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NUCLEIC ACID DELIVERY: REPORTS FROM THE SEARCH OF THE MAGIC BULLET

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"We might anticipate the in vitro culture of germ cells and such manipulations as the interchange of chromosomes and segments. The ultimate application of molecular biology would be the direct control of nucleotide sequences in human chromosomes, coupled with recognition, selection and integration of desired genes."

Joshua Lederberg, 1963

(Nobel Prize in Physiology or Medicine 1958)

To my parents

ABSTRACT

Gene therapy is regarded as one of the most promising therapeutic approaches, since it has the potential to treat disorders by correcting malformations at the nucleic acids level rather than proteins, as opposed to conventional medicine. However, for non-viral gene therapy to successfully fulfill the requirements associated to the "magic bullet" has proven difficult.

This thesis aimed at the development of non-viral nanovectors of different nature to transport plasmid DNA and single-stranded splice-switching oligonucleotides while shedding some light on the following aspects: (1) the interaction carrier–nucleic acid; (2) relevant assets of the vector for efficient delivery; and (3) relatable features of the nucleic acid particles for *in vivo* delivery.

In our first study we compared a series of systematically modified spermines to evaluate the contribution of the lipophilic component of the carrier to the nanocomplexes formulation. We have observed that all alkylated spermines protected DNA against DNase I degradation, and that this protection directly correlated with the length of the aliphatic component. Also toxicity directly correlated to the length of the fatty acid. Further characterization studies suggested the shortest lipospermines (butanoyl- and decanoylspermine) as the two most suitable candidates for *in vivo* delivery (intramuscular and intradermal).

More than 90% of human genome undergoes alternative splice-switching, and numerous disorders impart from malfunctions at this level. These can be corrected by splice-switching (SSO) or antisense oligonucleotides (ASO). In an optimization study for *in vitro* ASO delivery, we evaluated different amino acid-modified polyethylenimine (PEI). We found that PEI modified with amino acids of equal hydrophobic nature (and to an equal extent) still resulted in carriers with very different properties. One amino acid modification in particular (tyrosine-modified PEI) showed significant improvement of ASO activity, in a splice-correction context. Also the extent of this modification was proven to significantly decrease the vehicle's delivery efficiency. These findings suggested

the existence of parameters, other than the hydrophilic/hydrophobic balance of the carrier, relevant for the interaction with the nucleic acids and also for the activity of the ensuing particles.

In our third study, by stearylation of the cell-penetrating peptide (CPP) $(RxR)_4$ we were able to successfully deliver DNA and ASO. We showed that the stearyl-$(RxR)_4$ was significantly more effective than its parental form for DNA and ASO delivery. Our results suggest that the stearic acid-modification contributes to enhanced endosomal escape. Importantly, stearylation of another commonly used CPP – Arginine 9 – did not result in increased activity, supporting the earlier findings that properties such as length, composition and three-dimensional structure of the carrier are all determinant factors for the activity of the nucleic acid-containing particles.

Finally, we evaluated a novel class of carriers that share similarities with arginine and histidine-based peptides – D-diaminopropionic acid-based peptides ($Dapa_8$). Dapa have several attractive properties for gene delivery, with the advantage that they permit tailored-design and facilitate the manipulation of the delivery properties of the peptide. All peptides interacted with plasmid DNA and provided protection against DNase I degradation, at concentrations that did not decrease cell viability. Interestingly, all fatty acid-conjugates, including the palmitoyl-$Dapa_8$, formed stable and well defined nanoparticles, with an average diameter between 120 and 160 nm. However, further modifications or *de novo* peptide design are required to achieve efficient DNA delivery.

LIST OF PUBLICATIONS

I. **Viola JR**, Leijonmarck H, Simonson OE, Oprea II, Frithiof R, Purhonen P, Moreno PM, Lundin KE, Strömberg R, Smith CIE, *Fatty acid-spermine conjugates as DNA carriers for nonviral* in vivo *gene delivery*, Gene Ther. 2009 Dec; 16(12):1429-40.

II. Zaghloul EM*, **Viola JR***, Zuber G, Smith CI, Lundin KE, *Formulation and delivery of splice-correction antisense oligonucleotide by amino acid modified polyethylenimine*, Mol Pharm. 2010 Jun 7;7(3):652-63 (* *contributed equally*).

III. Lehto T, Abes R, Oskolkov N, Suhorutsenko J, Copolovici DM, Mäger I, **Viola JR**, Simonson OE, Ezzat K, Guterstam P, Eriste E, Smith CI, Lebleu B, Samir EL Andaloussi, Langel U, *Delivery of nucleic acids with stearylated $(RxR)_4$ peptide using a non-covalent co-incubation strategy*, J Control Release. 2010 Jan 4; 141(1): 42-51.

IV. **Viola JR**, Murtola M, Simonson OE, EL Andaloussi S, Strömberg R, Smith CIE, *Diaminopropionic acid peptides: biophysical characterization studies for DNA delivery*, manuscript in preparation.

Other publications by the author, not included in the thesis:

Pandey MK, Yang K, Pei C, Sharma PK, **Viola JR**, Stromberg R, Kumar J, Parmar VS, Watterson AC, *Design and biocatalytic synthesis of pluronic-based nanomicellar self-assembly systems for drug encapsulation applications*, J Macromol SCI A. 2010; 47, 788-793.

Oprea II, Simonson OE, Moreno PM, **Viola JR**, Lundin KE, Smith CI, *Temperature-assisted cyclic hybridization (TACH): an improved method for supercoiled DNA hybridization*, Mol Biotechnol. 2010 Jun; 45(2): 171-9.

Viola JR, EL-Andaloussi S, Oprea II, Smith CI, *Non-viral nanovectors for gene delivery: factors that govern successful therapeutics,* Expert Opin Drug Deliv. 2010 Jun; 7(6): 721-35. Review.

EL Andaloussi S, Lehto T, Mäger I, Rosenthal-Aizman K, Oprea II, Simonson OE, Sork H, Ezzat K, Copolovici DM, Kurrikoff K, **Viola JR**, Zaghloul EM, Sillard R, Johansson HJ, Said Hassane F, Guterstam P, Suhorutsenko J, Moreno PM, Oskolkov N, Hälldin J, Tedebark U, Metspalu A, Lebleu B, Lehtiö J, Smith CI, Langel U, Design of a peptide-based vector, *PepFect6, for efficient delivery of siRNA in cell culture and systemically* in vivo, Nucleic Acids Res. 2011 May 1; 39(9): 3972-3987.

Ezzat K, EL Andaloussi S, Zaghloul EM, Lehto T, Lindberg S, Moreno PMD, **Viola JR**, Magdy T, Abdo R, Guterstam P, Sillard R, Hammond SM, Wood MJA, Arzumanov AA, Gait MJ, Smith CIE, Hällbrink M,Langel U, PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation, Nucleic Acids Res. 2011 July 1; 39 (12): 5284-98.

Lehto T, Simonson OE, Mäger I, Ezzat K, Sork H, Copolovici DM, **Viola JR**, Zaghloul EM, Lundin P, Moreno PMD, Mäe M, Oskolkov N, Suhorutšenko J, Smith CIE, EL Andaloussi S, *A peptide-based vector for efficient gene transfer* in vitro *and* in vivo*, Mol Ther*, in press.

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LIST OF ABBREVIATIONS

AAV	Adenoassociated virus
Arg9	Arginine 9 peptide
ASO	Antisense oligonucleotides
BHK	Baby hamster kidney cells
CHO	Chinese hamster ovary cells
CI	Cell index
CMC	Critical micellar concentration
CPP	Cell-penetrating peptides
$Dapa_8$	D-Diaminopropionic acid-based peptides
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
EPR	Enhanced permeability retention effect
EtBr	Ethidium bromide
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HBSS	Hanks buffered salt solution
HepG2	Human hepatocellular cells
HEK	Human embryonic kidney cells
LDH	Lactate dehydrogenase
LNA	Lock nucleic acids
MTS	Membrane translocating sequences
NLS	Nuclear localization sequences
PCS	Photon correlation spectroscopy
PEG	Poly(ethylene glycol)
PEI	Polyethylenimine
PEIF	Phenylalanine-modified polyethylenimine
PEIL	Leucine-modified polyethylenimine
PEO	Poly(ethylene oxide)
PEIW	Tryptophan-modified polyethylenimine
PEIY	Tyrosine-modified polyethylenimine
PMO	Phosphorodiamidate morpholino oligonucleotides

PNA	Peptide nucleic acid
PPO	Poly(propylene oxide)
PTD	Protein transduction domains
QLS	Quasi-elastic light scattering
RES	Reticulo endothelial system
RGD	Arginine-Glycine-Apartate tripeptide
RISC	RNA-induced silencing complexes
RNA	Ribonucleic acid
SSO	Splice-switching oligonucleotides
Tat	Transcription-transactivating protein of HIV
U2-OS	Osteosarcoma cell line
iRNA	Interference RNA
mRNA	Messenger RNA
miRNA	MicroRNA
pDNA	Plasmid DNA

1 INTRODUCTION

1.1 GENE THERAPY: A BRIEF OVERVIEW

Gene delivery as a therapeutic approach aims at permanent, or transient, correction of a gene defect by intracellular delivery of nucleic acids. Conventionally, non-viral gene therapy refers to plasmid DNA delivery into cells to correct loss of function, thereby treating or alleviating symptoms of a disease. However, recent developments made the use of different nucleic acids, namely RNAs and short single-stranded oligonucleotides, possible, and more promising and appealing tools. In addition to gain of function, these strategies can also be used to turn off or down-regulate undesired genes, and as their mechanism of action differs from that of plasmid DNA, these approaches may be referred to as nucleic acid (as opposed to gene) therapy. Initially thought as a regimen for inherited genetic diseases, presently, the applicability of gene therapy has been largely widened, also owing to the novel array of tools. Since the first legal human clinical trial in 1989 (Rosenberg et al., 1990), numerous trials took place designed to establish feasibility and safety, to demonstrate the expression of therapeutic proteins *in vivo* by the genes transferred and, in some instances, to show therapeutic benefit. Eye diseases (Campochiaro et al., 2006; Chevez-Barrios et al., 2005), severe combined immunodeficiency (Cavazzana-Calvo et al., 2000; Gaspar et al., 2006), several types of tumors (Braybrooke et al., 2005; Davis et al., 2010; Rainov, 2000; Wierda et al., 2010), cystic fibrosis (Hyde et al., 2000; Moss et al., 2004), Duchenne's muscle dystrophy (Catlin et al., 2011b; Kinali et al., 2009; van Deutekom, 2005) and other muscle wasting disorders (Stedman et al., 2000), and neural malfunctions (Parkinson's Disease (Bankiewicz et al., 2006; Kaplitt et al., 2007), Alzheimer's Disease (Tuszynski et al., 2005) and Huntington's Disease (Bloch et al., 2004)) are the most common syndromes where gene therapy has been applied in the clinic.

Owing to numerous physiological barriers posed *in vivo*, a transport vehicle is often required to secure the delivery of the therapeutic agent. Provided that the carrier device is individually tailored, with respect to targeting and

pharmacokinetics, this tool holds the potential of having an important impact on the treatment of a large range of monogenic as well as acquired disorders. In fact the concept of a "personalized and targeted drug" dates back to the late nineteenth century and it is inherent to the concept of Paul Ehrlich's "magic bullet" (Bosch and Rosich, 2008). While searching for a cure to syphilis, in early experiments with methylene blue, Ehrlich observed that when administered through the auricular vein this dye preferentially accumulated in certain animal tissues – ganglion cells. He, thus, speculated about the existence of a dye with the ability to specifically stain pathogenic microorganisms, and eradicate them without harming the host organism. This marks the beginning of the perpetual search for the *magische Kugel* (German for "magic bullet"): an ideal therapeutic agent, with high specificity against the target tissue/organism, which translates into significant activity at low concentrations and minimum toxicity, and side-effects, for the host, including development of resistance against the drug.

For gene therapy the road that leads to this "holy grail" has, so far, been a roller coaster. Viruses have developed a number of ways to invade host cells and few are even *eximius* in integrating their genetic information into the host's genome. For this reason, modified viruses were suggested as delivery vectors (Rogers and Pfuderer, 1968) and, owing to their high efficiency, they are preferred over alternative carriers, being enrolled in approximately 70% of the clinical trials. Modifications are required to avoid possible immunological responses, albeit viral vehicles are often highly immunogenic, especially upon readministration. After years of research, in April 2000, Cavazzana-Calvo and collaborators communicated the 10 months follow-up of eleven treated patients suffering from X-linked severe combined immunodeficiency, and what was then believed to be the first significant clinical step forward for gene therapy: "*T, B, and NK cell counts and function, including antigen-specific responses, were comparable to those of age-matched controls. Thus, gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit*" (Cavazzana-Calvo et al., 2000). Unfortunately, in 2003, this clinical trial revealed otherwise, and the major drawback of viral vectors surfaced: random genomic integration possibly

leading to mutation (Cavazzana-Calvo et al., 2000; Hacein-Bey-Abina et al., 2002; Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). These adverse events had repercussions at the level of regulatory agencies that put a temporary hold on new ongoing trials. Later, in 2007, a patient died for the first time using an adenoassociated virus (AAV). The same year, Cavazzana-Calvo and colleagues treated an 18-year-old patient who had HbE/β-thalassaemia, initiating the first human gene-transfer trial for hemoglobin disorder using a lentiviral vector (Cavazzana-Calvo et al., 2010). Twenty years after the first human gene therapy clinical trial, the 20 months follow-up report of the treated β-thalassaemia patient made the scientific community believe, once more, in a significant step forward for gene therapy, in general, and for treatment of hemoglobin disorders in particular.

Nevertheless, and despite the first commercial gene-based drug made available in China (Peng, 2005; Wilson, 2005), the "magic bullet" has yet to be found and a gene transfer system built on highly defined components and low immunogenicity is still warranted. In addition to possible random genomic integration and immune responses, viral vectors present other disadvantages, such as limited capacity concerning the size of the gene to be transferred and the numerous empty and defective viral particles present in viral supernatant (while intact particles make up merely a minor proportion). Table 1, in the next page, summarizes the characteristics of the most widely used viral vectors.

Conversely to viruses, non-viral vectors can offer the required safety, and well-defined composition. Physical approaches, such as electroporation or magnetofection, have been demonstrated to be highly efficient in targeting several tissues, especially skeletal muscle or cancer tissues (Gehl, 2008; Lu et al., 2009; Mir et al., 2005; van Drunen Littel-van den Hurk et al., 2008). Yet, all the electrical equipments used in these methods present some inconveniences. Nano-strategies such as inorganic particles (i.e., gold (DeLong et al., 2009; Lin et al., 2009) and silica-based systems (Jin et al., 2009)) and chemical vectors are thus an alternative.

Table 1. Commonly used viral vectors (Raty et al., 2008)

Vector	*Advantages*	*Limitations*
Adenovirus *(Adenoviridae)*	High efficiency Transduces quiescent and dividing cells > 30kb transgene capacity Easy to produce in high titers	Coxsackie adenovirus receptor-dependent transduction Immunogenic Existing humoral response to certain serotypes
Murine leukaemia virus *(Retroviridae)*	Broad tropism Low immunogenicity Stable integration	Insertional mutagenesis Unable to transduce quiescent cells Inactivation by serum
Lentivirus *(Retroviridae)*	Low immunogenicity Stable integration in quiescent cells	Insertional mutagenesis Potential risk of recombination of pathogenic vector (HIV)
Adenoassociated virus *(Parvoviridae)*	Transduces quiescent and dividing cells Very long expression time Non-pathogenic, low immunogenicity Broad tropism	Very small transgene capacity Possible insertional mutagenesis
Semliki forest virus *(Alphaviridae)*	High titer Broad host range Efficient transgene expression	Low transgene capacity High cytotoxicity Short term expression
Herpes simplex-1 *(Alphaherpesviridae)*	Broad host range High titer Large transgene capacity	Latent wild type-viral activation risk Antigenic
Autographa californica multicapsid nucleopolyhedrovirus *(Baculoviridae)*	High titer Large transgene capacity Easy production Non-pathogenic	Limited transduction Production in insect cells Unstable genome

1.2 DNA, RNA AND SINGLE-STRANDED OLIGONUCLEOTIDES: DIFFERENT STRATEGIES FOR DIFFERENT APPLICATIONS

Traditionally, in the case of loss of function, gene therapy aims at correcting the levels of the target protein by repairing the defective gene or by substitution through the addition of a wild-type (non-mutated) copy, for example by introducing plasmid DNA (pDNA) vector carrying the native form of the gene. However, the complexity of the course of protein synthesis allows for a number of approaches that act on different stages of the process (Figure 1). For the protein synthesis a messenger RNA (mRNA), encoding the genetic information, is required, and it is synthesized in the nucleus by the process of transcription. Thus pDNA-based gene delivery acts at an early stage of the protein synthesis and in order for transcription to take place the plasmid needs to target the cell nucleus.

During transcription a nuclear RNA, precursor of the messenger RNA (pre-mRNA), containing the same nucleotide sequence as the DNA coding sequence, is synthesized. The pre-mRNA is further processed, by the splicing mechanism, to generate the mature mRNA consisting of nucleotide sequences that are translated into protein (exons). Alternative splicing of introns (non-translated sequences of nucleotides) within a gene can modulate gene expression and generate a large variety of protein sequences translated from a single gene. Splicing is determined by specific intronic sequences such as the acceptor (or 3' splice site) and donor (or 5' splice) sites at either end of the intron as well as a branch point site. Point mutations at the DNA level or errors occurring during transcription can result in a "cryptic splice site" within the transcript that is normally not spliced. As a consequence, the mRNA lacks part of the coding exon, which is likely to translate into an abnormal protein, found in e.g. β-thalassaemia (Cavazzana-Calvo et al., 2010), cystic fibrosis (Kerem, 2005) or Duchenne muscle dystrophy (Bartlett et al., 2000; Magri et al., 2011). In these cases, the use of antisense (ASO), or splice-switching (SSOs) oligonucleotides as blocking agents, to redirect the splice back to the correct splice-sites (Friedman et al., 1999) or to skip exons that contain mutations (Aartsma-Rus and van Ommen, 2007), is a promising approach (Figure 2). Owing to the size of cargo, plasmid delivery is more cumbersome than that of short oligonucleotides. 95-100% of human pre-mRNAs have alternative splice forms (Nilsen and Graveley, 2010) and several disorders impart from malfunctions at this level (Faustino and Cooper, 2003; Mironov et al., 1999), making this approach appealing over that of DNA delivery.

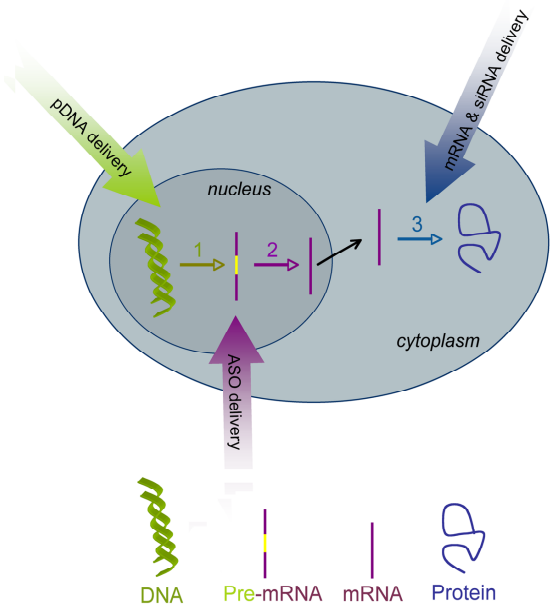


Figure 1. Stages of protein synthesis where gene delivery can be active. **1** – Transcription; **2** – splicing; **3** – Translation.

Conversely, also to treat loss of function, gene therapy can also be based on the delivery of mRNA (Kormann MHG, 2009). In this case, the therapeutic agent acts in the cytoplasm, at the translational level.

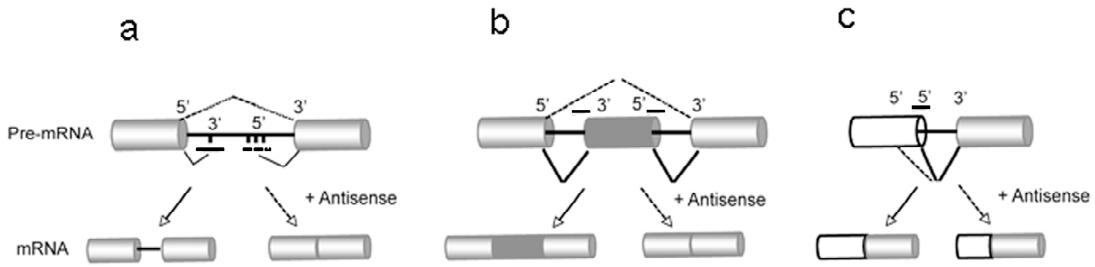


Figure 2. Schematic representation of splice modification modulated by oligonucleoties. Aberrant splicing is prevented by restoration of correct splicing by oligonucleotides (short black bars) that block aberrant cryptic splice sites, either 5' or 3' (**a**). Alternatively, oligonucleotides can induce skipping of a normal exon (gray) (**b**) or force the selection of an alternative 5' splice site (**c**) by targeting the correct splice sites. (Sazani and Kole, 2003)

For the cases of gain of function mutations, such as those introduced by a viral organism, approaches that apply the mechanism first described by Craig C. Mello and Andrew Z. Fire are very suitable (Fire et al., 1998), such as small interference RNA (siRNA) or small non-coding RNAs (in particular microRNA, miRNA). siRNA is specifically directed against the mRNA of the target protein by having a complementary sequence. After being processed by Dicer and associated to the RNA-induced silencing complexes (RISCs) the antisense strands of siRNA guide the RISCs to the complementary RNA sequences, which are subsequently cleaved. MicroRNAs are a class of endogenous, non-coding, single-stranded RNAs, typically 19 – 25 nucleotides long. Unlike iRNA, miRNA negatively regulates gene expression through two mechanisms, depending on the degree of complementarity towards its mRNA target. In plants, perfect, or nearly perfect, complementarity induces the cleavage of the mRNA target, and subsequent degradation, whereas in metazoans miRNAs usually bind to partially complementary regions, thereby inducing a decrease in protein levels independent of mRNA cleavage. Interestingly, this class of gene regulators has been shown to play pivotal roles in crucial biological processes, such as development, cell growth, differentiation and apoptosis. These non-coding

RNAs opened yet another window to a new generation of gene-manipulating tools: by the use of antimirs (short oligonucleotides that bind miRNAs) it is possible to upregulate the expression of proteins, an approach that has been explored as anticancer therapy (Krutzfeldt et al., 2005).

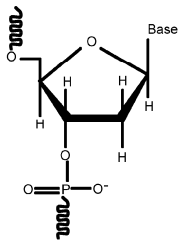
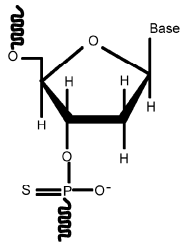
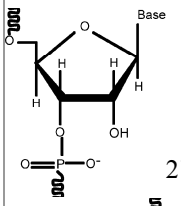
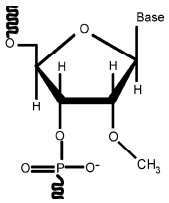
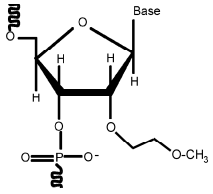
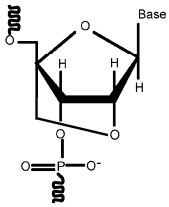
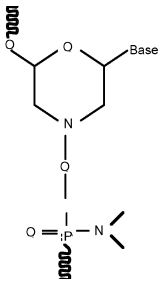
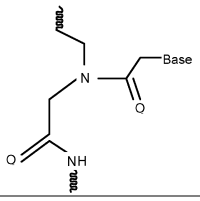
Finally, it is important to recognize that all the different nucleic acids that have just been described have distinctive properties. For polymer/peptide-based delivery not only the length but also the composition and three-dimensional structure of the nucleic acids are key aspects for the interaction between a carrier and the nucleic acid. Therefore, the transport vehicle should be designed accordingly to the strategy to be used.

1.2.1 Nucleic acid analogues in gene therapy

One of the major challenges for oligonucleotide-mediated gene therapy is the stability. Nucleic acids in their natural form, as phosphodiesters, are rapidly hydrolyzed by nucleases present in the blood. This is one of the defence mechanisms of the living organism, for instance against pathogenic genetic material. Recognition by degrading enzymes can be avoided by modification of the nucleic acid at the level of the phosphodiester backbone, nucleobase or sugar ring. The ensuing nucleic acid analogues have different properties from their parental molecules, such as distinct thermal stability (assessed by their melting temperature, T_m) or binding affinity towards other nucleic acids (RNA or DNA). In addition to increased plasma half-life, manipulation of these idiosyncrasies allows for an optimization of an antisense activity, by modulation of base-pairing affinity – a critical step in an antisense strategy. These are, thus, meaningful assets for gene therapy.

DNA analogues are commonly classified into three different generations, and their chemical modifications and characteristics are summarized in Table 2, in the next page.

Table 2. Different nucleic acids used in gene therapy approaches. Advantages and limitations of nucleic acids analogues in relation to their parental molecules (DNA and RNA) and different generations.

	First generation	*Second generation*	*Third generation*
	Phosphodiester (DNA)   Phosphorothioate	RNA  2'-*O*-methyl  2'-*O*-methoxy-ethyl 	LNA  Morpholino  PNA 
Advantages	(i) Oxygen substitution increases biologic stability: half-life is extended up to 10h (Campbell et al., 1990; Goodarzi et al., 1992; Hoke et al., 1991); (ii) Water soluble (charged molecules); (iii) Activates RNase H.	(i) Less toxic than 1st generation; (ii) Increased T_m (tighter binding); (iii) No RNase H activity (not required for splice-switching).	(i) More diverse: modifications of sugar ring and/or phosphodiester backbone; (ii) Improved T_m and nuclease resistance; (iii) Morpholinos: cost-effective; (iv) PNA: no electrostatic repulsions.
Side-effects	(i) Increases non-specific association with plasma proteins (Brown et al., 1994); (ii) May activate complement (Henry et al., 1997; Levin, 1999); (iii) T_m decreases 0.5ºC per nucleotide (Crooke, 2000).	(i) For RNase H activity gapmers are needed (Monia et al., 1993).	(i) PNAs are sensitive to ionic concentrations – do not hybridize well under physiological conditions

1.3 CHEMICAL VECTOR-BASED GENE DELIVERY

1.3.1 Chemical vectors: the interaction between carrier and nucleic acid

With the exception of hydrodynamic injections (Zhang et al., 2010), wherein the conditions are very special, naked pDNA is normally not capable of inducing relevant therapeutic effects when systemically administered *in vivo*. This is especially owing to enzymes existent in serum, i.e. nucleases, DNases and phosphodiesterases, which recognize and degrade natural nucleic acids. Chemically-modified oligonucleotides, on the other hand, show variable degrees of resistance (Heemskerk et al., 2009b; Sazani et al., 2002). Following introduction into the systemic circulation or local tissue, the oligonucleotides have a passive initial distribution, and therefore, notwithstanding the activity, excessive quantities are required. *In vivo* usage of large quantities of an active agent is not cost-effective and is often associated with toxicity. On these grounds, the therapeutic nucleic acid resembles any other drug, requiring stability against degradation as much as targeting function. Using the words of others, there are three limitations hindering gene therapy applications; 'delivery, delivery, delivery' (Greco et al., 2002)!

Chemical vectors refer to chemical-based, non-viral delivery systems, where peptides, lipids or polymers transport the nucleic acid. Owing to their phosphate backbone nucleic acids are negatively charged molecules, which can mediate electrostatic interactions (Bloomfield, 1996). In 1997, Bloomfield defined DNA condensation as the collapse of extended DNA chains into compact, ordered particles containing only one or few molecules (Bloomfield, 1997). Typically, this process results from neutralization of negative charges of the DNA phosphate groups with transition into the ordered phase occurring when 90% of the negative charges of the DNA phosphate groups are neutralized (Bloomfield, 1997). Classical DNA condensing strategies frequently rely on the use of cationic peptides or polymers, either natural or synthetic, although the interaction between carrier and nucleic acid is far more complex. Often DNA aggregates, large undefined structures containing several DNA molecules, are formed in

addition to well-defined and ordered particles. This is a common phenomenon, and it highlights the delicate balance that this interaction requires, not only for structural but also for functional purposes. DNA aggregates are frequently formed when using highly cationic molecules, such as polylysine or polyethylenimine (PEI) that can interact with different DNA chains thereby increasing the number of molecules per condensate and, consequently, the diameter. Moreover, highly cationic polymers will impair to a greater extent the DNA unpacking which is critical for its activity.

Interactions between the vector and the nucleic acid are not only of electrostatic nature, since also hydrophobic interactions take place between the nucleobases of DNA and the polymer (Geall and Blagbrough, 2000; Tuszynski et al., 2005). Combined with cationic contributions, these weaker bonds are likely tuning the interaction and can result in improved carriers (Alshamsan et al., 2009; Ghonaim et al., 2010; Mae et al., 2009). Together with the electrostatic binding hydrophilic interactions (through hydrogen bonds) might also have relevant contributions for the resultant DNA condensates. This is the case for DNA particles resulting from the addition of chitosan or carriers with amphiphilic properties, as for example poloxamer block copolymers, often referred to as Pluronics® (their tradename), which do not hold any charges.

Peptides or polymers that can interact with nucleic acids in a weaker fashion have recently become more popular also for the reason that these are likely to be less toxic (highly positively charged particles can induce complement activation and binding of immunoglobulins, and subsequent cytokine release). More importantly, complexes assembled by weaker interactions facilitate the release of the nucleic acid, which is required for activity. Supporting this theory is the report by Strand and colleagues where by manipulating the length and the degree of substitution of different chitosans the authors showed the feasibility to modulate the interactions between carrier and DNA, thereby reaching an optimized balance between the nanocomplexes stability and unpacking (Strand et al., 2010).

Polymers like poloxamers, composed of sequential blocks and with amphiphilic characteristics, are attractive agents mainly for the reasons that: (1) they present self-assembling properties (Kabanov et al., 2005; Santander-Ortega et al., 2006) that allow for incorporation of drugs into the micelles, and (2) the wide range of possible combinations of their monomeric units [poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO)], which confer significantly different characteristics to the polymers belonging to this class of molecules. More specifically, in the case of poloxamers, varying the number of the hydrophilic moiety in relation to its hydrophobic counterpart permits manipulation of properties like solubility and critical micellar concentration (CMC). Depending on the temperature conditions and the concentration of the polymer, different types of micelles can be formed: spherical, cylindrical and lamellar (Clover and Hammouda, 2009). Manipulation of such properties allows these polymers to be tailored according to specific applications (Batrakova and Kabanov, 2008; Kabanov et al., 2005; McIlroy et al., 2009; Roques et al., 2009; Santander-Ortega et al., 2006).

Overall, independently of the type of interaction, the key factors for an efficient DNA delivery system are (1) to confer DNA protection, (2) to allow formation of small well-defined particles (preferably 100-200 nm) that are efficiently internalized by cells and, importantly, (3) to display a moderate binding affinity allowing liberation and subsequent activity of the nucleic acid inside cells.

1.3.2 The "Trojan horse" of gene delivery: how to surpass the physiological barriers

The major shortcoming of non-viral nanovectors is the poor overcome of the physiological barriers, which translates into low efficiency. The *in vivo* system harbours a series of barriers (Figure 3) for non-self entities, such as bacteria or viruses, to cease their progression and possible detrimental effects. These entail extracellular obstacles, such as clearance mechanisms (by the reticulo-endothelial system, RES, or the kidneys) and nucleic acids degrading enzymes, but also more critical boundaries, cellular related. In particular, by a tight control of cellular uptake, fate (endosome/lysosome *versus* cytoplasm), and nuclear entry, the living organism poses a serious challenge to this therapeutic approach.

In this respect, learning from viruses and mimicking some of their properties and strategies have contributed to considerable advances, as described bellow. Table 3 summarizes the strategies currently used in gene therapy approaches to overcome the physiological barriers aforementioned.

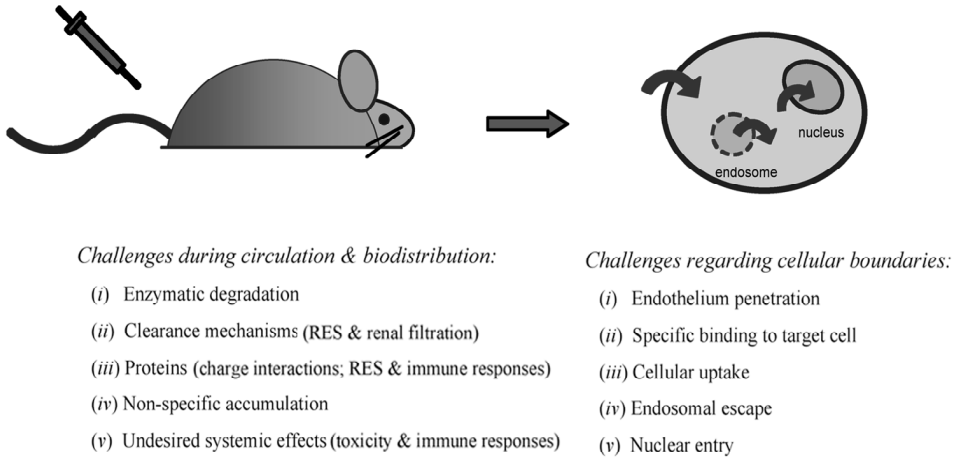


Figure 3. Schematic representation of the different barriers posed by *in vivo* conditions to gene drug delivery. Once in contact with blood, nucleic acids are exposed to enzymatic degradation and their delivery is threaten by clearance mechanisms and interaction with serum proteins. Along the process, undesired side-effects such as immune responses or toxicity should be avoided. Nucleic acid therapeutics that are not degraded nor cleared often undergo passive and continuous circulation, which represents a hurdle for specific delivery. Once the nucleic acid reaches the target cell additional barriers need to be overcome: cellular uptake, endosomal escape and dissociation of the nucleic acid from its carrier.

Upon systemic administration, current vectors have a passive biodistribution and increased circulation time is important not only because it allows for an even distribution but also for optimal tissue targeting of the therapeutic agent. Once in the blood, the nucleic acid is immediately degraded by nucleases and hence protection is required. In addition, interaction with blood proteins is yet another probable limitation for the delivery of the drug carrier, if its surface charge is not neutral or slightly negative. Positively charged nanoparticles will be rapidly cleared by the mononuclear phagocyte system, as they interact with proteins of opposite charge thereby forming large aggregates (Alexis et al., 2008). The carrier for the therapeutic nucleic acid should, therefore, copy the famous strategy of the horse of Troy; the cargo solely reaches its target site if remained unknown to

the living system. To address the aforementioned serum stability concerns current non-viral nano-strategies rely on formulations with chemical vectors, to provide stability and to prevent the nanoparticles from being recognized by the *in vivo* system as cell debris or pathogens and thus cleared. To avoid interactions the particles should be slightly negatively charged or neutral (Liu et al., 2003; Nyunt et al., 2009; Wu et al., 2001).

Table 3. Common strategies used in gene therapy approaches to overcome the *in vivo* barriers to the delivery process.

Challenges	
Circulation and biodistribution	
Limitations	*Approach*
Enzymatic degradation	Protection provided by nonviral vector
Interaction with serum-proteins Body clearance mechanisms Undesired systemic effects (toxicity, immune responses	Inert system – PEGylation and similar approaches
Cellular boundaries	
Limitations	*Approach*
Endothelium penetration	Leaky vasculature (tumors) or liver
(specific) Cellular uptake	Cell targeting receptor-mediated (targeting entity) Peptide/polymers able to cross the membrane
Endosomal escape	Fusogenic or endosome-destabilizing peptide/polymer
Dissociation of nucleic acid from vector	Stimuli-cleavable polymers for nucleic acid release in cytoplasm
Nuclear entry	Nuclear targeting entities

If not neutral or with a discrete charge, nanoparticles can also interact with erythrocytes and induce hemagglutination (Harris et al., 2009; Malek et al., 2009; Merdan et al., 2005; Trubetskoy et al., 2003). As earlier mentioned, aggregation is a recurrent issue in vector formulation and one possible way to circumvent this hurdle is by the inclusion of "stabilizers". However, prolongation of the circulation time of the drug seems to require an additional element to provide for steric hindrance, that is modification of the surface of polyplexes with

hydrophilic polymers. Poly(ethylene glycol) (PEG) has been extensively used in drug delivery for stability purposes, in particular since the year of 1990, when prolongation of the circulation time of liposomes using "amphipathic poly(ethylene glycols)" was reported (Klibanov et al., 1990). Currently, the selection of PEG molecules is rather wide – an assortment composed of different sizes and structures. Additionally, the PEGylation effect is also dependent on the peptide/polymer/liposome, the density of PEG grafting, type of nucleic acid and environment (Beyerle et al., 2009) but generally, most studies show enhanced circulation with PEG of molecular weights of 550, 2000 and 5000 (Faure et al., 2009; Hatakeyama et al., 2007; Kojima et al., 2010; Morille et al.). Albeit PEG being the most commonly used, there are alternative approaches to address the problems associated with systemic administration and circulation of non-viral vectors: (*i*) polyacrylic acid added to pre-formed DNA/PEI complexes (Trubetskoy et al., 2003), (*ii*) poly-E, a peptidic sequence composed of polyglutamic acid (Harris et al., 2009), (*iii*) N-(2-hydroxypropyl) methacrylamide (PHPMA) (Oupicky et al., 2002), and (*iv*) cholesterol (Akinc et al., 2009; Soutschek et al., 2004).

Regarding local-regional administration, generally the dilution effect that is present in the bloodstream is either prevented or decreased by the microenvironment created by the injection volume, as is the case of intradermal or intramuscular injections. Through this type of approach the vector also avoids direct interactions with blood proteins decreasing the probability of particle clearance. The most prevalent local application sites include the muscle, airways, tumors and eye. Of particular interest for this thesis is the muscular application. Muscular transgene expression may serve several purposes such as DNA vaccination (McIlroy et al., 2009), production and secretion of an active protein with systemic effects (Lavigne et al., 2008; Richard et al., 2005b) and treatment of muscle-specific diseases (Heemskerk et al., 2009a; Richard et al., 2005a; Rodino-Klapac et al., 2009; Zhang et al., 2010). Although local injections do not present the same type of barriers as the intravenous administration, most of the systems that have been used are either PEG-modified or contain similar large polymers that can provide steric hindrance of some sort (Avgoustakis, 2004; Chang et al., 2007).

It is, however, important to realize that drug particles with prolonged half-life and negligible cell uptake are of limited interest, for the reason that they are inactive. Stable carriers lacking targeting functions frequently locate to the lungs, liver, spleen, bone marrow or kidney. Reasons for these preferences are related to the reticulo-endothelial system (RES): liver, spleen, kidney and lungs are all RES-rich tissues (Kasuya and Kuroda, 2009). In addition, the lungs are likely the first organs to be reached by the nanoparticles and adsorption to plasma proteins results in agglomeration, thereby trapping the particles in the vascular beds (Alexis et al., 2008). It is also known that liver, spleen and bone marrow are surrounded by a discontinuous endothelium that may facilitate uptake. In order to avoid destruction in the RES, targeted delivery to specific tissues is preferable. Interestingly, a number of nanocarriers provided with stealth-like shield effects were shown to accumulate preferably in highly vascularized tissues, a phenomenon referred to as enhanced permeability and retention (EPR) effect (Brannon-Peppas and Blanchette, 2004; Chisholm et al., 2009; Fenske and Cullis, 2008; Matsumura and Maeda, 1986). As a consequence, these nanovectors favor tumor targeting, where blood vessels also often show increased permeability. Nevertheless, unambiguous targeting is still to be accomplished and, importantly, directed delivery reduces the dose requirements and hence possible side effects. Learning from viruses, several approaches have made use of targeting entities to increase efficiency of the therapy by specific cell uptake. Although most commonly used targeting entities consist of proteins and peptides, nucleic acids and carbohydrates have also been utilized (Table 4).

One advantage of this non-viral strategy over that of viruses, is that the present chemical and biochemical state-of-art allow for tailoring and chemical synthesis of several non-naturally-occurring targeting molecules (Mukthavaram et al., 2009). These hold the promise of achieving higher specificity than natural occurring molecules.

Table 4. Nature of functional entities used for cellular or intracellular targeting.

Nature of the functional entity	*Examples*
Protein	Transferrin (Ogris and Wagner, 2002; Russ et al., 2010) Lactoferrin (Huang et al., 2009a; Huang et al., 2009b; Huang et al., 2009c; Weeke-Klimp et al., 2007)
Peptide	Integrin binding peptide RGD* (Chen et al., 2010; Li et al., 2010; Pike and Ghandehari, 2009) SV40 virus NLS# (Branden et al., 1999; Miller and Dean, 2009)
Carbohydrate	Galactose (Kasuya and Kuroda, 2009; Letrou-Bonneval et al., 2008; Mukthavaram et al., 2009) Asialofetuin (Arangoa et al., 2003; Diez et al., 2009)
Nucleic acid	Aptamer (Dhar et al., 2008; McNamara et al., 2006; Zhou et al., 2008) m_3G-Cap NLS# (Moreno et al., 2009)
Lipid	Cholesterol§ (Soutschek et al., 2004)

***** Tripeptide Arginine-Glycine-Aspartate

NLS, nuclear localization signal

§ Cholesterol can be used for liver targeting by binding to human serum albumin or high density lipoprotein. Such approaches have been reported for delivery of siRNA (Soutschek et al., 2004) as well as antisense oligonucleotides (Krutzfeldt et al., 2005) using chemically coupled cholesterol. Size limitations may apply.

Importantly, the contribution of the therapeutic nucleic acid *per se* to cell or tissue specificity should not be neglected nor underestimated, as well as its influence at the gene expression level. As shown by Christine Wooddell *et al.* pDNA vector can be designed and optimized for enhanced and long term tissue-specific gene expression, based on the use of tissue-specific promoters and binding elements (Wooddell et al., 2008). Analogously, Luigi Naldini's laboratory used the fact that microRNAs are tissue-specific to direct gene expression (Brown et al., 2007; Brown and Naldini, 2009). By introducing the corresponding targeting sequences of the tissue-specific microRNA into the pDNA construct, the vector is consequently equipped with an active "detargeting" component. Cellular uptake may yet be improved through the use of vectors that posses a good ability to cross the cell membrane, such as amphiphilic peptides or polymers or cell-penetrating peptides. Cell-penetrating peptides, or CPPs, in particular, have been extensively studied as carriers not only for different types of nucleic acids (Abes et al., 2008; Crombez and Divita, 2011; Ezzat et al., 2011; Lehto et al.; Morris et al., 2007) but

also for proteins (Morris et al., 2001; Schwarze et al., 1999). This class of delivery vector is described in more detail in the next section.

Size-wise DNA nanoparticles are comparable to viruses and their mechanism of cell internalization is likely to be common, via the endocytic pathway (Love et al., 2010; Lundin et al., 2008). As the endosomal vesicle travels deeper into the cytoplasm, and closer to the nucleus, its pH significantly drops, endangering the integrity of the therapeutic nucleic acid. Current nano-strategies for endosomal escape involve the use of fusogenic (Akita et al., 2010; Farhood et al., 1995; Wagner et al., 1992) or endosome-destabilizing peptides or polymers (Andaloussi et al., 2011; Boussif et al., 1996). These are pH responsive carriers, with a pKa value typically close to 6, which induce the destabilization of the endosome by induction of an osmotic unbalance, as a consequence of their protonation. This effect is commonly known as "proton sponge". Chloroquine is a 4-aminoquinolone compound that was soon after the discovery of its properties (related to its anti-malarial effects) used to improve the delivery of vectors *in vitro* (Zenke et al., 1990). Howbeit, its application in gene delivery is hampered by its high toxicity. Presently, chloroquine is employed in transfection studies to determine whether the activity observed by a certain delivery system imparts from endosomal entrapment. Viable strategies to circumvent this limitation, nowadays include the use of the polymer PEI (Boussif et al., 1996) and histidine-rich peptides (Langlet-Bertin et al., 2010; Midoux and Monsigny, 1999), equally known to exert a "proton sponge" effect owing to their pKa value. More elegant approaches include the conjugation of fluoroquine groups, and derivatives thereof, to the delivery system (Andaloussi et al., 2011; Cheng et al., 2006). To be active, the nucleic acid must dissociate from the peptide or polymer. It is reasonable to hypothesize that the binding between carrier and nucleic acid would be vulnerable after the endosomal escape. Nevertheless, approaches to aid at this dissociation process comprise stimuli-cleavable peptides/polymers, for instance containing disulfide bridges that are susceptible to cleavage by enzymes present in the cytoplasm (McKenzie et al., 2000). Ultimately, for the case of pDNA or splice-switching oligonucleotides, the nucleus is the final cellular boundary. Pores in the nuclear

membrane are very selective and transport across such membrane is seldom passive (Paine et al., 1975; Pante and Kann, 2002). Ways to circumvent this barrier usually rely on the modification of the vector by addition of nuclear targeting entities, such as nuclear ligand sequences (NLS) (Ludtke et al., 1999; Moreno et al., 2009; Sebestyen et al., 1998).

1.3.3 Cell-penetrating peptides as chemical vectors: a brief synopsis on the CPP history

The term "cell-penetrating peptide" was first introduced in 1998, by the group of Langel, were they reported on a chimeric peptide vector – Transportan (TP10) – derived from the N-terminal of the neuropeptide galanin linked to mastoparan (a peptide present in wasp venom) (Langel, 2006; Pooga et al., 1998). Cell-penetrating peptides (CPPs), protein transduction domains (PTDs) and membrane translocating sequences (MTS) are merely different ways to address the same class of peptides, which, interestingly, has its origins in viral proteins (supporting the theory of viral mimicry). The exact definition of CPPs is yet to be described, much owing to the remaining uncertainties concerning their cell internalization mechanism. There is, however, a current understanding of CPPs: these are relatively "*short peptides, 5–40 aa, with the ability to gain access to the cell interior by means of different mechanisms, mainly including endocytosis, and with the capacity to promote the intracellular delivery of covalently or noncovalently conjugated bioactive cargoes*" (Langel, 2006).

The *Era* of the CPPs began in 1988 with the discovery of the shuttling properties of the transcription-transactivating (Tat) protein of HIV-1 that was shown to enter cells and translocate into the nucleus (Frankel and Pabo, 1988; Green and Loewenstein, 1988). Later, in 1991, Alain Prochiantz and collaborators reported on the cell internalization of the homeodomain of Antennapedia (a Drosophila DNA binding-protein) (Joliot et al., 1991). Further investigations led to the identification of the peptide sequence responsible for membrane translocation (Derossi et al., 1994), later named Penetratin. Penetratin is therefore the first PTD and consists of a 16-mer peptide derived from the third helix of the

homeodomain Antennapedia. In 1997, the group of Lebleu identified the minimal sequence of Tat required for cellular uptake (Vives et al., 1997). Other historical remarks for the CPP world include the delivery of nucleic acids by a non-covalent strategy, in 1997, by the groups of Heitz and Divita (Morris et al., 1997), and the first proof-of-concept of the *in vivo* application of these vectors for the delivery of peptides and proteins, by the team led by Dowdy, in 1999 (Schwarze et al., 1999). Presently, there is a vast number of CPPs; their popularity is mainly due to their relatively low toxicity and to their limitless cargo application (small peptides, large proteins and different types of nucleic acids).

Their mechanism of cell-uptake, however, remains controversial. Most reports support an endocytic pathway, but few studies point out for an endosomal-independent route and involving trans-membrane potential (Alves et al., 2010; Crombez et al., 2009; Morris et al., 2008). Various parameters seem to affect the cell internalization of CPPs: (i) the secondary structures of CPP, (ii) the nature and concentration of the cargo, (iii) the ability of the CPP to interact with the cell surface and membrane lipids, and (iv) the type of cell and its membrane composition (Alves et al., 2010; Raagel et al., 2010). For most cases where short oligonucleotides compose the cargo an energy-dependent endocytic route has been suggested (Lundin et al., 2008). Unlike the majority of chemical vectors, most CPP strategies rely on a covalent linkage to the cargo (Hassane et al., 2009; Ivanova et al., 2008; Saleh et al., 2010; Schwarze et al., 1999), although recently successful non-covalent applications have increased (Andaloussi et al., 2011; Lehto et al.; Morris et al., 2001; Morris et al., 1997). CPPs have now been used to deliver an array of different nucleic acids: oligonucleotides (PNA and PMOs, few currently being evaluated in the clinic (Moulton and Moulton, 2010)), siRNA (Andaloussi et al., 2011; Schwarze et al., 1999) and DNA (Lehto et al.).

Of particular interest for this thesis is the arginine-rich derivative peptide $(RxR)_4$ (R= arginine, x=aminohexanoic spacer). The most commonly used subgroup of CPPs consists of arginine-rich peptides (Akita et al., 2010; Futaki et al., 2001; Hatakeyama et al., 2009; Mitchell et al., 2000; Saleh et al., 2010). The guanidinium headgroups of the arginines are believed to be critical for the transport across

the cell membrane, whereas the length of the side chain, and the spacing and composition of the backbone can vary (Wender et al., 2000). Both the guadinine content and the length of the arginine polymer seem to affect the cellular uptake, which increases with the number of arginine residues. However, polymers longer than 15-mers are not attractive for the fact that they exhibit cellular cytotoxic properties and interact with serum proteins. The contribution of the guanidinium headgroup for the transport is propped up by the observation that short arginine oligomers entered cells more rapidly than their lysine, histidine and ornithine counterparts (Mitchell et al., 2000). Furthermore, additional studies on a series of polyguanidine peptoids differing in the length and flexibility of their side chains also support the importance of this group for cell internalization. Interestingly, the ability of these peptoids to enter cells improved with the increase in length of the side chain, suggesting that increased flexibility of the side chain might allow a higher percentage of guanidines to simultaneously reach the cell membrane. Based on these observations, and a number of computer peptide-modeling studies, the group led by Wender, ran a comparative study to analyze the influence of backbone spacing for cell uptake (Rothbard et al., 2002). One of the most efficient arginine-rich peptides contained a 6-aminohexanoic spacer – $(RxR)_4$ peptide – which has, already, been shown to be successful in the delivery of diverse nucleic acids (Amantana et al., 2007; Marshall et al., 2007). The combination of this type of systematic and comparative studies with those on cellular-internalization mechanisms of the nucleic acid nanoparticles contribute for a better understanding of the delivery process, in general, and for the carrier design and behavior predictions, in particular.

2 AIMS

The potential of gene therapy has never been questioned. However, to accomplish treatment by a therapeutic approach directed to the disease's site of origin requires a clear comprehension of the delivery process as well as carrier design in relation to its cargo. With the presented thesis I aimed at a better understanding of the interaction between amphiphilic peptide-based vectors and nucleic acids (namely, DNA and oligonucleotides) and the properties of the ensuing nanoparticles in connection to their activity.

More specifically, the aims of this thesis were:

- To analyze the influence of the length of single chain fatty acids conjugated to spermine in relation to the biophysical properties of the resulting DNA nanoparticles and their delivery ability. (***Paper I***)
- To evaluate the effect of different amino acid modifications on the ability of PEI to deliver splice-switching oligonucleotides and establish possible relations between the amino acid modifications, the properties of the nanocarriers and their activity. (***Paper II***)
- To assess the repercussions of stearyl-conjugation onto the cell-penetrating peptides Arginine 9 and $(RxR)_4$ and elucidate the stearic acid contributions to increased activity of stearyl-$(RxR)_4$. (***Paper III***)
- To appraise DNA delivery by a novel class of peptides, D-diaminopropionic acids-based, that share similarities with those that are arginine and histidine-based. (***Manuscript IV***)

3 METHODOLOGY

3.1 FORMULATION OF NUCLEIC ACIDS

For *papers I*, *II* and *manuscript IV*, formulations of DNA and oligonucleotide nanoparticles were prepared by equivolumetric mixing of the nucleic acid at the desired concentration with the corresponding carrier (fatty acid-spermine, PEIY or $Dapa_8$ peptide conjugate) at various concentrations corresponding to the different charge ratios (NH_3^+/PO_4^-). For transfection purposes in particular for the case of antisense oligonucleotides (ASO), the nanoparticles were left at room temperature (RT) for 30 minutes, to allow the mixture to equilibrate, before addition to serum-free Dulbecco's modified Eagle's medium (DMEM).

For *paper III*, the nucleic acids were first added to milli Q water and thereafter the calculated amount of CPPs was mixed to achieve the desired charge ratio. Nanocomplexes were prepared in $1/10^{th}$ of the final volume and left to equilibrate at RT for one hour, before diluting the solution in serum-free DMEM for cell transfections purposes.

3.2 EVALUATION OF THE DEGREE OF DNA CONDENSATION AND PROTECTION.

3.2.1 Gel retardation assay

Interaction between the gene delivery vector and the nucleic acid can be detected by an electrophoretic mobility-shift assay, being identified as a retardation in the pDNA migration. This retardation is typical for the formation of larger particles as compared to pDNA alone. For *paper I* and *manuscript VI*, DNA interaction was analyzed by an electrophoretic assay, in 0.8% agarose gel (in 40 mM Tris-acetate, 20 mM sodium acetate and 1 mM EDTA - TAE buffer – pH 7.8). DNA nanoparticles were prepared in 5.45% mannitol solution at a DNA concentration of 0.1 μg/μl. DNA complexes were prepared at different charge ratios by varying the concentrations of fatty acid spermine conjugate.

3.2.2 Ethidium bromide exclusion assay

Ethidium bromide (EtBr) is a DNA intercalating dye that when bound, and under ultra violet light, fluoresces with an orange colour. This property allows for detection of DNA that is available for the intercalation by EtBr. In *paper III*, this technique was used to assess the DNA degree of condensation by the CPP carriers. DNA complexes were prepared and let to equilibrate for 1h at RT, time after which EtBr solution was added, up to a final concentration of 400nM. Fluorescence was measured in black 96-well plates, 10 min after the EtBr addition, at λex = 518 nm and λem = 605 nm. Results are presented as relative fluorescence, with reference to the fluorescence emitted by the intercalation of EtBr with naked DNA.

3.2.3 DNase I protection and serum stability assays

DNase, or deoxyribonuclease, is a class of enzymes that catalyzes the hydrolytic cleavage of phosphodiester linkages in the DNA backbone. The most common enzyme is the restriction enzyme DNase I, an endonuclease that cleaves nucleic acids starting in the middle of the strand, preferentially at phosphodiester linkages adjacent to a pyrimidine nucleotide. DNase I recognizes single-stranded DNA as well as double-stranded. DNases are present in human serum (Wroblewski and Bodansky, 1950) and compose one of the physiological barriers imposed by the living organism to gene drug delivery. By subjecting DNA nanoparticles to the activity of this enzyme it is possible to evaluate the protection provided by the carrier to the nucleic acid, by analyzing the intact material.

For this assay, in *paper I* and *manuscript IV*, DNA complexes were first incubated with 5 U DNase I/µg of DNA for 15 min. Reaction was stopped by addition of EDTA up to 0.05 M, pH 8.0. EDTA is known to complex with ions that are required for the activity of the enzyme. Sodium dodecyl sulfate (SDS) was then added, up to 0.1% (w/v) concentration, to induce the complete release of DNA from the positively charged carrier (by ion competition), and facilitate the gel analysis. DNA was further purified using PCR purification kit (Qiagen)

before being submitted to electrophoresis in agarose gel containing ethidium bromide.

An alternative to DNase I digestion, and that better mimics the *in vivo* scenario, is to submit the DNA nanoparticles to serum conditions. In *paper III*, the stability of the DNA/$(RxR)_4$ particles was evaluated by the presence of serum, in different concentrations (5, 10 and 50%). Samples were incubated during different times (0, 1, 4 and 24h) and thereafter analyzed in agarose gel (2%), containing EtBr.

3.3 EVALUATION OF OLIGONUCLEOTIDE POLYPLEX STABILITY: POLYANION COMPETITION ASSAY.

Relative stability of oligo polyplexes can be evaluated by addition of anionic molecules that can compete with the carrier for the nucleic acid binding availability. The stability is higher when increased concentrations of the competing anion are required.

In *paper II*, the stability of ASO complexes was evaluated by following the dissociation of the oligonucleotides from the polyplexes in the presence of heparin. All nanocomplexes were prepared at a charge ratio of 20:1 and were incubated for 15 min, at 37 °C in presence of heparin sodium over a range of concentrations. Samples were thereafter analyzed in 1.5% agarose gel and visualized by staining with sybergreen II. At certain heparin concentration, the percentage of ASO released was calculated by normalization against nonformulated ASO.

3.4 TOXICOLOGICAL ASSESSMENT OF NUCLEIC ACID POLYPLEXES

In vitro cytotoxicity assays are used for general screening of chemicals and can be used to predict human toxicity (Clemedson and Ekwall, 1999; Scheers et al., 2001). Cytotoxicity assays based on different mechanisms of action will provide information on the toxic properties of the drug in relation to the specific pathway that is being assessed. Therefore, the same agent may result in different degrees of toxicity (differ in Ec_{50}) depending on the assay employed (Fotakis and Timbrell, 2006).

3.4.1 MTS/WST-1 assays

With these assays cell cytotoxicity is directly correlated to the amount of formazan dye formed, resultant from the cleavage of tetrazolium salts by mitochondrial dehydrogenases. For all papers and manuscript composing this thesis, the cytotoxicity of the different nanoparticles was assessed according to the protocol provided by the manufacturer. In brief cells were seeded in 96-well plates and maintained in DMEM supplemented with 10% FBS (Fetal Bovine Serum) 24h before transfection. The culture medium was replaced by serum-free DMEM including various concentrations of nucleic acids alone or formulated as previously described. After 4h incubation at 37^{o}C, the cell medium was replaced by DMEM supplemented with 10% FBS. Cells were further incubated for 21h. The number of surviving cells was determined by the WST-1/MTS assay. Cell proliferation was expressed as the ratio of the A_{450} of treated cells to that of the untreated cells. Visual inspection of the cells correlated well to the absorbance reading.

3.4.2 xCELLigence System (Roche)

The xCELLigence system measures electrical impedance across interdigitaded micro-electrodes that are placed on the bottom of tissue culture E-Plates (96-well plates) (Xing et al., 2005). The presence of the cells on top of the electrodes will affect the local ionic environment at the electrode/solution interface, leading to an increase in the electrode impedance. The impedance also depends on the quality of the cell interaction with the electrodes, and variations in cell morphology will cause a change in electrode impedance, which is displayed as cell index (CI) values. This approach can be used to assess cell viability, number, morphology, and adhesion degree in a number of cell-based assays. In comparison to other cytotoxic methods, the xCELLigence system holds the advantage of monitoring cellular events in real time without the need of labels.
In *paper II*, xCELLigence system was used to assess the cytotoxic properties of PEIY and PEIY/ASO complexes and to complement the information provided

by the WST-1 proliferation assay. Briefly, 1.5×10^4 and 3×10^5 BHK and HeLa705 cells, respectively, were seeded in the E-plate and cultured for approximately 20 h at 37 °C, 5% CO2/air, before the addition of PEIY or PEIY/ASO. Changes in the cell status were monitored and quantified by detecting sensor electrical impedance every 15 min during 45 h. CI was derived to represent the cell proliferation based on the measured electrical impedance. The presented "normalized cell index" results from the ratio between the cell index at the time point immediately before the addition of the PEIY or PEIY/ASO and that at the time point 0 h.

3.4.3 LDH leakage assay

Lactate dehydrogenase (LDH) is an enzyme that exists in the cytoplasm of cells and catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. The LDH leakage assay measures the activity of the enzyme in extracellular environment (Decker and Lohmann-Matthes, 1988); It is, therefore, an indicative of cell death due to membrane disruption.

For paper III, cells were treated in an analogous way as for MTS assay, with untreated cell defined as 0% leakage and lysed cells as 100%.

3.5 BIOPHYSICAL CHARACTERIZATION STUDIES: SIZE AND ZETA POTENTIAL OF NUCLEIC ACID-CONTAINING PARTICLES

3.5.1 Dynamic light scattering

Dynamic light scattering (DLS), also referred as photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QLS), is a technique used to measure particle size, typically in the sub micron region. In fact, DLS measures Brownian motion and relates this to the particle size, translating it into hydrodynamic diameter. The technique assumes that the particles have a spherical shape, and their size relates to how the particles diffuse within a fluid. Thus, parameters such as the surface structure of the particles, and concentration and type of the ions present in solution influence the result. This means that the

size can be larger than measured by electron microscopy, for example, where the particle is removed from its native environment.

Stability and hydrodynamic mean diameter of the DNA nanoparticles were determined by dynamic light scattering studies using a Zetasizer Nano ZS apparatus (Malvern Instruments, United Kingdom). When in mannitol solution (5.45%), the solvent was introduced in the system as a complex solvent and measurements were done using a refractive index of 1.338 (*paper I* and *manuscript IV*). For *paper II*, particle formulation followed the same protocol as for *in vitro* transfections, with a final concentration of 0.1 μM, and, accordingly, dilution was performed in FBS supplemented medium. In order to avoid light absorbance, experiments were performed in Opti-MEM® medium. Specifications of measurements inherent to the medium used followed those previously reported (Creusat and Zuber, 2008). For *paper III*, pDNA complexes resulting from the addition of stearyl-$(RxR)_4$ were formulated according to the protocol for *in vitro* transfection. Briefly, pDNA was formulated in deionized water and after 30 min incubation at RT, the polyplexes were diluted Opti-MEM® into a final volume 200 μl. All DLS measurements were conducted in disposable low volume cuvettes. The results were based in three measurements from three independent samples. All data were converted to "relative by intensity" plots from where the mean hydrodynamic diameter was derived.

3.5.2 Zeta potential

Measurement of zeta potential is needed to assist in the formulation of stable products, namely in drug formulation, to assess the charge stability of a disperse system. When dispersed in an aqueous system most particles have a surface charge that results from either ionization of surface groups or adsorption of charged species. These surface charges act on the distribution of the surrounding ions, resulting in a layer around the particle, different from that of the bulk solution, which moves as part of the particles. The zeta potential is defined as the potential at the point in this layer where it moves past the bulk solution, and the charge at this plane is sensitive to the concentration and type of ions in solution. Zeta potential is measured by applying an electric field across the

dispersion, using the technique of laser Doppler anemometry. Particles within the dispersion with a zeta potential will migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. As the particles move in the electric field they cause a shift in the incident laser beam. The frequency of this shift is measured as the particle mobility and is converted into zeta potential by the application of Smoluchowski or Huckel theories, and by taking into account the dispersant viscosity. When measuring zeta potential in a complex system (such as one relying on non-covalent interactions, for instance nucleic acid polyplexes) there are a number of considerations to account for: (*i*) zeta potential may, or may not, be related to the surface charge; (*ii*) it can even be of opposite charge sign to the surface charge, and, most importantly, (*iii*) it is the zeta potential, and not the surface charge, that controls charge interactions.

For *paper I*, zeta potential was measured in a Zetasizer Nano ZS apparatus (Malvern Instruments, United Kingdom), in a single use folded capillary cell, in 0.1μM potassium chloride solution.

3.6 GENE EXPRESSION STUDIES

3.6.1 *In vitro* gene expression

In vitro transfections are an easy and fast method to assess the delivery capacity of nucleic acid carriers. Conditions such as the transfection media, cells type and density, and concentration of the nucleic acid and delivery vector are usually relevant and can strongly influence the delivery efficiency. For these reasons often different delivery systems have different transfection protocols. For *paper I* and *manuscript IV* the protocol used for pDNA transfection was the following: one day prior to transfection, BHK (*paper I*) and U2-OS cells (human osteosarcoma) (*manuscript IV*) were seeded at a density of 50 000 cells per well in 24-well plates, at a final volume of 500 μl. DNA was formulated with the different peptides at ratios ranging from 0.3:1 to 20:1, with final DNA concentrations between 0.5-2 μg per well. DNA complexes were prepared in 300 μl volume, either in serum-free DMEM, Hank's Buffered Salt Solution (HBSS) or mannitol iso-tonic solution, and added to the cells after 20 minutes

incubation at RT. After 4h the cells' medium was replaced by serum-supplemented DMEM. Cells were harvested 48 hours post-transfection and the cell lysate was analyzed in a luminometer for Luciferase activity. Luciferase activity was measured using Promega's luciferase assay system on a 96 microplate luminometer (Fluostar optima) and normalized to protein content (measured by a micro BSA protein assay kit, by Promega). Lipofectamine® (Invitrogen) was used as a positive control and transfections were performed according to the protocol provided by the manufacturer.

For *paper III*, additional cells were used, namely Chinese hamster ovary (CHO) and Human embryonic kidney (HEK) and the charge ratios of CPP/plasmid complexes used were 1:1, 2:1, 3:1 and 5:1.

For studies on oligonucleotide delivery (*paper II* and *paper III*) the cells used have been developed in the laboratory of Dr. R. Kole (Kang et al., 1998). These modified HeLa705 allow for a reliable assessment of nuclear delivery of antisense oligonucleotides by a positive Luciferase activity read-out. HeLa cells have been stably transfected with a coding sequence of Luciferase reporter gene that is interrupted by the human β-globin thalassemic intron 2, which carries a cryptic splice site. The aberrant splice site prevents removal of the intron, thereby resulting in defective splicing and consequent dysfunctional protein. Blocking the mutation with antisense splice-switching oligonucleotides corrects the splicing and results in translated mRNA corresponding to the active form of the protein. Apart from the cell type, the oligonucleotide transfections followed the same protocol as for plasmid delivery.

3.6.2 *In vivo* gene expression

Muscle and skin are attractive target tissues for DNA vaccination purposes. Additionally, the muscle also offers the possibility to generate gene expression of a protein with a systemic effect, such as neurotrophic factors. In *paper I* and *manuscript IV,* we have tested the different plasmid DNA complexes for their *in vivo* delivery by local administration into the dermis and the *M. tibialis anterior*. All animal experiments were approved by The Swedish Local Board for Laboratory Animals.

Briefly, male BALB/c mice aged 10-13 weeks were first anaesthetized with isoflurane gas (400 ml air flow and 4% isoflurane) and kept under anesthesia (220 ml air flow and 2.2% isoflurane) during the administration procedure. 50 µl containing 5 µg DNA, either of pDNA or pDNA-nanocomplexes, were injected intradermally (i.d.) or intramuscularly (i.m.). At least 4 replicates for each formulation were used for both intramuscular and intradermal studies. Gene expression was assessed by imaging of the reporter gene (firefly luciferase) expression. On days 1, 3, and 7 after injections the mice were anaesthetized and injected intraperitoneally (i.p.) with 150 mg/kg (~3 mg/mouse) of D-Luciferin (Xenogen, Alameda, CA). Light signals (CCD) images were obtained using a cooled IVIS CCD camera (Xenogen, Alameda, CA), and images were analyzed with IGOR-PRO Living Image Software, which generates a pseudo-image with an adjustable color scale. The maximum photon/second of acquisition/cm2pixel/steridian was determined within a region of interest to be the most consistent measure for comparative analysis. In general, acquisition times ranged from three to five min.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Polyamines (spermidine, spermine and diamine putrescine) are naturally occurring molecules involved in many cellular processes, including chromatin condensation and DNA maintenance (Childs et al., 2003). Nevertheless, and despite the fact that the spatial molecular arrangement in between their positive charges fits perfectly with that in between the phosphates in the DNA molecule, suggesting an ideal interaction fit, spermine was shown to bind poorly to DNA resulting in no DNA protection against nuclease degradation (D'Agostino et al., 2005). Notwithstanding the extensive investigation on different types of lipospermines (Ahmed et al., 2006; Blagbrough et al., 2003; Gaucheron et al., 2001; Hosseinkhani and Tabata, 2006; Kichler et al., 1998; Tuszynski et al., 2005) these typically either contain double conjugations or have sixteen, or longer, hydrocarbon chains, which makes the fatty acid-spermines herein developed novel. Importantly, although DNA binding affinity has been suggested to be a function of both charge and hydrophobicity (Geall et al., 2002; Geall and Blagbrough, 2000) to what degree this affinity is beneficial for gene delivery is not clear. In the present paper we aimed at the evaluation of the hydrophobic contributions of fatty acid-spermine conjugates to DNA binding and the properties of the DNA nanoparticles. We have, therefore, introduced six amphiphilic molecules – fatty acid spermine conjugates (lipospermines) – that we have characterized as DNA delivery vectors in a comparative study where we differ the length and saturation level of the alkyl chain in well-defined and well-characterized lipospermines.

A series of studies, to assess DNA condensation and protection properties as well as biophysical and morphological features of the different DNA nanoparticles, allowed for the investigation of the influence of the length of single fatty acids conjugated to spermine in relation to the biophysical properties of the resulting DNA nanoparticles and their delivery ability. Additionally, these studies led to the finding of the most suitable candidates for *in vivo* gene delivery. Although steadily increasing, the number of reports with *in vivo* gene

expression data is hitherto behind its *in vitro* counterpart and correlation studies between the biophysical properties of the DNA particles and their delivery aptitude are few.

We showed that an increase in the length of the hydrocarbon chain resulted in enhanced gel retardation of DNA migration, which could indicate higher affinity. However, newly formed particles also became larger as the molecular weight of the spermine conjugates increases, contributing to lower gel mobility. Results from DNase I protection assay suggested that protection of the DNA, at equal concentrations, was more prominent with lipospermines having longer hydrocarbon chains and that the degree of saturation affects these properties – a trend also observed by Abbasi *et al.,* while using lipid-substituted poly-L-lysine (PLL) (Abbasi et al., 2008). Further studies on zeta potential also supported the contribution of the hydrophobic interactions to the formulation of DNA nanoparticles: regardless of the fact that all lipospermine have the same formal charge (+3), the pDNA complexes showed a fairly large range of potentials for the different nanocomplexes formulated at the same charge ratio. These findings suggested that, when using equal amounts of carrier in relation to DNA, the number of lipospermine molecules per DNA complex differs between the diverse DNA nanoparticles. The variation found could be attributed to the differences in the carbon chain for the butanoyl- and decanoylspermines with the longer chain presenting a more negative potential. Hence, interaction of butanoylspermine with plasmid DNA appears closer to a pure cationic interaction: the zeta potential of butanoylspermine/DNA complexes presented values close to neutralization with no significant changes within the range of studied concentrations. Also, the behavior expressed by butanoylspermine/DNA nanoparticles in the dynamic light scattering studies is typical for DNA complexes formed by cationic peptides: the improvement of the quality of the DNA formulation within a short range of charge ratios tested (from 0.6 – 2:1), as a consequence of the increase in concentration.

Conversely to butanoylspermine, decanoylspermine-derived particles displayed a clear concentration-dependent behavior with DNA complexes formed at higher

charge ratios resulting in more stable and homogenous particle population. These observations suggested a more balanced contribution from both charged and hydrophobic components towards the properties of the nanoparticles.

Butanoyl- and decanoylspermine were among the conjugates that displayed the lowest *in vitro* cytotoxicity. Cytotoxic evaluation of lipospermines showed that an increase in the length of the hydrocarbon chain induced increased toxicity, which is not unexpected. It is well known that longer aliphatic chains grant the molecules more detergent-like properties and could, thus, increase cell membrane disruption and induce subsequent cell death. On the other hand, inclusion of lipid moieties in the gene carrier could be advantageous to facilitate cell entry.

Owing to their degree of toxicity, palmitoyl and oleyl-derivatives were excluded from the *in vivo* studies. Linoleyl and linolenoyl-derivatives, on the other hand, underwent oxidation after repeated handling, and air exposure, and were excluded from further characterization studies. Butanoyl and decanoylspermine were, therefore, tested as DNA carriers for *in vivo* gene delivery in the skin and muscle. Although, at the used concentrations, both considerably improved gene expression in relation to naked plasmid, only butanoylspermine induced significant increases. Addressing the correlation between the properties of the DNA particles and their *in vivo* delivery ability, we observed that common features of these DNA nanoparticles were their average size range (100 – 200 nm), their moderate binding affinity (as showed by the gel retardation assay) and their neutral, or slightly negative, zeta potential.

Delivery systems that are efficient in the skeletal muscle or skin may be used for the purpose of DNA vaccine development. In addition, in the muscle they can also be of importance to progress in the treatment of disorders caused by lack of an active protein, that has a systemic effect and can be produced in the muscle and thereafter secreted, e.g. Anderson-Fabry disease. Lavigne *et al.,* have designed a chimeric vector to target skeletal muscle (Lavigne et al., 2008), however, the dose of DNA used was twice as high as that used in this work. DNA

complexed with these novel short lipospermines increased luciferase activity by a factor of 10 over that of naked pDNA. These results are comparable to some of the work done with the pluronics block coplymers, which have been, and continue to be, extensively explored (Bello-Roufai et al., 2007; Gaymalov et al., 2009; Pomel et al., 2008). Much alike these, as well as other vehicles, *in vitro* transfection activity of the lipospermines was poor (Chang et al., 2007; Pomel et al., 2008). Uptake studies, as well as intracellular localization, could be useful to determine the reason for such a phenomenon. However, due to their *in vivo* gene expression the most likely cause for the observed poor *in vitro* gene expression is hindered cell uptake. In view of the fact that plasmid DNA, alone, has been used for intramuscular gene delivery and proven very efficient (Wolff and Budker, 2005), in a therapeutic setup, a ten-fold enhancement of the protein expression, similar to what is seen with butanoylspermine/DNA formulations, could mean a substantially reduced administration dose.

4.2 PAPER II

PEI is an organic macromolecule with a high cationic charge-density, where every third atom is an amino nitrogen that can be protonated. This property is responsible for the protonation of PEI at low pH conditions, such as those found in the intracellular endosome. Therefore, upon endocytosis-mediated cellular uptake, PEI induces an osmotic unbalance inside the vesicle, thereby causing endosomal burst (and consequent release of its DNA cargo). First reported as an efficient transfection agent in 1995 (Boussif et al., 1995), PEI has since been used in a number of studies, modified in different manners to deliver diverse nucleic acids (Kichler, 2004; Malek et al., 2009; Merdan et al., 2005).Although unmodified PEI has been shown to be very efficient for plasmid delivery (Lemkine and Demeneix, 2001), modifications were required for its application for short oligonucleotides (Alshamsan et al., 2009; Creusat and Zuber, 2008). A possible explanation might be that the interaction between oligonucleotides and PEI is weak and does not support competition by other polyanions, causing the polyplex to dissociate upon contact with polyanions at the cell surface or present in solution. In the present study we

have modified PEI by introducing amino acid moieties: leucine (L), phenylalanine (F), tryptophan (W), and tyrosine (Y). Since these amino acids are known to constitute the core of globular proteins and aid to their stability, we hypothesize that these amino acid-conjugated PEI molecules could result in relatively stable and non-toxic oligonucleotide particles. Importantly, these conjugates were shown to maintain their "proton-sponge" property and the tyrosine-modified PEI (PEIY) was shown to very efficiently deliver siRNA (Creusat and Zuber, 2008). Herein, we sought to investigate if the dissimilarities between siRNA and antisense 2'-*O*-methyl phosphorotioate-modified RNA (ASO) could have significant differences on the nucleic acid/PEI interaction and whether this would translate into activity. Moreover, we set to analyze some of the properties of the nucleic acid particles resulting from the different modified PEIs in relation to their activity.

Analogously to the study by Creusat *et al.* on siRNA delivery, tyrosine-modified PEI was the conjugate that resulted in higher activity and significantly enhanced ASO delivery. When formulated with ASO at charge ratios of 20:1, or higher, PEIY induced a minimum of 80% splice correction of the defective transcript, in this case the Luciferase gene. A 20:1 ratio was preferred by its high activity and low cyotoxic effects. Importantly, this correction level was significantly higher than that resulting from the commercial transfection reagent Lipofectamine, and enhanced compared to similar reports (Hassane et al., 2009; Kang et al., 1999). Also the correction at the protein level was remarkable, 450-fold for a concentration of 1μM ASO and approximately 120-fold for 0.025μM ASO. Different delivery capacities correlate to different particle features and herein we analyzed the stability (upon anion competition) and the mean hydrodynamic diameter of the diverse ASO nanocomplexes. Our results support the initial hypothesis that the interaction between ASO and PEI is weak, as shown by the dissociation of the complexes upon addition of serum-containing medium (by the dynamic light scattering studies) and the low concentration of heparin required to release the ASO in the polyanion competition assay. This is most likely the reason for poor transfection activity, although complementary studies would be required to

strengthen this argument. Similar behavior was shown by ASO/PEIL particles. Conversely, ASO complexes resulting from PEIW and PEIF even at the highest concentrations of heparin herein used were not able to release more than 50% of the nucleic acid, suggesting a very high stability and a resistance to dissociation. Stability is known to play an important role in any delivery system, as the cargo must be protected from degradation. Nevertheless, the drug must, simultaneously, be able to release its active component, in this case the nucleic acid, once it reaches its target site. Consequently, carriers that strongly bind to their cargos may hamper their activity by creating shell-like particles unable to dissociate.

DLS studies revealed that both PEIY and PEIW had self-assembly properties, but PEIY formed significantly smaller particles (both with and without oligonucleotides), also in relation to PEIF. Since splice-switching takes place in the nucleus, it is possible that the particle size influences their activity.

The discrepancy of the PEIW and PEIF polymers in relation to the delivery of the two types of oligonucleotides (siRNA *versus* antisense 2'-*O*-methyl phosphorotioate-modified RNA) is not surprising since these two strategies rely on oligonucleotides of unequal chemistry as well as on delivery into different intracellular compartments (Laufer et al., 2009).

In conclusion, we have evaluated several amino acid modified-PEIs for the delivery of ASOs and identified tyrosine-conjugated PEI as a potent carrier for antisense 2'-*O*-methyl phosphorotioate-modified RNA. At optimal conditions, formulated at the charge ratio of 20:1 and in presence of serum, polyplexes of PEIY/ASO induced approximately 85% correction of aberrant mRNA and an increase in protein activity of 450-fold. We have also verified the relevance of hydrophobic contributions for the stability and efficiency of these polyplexes. In addition, we were able to show correlations between the size and stability of the ASO complexes and their activity.

4.3 PAPER III

Arginine-rich peptides are one of the most widely used classes of cell-penetrating peptides. Following the initial findings of Wender *et al*. on the delivery abilities of $(RxR)_4$ (Rothbard et al., 2002), an oligoarginine analogue, this CPP has been used for *in vitro* and *in vivo* delivery of splice-switching oligonucleotides (SSOs) (Abes et al., 2008; Fletcher et al., 2007). In recent years, hydrophobic modifications, in particular stearylation, has gained attention by improving the delivery abilities of the carrier not only for short oligonucleotides but also for plasmid DNA. Herein we set to investigate the effect of stearylation to the capacitiy of $(RxR)_4$ peptide to deliver DNA and SSOs by a non-covalent strategy, and to identify the delivery stages to which this modification has proven to be a useful asset. Additionally, we have compared the stearylation effect of $(RxR)_4$ peptide to that of another arginine-rich peptide, Arginine 9 (Arg9).

Since $(RxR)_4$ peptide had not been used for plasmid delivery we first analyzed its ability to condense and protect DNA. It was clear from the EtBr exclusion assay that the DNA was less available to bind the dye when co-incubated with $(RxR)_4$ or steraylated $(RxR)_4$ in comparison to stearyl-Arg9, suggesting a stronger binding to the $(RxR)_4$ derivatives. The formation of DNA particles was further confirmed by DLS studies, where stearyl-$(RxR)_4$ was shown to form particles with hydrodynamic average diameter of 450 nm (3:1 charge ratio) and of 370 nm (1.5:1 charge ratio). Interestingly, the difference in DNA transfection efficiency between the two stearylated peptides, $(RxR)_4$ and Arg9, was striking, with the stearyl-$(RxR)_4$ exhibiting approximately 170-fold increase in luciferase expression whereas stearyl-Arg9 resulted in very low expression. The addition of chloroquine (an endosomal disruption agent) to the transfection protocol suggested endosomal entrapment as the most plausible reason for the low expression values by the Arg9 peptide. While stearyl-$(RxR)_4$ already significantly improved DNA delivery and expression in relation to its parental form, the presence of chloroquine further enhanced Luciferase expression,

thereby suggesting room for improvement regarding the peptide design in relation to this physiological barrier. Nevertheless, taking into consideration that the difference of arginines between the two CPPs is not significant, and that both were conjugated to stearic acid, the remarkable divergence in gene expression underlines the relevance of the detailed structure of the carrier for the delivery.

Notwithstanding the lower DNA expression resulting from stearyl-$(RxR)_4$/DNA complexes in comparison to the commercial transfection reagent lipofectamine^{TM} 2000, the cytotoxic properties of the CPP were still significantly lower, giving it an advantage in relation to the lipid agent. Furthermore, transfection studies with plasmid encoding for green fluorescent protein (GFP) revealed that stearyl-$(RxR)_4$ is able to transfect whole cell population whereas lipofectamine^{TM} 2000 transfects part of the population, although capable of higher per cell transfection efficiency (with the GFP signal being stronger per cell than that of stearyl-$(RxR)_4$ transfected populations).

Since $(RxR)_4$ has been used for SSOs, although in covalent strategies, we sought to investigate the stearic acid-modified CPP for oligonucleotide delivery but in a non-covalent approach. Unmodified $(RxR)_4$, and stearyl-Arg9, did not result in splice-correction but the stearylated $(RxR)_4$ was able to induce almost 20-fold increase in Luciferase expression, when combined with SSOs at molar ratio 3 and in the absence of serum. Moreover, in relation to covalent strategies, the quantities of SSOs used in the present study were approximately ten times lower and resulted in comparable splice-correction levels. Uptake studies conducted with Cy5-labeled oligonucleotides showed no differences in cell uptake between the parental and the modified CPP, thereby suggesting that this was not hindering $(RxR)_4$ activity. The mechanism by which stearylation increases cytoplasmatic and nuclear delivery is not yet clear. However, this modification renders the peptide more hydrophobic and enables enhanced condensation of plasmid and oligonucleotides, presumably facilitating more stable nanoparticle formation. Probably, and also as suggested by our studies, stearic acid modification facilitates the peptide interplay with endosomal membranes, thus enabling the endosomal escape.

4.4 MANUSCRIPT IV

D-diaminopropionic acid-based peptides, $Dapa_8$, belong to a novel class of molecules that share similarities with arginine and histidine-based peptides. Dapa has an intermediate pKa (lower than arginine but higher than histidine), which makes them interesting constituents in peptides for nucleic acid delivery. In addition, polymers of D-diaminopropionic acids are more resistant to degradation, which is advantageous for *in vivo* delivery. Thus, herein we have explored a new class of cationic peptides, containing D-diaminopropionic acids, as potential DNA delivery agents. For the reason that the inclusion of hydrophobic moieties into peptides has been reported to contribute to the interaction with nucleic acids and also to improve cellular uptake and endosomal escape (Lehto et al., 2009; Mae et al., 2009), we have conjugated a series of different fatty acids to $Dapa_8$ peptides to generate a library of five alkyl-conjugated $Dapa_8$ peptides.

We first verified possible interactions between DNA and $Dapa_8$ peptides by an electrophoresis shift-assay that showed that all $Dapa_8$ peptides interacted with plasmid DNA. Similar to fatty acid-spermine conjugates (Viola et al., 2009), this interaction was concentration dependent and was more evident with increasing length of the alkyl chains. The formation of DNA particles was confirmed by DLS studies. In addition, DLS allowed the assessment of biophysical properties, such as size and over time stability, of the nanoparticles formed upon interaction with the different peptides. Interestingly, the tendency was that short fatty acid-conjugated $Dapa_8$ formed smaller particles and, in general, the particle size decreased with increasing peptide concentrations. All DNA complexes were characterized by an average diameter between 75 and 170 nm and were stable over 24h. Surprisingly, even palmitoyl-conjugated $Dapa_8$ formed stable and well-defined particles (with an homogeneous distribution), whereas palmitoyl-conjugated spermines (Viola et al., 2009) resulted in a heterogeneous and large-sized population.

An MTT-based cytotoxicity assay further supported the potential use of these peptides as carriers. Within the evaluated concentration range, none of the peptides affected cell viability of Baby Hamster Kidney (BHK) cells. In contrast, the previously investigated lipid-spermines significantly decreased cell viability when conjugated to oleyl and palmitoyl, the longest fatty acids used in these studies. Interestingly, di-substituted spermines used for siRNA delivery appeared to behave in an opposite manner: decanoyl, lauroyl and myristoyl, the shorter chains, resulted in the lowest cell viability (less than 20%) (Soltan et al., 2009). For carrier design purposes these studies underscore the significance of cargo used (DNA *versus* siRNA) and, importantly, the number, and position, of the fatty acid conjugation sites (N-terminal *versus* N^4 and N^9).

Unfortunately, none of the $Dapa_8$ conjugates was able to induce *in vitro* Luciferase expression. Identification of the delivery stage that the carriers could not surmount (cell uptake or endosomal escape) can be informative to improve the activity of the peptides.

However, since other delivery systems have also been shown to have little *in vitro* activity but are efficient when locally administered *in vivo* (Pomel et al., 2008; Viola et al., 2009), we have investigated the $Dapa_8$ conjugates for *in vivo* delivery (in the muscle *tibialis anterior* and in the dermis). All peptide/plasmid particles resulted in a level of Luciferase expression comparable to that of naked plasmid, when formulated at charge ratios between 0.3:1 and 2:1, hereby not improving DNA delivery. Intramuscular injections share similarities with hydrodynamic injections regarding the high volume that is confined in the compartment and the local pressure that is thereby built up inside the muscle. In the case of hydrodynamic injections, uptake of different molecules (nucleic acids, proteins, peptides and inert polymers) by hepatocytes has been reported to differ between molecules within the same range of particle size (Sebestyen et al., 2006). Particle sizes for the $Dapa_8$ peptides and the fatty acid-spermines are within the same range, with $Dapa_8$ derivatives forming a smaller-sized and more homogeneous particle population. Both these different classes of potential carriers failed to enhance gene expression *in vitro* whereas two spermine derivatives significantly

enhanced Luciferase expression in mice. Although DNA compacted into nanoparticles may in many cases be taken up more readily by cells as compared to its naked counterpart, it is clear that the particle size is not a determinant factor for efficient delivery. Moreover, in view of the fact that Arg_8 mediates *in vitro* gene expression and that fatty acylation enhances its activity (Khalil et al., 2004), it is surprising that, owing to their similarity, neither $Dapa_8$ peptides nor spermine derivatives were able to comparable DNA delivery and expression. Regardless of the possible "proton sponge" effect from the $Dapa_8$, the structural difference between this peptide and Arg_8 is unambiguously the dominating factor to determine its efficiency as a DNA delivery vehicle. Further investigation, such as cellular uptake studies or assessment of the stability of the complexes in transfection medium, may aid to the identification of the major limitation of these peptides. Nevertheless, additional modifications, or possibly *de novo* synthesis design, are required to accomplish efficient DNA delivery.

5 CONCLUSION AND FUTURE PERSPECTIVES

In 1973, Graham and van der Eb reported on successful delivery of plasmid DNA into cells by using calcium phosphate as "transfecting agent" (Graham and van der Eb, 1973). Presently, there are still studies relying on this first approach, refining the early strategy with the current knowledge on gene delivery (Giger EV, J Control release 2011; Liu Y, Wang T, Int J Nanomedicine, 2011). Interaction between cationic peptides and nucleic acids is still often considered to be mainly electrostatic. This general belief is based on the observation that DNA condensation – the collapse of extended DNA chains into compact particles – typically results from neutralization of the negative charges of the DNA phosphate groups. This finding paved the way for the development of numerous delivery vehicles with high-density charge, like the well-known cationic PEI or polylysine. Nevertheless, soon it was found that increasing the concentrations of the cationic transfection agents often inhibited gene expression, likely imparted by a tight binding between carrier and nucleic acid. This subsequent non-dissociation of the nucleic acid, required for its activity, has been confirmed herein for the case of tryptophan and phenylalanine-modified PEIs (*paper II*).

Hydrophobic interactions involving DNA have been reported (Geal AJ, 2000; Patel and Anchordoquy, 2005) and a number of studies have used lipophilic moiety-conjugation to improve the activity of the parental carrier. These hydrophobic modifications can either increase the binding affinity of the carrier towards the nucleic acid (such as the case of lipospermines, in *paper I*) or decrease the electrostatic contributions towards a more moderate interplay carrier-nucleic acid (such as the case of the amino acid modification in PEI, in *paper II*). In addition to modulate this interaction, fatty acid-modifications can influence the cellular uptake as well as the endosomal escape of the nucleic acid drug, by interacting with cell membranes (as suggested in *paper III* in regards to stearyl-modification of $(RxR)_4$ peptide). However, this ability often results in increased toxic properties of the delivery system (as seen for the case of lipospermines, in

paper I), and therefore the lipophilic modification needs to be balanced towards other properties of the delivery system. An alternative to fatty acid-modifications is the inclusion of hydrophobic moieties, such the amino acids approach used in *paper II*, or the substitution of few positively charged groups by more inert components, such as glucose (Strand et al., 2010).

Several observations underscored the relevance of both components, hydrophobic and cationic, of the carrier. With regards to fatty acid-modified spermine (*paper I*), longer fatty acid tails seemed to result in enhanced DNA interaction, as reflected by greater retardation of the pDNA migration in agarose gel-shift assays. This apparent increased affinity was however not paralleled by improved *in vivo* activity. Similarly, highlighting the relevance of the cationic component and/or the peptide structure, our *paper III* showed that different CPPs when equally modified with stearyl moiety did not exhibit the same increased efficiency. Additionally, by comparing our findings in *paper I* to those of Soltan *et al.* (Soltan et al., 2009), also the cargo (DNA *versus* siRNA) as well as the number, and position, of the conjugation sites (N-terminal *versus* N^4 and N^9) seem to play a role in the properties and activity of nucleic acid particles.

The interaction between chemical vectors and nucleic acids is, thus, complex and progress with regards to from-scratch-design carriers requires further knowledge on the crucial properties of the vector and the ensuing particles. On the other hand, mechanisms of cell internalization and intracellular localization of nanovectors are presently better understood, and with the development of microscopic technologies, of the conjugation and synthesis chemistry, and the number of dyes today available these assessments are more reliable and informative. However, it is important to keep in mind that cell and *in vivo* imaging systems currently accessible often require the use of fluorescent dyes that are likely to influence the results to an unknown extent. Nevertheless, such studies could aid to ascertain the reason behind the unsuccessful DNA delivery by the $Dapa_8$ conjugates (*manuscript IV*). Howbeit, if the limitation was poor cellular uptake there are no known guidelines regarding peptide structure or composition that can assist on the improvement of vector design at this level. In

this respect, structural studies and *in silico* modelling, despite their limitations, are still powerful tools capable of providing significant assistance in the development of vector design.

For *in vivo* gene delivery it is important to acknowledge the relevance of the design of the nucleic acid *per se*. As reported by the laboratory of Naldini (Brown et al., 2007) and Wolff (Wooddell et al., 2008) the nucleic acid, microRNAs and pDNA, respectively, can be used for tissue specific targeting. In particular, for the case of pDNA, the vector can be designed to include tissue specific promoters and enhancers to allow for high gene expression levels for longer time periods. Additional improvements include the use of chromatin insulators (Baum et al., 2003; Pikaart et al., 1998) and the removal of umethylated cytosine-guanine (CpG) motifs, which were shown to be immunostimulatory (Sato et al., 1996).

Interestingly, albeit their differences, butanoyl- and decanoylspermines when formulated with DNA at charge ratios of 1:1 presented similar features, namely: neutral surface charge, average diameter bellow 200 nm and a moderate binding towards DNA (showed by the low DNA gel retardation). These characteristics have been previously observed in other systems that also resulted in successful *in vivo* gene delivery (reviewed by (Viola et al., 2010)).

Although extrapolations concerning chemical vector modifications may not be transferred between peptides of different nature, several and methodical peptide-modification studies add to the general knowledge in gene delivery and improvement of that particular vector. This PhD thesis hopefully contributes, in a more widespread manner, to the understanding of vector design and, particularly, to the elucidation of lipospermines, PEI and arginine-rich CPPs as delivery vehicles for DNA and oligonucleotides.

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I believe every piece of work is the outcome of a journey comparable to the Nature's life cycle: a seed is first born, and soil must be made available, to allow it to grow and develop. There are a series of Seasons that the young plant must live through; Spring is the most longed for, as it follows the footprints of the roughest (and in Sweden also the longest!) season – the Winter! Some seeds travel farther than others; not all seeds grow; and not all grown seeds develop into a strong tree. The outgrowth is a consequence of a conjunction of circumstances. Here, I would like to thank a number of people that most significantly contributed to the conjecture of circumstances that made this thesis possible.

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7 REFERENCES

Aartsma-Rus, A., and van Ommen, G.J. (2007). Antisense-mediated exon skipping: a versatile tool with therapeutic and research applications. RNA *13*, 1609-1624.

Abbasi, M., Uludag, H., Incani, V., Hsu, C.Y., and Jeffery, A. (2008). Further investigation of lipid-substituted poly(L-Lysine) polymers for transfection of human skin fibroblasts. Biomacromolecules *9*, 1618-1630.

Abes, R., Moulton, H.M., Clair, P., Yang, S.T., Abes, S., Melikov, K., Prevot, P., Youngblood, D.S., Iversen, P.L., Chernomordik, L.V.*, et al.* (2008). Delivery of steric block morpholino oligomers by (R-X-R)4 peptides: structure-activity studies. Nucleic Acids Res *36*, 6343-6354.

Ahmed, O.A., Pourzand, C., and Blagbrough, I.S. (2006). Varying the unsaturation in N4,N9-dioctadecanoyl spermines: nonviral lipopolyamine vectors for more efficient plasmid DNA formulation. Pharm Res *23*, 31-40.

Akinc, A., Goldberg, M., Qin, J., Dorkin, J.R., Gamba-Vitalo, C., Maier, M., Jayaprakash, K.N., Jayaraman, M., Rajeev, K.G., Manoharan, M.*, et al.* (2009). Development of lipidoid-siRNA formulations for systemic delivery to the liver. Mol Ther *17*, 872-879.

Akita, H., Kogure, K., Moriguchi, R., Nakamura, Y., Higashi, T., Nakamura, T., Serada, S., Fujimoto, M., Naka, T., Futaki, S.*, et al.* (2010). Nanoparticles for ex vivo siRNA delivery to dendritic cells for cancer vaccines: programmed endosomal escape and dissociation. J Control Release *143*, 311-317.

Alexis, F., Pridgen, E., Molnar, L.K., and Farokhzad, O.C. (2008). Factors affecting the clearance and biodistribution of polymeric nanoparticles. Mol Pharm *5*, 505-515.

Alshamsan, A., Haddadi, A., Incani, V., Samuel, J., Lavasanifar, A., and Uludag, H. (2009). Formulation and delivery of siRNA by oleic acid and stearic acid modified polyethylenimine. Mol Pharm *6*, 121-133.

Alves, I.D., Jiao, C.Y., Aubry, S., Aussedat, B., Burlina, F., Chassaing, G., and Sagan, S. (2010). Cell biology meets biophysics to unveil the different mechanisms of penetratin internalization in cells. Biochim Biophys Acta *1798*, 2231-2239.

Amantana, A., Moulton, H.M., Cate, M.L., Reddy, M.T., Whitehead, T., Hassinger, J.N., Youngblood, D.S., and Iversen, P.L. (2007). Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate. Bioconjug Chem *18*, 1325-1331.

Andaloussi, S.E., Lehto, T., Mager, I., Rosenthal-Aizman, K., Oprea, II, Simonson, O.E., Sork, H., Ezzat, K., Copolovici, D.M., Kurrikoff, K.*, et al.* (2011). Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. Nucleic Acids Res *39*, 3972-3987.

Arangoa, M.A., Duzgunes, N., and Tros de Ilarduya, C. (2003). Increased receptor-mediated gene delivery to the liver by protamine-enhanced-asialofetuin-lipoplexes. Gene Ther *10*, 5-14.

Avgoustakis, K. (2004). Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: preparation, properties and possible applications in drug delivery. Curr Drug Deliv *1*, 321-333.

Bankiewicz, K.S., Forsayeth, J., Eberling, J.L., Sanchez-Pernaute, R., Pivirotto, P., Bringas, J., Herscovitch, P., Carson, R.E., Eckelman, W., Reutter, B.*, et al.* (2006). Long-term clinical improvement in MPTP-lesioned primates after gene therapy with AAV-hAADC. Mol Ther *14*, 564-570.

Bartlett, R.J., Stockinger, S., Denis, M.M., Bartlett, W.T., Inverardi, L., Le, T.T., thi Man, N., Morris, G.E., Bogan, D.J., Metcalf-Bogan, J.*, et al.* (2000). In vivo targeted repair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide. Nat Biotechnol *18*, 615-622.

Batrakova, E.V., and Kabanov, A.V. (2008). Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. J Control Release *130*, 98-106.

Baum, C., Dullmann, J., Li, Z., Fehse, B., Meyer, J., Williams, D.A., and von Kalle, C. (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. Blood *101*, 2099-2114.
Bello-Roufai, M., Lambert, O., and Pitard, B. (2007). Relationships between the physicochemical properties of an amphiphilic triblock copolymers/DNA complexes and their intramuscular transfection efficiency. Nucleic Acids Res *35*, 728-739.
Beyerle, A., Merkel, O., Stoeger, T., and Kissel, T. (2009). PEGylation affects cytotoxicity and cell-compatibility of poly(ethylene imine) for lung application: structure-function relationships. Toxicol Appl Pharmacol *242*, 146-154.
Blagbrough, I.S., Geall, A.J., and Neal, A.P. (2003). Polyamines and novel polyamine conjugates interact with DNA in ways that can be exploited in non-viral gene therapy. Biochem Soc Trans *31*, 397-406.
Bloch, J., Bachoud-Levi, A.C., Deglon, N., Lefaucheur, J.P., Winkel, L., Palfi, S., Nguyen, J.P., Bourdet, C., Gaura, V., Remy, P.*, et al.* (2004). Neuroprotective gene therapy for Huntington's disease, using polymer-encapsulated cells engineered to secrete human ciliary neurotrophic factor: results of a phase I study. Hum Gene Ther *15*, 968-975.
Bloomfield, V.A. (1996). DNA condensation. Curr Opin Struct Biol *6*, 334-341.
Bloomfield, V.A. (1997). DNA condensation by multivalent cations. Biopolymers *44*, 269-282.
Bosch, F., and Rosich, L. (2008). The contributions of Paul Ehrlich to pharmacology: a tribute on the occasion of the centenary of his Nobel Prize. Pharmacology *82*, 171-179.
Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., and Behr, J.P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A *92*, 7297-7301.
Boussif, O., Zanta, M.A., and Behr, J.P. (1996). Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold. Gene Ther *3*, 1074-1080.
Branden, L.J., Mohamed, A.J., and Smith, C.I. (1999). A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. Nat Biotechnol *17*, 784-787.
Brannon-Peppas, L., and Blanchette, J.O. (2004). Nanoparticle and targeted systems for cancer therapy. Adv Drug Deliv Rev *56*, 1649-1659.
Braybrooke, J.P., Slade, A., Deplanque, G., Harrop, R., Madhusudan, S., Forster, M.D., Gibson, R., Makris, A., Talbot, D.C., Steiner, J.*, et al.* (2005). Phase I study of MetXia-P450 gene therapy and oral cyclophosphamide for patients with advanced breast cancer or melanoma. Clin Cancer Res *11*, 1512-1520.
Brown, B.D., Gentner, B., Cantore, A., Colleoni, S., Amendola, M., Zingale, A., Baccarini, A., Lazzari, G., Galli, C., and Naldini, L. (2007). Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat Biotechnol *25*, 1457-1467.
Brown, B.D., and Naldini, L. (2009). Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. Nat Rev Genet *10*, 578-585.
Brown, D.A., Kang, S.H., Gryaznov, S.M., DeDionisio, L., Heidenreich, O., Sullivan, S., Xu, X., and Nerenberg, M.I. (1994). Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. J Biol Chem *269*, 26801-26805.
Campbell, J.M., Bacon, T.A., and Wickstrom, E. (1990). Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. J Biochem Biophys Methods *20*, 259-267.
Campochiaro, P.A., Nguyen, Q.D., Shah, S.M., Klein, M.L., Holz, E., Frank, R.N., Saperstein, D.A., Gupta, A., Stout, J.T., Macko, J.*, et al.* (2006). Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. Hum Gene Ther *17*, 167-176.
Catlin, N., Bettelheim, K., and Henderson, I. (2011). Individual patient (n=1) "trials" in Duchenne dystrophy. Neuromuscul Disord *21*, 525-526.
Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L.*, et al.* (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science *288*, 669-672.
Cavazzana-Calvo, M., Payen, E., Negre, O., Wang, G., Hehir, K., Fusil, F., Down, J., Denaro, M., Brady, T., Westerman, K.*, et al.* (2010). Transfusion independence and

HMGA2 activation after gene therapy of human beta-thalassaemia. Nature *467*, 318-322.
Chang, C.W., Choi, D., Kim, W.J., Yockman, J.W., Christensen, L.V., Kim, Y.H., and Kim, S.W. (2007). Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. J Control Release *118*, 245-253.
Chen, W., Jarzyna, P.A., van Tilborg, G.A., Nguyen, V.A., Cormode, D.P., Klink, A., Griffioen, A.W., Randolph, G.J., Fisher, E.A., Mulder, W.J.*, et al.* (2010). RGD peptide functionalized and reconstituted high-density lipoprotein nanoparticles as a versatile and multimodal tumor targeting molecular imaging probe. FASEB J *24*, 1689-1699.
Cheng, J., Zeidan, R., Mishra, S., Liu, A., Pun, S.H., Kulkarni, R.P., Jensen, G.S., Bellocq, N.C., and Davis, M.E. (2006). Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery. J Med Chem *49*, 6522-6531.
Chevez-Barrios, P., Chintagumpala, M., Mieler, W., Paysse, E., Boniuk, M., Kozinetz, C., Hurwitz, M.Y., and Hurwitz, R.L. (2005). Response of retinoblastoma with vitreous tumor seeding to adenovirus-mediated delivery of thymidine kinase followed by ganciclovir. J Clin Oncol *23*, 7927-7935.
Childs, A.C., Mehta, D.J., and Gerner, E.W. (2003). Polyamine-dependent gene expression. Cell Mol Life Sci *60*, 1394-1406.
Chisholm, E.J., Vassaux, G., Martin-Duque, P., Chevre, R., Lambert, O., Pitard, B., Merron, A., Weeks, M., Burnet, J., Peerlinck, I.*, et al.* (2009). Cancer-specific transgene expression mediated by systemic injection of nanoparticles. Cancer Res *69*, 2655-2662.
Clemedson, C., and Ekwall, B. (1999). Overview of the Final MEIC Results: I. The In Vitro--In Vitro Evaluation. Toxicol In Vitro *13*, 657-663.
Clover, B., and Hammouda, B. (2009). SANS from P85/Water-d under Pressure. Langmuir.
Creusat, G., and Zuber, G. (2008). Self-assembling polyethylenimine derivatives mediate efficient siRNA delivery in mammalian cells. Chembiochem *9*, 2787-2789.
Crombez, L., Aldrian-Herrada, G., Konate, K., Nguyen, Q.N., McMaster, G.K., Brasseur, R., Heitz, F., and Divita, G. (2009). A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. Mol Ther *17*, 95-103.
Crombez, L., and Divita, G. (2011). A non-covalent peptide-based strategy for siRNA delivery. Methods Mol Biol *683*, 349-360.
Crooke, S.T. (2000). Progress in antisense technology: the end of the beginning. Methods Enzymol *313*, 3-45.
D'Agostino, L., di Pietro, M., and Di Luccia, A. (2005). Nuclear aggregates of polyamines are supramolecular structures that play a crucial role in genomic DNA protection and conformation. FEBS J *272*, 3777-3787.
Davis, J.L., Theoret, M.R., Zheng, Z., Lamers, C.H., Rosenberg, S.A., and Morgan, R.A. (2010). Development of human anti-murine T-cell receptor antibodies in both responding and nonresponding patients enrolled in TCR gene therapy trials. Clin Cancer Res *16*, 5852-5861.
Decker, T., and Lohmann-Matthes, M.L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods *115*, 61-69.
DeLong, R.K., Akhtar, U., Sallee, M., Parker, B., Barber, S., Zhang, J., Craig, M., Garrad, R., Hickey, A.J., and Engstrom, E. (2009). Characterization and performance of nucleic acid nanoparticles combined with protamine and gold. Biomaterials *30*, 6451-6459.
Derossi, D., Joliot, A.H., Chassaing, G., and Prochiantz, A. (1994). The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem *269*, 10444-10450.
Dhar, S., Gu, F.X., Langer, R., Farokhzad, O.C., and Lippard, S.J. (2008). Targeted delivery of cisplatin to prostate cancer cells by aptamer functionalized Pt(IV) prodrug-PLGA-PEG nanoparticles. Proc Natl Acad Sci U S A *105*, 17356-17361.

Diez, S., Navarro, G., and de, I.C.T. (2009). In vivo targeted gene delivery by cationic nanoparticles for treatment of hepatocellular carcinoma. J Gene Med *11*, 38-45.
Ezzat, K., El Andaloussi, S., Zaghloul, E.M., Lehto, T., Lindberg, S., Moreno, P.M., Viola, J.R., Magdy, T., Abdo, R., Guterstam, P*., et al.* (2011). PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. Nucleic Acids Res *39*, 5284-5298.
Farhood, H., Serbina, N., and Huang, L. (1995). The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim Biophys Acta *1235*, 289-295.
Faure, A.C., Dufort, S., Josserand, V., Perriat, P., Coll, J.L., Roux, S., and Tillement, O. (2009). Control of the in vivo biodistribution of hybrid nanoparticles with different poly(ethylene glycol) coatings. Small *5*, 2565-2575.
Faustino, N.A., and Cooper, T.A. (2003). Pre-mRNA splicing and human disease. Genes Dev *17*, 419-437.
Fenske, D.B., and Cullis, P.R. (2008). Liposomal nanomedicines. Expert Opin Drug Deliv *5*, 25-44.
Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.
Fletcher, S., Honeyman, K., Fall, A.M., Harding, P.L., Johnsen, R.D., Steinhaus, J.P., Moulton, H.M., Iversen, P.L., and Wilton, S.D. (2007). Morpholino oligomer-mediated exon skipping averts the onset of dystrophic pathology in the mdx mouse. Mol Ther *15*, 1587-1592.
Fotakis, G., and Timbrell, J.A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett *160*, 171-177.
Frankel, A.D., and Pabo, C.O. (1988). Cellular uptake of the tat protein from human immunodeficiency virus. Cell *55*, 1189-1193.
Friedman, K.J., Kole, J., Cohn, J.A., Knowles, M.R., Silverman, L.M., and Kole, R. (1999). Correction of aberrant splicing of the cystic fibrosis transmembrane conductance regulator (CFTR) gene by antisense oligonucleotides. J Biol Chem *274*, 36193-36199.
Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001). Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J Biol Chem *276*, 5836-5840.
Gaspar, H.B., Bjorkegren, E., Parsley, K., Gilmour, K.C., King, D., Sinclair, J., Zhang, F., Giannakopoulos, A., Adams, S., Fairbanks, L.D*., et al.* (2006). Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. Mol Ther *14*, 505-513.
Gaucheron, J., Santaella, C., and Vierling, P. (2001). Highly fluorinated lipospermines for gene transfer: synthesis and evaluation of their in vitro transfection efficiency. Bioconjug Chem *12*, 114-128.
Gaymalov, Z.Z., Yang, Z., Pisarev, V.M., Alakhov, V.Y., and Kabanov, A.V. (2009). The effect of the nonionic block copolymer pluronic P85 on gene expression in mouse muscle and antigen-presenting cells. Biomaterials *30*, 1232-1245.
Geal AJ, B.I. (2000). Homologation of polyamines in the rapid synthesis of lipospermine conjugates and related lipoplexes. Tetrahedron *56*, 2449-2460.
Geall, A.J., Al-Hadithi, D., and Blagbrough, I.S. (2002). Efficient calf thymus DNA condensation upon binding with novel bile acid polyamine amides. Bioconjug Chem *13*, 481-490.
Geall, A.J., and Blagbrough, I.S. (2000). Homologation of polyamines in the rapid synthesis of lipospermine conjugates and related lipoplexes. Tetrahedron *56*, 2449-2460.
Gehl, J. (2008). Electroporation for drug and gene delivery in the clinic: doctors go electric. Methods Mol Biol *423*, 351-359.
Ghonaim, H.M., Li, S., and Blagbrough, I.S. (2010). N1,N12-Diacyl spermines: SAR studies on non-viral lipopolyamine vectors for plasmid DNA and siRNA formulation. Pharm Res *27*, 17-29.

Goodarzi, G., Watabe, M., and Watabe, K. (1992). Organ distribution and stability of phosphorothioated oligodeoxyribonucleotides in mice. Biopharm Drug Dispos *13*, 221-227.
Graham, F.L., and van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*, 456-467.
Greco, O., Scott, S.D., Marples, B., and Dachs, G.U. (2002). Cancer gene therapy: 'delivery, delivery, delivery'. Front Biosci *7*, d1516-1524.
Green, M., and Loewenstein, P.M. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein. Cell *55*, 1179-1188.
Hacein-Bey-Abina, S., Fischer, A., and Cavazzana-Calvo, M. (2002). Gene therapy of X-linked severe combined immunodeficiency. Int J Hematol *76*, 295-298.
Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M*., et al.* (2003a). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med *348*, 255-256.
Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E*., et al.* (2003b). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science *302*, 415-419.
Harris, T.J., Green, J.J., Fung, P.W., Langer, R., Anderson, D.G., and Bhatia, S.N. (2009). Tissue-specific gene delivery via nanoparticle coating. Biomaterials *31*, 998-1006.
Hassane, F.S., Ivanova, G.D., Bolewska-Pedyczak, E., Abes, R., Arzumanov, A.A., Gait, M.J., Lebleu, B., and Gariepy, J. (2009). A peptide-based dendrimer that enhances the splice-redirecting activity of PNA conjugates in cells. Bioconjug Chem *20*, 1523-1530.
Hatakeyama, H., Akita, H., Kogure, K., Oishi, M., Nagasaki, Y., Kihira, Y., Ueno, M., Kobayashi, H., Kikuchi, H., and Harashima, H. (2007). Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid. Gene Ther *14*, 68-77.
Hatakeyama, H., Ito, E., Akita, H., Oishi, M., Nagasaki, Y., Futaki, S., and Harashima, H. (2009). A pH-sensitive fusogenic peptide facilitates endosomal escape and greatly enhances the gene silencing of siRNA-containing nanoparticles in vitro and in vivo. J Control Release *139*, 127-132.
Heemskerk, H., de Winter, C.L., van Ommen, G.J., van Deutekom, J.C., and Aartsma-Rus, A. (2009a). Development of antisense-mediated exon skipping as a treatment for duchenne muscular dystrophy. Ann N Y Acad Sci *1175*, 71-79.
Heemskerk, H.A., de Winter, C.L., de Kimpe, S.J., van Kuik-Romeijn, P., Heuvelmans, N., Platenburg, G.J., van Ommen, G.J., van Deutekom, J.C., and Aartsma-Rus, A. (2009b). In vivo comparison of 2'-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. J Gene Med *11*, 257-266.
Henry, S.P., Monteith, D., Bennett, F., and Levin, A.A. (1997). Toxicological and pharmacokinetic properties of chemically modified antisense oligonucleotide inhibitors of PKC-alpha and C-raf kinase. Anticancer Drug Des *12*, 409-420.
Hoke, G.D., Draper, K., Freier, S.M., Gonzalez, C., Driver, V.B., Zounes, M.C., and Ecker, D.J. (1991). Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection. Nucleic Acids Res *19*, 5743-5748.
Hosseinkhani, H., and Tabata, Y. (2006). Self assembly of DNA nanoparticles with polycations for the delivery of genetic materials into cells. J Nanosci Nanotechnol *6*, 2320-2328.
Huang, R., Han, L., Li, J., Ren, F., Ke, W., Jiang, C., and Pei, Y. (2009a). Neuroprotection in a 6-hydroxydopamine-lesioned Parkinson model using lactoferrin-modified nanoparticles. J Gene Med *11*, 754-763.
Huang, R., Ke, W., Han, L., Liu, Y., Shao, K., Ye, L., Lou, J., Jiang, C., and Pei, Y. (2009b). Brain-targeting mechanisms of lactoferrin-modified DNA-loaded nanoparticles. J Cereb Blood Flow Metab *29*, 1914-1923.

Huang, R., Ke, W., Liu, Y., Wu, D., Feng, L., Jiang, C., and Pei, Y. (2009c). Gene therapy using lactoferrin-modified nanoparticles in a rotenone-induced chronic Parkinson model. J Neurol Sci.
Hyde, S.C., Southern, K.W., Gileadi, U., Fitzjohn, E.M., Mofford, K.A., Waddell, B.E., Gooi, H.C., Goddard, C.A., Hannavy, K., Smyth, S.E., *et al.* (2000). Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. Gene Ther *7*, 1156-1165.
Ivanova, G.D., Arzumanov, A., Abes, R., Yin, H., Wood, M.J., Lebleu, B., and Gait, M.J. (2008). Improved cell-penetrating peptide-PNA conjugates for splicing redirection in HeLa cells and exon skipping in mdx mouse muscle. Nucleic Acids Res *36*, 6418-6428.
Jin, S., Leach, J.C., and Ye, K. (2009). Nanoparticle-mediated gene delivery. Methods Mol Biol *544*, 547-557.
Joliot, A., Pernelle, C., Deagostini-Bazin, H., and Prochiantz, A. (1991). Antennapedia homeobox peptide regulates neural morphogenesis. Proc Natl Acad Sci U S A *88*, 1864-1868.
Kabanov, A., Zhu, J., and Alakhov, V. (2005). Pluronic block copolymers for gene delivery. Adv Genet *53*, 231-261.
Kang, S.H., Cho, M.J., and Kole, R. (1998). Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. Biochemistry *37*, 6235-6239.
Kang, S.H., Zirbes, E.L., and Kole, R. (1999). Delivery of antisense oligonucleotides and plasmid DNA with various carrier agents. Antisense Nucleic Acid Drug Dev *9*, 497-505.
Kaplitt, M.G., Feigin, A., Tang, C., Fitzsimons, H.L., Mattis, P., Lawlor, P.A., Bland, R.J., Young, D., Strybing, K., Eidelberg, D., *et al.* (2007). Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. Lancet *369*, 2097-2105.
Kasuya, T., and Kuroda, S. (2009). Nanoparticles for human liver-specific drug and gene delivery systems: in vitro and in vivo advances. Expert Opin Drug Deliv *6*, 39-52.
Kerem, E. (2005). Pharmacological induction of CFTR function in patients with cystic fibrosis: mutation-specific therapy. Pediatr Pulmonol *40*, 183-196.
Khalil, I.A., Futaki, S., Niwa, M., Baba, Y., Kaji, N., Kamiya, H., and Harashima, H. (2004). Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: enhanced cellular association by hydrophobic core formation. Gene Ther *11*, 636-644.
Kichler, A. (2004). Gene transfer with modified polyethylenimines. J Gene Med *6 Suppl 1*, S3-10.
Kichler, A., Zauner, W., Ogris, M., and Wagner, E. (1998). Influence of the DNA complexation medium on the transfection efficiency of lipospermine/DNA particles. Gene Ther *5*, 855-860.
Kinali, M., Arechavala-Gomeza, V., Feng, L., Cirak, S., Hunt, D., Adkin, C., Guglieri, M., Ashton, E., Abbs, S., Nihoyannopoulos, P., *et al.* (2009). Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. Lancet Neurol *8*, 918-928.
Klibanov, A.L., Maruyama, K., Torchilin, V.P., and Huang, L. (1990). Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett *268*, 235-237.
Kojima, C., Regino, C., Umeda, Y., Kobayashi, H., and Kono, K. (2010). Influence of dendrimer generation and polyethylene glycol length on the biodistribution of PEGylated dendrimers. Int J Pharm *383*, 293-296.
Kormann MHG, A.M., Nica G et al. (2009). Pulmonary messenger RNA application prevents fatal respiratory distress in surfactant protein B deficient mice (Abstract). Hum Gene Ther *20(11)*, 1409-1410.
Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K.G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. Nature *438*, 685-689.
Langel, Ü., ed. (2006). Preface in: Handbook of Cell-Penetrating Peptides, 2nd edn (Boca Raton, London, New York, CRC Press/Taylor & Francis).

Langlet-Bertin, B., Leborgne, C., Scherman, D., Bechinger, B., Mason, A.J., and Kichler, A. (2010). Design and evaluation of histidine-rich amphipathic peptides for siRNA delivery. Pharm Res *27*, 1426-1436.
Laufer, S.D., Recke, A.L., Veldhoen, S., Trampe, A., and Restle, T. (2009). Noncovalent peptide-mediated delivery of chemically modified steric block oligonucleotides promotes splice correction: quantitative analysis of uptake and biological effect. Oligonucleotides *19*, 63-80.
Lavigne, M.D., Yates, L., Coxhead, P., and Gorecki, D.C. (2008). Nuclear-targeted chimeric vector enhancing nonviral gene transfer into skeletal muscle of Fabry mice in vivo. FASEB J *22*, 2097-2107.
Lehto, T., Abes, R., Oskolkov, N., Suhorutsenko, J., Copolovici, D.M., Mager, I., Viola, J.R., Simonson, O.E., Ezzat, K., Guterstam, P.*, et al.* (2009). Delivery of nucleic acids with a stearylated (RxR)4 peptide using a non-covalent co-incubation strategy. J Control Release *141*, 42-51.
Lehto, T., Simonson, O.E., Mager, I., Ezzat, K., Sork, H., Copolovici, D.M., Viola, J.R., Zaghloul, E.M., Lundin, P., Moreno, P.M.*, et al.* A Peptide-based Vector for Efficient Gene Transfer In Vitro and In Vivo. Mol Ther *in press*.
Lemkine, G.F., and Demeneix, B.A. (2001). Polyethylenimines for in vivo gene delivery. Curr Opin Mol Ther *3*, 178-182.
Letrou-Bonneval, E., Chevre, R., Lambert, O., Costet, P., Andre, C., Tellier, C., and Pitard, B. (2008). Galactosylated multimodular lipoplexes for specific gene transfer into primary hepatocytes. J Gene Med *10*, 1198-1209.
Levin, A.A. (1999). A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim Biophys Acta *1489*, 69-84.
Li, Z., Huang, P., Zhang, X., Lin, J., Yang, S., Liu, B., Gao, F., Xi, P., Ren, Q., and Cui, D. (2010). RGD-conjugated dendrimer-modified gold nanorods for in vivo tumor targeting and photothermal therapy. Mol Pharm *7*, 94-104.
Lin, Y.C., Yu, B.Y., Lin, W.C., Lee, S.H., Kuo, C.H., and Shyue, J.J. (2009). Tailoring the surface potential of gold nanoparticles with self-assembled monolayers with mixed functional groups. J Colloid Interface Sci *340*, 126-130.
Liu, L., Zern, M.A., Lizarzaburu, M.E., Nantz, M.H., and Wu, J. (2003). Poly(cationic lipid)-mediated in vivo gene delivery to mouse liver. Gene Ther *10*, 180-187.
Love, K.T., Mahon, K.P., Levins, C.G., Whitehead, K.A., Querbes, W., Dorkin, J.R., Qin, J., Cantley, W., Qin, L.L., Racie, T.*, et al.* (2010). Lipid-like materials for low-dose, in vivo gene silencing. Proc Natl Acad Sci U S A *107*, 1864-1869.
Lu, X., Sankin, G., Pua, E.C., Madden, J., and Zhong, P. (2009). Activation of transgene expression in skeletal muscle by focused ultrasound. Biochem Biophys Res Commun *379*, 428-433.
Ludtke, J.J., Zhang, G., Sebestyen, M.G., and Wolff, J.A. (1999). A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. J Cell Sci *112 (Pt 12)*, 2033-2041.
Lundin, P., Johansson, H., Guterstam, P., Holm, T., Hansen, M., Langel, U., and S, E.L.A. (2008). Distinct uptake routes of cell-penetrating peptide conjugates. Bioconjug Chem *19*, 2535-2542.
Mae, M., El Andaloussi, S., Lundin, P., Oskolkov, N., Johansson, H.J., Guterstam, P., and Langel, U. (2009). A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy. J Control Release *134*, 221-227.
Magri, F., Del Bo, R., D'Angelo, M.G., Govoni, A., Ghezzi, S., Gandossini, S., Sciacco, M., Ciscato, P., Bordoni, A., Tedeschi, S.*, et al.* (2011). Clinical and molecular characterization of a cohort of patients with novel nucleotide alterations of the Dystrophin gene detected by direct sequencing. BMC Med Genet *12*, 37.
Malek, A., Merkel, O., Fink, L., Czubayko, F., Kissel, T., and Aigner, A. (2009). In vivo pharmacokinetics, tissue distribution and underlying mechanisms of various PEI(-PEG)/siRNA complexes. Toxicol Appl Pharmacol *236*, 97-108.
Marshall, N.B., Oda, S.K., London, C.A., Moulton, H.M., Iversen, P.L., Kerkvliet, N.I., and Mourich, D.V. (2007). Arginine-rich cell-penetrating peptides facilitate delivery of antisense oligomers into murine leukocytes and alter pre-mRNA splicing. J Immunol Methods *325*, 114-126.

Matsumura, Y., and Maeda, H. (1986). A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res *46*, 6387-6392.
McIlroy, D., Barteau, B., Cany, J., Richard, P., Gourden, C., Conchon, S., and Pitard, B. (2009). DNA/amphiphilic block copolymer nanospheres promote low-dose DNA vaccination. Mol Ther *17*, 1473-1481.
McKenzie, D.L., Kwok, K.Y., and Rice, K.G. (2000). A potent new class of reductively activated peptide gene delivery agents. J Biol Chem *275*, 9970-9977.
McNamara, J.O., 2nd, Andrechek, E.R., Wang, Y., Viles, K.D., Rempel, R.E., Gilboa, E., Sullenger, B.A., and Giangrande, P.H. (2006). Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol *24*, 1005-1015.
Merdan, T., Kunath, K., Petersen, H., Bakowsky, U., Voigt, K.H., Kopecek, J., and Kissel, T. (2005). PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice. Bioconjug Chem *16*, 785-792.
Midoux, P., and Monsigny, M. (1999). Efficient gene transfer by histidylated polylysine/pDNA complexes. Bioconjug Chem *10*, 406-411.
Miller, A.M., and Dean, D.A. (2009). Tissue-specific and transcription factor-mediated nuclear entry of DNA. Adv Drug Deliv Rev *61*, 603-613.
Mir, L.M., Moller, P.H., Andre, F., and Gehl, J. (2005). Electric pulse-mediated gene delivery to various animal tissues. Adv Genet *54*, 83-114.
Mironov, A.A., Fickett, J.W., and Gelfand, M.S. (1999). Frequent alternative splicing of human genes. Genome Res *9*, 1288-1293.
Mitchell, D.J., Kim, D.T., Steinman, L., Fathman, C.G., and Rothbard, J.B. (2000). Polyarginine enters cells more efficiently than other polycationic homopolymers. J Pept Res *56*, 318-325.
Monia, B.P., Lesnik, E.A., Gonzalez, C., Lima, W.F., McGee, D., Guinosso, C.J., Kawasaki, A.M., Cook, P.D., and Freier, S.M. (1993). Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. J Biol Chem *268*, 14514-14522.
Moreno, P.M., Wenska, M., Lundin, K.E., Wrange, O., Stromberg, R., and Smith, C.I. (2009). A synthetic snRNA m3G-CAP enhances nuclear delivery of exogenous proteins and nucleic acids. Nucleic Acids Res *37*, 1925-1935.
Morille, M., Montier, T., Legras, P., Carmoy, N., Brodin, P., Pitard, B., Benoit, J.P., and Passirani, C. (2010). Long-circulating DNA lipid nanocapsules as new vector for passive tumor targeting. Biomaterials *31*, 321-329.
Morris, M.C., Depollier, J., Mery, J., Heitz, F., and Divita, G. (2001). A peptide carrier for the delivery of biologically active proteins into mammalian cells. Nat Biotechnol *19*, 1173-1176.
Morris, M.C., Deshayes, S., Heitz, F., and Divita, G. (2008). Cell-penetrating peptides: from molecular mechanisms to therapeutics. Biol Cell *100*, 201-217.
Morris, M.C., Gros, E., Aldrian-Herrada, G., Choob, M., Archdeacon, J., Heitz, F., and Divita, G. (2007). A non-covalent peptide-based carrier for in vivo delivery of DNA mimics. Nucleic Acids Res *35*, e49.
Morris, M.C., Vidal, P., Chaloin, L., Heitz, F., and Divita, G. (1997). A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. Nucleic Acids Res *25*, 2730-2736.
Moss, R.B., Rodman, D., Spencer, L.T., Aitken, M.L., Zeitlin, P.L., Waltz, D., Milla, C., Brody, A.S., Clancy, J.P., Ramsey, B.*, et al.* (2004). Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. Chest *125*, 509-521.
Moulton, H.M., and Moulton, J.D. (2010). Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy. Biochim Biophys Acta *1798*, 2296-2303.
Mukthavaram, R., Marepally, S., Venkata, M.Y., Vegi, G.N., Sistla, R., and Chaudhuri, A. (2009). Cationic glycolipids with cyclic and open galactose head groups for the selective targeting of genes to mouse liver. Biomaterials *30*, 2369-2384.
Nilsen, T.W., and Graveley, B.R. (2010). Expansion of the eukaryotic proteome by alternative splicing. Nature *463*, 457-463.

Nyunt, M.T., Dicus, C.W., Cui, Y.Y., Yappert, M.C., Huser, T.R., Nantz, M.H., and Wu, J. (2009). Physico-chemical characterization of polylipid nanoparticles for gene delivery to the liver. Bioconjug Chem *20*, 2047-2054.
Ogris, M., and Wagner, E. (2002). Tumor-targeted gene transfer with DNA polyplexes. Somat Cell Mol Genet *27*, 85-95.
Oupicky, D., Ogris, M., Howard, K.A., Dash, P.R., Ulbrich, K., and Seymour, L.W. (2002). Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. Mol Ther *5*, 463-472.
Paine, P.L., Moore, L.C., and Horowitz, S.B. (1975). Nuclear envelope permeability. Nature *254*, 109-114.
Pante, N., and Kann, M. (2002). Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. Mol Biol Cell *13*, 425-434.
Patel, M.M., and Anchordoquy, T.J. (2005). Contribution of hydrophobicity to thermodynamics of ligand-DNA binding and DNA collapse. Biophys J *88*, 2089-2103.
Peng, Z. (2005). Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Hum Gene Ther *16*, 1016-1027.
Pikaart, M.J., Recillas-Targa, F., and Felsenfeld, G. (1998). Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. Genes Dev *12*, 2852-2862.
Pike, D.B., and Ghandehari, H. (2009). HPMA copolymer-cyclic RGD conjugates for tumor targeting. Adv Drug Deliv Rev.
Pomel, C., Leborgne, C., Cheradame, H., Scherman, D., Kichler, A., and Guegan, P. (2008). Synthesis and evaluation of amphiphilic poly(tetrahydrofuran-b-ethylene oxide) copolymers for DNA delivery into skeletal muscle. Pharm Res *25*, 2963-2971.
Pooga, M., Soomets, U., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J.X., Xu, X.J., Wiesenfeld-Hallin, Z.*, et al.* (1998). Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. Nat Biotechnol *16*, 857-861.
Raagel, H., Saalik, P., and Pooga, M. (2010). Peptide-mediated protein delivery-which pathways are penetrable? Biochim Biophys Acta *1798*, 2240-2248.
Rainov, N.G. (2000). A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. Hum Gene Ther *11*, 2389-2401.
Raty, J.K., Pikkarainen, J.T., Wirth, T., and Yla-Herttuala, S. (2008). Gene therapy: the first approved gene-based medicines, molecular mechanisms and clinical indications. Curr Mol Pharmacol *1*, 13-23.
Richard, P., Bossard, F., Desigaux, L., Lanctin, C., Bello-Roufai, M., and Pitard, B. (2005a). Amphiphilic block copolymers promote gene delivery in vivo to pathological skeletal muscles. Hum Gene Ther *16*, 1318-1324.
Richard, P., Pollard, H., Lanctin, C., Bello-Roufai, M., Desigaux, L., Escande, D., and Pitard, B. (2005b). Inducible production of erythropoietin using intramuscular injection of block copolymer/DNA formulation. J Gene Med *7*, 80-86.
Rodino-Klapac, L.R., Montgomery, C.L., Bremer, W.G., Shontz, K.M., Malik, V., Davis, N., Sprinkle, S., Campbell, K.J., Sahenk, Z., Clark, K.R.*, et al.* (2009). Persistent Expression of FLAG-tagged Micro dystrophin in Nonhuman Primates Following Intramuscular and Vascular Delivery. Mol Ther *18*, 109-117.
Rogers, S., and Pfuderer, P. (1968). Use of viruses as carriers of added genetic information. Nature *219*, 749-751.
Roques, C., Bouchemal, K., Ponchel, G., Fromes, Y., and Fattal, E. (2009). Parameters affecting organization and transfection efficiency of amphiphilic copolymers/DNA carriers. J Control Release *138*, 71-77.
Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T., Yang, J.C., Topalian, S.L.*, et al.* (1990). Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med *323*, 570-578.
Rothbard, J.B., Kreider, E., VanDeusen, C.L., Wright, L., Wylie, B.L., and Wender, P.A. (2002). Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake. J Med Chem *45*, 3612-3618.

Russ, V., Frohlich, T., Li, Y., Halama, A., Ogris, M., and Wagner, E. (2010). Improved in vivo gene transfer into tumor tissue by stabilization of pseudodendritic oligoethylenimine-based polyplexes. J Gene Med *12*, 180-193.
Saleh, A.F., Arzumanov, A., Abes, R., Owen, D., Lebleu, B., and Gait, M.J. (2010). Synthesis and splice-redirecting activity of branched, arginine-rich peptide dendrimer conjugates of peptide nucleic acid oligonucleotides. Bioconjug Chem *21*, 1902-1911.
Santander-Ortega, M.J., Jodar-Reyes, A.B., Csaba, N., Bastos-Gonzalez, D., and Ortega-Vinuesa, J.L. (2006). Colloidal stability of pluronic F68-coated PLGA nanoparticles: a variety of stabilisation mechanisms. J Colloid Interface Sci *302*, 522-529.
Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.D., Silverman, G.J., Lotz, M., Carson, D.A., and Raz, E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science *273*, 352-354.
Sazani, P., Gemignani, F., Kang, S.H., Maier, M.A., Manoharan, M., Persmark, M., Bortner, D., and Kole, R. (2002). Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. Nat Biotechnol *20*, 1228-1233.
Sazani, P., and Kole, R. (2003). Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. J Clin Invest *112*, 481-486.
Scheers, E.M., Ekwall, B., and Dierickx, P.J. (2001). In vitro long-term cytotoxicity testing of 27 MEIC chemicals on Hep G2 cells and comparison with acute human toxicity data. Toxicol In Vitro *15*, 153-161.
Schwarze, S.R., Ho, A., Vocero-Akbani, A., and Dowdy, S.F. (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse. Science *285*, 1569-1572.
Sebestyen, M.G., Budker, V.G., Budker, T., Subbotin, V.M., Zhang, G., Monahan, S.D., Lewis, D.L., Wong, S.C., Hagstrom, J.E., and Wolff, J.A. (2006). Mechanism of plasmid delivery by hydrodynamic tail vein injection. I. Hepatocyte uptake of various molecules. J Gene Med *8*, 852-873.
Sebestyen, M.G., Ludtke, J.J., Bassik, M.C., Zhang, G., Budker, V., Lukhtanov, E.A., Hagstrom, J.E., and Wolff, J.A. (1998). DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. Nat Biotechnol *16*, 80-85.
Soltan, M.K., Ghonaim, H.M., El Sadek, M., Kull, M.A., El-aziz, L.A., and Blagbrough, I.S. (2009). Design and synthesis of N4,N9-disubstituted spermines for non-viral siRNA delivery--structure-activity relationship studies of siFection efficiency versus toxicity. Pharm Res *26*, 286-295.
Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J.*, et al.* (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature *432*, 173-178.
Stedman, H., Wilson, J.M., Finke, R., Kleckner, A.L., and Mendell, J. (2000). Phase I clinical trial utilizing gene therapy for limb girdle muscular dystrophy: alpha-, beta-, gamma-, or delta-sarcoglycan gene delivered with intramuscular instillations of adeno-associated vectors. Hum Gene Ther *11*, 777-790.
Strand, S.P., Lelu, S., Reitan, N.K., de Lange Davies, C., Artursson, P., and Varum, K.M. (2010). Molecular design of chitosan gene delivery systems with an optimized balance between polyplex stability and polyplex unpacking. Biomaterials *31*, 975-987.
Trubetskoy, V.S., Wong, S.C., Subbotin, V., Budker, V.G., Loomis, A., Hagstrom, J.E., and Wolff, J.A. (2003). Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery. Gene Ther *10*, 261-271.
Tuszynski, M.H., Thal, L., Pay, M., Salmon, D.P., U, H.S., Bakay, R., Patel, P., Blesch, A., Vahlsing, H.L., Ho, G.*, et al.* (2005). A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat Med *11*, 551-555.
Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M.L. (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc Natl Acad Sci U S A *89*, 7934-7938.
van Deutekom, J.C. (2005). Gene therapy: the 'pro-sense' approach to Duchenne muscular dystrophy. Eur J Hum Genet *13*, 518-519.
van Drunen Littel-van den Hurk, S., Luxembourg, A., Ellefsen, B., Wilson, D., Ubach, A., Hannaman, D., and van den Hurk, J.V. (2008). Electroporation-based DNA transfer

enhances gene expression and immune responses to DNA vaccines in cattle. Vaccine *26*, 5503-5509.
Weeke-Klimp, A.H., Bartsch, M., Morselt, H.W., Van Veen-Hof, I., Meijer, D.K., Scherphof, G.L., and Kamps, J.A. (2007). Targeting of stabilized plasmid lipid particles to hepatocytes in vivo by means of coupled lactoferrin. J Drug Target *15*, 585-594.
Wender, P.A., Mitchell, D.J., Pattabiraman, K., Pelkey, E.T., Steinman, L., and Rothbard, J.B. (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc Natl Acad Sci U S A *97*, 13003-13008.
Wierda, W.G., Castro, J.E., Aguillon, R., Sampath, D., Jalayer, A., McMannis, J., Prussak, C.E., Keating, M., and Kipps, T.J. (2010). A phase I study of immune gene therapy for patients with CLL using a membrane-stable, humanized CD154. Leukemia *24*, 1893-1900.
Wilson, J.M. (2005). Gendicine: the first commercial gene therapy product. Hum Gene Ther *16*, 1014-1015.
Viola, J.R., El-Andaloussi, S., Oprea, II, and Smith, C.I. (2010). Non-viral nanovectors for gene delivery: factors that govern successful therapeutics. Expert Opin Drug Deliv *7*, 721-735.
Viola, J.R., Leijonmarck, H., Simonson, O.E., Oprea, II, Frithiof, R., Purhonen, P., Moreno, P.M., Lundin, K.E., Stromberg, R., and Smith, C.I. (2009). Fatty acid-spermine conjugates as DNA carriers for nonviral in vivo gene delivery. Gene Ther *16*, 1429-1440.
Vives, E., Brodin, P., and Lebleu, B. (1997). A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem *272*, 16010-16017.
Wolff, J.A., and Budker, V. (2005). The mechanism of naked DNA uptake and expression. Adv Genet *54*, 3-20.
Wooddell, C.I., Reppen, T., Wolff, J.A., and Herweijer, H. (2008). Sustained liver-specific transgene expression from the albumin promoter in mice following hydrodynamic plasmid DNA delivery. J Gene Med *10*, 551-563.
Wroblewski, F., and Bodansky, O. (1950). Presence of desoxyribonuclease activity in human serum. Proc Soc Exp Biol Med *74*, 443-445.
Wu, J., Lizarzaburu, M.E., Kurth, M.J., Liu, L., Wege, H., Zern, M.A., and Nantz, M.H. (2001). Cationic lipid polymerization as a novel approach for constructing new DNA delivery agents. Bioconjug Chem *12*, 251-257.
Xing, J.Z., Zhu, L., Jackson, J.A., Gabos, S., Sun, X.J., Wang, X.B., and Xu, X. (2005). Dynamic monitoring of cytotoxicity on microelectronic sensors. Chem Res Toxicol *18*, 154-161.
Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M.L. (1990). Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. Proc Natl Acad Sci U S A *87*, 3655-3659.
Zhang, G., Wooddell, C.I., Hegge, J.O., Griffin, J.B., Huss, T., Braun, S., and Wolff, J.A. (2010). Functional Efficacy of Dystrophin Expression from Plasmids Delivered to mdx Mice by Hydrodynamic Limb Vein Injection. Hum Gene Ther *21*, 221-237.
Zhou, J., Li, H., Li, S., Zaia, J., and Rossi, J.J. (2008). Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. Mol Ther *16*, 1481-1489.