DNA-Transporting Nanoparticles

Design and *in vitro* evaluation of DNA and formulation for non-viral gene delivery

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DNA-Transporterende Nanodeeltjes

Ontwerp en *in vitro* evaluatie van DNA en formulering voor niet-virale genafgifte

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 11 oktober 2010 des middags te 2.30 uur

door

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geboren op 14 januari 1979 te Tilburg

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“Het kunstzinnige of op de zaken vooruitlopende voorstellingsvermogen negeert en vat samen, laat perioden van verveling weg en richt onze aandacht op cruciale ogenblikken om aldus, zonder te liegen of te verfraaien, het leven een intensiteit en een samenhang te verlenen die het in de afleidende onoverzichtelijkheid van het moment zelf ontbeert.”

- Alain de Botton, The Art of Travel, 2002-

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General introduction

The concept of gene therapy

DNA contains the genetic instructions for development and functioning of cells, and at a different hierarchy, of organisms. A defective or missing gene can lead to a lack of production of essential proteins or production of non-functional proteins, which may cause diseases either by loss-of-function or by gain-of-function. Conventional treatments are based on treatment of symptoms or interference at the protein level. The aim of gene therapy is to interfere at the level of DNA, by replacing defective genes, introducing new genes or changing the expression of a person's genes in order to treat, cure and ultimately prevent a disease.

The basic idea of gene therapy is the successful introduction of therapeutic DNA into target cells, followed by transcription of the DNA into messenger RNA (mRNA) and subsequent translation of this mRNA into the proteins that should elicit the intended therapeutic effect (see Figure 1). The effect may be restoration of normal protein production (in case of hereditary diseases), addition of new functional proteins (for acquired diseases), induction of cell death (in case of cancer therapy) or induction of immune response (for vaccination purposes). The therapeutic aim and the structure of the required protein can easily be adjusted by changing the DNA sequence. A wide range of therapeutic DNA sequences are being investigated; the main gene types currently under investigation in clinical trials are depicted in Figure 1. The top three target diseases addressed by gene therapy clinical trials are cancer (64.5 %), cardiovascular diseases (8.7 %) and inherited monogenic diseases (7.9 %) [1]. All strategies are directed at somatic gene therapy, meaning that germ line cells are not modified and genetic modifications are not transferred to subsequent generations.

Figure 1. *The concept and application of gene therapy. The main types of expressed proteins investigated in gene therapy clinical trials are summarized in the diagram on the right (reprinted from [1] with permission).*

A prerequisite for gene therapy: gene delivery

The site of action of therapeutic DNA is within the nuclei of target cells where the machinery resides that can transcribe DNA sequences into mRNA. Success of gene therapy is therefore dependent on successful delivery of DNA from the site of administration into cell nuclei (Figure 2).

The produced mRNA is exported from the nucleus into the cell cytoplasm, where it is translated into a protein.

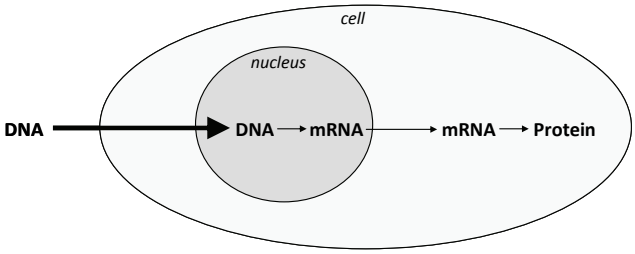

Figure 2. *Gene therapy requires delivery of DNA into cell nuclei.*

Viral gene delivery

Naturally occurring and highly efficient gene delivery systems are viruses. By replacing the genetic material of viruses by therapeutic genes, they can be used as efficient vectors for gene therapy. The most common viral vectors are derived from retroviruses, adenoviruses or adeno-associated viruses (AAV). Integration of retroviral DNA (and to a lesser extent of AAV DNA) into the host cell genome enables persistent transgene expression; this at the cost of risks of insertional mutagenesis. Although most children enrolled in trials for treatment of human severe combined immunodeficiency (SCID)-XI by reinfusion with retrovirus-transduced haematopoietic stem cells experienced substantial improvement, five out of twenty children developed leukaemia, caused by insertion of DNA near or in an oncogene [2,3]. Adenoviruses are non-integrating viruses that are extremely efficient in delivering double stranded DNA, but their viral capsids induce a potent immune response causing both severe adverse events and short-lived expression [4]. AAV, being non-pathogenic and non-inflammatory, represents a virus with a favorable safety profile but has the disadvantage of small packaging capacity. Although viral delivery remains the most efficient method and constitutes the majority of clinical trials (66.2 % [1]), issues of safety, limited possibilities for targeting and limited loading capacity justify the search for synthetic (or non-viral) alternatives.

Non-viral gene delivery

Naked DNA delivery

Systemic administration of 'naked' DNA molecules faces problems of rapid clearance, unfavorable distribution, degradation by nucleic acid degrading enzymes (nucleases) and inefficient cellular uptake. By using pressurized injections of large volumes of DNA sample into blood vessels temporary permeabilization of the capillary epithelium is obtained, which leads to increased

uptake [5]. This method of hydrodynamic delivery proved highly efficient in animals. Considering that a systemic bolus injection of large fluid volumes causes (temporary) cardiac dysfunction, clinical applicability of hydrodynamic gene delivery will be limited to local injections [5]. Other methods of naked DNA delivery include electroporation [6], tattooing [7,8], administration by gene gun [9,10], all of which involve local administration.

Carrier-mediated delivery

Non-viral gene delivery systems are investigated as safer and flexible alternatives to viral delivery vectors. The basic principle of non-viral carriers is packaging of DNA into small particles such that the DNA is protected against degradation and is more efficiently transported towards and into target cells. Formation of these particles is in most cases based on electrostatic interactions of cationic compounds with anionic plasmid DNA, which results in collapse of DNA into small, positively charged particles. An important feature of non-viral delivery systems is their versatility: possibilities for variation of backbone and functionalization of carrier compounds permit tailoring of systems to specific needs. Indeed a wide variety of compounds has been and continues to be investigated for their potential of safe and efficient gene delivery, including lipid-based vectors, polymers, dendrimers, (poly)peptides, and nanoparticles, as extensively reviewed by Mintzner *et al.* [11]. Despite these numerous efforts, non-viral delivery research is largely restricted to preclinical phases, mostly due to inefficiency of delivery of the present generation of vectors.

Progress in non-viral gene delivery

First generation studies

Initial studies aimed at proving the concept of expressing exogenous DNA in host cells. In 1979, Mulligan *et al.* reported the first study describing successful transfection of animal cells with recombinant plasmid DNA using the calcium phosphate transfection technology [12]. By 1980 successful *in vitro* delivery of DNA by liposome-mediated gene transfer had been demonstrated [13], followed by polylysine (pLL)-based transfection in the late 1980s [14] and polyethyleneimine (pEI)-based delivery in the 1990s [15]. Based on these initial findings, the search for alternative delivery agents has moved into various directions and is ever expanding (depicted in Figure 3). Clearly, the aim and type of improvements that are currently being explored have become highly diverse. To facilitate description of the progress made in the past decades, a categorization is made based on the four major problems that hamper successful non-viral gene delivery: toxicity, short-lived expression, heterogeneity of expression and low efficiency. Figure 3 provides a schematic overview of how research progressed from the initial concept to recognition of problems, subsequent elucidation of the mechanistic basis for these problems and strategies being explored to overcome the encountered problem. The progress made in each of the problem categories depicted in Figure

3 will be further explained briefly below. The discussed functionalizations are applied to DNA-Transporting Nanoparticles prepared with reagents including pEI (-derivatives), polyamines, polyamidoamines, chitosan (-derivatives), lipids, lipid/polymer hybrids, peptides, proteins, polymethacrylates, polyphosphazenes, dendrimers, and others (for a comprehensive and recent review see [11]).

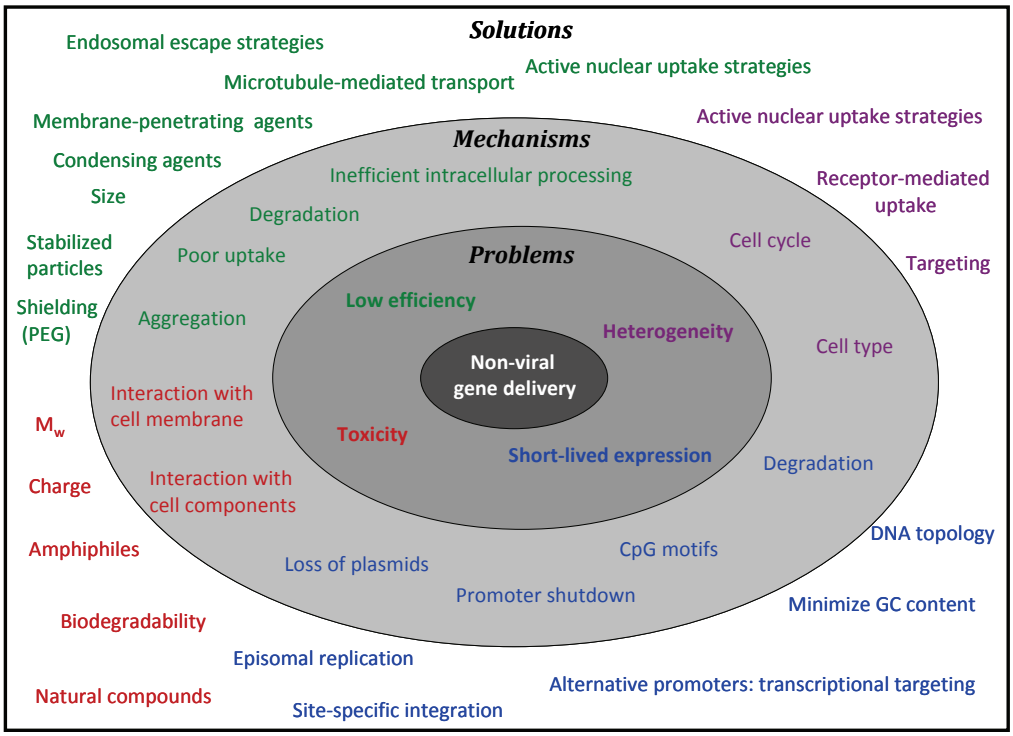

Figure 3. *Progress in gene delivery research. Inside- out: the concept of transgene expression upon non-virally delivered DNA in target cells (centre), the problems encountered (second ring), the mechanistic explanation for these problems (third ring) and the solutions investigated to improve success of non-viral gene delivery (outer part). The problems together with their mechanistic explanations and explored solutions are categorized into four groups: toxicity (red), short-lived expression (blue), heterogeneity (purple) and low efficiency (green) and serve as a basis for the explanation given in the text.*

Toxicity

As previously mentioned, the majority of non-viral gene delivery strategies rely on electrostatic interaction of positively charged carrier molecules with DNA. When an excess of carrier is used, positively charged particles are formed. The cationic nature of the formed particles facilitates binding to and uptake by cells through interaction with negatively charged proteoglycans present on the outer cell membrane [16]. However, the same interaction is responsible for cytotoxic effects as strong interaction of cationic polymers or lipids to outer and inner cell membranes affects the integrity of cell membranes [17-21]. Additionally, interaction of cations with negatively charged cell

components causes toxicity: binding to cellular proteins, RNA and DNA has been shown to affect natural protein expression and function and cell cycling [19,22-25]. Charge density, molecular weight, presence of free carrier and lack of biodegradability leading to intracellular accumulation of carrier molecules are important factors influencing toxicity [19,26-30] and are topics of investigation in new carrier development [27,31-40].

Short lived expression

An important cause for short-lived expression is related to the sequence of the DNA used. The mammalian genome differs from bacterial and viral genomes with regard to abundance and methylation state of CpG motifs [41,42]. Plasmids used for non-viral gene delivery often contain a strong viral promoter to drive efficient expression of the transgene, as well as bacterial DNA that serves a function in the plasmid production process. The high frequency of unmethylated CpG-motifs in these viral and bacterial sequences has several consequences. *De novo* methylation of unmethylated CpG in target cells leads to transcriptional interference and silencing of gene expression [43]. Recognition of unmethylated CpG motifs triggers the immune system, causing loss of expression through cytokine-mediated promoter shutdown and elimination of transgene expressing cells through apoptosis, innate and adaptive immune responses [42]. Designing plasmids with minimal contents of CpG is a useful strategy to reduce immunostimulation as well as to increase and prolong transgene expression [44-46].

Another factor determining the duration of expression is the loss of plasmids from cells by partitioning to non-nuclear compartments, destruction by intracellular nucleases and gradual dilution of the total number of plasmids over an increasing number of cells following each cell division. Efforts to prevent loss of plasmids from nuclei and prolong gene expression are directed at development of plasmids that can integrate site-specifically into chromosomal DNA [47-49] and plasmids that can replicate extrachromosomally (episomal plasmids) [50-53]. Nuclease sensitivity of DNA (in the absence of protective carrier reagents) is dependent on its structural characteristics. Plasmid DNA can exist in a supercoiled, open-circular or linear form and this topology is related to transfection efficiency. Although some controversy [54-57] remains regarding the optimal topoisoform for maximal transfection efficiency, the compact, supercoiled is mostly considered superior for gene delivery purposes [55,57-59]. The supercoiled isoform offers advantages of increased stability, smaller particle sizes and preferential intracellular trafficking towards the perinuclear region [57,58,60,61].

Heterogeneity

Transgene expression levels vary greatly among different cell types but also among cells within populations of single cell types. Cell types differ in surface area, charge (density), presence of surface receptors, rate of endocytosis, metabolic activity, proliferation state and cell cycling times, all of which affect their response to transfection. However, even within cultured populations of

single cell types heterogeneity in transgene expression is observed [62,63]. Heterogeneity patterns in adherent cell lines were found to be related to the microenvironment and cell state (e.g. local cell density, cell size, position within a population, mitotic and apoptotic states) [62]. One parameter of special interest for gene delivery is the effect of mitosis. The nuclear envelope temporarily breaks down during mitosis, and when it is reformed after mitosis, DNA (-complexes) can be enclosed in the nucleus. Transgene expression has indeed been related to cell cycle phases in several studies [64-66]. However, most tissues targeted by gene therapy are slow- to non-dividing and require alternative routes of nucleocytoplasmic shuttling of DNA. Gene delivery is therefore confined to employing active transport mechanisms, most commonly involving transport via nuclear pore complexes (NPC). NPC allow passive diffusion of proteins up to 40 kDa, or particles of approximately 9 nm or DNA molecules of 210-350 bp [67-70]. These limits are well below sizes of DNA-complexes (~100-500 nm) and of plasmids (~6000 bp) generally used for gene delivery studies. Solutions to overcome the nuclear barrier are sought in substantial reduction of the size of DNA (-complexes) and functionalization of DNA or carrier with molecules known to exploit active nuclear transport mechanisms, such as motifs from nuclear proteins [71-74] , viral structures [68,75-82] and glucocorticoids [83-85].

Low efficiency

Overall efficiency of gene delivery is determined by the efficiency of DNA-Transporting Nanoparticles in each consecutive step in the delivery process: transport towards target cells, uptake into target cells, release into cytosol, trafficking towards and into the nucleus and finally transcription and translation (schematically represented in Figure 4 A).

Efficient transport towards target cells is challenged by instability of complexes leading to pre-term dissociation of complexes (and subsequent degradation of DNA). Additionally, the positive charge of polyplexes and lipoplexes causes them to interact with blood components (e.g. albumin) and/or cells. The resulting aggregates are phagocytosed e.g. by Kupffer cells in the liver, leading to rapid clearance. Additionally, formation of large aggregates leads to accumulation in the lungs [86]. Efforts to prevent this are directed at development of small particles that are stable in the presence of salts and proteins based on cross linking and shielding with polyethyleneglycol (PEG) [87].

Cellular uptake of gene delivery particles is dependent on their charge, size, shape and targeting ligands. Polyplexes and lipoplexes interact with the negatively charged cell membrane via their positive charge, leading to endocytosis [16]. Important relations between particle size and uptake route, and of uptake route and transgene expression levels have been reported, but this relation is cell-type dependent and studies addressing the direct relation between size of DNA-containing particles and expression efficiency are lacking to date [88-93]. Based on quantification of intracellularly delivered DNA, it is unlikely that cellular uptake represents the limiting step in the delivery process [94-97].

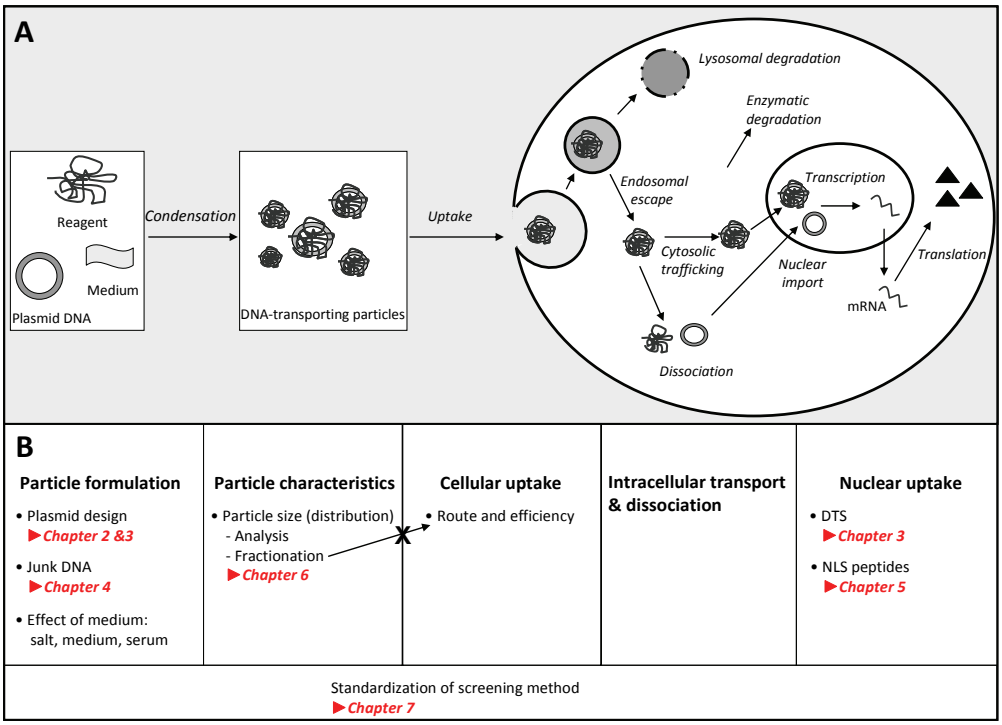

Figure 4. *Schematic presentation of gene delivery (A) and interventions studied in this thesis (B).*

Depending on the uptake route, particles end up in certain vesicles being either acidifying or non-acidifying vesicles, each with distinct properties [98]. In the clathrin-mediated pathway, vesicles interact with early endosomes, subsequently late endosomes and finally lysosomes. Particles should escape this route in time before lysosomal degradation occurs. Characteristic for the clathrin-mediated pathway is a gradual pH-reduction, which can be exploited by incorporating so-called proton-sponges or pH-sensitive membrane-disrupting peptides into delivery systems. The proton-sponge theory is based on basic groups with a pKa between 5 - 7.4 in the condensing polymer that become charged upon acidification, causing influx of protons, counter ions and subsequently of water which causes vesicles to swell und ultimately explode [15,99,100]. However, validity of this theory remains a matter of debate in the field [101-104]. Alternatively, particles can exploit pH-dependent membrane-destabilizing or membrane-penetrating moieties to escape from various types of vesicles [105,106].

Once released into the cytosol, the DNA must be transported towards the nucleus. The cytoplasm is a highly viscous and crowded environment containing organelles, proteins and RNA , which limits the diffusion of macromolecules and particles [69,107-109]. Reduction of size and shielding of particles are thought to facilitate trafficking. Additionally, active transport along microtubules has been described to be important for efficient intracellular transport of non-viral gene delivery systems [72,110-112].

As previously mentioned, uptake of macromolecules and particles into the nucleus is seriously challenged by the size-restrictions inherent to the dimensions of nuclear pore complexes. The finding by Capecchi *et al.* [113] that microinjection of plasmid DNA directly into the nucleus results in transgene expression in 50-100 % of the cells whereas cytoplasmic injection results in <0.01 % of transgene expressing cells clearly indicates that transport into the nucleus is a major bottleneck. Active nuclear uptake strategies therefore hold a prominent position in the development of more efficient gene delivery reagents. Such strategies can be based on incorporation of DNA Nuclear Targeting Sequences (DTS) within the DNA molecule [114] or coupling of Nuclear Localization Signals (NLS) to either DNA or carrier [115]. Alternatively, methods to increase nuclear uptake of macromolecules via modification of nuclear pore complexes are being explored. The NPC permeability increasing effect of the amphiphatic alcohol trans-cyclohexane-1,2-diol [116] and the NPC dilating effect described for dexamethasone [117] could prove valuable for enabling passage of large DNA (-containing particles) into the nucleus.

The delivery process ends inside the nucleus, the site of action for therapeutic DNA. Although transcription of plasmid in the presence of carrier molecules has been reported [118], the general thought is that dissociation of complexes is beneficial, if not crucial for efficient transcription. At this point it should be mentioned that the ideal time and place of DNA release (prior to or after nuclear delivery) is unknown. Once delivered into the cell nuclei, efficiency and specificity of transcription are determined by the promoter and enhancer present in the plasmid and can be tailored through plasmid design [119].

Mechanistic studies regarding each of these individual steps are crucial to understand the fundamental processes and to move towards rational design of delivery agents as alternatives to viruses. Recent technological developments including improved cellular subfractionation methods, advanced microscopic techniques (reviewed in [120,121]) and quantitative PCR have extended the toolbox and have enabled to study cellular uptake pathways, endosomal transport [101,122], intracellular trafficking [93,108,123], cytosolic degradation [124], nuclear uptake [88,94,125,126] and efficiency of transcription/translation [95,118]. Ongoing development of new (analytical) techniques and methods in the field of gene delivery will yield important findings and drive the rational design of non-viral gene delivery vectors in the near future.

To summarize, progress in gene delivery research in the past decades has provided proof of the concept of expressing exogenous DNA in cells and has subsequently revealed the barriers that need to be overcome towards clinical realization of non-viral gene therapy. In response to the identified barriers, a wide range of possibilities for optimization of DNA, carrier and formulation to improve processes at the extracellular, intracellular or even intranuclear level has opened up. A selection of these possibilities is explored in this thesis.

Aim and outline of this thesis

The aim of this thesis is to rationally design DNA-Transporting Nanoparticles, focusing on three different levels: (1) design of plasmids to increase safety, efficiency and duration of transgene expression, (2) design of formulations to increase efficiency of transfection and (3) development of methods to allow better analysis of DNA-Transporting Nanoparticles. A schematic outline of this thesis is given in Figure 4.

Design of DNA

Chapter 2 reviews the literature for improvements in gene delivery based on plasmid optimization. Careful selection of DNA promoter and enhancer sequences can be exploited to augment transcription levels, to target transcription to specific tissues or diseased cells or to achieve stimulus-responsive transcription. Additionally, introduction of specific sequences in the plasmid backbone can enable plasmid retention in the target cell and prolonged expression. Exclusion of immunostimulatory sequences renders formulations less immunogenic. Significant improvement can be expected for each of these examples, but in all cases success is dependent on efficient delivery. Interestingly, modification of the plasmid backbone by inserting sequences that serve as binding sites for transcription factors has been described to facilitate trafficking of plasmids from the cell cytosol into the nucleus. This modification to improve nuclear delivery is the topic of **chapter 3**. A selection of sequences reported to function as so-called DNA nuclear Targeting Sequences (DTS) are cloned into reporter plasmids and their effect on transgene expression and nuclear uptake is evaluated.

Design of formulations

A second approach to improve nuclear uptake of plasmid DNA is explored in **Chapter 4**. This approach involves modification of plasmid DNA via non-covalent interactions with nuclear localization signal (NLS)-bearing peptides.

A striking difference in the number of plasmids required to be delivered in order to achieve transgene expression is observed when comparing viral and non-viral gene delivery. **Chapter 5** describes variations in formulations of electrostatically formed DNA-Transporting Nanoparticles such that the dose of active plasmid DNA can be reduced while maintaining high expression levels. By replacing active DNA with inactive DNA, particle characteristics remain constant while the dose of active DNA can be drastically reduced.

Development of methods

Particle size is an important factor determining the efficiency and route of uptake of nanoparticles. Methods to characterize and control particle sizes under relevant conditions are therefore essential. **Chapter 6** describes the development of a new method for analysis of particle size distributions based on flow cytometry. The combination of single particle analysis, multi-parametric detection and possibility of sorting inspired us to investigate application of this method

to analyze particles size distributions (as opposed to batch analysis methods), to study particle sizes in the presence of background matter present in biological media and to sort heterogeneous samples into populations of well-defined sizes.

One important aspect that should not be ignored in screening for candidate reagents for gene delivery is that the outcome of transfection studies is highly dependent on the conditions chosen. Performing studies in various settings, using various testing conditions and by comparing to various reference reagents complicates interpretation and comparison of results. Reaching a consensus would enable placing new results into the context of previous findings and estimate the overall contribution to the improvement of non-viral gene delivery. In **chapter 7** the sensitivity of transfection outcomes on testing conditions chosen is illustrated, and a screening protocol is proposed with the aim of standardization within the field.

Chapter 8 provides a summarizing discussion of the work described in this thesis. Additionally, three strategic directions for future research on gene delivery are proposed.

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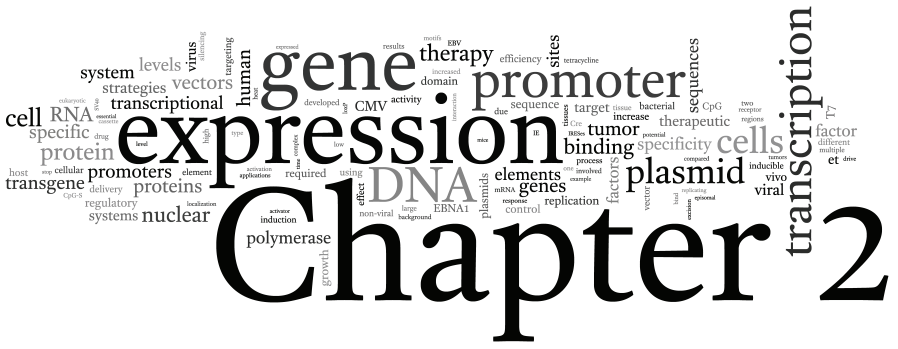

Plasmid engineering for controlled and sustained gene expression for non-viral gene therapy

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Abstract

Gene therapy requires the introduction of genetic material in diseased cells with the aim to treat or ultimately cure a disease. Since the start of gene therapy clinical trials in 1990, gene therapy has proven to be possible, but studies to date have highlighted the difficulty of achieving efficient, specific and long-term transgene expression. Efforts to improve gene therapy strategies over the past years were mainly aimed at solving the problem of delivery, without paying much attention to the optimization of the expression cassette. With the current understanding of the eukaryotic transcription machinery and the advanced molecular biology techniques at our disposition it has now become possible to create custom-made transgene expression cassettes optimized for gene therapy applications. In this review, we will discuss several strategies that have been explored to improve the level and duration of transgene expression, to increase control over expression or to restrict transgene expression to specific cell types or tissues. Although still in its infancy, such strategies will eventually lead to improvement of non-viral gene therapy and expansion of the range of possible therapeutic applications.

Introduction

Gene therapy can be defined as an approach to treat, cure, or ultimately prevent a disease by replacing defective genes, introducing new genes or by changing the expression of a person's genes. This concept was first proposed in 1972 [1], but to date clinical applications remain few due to inefficiency of gene delivery. Over the past years much effort has been made to develop strategies for effective delivery of DNA to the nucleus of target cells. Although viral vectors have been widely examined [2] and are still regarded the most efficient, their utility is limited due to safety issues, DNA loading capacity and difficulties in scale-up production [3]. Alternatively, non-viral delivery strategies have been developed including physical delivery of naked DNA and gene delivery using chemical carriers such as cationic polymers, lipids, detergents and peptide-based technologies, which have been reviewed elsewhere [4-9]. Although many reviews about non-viral gene delivery focus on optimizing the carrier and its entry mechanisms into the cell, not much attention has been given to the plasmid or DNA part of the non-viral carrier. Nevertheless, optimization of the plasmid vector can lead to increased or prolonged levels of expression and may therefore play an important role in compensating for the limited transfection efficiency achieved with most non-viral carriers. Moreover, plasmid engineering can be used to increase levels of specificity and control over protein expression. This is referred to as *transcriptional targeting* or *transcriptional control*. In this review, we focus on progress made in plasmid optimization over the past years and its value for non-viral gene therapy. The subject will be discussed systematically, starting with a description of the minimal requirements for expression of exogenous DNA and subsequently expanding the system with strategies aiming at improvements towards nuclear uptake, restriction of expression to target cells, external control over expression, prolongation of expression and ending with an overview of diverse strategies for further customization.

Minimal requirements for expression of exogenous DNA in eukaryotic cells

In gene therapy, the host transcription machinery is exploited for expression of exogenous DNA. This requires a thorough understanding of the mechanism of transcription and identification of the essential features necessary for transgene expression. Although there is still much to learn about the exact mechanisms underlying transcriptional control of a gene (for a recent review, see Orphanides [10]), current knowledge warrants rational design of exogenous DNA expression cassettes. The minimal requirements for plasmid production by replication in a prokaryotic host and expression of the therapeutic gene in eukaryotes are summarized in Table 1.

The process leading from a gene to a functional protein in eukaryotes includes transcription of the gene to primary RNA by RNA polymerase II, processing of primary RNA to mRNA, export of mRNA to the cytoplasm and translation of mRNA into a protein. In most cases, initiation of transcription is the most important point of control [11]. This will therefore be the main focus of this review.

Table 1. *Overview of essential elements required for plasmid vector expression with their definitions and functions.*

Element	Definition	Function
Plasmid vector		
Requirements for plasmid production in bacteria		
Origin of replication (ori)	A specific DNA sequence of 50-100 base pairs to which the bacterial host-cell enzymes bind, initiating and regulating replication	Plasmid replication
Selection marker	DNA sequence encoding a protein that provides bacteria with a certain selectable characteristic	• Selection of bacteria containing plasmid of interest • Enables pressurization for plasmid maintenance
Requirements for functionality in eukaryotes		
Promoter	Shortest DNA sequence at which RNA polymerase II can initiate transcription	• Position the start site for RNA synthesis • Regulate frequency of transcriptional initiation
Enhancer	DNA sequences to which gene activator proteins bind resulting in interaction with a promoter	Enhance a cell's capacity to transcribe a gene with greater efficiency and greater sensitivity to changes in the environment
PolyA signal	A recognition site existing of AAUAAA-hexamer positioned 10-30 nucleotides upstream the 3´end and a GU or U rich element located maximally 30 nucleotides downstream of the 3´end	Induces 3´end capping
Intron	Intervening non-coding DNA	• protect transcripts against rapid degradation • promote export of mRNA
Stop signal	DNA sequence at which RNA polymerase II is forced to stop and dissociate from the DNA	Termination of transcription and dissociation of RNA polymerase II
Coding DNA	DNA encoding a therapeutic protein	Therapeutic effect
Host cell		
RNA-polymerase II	Enzyme that performs transcription of DNA to RNA	Transcription of DNA to RNA
Poly-A polymerase	Enzyme that adds ~200 A nucleotides to the 3´ end	Provide synthesized RNA strand with the 3'end that protects it against nuclear degradation.
General transcription factors	Proteins that assemble on all promoters used by RNA polymerase II	• Help to position RNA polymerase II at promoter • Aid in separating the two strands of DNA • Release RNA polymerase II from promoter once transcription has begun
Luxury transcription factors	Gene regulatory proteins that bind to regulatory sequences other than the promoter	Regulation of transcription is dependent on cell type and/or environmental influences

Transcription is a complex process requiring many proteins (>100 individual subunits) to assemble at gene control regions (see Figure 1). A gene control region is defined as the whole expanse of DNA involved in regulating transcription of a gene, including the promoter and all regulatory sequences to which gene regulatory proteins bind (i.e. enhancers, silencers). Transcription regulating proteins can be divided into general transcription factors and additional (luxury) transcription factors. General transcription factors must form a preinitiation complex whose function is to unwind the DNA-helix, separate the DNA strands for use as a template and enable RNA polymerase II to take up its position so that mRNA synthesis can start. This complex is already capable of inducing transcription at a slow rate, but additional factors are required for high-level synthesis and specificity. These factors are inducible and enable transcription to speed up or slow down in response to cellular signals. Gene regulatory proteins function as regulatory units that are used to generate complexes whose function depends on the final assembly and composition of all the individual components [11]. Many gene regulatory proteins bind to DNA as either homodimers or heterodimers. This mixing of protein subunits allows for formation of many different proteins with varying DNA binding specificities [11].

Formation of the preinitiation complex and initiation of transcription is followed by the elongation phase, during which transcription continues. An important aspect within the elongation phase is the processing of a primary RNA transcript to mRNA by removal of intron sequences (splicing), 5´end capping and polyadenylation on the 3´end [11,12]. Proper capping is essential as it allows the cell to assess whether both ends of an mRNA molecule are present (indicative of an intact message) before exporting it from the nucleus to the cytoplasm. The enzymes involved in 5´end capping act in a sequence-independent fashion, whereas the enzymes responsible for 3´end modification require the presence of a consensus sequence for recognition (see Table I). The actual polyadenylation is performed by the enzyme poly-A polymerase, that in contrast to other RNA polymerases does not require a template for its action, hence the poly-A tail is not encoded in the DNA.

RNA polymerase II moves along the sense strand until it reaches a termination signal where it is forced to stop and dissociate from the DNA. The simplest example of a stop signal is a palindromic GC-rich region followed by an AT-rich region, but other stop signals exist, that may or may not require additional factors to terminate the transcription process [11,12].

Complexity of the eukaryotic transcription machinery may at first have seemed an obstacle impairing transgene expression. However, this review attempts to illustrate how it can be exploited to elegantly modulate expression of exogenous DNA, thereby significantly contributing to improvement of non-viral gene therapy and expanding the range of possible therapeutic applications. Once the basic fulfillments are met to have the exogenous DNA participate in the transcriptional process, additional features can be introduced to tailor the expression profile to one's needs. By interfering at the level of transcription initiation, increased efficiency, specificity and duration of expression can be established.

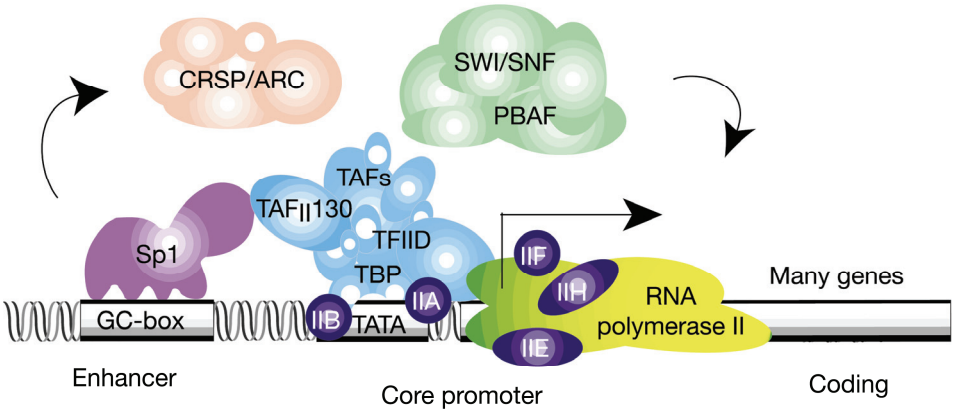


Figure 1. *A simplified schematic model of the eukaryotic transcriptional apparatus. It comprises three broad classes of multi-subunit assemblies: (1) the RNA polymerase II core complex and associated general transcription factors (TFIIA, -B, -D, -E, -F and –H), (2) multi-subunit cofactors (mediator, CRSP, TRAP, and others) and (3) various chromatin modifying or remodeling complexes (SWI/SNF, PBAF, ACF, NURF and RSF). Adapted from Levine et al.* [13].

Intracellular targeting: DNA nuclear targeting sequences

In gene therapy settings, plasmids generally rely on breakdown of the nuclear envelope during cell division for nuclear entry. However, since many cells targeted in gene therapy do not divide or divide very slowly, nuclear entry is a major limiting step in achieving gene expression [14]. Nuclear uptake has been described to occur spontaneously *in vivo* when exceeding a certain threshold (>1,000,000 plasmids/cell), possibly due to mass action [15], but this clearly does not reflect a realistic nor desirable situation.

In the absence of mitosis, plasmids have to be imported via the nuclear pore complex (NPC) in order to obtain transgene expression. This can be achieved by attaching proteins or synthetic peptides containing nuclear localization signals (NLS) to the plasmids. However, no consensus has been reached as towards their beneficial effects [16]. More recently, approaches have been described based on incorporation of peptide/protein structures that allow alternative interactions with a host cell's nuclear import mechanisms, such as targeting the importin β nuclear import receptor [17] and the use of steroid receptors as shuttles to facilitate nuclear import [16,18]. Both methods described above require the covalent or non-covalent attachment of peptides or proteins to DNA which is, from a pharmaceutical point of view, undesirable.

A perhaps more elegant way of enhancing nuclear uptake lacking the necessity for coupling of peptides/proteins to the DNA is the incorporation of DNA-sequences that are recognized by endogenous transcription factors involved in nuclear import. Such DNA nuclear targeting

sequences (DTS) [16] might facilitate nuclear localization by inducing (partial) coating of the plasmid with NLS-containing proteins and subsequent binding to importins.

Simian virus 40 (SV40), a virus whose regulatory sequences are often used to drive expression of plasmids, was shown to elicit a nuclear localization effect [14]. Analysis of the sequence requirements for nuclear import has led to the identification of a 72-bp repeat within the SV40 enhancer [14]. The nuclear localization effect of this 72-bp repeat has been demonstrated both *in vitro*, in various cell types including epithelial, endothelial and smooth muscle cells from a variety of organisms [19], as well as *in vivo* [20,21].

The 72-bp element increased cytomegalovirus (CMV)-promoter driven gene expression by as much as 20-fold after 7 days in murine tibialis muscle *in vivo* [20]. Since the CMV promoter is very strong, this effect is likely to be ascribed to enhancement of nuclear localization rather than further enhancement of transcriptional activity [20]. This hypothesis is further supported by the observation that expression of plasmids including the SV40 72-bp repeat is increased in comparison to plasmids lacking this sequence in post-mitotic cells but not in dividing tumor tissue [20]. SV40 contains numerous binding sites for different general transcription factors; it is thought that binding of NLS-containing transcription factors accounts for the nuclear localization [14,16]. However, several other viral (CMV, Rous sarcoma virus (RSV)) and cellular promoters/enhancers that contain similar binding sites tested negative for this effect [14,16]. What distinguishes SV40 from these other structures is not yet fully understood. One possible explanation is that some, but not all transcription factors are able to induce nuclear uptake. It is thought to be essential that the NLS and the DNA-binding domain within the transcription factor are sufficiently separated both functionally and spatially [16].

Other sequences proposed to have nuclear targeting capacity include nuclear factor-κB (NF-κB) binding sites [22] and the origin of plasmid replication (oriP) sequence from the Epstein-Barr virus (EBV) [16,23]. Additionally, sequences have been identified to act as DTS in a cell-specific manner, including the smooth muscle gamma actin (SMGA) [24] and flk-1 promoter [16] with specificity for smooth muscle cells and endothelium, respectively. When using viral elements, possible risks of immunogenicity due to high CpG content should not be neglected (see also under Sustained expression).

Rather than attempting to overcome the barrier of nuclear uptake through utilization of endogenous proteins for nuclear import, gene therapy strategies have been developed to avoid this barrier by ways of cytoplasmic expression. All expression systems described so far rely on the endogenous, nuclear transcriptional machinery of the cell for expression of the transgene. In contrast, Gao *et al.* [25] developed a strategy in which the transgene is co-delivered with an exogenous transcription machinery which is insensitive to the endogenous regulatory mechanism. This exogenous machinery consists of a bacteriophage T7 RNA polymerase driving transcription of a gene controlled by the T7 promoter. Importantly, phage RNA polymerases exhibit striking specificity for their promoters [26]. Several complications are involved when using the exogenous

transcription machinery. First, since localization of T7 RNA polymerase and therefore transcription of the foreign gene is restricted to the cytoplasm, transcripts will not be capped properly. To achieve efficient translation of the uncapped transcript, an internal ribosome entry site (IRES) of the encephalomyocarditis virus was therefore inserted into the 5´ untranslated region. In most eukaryotes, translation of mRNA requires the presence of an initiation codon that can be recognized by ribosomes. However, in some viral mRNA, ribosomes start translation at internal sites in the mRNA. These internal ribosomal entry sites can be shuttled from their viral settings to unrelated genes to enable expression of proteins in the absence of a functional initiation codon. Using these constructs, significant levels of reporter gene expression were observed in a variety of mammalian cells. A limitation of this expression system is the rapid turnover of T7 polymerase enzyme, which means that expression for long periods of time cannot be sustained. To establish a continuous supply of the polymerase, Brisson *et al.* [27] developed an "autogene" (pCMV/T7-T7pol) consisting of the T7 polymerase gene driven by a CMV and T7 promoter (see Figure 2).

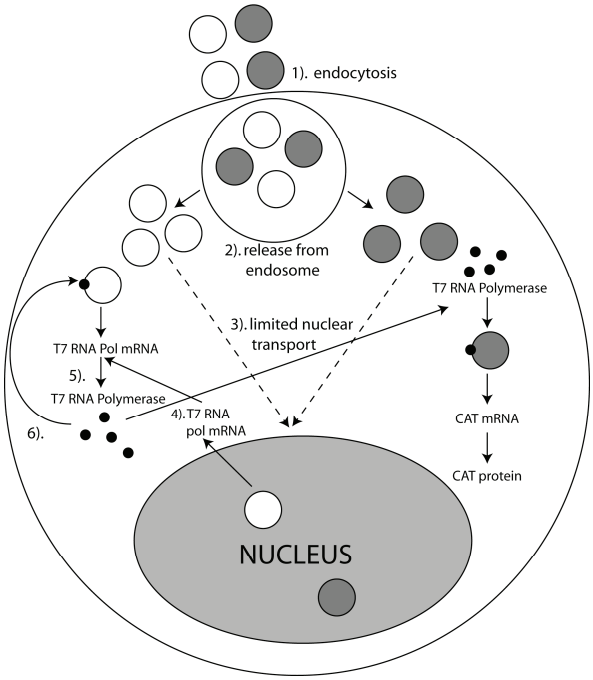


Figure 2. *Proposed mechanism of action of the pCMV/T7-T7pol autogene. Co-delivery and endocytosis of pCMV/T7-T7pol (large white circles) and pT7-CAT (large grey circles). A small portion of pCMV/T7-T7pol is translocated into the nucleus and transcribed by RNA polymerase II to generate T7 RNA polymerase mRNA. In the cytoplasm, T7 RNA polymerase mRNA is translated into T7 RNA polymerase (small black circles) which can then drive further T7 RNA polymerase expression in the cytoplasm by way of the T7 promoter on pCMV/T7-T7pol and simultaneous expression of the chloramphenicol acetyltransferase (CAT) gene located on pT7-CAT. This way, pCMV/T7-T7pol requires no addition of exogenous T7 RNA polymerase and takes advantage of the excess DNA remaining in the cytoplasm due to inefficient nuclear import. Adapted from Brisson et al. [27] with permission.*

The CMV promoter is used to drive the first round of expression of T7 RNA polymerase, which can then act on the T7 promoter to drive expression of both new T7 RNA polymerase and the target gene. This way, no co-delivery of the T7 RNA polymerase enzyme is required, which can be very expensive and potentially immunogenic. Obviously, in this model some nuclear uptake is essential. However, the required amount is thought to be much less than in "classic" nuclear expression models and therefore easier to achieve. The new T7 RNA autogene was shown to induce higher, more sustained levels of reporter gene expression than observed with the autogene lacking the CMV promoter or with nuclear expression systems in which a reporter gene was driven by a CMV promoter only. Additionally, pCMV/T7-T7pol is easily amplified and purified from bacteria using standard methods, possibly because binding of T7 RNA polymerase to the T7 promoter is sterically hindered by CMV promoter-induced conformational changes [27]. It is noteworthy that no detectable quantities of antibodies against T7 RNA polymerase were generated in mice upon direct injection of a T7 system, as reported by Chen *et al.* [28].

Transcriptional targeting: restricting transgene expression to specific cells or tissues

When the first expression cassettes for gene therapy were developed, viral elements were used to drive expression of the foreign gene. Among the strongest promoters identified to date are the CMV immediate early (CMV IE) promoter/enhancer, RSV long terminal repeat (RSV-LTR), and SV40 regulatory sequences. Although the viral promoters are still considered strong relative to the cellular promoters applied more recently, their popularity has diminished considerably in the past years. Reasons include their lack of specificity and the observation that structural differences between host and foreign DNA are registered by the host and provoke immunostimulatory and silencing effects resulting in inactivation (see below). Therefore, a need arose for non-viral, cellular gene regulatory regions that are not as easily inactivated. In addition, targeting of genetic medicines to specific cells is often required to prevent toxicity to healthy cells (especially to liver and bone marrow cells [29]) and to decrease the required dose. Differential gene expression among cell types and environmental conditions is physiologically possible because different genes are driven by different (combinations) of promoter and enhancer sequences and each of these regulatory sequences contain binding sites for multiple transcription factors. Changing the set of transcription factors within a cell leads to activation of a different set of genes ultimately leading to a change in a cell's protein expression profile [11]. Selective expression of transgenes in specific cells or tissues can be achieved by constructing DNA expression cassettes that contain regulatory regions that are recognized by transcription factors specifically present or selectively expressed by the target cell population. This so-called transcriptional targeting can be based on tissue specificity where transcription is directed specifically among healthy tissues, and on tumor specificity by using elements that are active selectively in tumor cells due to aberrant gene expression or tumor biology.

Tissue specific promoters

There is an increasing list of well-characterized regulatory elements controlling cell type specific expression, with target tissues including the pancreas [30-32], breast [30,31,33-35], bone [29,30,36-38], brain [30,31,36,39], melanocytes [29-31,34,36,40-43], kidney [31], bladder [31], prostate [29-31,36,40,44], testes [31], connective tissue [31], muscle [31,36,37,42], endothelium [29,30,36,45-62], liver [30,31,34,36,40], GI-tract [57], lung [30,31,36], epidermis [63], thyroid [39], hematopoietic cells [30,31,34] and ovary [29,37]. Tissue specific promoters display a natural activity in normal tissues without discriminating diseased from healthy cells. Therefore, in cases where toxic genes are to be expressed, their utility as such is limited to dispensable tissues such as melanocytes, prostate, breasts, endocrine and exocrine tissues [30]. Combining tissue specific promoters with additional targeting moieties can further increase their utility. For example, combination of tissue and tumor specific promoters may enable targeting of specific cells/malignancies within non-dispensable tissues. A complication involved when using cellular regulatory elements is their low activity per se. To obtain a therapeutic effect, usually combination with a strong promoter element (either viral or cellular) is required.

Tumor specific promoters

One of the most important factors limiting success of conventional cancer therapy strategies is their lack of tumor selectivity. Available cytotoxic agents have been reported to have a cancer-to-normal cell therapeutic ratio as low as 2:1 to 6:1. Novel approaches using gene therapy strategies have been reported to succeed in increasing this ratio to 10,000:1 [30]. Minimizing or excluding inappropriate expression in surrounding non-target cells is of great importance for limiting adverse effects and therefore for increasing the therapeutic index. Considering the complexity of the biological nature of tumors, the genetic alterations that lead to the malignant phenotype of tumor cells and the high genetic mutation rates, it is difficult to address universal tumor features. However, an increasing number of key-events involved in the process of tumorigenesis and characteristics of tumor tissues are being discovered. Employing regulatory elements that direct transcription in response to these key-events may enable targeting of gene expression to tumors of various origins (see Table 2). Design of tumor-specific expression vectors can be based on aberrant gene expression profiles in tumor cells or on typical tumor biology. Aberrant gene expression may be a matter of mutations to certain genes, or errors in the process of their transcription. In other cases, the genetic expression pattern is affected, for example re-expression of embryonic genes or expression of viral genes may occur. To date, no genes have been identified to be completely restricted to tumor cells; when referring to genes as “tumor specific” it is meant that they are found at much higher levels in tumor cells than in normal cells. Tumor growth has several biological consequences, affecting vasculature and metabolism. Fast-growing tumors are often poorly vascularized, this poor vascularization in combination with the solid character of the tumor tissue results in a high interstitial and low intravascular pressure, leading to a decrease in nutrient supply and ultimately necrosis of the tumor core. Typical hallmarks of the microenvironment of such

tumors consequentially are glucose deprivation, chronic anoxia/hypoxia and acidosis [34,64,65]. These conditions cause activation of a number of so-called stress proteins, which is thought to be an adaptive response evolved to protect cells against stress-induced cell death. The obtained cell survival is required for tissue preservation and organ protection in cases where normal cells are

Table 2. *Tumor specific expression.*

Key-event	Application in gene therapy	Reference
Aberrant gene expression		
Oncofoetal genes	AFP	[29-31,34,36,40,66]
	CEA	[29-31,34,36,40,43]
Oncogenes	ErbB2 (ErbB3, ErbB4)	[29,30,34,36,40]
	c-Myc/Myc-Max responsive elements	[34]
	MUC1/DF3	[29,36,37,40,43]
	Ras	[29]
	EGFR (ErbB1)	[29]
	FGFR	[29]
	PIK3	[29]
	Fms	[29]
	Akt2	[29,67]
Cell cycle-regulated genes	Cyclin A, Cdc25C	[36]
	Heparanase	[30]
	RhoC	[30]
	Fibronectin	[30]
	Thymosin β4	[30]
	Endoglin (CD105)	[30]
	Integrins; αV-B3 integrin	[30]
	c-Myc, Cdc2, E2F-1	[29,30,36,43]
Immortalization	Telomerase (hTERT gene)	[29,30,36,43,68]
Tumor biology		
Angiogenesis	E-selectin, Le(a) and Le(x)	[29,30,36,43,61]
	Endoglin (CD105)	[30,36,49]
	VEGF	[29,30,47,54,60,61]
	Human/murine (prepro)-endothelin-1	[30,50,59,61]
	Tie-2	[49,61,68]
Pathogenic vasculature	HRE; HIF-1 target genes (including VEGF, erythropoietin, LDH)	[30,36,47,60,69-71]
	Grp78(BIP)	[30,34,36,64,65]
Impaired glucose metabolism	Hexokinase II	[30,36]
Diverse		
	hPRL, ALA, BLG, Osteocalcin, SLPI, L-plastin, GRP, AVP, K-Ras, SI, HCG, MK, HAFR gene promoter (P1), Cyclin D1, FGFR, CA125 antigen, inhibin/activin, hyaluronan receptor, trypsin inhibitor, metalloprotease pump I, UPA, LDH gene-HRE, Cox-1, Cox-2	[29,30,34,36-38,40,57,72-74]

Abbreviations: AFP: α-fetoprotein; ALA: human α-lactalbumin; AVP: vasopressin; BLG: bovine β-lactoglobulin; CEA: carcinoembryonic antigen; Cox: cyclooxygenase; FLT-1: fms-like tyrosine kinase-1; EGFR: endothelial growth factor receptor; FGFR: fibroblast growth factor receptor; GRP: gastrin-releasing peptide; Grp: glucose regulated protein; HAFR: human α-folate receptor; HCG: human chorionic gonadotropin; HIF: hypoxia inducible factor; hPRL: human prolactine; HRE: hypoxia responsive element; hTERT: human telomerase reverse transcriptase; ICAM-2: intracellular adhesion molecule 2; KDR: kinase-like domain receptor (human homologue of flk-1); LDH: lactate dehydrogenase; MK: midkine; MMTV: mouse mammary tumor virus; MUC: mucine; PECAM-1: platelet endothelial cell adhesion molecule-1; PIK: 3 phosphoinositide-dependent protein kinase; SI: sucrase-isomaltase; SLPI: secretory leukoprotease inhibitor; tie: tyrosine kinase with immunoglobulin and epidermal growth factor homology domains; tPA: tissue-type plasminogen activator; VCAM: vascular cell adhesion molecule; VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor; vWF: von Willebrand factor; WAP: whey acidic protein

exposed to pathological conditions, but also occurs in neoplastic cells where it can lead to cancer progression, drug resistance and protection of cancer cells from immune surveillance. Indeed, a correlation between elevated levels of stress proteins in a variety of cancer cell lines with malignancy was observed [65]. Therefore, recruitment of the transcriptional control elements of stress proteins to target cancer cells is an interesting topic for investigation. Successful results have been obtained both *in vitro* and *in vivo* with the glucose-regulated protein 78 (Grp78) promoter [64,65] and with promoters containing hypoxia responsive elements (HREs), of which the vascular endothelial growth factor (VEGF) promoter is most widely investigated [47,54,60,69,70,75].

When a tumor reaches a size of ~1 mm^3, tumor cells induce proliferation of (myo)fibroblasts and endothelial tissue. This process is mediated by several growth factors (i.e. transforming growth factor α (TGF-α), basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF)) which stimulate proliferation of stromal cells. This process of neovascularization is named tumor angiogenesis and of great importance for the growth and development of both primary tumors and metastases [76]. Because of its major role in regulating important biological processes in tumor angiogenesis, vascular endothelium can be considered as a suitable target for cancer gene therapy for tumors of diverse origins. Tumor endothelium can be distinguished from normal vasculature through the expression of specific membrane-associated receptors, adhesion molecules and other proteins, and by the high proportion of proliferating cells [36]. Advantages of targeting tumor vasculature include its readily accessibility to systemically administered drugs due to its large surface area and proximity to the circulation, and the comparative homogeneity of this biological hallmark among different tumor types [36,61]. From a delivery point of view, enhanced vascular permeability and angiogenesis and the consequential retention of macromolecules can be used for passive targeting strategies.

Transcriptional control: controlling expression in space and time

Some gene therapeutic applications require tight control over the level of transgene expression to prevent expression-induced toxicity. For example, the use of gene therapy to restore insulin production in insulin-dependent diabetes mellitus requires tight control of transgene expression in response to blood glucose levels. Temporal and spatial restriction of expression aims at adding levels of specificity and maximizing the ratio of expression levels in the induced state to background levels.

Physical induction

One of the most striking examples of selective and inducible transcriptional regulation observed in eukaryotic cells is the induction of so-called heat shock proteins (hsps) in response to exposure to super-optimal temperatures. The most prominent heat shock protein is hsp70 and its expression is mediated mainly by interaction of heat shock factor 1 (HSF1) with heat shock elements (HSEs) present in the hsp70 promoter [77]. Binding of HSF1 to HSEs leads to release of RNA polymerase that is normally stably bound proximally to the hsp70 promoter and enables initiation of

transcription[77]. Huang *et al.* [78] used a 400 bp hsp70 promoter driving expression of the GFP reporter gene or the cytokines TNF-α or IL12 engineered into an adenoviral vector. They demonstrate that moderate hyperthermia (39-43 °C) for relatively short periods of time (20-60 minutes) efficiently activates gene expression (GFP, TNF-α or IL12) driven by the hsp70 promoter both *in vitro* and *in vivo*. Promoter activity starts at 39 °C with an optimum at 42-43 °C and decreasing efficiency at higher temperatures due to cell death. Expression starts ~3 hours after hyperthermia treatment, peaks at 18-24 hours and drops back to background levels after 72 hours. Additional *in vitro* studies involving cytokine gene expression show an induction of $>6.8 \cdot 10^5$ and >13,600 over background expression levels for TNF-α and IL12, respectively. Importantly, cytokine levels in non-heated control cells are below the detection limits and equal those in non-transfected cells. Observations of low leakiness and inducible expression were confirmed *in vivo*.

Advantages of heat-responsive promoters include low background expression with high inducibility, low leakiness and convenience and safety of the stimulus. Disadvantageous is that conventional heat-treatment has a rather poor resolution (localization within an order of cm [78]) and succeeds in heating to appropriate temperatures only the extremities, ovaries, brain, breast, prostate, head and neck. Furthermore, growth conditions may vary within a tumor and this could affect transgene expression. Appropriate measures must be taken to adjust heat treatment such that the desired effect is obtained in all tumor areas. Recently, advanced heating techniques based on MRI-guided Focused Ultrasound heating have been described that may offer a solution to these problems [77].

Responsiveness of the hsp70 promoter to various environmental and physiological signals, (i.e. several transcription factors including CTF, SP1, ATF/CREB, hypoxia, acidosis, energy depletion, oxidative stress, cytokines, certain toxic compounds, ischemia, UV radiation [77,78]) provides potential pathways to further modulate promoter activity, but at the same time complicates predictability and control. For example, even inflammation or fever may unwantedly activate heat-responsive transcription elements in the absence of heat-treatment [77,78]. The hsp70 promoter is repressed by wild-type tumor suppressor gene p53, but overexpressed in many tumor cells with defective p53 function [79]. The combined inducibility by heat, hypoxia and p53 mutations potentiates efficient transcriptional targeting of tumor cells.

An important feature that requires further investigation is the risk of developing thermotolerance. Cells exposed to elevated temperatures become temporarily resistant to a subsequent heat shock. Thermotolerance is transient in nature and its magnitude and duration depend on the severity of the initial heat dose. Manipulation of the hsp70 promoter may solve this problem; a hsp70 promoter modified to contain extra HSE was shown to be more active than the unmodified promoter in thermotolerant cells [77].

Several promoters displaying radiation-responsiveness have been proposed, including the early growth response-1 gene (Egr-1) [80-83], wild-type p53-activated fragment 1 (WAF1) [84,85] and recA [86]. The first promoter tested was the Egr-1 promoter. Egr-1 driven expression of TNF-α has been

shown to result in increased tumor growth inhibition compared with radiation-treatment alone. CArG-elements ($CA(A/T)_6GG$) within this promoter were identified to account for radiation-responsiveness and synthetic radio-inducible promoters were developed exploiting multiple CArG-elements as enhancers for other (stronger) promoters such as CMV. Multiplication of CArG-elements improved inducibility while decreasing leakiness. Importantly, activation of Egr-1 is predominantly p53-independent in contrast to other radio-inducible promoters, decreasing the likelihood of tumor-dependent interference with expression.

Expression levels obtained with the WAF1 promoter were found to equal or even exceed those observed for other radiation-inducible promoters [84,85]. Worthington *et al*. [85] performed an *ex vivo* experiment in which the WAF1 promoter was used to drive expression of the human inducible NO-synthetase (iNOS) gene in rat arteries. iNOS expression was observed to be induced 5-fold 8 hours after exposure to 4 Gy radiation in rat tail artery segments. WAF1 driven iNOS expression resulted in full relaxation in artery segments that were preconstricted with phenylephrine 1 hour after exposure to 4 Gy X-rays; this effect was reversible. Next, an experiment was performed where WAF1/iNOS was injected directly into two different tumor types (RIF-1 and HT29) in mice [84]. The WAF1 promoter was induced by an initial X-ray dose of 4 Gy followed, 8 hours later, by treatment doses of 10 or 20 Gy. Examination of the mice tissue revealed that 48 hours after intratumoral injection, vector sequences were detected in all tissues tested, indicating that substantial leaking of the system from tumor tissue to non-target tissue occurs. Importantly, no significant increase in iNOS levels was observed in any of the tissues other than the tumor and surrounding dermal tissue that had been exposed to the irradiation. Induction of iNOS levels was 2.1- and 3.3-fold in RIF-1 and HT29, respectively, when compared to control non-tranfected tumors. When compared to transfected but non-irradiated tumor cells, induction was 3- and 1.6-fold, respectively. A problem involved when using the WAF1 promoter is that expression does not occur in a straightforward dose-dependent fashion and the optimal radiation dose appears to be dependent on cell-type and transgene used. Upregulation of WAF1 in hypoxic conditions may be advantageous for radiotreatment of tumors as hypoxia often hampers effectiveness of regular radiotherapy.

The major advantage of radiation-induced transcription is the high level of precision of target area selection. Radiation therapy can reach precise localization within an order of millimeters. Limitations to radiation-inducible promoters include leakiness and the carcinogenic nature of the stimulus, which restricts applications to cancer treatment. Additional research is required to establish optimal radiation dosing schemes and relevance thereof in clinical settings.

Chemical induction

Pharmacological regulation of transcription is desirable when the aim is to drive long-term expression of transgenes as it allows (1) titration of protein into the therapeutic window, (2) dose-adjustment, (3) reversibility/termination of therapy and (4) fluctuating daily dosing regimens relevant for many proteins (see Figure 3 A) [87,88].

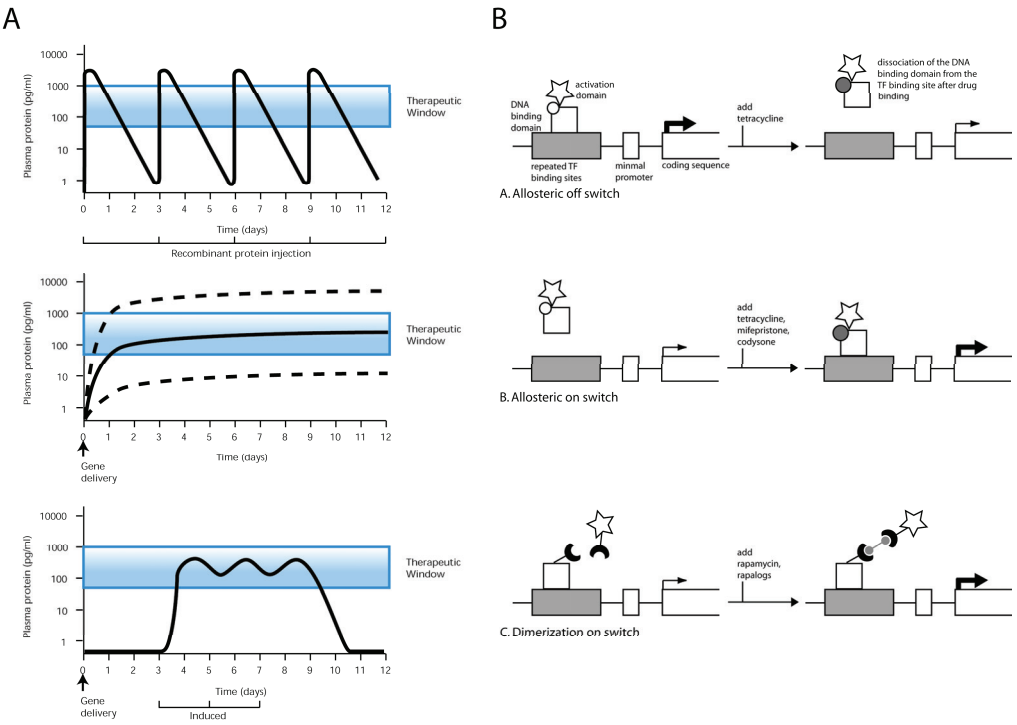


Figure 3. *Chemical induction of gene expression. A: Hypothetical protein plasma levels after (top) intravenous injection of recombinant protein, (middle) gene therapy using non-inducible expression systems, (bottom) gene therapy using inducible expression systems. Adapted from Clackson* [88] *with permission. B: Schematic illustration of the different types of chemically inducible gene expression systems. Adapted from Harrington et al.* [89] *with permission.*

The key element of chemically inducible systems is a modulator-controlled transactivator consisting of (1) a DNA binding domain (DBD) that can recognize and bind to specific cis-elements within the promoter, (2) a transcription activation domain (AD), and (3) a binding site for the modulator (chemical/drug) [90].

Recruitment of the AD to the promoter may be based either on allosteric interaction or dimerization (see Figure 3 B). In case of allosteric interaction, a specific drug-controlled DNA-binding domain is fused to a heterologous transcriptional activation domain. This fusion-control element is placed upstream and interacts with the promoter only in the presence of the drug of concern, allowing transcription to be initiated.

The first and most frequently described allosteric interaction system is a tetracycline (Tet) (or analogue) dependent gene expression system, that comes in two variants: a system in which transcription is either suppressed (Tet-Off) or inititated (Tet-On) upon adding the tetracycline drug. The natural Tet-controlled DBD of the *Escherichia coli* (*E. coli)* Tet repressor (TetR) is fused to a heterologous AD (e.g. virion protein 16 (VP16)) to produce the tetracycline transactivator tTA. This

tTA activates transcription upon binding to Tet operator (TetO) sequences upstream of target genes. However, when tetracycline or an analogue (e.g. doxycycline) is present, DNA binding and hence transcription is abolished due to binding of the drug to TetR. Initial reports describe low-level background expression in the presence of Tet, with an up to 100,000-fold increase upon Tet withdrawal depending on the cell clone used [87,89,91]. However, subsequent studies have never succeeded in achieving such extreme induction ratios, largely due to high background expression levels [89]. Drawbacks of the Tet-Off approach include the necessity of long-term administration of tetracycline (analogues) and the slow onset of induction due to dependence on tetracycline (analogue) clearance [92]. Wilson *et al.* have proposed utilization of the Tet-Off based gene expression system for treatment of diabetes by establishing constant background insulin replacement with infrequent adjustments to this basal expression level upon tetracycline administration to allow anticipation to illness, diet, exercise etc. [93]. They observed repression of proinsulin secretion in a dose-dependent and reversible fashion upon addition of tetracycline following transfection of murine and human myoblasts with a tetracycline-repressible transactivator and an insulin plasmid containing a tetracycline-responsive element upstream of a minimal CMV promoter. Functionality of this approach was also confirmed *in vivo* following intramuscular plasmid injection and oral tetracycline administration in rats. Baron *et al.* describe optimization of Tet-regulated systems that show reduced toxicity by modification and screening of tetracycline-controlled tTAs to eliminate potential interaction sites for various cellular transcription factors and sites that may elicit a cellular immune response [94].

Two strategies to change the Tet-system from an off- to an on-system have been investigated. The first involves fusing TetR to a strong transcriptional repression domain, enabling de-repression of transcription upon drug binding. This was reported to result in a Tet-mediated reporter induction of up to 50-fold. The second approach aims at producing a true on-switch mechanism and employs a mutant TetR that only binds DNA in the *presence* of Tet [87,88]. Unfortunately, reversal of response type is accompanied by decreased binding affinity of TetR for its inductor and increased background expression levels. Several mutant forms have been screened to identify those with minimal background activity combined with acceptable binding affinity [92,95]. For mutant TetR systems, induction ratios of up to 10,000-fold have been reported [87]. Another approach to reduce background expression has been described in which the Tet-On system is used in combination with a tetracycline-controlled transcriptional silencer (tTS). In the absence of tetracycline, the tTS interacts with TetO sequences to suppress transcription. However, when Tet is present in sufficient concentrations, conformational changes in tTS lead to dissociation of tTS from TetO allowing the reverse transcriptional activator to bind and activate transcription [92]. This approach requires efficient delivery of three separate vectors and unless these can be integrated into a single plasmid construct, it is not very attractive for use in gene therapy.

In antiprogestin-regulated gene expression systems, truncated forms of the human progesterone receptor ligand binding domain (PR-LBD) are linked to specific DNA-binding and transcription

activator domains to form a chimeric protein that functions as an antiprogestin-responsive transcription factor. The PR-LBD is modified such that it can no longer bind progesterone or any other known endogenous steroid but can be selectively activated by progesterone antagonists such as mifepristone (Mfp). Non-viral gene delivery of an Mfp-controlled system in mice resulted in an average multitude of transgene induction of 14-19-fold in response to Mfp. This effect could be reached repeatedly over a period of ~ three weeks [96]. Other studies report up to 200-fold induction ratios (depending on the minimal promoter used) both in cell culture and in animals[87,97].

To avoid problems of interference from endogenous hormones involved when employing human/mammalian steroid hormones and their receptors, the use of the prokaryotic steroid ecdysone (Ec) (or its synthetic analogue, muristerone A) and its nuclear receptor was proposed. Fusing the *Drosophila melanogaster* Ec receptor Ec-binding domain to heterologous DBDs and ADs was shown to permit Ec-dependent activation of transcription. These systems did not respond to a series of endogenous human steroids. In vitro, low background activity combined with induction ratios of up to 10,000-fold were observed [87].

Many tumors develop anti-cancer drug resistance through overexpression of the multidrug resistance (Mdr)-1 gene. Since the Mdr1 promoter is inducible by cytostatic agents such as doxorubicin, vincristine and taxol, it can be employed for pharmacological upregulation of transgene expression to potentiate chemotherapeutic effects [98-100].

Only recently, a novel form of chemical induction was described, in which a gas is used as the inductor for expression of transgenes [101,102]. Weber *et al.* [101] developed a system derived from the fungus *Aspergillus nidulans* based on the enzymatic machinery that regulates conversion of ethanol to acetyl coenzyme A in response to acetaldehyde. They developed an inducible promoter consisting of a minimal CMV promoter and five operators each containing binding sites for the transcriptional activator. Transgene expression obtained with this construct was regulated tightly by the gaseous acetaldehyde. *In vitro* expression levels correlated linearly with gas concentration and reached an optimum at gas levels that were below the no-observed-effect level (NOEL) of 152 ppm as declared by the World Health Organization (Health and Safety Guide 90, 1995). However, *in vivo* no linear relation between acetaldehyde levels and gene expression was observed. Moreover, since gas concentrations of >1,000 ppm are likely to be required for optimal induction, strategies augmenting transactivation need to be developed to make the system function at gas levels below the NOEL, which will be essential for clinical application. Limitations to this system include the unpredictability of the influence of endogenous acetaldehyde and possible immunostimulation in response to the fungal elements [90].

Chemical dimerization-controlled transcription requires co-expression of two fusion proteins that each contain a drug-binding domain. This domain is fused to a DNA-binding domain in one protein and to a transcriptional activation domain in the other. Additionally, the target gene construct is introduced consisting of the therapeutic gene driven by a promoter that contains binding sites for the DBD. Administration of a drug able to cross-link both fusion proteins through their drug-binding

domains, results in the formation of a functional transcription factor. This results in recruitment of the AD to the promoter and transcription initiation, respectively. The bivalent drug may be homodimeric (binding equal domains) or heterodimeric (binding different domains). Heterodimeric drugs are usually preferred as in case of homodimerization, non-productive DBD-DBD or AD-AD homodimers can be formed in addition to the functional DBD-AD heterodimers. A well-described example of a heterodimeric drug is rapamycin, an orally bioavailable drug that mediates the formation of dimers between the human proteins FKBP (FK506 binding protein) and FRAP (FKBP12 rapamycin-associated protein). Low background expression levels and high inducibility (over 10,000-fold *ex vivo*) were observed employing the rapamycin-regulated strategy [87]. Expression levels comparable to those obtained with the CMV promoter have been reported [103]. Efforts have been made to modify the system to function with non-immunosuppressive analogues of rapamycin ('rapalogs') [103].

The highly modular character of dimerizer-regulated systems facilitates incorporation of a wide range of DBDs, ADs, drug binding domains and development of alternative dimerizer drugs allowing practically unlimited opportunities for optimization. For a review on progress made with dimerizer-based strategies, the reader is referred to Pollock and Clackson [103].

Despite the promise of pharmacological regulation of transgene expression, there are some less elegant sides of chemically inducible systems. Although the transcriptional control elements can be modified to optimize and target expression, no control over the fate of the inducing drug exists and many non-target cells will be unnecessarily exposed to this drug. Another disadvantage is the requirement of multiple constructs, meaning that effect is only obtained in those cells that receive all of the essential elements. To increase efficiency and to minimize burdening of the delivery system, efforts must be made to combine the different elements into a single construct.

Sustained expression

A serious issue currently limiting the widespread use of non-viral gene therapy vectors is their transient nature of transgene expression. Expression is decreased in time due to several mechanisms including potential loss by recombination or destruction by nucleases [31,104] and partitioning to non-nuclear compartments [104]. Also, in dividing cells a logarithmic decrease in the percentage of transfected cells during replication of the target population occurs since the plasmids do not replicate while the cells do [31,104]. Finally, recognition and subsequent silencing of foreign DNA *in vivo* impedes persistent transgene expression. Diverse strategies have been developed to overcome one or more of these problems to increase duration of expression.

Integrating expression vectors

One solution to allow for replication of the delivered gene is integration of the gene into the host genome. Enzymes that are capable of inserting foreign DNA into the host genome include viral integrases, (site-specific) recombinases and transposases [105]. Among these systems, viral systems,

especially retroviruses, integrate at relatively high frequencies. However, loading capacity is limited and lack of site-specificity increases the risk of insertional mutagenenis [105]. It was recently discovered that retroviruses and recombinant adeno-associated virus (rAAV) vectors actually integrate preferentially into transcriptionally active regions rather than randomly [106]. For example, murine leukaemia virus (MLV), human immunodeficiency virus (HIV) and adeno-associated virus (AAV) have been observed to preferably integrate into genes rather than non-genomic regions, and AAV has been associated with deletions and rearrangements within host DNA [106-108]. In this light, recombinases and transposases may offer a safer alternative. Site-specific recombinases are enzymes that catalyze DNA strand exchange between stretches of DNA that are homologous only to a limited degree [105]. Recombinase proteins bind covalently to recognition sites within the DNA, cleave its backbone, exchange the double-stranded DNA segments and finally religate the DNA. Some recombinases function independently, others require the help of additional proteins. Two of the most widely described site-specific recombinases are cyclization recombination recombinase (Cre) and ϕC31 integrase, enzymes derived from *E. coli* bacteriophage P1 and *Streptomyces* phage ϕC31, respectively. Integration is possible through interaction with so-called "pseudo sites" that resemble the original recombination sites that are absent in the human genome [108]. Cre-mediated integration is reversible, hence unstable and inefficient, due to the fact that excision is favored over integration. The explanation for this is that *loxP* (Locus of Crossing over of P1 phage) sites remain unaltered and are therefore still substrates for excision/integration reactions, until finally all *loxP* sites are removed by excision. An advantage of Cre is its remarkable site-specificity. However, this at the same time represents an obstacle as extensive manipulation to Cre is required to obtain a broader specificity to make it react with pseudo target sites within the host-genome. Since ϕC31 displays a greater affinity for and recognizes more (at least 11) pseudo target sites within the human genome, it does allow stable integration of DNA herein with greater efficiency (24-56 %) [108]. However, its lower degree of specificity requires additional research on potential target sites to assess risks of insertional mutagenesis [105]. Individual recombinases can be further optimized through a process of directed evolution: random mutations are introduced and screened for improved efficiency and specificity [108]. Furthermore, Buchholz *et al.* describe customization of the Cre-like recombinase flippase (FLP) to adjust its temperature optimum from 30 °C to 37 °C to make it useful for application in human gene therapy applications [109].

Transposases are enzymes that can transfer discrete segments of DNA (transposons) from one molecule to another through a cut-and-paste process [107]. Transposases are abundant in prokaryotes and insects, but have no equivalent in vertebrates. Transposons originating from non-vertebrates are functional in human, albeit at low efficiency. However, with the reconstruction of the so-called *Sleeping Beauty* (SB) transposon, a vertebrate-derived DNA-transposon was created that displays much higher activity than any other transposon in a wide range of vertebrates [107]. SB transposon consists of the transposable gene flanked by two terminal inverted repeats (IRs) that contain binding sites for the transposase. Binding of transposase to these sites is followed by

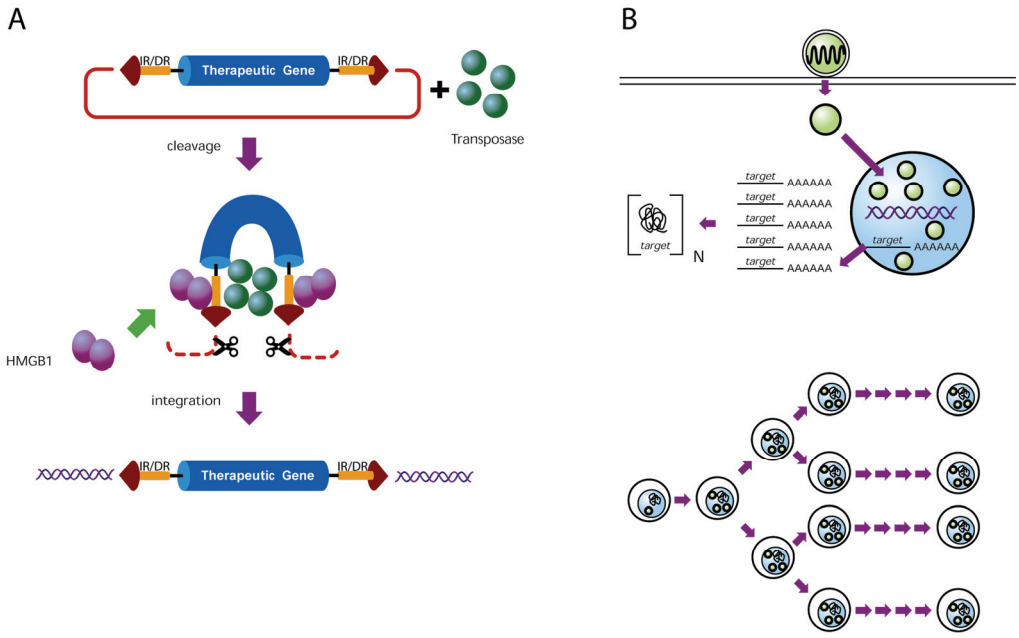


Figure 4. *Strategies for sustained gene expression. A: The Sleeping Beauty transposable element and its transposition. A plasmid in which the transposase gene is replaced by a therapeutic gene (transposon) and the transposase are supplied in trans. The transposase binds to its binding sites within the IR/DR repeats flanking the therapeutic gene and, together with host factors such as HMGB1, a synaptic complex is formed, in which the ends of the transposon are paired. After excision of the transposon from the plasmid, it can be integrated into a new location. B: Episomally replicating vectors. Replicating episomal plasmids yield high levels of target gene expression through several mechanisms: vector replication leads to accumulation of multiple copies of the episomal plasmids; increased copy numbers of plasmids lead to increased levels of target gene mRNA, and consequently increased levels of target gene protein; efficient vertical transfer of the episomes during cell division results in maintenance of high-level gene expression.*

formation of a synaptic complex, excision of the DNA from the donor site and reintegration at a target site, respectively (see Figure 4 A). Formation of the synaptic complex requires interaction with the host protein HMGB1 (high mobility group box 1), a non-histone protein associated with eukaryotic chromatin that serves as a cofactor for SB transposase [107]. SB transposase is highly specific; no cross-mobilization is observed among closely related transposons [107]. In its original form, maximum delivery capacity of SB is limited to 10 kb, with each kilobase increase the efficiency decreases exponentially with ~30 %. However, flanking of transgenes with two complete SB elements has been shown to increase these size limits considerably [107]. Analysis of insertion sites in human cells revealed that SB, unlike diverse viruses, shows no preference for insertion into genic regions over non-genic regions, suggesting that SB transposition may offer a safer alternative for stable expression compared to viral integration. From several *in vivo* studies it appeared that long-term expression could be obtained (>5 months), whereas no cytotoxic effects, nor immune

responses, nor liver tumors were developed [110-112]. These results illustrate the potential of SB for use in gene therapy settings. However, the remaining risk of insertional mutagenesis should not be ignored. Future directions for integrating strategies will be based on strategies allowing for targeted integration (site-directed genome modification). This can be obtained by alterations within the DNA sequence or through the use of accessory proteins [105,113].

Episomally replicating vectors

An approach that does not require integration of the gene into the genome and therefore avoids risks of insertional mutagenesis is the use of autonomously replicating plasmids or episomes (see Figure 4 B). In episomally replicating plasmids, sequences from (generally) viral DNA are incorporated that enable the plasmid to replicate extrachromosomally. There are several advantages over integrating systems: (1) the transgene cannot be interrupted or subjected to regulatory constraints which often occur from integration into cellular DNA [33,114]; (2) higher transfection efficiency can be obtained than with chromosome-integrating plasmids [33,114]; (3) episomes display a low mutation rate and tend not to rearrange [33]; and (4) episomally replicating systems have the ability to transfer large amounts of DNA. For instance, systems based on the Epstein-Barr virus (EBV) viral replication and retention components can carry up to 185 kb of viral DNA [33,114] and allow replication in both eukaryotic and prokaryotic cells, which enables easy shuttling among these host cells [33,114].

Constructs from several viruses including EBV, BK virus (BKV), SV40 and bovine papilloma virus 1 (BPV-1) have been investigated as episomal expression vector candidates [31,114]. In general, the main problem with episomal expression vectors is the requirement of *trans*-acting factors, which are often associated with risk of transformation. This is especially the case for vectors based on polyomaviruses (i.e. BKV, SV40) where the *trans*-acting factor is a large T antigen (Tag). Large T antigens were shown to have numerous unacceptable properties including the ability to bind the tumor suppressor gene p53, to induce chromosomal aberrations and to influence cellular gene expression by interfering with cellular transcription factors [114]. In this respect, strategies based on EBV are considered to be relatively harmless. Additionally, EBV displays a low mutation frequency and is capable of carrying large amounts of DNA [114]. Most progress so far has indeed been made with EBV and therefore this system will be discussed in more detail.

EBV contains two elements that enable stable episomal maintenance of the viral DNA in the host cell: oriP, required *in cis*, and the EBV nuclear antigen 1 (*EBNA1*) early gene, required *in trans* [33,114-116]. OriP contains two regions being the family of repeats (FR) and the dyad symmetry (DS) element, containing 20 and 4 binding sites for EBNA1, respectively [114]. EBNA1 has been reported to facilitate nuclear localization of the plasmid [115]. Expression of the *EBNA1* gene is followed by binding of EBNA1 as a homodimer to oriP and recognition of the bound oriP-site as a functional DNA origin by human cells [31,114]. The EBNA1 dimer/oriP complex then serves several functions: replication, maintenance and transcription.

The actual role of EBNA1 in *replication* is still under discussion. Binding of EBNA1 dimers to the DS within oriP is thought to result in a structural distortion which is important for the initiation of DNA-synthesis. Importantly, the FR contains a replication fork barrier that forces unidirectional replication and thereby reduces risks of mutation and rearrangements [114]. Conversely, some groups have reported an EBNA1-*in*dependent synthesis of oriP-containing plasmids [114]. Despite the ambiguity of the exact mechanism of replication, EBV vectors replicate once per cell cycle in synchrony with the host chromosomes [114].

In addition to inducing plasmid replication, EBNA1 facilitates *binding of the plasmid to the nuclear matrix*: binding of EBNA1 both to the FR on the plasmid and to chromosomal elements mediates physical association of the plasmid with the host chromosomes in order to retain the plasmid [114,115]. Also, a cellular protein EBP2 (EBNA1- binding protein 2) has been identified which is thought to play a role in segregation of the episomes during cell division [114].

EBNA1 is thought to mediate *transcriptional upregulation* upon binding to the FR through a mechanism that has not yet been revealed [114,115]. This effect seems dependent on the cell type used and on the promoter within the plasmid [114].

Stable replication of EBV-derived episomal vectors was shown in a variety of mammalian cells, including human epithelial, fibroblast, and lymphoma cells, as well as monkey and dog cell lines[114]. Cui *et al.* report highly efficient gene transfer of EBV-derived plasmid vectors containing the *EBNA1* gene and the oriP sequence *in vivo* [115]. Both EBV-derived and conventional plasmid vectors encoding luciferase or β-galactosidase (β-gal) as markers were constructed and injected into the tail vein of mice using the hydrodynamic pressure method of transfection. Subsequently, gene expression was measured in the liver over time. For the conventional luciferase-expression vector, a maximum level of protein expression was measured at 8 hours post-transfection, declining 4.8-fold during the following 16 hours. In comparison, transfection with the EBV-based luciferase-expression vector resulted in an approximately 1.7-fold and 7.2-fold higher expression at 8 and 24 hours after transfection, respectively. Similar results were observed for the β-gal-expression vectors.

High transfection efficiency, long-term expression, capacity to carry large amounts of DNA and low mutation and rearrangement rates all contribute to the potential success of EBV-based vectors in non-viral gene therapy. However, although in most cases stable episomal maintenance of EBV-derived vectors is obtained, in some cases integration in the host chromosome and rearrangements within the vector have been reported [114]. This effect seems dependent on the cell line used, and will require thorough research before EBV-based vectors can be used safely in humans. Another important issue is the observation that plasmid copy numbers vary considerably among different cell lines, ranging from 5 to 100 [114]. This merits attention since the plasmid copy number within a cell is determinative for both therapeutic and possibly toxic effects.

Finally, risks of oncogenicity should be examined more carefully. For EBV, the viral *EBNA2* gene is considered to be mainly responsible for oncogenicity, and therefore EBNA1 was thought to be

innocent. Nonetheless, results of some *in vitro* and *in vivo* experiments put this assumed innocence in a new light. For instance, EBNA1 was shown to be able to bind to RNA *in vitro* and it may therefore influence expression at the post-transcriptional level [114]. Also, it was suggested that EBNA1 might interact with the c-*Myc* gene which may result in deregulation of this proto-oncogene. *In vivo* results are contradictory. EBNA1 expression appeared to predispose B cells to lymphoma in transgenic mice in a similar fashion as transgenic c-*Myc* expression does, implying oncogenicity of EBNA1 in mice. On the other hand, in other *in vivo* experiments where mice were transfected with EBV-based vectors using lipofection, no pathological changes were observed [114]. Obviously, additional experiments should be performed to ascertain safety of EBNA1 use.

The episomal vectors described above all require at least one viral gene product, such as EBNA1 or Tag in case of EBV or SV40, respectively [117]. The potential immunostimulatory and transforming properties of virally encoded proteins impede application of these systems for human gene therapy. Alternatively, mammalian scaffold/matrix attachment regions (S/MARs) have been identified that can be incorporated into circular non-viral vectors to replace the transacting viral gene products. S/MARs are cis-acting elements consisting of 100-1000 bp AT-rich regions lacking a defined consensus sequence [117]. The S/MAR element enables interaction of the plasmid with components of the nuclear matrix, allowing for co-segregation of the plasmid with chromosomes during mitosis. Additionally, it is thought that this interaction facilitates plasmid replication by bringing the plasmid into contact with the host replication machinery [108].

Piechaczek *et al.* [118] developed an episomally replicating expression vector in which the SV40-ori sequence was used together with the S/MAR from human β-interferon gene cluster (replacing the viral Tag protein). This construct was demonstrated to replicate episomally in CHO-cells and to provide stable expression over more than 100 cell divisions in the absence of selective pressure. Ehrhardt *et al.* [117] developed a vector in which the CMV promoter was replaced by a cellular promoter to eliminate potential silencing effects induced by non-mammalian sequences. They incorporated an S/MAR sequence derived from the chicken lysozyme locus (ChMAR) as *cis*-acting elements within a non-viral plasmid vector encoding the human coagulation factor IX (hFIX). *In vivo*, 5-fold induction of hFIX expression levels were observed for the S/MAR containing plasmid when compared to plasmids lacking the S/MAR for up to 1 year after transfection.

In addition, other vectors are also being explored that are worth mentioning: yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), human artificial chromosomes (HACs) or other mammalian artificial episomal chromosomes (MAECs) and chimeric systems thereof. Constructs have been designed in which the oriP/EBNA1 module is inserted into YACs and BACs to function as an alternative to the centromere. The resulting molecules were shown to form large circular episomes capable of stable and persistent expression [119]. As these artificial chromosomes are designed to carry large DNA inserts, these systems are not very suitable for the use as vectors in gene therapy applications where in most cases only a few genes need to be expressed. Delivery of chromosomal vectors is relatively complicated, particularly since their large

size (1-2 µm), in comparison to plasmids (sizes generally within the nm-range in condensed form), hampers cellular uptake via the endocytic route [120]. Additionally, the structural stability of chromosomal vectors containing both DNA and proteins is inferior to that of pure plasmid DNA [120].

Altogether, extrachromosomally replicating plasmid vectors have great potential for application in gene therapy by increasing the efficiency of transfection and longevity of gene expression. Especially in the case of cancer treatment where dividing tumor cells are involved, maintenance and vertical transfer of the therapeutic plasmid will be crucial for the duration of the effect and therefore for success of treatment. Retention rates of 92-98 % have also been reported to be typical for EBV-derived vectors in the absence of selection pressure [114]. Although this does mean that retention is still imperfect and loss over time will remain an issue, these replicating plasmid vectors can be considered far superior compared to conventional non-replicating plasmids.

Preventing gene silencing

Despite the use of integrating or episomally replicating vectors, sustained expression of transgenes may be hampered by gene silencing [121]. Although the exact mechanism of silencing has not yet been elucidated, several possibilities have been coined and are worth mentioning. The most striking example of gene silencing is the increasing number of experiments showing that the CMV promoter, regarded as one of the strongest promoters, is frequently shut-down *in vivo* [122-124]. This is thought to be caused by its DNA characteristics being aberrant from those of eukaryotic DNA. Mammalian genomes differ from bacterial and viral genomes with regard to abundance and methylation state of CpG motifs. The mammalian genome contains a CG frequency of approximately 1:64, which is much lower than the expected frequency of 1:16, as observed in bacteria [125]. Whereas within bacterial and viral DNA CpG motifs remain in an unmethylated state, approximately 70 % of CpGs are methylated in the mammalian genome, leaving unmethylated only those CpG islands associated with genes in the germline or located within the promoter regions of transcriptionally active genes [123].

The high frequency of unmethylated CpG motifs present in bacterial and viral DNA has several consequences. In eukaryotic cells, these CpG motifs are *de novo* methylated, a process that is possibly triggered by transcription itself [126]. The process of methylation serves a physiological function in vertebrates in normal development: X-chromosome inactivation, imprinting and silencing parasitic DNA transcription [123]. Methylation may interfere with binding of transcription factors and therefore block initiation of transcription [122,124,127]. Additionally, methylated CpG basepairs can be recognized and bound by certain cellular proteins, including methyl-CpG-binding proteins (MeCP) MeCP1 and MeCP2. These proteins may compete with transcription factors and/or recruit histone deacetylase activity, which could account for the reorganization of DNA into tightly packed chromatin structures incompatible with transcription [122,124]. The efficiency with which methylation suppresses transcription may be dependent on the position of the methyl groups within the promoter region, density of the methyl groups and the strength of the promoter [124].

Methylation of CpG islands located downstream of an active promoter does not block elongation [122]. On the other hand, when methylation of CpG motifs induces a condensation process, this might spread out to the vicinity of the transgene and silence it.

Another implication is the profound stimulation of the immune system associated with CpG motifs. The sequence requirement for eliciting an immune response is a central 5'-CG-3' motif; the most active sequence in humans is GTCGTT and this stimulatory CpG motif (CpG-S) acts as a "danger signal" that is recognized by "pattern recognition receptors" present on immune cells. The high content of CpG-S in the unmethylated state is typical of bacterial and of some viral genomes (including CMV). Unmethylated CpG-S triggers cells of both the innate and the adaptive immune system, which has consequences regarding expression and toxicity. Gene expression can be decreased through several mechanisms, including cytokine-mediated promoter shutdown and elimination of the expressing cell through apoptosis, innate or adaptive immune responses [125].

When constructing a plasmid vector, several strategies can be used to reduce the immunostimulatory effects of CpG-S motifs: (1) methylation, (2) addition of neutralizing CpG motifs (CpG-N), and (3) elimination of CpG-S [125,128]. However, as described above, methylation of essential CpG motifs within regulatory elements can also result in a drastic decrease in gene expression. CpG sequences preceded by a cytosine (C) and/or followed by a guanine (G) are found to be able to neutralize the immune activating properties of CpG-S motifs, hence the name neutralizing CpG (CpG-N) [129,130]. The net stimulatory potency of a particular sequence appears to depend on the overall ratio of CpG-S to CpG-N motifs. From experiments in mice it was concluded that immunostimulation can be inhibited by CpG-N sequences placed in *cis* and not too far from the CpG-S sequence and through a non-sequence specific effect of *trans* CpG-N sequences. Reducing the CpG-S content of a plasmid does not only result in decreased cytokine production, but very interestingly has also been reported to increase and prolong expression *in vitro* and *in vivo* in mice [131,132]. Taken together, the CpG content and methylation state should be taken into consideration when choosing a regulatory element, with preference for the lowest content of CpG-S. It should be examined whether additional danger signals exist comparable to the CpG-S motifs.

Plasmids constructs used for gene therapy often combine bacterial and eukaryotic DNA elements. Within the nucleus of eukaryotic cells, the transgene is expressed, whereas the bacterial backbone remains inactive. It has been hypothesized that transcriptional activity is related to chromatin structure. In the process of transcription, certain components acetylate histones 3 and 4, resulting in loosening of their binding to DNA and formation of euchromatin. As bacterial DNA is not involved in transcription complexes, histones 3 and 4 may remain unacetylated, leaving the DNA in the more condensed heterochromatin state. In the absence of so-called insulators between euchromatin and heterochromatin regions, heterochromatin might spread into euchromatin in its vicinity resulting in transgene silencing [121]. The role of bacterial DNA in transgene silencing has been examined by Chen *et al.* [121]. They conclude that for silencing to occur, covalent connection of bacterial DNA to the transgene is essential. The silencing effect was observed in both circular and

linear DNA and was found to be independent of specific bacterial DNA sequences and reporter and promoter/enhancer. Exclusion of bacterial DNA resulted in 2- to 3-log higher expression levels in murine livers.

Further customizations

Several strategies have been developed to further customize transgene expression, for example to increase strength, specificity, efficiency or to decrease size or leakiness.

To increase promoter efficiency, all regions within a promoter that do not contribute to its transcriptional strength or specificity should be identified and eliminated. On the other hand, functional elements can be multimerized to an optimum, as described for CArG-elements in the Egr-1 promoter. However, this approach may not be applicable to all promoters and requires time-consuming empirical optimization of each individual promoter [36].

Another, less extensively described, strategy involves increasing promoter strength by activating point mutations [36]. Substituting one G-to-A at nucleotide –119 was shown to significantly increase activity of the human α-fetoprotein (AFP) promoter [133]. However, such possibilities for modifications have only been found coincidentally in few cases.

Promoter activity can also be altered by modifications in DNA sequences (spacers) that separate the individual consensus sequences of promoters. Within these areas it is not so much the sequence per se that is of importance, but the DNA structure resulting from this sequence [134]. Jensen *et al.* [135] constructed a library of 38 mutant promoters and measured a range of relative expression strengths varying from 0.3 to 2,000, covered in small increments. Mutants were designed to contain the known consensus sequences as in the wild-type form, while the sequences of the separating spacers were randomized. However, retrospectively it appeared that all promoters with activities <5 had changes either in the consensus sequence or in the length of the spacer between –35 and –10 sequences. Nonetheless, promoters devoid of these features still had activities varying from 5 to 2,050, indicating that a 400-fold variation in promoter activity can be obtained by spacer randomization. Furthermore, the slight increment at which promoter activity is increased allows fine-tuning to an almost infinitive degree. It should be realized that ranking is dependent on the gene studied and the type of cell culture used. For an optimized method for generating promoter libraries the reader is referred to Solem *et al.* [134].

Yet another strategy is based on constructing “chimeric promoters” that combine the transcription regulatory elements of different promoters eliciting specificity for the same tissue or eliciting different specificity patterns (for example tissue-specificity and tumor-specificity) to further restrict expression to certain target cells. By screening random combinations, optimal constructs can be identified. For example, a range of muscle specific promoters was constructed by assembling 5-20 DNA elements involved in muscle-specific transcriptional activation in a random order and linking them to a minimal chicken α-actin promoter. Remarkably, when tested in

differentiating muscle cells in culture, one of the combinations was shown to be 6-fold more active than the strong CMV IE promoter/enhancer [36].

Positive feedback loops

Both strength and specificity of expression systems can be increased significantly by incorporation of a positive feedback loop. Generally, a promoter eliciting certain specificity is used to drive expression of both the desired effector gene and a strong artificial transcriptional activator. This transcriptional activator then upregulates transcription through interaction with appropriate binding sites within the promoter. In most cases, the transcriptional activator is a chimeric protein consisting of a DNA-binding domain fused to a transcriptional activation domain and is therefore also referred to as recombinant transcriptional activator (RTA) [36].

Nettelbeck *et al.* demonstrated enhancement of the very weak but highly specific von Willebrand factor (vWF) and sucrase-isomaltase (SI) promoter in a positive feedback-loop approach [57]. The vWF promoter exhibits a particularly high degree of specificity for endothelial cells when compared to other endothelial-specific promoters (e.g. PECAM-1/CD31, flk-1/KDR); the SI promoter is highly specific for intestinal cells and gastrointestinal tumors. Here, the RTA is a VP16-LexA fusion protein consisting of the DNA-binding domain of LexA and the transcriptional activation domain VP16 of the herpes simplex virus. VP16-LexA exerts its stimulatory effect through LexA binding sites introduced into the promoter (see Figure 5 A). Two approaches were examined, both using a cell type specific promoter (either the vWF promoter or the SI promoter) to drive transcription of the reporter/effector gene, but one employing a second cell type specific promoter to control the RTA and the other employing an IRES for this purpose. The construct using the two cell type specific promoters proved the most successful and exhibited a 20- to 169-fold enhancement while retaining a 30- to >1,000-fold cell type specificity when compared to a normal vWF-promoter. Similarly, for the SI promoter a 14- to 37-fold enhancement was observed while specificity was retained.

Although particularly useful for weak promoters, transcriptional feedback amplification also proves useful for strong promoters. Emiliusen *et al.* first screened different elements of the strong human tyrosinase promoter to select one with the highest level of specificity regardless of its activity and then successfully increased expression by using a feedback loop [136].

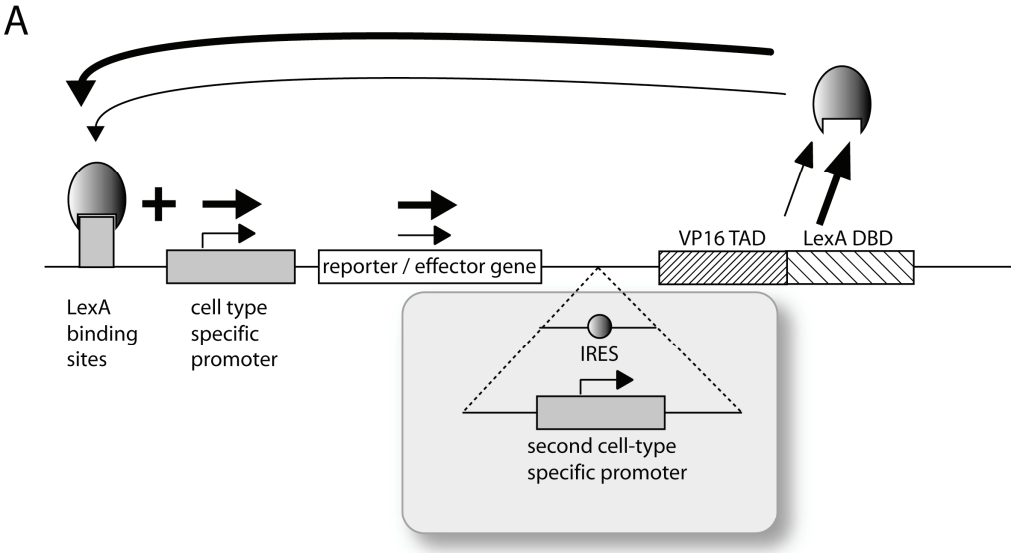
Gene excision by the Cre/loxP system

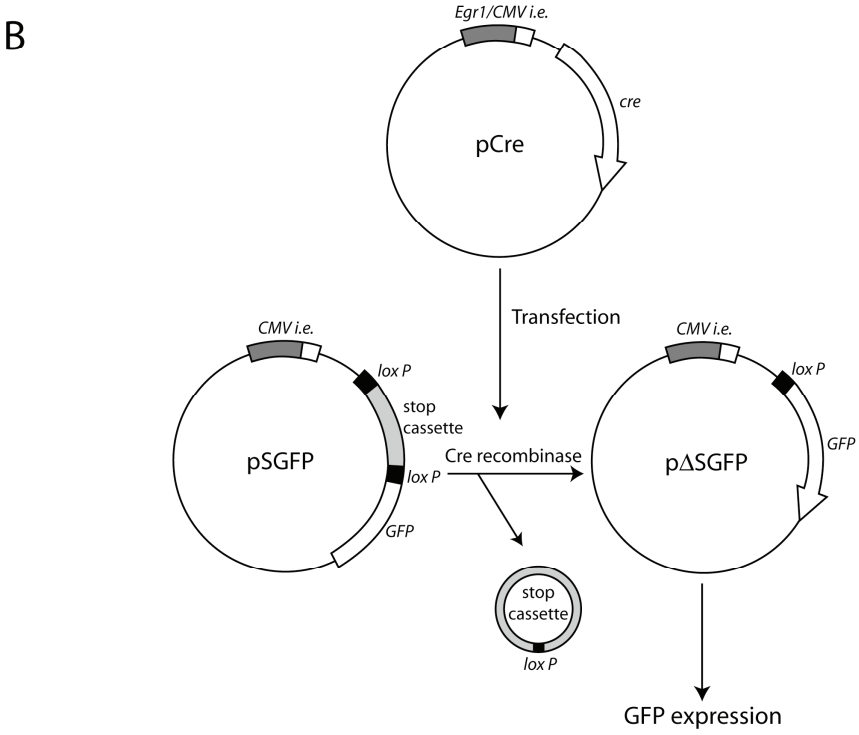
In cases where only temporary protein production is required, it may be desirable to definitively switch on or off gene expression upon an external signal. One method to do so is by excision of specific DNA sequences by recombinases.

Cre is a recombinase derived from *E. coli* phage P1 [137] that excises DNA fragments flanked by *loxP* sites. The DNA is excised as a circular molecule, leaving a 34 bp *loxP* site on each reaction product [138]. For gene therapy purposes, the Cre/*loxP* system could be used to specifically delete DNA sequences within plasmid vectors on command. When a therapeutic gene is expressed from a

plasmid in which it is flanked by *loxP* sites, its expression could be terminated by administration of a plasmid encoding Cre. As it is rather difficult to deliver plasmid encoding Cre recombinase to all cells expressing the therapeutic gene, it is very likely that this system will generate only partial shutdown of therapeutic gene expression. The system can also be inverted to switch *on* expression of a therapeutic gene. In this case, a "silenced" plasmid is constructed in which a *loxP* flanked stop cassette is placed amidst a promoter and the therapeutic gene, thereby preventing transcription of the transgene. The stop cassette is then excised from the plasmid upon Cre-expression, leaving a plasmid (that is no longer silenced) with the promoter driving expression of the therapeutic gene. Scott *et al.* combined this Cre/*loxP* system with a radio responsive promoter to obtain a radiation-controlled molecular switch [80,82,139]. One plasmid was constructed containing a CMV IE promoter separated from a reporter (or therapeutic) gene by a *loxP*-flanked stop cassette. In a second plasmid, the Cre recombinase coding sequence is placed under the control of the Egr-1 radiation responsive enhancer combined with a CMV IE promoter. When exposed to radiation, the Egr-1/CMV IE promoter starts transcription of the Cre recombinase coding sequence. In turn, the expressed Cre excises the stop cassette from the second plasmid and the CMV IE promoter can drive transcription of the reporter or therapeutic gene (see Figure 5 B). With GFP as the reporter gene, the system incorporating the Cre/*loxP* mechanism resulted in approximately a 14.4-fold higher fluorescence when compared to a system in which GFP was directly controlled by the Egr-1/CMV IE promoter. When the herpes simplex virus thymidine kinase (HSVtk) gene was used to mediate cell killing, the switch-incorporated system resulted in almost the same increase in sensitivity to ganciclovir as that achieved by a system in which HSVtk was directly controlled by a CMV IE promoter. Also, 3-fold more growth inhibition was accomplished when compared to a system in which the Egr-1/CMV IE promoter directly controlled HSVtk. Importantly, for the switch-incorporated system a 1 Gy radiation dose achieved cell growth inhibition equivalent to that of a 3 Gy dose for systems without the switch [82].

Next page: Figure 5. *A: Positive feedback loop. Schematic illustration of the positive feedback loop mechanism. Within target cells, the cell type specific promoter will initiate the first round of expression (thin arrows), leading to simultaneous expression of both the reporter/effector gene and the VP16-LexA fusion protein. Interaction of the VP16-LexA protein with the LexA-binding sites upstream of the first cell type specific promoter then results in transactivation and enhancement of transcription (thick arrow). Co-expression of the two separate genes can be realized by using either an IRES or two internal promoters. Adapted from Nettelbeck et al.[57] with permission. B: Cre/loxP gene switch system. After co-transfection of the plasmids pcre and pSGFP, cre recombinase is expressed from the plasmid pcre and excises the stop cassette from pSGFP via the loxP sites. The pSGFP plasmid that is generated expresses the GFP gene driven by the CMV IE promoter/enhancer. Adapted from Scott et al. [82] with permission.*


A
VP16 TAD
LexA DBD
reporter / effector gene
LexA
binding
sites
cell type
specific
promoter
IRES
second cell-type
specific promoter


B
Egr1/CMV i.e.
cre
pCre
Transfection
CMV i.e.
lox P
stop
cassette
pSGFP
lox P
GFP
Cre recombinase
CMV i.e.
lox P
GFP
pΔSGFP
stop
cassette
lox P
GFP expression

All these results highlight the benefits of incorporating the Cre/*loxP* system, especially when a highly specific but relatively weak promoter is to be used. Cre/*loxP* can increase specificity and activity while leakiness is expected to be low because the therapeutic gene is silenced by a stop cassette in the absence of Cre. In directly inducible systems based on hybridization of inducible regulatory elements with strong constitutive promoter elements, strength of the constitutive promoter is reduced as a compromise to increase specificity and reduce leakiness. The great advantage of the Cre/*loxP* system is that the strong CMV IE promoter can be used to its full potential, while at the same time expression is under control of an external signal. Additional issues to be addressed to make this vector system of practical use include engineering the system into one single plasmid and examination whether the excised circular DNA molecule and the *loxP* site in the therapeutic plasmid that remains after excision are in any way harmful.

A perhaps more substantial point of concern is the finding that sequences exist in human and mouse genomes that despite being divergent from *loxP* are capable of supporting Cre-mediated recombination [137]. These so-called *pseudo-lox* sites are shown to support recombination at up to 100 % of the efficiency of native *loxP* sites when tested in bacterial assays and also to support Cre-mediated integration and excision in a human cell environment. This observation might complicate application of Cre in gene therapy strategies, as it implies that administration of Cre could possibly affect the host's genome. However, additional work is required to determine whether the efficiency of Cre-mediated deletion is dependent on features like chromatin structure, transcription rate or DNA-methylation. This information is required to estimate the actual effect of Cre when expressed in human cells and consequently to determine whether it will be useful for gene therapy applications.

The use of DNA excision is not limited to creating gene switches, but perhaps more importantly allows removal of bacterial plasmid components that are required for replication and selection in bacteria during the production process, but are unnecessary (and often undesired) for expression in human cells. Riu *et al.* [140] describe the excision of the purified transgene expression cassette from plasmids *in vivo*. They constructed plasmids in which the transgene expression cassette was flanked by two I-*Sce*I (*Saccharomyces cerevisiae* mitochondrial endonuclease) recognition sites and co-injected these with plasmids encoding I-*Sce*I cDNA into mouse liver. *In vivo*, I-*Sce*I expression leads to excision of a linear purified expression cassette, free of bacterial DNA. The two free double-stranded DNA ends then ligate intermolecularly to form large concatemers or intramolecularly to form circular DNA molecules, the latter being reported as the preferred route[141]. Such structures have been shown to reside extrachromosomally and remain active for several months [142]. As previously described, bacterial DNA has a silencing effect on transgene expression when covalently attached to the transgene. Riu *et al.* [140] show that removal of this covalent linkage results in a significant increase in both level and persistence of expression. I-*Sce*I is regarded as a suitable and safe endonuclease as it is highly specific and has not been reported to

cleave in human genomic DNA. In addition, much experience with its use in cells from a variety of organisms is on hand [140].

Expression of multiple genes combined in a single plasmid

Many of the more advanced gene therapy strategies described require co-expression of multiple genes. To limit burdening of the delivery vector and to guarantee successful co-transfection of the required genes, it is favorable to combine the transgenes in a single plasmid construct rather than co-delivering multiple plasmids. Development of strategies to succeed in co-expression of two or more genes from a single construct (bi- or polycistronic vectors, respectively) will therefore be of increasing importance. The diverse strategies now available have been reviewed by De Felipe [143]. Important aspects that need to be considered when choosing a strategy are the size of the element, coordination of expression of the multiple genes and control over the relative expression patterns. Ideally, the multiple genes are expressed equally and predictably. This can best be achieved when using a single open reading frame (ORF). However, this strategy is often not suitable as the expression of multiple proteins in a single ORF results in physically linked proteins that may not always be functional. In creating polycistronic vectors, most experience is based on the use of IRESes and until now this still represents the best available way to ensure co-expression of multiple genes in a single plasmid construct (successful co-expression has been reported in >90% of cells). IRESes also enable translation from RNAs produced by RNA polymerases other than RNA polymerase II, as is the case for the cytoplasmic expression strategy based on bacteriophage T7 RNA polymerase as described previously.

A disadvantage of IRESes is that expression levels of the genes upstream and downstream of the IRES are unequal, with the downstream gene being expressed at significantly lower levels and the exact balance being dependent on cell type and transgenes involved. Another disadvantage is the relatively large size of IRESes (~ 0.5 kb) compared to some other elements. However, the isolation of mini-IRES sequences of less than 0.1kb may offer a solution [144]. Some IRESes require the presence of part of the N-terminus of the original viral protein they belonged to for their full activity. This is undesirable as it means that additional viral sequences must be incorporated in the plasmid and a small part of the viral protein will consequently be present in the translated transgene product. Traditionally, IRESes from viral genes were used, and these lack the possibilities for specificity or regulation of expression. Some of the advanced gene therapy strategies previously described rely on individually controlled expression of multiple genes and incorporation of internal promoters has long been regarded as the only strategy suitable for this purpose. Interestingly, the discovery of certain cellular IRESes sheds a new light on this issue. It has been discovered that these IRESes require IRES trans-acting factors (ITAFs) present in the host cell for their activity. Similar to promoter specificity relying on the presence of different sets of transcription factors, IRESes display specificity based on specific interactions with different ITAFs. IRESes seem to

resemble promoters regarding specificity and inducibility: they have been found in mRNAs encoding growth factors, oncogenes, proteins involved in apoptosis and cell proliferation and also in mRNAs corresponding to stress proteins (formed in response to hypoxia, heat etc.).

With the increasing range of methods becoming available for establishing co-expression of multiple genes, one is given the opportunity to carefully select an element with optimal characteristics for the designed expression cassette.

Conclusion

Gene therapy has the potential to treat a great variety of severe diseases, including genetic disorders and cancer, but to date clinical applications have remained few due to inefficiency of delivery and expression. Although efficient delivery of genes to the required cell population is a critical aspect and still leaves much for improvement, also optimizing the plasmid vector can lead to increased or prolonged levels of expression and may therefore play an important role in compensating the limited transfection efficiency achieved with most non-viral carriers. In this review we have attempted to give an overview of the work that has been done on optimizing plasmid vectors for gene therapy applications.

To date, the most frequently used expression vectors in non-viral gene delivery systems make use of viral elements (promoters/enhancers) to drive the expression of the transgene. Viral-based expression vectors have established proof of principle, but due to immunostimulatory and silencing effects provoked in host cells, it is predicted that they will eventually be outlasted by their cell-based counterparts. Moreover, cellular promoters offer the opportunity for transcriptional targeting, which will contribute to safe and efficient *in vivo* human gene therapy due to restricted expression of transgenes in target tissue. Together with the use of increasingly potent therapeutic genes (e.g. suicide genes) developed to compensate for inefficient gene delivery, a need is generated for improved targeting strategies. In this light, tumor-specific expression strategies will especially prove useful to restrict cytotoxic gene expression to malignant cells or tissues.

However, it seems that with increasing specificity comes decreasing strength. It will therefore be essential to either further increase specificity or increase strength of weak but highly specific regulatory elements. Technologies incorporating positive feedback loops are estimated to contribute significantly to this purpose.

Additional layers of specificity can be offered by externally inducible systems. These will not only prove useful in further restricting expression to target tissues, but will be indispensable for treatment of diseases that require synthesis of proteins within a small therapeutic range, such as diabetes. When using physical stimuli to induce expression one has the advantage of being able to target both gene expression and the inducing stimulus to the target issue. In case of drug-inducible gene regulation, the ease of oral administration with which expression of a therapeutic protein can be induced is a great advantage. Furthermore, the extreme diversity of chemically inducible

systems offers the opportunity of fine-tuning expression patterns to meet pharmacological/pharmacokinetic requirements.

Another drawback of currently used non-viral gene delivery systems is the transient nature of gene expression. This problem may however be solved in the nearby future. Systems incorporating sequences that allow replication and maintenance, such as episomally replicating vectors, might succeed in establishing stable transgene expression over prolonged periods of time.

Interesting results are expected of so-called auto-regulating plasmid vectors. These strategies are based on the incorporation of genes that encode cofactors required for transcription of the plasmid (e.g. transcription factors, polymerase) within the plasmid such that its expression is less dependent on host factors.

Considering the strategies described in this review, optimism is justified that eventually expression cassettes can be created in which relevant parameters for transgene expression (e.g. promoter strength, specificity, leakiness, inducibility, efficiency, safety, duration of expression, kinetics and possibility of termination) are carefully balanced to meet requirements for clinical application and ultimately to realize human gene therapy.

ABBREVIATIONS: AAV, adeno-associated virus; ACF, ATP-utilizing chromatin assembly and remodeling factor; AD, activation domain; AFP, a-fetoprotein; ATF/CREB, activating transcription factor/cAMP-responsive element binding protein; BAC, bacterial artificial chromosome; bFGF, basic fibroblast growth factor; BKV, BK virus; BPV-1, bovine papilloma virus 1; CArG, CA(A/T)6GG; CAT, chloramphenicol acetyltransferase; ChMAR, chicken lysozyme MAR; CMV, cytomegalovirus; CMV IE, cytomegalovirus immediate early; CpG-N, neutralizing CpG motif; CpG-S, stimulatory CpG motif; Cre, cyclization recombination recombinase; CRSP, cofactor required for Sp1; CTF, CCAAT transcription factor; DBD, DNA binding domain; DR, direct repeat; DS, dyad symmetry; DTS, DNA nuclear targeting sequence; EBNA1, EBV nuclear antigen 1; EBP2, EBNA1-binding protein 2; EBV, EpsteinYBarr virus; Ec, ecdysone; Egr-1, early growth response-1 gene; FKBP, FK506 binding protein; FLP, flippase; FR, family of repeats; FRAP, FKBP12 rapamycin-associated protein; GFP, green fluorescent protein; Grp78, glucose-regulated protein 78; HAC, human artificial chromosome; hFIX, human coagulation factor IX; HIV, human immunodeficiency virus; HMGB1, high-mobility group box 1; HRE, hypoxia responsive element; HSE, heat shock element; HSF1, heat shock factor 1; hsp, heat shock protein; HSVtk, herpes simplex virus thymidine kinase; IL-12, interleukin-12; iNOS, inducible NO synthetase; IR, inverted repeat; IRES, internal ribosome entry site; I-SceI, Saccharomyces cerevisiae mitochondrial endonuclease; ITAF, IRES trans-acting factor; KDR, kinase-like domain receptor; loxP, Locus of Crossing over of P1 phage; MAEC, mammalian artificial episomal chromosome; Mdr, multidrug resistance; MeCP, methyl-CpG-binding proteins; Mfp, mifepristone; MLV, murine leukemia virus; MRI, magnetic resonance imaging; NF-kB, nuclear factor-kB; NLS, nuclear localization signal; NOEL, no-observed-effect level; NPC, nuclear pore complex; NURF, nucleosome-remodeling factor; ORF, open reading frame; oriP, origin of plasmid replication; PBAF, polybromo BRG1-associated factor; PDGF, platelet derived growth factor; PECAM-1/CD31, platelet-endothelial cell adhesion molecule-1/CD31; PR-LBD, progesterone receptor ligand binding domain; rAAV, recombinant adeno-associated virus; RSF, remodeling and spacing factor; RSV, Rous sarcoma virus; RSV-LTR, Rous sarcoma virus long terminal repeat;

RTA, recombinant transcriptional activator; S/MAR, scaffold/matrix attachment region; SB, Sleeping Beauty; SI, sucrase-isomaltase; SMGA, smooth muscle gamma actin; SV40, simian virus 40; SWI/SNF, switching/sucrose non-fermenting; Tag, large T antigen; Tet, tetracycline; TetO, Tet operator; TetR, Tet repressor; TGF-a, transforming growth factor a; TNFa, tumor necrosis factor a; TRAP, thyroid hormone receptor-associated protein; tTA, tetracycline transactivator; tTS, tetracycline transcriptional silencer; VEGF, vascular endothelial growth factor; VP16, virion protein 16; vWF, von Willebrand factor; WAF1, wild-type p53-activated fragment 1; YAC, yeast artificial chromosome; b-gal, b-galactosidase.

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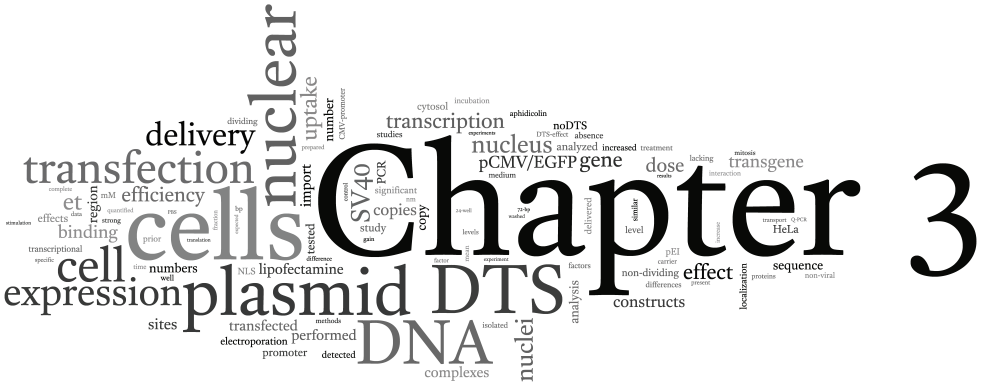

DNA nuclear Targeting Sequences for non-viral gene delivery

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Abstract

Plasmid DNA can interact with cytoplasmic transcription factors via specific binding regions in the plasmid, so called DNA nuclear Targeting Sequences (DTS). It has been postulated that this interaction can play an important role in non-viral gene delivery, via nuclear localization signals (NLS) in the transcription factors that facilitate import of the complexes into the nucleus. The aim of this study was to evaluate if modification of plasmids with these DNA nuclear Targeting Sequences (DTS) could indeed improve efficiency of non-viral gene delivery. A set of DTS were identified and cloned into EGFP-reporter plasmids controlled by the CMV-promoter. It was shown that neither of these DTS increased transgene expression. We varied several parameters (mitotic activity, applied dose and delivery strategy), but we were unable to detect a DTS effect using transgene expression as readout. Transgene expression was shown to be upregulated after stimulation with TNF-α. But, since this effect was similar for constructs with NFκB binding sites and constructs with unrelated DTS, this effect is ascribed to non-specific upregulation of transcription rather than enhanced nuclear import. To discriminate between differences in transgene expression of the used plasmids and their actual nuclear import, we quantified nuclear translocation of the plasmid by performing quantitative PCR on isolated cell nuclei. Nuclear copy numbers of plasmids containing or lacking a DTS did not differ significantly after lipofectamine-based transfection in dividing and non-dividing cells. To conclude, no beneficial effects of DTS on gene expression or nuclear uptake were observed in this study.

Introduction

Non-viral gene therapy faces several challenges at the level of delivery. It has been demonstrated extensively that DNA-containing particles, either polymer- [1-4], lipid- [5-7], or peptide- [8-10] based, can be taken up by cells. However, the success of non-viral gene delivery is often limited due to failure of one of the subsequent delivery steps, as only a fraction of the cells actually express the delivered transgene. This indicates that the delivery hurdle is at the level of inefficient intracellular processing. Several barriers have been described and studied, including failure to escape from vesicular structures [11,12], lysosomal degradation, enzymatic degradation in the cytosol [13], entrapment in the highly viscous and crowded cytosol [14,15], lack of transport towards the nucleus[16-19] and uptake into the nucleus [20-25], and finally inefficiency of transcription and/or translation [26-29]. Although optimization at each of these steps will contribute to the improvement of overall delivery efficiency, most gain can be made by optimizing delivery at the bottleneck. Capecchi *et al.*[30] showed that microinjection of plasmid DNA directly into the nucleus resulted in transgene expression in 50-100 % of the cells whereas cytoplasmic injection resulted in <0.01 % of transgene expressing cells. This observation clearly indicates that transport into the nucleus is the major bottleneck for successful non-viral gene delivery.

The nucleus is a double membrane organelle that is well-separated from the rest of the cell. Exchange of molecules requires transport via one of the approximately 2,000 Nuclear Pore Complexes (NPC) per nucleus [31]. NPC allow passive diffusion of proteins up to 40 kDa, or particles of approximately 9 nm or DNA molecules of 210-350 bp [14,32-34]. These limits are well below sizes of DNA-complexes (~100-500 nm) and of plasmids (~6,000 bp) generally used for gene delivery studies. When the nuclear envelope temporarily breaks down during mitosis, DNA (-complexes) can gain access to the nucleus and transgene expression has indeed been related to cell cycle phases in several studies [35-37]. However, most tissues targeted by gene therapy are slow- to non-dividing and require alternative routes of cytonucleoplasmic shuttling of DNA. Gene delivery is therefore confined to employing active transport mechanisms that induce widening of the NPC.

Large macromolecules such as viral proteins and host proteins with a function in the nucleus (i.e. heterogeneous nuclear ribonucleoprotein A1 (hnRNP), transcription factors, polymerases) are able to gain access to the nucleus. These proteins were discovered to have consensus sequences, called nuclear localization signals (NLS), which can interact with importin-α, importin-β or transportin. The formed complexes are then docked to the NPC and the protein is shuttled to the inner membrane of the nucleus. Many efforts have been made to mimic this process by incorporating NLS in gene delivery complexes; mostly by using the consensus sequence NLS of the Simian Virus 40 (SV40) large T antigen [23]. Strategies tested include coupling of NLS peptides covalently [20,38] or non-covalently to DNA or to the carrier system. Unfortunately, success has been highly variable and generally disappointing [20,21,32,38-46]. When incorporating NLS, one should consider costs and complexity of coupling chemistry, but also detrimental effects at the level of transcriptional

interference, preterm dissociation of NLS from the DNA to be delivered and alterations in the physicochemical characteristics (see Chapter 4 of this thesis).

An elegant strategy to bypass these issues was first presented by Dean *et al.* Based on the finding by Graessmann *et al.* [47] that an enhancer region within the SV40 viral DNA facilitated nuclear import, Dean *et al.* examined usability of this sequence for non-viral gene delivery and named it DNA nuclear targeting Sequence (DTS) [22,48-50]. In subsequent studies, it was found that the absence or presence of the SV40 origin of replication and early promoter region was critical for nuclear uptake of naked plasmids from cytosol in the absence of cell division [48] and a 72-bp region was identified to be sufficient to establish this effect [49]. The effect was shown to be sequence specific and two strong promoter or enhancer regions other than SV40 (CMV immediate-early promoter/enhancer and RSV-LTR) proved incapable of inducing nuclear import of plasmid in non-dividing cells [49]. Inhibitor studies revealed that plasmid import occurred via the same pathway as signal-mediated protein import and that transcriptional activity of the cell was required for the process to occur [48]. Based on these findings, the following mechanism of DTS-mediated nuclear uptake was postulated: (1) transcription factors (TF) present in the cytosol bind to their recognition sequences present in the plasmid via their DNA-binding domain; (2) NLS naturally present in TF interact with importin-α and subsequently with importin-β leading to active transport towards the nucleus and docking of the TF/plasmid complex to the NPC [22].

After establishing this principle, several applications and variations have been studied. The DTS-effect was shown to occur in several cell types upon microinjection, electroporation and transfection [48-50] and was confirmed *in vivo* [51-53]. By replacing the heterogeneous SV40 sequence that is responsive to various TFs, including NFkB and SP1, by consensus sequences for nuclear factor kappa B (NFκB) [54-56] or Glucocorticoid Responsive Elements (GREs) [57], plasmid import could be mediated via a specific pathway and be stimulated via TNF-α or corticosteroids, respectively. By using a sequence responsive to a specific transcription factor overexpressed in target tissue (i.e. smooth muscle specific transcription factor SRF), nuclear uptake and therefore transgene expression was restricted to target cells (i.e. smooth muscle cells) [50,51,53,58,59].

In this study we aimed to further optimize the DTS-effect for non-viral gene delivery. A range of constructs with various DTS based on the heterogeneous SV40 DTS, NFκB and GRE consensus sequences was prepared and tested for their relative potential to enhance transfection efficiency. Our next questions involved the relation between carrier type and the DTS effect, as we hypothesize that for the DTS-effect to occur, the DTS must be available within the cytosol, requiring dissociation from its carrier. To study this effect, we compared results upon electroporation (no carrier), transfection with lipofectamine (dissociates prior to nuclear uptake [6]) and transfection with 22 kDA linear pEI (shown to deliver DNA into the nucleus as intact complexes [54,60]). As we did not observe any significant differences between the constructs tested under any circumstance tested, we continued to explore the reason for this discrepancy with results published by others. To

be able to distinguish effects at the level of transcription and of translocation, we quantified nuclear uptake of plasmids by quantitative PCR on isolated cell nuclei.

Materials and methods

Materials

All chemicals and reagents were purchased from Sigma (St. Louis, USA) unless stated otherwise. Other chemicals used: Hepes (99 %) and magnesium chloride, $MgCl_2 \cdot 6H_2O$ (Acros Organics BVBA, Geel, Belgium); sodium chloride and ethanol (Merck, Darmstadt, Germany); potassium chloride (ICN Biomedicals, Aurora, USA); diethylether (Biosolve, Valkenswaard, The Netherlands); phosphate-buffered saline (PBS) (B. Braun Melsungen AG, Melsungen); fetal bovine serum (FBS; Integro, Zaandam, The Netherlands), Trypsin/EDTA 10×, Plain DMEM (Dulbecco's modification of Eagle's medium, with 3.7 g/l sodium bicarbonate, 1 g/l l-glucose, L-glutamine) and antibiotics/antimycotics (penicillin, streptomycin sulphate, amphotericin B; PAA Laboratories GmbH, Pasching, Austria), TNF-α (rh TNF-α; R&D Systems, Minneapolis, USA).

TOPO TA Cloning Kit, TOP10 *E. Coli* bacteria, propidium iodide (PI), Lipofectamine™2000 (lipofectamine), Opti-MEM® I Reduced Serum Medium (optimem), UltraPure™ Buffer-Saturated Phenol (TE-saturated phenol) and UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) were purchased from Invitrogen (Oregon, USA).

All restriction enzymes and other DNA-modifying enzymes, RNase (RNase I, DNase ad protease free), Proteinase K (recombinant, PCR grade) and DNA-ladders were purchased from Fermentas (St. Leon-Rot, Germany). The oligonucleotides used for PCR reactions, sequencing and cloning were synthesized by Eurogentec S.A. (Seraing, Belgium). NucleoSpin Extract II DNA kits and Nucleobond PC Endotoxin Free kits for plasmid purification were obtained from Bioke (Macherey-Nagel, Bioke, Leiden, The Netherlands). LabelIT Cy5 Nucleic Acid Labeling Kit was purchased from Mirus Bio, Madison, WI, USA.

Cloning of plasmid constructs

Plasmid pCMV/EGFP was constructed from pShooter (Invitrogen, Oregon, USA): the EGFP coding sequence from pEGFP-N1 (bp 613–1410; Clontech, Saint-Germain-en-Laye, France) was amplified by PCR and cloned into the multiple cloning site of pShooter from which the nuclear localization signal (NLS)-tag coding sequence was removed with NotI and XbaI restriction enzymes.

pCMV/EGFP was digested with DraIII and KasI to remove the complete SV40 ori and enhancer region. After purification from a 1 % agarose gel, vector DNA was ligated to a piece of synthetic DNA obtained by annealing complementary oligonucleotides 1 (Table 1) and ligation product was transformed into *E. coli* TOP10 cells by standard heat-shock procedure. Plasmids were isolated

using Nucleobond PC Endotoxin Free kits and identities were confirmed by restriction analysis and sequencing.

The resulting plasmid, pCMV/EGFP_noDTS_Sp1 was further digested with BamHI and HindIII. The large fragment was purified from a 1 % agarose gel and either blunted and self-ligated to result in plasmid pCMV/EGFP_noDTS or ligated to pieces of synthetic DNA obtained by annealing complementary oligonucleotides 2-6 (Table 1). Ligation products were transformed into *E. Coli* Top10 bacteria and plasmids were isolated using Nucleobond PC Endotoxin Free kits. Plasmids were quantified by 260 nm absorbance measurement and plasmid identity and quality were analyzed on 1 % agarose gels before and after digestion and 260/280 ratio measurements. Sequences of all plasmid constructs were verified by sequencing using oligo's 7 (Table 1) as primers (outsourced to Baseclear, Leiden, The Netherlands).

Table 1. *Sequences of oligonucleotides used for preparation of new constructs (BamHI and HindIII).*

1. Cloning
5'- GTGGGATCCCTAACTCCGCCCAGAAGCTTG-3'
3'-CATCACCCTAGGGATTGAGGCGGGTCTTCGAA -5'
2. 72-bp-repeat SV40 (based on [61, #28])
5'- GGATCCGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAAAGCTTA-3'
3'-ACCTAGGCCACACCTTTCAGGGGTCCGAGGGGTCGTCCGTCTTCATACGTTTCGTACGTAGAGTTAATCAGTCGTTGGTTTCGAA -5'
3. 2xNFkB
5'- GGATCCTGGGGACTTTCCGCTGGGGACTTTCCGCAAGCTTA-3'
3'-ACCTAGGACCCCTGAAAGGCGACCCCTGAAAGGCGTTCGAA -5'
4. 3xNFkB
5'- GGATCCTGGGGACTTTCCGCTGGGGACTTTCCGCTGGGGACTTTCCGCAAGCTTA-3'
3'-ACCTAGGACCCCTGAAAGGCGACCCCTGAAAGGCGACCCCTGAAAGGCGTTCGAA -5'
5. 5xNFkB (based on [55,62])
5'- GGATCCTGGGGACTTTCCGCTGGGGACTTTCCGCTGGGGACTTTCCGCTGGGGACTTTCCGCTGGGGACTTTCCGCAAGCTTA-3'
3'-ACCTAGGACCCCTGAAAGGCGACCCCTGAAAGGCGACCCCTGAAAGGCGACCCCTGAAAGGCGACCCCTGAAAGGCGTTCGAA -5'
6. GRE (based on [57,62])
5'- GGATCCGGTACATTTTGTTCTAGAACAAAATGTACCGGTACATTTTGTTCTGGTACATTTTGTTCTAAGCTTA-3'
3'-ACCTAGGCCATGTAAAACAAGATCTTGTTTTACATGGCCATGTAAAACAAGACCATGTAAAACAAGATTCGAA -5'
7. Sequencing Kas to Dra
5'-GCCCGCTCCTTTCGCTTTCT-3'
3'-GACAACGTCGAGCACAGCT-5'

DNA labeling

Plasmids were covalently labeled with Cy5 according to a modified version of the manufacturer's protocol: DNA was incubated overnight with a labeling mixture at room temperature and purified using ethanol precipitation. DNA was quantified and checked for purity by measuring the absorbance at 260 and 280 nm in a Nanodrop Spectrophotometer (Wilmington, DE USA). On average, 1 Cy5-molecule was bound per 150 bp, as calculated according to the manufacturer's instructions.

Cell culture

Human epithelial ovarian carcinoma cells (HeLa) and human epidermoid carcinoma cells (A431) were grown in DMEM supplemented with antibiotics/antimycotics and 10 % or 7.5 % heat-inactivated FBS, respectively. Cells were maintained at 37 °C in a 5 % CO_2 humidified air

atmosphere and split twice weekly. Both cell lines were kindly given by the Institute of Biomembranes (Utrecht University, The Netherlands). Cells were confirmed to be free from mycoplasm by periodical testing with a MycoAlert® Mycoplasma Detection Kit (Lonza, Verviers, Belgium).

Preparation of the complexes

pEI-polyplexes were prepared at an N/P ratio of 6 by adding 4 volumes of pEI (Exgen500; MBI Fermentas, St Leon-Rot, Germany) solution to 1 volume of a 50 µg/ml DNA-solution, mixing by pipetting and incubating 30 min at room temperature. pEI-polyplexes were prepared in Hepes-buffered saline (20 mM Hepes, 150 mM NaCl; pH 7.4 (HBS). Lipofectamine-complexes were prepared at a ratio of 2 µl lipofectamine per 1 µg DNA by adding 4 volumes of lipofectamine solution to 1 volume of a 50 µg/ml DNA-solution (both diluted in optimem), mixing by pipetting and incubating 30 min at room temperature.

Transfection

For studies in dividing cells, 40,000 (HeLa) cells were seeded per well into 24-well tissue culture plates 24 h prior to transfection, such that 60–70 % confluency was reached on the day of transfection. For studies in non-dividing cells 40,000 (HeLa) cells were seeded per well into 24-well tissue culture plates 48 h prior to transfection and medium was replaced by complete medium supplemented with 15 µM aphidicolin. Cells were continuously exposed to aphidicolin from at least 16 hours prior to transfection until time of analysis.

Immediately prior to transfection the culture medium was refreshed with 500 µl DMEM medium, supplemented with 10 % FBS (and 15 µM aphidicolin where applicable). 100 µl of the polyplex or lipoplex samples (corresponding to 1 µg DNA/well) was added per well and after 4 h incubation, medium was replaced with fresh DMEM supplemented with 10 % FBS (and 15 µM aphidicolin where applicable). Cells were incubated for indicated times at 37 °C in a 5 % CO_2 humidified air atmosphere until analysis. Experiments were performed in triplicate.

Electroporation

Cells were harvested by trypsin/EDTA (T/E) treatment, counted and diluted to $1.1 \cdot 10^6$ cells/ml in 20 mM Hepes in PBS (pH 7.4). DNA was diluted to 2 µg DNA (or as indicated) into 25 µl Hepes buffer. 25 µl DNA solution was added to 225 µl cell suspension and incubated on ice for 10 min. After addition of 250 µl Hepesbuffer, the sample is transferred into an electroporation cuvette and electroporation was performed 250 mV for 7 msec (Electro Square Porator ECM830 with Safety Stand 630B and Electroporation Cuvettes Plus no 640 4mm GAP cuvettes; BTX, San Diego, USA). Cells were left to equilibrate for at least 10 minutes, transferred into an Eppendorf tube and mixed with 500 µl completed medium. From this, a suitable volume was transferred into a 24-well plate and incubated for indicated times at 37 °C in a 5 % CO_2 humidified air atmosphere until analysis.

Cell cycle analysis

To confirm arrest of cells in the S-phase cell cycle analysis was performed based on flow cytometric analysis of cell DNA content following cell staining with propidium iodide (see Figure 1). After synchronization treatment, the cells were washed two times with PBS to ensure removal of all dead cells, harvested and fixed. Fixation was achieved by incubating the cells in a 300 µl PBS/700 µl ethanol solution at -20 °C for at least 1 hour. After fixation the ethanol/cell solution was underlayered with 1 ml FCS before being spinned down and resuspended in 500 µl (for $1 \cdot 10^6$cells) PBS containing PI and RNase (930 µl PBS, 50 µl PI (1 mg/ml), and 20 µl RNAse (5 mg/ml)). After 1 hour incubation at room temperature the cells were analyzed with a FACSCantoII cytometer (Becton and Dickinson, Mountain View, CA, USA) equipped with a 488 nm 20 mW Solid State diode laser and a 633 nm 20 mW HeNe laser. 10,000 cells were recorded per sample and cell cycle analysis of DNA profiles was performed using Cylchred software, available from Cytonet UK.

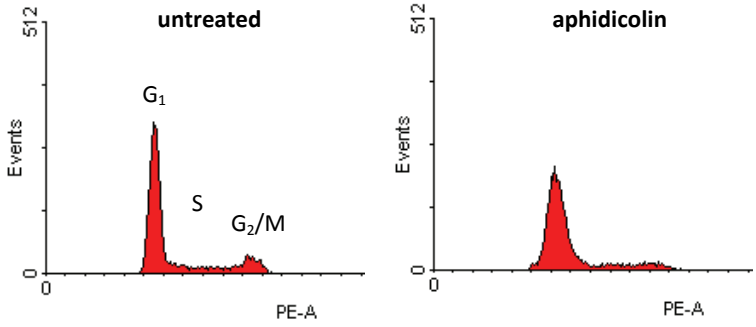


Figure 1. *Arrest of HeLa cells in S-phase by treatment with 15 µM aphidicolin.*

Stimulation of NFκB

Cells were incubated for 3 h with poly- or lipoplexes after which the medium was replaced by 500 µl complete medium supplemented with 25 ng/ml TNF-α. After another 3 h incubation cells were harvested and analyzed.

Flow cytometry

48 h after transfection, cells were washed, trypsinized and resuspended in DMEM supplemented with 10 % FBS to inactivate the trypsin. Cells were transferred into round-bottom 96-well plates and centrifuged for 5 min at 250 x g at 4 °C. Medium was removed and cells were resuspended in 200 µl phosphate-buffered albumin (PBA; 1 %, w/v albumin in PBS). Immediately prior to measurement, 20 µl of a PI solution (10 µg/ml in water) was added for live/dead cell discrimination. Flow cytometric analysis was performed on a FACSCantoII (Becton and Dickinson, Mountain View, CA, USA) equipped with a 488 nm 20 mW Solid State diode laser and a 633 nm 20 mW HeNe laser. 10,000 cells were recorded per sample to determine EGFP expression (FITC-channel) and PI-staining (PE-channel).

Quantification of nuclear localization by cell fractionation and Q-PCR

Quantification of plasmid copies delivered to nuclei was performed based on a method developed by Cohen *et al.* [63]. Transfection procedures were similar as described above, but were upscaled to T75 culture flasks per sample: $1.8 \cdot 10^6$ cells were seeded per flask and transfections were performed at a dose of 18 µg per flask.

Isolation of cell nuclei

24 h after transfection, cells were harvested and nuclei were isolated by swelling in 1 ml hypotonic buffer (10 mM PIPES pH 7.4, 1 mM DTT, 2 mM MgCl2, 10 mM KCl) for 25 minutes followed by lysis in a Dounce homogenizer (Wheaton, VWR International BV, Amsterdam, The Netherlands) with 50 strokes of the tight pestle. Cell lysates were mixed with equal volumes of 50% iodixanol (Optiprep Density Gradient Medium; Sigma-Aldrich, St. Louis, USA) in isotonic buffer (10 mM Na_2HPO_4, 1 mM $MgCl_2$, 130 mM KCl, pH 7.4). Single Particle Optical Sensing (SPOS) was used to confirm that cells were lysed and nuclei were intact.

A three-step discontinuous iodixanol gradient was prepared by first underlaying 3 ml of 30 % and 35 % iodixanol in isotonic buffer and then carefully adding the cell lysate (in 25 % iodixanol) on top. After spinning for 40 minutes at 10,000 x g at 4 °C in a swing-out rotor (Beckman OptimaTM LE-80K Ultracentrifuge with SW41-TI rotor, Beckman Coulter B.V., Woerden, The Netherlands), nuclei were recovered from the 30/35 % interface with a 18-gauge needle (Sterican 18 G x 1 ½"; B. Braun Melsungen AG, Melsungen, Germany).

Total DNA extraction

Isolated nuclei were lysed by treatment with 0.5 % SDS, 100 µg/ml proteinase K and 20 µg/ml RNase for 5 h at 50 °C. Total DNA was recovered by subsequent extractions with equal volumes of TE-saturated phenol (1x) and phenol:chloroform:isoamyl alcohol 25:24:1 (2x) followed by washes with water-saturated ether (2x). DNA was precipitated from the final water phase with 1/10 V sodium acetate (3 M, pH 5.2) and 2 V ice-cold 96 % ethanol incubated overnight at -20 °C. After washing the DNA pellet with 70 % ethanol, the pellet was air-dried and dissolved in MilliQ-water. Samples were used directly or stored at -20 °C.

Quantification of plasmid and genomic actin DNA copies by Q-PCR

Actin copies were quantified using Taqman Gene Expression Assay Hs03023880_g1 (Applied Biosystems, Foster City, USA) which contains primers and a FAM-labeled TaqMan MGB probe to amplify human genomic β-actin DNA. PCR reaction mixtures were prepared containing 5 µl DNA (2-2,000 fg), 1 µl Taqman gene Expression Assay, 10 µl 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, USA) and MilliQ-water added to a final volume of 20 µl. PCR was performed using a two-temperature thermal cycling program consisting of 15 s at 95 °C and 60 s at 60 °C for 40 cycles. A standard curve of plasmid pAct, which contains the human β-actin sequence

(pCMV-Sport6 containing the complete CDS for Homo sapiens actin, beta, mRNA (cDNA clone MGC:5475 IMAGE:3451917); Invitrogen, Oregon, USA) was used to calibrate the PCR system.

Plasmid DNA was quantified with a SYBR Green assay using a three-temperature thermal cycling program consisting of 10 s at 95 °C, 10 s at 61 °C and 30 s at 72 °C for 40 cycles. Primers were designed to specifically amplify a 116 bp region from the ampicillin CDS using VectorNTI (Invitrogen) and Primer-BLAST (available from NCBI) software and are of the following sequence: forward 5'-TCC-ATA-GTT-GCC-TGA-CTC-CC-3' and reverse 5'-GAT-AAA-TCT-GGA-GCC-CGT-GA-3'. PCR reaction mixtures consisted of 5 µl DNA (2-2,000 fg), 250 nM forward primer, 250 nM reverse primer, 10 µl iQ SYBR Green Supermix (Bio-Rad, Hercules, USA) and MilliQ-water added to a final volume of 20 µl. A standard curve of known amounts of plasmid pAct was used to calibrate the PCR system.

Since 2 actin copies are present per genome per cell, the number of plasmids per nucleus was calculated as # plasmid copies/ (# actin copies/2).

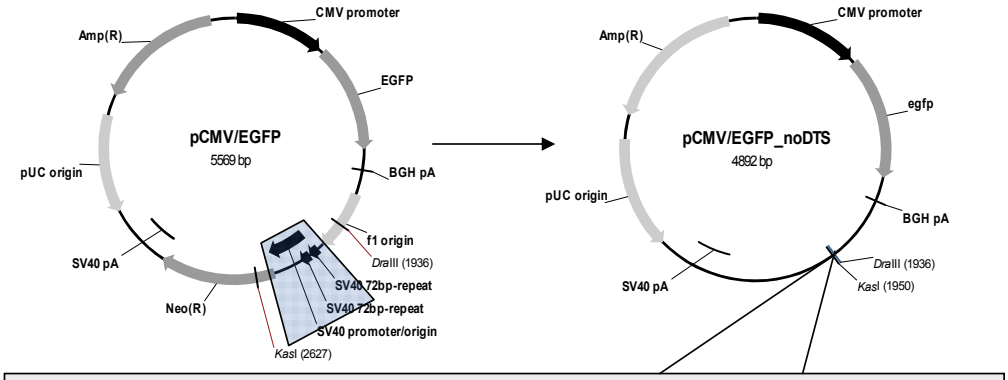
Experimental setup and statistical analyses

On each experimental day, three independent plasmid/carrier preparations were made and tested on the same batch of cells.

Results

Design and cloning of plasmid constructs

To study and compare effects of various DTS on gene delivery efficiency, DTS were identified from literature [48,49,55,57,62] and a range of plasmid constructs were prepared as described in the materials and methods section. The SV40 origin/promoter sequence was excised from pCMV/EGFP to obtain the control plasmid pCMV/EGFP_noDTS. Subsequently, the SV40 72-bp repeat, a repetition of 2, 3 or 5 NfκB consensus binding sites, or a glucocorticoid responsive element were inserted at the position where the SV40 ori/promoter sequence previously was located (see Figure 2). Successful modifications were confirmed by sequencing.



Inserts:

- 72bp region SV40
  GGATCCGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAAAGCTT
- 2-3-5x NFκB
  GGATCC (TGGGGACTTTCCGC)$_{2-3-5}$ AAGCTT
- GRE
  GGATCCGGTACATTTTGTTCTAGAACAAAATGTACCGGTACATTTTGTTCTGGTACATTTTGTTCTAAGCTT

Figure 2. *Design and cloning of plasmid constructs.*

Effect of DNA nuclear Targeting Sequences on transfection efficiency

HeLa cells were transfected with plasmids containing the complete SV40 promoter/origin region, plasmids from which this complete region was deleted and plasmids in which a DTS region was inserted: 72-bp enhancer region of SV40, 2-3-5x NFkB binding sites or a Glucocorticoid Responsive Element (GRE). Gene expression was analyzed 6 h after the start of transfection to minimize effects of mitosis. As shown in Figure 3, no significant differences between the plasmid lacking a DTS

sequence and any of the constructs containing a DTS was observed. This means that presence or absence of a DTS does not affect the number of cells that is successfully transfected. Since it is possible that the DTS did have an effect on the amount of transgene expressed per successfully transfected cell, we also looked at the Mean Fluorescence Intensity (MFI) but no differences were observed (data not shown). Next, we tested if TNF-α stimulation could induce uptake of the NFκB-based constructs. Treatment with TNF-α induces activation of NFκB leading to nuclear translocation of NFκB. It is therefore expected that plasmids containing NFκB- binding sites are preferentially shuttled into the nucleus. Figure 3 B shows that transfection efficiency for the NFκB-plasmids was indeed enhanced (again, similar trends were observed when analyzing MFI). However, transfection efficiencies were increased to a similar extent for all other constructs tested. Stimulation of gene expression was similar for plasmids containing the full SV40 promoter/ori region, the minimized 72-bp SV40 region or plasmids from which the entire SV40 regulatory region was removed (see Figure 3 A). Lastly, a plasmid from which the SV40 region was replaced by a Glucocorticoid Responsive Element (GRE) responded similarly to TNF-α treatment as the plasmids containing several NFκB binding sites (Figure 3 C).

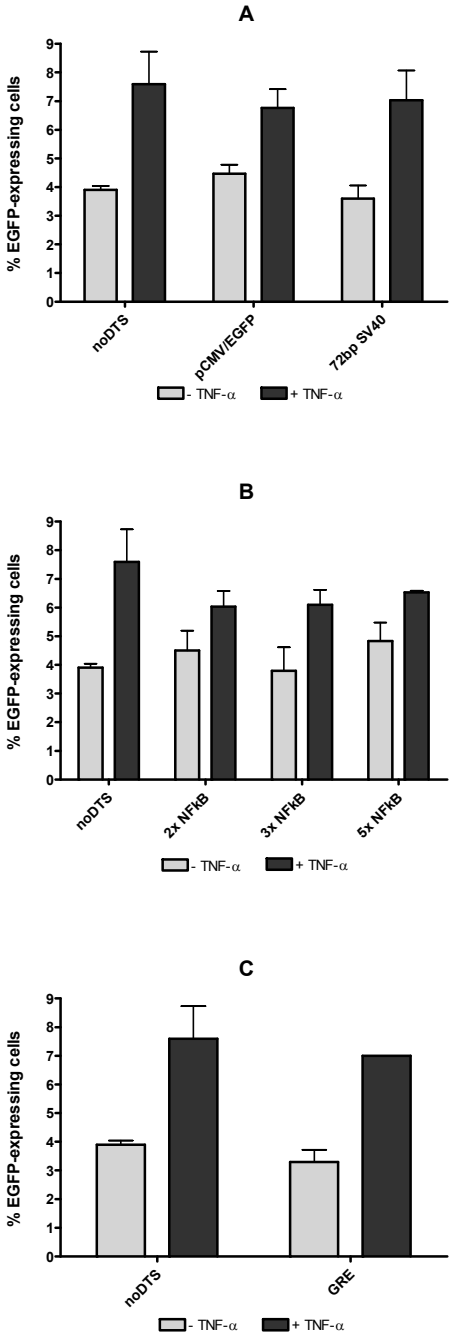
These results show that the stimulation induced by TNF-α is unspecific and is most likely related to upregulation of transcriptional processes. The plasmids tested are all under transcriptional control of the CMV-promoter and this promoter contains 3 NFκB- binding sites, which may explain the transcriptional activation upon TNF-α stimulation. Interestingly, despite the presence of these NFκB sites, the CMV-promoter itself was previously described not to be involved in nuclear import processes[49,54].

Since we did not manage to achieve DTS-mediated increase in transfection efficiency with any of the constructs tested, we continued to examine the parameters that could be critical in obtaining sequence-specific nuclear uptake. For this, we selected the pCMV/EGFP (DTS) and pCMV/EGFP_noDTS (noDTS) for proof of principle studies.

Effect of delivery strategy on DTS effect

The suggested mechanism of DTS-induced nuclear uptake is sequence-specific binding of NLS-containing transcription factors to plasmid DNA that is delivered into the cytosol, followed by import of the formed TF/plasmid complexes into the nucleus. This implies that the plasmid is delivered into the cytosol and that the DNA is available for interaction with those proteins. It is therefore expected that escape from vesicles and (partial) dissociation from the carrier is essential for the DTS effect to occur. To test if the type of delivery affected occurrence of the DTS-effect, three methods to introduce plasmid into cells were tested. Lipofectamine was used as a transfection reagent which has been described to release DNA prior to nuclear uptake and transcription [6]. In contrast, 22 kDa linear pEI is thought to deliver DNA into nuclei as intact complexes [54,60,64] and has even been described to allow transcription to occur while still bound to

Figure 3. *The effect of various DTS on transfection efficiency. % EGFP-expressing cells after transfection with SV40-based DTS (A), NFκB DTS (B) and a GRE DTS (C). HeLa cells were transfected with the indicated plasmids and lipofectamine for 4h in the presence of serum, washed and analyzed 6 h after the onset of transfection. Cells were non-stimulated (light grey bars) or stimulated by incubation with 25 ng/ml TNF-α during time of transfection (dark grey bars). Data are presented as mean ± SD (n=3). One-way ANOVA followed by post hoc analysis with Bonferroni correction was performed on the expression data of each plasmid. None of the plasmids differed significantly from plasmid noDTS either in the presence or absence of TNF-α.*



the plasmid [26]. Electroporation was used to introduce naked DNA directly into the cytosol, bypassing vesicular uptake and release and unpackaging of complexes. Figure 4 shows that regardless of the delivery strategy chosen, no differences in transgene expression efficiency are observed for plasmids containing or lacking a DTS. Neither the % of transfected cells nor the mean EGFP expression per cell differed significantly for plasmids containing or lacking the DTS.

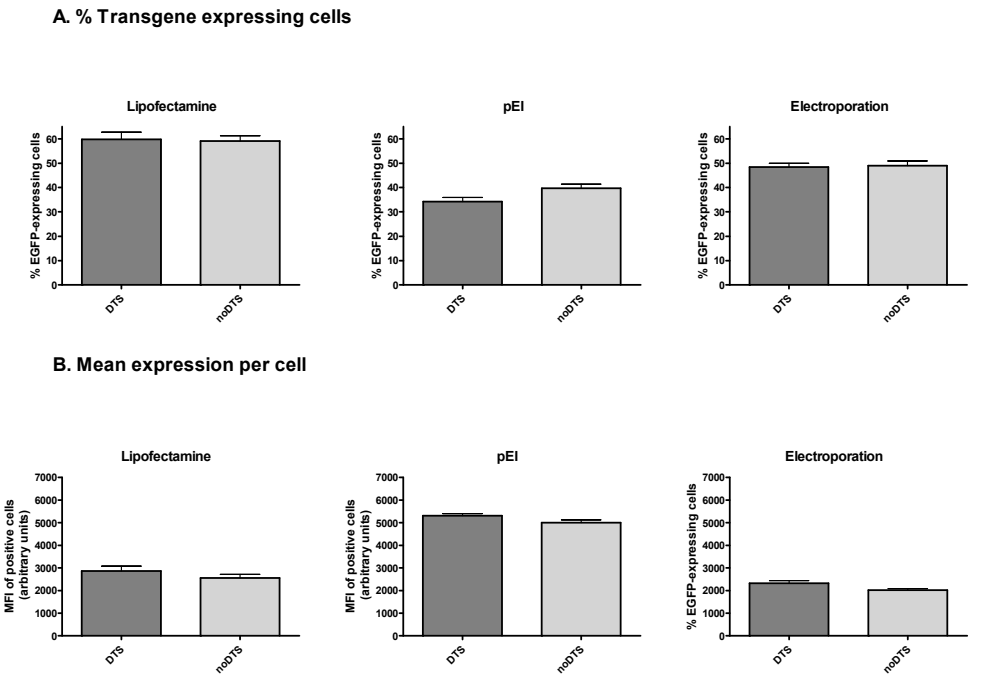


Figure 4. *Dependency of the DTS-effect on delivery strategies. % of EGFP-expressing cells (A) and mean expression per cell (B) after transfection with lipofectamine (left), 22 kDa linear pEI (middle) or after electroporation (right) of plasmids with DTS (pCMV/EGFP; dark grey bar) versus plasmids without DTS (pCMV/EGFP_noDTS; light grey bar). Cells were transfected at a dose of 1 µg DNA/ well in a 24-well plate with lipofectamine or pEI for 4 h in the presence of serum, washed and analyzed after 48 h or electroporated at a dose of 2 µg/2.25·10^5 cells and analyzed after 24 h. Data are represented as mean ± SD of 3 independent measurements. MFI: mean fluorescence intensity.*

Dependency of the DTS effect on mitosis

In the previous experiments, transfections were performed in dividing cells. Possibly, benefits in increased active uptake are outweighed by bulk access to the nucleus when the nuclear envelope is temporarily broken down at the time of mitosis. The shortest incubation time tested was 6 h which is well below the cycling time of HeLa cells. Nevertheless, due to the asynchronous nature of cultured cells, a fraction of cells will still be undergoing mitosis within this interval. To minimize this

fraction even further, cells were incubated with aphidicolin, a reagent that arrests cells in S-phase by interfering at the level of DNA-synthesis and with relatively mild cytotoxicity [65]. Results obtained in HeLa cells after lipofectamine-based transfections of plasmids containing or lacking the SV40 DTS are shown in Figure 5 (left panel). Again, no significant difference between the two plasmid constructs is observed, regardless of incubation time and most importantly no effect was observed in non-dividing cells. Similar results were obtained in a second human cell line, A431 (Figure 5, right panel).

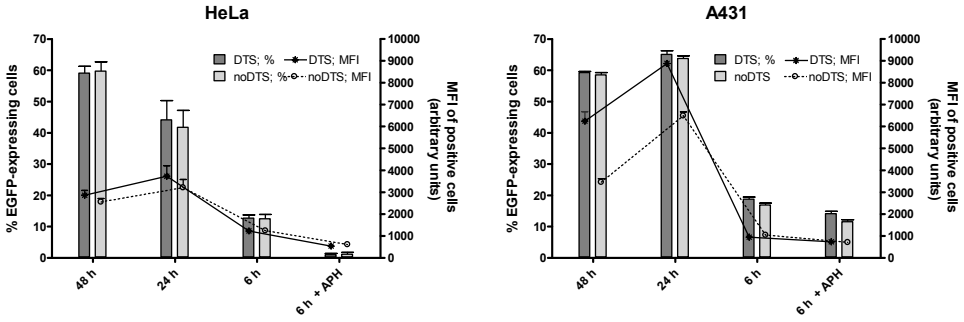


Figure 5. *Dependency of the DTS-effect on mitosis. % of EGFP-expressing cells (bars; left y-axis) and MFI of positive cells (lines; right y-axis) after transfection of HeLa cells (left panel) or A431 cells (right panel) with plasmids with DTS (pCMV/EGFP; dark grey bar and asterix) versus plasmids without DTS (pCMV/EGFP_noDTS; light grey bar and open circle). Cells were transfected at a dose of 1 µg DNA/ well in a 24-well plate with lipofectamine for 4 h in the presence of serum, washed and analyzed at the indicated timepoints after onset of transfection. As a model for non-dividing cells, cells were arrested at the S-phase by treatment with 15 µM aphidicolin starting 24 h prior to transfection and continuously throughout the experiment. Data are represented as mean ± SD of 3 independent measurements.*

Can the DTS effect be outweighed by the amount of plasmids delivered into the cytosol?

Dean *et al.* [22] argued that an absolute dependence on the presence of a DTS for nuclear import is not always existent. They hypothesize that "It is likely that when the cytoplasm becomes filled with large concentrations of plasmids, at least some make their way to the nuclear envelope and be imported into the nucleus independent of any DTS." [22]. In support of this hypothesis, Utvik *et al.* [66] have reported a threshold level for plasmid copies above which nuclear uptake occurs. When injecting 10^5 or less plasmid copies into cytoplasmically into single muscle cells of intact mice, no expression was observed, whereas injection of 10^6 or more resulted in transgene expression in the absence of cell division, and in the absence of any active nuclear targeting strategy.

In our transfection experiments a dose of 1 µg DNA per well of a 24-well plate is applied, which corresponds to roughly $2 \cdot 10^6$ plasmids/cell. It is not expected that 100 % of the applied dose is taken up by cells and therefore it is unlikely that under the conditions used the threshold reported by Utvik *et al.* [66] is exceeded. Nevertheless, it is unknown if the reported threshold applies to

different cell types. Therefore, to fully exclude the phenomenon of mass action, an experiment was performed in which the numbers of plasmids dosed per cell are decreased and both the actual amount delivered into the cells and the transgene expression are monitored. In the first experiment, HeLa cells were electroporated with various amounts of plasmids with or without DTS and gene expression was measured 24 h after electroporation (see Figure 6 A). Transfection efficiency was similar for the two plasmid constructs at all amounts tested. Up to a dose of 1 µg, each two-fold increase in applied dose resulted in a two-fold increase in the % successfully transfected cells. At doses exceeding 1 µg, a saturation effect was observed and a maximum number of transgene expressing cells of approximately 50 % was reached. When looking at the mean expression per cell, again a dose-response relation is observed. However, the expression per cell increases only by a factor two upon a 16-fold increase in dose. This shows that once a cell is successfully transfected, the number of plasmids transfected is not an important parameter for overall transfection efficiency. Possibly, transcription and/or translation processes become rate-limiting at this stage. A second experiment was performed in which cells were treated similarly, but gene expression was measured 4 h after electroporation to minimize effects of cell division (see Figure 6 B). Since the proportion of cells passing through mitosis in the course of the experiment is largely reduced, DTS-mediated nuclear import was expected to become more apparent. The results show that very little cells (<3 %) were transfected, independent of the presence of the DTS. This indicates that the DTS was inefficient in mediating active nuclear uptake and subsequent transgene expression in the large fraction of non-dividing cells within the total cell population. Similar to the trends observed after 24 h incubation, DNA dose predominantly affected the % of transfected cells and to a much lesser extent the expression per cell.

In these experiments, the highest plasmid dose used was 2 µg DNA/ $2.25 \cdot 10^5$ cells, which corresponds to delivery of maximally $1.5 \cdot 10^6$ plasmids/cell. This dose is close to the critical dose of $1 \cdot 10^6$ described by Utvik *et al.* , but is most likely a gross overestimation of the actual amount of plasmids delivered. To check this, it was quantified how much of the applied dose was actually delivered into the cells after electroporation by measuring plasmid copy numbers relative to actin copy numbers by Q-PCR on cell lysates. After electroporation of 1.8 µg/ $2.25 \cdot 10^5$ cells (corresponding to $1.3 \cdot 10^6$ plasmids/cell), an average plasmid copy numbers/ cell of 1.5 (± 0.6) $\cdot 10^3$ was detected. This confirms that the number of plasmids that reached the cell cytosol in these experiments was well below the levels described by Utvik *et al.* making it unlikely that the DTS-effect was masked by unspecific nuclear uptake due to overloading of the cytosol.

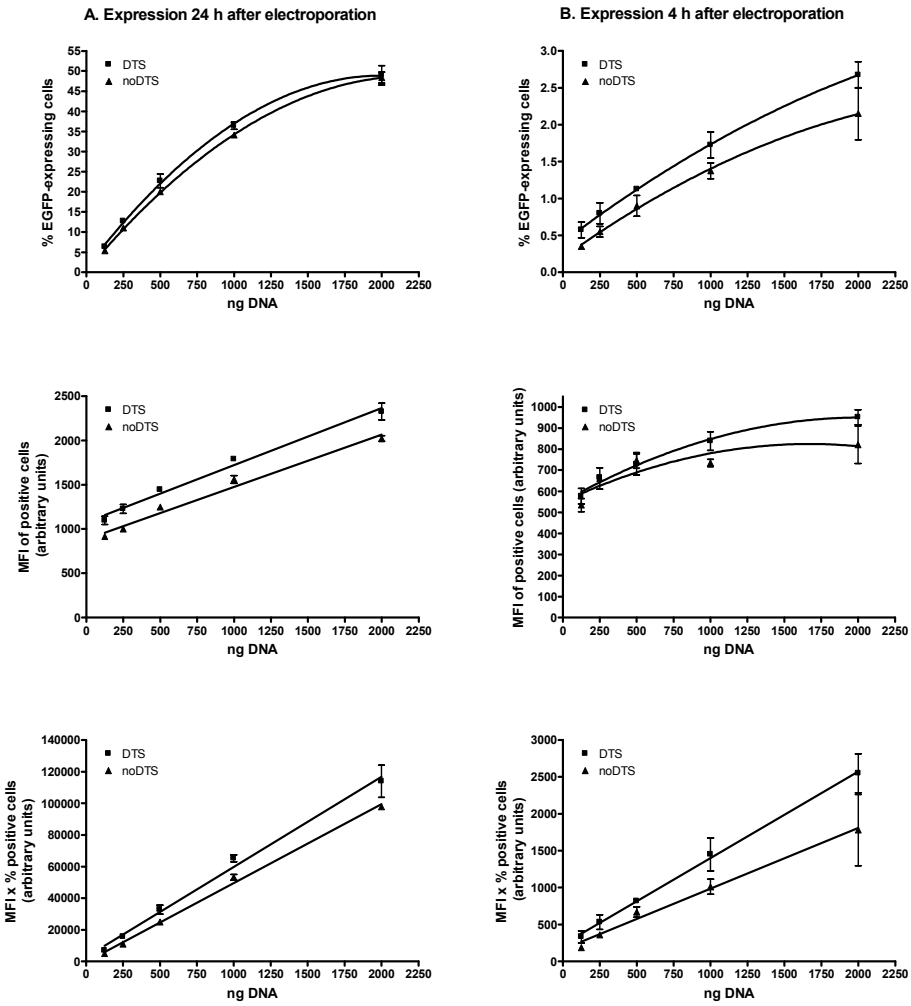


Figure 6. *Dose-dependent gene expression upon electroporation. HeLa cells were electroporated with varying doses of plasmids containing or lacking a DTS and analyzed for EGFP-expression by flow cytometry either 24 h (A) or 4 h (B) after electroporation. Gene expression drops with decreasing plasmid amounts regardless of the presence or absence of a DTS.*

Is the DTS effect observed at the level of localization rather than expression?

The efficiency of nuclear delivery for plasmids containing or lacking a DTS was evaluated by isolating nuclei from transfected cells and quantification of plasmid copies by Q-PCR. The number of genomic actin copies was determined for use as an internal standard representing the number of cells (cell nuclei). Hypotonic swelling followed by Dounce homogenization rather than detergent-based cell lysis was used for cell fractionation as this was previously described to yield a pure

nuclear fraction with minimal contamination of cytoplasmic components [63]. First, Q-PCR was performed on total cell lysates to determine the efficiency of uptake into cells. After incubation of cells with complexes for 4 h, and an additional hour in the absence of complexes, cells were harvested and analyzed. As shown in Table 2, transfection of pCMV/EGFP with lipofectamine resulted in 5.4 (± 1.8)·10^2 copies per cell in dividing cells (versus 3.4 (± 1.7)·10^2 for pCMV/EGFP_noDTS, difference is not significant). This number remained constant up to 24 h after transfection, indicating that little intracellular elimination occurred. Similarly, Ruponen *et al.* [67] found little elimination of plasmid DNA within 24 h after transfection with cationic liposomes.

Next, *nuclei* were isolated from transfected cells and plasmid copy numbers/nucleus were determined (see Table 2). The number of plasmids per nucleus 24 h after transfection was 1.8 (± 0.8)·10^2 (versus 2.4 (± 1.6)·10^2 for pCMV/EGFP_noDTS, difference is not significant), corresponding to ~40 % of total cellular plasmid DNA. During the 24 h incubation, cells will have undergone at least one mitotic event during which plasmids gain bulk access into the nuclei which may explain the high % found in nuclei. The situation in non-dividing cells was rather different. 24 h after transfection of aphidicolin-arrested cells, 1.7 (± 0.6)·10^3 plasmid copies were detected per cell (compared to 5.4 ± 1.8)·10^3 for pCMV/EGFP_noDTS, difference is not significant). Treatment of cells with aphidicolin has previously been described not to affect the rate of uptake of lipid/DNA complexes [37]. The increased number of copies detected per cell as compared to dividing cells probably simply reflects the lower number of cells present in the absence of division. More importantly, the number of plasmid copies detected per nucleus was decreased to 2-4 % of total cellular plasmid DNA. This confirms that in the absence of mitosis, transport of DNA into the nucleus is severely diminished (although not completely abolished). The difference between copy numbers detected per nucleus after transfection with plasmids with or without DTS was not significant (7.5 (± 2.7)·10^1 versus 3.4 (± 1.1)·10^1).

When comparing pEI and lipofectamine, large differences in detected copy numbers were observed. At times of 5-24 h after the start of transfection, roughly 2·10^4 plasmids were detected per cell in dividing cells when pEI was used as delivery agent. This is two orders of magnitude more than observed for lipofectamine. Again, copy numbers per cell were increased in aphidicolin-arrested cells. While collecting nuclei of pEI-transfected cells after ultracentrifugation through a discontinuous iodixanol gradient, we noticed the presence of aggregates at the 30/35 % interface which we did not observe during processing of lipofectamine-transfected cells. This is probably due to adherence of extranuclear plasmid DNA (-complexes) to the nuclei followed by aggregation and indicates that in our hands the method failed to yield pure nuclei devoid of extranuclear plasmid (-complexes) in case of pEI-transfected cells. Analysis of the nuclear fractions showed that nuclear copy numbers did not differ from- or even outnumbered- total cellular copy numbers, which further supports our assumption that we failed to isolate clean nuclei. At the time of this writing efforts to further optimize and validate the method were still ongoing. Results obtained for pEI-based delivery therefore remain elusive. Nevertheless, in the experiments performed with

lipofectamine no significant enhancement of plasmid nuclear localization that could be ascribed to the DTS effect could be detected.

Table 2. *Cellular and nuclear delivery of plasmids with and without DTS in dividing and aphidicolin-arrested cells. The number of plasmid copies per cell or per nucleus was determined after transfection with 22 kDa linear pEI or lipofectamine. Cells were transfected at a dose of 18 µg DNA/ T75-flask (or 2.3 µg/well in 6-well format) with lipofectamine or pEI for 4 h in the presence of serum, washed and analyzed after 24 h (or 5 h). As a model for non-dividing cells, cells were arrested at the S-phase by treatment with 15 µM aphidicolin starting 24 h prior to transfection and continuously throughout the experiment. Data are represented as mean ± SD of 3 independent measurements.*

		l-pEI		lipofectamine	
		+DTS	-DTS	+DTS	-DTS
Dividing cells					
4+1 h	# plasmids/cell	$2.7 \pm 0.8 \times 10^4$	$2.7 \pm 1.3 \times 10^4$	$5.4 \pm 1.8 \times 10^2$	$3.4 \pm 1.7 \times 10^2$
24 h	# plasmids/cell (C)	$1.4 \pm 0.2 \times 10^4$	$1.9 \pm 0.5 \times 10^4$	$4.9 \pm 2.8 \times 10^2$	$2.8 \pm 0.9 \times 10^2$
24 h	# plasmids/nucleus (N)	$2.1 \pm 0.3 \times 10^4$	$8.0 \pm 6.2 \times 10^4$	$1.8 \pm 0.8 \times 10^2$	$2.4 \pm 1.6 \times 10^2$
Aphidicolin-arrested cells					
24 h	# plasmids/cell (C)	$9.8 \pm 3.9 \times 10^4$	$2.3 \pm 1.3 \times 10^5$	$1.7 \pm 0.6 \times 10^3$	$5.4 \pm 1.8 \times 10^2$
24 h	# plasmids/nucleus (N)	$7.4 \pm 5.3 \times 10^4$	$1.3 \pm 0.2 \times 10^5$	$7.5 \pm 2.7 \times 10^1$	$3.4 \pm 1.1 \times 10^1$

Discussion

In this paper the relevance of DNA nuclear Targeting Sequences for improving efficiency of non-viral gene delivery was evaluated. Several DTS were identified from literature and cloned downstream of an EGFP coding sequence that was under the control of the CMV-promoter. It was shown that insertion of either the full-length SV40 DTS (372 bp promoter/origin region), the partial SV40 DTS (72-bp enhancer region), repetitive NFκB binding sites or a Glucocorticoid Responsive Element did not increase transgene expression. Additionally, preliminary findings showed that cloning of multiple repeats of the SV40 DTS had no beneficial effect on transfection efficiency (data not shown). It was shown that expression levels could be augmented by activation of the NFκB-pathway through TNF-α stimulation. However, this effect was not restricted to plasmids containing NFκB inserts, but also observed to a similar extent for all the other plasmids tested. This indicates that the effect is more likely related to unspecific transcriptional upregulation rather than NFκB specific nuclear import. In the plasmids used in this study, transcription is driven by the CMV promoter, which contains 3 NFκB binding sites and could therefore be upregulated by TNF-α induced NFκB activation. Interestingly, these sites seem to have no role in nuclear targeting as Dean *et al.* [49] showed unability of the CMV promoter to direct nuclear localization of plasmid after cytoplasmic microinjection. The presence of NFκB sites and their apparent unability to function as DTS remains unaddressed in his paper. In two papers by Gonçalves *et al.* and Breuzard *et al.*, the importance of spacing between transcription factor binding sites is addressed and it is suggested that the separation between the individual NFκB sites (142 bp and 152 bp) within the CMV-

promoter are not optimal to mediate NFκB-mediated nuclear import, but would be able to induce transcriptional enhancement [54,68].

It was shown that the presence of the full-length SV40 DTS did not enhance transfection efficiency regardless of mitotic activity of cells. We aimed to compare three delivery methods to gain insight into whether the DTS is available for interaction with transcription factors after delivery into the cell. Contrary to the situation after electroporation, after transfection with lipids or polymers the DNA is present in a condensed form and we hypothesized that this might interfere with binding of transcription factors. However, no DTS effect was observed for any of the delivery methods chosen (condensed/not-condensed). Dose-response experiments excluded the possibility that relatively small increments in specific nuclear uptake were outweighed by overloading the cytoplasm with plasmid DNA which could potentially lead to unspecific nuclear uptake. Finally, it was evaluated whether effects of DTS could be detected at the level of translocation rather than expression. Results from quantitative PCR on nuclear fractions indicate that nuclear localization of plasmids was not facilitated by the presence of the full-length SV40 DTS.

Overall, the beneficial effects of DTS on transfection efficiency were much less than we expected based on findings from literature. Prasad *et al.* [69] also question the effect of an SV40 DTS on transfection efficiency of CMV-plasmids. In their study, constructs with no SV40DTS, the complete SV40 DTS or the 72-bp repeat were used to perform transient gene expression assays in various cell types. It was observed that gene expression from DTS-containing plasmids compared to expression from plasmids lacking a DTS varied with cell type and plasmid backbone used. In support of our data, they did not observe any beneficial effects in HeLa-cells. The authors conclude that effects of the SV40 DTS on overall gene expression are only weakly dependent on facilitated nuclear import. Other factors governing overall transfection efficiency would be enhancement of transcription and replication of plasmids containing the SV40 origin of replication in SV40-transformed cells such as COS-7. Unfortunately, no data regarding nuclear localization efficiency are included and therefore these statements remain speculative.

In our study, we attempted to study the effect of DTS at the level of nuclear localization efficiency by performing quantitative PCR on isolated nuclei. Studies presenting data on quantification of intranuclear plasmids are still limited, although important progress has been made in the past years. Various methods have been described; including microscopic analysis based on FISH-detection [48,70], sometimes combined with FRET or FRAP effects[54]. Drawbacks of these techniques are the need for fluorescent labeling and consequent artifacts (especially if DNA is labeled prior to transfection [71]), detection limits, resolving power to discriminate internal from adhered DNA and poor statistical power related to the low number of cells analyzed. Alternatively, methods based on cell fractionation and quantification of DNA in the nuclear fraction have been described. Isolated nuclei were previously analyzed by flow cytometry [24,72,73], Southern blotting [74] or quantitative PCR [27,63,74,75]. Analysis by flow cytometry seems the fastest and simplest option, but requires fluorescent labeling of DNA and is complicated by low yields of isolated nuclei [76] and

leakage of compounds out of the nuclei [24]. Q-PCR offers a sensitive and quantitative analysis on non-modified plasmid DNA and was chosen here. A general concern in cell fractionation is cross-contamination among fractions, in our case contamination of the nuclear fractions with extranuclear DNA(-complexes). The majority of cell fractionations is performed after cell lysis with detergents [24,27,72-75]. Cohen *et al.* [63] showed that detergent-lysis induces aggregation of nuclei and adsorption of DNA(-complexes) to the outer membrane of the cell nuclei. Moreover, they show that this plaque could not be sufficiently removed by treatment with competitive agents (pASPA, DNA) or restriction enzymes. They developed an alternative method where cells are lysed by hypotonic swelling followed by Dounce homogenization which is described to yield clean nuclei largely free from plaque.

Using this method, we found nuclear plasmid copy numbers of 1.8 (± 0.8)·10^2 and 2.4 (± 1.6)·10^2 after lipofectamine-based transfection of dividing cells with pCMV/EGFP and pCMV/EGFP_noDTS, respectively. We used a dose of 18 µg DNA/T75 flask seeded with 1.8·10^6 cells and taking into account the doubling time of cells, this corresponds to a dose of roughly 8·10^5 plasmid copies/cell. In comparison, Cohen *et al.* [63] detected approximately 1·10^3 plasmid copies per nucleus after transfection with either linear pEI or lipofectamine at a dose of 1·10^5 plasmid copies per cell. Tachibana *et al.* [74] report nuclear delivery of 1·10^4 plasmid copies per nucleus after transfection with cationic liposomes and a dose of 20 µg/ 5·10^6 cells, which is slightly less than doses used by us. Hama *et al.* [75] recovered 1.7·10^4 plasmid copies per nucleus from cells transfected with lipofectamine at a dose of 2.5 µg/ 2.5·10^5 cells. The latter two studies used detergent-lysis which could explain the higher copy numbers found. James and Giorgio [72] used flow cytometric analysis of fluorescence in isolated nuclei and found 1,250 pCMV-plasmids per nucleus in Hela cells 4 h post-transfection with cationic liposomes (dose 1.25 µg/ 2·10^5 cells). Breuzard *et al.* [54], who used microscopic analysis for quantification of nuclear delivery, report lower numbers: approximately 368 copies/nucleus in cells transfected with pCMV/luc complexed with linear pEI (dose 0.5 µg/1·10^4 cells). These data show that copy numbers found by us after transfection in dividing cells are within the expected range (at the lower end). In non-dividing cells we detected 7.5 (± 2.7)·10^1 versus 3.4 (± 1.1)·10^1 plasmid copies/nucleus for pCMV/EGFP and pCMV/EGFP_noDTS, respectively. To our knowledge, no previous attempts have been described to quantify nuclear uptake in aphidicolin-arrested cells. The difference in plasmid copy numbers/nucleus resulting from transfection of either dividing or non-dividing cells with plasmid containing or lacking the SV40 DTS was not significant. Previous studies investigating the role of DTS in gene delivery relied on microscopic techniques to evaluate nuclear uptake. Discrepancies between findings regarding the effect of DTS on nuclear localization might be inherent to differences in methods chosen. At this stage, the presence of extranuclear plaque cannot be fully excluded and this could obscure subtle differences in intranuclear copy numbers. Additionally, it should be mentioned that the method used in this study is based on batch analysis of DNA in pooled nuclear fractions, leaving heterogeneity among cells within a population unconsidered. Using microscopic techniques offers the ability to study

individual cells and take into account heterogeneity by quantifying both the number of plasmid-positive nuclei and ranking the signal within these nuclei.

Nevertheless, even if small increments in nuclear uptake were overlooked by us, this would not change the observation that transgene expression was unaffected by the presence of a DTS. We conclude that the DTS-effect may occur at the level of nuclear import but is obscured by effects on any of the other steps involved (i.e. transcription, translation). In our study, plasmids in which transcription was controlled by the strong CMV-promoter were used to ensure detectable expression levels in poorly transfectable non-dividing cells. We propose the following hypothesis, which is schematically presented in Figure 7. When using a strong promoter, the bottleneck is at the (post-)transcriptional level and increments in pre-transcriptional processes, including nuclear uptake, do not affect overall gene expression levels. In contrast, when using a weak promoter, the transcription machinery is not saturated and increases in nuclear delivery of plasmids lead to increased transcription and translation (until translation is saturated), hence increased transgene expression.

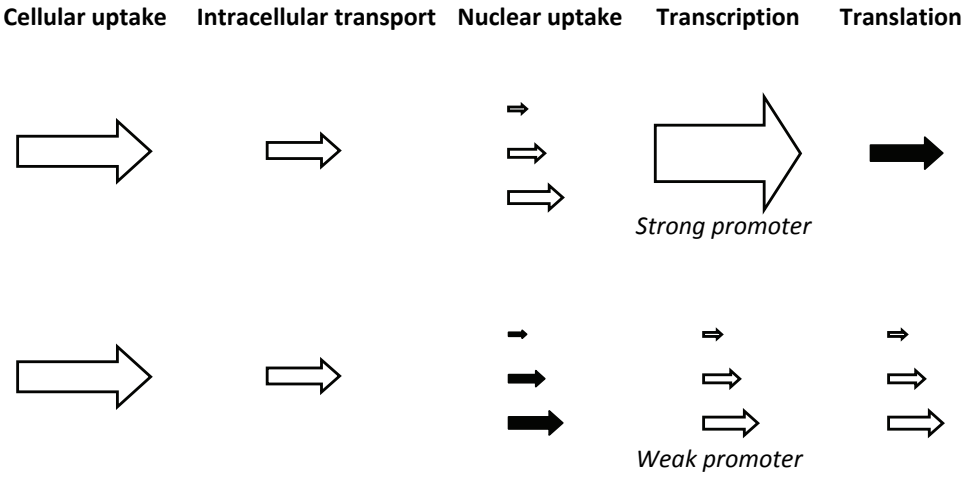


Figure 7. *Schematic presentation of hypothesized bottlenecks (black arrows) for transgene expression of plasmid driven by a strong or weak promoter.*

In support of this hypothesis, a threshold for nuclear delivery of plasmids has been described above which additional plasmids no longer result in increased expression [63,74]. This means that above this threshold, processes following nuclear uptake become the limiting factor, possibly subnuclear disposition or translation [27].

The presented hypothesis also explains discrepancies between our results and findings by others. For example, Gonçalves *et al.* [68] and Breuzard *et al.* [54] describe the optimization of an extended NFκB DNA binding site and show that insertion of these NFκB binding sites into pTal, a plasmid in

which luciferase is driven by a minimal promoter, increased transgene expression levels 1,600-fold and an additional 8-fold after stimulation with TNF-α as compared to the control plasmid. Expression levels obtained with the modified plasmid reached similar levels as an unrelated plasmid in which luciferase was under control of the strong CMV-promoter. When looking at nuclear delivery, the NFκB-modified plasmid was reported to deliver 6-fold more copies than the control vector, and 4-fold more than the CMV-luc plasmid (5 h after transfection of TNF-α stimulated HeLa cells). No constructs in which NFκB binding sites were combined with the CMV-promoter were tested. Nevertheless, the observation that much less CMV-luc is delivered into nuclei than the NFκB-modified plasmid although expression levels are similar, supports our findings/ hypothesis.

Similarly, Mesika *et al.* [55] showed a 12-fold increase in transfection efficiency after insertion of repetitive NFκB binding sites into a construct in which expression is under the control of the SV40 promoter, which is considerably weaker than the CMV promoter [77].

In case of Thanaketpaisarn *et al.* [78] increased expression is in fact observed upon insertion of NFκB binding sites into a CMV-driven plasmid. Possibly, this is due to the position of the NFκB-sites within the plasmid. DTS can elicit two effects: one is promoting nuclear uptake and is thought to be position-independent, whereas the other one is transcriptional enhancement which is position-dependent [54]. Thanaketpaisarn *et al.* have cloned the NFκB-sites directly upstream the CMV-promoter and they may therefore be involved in direct enhancement of CMV-driven transcription whereas in our constructs the DTS were cloned downstream of the coding sequence. Indeed, the authors mention in their discussion that no significant difference in amount of plasmid in the nuclear fraction was found.

One discrepancy however remains unexplained. Vacik *et al.* [50] report differences in transgene expression between CMV-driven plasmids with versus without an SV40 DTS upon transfection of aphidicolin-arrested CV1 (and HeLa) cells with lipofectin. Since this setup closely resembled ours, we contacted the authors for comment but received no response.

Conclusion and prospects

Although DNA nuclear Targeting Sequences can increase nuclear delivery of plasmids, no significant benefits for overall transgene expression are obtained if combined with strong promoters. The added value of DTS may lie in compensating for weak promoters. Weak promoters can be a useful alternative to the strong CMV promoter to minimize immunological responses and promoter shutdown *in vivo*, and for transcriptional targeting (see chapter 2).

Besides their relevance for improving efficiency of non-viral gene delivery, DTS are of value from a mechanistic point of view to gain insight into cytonucleoplasmic transport of macromolecules. In this respect, focusing on well-defined homogeneous DTS is preferred over heterogeneous DTS such as the SV40 enhancer region. Using such homogeneous DTS, research should be directed at

studying the nature and kinetics of protein-DNA interaction. Firstly, it would be valuable to identify which proteins interact with a DTS and which of these proteins are able to fulfill a role in facilitating nuclear import of the DTS-containing plasmid. Not all proteins that contain a DNA binding domain and an NLS can act to shuttle DNA into the nucleus and it is thought that the overall organization and structure of the DNA-TF complex might be important (for example, spacing between the DNA binding domain and the NLS). Subsequently, efforts to optimize interaction between the protein and DNA could be made. Studies performed by Gonçalves *et al.* [68] show that optimization of the DTS sequence is important and that spacing between repetitive binding sites has a strong effect on transfection efficiency.

Secondly, it would be interesting to study if and how this interaction takes place with plasmid DNA while complexed with its carrier inside the cell cytosol. Gonçalves *et al.* [68] performed a cross-linked immunoprecipitation assay with antibodies directed against NFκB and found that plasmid DNA was present in the immunocomplexes, indicating cytoplasmic interaction of plasmid with NFkB. However, it remains elusive whether this interaction occurred in the presence of carrier, as the carrier is likely to dissociate during the cell work-up which includes triton and SDS treatments. In our hands treatment with these detergents disassembled plasmid/pEI complexes (not shown). Future studies regarding the kinetics of vector disassembly, the kinetics of transcription factor activation (under normal conditions, in diseased tissues and in response to drugs) and binding of those transcription factors to DTS within the plasmid 'to be delivered' will help to understand and exploit processes by which plasmid DNA can gain access to the cell nucleus.

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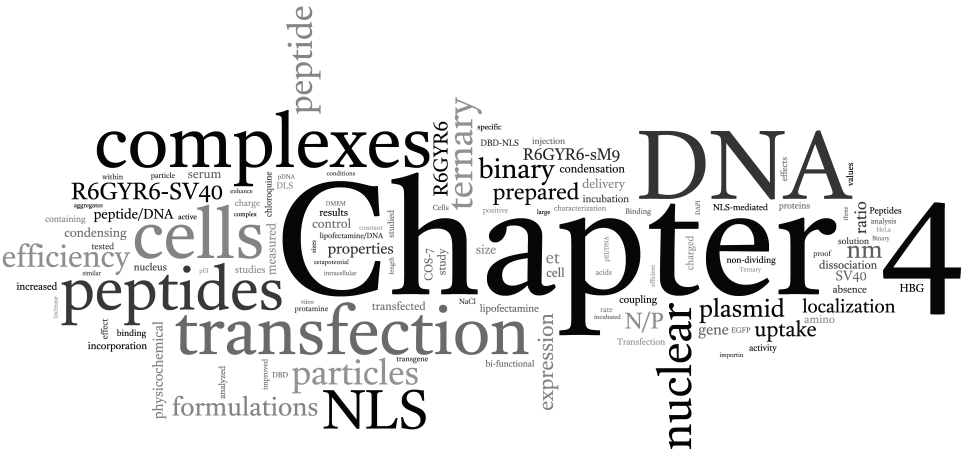

Bi-functional peptides with DNA Binding Domain and NLS enhance transfection efficiency in an NLS-independent way

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Abstract

The objective of this study is to target DNA within DNA-Transporting Nanoparticles to the nucleus by incorporation of bi-functional peptides consisting of a DNA Binding Domain (DBD) and a nuclear localization signal (NLS). Peptides were designed to contain a protamine-derived DBD (R6GYR6) and either a charged SV40 NLS (PKKKRKV) or a neutral sM9-derived NLS (YNNQSSNFGPMKC). Peptides were shown to deliver DNA, but transfection activity required combination with a second delivery agent. Incorporation of peptides in lipofectamine-, but not pEI-mediated complexes improved transfection efficiency up to 10-fold. The effect was also observed for DBD-peptides lacking an NLS. Peptides were unable to mediate transfection in non-dividing cells. The improvement of transfection activity therefore seems to reflect changes in physicochemical properties of the constructed particles that enhance cellular processing at another level than specific NLS-mediated nuclear uptake.

Introduction

Gene therapy requires the introduction of foreign DNA into cells with the aim of treating, or ultimately curing a disease. Nuclear uptake is considered a major barrier to the efficiency of non-viral gene delivery. Microinjection studies have revealed a >100-fold difference in gene expression upon direct injection into the nucleus versus cytoplasmic injection [1,2]. In the absence of cell division, transport of molecules from cytoplasm to nucleus is restricted to molecules <9 nm that can diffuse freely through the nuclear pore complex (NPC) [3] or molecules with a theoretical maximum of ~39 nm [4] (an experimental upper limit of ~28 nm was reported for non-deformable cargo [5]) that exploit active transport mechanisms. Cyto-nucleoplasmic trafficking events largely depend on the importin-mediated pathway, involving recognition of nuclear localization signals within the cargo molecule by the adaptor protein importin α, subsequent binding of the cargo/importin α complex to importin β and, lastly, docking of the entire assembly to the NPC (reviewed in [6]).

Over the past years many strategies have been developed to enhance nuclear uptake of exogenous DNA by mimicking nuclear trafficking of viral genomes or nuclear proteins. Efforts to transport DNA into the nucleus via the importin-mediated pathway have been directed at various stages within the pathway (see schematic representation in Figure 1), but are predominantly focused on modification of DNA (containing particles) with NLS. However, localization studies with various proteins and DNA constructs modified with NLS have stressed that the effect of an NLS is strongly influenced by the cargo to which it is attached. Several non-karyophilic proteins including BSA, ferritin and IgM were shown to be effectively delivered into the nucleus upon modification with NLS [7,8]. Despite general successes obtained with NLS-bearing proteins, nuclear localization of DNA-NLS conjugates has been less evident.

Strategies for NLS-mediated nuclear delivery of DNA vary in the type of NLS, its flanking regions and in the coupling method chosen and have produced diverse results and insights. Initial efforts involved association of nuclear proteins to DNA, but later approaches used minimal NLS peptides instead of complete proteins to avoid difficulties encountered when developing proteins for pharmaceutical purposes (i.e. difficulties in production, stability and immunogenicity).

Sebestyen *et al.* [9] investigated effects of SV40 large T antigen nuclear localization signals (CGYGPKKKRKVGG) covalently coupled to DNA and found that coupling of >100 NLS resulted in nuclear accumulation of the DNA in digitonin-permeabilized cells (but not in microinjected cells). Zanta *et al.* [10] reported 10-1,000 fold transfection enhancements upon modification of a linear capped DNA molecule with a single NLS (NH2-PKKKRKVEDPYC). Contrarily, a study by Ciolina *et al.*[11] points out that although NLS coupled to plasmid specifically interacted with the NLS-receptor importin α, no improved nuclear uptake of plasmid DNA was observed after microinjection of peptide/DNA conjugates. An increase in gene expression was observed under some conditions, but similar results were reported to be observed for a mutant NLS. Additionally, Van der Aa *et al.* [12]

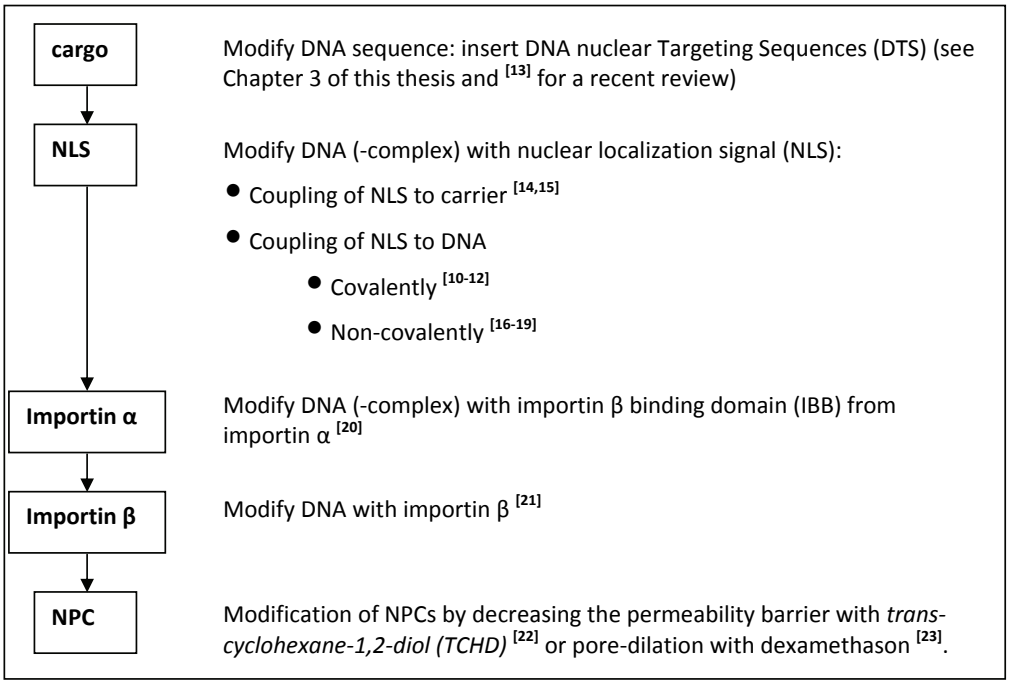


Figure 1. *Schematic overview of strategies to improve nuclear uptake of DNA for gene therapy*

observed that covalent coupling of a single NLS (NH2-PKKKRKVEDPYC) to linear capped DNA was neither able to improve transfection efficiency of cationic polymers nor the nuclear import of the DNA constructs in digitonin-permeabilized cells. Furthermore, covalent coupling of NLS to DNA has the potential drawback to interfere with transcription. An optimum number of NLS has to be found below which no nuclear accumulation is established and above which transcription is seriously hampered [9].

An approach that avoids perturbing modification of DNA is the conjugation of NLS peptides to the delivery vector rather than to the DNA [14,15,24,25]. However, this requires nuclear delivery of intact complexes. Since it is still unknown whether the nuclear entry of intact complexes is preferred over entry of (released) naked DNA, strategies for nuclear uptake of DNA after condensing agent/DNA disassembly cover a substantial part of investigations.

In addition to covalent conjugation of NLS to either the condensing agent or DNA, non-covalent approaches for complexation of NLS with DNA have been explored. These rely on sequence-specific interaction as is the case for PNA-clamps [16] or random binding, mostly based on electrostatic interactions [18,19,26-33]. Ludtke *et al.* [18] showed that conjugation of 8 separate nuclear localization signals (CKKKSSSDDEATADSQHSTPPKKKRKVEDPKDFPSELLS) to 350-1500 bp linear DNA molecules through biotin-streptavidin interaction induced nuclear localization and transgene expression in microinjected living cells[18]. Improved transfection efficiencies were also reported upon random

electrostatic coupling of peptides containing the M9-derived NLS [19], TAT oligomers [32] and protamine [34].

In the present study, we investigate the use of non-covalent attachments of NLS directly to DNA via major groove-binding peptide-clamps. For this purpose, bi-functional peptides were designed to contain a DNA-binding domain (DBD) and an NLS. The DBD was derived from protamine, a protein naturally occurring in sperm and known for its high affinity for DNA. Brewer *et al.* [35] studied the kinetics of DNA condensation and decondensation by subsets of the protamine 1 (P1) DNA binding domain. They identified the number of clustered arginine residues as the most important factor affecting condensation and stability, with dissociation rates increasing at least three orders of magnitude upon increasing from a single to two or more arginine domains. Additional stability could be obtained by separating individual binding domains by tyrosine or phenylalanine residues, which could function as hinges allowing more favorable wrapping of the peptides around the major groove of the double helix (see Figure 2). Based on the results reported by Brewer *et al.* [35], we selected RRRRRRGYRRRRRR (R6GYR6) as a DNA-binding domain for our study. Being 14 amino acids in length, this peptide also fulfils the requirement of >6-10 amino acids needed for compaction of DNA into stable particles [36].

We have included two different types of NLS in this study: a classical SV40-derived NLS and a non-classical NLS derived from the M9 sequence of heterogeneous nuclear ribonucleoprotein (hnRNP). The SV40 NLS (PKKKRKV) contains positively charged amino acids and may therefore interact with DNA itself, which potentially decreases its availability for the importin α receptor. Therefore we also included a non-classical and neutral NLS called sM9 (YNNQSSNFGPMK), which is a shortened version of M9 containing the 12 amino acids thought responsible for transportin binding based on molecular modeling. sM9 was previously observed to facilitate nuclear uptake of Texas Red-labeled BSA in digitonin-permeabilized cells [15].

The aim of this study is to investigate the use of these new bi-functional DBD-NLS peptides for gene delivery. First, we evaluated the binding and condensing properties of the peptides by Surface Plasmon Resonance (SPR) and Dynamic Light Scattering (DLS). Next, transfection efficiencies and cellular uptake of binary (peptide / DNA) and ternary (peptide / DNA / condensing agent) peptide-containing formulations were studied. The effect of peptides to mediate transfection in non-dividing cells was studied in aphidicolin-arrested cells.

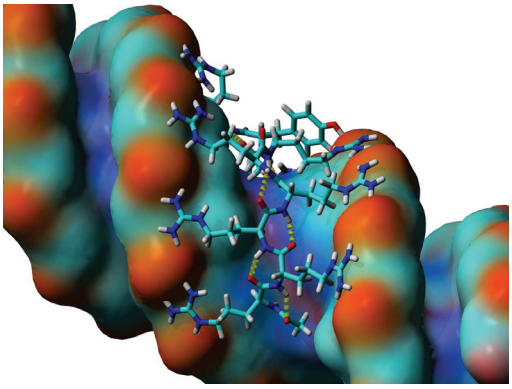


Figure 2. *Binding of R6GYR6 into the major groove of the DNA helix.*

Materials and methods

Material

Peptides were ordered with the C-terminus amidated and the N-terminus acetylated at >95 % purity from Caslo (Denmark): RRRRRRGYRRRRRR (R6GYR6), PKKKRKVGGSRRRRRRGYRRRRRR (R6GYR6-SV40) and YNNQSSNFGPMKCGGSRRRRRRGYRRRRRR (R6GYR6-sM9).

pCMV-LacZ plasmid DNA was purchased from the Plasmid Factory, Bielefeld, Germany. Plasmid pCMV-EGFP was constructed from pShooter (Invitrogen, Oregon, USA): the EGFP coding sequence from pEGFP-N1 (bp 613–1410; Clontech, Saint-Germain-en-Laye, France) was amplified by PCR and cloned into the multiple cloning site of pShooter from which the nuclear localization signal (NLS)-tag coding sequence was removed by digestion with NotI and XbaI. pCMV-LacZ and pCMV-EGFP are expression plasmids encoding for β-galactosidase and enhanced green fluorescent protein (EGFP), respectively, under the transcriptional control of the human cytomegalovirus promoter (CMV).

Oligonucleotides were synthesized by Eurogentec S.A. (Seraing, Belgium). SA Sensor Chips were purchased from Biacore, Sweden.

Exgen 500 (22 kDa l-pEI) was purchased from Fermentas, St. Leon-Roth, Germany. LipofectamineTM 2000 (lipofectamine), propidium iodide (PI) and DAPI were purchased from Invitrogen, Breda, The Netherlands. Plain DMEM (Dulbecco's modification of Eagle's medium, with 3.7 g/l sodium bicarbonate, 1 g/l l-glucose, l-glutamine), RPMI 1640, antibiotics/antimycotics (penicillin, streptomycin sulphate, amphotericin B), 200 mM L-glutamine solution (100x), Foetal Bovine Serum (FBS) and phosphate buffered saline (PBS) were purchased from PAA Laboratories GmbH, Pasching, Austria). LabelIT Fluorescein and Cy5 Nucleic Acid Labeling Kits were purchased from Mirus Bio, Madison, WI, USA. Other chemicals used: NaCl, D-Glucose, bovine serum albumine (BSA), Tris(hydroxymethyl)-aminomethane (Tris), Triton X-100, o-nitrophenyl-β-d-galactopyranoside (ONPG), β-galactosidase, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) and N-methyl dibenzopyrazine methylsulfate (PMS) (Sigma-Aldrich, Zwijndrecht, The Netherlands); Hepes (Acros Organics, Tilburg, The Netherlands). All buffers and reversed osmosis water were filtered through 0.2 µm filters prior to use.

Surface Plasmon Resonance

Experiments were performed on a BIAcore3000 apparatus with SA sensor chips consisting of streptavidin covalently immobolized on a carboxymethylated dextran matrix. SA chips were preconditioned with 50 mM NaOH in 1 M NaCl followed by overnight stabilization with DNA binding buffer (10 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA). A 19 bp biotinylated oligomer was hybridized with an excess of 19 bp complementary oligomer in a PCR apparatus and the resulting ds-oligomer was immobilized on the SA-chip to 400 RU. The immobilized chip was then double

primed with, and put on running buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.01 % Polysorbate 20). This running buffer was also used during dissociation measurements.

Peptides were diluted in sample injection buffer (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.01 % Polysorbate 20) and stored at 8 ^{o}C before injection. Injections were done with 250 µl sample injection at a flow-rate of 50 µl/min, dissociation-rates were measured for 1,500 seconds. Previous to each new peptide measured three 60 nM R_6GYR_6 stability injections were done. Before samples were injected a saturation injection was done at 500 nM for the loose anchor peptides and at 50 nM for the NLS anchor peptide conjugates. After each injection the chip surface was regenerated with 1 M NaCl. Injected peptide concentrations were 100-200-300-400-500 nM for R_6GYR_6, R_6GGR_6 and R_6YGR_6 and 10-20-30-40-50 nM for R_6GYR_6-SV40 and R_6GYR_6-SM9. Dissociation rate constants were determined by applying a separate and global k_d fit to reference-subtracted sensorgrams using BIAevaluation software 3.0 was used.

Dynamic light scattering

Particle size of the polyplexes was measured in HBG (viscosity 1.145 cP, refractive index 1.3402) with Dynamic Light Scattering (DLS) on an ALV CGS-3 system (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water bath) set at 25 °C. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles (Z_h) and the particle size distribution (polydispersity index, PDI) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by the cumulants method (fitting a single exponential to the correlation function to obtain the mean size (Zave) and polydispersity index (PDI)). The diffusion coefficients calculated from the measured autocorrelation functions were related to the hydrodynamic radius of the particles via the Stokes-Einstein equation, $Z_h = (k_BTq^2)/(3\pi\eta\Gamma)$, where Z_h is the hydrodynamic radius of the particles, k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, Γ is the decay rate, and q is the scattering vector ($q= 4\pi n \sin(\Phi/2))/ \lambda$), in which n is the refractive index of the solution, Φ is the scattering angle, and λ is the wavelength of the incident laser light. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90 ° angle. The system was calibrated with 200 nm polystyrene latex standard beads (Duke Scientific, Leusden, The Netherlands).

Zetapotential measurements

The surface charge of the polyplexes was measured in HBG (viscosity 1.145 cP, dielectric constant 79) using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) with universal ZEN 1002 'dip' cells and DTS (Nano) software (version 4.20). The Helmholtz–Smoluchowski equation was used for converting electrophoretic mobilities into zeta potentials. DTS 1050 latex beads (Zeta Potential Transfer Standard, Malvern Instruments, Malvern, UK) were used as a test standard.

Cell culture

COS-7 African Green monkey kidney cells and human epithelial ovarian carcinoma cells (HeLa) were grown in DMEM supplemented with antibiotics/antimycotics and 5 % or 10 % heat-inactivated FBS, respectively. The original cell lines originates from American Type Culture Collection (ATCC, Maryland, USA). Cells were maintained at 37 °C in a 5 % CO_2 humidified air atmosphere and split twice weekly. Cells were confirmed to be free from mycoplasm by periodical testing with a MycoAlert® Mycoplasma Detection Kit (Lonza, Verviers, Belgium).

Complex formation

Binary complexes were prepared by adding 4 volumes of peptide solution (various concentrations in HBG) to 1 volume of plasmid solution (50 µg/ml in HBG), mixing immediately by pipetting up and down 10x and incubating 30 min at room temperature. Ternary complexes were prepared in two steps: 1 volume DNA solution was first complexed with 2 volumes of peptide solution (2x concentrated compared to solutions used for binary complexes) and after 30 min incubation 2 volumes of 22 kDa l-pEI (l-pEI/DNA N/P ratio 6) or lipofectamine (Lipofectamine/ DNA ratio: 2.5 µl/µg DNA) were added and incubated another 30 minutes. Complexes were prepared freshly and in triplo for each experiment.

Transfection

For flow cytometry analysis 40,000 cells were seeded per well into 24-well plates 24 h prior to transfection, such that ~80 % confluency was reached on the day of transfection. Immediately prior to transfection the culture medium was refreshed with 500 µl DMEM supplemented with 5 % FBS. 100 µl sample (corresponding to 1 µg DNA/well) was added per well and after 4 h incubation, medium was replaced with fresh DMEM supplemented with 5 % FBS. Cells were incubated for 48 h at 37 °C in a 5 % CO_2 humidified air atmosphere until analysis. Where indicated, cells were transfected in the presence of 100 µM chloroquine.

Cell synchronization

COS-7 cells were synchronized by continuous incubation with 15 µM aphidicolin (Aphidicolin from Nigrospora sphaerica, >98 %; Sigma-Aldrich, St Louis, USA) from 24 h prior to transfection until time of analysis.

Flow cytometry

At the indicated time after transfection, cells were washed, trypsinized and resuspended in DMEM supplemented with 10 % FBS to inactivate the trypsine. Cells were transferred into round-bottom 96-well plates and centrifuged for 5 min at 250x g at 4 °C. Medium was removed and cells were resuspended in 200 µl phosphate-buffered albumine (PBA; 1 %, w/v albumine in PBS). Immediately prior to measurement, 20 µl PI solution (10 µg/ml in water) was added for live/dead cell discrimination. Flow cytometric analysis was performed on a FACSCantoII (Becton and

Dickinson, Mountain View, CA, USA) equipped with a 488 nm 20 mW Solid State diode laser and a 633 nm 20 mW HeNe laser. 10,000 cells were recorded per sample to determine EGFP expression (FITC-channel) and PI-staining (PE-channel).

Microscopy

Widefield fluorescence and multiphoton microscopy were used to study localization of particles in 4 % paraformaldehyde-fixed cells. HeLa cells were seeded at 8,000 cells/well in 16-well glass chamber slides (LabTek, Nunc, Rochester, NY, USA) 48 h prior to transfection. Complex formation and transfection was performed as described above, but plasmid DNA covalently labelled with FITC or Cy5 (Mirus LabelIT) was used. 4 h after transfection, cells were washed with PBS and fixed with 4% formaldehyde. Nuclei were stained with DAPI (Invitrogen) and slides were mounted with FluorSave™ Reagent (Calbiochem, San Diego, USA). Fluorescent signals were visualized using a multiphoton system (Bio-Rad, Hemel Hempstead, UK). DAPI was excited by multiphoton excitation at 780 nm using a mode-locked Titanium:Sapphire laser (Tsunami; Spectra-Physics, San Jose, CA, USA) pumped by a 10-W solid state laser (Millennia Xs; Spectra Physics). Cy5, FITC and Rhodamine were excited by confocal lasers. Samples were examined using a TE200 inverted microscope using a 60x/1.4 oil objective (Nikon, Tokyo, Japan) and images were processed in ImageJ. Epifluorescence Microsopy was performed using a Nikon Eclipse TE-2000 inverted microscope (Nikon, Tokyo, Japan).

Results

Interaction of peptides with DNA

Binding kinetics of peptides and oligonucleotides were studied by performing Surface Plasmon Resonance (SPR) measurements. Biotinylated oligonucleotides were immobilized on sensor chips with pre-immobilized Streptavidin and peptides were injected after which the dissociation rate was determined. The dissociation rate constant (k_d) was selected as the most relevant parameter since it reflects peptide/DNA interaction after administration whereas association rates are only important in the process of peptide/DNA complex preparation. The k_d and calculated half-life values ($t_{1/2}$) of peptides are shown in Table 1. At pH 7.5, half-life values were within 11-32 minutes for all three peptides. R6GYR6-SV40 had a slower dissociation rate than the anchor peptide alone which could be related to the five additional positive charges present in the SV40 NLS. R6GYR6-sM9, though containing only one additional charged amino acid, was observed to have a similar dissociation rate as R6GYR6-SV40.

Next, it was examined whether the positively charged peptides exhibit condensing activity by incubating plasmid DNA with varying amounts of peptide and measuring resulting particle sizes and zetapotentials (see Figure 3). These measurements show that DNA containing nanoparticles can be formed with the DBD-NLS peptides. The DNA Binding Domain R6GYR6 itself was inefficient in

Table 1. *Dissociation constants and calculated half-life values for peptide/DNA interactions as determined by Surface Plasmon Resonance.*

	R6GYR6		R6GYR6-SV40		R6GYR6-sM9	
	k_d (s^{-1})	$t_{1/2}$ (min)	k_d (s^{-1})	$t_{1/2}$ (min)	k_d (s^{-1})	$t_{1/2}$ (min)
pH 7.5	$1.1E^{-3}$	11	$3.8E^{-4}$	30	$3.7E^{-4}$	32

condensing plasmid DNA. In the absence of peptide, count rates were too low to reliably measure particle sizes. Count rates increased to >500 kcps from peptide/DNA N/P ratio 0.5 and above. Particle sizes measured were approximately 400 nm and were unaffected by increasing N/P ratios. Increasing the peptide to DNA ratio up to N/P 40 resulted in less negative zetapotentials, but did not result in positively charged particles. The DBD-NLS peptide R6GYR6-SV40 was able to condense particles at N/P ratios of 1.5 and above, reaching sizes of 100-250 nm. At small peptide/DNA excesses (N/P 5 and above), positive zetapotentials were reached. With a diameter of ~100 nm and zetapotential of approximately +22 mV, R6GYR6-SV40/plasmid particles are expected to have good transfection ability. Unexpectedly, the DBD-NLS peptide R6GYR6-sM9 closely resembled R6GYR6-SV40 in its ability to condensate DNA. Despite its lower charge density, R6GYR6-sM9 was able to form particles at N/P ratios > 1 reaching a stable size of ~70 nm and zetapotential of approximately +22 mV from N/P 5 and up.

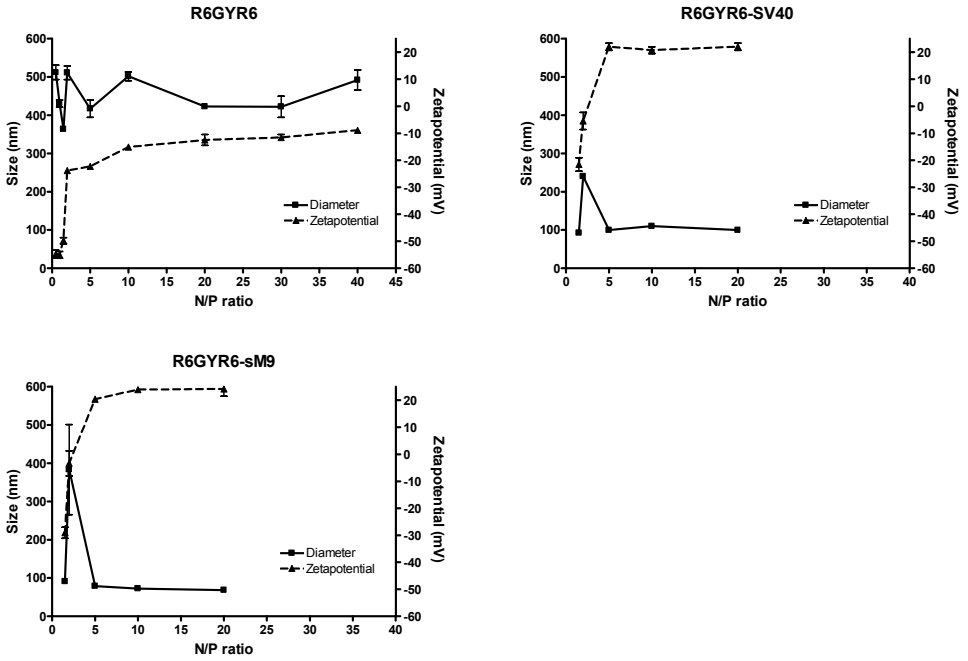


Figure 3. *Ratio-dependent particle size and zetapotential of binary complexes. Binary complexes of R6GYR6 (left), R6GYR6-SV40 (middle) or R6GYR6-sM9 (right) and plasmid were prepared in HBG at various N/P ratios (n=3) and analyzed 30 minutes after complexation. Formulations for which <100 kcps were detected by DLS are omitted from the graph.*

The differences in condensing behavior of the peptides are probably due to different lengths of positively charged amino acid stretches: the short length of R6GYR6 impedes efficient condensation whereas the increased length of R6GYR6-SV40 and R6GYR6-sM9 results in efficient condensation of plasmid DNA. The lack of difference between R6GYR6-SV40 and R6GYR6-sM9 indicates that the additional charged amino acids in R6GYR6-SV40 are not critical for DNA condensation.

In vitro transfection efficiency of binary complexes

Next the transfection efficiency of binary complexes with suitable physicochemical properties was tested. Figure 4 A shows that peptide/DNA complexes only showed marginal transfection of COS-7 cells *in vitro* (<1 %) compared to free pDNA. Possible explanations for this finding are instability of the particles in biological fluids or insufficient condensation of the DNA leading to inefficient particle uptake and/or susceptibility to DNAses.

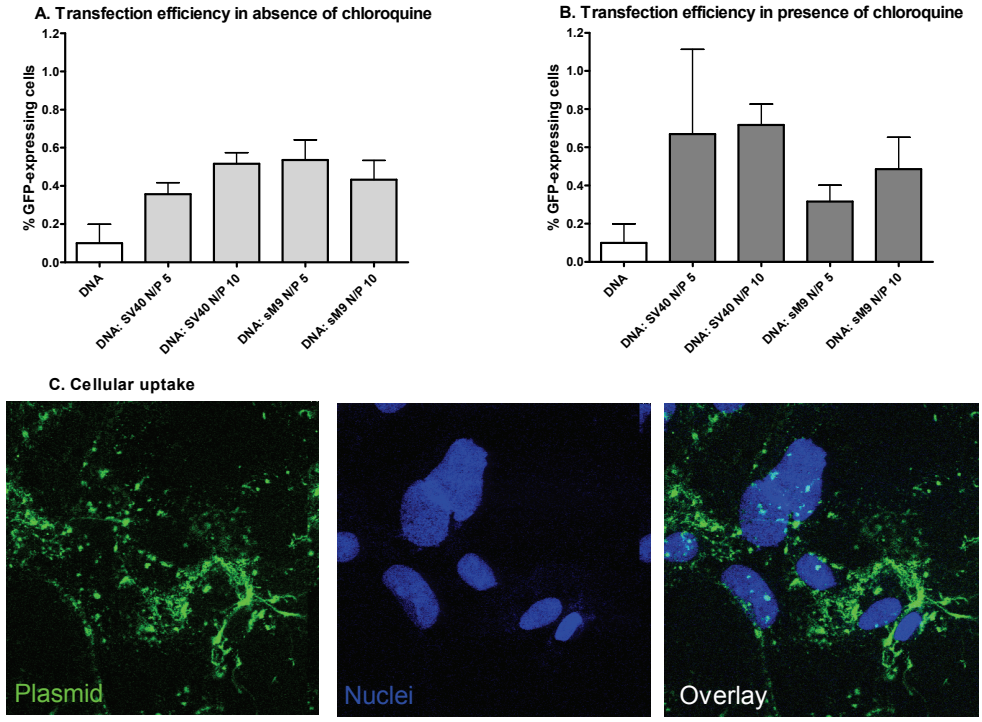


Figure 4. *Transfection efficiency and cellular uptake of binary complexes. COS-7 cells were incubated for 4 h in the presence of serum and either in the absence (A) or presence (B) of chloroquine with binary complexes of R6GYR6-SV40 or R6GYR6-sM9 and pEGFP-C1 prepared at N/P ratio 5 or 10. EGFP expression was measured with flow cytometry 48 h after transfection. Data are presented as mean+SD. (C) Confocal images of uptake of binary R6GYR6-SV40/pLacZ (N/P=10) particles in HeLa cells. pLacZ was labeled with FITC (depicted in green) and nuclei were stained with DAPI (depicted in blue). Cells were fixed with 4 % formaldehyde after 4 h incubation with the complexes. (HeLa cells were used for better imaging; uptake in COS-7 was confirmed).*

To test if binary particles are taken up by cells, cells were transfected with FITC-labeled DNA condensed with R6GYR6-SV40 N/P=10 and examined with multifoton microscopy (Figure 4 C).

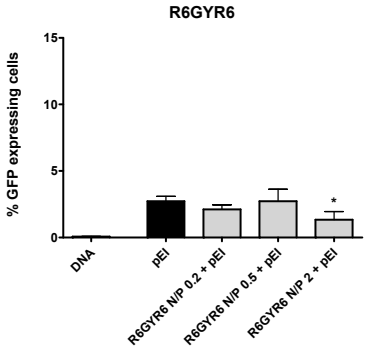
The results confirm that the plasmid DNA was internalized into cells. The lack of transfection activity is therefore thought to be at the level of intracellular processing. One possible explanation would be that binary particles enter cells, but are inefficient in escaping from the endosomes. To induce endosomal escape, cells were transfected with binary particles in the presence of chloroquine (see Figure 4 B). Upon treatment with chloroquine, a slight but non-relevant increase in peptide-mediated transfection efficiency was observed (% transfected cells remains <1 %). Altogether the results in Figure 4 indicate that peptides can deliver plasmid DNA into cells, but are unable to induce efficient transgene expression regardless of endosomal escape.

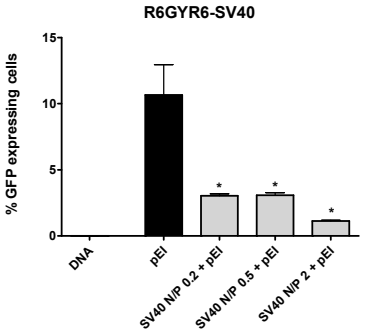
In vitro transfection efficiency of ternary complexes

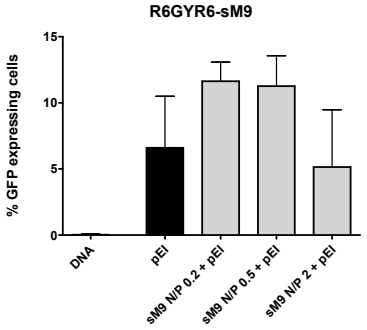
To test if peptides are able to enhance transfection efficiency of polyplexes or lipoplexes in which the DNA is better protected, ternary complexes were prepared and tested (see Figure 5 and 6). Ternary complexes were prepared by pre-incubating DNA with peptide prior to addition of 22 kDa l-pEI or lipofectamine. Total amount of DNA and of condensing agent were kept constant (pEI/DNA N/P=6; lipofectamine 2.5 µl/µg DNA), while the amount of peptide was varied (expressed as peptide/DNA N/P ratio).

As shown in Figure 5, R6GYR6 nor the NLS-containing peptides R6GYR6-SV40 and R6GYR6-sM9 gave a significant enhancement of pEI-mediated transfection. Transfection efficiency of pEI control formulations was between 3-10 %, which is quite low, but common for pEI-polyplexes prepared in low-salt buffers. Preparation in high-salt buffers leads to higher transfection, but under these conditions polyplexes are aggregated, which is undesirable for gene therapy purposes. Here, we wanted to investigate if efficiency of the small pEI/DNA complexes could be improved by incorporation of an active nuclear targeting strategy. Preparation of binary pEI/DNA complexes resulted in particles of approximately 80 nm with a positive zetapotential of ~25 mV. The physicochemical properties of the ternary DA/peptide/pEI complexes were similar to those of binary complexes (see Figure 5, table inset). One exception was the ternary formulation of R6GYR6 incorporated at a peptide/DNA N/P ratio of 2, which gave large aggregates.

Binary lipofectamine/DNA formualtions transfected ~ 2 % of cells. Lipofectamine was used at a ratio of 2.5 µl/µg DNA, which is the expected optimal ratio according to the manufacturer. Preparation of complexes in Optimem instead of HBG yields greatly increased transfection, but under these conditions large aggregates are formed (see Chapter 7). Pre-incubating DNA with peptides prior to complexation with lipofectamine in HBG greatly augmented transfection efficiency (see Figure 6). This effect was dependent on the type of peptide and of the amount of peptide used, with a maximal increase of approximately 10-fold observed for R6GYR6-SV40 incorporated at an N/P ratio of 1. However, the peptide-mediated increase was not NLS related, as



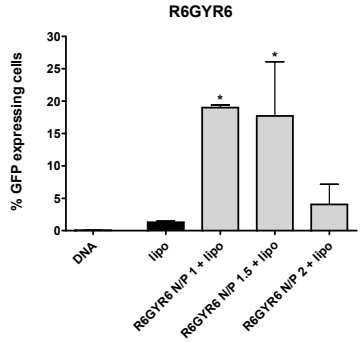


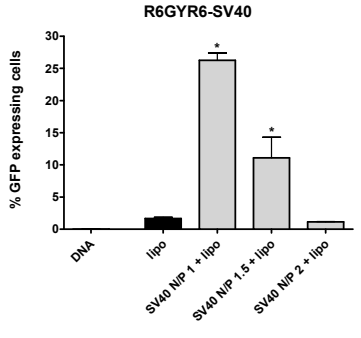


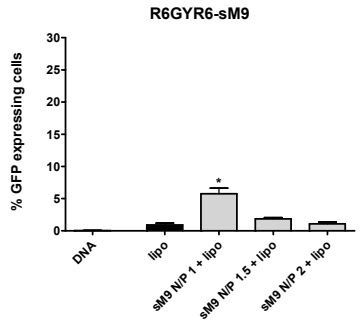
Peptide	N/P	Zave (nm)	PDI	ZP (mV)
-		79 ± 6	0.16 ± 0.03	25 ± 4
R6GYR6	0.2	68 ± 1	0.14 ± 0.01	21 ± 1
	0.5	71 ± 3	0.14 ± 0.02	23 ± 1
	2	*1050 ± 90*	0.1 ±0.1	30 ± 1
R6GYR6-SV40	0.2	67 ± 4	0.16 ± 0.01	22 ± 1
	0.5	73 ± 1	0.14 ± 0.01	21 ± 3
	2	99 ± 1	0.10 ± 0.02	28 ± 1
R6GYR6-sM9	0.2	65 ± 1	0.21 ± 0.01	21 ± 3
	0.5	66 ± 1	0.22 ± 0.02	23.8 ± 0.5
	2	95 ± 6	0.22 ± 0.03	27.2 ± 0.3

Figure 5. *Transfection efficiency and characterization of ternary pEI-based complexes. COS-7 cells were incubated with ternary particles for 4 h in the presence of serum and analyzed for EGFP expression by flow cytometry 48h after transfection. Ternary complexes were prepared in HBG as described in the materials and methods section. Control formulations were prepared as binary complexes by incubating plasmid with l-pEI (N/P=6) for 30 minutes. Data are presented as mean+SD of three independent experiments measured in triplicate. * indicates formulations that differed significantly from binary pEI formulations (p<0.05). Inset: characterization of control formulation and ternary complexes by DLS and zetasizer. Data are presented as mean±SD of triplicates and aggregates that can not be accurately sized by DLS are presented in italic.*

incorporation of the DBD peptide R6GYR6 also enhanced lipofectamine-based transfection. When looking at the characterization data (Figure 6, table inset), it is seen that the physicochemical properties of lipofectamine-based complexes were substantially altered upon incorporation of peptides, both with regard to size and charge. Binary lipofectamine/DNA complexes were observed to have a highly negative zetapotential of ~-40 mV. This negative charge is an uncommon property for a gene delivery complex, but has previously been reported for similar lipofectamine/DNA complexes elsewhere[37-39]. Pre-incubation of DNA with peptides was observed to invert zetapotentials to positive values, which would partially explain the increased transfection activity of the ternary complexes. A second parameter of interest is the size of the complexes. The size of lipofectamine/DNA complexes was approximately 135 nm but proved highly sensitive to pre-incubation with peptides. Mixing DNA with R6GYR6 prior to condensation with lipofectamine







Peptide	N/P	Zave (nm)	PDI	ZP (mV)
-		134 ± 1	0.14 ± 0.02	-41 ± 2
R6GYR6	1	*1670 ± 50*	1	13.0 ± 0.5
	1.5	*800 ± 100*	0.3 ± 0.3	19 ± 1
	2	*890 ± 40*	0.33 ± 0.04	20 ± 1
R6GYR6-SV40	1	*1800 ± 200*	0.6 ± 0.3	24 ± 1
	1.5	180 ± 10	0.09 ± 0.01	26 ± 1
	2	135 ± 4	0.13 ± 0.02	25 ± 1
R6GYR6-sM9	1	175 ± 7	0.21 ± 0.03	20.8 ± 0.4
	1.5	400 ± 200	0.5 ± 0.2	20 ± 2
	2	100 ± 4	0.22 ± 0.01	33 ± 1

Figure 6. *Transfection efficiency and characterization of ternary lipofectamine-based complexes. COS-7 cells were incubated with ternary particles for 4 h in the presence of serum and analyzed for EGFP expression by flow cytometry 48 h after transfection. Ternary complexes were prepared in HBG as described in the materials and methods section. Control formulations were prepared as binary complexes by incubating plasmid with lipofectamine (2.5 µl/µg DNA) for 30 minutes. Data are presented as mean+SD of three independent experiments measured in triplicate. * indicates formulations that differed significantly from binary pEI formulations (p<0.05). Inset: characterization of control formulation and ternary complexes by DLS and zetasizer. Data are presented as mean±SD of triplicates and aggregates that can not be accurately sized by DLS are presented in italic.*

resulted in complexes exceeding 800 nm. For R6GYR6-SV40 and R6GYR6-sM9, particle sizes were also affected, depending on the peptide:DNA ratio used. The increased transfection activity of part of the ternary DNA/peptide/lipofectamine formulations most likely reflects their altered physicochemical properties rather than specific NLS-mediated improvement.

Peptides in ternary complexes do not mediate active nuclear uptake

To examine if improved active nuclear uptake did play a role in the observed enhancement of transfection efficiency for the ternary lipofectamine-based formulations, transfection efficiency was studied in non-dividing cells. As a model for non-dividing cells, COS-7 cells were arrested in S-phase by continuous treatment with aphidicolin from 24 h prior to transfection until time of

analysis. No transgene expression was observed for any of the formulations tested (data not shown). The ability of the peptides to deliver plasmid DNA into cell nuclei was further evaluated by microscopic analysis of cells that were transfected with ternary and binary (control) complexes prepared with Cy-5 labeled plasmid DNA. Cells were not treated with aphidicolin, but analyzed after a short incubation of 4 h to minimize effects of cell division. The micrographs (Figure 7 A-D) show that lipofectamin-based complexes were present as large aggregates, corresponding to the characterization data. DNA appeared to be largely present bound to the outside of the cells, and internalized DNA was primarily localized inside the cytosol. In comparison to binary lipofectamine/DNA complexes, ternary peptide-containing complexes appeared to deliver more DNA, but no relevant differences in intracellular distribution between control and peptide-containing formulations was seen. For comparison, micrographs of cells transfected with non-aggregated pEI-based particles are shown (Figure 7 E and F). Again, no difference in localization pattern was observed between control pEI/DNA transfected cells and cells transfected with ternary complexes containing an NLS-peptide.

Conclusion an discussion

In this study the potential of bi-functional peptides consisting of a DNA Binding Domain and an NLS for improving efficiency of non-viral gene delivery was investigated. A strategy of non-covalent coupling of NLS was chosen to prevent transcriptional interference associated with covalent coupling strategies. The peptides were shown to bind to DNA and to display dissociation half life values of 11-32 minutes under *in vitro* conditions. Although the required time for an NLS to reach the nucleus once inside a cell has never been determined precisely, the general thought is that most NLS peptides are localized inside the nucleus within 30 and 120 minutes [40]. The observed half-life values of 30 minutes for DBD-NLS peptides are therefore considered to be in a favorable range: since plasmids will be covered with multiple peptides, NLS-peptides will remain present throughout the 30-120 minutes of intracellular trafficking and eventual dissociation of peptides from DNA will allow transcription.

Mixing of plasmid DNA with R6GYR6, R6GYR6-SV40 and R6GYR6-sM9 resulted in formation of submicron particles. Previously it was reported that compaction of DNA into stable microparticulate structures by oligopeptides suitable for gene therapy requires a minimum chain length of 6-10 cationic amino acids [36]. Here, the 14 amino acid DBD-peptide was able to form particles of around 400 nm, but could not confer positive charge to the complex, probably because the relatively short length was insufficient for efficient condensation. Both DBD-NLS peptides proved capable of condensing plasmids into ~100 nm particles with a positive charge of ~20 mV at low peptide/DNA ratios. Despite these favorable characteristics, poor transgene expression was obtained upon incubation of any of the complexes with COS-7 cells, regardless of stimulation of endosomal escape with chloroquine.

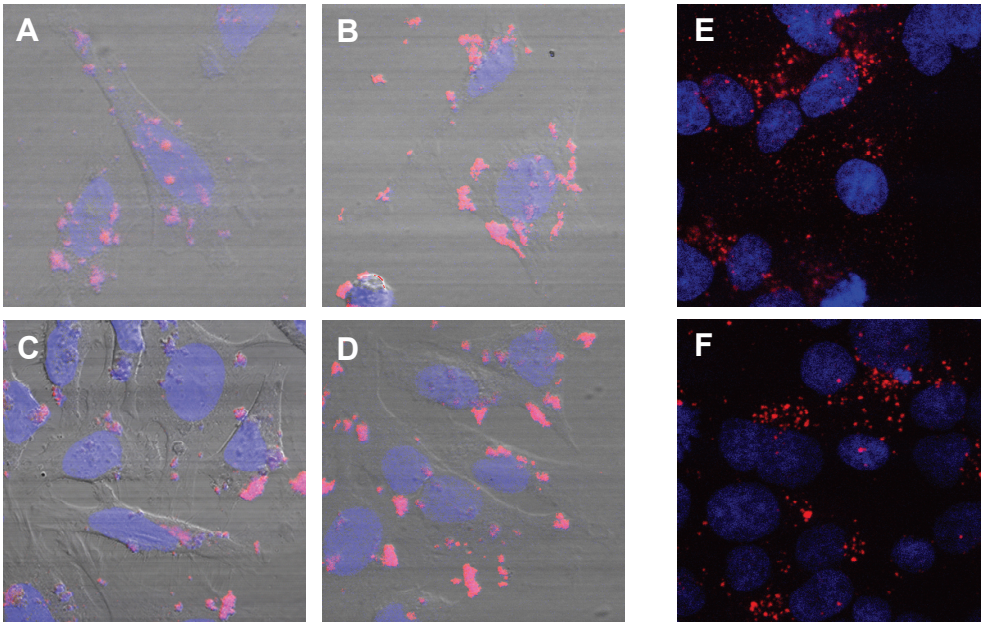


Figure 7. *Localization of plasmid DNA after transfection of ternary lipofectamine-based (A-D) and pEI-based (E-F) particles. Ternary complexes were prepared with Cy5-labeled plasmid (red) and the indicated peptide at an N/P ratio of 1 as described in the materials and methods section. After 4h incubation of cells with particles in the presence of serum, cells were washed, fixed, stained with DAPI nuclear stain (blue) and analyzed by multiphoton/confocal microscopy. A-D: A:control lipofectamine/DNA; B: ternary complexes prepared with R6GYR6; C: ternary complexes prepared with R6GYR6-SV40; D: ternary complexes prepared with R6GYR6-sM9. E-F: E: control pEI/DNA; F: ternary complexes prepared with R6GYR6-SV40. (HeLa cells were used for better imaging results).*

Transfection with similar particles has been examined by others, with varying outcomes. Duvshani-Eshet *et al.* [29] tested the ability of bi-functional peptides consisting of a cell-penetrating peptide (CPP) domain and the SV40 NLS to improve transfection. Peptides resulted in a peptide/DNA ratio dependent increase in transgene expression in comparison to naked DNA, but in this setup expression levels did not exceed 10^3 RLU/mg protein, which was at least 100-fold lower than values obtained with standard pEI or cationic liposomes. Trabulo *et al.* [33] used the same construct and report preparation of binary complexes 1,900-2,900 nm in size that were shown to transfect up to 50 % of treated cells (in absence of serum). Rudolph *et al.* [32] used dimers, trimers or tetramers of Tat to condense pDNA into ~700 nm complexes and observed successful transfection which could be further augmented upon incubation with chloroquine (all in absence of serum). In contrast, Collins *et al.* [41] report absence of gene expression after transfection with binary complexes of pDNA and the SV40 NLS or even protamine (tested in absence of serum). Additionally, Bremner *et al.* [17] found that a peptide based on human T cell leukaemia virus type 1

(HTLV), but not an SV40 NLS, an extended SV40 NLS, nor M9 peptide were able to induce gene expression, regardless of chloroquine treatment.

The discrepancy in these results may be related to the different characteristics of the peptides. Possibly, the cell penetrating domain of the peptides used by Duvshani, Trabulo and Rudolph is crucial in achieving efficient gene expression. It should however also be mentioned that these studies used fairly large complexes and were performed in the absence of serum, both factors being known to facilitate *in vitro* transfection. Binary particles described in this chapter were small and were tested in the presence of serum, which is expected to more closely mimic the *in vivo* situation.

Our next effort was to test if DBD-NLS peptides could enhance transfection efficiency of common polymer- or lipid-based formulations. Preparation of ternary DNA/peptide/pEI complexes gave positively charged particles of approximately 80 nm, but incorporation of peptides had no beneficial effect over control DNA/pEI formulations with regard to transgene expression levels or intracellular localization of plasmid DNA. This lack of NLS-effect could be due to intracellular dissociation of the peptide and/or of pEI, a process which was not addressed in this study and requires further investigation. Lipofectamine-based complexes proved highly sensitive to the type and amount of peptide incorporated. Under certain conditions transfection activity was substantially increased, but these improvements appeared to be related to changes in physicochemical properties rather than to specific effects of the peptides. Absence of a specific NLS-effect for these formulations was further confirmed by the inability of the peptides to mediate transfection in non-dividing cells, which suggest that the effect is not at the level of active nuclear import. Additionally, microscopy studies revealed increased cellular uptake, but no nuclear accumulation induced by the peptides.

The strategy to combine bi-functional NLS-containing peptides and polymers or, in most cases, lipids was also explored by others. In agreement with our findings, several of these studies conclude that the NLS-domain had no added value. Increases in transfection efficiency were attributed to functionalities of the other domains of the peptides or to altered physicochemical properties [20,26,33].

In contrast, several other studies do claim an NLS-effect for ternary (or quaternary) formulations. Incorporation of Tat-peptides in polymer- and lipid-based complexes was shown to improve transfection efficiency in dividing cells up to 100-fold compared to binary polymer/DNA or lipid/DNA formulations [32]. Based on the finding that efficiency was still improved 40-fold in aphidicolin-arrested cells the authors conclude that Tat facilitated active nuclear uptake. However, the fact that the contribution of the peptides is larger in dividing than in non-dividing cells does not support this conclusion. Moreover, no direct proof of enhanced nuclear localization is supplied. As data regarding physicochemical properties of the ternary complexes are lacking, it can not be excluded that the observed effects are related to physicochemical properties. Collins *et al.* [41] studied the effect of SV40, a mutant version of SV40 (mSV40) and protamine on lipofection of

dividing and non-dividing cells. Results obtained in serum-starved MCF7 cells showed decreased transfection levels for control formulations and formulations containing mSV40 or protamine, whereas transfection levels of SV40 NLS-containing formulations remained constant in dividing and serum-starved cells. This provides indirect proof of NLS-mediated nuclear localization of pDNA. Direct proof of NLS peptide-mediated nuclear localization was shown by Subramanian *et al.* [19]. A bi-functional peptide consisting of a cationic DBD and the non-classical NLS M9 was mixed with fluorescently labeled pDNA and shown to localize the plasmid into the nucleus of digitonin-permeabilized cells. Transfection efficiency of lipid/DNA formulations was increased upon incorporation of the peptide construct, but localization of ternary complexes was not studied.

Conclusion

Based on the preliminary findings described in this chapter, there is no indication that non-covalent incorporation of NLS-peptides enhances transfection efficiency through specific NLS-mediated nuclear delivery of DNA. At the most, these peptides in ternary complexes may prove valuable for increasing transfection efficiency through improved physicochemical properties such as size, charge and strength of condensation (related to availability of DNA for transcription). Similarly to what is presented in this manuscript, many studies report unspecific enhancement of transfection unrelated to NLS effects. These enhancements are attributed to characteristics of other domains within constructs tested (such as cell penetrating peptides and condensing agents [26,33]) or changes in physicochemical properties induced by incorporation of peptides in ternary complexes (as reported here and by others [20]). Those studies that do report successful NLS-mediated enhancements upon electrostatic coupling mostly lack convincing direct proof and/or do not exclude the alternative effects described above. Additional research is therefore required to distinguish these effects from specific NLS-mediated nuclear delivery. Future investigations should be directed at obtaining direct proof of NLS-effects based on quantification of nuclear uptake and assessment of interaction with importin receptors to support indirect proof from transfection readouts.

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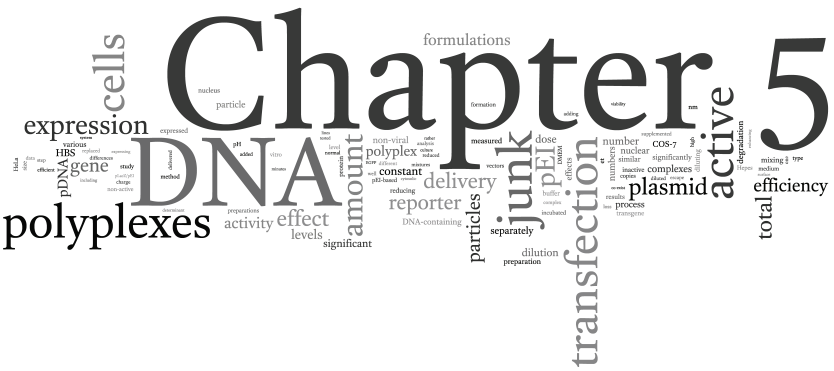

Junk DNA enhances pEI-based non-viral gene delivery

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Abstract

Gene therapy aims at delivering exogenous DNA into the nuclei of target cells to establish expression of a therapeutic protein. Non-viral gene delivery is examined as a safer alternative to viral approaches, but is presently characterized by a low efficiency. In the past years several non-viral delivery strategies have been developed, including cationic polymer-based delivery. One of the most described and most active polymers is linear pEI. This study addresses questions regarding formulating highly efficient pEI based polyplexes. By mixing reporter plasmid DNA with non-coding junk DNA it was shown that the amount of reporter plasmid can be significantly decreased in linear pEI-based transfection while maintaining transfer activity. Junk DNA maximally exerts its function when co-delivered with active DNA within the same pEI-complexes rather than co-delivery of distinct junk DNA/pEI and active DNA/pEI complexes. We conclude that not the total amount of active DNA, but rather the total amount of active DNA-containing particles is the limiting factor in pEI-mediated transfection.

Introduction

Gene therapy requires introducing exogenous DNA into the nuclei of target cells to express a therapeutic protein with the aim of treating, or ultimately curing a disease. Although this concept was first proposed already in 1972 [1], practical applications remain few due, among other reasons, to inefficiency of delivery.

Viral gene delivery is still regarded as the most efficient [2]. Viruses can be considered as natural gene delivery vectors that have evolved to be extremely efficient in targeting and infecting cells, and having their genome expressed or even reproduced. On the contrary, evolution of non-viral vectors has only just begun. Research performed in the last decades has resulted in identification of several important barriers and requirements in the process of gene delivery, including: (1) protection of DNA against degradation and circumvention of protein adsorption onto particles, (2) interaction with cell membrane and cellular uptake, (3) translocation from vesicular structures to cytosol, thereby escaping lysosomal degradation, (4) cytosolic trafficking and protection against degradation by cytosolic nucleases, (5) nuclear import, (6) transcription and (7) translation (see Figure 1). Inefficiency at each of these individual steps may greatly impair the overall transfection activity and therefore must be evaluated and optimized in order to make non-viral gene delivery vectors an alternative for viruses. Nevertheless, attention for the less efficient non-viral carrier systems is justified as they offer biocompatibility, ease of production and scaling up, and they can be tailored to specific requirements based on the selected application.

Over the past years, many efforts have been made to develop non-viral delivery strategies, including lipid-, polymer- and peptide- or protein-based carrier systems (for review refer to [3]). Among the best characterized and most frequently used polymeric carriers are the polyethylenimines (pEI), which are generally considered as a golden standard for non-viral gene transfection. pEI-based transfection was first described by Boussif *et al.* [4] and since then numerous efforts have been made to gain insights into the mechanism of action [5-18] and to further optimize this polymer [19-24].

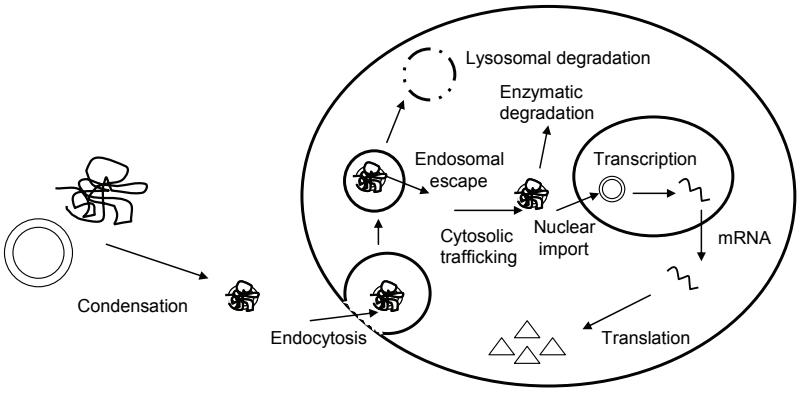


Figure 1. *Gene delivery is a multistep process.*

Recently, research was performed to identify the cause of difference in efficiency between viral vectors and non-viral formulations. It appears that viral vectors are more efficient in establishing gene expression despite delivering lower copy numbers of DNA. Adenovirus was described to deliver approximately 3 orders of magnitude less plasmid copies into the nuclei of dividing cells than 25 kDa pEI whereas gene expression was 2 orders of magnitude higher [25]. Similarly, it was reported that lipofectamine requires 3-4 orders of magnitude more plasmid copies to be delivered in dividing cells to achieve similar transfection activity as adenovirus [26,27].

In this study we chose linear 22 kDa pEI as a model system to study transfection efficiency (based on gene expression levels) and the discrepancy between the extremely high numbers of plasmids that need to be delivered in comparison to viral delivery to obtain gene expression. We present a method to increase the efficiency of active plasmid delivery by incorporating inactive junk DNA as carrier material. The dose of active DNA could be decreased 16-fold while transfection levels maintained high (27 % of the original levels). By comparing preparations in which active and junk DNA are mixed prior to complexation with mixtures of separately prepared particles containing either active or junk DNA we show for the first time that junk DNA maximally exerts its function when incorporated within the active complexes. This finding stresses the importance of particle composition and opens the way to rationally design multi-component particles.

Materials and methods

Material

Linear poly(ethyleneimine) (PEI), ExGen 500, was purchased from MBI Fermentas (St Leon-Rot, Germany). Plasmids pCMV/LacZ was purchased from the Plasmid Factory (Bielefeld, Germany). Plasmid pCMV/EGFP was constructed from pShooter (Invitrogen, Breda, The Netherlands): the EGFP coding sequence from pEGFP-N1 (bp 613-1410; Clontech, Saint-Germain-en-Laye, France) was amplified by PCR and cloned into the multiple cloning site of pShooter from which the nuclear localization signal (NLS)-tag coding sequence was removed by restriction with NotI and XbaI. pCMV/LacZ and pCMV/EGFP are expression plasmids encoding for β-galactosidase and enhanced green fluorescent protein (EGFP), respectively, under the transcriptional control of the human cytomegalovirus promoter (CMV). pUC18/19 plasmids were obtained from Invitrogen (Breda, The Netherlands). pUC18/19 vectors are small, high copy number, *E.coli* plasmids that do not encode reporter genes. Other chemicals used: Hepes (99 %) and magnesium chloride, $MgCl_2.6H_2O$ (Acros Organics BVBA, Geel, Belgium); *o*-nitrophenyl-*ß-D*-galactopyranoside (ONPG), ß-galactosidase, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) and N-methyl dibenzopyrazine methylsulfate (PMS) (Sigma, Zwijndrecht, The Netherlands); Tris(hydroxymethyl)-aminomethane (Tris) and sodium chloride (Merck, Darmstadt, Germany); Triton X-100 (BDH, England); RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria);

phosphate buffered saline (PBS) (B. Braun Melsungen AG, Melsungen); Fetal Bovine Serum (FBS; Integro, Zaandam, The Netherlands), Trypsin/EDTA 10x, Plain DMEM (Dulbecco's modification of Eagle's medium, with 3.7 g/l sodium bicarbonate, 1 g/l L-glucose, L-Glutamine) and antibiotics/antimycotics (penicillin, streptomycin sulphate, amphotericin B; PAA Laboratories GmbH, Pasching, Austria), propidium iodide (PI) (Invitrogen, Breda, The Netherlands).

COS-7 African Green monkey kidney cells were provided by Prof. J.C. Clevers (Department of Immunology, Academic Hospital Utrecht, The Netherlands). The original cell line originates from American Type Culture Collection (ATCC, CRL 1651, USA). HeLa cells were a generous gift from Rob Roovers (Department of Cellular Architecture and Dynamics, Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands).

Preparation of the complexes

Polyplexes were prepared by adding 4 volumes of polymer solution to 1 volume of a 50 µg/ml DNA-solution (both diluted in hepes-buffered saline: 20 mM Hepes, 150 mM NaCl; pH 7.4 (HBS)), mixing by pipetting and incubating 30 minutes at room temperature. Total volume of polyplex dispersion and N/P ratio are kept constant (N/P=6 for Exgen) for all formulations. The amount of reporter DNA is varied from 100-0 % while total DNA is kept constant by adding junk DNA. Reporter and junk DNA are either mixed prior to polyplex formation (pReporter+pJunk/pEI) or after preparing separate polyplexes (pReporter/pEI + pJunk/pEI). As a control, pReporter/pEI polyplexes are diluted with HBS to contain equivalent amounts of pReporter per volume as the reporter/junk DNA mixtures (also see Figure 2).

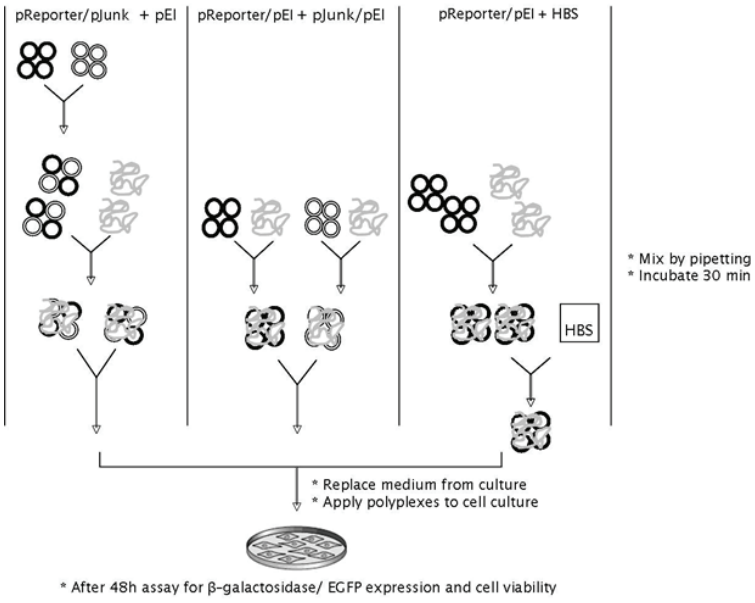


Figure 2. *Schematic representation of polyplex preparation and transfection.*

Characterization of the complexes

Particle size of the polyplexes was measured in 20 mM Hepes, pH 7.4 (viscosity 0.89 cP, refractive index 1.333) with Dynamic Light Scattering (DLS) on an ALV CGS-3 system (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22mW He-Ne laser operating at 632.8nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water bath) set at 25 °C. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles (*Z*h) and the particle size distribution (polydispersity index, PDI) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by the cumulants method and the diffusion coefficients calculated from the measured autocorrelation functions were related to the hydrodynamic radius of the particles via the Stokes-Einstein equation, $Z_h = (k_B T q2)/(3\pi\eta\Gamma)$, where Z_h is the hydrodynamic radius of the particles, k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, Γ is the decay rate, and q is the scattering vector ($q= 4\pi n \sin(\Phi/2))/ \lambda$), in which n is the refractive index of the solution, Φ is the scattering angle, and λ is the wavelength of the incident laser light. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle. The system was calibrated with 200 nm polystyrene latex standard beads (Duke Scientific, Leusden, The Netherlands).

The surface charge of the polyplexes was measured in 20 mM Hepes, pH 7.4 (viscosity 0.89 cP, dielectric constant 79) using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) with universal ZEN 1002 'dip' cells and DTS (Nano) software (version 4.20). The Helmholtz-Smoluchowski equation was used for converting electrophoretic mobilities into zeta potentials. The system was calibrated with DTS 1050 latex beads (Zeta Potential Transfer Standard, Malvern Instruments, Malvern, UK).

Cell culture and in vitro transfection

COS-7 African Green monkey cells and HeLa cells were grown in DMEM supplemented with antibiotics/antimycotics and 5 % and 10 % heat-inactivated fetal bovine serum, respectively. Cells were maintained at 37 °C in a 5 % CO_2 humidified air atmosphere and split twice weekly.

For ONPG and XTT assays, 10,000 (COS-7) or 6,000 (HeLa) cells were seeded per well into 96-well tissue culture plates 24 hours prior to transfection, such that 60–70 % confluency was reached on the day of transfection. Immediately prior to transfection the culture medium was refreshed with 100 µl DMEM medium, supplemented with twice the normal % of FCS. 100 µl of the polyplex samples (corresponding to 1 µg DNA/well) was added per well and after 1 h incubation, medium was replaced with fresh DMEM supplemented with the normal % of FCS.

For flow cytometry analysis, 40,000 (COS-7) or 30,000 (HeLa) cells were seeded per well into 24-well tissue culture plates 24 hours prior to transfection, such that 60–70 % confluency was reached on the day of transfection. Immediately prior to transfection the culture medium was refreshed with 500 µl DMEM medium, supplemented with twice the normal % of FCS. 500 µl of the polyplex

samples (corresponding to 5 µg DNA/well) was added per well and after 1 h incubation, medium was replaced with fresh DMEM supplemented with the normal % of FCS.

Cells were incubated another 48 h at 37 °C in a 5 % CO_2 humidified air atmosphere until analysis. Experiments were performed in triplicate.

ONPG-assay

48 h after transfection, cells were washed 1x with 100 µl icecold PBS and lysed with 20 µl lysisbuffer (50 mM Tris/HCl buffer (pH 8.0), 150 mM NaCl and 1 % Triton X-100) during 20 minutes at 4 °C. Next, 180 µl ONPG-staining solution (18.5 ml PBS, 200 µl 0.1 M $MgCl_2$-solution and 1.35 ml 10 mg/ml ONPG-solution in PBS) was added and enzyme activity of ß-galactosidase was determined by deriving the kinetic velocity of ONPG-conversion by measuring absorbance at 415/490 nm at time intervals of 1 minute during 40 minutes.

XTT-assay

Cell viability was determined using an XTT colorimetric assay based on cleavage of a tetrazolium reagent to form an orange formazan dye, which is indicative for metabolic activity. 48 h after transfection, 50 µl XTT-solution (25 µM PMS and 1 mg/ml XTT in plain RPMI 1640) was added per well and incubated for 1 h at 37°C in a CO_2-incubator. Absorbance was measured at 490 nm with a reference wavelength of 655 nm. Cell viability was expressed as the relative metabolic activity normalized against HBS-treated cells.

Flow cytometry

48 h after transfection, cells were washed, trypsinized and resuspended in 500 µl DMEM supplemented with 5 % FCS to inactivate the trypsine. Cells were transferred into FACS-tubes and centrifuged for 5 minutes at 250 g at 4 °C. Medium was removed and cells were resuspended in 300 µl phosphate-buffered albumine (PBA; 1 % w/v albumine in PBS). Immediately prior to measurement, 20 µl propidium iodide solution (PI; 1 µg/ml in water) was added for live/dead cell discrimination. Flow cytometric analysis was performed on a FACScalibur (Becton and Dickinson, Mountain View, CA, USA) using a 15 mW 488 nm, air-cooled argon-ion laser and data were analyzed using Summit® software (DakoCytomation, Fort Collins, CO, USA). 10,000 cells were recorded per sample to determine EGFP-expression (FL1-channel) and PI-staining (FL3-channel).

Experimental setup and statistical analyses

On each experimental day, three independent plasmid/pEI preparations were made and tested on the same batch of cells. Experiments were repeated at least on two different days. For the statistical analyses, we considered the results obtained for each polyplex preparation as an independent sample. Expression levels obtained were normalized against those obtained with polyplexes containing 100 % active plasmid DNA. First, the expression data were analyzed by two-way ANOVA with type of preparation and concentration as fixed factors. Subsequently one-way

ANOVA followed by post-hoc analyses with Bonferroni correction was performed on the expression data of each polyplex dilution separately. A similar statistical analysis was done on the viability data. A p-value <0.05 was considered significant.

Results

Formulations containing junk DNA are physicochemically equivalent to normal pDNA polyplexes

Mean diameters and zeta potentials of polyplexes prepared with either reporter pDNA or junk pDNA or a mixture of both were measured (Table 1) to evaluate whether physicochemical properties of the various formulations are equivalent. Particles were prepared at an N/P ratio of 6, which was earlier found to be optimal for pEI-based transfections (data not shown). Complexes prepared in low-salt buffer (20 mM Hepes, pH 7.4) were approximately 70 nm in diameter with a positive surface charge of approximately 25 mV, which remained stable up to 4 h after preparation. No significant differences were observed between size and charge of the various formulations tested. When complexes were prepared in high-salt buffer (20 mM Hepes, 150 mM NaCl, pH 7.4), the size increased substantially in time, reaching >500 nm 30 minutes after preparation (data not shown). The effect of salt on pEI-based polyplexes is a well-known phenomenon and the size increase accounts for increased transfection activity *in vitro* [17].

Table 1. *Size, polydispersity and surface charge (zeta potential) of polyplex formulations (n=3)*[a]

Formulation	Z_{ave} (nm)	PDI	Zeta potential (mV)
pCMV-LacZ/pEI	65 ± 1	0.18 ± 0.02	26 ± 2
pUC/pEI	72 ± 2	0.36 ± 0.07	25 ± 3
(pCMV-LacZ+pUC)/pEI	69 ± 2	0.24 ± 0.03	24 ± 6

[a] *low salt buffer, 20 mM Hepes, pH 7.4*

Incorporation of junk DNA into polyplexes increases efficiency of transfection

First, a dose-response curve was measured by adding a range of dilutions of polyplexes in HBS (pLacZ/pEI + HBS) to COS-7 cells and measuring expression levels (Figure 3 A, black bars). When decreasing the total amount of polyplexes incubated with the cells, transfection efficiency drops more than proportionally: at 25 % of the initial dose, only approximately 10 % of the expression level is achieved.

Another method to vary the amount of reporter pDNA incubated with the cells is to dilute it with junk DNA prior to complex formation (pLacZ/pUC + pEI). Using this method allows keeping the total amount of polyplexes, dose of pEI and total amount of DNA incubated with the cells constant.

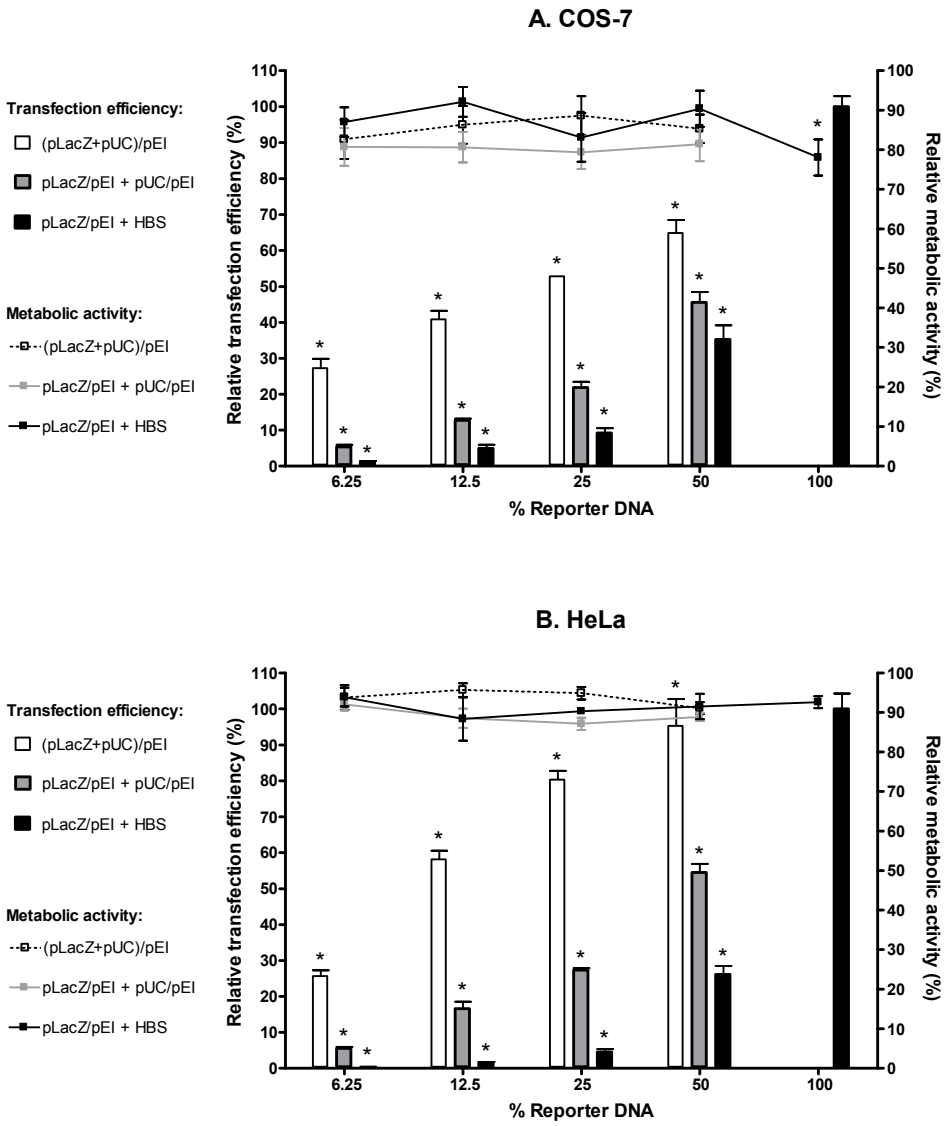


Figure 3. *In vitro β-galactosidase expression (bars) and cell viability (lines) after transfection of COS-7 cells (A) and HeLa cells (B) with pDNA/pEI polyplexes. Reporter DNA dose was varied by diluting polyplexes in HBS (black bars), by diluting reporter pDNA with junk pDNA prior to complex formation (white bars) or by mixing reporter pDNA polyplexes with junk pDNA polyplexes (grey bars). In the latter 2 cases, total DNA was kept constant at 1 µg/well. Transfection efficiency is expressed relative to the 100 % point. Values are expressed as mean + SEM. Differences between the three polyplex preparations are significant (A: $F(2,162) = 199.028$, $p<0.000$ (n=15); B: $F(2,24)=470.558$, $p<0.000$ (n=3)). Importantly, the interaction between the type of polyplex preparation and the dilution was significant (A: $F(6,162) = 3.186$, $p<0.01$; B: $F(6,24) = 18.348$, $p<0.000$), indicating different behavior of the various formulations upon dilution. Differences between the three preparations are significant for all dilutions in both cell lines (indicated with * in graphs; $p<0.05$).*

When this method was applied, a remarkable effect on transfection efficiency was observed (Figure 3 A, white bars). The amount of active DNA could be reduced to 6.25 % while maintaining transfection efficiency at 27 % of the original value. For comparison, reducing the amount of polyplexes to 6.25 % in HBS resulted in complete loss of transfection activity.

Similar findings were made by Kichler *et al.* [28], who hypothesize that the observed effect of junk DNA is related to endosomal escape. Based on the fact that adding junk DNA allows decreasing the amount of active DNA, while keeping the amount of pEI constant, they conclude that the total amount of pEI is the determinant. A certain threshold amount of pEI would be required for its proton sponge activity [4] leading to endosomal escape.

To test this hypothesis, we evaluated a third method to vary the amount of reporter DNA in which active DNA -containing polyplexes were diluted with junk DNA-containing polyplexes (pLacZ/pEI + pUC/pEI; Figure 3 A, grey bars). Like for the previous method, the total amount of polyplexes, dose of pEI and total DNA are kept constant but in this case part of the polyplexes are inactive. Size and charge are similar to the earlier used dispersions (Table 1). Dilution of reporter pDNA-polyplexes with separately prepared junk pDNA particles results in a loss of transfection activity that is intermediate between the previous two methods. The effect of separately added junk DNA particles is considerably less than the effect of junk DNA present within active particles, which indicates that the total amount of pEI incubated with the cells is not the major determinant. Rather, it means that not the total amount of active DNA, nor the total amount of pEI, but the total number of active DNA-containing particles is essential.

Similar effects of dilution in HBS or with junk DNA were observed in HeLa cells (Figure 3 B). In this cell type a saturation level could be reached by mixing reporter plasmid with junk plasmid prior to complex formation.

Toxicity of the formulations was also assessed by measuring metabolic activity based on the XTT-assay (Figure 3, line graphs). All formulations caused a loss in metabolic activity of approximately 10-20 %, with the highest toxicity observed at the highest dose of polyplexes (significantly different from lower doses, $p<0.05$). Two-way ANOVA followed by post-hoc analysis with Bonferroni correction revealed that toxicity of polyplexes in which active and junk DNA co-exist does not differ significantly from that of mixtures of separately prepared active and junk DNA-containing polyplexes. The absence of significant differences in toxicity observed for the various formulations with equal amounts of pEI excludes an effect of reduced cell numbers on transfection efficiencies.

Increased transfection efficiency is not related to a specific type of junk DNA

In the previous experiments, pUC18 or pUC19 plasmids were used as junk DNA. pUC plasmids are small prokaryotic plasmids (2686 bp) that do not contain any expression elements that are functional in mammalian cells. To test if the observed effects are related to the presence of these specific plasmids, experiments were repeated with a eukaryotic reporterless vector. For this

purpose, the empty pShooter plasmid (pCMV/myc/nuc, 5000 bp) was used which contains functional promoter and enhancer regions, but lacks a reporter gene coding sequence. As shown in Figure 4, incorporation of pShooter (pSho) gives similar effects as those obtained with the pUC vectors. Again, reducing the amount of active DNA was related to a non-proportional decrease in transfection efficiency when pShooter was co-delivered with pLacZ within the same complexes. In this case, transfection efficiencies resulting from co-delivery of separately prepared pLacZ/pEI complexes and pSho/pEI complexes did not differ significantly from pLacZ/pEI polyplexes diluted in HBS, except at 50 % reporter DNA. These results show that the observed effects are not related to specific sequences or size of the junk DNA, but rather are a non-specific effect.

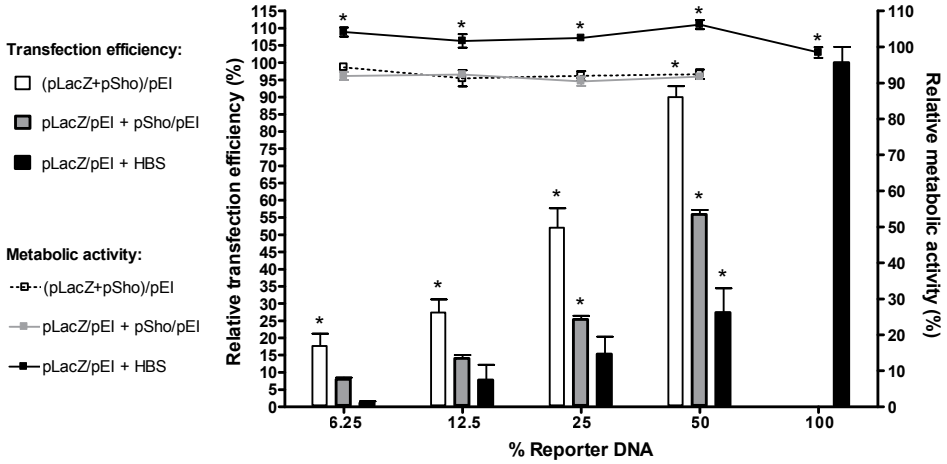
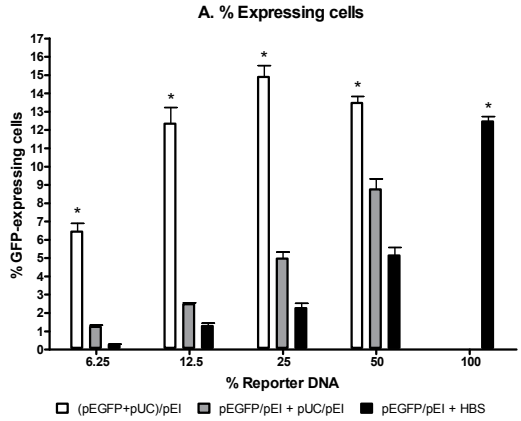


Figure 4. *In vitro β-galactosidase expression (bars) and cell viability (lines) after transfection of COS-7 cells with pDNA/pEI polyplexes. Reporter DNA dose was varied by diluting polyplexes in HBS (black bars), by diluting reporter pDNA with junk pDNA prior to complex formation (white bars) or by mixing reporter pDNA polyplexes with junk pDNA polyplexes (grey bars). In the latter 2 cases, total DNA was kept constant at 1 µg/well. Transfection efficiency is expressed relative to the 100 % point. Values are expressed as mean + SEM. Differences between the three polyplex preparations are significant ($F(2,58) = 81.666$, $p<0.000$ ($n=6$)). Importantly, the interaction between the type of polyplex preparation and the dilution was significant ($F(6,58) = 8.083$, $p<0.000$), indicating different behavior of the various formulations upon dilution. Differences between (pLacZ+pSho)/pEI and the other two formulations are significant at all dilutions; differences between (pLacZ/pEI + pSho/pEI) and (pLacZ + HBS) are only significant at 50 % reporter DNA (indicated with * in graphs; $p<0.05$).*

Junk DNA affects the number of expressing cells rather than expression per cell

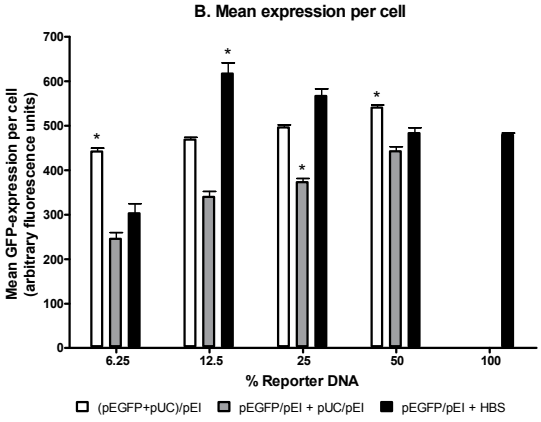
To discriminate between effects at the levels of expression per cell or of the number of expressing cells, we transfected COS-7 cells with polyplexes containing plasmids encoding for EGFP. Similar formulations were prepared as for the previous experiments: pEGFP/pEI polyplexes were diluted either in HBS or with pUC/pEI polyplexes, or pEGFP was first mixed with pUC and then

condensed with pEI. Transfection efficiency was measured with flow cytometry, allowing analysis at the single cell level. The results show that mixing of junk DNA with reporter DNA primarily affects the number of cells that express EGFP, whereas intracellular expression levels remain mostly unaffected (Figure 5). These results imply that reducing the number of active DNA-containing particles directly reduces the probability of successfully transfecting cells, whereas once a cell has been successfully transfected the amount of active DNA is not critical for the protein expression level in a cell. By diluting active DNA with junk DNA prior to complex formation, the amount of active DNA can be reduced without reducing the total number of DNA-containing particles resulting in both constant percentages of cells that can be successfully transfected and constant protein expression levels.



A: % EGFP-expressing cells:

*The three polyplex preparations differ significantly (F(2,65) = 84.501, p<0.000). Interaction between the type of polyplex preparation and the dilution was found to be significant F(6,65)= 2.470, p<0.05. Formulations indicated with a * in the graph differ significantly from the other groups (p<0.05).*



B: Mean expression:

*The three polyplex preparations differ significantly F(2,65) = 30.796, p<0.000). Interaction between the type of polyplex preparation and the dilution was found to be significant F(6,65)= 5.974, p<0.000. Formulations indicated with a * in the graph differ significantly from the other groups (p<0.05).*

Figure 5. *In vitro EGFP expression after transfection of COS-7 cells with pDNA/pEI polyplexes. Reporter DNA dose was varied by diluting polyplexes in HBS (black bars), by diluting reporter pDNA with junk pDNA prior to complex formation (white bars) or by mixing reporter pDNA polyplexes with junk pDNA polyplexes (grey bars). In the latter 2 cases, total DNA was kept constant at 5 µg/well. Transfection efficiency is expressed relative to the 100 % point. Values are expressed as mean + SEM (n=6).*

Discussion

Under standard transfection conditions used to study non-viral gene delivery *in vitro*, extremely high numbers of plasmid copies are incubated with cells. In general, 0.2-1 µg DNA is used to transfect ~20,000 cells, which corresponds to roughly 10-50 pg plasmid, or 1.5-7.5·10^6 plasmid copies, per cell. For comparison, a human cell contains 7.1 pg DNA per cell [29]. Viral gene delivery requires less DNA to be delivered: adenovirus-based delivery achieves similar expression levels as lipofectamine or pEI at 3 orders of magnitude less gene copies delivered [25-27]. The aim of our study was to examine this disproportional relation between plasmid dose and transgene expression levels. We used linear pEI as a model system and studied the effect of plasmid dose, pEI dose and of the amount of particles. A steep decrease in transfection activity was observed by reducing the overall amount of polyplexes upon dilution in buffer. Strikingly, when keeping the number of polyplexes constant but varying the dose of active DNA by diluting it with inactive junk DNA prior to complexation, expression levels remained high. A decrease in active DNA from 1 µg to 0.0625 µg per well led to a complete loss in gene expression upon dilution of polyplexes in buffer, but active DNA could be diluted to 0.0625 µg with junk DNA while maintaining nearly 30 % of the original expression levels. We found this effect to be consistent for various reporter genes (pCMV/LacZ, pEGFP), junk DNA plasmids (pUC18/19, pShooter-GFP) and cell lines tested (COS-7, HeLa) and similar findings were also observed elsewhere [28,30-32]. Additionally, the use of junk DNA to enhance silencing effects in siRNA delivery has also been described [33,34]. The mechanism behind this effect however remains open to speculation. By choosing a rational experimental setup that allowed us to discriminate between effects related to junk DNA incorporated within active complexes or co-delivery of distinct junk DNA/pEI and active DNA/pEI complexes, we obtained new insights that significantly contribute to the exclusion of suggested hypotheses. To address the options that might explain the non-linearity in dose-response effects, all steps involved in gene delivery (Figure 1) must be considered.

Physicochemical properties of polyplexes

The first step consists of formulating the DNA of interest such that the DNA is condensed into a small, positively-charged stable particle in order to facilitate transport, protection against degradation, and interaction with the cell membrane of target cells. As the various formulations we tested in our study were equivalent regarding size and surface charge, it is unlikely that this step contributes to the effect of junk DNA.

Interaction with the cell membrane and cellular uptake

The second step involves interaction with the membrane and uptake into target cells. Polyanionic glycosamineglycans (GAGs) present at the cell surface have been reported to inhibit cation-mediated gene transfer [35]. This inhibition process could possibly become ‘saturated’ by adding excess polyplexes or polyplexes containing non-active junk DNA. However, in this case one

would not expect the differences in transfection efficiency between formulations containing active and junk DNA within one particle and those consisting of separate active and non-active DNA-containing particles that we observed. Additionally, a critical role of GAGs was excluded by Kichler *et al.* [28], who observed similar transfection effects in both normal and GAG-deficient mutant cells. Theoretically, the difference between formulations of separately prepared active and inactive particles and those in which active and non-active DNA co-exist within one particle could be due to competition at the level of cellular uptake. However, there is no reason to believe in preferential uptake of one particle over another otherwise physicochemically equivalent particle. As the amount of plasmids entering cells after non-viral transfection is roughly within the order of 10^4 [31,32], one would statistically expect to end up with similar amounts of active and non-active plasmid copies for both formulations.

Endosomal escape

The next step in the delivery process is the timely escape from endocytic vesicles before their maturation into lysosomes where degradation occurs. Endosomal escape is regarded as a critical factor for efficient gene delivery and the success of polyethylenimines is often ascribed to their endosome-buffering capacity that leads to osmotic swelling and consequential bursting of the endosome [4,6,28,36,37]. This so-called proton-sponge theory has been topic of elaborate investigation but remains under debate [5,12,38-40]. Several studies have described the beneficial effect of excess pEI on transfection activity. Removal of free pEI from pEI-based polyplex formulations results in decreased transfection efficiency (and toxicity) whereas addition of excess pEI to pEI- or polylysine-based transfection assays enhances transgene expression levels [6,37]. This led to the hypothesis that a certain threshold amount of pEI is essential for its proton sponge activity. This hypothesis was supported by their finding that dilution of polyplexes in buffer results in loss of transfection efficiency whereas the dose of active DNA could be reduced (by mixing it with non-active DNA) without losing transfection activity as long as the amount of pEI remained constant [28]. However, in our study we observe that when transfecting cells with mixtures of separately prepared polyplexes containing either active or non-active DNA transfection levels are considerably lower than after transfection with polyplexes in which active and inactive DNA are combined, despite the total amount of pEI being equal. This shows that the total amount of pEI is not the major determinant. It is recognized that transgene expression levels obtained upon transfection with mixtures of active polyplexes and inactive polyplexes are higher than those achieved after plain dilution of polyplexes in buffer. This might indicate that the amount of pEI plays a role, however this would still be inferior to the effect caused by co-existence of active and non-active DNA within single particles. A possible explanation could also be that exchange of plasmid occurs to some extent between the separately prepared complexes after mixing. Based on our findings we conclude that it is not the total amount of active plasmid DNA, nor the total amount of pEI, but the total number of active DNA-containing particles is the major determinant. Additional reasons to doubt the prominent role

of pEI-amount are reports of similar effects obtained with carriers without proton sponge activity[32] and of enhanced transgene expression of cytoplasmically microinjected reporter DNA upon co-injection of pUC DNA [41].

Cytosolic trafficking and nuclear import

The next important step after endosomal escape is cytosolic trafficking. Translocation of non-viral transfection systems from cytosol into the nucleus is a highly inefficient process, especially in non-dividing cells. Most *in vitro* transfection studies are performed by transfecting dividing cells and analyzing gene expression 24-48 hours post-transfection. During this incubation time, cells have undergone at least 1 (but more likely 2 or 3) cell cycle(s) including mitosis during which the nuclear envelope is disrupted. This enables plasmids or polyplexes to enter the nucleus and overshadows the difficulty of actively delivering particles into intact nuclei via the nuclear pores. Pollard *et al.* have studied transport of naked plasmid and pEI-complexed plasmids in the absence of cell division after cytosolic injection [42]. They observed that only 1 in 100 pEI-based polyplexes reaches the nucleus after cytosolic micro-injection, confirming the inefficiency of this transport process. Additionally, expression efficiency was studied upon nuclear injection of various plasmid numbers showing that a threshold of ~10^3 copies must be injected in order to achieve a 100 % chance of transfection. Decreasing the amount of injected DNA below this threshold results in a decrease in number of transgene expressing cells. Both of these findings could explain our finding that the total number of active DNA-containing particles, but not the total amount of DNA (per particle) is the major determinant in transfection efficiency in dividing cells.

Cytosolic degradation

Besides inefficient active nuclear translocation, degradation by nucleases present in the cytosol can be a limiting factor for gene delivery [43]. It is possible that the enhancing effect of co-delivering junk DNA with active DNA is related to competition of junk DNA with active DNA for nucleases or even a saturation of nucleases. This hypothesis could explain the observation that co-injection of pUC DNA with reporter DNA into the cytosol enhances reporter gene expression [41]. Nevertheless it seems unlikely that reduced degradation of active DNA is the key effect. First, this would not explain the differences observed for polyplexes in which active and inactive DNA co-exist and those prepared separately with either DNA. Second, the enhanced silencing effect of junk DNA for siRNA delivery could not be related to this process as RNA and DNA are degraded by different enzymes. Third, Kichler *et al*. showed that the junk DNA could also, to some extent, be replaced by another polyanion (polyglutamic acid) which is insensitive to DNases [28]. It should be mentioned that upon higher dilution factors of active DNA, the effect of polyglutamic does become less pronounced than for junk DNA, indicating that competition for degradation could be involved as a secondary process.

Intranuclear processing

Steps that must occur after nuclear delivery include trafficking of the DNA to transcriptionally active regions inside the nucleus, followed by transcription into mRNA and finally translation of mRNA into protein. These steps have long been undervalued, but have recently gained attention in the field [44-46]. Moriguchi *et al.* report that no significant differences are measured between nuclear plasmid numbers between formulations with or without junk DNA and ascribe the observed enhancing effect of junk DNA at post-nuclear events [32]. They hypothesize that plasmid DNA inside the nucleus could become unavailable for transcription due to binding by histones and condensation into heterochromatin, or due to methylation processes. The latter process could be saturated by increasing plasmid numbers in the nucleus, as is the case when using junk DNA. Carpentier *et al.* have studied nuclear plasmid numbers in sorted populations of transgene expressing and non-expressing cells. They found that despite copy numbers in expressing cells are 5-6 fold higher, still a substantial amount of plasmids can be found in non-expressing cells (550 versus 1,850 nuclear copy numbers per cell). This indicates that transgene expression efficiency is not only affected by delivery processes, but also to a great extent by transcriptional competency of the delivered plasmid [31]. Dose-response experiments show that (at the plasmid doses tested) no saturation of mRNA production occurred, whereas protein production was shown to decrease at high doses. Carpentier *et al.* therefore hypothesize that the enhancing effect of adding junk DNA is related to limitations at the level of translation.

Conclusion

To conclude, we and others have observed a substantial enhancing effect of co-delivering junk DNA on transgene expression or silencing upon plasmid DNA or siRNA delivery. This effect is consistent in many cell lines and for many different reporter constructs. The effect is observed for various carrier systems, including pEI and lipid-based carriers. The effect requires that the junk DNA is co-complexed with the DNA (or RNA) of interest within the same particles. The effect is also observed upon replacing junk DNA with another polyanion: polyglutamic acid.

Many explanations and hypotheses have been proposed and have been discussed here. We believe that an effect of the total amount of pEI can be excluded based on our findings that transfection activity of mixtures of separately prepared polyplexes containing either active or inactive DNA is inferior to that of polyplexes in which the two plasmids co-exist, whereas the total amount of pEI is equal. Additionally, the fact that junk DNA can be replaced by polyglutamic acid makes it unlikely that the enhancing effect is at the level of enzymatic degradation (DNases) or inactivation (methylation).

We conclude that incorporation of junk DNA in polyplexes enhances transfection efficiency by increasing the total number of active DNA-containing particles, which has a beneficial effect on the stochastic process of active particles reaching the final destination: the nucleus. Once inside the

nucleus, it may be beneficial that the number of transcriptionally active plasmids delivered is reduced due to the presence of junk DNA within the complexes to stay below transcriptional and/or translation saturation levels. Importantly, the fact that co-existence of junk and active DNA within a particle is crucial indicates that at the rate-limiting steps in the process of gene delivery are underwent by intact polypexes rather than dissociated plasmids. Additionally, it suggests that very few polyplexes pass the rate-limiting step, because the difference in transfection efficiency between formulations in which active and junk DNA co-exist within one particle and those in which both plasmids are formulated separately would be outweighed by the numbers.

The finding that part of the plasmid DNA can be replaced without loss in transfection activity raises possibilities to further tailor particle composition, such that other functionalities can be incorporated (i.e. second reporter plasmid) or that part of the DNA can be replaced by chemical compounds, reducing costs and improving the safety profile by reducing immunogenicity (by reducing immunostimulatory DNA and endotoxin impurities).

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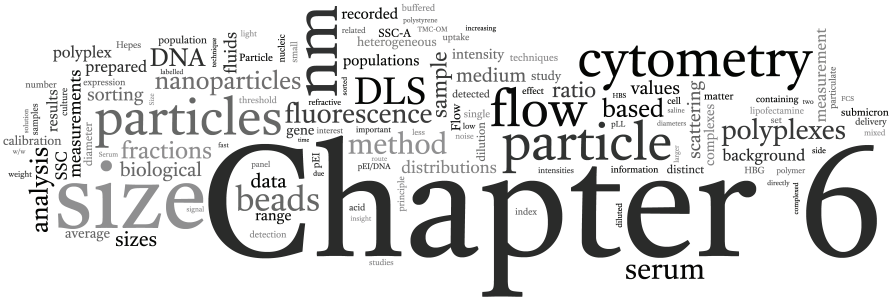

Flow cytometry for rapid size determination and sorting of nucleic acid containing nanoparticles in biological fluids

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Abstract

A method based on flow cytometry was developed which allows measurement of particle size distributions of nanoparticles directly in biological fluids and preparative sorting into distinct size fractions. Fluorescently labelled beads of distinct sizes (0.1 – 2 μm) were used to establish a correlation between diameter and side scattering intensity (SSC). Simultaneous detection of fluorescence and SSC allowed us to set a threshold on fluorescence thereby providing the possibility to distinguish nanoparticles of interest from other particulate matter (e.g. low density lipoproteins or other serum components) which is frequently present in biological fluids. Finally, a proof of principle was established for sorting a heterogeneous submicron particle population into separate size fractions.

Introduction

The development of nanomedicines is accompanied by an increasing demand for sizing techniques in the nanoscale range. In the field of non-viral gene delivery numerous efforts have been and continue to be made on the development of nucleic acid (NA)-transporting nanoparticles. Sizing of NA-transporting nanoparticles is highly important not only for quality control purposes in the end stage, but also in the development phase where biological effects (and risks) of different particle size classes remain to be addressed. For example, when designing DNA-containing particles for non-viral gene delivery, size is of utmost importance for cellular processing, with uptake routes and gene expression efficiency both depending on size [1-4]. Rejman *et al.* have demonstrated a direct relation between particle size and uptake route, with beads below 200 nm being taken up exclusively via clathrin mediated pathways and beads greater than 200 nm to an increasing extent via the caveolar route [3]. Additionally, it has been shown that in the case of lipid/DNA complexes (lipoplexes), clathrin-dependent pathways are exclusively involved in uptake and gene expression whereas in case of polymer/DNA complexes (polyplexes), both internalization pathways occur but only the caveolar route results in expression of the delivered DNA [2,4]. All these studies illustrate the importance of size and of uptake route. However, no study has been performed to establish a direct, detailed relation between size of gene delivery vectors under *in vitro* transfection conditions and uptake route and subsequent expression efficiency.

One reason why these studies are lacking is that methods currently available to measure particle size do not allow reliable measurements in the presence of small particulate contaminations present in biological fluids (e.g. serum) [5]. Serum is a complex fluid containing (amongst other) serum albumin, lipoproteins (HDL & LDL), immuno-, γ- and macrogobulins, and oleic acid. This heterogeneous nature can give rise to significant background signals, whereas sizing techniques based on conventional light scattering require minimum levels of background noise for generation of accurate results. Moreover, samples of polymer/DNA complexes are typically dilute in nature and often contain large fractions of free polymer [6], which can interact with serum proteins and form aggregates that strongly interfere with size measurements by conventional light scattering techniques [7]. With imaging techniques such as electron microscopy and atomic force microscopy individual particles can be studied, however, these techniques are laborious and have poor statistical power. Imaging is particularly valuable to obtain information regarding particle morphology, but when deriving sizes from images one should be aware of the risk for artefacts.

An even more important limiting factor is that there are no suitable methods available for preparative separation of heterogeneous particle populations into distinct size fractions in the range between 100-2,000 nm. Two closely related fractionation techniques are Field-flow fractionation (FFF) and Split-flow thin (SPLITT) fractionation. FFF is currently restricted to analytical applications whereas SPLITT can be used for preparative purposes, but only allows splitting a population in two fractions per run [8,9]. Submicron particles can be separated with gradient

ultracentrifugation, but this method is laborious, time-consuming, scale-restricted and requires purification (dialysis) and offline analysis of the fractions [10]. Additionally, the effect of shear force on particle stability should be assessed. Also stability and aggregation in biological fluids can not be studied.

This manuscript describes a method based on flow cytometry that allows high-resolution size distribution analysis of multimodal populations directly in biological fluids and a proof of principle for preparative fractionation of polydisperse samples in the submicron range. Flow cytometry is a well established technique that integrates light scattering and fluorescence measurements to gather information regarding size, shape, morphology of cells and presence and intensity of diverse fluorescent signals (indicative of molecular expression based on immunocytochemistry). Flow cytometry was originally developed as a tool to study cells, which have typical sizes of 2-120 µm (with a majority being 10-20 µm), and attempts to apply this method to submicron particles are scarce. Recently this technique was exploited to study submicron matter, including unilamellar synthetic vesicles [11], liposomes [12] and viral particles [13,14]. In this manuscript we apply flow cytometry for studying nucleic acid containing nanoparticles. With the method presented here, new insights can be obtained regarding the behavior of a variety of NA-transporting nanoparticles under physiological conditions which are critical in understanding the process of non-viral gene delivery and contribute to predictability of *in vivo* behavior.

Materials and methods

Material

pCMV-lacZ plasmid DNA was purchased from the Plasmid Factory, Bielefeld, Germany. Exgen 500 was purchased from Fermentas, St. Leon-Roth, Germany. LipofectamineTM 2000 (lipofectamine) was purchased from Invitrogen, Breda, The Netherlands. Poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) and O-methylated N,N,N-trimethylated chitosan with a degree of quatenization of 45 % (TMC-OM DQ45 %) were synthesized as previously described [15,16]. Poly-ʟ-lysine (pLL) (mol wt 4,000) and poly-ʟ-lysine (mol wt 28,200), NaCl and D-Glucose were purchased from Sigma-Aldrich, Buchs SG, Switzerland. Hepes was purchased from Acros Organics, Tilburg, The Netherlands. DMEM containing 3.7 g/l NaHCO3, 0.11 g/l sodium pyruvate and 4.5 g/l glucose (Gibco BRL, Breda, The Netherlands) was supplemented with antibiotics/antimycotics solution (100x): 10,000 IU/ml penicillin G sodium, 10 mg/ml streptomycin sulphate and 25 µg/ml amphotericin B in 0.85 % saline (Gibco BRL, Breda, The Netherlands), 200 mM L-glutamine solution (100x; Gibco BRL, Breda, The Netherlands) and Foetal Bovine Serum, FBS (Integro, Zaandam, The Netherlands), sterile and heat inactivated. Phosphate buffered saline was purchased from B-Braun, Melsungen, Germany. FluoSpheres Size Kit #2, carboxylate-modified microspheres of 0.02-0.1-0.2-0.5-1.0-2.0 µm, yellow-green fluorescent (505/515), was purchased from Molecular Probes, Invitrogen, Breda, The Netherlands. LabelIT Fluorescein Nucleic Acid

Labeling Kits were purchased from Mirus Bio, Madison, WI, USA. All buffers and reversed osmosis water were filtered through 0.2 µm filters prior to use.

Fluorescent labeling of plasmid DNA

pCMV-lacZ was covalently labeled with fluorescein according to a modified version of the manufacturer's protocol: DNA was incubated overnight with a labeling mixture at room temperature and purified using ethanol precipitation. DNA was quantified and checked for purity by measuring the absorbance at 260 and 280 nm in a Nanodrop Spectrophotometer (Wilmington, DE USA). On average, 1 fluorescein molecule was bound per 150 bp (corresponding to ~47 fluorescein molecules per plasmid), as calculated according to the manufacturer's instructions.

Preparation of DNA-containing nanoparticles

Complexes were prepared by mixing 1 volume of fluorescein-labeled plasmid solution (50 µg/ml) with 4 volumes of polymer/ lipid solution and incubating 30 min at room temperature. Solutions were prepared either in Hepes buffered glucose (HBG; 20 mM Hepes, 5 % (w/w) glucose, pH 7.4) or Hepes buffered saline (HBS; 20 mM Hepes, 150 mM NaCl, pH 7.4). Polyplexes were prepared freshly for each experiment. The chosen ratios of polymer/lipid to DNA were either based on recommendations by the manufacturers (linear pEI, lipofectamine) or considered optimal based on previous experiments in our lab (pDMAEMA, pLL, TMC-OM 45 %). pEI was used at a nitrogen to phosphate (N/P) ratio of 6, pDMAEMA at N/P=5 [15], pLL at w/w ratio 5 [15], TMC-OM 45 % at w/w ratio 12 (unpublished results) and lipofectamine at a ratio of either 2.5 µl lipofectamine/ µg DNA (as recommended by the manufacturer) or 10 µl lipofectamine/ µg DNA (excess resulting in positively charged complexes).

Size measurement by Dynamic Light Scattering and Single Particle Optical Sensing

Particle size of the polyplexes was measured in 20 mM Hepes, pH 7.4 or HBS (viscosity 0.89 cP, refractive index 1.333) or HBG (viscosity 1.145 cP, refractive index 1.3402) with Dynamic Light Scattering (DLS) on an ALV CGS-3 system (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water bath) set at 25 °C. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles (Z_h) and the particle size distribution (polydispersity index, PDI) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by the cumulants method (fitting a single exponential to the correlation function to obtain the mean size (Zave) and polydispersity index (PDI)) and CONTIN (to fit a multiple exponential to the correlation function to obtain particle size distributions). The diffusion coefficients calculated from the measured autocorrelation functions were related to the hydrodynamic radius of the particles via the Stokes-Einstein equation,

$Z_h = (k_B T q^2)/(3\pi\eta\Gamma)$, where Z_h is the hydrodynamic radius of the particles, k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, Γ is the decay rate, and q is the

scattering vector ($q= 4\pi n \sin(\Phi/2))/ \lambda$), in which n is the refractive index of the solution, Φ is the scattering angle, and λ is the wavelength of the incident laser light. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle. The system was calibrated with 200 nm polystyrene latex standard beads (Duke Scientific, Leusden, The Netherlands).

Diameters of the fluorescent beads were confirmed by DLS (100, 210 and 500 nm bead) or Single Particle Optical Sensing (SPOS) (1,100 and 2,000 nm beads) using an Accusizer-780 A auto diluter (Particle Sizing System, Santa Barbara, USA) calibrated with polystyrene latex beads of defined size.

Flow cytometry analysis

Flow cytometry was performed using a FACSCalibur (Becton and Dickinson, Mountain View, CA, USA) benchtop flow cytometer equipped with an air-cooled Argon-ion laser at 488 nm and various flow speed controls or a FACSCanto II or FACSAria Cell Sorter (Becton and Dickinson, Mountain View, CA, USA) equipped with three air-cooled lasers at 488, 633 and 405 nm. Side scatter outcome was set to logarithmic and detected at a scattering angle of 90 ° with a threshold set on FL-1 (set to linear amplification) to exclude side scatter values from non-fluorescent particulate matter. Flow rates and/or dilutions were chosen such that less than 2,000 events/s were recorded to prevent coincidence. For each measurement a total number of 10,000 events were recorded.

Data processing

FACSCalibur data were processed with Summit® software (DakoCytomation, Fort Collins, CO, USA) and FACSCanto II and FACSAria data with FACSDiva software (Becton and Dickinson, Mountain View, CA, USA) or FCS Express (www.denovosoftware.com). For calibration, histograms of SSC-values were plotted and the geometric means of each peak corresponding to a bead size were derived. Bead sizes (y) and geometric means of SSC-values (x) were then plotted in Graphpad Prism and fitted with nonlinear regression using: $y=a+bx^c$ (1) as described previously [12].

For size analysis of samples, raw data were converted to ASCI using the program MFI and FCSExtract for data obtained with the FACSCalibur data and FACSAria, respectively. Data were converted from 10-bit (1024 channels) to log4-scale using: $y=10(x/256)$ (2). Data from channel 0 were excluded from the analysis. The resulting log4-scale values were then converted to sizes by applying (1).

Results

Size determination of nanoparticles with flow cytometry: proof of concept

First, we examined whether side scattering (SSC) values measured by flow cytometry could be correlated to sizes within the range of 100-2,000 nm using fluorescently labelled polystyrene beads of distinct sizes. Sizes of the beads used for calibration were analyzed by Dynamic Light Scattering (DLS) and Single Particle Optical Sensing (SPOS) for nanosized and low-micrometer sized beads

respectively, as shown in Table 1. With flow cytometry, scattering intensities detected at 90 ° were recorded for each batch of the calibration beads. Flow cytometry parameter settings were adjusted such that background noise is negligible (by adjusting threshold on fluorescence detected in Fl-1), and detected fluorescence and SSC are within scale (by adjusting detector amplitudes). Flow speed and dilution of beads were adjusted until <2,000 events/ second were recorded in order to prevent coincidence (simultaneous detection of >1 particle) and SSC-A values were recorded (10,000 events per measurement). Geometric mean of SSC-A values and diameters were plotted and a calibration curve was fitted according to equation $y = a + bx^c$ [12], resulting in an R^2= 0.9997 (Figure 1 A).

Table 1. *Diameters of fluorescein-labelled calibration beads as determined by Dynamic Light Scattering (100-210-500 nm) or Single Particle Optical Sensing (1,100-2,000 nm) (n=3).*

Bead (nm)	**Experimental bead size (nm)**	
	Z_{ave} (nm)	**PDI**
100	111 (±2)	0.02 (±0.02)
210	221 (±5)	0.05 (±0.02)
500	504 (±5)	0.10 (±0.05)
	Volume-weighted mean size (µm)	
1100	1.06 (± 0.01)	
2000	1.97 (± 0.01)	

Reproducibility of the measurement was confirmed by performing three independent calibrations resulting in SSC-A values of 2.6 ± 0.6 (100 nm), 25.4 ± 0.4 (200 nm), 141 ± 5 (500 nm), 781 ± 12 (1,000 nm), 3216 ± 101 (2,000 nm). Diluting beads in phosphate buffered saline (PBS), Hepes buffered saline (HBS) or Hepes buffered glucose (HBG) gave similar results (data not shown), indicating that calibration is insensitive to changes in the dispersion medium (as long as no aggregation is induced).

Next, we applied the method to a typical sample of nucleic acid containing nanoparticles. A polyplex sample was prepared by mixing linear 22 kDa poly(ethylene imine) (pEI) with fluorescein-labelled plasmid at an N/P ratio of 6. The sample was diluted in low-ionic strength buffer (HBG) and analyzed by DLS and flow cytometry, as shown in Figure 1 B. The histogram obtained by flow cytometry revealed a highly heterogeneous population of polyplexes with diameters ranging from <100 nm to 300 nm with a peak frequency between 100-120 nm. Conversion of the geometric mean of SSC-A to size using (equation 1) gives an average polyplex diameter of 117 nm, which is close to the Z-average found by DLS (125 nm).

It is frequently described in literature that the size of polyplexes is affected by the composition and pH of the buffer in which they are prepared and the medium in which they are dispersed[1,17-20]. High ionic strength causes aggregation of pEI/DNA polyplexes due to reduced electrostatic repulsion between particles [17]. This can be explained by the basic physicochemical principle that upon increasing ionic strength the double layer surrounding the charged polyplexes is compressed, leading to increased interparticle attractions and aggregation. Here we evaluated this effect of

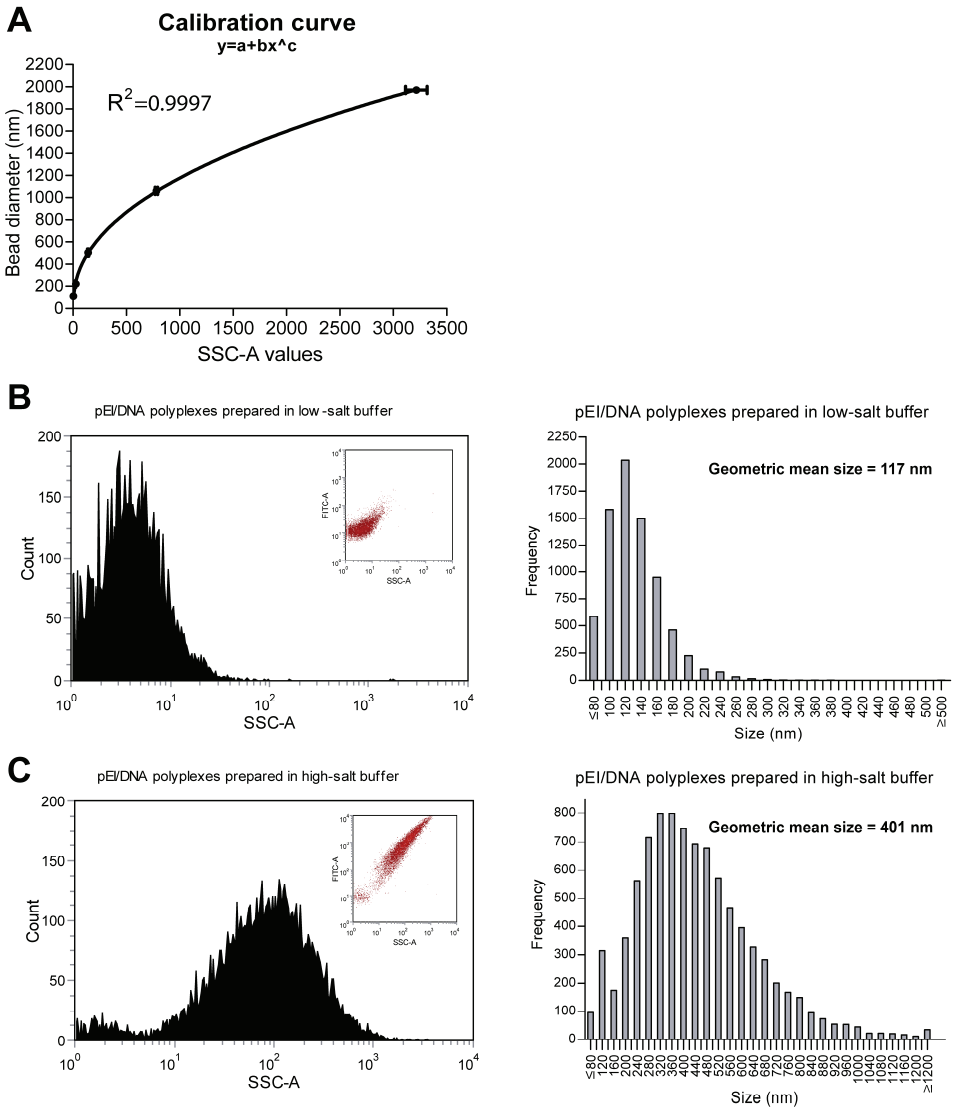


Figure 1. *Flow cytometry for particle size (distribution) analysis.*

(A) Calibration: SSC-A values are recorded for each of the calibration beads and geometric mean SSC-A values are related to diameters using the formula $y = a + bx^c$ *(a= 18.39; b= 52.36; c= 0.4482). For sake of convenience the variable is plotted on the X-axis.*

(B) and (C) Size analysis of pEI/DNA polyplexes. A histogram of side scattering intensities is recorded and converted to a histogram of diameters using the calibration curve. The insets show dot plots of SSC-A (indicative of size) against FITC-A (fluorescence intensity of the particles). (B): Analysis of pEI/DNA polyplexes prepared in HBG; (C): Analysis of pEI/DNA polyplexes prepared in HBS.

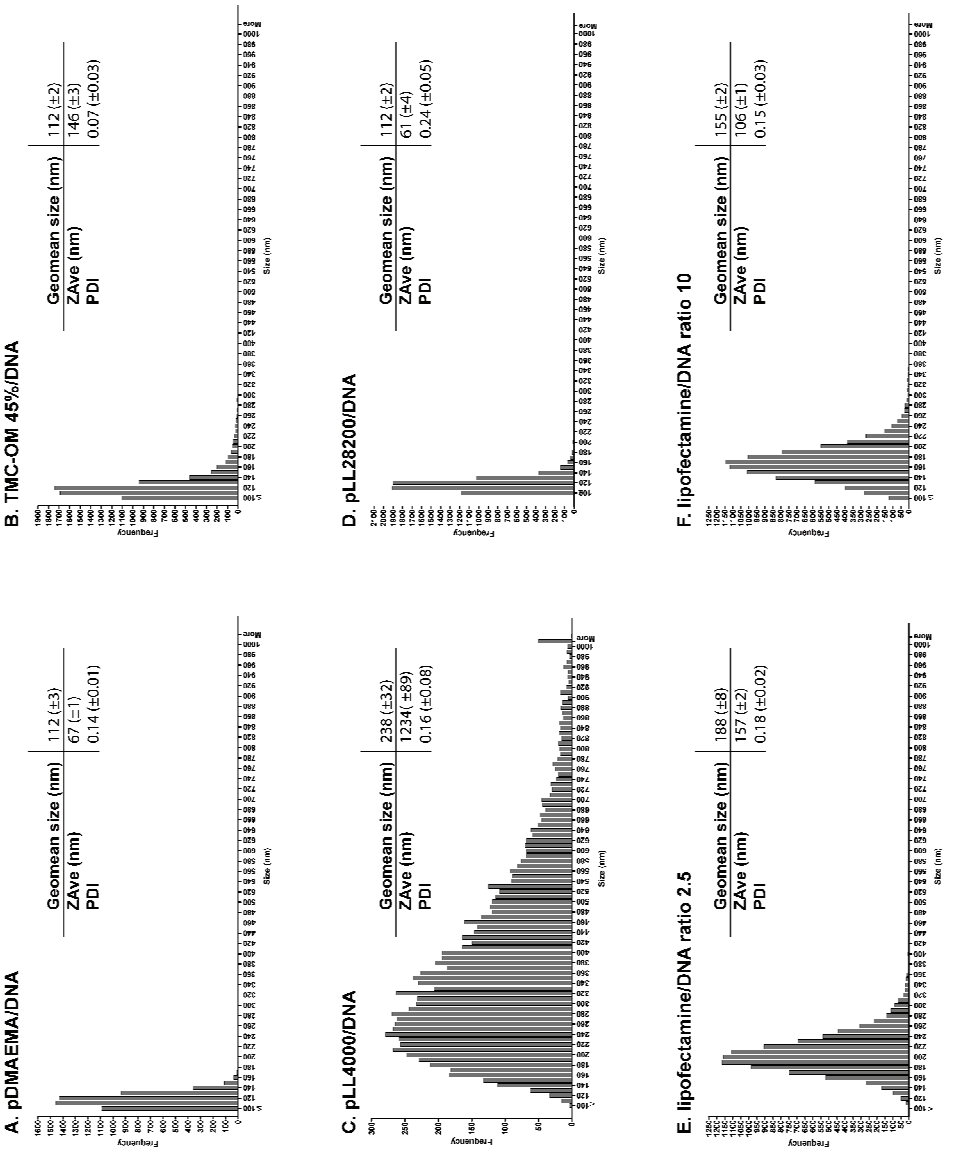
electrolytes on particle size distribution by testing polyplex formulations prepared either in low- (hepes buffered glucose; HBG) or high-salt buffer (hepes buffered saline; HBS). From conventional DLS measurements it is seen that the average size of polyplexes increases to highly polydisperse populations >500 nm when using high-salt buffers. Similarly, from our flow cytometry data an average size of 117 versus 401 nm is obtained for low- and high-salt buffers, respectively (Figure 1 C). From the distribution data obtained with flow cytometry, it can be derived *how* the average particle size is affected, which can be valuable in the case where formation of few aggregates occurs rather than an overall shift in particle sizes.

These results show that flow cytometry can be used for sizing in the range of 100-2,000 nm and can be applied for characterization of NA-transporting nanoparticles.

Size analysis of DNA-containing nanoparticles by DLS and flow cytometry

Figure 2 shows the characterization of various DNA-containing particles. The gene delivery reagents used have been described in literature (low- and high- molecular weight poly-L-lysine (pLL) and lipofectamine) and/or developed in our lab (pDMAEMA, TMC-OM 45 % [15,16]). All formulations are based on electrostatic interaction between the cationic polymer or lipid with negatively charged DNA, but the resulting particles have very different characteristics and transfection efficiencies. The DLS data already disclosed the general differences in hydrodynamic diameters, but did not give insight in size distributions which is in particular relevant when one is dealing with samples with broad size distributions. On the other hand, when combined with flow cytometric analysis, a good insight in sample composition was obtained. For example, pDMAEMA-based complexes were found to be very small (<100 nm) with a narrow size distribution (all <200 nm), whereas TMC-OM 45 % were on average small, but slightly more heterogeneous (ranging to 250 nm). Lipofectamine-based complexes were significantly larger and had an intermediate size distribution (100-400 nm). When looking at polylysine-based complexes, we observed that low-molecular weight pLL was much less efficient in condensing DNA than high-molecular weight pLL resulting in much larger particles with a very broad distribution (geomean size 283 ± 32 with a range from 100-1,000 nm, compared to a geomean size of 112 ± 2 with all sizes ≤200 nm for high-molecular weight pLL). DLS failed to characterize this population due to the presence of large complexes, which causes bias of the Z_{ave} towards higher values.

Next page: Figure 2. *Characterization of various DNA-containing nanoparticles by DLS and flow cytometry. Histograms show size (diameter) distributions derived from flow cytometry measurements and insets give average particle diameters based on flow cytometry (geomean size) and average particle diameter and polydispersity based on DLS measurement (Z_{ave} and PDI). Polyplexes were prepared in HBG at a final DNA concentration of 10 µg/ml and measured undiluted by DLS. For flow cytometry polyplexes were diluted until < 2,000 events/s were recorded to prevent coincidence. (A): DNA complexed with pDMAEMA at a N/P ratio of 5.; (B): DNA complexed with TMC-OM DQ45 % at a polymer: DNA w/w ratio of 12; (C): DNA complexed with low molecular weight poly-L-lysine (pLL_{4000}) at a polymer: DNA w/w ratio of 5; (D): DNA complexed with high molecular weight poly-L-lysine (pLL_{28400}) at a polymer: DNA w/w ratio of 5; (E): DNA complexed with lipofectamine at a ratio of 2.5 µl/µg DNA; (F): DNA complexed with lipofectamine at a ratio of 10 µl/µg DNA.*



A comparison of distribution data acquired by DLS versus flow cytometry

The present results show an important difference in information arising from either DLS or flow cytometry: whereas DLS generated data are based on the overall population in the sample container, flow cytometry provides information at the single particle level. An important question in non-viral gene delivery is which particle size results in the most efficient cellular uptake,

processing and finally gene expression. In this case, more relevant information can be derived from number distributions than from weight or volume distributions. When a larger average particle size is the result of formation of few aggregates, the *in vitro* transgene expression may be the result of the remainder of small particles or of the aggregates, neither of which are properly reflected by the average particle size.

DLS measures intensity fluctuations of scattered light as a function of particle size. Since this intensity is proportional to the sixth power of the particle radius, it is highly biased towards larger particles [21]. Our method based on flow cytometry is based on single particle detection from which a number distribution can be directly generated and might therefore provide more insight into the actual composition of (highly) polydisperse polyplex samples than the conventionally used DLS technique. To illustrate the difference in size resolution between both techniques, an experiment was performed where polystyrene beads of 200 and 500 nm were mixed at fixed number ratios and measured by DLS and flow cytometry (Figure 3). DLS (using CONTIN to fit a multiple exponential to the correlation function) was not able to recognize separate populations at any of the ratios tested and generated an overall average diameter (227 nm when 200 and 500 nm beads are mixed at a 1,000:1 ratio and increasing to 367 nm for a 1:1 mixture). Flow cytometry on the other hand succeeded in identifying both populations even at ratio's of 1,000:1 (observed ratio 649:1). Strikingly, even when 200 and 500 nm beads were mixed at a 1:1 number ratio PDIs calculated from DLS-measurements were <0.2, a value which is generally regarded as a cut-off for an acceptable polydispersity index. This illustrates that characterization of polyplexes, or any heterogeneous sample for that matter, solely by standard DLS can be misleading and additional analysis by flow cytometry offers a fast and simple method to obtain a more realistic representation of sample size distribution.

Characterization of nanoparticles in biological fluids

The improved resolving power and ability to generate number distributions in flow cytometry are important parameters when studying polyplex size as they can give detailed insight into effects of salts and components in biological fluids and allow recognizing multimodal distributions. A unique advantage of using flow cytometry is that scattering and fluorescence intensity can be recorded simultaneously. The fluorescence can be used as an inclusion criterion, allowing exclusion of unlabeled particulate matter while including similarly sized labelled particles of interest in the measurement. This is of special interest when size of particles needs to be measured in biological fluids, as these often contain large proteins and particulate matter such as serum albumin, low density lipoproteins (LDL), high density lipoproteins (HDL), globulins, heparin and chylomicrons.

Serum components have been described to affect nucleic acid containing particles in various ways. Firstly, serum proteins can adsorb to the positively charged particles leading to changes in size (aggregation), zetapotential and cellular uptake [22-24]. Secondly, dissociation of the complexes

followed by release and degradation of the nucleic acids can occur [22,24,25]. Thirdly, the large fraction of polymer that is present in free form [6] can interact with negatively charged serum proteins and form particles [7].

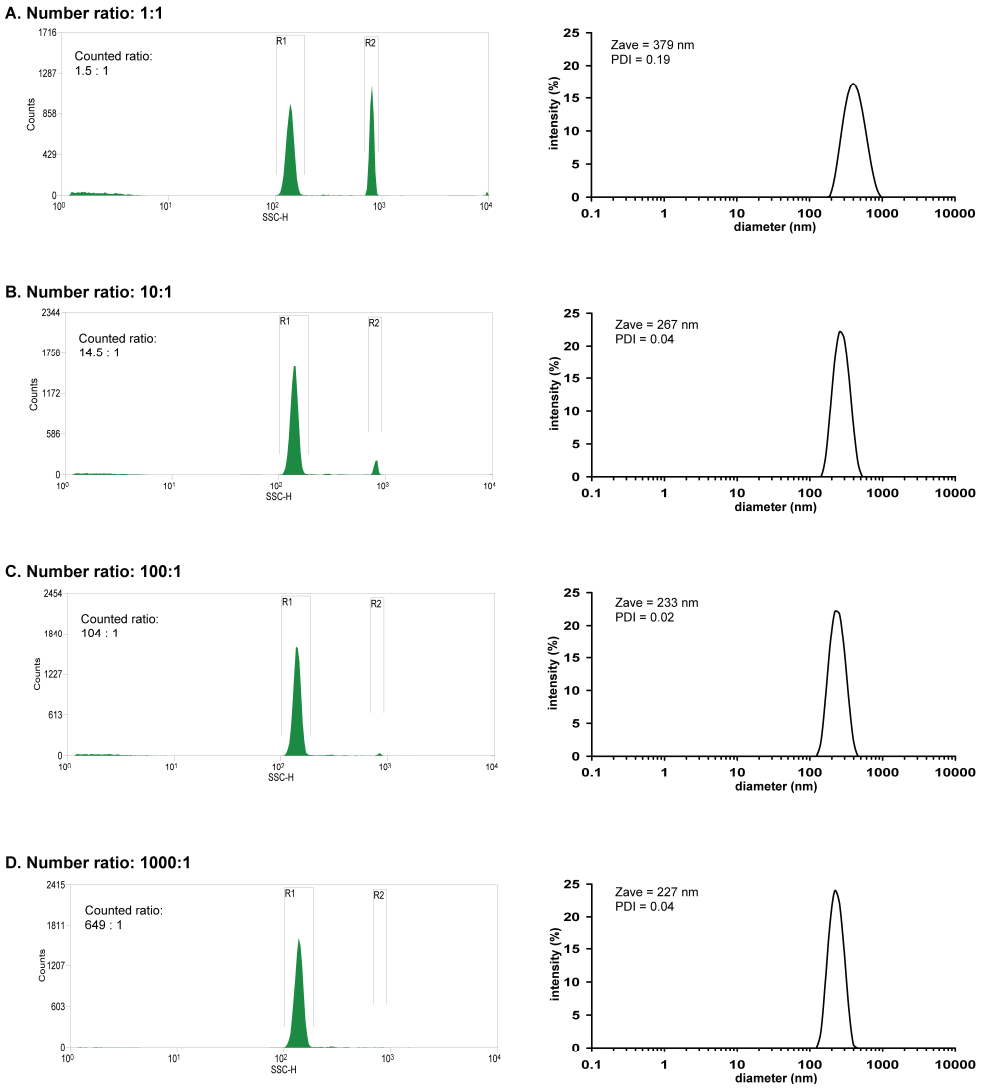


Figure 3. *Resolution of flow cytometry versus DLS. 200 and 500 nm beads were mixed in various number ratios and mixtures were measured by flow cytometry (left) and DLS (right). A.) Number ratio 1:1; B.) Number ratio 10:1; C.) Number ratio 100:1 and D.) Number ratio 1000:1.*

Serum background signal interferes with nanoparticles size analysis by DLS

The effect of serum on DLS measurements for size analysis of polyplexes was evaluated, as shown in Figure 4. Measurement of pEI/DNA polyplexes in buffer resulted in a monomodal size distribution with a clear peak at a diameter of approximately 100 nm (Figure 4 A, left panel; ~150 kilocounts per second (kcps)). However, when these complexes were mixed with serum-supplemented cell culture medium, a multimodal distribution is observed with peaks that could not be clearly resolved (Figure 4 A, right panel; ~700 kcps). Moreover, it is unclear which peak represents the polyplexes. Background measurements for serum-containing cell culture medium and 100 % serum were also recorded (Figure 4 B) and reveal a significant background signal with the presence of two peaks around 10 and 50 nm. It should be noted that nucleic acid complexes typically prepared for *in vitro* studies are of low concentration and are measured near the detection limits of DLS. Count rates for standard pEI/DNA complexes prepared at an optimal N/P ratio of 6 are typically 100-200 kcps at maximum laser power. As indicated in Figure 4 B, serum (-containing medium) gave significant background noise under these circumstances and this noise is also clearly visible in the recordings shown in Figure 4 A.

Lastly, we evaluated the effect of mixing free pEI with serum-containing medium (Figure 4 C). Cell culture studies are normally performed at conditions using 10-20 % FCS and at these concentrations formation of particles of approximately 200 nm in size were detected due to interaction between free pEI and serum components.

Based on the data presented in Figure 4 it can be concluded that reliable size analysis of NA-transporting nanoparticles in biological fluids cannot be achieved because measurements are seriously hampered by presence of disturbing background signals that cannot be distinguished from the signal of the particles of interest. These results illustrate the need for a more robust technique for particle size measurements in biological (or simulated) media.

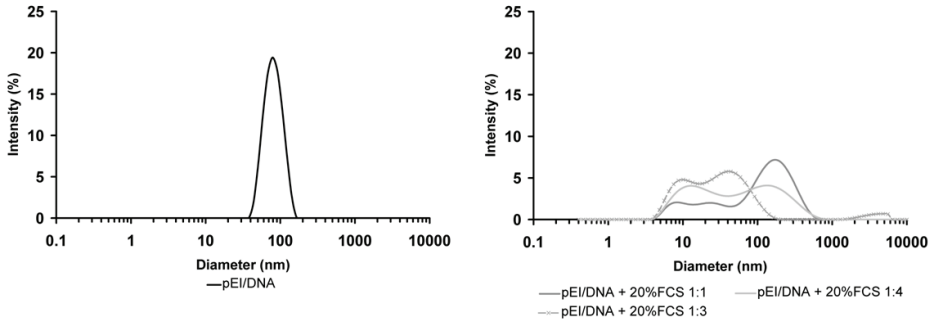
Next page: Figure 4. *Complications of DLS measurements of nucleic acid containing particles in serum. Contin analysis was performed to obtain DLS size distributions of:*

(A): Polyplexes in absence (left) or presence (right) of serum. Polyplexes were prepared in Hepesbuffer and measured directly or after dilution in cell culture medium supplemented with 20 % serum.

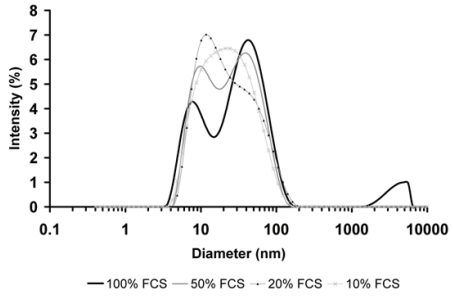
(B): Serum or serum-supplemented cell culture medium.

(C): Free pEI mixed with serum or serum-supplemented medium. pEI was diluted to a concentration equivalent to that in polyplex samples and then mixed with an equal volume of serum(-supplemented medium). Signals recorded in the absence of pEI are included for reference.

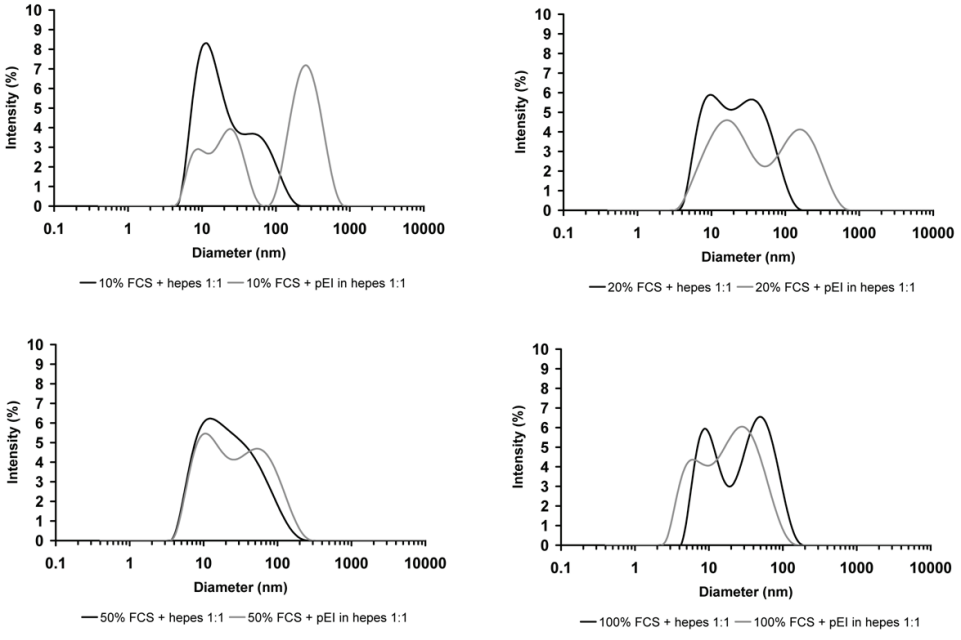
A. pEI/DNA polyplexes in Hepesbuffer (left) and dilutions in DMEM +20% FCS (right)



B. 10-100% FCS in DMEM



C. 10-100% FCS in DMEM mixed 1:1 with Hepesbuffer or free pEI in Hepesbuffer



Exclusion of serum background signal enables nanoparticles size analysis by flow cytometry

Next, we used our new method to study particle size of polyplexes in serum-supplemented cell culture medium by discriminating side scattering resulting from fluorescently labelled particles from those of background material. Figure 5 illustrates the principle of the measurement as shown for detection of beads in 100 % FCS. Measurement of a sample of 200 nm beads diluted in 100 % FCS using normal settings gives rise to the presence of two distinct populations that differ both in side scattering intensity (size) and fluorescence intensity (Figure 5 A, left panel). Although these populations could already be resolved based on their SSC values, the resolving power based on their fluorescence intensities is much stronger. By increasing the threshold on the FL1-channel (the channel in which fluorescein is detected), the signal resulting from serum was successfully excluded (Figure 5 A, right panel). At these settings, less than 50 events/ second were recorded for background serum measurements, which can be considered negligible. This resolution based on fluorescence becomes crucial in case of smaller particles, as illustrated for 100 nm beads diluted in 100 % FCS in Figure 5 B. In this case, resolution of the two populations based on SSC intensities is no longer possible. However, based on differences in fluorescence intensities the 100 nm beads can be isolated from the overall population. After applying the threshold, serum particles were excluded and only the beads were still detected (Figure 5 B, right panel). Obviously, the distinction of serum background noise becomes increasingly straightforward for particles that are larger and/or have higher fluorescence intensity.

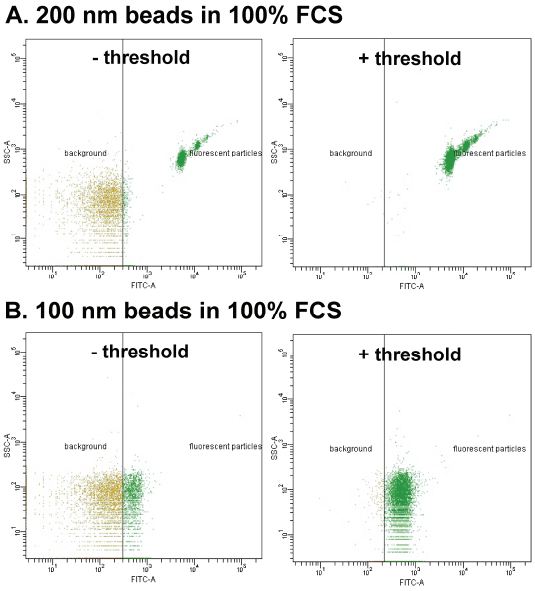


Figure 5. *Principle of particle size analysis in the presence of serum using flow cytometry. 200 nm beads (A) or 100 nm beads (B) were diluted in 100 % serum (FCS) and measured with or without a threshold on fluorescence. Data are plotted as scatterplots plotting intensities measured in channel FL1 (fluorescein; x-axis) against SSC-A (size; y-axis).*

Using this principle, pEI/DNA polyplex sizes were measured in the presence of serum (Figure 6). These results show a gradual increase in size upon incubation in serum-containing medium, but importantly, the effect is much smaller than we expected and no large aggregates were observed. Background measurements performed for mixtures of free pEI with serum-containing medium resulted in less than 50 events/ second, similar to plain serum measurements. This finding reflects the ability of flow cytometry to exclude (aggregated) particulate matter other than the nanoparticles from the measurement, thereby preventing overestimation of particle sizes of particles of interest.

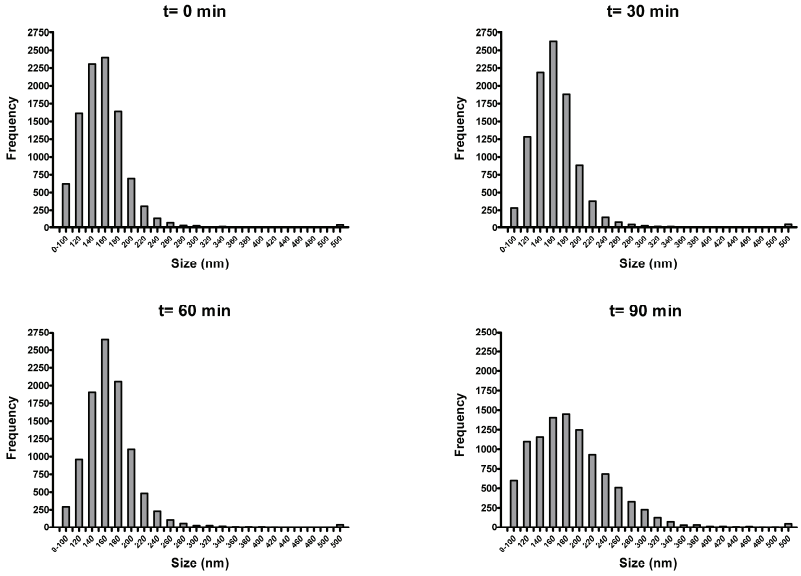


Figure 6. *Stability of polyplexes in biological fluids. Polyplexes were prepared in HBG and subsequently incubated in cell culture medium supplemented with 10 % Fetal Calf Serum. A threshold on fluorescence was set to exclude background signal of the medium. Size distributions were measured at time points 0, 30, 60 and 90 minutes after adding medium.*

Fluorescence-Activated Particle Sorting (FAPS)

A powerful and frequently used application of flow cytometry is Fluorescence-Activated Cell Sorting (FACS). We investigated whether the sorting method is also applicable to particles in the submicron range. The results given in Figure 7 A show that a mixture of beads ranging from 200-2,000 nm was successfully sorted into 4 defined size fractions. Figure 7 B shows that a heterogeneous polyplex sample could also be successfully separated into two fractions. Re-analysis of both fractions gave SSC-A values that correspond to those around which the gates had been set, indicating that sorting was performed correctly and that the particle characteristics were not

significantly altered during the sorting process. From these results it can be concluded that sorting is only feasible for populations with distinct SSC-A values and that performance increases with increasing sizes (in the range under investigation) selected. Nevertheless, this experiment shows a proof of principle of Fluorescence-Activated Particle Sorting (FAPS). In principle, sorting a heterogeneous polyplex sample into two distinct fractions of ~200 nm and ~500 nm would allow performing *in vitro* studies in which effect of polyplexes could be directly related to particle size. However, subsequent use of sorted polyplex fractions appeared not practically feasible due to the extensive dilution of sample during the sorting process. At this point it should be noted that use of dilute sorted fractions is feasible for particles that (unlike our particles) allow concentration after sorting. The dilution occurs because sorting requires formation of droplets with on average one particle per 3 droplets. Therefore the dilution factor is directly related to the size of the droplets formed, hence to the diameter of the nozzle inside the FACS sorter. Decreasing this nozzle diameter may offer a solution in obtaining less diluted sample fractions.

Discussion

In this study we successfully developed a method for particle size analysis that combines fast measurement of complete populations with information at the single particle level. We applied this method to study nanoparticles used in the field of gene delivery, but it is generally applicable to any set of nanoparticles that is fluorescently labelled. The method is valid within the nanoscale range, with a lower limit around 100 nm. Below this size, calibration is complicated due to insufficient sensitivity of the SSC-detectors currently used in flow cytometry equipment. Additionally, it can be seen from the calibration curve that for the small size range the SSC values increase steeply with size and therefore are less robust under those conditions. Recently, efforts have been made to develop equipment specialized towards submicron particle analysis as an alternative to commercially available flow cytometers [14,26].

An important advantage of the method presented here over existing nanoparticle sizing methods such as dynamic light scattering and single-particle optical sensing is the potential to measure nanoscale particles in the presence of other particulate matter which is often present in biological fluids. By adding fluorescence intensity as an inclusion criterion, this method can easily distinguish particles of interest from equally sized background noise. This strategy allowed us for the first time to measure polyplex size distributions in the presence of serum in cell culture medium and under conditions prevailing during transfection studies. The extra feature of fluorescence detection distinguishes this method from conventional scattering-based techniques such as DLS and single particle optical sensing. A disadvantage of introducing fluorescence measurements in particle size determination is the inherent requirement for sample labelling. A general concern when using labelling is that the presence of fluorescence labels may influence particle characteristics. In our study, data obtained with DLS showed similar size profiles for labelled and unlabeled polyplexes in

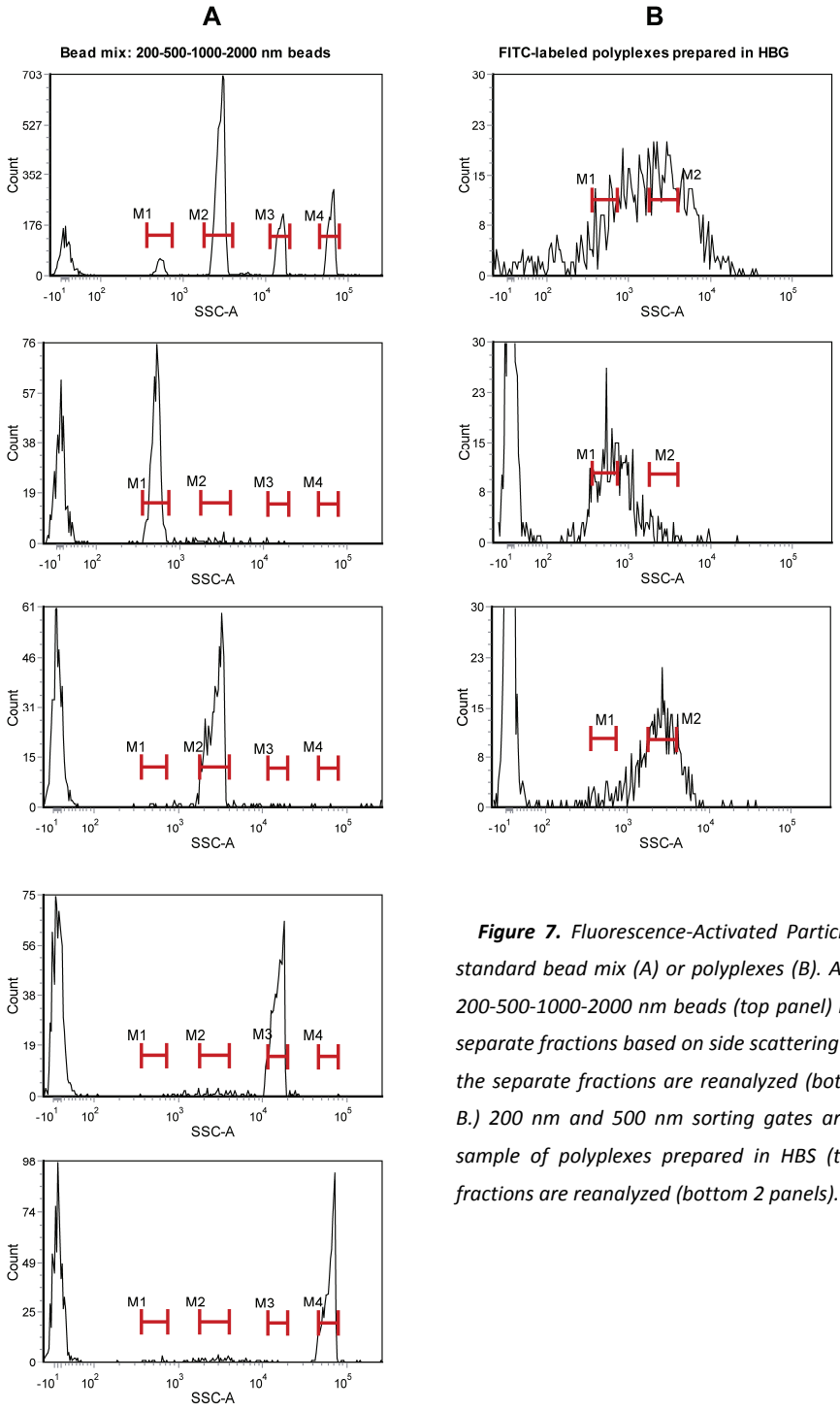


Figure 7. *Fluorescence-Activated Particle Sorting of a standard bead mix (A) or polyplexes (B). A.) A mixture of 200-500-1000-2000 nm beads (top panel) is sorted into 4 separate fractions based on side scattering intensities and the separate fractions are reanalyzed (bottom 4 panels); B.) 200 nm and 500 nm sorting gates are applied to a sample of polyplexes prepared in HBS (top panel) and fractions are reanalyzed (bottom 2 panels).*

buffer media (data not shown). Thus, the effect of the fluorescent label on polyplex characteristics is expected to be small. However, the effect of labelling on particle characteristics should be evaluated for the nanoparticle of interest.

Another advantage of the flow cytometry based described method is the completeness of information that can be obtained. Whereas DLS generates data based on the overall population present in the sample container, flow cytometry records single particles. These parameters are important when studying polydisperse samples under physiological circumstances as they give detailed insight into effects of salts and components in biological fluids and allow recognizing multimodal distributions. Flow cytometry is therefore more suitable for measurement of number distributions and provides better size resolution based on direct, single particle measurement instead of deriving them through deconvolution strategies as for DLS.

The principle of the size measurement is comparison of SSC values of unknown particles to those of beads of distinct sizes. This is valid when assuming that SSC is only related to size. However, SSC is also affected by a.o. refractive index and geometry of particles [11,21]. In the present study, irregularly shaped polyplexes are compared to spherical polystyrene beads and it is recognized that conversion of SSC to size is affected by the differences in RI and surface properties. Therefore it cannot be claimed that the flow cytometry derived sizes found are the true absolute sizes of the particles. As determination of refractive indices of polyplexes is not straightforward, estimations from literature are often used. In case of polyplexes, refractive indices of 1.59 have been reported [27-29], which closely match the value of 1.60 reported for polystyrene [30]. Therefore, we expect the deviation in relative refractive index to be small. We therefore believe that flow cytometry analysis can be used to obtain a realistic insight in size distributions and offers a fast and valuable analysis tool supplementary to conventionally used DLS.

The method presented here requires limited time and efforts for development and optimization: to implement this technique, flow cytometry parameter settings need to be established such that background noise is negligible, and detected fluorescence and SSC are within scale. Additionally, it is recommended to perform a calibration for each experiment. Once settings have been determined, measurements are fast and data handling is straightforward. Settings should be adjusted such that single particles are recorded. Most samples are more concentrated than the flow cytometer can handle and therefore require (stability upon) dilution. This means that sample consumption is very small, which is beneficial.

Since flow cytometry was originally developed as a tool to study cells (with typical sizes of 20-50 µm), flow cytometers are not necessarily equipped with optimal detectors for studying particles in the submicron range. Use of these instruments for the method described in this paper therefore poses specific requirements on the instrument: sensitivity of fluorescence and of SSC detectors are critical. Insufficient fluorescence sensitivity results in detection of an incomplete population, with the smallest particles (with the least fluorescence) below detection limit (or threshold). It should however be realized that this type of results leads to particle sizes biased towards larger particles.

Moreover, it should be stressed that the requirements for sensitivity are related to the labeling intensity of the sample. It is therefore advisable to carefully look at the scatter plot of SSC versus fluorescence intensity to evaluate whether the complete population of particles is being detected and to optimize labeling (or equipment) accordingly.

Our ultimate goal was to sort a heterogeneous polyplex sample into fractions of distinct sizes that could be used for *in vitro* studies. As shown, sorting of submicron particles was possible. Nevertheless, subsequent use of sorted fractions appeared not practically feasible due to the extensive dilution of the sample during the sorting process. At this point it should be noted that use of dilute sorted fractions is feasible for particles that (unlike our particles) allow concentration after sorting. The FACSAria used in this study was equipped with a nozzle with a diameter of 70 µm. The smallest nozzles available for FACS machines at present is 50 µm, which would reduce droplet size (hence dilution factor) by a factor of 2. If manufacturers would be able to equip machines with even smaller sized nozzles, dilution factors could potentially be decreased to acceptable values and allow a first method for sorting of heterogeneous particle populations in the submicron range. By incubating the separated size fractions with cells the direct relation between polyplex particle size, cellular uptake route, subsequent processing and ultimately gene expression could be studied for the first time. This would significantly contribute to the rational design of more efficient gene delivery vectors.

Conclusion

In conclusion, the method based on flow cytometry presented in this paper offers a fast and reliable method to study the particle size of nanoparticles in biological fluids. With this method, more detailed information regarding particle populations can be obtained than with conventional light scattering techniques such as DLS. At the same time, it offers a fast alternative to image-based single particle analysis with less risk for bias. Added values of this method are the ease of use, speed of measurement, completeness and reliability/reproducibility of data and the possibility to measure in the presence of particulate matter often present in biological fluids without interfering with the analysis of the desired collection of nanoparticles. Moreover, when technological modifications can be made to the currently used flow cytometers, this technique might be further developed as a first method to sort heterogeneous particle populations into distinct size fractions in the nanoscale range without extensive dilution.

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Towards a standard protocol for *in vitro* transfection screenings of non-viral gene delivery systems

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Submitted for publication

Abstract

Screening of new gene delivery candidates regarding transfection efficiency and toxicity is usually performed by reading out transgene expression levels relative to a reference formulation after *in vitro* transfection. However, over the years and among different laboratories, this screening has been performed in a variety of cell lines, using a variety of conditions and read-out systems, and by comparison to a variety of reference formulations. This makes a direct comparison of results difficult, if not impossible. Reaching a consensus would enable placing new results into context of previous findings and estimate the overall contribution to the improvement of non-viral gene delivery. In this paper we illustrate the sensitivity of transfection outcomes on testing conditions chosen, and propose a screening protocol with the aim of standardization within the field.

Introduction

Since gene therapy was first proposed in 1972 [1], numerous efforts have been made and many papers have been published on the topic of gene delivery. Non-viral gene delivery strategies are under investigation as safer and more versatile alternatives to viruses. One of the most efficient synthetic reagents is polyethylenimine (pEI), a polymer first proposed as a DNA delivery agent by Boussif *et al.* in 1995 [2]. Despite its efficiency, therapeutic application is limited because of toxicity (especially for high molecular weight pEIs) and lack of biodegradability. Many alternatives in the form of other polymers, lipids and peptides have been described, with varying degrees of success. Nevertheless, insight into structure-effect relations is still limited.

Current research in gene delivery can be categorized into mechanistic studies aimed at gaining insight in (steps in) the delivery process itself and into the development and screening of candidate reagents. Mechanistic studies are crucial to understand the fundamental processes and to move towards rational design of delivery agents. However, such studies require sophisticated, laborious and expensive techniques (e.g. advanced microscopic techniques, cellular subfractionation, quantitative PCR) and it is not feasible to apply them to each and every reagent under investigation. Preselecting potential candidates through screening in relatively simple cost- and time-effective experiments is therefore a prerequisite.

Based on early findings, the search for alternative delivery agents has moved into various directions and is ever expanding. Improvements are looked for at the level of biocompatibility (i.e. trends towards low molecular weight [3,4], naturally occurring [5,6] or biodegradable compounds [7-10]), cellular uptake (modification of charge, functionalization with targeting ligands or membrane-permeant moieties [11-13]), endosomal escape (membrane disrupting agents, proton sponges [14]), cytosolic transport (PEGylation, dynein-binding peptides [15]), vector unpackaging (biodegradable, reducible or bioresponsive compounds [16]) or nuclear uptake (nuclear localization signals, steroids[17]). These functionalizations are applied to reagents including pEI (-derivatives), polyamines, polyamidoamines, chitosan (-derivatives), lipids, lipid/polymer hybrids, peptides, proteins, polymethacrylates, polyphosphazenes, dendrimers, and others (for a recent review see [18]). This type of research has lead to important findings and discovery of potential reagents. Unfortunately, absence of consensus regarding screening methods complicates comparison of these individual strategies to one another and therefore possibilities for recognition of structure-effect relations can not be utilized maximally.

An illustrative example of the impact of methodology on experimental outcomes is given by publication of two papers investigating the nuclear localisation signal (NLS) bearing cell-permeable peptide (CPP) $S4_{13}$-PV in two consecutive issues within the same volume of the Journal of Gene Medicine. The first paper describes inefficient transfection upon incubation of cells with complexes of peptide (ALWKTLLKKVLKAPKKKRKV) and plasmid (pGL3-luc) prepared at a peptide/DNA ratio of 10 [19]. Expression levels reported were <100 Relative Light Units/mg protein, corresponding to a

Table 1. *Parameters of variation in in vitro transfection screenings extracted from literature [21-44].*

Parameter	times used
Cell type	
HeLa	6
HEK293T	3
HEK293/ A549/CHO/ bone marrow-derived mesenchymal stem cells/ NIH-3T3/ MCF-7/ HepG2	2
bEnd-3/ HDFs/ MA/K562/ HT29/ HT1080/ B16F10/ bone marrow dendritic cells/ primary myoblasts/ CHO-K1/ Calu-3/COS/COS-7/mouse spleen-derived APC/ BHK-21/ SK-N-BE(2)C/ mNPC	1
Positive control	
25 kDa b-pEI	7
Lipofectamine™2000	6
Lipofectin	5
pLL/ Lipofectamine™	2
22 kDa l-pEI/ 0.8 kDa b-pEI/pEI1800/pEI/Superfect/ GenePorter™/ pDMAEMA/DOTAP	1
No external reference	4
Buffers used for complexation	
20 mM Hepes pH 7.4	3
PBS	2
10 mM Hepes / 10 mM Hepes, pH 7.4 / 10 mM Hepes, 150 mM NaCl, pH 7.4 / 10 mM Hepes, 1mM NaCl, pH 7.4 / 10 mM Hepes, 5% glucose, pH 7.4 / 20 mM Hepes / 20 mM Hepes pH 7.2/ HBS pH 7.4/ 5% glucose / 150 mM NaCl / α-MEM/ MQ-water/ DMEM	1
Unclear	5
Dose (µg DNA/cm^2)	
1/ 0.5/ 0.2	4
0.25/ unclear	2
3.1/2/ 1.6/ 0.75/ 0.4/ 0.1	1
Cell confluency	
70%	3
60-70%/ 70-80%/ 80% / unclear	1
Reported as # of cells/ well	17
Incubation of complexes with cells (h)	
4	15
3	4
2	2
1/ 5 / 48	1
Incubation medium	
Without serum	12
With serum	6
With and without serum	4
Unclear	2
Expression readout	
Luciferase	15
(E)GFP	5
B-galactosidase	3
Silencing	1
Toxicity readout	
MTT/XTT	13
WST-1	2
LDH/ Resazurin reduction assay/ MTS proliferation assay/ NADH Tetra Color ONE cell proliferation assay system/crystal violet assay	1
None	5
Physicochemical characterization	
Dynamic Light Scattering (DLS)	19
Zetapotential	14
Gel retardation	14
Fluorescence displacement assay	5
Microscopy(TEM/SEM/AFM)	5
Nuclease resistance assay	4

170-fold lower efficiency than Lipofectamine-mediated transfection. In the second paper, complexes of peptide (ALWKTLLKKVLKAPKKKRKVC) and plasmid (pEGFP-C1) prepared at a peptide/DNA charge ratio of 10 were reported to efficiently transfect ~50 % of treated cells, which was slightly more than observed in case of Lipofectamine (~40 % transfected cells) [20]. These papers differed with regard to cell type (BHK versus HeLa), DNA dose (30 µg/well in 6-well format versus unclear dose), read-out assay (luciferase versus EGFP), time of incubation of complexes with cells (24 h versus 4 h) and time between transfection and analysis (24 h versus 48 h).

To further illustrate variability within the field, a simple literature extraction was performed. At the time of writing, 3,571 articles have been published in the Journal of Controlled Release, of which 486 were identified in Pubmed to deal with gene delivery. After excluding reviews and work on oligonucleotides, siRNA and viral gene delivery this number was reduced to 300. Of these 300 papers, approximately half describe *in vitro* screenings of reagents for improved gene delivery (the remainder are focussed on targeting, tissue engineering, mechanistic studies, method development, viral/non-viral hybrids or were excluded because of lacking *in vitro* transfection data or irrelevance for other reasons). From this collection, the 24 papers most recently published (between January 2009 and May 2010) were screened for methods and variables used to study transfection, resulting in Table 1. This small selection of literature on the topic already illustrates the issue of variability in screening methods used and is representative for findings in many other journals.

The aim of this paper is to illustrate the variability in screening procedures and sensitivity of transfection outcomes to testing conditions and to propose a standardized screening protocol that might contribute to facilitate research in gene delivery through improved comparability between studies and laboratories. We specifically do not aim at optimizing transfection parameters for the purpose of maximizing protein expression by transfected cells as this will lead to unique protocols for each transfection reagent and for each cell type. For this type of information the reader is referred to product manuals supplied by manufacturers of transfection reagents. This manuscript is aiming at scientists working on the development of new synthetic gene delivery systems and looking for a standardized protocol for *in vitro* gene transfection.

Based on the literature extraction, parameters influencing transfection outcomes are identified (see Figure 1) and discussed. Effects of reagent:DNA ratio, cell confluency, dose, incubation time and read-out assay on transfection outcomes are investigated and optimized. Subsequently, the effect of buffers and serum on particle size (stability) and transfection efficiency is shown. Additionally, the variability among commonly used reference reagents (positive controls) is discussed. Finally, transfection efficiencies are compared in a selection of dividing cells as well as in non-dividing cells. For transparency of results, studies were not performed with a novel reagent, but with a selection of reagents that are commonly used and commercially available to all gene delivery research laboratories. Using these reagents, a set of data is supplied disclosing sensitivity of transfection studies, possible artefacts and biased conclusions. Based on our dataset, we would

like to suggest a common standard to be included in gene delivery research that allows easier comparisons/ data extrapolation between labs in the future.

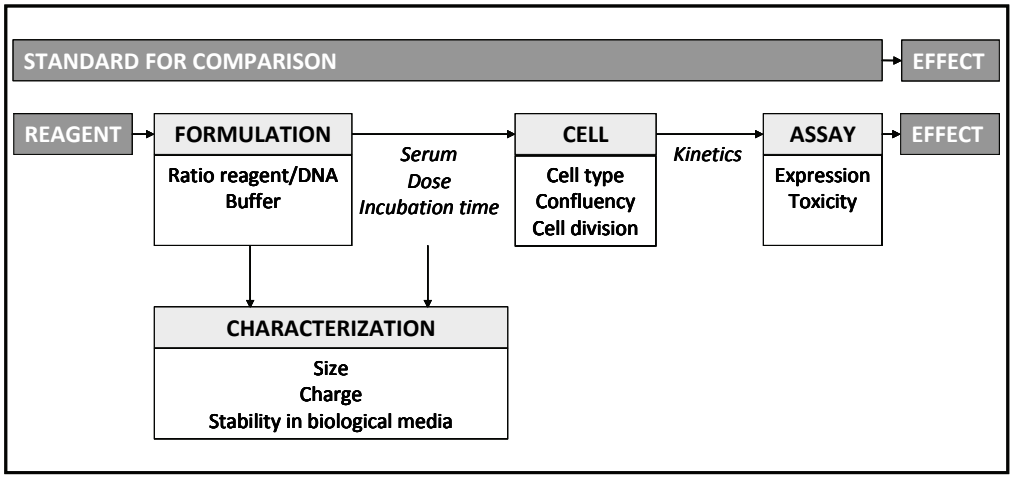


Figure 1. *Schematic overview of parameters affecting transfection efficiency.*

Discussion of parameters affecting transfection

Cell type dependency

The majority of transfection studies investigating the potential of gene delivery are performed in adherent monolayer cell culture systems. An extensive number of different cell types have been used, which vary in species (e.g. human, murine, rat or monkey), tissue type (e.g. liver, kidney, endothelium, muscle) and proliferation characteristics (e.g. cancer cell lines, virally-transformed cell lines, primary cells). Typical examples of model cell lines often used in gene delivery research include HeLa, HEK-293 (with or without transformation with SV40 large T Antigen) and COS-7. Transfection efficiencies are highly variable among different cell lines. A clear example of this cell type dependency was observed upon transfection with pDMAEMA, a polymer for gene delivery developed in our laboratory [45,46]. This polymer proved highly efficient in transfecting COS-7 cells, but failed to achieve transgene expression in HeLa cells (see Figure 2).

In this paper, transfection efficiencies of commonly used reference reagents will be tested in four different cell lines. HeLa and COS-7 were selected as typical model cell lines with HeLa being the first and most used cell line in research and COS-7 being a cell line often used in gene delivery studies for its ease of transfection. In addition to these model cell lines, we selected OVCAR-3 (an ovarian carcinoma cell line) and HUVEC (used as a model for angiogenesis and vascular pathology) to represent cell lines relevant for a possible *in vivo* application.

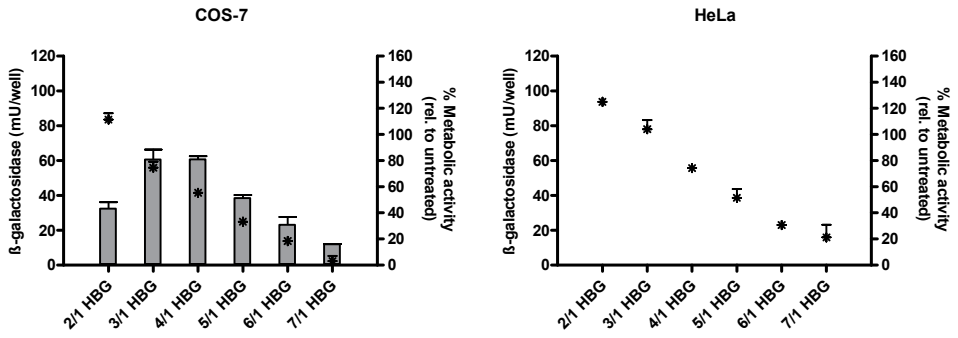


Figure 2. *Cell type dependent transfection by pDMAEMA. COS-7 (left) and HeLa (right) cells were transfected with pDMAEMA/pCMV-LacZ complexes prepared at various N/P ratios in Hepes buffered glucose. Complexes were incubated with cells for 4 h in the presence of 10 % serum and analyzed for gene expression (bars; left y-axis) and metabolic activity (asterices; right y-axis) 48 h after transfection.*

Dose of DNA per cell

The final dose of DNA reached per cell in *in vitro* experiments is determined by the number of cells per well (commonly expressed as % confluency), the dose applied per well and the time during which complexes are incubated with cells. Most transfection experiments are performed at a confluency of 60-80 %. In contrast, large variations in incubation times and DNA dose are used. Incubation of complexes with cells ranges from 1 h to continuous exposure until time of analysis and DNA doses vary from 0.2 to 1 µg DNA/well (96-well format). Increasing incubation time and/or dose can affect transfection outcomes in various ways: (1) more complexes can be processed by the cells leading to increased transfection, (2) longer exposure and uptake of more complexes can result in increased toxicity, (3) more particles reach the bottom of the wells where the cells are attached [47]. It is therefore important to find the suitable dose and incubation time that minimizes bias due to different sedimentation times of variously sized complexes while maximizing gene expression and minimizing toxicity.

Readout assays

Transfection efficiency is often determined with plasmids containing reporter genes under the control of strong, constitutively active promoters (e.g. cytomegalovirus (CMV)). Read-out of transgene expression can be direct (detection of EGFP) or indirect based on enzymatic conversion of an added substrate to a colored (conversion of ONPG by β-galactosidase) or luminescent (conversion of luciferin by luciferase) product. Detection of the fluorescent protein or colored/luminescent products can be at the single cell level (flow cytometric or microscopic analysis) or a batch analysis (detection of collective protein/ product from all cells in a well). Each assay is characterized by differences in sensitivity, signal-to-noise ratio, kinetics of expression and analytical techniques required for detection. In this paper, three of the most commonly used

reporter gene assays will be compared: luciferase, EGFP and β-galactosidase. Similarly, toxic effects of formulations can be studied with various assays. Since toxicity screening is not the major topic of attention in this paper, cell viability measurements are limited to the most widely used assay: detection of dehydrogenase activity in active mitochondria by the MTT/XTT assay. This assay belongs to the category of functional viability assays, as opposed to membrane integrity assays such as the lactate dehydrogenase (LDH) assay. The LDH assay is based on detection of LDH released from cells with compromised membranes and since the half-life of released LDH is approximately 9-10 hours [48], this assay is particularly suitable to study immediate toxicity. MTT/XTT assays are useful to study toxicity and efficiency of gene delivery in parallel at later timepoints after treatment.

Reference reagents/ positive controls

In literature, a great variety of control reagents included for estimation of transfection efficiency of a compound under investigation are described, including naked plasmid DNA, 22 kDa linear pEI (or linear pEI of other molecular weights), 25 kDa b-pEI (or branched pEI of other molecular weights), Lipofectamine, Lipofectin, Superfect, GenePorter, Fugene, pDMAEMA, polylysine, DOTAP. In studies aiming at improving a certain polymer through for example modification with polyethyleneglycol (PEG) or targeting ligands, or by modifying molecular weight, crosslinking or charge density, the unmodified polymer is usually included for reference. Including independent reference reagents is however valuable to enable comparison among experiments and laboratories. Here, we select three frequently described reference reagents, being 22 kDa l-pEI, 25 kDa b-pEI, Lipofectamine™2000 and naked plasmid DNA and compare their transfection efficiencies regarding gene expression and toxicity in one experiment. In this experiment, the effects of frequently used dispersion media (see Table 1) in which complexes are prepared will also be evaluated. Ionic strength is known to affect complex formation and particle aggregation [47,49]. Effects of dispersion media on transfection efficiency have been described in several cases [47,50] but are often unrecognized or undervalued. In this paper, Hepesbuffer (being the most commonly used) is chosen and supplemented with either NaCl (high ionic strength) or glucose (low ionic strength) for isotonicity reasons. The effect of serum is important to predict *in vivo* potential of new reagents developed for systemic administration and will therefore also be addressed here.

Physicochemical characterization

When developing reagents for non-viral gene delivery, the ideal scenario is to have well-defined nanoparticles, preferably <100 nm, which are stable in physiological media and efficiently transfect cells with minimal toxicity. In practice, DNA-complexes are mostly formed via electrostatic interaction with positively charged polymers, lipids or peptides. Characteristics of the resulting particles depend on the ratio of cationic reagent to negatively charged DNA. Increasing charge ratios >1 results in decreasing particle sizes (measured by DLS or microscopic techniques such as

TEM, SEM and AFM), increasing zetapotentials, altered electrophoretic mobility (measured in gel retardation assays), increased compaction (as measured by fluorescence displacement assays) and increased protection of DNA against degradation (measured in nuclease resistance assays) until an optimum is reached. Usually this optimum is reached under conditions of considerable excess of the cationic delivery agent. The upper limit of reagent:DNA ratios is determined by the cytotoxicity of the formulations. It is therefore very important to optimize reagent:DNA ratios based on particle characteristics as well as cellular effects. The optimal ratio is that at which small stable particles are formed and the best activity/toxicity profile is obtained. In this paper, characterization of formulations is performed by two of the most commonly used methods, being Dynamic Light Scattering (DLS) and zetapotential measurements. Additionally, their stability upon exposure to physiological salt concentrations and biological media is studied. The latter also requires measurement in the presence of serum. However, methods to measure submicron particles in the presence of serum are still limited. The heterogeneous nature of serum can give rise to significant background signal, whereas sizing techniques based on conventional light scattering (i.e. DLS) require minimum levels of background noise for generation of accurate results. Moreover, samples of polymer/DNA complexes are typically dilute in nature and often contain large fractions of free polymer [51], which can interact with serum proteins and form aggregates that strongly interfere with size measurements by conventional light scattering techniques [45,52]. Recently, in our lab we developed a high throughput method based on flow cytometry which allows measurement of particle size distributions of submicron particles (>100 nm) directly in biological fluids based on simultaneous detection of fluorescence and side scatter intensity (SSC)[52]. This method will be used to study effect of serum on particle size distributions and the obtained results will be related to transfection outcomes.

Materials and methods

Material

pCMV-LacZ and pCMV-Luc plasmid DNA was purchased from the Plasmid Factory, Bielefeld, Germany. Plasmid pCMV-EGFP was constructed from pShooter (Invitrogen, Oregon, USA): the EGFP coding sequence from pEGFP-N1 (bp 613–1410; Clontech, Saint-Germain-en-Laye, France) was amplified by PCR and cloned into the multiple cloning site of pShooter from which the nuclear localization signal (NLS)-tag coding sequence was removed by restriction with NotI and XbaI. pCMV-LacZ, pCMV-luc and pCMV-EGFP are expression plasmids encoding for β-galactosidase, firefly luciferase and enhanced green fluorescent protein (EGFP), respectively, under the transcriptional control of the human cytomegalovirus promoter (CMV).

Exgen 500 (22 kDa l-pEI) was purchased from Fermentas, St. Leon-Roth, Germany. LipofectamineTM 2000 (lipofectamine), OPTI-MEM I (Optimem) and propidium iodide (PI) were

purchased from Invitrogen, Breda, The Netherlands. Poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA, M_n 150 kDa/ M_w 2,600 kDa) was synthesized in house as previously described [46]. DMEM (Dulbecco's modification of Eagle's medium, with 3.7 g/l sodium bicarbonate, 1 g/l l-glucose, l-glutamine), RPMI 1640, antibiotics/antimycotics (penicillin, streptomycin sulphate, amphotericin B), 200 mM L-glutamine solution (100x), Foetal Bovine Serum (FBS) and phosphate buffered saline (PBS) were purchased from PAA Laboratories GmbH, Pasching, Austria). EGM™-2 endothelial cell growth medium-2, EGM-2 bullet kits and MycoAlert® Mycoplasma Detection Kits were purchased from Lonza (Verviers, Belgium). LabelIT Fluorescein and Cy5 Nucleic Acid Labeling Kits were purchased from Mirus Bio, Madison, WI, USA. Luciferase assay reagent and Reporter Lysis Buffer 5x were obtained from Promega (Leiden, The Netherlands). Micro BCA Protein Assay kits were purchased from Pierce (Perbio Science, Etten-Leur, The Netherlands). All other chemicals were bought from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Preparation of polyplexes and lipoplexes

Complexes were prepared by adding 4 volumes of polymer/ lipid solution to 1 volume of plasmid solution (50 µg/ml) and mixing immediately by pipetting up and down 10 x followed by 30 min incubation at room temperature. Solutions were prepared either in Hepes buffered glucose (HBG; 20 mM Hepes, 5 % (w/w) glucose, pH 7.4), Hepes buffered saline (HBS; 20 mM Hepes, 130 mM NaCl, pH 7.4) or Optimem. Optimem is composed of Eagle's Minimal Essential Medium, buffered with Hepes and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine or GLUTAMAX, trace elements, growth factors, insulin and transferrin (total protein level 15 µg/ml), phenol red and CaCl2 (99.9 mg/l) [53]. For each experiment, independent plasmid/carrier preparations were prepared freshly in triplicate. The ratios of polymer/lipid to DNA were optimized based on transfection outcomes and size measurements. N/P ratios indicate the molar ratio of nitrogens within polymers to phosphates in DNA.

Size measurement by Dynamic Light Scattering (DLS)

Particle size of the polyplexes was measured in 150 mM NaCl, HBS (viscosity 0.89 cP, refractive index 1.333), HBG (viscosity 1.145 cP, refractive index 1.3402) or Optimem or DMEM (viscosity 0.8228 cP, refractive index 1.4), as indicated, with Dynamic Light Scattering (DLS) on an ALV CGS-3 system (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22mW He-Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator and a temperature controller (Julabo water bath) set at 25 °C. Time correlation functions were analyzed to obtain the hydrodynamic diameter of the particles (Z_h) and the particle size distribution (polydispersity index, PDI) using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by the cumulants method (fitting a single exponential to the correlation function to obtain the mean size (Zave) and polydispersity index (PDI)) and CONTIN (to fit a multiple exponential to the correlation function to obtain particle size distributions). The diffusion coefficients calculated from the measured autocorrelation functions were related to the

hydrodynamic radius of the particles via the Stokes-Einstein equation, $Z_h = (k_B T q^2)/(3\pi\eta\Gamma)$, where Z_h is the hydrodynamic radius of the particles, k_B is the Boltzmann constant, T is the absolute temperature, η is the solvent viscosity, Γ is the decay rate, and q is the scattering vector ($q= 4\pi n \sin(\Phi/2))/ \lambda$), in which n is the refractive index of the solution, Φ is the scattering angle, and λ is the wavelength of the incident laser light. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle. The system was calibrated with 200 nm polystyrene latex standard beads (Duke Scientific, Leusden, The Netherlands).

Zetapotential measurements

The surface charge of the polyplexes was measured in 20 mM Hepes, pH 7.4 (viscosity 0.89 cP, dielectric constant 79) using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, UK) with universal ZEN 1002 'dip' cells and DTS (Nano) software (version 4.20). The Helmholtz–Smoluchowski equation was used for converting electrophoretic mobilities into zeta potentials. The system was calibrated with DTS 1050 latex beads (Zeta Potential Transfer Standard, Malvern Instruments, Malvern, UK).

Flow cytometry for analysis of particle size distribution in serum-containing medium

Particle size distributions in serum-containing medium were determined as previously described [52]. In short, reagent/DNA complexes were prepared with FITC-labelled plasmid DNA and Side scatter (SSC) values measured by flow cytometry were converted to size based on a calibration curve obtained with standard FITC-labelled polystyrene beads of 100-2000 nm. A threshold set on FL-1 (set to linear amplification) was used to exclude side scatter values from non-fluorescent particulate matter.

Cell culture

Human epithelial ovarian carcinoma cells (HeLa) were grown in DMEM supplemented with antibiotics/antimycotics and 10 % FBS. COS-7 African Green monkey kidney cells were cultured in DMEM supplemented with antibiotics/antimycotics and 5 % FBS. OVCAR-3 cells were cultured in RPMI1640 supplemented with antibiotics/antimycotics, 20 % FBS, sodium pyruvate (1 mM), Hepes (10 mM), bovine insuline (0.01 mg/ml) and glucose (5.4 g/l). These three cell lines were originally obtained form the American Type Culture Collection (ATCC, Maryland, USA).

HUVEC human umbilical vein endothelial cells (HUVECs) originate from Glycotech Corporation, (Rockville Maryland, USA, cat. No. 41-001). HUVECs were cultured in EGM™-2 endothelial cell growth medium-2 consisting of EBM-2 medium supplemented with an EGM-2 bullet kit and used to a maximal passage number of 7.

Cells were maintained at 37 °C in a 5 % CO_2 humidified air atmosphere and split once (OVCAR-3) or twice (HeLa, COS-7, HUVEC) weekly. Cells were confirmed to be free from mycoplasm by periodical testing with a MycoAlert® Mycoplasma Detection Kit.

Transfection

For ONPG and XTT assays cells were seeded into 96-well tissue culture plates 24 h prior to transfection, such that ~80 % confluency was reached on the day of transfection. This corresponded to 8,000 (HeLa), 10,000 (COS-7), 20,000 (OVCAR-3) or 5,000 (HUVEC) cells/well. For flow cytometry analysis, 59,000 (HeLa) cells were seeded per well into 24-well tissue culture plates 24 h prior to transfection, such that ~80 % confluency was reached on the day of transfection. For studies in non-dividing cells 8,000 (HeLa) cells were seeded per well into 96-well tissue culture plates 48 h prior to transfection and medium was replaced by complete medium supplemented with 15 µM aphidicolin 24 h prior to transfection. Cells were continuously exposed to aphidicolin from at least 16 hours prior to transfection until time of analysis.

Immediately prior to transfection the culture medium was refreshed with 100 µl (ONPG/luciferase/XTT assay) or 600 µl (EGFP assay) DMEM supplemented with 10 % FBS (and 15 µM aphidicolin where applicable). 25 µl (ONPG/luciferase/XTT assay) or 150 µl (EGFP assay) of the polyplex or lipoplex samples (corresponding to 0.25 µg DNA/well in 96-well format and 1.5 µg DNA/well in 24-well format) was added per well and after 4 h incubation, medium was replaced with fresh DMEM supplemented with 10 % FBS (and 15 µM aphidicolin where applicable). Cells were incubated for indicated times at 37 °C in a 5 % CO_2 humidified air atmosphere until analysis. Experiments were performed in triplicate.

Cell cycle analysis

To confirm arrest of cells in the S-phase, cell cycle analysis was performed based on flow cytometric analysis of cell DNA content following cell staining with propidium iodide (see Figure 3). After synchronization treatment, the cells were washed two times with PBS to ensure removal of all dead cells, harvested and fixed. Fixation was achieved by incubating the cells in a 300 µl PBS/700 µl ethanol solution at -20 °C for at least 1 hour. After fixation the ethanol/cell solution was underlayered with 1 ml FCS before being spinned down and resuspended in 500 µl (for $1 \cdot 10^6$ cells) PBS containing PI and RNase (930 µl PBS, 50 µl PI (1 mg/ml), and 20 µl RNAse (5 mg/ml)). After 1 h incubation at room temperature the cells were analyzed with a FACSCantoII cytometer (Becton and Dickinson, Mountain View, CA, USA) equipped with a 488 nm 20 mW Solid State diode laser and a 633 nm 20 mW HeNe laser. 10,000 cells were recorded per sample and cell cycle analysis of DNA profiles was performed using Cylchred software, available from Cytonet UK.

β-galactosidase assay

At the indicated time after transfection, cells were washed 1x with 100 µl ice-cold PBS and lysed with 20 µl lysisbuffer (50 mM Tris/HCl buffer (pH 8.0), 150 mM NaCl and 1 % Triton X-100) during 20 min at 4 °C. Next, 180 µl ONPG-staining solution (18.5 ml PBS, 200 µl 0.1M $MgCl_2$-solution and 1.35 ml 10 mg/ml ONPG-solution in PBS) was added and enzyme activity of β-galactosidase was determined by measuring absorbance at 405 nm relative to the absorbance at 655 nm after 30 min incubation at 37 °C. Expression data were corrected for background values of untreated cells.

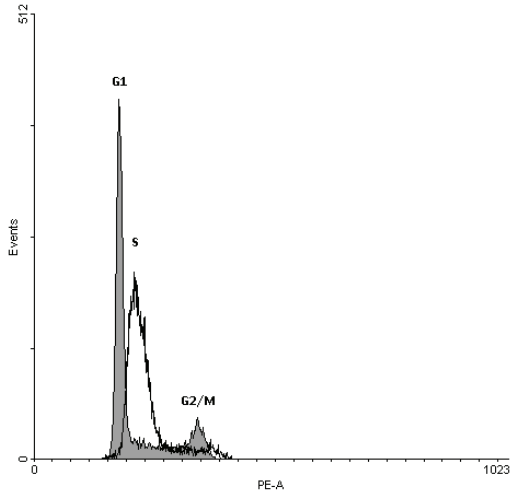


Figure 3. *Arrest of HeLa cells in S-phase by treatment with 15 µM aphidicolin. Untreated cells (grey) and cells incubated for 24 h with 15 µM aphidicolin (transparent overlay) were fixed, stained with PI and analyzed for total DNA content by flow cytometry.*

Luciferase and BCA assay

Experiments were performed in duplicate in two separate 96-well plates. One series was used for luciferase assay and one for protein determination. For the luciferase assay, at the indicated time after transfection, cells were washed 1x with 100 µl icecold PBS and lysed with 50 µl lysis buffer (Reporter Lysis Buffer 5x, diluted with MilliQ water). A freeze-thaw cycle was performed by incubating samples for 1 h at -80 °C. Next, 5 µl cell lysate was mixed with 10 µl Luciferase Assay Reagent and after 2 seconds luminescence was measured over 10 seconds using a Mithras LB 940 Multilabel Reader (Berthold Technologies, Bad Wildbad, Germany). For the BCA assay, after the indicated time after transfection, cells were washed 1x with 100 µl ice-cold PBS and lysed with 50 µl lysis buffer (0.1 M NaOH). Then, 5 µl cell lysate was added to 145 µl PBS and total protein content was determined using a Micro BCA Protein Assay kit according to the manufacturer's instructions. Results were corrected for background values and expressed as relative light units (RLU) per µg cellular protein.

Flow cytometry

At the indicated time after transfection, cells were washed, trypsinized and resuspended in DMEM supplemented with 10 % FBS to inactivate the trypsin. Cells were transferred into round-bottom 96-well plates and centrifuged for 5 min at 250 x g at 4 °C. Medium was removed and cells were resuspended in 200 µl phosphate-buffered albumin (PBA; 1 %, w/v albumin in PBS).

Immediately prior to measurement, 20 µl PI solution (10 µg/ml in water) was added for live/dead cell discrimination. Flow cytometric analysis was performed on a FACSCantoII (Becton and Dickinson, Mountain View, CA, USA) equipped with a 488 nm 20 mW Solid State diode laser and a 633 nm 20 mW HeNe laser. 10,000 cells were recorded per sample to determine EGFP expression (FITC-channel) and PI-staining (PE-channel).

Mitochondrial activity (XTT)

Cell viability was determined using an XTT colorimetric assay based on cleavage of a tetrazolium reagent to form an orange formazan dye, which is indicative for metabolic activity. 48 h after transfection, medium was replaced (to correct for evaporation) and after 2 h 50 µl XTT-solution (25 µM PMS and 1 mg/ml XTT in plain RPMI 1640) was added per well and incubated for 1 h at 37 °C in a CO_2-incubator. Absorbance was measured at 490 nm with a reference wavelength of 655 nm. Cell viability was expressed as the relative metabolic activity normalized against HBS-treated cells.

Results

Optimization of transfection screening protocol

Optimization of reagent: DNA ratio

First, the optimal reagent:DNA ratios for the selected reagents were determined. 22 kDa linear pEI (l-pEI) and lipofectamine were diluted and mixed with plasmid DNA in the dispersion media recommended by the manufacturer (150 mM NaCl and Optimem, respectively). For 25 kDa branched pEI (b-pEI), HBS was used which we previously found to be optimal.

Both l-pEI and lipofectamine gave highly aggregated complexes when used according to the manufacturer's instructions (Figure 4). As in these cases ratio optimization based on physicochemical properties is not possible, ratios at which the best expression:toxicity ratio was observed were chosen (linear pEI: N/P 6; lipofectamine: 2 µl/µg DNA). For b-pEI, particle size was monitored as a function of N/P ratio and it was observed that at N/P ratios >3.75 small particles of ~200 nm were formed in HBS. Combining this finding with the transfection outcomes, N/P 5 was selected to give the highest gene expression and the lowest toxicity.

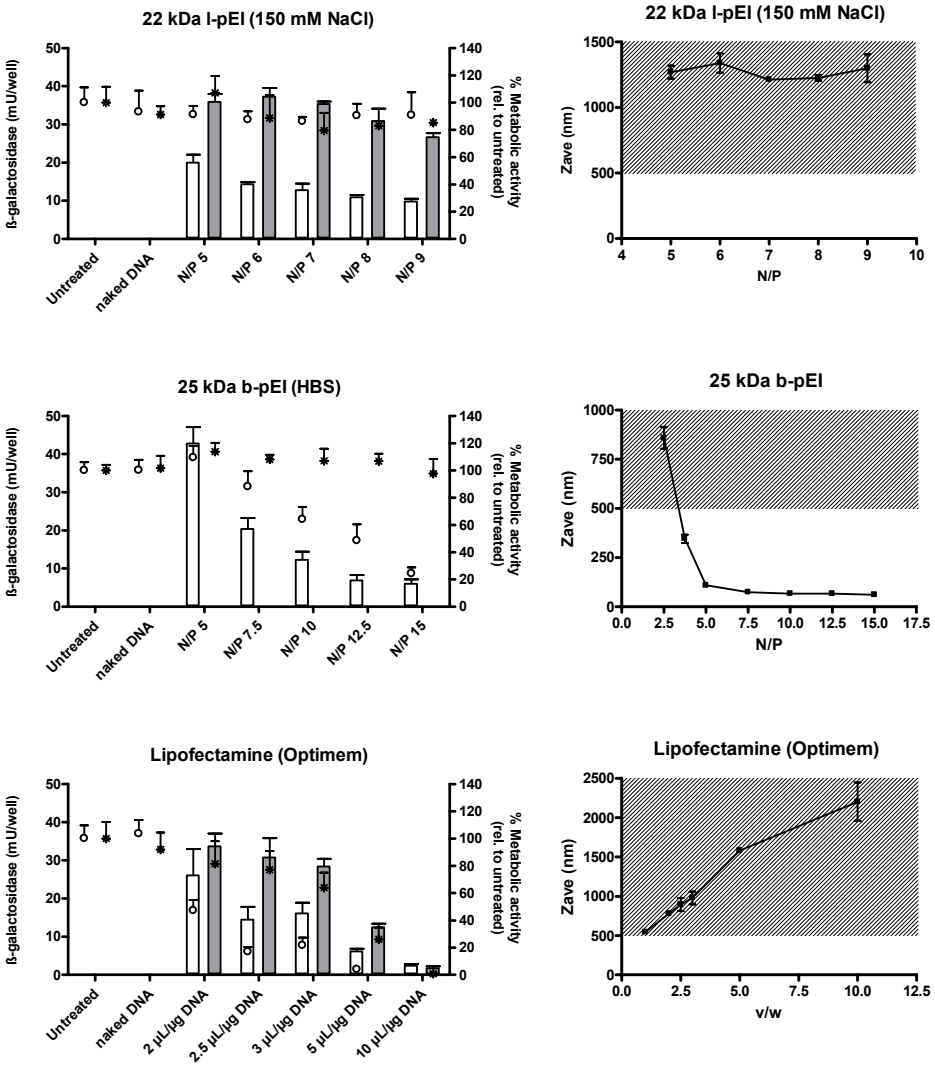


Figure 4. *Optimization of reagent:DNA ratio based on in vitro transfection and particle size analysis. l-pEI and lipofectamine were diluted and mixed with pCMV-LacZ plasmid in the dispersion medium recommended by the manufacturer; b-pEI was diluted and mixed with pCMV-LacZ plasmid in HBS (as was found optimal in our lab). Left graphs: gene expression (bars) and cell viability (circle and asterix) after transfection of Hela cells in the absence (white bar/circle) or presence (grey bar/asterix) of 10 % serum with complexes prepared in the indicated dispersion medium. Gene expression and cell viability were measured 48 h after transfection. Data are expressed as mean+SD. Right graphs: hydrodynamic diameter of complexes prepared in the indicated dispersion medium measured by DLS 30 minutes after complexation. Dashed area indicates aggregated particles that cannot be accurately sized by DLS.*

Cell confluency

To determine the effect of cell confluency on the level of transgene expression and cell viability, cells were seeded at densities ranging from 2,000-12,000 cells/well (96-well format) and transfected with l-pEI/DNA complexes of fixed composition. 48 h after transfection, transgene expression and metabolic activity were analyzed. Figure 5 shows a clear dose-response relation both regarding gene expression and metabolic activity at densities up to 8,000 cells/well (corresponding to 80 % confluency at time of transfection). At densities below 8,000 cells/well, cell viability drops below 80 % of untreated cells. Seeding >8,000 cells/well did not give a further increase in gene expression, possibly due to the observed relative decrease in metabolic activity. Therefore, a density of 8,000 cells/well (96-well format) was selected to be optimal for transfection studies in HeLa cells.

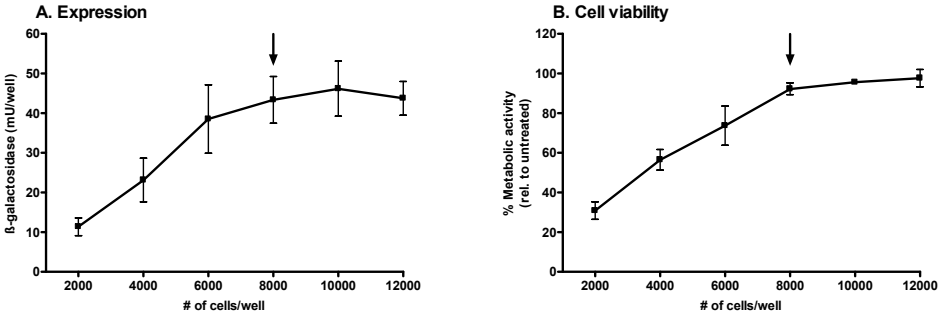


Figure 5. *Effect of confluency on gene expression (A) and cell viability (B). HeLa cells were seeded at densities ranging from 2,000 to 12,000 cells per well (96-well format) and after 24 h transfected in the presence of 10 % serum. l-pEI/pCMV-LacZ complexes were prepared in HBS at an N/P ratio of 6 and incubated 4 h with the cells at a dose of 0.25 µg DNA/well. Gene expression and metabolic activity were analyzed 48 h after transfection. Metabolic activity is expressed relative to buffer-treated cells seeded at corresponding densities. Data are expressed as mean+SD. Arrows indicate the selected confluency for further experiments.*

DNA dose and incubation time

Next, DNA dose and incubation time were optimized. Optimizations were performed for all reagents and representative examples are shown in Figure 6 (data in other buffers and in absence of serum are available in the Supplementary information, Figure S1).

After one hour incubation of l-pEI/DNA or lipofectamine/DNA with cells, an expected dose-response relation was observed, with the highest dose giving the highest expression levels. Maximal expression was however observed after an incubation time of four hours, at which time the dose-response relation inverted and the lowest dose of 0.25 µg/well (96-well format) gave the best transfection. Surprisingly, this finding was not accompanied by differences in cell viability between the tested doses. It is however possible that toxicity occurred at a functional level left

undetected by the XTT assay, for example by carrier-mediated interference of cellular mRNA or protein expression [54]. At increasing incubation time, dose-dependent toxic effects started to dominate. For b-pEI, expression levels increased with increasing incubation time and/or dose, but expression levels remained relatively low at all times (<10 %). Inefficient transfection of b-pEI in the presence of serum was also observed in Figure 3. A positive effect of (mild) toxicity on transfection efficiency has previously been suggested and could explain the transfection behavior of b-pEI observed here [46]. Incubation of cells with 0.25-0.5 or 1 µg naked plasmid DNA for 1-24 h did not give any gene expression under any of the circumstances tested (data available in Supplementary information, Figure S1 D).

Read-out and kinetics

To compare results of transfections with three common read-out systems, Hela cells were transfected with either pCMV-LacZ, pCMV-luc or pCMV-EGFP and analyzed at several timepoints after transfection. Data obtained for l-pEI- and b-pEI-based polyplexes prepared in HBS and lipofectamine/DNA complexes prepared in Optimem are shown in Figure 7 (results for other buffers and in the absence of serum are shown in the Supplementary information, Figure S2). The three readout systems did reveal differences in onset and peak of expression levels, but the overall observed trends were similar.

Table 2. *Characteristics of reporter gene assays (partially based on [55]).*

Advantages	Disadvantages
β-galactosidase + ONPG → colorimetric detection	
+ Cheap and easy + Variety of substrates available allowing different and more sensitive analysis: fluorescence (FGD), luminescence (1,2-dioxetane-β-gal) + Possibility for histochemical staining → single cell analysis + Stable reporter protein + *In vitro* and *in vivo*	- Slower onset and peak of expression detection - Lower sensitivity (when using non-luminescent assays) - Background signal from endogenous β–galactosidase in some cell types (NB *in vivo)*
Luciferase + luciferin → luminescence detection	
+ Fast onset and peak of expression detection + Sensitivity + *In vitro* and *in vivo*	- Expensive - No histology possible - Only batch analysis possible - Relatively labile reporter protein
EGFP → fluorescence detection	
+ No substrate required + Cheap, easy + Analysis at single cell level and batch analysis possible + Allows various types of analysis: flow cytometry, microscopy or fluorimetric + *In vitro* and *in vivo*	- Relative insensitivity of fluorimetric assay makes it less suitable for high throughput screening - Microscopy and flow cytometry require expensive equipment and are less suitable for high-throughput screening

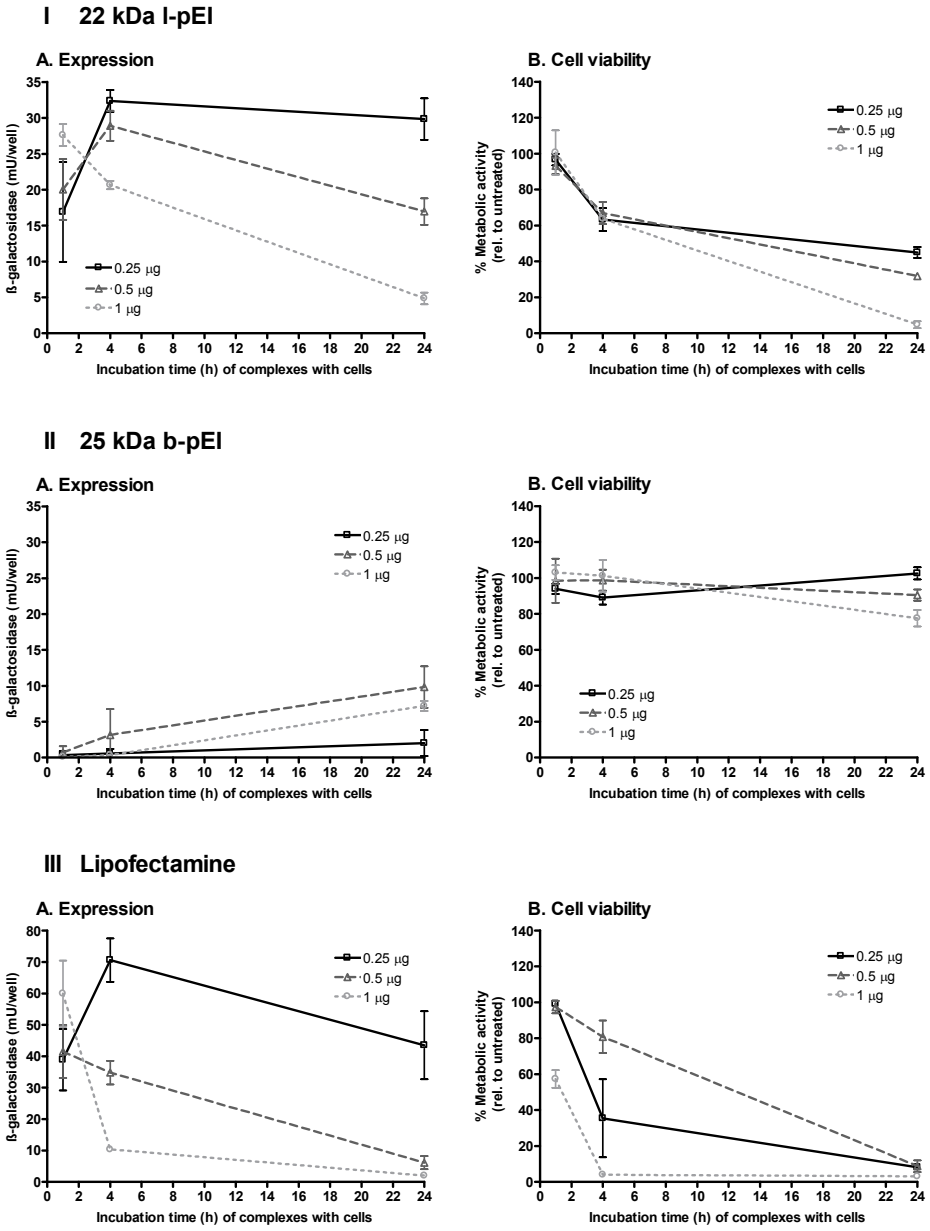


Figure 6. *Effect of DNA dose and incubation time on gene expression (A) and cell viability (B). HeLa cells were transfected in the presence of 10 % serum with l-pEI/DNA polyplexes prepared in HBS (I), b-pEI/DNA polyplexes prepared in HBS (II) or lipofectamine/DNA prepared in optimem (III). Gene expression and cell viability were measured 48h after transfection. Cell viability is expressed as the metabolic activity relative to that of untreated cells at the corresponding time. Data are expressed as mean+SD.*

The β-galactosidase assay had a slower onset (~8 h) and peak (48 h) than either EGFP or luciferase (onset 4 h; maximum at 24-48 h). As expected, the luciferase assay had the lowest detection limit. This was the only assay in which transfection with b-pEI in the presence of serum could be detected, but levels were very low (approximately 3 orders of magnitude lower than for l-pEI and lipofectamine). Regardless of the assay chosen, transfection efficiency would be ranked l-pEI ≥ lipofectamine >>> b-pEI. Since the reporter gene assay proved not crucial for the experimental outcome, the choice for a system can be made based on other parameters as listed in Table 2. In our lab, for reasons of extensive experience, β-galactosidase is chosen for general screening purposes while EGFP and luciferase assays can be employed for follow-up studies.

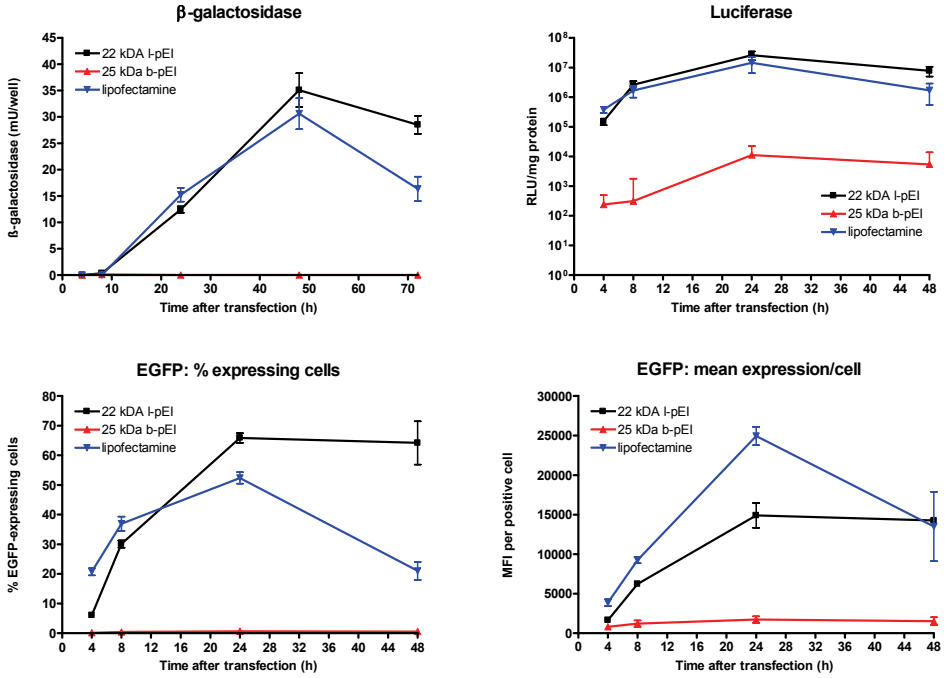


Figure 7. *Kinetics of gene expression for various read-out systems. HeLa cells were transfected with indicated complexes containing either pCMV-LacZ (A), pCMV-Luc (B) or pCMV-EGFP (C and D) and expression was measured in the presence of 10 % serum at the indicated timepoints post-transfection. 22 kDa l-pEI and 25 kDa b-pEI complexes were prepared in HBS and, lipofectamine complexes in Optimem. Complexes were incubated 4 h with cells (0.25 µg DNA/well in 96-well format for ß-galactosidase and luciferase assays; 1.5 µg/well in 24-well format for EGFP assay). Data are expressed as mean+SD.*

Effects of buffers and serum

Effect of buffers on particle size

Complexes of l-pEI/DNA, b-pEI/DNA and lipofectamine/DNA were prepared in three different buffers: 20 mM hepes buffer supplemented with either 130 mM NaCl (HBS) or 5 % glucose (HBG) to reach isotonicity or Optimem (for composition see materials and methods section), the recommended buffer for preparation of lipofectamine/DNA complexes. Table 3 shows particles sizes and zetapotentials of the investigated formulations and their stability in time. Preparation of l-pEI/DNA complexes in HBG resulted in stable particles of ~80 nm with a zetapotential between +20 and +30 mV. Particles prepared in either HBS or Optimem were observed to be highly aggregated. Complexation of DNA with b-pEI was found to be less affected by ionic strength (in agreement with other studies [56]). Preparation in HBG gave particles of ~65 nm with a zetapotential between +25 and +30 mV (see Table 3). In HBS, particles of ~200 nm were formed that gradually increase in size in time. In Optimem on the other hand, particles appeared to be highly aggregated. Lipofectamine formed highly aggregated complexes upon incubation with DNA in Optimem. Complexation in either HBG or HBS gave small particles <200 nm. Interestingly, when prepared in HBG at the ratio found to be optimal in Optimem (2 µl lipofectamine/ 1 µg DNA), lipofectamine formed complexes with a highly negative zetapotential (~-48 mV).

Table 3. *Particle size, PDI and zetapotential of complexes used for transfection studies in HeLa cells. Complexes were prepared as described in materials and methods in the indicated dispersion medium and analyzed undiluted 30 min, 4 h and 24 h after complexation. Particles aggregated to size exceeding the detection limit of DLS are shown in italic.*

		HBG			HBS		Optimem	
Reagent	**Time**	**Zave (nm)**	**PDI**	**ZP (mV)**	**Zave (nm)**	**PDI**	**Zave (nm)**	**PDI**
22 kDa l-pEI	30 min	88 (9)	0.11 (0.03)	28 (1)	*1490 (30)*	*0.3 (0.3)*	*940 (30)*	*0.2 (0.2)*
	4 h	70 (10)	0.2 (0.1)	21 (2)	*3100 (300)*	*0.7 (0.5)*	*1730 (50)*	*1*
	24 h	89 (4)	0.19 (0.03)	25 (3)	*1900 (900)*	*0.6 (0.4)*	*1900 (600)*	*0.5 (0.5)*
25 kDa b-pEI	30 min	62 (4)	0.20 (0.02)	28 (2)	202 (5)	0.08 (0.04)	*940 (40)*	*0.2 (0.3)*
	4 h	65 (4)	0.20 (0.03)	25 (1)	290 (10)	0.08 (0.08)	*1600 (100)*	*0.7 (0.5)*
	24 h	66 (4)	0.18 (0.01)	28 (1)	487 (6)	0.13 (0.06)	*1280 (70)*	*0.7 (0.6)*
Lipofectamine	30 min	122 (1)	0.10 (0.04)	-48 (1)	173 (3)	0.20 (0.03)	*840 (10)*	*0.19 (0.07)*
	4 h	124 (2)	0.17 (0.01)	-48 (2)	181 (2)	0.18 (0.03)	*850 (10)*	*0.2 (0.1)*
	24 h	129 (3)	0.12 (0.05)	-50 (2)	201 (4)	0.11 (0.09)	*700 (300)*	*1*

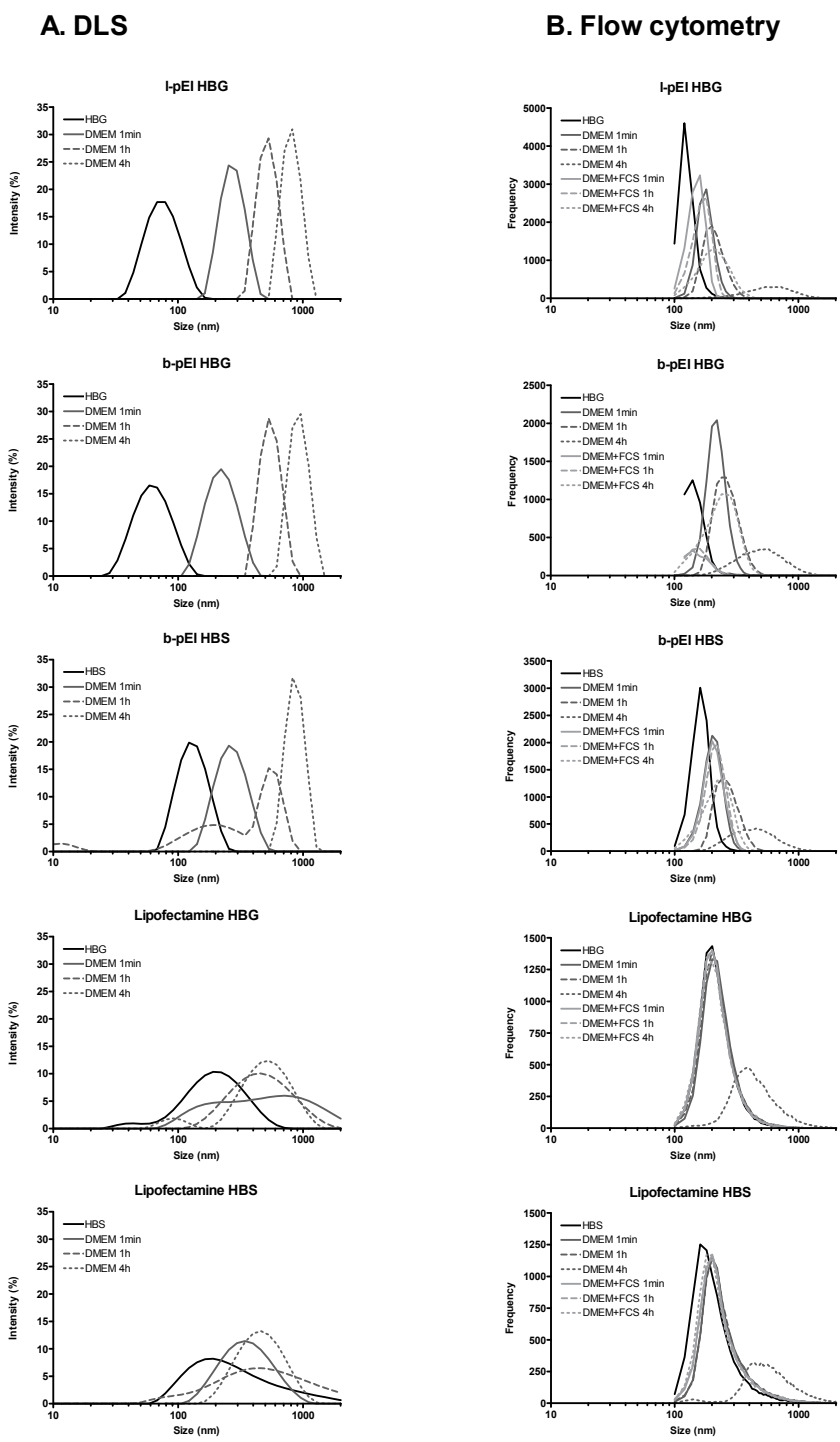
Effect of medium and serum on particle size

Next, the effect of culture medium with or without serum on particle size distributions was investigated. Figure 8 A shows size distributions of the indicated formulations upon incubation in DMEM as measured by DLS (only formulations that were not already aggregated in buffers are presented). All formulations showed a gradual increase in particle size in time, reaching ~1,000 nm for pEI-based formulations. In case of lipofectamine, sizes increased to ~600 nm in DMEM. The

lower intensity of the DLS-signal combined with the broadness of the distribution may reflect destabilization of the particles.

Using a flow cytometry-based method recently developed in our laboratory, particle size distributions upon incubation in DMEM with or without 10 % serum were studied (see Figure 8 B). Results obtained in DMEM correlate with those observed in DLS measurements; again a gradual increase in size is observed in time. The absolute sizes found by flow cytometry in general were smaller than those measured with DLS, which can be explained by the fact that DLS-derived diameters are highly biased towards large particles within a heterogeneous sample whereas flow cytometry analyzes single particles. Measurements in serum-containing medium revealed much less change in particle sizes than observed in DMEM without serum. pEI-based complexes showed an increase from ~100 nm to 200-300 nm after 4 h incubation whereas size of lipofectamine complexes was not affected at all. An explanation for the effects of DMEM without serum is that salt present in the medium induces aggregation caused by reduced electrostatic repulsion between particles. When using serum-supplemented DMEM, adsorption of negatively charged serum proteins onto the positively charged particles may lead to shielding of particles, thereby preventing severe aggregation [47].

Next page: Figure 8. *Particle size distribution upon incubation in cell culture medium. Complexes were prepared in the indicated dispersion medium and after 30 min incubation diluted in cell culture medium with or without 10 % serum. Size distributions were measured at indicated times after mixing with DLS (A; buffer and medium without serum) and flow cytometry (B; buffer, medium without and with serum).*


A. DLS
B. Flow cytometry
l-pEI HBG
b-pEI HBG
b-pEI HBS
Lipofectamine HBG
Lipofectamine HBS
HBG
HBS
DMEM 1min
DMEM 1h
DMEM 4h
DMEM+FCS 1min
DMEM+FCS 1h
DMEM+FCS 4h
Intensity (%)
Frequency
Size (nm)

Effect of buffers and media on transfection efficiency

Formulations of l-pEI, b-pEI and lipofectamine prepared in HBG, HBS and Optimem were compared regarding transfection efficiency and toxicity (see Figure 9). Dilutions of naked plasmid DNA in corresponding buffers were included for reference and were once more observed to lack transfection efficiency (as previously described).

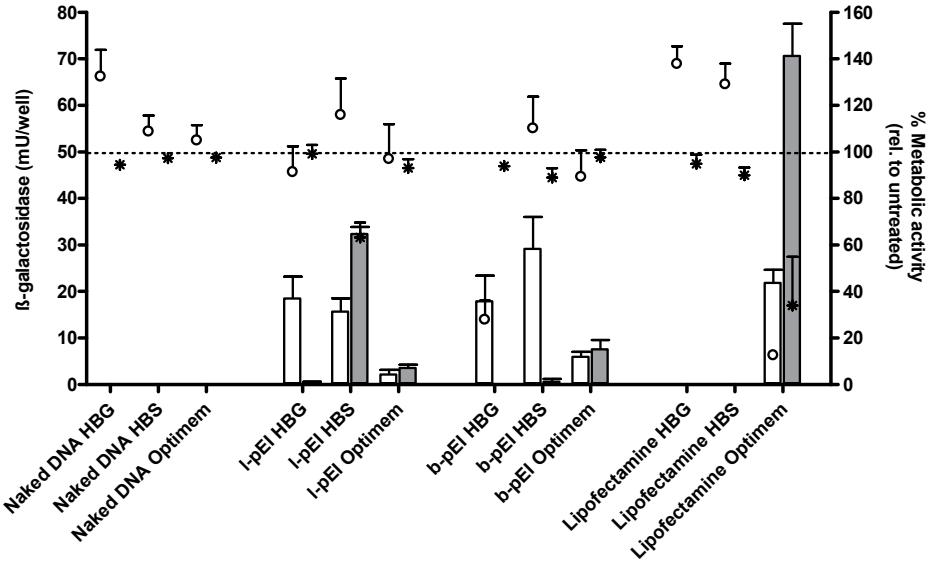


Figure 9. *Gene expression and cell viability obtained after transfection of HeLa cells with frequently used control formulations prepared in various buffers. Gene expression (bars) and cell viability (circle and asterix) after transfection of HeLa cells in the absence (white bar/circle) or presence (grey bar/asterix) of 10 % serum with complexes prepared in the indicated dispersion medium. Complexes were incubated 4 h with cells (0.25 µg DNA/well) and gene expression and cell viability were measured 48 h after transfection. Dashed line indicates 100 % viability.*

Transfection efficiencies of l-pEI, b-pEI and lipofectamine all proved highly sensitive to the buffer in which complexes were prepared as well as the presence of serum during incubation with cells. When looking at l-pEI, the highest transfection efficiency was observed for complexes prepared in HBS. These complexes performed a factor two better in the presence than in the absence of serum. On the contrary, complexes of l-pEI prepared in HBG only transfected in the absence of serum. Transfection efficiency of complexes prepared in Optimem was present but low, regardless of the presence of serum. Toxicity of all l-pEI-based formulations was negligible, except for complexes prepared in HBS (~60 % metabolic activity compared to untreated cells), the most efficient l-pEI formulation regarding gene expression.

Formulations of b-pEI prepared in HBG and HBS both transfected cells well in the absence, but not in the presence of serum. Preparation in Optimem resulted in low expression levels regardless of the absence or presence of serum. Significant toxicity was only observed upon transfection with HBG-formulations in the absence of serum (~30 % metabolic activity compared to untreated cells).

When using lipofectamine successful transfection was solely obtained for particles prepared in Optimem. Expression levels observed in the presence of serum were the highest among all formulations tested, but were accompanied by high toxicity (<40 % metabolic activity compared to untreated cells).

When comparing these transfection outcomes to the characterization results (obtained by flow cytometry), a tendency towards increased expression levels upon aggregation was recognized (see Figure 10). L-pEI complexes prepared in HBS or Optimem were >500 nm and yielded transgene expression both in the presence and absence of serum. Formulations prepared in HBG were ~80 nm and remained relatively small (mostly <300 nm) upon incubation in serum-containing medium, under which conditions no expression was detected. Incubation of these particles in serum-free medium however led to an increase in size >500 nm accompanied by successful transfection. Similarly, b-pEI formulations that were ~60 nm (HBG) or ~200 nm (HBS) in buffers and remained small (mostly <300 nm) upon incubation in serum-containing medium lacked transfection efficiency. Incubation of these particles in medium without serum or preparation of b-pEI/DNA complexes in Optimem yielded larger particles and successful transfection. Lipofectamine behaved somewhat differently, as small particles prepared in HBS and HBG did gain size upon incubation in serum-free medium, but lacked transfection efficiency. However, this may be explained by the highly negative zetapotential observed in HBG (see Table 3).

The preliminary size-effect plot shown in Figure 10 can be explained by mixed effects of sedimentation and optimal cellular processing. Promotion of *in vitro* transfection activity by the formation of aggregates of polyplexes has previously been described and was suggested to result from sedimentation followed by increased interaction with cell membranes and subsequent uptake[47,57,58]. Below the apparent optimum of 500 nm, particles lack (*in vitro*) efficiency due to limited sedimentation onto the cell monolayer, whereas at much larger sizes efficiency of cellular uptake and processing become limiting.

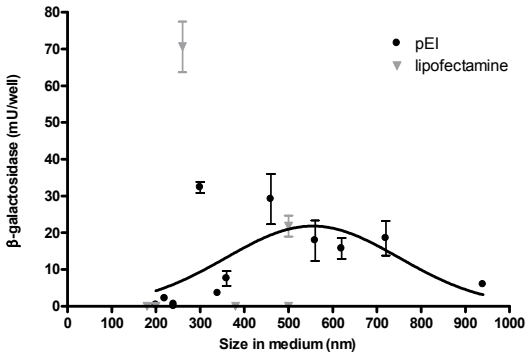


Figure 10. *Relation between size of complexes in medium and observed transfection efficiency. Sizes of complexes in DMEM and DMEM supplemented with 10 % serum were plotted against observed transfection efficiencies for the corresponding formulations. Results for l-pEI and b-pEI are pooled and depicted as black filled circles; lipofectamine was depicted separately in grey triangles.*

Cell type dependency

Figure 11 shows results obtained upon transfection of HeLa, COS-7, OVCAR-3 and HUVEC cells (see Table 4 for characteristics) with l-pEI/DNA complexes. Results obtained in absence of serum and for other reagents are available in the Supplementary information (Figure S3).

Obviously, overall expression levels varied greatly among these cell lines with transfection efficiency in COS-7 > OVCAR-3 > HeLa >>> HUVEC. One of the reasons why high expression levels are observed in COS-7 may be that COS-7 cells constitutively express the Simian Virus (SV40) large T antigen. Interaction of this antigen with SV40 origin of replication regions often present in reporter plasmids leads to plasmid replication [59]. Transfection efficiencies obtained in COS-7 are therefore likely to be an overestimation of actual transfection in normal tissues. In contrast, no transgene expression was observed for any of the tested formulations in HUVEC. Expression levels in HeLa and OVCAR-3 were observed to be intermediate between COS-7 and HUVEC. Optimal N/P ratios were observed to be dependent on both cell type and buffer used, indicating that ratios of reagent/DNA can be further optimized for a particular cell line.

Altogether, these results illustrate the variability among cell lines and illustrate the value of performing screenings in a selection of cell lines.

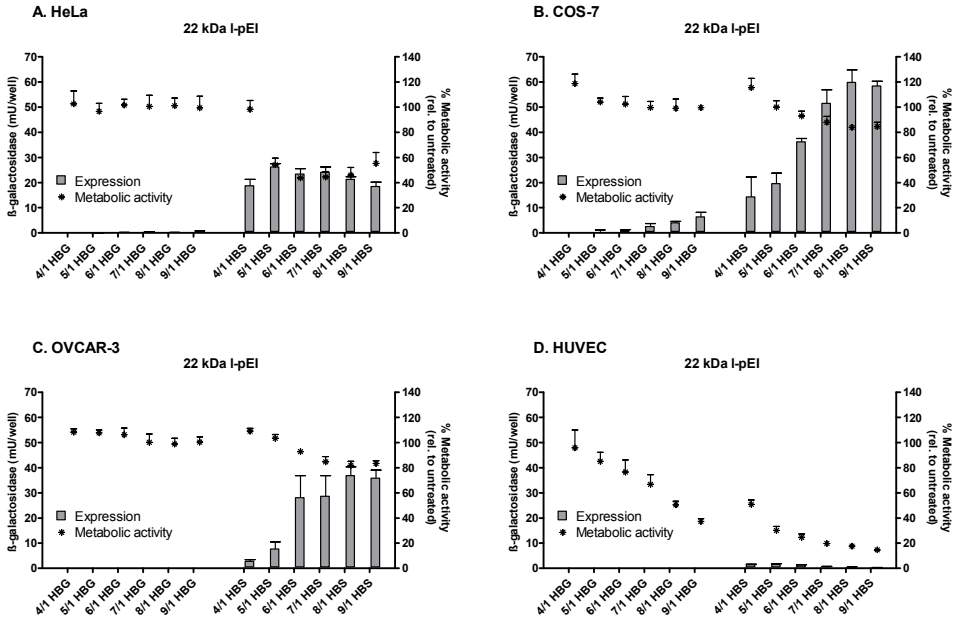


Figure 11. *Transfection efficiency in various cell lines. HeLa (A), COS-7 (B), OVCAR-3 (C) and HUVEC (D) cells were transfected with 22 kDa l-pEI/pCMV-LacZ complexes prepared at various N/P ratios in HBG or HBS. Complexes were incubated with cells for 4 h in the presence of 10 % serum and analyzed for gene expression (bars; left y-axis) and metabolic activity (asterices; right y-axis) 48 h after transfection.*

Table 4. *Characteristics of cell lines.*

Cells	Characteristics
HeLa	Human cervical cancer cells, model cell line, fast-dividing, intermediate-easy to transfect
COS-7	African green monkey kidney cells, fast-dividing, easy to transfect, SV40-immortalized
OVCAR-3	Human ovarian carcinoma cell line, slow-dividing cell line
HUVEC	Human umbilical vein endothelial cells, primary cells

Dependency on cell division

As a model for studying transfection efficiency in non-dividing cells, HeLa cells were arrested in S-phase by treatment with aphidicolin, an inhibitor of DNA polymerase α.

Figure 12 shows results obtained in HeLa cells transfected in the absence (A) or presence (B) of 15 µM aphidicolin. Cell division was observed to be a major determinant in transfection efficiency. Overall expression levels dropped roughly 10-fold for l-pEI complexes and to 0 for b-pEI and lipofectamine. Clearly, transfection of non-dividing cells represents a major bottleneck and should be part of screening procedures for gene delivery agents with final applications *in vivo*.

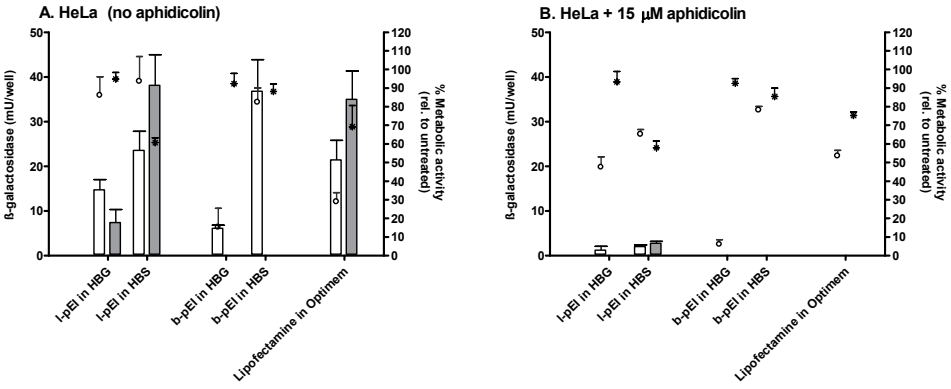


Figure 12. *Transfection efficiency in dividing versus non-dividing cells. Transfection of dividing cells was performed in HeLa cells in the absence of aphidicolin (A) and transfection in non-dividing cells was studied by transfecting HeLa cells in the presence of 15 µM aphidicolin, a reagent which arrests cells in S-phase (B). Cells were transfected with the indicated formulations (0.25 µg DNA/well in 96-well format) for 4 h in the absence (white bar/circle) or presence of serum (grey bar/asterix) and analyzed for gene expression (bars) and metabolic activity (circle and asterix) 48 h after transfection.*

Discussion

In this paper sensitivity of transfection outcomes to various parameters identified from previous gene delivery studies was illustrated. Parameters studied included cell confluency, dose and incubation time, expression read-out assays, kinetics of expression and data presentation, particle formulation, characterization and stability in biological media, celltype and mitotic activity. In this section the outcomes will be discussed and optimal parameters will be selected to form a standardized protocol.

Optimization of the transfection screening protocol

Cells require a certain confluency to grow and perform naturally, and too low confluencies lead to decreased growth rates and increased sensitivity to treatment. When confluence is chosen too high on the other hand, cells become (over)confluent throughout the assay, potentially causing cell cycle arrest, decreased metabolic activity and detaching of cells. In our study, seeding HeLa cells at a density of 8,000 cells/0.32 cm^2 resulting in a confluency of ~80 % at the time of transfection was found to be optimal.

The amount of DNA to incubate with cells should be chosen such that expression can be detected while toxicity remains limited. Based on our findings, a dose of 0.25 µg/well in 96-well format was considered optimal. Increasing the dose of DNA may yield higher expression levels, but at the cost of increased toxicity (unless incubation times are kept very short) and potential loss of discriminative power. At high doses of plasmids in which transcription is controlled by a strong promoter (such as the CMV promoter) the system can become saturated, possibly due to saturation of transcription and/or translation [60]. To place chosen doses into perspective, a human cell contains 7.1 pg chromosomal DNA. A dose of 0.25 µg per 8,000 cells seeded per well corresponds to a dose of 31 pg DNA/cell. Assuming that approximately 10 % of the applied dose is taken up by cells [61], this translates into delivery of a mass of exogenous DNA corresponding to ~40 % of a cells chromosomal DNA.

Similarly, the optimal time of incubation of complexes with cells should balance detectable expression with limited toxicity. An additional factor to take into account is the time that particles require to reach the cells attached at the bottom of the wells. Electrostatic interaction of DNA with cationic polymers, lipids or peptides generally yields relatively polydisperse populations containing both well-defined particles and aggregates. Moreover, this process is highly sensitive to the dispersion medium in which it takes place. According to the theory developed by Derjaguin and Landau, and Verwey and Overbeek (known as the DLVO theory), the forces on colloidal particles in dispersion are the resultant of electrostatic repulsion and van der Waal's attraction [62]. An increase in the ionic strength of the dispersion medium leads to decreased electrostatic repulsion and subsequent agglomeration of particles. Faster sedimentation of larger particles may lead to increased uptake and gene expression [47] in an *in vitro* setup and cause them to play a dominant role in transfection outcomes when using short incubation times. This phenomenon should be

considered as experimental bias and must be taken into account when interpreting data and drawing conclusions regarding the potential of reagents for *in vivo* gene delivery where sedimentation is irrelevant and aggregation is undesired. Based on optimization studies performed with larger l-pEI/DNA particles prepared in HBS and smaller l-pEI/DNA particles prepared in HBG, we selected 4 h as the optimal incubation time. Decreasing the incubation time increases risk of predominant effects of aggregates whereas prolonged incubation induces more toxicity.

Selection of a readout assay

Several reporter gene assays are available for analysis of transfection efficiencies. Here, we compared a β-galactosidase assay with ONPG as substrate, a firefly luciferase assay and an EGFP-based assay analyzed by flow cytometry. The choice for a reporter gene assay proved not crucial for the experimental outcome and can therefore be made based on other parameters (see Table 2). An important advantage of using EGFP is that transfected cells can be analyzed by flow cytometry, which allows gathering information regarding the % of gene expressing cells as well as the mean expression per cell (as shown in Figure 7 C and D in this paper). Thereby it is possible to discriminate whether transfection efficiency is due to a small population of cells expressing high levels of protein or due to many cells expressing low levels of proteins. This information gives insight into the mechanism of transfection. In addition, with EGFP it is possible to measure transgene expression in living cells without requirement for substrates. β-galactosidase and luciferase reporter proteins can only be detected indirectly by conversion of an added substrate into a colored, fluorescent or luminescent product. An advantage of enzyme/substrate based detection is that sensitivity can be improved by increasing substrate incubation time. Moreover, these assays are better suited for high-throughput screenings.

Data presentation

Perhaps more important than the choice of the assay is the choice of data presentation. Expression and toxicity can be expressed as absolute values or calculated relative to control values. In case of toxicity data, untreated cells serve as a negative control representing 100 % cell viability. Since this value is known and fixed, data can be presented relative to negative control values. In contrast, expression data must be compared to a positive control. Since expression obtained with control formulations is highly dependent on the type of reagent, buffers and conditions used, it is not preferable to present expression data calculated relative to control values. Alternatively, showing the absolute expression data of both test formulations and control formulations allows interpretation of the overall assay while maintaining the desired possibility of comparing samples to controls.

Choosing the formulation

A well-known critical parameter for the preparation of particles based on electrostatic interactions is the ratio of cationic reagent to negatively charged DNA. A range of ratios must first be screened to identify the minimal ratio at which DNA is condensed into small positively charged particles and is protected against degradation. Next, the optimal ratio to obtain maximal gene expression and minimal toxicity must be found in transfection studies. Usually, an excess of cationic reagent is required to obtain stable particles and to achieve gene expression. In addition to the characteristics of the reagent and the ratio of reagent to DNA, the dispersion medium in which particles are prepared affects particle size (stability) and transfection efficiency and should be carefully chosen. Gene delivery reagents are developed for therapeutic purposes and formulations should ultimately be regarded as pharmaceutical products. From this point of view, formulations should be prepared in uncomplicated well-defined dispersion medium. Preparation of complexes in cell culture medium or minimal media such as Optimem is therefore not preferred. Low ionic strength buffers supplemented with glucose at iso-osmotic concentrations (such as HBG) are recommended for the formation of electrostatic complexes. To evaluate effects of ionic strength on a formulation's characteristics (and stability), additional formulations prepared in high ionic strength buffers can be tested.

Characterization of formulations

Effects of particle characteristics such as size and surface charge are well-known determinants in cell transfection. Additionally, it is known that particle characteristics are not only dependent on the reagent and DNA used, but are also sensitive to dispersion media (i.e. ionic strength) and subject to changes upon incubation in physiological media. These phenomena should not be ignored and the importance of standardizing and disclosing conditions under which complexes are being prepared is stressed here. Additionally, performing characterization under both realistic and rational conditions requires attention. Quite frequently characterization studies are performed in plain buffers (or even water) and that buffer compositions are changed for *in vitro* studies for isotonicity reasons. The effect of ionic strength on particle size shown here and by others [47,56] indicates that characterization studies can only be linked to *in vitro* results when in both cases complexes are prepared in corresponding dispersion media. At the same time, compatibility of dispersion media with analytical techniques should be considered. For example, measuring zetapotentials in aqueous media without stabilizing and defining pH creates meaningless data. The high conductivity of high ionic strength media causes substantial heat development and complicates obtaining reliable data. Therefore, most reliable results are expected to be obtained upon analysis in low ionic strength buffers.

Another point of consideration is optimization and characterization of formulations with respect to the intended route of administration and target tissue. Depending on the delivery route and target tissue, particles will have to meet certain criteria. These will be less strict in case of local

delivery, where fewer barriers are encountered between the site of administration and the site of action. Most approaches, however, will depend on intravenous administration. For these cases, investigation of the behavior of particles regarding physicochemical properties and stability, as well as transfection efficiency and toxicity upon incubation in biological media is crucial to estimate potential for *in vivo* delivery. Analysis of size (distributions) in the presence of serum should therefore always be included in studies. Recent developments of new methods based on fluorescence fluctuation spectroscopy [63], flow cytometry [52] and fluorescence nanoparticles tracking analysis by Nanosight [64] now allow reliable assessment of particle sizes in heterogeneous samples and should become routine in gene delivery screenings.

Selection of reference reagents

Importantly, interpretation of transfection efficiency data of new reagents requires comparison to reference reagents. Although a seemingly trivial issue, selection of a suitable reference reagent is a difficult task. As shown in this paper, large variability in transfection efficiencies among commonly used reference reagents exists. Moreover, transfection efficiencies of these reagents are highly dependent on experimental conditions chosen, such as the composition of buffers and medium. These two aspects complicate interpretation of results described in literature which have been obtained using various references, formulation parameters and conditions, especially if detailed descriptions are lacking and data are expressed relative to controls. Reaching consensus with regard to the reference reagent to be used as a positive control would create transparency. Ideally, this standard formulation would be a well-defined and readily available compound that has the ability to condense DNA into well-defined small particles which are stable in biological media and can induce moderate but robust gene expression at low toxicity in the presence and absence of serum. Unfortunately, none of the reagents tested here meets the requirement of generating small particles that remain stable in biological fluids. Based on our findings, we have the opinion that 22 kDa l-pEI represents the most useful reference reagent as it was shown to work both in HBG and HBS and both in the presence and absence of serum (depending on the buffer though). Limitations of b-pEI are its inefficiency in transfecting cells in the presence of serum. A disadvantage of lipofectamine is that transfection was only efficient when large complexes were formed in Optimem. Naked plasmid DNA was shown not to transfect cells under any of the conditions that we tested and only has value as a negative control. Considering the relation between ionic strength, particle size and transfection outcomes, screening reagents in both HBS and HBG is valuable.

Selection of cell models

A great advantage of gene therapy is that it can be relatively easily tailored to a specific disease by altering the therapeutic DNA. It is valuable to perform studies in cell types and under conditions relevant for a specific disease when developing gene delivery strategies for a specific application.

To give a few examples, Calu-3 cells serve as a model for cystic fibrosis, HEK293 for kidney disease, HUVEC for angiogenesis, OVCAR for ovarian carcinoma. Nevertheless, including a standard model cell line would be useful for primary screenings and to estimate relative potencies of new reagents in comparison to already existing compounds. For this purpose, we suggest to include transfection experiments in HeLa cells, which is a commonly used and readily available cell line.

Although *in vitro*/*in vivo* correlation continues to be a matter of debate, some useful efforts to maximize predictive value of *in vitro* transfection studies can be made, including screening in various cell types with different characteristics and screening in non-dividing cells. The effect of serum is important and should always be assessed in order to predict *in vivo* potential of new reagents. An important discrepancy between cell lines and the *in vivo* situation is the proliferation state of cells. Cells in culture divide rapidly (doubling times of ~24 h) whereas cells in tissues, the targets for gene therapy, are mostly quiescent. This has a huge impact on transfection outcomes because temporary breakdown of the nuclear envelope during mitosis allows bulk access of DNA (-complexes) to the nucleus whereas active import via nuclear pore complexes is essential in absence of mitosis. The ultimate test would be to study transfection *in vivo*. However, additional barriers would be encountered such as stability in blood and biodistribution. *In vitro* models are necessary to gain insight into the potential of gene delivery systems to transfect cells in the absence of mitosis. Several methods are available to arrest cells in a certain phase within the cell cycle, including serum deprivation, thymidine block, synchronization with hydroxyurea, lovastatin etc. In our lab, arresting HeLa cells in S-phase with aphidicolin proved most useful as it combined efficient (unlike serum deprivation) and continuous synchronization with acceptable toxicity (unlike hydroxyurea).

Suggested protocol and conclusion

This paper highlights the sensitivity of transfection experiments, thereby stressing the importance for assay standardization within the field of gene delivery. Based on our findings, we suggest the standard protocol as shown in Box 1. The scope of this paper is limited to non-viral gene delivery of plasmid DNA, but a similar approach would be valuable for studies on siRNA delivery.

The proposed screening protocol offers a useful starting point for evaluating the potential of novel gene delivery reagents. Based on outcomes of this screening, directions for follow-up studies can be decided upon. If characterization studies in buffers and physiological media reveal insufficient colloidal stability, a primary focus on improving particle properties is required before moving to cell (and *in vivo*) studies. In case of unfavorable activity/toxicity ratios, subsequent efforts can be made to modify the molecular structure of the reagent and/or removing excess free reagent. Stable particles that exhibit limited toxicity can be selected for mechanistic follow-up studies. Understanding what makes one reagent successful whereas another fails to mediate

transgene expression will contribute to the rational design of novel formulations. Recent technological developments including improved cellular subfractionation methods, advanced microscopic techniques (reviewed in [65,66]) and quantitative PCR have extended the toolbox and enable studying cellular uptake pathways, endosomal transport [67,68], intracellular trafficking [69-71], cytosolic degradation [72], nuclear uptake [61,73-75] and efficiency of transcription/translation [76,77]. Progressive implementation of these techniques and methods in the field of gene delivery is expected to yield important findings and substantial progress. However, as these studies require sophisticated, laborious and expensive techniques it is not feasible to apply them to each and every reagent under investigation. The screening protocol suggested in this paper provides a relatively simple, cost- and time-effective procedure for the pre-selection of potential candidate reagents. Reaching consensus on screening procedures within the field will enable placing individual results into a broader context, thereby accelerating the identification of structure/activity relations and facilitating to estimate significance of new findings to progress in non-viral gene delivery.

Next page: Box 1. *Suggested standard protocol for in vitro transfection screenings of non-viral gene delivery systems. For detailed procedures the reader is referred to the materials and methods section.*

STANDARD PROTOCOL

Particle preparation

- Prepare working stocks of reagent (vary concentration to vary reagent:DNA ratios) and of plasmid DNA in both HBG and HBS. Prepare polyplexes by adding 4 volumes of reagent to 1 volume of plasmid DNA (50 µg/ml) and mix by pipetting 10x. Incubate 30 minutes at room temperature.
- Optimize reagent/DNA ratios based on characterization and transfection efficiency.
- Reference formulation/ positive control: 22 kDa l-pEI/DNA at N/P=6 in HBG and HBS.

Characterization

- Measure size and stability of formulations in HBG and HBS.
- Measure zetapotential in HBG.
- Evaluate effect of medium with and without serum on particle size (e.g. by flow cytometry or single-particle tracking analysis).

Cell transfection

- Seeding: 24 h prior to experiment, seed cells to reach 80% confluency on day of transfection.
- Treatment: immediately prior to transfection replace medium with medium with and without serum. Dropwisely add 25 µL sample per well (DNA dose: 0.25 µg/well in 96-well format). After 4 h incubation replace medium with normal cell culture medium.
- Selection of cell type:
  - HeLa
  - Additional model cell line(s)
  - Cell line relevant for disease/application studied
  - Model for non-dividing cells (i.e. aphidicolin-arrested cells, primary cells)

Readout and data presentation

- Expression assay: Use expression plasmids with the CMV promoter and an optional reporter gene (readout: β-galactosidase 48 h/luciferase 24 h/EGFP 24 h post-transfection). Present absolute data (i.e. mU/well) for samples and positive controls.
- Toxicity assay: i.e. XTT. Present data as % metabolic activity relative to untreated cells.

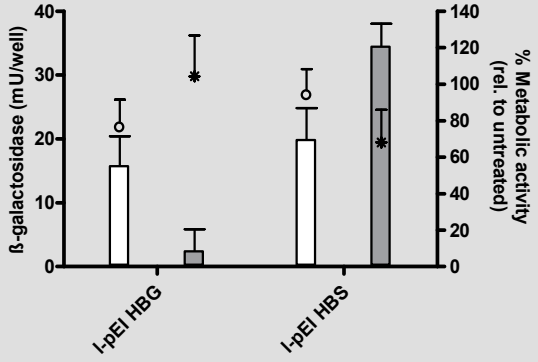


Figure 13. *Gene expression and cell viability obtained using the suggested standard protocol. Gene expression (bars) and cell viability (circle and asterix) after transfection of HeLa cells in the absence (white bar/circle) or presence (grey bar/asterix) of 10 % serum with complexes prepared in the indicated dispersion medium. Complexes were incubated 4h with cells (0.25 µg DNA/well) and gene expression and cell viability were measured 48h after transfection. Means of 6 independent experiments performed in triplicate are pooled and presented as mean+SD.*

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Supplementary information to:

Towards a standard protocol for in vitro transfection screenings of non-viral gene delivery systems

Contents:

Fig S1 A-D Effect of DNA dose and incubation time on gene expression and cell viability of formulations of 22 kDa l-pEI (S1 A), 25 kDa b-pEI (S1 B), lipofectamine (S1 C) and naked plasmid DNA (S1D) prepared in HBG (A), HBS (B) or optimem (C). HeLa cells were transfected in the absence or presence of 10 % serum and gene expression and cell viability were measured 48h after transfection. Cell viability is expressed as the metabolic activity relative to that of untreated cells at the corresponding time. Data are expressed as mean±SD. (*Supplementary to Chapter 7, Figure 6)*

Fig S2 A-D Kinetics of gene expression for various read-out systems. HeLa cells were transfected with indicated complexes containing either pCMV-LacZ (S2 A), pCMV-Luc (S2 B) or pCMV-EGFP (S2 C and D) and expression was measured in the absence or presence of 10 % serum at the indicated timepoints post-transfection. Complexes were incubated 4 h with cells (0.25 µg DNA/well in 96-well format for β-galactosidase and luciferase assays; 1.5 µg/well in 24-well format for EGFP assay). Data are expressed as mean±SD. (*Supplementary to Chapter 7, Figure 7)*

Fig S3 A-D Transfection efficiency in various cell lines. HeLa , COS-7, OVCAR-3 and HUVEC cells were transfected with 22 kDa l-pEI (S3 A), 25 kDa b-pEI (S3 B), lipofectamine (S3 C) and pDMAEMA (S3 D) in indicated buffers. Complexes were incubated with cells for 4 h in the absence or presence of 10 % serum and analyzed for gene expression and cell viability 48 h after transfection. (*Supplementary to Chapter 7, Figure 11)*

Fig. S1A: 22 kDa I-pEI

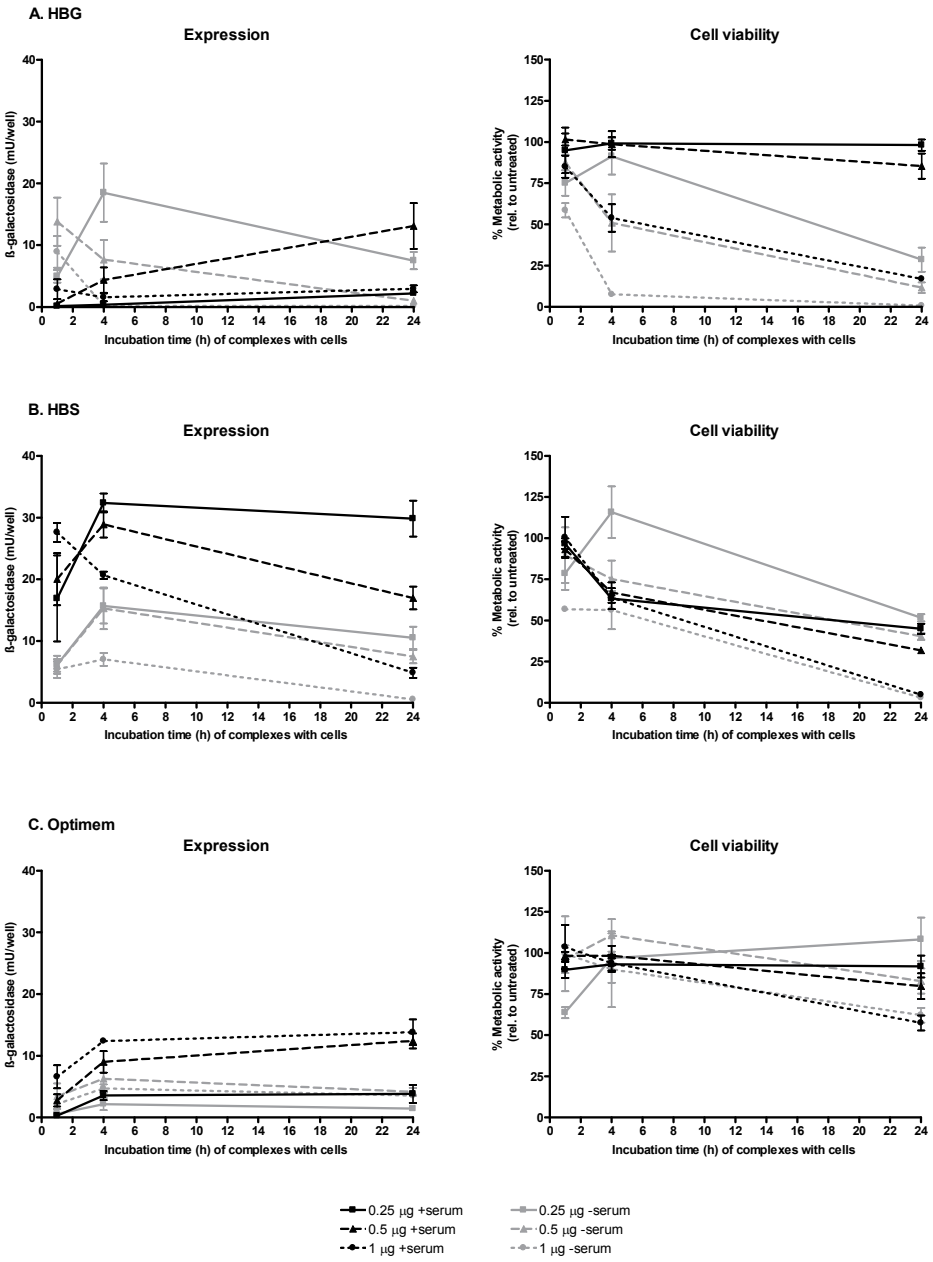


Fig. S1B: 25 kDa b-pEI

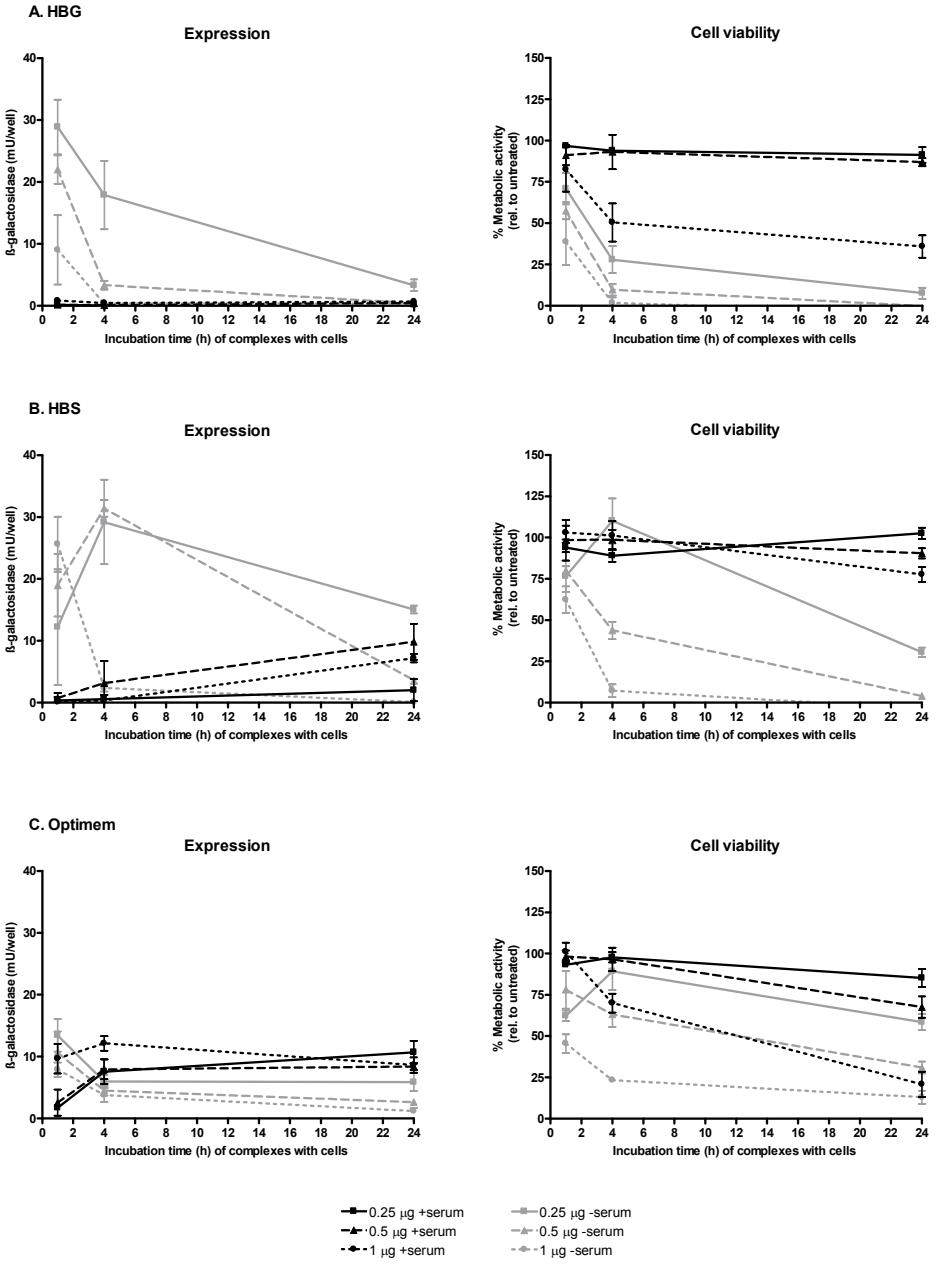


Fig. S1C: lipofectamine

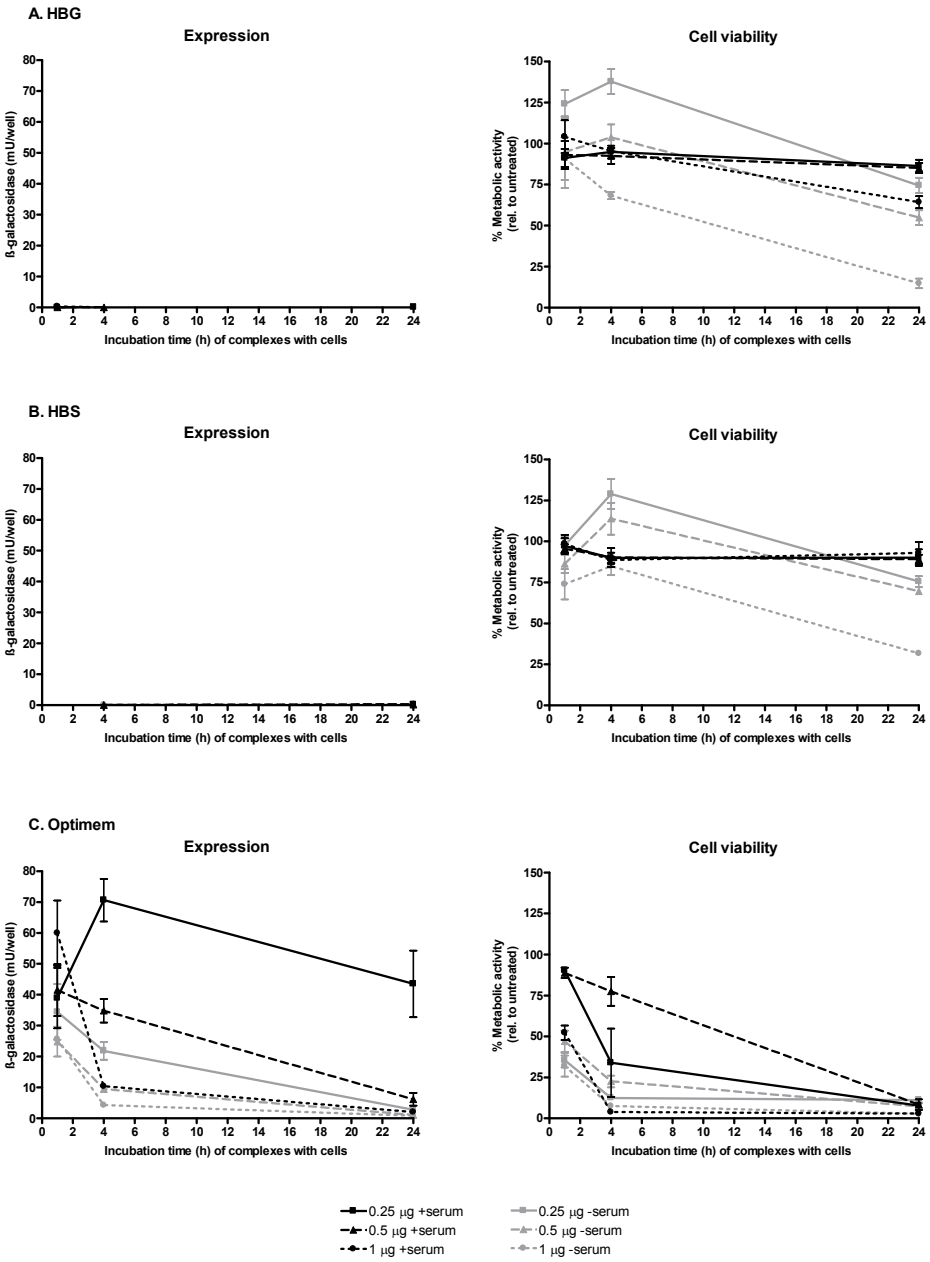


Fig. S1D: naked plasmid

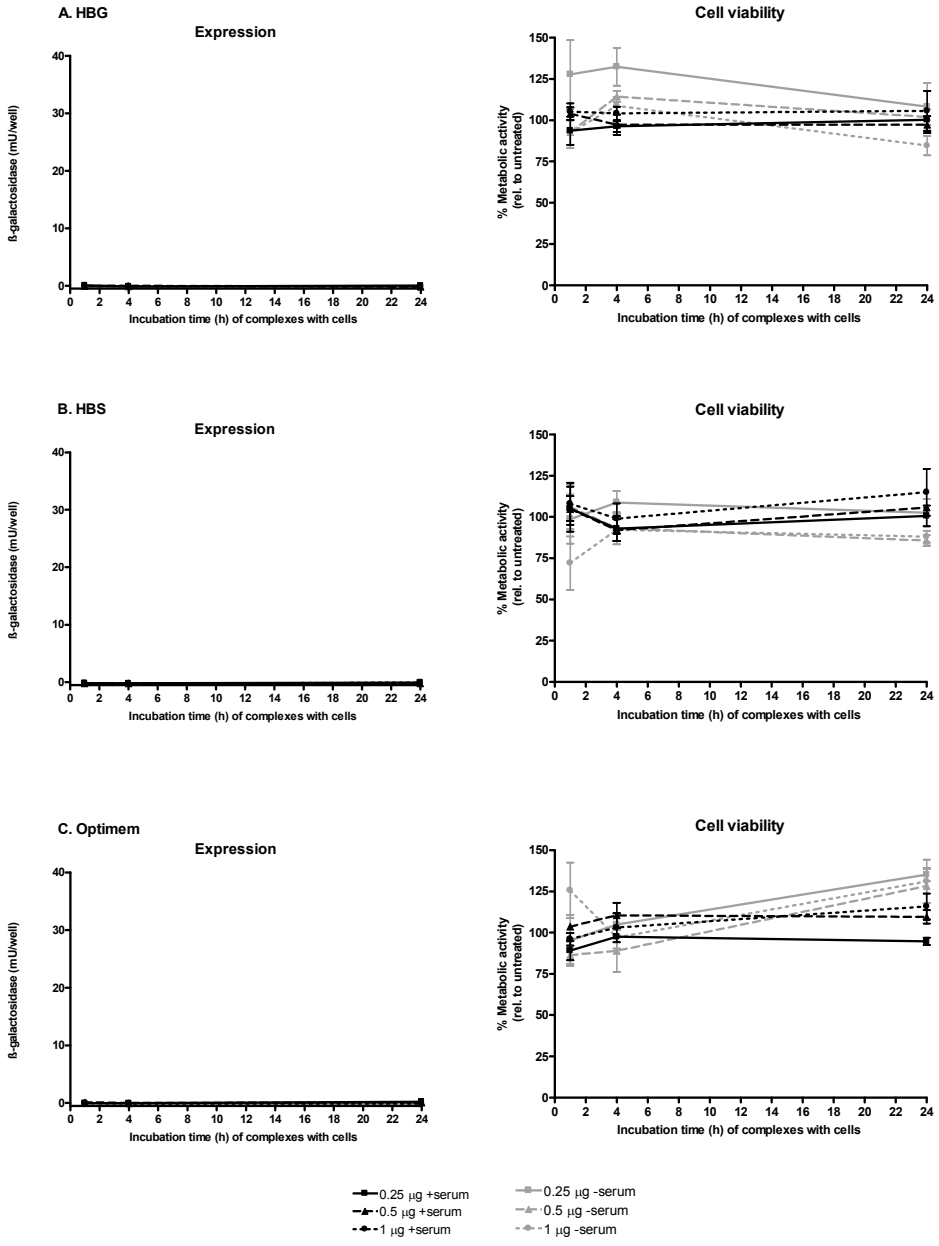


Fig. S2A Kinetics of expression: β-galactosidase

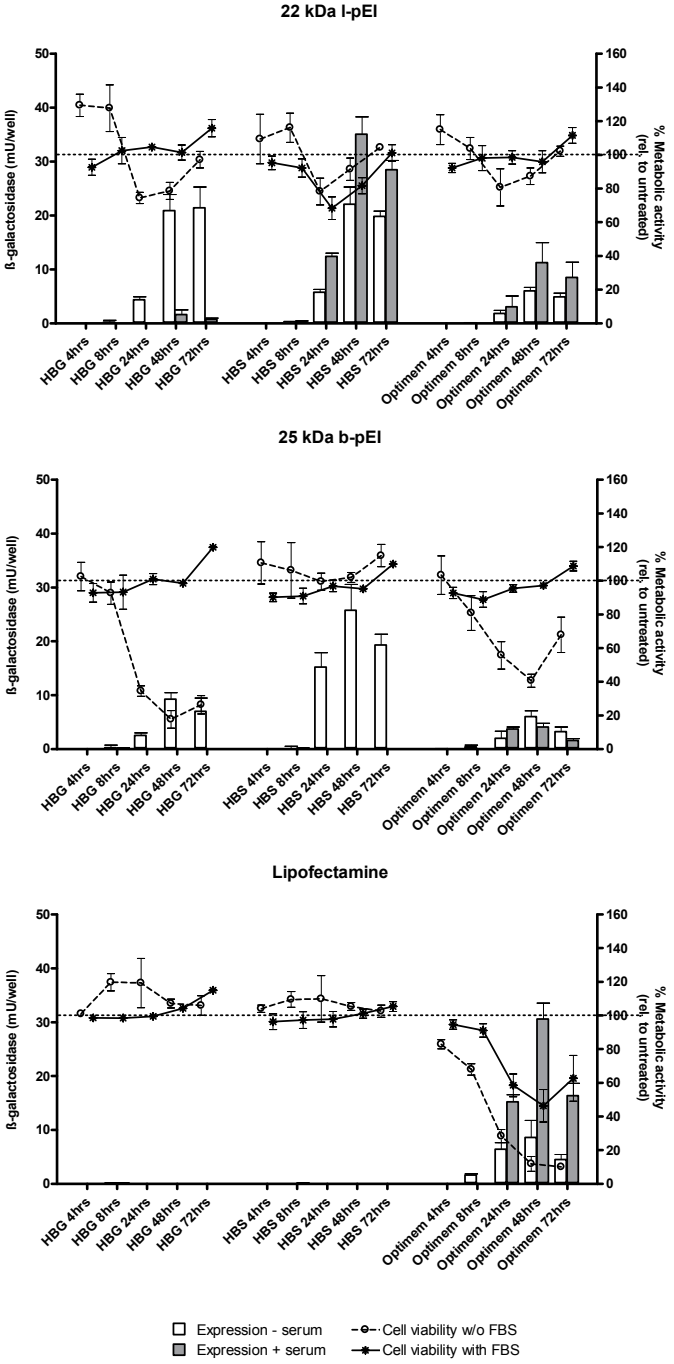


Fig. S2B Kinetics of expression: luciferase

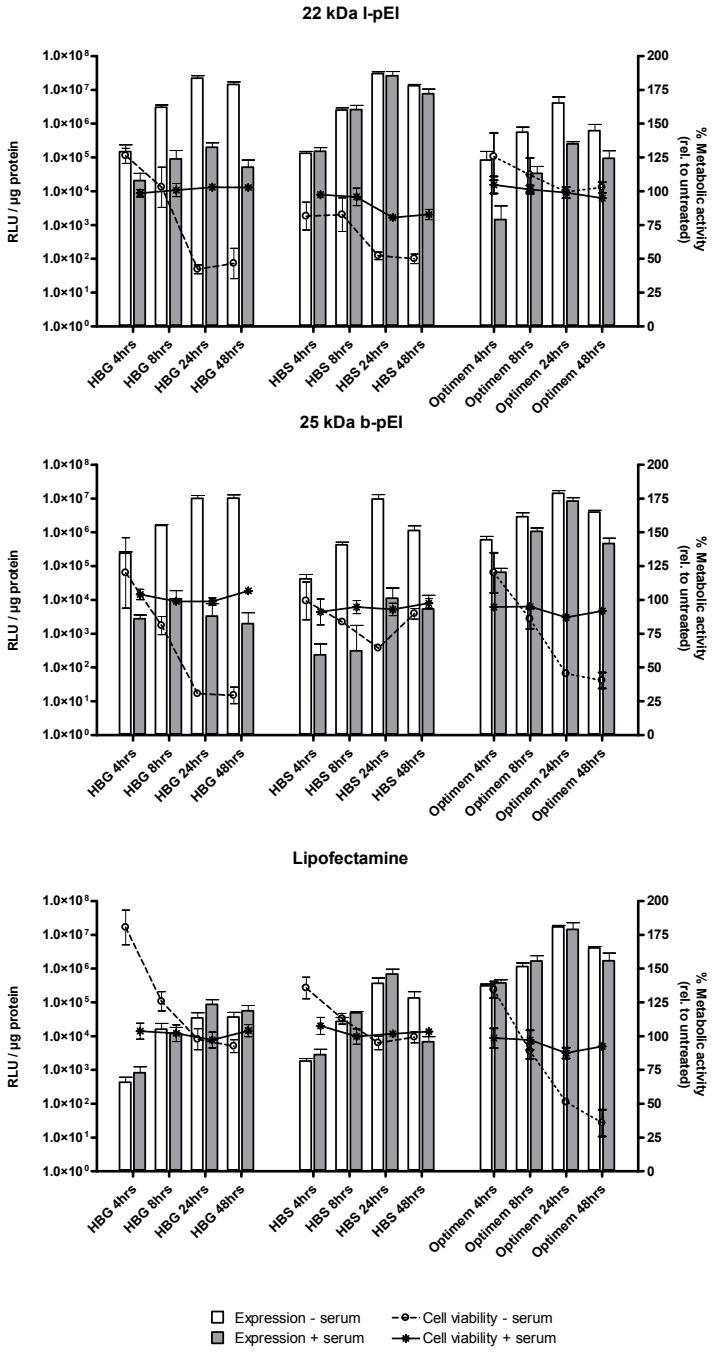


Fig. S2C Kinetics of expression: EGFP %expressing cells

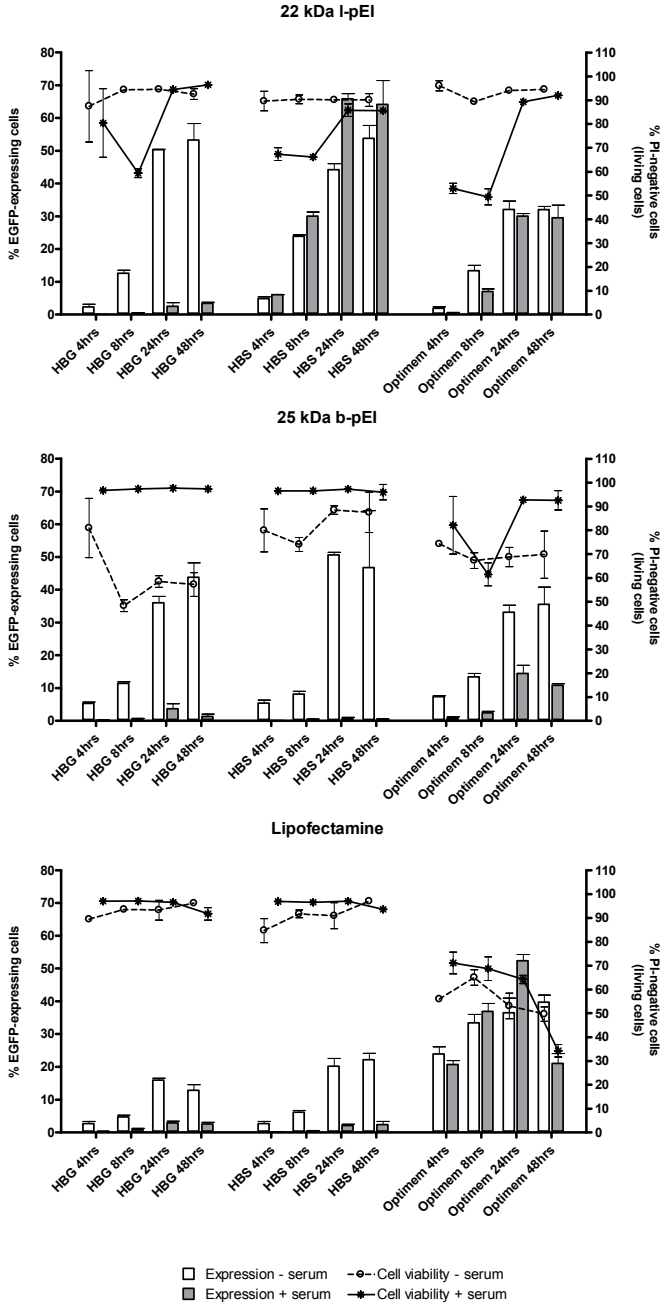
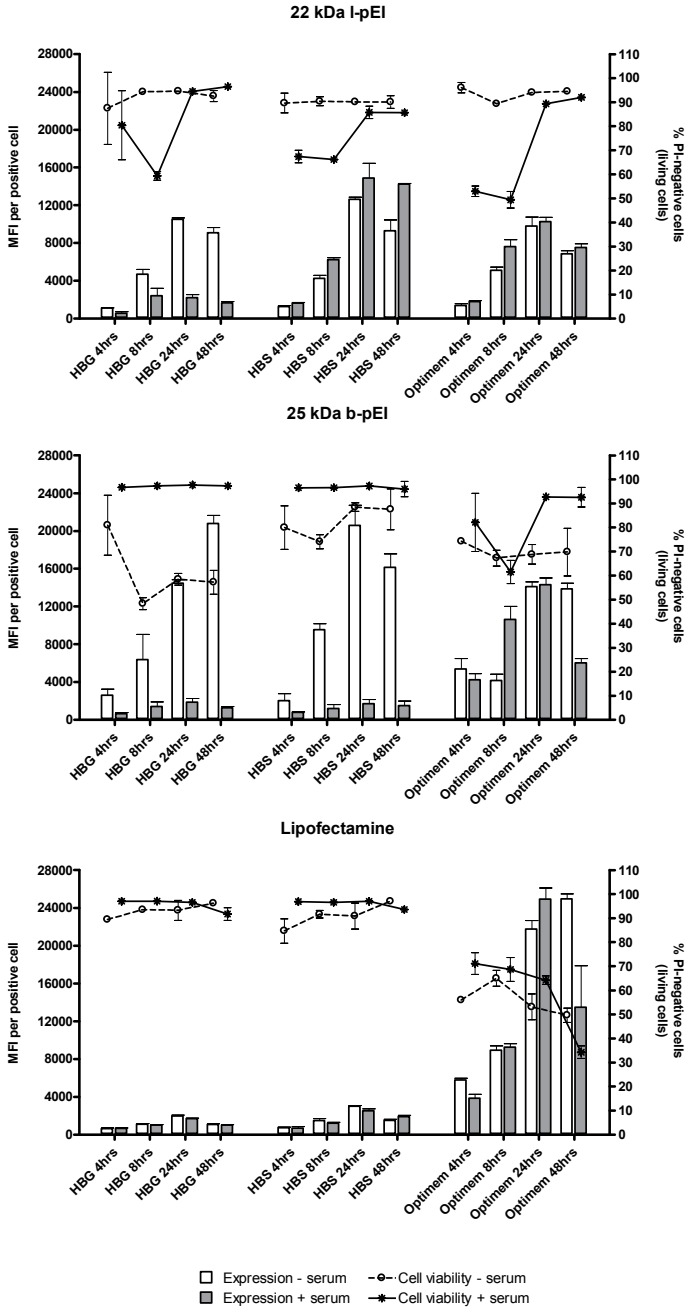


Fig. S2D Kinetics of expression: EGFP MFI per expressing cell



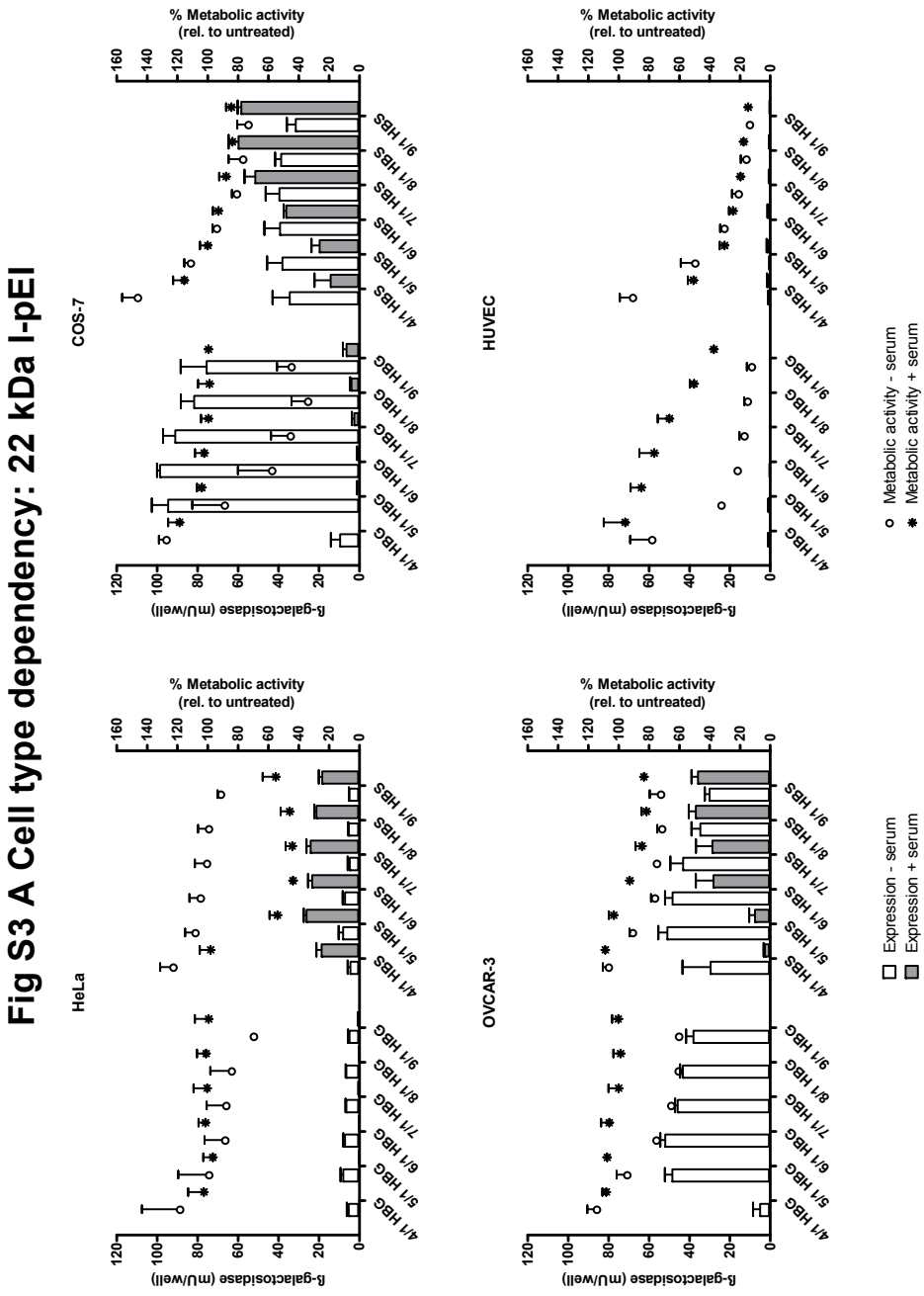


Fig S3 A Cell type dependency: 22 kDa l-pEI
COS-7
HUVEC
HeLa
OVCAR-3
% Metabolic activity (rel. to untreated)
ß-galactosidase (mU/well)
4/1 HBG
5/1 HBG
6/1 HBG
7/1 HBG
8/1 HBG
9/1 HBG
4/1 HBS
5/1 HBS
6/1 HBS
7/1 HBS
8/1 HBS
9/1 HBS
Metabolic activity - serum
Metabolic activity + serum
Expression - serum
Expression + serum

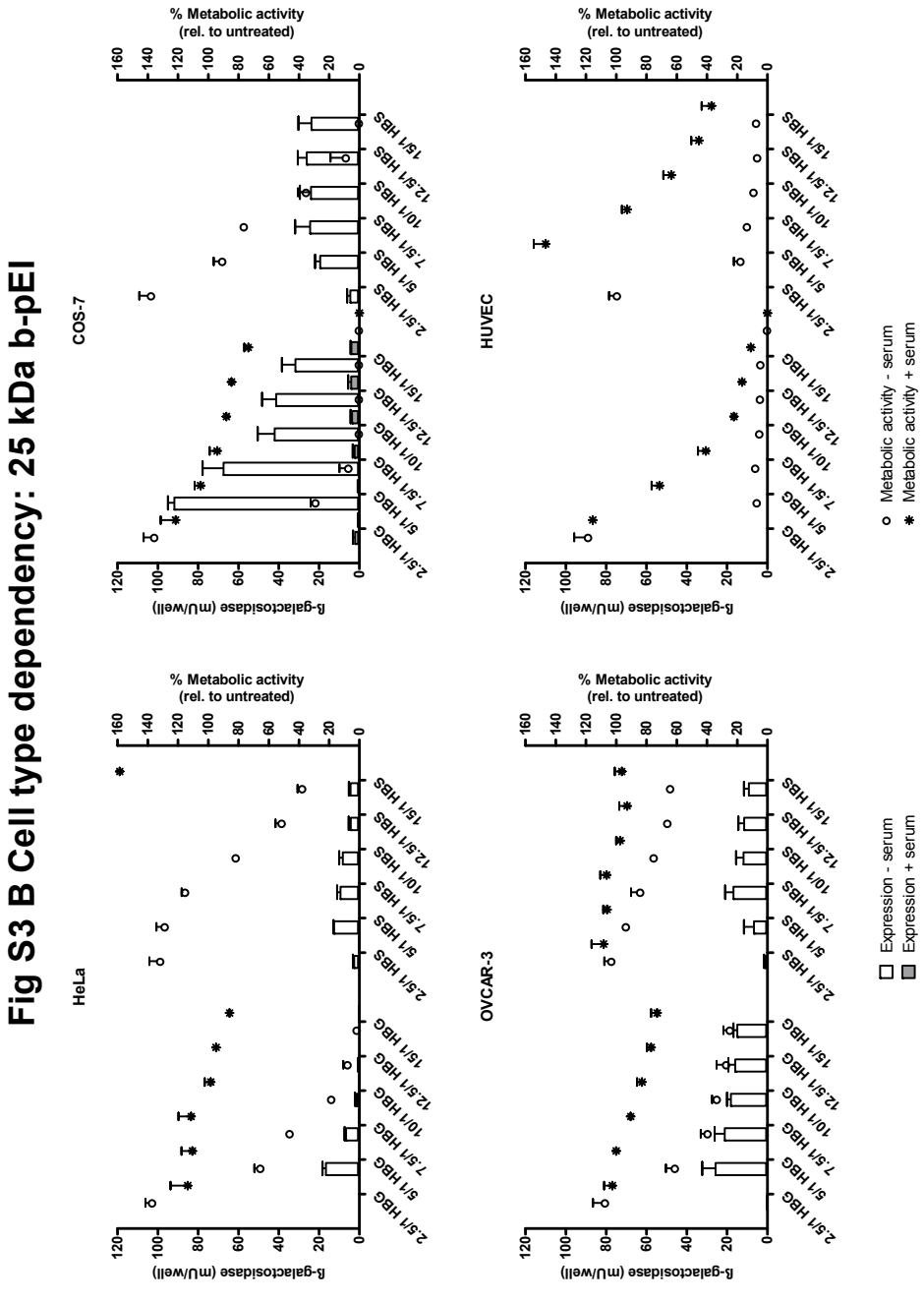


Fig S3 B Cell type dependency: 25 kDa b-pEI
COS-7
HUVEC
HeLa
OVCAR-3
% Metabolic activity (rel. to untreated)
ß-galactosidase (mU/well)
2.5/1 HBG
5/1 HBG
7.5/1 HBG
10/1 HBG
12.5/1 HBG
15/1 HBG
2.5/1 HBS
5/1 HBS
7.5/1 HBS
10/1 HBS
12.5/1 HBS
15/1 HBS
Metabolic activity - serum
Metabolic activity + serum
Expression - serum
Expression + serum

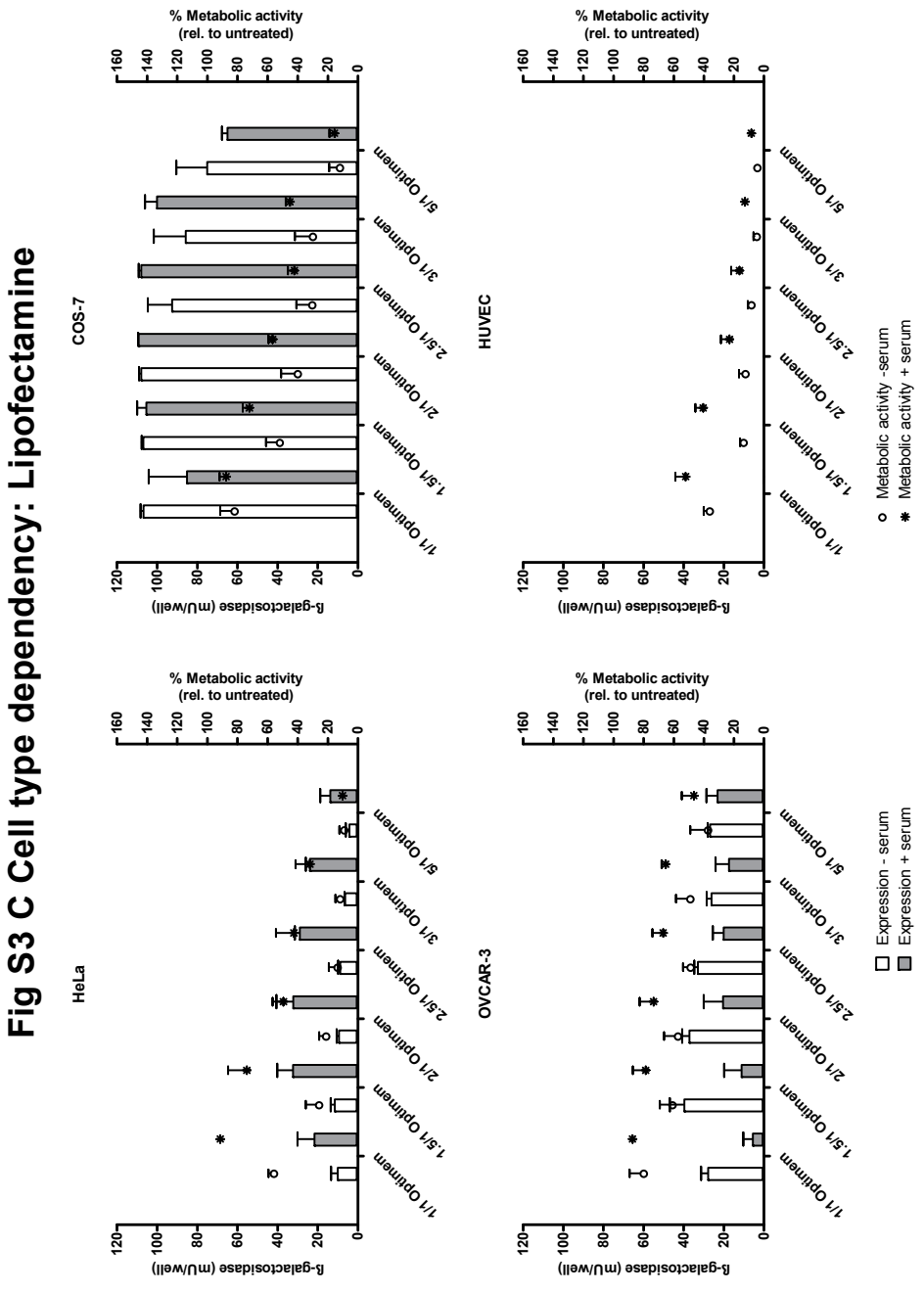


Fig S3 C Cell type dependency: Lipofectamine
COS-7
HUVEC
HeLa
OVCAR-3
% Metabolic activity
(rel. to untreated)
ß-galactosidase (mU/well)
1/1 Optimem
1.5/1 Optimem
2/1 Optimem
2.5/1 Optimem
3/1 Optimem
5/1 Optimem
Metabolic activity -serum
Metabolic activity + serum
Expression - serum
Expression + serum

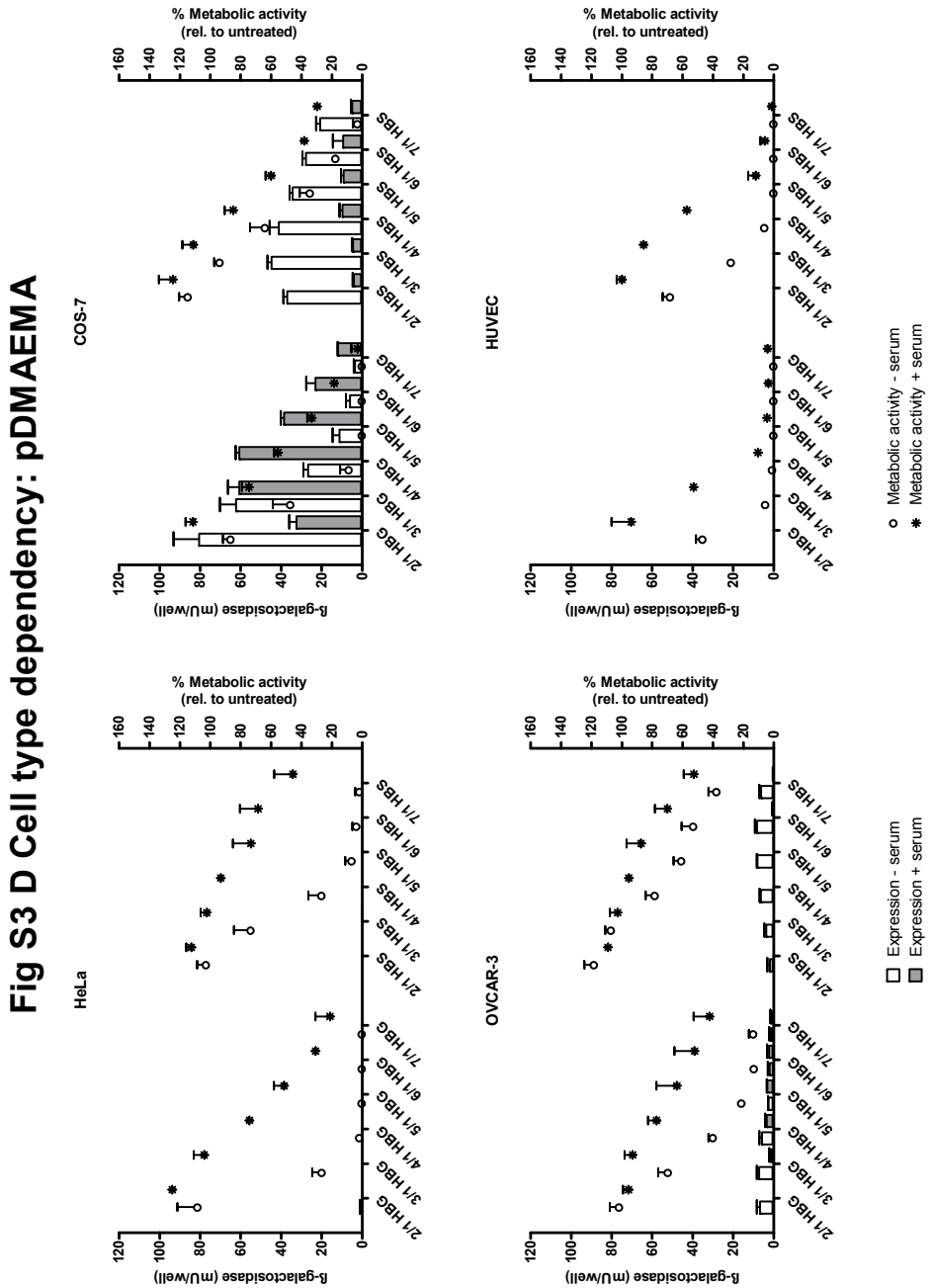
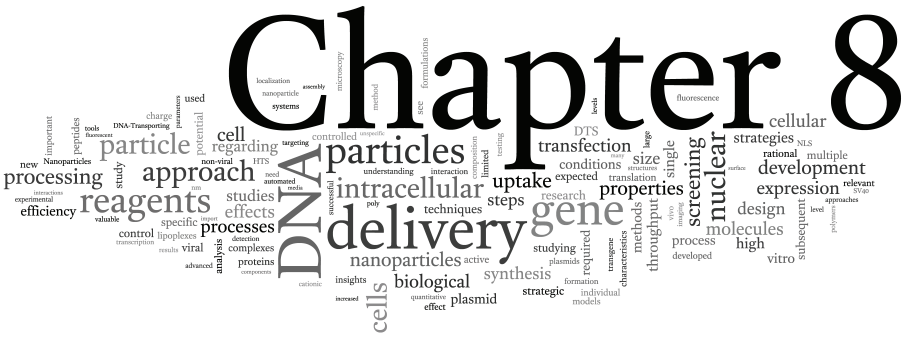


Fig S3 D Cell type dependency: pDMAEMA
COS-7
HUVEC
HeLa
OVCAR-3
% Metabolic activity
(rel. to untreated)
ß-galactosidase (mU/well)
2/1 HBG
3/1 HBG
4/1 HBG
5/1 HBG
6/1 HBG
7/1 HBG
2/1 HBS
3/1 HBS
4/1 HBS
5/1 HBS
6/1 HBS
7/1 HBS
Metabolic activity - serum
Metabolic activity + serum
Expression - serum
Expression + serum

Chapter 8



Summarizing discussion and perspectives

Summarizing discussion

Gene therapy is an interesting approach to treat various diseases through delivery of therapeutic protein-encoding DNA into target cells. After initial proof of concept was obtained, many hurdles appeared (as discussed in **chapter 1**). Although these hurdles have delayed the realization of therapeutic applications, research over the last years has resulted in important insights into the cellular processes required for non-viral gene delivery and expression. Additionally, novel technologies and methods have been developed to study the complex processes involved. Further improvement can be made through rational design of plasmid and formulation, and by implementing new methodologies that allow increasingly realistic and relevant analysis of extracellular and intracellular behavior of formulations.

Chapter 2 reviews the possibilities for improving non-viral gene delivery by rational design of plasmid DNA. Three feasible strategies from which a significant improvement in control and level of transgene expression can be expected are highlighted in this summary. First, replacement of constitutively active promoter (and enhancer) regions by regulatory elements active in specific cell types or under specific conditions adds an extra level of control, hence safety. Second, minimizing viral and prokaryotic DNA sequences through replacement of viral promoters and removal of bacterial elements required for production decreases immunogenicity and gene silencing, thereby postponing loss of expression. Third, strategies based on site-specific integration of therapeutic DNA into genomes or on extrachromosomal replication of plasmids deserve attention and should be further explored to enable safe and stable transgene expression. Although valuable improvements can be expected from such plasmid optimizations, they can only be exploited after the problem of delivery is solved. One exception of plasmid engineering that affects delivery is represented by the modification of plasmid with DNA sequences that are recognized by endogenous transcription factors involved in nuclear import. These DNA nuclear Targeting Sequences (DTS) would facilitate nuclear localization by inducing (partial) coating of plasmid DNA with NLS-containing proteins and subsequent binding to importins.

The potential of such DTS to enhance transfection efficiency *in vitro* was investigated in **chapter 3**. Several DTS were identified from literature and cloned downstream of an EGFP coding sequence that was under the control of the strong CMV-promoter. It was shown that insertion of neither the full-length SV40 DTS (372 bp promoter and origin region), nor the partial SV40 DTS (72-bp enhancer region), nor repetitive NFκB binding sites nor a Glucocorticoid Responsive Element increased transgene expression in dividing and non-dividing cells. We hypothesized that the method of DNA delivery would affect the availability of DTS for interaction with cytosolic proteins. To study this effect, plasmids containing or lacking DTS were administered by electroporation (no carrier), transfection with lipofectamine (thought to dissociate prior to nuclear uptake) and transfection with 22-kDA linear pEI (shown to deliver DNA into the nucleus as intact complexes). No DTS effect was observed regardless of the delivery method. Dose-response experiments were performed to

exclude the possibility that relatively small increments in specific nuclear uptake were outweighed by overloading the cytoplasm with plasmid DNA which could lead to unspecific nuclear uptake. Results from quantitative PCR on isolated nuclei indicated that nuclear localization of plasmids after lipofectamine-based transfection in dividing and non-dividing cells was not facilitated by the presence of the full-length SV40 DTS. In contrast, others did observe DTS-mediated increases in nuclear uptake as analyzed by microscopic techniques. The inability of DTS to increase expression levels driven by a strong CMV-promoter suggests that, regardless of whether nuclear localization is facilitated, a bottleneck exists downstream of nuclear delivery (i.e. transcription and/or translation). Future studies could be conducted to evaluate the potential value of DTS to compensate for weak (but specific) promoters.

In **chapter 4** the effect of coupling nuclear localization signals (NLS) to DNA was investigated. To avoid interference of modifications with transcriptional processes, a non-covalent coupling strategy was chosen. Bi-functional peptides containing a protamine-derived DNA Binding Domain (DBD) and an NLS derived from either the SV40 large T antigen (SV40) or the heterogeneous nuclear ribonucleoprotein A1 (sM9) were designed and used to prepare binary peptide/DNA or ternary peptide/DNA/condensing agent complexes. Binary complexes were unable to mediate transgene expression, despite the ability of DBD-NLS peptides to condense and deliver DNA into cells. Although peptides did not enhance pEI-based transfection, incorporation of peptides in lipofectamine-based complexes led to significantly increased transfection. This effect was shown to be NLS-independent and was attributed to altered physicochemical properties of the lipoplexes. The unspecific enhancement of transfection induced by combination of peptides with other delivery strategies described here and also by others raises questions regarding NLS strategies and general formulation issues. Taking into account that many improvements in transgene expression upon incorporation of non-covalently coupled NLS peptides described in literature are not related to nuclear uptake, factual proof is limited to a few publications. Covalent coupling strategies on the other hand have provided proof of NLS-mediated nuclear import, but the high coupling ratios required were shown to impede transcription. Stable and controlled modification of DNA with multiple NLS seems essential for successful NLS-mediated nuclear import of plasmids. Site-specific coupling of multiple NLS via PNA-clamps might be an interesting approach worthwhile investigating. The synergistic effect of peptides and lipids can be further exploited to maximize lipid-based transfection while keeping total lipid doses (and toxicity) low. When studying specific interventions (e.g. NLS), secondary effects on relevant parameters such as size and charge should be acknowledged to recognize and prevent confounding. Moreover, it emphasizes the need for better controlled particle formation.

When comparing viruses to non-viral gene delivery, a large discrepancy exists between the numbers of gene copies that need to be delivered to achieve similar transgene expression levels. **Chapter 5** describes an approach to increase efficiency of pEI-based transfection. It was shown that the amount of active DNA could be reduced substantially while maintaining transfection activity by

replacing active DNA with non-coding junk DNA. The effect was maximal when both DNA types were co-delivered within the same pEI complexes. The results indicate that not the total amount of DNA, but the number of active DNA-containing particles is the critical factor in determining transfection efficiency. This finding advocates the development of particles containing single active DNA molecules. Alternatively, it means that multiple functionalities can easily be combined within polyplexes without loss of efficiency of the individual components. For example, multiple short-hairpin RNA-encoding plasmids could be co-delivered to silence multiple targets at once. Additionally, active DNA can be partially replaced by junk DNA or other polyanionic excipients, reducing costs and improving the safety profile by reducing immunogenicity (by reducing immunostimulatory DNA and endotoxin impurities).

Preparation of DNA-Transporting Nanoparticles by electrostatic interaction of cationic reagents with anionic DNA generally yields heterogeneously sized populations. Additionally, size of such poly- and lipoplexes is affected by exposure to salts and proteins. Size matters both in the development phase where biological effects (such as cellular uptake) of different particle size classes remain to be established, as well as for quality control purposes. Analytical tools are therefore required to characterize particles under physiologically relevant conditions. In **chapter 6** a new method based on flow cytometry was developed which allows measurement of particle size distributions of nanoparticles directly in biological fluids. Fluorescently labeled beads of distinct sizes (0.1 – 2 µm) were used to establish a correlation between diameter and side scattering intensity (SSC). Simultaneous detection of fluorescence and SSC provided the possibility to distinguish fluorescently labeled nanoparticles of interest from other particulate matter (e.g. low density lipoproteins or other serum components) frequently present in biological fluids. The presented method can be used for fast screening of colloidal stability of DNA-Transporting Nanoparticles in various media and the obtained size distributions offer additional information to the average particle sizes generated by conventional DLS measurements. The recent introduction of fluorescence nanoparticles tracking analysis by Nanosight will enable further validation of the method. It is expected that flow cytometry and Nanosight can be used as complementary tools with one providing a fast screening tool and the other providing in-depth analysis of absolute size of selected samples. Future efforts could be made to further exploit the multi-parameter characterization by flow cytometry for studying multi-component structures (such as ternary formulations) or association of individually labeled molecules (such as DNA and polymer) based on simultaneous detection of multiple fluorophores. Our study also included an effort to sort a heterogeneous submicron particle population into subpopulations of separate sizes which we aimed to use for subsequent cell studies. Although a proof of principle for sorting was obtained, further practical application was hampered through technological limitations of the equipment as sorting led to extensive sample dilution. It is hoped that companies developing flow cytometers recognize the demand for applications in the submicron range. Significant contributions could be

expected from the development of specialized equipment with increasingly sensitive side, and preferably forward, scatter detectors as well as tailored fluidics and nozzle sizes.

Testing of new gene delivery reagents is usually performed by reading out transgene expression levels relative to a reference formulation after *in vitro* transfection. Over the years and among different laboratories, such screening has been performed in a variety of cell lines, using a variety of conditions, using a variety of read-out systems and by comparison to a variety of reference formulations. In **chapter 7** the effects of a large number of variables on transfection efficiency was investigated. Based on this dataset, a screening protocol is suggested with the aim of standardization within the field.

Perspectives

The focus of this thesis, and of this section, is on *in vitro* processes, including the behavior of DNA Transporting Nanoparticles in biological media and inside cells. To achieve gene therapy as the ultimate goal, an important translation to *in vivo* situations will be required. In contrast to the ready and direct access of particles to cells under *in vitro* testing conditions, particles administered *in vivo* have to travel a complex route before arriving at the target cells. In order to reach the cells of interest, particles should be modified to reduce interaction with proteins and non-target cells, tailored to display favorable biodistribution and kinetic profiles, and equipped with targeting strategies. This places extra demands for the design of gene delivery systems, but also stresses the need for *in vitro* models that resemble *in vivo* conditions more closely. Valuable efforts in this direction include the use of three-dimensional cell culture systems, multilayer cell cultures and *ex vivo* tissue models [1,2].

Although consideration of extracellular barriers is indicated, the primary bottlenecks for non-viral gene delivery exist at the level of intracellular processing. Understanding of cellular uptake routes, trafficking through the cytoplasm and nuclear uptake are essential for the development of efficient gene delivery formulations. This thesis describes efforts in these directions to improve efficiency and understanding of gene delivery through interventions at several levels. Many different strategies and modifications of delivery reagents are at hand and may provide incremental improvements, but to exploit and understand their full potential I am of the opinion that some fundamental aspects need to be resolved.

Knowledge regarding complex biological processes is still limited and studying processes ranging from behavior of particles in extracellular fluids to intracellular processing qualitatively and quantitatively remains a technological challenge. Nevertheless, such information is crucial for true rational design of gene delivery systems.

The majority of gene delivery systems involve particles that are formed through electrostatic interactions, yielding heterogeneous populations of poorly controlled size, shape and composition. Before dressing up such systems with targeting ligands and strategies to improve intracellular

processing, it is important to gain control over particle architecture and stability and understanding of effects of particle properties on biological effects.

Options for biomaterials and structural modifications are continuously expanding, generating extensive collections of potential gene delivery reagents. Sensitivity of particle formation and stability as well as subsequent biological effects to testing conditions chosen (and limited understanding thereof) should be taken into account when testing these reagents. The vast numbers of reagents and parameters required to be included in experiments ask for a systematic, large-scale approach.

In the light of these three fundamental issues and recent technological advances, three strategic directions for future research on non-viral gene delivery are proposed. Additionally, suggestions for exchange and cooperation between these strategic paths are given.

Strategy 1: Rational design based on intracellular pharmacokinetics and pharmacodynamics

► *Gaining knowledge of intracellular pharmacokinetics/pharmacodynamics (i.c. PK/PD) enables true rational design of formulations for gene delivery.*

Research in gene delivery has developed from proof-of-concept into various distinct directions (see Chapter 1). Initial proof-of-concept studies revealed problems of toxicity, short-lived expression, heterogeneity and inefficiency of delivery. Subsequent research aimed at finding mechanistic explanations for these issues and provided insights regarding clearance, gene silencing, cell type and cycle dependency and interference with cellular functions. Questions regarding intracellular processing were more difficult to address, as tools to study and quantify processes at the single cell, organelle or even molecule level were largely inaccessible at the time. Information regarding the individual steps in the process of gene delivery is crucial for understanding the (lack of) success of studied reagents and for the rational design of novel reagents. Questions regarding the fate of nanoparticles inside cells have driven the development of novel tools and methods which now offer opportunities to identify bottlenecks.

Although efforts to elucidate the intracellular fate of DNA in cells through classical cell fractionation methods followed by high-sensitivity quantitative PCR analysis [3-9] deserve attention, a prominent role for fluorescence microscopy techniques is expected. The principle of fluorescence microscopy is the detection of fluorescently labeled molecules or particles in media or cells. Most applications involve fixation of treated cells followed by additional staining to visualize relevant cellular structures and imaging. With this procedure, static information regarding cellular uptake and distribution is obtained with a resolution limit of approximately 170 nm. Recent advances in sensitivity and resolution of imaging techniques, advances in resolution and speed of digital

detection and recording, and expanding possibilities for labeling of molecules and cellular structures or processes permit new possibilities.

Resolution for measuring co-existence of molecules in particles is improved considerably by exploiting Fluorescence Resonance Energy Transfer (FRET) between donor and acceptor molecules. Since this effect only occurs when the distance between fluorophores is <10 nm, co-localization (as an indication for association) can be studied with high precision. Although the various topologies for plasmid DNA and the random formation of poly- and lipoplexes complicate proper positioning of fluorophores as required for FRET, some successful results have been reported to study the stability and unpackaging of plasmid DNA from complexes inside cells [10-14]. Dynamic information regarding motility, co-localization and degradation can be obtained through techniques such as Fluorescence Recovery After Photobleaching (FRAP), Fluorescence Correlation Spectroscopy (FCS) and Single Particle Tracking (SPT). FRAP measurements rely on movement of fluorescent molecules into pre-bleached areas and are suitable for measuring diffusion coefficients in the range of 0.1-100 $\mu m^2/s$ [15]. In FCS (or a variant called Fluorescence Fluctuation Spectroscopy), fluctuation patterns related to movement of one or multiple fluorophores in a certain detection volume are processed through correlation (or fluctuation) analysis and diffusion coefficients >100 $\mu m^2/s$ can be measured [15]. Both techniques have provided valuable information regarding degradation, dissociation and trafficking of overall populations of DTN in media and inside cells [11-13,16-21]. SPT represents a powerful tool to study movement of individual complexes/particles intracellularly (diffusion coefficients 0.0001-10 $\mu m^2/s$) [15] and is expected to pay significant contribution to the understanding of intracellular processing of DNA-Transporting Nanoparticles.

Besides relevant technical aspects such as development of scanning laser modules and improved camera sensitivity and integration times, characteristics of fluorescent labeling play an important role. In case of FRET, the broad emission spectra typical for organic dyes can cause spectral overlap and crosstalk. In case of FCS, fluctuation measurements suffer from photobleaching. The development of quantum dots (QD) and the less toxic nanodiamonds which are characterized by photostability and narrow emission spectra may prove valuable solutions [11-13]. Since the most relevant insights are ultimately expected from live cell imaging, the demand for methods to visualize cellular structures/organelles or processes in living cells increases. As an alternative to the immunostaining procedures generally used in fixed cells, fluorescent tracers can be used. A promising approach is the use of transgenic techniques to create cells that produce their own proteins as fluorescent chimeric molecules (e.g. actin-GFP expressing cells). This approach is highlighted here as this minimizes interference of labeling with processes and particles studied.

Steps in the process towards successful gene expression that are left uncovered by fluorescence tools are transcription and translation. An original and valuable approach to address efficiencies at these levels was developed in the laboratory of Harashima [4]. By quantifying nuclear plasmid copies (by Q-PCR on isolated nuclei), cellular mRNA levels (by RT-PCR) and protein expression, they could calculate transcription efficiency (mRNA/nuclear plasmid copies) and translation efficiency

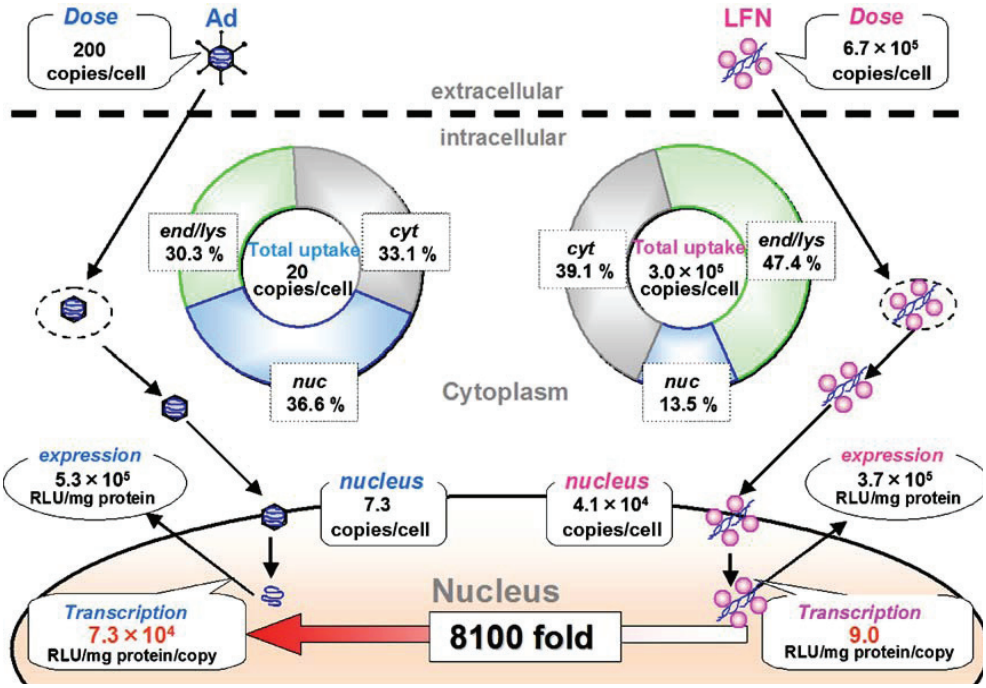


Figure 1. *Schematic overview of the quantitative comparison of gene delivery by adenovirus versus lipofectamine. Reprinted by permission from Macmillan Publishers Ltd: Molecular Therapy [5], copyright 2006.*

(protein/mRNA). This approach led to new and unexpected findings. Upon comparison of lipoplexes and adenovirus it was observed that nuclear delivery and transcription efficiency were only slightly less efficient for lipoplexes (in dividing cells), whereas translation efficiency was approximately 500-fold lower. This was ascribed to translational interference caused by electrostatic interaction of lipofectamine with mRNA and stresses the importance of including post-nuclear delivery processes in gene delivery studies.

Implementing the recently developed tools based on microscopy and PCR will enable acquisition of mechanistic insights regarding practically each individual step in the intracellular processing of DNA and reagents. Insights in bottlenecks for specific reagents can be obtained and reagents can subsequently be modified based on these findings. Importantly, this approach generates build-up of mechanistic insights that can be broadly applied for rational design of reagents and particles for intracellular delivery. Significant progress regarding method development and subsequent evaluation of intracellular processing has been made in the past years. Nevertheless, studies were mainly focused on single steps within the overall process leaving unacknowledged the possibility that improvements at one stage can have detrimental effects on preceding or subsequent steps. The next challenge will be to move from studies addressing single steps (e.g. nuclear uptake or

endosomal escape) to an integrated intracellular kinetics model as proposed by Akita and Harashima[22]. Based on results from quantitative microscopy and PCR studies on lipofectamine versus adenovirus-mediated gene delivery rate-limiting steps were identified (see Figure 1) and used to develop "Programmed Packaging" devices equipped with PEG and targeting ligands to reach target cells, octa-arginines to facilitate macropinocytosis, fusogenic lipids for endosomal escape and nuclear localization signals for active nuclear import.

A limitation of this integral approach is that it is time-consuming and laborious and therefore not widely applicable for testing of novel reagents. Pre-selection of potential candidates is a prerequisite for its feasibility. Validation of methods is an important point of attention to avoid noise resulting from suboptimal methods (e.g. fixation artifacts) and conclusions derived from indirect evidence. Implementation of this approach in laboratories investigating gene delivery is difficult as techniques require both advanced and expensive equipment and skilled operators. Substantial knowledge and expertise in cell biology, physics and optics is required, and must be obtained either through attraction of new personnel, close collaboration or retraining of current personnel with background in pharmaceutical sciences. The scope of this line of research will be largely (if not uniquely) restricted to *in vitro* analysis and may lack translation to *in vivo* results.

The key feature of this strategic direction is rational design based on increased understanding of complex biological processes. Important progress should come from integrated studies addressing multiple steps in parallel that allow appreciation of improvements at intermediate steps. Two topics for future investigation are highlighted here: size effects and optimal location and timing for dissociation. Differential size optima for uptake, cytosolic transport and nuclear uptake are likely to exist and advocate the development of strategies based on sequential unpackaging of highly condensed DNA particles from larger particles. Establishment of the ideal place and time for DNA release would enable rational application of nuclear uptake strategies to either DNA or carrier systems.

Challenges to this approach include:

- The implementation of advanced, expensive equipment and methods for qualitative and quantitative studies in single cells in conventional drug delivery laboratories.
- The closure of the existing expertise gap between conventional pharmaceutical sciences and fields of cell biology, physics and optics.
- Acceleration of research through adequate pre-selection of interesting reagents and translation of methods to high throughput processing.
- Availability of well-defined and characterized particles to enable unbiased assessment of relations of specific structures or properties and effects.

Strategy 2: Optimization of particle design

► *Development of particles of controlled size, shape and composition provides pharmaceutically relevant formulations and enables to study the biological effects of well characterized particles and subsequent optimization.*

The majority of gene delivery strategies employ polyplexes or lipoplexes formed through electrostatic interaction of a cationic reagent with anionic DNA. Such particles are formed in a random fashion leading to large multimolecular and polydisperse complexes. The actual composition regarding number of reagent molecules and DNA per particle is largely unknown and difficult to control. Excess reagent is usually required to push the equilibrium towards DNA condensation and this free fraction is applied together with the particles of interest, thereby contributing to toxicity. Upon exposure to salts and proteins, the physicochemical properties of the complexes may change dramatically (see Chapter 7). Control over particle composition is further complicated when complexes of increased complexity consisting of multiple components are designed (see Chapter 4).

The heterogeneous, ill-defined and environment-sensitive character of poly- and lipoplexes poorly resembles the highly organized virus particles. The central topic of this second strategic direction is the organization of DNA-Transporting Nanoparticles. Instead of using random and unspecific electrostatic interaction, particles of well-defined size, shape and charge are designed. The relation between biological processing and particle characteristics can be studied and applied for various purposes requiring intracellular drug delivery. Charge of particles will be designed such that efficient uptake is combined with limited unspecific interactions with extracellular components and negatively charged cellular components such as RNA.

In case of poly- and lipoplexes, particle formation and resulting characteristics are largely dependent on the DNA itself. Alternatively, particles can be prepared for which the dimensions are controlled by the manufacturing process independent of the presence of DNA. The DNA can be incorporated by embedding in (biodegradable) polymeric matrices such as PLGA [23], gelatin [24-26] or chitosan [27]. Alternatively, layer-by-layer coated particles can be prepared consisting of a core particle of (for example) polymer [28,29], solid lipid [30] or gold [31,32] and subsequent coatings with (functionalized) polymers and DNA. More recently, carbon nanotubes [33] and DNA-encapsulating nanocapsules [34] were added to the range of potential gene delivery reagents. These technologies enable the production of particles with controlled and predictable properties, which is important for batch-to-batch consistency and predictable biological effects (both regarding activity and toxicity). The composition of the nanoparticles can be varied to introduce biodegradability and suitable release profiles. The relation between size and shape of micro- and nanoparticles and cellular uptake has been described and can be further evaluated and exploited for intracellular drug delivery. A recent study illustrating the potential of this approach was conducted by Xu *et*

al.[35]. They developed layered double hydroxide (LDH; a natural mineral) nanoparticles (see Figure 2) and studied the subcellular compartment targeting. LDH nanoparticles shaped as hexagonal sheets (50-150 nm wide and 10-20 nm thick) remained in the cytoplasm, whereas a rod-shaped variant (30-60 nm wide and 100-200 nm long) was reported to quickly (within 3 h) localize into cell nuclei.

Surface charge of nanoparticles can be modified to balance uptake efficiency versus unspecific interactions with protein and non-target cells (charge neutralization, shielding with PEG or introduction of hydroxyl groups [36]). Coating of particles can be chosen to control solubility (charge, hydrophilicity) and interactions with cell membranes (charge). Surfaces can be further modified with ligands with the purpose of targeting or improved cellular uptake (cell penetrating peptides) and intracellular processing (nuclear localization signals). Interestingly, not only the charge and chemical properties of surface modifications, but also the spatial arrangement of surface ligands were recently shown to affect cell membrane penetration [37].

In addition to the characteristics of the overall nanoparticles, it could be worthwhile to pay attention to the stoichiometry of DNA and to site-specific interaction of delivery reagents with DNA molecules. The importance of the number of active DNA-containing particles rather than total dose of DNA shown in chapter 5 of this thesis suggests that development of particles containing single DNA molecules would be valuable. In fact, when looking at viruses, not only size and shape of capsids are well-defined, the DNA/RNA itself is also packaged in an organized fashion such that each virus particle contains single copies of the required gene(s). Efforts to obtain collapse of single

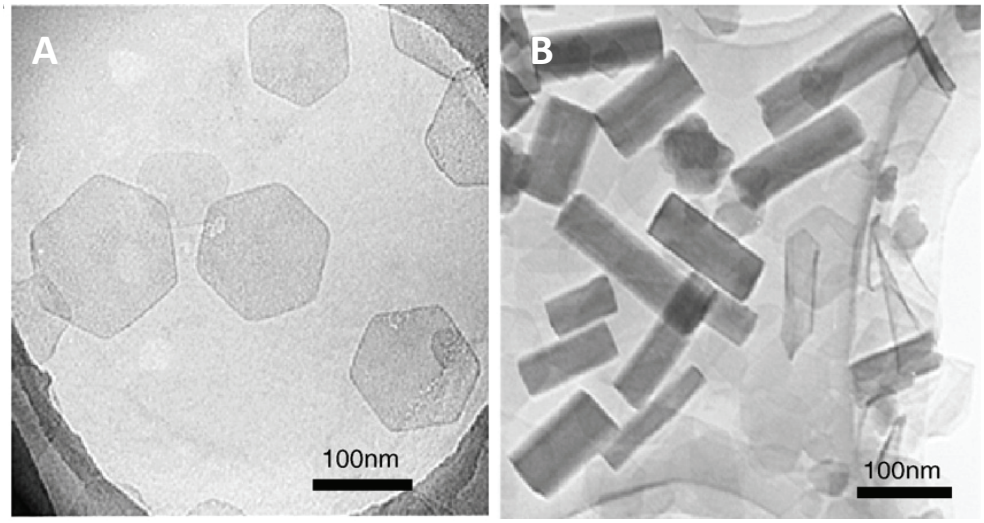


Figure 2. *Transmission electron microscopy images of layered double hydroxide nanoparticles prepared as hexagonal sheets (A) or rods (B). Reprinted from J Control Release , 130/1, Xu ZP et al., Subcellular compartment targeting of layered double hydroxide nanoparticles, 86-94, Copyright 2008, with permission from Elsevier [35].*

DNA molecules into very small structures using non-viral strategies have received interest, but remain relatively scarce. Monomolecular collapse of plasmid DNA into virus-like particles was accomplished by Blessing *et al.* [38]. Condensation of single plasmids was achieved by mixing DNA with cationic detergents below the critical micelle concentration and subsequent stabilization of the particles by cysteine-mediated detergent dimerization. This concept was further explored and optimized by others [39,40], but in all cases *in vitro* transfection required incorporation of the monomolecular DNA nanoparticles into larger structures to mediate efficient cellular uptake. Existence of different size optima for individual process steps should be further explored and may require development of particles that unpack gradually upon biological stimuli.

Viruses can have yet another level of control over DNA incorporation into virus particles. To be able to distinguish host DNA from viral gene copies that need to be encapsulated, assembly proteins recognize specific (RNA or) DNA sequences in the viral genetic material. Binding of coat proteins to these packaging signals initiates assembly of the viral capsid. Sequence specific encapsidation of synthetic nanoparticles in viral protein shells has been accomplished [41] (see Figure 3) and it would be interesting to further explore this concept for DNA-containing nanoparticles for gene delivery. Incorporation of the viral encapsidation signal of SV40 (ses) in plasmids might represents another interesting approach to initiate sequence-specific assembly of viral capsids [42,43].

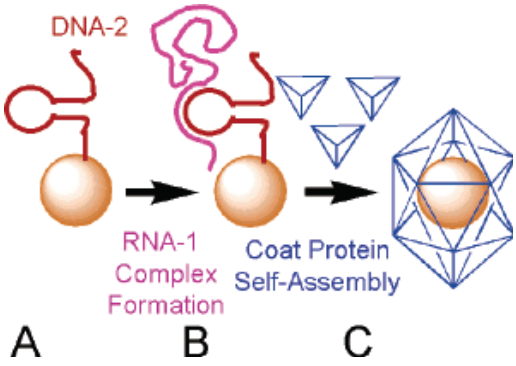


Figure 3. *Sequence specific initiation of viral capsid assembly around a gold nanoparticle. The DNA-tag on the gold particle is bound by viral RNA and forms the origin of assembly (OAS). Viral coat proteins recognize the OAS and polymerize around the particle. Reprinted with permission from [41]. Copyright 2006 American Chemical Society.*

Key to this second strategic line of research are tailorable and predictive nanoparticle formation, and advanced characterization techniques. Reproducible control over basic particle properties will enable to study structure-activity relations and understanding of the impact of surface properties on (intra)cellular processing. Specific functionalizations can be introduced without inducing large changes in overall particle characteristics. Since ligand-mediated effects (e.g. NLS-mediated nuclear import) are often dependent on the cargo, control over this cargo is essential to study the actual effects of these ligands rather than indirect effects due to altered cargo properties. Analysis of

condensation of single DNA molecules and subsequent cellular processing requires extremely sensitive detection methods. Strategies will depend on gaining additional insight into organization and highly efficient genome packaging strategies of viruses at the molecular level.

Challenges to this strategic direction include:

- The continuous development of technologies for predictable and reproducible particle preparation at the nanoscale range.
- To gain insight into alterations of particle (surface) properties upon exposure to biological media and to address these in models aiming to relate particle properties to biological effects.
- Analysis of effects of particle properties at individual steps rather than the overall delivery process.

Strategy 3: High throughput screening (HTS) of reagents

► *Parallel screening of large libraries of candidate reagents under many conditions enables identification of (subtle) structure/activity relations and increases the probability of identifying successful molecules.*

As described in this thesis, research regarding gene delivery reagents has become highly diverse and several key requirements such as low toxicity, biodegradability, successful cellular delivery and intracellular routing have been identified. Development of reagents meeting these criteria requires rational design of key properties of molecules and systematic analysis thereof. Conventional approaches are based on studying small sets of reagents with few variations under a limited set of experimental conditions. This approach is not only costly and time-consuming, but is prone to inter-experimental variability. Moreover, as each variable and condition affects many other variables as well as the overall outcome (as illustrated in Chapter 7), limited experimental setups are inadequate to obtain comprehensive insights and derive structure-activity relationships.

Recent developments in chemical synthesis, technology and informatics now allow for an alternative approach. The combination of combinatorial chemistry, robotics and computational modeling enables to investigate large libraries of synthetic polymers/lipids/peptides in a single high throughput experiment covering many parameters at once. In this setup experiment-to-experiment inconsistencies are avoided such that all reagents and parameters can be studied under equal experimental conditions. Large databases are rapidly generated from which promising reagents can be selected for in-depth follow-up studies and optimizations, thereby reducing time spent on search for lead compounds in conventional approaches.

The principle setup of high throughput screenings consists of the following steps: (1) design of experimental settings and library based on previous knowledge, (2) performing the high

throughput experiment, (3) validation of experimental outcomes through informatics and statistics, (4) validation of selected outcomes through conventional (non-high throughput) experiments [44]. After identifying trends from this first round, consecutive runs can be added for incremental optimizations.

Prerequisite for the success of this approach is the ability to create multiparameter libraries, design of an adequate testing protocol, technologies for automated synthesis, formulation and *in vitro* testing and development of informatics and advanced statistical methods that allow processing and correct interpretation of vast amounts of data generated. The latter involve disciplines distant from pharmaceutics and will not be discussed here. Creation of a suitable library requires the ability to synthesize sets of reagents which share certain properties while introducing variations in other properties in a predictable and systematic fashion. Controllable synthesis processes include solid-phase peptide synthesis, synthesis of polymers via Michael addition reactions and synthesis through click-chemistry. High throughput synthesis and processing however sets additional limits to starting materials and synthetic processes as automated processing requires fluids of low viscosity, whereas high-molecular weight materials are often characterized by high viscosities and reduced solubility [45]. Fully-automated high throughput screening furthermore requires compatibility of synthesis conditions with subsequent *in vitro* testings, limiting applications to syntheses performed under mild conditions without formation of by-products necessitating additional purification steps. Since gene delivery requires particles, besides chemical synthesis a need exists for combinatorial techniques to produce nano- (or micro-) particles of DNA and reagent. Controlled complexation and monitoring of physicochemical properties is essential to limit artifacts of size-induced enhancement of *in vitro* gene expression that is not relevant for *in vivo* conditions.

The high throughput synthesis and screening of poly (β-amino esters) for DNA delivery and more recently of lipidoids for siRNA delivery developed in the laboratory of Langer [24,46-53] (see Figure 4) and of biodegradable pEI derivatives [54] are successful examples. Approaches exploiting combinatorial synthesis followed by semi-automatic or manual testing for gene delivery purposes have been described for cationic N-substituted glycine oligomers [55] and a library of cationic polymers obtained by ring-opening polymerization of the epoxide groups of diglycidyl ethers and the amines of (poly)amines [56]. The approach is suitable for certain polymers, peptides and lipids, but is less obvious for reagents synthesized through uncontrollable processes, requiring purification or requiring complicated formulations such as liposomes. Nevertheless, Yingyongnarongkul *et al.* report combinatorial synthesis of polyamino-based cationic lipids that were subsequently processed to liposomes and used for transfection studies [57,58].

Critical for the success of this approach is the development of an adequate screening method for analyzing transfection efficiency. Inappropriate screening models will generate artifacts (see Chapter 7) and cause selection of reagents based on the wrong criteria. Limiting screening to a single readout parameter such as gene expression will provide a strict selection of reagents that

have favorable characteristics for the complete process, but leave potential reagents that have a limitation in one or more steps in the process unrecognized. Acquisition of mechanistic insights is limited when using this approach (depending on the elaborateness of the *in vitro* screening). High throughput screening of intracellular processing is complicated for reasons of limited space and resolution. Important progress can be expected from developments in high throughput fluorescence microscopy. At present, this technology can already be successfully applied for studying gene expression/silencing, cell viability and co-localization and its application for studying gene delivery is currently being explored in our laboratory. As more and more advanced fluorescence microscopy techniques such as FRET, FRAP and FCS are expected to become available for high throughput imaging, studying individual steps in the process of gene delivery will become a realistic option. It should be mentioned that tracking of intracellular processing necessitates modification of molecules with tags, mostly fluorescent labels. An important contribution should therefore come from development of bright, quantitative and reproducible labeling methods that interfere as little as possible with both the biological process and the molecule or particle studied.

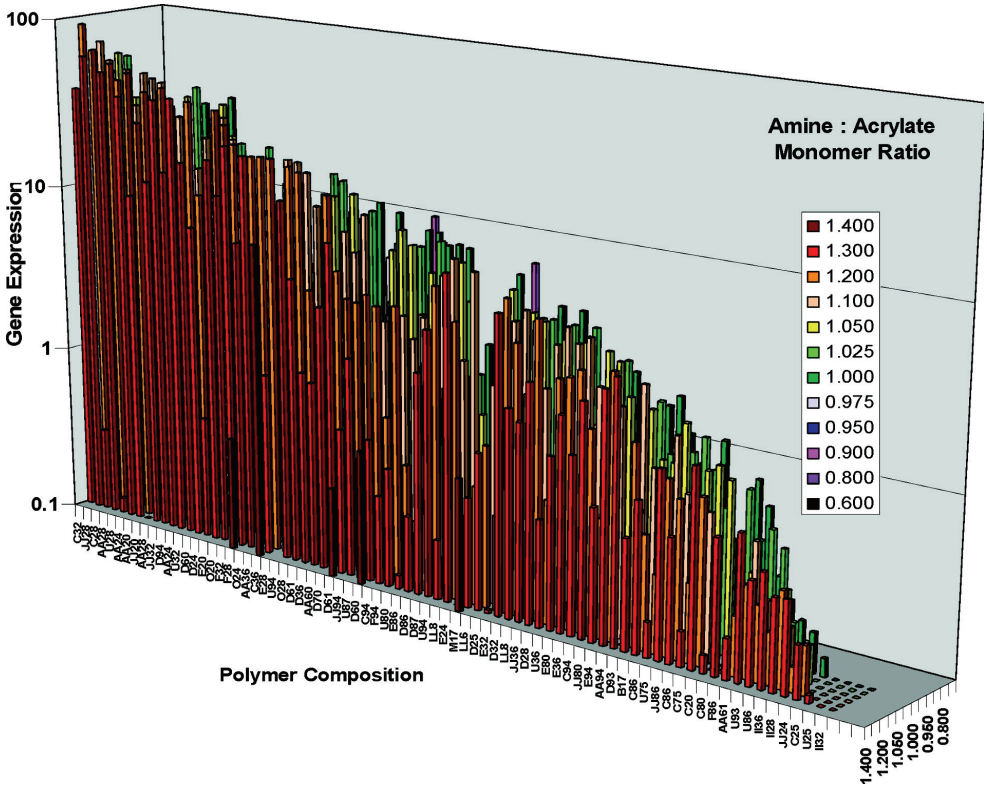


Figure 4. *A high throughput approach allows screening of hundreds of parameters in a single experiment and rapid identification of structure-activity relationships. Reprinted by permission from Macmillan Publishers Ltd: Mol Ther [48], copyright 2005.*

A limitation of this approach is that screening relies fully on *in vitro* setup and results potentially lack translation into and relevance for *in vivo* results.

Improved chemistry enables controlled synthesis of polymers of defined size, architecture and composition. Extensive possibilities for variations in reagents and variability in experimental parameters make high-throughput synthesis and screening a time- and cost-effective approach which increases the probability of identifying promising reagents from diverse libraries. This strategic line of research enables studying important characteristics of polymers, including molecular weight, charge density, structure of the backbone, biodegradability and bio-responsiveness and to identify structure-activity relationships.

Challenges to this strategic direction include:

- Design of reagent libraries and synthesis protocols that are compatible with automatic handling devices.
- Development of methods that allow automated controlled particle formation and monitoring.
- Development of automated procedures to produce and test formulations of increased complexity.
- Development of an adequate, standardized screening protocol based on consensus in the field. Special attention should be paid to include models for non-dividing cells.
- Black box approach studying gene expression as final outcome should make way to approaches studying functional role of delivery reagents in biological processes. This poses extra demands for image acquisition and image analysis. Live cell imaging requires hardware-driven microscopes in which acquisition settings are automatically adjusted based on feedback from online analysis to maintain focus and optimal sensitivity. Important advances are expected once powerful imaging techniques such as FRET, FRAP and FCS can be automated.
- Advances in automated image processing, datamining and storage of immense databases should be adopted from other disciplines for successful implementation of HTS.

Concluding remark

All three strategic lines of research offer valuable opportunities. HTS technologies facilitates rapid identification of high potential gene delivery candidates, intracellular PK/PD studies provide fundamental insights applicable to both other research lines (and research beyond gene delivery) and focus on stable nanoparticle design will generate a platform of new nanoparticles applicable for various types of intracellular drug delivery. Interactions between the three disciplines will have a synergistic effect on development of DNA-Transporting Nanoparticles (see Figure 5). Candidate reagents identified rapidly by the HTS approach can be further studied in integrated intracellular

kinetics models and may accelerate discovery of new molecular structure/activity relations at intracellular processing steps. Communication of new methodologies developed in the field of intracellular PK/PD studies can accelerate their translation into HTS approaches and progress from black box to advanced screening models. Material characteristics studies by HTS can be used to tune the composition of matrix and surface coatings of stable nanoparticles. Technologies for controlled nanoparticle preparation and monitoring should be implemented in HTS approaches to improve reliability of screening procedures. Close collaboration between groups working on intracellular PK/PD models and on nanoparticle design will enable an iterative process of finding optimal properties for individual process steps and subsequent optimization of nanoparticle architecture. Altogether, these approaches should enable the development of increasingly sophisticated DNA-Transporting Nanoparticles that are capable of overcoming the multiple barriers involved in non-viral gene delivery.

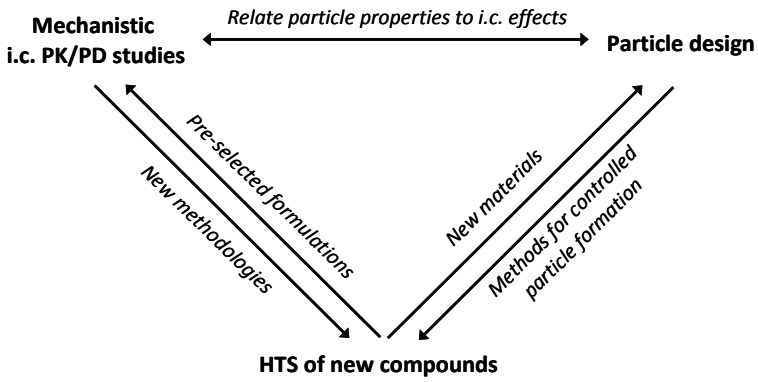


Figure 5. *Diagram of the three described strategic directions and potential synergistic interactions.*

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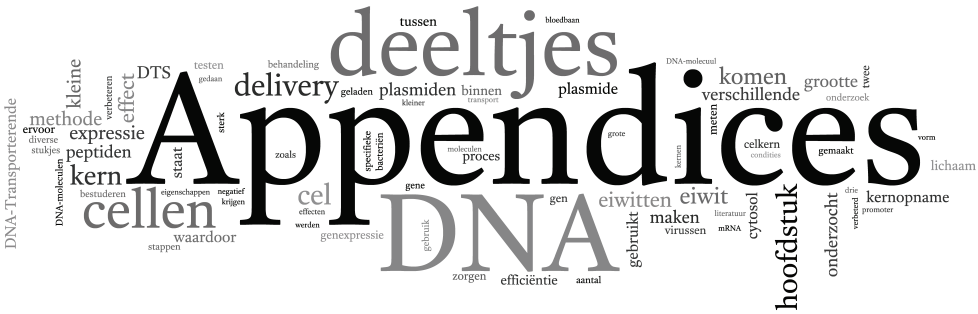


Nederlandse samenvatting voor niet-ingewijden

List of publications

Curriculum Vitae

Gentherapie: DNA als geneesmiddel

Onze erfelijke informatie ligt opgeslagen in het DNA. In mensen en veel andere organismen is het DNA aanwezig als chromosomen in de kernen van cellen. Elk chromosoom bevat meerdere genen, en elk gen bevat een code (DNA sequentie) die vertaald kan worden in eiwitten. Dit gebeurt in twee stappen: de eerste stap is de vertaling van DNA naar messenger RNA (mRNA) en heet transcriptie; de tweede stap is de vertaling van het mRNA naar de aminozuren die samen een eiwit vormen, dit proces heet translatie. De vele genen in onze cellen zorgen ervoor dat een heel scala aan eiwitten aangemaakt wordt (eiwitexpressie) met een scala aan functies die samen zorgen voor het functioneren van onze cellen, organen en uiteindelijk ons hele lichaam.

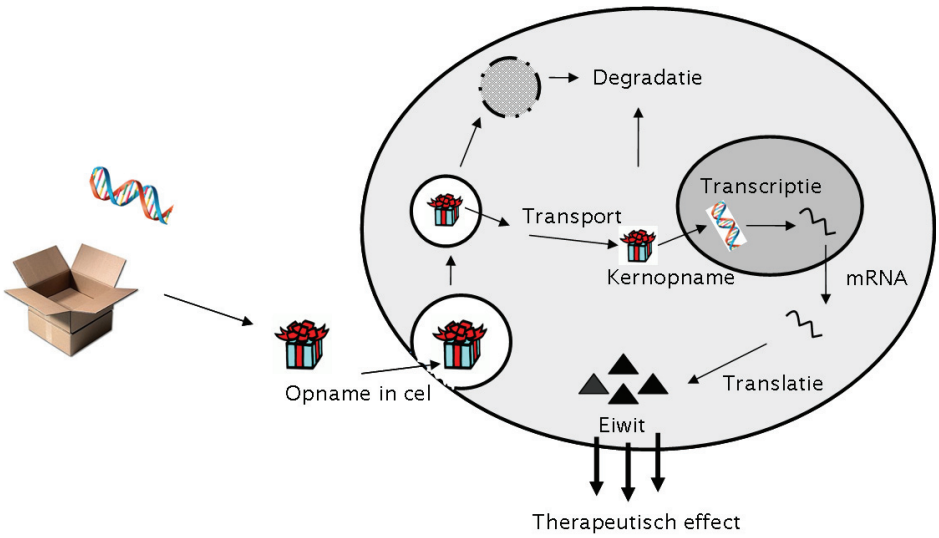
Het principe van gentherapie is het inbrengen van nieuw genetisch materiaal in cellen met als doel om veranderingen aan te brengen in de eiwitexpressie van deze cellen. Er kunnen verschillende soorten genetisch materiaal toegediend worden, maar dit proefschrift beperkt zich tot het gebruik van DNA. Het doel is dan om DNA in cellen te brengen zodat een eiwit met therapeutische werking tot expressie wordt gebracht. Dit eiwit kan corrigeren voor een bestaand, maar defect eiwit, of dienen als supplement voor een ontbrekend eiwit (bijvoorbeeld voor de behandeling van cystic fibrosis waarbij een ontbrekend eiwit de vorming van taai slijm in de luchtwegen veroorzaakt). Ook kan een eiwit worden gekozen dat een giftig effect teweeg brengt en ervoor kan zorgen dat de cellen (en hun omgeving) vernietigd worden. Deze zogenaamde “suicide gene therapy” kan gebruikt worden voor het bestrijden van kankercellen. Daarnaast kan DNA gebruikt worden dat codeert voor eiwitten die als vaccin werken. Overigens is het belangrijk om onderscheid te maken tussen twee vormen van gentherapie: somatische gentherapie en kiembaan-gentherapie. In Nederland is alleen de somatische variant toegestaan waarbij alleen lichaamscellen (en geen geslachtscellen) betrokken zijn en er geen sprake is van effecten op het nageslacht.

Huidige status

Momenteel zijn er slechts twee gentherapie-producten op de markt: Gendicine™ en Oncorine™ zijn beide in China geregistreerd voor de behandeling van hoofd-hals kanker. Een Nederlands bedrijf (Amsterdam Molecular Therapeutics) heeft in januari 2010 een aanvraag ingediend voor registratie van het middel Glybera® voor de behandeling van de zeldzame stofwisselingsziekte lipoproteine lipase deficiëntie. In totaal lopen er momenteel ongeveer 1600 klinische studies, waarvan het merendeel fase I studies zijn waarin de veiligheid getoetst wordt (ongeveer 1000) en een klein aantal fase III heeft bereikt waarin de werkzaamheid getest wordt (ongeveer 60). De top drie aandoeningen die onderzocht worden, zijn kanker (meer dan de helft), cardiovasculaire aandoeningen en erfelijke ziekten.

Drie problemen: delivery, delivery, en delivery

Wat is de reden dat er ondanks het vele onderzoek dat sinds de jaren ‘70 is verricht slechts twee gentherapeutica geregistreerd zijn? Een onderzoeker uit het veld, David Baltimore, heeft eens gezegd dat er drie problemen zijn: delivery, delivery en delivery. Met delivery wordt bedoeld het afleveren van een geneesmiddel op de plek van bestemming. In geval van gene delivery is de plek van bestemming de kernen van cellen in een bepaald weefsel, bijvoorbeeld een tumor. Dit betekent dat het DNA vanuit de bloedbaan naar het juiste weefsel moet komen, daar in de cellen terecht moet komen en binnen de cel een weg naar de kern moet afleggen. Dit is een complex proces waarbij vele barrières overwonnen moeten worden (zie Figuur 1). Allereerst moet het DNA in kleine deeltjes verpakt worden om afbraak (degradatie) van DNA door enzymen (nucleasen) in de bloedbaan en binnen de cel te voorkomen. Naast bescherming heeft het verpakkingsmateriaal (carrier of delivery agent) als doel om de grote negatief geladen DNA-moleculen in kleine positief geladen deeltjes te verpakken, wat ervoor zorgt dat de deeltjes beter door de bloedbaan kunnen bewegen en beter door cellen opgenomen kunnen worden. De positieve lading van de deeltjes zorgt dat ze gemakkelijk aan de negatief geladen buitenkant (membraan) van cellen plakken en vervolgens in blaasjes de cel binnengaan. Deze opname in cellen heet endocytose en is van meerdere factoren afhankelijk, waaronder de grootte van de deeltjes. Eenmaal in de cel moeten de DNA-Transporterende deeltjes uit de blaasjes vrijkomen. Ze komen dan terecht in het cytosol van de cel, wat erg stroperig (viskeus) is en vol zit met eiwitten en organellen. De deeltjes moeten door dit cytosol naar de celkern bewegen, en uiteindelijk in de kern terecht komen. De kern is echter goed afgeschermd van het cytosol en transport tussen het cytosol en de kern kan alleen via kleine



Figuur 1. *Gene delivery: een schematische weergave van de opeenvolgende stappen die nodig zijn om een therapeutisch gen tot expressie te krijgen.*

kanalen, zogenaamde kernporie complexen (nuclear pore complexes). Deze kanalen laten deeltjes tot ongeveer 9 nanometer (dit is een miljoen keer kleiner dan een millimeter) door, wat vele malen kleiner is dan de afmetingen van de DNA-Transporterende deeltjes, of van het DNA-molecuul zelf. Toch moeten er soms grotere moleculen vanuit het cytosol naar de kern getransporteerd worden en deze moleculen hebben een signaal waardoor de kernporiën weten dat ze moeten verwijden om het molecuul binnen te laten. In dat geval kunnen echter nog steeds maar deeltjes tot ongeveer 39 nanometer door de poriën, waardoor het een groot probleem (uitdaging) blijft om grote vreemde DNA-moleculen naar binnen te krijgen. Eenmaal in de celkern moet het DNA terecht komen bij de moleculen die de genetische code kunnen omzetten in mRNA. Tot slot moet het mRNA uit de kern getransporteerd worden en in het cytosol omzet worden in het gewenste eiwit dat een therapeutisch effect geeft.

Het doorlopen van deze route wordt nog eens bemoeilijkt omdat het lichaam er alles aan doet om te voorkomen dat er vreemd DNA in celkernen terecht komt. Aangezien DNA van nature uitsluitend in celkernen (en mitochondriën) voorkomt, wordt alle DNA daarbuiten geïnterpreteerd als een gevaar. Het lichaam kan dit zien als DNA dat is vrijgekomen uit afgestorven cellen of afkomstig uit aan ziekteverwekkende indringers zoals virussen of bacteriën en het vervolgens opruimen met behulp van nucleases of het afweersysteem.

Natuurtalenten: virussen

Virussen zijn al tijden in staat om hun genetisch materiaal in een gastheer tot expressie te laten komen. Een voor de hand liggende strategie voor gene delivery is dus om virusdeeltjes als verpakking te gebruiken waarbij het DNA van het virus vervangen wordt door het therapeutische DNA. Dit blijkt een erg efficiënte strategie, maar helaas niet zonder gevaren. Het lichaam kan de virusdeeltjes herkennen waardoor een afweerreactie wordt opgewekt. Of de virussen behouden onverhoopt iets van hun ziekteverwekkende eigenschappen. Sommige virussen laten hun DNA in het chromosomale DNA inbouwen. Als dit op de verkeerde plekken gebeurt, kunnen kankergenen worden geactiveerd. Een klasse virussen die relatief veilig is, heeft weer als nadeel dat er maar weinig DNA in past. Daarom wordt er veel onderzoek gedaan naar veiligere alternatieven in de vorm van synthetische, of niet-virale gene delivery strategieën.

Niet-virale gene delivery

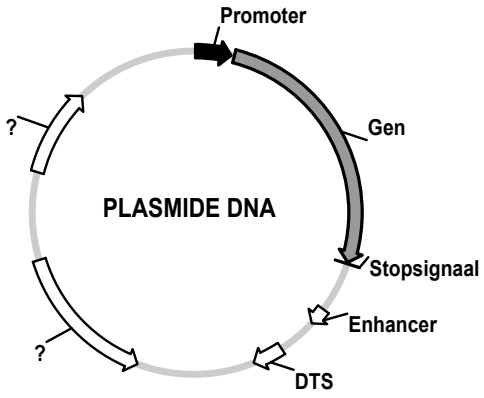
In 1979 werd de eerste studie gepubliceerd waarin men erin was geslaagd om een vreemd gen zonder gebruik van virussen tot expressie te laten komen in dierlijke cellen. In de volgende decennia werden diverse systemen ontwikkeld om DNA in te pakken en in cellen tot expressie te laten komen (transfecteren). Alhoewel het concept bewezen was, kwamen ook steeds meer problemen aan het licht. Het DNA bleek maar in heel weinig gevallen tot expressie te komen (inefficiënt), maar kort effect te hebben (tijdelijk effect), alleen in specifieke cellen en

omstandigheden te werken (heterogeniteit) en de DNA-Transporterende deeltjes bleken schadelijke effecten te hebben (toxiciteit). Daarnaast liep men aan tegen technologische beperkingen om het proces van begin tot eind te kunnen bestuderen, waardoor men lang in het duister tastte bij welke stap het misging. Aangezien een cel slechts ongeveer 20 micrometer (een duizendste van een millimeter) groot is, zijn er zeer ingewikkelde en gevoelige technieken nodig om te kunnen zien wat hierbinnen gebeurt.

Onderzocht in dit proefschrift

Bij het samenstellen en testen van DNA-Transporterende deeltjes kunnen diverse aspecten onderzocht en verbeterd worden. In dit proefschrift wordt aandacht besteed aan het ontwerp van het DNA (hoofdstuk 2 en 3), de deeltjes waarin het DNA verpakt wordt (hoofdstuk 4 en 5) en aan methoden die relevant zijn bij het bestuderen van de eigenschappen en werkzaamheid van de gemaakte deeltjes (hoofdstuk 6 en 7). Tot slot worden aanbevelingen gedaan voor toekomstig onderzoek (hoofdstuk 8).

In hoofdstuk 2 wordt aan de hand van een uitgebreide literatuurstudie onderzocht wat er verbeterd kan worden door zorgvuldig ontwerp van het DNA-molecuul. Om te begrijpen hoe we zelf DNA kunnen ontwerpen, is het handig om eerst kort te vertellen hoe DNA in het lab gemaakt wordt. Hiervoor maken we gebruik van bacteriën. Bacteriën hebben de bijzondere eigenschap dat ze naast hun chromosomen ook over speciaal cirkelvormig DNA (plasmiden) kunnen beschikken. Dit plasmide DNA kunnen ze onderling uitwisselen om zich zo beter aan hun omgeving aan te kunnen passen. In het lab kunnen we dit cirkelvormige DNA openknippen en er nieuwe stukken DNA inplakken om zo een DNA-molecuul naar wens te maken. Vervolgens stoppen we dit nieuwe plasmide weer in bacteriën en laten we ze zich vermenigvuldigen, waarbij ook het plasmide vermenigvuldigd wordt. Uiteindelijk worden de bacteriën gedood en de plasmiden opgezuiverd. In Figuur 2 staat een schematische weergave van een plasmide, met daarin allerlei functionele eenheden die hierna besproken worden.



Figuur 2. *Schematische weergave van plasmide DNA. Minimaal zijn nodig een promoter, een gen en een stopsignaal. Daarnaast kunnen extra stukjes DNAtoegevoegd worden, zoals bijvoorbeeld enhancers en DNA nuclear Targeting Sequences (zie Hoofdstuk 3). Ook kunnen ongewenste stukken DNA verwijderd worden door te knippen en plakken met speciale enzymen..*

Om DNA als geneesmiddel te laten werken, moet het in de cellen afgelezen kunnen worden (tot expressie kunnen komen) en hiervoor zijn minimaal drie dingen nodig: een startsignaal (promoter) zodat de cel weet waar het moet beginnen met lezen, gevolgd door (2) het gen dat codeert voor het gewenste therapeutische eiwit en (3) het stopsignaal dat aangeeft waar de cel moet stoppen met aflezen. Daarnaast kunnen sequenties ingebouwd worden die het transcriptieproces *efficiënter* maken (enhancers) of de *werkzaamheid verlengen*. Ook kan ervoor gezorgd worden dat het DNA alleen in bepaalde cellen van het lichaam tot expressie komt, waardoor de *specificiteit en daarmee de veiligheid* van de behandeling wordt vergroot. Cellen spreken als het ware verschillende talen en door de boodschap in een specifieke taal aan te bieden, kan gezorgd worden dat de boodschap alleen door de gewenste cellen begrepen kan worden. Anderzijds zijn er ook stukjes DNA die een negatief effect hebben, zoals elementen afkomstig uit viraal of bacterieel DNA. Dit DNA verschil van ons DNA omdat de frequentie waarin elk van de vier baseparen A, T, G en C voorkomt sterk verschilt. Het lichaam herkent dit patroon, waarna het afweersysteem ingeschakeld wordt en het DNA geïnactiveerd wordt. Om te *voorkomen dat therapeutisch DNA te snel uitgeschakeld wordt*, moet dus zo min mogelijk viraal/bacterieel DNA gebruikt worden. Kortom, door zorgvuldig ontwerp van het DNA-molecuul kan gentherapie efficiënter, veiliger en langer werkzaam gemaakt worden.

De bovenstaande voorbeelden van DNA design kunnen weliswaar belangrijke verbeteringen geven, maar werken alleen wanneer het DNA eenmaal succesvol in de kern is afgeleverd. Er is in de literatuur echter ook een vorm van DNA design beschreven dat een effect zou hebben op het delivery proces zelf. **In hoofdstuk 3 wordt onderzocht of bepaalde stukjes DNA het transport van het cytosol naar de celkern kunnen verbeteren.** Het idee is als volgt: in de cel komen eiwitten voor die een signaal bevatten waardoor ze in staat zijn naar de kern te transporteren. Een deel van deze eiwitten is daarnaast in staat om aan bepaalde DNA-sequenties te binden. Door deze sequenties in te bouwen in het DNA-molecuul dat we willen afleveren, zou deze binnen de cel aan de betreffende eiwitten kunnen binden en met deze eiwitten kunnen meeliften de kern in.

In de literatuur zijn enkele voorbeelden van zulke stukjes DNA, genaamd DNA nuclear Targeting Sequences (DTS) beschreven. Om het effect van deze DTS te testen, hebben we gebruik gemaakt van kweekcellen en van plasmide DNA dat codeert voor een groengekleurd eiwit. Zo konden we eenvoudig de groenkleuring van de cellen meten als maat voor de efficiëntie van genexpressie. We zagen echter geen verschil in efficiëntie tussen DNA met of zonder een DTS. Hierbij maakte het niet uit welke type cellen we gebruikten en of de cellen wel of niet konden delen. Ook werd de hypothese getest dat de methode van genafgifte in de cel van invloed zou kunnen zijn op het optreden van het DTS-effect. De verwachting was dat bij afgifte van vrij DNA in het cytosol makkelijker binding tussen de relevante transport-eiwitten en de DTS in het DNA zou kunnen optreden, terwijl dragersystemen waarvan beschreven is dat ze het DNA in intacte deeltjes de kern in transporteren deze interactie zouden remmen. Het DTS-effect werd echter in geen van beide gevallen waargenomen.

In deze studies keken we naar de uiteindelijke expressie van eiwit, maar zoals weergegeven in Figuur 1 zitten er nog een aantal stappen tussen kernopname en eiwitproductie. Om zuiver naar het effect van een DTS op de kernopname te kijken, hebben we een methode opgezet om de hoeveelheid DNA die per celkern werd afgeleverd te meten. Dit deden we door de cellen na behandeling kapot te maken, de kernen eruit te halen en met behulp van Q-PCR het aantal afgeleverde DNA-moleculen ten opzichte van chromosomaal DNA (als maat voor het aantal kernen) te meten. Q-PCR staat voor kwantitatieve polymerase-kettingreactie en is een methode waarmee kleine hoeveelheden DNA vermenigvuldigd kunnen worden tot hoeveelheden die we kunnen meten. Echter, ook in deze experimenten zagen we geen verschil tussen DNA met of zonder DTS. In de literatuur zijn wel verschillen in kernopname tussen plasmiden met of zonder DTS beschreven, waarbij de kernopname met behulp van microscopische metingen werd bepaald. We kunnen op dit moment niet uitsluiten dat de verschillen in waarneming veroorzaakt zijn door de gekozen methode.

Kortom: ofwel zijn DTS niet in staat de kernopname te verbeteren, ofwel zorgen ze voor een kleine verhoging van het aantallen plasmiden dat de kern bereikt, maar leidt dit alsnog niet tot meer genexpressie. Een mogelijke verklaring voor dit laatste is dat een proces na kernopname (dus transcriptie of translatie) snelheidsbepalend is. Voor onze experimenten is een plasmide gebruikt met een sterke virale promoter; vervolgstudies zullen moeten uitwijzen of DTS wel toegevoegde waarde hebben indien plasmiden met een zwakke (maar specifieke) promoter.

In hoofdstuk 4 wordt onderzocht of het transport naar de kern verbeterd kan worden door de signalen die eiwitten gebruiken in DNA-Transporterende deeltjes in te bouwen. Zoals eerder genoemd, zijn er in de cel eiwitten die een signaal bevatten waardoor ze via actieve opname de celkern in kunnen transporteren. Deze signalen worden Nuclear Localization Signals (NLS) genoemd. In dit hoofdstuk wordt onderzocht of het koppelen van deze kleine stukjes eiwit (peptiden) aan plasmide DNA ervoor kan zorgen dat het DNA de kern in kan. Om het NLS aan het plasmide te koppelen, hebben we bi-functionele peptiden ontworpen die uit een DNA-bindend domein (DBD) en een NLS bestaan. Deze DBD-NLS peptiden bleken in staat om met DNA kleine deeltjes te vormen en deze in cellen af te leveren, maar dit resulteerde niet in genexpressie.

Vervolgens hebben we gekeken of de peptiden de efficiëntie van twee andere veel gebruikte dragersystemen (lipofectamine en polyethyleneimine, kortweg pEI) konden verbeteren. Dit gaf een wisselend effect: pEI was niet gebaat bij toevoeging van de peptiden, maar de efficiëntie van een lipofectamine bleek sterk verhoogd te worden na inmenging van de peptiden. In dit geval bleken echter belangrijke eigenschappen van de deeltjes zoals de grootte en lading zeer sterk veranderd te zijn, wat ook een verklaring kan zijn voor de toegenomen efficiëntie. Dit vermoeden werd bevestigd door het onvermogen van de deeltjes om DNA tot expressie te brengen in niet-delende cellen en omdat er niet meer deeltjes in celkernen terugvonden.

Kortom, de geteste peptiden bleken niet in staat om het transport van DNA-moleculen naar de kern te verbeteren. Inmiddels zijn in de literatuur meerdere vergelijkbare voorbeelden beschreven waarin het inmengen van peptiden weliswaar meer genexpressie gaf, maar niet door verbeterde kernopname. Anderzijds zijn er studies die aantonen dat NLS-peptiden wel verhoogde kernopname kunnen geven als ze op een andere manier aan het DNA gekoppeld worden dan hier getest werd. Deze koppelingsmethode heeft echter weer als nadeel dat de peptiden niet meer van het DNA loslaten, waardoor de cellen het DNA niet meer kunnen aflezen. Een interessante richting voor vervolgonderzoek zou zijn om de NLS-peptiden wel volgens deze methode te koppelen, maar alleen op plekken in het plasmide DNA die niet afgelezen hoeven te worden.

In hoofdstuk 5 wordt onderzocht hoeveel DNA-moleculen per deeltje ingepakt moeten worden. Een virus heeft aan één genkopie per deeltje genoeg, maar bij het maken van niet-virale DNA-Transporterende deeltjes worden vele plasmiden tegelijk verpakt. Om te testen of dit wel zinvol is, hebben we deeltjes gemaakt met verschillende verhoudingen van actieve plasmiden met gen en inactieve plasmiden zonder gen (junk DNA). Het bleek dat een substantieel deel van het actieve DNA vervangen kon worden zonder evenredig verlies in genexpressie. Dit effect trad vooral op wanneer de twee soorten DNA samen in hetzelfde deeltje afgeleverd werden, en in veel mindere mate wanneer los gevormde deeltjes van elk type DNA toegediend werden. De resultaten tonen aan dat niet de totale hoeveelheid DNA, maar het totale aantal actief DNA-bevattende deeltjes een kritieke factor is voor de efficiëntie. Deze bevinding pleit voor de ontwikkeling van deeltjes waarin een enkel DNA molecuul verwerkt zit in plaats van complexen op basis van vele DNA-moleculen. Anderzijds zouden meerdere plasmiden tegelijkertijd afgeleverd kunnen worden om verschillende therapeutische effecten te combineren, zonder verlies aan activiteit van de individuele componenten. Daarnaast kan het vervangen van actief DNA door junk DNA of andere grote negatief geladen hulpstoffen de kosten verlagen en het veiligheidsprofiel verbeteren.

In hoofdstuk 6 wordt een methode beschreven om de deeltjesgrootte van DNA-Transporterende deeltjes te bepalen. De grootte van de deeltjes is een belangrijke parameter omdat grootte gerelateerd is met allerlei stappen in het delivery proces: deeltjes moeten klein genoeg zijn om ze in de bloedbaan te kunnen spuiten en moeten klein blijven om geen verstoppingen in kleine bloedvaten te veroorzaken. Eenmaal bij de cellen aangekomen, bepaalt de grootte van de deeltjes (o.a.) via welke route en hoe efficiënt ze door de cel opgenomen worden.

Bij het maken van DNA-Transporterende deeltjes worden weliswaar kleine positief geladen deeltjes gevormd, maar de gevormde deeltjes verschillen onderling sterk in grootte. Daarnaast wordt de grootte van de deeltjes beïnvloed door blootstelling aan zouten en eiwitten, die veelvuldig voorkomen in het lichaam. Het is daarom van belang om methoden te hebben waarmee deeltjes onder deze condities gekarakteriseerd kunnen worden. In hoofdstuk 6 wordt hiervoor een nieuwe methode ontwikkeld waarbij we gebruik maken van een flow cytometer (ook wel FACS

genoemd), een apparaat dat je zou kunnen zien als een hele kleine M&M-sorteerder. Dit apparaat kan tegelijkertijd de afmetingen en vorm (wel of geen pinda) en de kleur van deeltjes meten en de deeltjes op basis van deze eigenschappen sorteren. Met behulp van beads gelabeld met een fluorescente kleurstof van verschillende groottes (0.1 – 2 micrometer) kon het apparaat geijkt worden om deeltjes kleiner dan een micrometer te meten. Door de deeltjes waarin we geïnteresseerd waren een kleur te geven, konden deze onderscheiden worden van niet-gekleurde deeltjes die vaak van nature aanwezig zijn in biologische vloeistoffen. De ontwikkelde methode kan gebruikt worden voor een snelle screening van de stabiliteit van DNA-Transporterende nanodeeltjes in diverse vloeistoffen. Zoals beschreven kan een FACS ook deeltjes sorteren. We hebben geprobeerd om hiermee DNA-Transporterende Nanodeeltjes in verschillende groottes te sorteren, zodat we er vervolgens celopnamestudies mee zouden kunnen doen. Alhoewel het sorteren lukte, konden we geen vervolgstudies doen omdat de deeltjes tijdens het sorteerproces zeer sterk verdund werden. Flow cytometers zijn oorspronkelijk ontworpen om cellen te bestuderen en wellicht worden de mogelijkheden verbeterd als bedrijven de vraag naar toepassingen voor kleinere deeltjes herkennen en aangepaste apparatuur ontwikkelen.

In hoofdstuk 7 wordt het effect van allerlei experimentele condities op het uitlezen van genexpressie onderzocht en wordt een voorstel voor een standaard protocol voor het testen van nieuwe middelen gedaan. In de afgelopen jaren en in verschillende laboratoria wordt het testen van genafgiftesystemen in allerlei verschillende celtypes, onder een variëteit aan condities, met diverse uitleessystemen en ten opzichte van allerlei verschillende referenties uitgevoerd. In dit hoofdstuk worden de effecten van diverse parameters op de gemeten efficiëntie onderzocht. Op basis van deze dataset wordt een screening protocol voorgesteld met het doel om tot standaardisatie binnen het veld te komen. Dit zou het mogelijk maken om resultaten van verschillende laboratoria beter te kunnen vergelijken en interpreteren.

Tot slot worden in hoofdstuk 8 aanbevelingen gedaan voor toekomstig onderzoek naar niet-virale genafgifte. Hierboven staan al enkele specifieke suggesties voor specifieke vervolgexperimenten, maar in dit hoofdstuk beschrijf ik drie strategische richtingen voor vervolgonderzoek in het algemeen: (1) nadruk op het bestuderen van kwalitatieve en kwantitatieve processen binnen de cel om zo meer inzicht te krijgen in de losse stappen van het delivery proces, (2) nadruk op het kunnen produceren van nanodeeltjes waarvan de grootte, vorm en samenstelling goed gecontroleerd kunnen worden als alternatief voor de willekeurig gevormde deeltjes die nu veelal gebruikt worden en (3) met behulp van automatisering grote aantallen middelen en condities tegelijkertijd testen om versneld inzicht te krijgen welke middelen potentie hebben voor efficiënte genafgifte. Hopelijk zullen deze benaderingen het mogelijk gaan maken om gericht DNA-Transporterende Nanodeeltjes te ontwikkelen die de vele barrières in niet-virale genafgifte stapsgewijs kunnen overwinnen.

List of publications

van Gaal EVB, Oosting RS, van Eijk R, Bakowska M, Feyen D, Kok RJ, Hennink WE, Crommelin DJA, Mastrobattista E. DNA nuclear Targeting Sequences for non-viral gene delivery. *Manuscript in preparation.*

van Gaal EVB, van Eijk R, Oosting RS, Kok RJ, Hennink WE, Crommelin DJA, Mastrobattista E. Towards a standard protocol for *in vitro* transfection screenings of non-viral gene delivery systems. *Submitted for publication.*

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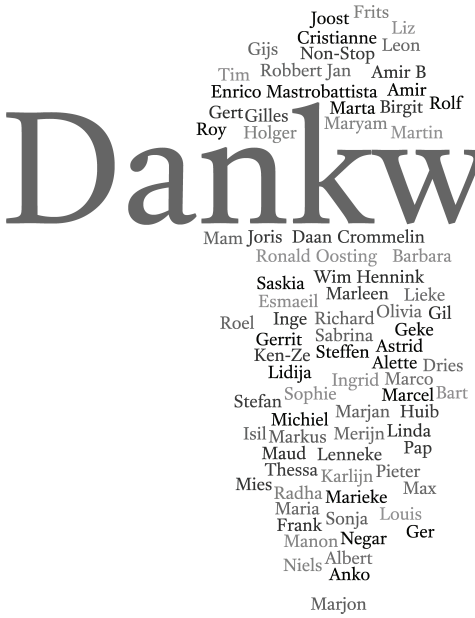
Curriculum Vitae

Ethlinn van Gaal was born in Tilburg, The Netherlands, on January 14th 1979. In 1997 she graduated from the Elzendaal College secondary school in Boxmeer and started her study Pharmacy at Utrecht University. In 2002 she visited the Victorian School of Pharmacy at Monash University in Melbourne, Australia, for a 6-month research project on non-viral gene delivery under the supervision of prof. dr. Colin Pouton, dr. Richard Prankerd and dr. Jalal Jazayeri. Between 2003 and 2005, Ethlinn did internships at several community and hospital pharmacies and at Astellas Pharma (Leiderdorp, The Netherlands) for her masters degree. In April 2005 she obtained her masters degree with a specialization in Drug Development and became a licensed pharmacist. Ethlinn worked as a junior lecturer teaching Pharmaceutical Analysis at Utrecht University and as a formulation scientist/production pharmacist at Enceladus before starting her PhD project in October 2005 at the department of Pharmaceutics at the Utrecht Institute for Pharmaceutical Sciences (UIPS). Under the supervision of prof. dr. Daan Crommelin, prof. dr. ir. Wim Hennink, dr. Enrico Mastrobattista and dr. Ronald Oosting she worked on the design and *in vitro* evaluation of DNA-Transporting Nanoparticles for non-viral gene delivery, which resulted in this thesis. During her PhD studentship, she was a member of the UIPS PhD Education Committee, and of the organizing committee of the UIPS PhD symposium "Hora est, what's next?" and the 7th biennial Global Pharmaceutics Education Network conference held in Leuven, 2008.

Ethlinn is currently working as a postdoctoral researcher at the department of Pharmaceutics on a project focusing on the industrial exploitation of core crosslinked polymeric micelles, financed by MEDITRANS, an Integrated Project funded by the European Commission under the "nanotechnologies and nano-sciences, knowledge-based multifunctional materials and new production processes an devices" (NMP), thematic priority of the Sixth Framework Program.

Dankwoord



“Het leven draait niet om winnen maar om verliezen,
en wie dat het beste kan, die sterft straks als een gelukkig man”
-Leon Giesen-

Hèhè, het zit erop! Dit boekje is een feit en de weg er naar toe is ten einde. Het was een intensieve tijd waarin ik ontzettend veel geleerd heb en veel pieken en dalen heb doorgemaakt. Iedereen die hieraan heeft bijgedragen wil ik op deze plek bedanken!

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Nog even volhouden en dan kun je weer opladen tot de enige echte Ellen met grote glimlach en bruisend van energie, verhalen, plannen, concertagenda's etc. etc. etc.…ik verheug me er nu al op!

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En dan jij Merijn, lieve Merijn. Jij hebt dit project van het begin tot de laatste minuut, en van binnen en van buiten meegemaakt en bent een onmisbare steun voor me geweest. Ik heb me wel duizenden malen afgevraagd of ik alles anders zou doen als ik opnieuw kon beginnen, maar er is één reden waarom ik dat nooit zou doen en dat ben jij!

Het is een dag in oktober, dat is helemaal waar
Maar het voelt als nieuwjaar,

Gelukkig nieuwjaar!

 Ethlinn