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Research paper

Polyethylenimine PEI F25-LMW allows the long-term storage of frozen complexes as fully active reagents in siRNA-mediated gene targeting and DNA delivery

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Abstract

Background: Polyethylenimines (PEIs) are synthetic, charged polymers which function as transfection reagents based on their ability to compact DNA into complexes. Recently, PEI-mediated delivery of nucleic acids has been extended towards small interfering RNAs (siRNAs) which are instrumental in the induction of RNA interference (RNAi). Since RNAi represents a powerful method for specific gene silencing, the PEI-based delivery of siRNAs is a promising tool for novel putative therapeutic strategies. *Aim:* For therapeutic use, major requirements are the development of formulations which (i) are sufficiently stable in the presence of serum, and which can be (ii) easily and reproducibly manufactured and (iii) stored for a prolonged time with full retention of their integrity and bioactivity. In this paper, we explore the potential of PEI F25-LMW, a low-molecular weight PEI with superior transfection efficacy and low toxicity, towards these goals. *Results:* We have systematically analyzed and determined optimal DNA and siRNA complexation conditions with regard to various parameters including buffer concentration, ionic strength, pH and incubation time. As opposed to 22 kDa linear PEI (L-PEI), the low-molecular weight (4–10 kDa) PEI F25-LMW performs DNA transfection and siRNA gene targeting with identical efficacies in the presence of serum, thus emphasizing its usefulness in vivo. Furthermore, in contrast to other polyethylenimines, PEI F25-LMW-based DNA or siRNA complexes allow freeze/thawing and frozen storage for several months. Their activity is fully retained without requiring specific buffer conditions or the addition of any lyoprotectant. Physicochemical analysis and atomic force microscopy reveal a distinct size pattern with the presence of two complex subgroups and show that frozen PEI F25-LMW complexes remain stable with little increase in complex size, no changes regarding their zeta potential and cytotoxicity, and full retention of nucleic acid protection. *Conclusions:* Frozen PEI F25-LMW-based complexes represent efficient and stable ready-to-use formulations of DNA- or siRNA-based gene therapy products.

Keywords: RNAi; siRNAs; PEI; Polyethylenimine; Frozen complexes; Nucleic acid delivery

1. Introduction

Polyethylenimines (PEIs) are branched or linear synthetic polymers with the different available products characterized by their molecular weights, ranging from low-molecular weight (<1000 Da) to high-molecular weight PEIs (>1000 kDa). Due to a protonable amino group in every third position [1,2], they possess a high cationic

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charge density which leads to the condensation of nucleic acids into complexes. Since this complex formation allows the efficient cellular uptake through endocytosis, PEIs have been demonstrated to mediate effective in vitro and in vivo gene transfer in a variety of cell lines and in animals for DNA delivery (for review, see e.g., [3–7] and references therein).

More recently, the use of polyethylenimines or PEI derivatives has been extended towards the complexation and delivery of small RNA molecules including all-RNA ribozymes [8–10] and small interfering RNAs (siRNAs) ([11–16], see [17,18] for review). siRNAs are 21–25 nt double-stranded RNA molecules playing a pivotal role in RNA interference (RNAi), since RNAi is based on the incorporation of an siRNA into an 'RNA-induced silencing complex' (RISC), with the siRNA sequence-specifically guiding RISC to the target RNA and inducing endonucleolytic cleavage of the mRNA strand within the target site [19,20]. RNAi is commonly used as a powerful tool in biological and biomedical research providing the relatively easy reduction of the expression of a target gene in functional studies of various genes. This also offers the possibility of therapeutic intervention based on the targeting of pathologically relevant genes (see e.g., [17,21,22] for review).

One critical factor that determines the success of siRNA-based RNAi approaches is the ability to deliver intact siRNAs efficiently into the cells. While chemically unmodified RNA molecules are very instable and prone to rapid degradation, upon PEI complexation they are efficiently condensed and thus fully covered and protected against enzymatic or non-enzymatic degradation [8,11]. PEI-mediated cellular DNA or siRNA delivery is dependent on the PEI, on the complexation procedure and on the transfection conditions. Among others, this includes the PEI's molecular weight and degree of branching as well as the ionic strength of the complexation solution, the presence of serum proteins, the complexation time and the N/P ratio (defined as the ratio of the nitrogen atoms of PEI to nucleic acid phosphates in the complex). In general, low-molecular weight PEIs display higher transfection efficacy which may be due to a more efficient uptake of the complexes, a better intracellular release of the DNA and/or their generally lower in vitro cytotoxicity [23–28]. On the other hand, cytotoxicity and low transfection efficacy may, among others, rely on the formation of larger aggregates which prevents their endocytosis and, when formed on the cell surface, impairs membrane functions finally leading to cell necrosis [24]. For the commercially available low-molecular weight 22 kDa linear PEI (L-PEI), used as siRNA delivery reagent, several studies have shown contradicting results [11,12,29,30]. Additionally, to improve the biocompatibility and efficiency in vitro as well as to influence the biodistribution and circulation times in vivo, modifications have been introduced including PEGylation, i.e., grafting with the hydrophilic polymer poly(ethylene glycol) (for review, see [3,7,31,32]). While PEGylation has been shown to influence the size, zeta potential, morphology and stability of DNA and siRNA complexes as well as the efficacy of DNA or siRNA complexation, protection and delivery, data still seem to be somewhat contradictory and are strongly dependent on the PEI and its degree and pattern of PEGylation. Although it was hypothesized that PEGylation enhances siRNA release in the cytoplasm, leading to increased biological activity [33], other studies show that PEG grafting seems to exert negative effects on intracellular release of nucleic acids [34]. More recently, we have shown in several PEG-PEIs that, in contrast to DNA, PEG-PEIs are rather comparable to non-PEGylated PEIs with regard to siRNA transfection and toxicity in vitro [32], and siRNA delivery, complex circulation times, biodistribution and biocompatibility in vivo (Malek and Aigner, unpublished data), and previous in vivo studies have successfully utilized non-PEGylated PEIs for in vivo siRNA delivery ([11,12], Höbel et al., submitted for publication). Thus, it appears that for siRNA delivery non-PEGylated PEIs are well suited. More recently, we introduced the novel low-molecular weight polyethylenimine 'PEI F25-LMW' derived through the fractionation of a commercially available 25 kDa branched PEI by size exclusion chromatography. PEI F25-LMW demonstrates high DNA and siRNA delivery efficacies and low toxicity in various cell lines and under various conditions [35].

Non-viral vectors like polyethylenimines may offer promising strategies for the delivery of DNA or RNA molecules, but they often suffer from poor physical stability and comparably low transfection efficacy especially in the presence of serum, and their application as medical products will require the development of formulations which can be stored for a prolonged time. While freezing or freeze-drying may be the important strategies for preserving transfection efficacies of complexes, they so far require the complex formation under highly specific and restricted medium conditions, the addition of lyoprotectants and/or conjugate formation by chemical coupling of ligands (see e.g., [35–37] and references therein).

In this paper, we show that the DNA transfection efficacy as well as siRNA gene targeting efficiency of PEI F25-LMW complexes, but not of L-PEI complexes, is independent of the presence of serum, and explore the optimal complexation conditions for PEI F25-LMW. Furthermore, we introduce and characterize simple freezing of PEI F25-LMW-based DNA or siRNA complexes for the generation of efficient ready-to-use formulations of DNA- or siRNA-based gene therapy products.

2. Materials and methods

2.1. Preparation and storage of PEI/DNA and PEI/siRNA complexes

Polyethylenimine PEI F25-LMW was prepared as described previously [35]. Briefly, 100 mg 25 kDa

branched PEI (Al 25-kDa, Sigma–Aldrich, Taufkirchen, Germany) was fractionated by size exclusion chromatography on Sephadex G-50 fine resin (Amersham Biosciences, Freiburg, Germany) in 150 mM NaCl. One milliliter fractions were collected in an automated fraction collector (Amersham Biosciences), pooled and concentrated in an Amicon stirred cell with an Ultracel Amicon YM1 Ultrafiltration Disc, 1000 NMWL (Millipore, Eschborn, Germany). For the determination of PEI concentrations, 100 μl/well 0.02 M cupric acetate in 5% potassium acetate (pH 5.5) and 20 μl of the sample or a PEI standard of known concentration were mixed in a 96-well plate. The absorption at 630 nm was measured using an ELISA reader (Bio-Tek Instruments, Winooski, VT), and concentrations were determined by comparison with the standard curve. Low-molecular weight 22 kDa linear PEI (L-PEI) was purchased from Qbiogene (Wiesbaden, Germany). Chemically synthesized siRNA duplexes directed against luciferase (pGL3 and, as a negative control, pGL2) were purchased from MWG (Ebersberg, Germany). For transient DNA transfections, the pGL3-Control plasmid (Promega, Madison, WI) with a human SV-40 promoter driving luciferase expression was used for transient DNA transfections.

For PEI complexations, the optimal PEI/nucleic acid (i.e., DNA or siRNA) ratios were determined, which are expressed as PEI/DNA or PEI/RNA equivalents (N/P ratios) on the basis of PEI nitrogen per nucleic acid phosphate (1 μg of DNA or siRNA is 3 nmol of phosphate, and 1 μl 0.9 mg/ml PEI solution contains 10 nmol of amine nitrogen). Generally, 0.4–0.8 μg nucleic acids (DNA or siRNA) were dissolved in 40 μl of 150 mM NaCl buffered with 10 mM Hepes, pH 7.4, and in a separate vial appropriate amounts of PEI solution (5 μg PEI F25-LMW or 25 kDa PEI ($=N/P$ 33), or 1 μl L-PEI ($=N/P$ 5), respectively, per 0.5 μg nucleic acid) were dissolved in 40 μl of the same buffer. The PEI solution was then pipetted to the DNA or siRNA solution resulting in the desired N/P ratio. Alterations regarding the NaCl concentration, the Hepes concentration, the pH or the sequence of pipetting are indicated in the text and in the figures. After vortexing, the mixture was incubated for 1 h at room temperature unless stated otherwise. Complexes were then either used directly ('fresh complexes') or stored at r.t., 4 °C or frozen at −20 °C for 3 d unless indicated otherwise in the text and in the figures. TransIT TKO was purchased from Mirus (MoBiTec, Goettingen, Germany), and siRNA transfections were performed with 12.8 pmol/well siRNA according to the manufacturer's instructions.

2.2. Tissue culture and transfection of cells

Cell lines SKOV-3 (ovarian carcinoma) and PC-3 (prostate carcinoma) were obtained from the American type culture collection (ATCC/LGC Promochem, Wesel, Germany) and cultivated under standard conditions (37 °C, 5% CO_2) in IMDM (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS) unless indicated otherwise. SKOV-3 ovarian carcinoma cell lines with stable constitutive luciferase expression (SKOV-3/Luc) were generated as described previously [11]. For transfection, complexes were prepared according to the procedure described above. Cells were seeded at 4×10^4 cells/well in 24-well plates and were transfected in 1 ml medium in the presence of 10% FCS unless indicated otherwise with 0.5 μg PEI-complexed DNA or 0.8 μg siRNAs, either freshly prepared or stored, with the corresponding amounts of naked siRNA or pure PEI, or with TransIT TKO/siRNA as stated in the figures. The medium was left on the cells for the time period indicated in the experiments unless stated otherwise.

In general, experiments were performed at least three times with at least 2–4 wells per data point in each experiment. Exceptions included all assessments of DNA transfection or siRNA targeting efficacies upon freezing and/or storage of the frozen complexes which were done in more than five independent experiments. In all assays for targeting efficacy or cytotoxicity, normalization of the RLU (control siRNA treatment was set to 100% unless stated otherwise) or the cytotoxicity (total lysis was set to 100%) allowed the combination of the several independent experiments which is shown in the figures. When absolute values are presented, the figures show one representative experiment of $n = 3$–5 (see above).

2.3. Determination of luciferase activity

The luciferase activity was determined using the luciferase assay kit from Promega (Mannheim, Germany) according to the manufacturer's protocol. Briefly, the medium was aspirated and the cells were lysed in 100 μl lysis buffer. In a luminometer tube, 25 μl substrate was mixed with 10 μl lysate, and chemiluminescence was determined immediately in a luminometer (Berthold, Bad Wildbad, Germany).

2.4. Cytotoxicity assay

Cytotoxicity was determined 72 h post-transfection, unless indicated otherwise, by measurement of lactate dehydrogenase (LDH) release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega according to the manufacturer's protocol and as described previously [35]. Background values which include spontaneous LDH release from untreated cells were subtracted, and the numbers shown in the figures represent the percentage of maximum cytotoxicity which was determined after 100% cell lysis. Less than 10% LDH release were regarded as non-toxic effect level [38].

2.5. siRNA stability assay

The determination of in vitro siRNA stability was performed essentially as described previously [8,11]. Briefly,

2.5 μg PEI-complexed or free [^{32}P] end-labeled siRNA was incubated in 40 ng/ml RNase A at 37 °C. At the time points indicated, 1% sodium dodecyl sulfate in RNA loading buffer was added, mixtures were heat-denatured for 5 min at 65 °C and separated by 1% agarose gel electrophoresis, prior to blotting onto Hybond membranes, visualization by autoradiography and PhosphoImager- based quantitation.

2.6. Photon correlation spectroscopy (PCS) and laser doppler anemometry (LDA)

The hydrodynamic diameters of complexes containing nucleic acids (DNA or siRNA) at a concentration of 10 μg/ml, prepared and stored as described above, were determined by PCS using a Zetasizer Nano ZS from Malvern Instruments (Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 25 °C. Scattered light was detected at a 173° angle with laser attenuation and measurement position adjusted automatically by the Malvern software. Values given are the means ± standard deviation of three independent experiments with each experiment including three measurements of the same sample with at least 10 runs each, as determined by the zetasizer. The zeta potential of complexes with a nucleic acid concentration of 6.6 μg/ml was determined by LDA using a folded capillary electrophoresis cell of the Zetasizer Nano ZS at 25 °C, the light signal detected at a 17° angle. The average value was calculated with the data of three times 10 runs ± standard deviation.

2.7. Atomic force microscopy

DNA and siRNA complexes, prepared as described above, were directly transferred onto a silicon chip by dipping the chip into the complex solution. Atomic force microscopy was performed on a vibration-damped NanoWizard (JPK instruments, Berlin, Germany) as described in detail elsewhere [33,39]. Commercial pyramidal Si_3N_4 tips (NSC16 AlBS, Micromasch, Estonia) mounted to a cantilever with a length of 230 μm, a resonance frequency of about 170 kHz and a nominal force constant of about 40 N/m were used, and measurements were performed in intermittent contact mode to avoid damage of the sample. The scan speed was proportional to the scan size, and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the height signal in the retrace direction (512 × 512 pixel).

2.8. Statistics

Statistical analyses were performed by Student's *t*-test or Tukey's multiple comparison test using GraphPad Prism4, and significance levels are indicated in the figure legends.

3. Results

3.1. Serum-dependence of the DNA or siRNA transfection efficacy

The complex stability and biological efficacy of PEI/DNA or PEI/siRNA complexes can be impaired by proteins at higher concentrations in the transfection medium. Therefore, the serum-dependence of PEI-mediated DNA plasmid transfection was determined for PEI F25-LMW and compared to L-PEI. PEI/DNA complexes were formed under optimal conditions regarding *N*/*P* ratio and complexation buffer (10 mM Hepes/150 mM NaCl, see below), and were added to SKOV-3 ovarian carcinoma cells or PC-3 prostate carcinoma cells in the presence of 2%, 5%, or 10% FCS in the medium during the 5 h transfection period. In the case of L-PEI, a strong dependence of the transfection efficacy, as determined by the cellular luciferase activity after 48 h, was observed in both cell lines (Fig. 1b). In fact, in most experiments comparably little DNA transfection was determined in the presence of 10% FCS. In contrast, PEI F25-LMW-mediated DNA transfection was much more robust by being markedly less dependent on serum with only little (SKOV-3 cells) or no (PC-3 cells) differences in luciferase activities (Fig. 1a).

Since our more prominent goal is to use polyethylenimines for siRNA delivery, the experiment was repeated in a stably luciferase-expressing cell line derived from SKOV-3 cells [11]. Treatment of SKOV-3/Luc cells with PEI F25-LMW-complexed luciferase-specific siRNAs led to a marked ~80% reduction of luciferase expression compared to the same treatment with PEI F25-LMW-complexed, non-specific siRNAs. (Fig. 1c, left). Again, this effect was independent of the serum concentration in the transfection medium. Using L-PEI for siRNA delivery, identical results were obtained in the presence of 2% FCS, but not at 5% or 10% FCS, indicating, in these cells, a strong dependence of the gene targeting efficiency on the FCS concentration in the transfection medium (Fig. 1c, right). Furthermore, to exclude non-specific effects based on the action of the complexes or free polymers, untreated cells were compared to cells exposed to (i) PEI F25-LMW/non-specific siRNA, (ii) L-PEI/non-specific siRNA, (iii) PEI F25-LMW alone and (iv) L-PEI alone, using the same amounts as in the other experiments. The determination of the luciferase activity, which is an indicator of cell number and cell viability, revealed that neither the PEI complexes nor the PEIs alone exert any unspecific effects under the conditions employed in our experiments. Therefore, in all subsequent experiments we employed non-specific siRNA, PEI-complexed under the same conditions, as reference value/negative control. From these data we conclude that PEI F25-LMW represents a robust system for DNA or siRNA delivery, and further characterized the applicability of PEI F25-LMW-based complexes.

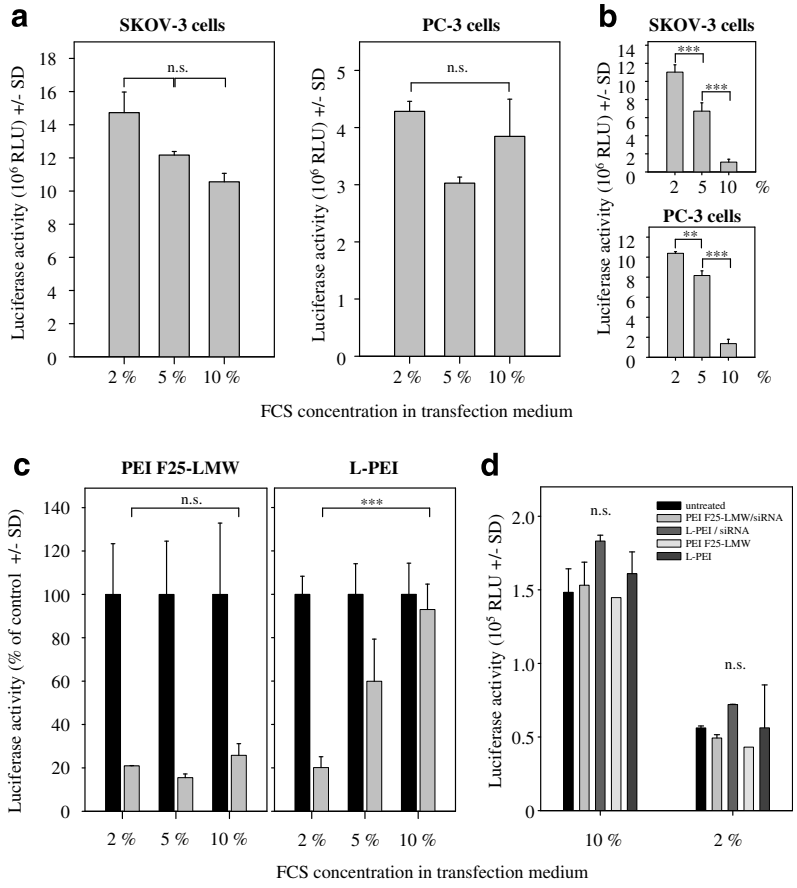


Fig. 1. Serum-dependence of PEI F25-LMW- and L-PEI-mediated DNA transfection and siRNA gene targeting efficacies. SKOV-3 and PC-3 cells were transfected with 0.5 μg luciferase plasmid DNA using PEI F25-LMW (a) or L-PEI (b) at different serum concentrations as indicated in the figures. (c) Stably luciferase-expressing SKOV-3/Luc cells were transfected with 0.8 μg PEI-complexed luciferase-specific (grey bars) or non-specific (black bars) siRNAs at the serum concentrations indicated using PEI F25-LMW (left) or L-PEI (right). RLUs from treatment with non-specific siRNAs were set to 100%. (d) The comparison of untreated SKOV-3/Luc cells with cells exposed to PEI F25-LMW/non-specific siRNA or L-PEI/non-specific siRNA complexes, or the PEIs alone, using the same amounts as in the other experiments, demonstrates the absence of non-specific effects of the complexes or the carriers. $^{*}p < 0.03$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Experimental details: complexation buffer 10 mM Hepes/150 mM NaCl (pH 7.4), 0.5 μg DNA or 0.8 μg siRNA, $N/P = 30$ (PEI F25-LMW) or $N/P = 5$ (L-PEI), complexation time 60 min, determination of chemiluminescence 48 h (DNA) or 72 h (siRNA) after transfection.

3.2. Optimization of PEI F25-LMW complexation

According to many PEI transfection standard protocols, the PEI complexation is performed for no longer than 10 min either in (10 mM Hepes-buffered) 150 mM NaCl or in 5% glucose. In this set of experiments, we determined optimal complexation conditions for PEI F25-LMW by altering various parameters. Regarding the ionic strength of the complexation solution, the complexation in 150 mM NaCl, buffered with 10 mM Hepes (pH 7.4) showed best results. In contrast, the reduction of NaCl concentrations through complexation in Hepes-buffered 15 mM NaCl resulted in very little luciferase activity (Fig. 2a). Concomitantly, gene targeting efficacies of PEI F25-LMW/siRNA complexes formed in Hepes-buffered 15 mM NaCl were poor while optimal conditions resulted in a robust downregulation of luciferase activity (Fig. 2d). Hepes buffering was not necessary for optimal complexation (Fig. 2e (left panel), 0 mM vs. 10 mM) but may stabilize optimal pH values (see below) and is therefore preferable. Gene targeting using TransIT-TKO/siRNA complexes led to comparable results with, however, the already smaller siRNA amounts (12.8 pmol). In contrast, naked siRNA did not exert any specific or non-specific decrease in luciferase activity even at high concentrations (Fig. 2e, right), indicating that PEI or another carrier is necessary for the induction of siRNA-mediated gene targeting. An increase in Hepes concentration beyond 10 mM led to the almost complete loss of PEI F25-LMW/siRNA-mediated gene targeting efficacy indicating that it is advantageous in SKOV-3 cells to limit Hepes to only very low concentrations (Fig. 2e, left panel). Finally, although many protocols explicitly state that the PEI solution needs to be pipetted to the nucleic acid solution (not vice versa) to initiate the complexation, the reversal of this sequence of pipetting led to no adverse effects and resulted in comparably high transfection efficacies in our experiments (data not shown).

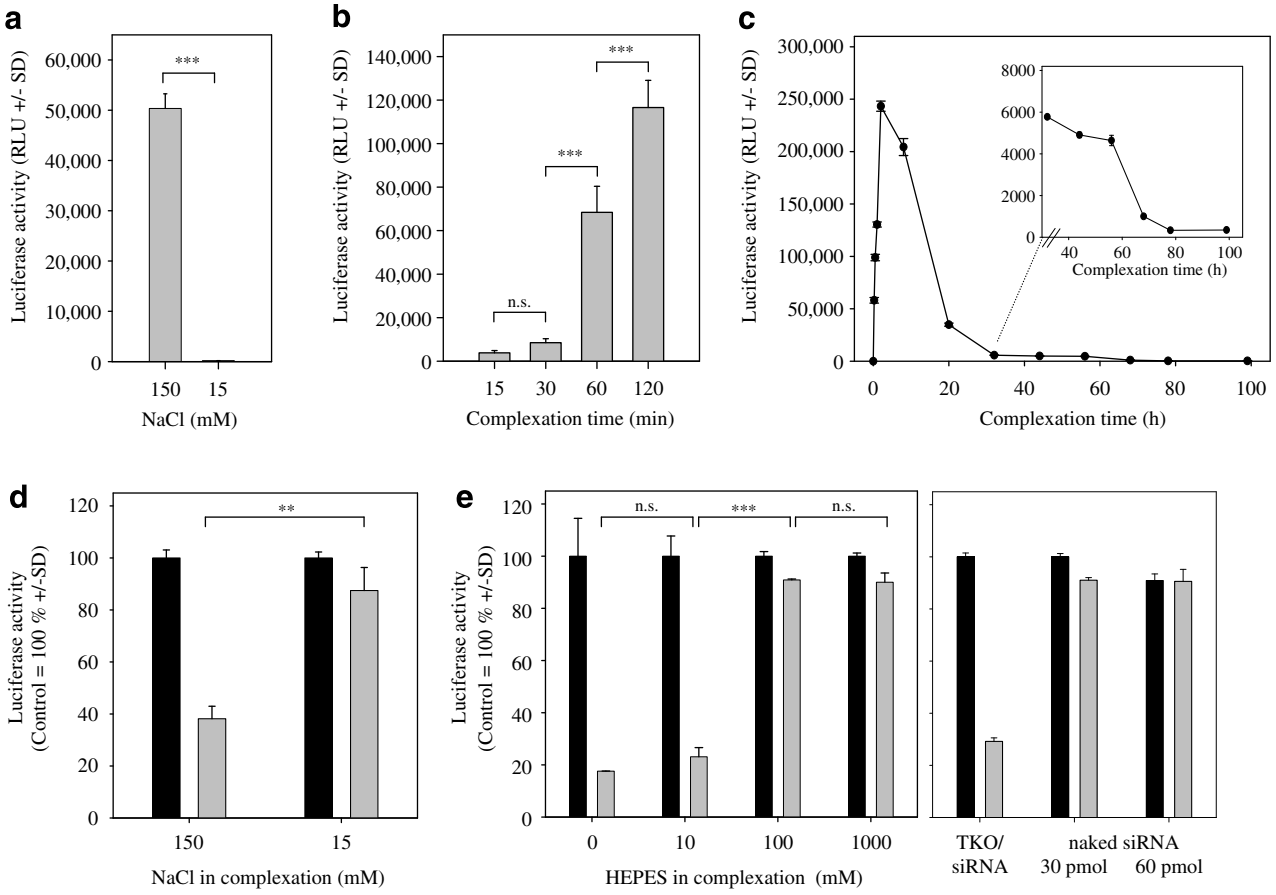


Fig. 2. Dependence of the PEI F25-LMW-mediated DNA transfection or siRNA gene targeting efficacy on the buffer conditions or on the complexation time. Complexation of 0.5 μg luciferase plasmid DNA was performed in Hepes-buffered 15 mM or 150 mM NaCl for 60 min (a) or in Hepes-buffered 150 mM NaCl for the indicated time periods (b and c) prior to transfection of SKOV-3 cells and determination of RLUs. (d) Dependence of PEI F25-LMW/siRNA gene targeting efficacy on complexation buffer conditions. 0.8 μg luciferase-specific (grey bars) or non-specific (black bars) siRNAs were complexed in 10 mM Hepes-buffered NaCl solutions as indicated (d) or in 150 mM NaCl with various Hepes concentrations (e, left panel). TransIT-TKO/siRNA served as positive control while naked siRNA at the same amounts used above did not exert any targeting efficacy (e, right panel). $^{*}p < 0.03$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant. Experimental details: complexation buffer 10 mM Hepes/150 mM NaCl (pH 7.4) (b, c, and e), 0.5 μg DNA (a–c) or 0.8 μg siRNA (d and e), $N/P = 30$, complexation time 60 min (a, d, and e), determination of chemiluminescence 24 h (DNA) or 48 h (siRNA) after transfection. RLUs from the treatment of stably luciferase-expressing SKOV-3/Luc cells upon treatment with non-specific siRNAs were set to 100%.

When comparing different complexation times, the transfection efficacy of complexes formed in the optimal 10 mM Hepes/150 mM NaCl buffer was found to be time-dependent: a prolonged complexation for 2 h rather than 15 min led to a marked ~10-fold increase in the transfection efficacy as determined by the luciferase activity of SKOV-3 cells, 48 h after administration of the PEI F25-LMW/luciferase DNA complexes (Fig. 2b). In fact, a sharp increase in transfection efficacy was already observed when the complexation time was extended from 30 min to 60 min, and almost doubled again for 2 h complex formation. In the subsequent experiments, we chose the 60 min complexation time in order to achieve comparably high transfection efficacies while avoiding time-consuming prolonged incubation steps. However, the storage of the complexes for longer time periods, i.e., beyond 2 h, at room temperature resulted in decreased transfection efficacies. While bioactivities were still 85% after 8 h, the transfection efficacy dropped substantially afterwards (Fig. 2c). Notably, however, very low residual activities were still observed as long as 68 h after the start of the complexation (Fig. 2c, inset). Nevertheless, these data demonstrate that PEI F25-LMW-based complexes cannot be stored at room temperature for a prolonged time while retaining transfection efficacy.

Finally, since the complex formation relies on electrostatic interactions between PEI and nucleic acids with the degree of PEI protonation being dependent on the H^+ concentration, the dependence of the gene targeting efficacy of PEI F25-LMW/siRNA complexes on the pH during complexation was studied. The variation of the pH revealed that no changes in PEI F25-LMW/siRNA-mediated gene targeting were observed when the complexation was performed between pH 6 and 9. In contrast, further lowering of the pH resulted in a partial (pH 5) or almost complete (pH 4) loss of complex bioactivity (Fig. 3a).

In a more detailed analysis, we studied this pH dependence at different N/P values. While gene targeting efficacies of complexes formed at pH 9 and pH 7 improved in

a similar manner upon the increase of N/P ratios with optimal conditions at $N/P = 66$ (pH 9) and at $N/P = 33$ or 66 (pH 7) (Fig. 3b, right and center panel), the variation of the N/P values had no positive effect when the complexation was performed at pH 4 (Fig. 3b, left panel). Taken together, these data show that a pH range between weakly acid and quite strongly basic conditions does not affect the PEI F25-LMW/siRNA complex formation, while acidification below pH 6 impairs the complexation.

3.3. Storage of PEI F25-LMW-based complexes

Usually, the PEI-based transfection of cells relies on the preparation of fresh complexes prior to transfection;

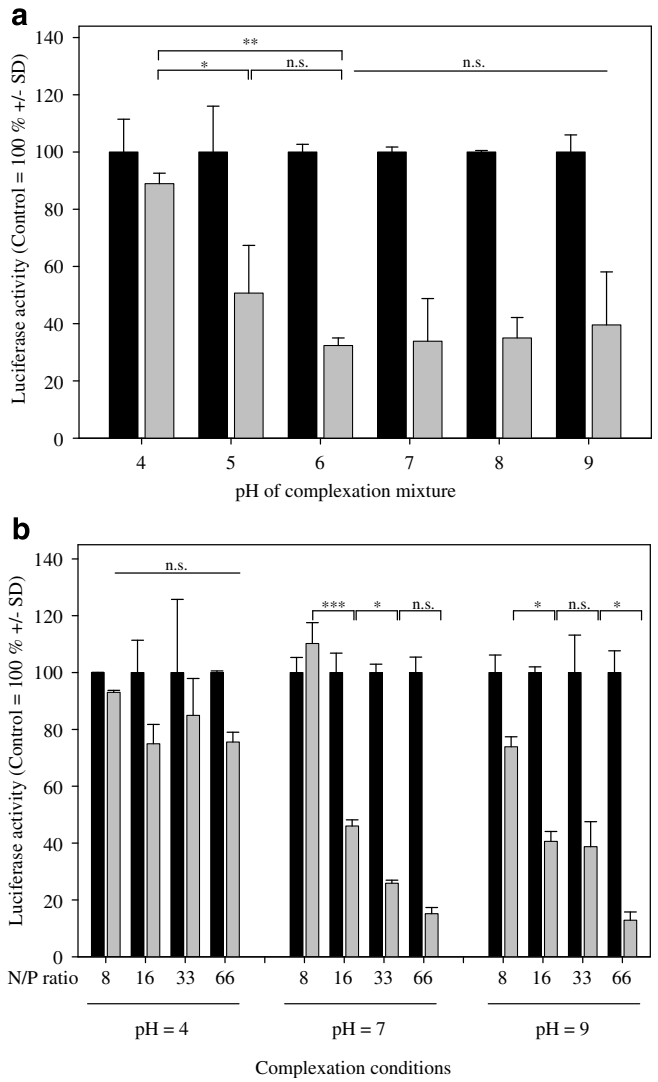


Fig. 3. Dependence of the PEI F25-LMW-mediated siRNA gene targeting efficacy on the buffer pH during complexation. Luciferase-specific (grey bars) or non-specific (black bars) siRNAs (0.8 μg) were complexed for 60 min in 10 mM Hepes/NaCl at the pH values indicated with $N/P = 33$ (a) or various N/P ratios (b). Chemiluminescence in stably luciferase-expressing SKOV-3/Luc cells was determined 48 h after transfection and RLUs from treatment with non-specific siRNAs were set to 100%. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant or no significant differences between any of the grey bars.

a process which is somewhat tedious and difficult to standardize. The aim of the next set of experiments was to identify conditions under which PEI/DNA complexes can be stored for a prolonged time while retaining transfection efficacy. Storage of PEI F25-LMW/luciferase plasmid complexes at 4 °C or at room temperature for 3 d led to a complete (r.t.) or almost complete (4 °C) loss of transfection efficacy as determined by cellular luciferase activity, 48 h after transfection with the respective complexes. Interestingly, however, when PEI F25-LMW/DNA complexes were kept at −20 °C for the same time, transfection efficacies were still high and identical to freshly prepared complexes (Fig. 4a), indicating that frozen PEI F25-LMW/DNA complexes remain intact. In contrast, L-PEI/DNA complexes did not allow freezing since the transfection efficacy was lost (data not shown). To distinguish more closely between different freezing conditions, PEI F25-LMW/DNA complexes were frozen and stored at −80 °C, frozen and stored at −20 °C, or were snap-frozen in liquid nitrogen and kept there for 3 d prior to transfection. Transfection efficacies were uniformly high for all frozen PEI F25-LMW/DNA complexes and no differences were observed in comparison to freshly prepared complexes (Fig. 4b). Additionally, prolonged storage for up to 4 months was tested and no decrease in transfection efficacy was detected (data not shown).

Likewise, the freezing of PEI F25-LMW/siRNA complexes completely retained siRNA-mediated gene targeting efficacy as compared to freshly prepared complexes. In fact, a ~70% downregulation of luciferase activity in endogenously luciferase-expressing SKOV-3/Luc cells was observed with fresh complexes as well as with complexes kept at −20 °C for 1 week, 4 weeks or 3 months (Fig. 5a, left). Storage of complexes at room temperature or 4 °C, however, led to complete loss of bioactivity. Extending these studies towards other PEIs, we detected similar targeting efficacies, despite some cytotoxicity, in frozen PEI/siRNA complexes based on the branched 25 kDa PEI, while freezing of L-PEI/siRNA complexes completely abolished their activity (Fig. 5a, right panels). From these data we conclude that PEI F25-LMW-based DNA or siRNA complexes can be stored frozen to retain their full complexation efficacies independent of the freezing conditions or the duration of storage.

3.4. Protection of siRNA molecules in PEI F25-LMW/siRNA complexes

Since serum contains RNases, which lead to a rapid degradation of siRNAs, the protection of siRNA molecules is instrumental for any successful transfection in the presence of serum or in vivo, and was therefore analyzed in the next set of experiments. Freshly prepared PEI F25-LMW complexes fully protected siRNAs from RNase A-mediated degradation for at least 4 h as indicated by the gel bands representing [^{32}P]-labeled, intact full-length siRNA

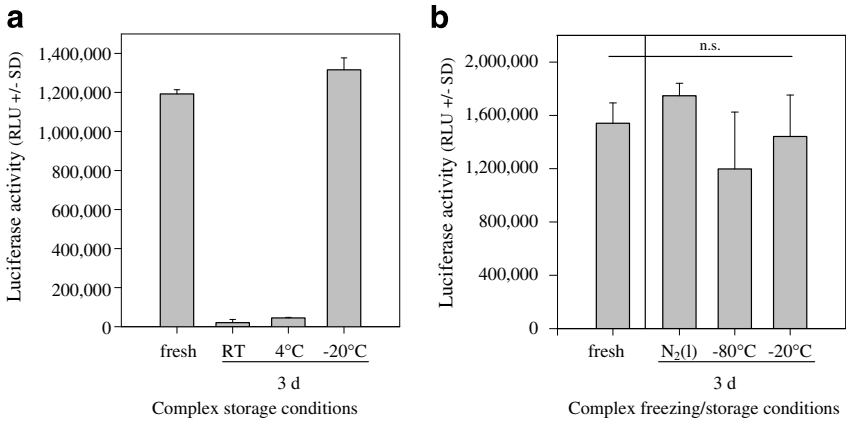


Fig. 4. Full conservation of transfection efficacy upon freezing of PEI F25-LMW/DNA complexes. (a) Complexes containing 0.5 μg DNA (complexation buffer 10 mM Hepes/150 mM NaCl (pH 7.4), $N/P = 30$, complexation time 60 min) were prepared freshly or stored as indicated in the figure prior to transfection of SKOV-3 cells and determination of RLUs 48 h after transfection. (b) The transfection efficacy is fully retained independent of the freezing procedure and frozen storage conditions for 3 d. n.s., no significant differences between any of the bars.

molecules, while non-complexed siRNA was rapidly degraded (Fig. 5b). More importantly, identical results were obtained from complexes kept at −20 °C for 1 week demonstrating that freezing/thawing and frozen storage does not impair the PEI F25-LMW-mediated siRNA stabilization/protection (Fig. 5b).

3.5. Cytotoxicity of PEI F25-LMW complexes

For certain PEIs and PEI-based complexes, their cytotoxicities represent major limitations with regard to their applicability in vitro and in vivo. To test whether the marked differences in the luciferase activity of cells treated with differently stored complexes (see Fig. 4a) are really based on variations in the transfection efficacy, or if they are mainly caused by differential induction of cell death, the cytotoxic effects of PEI F25-LMW/DNA and PEI F25-LMW/siRNA complexes stored under various conditions were measured. After 24 h, which represents the recommended assay conditions, cytotoxicities of the amounts used in the other experiments were close or below the limit of detection for naked siRNA as well as for L-PEI/siRNA and for PEI F25-LMW/siRNA complexes, and only TransIT-TKO/siRNA complexes showed a very low ~2% cytotoxicity (Fig. 6a). Decreasing the FCS concentration in the medium from 10% to 2% or increasing the time period of the complex treatment from 24 h to 72 h both led to an increase in cytotoxicity (Fig. 6a, right). To test our complexes more rigorously and to get higher numbers thus facilitating the comparisons in Fig. 6b–d, 72 h treatment at 2% FCS was selected for subsequent experiments with values of cytotoxicity being between 15% and 18%. More importantly, no major differences between different storage conditions were observed (Fig. 6b) indicating that cytotoxic effects do not influence the luciferase activity or the siRNA-mediated luciferase downregulation in stable SKOV-3/Luc cells. Likewise, cytotoxicities were identical in PEI F25-LMW/DNA complexes stored under different conditions (Fig. 6c and data not shown) although PEI F25-LMW/DNA complexes were generally less toxic (~50% less compared to PEI F25-LMW/siRNA complexes; Fig. 6d). From these data, we conclude that the differences in luciferase activity and siRNA-mediated luciferase gene targeting efficacy are based on variations in the transfection efficacies of the respective PEI F25-LMW complexes and do not reflect different cytotoxicities.

3.6. Physicochemical characterization of PEI F25-LMW complexes

To analyze in more detail which effects may lead to loss or retention of PEI F25-LMW-mediated DNA or siRNA transfection efficacy, the sizes and zeta potentials of complexes stored under various conditions were measured. The zeta potential remained constant at ~35–40 mV for all complexes upon 3 d storage at room temperature, 4 °C or −20 °C (Fig. 7a and data not shown). Likewise, the determination of the zeta potentials revealed no differences between PEI F25-LMW/DNA and PEI F25-LMW/siRNA complexes (Fig. 7b).

The determination of the hydrodynamic complex sizes of PEI F25-LMW complexes met serious problems since their polydispersities, as indicated by the polydispersity indexes, were too high for reliable measurements (PDI > 0.3). This was also true when buffer conditions for complexation were changed towards higher or lower complex concentrations, lower ionic strength than used for cell transfection, when the complexation time was varied or when the complexes were filtered or centrifuged prior to measurement (data not shown). However, although the polydispersity largely prevented the determination of precise values (as also indicated by the correlation function), PCS revealed nevertheless that fresh or frozen PEI F25-LMW complexes were never above a several hundred nm

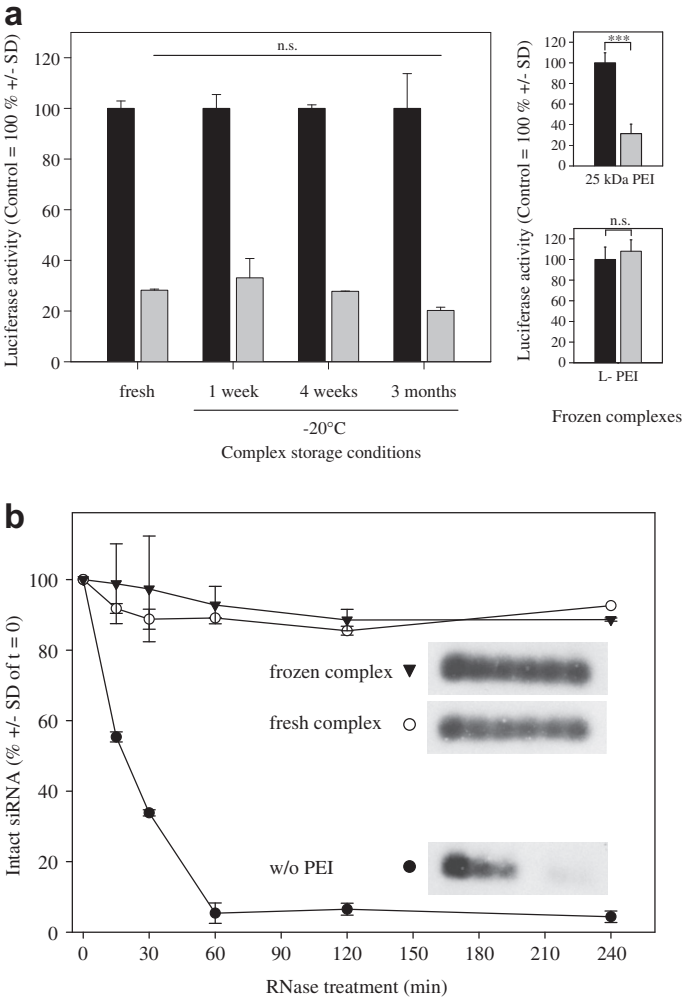


Fig. 5. Long-term conservation of PEI F25-LMW/siRNA-mediated gene targeting efficacy and siRNA protection upon freezing. (a, left) Complexes containing 0.8 μg specific (grey bars) or non-specific (black bars) siRNAs were prepared freshly (complexation buffer 10 mM Hepes/150 mM NaCl (pH 7.4), $N/P = 30$, complexation time 60 min) or stored at −20 °C as indicated in the figure prior to transfection of stably luciferase-expressing SKOV-3/Luc cells and determination of RLUs 72 h after transfection. n.s., no significant differences between any of the grey bars. (a, right) Assessment of targeting efficacies of complexes based on 25 kDa PEI (upper panel, $N/P = 33$) or L-PEI (lower panel, $N/P = 5$) prepared under the same conditions and frozen. $^{***}p < 0.001$, n.s., not significant. (b) Protection of PEI F25-LMW-complexed siRNAs against RNase A-mediated degradation. [^{32}P] end-labeled siRNA was left uncomplexed (black circles) or was complexed with PEI F25-LMW under the same conditions as described above with (black triangles) or without (open circles) subsequent freezing of the complex. Upon RNase treatment, samples were taken as indicated in the figure and subjected to agarose gel electrophoresis and autoradiography. Gel bands (see insert) representing full-length intact siRNA were quantitated by phosphoimaging and $t = 0$ was set to 100%.

range while the storage of PEI F25-LMW/siRNA or PEI F25-LMW/DNA complexes at room temperature or 4 °C resulted in aggregation with the formation of complexes well beyond 4 μm. In contrast, this aggregate formation was never observed upon freezing and storage of PEI F25-LMW complexes at −20 °C.

3.7. Atomic force microscopy

Alternatively, for the precise determination of their size, structure and integrity, PEI F25-LMW-based complexes were analyzed by atomic force microscopy (AFM). The high magnification revealed the formation of well-shaped, spherical and condensed PEI F25-LMW complexes with DNA or siRNA (Fig. 7c shows frozen PEI F25-LMW/DNA complexes as a representative example). The absence of visible free siRNA or DNA molecules indicated the complete complexation of the nucleic acid molecules. Otherwise, they would be visible as sticking out of the complex or as free molecules (see e.g., [42,43]). The further analysis of the complex sizes revealed the presence of complexes with two distinct size distributions (Fig. 7d). Small complexes were found with sizes in the range of ∼25–75 nm and large complexes in the range of ∼60–130 nm, and thus complexes are well suited for endocytotic uptake into cells. The percentage of the subpopulations was dependent on the nucleic acid complexed with siRNA leading to a smaller proportion of large complexes as compared to DNA. On the other hand, particle size analysis also revealed an increase by about 1/3 in the percentage of the large particles upon freezing for PEI/siRNA complexes, whereas for PEI/DNA complexes no significant change in

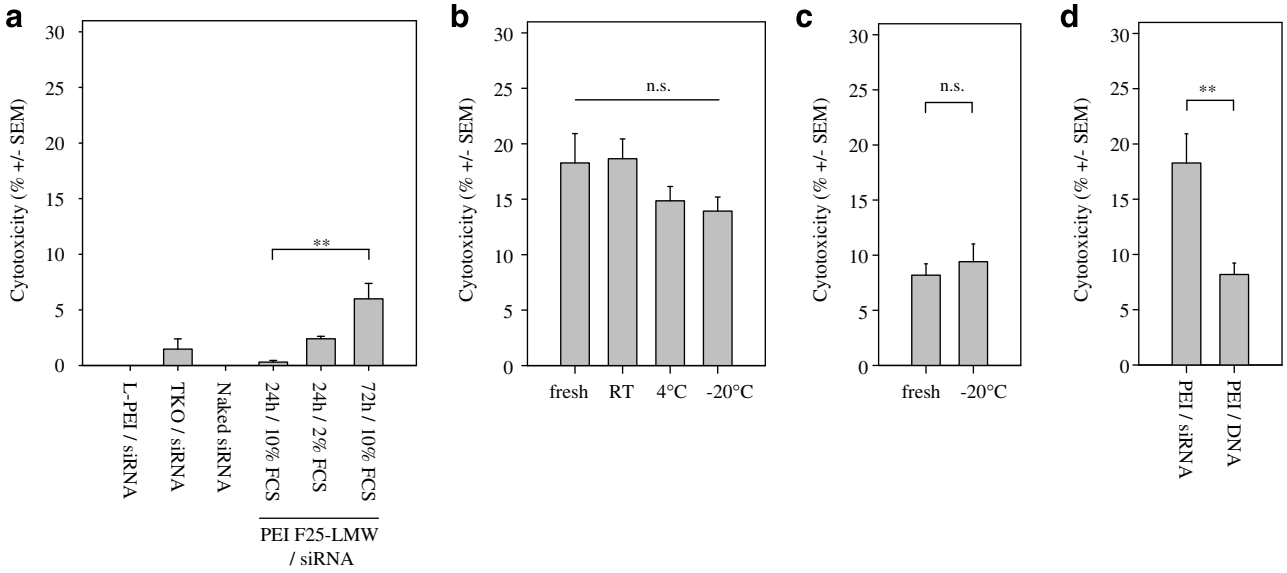
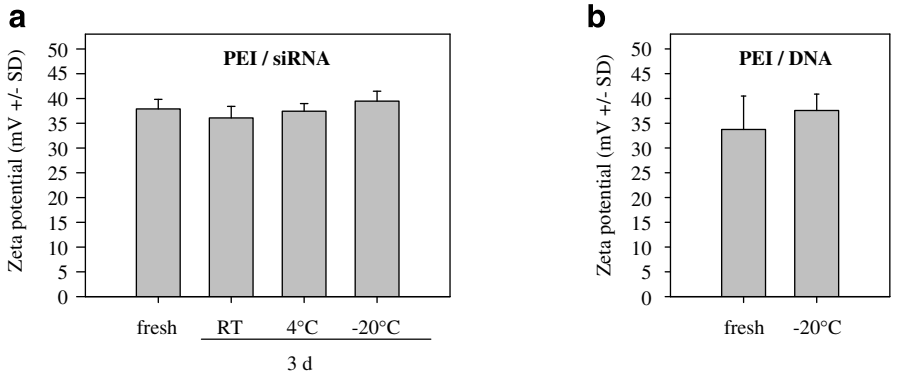
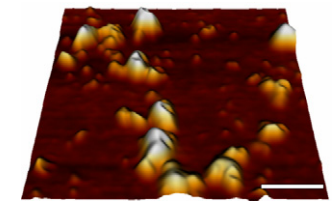


Fig. 6. (a) Cytotoxicities of siRNA, naked or formulated with L-PEI, PEI F25-LMW or TransIT-TKO, after 24 h in the presence of 10% FCS. Lower FCS concentration (2%) or longer incubation time (72 h) leads to increased toxicity of PEI F25-LMW/siRNA complexes (a, right bars). (b–d) Cytotoxicities of PEI F25-LMW/DNA and PEI F25-LMW/siRNA complexes after 72 h in the presence of 2% FCS. Complexes containing siRNA (b) or DNA (c) were prepared freshly or stored as indicated in the figure. (d) Direct comparison of fresh complexes containing siRNA (left) or DNA (right). $^{**}p < 0.01$, n.s., no significant differences between any of the bars. Experimental details: complexation buffer 10 mM Hepes/150 mM NaCl (pH 7.4), 0.5 μg DNA or 0.8 μg siRNA, $N/P = 30$ (PEI F25-LMW) or $N/P = 5$ (L-PEI), complexation time 60 min, determination of LDH release 72 h after transfection unless indicated otherwise.





Complex type + storage conditions	Size [nm] of large complexes		Size [nm] of small complexes	
PEI / siRNA fresh *1	99.2 ± 12.1	(18.83%)**	48.9 ± 9.7	(81.17%)
PEI / siRNA –20°C *2	113.8 ± 17.5	(25.93%)	64.7 ± 10.5	(74.07%)
PEI / DNA fresh *3	77.5 ± 13.4	(45.96%)	32.7 ± 8.7	(54.05%)
PEI / DNA –20°C *4	98.3 ± 16.2	(47.22%)	34.1 ± 9.4	(52.78%)

Fig. 7. Physicochemical analysis of PEI F25-LMW/DNA and PEI F25-LMW/siRNA complexes. Complexes containing 0.8 μg siRNA (a) or DNA (b) were prepared freshly (complexation buffer 10 mM Hepes/150 mM NaCl (pH 7.4), $N/P = 30$, complexation time 60 min) or stored as indicated in the figure prior to determination of the zeta potential. (c and d) Atomic force microscopy of PEI F25-LMW/DNA and PEI F25-LMW/siRNA complexes prepared as described above. (c) Representative picture of frozen PEI F25-LMW/DNA complexes. Bar: 100 nm. (d) AFM-based size determination of complex sizes ± SD, freshly prepared or stored frozen as indicated. Total numbers of the analyzed particles: 812 (*1), 923 (*2), 792 (*3), 656 (*4), respectively; **percentages of the total number of particles.

the ratio of small to large complexes was observed (Fig. 7d). Nevertheless, despite some changes in the fractions of the subpopulations upon freezing of the complexes, in both size groups an only slight increase of the complex sizes was detected and no aggregate formation was observed (Fig. 7d).

4. Discussion

Non-viral vectors like polyethylenimines offer promising strategies for the delivery of DNA or RNA molecules in gene therapy including novel gene-targeting approaches. One major problem is that they often suffer from poor physical stability leading to complex decomposition, loss of transfection efficiency and/or the tendency to aggregate ([7] for review), and thus require the preparation of fresh polyplexes directly prior to administration. For the application of non-viral oligonucleotide delivery vectors as medical products, however, it will be imperative to develop formulations which can be stored for a prolonged time and, upon reconstitution, provide ready-to-use reagents of reproducible quality. While freezing or freeze-drying represents important strategies towards this goal, several studies have shown that PEI-based complexes without protectant aggregate under these conditions (e.g., [36,40] and references therein). Thus, the maintenance of particle sizes and the preservation of bioactivity often require special precautions like complex formation under certain medium conditions, addition of lyoprotectants including sucrose, trehalose, mannitol or dextran [37,40–43], and/or the preparation of shielding conjugates by coupling of PEI, e.g., to PEG or melittin [44,45]. Previously, we have shown that complexes based on PEI F25-LMW can be lyophilised; however, retention of bioactivity was again strongly confined to very limited buffer conditions (5% glucose) [35].

In this paper, we demonstrate that complexes based on PEI F25-LMW, without any chemical modifications and formed under normal conditions, i.e., in saline, can be frozen with full retention of their transfection efficacy. Concomitantly, no changes in the zeta potential or in the complex sizes are observed, as opposed to storage in a non-frozen state (4 °C or room temperature) which leads to extensive aggregation and complete inactivation of the complexes. The latter finding is in agreement with the previous studies (see [7] for review) and indicates that (lack of) aggregation is critical for transfection efficacy. However, in other studies no direct correlation between particle size preservation and recovery of transfection was observed, and structural alterations other than changes in particle size have been suggested to contribute to gene delivery [41,46]. With this regard, it should be noted that freezing of PEI F25-LMW-based siRNA complexes in our experiments obviously did not impair the structural integrity since PEI F25-LMW-mediated siRNA protection against RNase A was also fully maintained upon freeze/thawing of the complexes. Importantly, freezing of PEI F25-LMW-based complexes did not require the addition of any lyoprotectant although the complexes were formed in 150 mM sodium chloride, and the biological activity was preserved independently of the freezing conditions and duration of storage. In fact, we show that PEI F25-LMW/DNA or PEI F25-LMW/siRNA complexes can be kept frozen for several months without the loss of transfection efficacy and thus may represent a long-term storage, ready-to-use formulation of medical gene therapy products. Thus, these complexes meet the criteria of easy and reproducible manufacturing as demonstrated, e.g., in the frame of this study with highly reproducible results from several investigators over more than 2 years with various batches of PEI F25-LMW and reproducible targeting efficacy also after prolonged storage. In this context, it should be noted that the degree of branching seems to play a major role, since complexes based on branched 25 kDa PEI allowed frozen storage as well while L-PEI/siRNA complexes completely lost their bioactivity upon freezing, thus indicating major structural differences between complexes based on branched or linear PEI, respectively.

The significance of complex sizes and their changes due to aggregation as at least one critical aspect in bioactivity is also emphasized by the dependence of the transfection efficacy on the complexation time. Our data support the notion that complexes over time increase in size due to aggregation. In this context, the presence of two rather distinct populations of complexes regarding their complex sizes is interesting to note. From the absolute numbers of our AFM measurements, it can be speculated that this effect may be based on the dimerization of the already existing complexes. In fact, particle size analysis revealed that for PEI/siRNA complexes the fraction of large particles increased upon freezing. This would indicate that complex 'growth'/aggregation does not necessarily involve merely the addition of new PEI and nucleic acid molecules but rather relies on the 'merger' of the already existing complexes, as suggested previously [47]. Independent of the mechanism, this aggregate formation will probably vary between different PEIs, thus emphasizing the necessity to determine the optimal complexation times for every PEI individually. This is further supported by the fact that PEI/DNA complexes have been shown to be internalized through different endocytosis pathways, dependent not only on the cell line, but also on the PEI [48]. Also, the poorer transfection efficacy of complexes prepared in low-salt buffers is in agreement with the earlier studies and may be explained by the general formation of smaller complexes under low ionic strength conditions, leading again to reduced cellular uptake (see above). Finally, when the aggregation further proceeds as detected in our experiments and shown in previous studies (see [7] for review) and resulting in large particles, the bioactivity sharply drops again, probably due to the formation of complexes which are too large for endocytosis. Thus, this model may explain the observed differences in transfection and gene targeting efficacies. Nevertheless, it should be noted that other studies indicate that the complex structure and stability rather than the degree of endocytosis and the cytoplasmic concentration is the decisive parameter regarding the transfection efficacy of a given polyethylenimine (see e.g., [49]).

Additionally, other complexation parameters may require individual optimization as well. The degree of protonation at a certain pH varies between different PEIs which may well result in differences in the pH dependence of the complexation. Our data are in agreement with Boussif et al. (1995) regarding the constantly high bioactivity of PEI complexes between pH 5 and 7 [1], but further extend this knowledge towards a broader pH range as well as towards PEI/siRNA complexes. More specifically, it should be noted that complex formation with PEI F25-LMW still occurs even at pH 9 indicating that very low degrees of protonation still seem to be sufficient. Unlike Boussif et al. [1], however, the order of adding the reagents for the complexation did not alter the complex properties in our experiments with PEI F25-LMW.

Another essential criterion for the use of polyplexes especially in vivo is the preservation of their stability and transfection efficacy in the presence of serum. In previous studies, it was found that PEI complexes, independent of the serum concentration and of the PEI used, were able to efficiently protect, e.g., small RNA molecules like ribozymes [8] or siRNAs [11,35] against RNase A- or FCS-mediated degradation indicating the formation of stable complexes. In contrast, it has been shown that efficient DNA transfection or siRNA delivery is markedly dependent on the PEI (see e.g., [35]) and that PEI-mediated in vitro gene delivery can be significantly lower in the presence of serum [50,51]. This latter finding is in agreement with our results for L-PEI complexes which indicate a 'serum dose effect' of transfection efficacy and may also explain previous contradicting results for L-PEI-mediated siRNA delivery [11,12,29,30]. However, unlike others [29,30] we did not observe major differences between L-PEI/DNA and L-PEI/siRNA complexes. The, compared to L-PEI, improved efficacy of PEI F25-LMW-based DNA or siRNA complexes in the presence of serum may be explained by the fact that L-PEI is linear while PEI F25-LMW is branched. This may influence the stability of the complexes formed.

5. Conclusion

The serum-independent, high efficiencies in DNA transfection and siRNA delivery in the absence of toxicity as well as the possibility for long-term storage in a frozen state indicate that PEI F25-LMW-based complexes, prepared under optimized conditions described in this paper, are attractive reagents for gene targeting and delivery.

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References

[1] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (16) (1995) 7297–7301.
[2] J.P. Behr, The proton sponge: a trick to enter cells the viruses did not exploit, Chimica 51 (1997) 34–36.
[3] A. Kichler, Gene transfer with modified polyethylenimines, J. Gene Med. 6 (Suppl. 1) (2004) S3–S10.
[4] E. Wagner, R. Kircheis, G.F. Walker, Targeted nucleic acid delivery into tumors: new avenues for cancer therapy, Biomed. Pharmacother. 58 (3) (2004) 152–161.
[5] T.G. Park, J.H. Jeong, S.W. Kim, Current status of polymeric gene delivery systems, Adv. Drug Deliv. Rev. 58 (4) (2006) 467–486.
[6] U. Lungwitz, M. Breunig, T. Blunk, A. Gopferich, Polyethylenimine-based non-viral gene delivery systems, Eur. J. Pharm. Biopharm. 60 (2) (2005) 247–266.
[7] M. Neu, D. Fischer, T. Kissel, Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives, J. Gene Med. 7 (8) (2005) 992–1009.
[8] A. Aigner, D. Fischer, T. Merdan, C. Brus, T. Kissel, F. Czubayko, Delivery of unmodified bioactive ribozymes by an RNA-stabilizing polyethylenimine (LMW-PEI) efficiently down-regulates gene expression, Gene Ther. 9 (24) (2002) 1700–1707.
[9] C. Brus, H. Petersen, A. Aigner, F. Czubayko, T. Kissel, Physicochemical and biological characterization of polyethylenimine-graft-poly(ethylene glycol) block copolymers as a delivery system for oligonucleotides and ribozymes, Bioconjug. Chem. 15 (4) (2004) 677–684.
[10] C. Brus, H. Petersen, A. Aigner, F. Czubayko, T. Kissel, Efficiency of polyethylenimines and polyethylenimine-graft-poly (ethylene glycol) block copolymers to protect oligonucleotides against enzymatic degradation, Eur. J. Pharm. Biopharm. 57 (3) (2004) 427–430.
[11] B. Urban-Klein, S. Werth, S. Abuharbeid, F. Czubayko, A. Aigner, RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo, Gene Ther. 12 (5) (2005) 461–466.
[12] M. Grzelinski, B. Urban-Klein, T. Martens, K. Lamszus, U. Bakowsky, S. Hobel, F. Czubayko, A. Aigner, RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs in vivo exerts antitumoral effects in glioblastoma xenografts, Hum. Gene Ther. 17 (7) (2006) 751–766.
[13] Q. Ge, L. Filip, A. Bai, T. Nguyen, H.N. Eisen, J. Chen, Inhibition of influenza virus production in virus-infected mice by RNA interference, Proc. Natl. Acad. Sci. USA 101 (23) (2004) 8676–8681.
[14] R.M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, G. Molema, P.Y. Lu, P.V. Scaria, M.C. Woodle, Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle, Nucleic Acids Res. 32 (19) (2004) e149.
[15] S.H. Kim, J.H. Jeong, S.H. Lee, S.W. Kim, T.G. Park, PEG conjugated VEGF siRNA for anti-angiogenic gene therapy, J. Control Release (2006).
[16] M. Thomas, J.J. Lu, Q. Ge, C. Zhang, J. Chen, A.M. Klibanov, Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung, Proc. Natl. Acad. Sci. USA 102 (16) (2005) 5679–5684.
[17] A. Aigner, Applications of RNA interference: current state and prospects for siRNA-based strategies in vivo, Appl. Microbiol. Biotechnol. (2007).

[18] A. Aigner, Nonviral in vivo delivery of therapeutic small interfering RNAs, Curr. Opin. Mol. Ther. 9 (4) (2007) 345–352.
[19] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (6836) (2001) 494–498.
[20] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 391 (6669) (1998) 806–811.
[21] M.A. Behlke, Progress towards in vivo use of siRNAs, Mol. Ther. 13 (4) (2006) 644–670.
[22] M. Meyer, E. Wagner, Recent developments in the application of plasmid DNA-based vectors and small interfering RNA therapeutics for cancer, Hum. Gene Ther. (2006).
[23] D. Fischer, R. Bhattacharya, B. Osburg, U. Bickel, Inhibition of monocyte adhesion on brain-derived endothelial cells by NF-kappaB decoy/polyethylenimine complexes, J. Gene Med. 7 (8) (2005) 1063–1076.
[24] D. Fischer, T. Bieber, Y. Li, H.P. Elsasser, T. Kissel, A novel nonviral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity, Pharm. Res. 16 (8) (1999) 1273–1279.
[25] D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis, Biomaterials 24 (7) (2003) 1121–1131.
[26] K. Kunath, A. von Harpe, D. Fischer, H. Petersen, U. Bickel, K. Voigt, T. Kissel, Low-molecular-weight polyethylenimine as a nonviral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine, J. Control Release 89 (1) (2003) 113–125.
[27] M. Ogris, P. Steinlein, M. Kursa, K. Mechtler, R. Kircheis, E. Wagner, The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells, Gene Ther. 5 (10) (1998) 1425–1433.
[28] L. Wightman, R. Kircheis, V. Rossler, S. Carotta, R. Ruzicka, M. Kursa, E. Wagner, Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo, J. Gene Med. 3 (4) (2001) 362–372.
[29] Z. Hassani, G.F. Lemkine, P. Erbacher, K. Palmier, G. Alfama, C. Giovannangeli, J.P. Behr, B.A. Demeneix, Lipid-mediated siRNA delivery down-regulates exogenous gene expression in the mouse brain at picomolar levels, J. Gene Med. 7 (2) (2005) 198–207.
[30] A.C. Richards Grayson, A.M. Doody, D. Putnam, Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro, Pharm. Res. (2006).
[31] M. Ogris, E. Wagner, Tumor-targeted gene transfer with DNA polyplexes, Somat. Cell Mol. Genet. 27 (1–6) (2002) 85–95.
[32] A. Malek, F. Czubayko, A. Aigner, PEG grafting of polyethylenimine (PEI) exerts different effects on DNA transfection and siRNA-induced gene targeting efficacy, J. Drug Target 16 (2) (2008) 124–139.
[33] S. Mao, M. Neu, O. Germershaus, O. Merkel, J. Sitterberg, U. Bakowsky, T. Kissel, Influence of polyethylene glycol chain length on the physicochemical and biological properties of poly(ethylene imine)-graft-poly(ethylene glycol) block copolymer/SiRNA polyplexes, Bioconjug. Chem. 17 (5) (2006) 1209–1218.
[34] V. Knorr, L. Allmendinger, G.F. Walker, F.F. Paintner, E. Wagner, An acetal-based PEGylation reagent for pH-sensitive shielding of DNA polyplexes, Bioconjug. Chem. 18 (4) (2007) 1218–1225.
[35] S. Werth, B. Urban-Klein, L. Dai, S. Hobel, M. Grzelinski, U. Bakowsky, F. Czubayko, A. Aigner, A low molecular weight fraction of polyethylenimine (PEI) displays increased transfection efficiency of DNA and siRNA in fresh or lyophilized complexes, J. Control Release 112 (2) (2006) 257–270.
[36] W.L. Hinrichs, F.A. Mancenido, N.N. Sanders, K. Braeckmans, S.C. De Smedt, J. Demeester, H.W. Frijlink, The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying, Int. J. Pharm. 311 (1–2) (2006) 237–244.
[37] C. Brus, E. Kleemann, A. Aigner, F. Czubayko, T. Kissel, Stabilization of oligonucleotide-polyethylenimine complexes by freeze-drying: physicochemical and biological characterization, J. Control Release 95 (1) (2004) 119–131.
[38] S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, M. Hashida, In vitro cytotoxicity of macromolecules in different cell culture systems, J. Control Release 34 (1995) 233–241.
[39] V. Oberle, U. Bakowsky, I.S. Zuhorn, D. Hoekstra, Lipoplex formation under equilibrium conditions reveals a three-step mechanism, Biophys. J. 79 (3) (2000) 1447–1454.
[40] T.J. Anchordoquy, T.K. Armstrong, M.C. Molina, Low molecular weight dextrans stabilize nonviral vectors during lyophilization at low osmolalities: concentrating suspensions by rehydration to reduced volumes, J. Pharm. Sci. 94 (6) (2005) 1226–1236.
[41] M.C. Molina, S.D. Allison, T.J. Anchordoquy, Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators, J. Pharm. Sci. 90 (10) (2001) 1445–1455.
[42] H. Talsma, J. Cherng, H. Lehrmann, M. Kursa, M. Ogris, W.E. Hennink, M. Cotten, E. Wagner, Stabilization of gene delivery systems by freeze-drying, Int. J. Pharm. 157 (2) (1997) 233–238.
[43] M. Reinisalo, A. Urtti, P. Honkakoski, Freeze-drying of cationic polymer DNA complexes enables their long-term storage and reverse transfection of post-mitotic cells, J. Control Release 110 (2) (2006) 437–443.
[44] M.C. Lenter, P. Garidel, J. Pelisek, E. Wagner, M. Ogris, Stabilized nonviral formulations for the delivery of MCP-1 gene into cells of the vasculoendothelial system, Pharm Res. 21 (4) (2004) 683–691.
[45] M. Kursa, G.F. Walker, V. Roessler, M. Ogris, W. Roedl, R. Kircheis, E. Wagner, Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer, Bioconjug. Chem. 14 (1) (2003) 222–231.
[46] E.K. Wasan, P. Harvie, K. Edwards, G. Karlsson, M.B. Bally, A multi-step lipid mixing assay to model structural changes in cationic lipoplexes used for in vitro transfection, Biochim. Biophys. Acta 1461 (1) (1999) 27–46.
[47] M.X. Tang, F.C. Szoka, The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes, Gene Ther. 4 (8) (1997) 823–832.
[48] K. von Gersdorff, N.N. Sanders, R. Vandenbroucke, S.C. De Smedt, E. Wagner, M. Ogris, The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type, Mol. Ther. 14 (5) (2006) 745–753.
[49] M. Breunig, U. Lungwitz, R. Liebl, J. Klar, B. Obermayer, T. Blunk, A. Goepferich, Mechanistic insights into linear polyethylenimine-mediated gene transfer, Biochim. Biophys. Acta 1770 (2) (2007) 196–205.
[50] W. Guo, R.J. Lee, Efficient gene delivery via non-covalent complexes of folic acid and polyethylenimine, J. Control Release 77 (1-2) (2001) 131–138.
[51] O. Boussif, M.A. Zanta, J.P. Behr, Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold, Gene Ther. 3 (12) (1996) 1074–1080.