International Journal of Pharmaceutics 390 (2010) 76–83

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Junk DNA enhances pEI-based non-viral gene delivery

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ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 15 May 2009
Accepted 25 August 2009
Available online 29 August 2009

Keywords:
Non-viral gene delivery
pEI
Junk DNA
Transfection
Plasmid

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 upon 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.

1. 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 (Friedmann and Roblin, 1972), practical applications remain few due, among other reasons, to inefficiency of delivery.

Viral gene delivery is still regarded as the most efficient (Walther and Stein, 2000). 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 Fig. 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 Mintzer and Simanek, 2009). 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. (1995) and since then numerous efforts have been made to gain insights into the mechanism of action (Akinc and Langer, 2002; Boeckle et al., 2004; Breunig et al., 2006; Breuzard et al., 2008; Brunner et al., 2002; Clamme et al., 2003; Erbacher et al., 2004; Forrest and Pack, 2002; Grosse et al., 2007; Itaka et al., 2004; Ogris et al., 1998; Rejman et al., 2006; Godbey et al., 1999b,d) and to further optimize this polymer (Godbey et al., 1999a,c; Neu et al., 2005; Ogris et al., 1999; Dunlap et al., 1997; Kircheis et al., 2001).

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

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doi:10.1016/j.ijpharm.2009.08.032

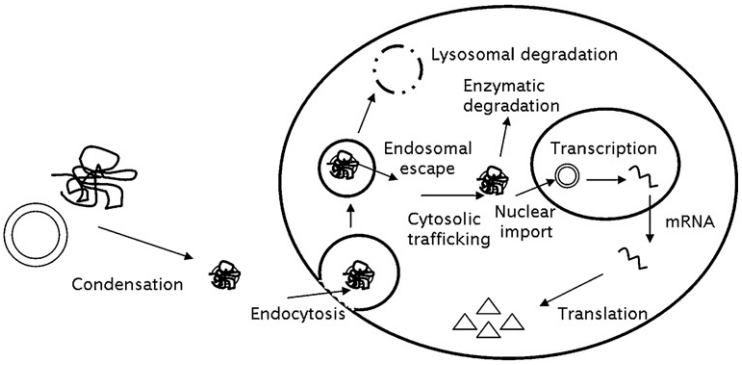

Fig. 1. Gene delivery is a multistep process.

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 (Varga et al., 2005). 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 (Hama et al., 2006, 2007).

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.

2. Materials and methods

2.1. Materials

Linear poly(ethyleneimine) (PEI), ExGen 500, was purchased from MBI Fermentas (St Leon-Rot, Germany). Plasmid 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, $MgCl_2{\cdot}6H_2O$ (Acros Organics BVBA, Geel, Belgium); *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, 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 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), 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).

2.2. Methods

2.2.1. 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 min 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 to 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 Fig. 2).

2.2.2. 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 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 and the diffusion coefficients calculated from the measured autocorrelation

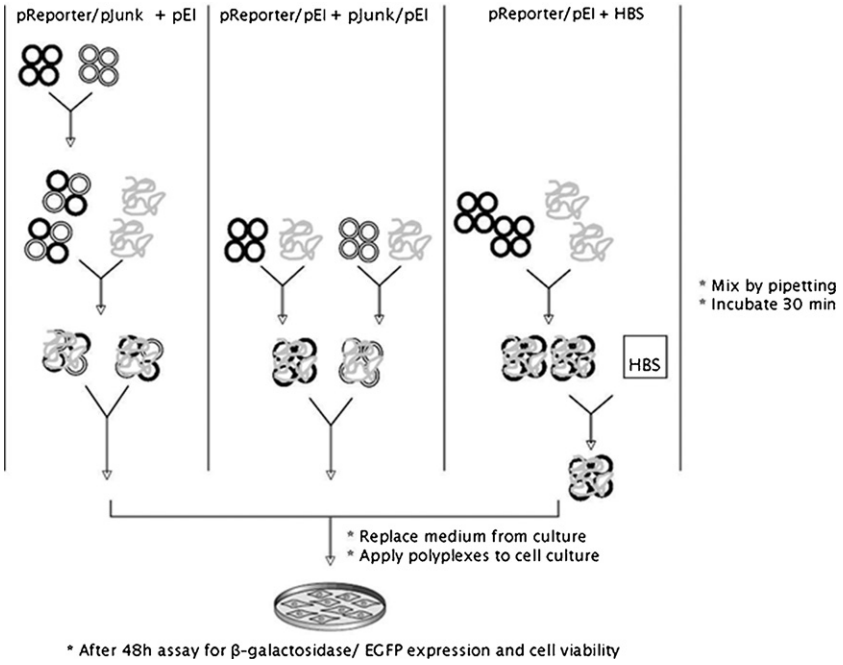

Fig. 2. Schematic representation of polyplex preparation and transfection.

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).

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).

2.2.3. 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 6000 (Hela) cells were seeded per well into 96-well tissue culture plates 24 h 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 h 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.

2.2.4. ONPG-assay

48 h after transfection, cells were washed 1× 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 min 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 β-galactosidase was determined by deriving the kinetic velocity of ONPG-conversion by measuring absorbance at 415/490 nm at time intervals of 1 min during 40 min.

2.2.5. 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.

2.2.6. 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 min 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

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.

and data were analyzed using Summit® software (Dako Cytomation, Fort Collins, CO, USA). 10,000 cells were recorded per sample to determine EGFP expression (FL1-channel) and PI-staining (FL3-channel).

2.2.7. 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 2 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.

3. Results

3.1. 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 min 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* (Ogris et al., 1998).

3.2. 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 (Fig. 3A, 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. When this method was applied, a

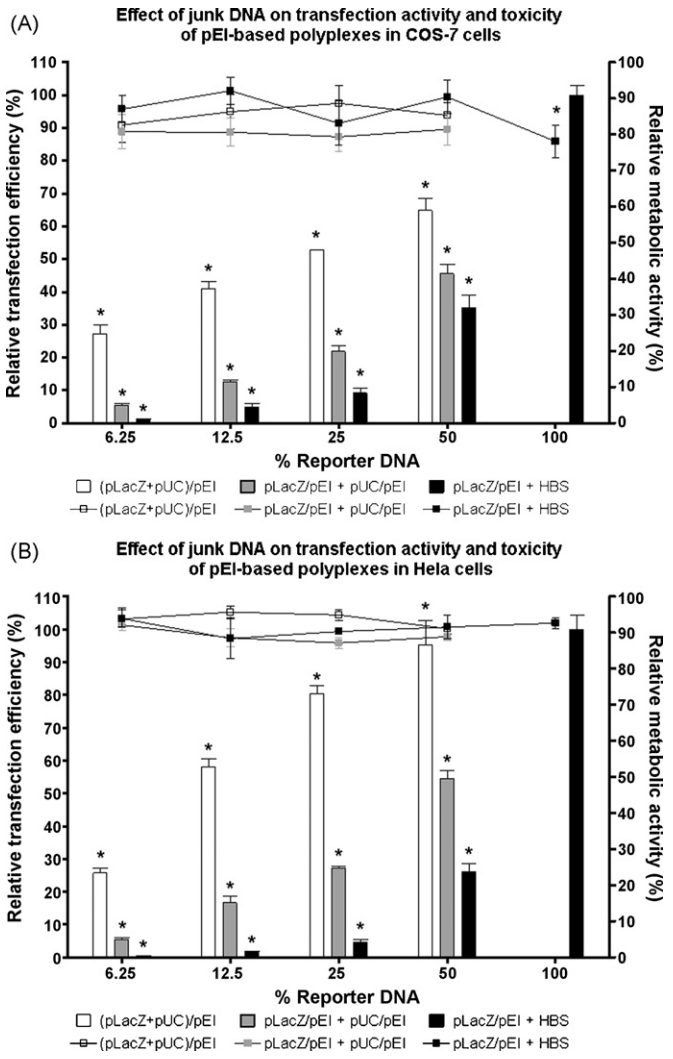

Fig. 3. *In vitro* β-galactosidase expression (bars) and cell viability (lines) after transfection of COS-7 cells (A) and Hela cells (B) with pDNA/pEI polyplexes. Reporter DNA dose was varied by diluting polyplexes in HBS (black bars), by diluting reporter pDNA with junk pDNA prior to complex formation (white bars) or by mixing reporter pDNA polyplexes with junk pDNA polyplexes (grey bars). In the latter two 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; $p < 0.05$).

remarkable effect on transfection efficiency was observed (Fig. 3A, 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. (2005), 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 (Boussif et al., 1995) 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; Fig. 3A, 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 (Fig. 3B). 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 (Fig. 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.

3.3. 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 Fig. 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.

3.4. 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

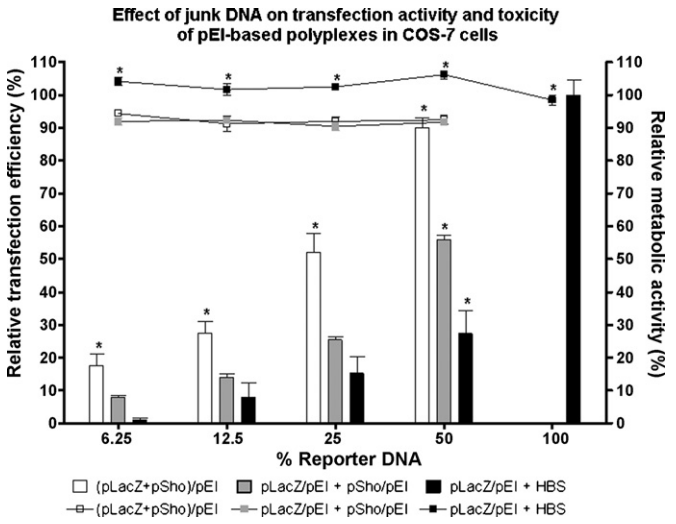

Fig. 4. *In vitro* β-galactosidase expression (bars) and cell viability (lines) after transfection of COS-7 cells with pDNA/pEI polyplexes. Reporter DNA dose was varied by diluting polyplexes in HBS (black bars), by diluting reporter pDNA with junk pDNA prior to complex formation (white bars) or by mixing 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; $p < 0.05$).

remain mostly unaffected (Fig. 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.

4. 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 $(1.5–7.5) \times 10^6$ plasmid copies, per cell. For comparison, a human cell contains 7.1 pg DNA per cell (Ausubel et al., 1990). 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 (Hama et al., 2006, 2007; Varga et al., 2005). 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 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

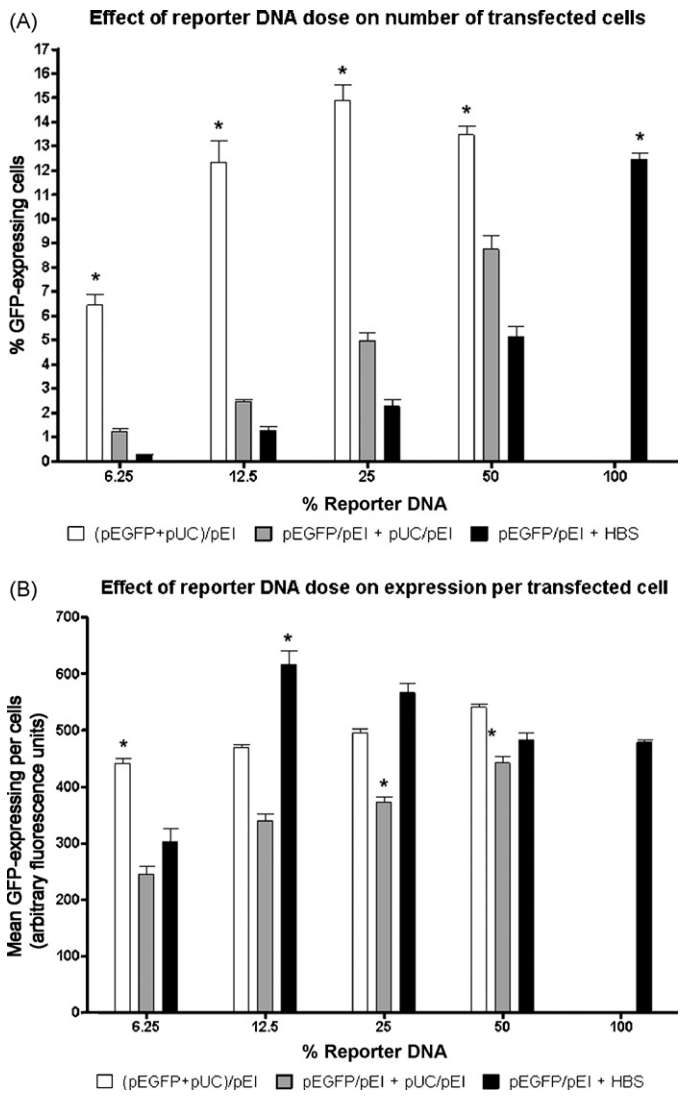

Fig. 5. *In vitro* EGFP expression after transfection of COS-7 cells with pDNA/pEI polyplexes. Reporter DNA dose was varied by diluting polyplexes in HBS (black bars), by diluting reporter pDNA with junk pDNA prior to complex formation (white bars) or by mixing reporter pDNA polyplexes with junk pDNA polyplexes (grey bars). In the latter two 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 ($n = 6$). (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 ($p < 0.05$).

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 (Barthel et al., 1993; Carpentier et al., 2007; Kichler et al., 2005; Moriguchi et al., 2006). Additionally, the use of junk DNA to enhance silencing effects in siRNA delivery has also been described (Schiffelers et al., 2004; Tagami et al., 2007). 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 (Fig. 1) must be considered.

4.1. 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.

4.2. 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 (Ruponen et al., 2004). 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. (2005), 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 (Carpentier et al., 2007; Moriguchi et al., 2006), one would statistically expect to end up with similar amounts of active and non-active plasmid copies for both formulations.

4.3. 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 (Akinc et al., 2005; Boeckle et al., 2004; Boussif et al., 1995; Kichler et al., 2001, 2005). This so-called proton sponge theory has been topic of elaborate investigation but remains under debate (Forrest and Pack, 2002; Funhoff et al., 2004; Godbey et al., 2000; Yang and May, 2008). 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 (Boeckle et al., 2004; Kichler et al., 2001). 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 (Kichler et al., 2005). 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 that 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 (Moriguchi et al., 2006) and of enhanced transgene expression of cytoplasmically microinjected reporter DNA upon co-injection of pUC DNA (Ludtke et al., 2002).

4.4. 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 h 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 (Pollard et al., 1998). 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 $\sim 10^3$ 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.

4.5. Cytosolic degradation

Besides inefficient active nuclear translocation, degradation by nucleases present in the cytosol can be a limiting factor for gene delivery (Lechardeur et al., 1999). 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 (Ludtke et al., 2002). 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 (Kichler et al., 2005). 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.

4.6. 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 (Bishop et al., 2006; Block et al., 2006; Van Gaal et al., 2006). 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 (Moriguchi et al., 2006). 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 1850 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 (Carpentier et al., 2007). 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.

4.7. 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 otherwise 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).

References

Akinc, A., Langer, R., 2002. Measuring the pH environment of DNA delivered using nonviral vectors: implications for lysosomal trafficking. Biotechnol. Bioeng. 78, 503–508.

Akinc, A., Thomas, M., Klibanov, A.M., Langer, R., 2005. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J. Gene Med. 7, 657–663.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.D., Struhl, K., 1990. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

Barthel, F., Remy, J.S., Loeffler, J.P., Behr, J.P., 1993. Gene transfer optimization with lipospermine-coated DNA. DNA Cell Biol. 12, 553–560.

Bishop, C.L., Ramalho, M., Nadkarni, N., May Kong, W., Higgins, C.F., Krauzewicz, N., 2006. Role for centromeric heterochromatin and PML nuclear bodies in the cellular response to foreign DNA. Mol. Cell Biol. 26, 2583–2594.

Block, G.J., Eskiw, C.H., Dellaire, G., Bazett-Jones, D.P., 2006. Transcriptional regulation is affected by subnuclear targeting of reporter plasmids to PML nuclear bodies. Mol. Cell Biol. 26, 8814–8825.

Boeckle, S., Von Gersdorff, K., Van Der Piepen, S., Culmsee, C., Wagner, E., Ogris, M., 2004. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. J. Gene Med. 6, 1102–1111.

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7301.

Breunig, M., Lungwitz, U., Liebl, R., Goepferich, A., 2006. Fluorescence resonance energy transfer: evaluation of the intracellular stability of polyplexes. Eur. J. Pharm. Biopharm. 63, 156–165.

Breuzard, G., Tertil, M., Goncalves, C., Cheradame, H., Geguan, P., Pichon, C., Midoux, P., 2008. Nuclear delivery of NFκB-assisted DNA/polymer complexes: plasmid DNA quantitation by confocal laser scanning microscopy and evidence of nuclear polyplexes by FRET imaging. Nucleic Acids Res. 36, e71.

Brunner, S., Furtbauer, E., Sauer, T., Kursa, M., Wagner, E., 2002. Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation. Mol. Ther. 5, 80–86.

Carpentier, E., Paris, S., Kamen, A.A., Durocher, Y., 2007. Limiting factors governing protein expression following polyethylenimine-mediated gene transfer in HEK293-EBNA1 cells. J. Biotechnol. 128, 268–280.

Clamme, J.P., Azoulay, J., Mely, Y., 2003. Monitoring of the formation and dissociation of polyethylenimine/DNA complexes by two photon fluorescence correlation spectroscopy. Biophys. J. 84, 1960–1968.

Dunlap, D.D., Maggi, A., Soria, M.R., Monaco, L., 1997. Nanoscopic structure of DNA condensed for gene delivery. Nucleic Acids Res. 25, 3095–3101.

Erbacher, P., Bettinger, T., Brion, E., Coll, J.L., Plank, C., Behr, J.P., Remy, J.S., 2004. Genuine DNA/polyethylenimine (PEI) complexes improve transfection properties and cell survival. J. Drug Target. 12, 223–236.

Forrest, M.L., Pack, D.W., 2002. On the kinetics of polyplex endocytic trafficking: implications for gene delivery vector design. Mol. Ther. 6, 57–66.

Friedmann, T., Roblin, R., 1972. Gene therapy for human genetic disease? Science 175, 949–955.

Funhoff, A.M., Van Nostrum, C.F., Koning, G.A., Schuurmans-Nieuwenbroek, N.M., Crommelin, D.J., Hennink, W.E., 2004. Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH. Biomacromolecules 5, 32–39.

Godbey, W.T., Wu, K.K., Hirasaki, G.J., Mikos, A.G., 1999a. Improved packing of poly(ethylenimine)/DNA complexes increases transfection efficiency. Gene. Ther. 6, 1380–1388.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999b. Poly(ethylenimine) and its role in gene delivery. J. Control Release 60, 149–160.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999c. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J. Biomed. Mater. Res. 45, 268–275.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999d. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc. Natl. Acad. Sci. U.S.A. 96, 5177–5181.

Godbey, W.T., Barry, M.A., Saggau, P., Wu, K.K., Mikos, A.G., 2000. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. J. Biomed. Mater. Res. 51, 321–328.

Grosse, S., Aron, Y., Thevenot, G., Monsigny, M., Fajac, I., 2007. Cytoskeletal involvement in the cellular trafficking of plasmid/PEI derivative complexes. J. Control Release 122, 111–117.

Hama, S., Akita, H., Ito, R., Mizuguchi, H., Hayakawa, T., Harashima, H., 2006. Quantitative comparison of intracellular trafficking and nuclear transcription between adenoviral and lipoplex systems. Mol. Ther. 13, 786–794.

Hama, S., Akita, H., Iida, S., Mizuguchi, H., Harashima, H., 2007. Quantitative and mechanism-based investigation of post-nuclear delivery events between adenovirus and lipoplex. Nucleic Acids Res. 35, 1533–1543.

Itaka, K., Harada, A., Yamasaki, Y., Nakamura, K., Kawaguchi, H., Kataoka, K., 2004. In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. J. Gene Med. 6, 76–84.

Kichler, A., Leborgne, C., Coeytaux, E., Danos, O., 2001. Polyethylenimine-mediated gene delivery: a mechanistic study. J. Gene Med. 3, 135–144.

Kichler, A., Leborgne, C., Danos, O., 2005. Dilution of reporter gene with stuffer DNA does not alter the transfection efficiency of polyethylenimines. J. Gene Med. 7, 1459–1467.

Kircheis, R., Wightman, L., Wagner, E., 2001. Design and gene delivery activity of modified polyethylenimines. Adv. Drug Deliv. Rev. 53, 341–358.

Lechardeur, D., Sohn, K.J., Haardt, M., Joshi, P.B., Monck, M., Graham, R.W., Beatty, B., Squire, J., O'brodovich, H., Lukacs, G.L., 1999. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther. 6, 482–497.

Ludtke, J.J., Sebestyen, M.G., Wolff, J.A., 2002. The effect of cell division on the cellular dynamics of microinjected DNA and dextran. Mol. Ther. 5, 579–588.

Mintzer, M.A., Simanek, E.E., 2009. Nonviral vectors for gene delivery. Chem. Rev. 109, 259–302.

Moriguchi, R., Kogure, K., Iwasa, A., Akita, H., Harashima, H., 2006. Non-linear pharmacodynamics in a non-viral gene delivery system: positive non-linear relationship between dose and transfection efficiency. J. Control Release 110, 605–609.

Neu, M., Fischer, D., Kissel, T., 2005. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. J. Gene Med. 7, 992–1009.

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., Wagner, E., 1998. The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. Gene Ther. 5, 1425–1433.

Ogris, M., Brunner, S., Schuller, S., Kircheis, R., Wagner, E., 1999. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 6, 595–605.

Pollard, H., Remy, J.S., Loussouarn, G., Demolombe, S., Behr, J.P., Escande, D., 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J. Biol. Chem. 273, 7507–7511.

Rejman, J., Conese, M., Hoekstra, D., 2006. Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis. J. Liposome Res. 16, 237–247.

Ruponen, M., Honkakoski, P., Tammi, M., Urtti, A., 2004. Cell-surface glycosaminoglycans inhibit cation-mediated gene transfer. J. Gene Med. 6, 405–414.

Schiffelers, R.M., Ansari, A., Xu, J., Zhou, Q., Tang, Q., Storm, G., Molema, G., Lu, P.Y., Scaria, P.V., Woodle, M.C., 2004. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res. 32, e149.

Tagami, T., Barichello, J.M., Kikuchi, H., Ishida, T., Kiwada, H., 2007. The gene-silencing effect of siRNA in cationic lipoplexes is enhanced by incorporating pDNA in the complex. Int. J. Pharm. 333, 62–69.

Van Gaal, E.V., Hennink, W.E., Crommelin, D.J., Mastrobattista, E., 2006. Plasmid engineering for controlled and sustained gene expression for nonviral gene therapy. Pharm. Res. 23, 1053–1074.

Varga, C.M., Tedford, N.C., Thomas, M., Klibanov, A.M., Griffith, L.G., Lauffenburger, D.A., 2005. Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. Gene Ther. 12, 1023–1032.

Walther, W., Stein, U., 2000. Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs 60, 249–271.

Yang, S., May, S., 2008. Release of cationic polymer–DNA complexes from the endosome: a theoretical investigation of the proton sponge hypothesis. J. Chem. Phys. 129, 185105.