually non-viral vectors are taken up by receptor-dependent endocytosis and employing or initiating this inherent process enables the delivery of particles safely, and leaves cellular membranes intact. However, the benefits of endosomal uptake are negated by endosomal escape. Several strategies have been used to meet this limitation, including the proton sponge strategy [66]. This relies on the presence of secondary and tertiary amines that have pKa values in a similar range as the endosomal and lysosomal pH, 5.5 – 6. During the acidification of endosomes, amines become protonated and this alters the osmolarity of the vesicles by increasing the influx of protons and counter-ions into the vesicles. This, in turn, leads to the uptake of additional water molecules, increasing osmotic pressure, swelling, and finally rupture of the vesicle. However, this classical proton sponge hypothesis has been recently challenged [67]. These authors did not observe the rupture of endosomes nor the release of intact vectors and nucleic acid complexes into the cytosol. Instead, these two components were released separately into the cytoplasm via local pores within the endosomal membrane. These intriguing results require further confirmation and could potentially offer a new means of designing efficient endosomal escapers.

In many cases, once the therapeutic genetic materials have gained access to the cytosol, whether associated with the delivery vehicle or not, their journey is almost complete. However, some nucleic acids, including pDNA molecules, must traverse the passage across the nuclear membranes, which is another critical intracellular barrier. It is commonly accepted that pDNA gains access to the nucleus during cell division, at which time the nuclear membrane breaks down. This is mainly supported by the fact that only molecules smaller than 9 nm and up to 40kDa are able to diffuse through the nuclear pore complex [68]. Nucleic acids have a much higher molecular weight and size and thus are not able to diffuse through the nuclear membrane. Although there are some strategies available to target the nuclear pore complex, nuclear uptake mechanisms in general have been poorly studied and no excellent methods currently exist to induce the uptake of genetic material. Improving this situation will require further insight.

1.2.2 Common cationic gene delivery reagents for *in vivo* administration

Cationic polymers offer a means to condense nucleic acids into a nanoparticle core. The most commonly used polymers are polyethyleneimine (PEI) [69], [70], poly L-lysine (PLL) [69], chitosan [71], [72], poly(2-N-(dimethyl-aminoethyl) methacrylate) (pDMAEMA) [73] and poly(amidoamine) (PAMAM) [74] [75] (Figure 1).

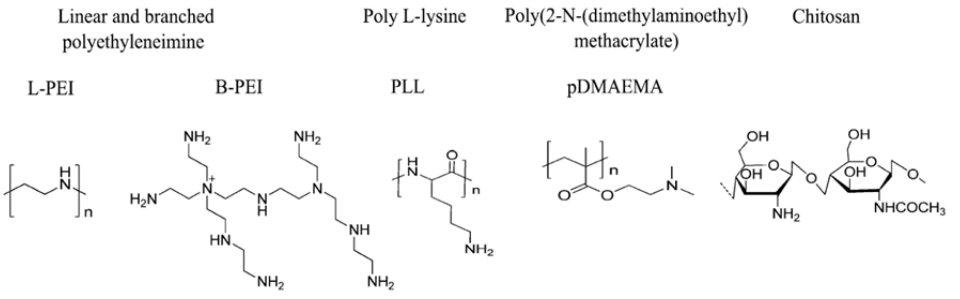

Figure 1. The most common cationic polymers used in gene delivery

Polyethyleneimine (Figure 1) is a polymer with high cationic charge-density potential, where every third atom is an amino nitrogen that can be protonated and it has been widely studied and used within gene delivery applications for two decades already [76]. PEI can be linear or branched with either a low or high molecular weight. The efficacy of gene delivery using low molecular weight PEI is moderate but is significantly less toxic than delivery using high molecular weight PEI. This polymer is able to condense and bind nucleic acids very efficiently and acts as a proton sponge within endosomes inside the cells, which is a useful property to induce endosomal escape when delivering nucleic acids into cells. PEI-mediated gene delivery has been reported in numerous applications, however, when utilizing it as a single delivery vector for systemic administration of pDNA, it induces significant gene expression in lungs [77]. These authors used a CMV-based luciferase encoding gene expression vector, L-PEI, that is able to induce gene expression starting from 10^3 (pDNA dose 20 μg) up to 10^7 (with pDNA dose 125 μg) in relative light units per milligram of total protein content (RLU/mg). Naked pDNA administration in lungs induced gene expression levels up to only 10^3 RLU/mg. It was concluded that this gene expression was specifically induced in lung alveolar tissues, however, high gene expression was induced in other organs as well [77].

Besides its use as a single-component gene delivery vector, a receptor binding ligand transferrin (Tf) has been incorporated into PEI/pDNA complexes which leads to enhancement of transgene expression in a subcutanous mouse Neuro2a model after systemic administration, mainly by masking the high positive charge of PEI/pDNA complexes [78]. Another example is PEGylated PEI-Tf/pDNA complexes, which, after systemic administration, enhanced gene induction in tumors. However, expression was also observed in different organs such as liver, lungs, and also near the injection site in tail [79]. Although PEGylation improved gene delivery in tumors and decreased it in lungs, it was obvious that complexes were still being taken up by non-tumor tissues – they could already transfect cells in tail and be taken up by lungs. This highlights the need to mask or shield the overall transfection potential of the cationic polymer so that it is indeed taken up or activated specifically in tumor tissues, thereby enabling efficient cancer gene therapy. Various ligand-targeting formulation

strategies for pDNA delivery have been compared, including the use of PEI and PEG [80]. The first approach involved covalent conjugation of a targeting-ligand to PEI and this construct was used to complex pDNA and followed by attaching a PEG molecule. In the second approach, pDNA is condensed by PEI and these complexes are functionalized with PEG followed by attachment of a ligand to the distal end of the PEG. This resulted in particles that have both PEG and the targeting ligands on their surface. In the third approach, complexes were prepared in a single step by condensing the pDNA with a mixture of PEI, PEG-PEI, and ligand-PEG-PEI. The third strategy, although it used a different ligand, provided both the most efficient and significantly higher levels of gene induction in tumor tissues, while gene expression in other tissues, such as lung, remained modest [80], [81], [82]. This intriguing result suggests that the most efficient and optimal gene induction occurs using a formulation technique where all components are able to simultaneously complex pDNA, and also turns out to be the simplest method to functionalize pDNA/PEI complex with both PEG and a targeting ligand.

Despite being very efficient in gene delivery, PEI-mediated systemic transfection has been associated with side-effects [83]. In this report [84], PEI/pDNA complexes were separated from excess free PEI and systemically delivered, thereby achieving reduced side-effects and reduced transfection efficacy as well. They then used stepwise addition of free PEI to purified complexes to optimize transfection efficiency while reducing side-effects. The authors of this study highlighted the importance of a free cationic fraction of the polymer. Nevertheless, PEI has successfully been used in several preclinical studies, and the formulations have been optimized and is now commercially available as an *in-vivo* gene delivery reagent [85].

Poly-L-Lysine or PLL has been extensively used for gene delivery. At physiological pH, the primary ε–amine group in lysine carries cationic charge and is the main source for nucleic acid complexation via electrostatic interactions [86]. Higher molecular weight PLL is capable of complexing pDNA at a charge ratio (CR) of one (CR1), whereas lower molecular weight PLL is four times less efficient and requires higher concentrations to achieve similar efficacy [87]. Although PLL/pDNA complexes form, they are considered to be toxic to cells, which significantly reduces their transfection potential. These limitations have been addressed to some degree using strategies such as PEGylation [88].

One interesting application for PLL in gene delivery platforms is TerplexDNA, a DNA delivery vector that consists of three components: (i) balanced hydrophobicity and net surface charge between stearyl-poly(l-lysine), which is needed to condense negatively charged nucleic acids; (ii) low density lipoprotein (LDL) as a targeting moiety, and (iii) nucleic acid [89], [90]. Systemic administration of Terplex DNA leads to significant enhancement of the half-life of pDNA compared with unformulated genetic material. Complexes distributed within several organs, mainly in lungs and kidneys, and gene expression was detected in liver, heart, and to a smaller extent in a lung [91]. The

authors also analyzed the potential of PLL alone and stearylated PLL to induce gene expression after systemic administration. Intriguingly, they did not detect any activity. It was also claimed, that Terplex DNA-induced gene expression is mediated via receptor-dependent endocytosis in hepatocytes, which express LDL receptors. These receptors are also expressed on the surface of both arterial endothelial cells and myocytes and the availability of receptor-mediated gene delivery in heart opened up an opportunity to use gene therapy in heart diseases [92], [93].

Poly(2-N(dimethylaminoethyl)methacrylate) or pDMAEMA (Figure 1) is considered to be a good alternative to PEI because of its reduced toxicity and significant buffering capacity. Its potential as a gene carrier vector was first studied in 1996 where it was compared with different cationic polymers. In this study it was found to have similar toxicity as the other polymers, however, it was reduced when bound with pDNA [94]. The reduced cytotoxicity, (IC50 ~ 40 μg/ml, whereas for PEI it is ~ 30 μg/ml [95]), good buffering capacity, simple and more controlled synthesis method by using radical polymerization of the corresponding vinyl monomer, makes pDMAEMA a good candidate vector for gene delivery [96]. Indeed, one advanced example of pDMAEMA mediated gene delivery is to brain after adding a targeting ligand to PEGylated pDMAEMA and pDNA complexes. This formulation induced gene expression in several organs, including brain after systemic administration of 50μg of complexed pDNA [97]. Although undecorated complexes distributed more in liver and in kidneys, the possibility to target complexes in brain and achieve gene expression clearly demonstrates the feasibility of this system.

Chitosan (Figure 1) is a biodegradable polysaccharide that is composed of d-glucosamine and N-acetyl-d-glucosamine linked by a (1→4) glycosidic linkage. Their cationic charge originates from primary amines and provides efficient DNA binding. The clear advantage that chitosan has over cationic polymers is its biodegradability, which leads to enhanced biocompatibility and reduced toxicity. Its molecular weight is very well correlated with the reduced size of their complexes with pDNA molecules – the smaller polymer molecular weight, the smaller complex size at the same CR [98]. Transfection efficacy depends not only on the size of the complexes, but also by their stability and pDNA release profile and together, these properties could contribute to transfection, thus, chitosan formulations with different molecular weights in an appropriate range should be considered [71].

Cationic lipids were first studied for their ability to transfect pDNA already in 1987 [99] and are now the most commonly used non-viral gene delivery reagents. They are able to complex pDNA, and the resultant lipoplexes are considered non-immunogenic due to a lack of immunogenic proteins. Furthermore, they are easily modified to obtain desired properties, such as size, surface charge, or specific targeting moieties. Although hundreds of lipids have been developed for gene delivery purposes, they share similar features: They consist of three basic domains (i) a hydrophilic positively-charged headgroup (ii) a hydrophobic domain (aliphatic chains), and (iii) a spacer linking both parts [100].

Usually, the hydrophilic headgroup is positively charged and interacts with and condenses nucleic acids electrostatically. Based on the structures of the hydrophilic headgroups, they can be grouped into six categories [101]:

1) quaternary ammonium headgroups,
2) amines (primary, secondary, or tertiary amines),
3) amino acids or peptides,
4) guanidine headgroups (monoguanidinium or conjugates of guanidinium and other headgroups),
5) heterocyclic headgroups,
6) unusual headgroups.

It is typical to first thoroughly study the structure-activity relationship of cationic lipids to find favorable parameters for transfection. Cationic lipids are used in combination with neutral helper lipids to form liposomes and pack DNA, which together form a DNA delivery system that meets the stringent criteria presented for non-viral gene delivery platforms. Cationic lipid based transfection shares many similar properties with cationic polymer-based systems. Transfection is dependent on the chemical structure of the cationic lipid (charge, linker and hydrophobic moiety), the CR between the cationic lipid and the DNA, the structure and proportion of the helper lipid in the complexes, the size and structure of the liposomes, and the total number of lipoplexes added to the cells. These properties, together with the chemical structure of the cationic lipid, determines the nanocomplex properties with nucleic acid and its transfection potential [102]. For example, a significant lipid-mediated hepatic siRNA delivery *in vivo* into non-human primates was reported already a decade ago [44], but significant improvements where achieved with ionizable cationic lipids as a key lipid components of stable nucleic acid lipid particles, which resulted in very efficient endogenous gene-silencing (at siRNA dose of 0.1 mg/kg) in hepatocytes of non-human primates [103].

1.2.3 Strategies for improvement for cancer gene delivery

As mentioned above, gene therapy has been considered to be a very potent strategy for cancer therapy, due to the possibility of addressing several targets simultaneously. Furthermore, tumors possess several properties that enable improved gene delivery.

In cancer gene therapy applications, genetic material is delivered to cells to [104]:

- restore/correct mutations,
- carry out gene expression modulation using RNAi,
- produce cytotoxic or prodrug activating gene products,
- reduce angiogenesis,
- increase immune response against tumor-cells.

The universal requirements for gene delivery vectors also apply for cancer gene therapy, such as binding of genetic material and shielding this from degradative enzymes. In addition to these common traits, cancer gene therapy products must be exclusively associated with the tumor and its cells, which means that delivery vectors must target cancer cells or have a specific mechanism that affects only cell-types associated with the tumor to reduce side-effects which are typically present when utilizing conventional cancer therapeutics. For the vector to reach the cancer cells, extra- and intracellular obstacles must be overcome. For that, a range of "smart" polymer based nanoparticles has been extensively developed and studied in recent years [104]. They consist of different modules to achieve most optimal parameters to mediate efficient, yet safe genetic material delivery in cancer cells.

Cationic gene delivery reagents have been improved using various modifications such as tuning the surface of the nanoparticles. One possibility to increase stability is the addition of a PEG molecule, which, as a hydrophilic polymer, increases the solubility of drugs. When incorporated into a nanocomplex surface, it forms a hydrophilic exterior that sterically inhibits the binding of serum proteins, reduces opsonization and reduces clearance by RES [105]. The main disadvantage of PEGylation is reduced binding with target molecules, which has been called the PEG dilemma [106]. This dilemma can be avoided by incorporating PEG molecules on the surface of the particles via cleavable linkers. These linkers can be sensitive to specific stimulus such as certain enzymes, pH, temperature, ultrasound, or radiation [107], [108].

Due to its intrinsic genetic variability and additional physiological barriers for drug delivery systems, the treatment of cancer is very challenging. Tumors have an altered microenvironment with a leaky vasculature, increased interstitial fluid pressure, a slightly acidic environment compared to other tissues, and poor lymphatic drainage. So far, the most successful strategy in preclinical studies has been a combined approach of chemotherapy with gene therapy approaches, provides a synergistic effect that may either increase drug efficacy and enhance gene therapy mediated bioeffect [104].

Various approaches are used to deliver nanoparticles into tumor tissues. The first is passive targeting [109], [110], which is based on the accumulation of nanoparticles within tumors due to incomplete tumor vasculature through enhanced permeability and a retention effect [111]. Adding an active targeting moiety that increases the binding affinity further increases the availability of nanoparticles within tumor tissues. These moieties could be proteins, e.g. antibodies and their fragments, peptides, small molecules, or carbohydrates [112]. One example is the targeted delivery of the P53 gene in tumors. TP53 is a tumor suppressor gene that encodes a protein critical for two of the pathways involved in regulating tumor cell growth – apoptosis and the regulation of angiogenesis. This gene becomes mutated in a wide variety of human tumors [113] and to correct this, a liposomal complex composed of pDNA that includes a correct version of TP53 is targeted to tumor cells by an anti-transferrin receptor single-chain antibody. This complex sensitizes tumor cells to conventional cancer

therapy and has moved from preclinical studies to clinical trials [114]. Another recent example of the delivery of TP53 using pDNA relies on a more distinct targeting strategy, where branched PEI and tuned ratios of PEG, histidine, and glutamic acid form complexes with the pDNA. These are then targeted to the tumor via its acidic microenvironment, wherein charge reversal of the complex occurs and promotes cellular internalization, endosomal destabilization, and finally gene expression of TP53 pDNA [115]. Several similar strategies have potential to improve the delivery of nanocomplexes to tumors [112].

One strategy to target nanocomplexes to tumor cells is using single chain antibody fragments, which are derived from antibodies but their affinity and specificity have remained. In one report cationic liposomes have been decorated with antitransferrin receptor single-chain antibody fragment (TfRscFv) and used for tumor specific gene delivery [116]. These liposomes have been exploited for gene delivery to express truncated retinoblastoma protein (RB94), that exerts potent tumor cell growth suppression [117]. Systemic administration of these targeted liposomes to enabled to express RB94 and sensitized tumors to chemotherapy, leading to reduction in tumor growth [116]. This potent gene-delivery platform in combination with chemotherapy has now entered to phase I clinical study [118].

Tumors need very good nutrient and oxygen supply for development and growth, therefore the neovascularization occurs rapidly and several growth factors are overexpressed in almost majority of cancer types. One main regulator of angiogenesis is vascular endothelial growth factor (VEGF), that is overexpressed in most tumors and is closely associated with tumor growth and metastasis [119]. This makes the targeting of angiogenesis and VEGF-associated pathways highly desirable. In numerous reports the expression of VEGF is targeted with RNAi for cancer therapy. For example, PEGylated PEI has been targeted to tumors via targeting peptides such as Arg-Gly-Asp (RGD) peptide [120] or Ala-Pro-Arg-Pro-Gly (APRPG) peptide [121] to deliver siRNA against VEGF. In both these studies the reduction in tumor vessel density and the inhibition of tumor growth was observed. APRPG is peptide that has identified via phage display technology using mouse angiogenesis model, where it adhered to neovascular endothelial cells [122]. Authors also applied liposomes encapsulating drug against angiogenic endothelial cells and decorated with APRPG peptide for antineovascular therapy which led to inhibited tumor growth.

1.3 Cell-penetrating peptides

Cell penetrating peptides (CPPs) are short, usually less than 30 amino acid-long cationic and/or amphipathic peptides that are able translocate cellular membranes and deliver a wide variety of cargo into intracellular compartments [123]. A concise definition of what constitutes a CPP is relatively difficult to write, however, it is clear that all CPPs share several common properties: (1) they are peptides that consist of natural or non-natural amino acids that have possibly been modified with chemical entities that improve their delivery properties, (2) conventional CPPs carry a positive net charge, (3) they are able to translocate over cellular membranes, (4) and most importantly, they are able to deliver a wide variety of cargo. CPPs have been applied in numerous studies for drug delivery *in vitro* and *in vivo*, and function not only as a single-component delivery vector, but also within comprehensive delivery platforms as a moiety to aid cellular translocation.

1.3.1 Overview of history and classification

The first evidence that proteins are taken up by cells was provided in 1988, when it was found that the trans-activator protein (TAT) from the human immunodeficiency virus type 1 (HIV-1) [124] is taken up by cells. It was found that only a portion of the 86 amino acid TAT protein was necessary for both cellular uptake and enzymatic activity. Further investigation of deletional mutants confirmed that TAT (37–57) entered cells [125]. This led to the discovery of a penetratin CPP in 1994 [126] and the later Tat peptide [127].

The development of a transportan peptide [128] and a study of its deletional analogs [129] underlies the beginning of second generation CPPs termed PepFects and NickFects. These have proven to be very efficient in the delivery of nucleic acids in a wide range of applications [130], [131], [132]. Another important milestone in the context of the work presented in this dissertation is the description of a non-covalent complex formation between CPPs and nucleic acids and their efficient internalization within cells. From a clinical point of view, one cannot overlook the development of the activatable CPP-based strategy (ACPP) by R. Tsien who used this approach in a preclinical study [133]. During the twenty years since CPPs were discovered, they have gained considerable attention by the scientific community, and, in a relatively short time, significant achievements have been made towards their clinical use, as evidenced by numerous studies of CPP-based applications in cell culture and also in preclinical disease models.

There are several ways of classifying CPPs and one of them is based on their origin. Firstly, peptides derived from proteins such as Tat [127] and penetratin [126]. Secondly, chimeric CPPs, which have been combined from sequences obtained from different proteins. Such combination with NLS and with hydrophobic domain that associates with cell membranes is Pep1 [134]. Another example is MPG, where signal peptide, taken from HIV glycoprotein 41 is also

combined with NLS [135]. Another noteworthy chimeric peptide is transportan which has 27 amino acids of which the 12 aminoterminal amino acids originate from the neuropeptide galanin and 14 amino acids at the carboxyl terminus originate from mastoparan, connected via one single lysine [128]. Beyond this are purely synthetic peptides such as the polyarginine family [136], [137], MAP [138] and CADY [139].

A more useful method of classifying CPPs uses the peptide sequence and their interactions with lipid membranes, where the distribution of charged and hydrophobic amino acids are taken into account [140]. This classification system defines three classes of CPPs:

(i) primary amphipathic, containing charged and hydrophobic regions in their primary sequence;
(ii) secondary amphipathic, becoming amphipathic by obtaining secondary structures like α-helix or β-sheet;
(iii) non-amphipathic, usually very cationic peptides.

Characteristics such as amphipathicity and cationicity are one of the key factors that not only determine how CPPs associate with cell membrane components and translocate into cells, but in how they form CPP/cargo complexes.

1.3.2 Uptake mechanism and endosomal release

It is commonly accepted that CPPs gain intracellular access via two main mechanisms – by either the endocytotic pathway or via direct penetration (Figure 2). The mechanism for the cellular entry of CPPs is not universal, and depends on the CPP sequence, concentration, or other factors. The types of CPPs that interact with lipids and thus possess high membrane activity might occasionally cause transient membrane damage. Nonetheless a variety of mechanisms have been proposed to explain how they translocate through cellular membranes. These depend on the type of CPP, its concentration, and even the nature and properties of the cellular membrane, however, in all cases mechanistic proof remains elusive.

Despite this, it is commonly accepted that when linked with high-molecular weight cargo molecule such as nucleic acids, endosomal uptake is preferred. Endocytosis could be subdivided as phagocytosis, associated with macrophages, or pinocytosis, which is present in all types of cells. The latter can be further subdivided based on the proteins that are involved in vesicule formation, the size of the endosome that forms and its intracellular fate via different types of endocytosis such as clathrin- or caveolae-mediated endocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis [141], [142], [143]. Although the above-mentioned mechanism is predominant, one cannot entirely exclude direct translocation of the CPP/cargo complexes. Receptor dependent endocytosis, for example, has been demonstrated for arginine rich peptides,

which are taken up by a chemokine receptor thereby stimulating macropinocytosis [144]. A chemokine receptor targeting ligand has been conjugated to the CPP Tat to make it more specific for cancer cells [145]. The involvement of receptor-mediated endocytosis has also been shown for CPP/oligonucleotide (ON) complexes, where participation of scavenger receptors mediate their uptake [146], [147].

Usually, CPP uptake mechanism studies are performed in cultured cells that are genetically highly identical and possess similar membrane components. Furthermore, the components present in the extracellular media are also well defined. A serious concern arises, when one would like to translate *in vitro* findings to *in vivo* conditions, where the uptake of CPPs could depend on the administration method (local or systemic). Hence, the properties of CPP and/or CPP-cargo complexes change due to the influence of unspecific interactions with extracellular components such as proteins and digestive enzymes. When administered systemically, interactions with the cells present in blood or that line blood vessels, could also have impact on CPP uptake in tissues. Taken together, the uptake mechanism by tissue cells could be different than the mechanisms described using cell culture models. Resolving this issue will require extensive studies using relevant models.

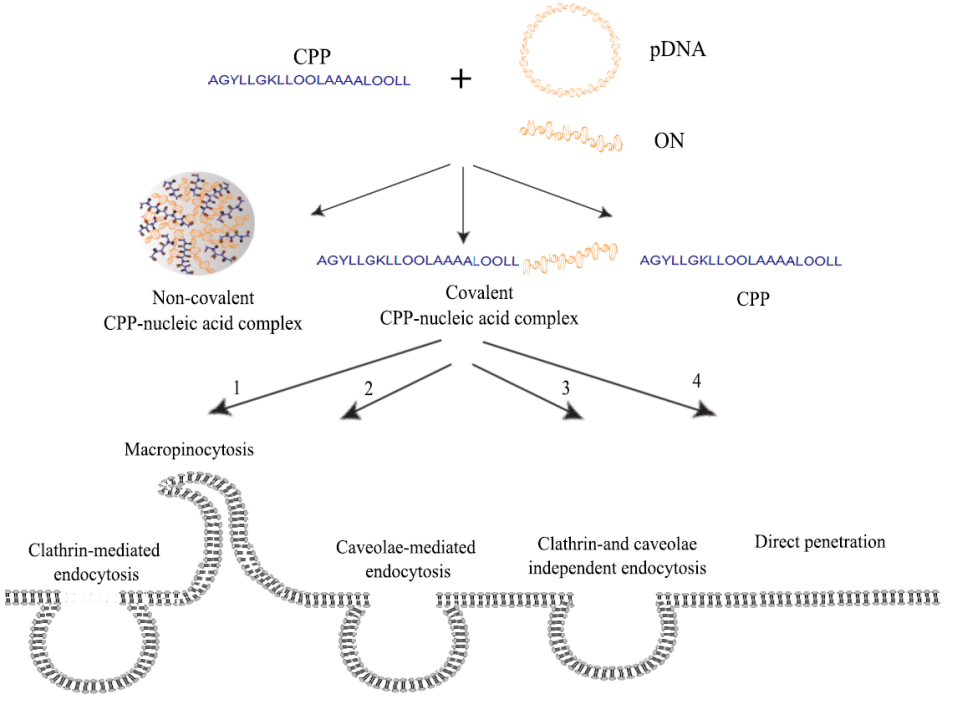

Figure 2. Overview of CPP/nucleic acid complex formation strategies and their possible cellular uptake route

1.3.3 CPP mediated nucleic acid delivery

Nucleic acid delivery is one of the most important applications of CPPs and many different peptide sequences used for this purpose appear in the literature. Various strategies are available to modify otherwise inefficient peptides to become more efficient in gene delivery, including the incorporation of CPPs into more comprehensive and efficient delivery systems [148].

First and foremost, CPP-mediated gene delivery is based on the cationic nature of CPPs which are able to neutralize the negative charge of DNA. The most common complexation strategy for intracellular CPP-mediated nucleic acid delivery is non-covalent formulation (Figure 2). Several studies have shown that charge neutralization alone is insufficient to form active transfection complexes, but it is enhanced by certain modifications that can significantly improve the condensation of nucleic acids. The resultant nano-sized (~100–300nm) particles are formed concurrently due to both electrostatic forces and hydrophobic interactions, thereby enabling efficient complex formation at lower peptide concentrations [149]. Less condensed complexes could be more sensitive to enzymatic attack, which could contribute to a decrease in transfection efficacy in the presence of serum proteins, which has been challenging for some gene delivery platforms. Another method of forming complexes is to covalently attach the nucleic acid molecule to the CPPs with a linker. The main advantage of covalent conjugation may be a more defined structures. However, this assessment is not always correct because CPP/nucleic acid covalent conjugates may also self-assemble into undefined nanostructures that could resemble non-covalent complexes. This approach is also not rational for larger nucleic acid molecules such as pDNA.

Now we present a selection from the numerous reports of CPP mediated pDNA delivery into cells that have resulted in a significant biological effect. One interesting library of CPPs has been reported by Kichler *et al*., where synthetic cationic amphipathic peptides have been modified with histidines [150]. It was shown already decades ago that using histidine mutations in a cationic peptide sequence can enhance gene transfection [151] and the main effect behind this is the pKa of the histidine imidazole group, which is ~6.0 and becomes cationic in a slightly acidic medium. Polyhistidines mediate an acid-dependent fusion and leakage of negatively charged liposomes [152], [153]. At the physiological pH histidine alone is not able to condense pDNA efficiently, therefore, it requires an extra mechanism for DNA binding. A number of authors have found that the mere presence of histidines is not sufficient, as demonstrated previously for polylysines – a certain positioning of histidines is required to form the polar face of the peptide α-helix [150]. The importance of the α–helical structure in DNA transfection was reported by Kuriyama *et al*., who obtained a 37 amino acid long peptide from human Factor VIII c-terminal domain to target phosphatidylserine, which is present in disturbed cellular membranes such as in cancer cells [154]. The peptide sequence was mutated to increase similarity with cationic amphiphilic α-helical oligopeptides. A peptide

with the same amino acid composition, but with a scrambled sequence was used as a control to confirm that an α-helical structure was not formed in the presence of membranes. The α-helical peptide was able to induce significant gene expression levels (comparable to the commercial gene delivery reagent, Lipofectin), while the negative control remained at baseline levels.

Another example of efficient pDNA delivery in cell culture was reported by Divita and colleagues, where they demonstrated that a hydrophobic fusion peptide constructed from HIV-1, gp41, and the hydrophilic nuclear localization sequence of SV40 large T antigen, MPG, can efficiently deliver pDNA into cells [155]. This peptide predominantly binds pDNA by exploiting electrostatic forces, however these are not sufficient to form complexes at CR2, where positive charges are already in excess. These authors claim that for complete complexation a so-called peptide-cage is formed at higher peptide concentrations and it is this cage that confers protection of the cargo molecule against enzymatic attack. Complexes with fully incorporated pDNA and a peptide cage are able to transfect cells and mediate gene expression comparable to LF2000. It is noteworthy that the gene expression obtained was not dependent on the peptide concentration – both CR5 and CR20 provided similar levels of luciferase activity. Besides the induced reporter gene activity, complexes were able to deliver pDNA with the full-length encoding of antisense cDNA for human cdc25C, which led to an efficient decrease in the target protein and a resulting bioeffect.

One common strategy to enhance the DNA binding ability of the peptide and form stable complexes is to introduce a hydrophobic moiety to the peptide sequence. Incorporation of an alkyl chain to the peptide mastoparan to obatin higher gene expression was evaluated already in 1999 [156]. By increasing lipophilicity, the authors were able to confer binding ability to mastoparan, which is otherwise unable to bind pDNA. The authors also showed that increasing the hydrophobicity increased or stabilized the presence of α-helical structure of the peptides and these peptides provide higher transfection. The benefit of incorporating a fatty acid into the peptide sequence has been shown in several studies [137], [157], [158] that delivered nucleic acids. It has also been previously reported that adding stearic acid to the CPP improves complex formation between CPP and nucleic acid and resulted in improved gene modulation at a reduced peptide concentration [159], [160]. This effect was confirmed by pDNA delivery, where improved complex formulation and enhanced gene expression levels were observed [161].

While CPPs have mainly been shown to be non-toxic [162], enzyme resistant, and provide efficient transfection in cell culture, their potential for *in vivo* delivery is s still under investigation. Many studies describe efficient *in vivo* pDNA delivery platforms, where CPPs have been utilized solely as a one moiety to increase cellular uptake. Several reports have shown that CPP can aid in local pDNA administration, yet, only a handful of works have achieved significant gene expression levels when systemically administering single-

component CPP/pDNA complexes. An overview of some of these studies is provided in Table 2.

CPPs have also been used as single-component systems to deliver siRNA in mice [163], [164]. Relatively efficient pDNA was achieved using either an intramuscular route [161] or using systemic administration [165], [166], [167]. The latter studies report the highest gene expression induction in lungs, with a profile very similar with other non-viral gene delivery reagents. In all of these studies a non-covalent complexation strategy has been used to form CPP and pDNA nanocomplexes. It is not entirely clear which properties from these nanocomplexes are responsible for efficient gene induction *in vivo*, however, the main parameters that determine the efficacy are probably the CPP sequence, the secondary structure it forms, membrane activity, nucleic acid condensation strength, physicochemical parameters, and stability against enzymatic digestion. Moreover, it has been shown for PEI [84], and recently for free polycations [168], that when non-covalent complexes form, the cationic delivery reagent is in excess and exists as a free fraction that is not incorporated into complexes with nucleic acids. This fraction is important for gene induction but also possibly mediates toxic side-effects. This makes the determination of parameters that are responsible for transfection efficacy even more difficult because delivery is not only mediated by the properties of the complexes that form, but possibly by the free cationic fraction and its properties. It is not clear if this could be the case for CPPs as well, but there are some indications that support this. Due to the multitude of possibilities, it is very difficult to optimize gene delivery vector and formulations for *in vivo* applications.

Table 2. Overview of CPPs for *in vivo* gene delivery.

Name	Sequence	Administration	Effect and pDNA dose	Refs
C-5H-Tat-5H-C	CHHHHHRKKRRQRRRRHHHHHC	Intrastriatum, intrathecal	Comparable to PEI; NP ratio 3, 10μg;	[169]
PF3	[a]AGYLLGKINLKALAALAKKIL	Intradermal	10x higher compared to naked pDNA; CR1; 1, 5 and 10μg of pDNA	[161]
PEG-POD	[b]CGGG(ARKKAAKA)$_4$	Subretinal	200–500x higher compared to naked pDNA; 1 and 2 μg of pDNA	[170]
PEG-POD	[b]CGGG(ARKKAAKA)$_4$	I.v.	In lungs, 50–100 times over pDNA; 40 μg pDNA	[170]
Tat 47-57	YGRKKRRQRRR	I.v.	No gene expression; 10 μg of pDNA	[171]
ppTG1	GLFKALLKLLKSLWKLLLKA	I.v.	5x10^5 RLU/mg in lungs, 50 μg pDNA	[165]
ppTG20	GLFRALLRLLRSLWRLLLRA	I.v.	2x10^6 RLU/mg in lungs, 50 μg pDNA	[165]
JTS1-K13	GLFEALLELLESLWELLLEAC CYKAKKKKKKKKWKKKKQS	I.v.	9x10^4 RLU/mg in lungs, 50μg pDNA	[165]

[a] Stearylation
[b] PEGylation

1.3.4 Tuning specificity of CPPs

As discussed above, CPPs are efficient delivery vectors that have a common property of being able to deliver macromolecular cargo into a very wide range of cells and tissues. This trait can be a drawback when developing delivery systems that target specific tissues or cell types. However, there are several strategies available to overcome this limitation – one of them is the activatable cell-penetrating peptide strategy (ACPP) developed and applied by R. Tsien *et al*. This technique is based on masking the cationic CPP's activity via an anionic peptide domain in a reversible manner [172]. This masking is detached via cleavage of protease-sensitive peptide linker that covalently conjugates the two domains together. Systemically administrating a masked conjugate does not induce any activity prior to deactivation. In his work Tsien has used enzyme sensitive activation of ACPP, which relies on matrix metalloproteinases (MMPs). These are enzymes that are needed to degrade extracellular matrix components during neoplastic growth and are therefore overexpressed in almost any types of tumor tissue [173]. When an MMP sensitive linker is cleaved, this leads to release of activated CPP with cargo molecule and enhanced cellular uptake [172]. Besides MMPs, other proteases has been harnessed using the ACPP strategy [174], [175].

Tumor-targeting peptides also increase the specificity of CPP-mediated cargo delivery [176]. Phage display technology has significantly contributed to a major portion of tumor homing peptides, including Arg-Gly-Asp or RGD which has high affinity for integrins [177]. Since its discovery, this peptide sequence has been studied, modified, and used in many applications for targeted drug delivery to tumors [178]. One recent report by our group investigated CPP-tumor homing peptide mediated drug delivery to glioma xenograft tumors. This study used phage-display technology to identify a glioblastoma homing peptide, gHo, which was further conjugated to a known CPP pVEC [179], [180]. However, targeting brain is considered one of the biggest challenges in the drug delivery field, due to the presence of the BBB, which is impermeable to the majority of therapeutics. Nevertheless, achievements have been made to aid delivery across the BBB. In one report, specific peptides are validated [181] and it has been confirmed, that one of these peptides, Angiopep-2 (ANG), crosses the BBB via receptor dependent transcytosis [182]. Its potential has been explored for dendrimer based gene delivery [183], as well as for drug delivery [184], [185].

2. AIMS OF THE STUDY

The main objective of the research presented in this dissertation is the development of an efficient CPP vector for systemic gene delivery applications. Various strategies were used to design peptides with improved usability for *in vivo* conditions and their delivery efficacy was studied in cell cultures, *in vivo* after systemic administration in both healthy and tumor-bearing mice. Specifically, the aims of this work are:

- Paper I: The main aim was to establish the potential of PF14 to deliver pDNA into cells. For that, CPP and pDNA complexes were formed, characterized and their uptake rate into cells was evaluated. We established the internalization route and then studied various aspects of induced bioactivity such as transfection rate in the presence of serum proteins, the percentage of transfected cells, and the toxicity profile.

- Paper II: Here we aimed to study the potential of CPP-based delivery of pDNA across the blood-brain barrier. For that, we designed a range of CPPs, including PF14 analogs, that were based on using two BBB targeting peptide sequences – a glioma-homing peptide and angiopep-2. To assess their gene delivery potential, we exploited an *in vitro* model of the BBB.

- Paper III: The aim here was to investigate the potential of PF14 to deliver pDNA and induce gene expression in different organs after systemic administration via the tail vein. For this we investigated if PEGylation can be used to shield the efficient and unspecific transfection profile of PF14 and improve its *in vivo* usability. We went on to study the potential of PEGylated PF14/pDNA nanocomplexes to reach into tumors and then become activated via cleavage of the PEG by enzymes that are overexpressed in tumors.

- Paper IV: Explore additional possibilities to further improve PF14 mediated *in vivo* gene delivery as a suitable alternative to PEGylation. Optimization of complex formulation was studied varying CPP/pDNA doses and modifying PF14 fatty acid content and charge to investigate the influence of these parameters on complex formation, stability against enzymatic attack, membrane activity, transfection in cell culture and on gene delivery potential after systemic administration.

3. METHODOLOGICAL CONSIDERATIONS

3.1 Peptide modification

All the peptide sequences that were studied in this dissertation are presented in Table 3. All of these are based on the PF14 peptide, which was first established by Ezzat *et al.* [186] to deliver ONs and is now further modified to achieve improved potential for gene delivery for *in vivo* applications.

In Paper I, we evaluated the potential of PF14 for pDNA transfection. For that, this peptide was non-covalently mixed with nucleic acid and resulted in the formation of nano-sized complexes that are taken up by cells via endocytosis, using scavenger receptors. At the same time nsPF14 (PF150), which lacks the N-terminal fatty acid modification, was not able to form sufficiently stable complexes. PF14 was able to induce significant gene expression levels in different cell-lines.

In Paper II, we studied the potential of CPP-based delivery of pDNA across the BBB. For that, we constructed a range of CPPs using two targeting sequences – glioma-homing peptide [179] and angiopep-2 [181] to target the BBB. gHo was conjugated to PF14, TP10 [187], stearyl-TP10 (PepFect3, PF3), PF28, SynB3 [188], [189] and stearyl-SynB3, to obtain PF31, PF33, PF34, PF35, PF36, and PF37, respectively. ANG was conjugated to a truncated PF14, resulting PF32. Both of these targeting peptides were conjugated to CPPs C-terminally.

To assess their gene delivery potential, we exploited an *in vitro* model for BBB.

In Paper III, after having established that PF14 is able to deliver pDNA efficiently in cell culture, we evaluated its transport potential *in vivo*, after systemic administration. This lead to the induction of significant gene expression in different tissues such as lungs, liver, and spleen. However, this efficient gene delivery is associated with acute side-effects and, therefore, we conclude that this delivery vector needs improvement. PEGylation has improved the properties of a wide variety of therapeutic molecules, and is also able to act as a biologically inert shield to sterically protect nanoparticles against unwanted interactions [190]. To apply this strategy, a series of PEGylated peptides (PF141-PF143) were designed followed by an assessment of their ability to form complexes and transfect cells. These peptides were able to completely mask the transfection potential of PF14. To restore the PF14 mediated transfection, peptides were PEGylated via a cleavable linker, which enables specific activation of complexes in tumor tissues. For this we exploited the MMP activation strategy, first presented by R. Tsien's group [172]. PEGylation was conjugated to peptides via an MMP enzyme specific cleavage site that consists of six amino acids (PF144-PF146). As a control, we used scrambled sequence that contained the same amino acid (PF147-PF149)

In Paper IV, we studied the formulation of PF14 based transfection complexes for the purpose of reducing previously reported side-effects *in vivo*. First,

we either decreased the charge by substituting Orn10 with Glu10 or increased the charge by substituting Ala15 and Ala16 with ornithines. These resulted in the peptides PF1451 and PF1450, respectively. Increasing the charge should enable one to decrease the concentration of peptide required to complex the same amount of nucleic acids. As a control we used PF6 which has been reported to have a high cationic charge density [191] Next, we either reduced (PF155) or decreased (PF161) the fatty acid length to determine if this property can influence the complexation and stability of peptide/pDNA complexes under *in vivo* conditions.

3.2 Peptide synthesis

All peptides used in this dissertation (Table 3) were synthesized using a solid-phase peptide synthesis (SPPS) strategy first introduced by Bruce Merrifield in 1963. This technique is based on the stepwise addition of protected amino acids to a growing peptide chain which is covalently linked to a solid phase resin [192]. In this work, synthesis of peptides was carried out using an automated peptide synthesizer using standard protocols for Fmoc solid-phase synthesis. We used rink-amide MBHA resin as a solid phase to obtain C-terminally amidated peptides. N-terminally stearylated peptides were prepared by treatment of peptidyl resin with 4 equiv. of stearic acid, 4 equiv. of HOBt/HBTU, and 8 equiv. of DIEA in DMF/DCM for 18 h. C-terminally PEGylated peptides were prepared by treatment of Rink-amide MBHA resin with 2 equiv. of Fmoc-PEGn-CH_2CH_2COOH, 2 equiv. of HOBt/HBTU and 4 equiv. of DIEA in DMF/DCM for 24 h which was followed by standard Fmoc peptide synthesis. The final cleavage of peptide chains from resin was performed using a 95% trifluoroacetic acid (TFA)/2.5% TIS/2.5% water for 2 h, at RT. Peptides were purified using a high-performance liquid chromatography (HPLC) using a 5−80% acetonitrile (0.1% TFA) gradient. The molecular weight of the peptides was analyzed by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectroscopy and the purity of each was >90%, as determined by analytical HPLC.

Table 3. Sequences used in thesis

CPP	Sequence	Reference
PF14	Stearyl-AGYLLGKLLOOLAAAALOOL-NH2	[186],[193]
PF31	Stearyl-AGYLLGKLLOOLAAAALOOLNHQQQNPHQPPM-NH2	[194]
PF32	Stearyl-LLOOLAAAALOOLTFFYGGSRGKRNNFKTEEY-NH2	[194]
PF33	AGYLLGKINLKALAALAKKILNHQQQNPHQPPM-NH2	[194]
PF34	Stearyl-AGYLLGKINLKALAALAKKILNHQQQNPHQPPM-NH2	[194]
PF35	Stearyl-WLKLWKKWLKLWNHQQQNPHQPPM-NH2	[194]
PF36	RRLSYSRRRFNHQQQNPHQPPM-NH2	[194]
PF37	Stearyl-RRLSYSRRRFNHQQQNPHQPPM-NH2	[194]
PF141	Stearyl-LLOOLAAAALOOLL-X-PEG600	[167]
PF142	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PEG1000	[167]
PF143	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PEG2000	[167]
PF144	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG600	[167]
PF145	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG1000	[167]
PF146	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-PLGLAG-PEG2000	[167]
PF147	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-LALGPG-PEG600	[167]
PF148	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-LALGPG-PEG1000	[167]
PF149	Stearyl-AGYLLGKLLOOLAAAALOOLL-X-LALGPG-PEG2000	[167]
PF150	AGYLLGKLLOOLAAAALOOL-NH2	[186],[193]
PF155	Decanyl-AGYLLGKLLOOLAAAALOOL-NH2	
PF161	Behenyl-AGYLLGKLLOOLAAAALOOL-NH2	
PF1450	Stearyl-AGYLLGKLLOOLAOOALOOL-NH2	
PF1451	Stearyl-AGYLLGKLLEOLAAAALOOL-NH2	
PF6	Stearyl-AGYLLGK[a]INLKALAALAKKIL-NH2	[164]

PF, PepFect; Stearyl, stearic acid; decanyl, decanylic acid; behenyl, behenylic acid; X, 6-aminohexanoic acid.

[a] four trifluoromethylquinoline moieties attached to succinylated lysine tree.

3.3 Complex formulation strategies

3.3.1 Non-covalent formulation strategy

There are two main strategies to form complexes between a peptide and its cargo: covalent conjugation and non-covalent formulation. The latter is beneficial for nucleic acids due to their negative net charge, which bind to cationic CPPs. Non-covalent complex formulation methods has been used to complex various peptides with nucleic acids, including pDNA [195], [135]. Besides electrostatic forces, complex formation and pDNA condensation is supported by hydrophobic interactions of peptide molecules which are able to increase peptide-mediated gene delivery [137], [159], [160].

Throughout the work presented in this dissertation, we have used non-covalent complex formation with the luciferase and EGFP encoding plasmids, pGL3, pLuc2, and pEGFP-C1, which were mixed with CPPs at different pDNA-to-peptide CRs, which were calculated theoretically, taking into account the positive charges of the peptide and negative charges of the pDNA. For *in vivo* studies, higher dose of pLuc2 (20 μg, if not indicated otherwise) was mixed with peptide at CR2 or CR4 in MQ water and the volume of complexes was kept constantly 100 μl. All complexes were incubated at room temperature (RT) for 40 minutes prior to their use in subsequent assays. Immediately before systemic administration, glucose was added to complexes to achieve an isotonic injection solution (5%) and injected.

3.3.2 Modification of PEGylation rate

In Paper III, to achieve tunable PEGylation, complexes were formed by varying the PEGylation rate which is defined as the amount of PF14 which is substituted with its PEGylated PF14 analogue. For example, a PEGylation rate of 20% represents complexes with 80% of PF14 and 20% corresponds to PEGylated PF14 analogue. To achieve this, PEGylated peptides were first mixed with pDNA followed by the addition of PF14 and incubation for 40 minutes at room temperature.

3.4 Characterization of formed complexes

We characterized the peptide/pDNA complexes using four different methods in parallel. Initially, the ability of cationic CPP to bind and neutralize pDNA was assessed with gel shift assay, the pDNA condensation rate was determined by an ethidium bromide (EtBr) exclusion assay. Both of these characterize interactions with pDNA but do not show if indeed nano-sized complexes are formed. To verify this, we used dynamic light scattering (DLS) to measure the hydrodynamic diameter in aqueous solution as well as the zeta potential of the preformed complexes. Last, but not least, we used a heparin displacement assay to compare the stability of the complexes.

3.4.1 DNA binding and condensation

DNA binding was first assessed using a gel retardation assay that is able to detect if the complexes that form are too large to migrate through agarose gel. If complete DNA binding occurs and complexes are formed, nucleic acid can be detected in the well, however, in the case of incomplete complexation a free fraction of pDNA able to migrate into the gel.

We analyzed the rate of DNA condensation by peptides using an EtBr exclusion assay. This assay is based on the ability of EtBr to interact with nucleic acids, and results in an increase of fluorescence. When pDNA is efficiently condensed, then interactions with EtBr are limited, which results in quenching of pDNA fluorescence.

3.4.2 Complex size and surface charge

To study the physicochemical parameters of CPP/pDNA complexes such as size and surface charge (zeta potential), we carried out DLS measurements using a Zetasizer Nano ZS apparatus. The DLS detects the size of particles from fluctuations in scattered light intensity due to the Brownian movement of the measured particles. This is a commonly applied technique to measure the average size of particles present in a solution [196].

Each charged particle in solution surrounded by an electrical double layer of ions and counter-ions. The potential that exists at this hydrodynamic boundary is known as the zeta potential – it is determined by electrophoresis of the sample and measuring the velocity of the particles using laser Doppler velocimetry [197]. The surface charge of the particles, together with its size, can determine not only its transfection ability, but also the biodistribution between tissues, and eventually, *in vivo* fate.

3.4.3 Heparin displacement assay

The heparin displacement assay we used is based on the ability of negatively charged heparin molecules to interact with positively charged cationic vector and displace the negatively charged pDNA from complexes. The concentration of heparin required to displace pDNA indicates the strength of CPP/pDNA complexes and can be monitored by either gel shift or an EtBr exclusion assay.

3.5 Cell cultures

The majority of cell-lines used in thesis (Table 4) are commonly utilized to study gene delivery potential of different vectors. The main optimization and bioactivity studies throughout this thesis have been made in CHO cells, however, it is essential to assess gene delivery efficacy in a variety of cell-lines

because transfection properties can vary depending on the cell-line type and its origin. In total, we used eleven different cell-lines that originate from human tissues or *mus musculus*, being cancer or non-cancer cell-lines. All cells were cultured at 37°C, 5% CO2 under a humidified environment with suitable media, supplemented with nutrients and antibiotics.

Table 4. Cell-lines used in thesis

Cell-line name		**Application**	**Paper**
HEK239	Human embryonic kidney cells	pDNA delivery efficiency	I
HT1080	Human fibrosarcoma cells	pDNA delivery efficiency	IV
U2OS	Human osteosarcoma cells	pDNA delivery efficiency	I
U87	Human glioblastoma cells	pDNA delivery, tumor induction	I, II, III
THP1	Human monocytic cells	Evaluation of immune response induced by CPPs and their complexes	I
RD4	Human rhabdomyosarcoma cells	pDNA delivery efficiency	I
CHO	Chinese hamster ovary cells	pDNA delivery efficiency, uptake studies, toxicity evaluation	I, III, IV
MEF	Mouse embryonic fibroblasts	pDNA delivery efficiency	I
mES	Mouse embryonic stem cells	pDNA delivery efficiency	I
N2a	Mouse neuroblastoma cells	pDNA delivery efficiency, tumor induction,	III
bEnd.3	Mouse brain endothelial cells	pDNA delivery in *in vitro* BBB models	II

3.6 Bioactivity of CPP/pDNA complexes in cell culture

3.6.1 Receptor dependent uptake and endosomal escape

We analyzed the intracellular delivery and uptake mechanism for PF14/pDNA complexes in Paper I using CHO cells (Table 4). Scavenger receptor mediated endocytosis was shown to be a prominent uptake mechanism for PF14/ON nanoparticles [146]. Furthermore, the affinity of this receptor to bind poly-anionic ligands has been shown earlier [198]. The effect of inhibiting scavenger receptors on the transfection efficacy of PF14 was evaluated using the specific inhibitory ligands, polyinosinic acid, fucoidan, and dextran sulfate. As a negative control, we used three structurally similar molecules that have no affinity

against receptors under the same experimental conditions: poly C, galactose, and chondroitin sulfate [146], [198], [199]. These results were corroborated using transmission electron microscopy, where we determined the intracellular localization of Nano-gold labelled pDNA complexed with PF14.

The extent of endosomal entrapment was evaluated using the endosomotropic agent chloroquine CQ in Papers I and IV, which acts as a weak base and adsorbs protons. This property inhibits acidification of endosomal compartments and results in the swelling and rupture of endosomes [200]. This leads to the release of endosomal content, including complexes, into the cytosol and an increase in transfection efficiency which could indicate the proportion of complexes entrapped in endosomes

3.6.2 Evaluation of gene delivery efficiency

The gene delivery efficiency of PF14/pDNA complexes was initially evaluated in Paper I. To characterize the transfection profile *in vitro*, we exploited different experimental strategies. We assessed the impact of the presence of serum proteins on transfection efficiency and evaluated the decay kinetics by analyzing the luciferase activity after PF14/pGL3 transfection at different time points (4–72 hours). To assess the ability of PF14 complexes to transfect the cell population at different confluences, an increasing range of cells were transfected, and the luciferase activity was measured. The delivery efficacy rate in the cell population was evaluated by delivering an EGFP encoding plasmid (pEGFP-C1) and measuring its expression in the cell population using fluorescence-activated cell sorting (FACS) analysis.

3.6.3 Toxicity profile and induction of innate immunity

To analyze the impact of peptide mediated transfection on the viability of cells, we used a colorimetric MTS cell viability assay in Papers I and III and a WST-1 cell proliferation assay in Paper II. These analyzes enable one to detect changes in the metabolic activity of cells after transfection and are based on mitochondrial dehydrogenases that reduce tetrazolium into a colored formazan product which is proportional to the number of viable cells.

A human acute monocytic leukemia cell-line (THP-1) was used as a model system for immune response studies *in vitro* because they are capable of producing cytokines IL-1 and TNF-a when differentiated into macrophage-like cells. They were used earlier to study the induction of immune response by CPPs and their complexes with nucleic acids [162]. In Paper I, IL-1 production after treatment of cells with PF14/pDNA complexes was analyzed using this model.

3.6.4 Gene delivery potential of CPP/pDNA complexes *in vitro* BBB model

To study the transport potential of CPPs over BBB, we used a Transwell™ apparatus. This consists of two compartments. The first is an apical compartment with a semi-permeable insert representing the blood site. This is where brain endothelial bEnd.3 cells were cultured. In the lower chamber we cultured glioblastoma U87 cells. All experiments were performed on bEnd.3 cell monolayer 10–14 days after cell seeding for the transfection assay. The media was changed in both the upper and lower chambers every 2–3 days. U87 cells were separately seeded and the experiments were carried out 3–4 days after seeding. On the day of each experiment, the bEnd.3 cell monolayers grown on semi-permeable inserts were placed on U87 seeded wells and transfected. Two hours after transfecting bEnd.3 cells, all inserts were removed from U87 wells and incubated for an additional 22 hours and thereafter we measured the luciferase activity.

3.7 Evaluation of CPP/pDNA complex interactions with blood components

The first interactions of any therapeutics after systemic administration occurs with blood components. Therefore, we evaluated interactions of complexes with different components such as RBCs, serum, and also more specific degradation of complexes by the enzymes DNase I and proteinase K.

3.7.1 Hemolysis

RBCs possess negative zeta potential, which is contributed by molecules on the cell surface that have the negative charge [61] and therefore these are one of the first cells that have possibility to interact with positively charged complexes in the bloodstream. As a measure of membrane activity and toxicity, we analyzed the hemolytic activity of both naked CPPs and CPP/pDNA complexes in Paper IV using a 2% mouse RBC solution. We expressed the results as the concentration of peptide, at which 50% of RBCs are lysed (hemolytic concentration 50, HC50).

3.7.2 Serum incubation

To evaluate the impact of serum on the size of complexes or their zeta potential and transfection efficiency, we incubated complexes with fetal bovine serum (FBS) and carried out either a gel shift assay, measured their size and surface charge or transfected cells after incubation of complexes in FBS.

3.7.3 Stability against enzymatic attack

One requirement for gene delivery vectors is their ability to protect their cargo from degradative enzymes such as nucleases. Delivery vectors should also be sufficiently stable against proteinase digestion. To assess these parameters, CPP/pDNA complexes were treated with DNase I and Proteinase K and further analyzed using either a gel shift assay, EtBr exclusion, or transfection with enzyme-treated complexes.

3.8 *In vivo* bioactivity of systemically administered CPP/pDNA complexes

All animal experiments and procedures presented in this dissertation were approved by the Estonian Laboratory Animal Ethics Committee (approval nos. 69 and 70, dated Feb 9, 2011).

3.8.1 Induction of tumor models

To analyze the biodistribution of CPP/pDNA complexes or their induced gene expression in tumors, we used subcutanous (s.c.) tumor models. We induced mouse Neuro2a tumors in BALB/c mice. Human tumor xenografts were induced in nude animals (Hsd: Athymic Nude-Foxn1nu female, 4–6 week old). The tumors were induced by resuspending 1×10^6 cells in 100 µl volume of ice-cold DMEM without any supplements. The xenografting was performed by implanting the cell suspension subcutaneously to the right flank. Upon the appearance of tumor growth, (tumor size of approximately 100 mm^3), mice were injected i.v. (via tail vein) with the peptide/pDNA complexes.

3.8.2 Biodistribution

The availability of complexes was determined 24 hours after administration. For that, PF14 and PEGylated PF14 peptides were complexed with Cy5-labelled pDNA. Complexes were administered i.v. via tail vein. After 24 hours, the mice were sacrificed and their tissues were collected and subjected to a homogenization protocol (provided in paragraph 3.8.4). We measured the fluorescence of Cy5-labelled pDNA within the tissue homogenates using a Spectra Max Gemini XS fluorometer. Three technical replicates were combined to find an average FU (fluorescence unit). The average FU values within each sample were normalized to the protein content to obtain the RFU/mg.

3.8.3 Pharmacokinetic profile

The stability of complexes in blood was studied using Cy5-labelled pDNA, which was mixed with the peptides and injected as described above. Blood was collected at different time points (0.5 h–6 h) from the saphenous vein and its fluorescence was measured. For data analysis, the fluorescence of the untreated samples was subtracted from the other samples. The resulting fluorescence values were normalized to the pDNA concentration which was determined using either naked pDNA or peptide/pDNA complex calibration curves.

3.8.4 Gene expression induction in different tissues

Throughout Papers III and IV, we measured luciferase gene expression in different tissues. For that, 24 hours after administration of complexes, mice were sacrificed using cervical dislocation, their organs were harvested and snap-frozen on dry ice. The tissues were homogenized using a Precellys®24-Dual homogenization system and lysed using 1 × Promega lysis buffer. The luciferase content was analyzed as described previously [201]. Briefly, homogenized tissues were thawed and 500 µl of Promega Reporter lysis 1 × buffer was added. The samples were subsequently vortexed for 15 minutes, subjected to 3 consecutive freeze–thaw cycles (liquid nitrogen and 37 °C water bath), centrifuged for 3 min at 10 000 g, at 4 °C; followed by the removal of supernatant, which was saved for later analysis. Thereafter, 500 µl of lysis buffer was again added to the pellet and the extraction process repeated (without freeze – thaw cycles). The second supernatant was combined with the first one and was subjected to luciferase activity or for fluorescence measurements. After measuring luciferase, the obtained LUs (light unit) out of three technical replicates was normalized to the protein content and the resultant RLU/mg were normalized to the corresponding tissue of the animals that received naked pDNA injection.

4. RESULTS AND DISCUSSION

4.1 Characterization of PF14 mediated gene delivery in cell culture (Paper I)

Our group has recently designed a new series of modified CPPs, PepFects, that have been shown to be efficient for the delivery of various nucleic acids [161], [186], [164],[202], [203]. Their shared properties, such as having a conjugated fatty acid moiety and cationic charge makes them good vectors to bind and condense nucleic acids into complexes (Figure 2 and 3). The main focus of the research presented here, was to determine whether PF14, which has previously been shown to be an efficient delivery vehicle for SCO [186] and siRNAs [146], could be utilized to deliver pDNA into cells.

First, we analyzed the ability of PF14 to form complexes with pDNA and it became clear that this peptide can indeed bind and condense pDNA into complexes that are relatively stable in the presence of a negatively charged competitor molecule, heparin. DLS measurements revealed that the average size of these complexes is between 130–180 nm and they possess a negative surface charge. In comparison, nsPF14, which lacks stearic acid modification, can also condense pDNA with a similar efficiency; however, the heparin displacement assay revealed that nsPF14 creates weaker complexes.

After confirming effective nanoparticle formation, we moved on to study the transfection efficacy of both peptides using a luciferase encoding plasmid DNA, pGL3. Complexes made with PF14 provide high luciferase levels in CHO cells, while nsPF14 complexes are not able to induce any gene expression (Figure 3). This was explained with uptake studies which revealed that nsPF14 is unable to deliver fluorescently labelled pDNA into cells over a range of CRs, yet PF14 is able to deliver pDNA into cells in a dose dependent manner. We propose that nsPF14 forms unstable complexes that dissociate before they can be taken up by cells. It has been shown that the delivery properties of different CPPs for oligonucleotides can be significantly improved when conjugated with fatty acids [204].

The potential of PF14 to deliver pDNA was studied in a variety of different cell-lines, including cancer and non-cancer cells. We found that PF14 complexes induce gene expression with similar efficacy as a commercial and commonly used cationic lipid based transfection reagent Lipofectamine™ 2000 (LF2000). Moreover, we observed significant gene expression in cell-lines that are considered relatively refractory to chemical transfection reagents. The gene expression was again comparable with LF2000.

The first indication that endocytotic pathways are utilized in the uptake of PF14/pDNA complexes came from the experiment where CQ incubation increased the transfection efficacy of PF14 complexes 10 fold. Endocytosis is commonly recognized as an main cellular entry route during CPP/cargo delivery, however, it is also agreed that the biggest intracellular bottleneck in this entry route is endosomal escape [205]. We used transmission electron micros-

copy to verify that endocytosis occurs, and these results indicate that caveolae-mediated endocytosis is the dominant entry route. Caveolar uptake has previously been shown to be involved in the cellular delivery of proteins by TP10, the predecessor peptide of PepFects [206]. Based on a previous report that the uptake of PF14/ON nanocomplexes are dependent on class A scavenger receptors (SCARA) [146], the inhibition of certain SCARA subtypes significantly decreased the delivery and expression of PF14/pDNA complexes. This suggests that this receptor mediates the uptake of PF14/pDNA nanoparticles via caveolae-mediated endocytosis. This is in line with reports that SCARAs are internalized via caveolae dependent endocytosis in macrophages [207]. Furthermore, SCARAs are associated with the uptake of negatively charged molecules [198], [199].

Regarding PF14 mediated gene delivery, one important feature is its ability to transfect a large population of cells in the presence of serum, which also does not significantly reduce gene expression. Complexes made with this peptide provide improved delivery efficiency compared with its predecessor, PF3. It is also noteworthy that PF14-mediated delivery and its efficiency are not associated with toxic side effects as assessed using the *in vitro* toxicity and immunogenicity assays. Taken together, this conclusively makes PF14 an efficient peptide for pDNA delivery into a wide variety of cells.

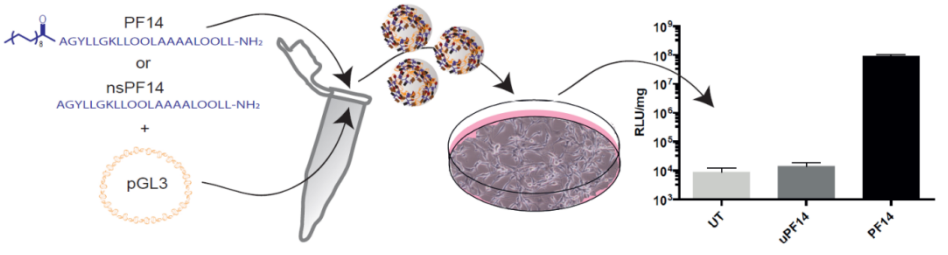

Figure 3. Noncovalent Pepfect14 and pDNA complexes mediate efficient gene delivery into cultured cells

4.2 CPP-based delivery of pDNA across the blood–brain barrier (Paper II)

In this study, we used a series of CPPs, including PF14 and its analogs, to deliver pDNA to brain cells in an *in vitro* BBB model (Figure 4). PF14 was conjugated with two different targeting peptides, including a glioma-homing peptide (gHo). The gHo peptide was identified as a glioma-specific homing peptide via an *in vitro* phage display technology and has previously been used as a targeting moiety in a glioma xenograft mouse model [179]. The other peptide conjugated with PF14 was angiopep-2 (ANG), which was derived from the Kunitz domains of aprotinin, a protease inhibitor, and this peptide may be a key

to unlocking receptor-mediated transport into the brain because ANG has previously been observed to demonstrate transcytosis via the low-density lipoprotein receptor-related protein 1 (LRP1) receptors on the BBB which results in its endocytosis into glioma cells via their LRP receptors [181], [183]. For reference, we present synthesized peptides in table 3.

In this BBB model, transfection was achieved by PF14 and its analogs, PF31 and PF32. PF31 carried the gHo targeting sequence, while PF32 is a truncated version of PF14 together with the ANG sequence. PF32 induced two times higher gene expression over PF14, and was the most successful candidate in this study (Figure 4). DLS measurements for PF32 and PF14 confirmed that both condense pDNA into nano-sized particles at CR2 and 3 with complex sizes ranging between 120–180 nm and with slightly negative or neutral surface charge. Both of these peptides were able to transfect cell-lines used in BBB model, the Transwell system, separately as well, however, PF32 delivered pDNA more efficiently in the bEend3 cell-line than the PF14 peptide. It also exhibited better gene induction in U87 cells in the Transwell system as well. Taken together, PF14 has potential to be modified with homing peptides without significantly compromising its delivery efficacy. Furthermore, PF14 and its analogs are potential gene carriers for *in vivo* gene delivery over the BBB.

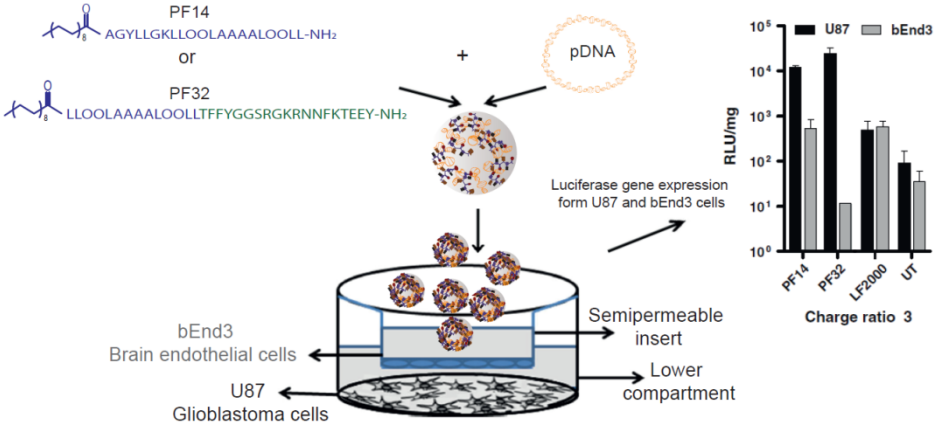

Figure 4. PF14 or PF14 modified with targeting peptide sequence are both efficient in transfecting cells over in *in vitro* BBB modelling system.

4.3 PF14 gene delivery potential and its improvements for *in vivo* applications (Paper III)

After confirming that PF14 is able to deliver pDNA, we proceeded to evaluate its potential to deliver genes under *in vivo* conditions. To assess this, PF14 was complexed with luciferase encoding pDNA (20 μg) and administered systemically via intravenous (i.v.) injection. PF14-mediated gene delivery induces luciferase expression in various organs, particularly in lungs, where luciferase levels reach up to 3 logs over control (naked pDNA levels). We went on to compare this expression with other CPPs and found that the delivery efficacy stayed in line with ppTG1 (Table 2), however, PF14 was able to achieve the same gene expression levels with a significantly lower dose of pDNA (20 μg vs. 50 μg). However, we also observed an occasional toxic event, where one animal out of fifteen died after minutes. This result highlights the need to further optimize PF14 mediate gene delivery for improved *in vivo* use.

PEGylation has been shown to mask the delivery activity of CPP [190], and is able to enhance the half-life of therapeutic molecules in the bloodstream by reducing interactions with blood-stream components. PEGylation also enhances passive tumor targeting [208]. To study the effect of PEGylation on gene delivery, we created PF14 analogues with C-terminally attached PEG molecules that had different molecular weights (600, 1000, and 2000, for PF141, PF142 and PF143, respectively). Their sequences are provided in table 3. First, we performed experiments to test if PF14 is still able to complex pDNA after PEGylation. We found that PF141 and PF143 were able to complex pDNA, but PF142 does not efficiently form complexes and requires higher concentrations for that. We then tested if PEGylated complexes are able to transfect cells and found that this ability was abolished, which suggests overly efficient masking by the PEG molecule.

We hypothesized that more favorable complex properties could be achieved with complexes formed with an optimal amount of PEG. To study this, we titrated the amount of PEGylated peptide in the complexes and studied both complex formation and their transfection ability. To describe the amount of PEGylated peptide within the complexes, we introduce the term PEGylation rate, which is defined as the amount of PF14 that is substituted with its PEGylated analog. For example, a PEGylation rate of 20% means that 20% of the cationic charges arise from the PEGylated peptide. Generally, the more PEGylated a peptide was, the lower was transfection ability of complexes. The size for all these complexes remained between 100–200 nm and displayed a positive surface charge

We also tested the biodistribution of complexes 24h after systemic administration and it was seen that PEGylation reduced the accumulation of complexes in lungs, compared with PF14/pDNA complexes. Moreover, PEGylated complexes remain in the blood stream longer than unmodified PF14, which probably interacted more with different components and tissues. When analyzing gene induction by PEGylated complexes in liver and lung, it became

evident that gene expression decreases with increasing PEGylation rate. Moreover, even at low PEGylation rates, these complexes are able to induce gene expression in tumors (but luciferase activity was still detected in lungs and liver).

In order to achieve tumor tissue specific gene delivery, we used a strategy reported earlier by Tsien [172], where tumor specific CPP activation is achieved via cleavage of a deactivating moiety, which in this work is represented by the PEG molecule (Table 3, Figure 5). The PEG is conjugated to the PF14 via short peptide sequence, that is substrate for MMP enzyme, which is overexpressed in tumors. Once the complex reaches in tumors, the PEG molecule can be cleaved, thereby restoring the ability to transfect cells. However, if the PEG shell is too dense, it might prevent interactions between the enzyme and its cleavage site. At the other extreme, complexes made with PF14 analogues with low PEGylation rates do not completely deactivate the complex. To find a suitable PEGylation rate, we evaluated complexes made with an increasing PEGylation rate in cell culture, where complexes were pretreated with MMP enzyme. We confirmed that complexes treated with the enzyme were able to induce gene expression, while complexes without MMP treatment and complexes made with an uncleavable control peptide induced gene expression only at a moderate level. The titration of PEGylation in complexes did not hinder MMP enzyme activity, at least not at the concentrations we tested, which is presumably much higher than that found in tumor tissues. We then proceeded to test how PEGylated PF14 complexes behave *in vivo*. For this, we evaluated the gene delivery potential of differently PEGylated complexes in mice bearing subcutanous Neuro2a tumors. Although gene expression was induced in tumors already at lower PEGylation rates, in this case the transgene was also delivered to lung tissue. At higher PEGylation rates, gene expression was not observed in any other tissue aside from the tumors. The most optimal gene expression was achieved with PF144, which has a smaller sized PEG and a PEGylation rate of 70%. PF144 provides the highest and the most specific gene induction. It is also noteworthy that none of the animals that received PEGylated complexes were observed to experience acute toxic effects. In conclusion, this strategy represents a simple, yet potent approach for tumor specific gene therapy.

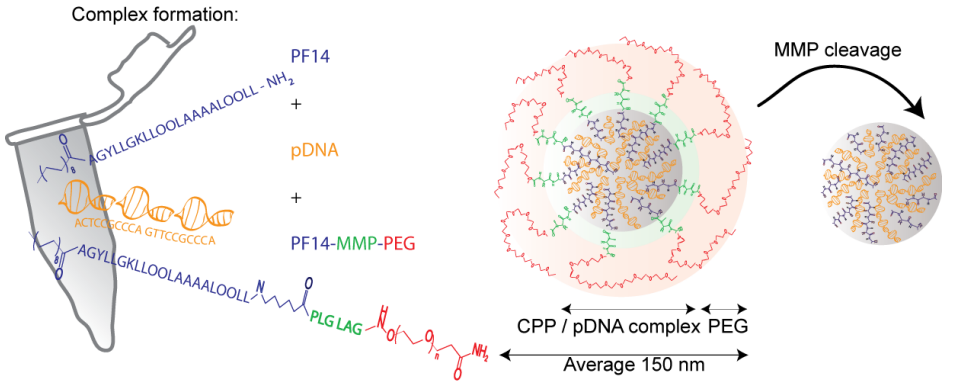

Figure 5. The complexation scheme of PEGylated MMP sensitive peptide/pDNA complex formation [167].

4.4 Formulation characteristics governing PF14 gene delivery *in vivo* (Paper IV)

This research addresses various parameters that can potentially influence the ability of CPP/pDNA complexes to transfect cells *in vivo*. In our previous work we observed occasional acute toxic effects associated with PF14 mediated gene delivery. We explored PEGylation as a potential solution, but found that this altered the transfection process itself. Knowing that the toxicity of cationic polymers are directly associated with the free fraction of polymer that is not bound to the DNA [84], [168], we hypothesized that toxic side-effects can be mitigated by increasing the amount of negatively charged nucleic acids in PF14 complexes (Figure 6).

DLS measurements revealed that increasing the nucleic acid concentration results in a sharp decrease in the surface charge. This indicates an absence of free peptide to supplement the positive surface charge. We assessed toxicity using hemolysis instead of more common *in vitro* toxicity assays because RBCs are one of the first cells that can interact with systemically administered complexes. Furthermore, hemolysis can indicate high membrane activity of CPP and its respective complexes. For PF14, the concentration needed to lyse 50% of cells (HC50) in this experimental setup was 0.66 μM. Adding nucleic acid increased the HC50 by about two fold, which indicates that a fraction of the peptide is complexed with pDNA which results in a reduction of hemolysis. Transfecting cells with complexes made at three increasing CRs clearly indicated that the excessive peptide concentration does not increase gene induction. Controversially, potential cytotoxicity can occur. This also applies for *in vivo* gene delivery, where complexes made at CR2 with high pDNA doses (50 μg) induce high, almost comparable transgene expression with the parent formulation (CR4 and 20 μg) but do not induce any occasional lethal effects. This was also corroborated by biodistribution studies, where optimized complexes dis-

played reduced accumulation in liver and lungs and accumulated more in bladder compared with the parent formulation. This reduced accumulation in lung tissues also occurred with PEGylated complexes, which highlight the possibility of using an optimized formulation as a good alternative to PEGylation (Figure 6).

To further investigate the possibility of reducing the free peptide fraction in the complexes, we synthesized several PF14 analogs (Table 3), with either an increased (PF1450) or decreased (PF1451) charge, or where the length of fatty acid chain had been shortened (PF155) or extended (PF161). We used PF6 as a control because it is a highly charged peptide at neutral pH [191]. All of these peptides, including the positive control Jet-PEI, were able to complex and condense pDNA, transfect it into cells at moderate (PF1451 and PF155) to high levels (PF1450, PF161, and PF6). Both peptides with a high charge content induced higher gene expression than PF14, while PF161 remained at the same level. All of these peptides formed nano-sized complexes, and typically had a positive surface charge. We observed that the surface charge was influenced more by the presence and length of the fatty acid. We also observed that the zeta potential increases with an increase in alkyl chain length. Next, we evaluated how well complexes made with these peptides provided protection against different enzymatic treatments. First, we observed that peptides with higher charge provided better DNA protection when subjected to DNAse attack. Proteinase K, which degrades the peptide was completely inefficient to degrade PF6 complexes, followed by PF161, PF14 and PF1450. PF6 also had reduced hemolytic activity, while other peptides stayed in a similar range with each other. When adding pDNA to complexes, the peptides with lower charge content were more tightly bound to complexes and their HC50 increased. This indicates the importance of the free peptide concentration.

When delivering pDNA systemically using these peptides, both PF161 and PF1450 were able to induce gene expression in liver and lungs, PF161 even provided similar levels as PF14. Intriguingly, PF6, which displayed excellent gene transfer activity in cell culture, was stable against enzymatic attack and displayed reduced hemolysis, was, nonetheless, also not able to induce any gene expression. PF1455, which has the highest membrane activity, also did not induce gene expression. We found that increased charge content and sufficient fatty acid length are both factors that simultaneously contribute to the stability of these complexes against enzymes, transfection in cell culture, and for sufficient membrane activity. Taken together, these parameters ought to be considered when optimizing CPP-based gene induction for *in vivo* gene therapy applications.

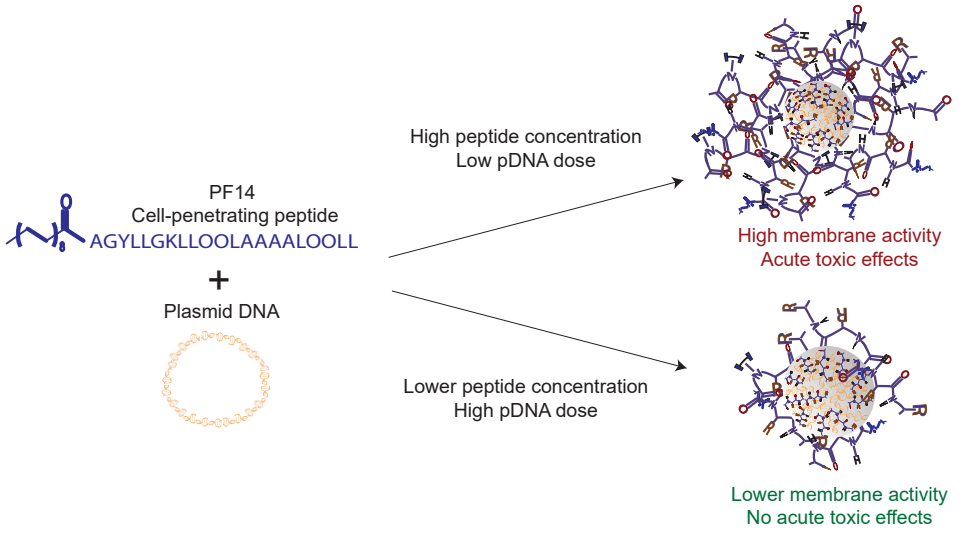

Figure 6. We suggest that, depending on the concentrations used, CPP/pDNA complexes can have a high concentration of free peptide, which can result in excess membrane activity or acute toxic effects. Increasing the pDNA dose enables it to bind more peptides from the solution and results in less toxicity.

5. CONCLUSIONS

The key findings from the research presented in this dissertation are reproduced below:

- **Paper I.** In this study we extrapolated the SCO-delivery properties of PepFect14 vector for the delivery of pDNA. This peptide efficiently formed nanoparticles with pDNA and induced efficient gene delivery in a variety of cell-lines, including primary cells. Moreover, we concluded that PF14/pDNA nanocomplexes were taken up by cells via class A scavenger receptors and caveolae-mediated endocytosis.

- **Paper II.** In this study we modified several CPPs, including PF14, to provide tumor homing or include targeting peptides and assessed their ability to deliver genes within an *in vitro* BBB model. Coupling of the ANG peptide to truncated PF14 resulted in an efficient peptide, PepFect32, which exhibited both the highest transcytosis across the BBB in the *in vitro* model and the highest transfection efficacy in glioma cells. We concluded that this is the most efficient peptide-based vector for pDNA delivery across the BBB in this *in vitro* model.

- **Paper III.** In this study we evaluated the effectiveness of the PF14 peptide to deliver pDNA into several tissues after systemic administration. Furthermore, we aimed to improve its *in vivo* usability via PEGylation, where PEG was attached to PF14 via a peptide linker that is cleaved by enzymes overexpressed in tumors. This strategy enabled us to induce gene expression specifically in tumors, concomitantly leaving gene expression at baseline levels in other tissues.

- **Paper IV.** In this study we explored additional strategies to improve PF14 mediated *in vivo* gene delivery and investigated various peptide parameters that could possibly influence the efficacy of gene induction *in vivo*. We characterized an optimal complex formulation that provided reduced side-effects while simultaneously leaving gene expression at high levels. By analyzing a range of PF14 analogs whose charge and fatty acid length were modified we found that both properties are simultaneously necessary for complex formation, stability against enzymatic attack, and gene delivery in both cell culture and after systemic administration *in vivo*.

In conclusion, the research presented in this dissertation found that the CPP, PF14, can be utilized as a potent gene delivery vehicle in cell culture. This CPP is efficient not only in regular cell-lines, but also in primary cells, and in specific application such as in the *in vitro* BBB model. We also found that several parameters need to be optimized during the process of translating efficient *in*

vitro gene delivery to a platform for pre-clinical systemic gene delivery. These include the properties of the nanocomplexes, which should be biocompatible and have a minimum number of undesirable interactions. At the same time, these nanocomplexes should have an extended half-life while circulating in blood and sufficient stability against enzymatic attack. Last but not least, after optimizing these properties the complexes must still be able to transfect cells and overcome intracellular barriers such as endosomal escape and nuclear entry. During this research we developed an optimized PF14 based gene delivery platform that can potentially be used to study gene therapy applications within relevant genetic disease models.

SUMMARY IN ESTONIAN

Rakku sisenevad peptiidid geeni transpordiks: transfektsioonist rakukultuuris geenitranspordiks *in vivo* tingimustes.

Viimaste aastakümnete saavutused inimese genoomi täielikus sekveneerimises ja personaalmeditsiini väljatöötamisel on võimaldanud identifitseerida seoseid geneetiliste mutatsioonide ning nende poolt põhjustatud vigase geeniekspressiooni vahel. See on võimaldanud nukleiinhapetel (NH) põhinevate ravistrateegiate arendamist, et reguleerida või parandada vigaseid geeniekspressiooni tasemeid rakkudes. NHde toimetamine rakkudesse eeldab mitmete rakuväliste ning rakusiseste barjääride ületamist. Rakkudesse viidav funktsionaalne geen sisestatakse tavaliselt bakteriaalse päritoluga plasmiidi (pDNA), kuid füsikokeemiliste omaduste tõttu on pDNA rakku sisenemine piiratud ning tema bioloogilise aktiivsuse saavutamiseks on vajalik kasutada transportvektoreid.

Geeni transportvektoreid on võimalik jagada vastavalt nende päritolule kaheks – viraalsed ning mitteviraalsed vektorid. Viraalsete vektorite põhiliseks eeliseks on efektiivsus, kuid patogeense päritolu tõttu on nende kasutamine alati seotud potentsiaalsete kõrval toimetega, lisaks, pole nad sobivad lühikeste sünteetiliste oligonukleotiidide (ON) transpordiks. See on viinud mitteviraalsete vektorite väljatöötamiseni, mis on tavaliselt lipiidsed või polümeersed katioonsed materjalid, mille põhiliseks omaduseks on kondenseerida pDNA nanosuurusteks kompleksideks. On näidatud, et sellised vektorid on suhteliselt ohutud, kuid mitte nii efektiivsed kui viraalsed vektorid.

Üks võimalikke lahendusi sellele kesksele probleemile võib olla kuni 30 aminohapet pikad peptiidsed ühendid, rakku sisenevad peptiidid (RSPd), mis suudavad rakkudesse viia väga erinevaid bioloogilisi ühendeid, seal hulgas NHsid. Positiivsed laengud ning hüdrofoobsed domeenid võimaldavad RSPdel kondenseerida NHsid nanosuurusteks osakesteks, mis seejärel sisenevad rakkudesse peamiselt endotsütoosi teel. RSPd on väga efektiivsed geneetilise materjali transportimisel rakukultuuris, kuid suurimaks väljakutseks on osutunud nende kasutatavus *in vivo*, kus lisaks rakusisestele takistusele tuleb RSP/NH kompleksidel vastu pidada vereringes leiduvatele lagundavatele ensüümidele, kõrval-efektide mõju organismile peab olema minimaalne ning lõpuks sihtmärkkoeni jõudes peavad olema säilinud omadused, mis võimaldavad rakke transfekteerida piisava efektiivsusega. Antud doktoritöö eesmärk on peptiididel põhineva transportsüsteemi kasutatavuse suurendamine *in vivo*, saavutamaks geeniekspressiooni, mis edasistes uuringutes võimaldaks rakendada RSP-de vahendatud geeniteraapiat haiguste ravimiseks loomses mudelis.

Esimese töö eesmärk on uurida, kas RSPd, PepFect14 (PF14), on võimalik kasutada pDNA transpordiks. Selgus, et PF14 on võimeline pDNAd kondenseerima negatiivse pinnalaenguga nanosuurusteks kompleksideks, mis on võimelised indutseerima kõrget geeniekspressiooni väga erinevates rakuliinides, seal hulgas raskesti transfekteeritavates primaarrakkudes. Sisenemisemehha-

nismide väljaselgitamisel selgus, et kompleksid kasutavad rakkudesse sisenemiseks klass A *scavenger* retseptoreid ning kaveoliin-vahendatud endotsütoosi.

Teises töös uuriti RSP/pDNA komplekside potentsiaali läbida hematoentsefaalset barjääri. Selleks sünteesiti rida RSPsid, seal hulgas PF14 põhinevaid analooge, mis sisaldasid hematoentsefaalses bärjääri spetsiifiliste retseptoritega seonduvaid peptiidseid järjestusi või glioblastoomi rakkudega seonduvaid peptiide. Nende analoogide pDNA transpordi potentsiaali uurimiseks rakendati hematoentsefaalbaarjääri rakukultuuri mudelit. Kõige efektiivseima geeniekspressiooni indutseeris PF14 analoog, teiseks efektiivseimaks oli PF14 ilma modifikatsioonita. Need tulemused näitasid, et PF14l põhinev geenitransport on efektiivne ka spetsiifilistes rakendustes ning paindlik modifikatsioonidele, säilitades ka siis oma aktiivsuse.

Kolmandas töös uuriti PF14ne potentsiaali transportida pDNAd *in vivo*. Süsteemse manustamise järgselt saavutati kõrge geenieksprесsioon kopsus, maksas ning ka põrnas. Akuutsete kõrvaltoimete esinemine viitas vajadusele optimeerida PF14-vahendatud geenitransporti. Lisaks oli eesmärgiks geeni transport spetsiifilistesse kudedesse, näiteks kasvajakoesse. Selle saavutamiseks PEGüleeriti (polüetüleenglükool ehk PEG molekuli lisamine) PF14 peptiid. See molekul võimaldab maskeerida peptiid/pDNA kompleks ebasoovitatavate interaktsioonide eest vereringes, suurendades sellega sobivust ning poolväärtusaega, lisaks, võimaldab PEG molekul maskeerida PF14 universaalse transfektsiooni potentsiaali kopsudesse, maksa ning ülejäänud kudedesse. Selleks, et kompleks oleks võimeline transfekteerima ainult kasvajakude, lisati PEG molekul läbi peptiidse järjestuse, mis on substraadiks tuumori koes üle-ekspresseeritud spetsiifilistele ensüümidele. Kui maskeeritud peptiid/pDNA kompleks akumuleerub passiivselt kasvajakoesse siis ensüüm lõikab antud järjestust, mille tulemusena PEG eemaldatakse, taastub PF14 transfektsiooni võime ning geen transporditakse kasvajakoes olevatesse rakkudesse. Kasutades PEGüleerimisel põhinevat aktiveeritavat süsteemi, saavutasime kasvajakoe spetsiifilise geeniekspressiooni, ilma akuutsete toksiliste kõrvalmõjudeta ning antud transportsüsteem on saadaval edasisteks eelkliinilisteks vähivastaste rakenduste uurimiseks.

Neljandas töös uuriti lisavõimalusi parandamaks PF14/pDNA transporti pärast süsteemset manustamist ilma PEGülatsioonita, mis vähendab üldiselt transfektsiooni. Selleks optimeeriti RSP ja pDNA vahelist suhet nii, et võimalikult suur osa peptiidist oleks pDNA vahendusel kompleksidesse seotud, kuna on varasemalt näidatud katioonsete polümeeride puhul, et kompleksidesse mitte seotud vaba fraktsioon polümeerist vahendab toksilisi kõrvalmõjusid. Samuti uurisime peptiidist tulenevaid omadusi, mis potentsiaalselt mõjutavad RSP/pDNA komplekside transfektsiooni rakkudesse. Selleks sünteesiti erinevad analoogid, milles oli muudetud katioonsete laengute hulk, teiseks, analoogid, mille rasvhappe jäägi pikkus oli muudetud. Nende mõjude uurimiseks kasutati erinevaid analüüsimeetodeid, mis võiks imiteerida *in vivo* tingimusi ning viimaks, hinnati nende peptiidide potentsiaali transportida pDNAd erinevatesse kudedesse pärast süsteemset manustamist.

Selgus, et pDNA doosi suurendamine katioonse peptiidi fraktsiooni potentsiaalseks vähendamiseks võimaldas indutseerida peaaegu sama kõrge geeniekspressiooni organites ilma toksiliste kõrvalmõjudeta. Teiseks, ilmnes, et peptiidi *in vivo* transfektiooni puhul on oluline samaaegselt nii piisav katioonne laeng kui ka rasvhappe jääk, mis võimaldavad efektiivselt moodustada komplekse RSP ja DNA vahel, suurendavad geenitranspordi efektiivsust rakukultuuris ning on piisavalt stabiilsed ensümaatilistele mõjutustele. Analoogid, mille katioonne laeng oli suurem või rasvhappe jääk pikem PF14st olid ainsana võimelised *in vivo* tingimustes geeniekspressiooni kudedes indutseerima.

Kokkuvõtteks, RSPd on efektiivsed NH transportvektorid rakkudesse, kuid nende rakendamine loomsetes mudelites nõuab mitmel juhul lisamodifikatsioone ning optimeerimist. Siiski, on neil potentsiaal saavutada efektiivsus geeniteraapia läbiviimiseks geenimutatsioonidest tulenevate haiguste raviks nagu seda on kasvajad.

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Careers and positions

01.2013–07.2015 University of Tartu, Specialist

Education

01.2013–...	University of Tartu, Faculty of Science and Technology, PhD Engineering and Technology, Biomedical Technology
2011–2013	University of Tartu, Faculty of Science and Technology, MSc Gene technology
2008–2011	University of Tartu, Faculty of Science and Technology, BSc Gene technology
2005–2008	Jõgeva secondary school

R&D related managerial and administrative work

2013−... Member of Estonian Biochemical Society

Science Awards and Recognitions

2013	Estonian Biochemical Society student research contest, Prize
2013	Estonian National Contest for University Students – bio- and environmentalsciences, master students – 1st prize (2013)

Publications

Veiman, Kadi-Liis; Künnapuu, Kadri; Lehto, Tõnis; Kiisholts, Kristina; Pärn, Kalle; Langel, Ülo; Kurrikoff, Kaido (2015). PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo. Journal of Controlled Release, 209, 238−247, j.jconrel.2015.04.038.

Kurrikoff, K.; Veiman, K.-L.; Langel, Ü. (2015). CPP-based delivery system for in vivo gene delivery (Methods in Molecular Biology). Methods in molecular biology, 1324, 339−347, 978-1-4939-2806-4_22.

Cerrato, Carmine; Veiman, Kadi-Liis; Langel, Ülo (2015). Advances in peptide delivery. In: Advances in the Discovery and Development of Peptide Therapeutics (160−171). Future Science Group.

Srimanee, Artita; Regberg, Jakob; Hallbrink, Mattias; Kurrikoff, Kaido; Veiman, Kadi-Liis; Vajragupta, Opa; Langel, Ülo (2014). Peptide-Based Delivery of Oligonucleotides Across Blood–Brain Barrier Model. International Journal of Peptide Research and Therapeutics, 20 (2), 169−178, s10989-013-9378-4.

Veiman, K.L.; Mäger, I.; Ezzat, K.; Margus, H.; Lehto, T.; Langel, K.; Kurrikoff, K.; Arukuusk, P.; Suhorutšenko, J.; Padari, K.; Pooga, M.; Lehto, T.; Langel, Ü. (2013). PepFect14 peptide vector for efficient gene delivery in cell cultures. Molecular Pharmaceutics, 10 (1), 199−210, mp3003557.

ELULOOKIRJELDUS

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Töökohad ja ametid

01.2013–07.2015	Tartu Ülikool, Spetsialist

Haridustee

01.2013–...	Tartu Ülikool, tehnoloogiainstituut, doktorantuur (tehnika- ja tehnoloogia, biomeditsiin)
2011–2013	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, MSc Geenitehnoloogia
2008–2011	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, BSc Geenitehnoloogia
2005–2008	Jõgeva Gümnaasium

Teadusorganisatsiooniline ja -administratiivne tegevus

2013−...	Eesti Biokeemia Seltsi liige

Teaduspreemiad ja tunnustused

2013	Eesti Biokeemia Seltsi üliõpilaste teadustööde konkursi Auhind
2013	Eesti üliõpilaste teadustööde riiklik konkurss, bio- ja keskkonnateadused, magistriõppe üliõpilased, I preemia

Publikatsioonid

Veiman, Kadi-Liis; Künnapuu, Kadri; Lehto, Tõnis; Kiisholts, Kristina; Pärn, Kalle; Langel, Ülo; Kurrikoff, Kaido (2015). PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo. Journal of Controlled Release, 209, 238−247, j.jconrel.2015.04.038.

Kurrikoff, K.; Veiman, K.-L.; Langel, Ü. (2015). CPP-based delivery system for in vivo gene delivery (Methods in Molecular Biology). Methods in molecular biology, 1324, 339−347, 978-1-4939-2806-4_22.

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