Original Article

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Plasmid linearization changes shape and efficiency of transfection complexes

Abstract: The ability to efficiently transfect plasmid DNA (pDNA) into eukaryotic cells has exerted major impact on scientific research in recent years, and translation to clinical application is ongoing, but challenging. In addition to the choice of the delivery vector, the topology of the DNA seems to be a key factor for efficient transfection. The nanostructured DNA/Vector complexes may differ in size, charge, and shape, for example. This study therefore investigated the transfection efficiency of circular versus linearized plasmid DNA using a GFP expressing vector with Lipofectamine 2000 and linear 25 kDa polyethyleneimine (PEI). Transfection efficiency and cytotoxicity were measured by flow cytometry and fluorescence microscopy. Shape was determined by transmission electron microscopy. Transfection agent concentrations were chosen below the toxicity level. We determined the optimal N/P ratio over 48 h by using two different concentrations of plasmid DNA. With the increase of DNA concentration and increasing N/P ratio, transfection efficiency also increased. Our results showed a better transfection efficiency with the circular compared to the linearized DNA, under the same experimental conditions for both Lipofectamine and PEI. In electron microscopy, there was a notable difference in the shape of the complexes: circular DNA had random coil appearance in well compacted, roughly spherical shape, while linearized DNA appeared as worm-like strands, both, when complexed with Lipofectamine or with polyethyleneimine. This generates the hypothesis that the shape of the transfection particle may be an important factor for successful gene transfer.

Keywords: cytotoxicity; DNA transfection; polyethyleneimine.

Introduction

The development of transfection techniques of DNA into eukaryotic cells has had a drastic impact on basic scientific research within the past decades such as to study the function of genes or gene products, by enhancing or inhibiting specific gene expression in cells and to produce recombinant proteins. Different purposes have even led to various clinical applications such as gene therapy (1) and DNA immunization (2). Application of nanotechnology in medicine, the emerging scientific field of nanomedicine, has yielded new synthetic transfection vectors for nucleic acids, leading to significant expansion of the potential for clinical applications. For the delivery of DNA to the target cell, several different delivery vehicles have been developed, including liposomes (3), polymers (4), dendrimers (5) or magnetic nanoparticles (6), and the advent of receptor-targeted delivery (7), smart nanomaterials (8, 9) and multifunctional smart nanosystems (10) expands our toolbox further. In addition to the gene delivery system, the structure of the DNA segment to be transfected plays also a key role for transfection efficiency. While DNA in the form of circular plasmids is often used even if the transfection target cell is eukaryotic, such circular plasmids are not the naturally occurring form of eukaryotic DNA, which typically is rather structured as a linear expression segment within a chromosome. While circular bacterial plasmids are easily produced in large quantities in bacterial culture, alternatives like linear expression systems or microcircle DNA (11) are thought to confer advantages including improved nuclear translocation (linear constructs) or absence of sequences that might lead to side effects, like non-methylated CpG, or antibacterial resistance. Shape and charge of the DNA/Vector construct may also play a role for cell uptake.

The focus of this study was first, to compare transfection efficiency of circular versus linear plasmid DNA, using cancer cells as target cells, with a cationic lipid system (Lipofectamine) or a cationic polymer (polyethyleneimine), and second, to develop hypotheses for potential differential efficiency. Lipofectamine is a standard pDNA

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transfection agent usable only for in vitro transfection (12, 13). Polyethylenimine (PEI) is a highly cationic polymer proven to be efficient and versatile for gene delivery in vitro and in vivo, and developed also with the goal of clinically applications (14). PEI is a highly cationic polymer available at different molecular weights and different molecule structures such as branched and linear versions. High molecular weight PEIs (800 kDa) have shown increased toxicity compared to low molecular weight and linear PEI (25 kDa), rendering the latter the preferred transfection agent (15). We therefore compared circular and linearized forms of a plasmid DNA regarding transfection efficiency with either Lipofectamine®2000 or linear 25 kDa PEI.

Materials and methods

Preparation of plasmid DNA

The pEGFP-C1 plasmid, encoding green fluorescent protein (Clontech) was used in this study for transfection. To linearize the plasmid, the restriction enzyme AseI, (New England BioLabs) was used for digestion (Figure 1). Twenty units of enzyme were used to cleave 2 μg of pDNA in 37°C for 15 min. The cut DNA was analyzed by 1.0% agarose gel and stained in ethidium bromide solution (0.5 μg/mL). Electrophoresis was carried out with a current of 80 V for 1 h in TAE running buffer.

Preparation of PEI solution

Linear 25 kDa PEI was purchased from Polyscience (Warrington, PA) and used to prepare a 1 mg/mL stock solution. To dissolve PEI, deionized H₂O was heated to ~80°C and mixed with 1 mg of PEI. In addition the solution was cooled down to room temperature. The pH was adjusted to pH 7.2 and the solution was filtered using a 0.22 μm filter (Merck Millipore). Stock solution of linear PEI were stored at −20°C and thawed and stored at 4°C while in use.

Cell culture

HeLa cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; catalog number ACC-57) and adapted to grow in Roswell Park Memorial Institute 1640 Medium (RPMI 1640 Medium; Invitrogen catalog number 31870-025). All cells used in the experiment were cultured in RPMI containing 10% fetal calf serum (FCS), 1% GlutaMax™, 1% P/S and 1% NEAA. Cultures were maintained at a temperature of 37°C in a humidified 5% CO₂ atmosphere.

Transfection procedures

All transfections were carried out in 24-well plates (Corning) using either Lipofectamine®2000 (Invitrogen) or linear 25 kDa PEI. HeLa cells were seeded at 3×10⁴ cells/well in 0.5 mL RPMI 1640+10% FCS, 24 h prior to transfection.

Figure 1  Transfection efficiency and cytotoxicity of linear 25 kDa PEI at varying N/P ratios and different DNA concentrations. pEGFP-C1 plasmid DNA 250 ng (A) and 500 ng (B) was complexed with the exact amount of PEI depending on the N/P ratio. (C) Fluorescence microscopy images showing PEI transfection at different N/P ratios.
For the transfections with linear PEI, circular and linearized plasmid DNA (0.25 μg or 0.5 μg/well) and the exact amount of PEI depending on the N/P ratio was separately added to 50 μL OPTI-MEM® Reduced-Serum Medium, briefly vortexed and kept at room temperature for 5 min. The 50 μL of plasmid DNA was added to the 50 μL PEI solution, briefly mixed and kept at room temperature for 10 min. For the transfections with Lipofectamine, circular and linearized plasmid pEGFP-C1 DNA (0.25 μg or 0.5 μg/well) and 0.5 μL of Lipofectamine was separately diluted into 50 μL OPTI-MEM and left at room temperature for 5 min. The 50 μL of plasmid DNA was added to the 50 μL Lipofectamine solution, briefly mixed and kept at room temperature for 5 min.

The culture medium was removed from each well containing PEI/DNA complexes. The cells were washed with phosphate buffered saline (PBS) and fresh RPMI medium without antibiotics was added. The 100 μL of PEI/DNA and DNA/Lipofectamine complexes was added drop-wise to the wells and the cells were incubated at 37°C.

The cells treated with PEI were washed after 5 h with PBS and fresh medium was added following incubation at 37°C for additional 43 h.

Transfection efficiency measurements

Transfection efficiency was determined by flow cytometry analysis 24 and 48 h post-transfection. Briefly, transfected cells were washed twice with ice-cold PBS and harvested by trypsinization. Cells were collected by centrifugation at 1000 rpm for 5 min at RT, the supernatant was removed, and the pellet was re-suspended in PBS containing 1% BSA at a concentration of 1×10^6 cells/mL. Percentage of GFP-expressing cells and mean fluorescence intensity (MFI) were detected by flow cytometry equipped with BD Accuri C6 (Becton Dickinson, San Jose, CA). Cytotoxicity was analyzed by adding 0.05 μg/mL 7-AAD (559925, BD pharimgen) to the samples 10 min before flow cytometry analysis.

Transmission electron microscopy

Transmission electron microscopy (TEM) was employed as imaging technique to visualize the circular and linearized plasmid DNA/Lipofectamine and DNA/PEI complexes in aqueous environment. Complexes using 500 ng DNA were placed on a copper grid covered with a nitroglycerin film coated with carbon. A staining agent was added (2% uranyl acetate).

Statistical analysis

The data for flow cytometry analysis were analyzed by FlowJo X. Standard statistics including calculation of means and standard deviations (SD) and Student’s t-test for group comparisons. In all experiments, p<0.05 was considered statistically significant.

Results

Transfection efficiency and cytotoxicity of linear 25 kDa PEI at varying N/P ratios

To determine the optimal N/P ratio of linear 25 kDa PEI regarding transfection efficiency, 250 ng as well as 500 ng of circular pEGFP-C1 DNA was complexed with the appropriate amount of PEI at N/P ratios of 5, 10, 15, 20, and 40. Transfection efficiency measurements were done by flow cytometry analysis. As shown in Figure 1, the percentage of GFP expressing cells increased with increasing N/P ratio and the higher DNA concentration. The maximal amount of nonviable cells was 2.5% after 48 h, revealing very low toxicity of the transfection agent.

![Figure 2](https://example.com/figure2.png)

Figure 2 Comparison of transfection efficiency and cytotoxicity of Lipofectamine and linear 25 kDa PEI. HeLa cells were plated at the density of 3×10^4 cells and transfected with 250 ng or 500 ng GFP plasmid DNA, complexed with Lipofectamine or PEI at N/P ratio 40. (A) GFP expression and cytotoxicity of transfected cells. (B) Mean fluorescence intensity of transfected cells. The data shown are the mean and SD from three different experiments. Two asterisks indicated p<0.01 and three p<0.001.
Comparison of transfection efficiency and cytotoxicity of linear 25 kDa PEI and Lipofectamine

For the further comparison of PEI with Lipofectamine, the N/P ratio 40 was chosen due to the highest transfection efficiency. The optimal transfection condition for Lipofectamine was determined by seeding in a 24-well plate 2×10^4, 3×10^4, 4×10^4, and 6×10^4 cells per well and complexing 250 and 500 ng circular plasmid DNA with 0.5, 1.0, and 1.5 μL Lipofectamine. The highest transfection efficiency was detected using 3×10^4 and 0.5 μL Lipofectamine for both 250 and 500 ng pDNA (data not shown).

After 48 h Lipofectamine displayed transfection efficiency superior to PEI (26% with 250 ng and 20% with 500 ng vs. 11% with 250 ng and 13% with 500 ng plasmid DNA, respectively). Although the percentage of GFP expressing cells was higher with Lipofectamine, the MFI decreased with increasing amount of DNA. The comparison of MFI of Lipofectamine with PEI N/P ratio 40 revealed much lower values at both DNA concentrations (Figure 2). Comparison of cytotoxicity showed no significant difference between the transfection agents.

Comparison of transfection efficiency and cytotoxicity of circular and linearized plasmid DNA

The circular DNA showed very efficient GFP expression for both Lipofectamine and PEI. For Lipofectamine the GFP expression of linearized pDNA compared with circular dropped 6.5 times down with 250 ng DNA after 48 h. An eight times higher GFP expression was detected in the case of PEI with 500 ng circular plasmid DNA. The MFI of linearized plasmid DNA transfected cells decreased for Lipofectamine approximately 6 times and 40 times for PEI (Figure 3).

Shape of the transfection nanoparticle in transmission electron microscopy

Figure 4 shows the transmission electron microscopy of the resulting transfection nanoparticles created from Lipofectamine and PEI, respectively, with circular and linear plasmids. Circular plasmids display, in the case of both complexing agents, a random coil structure in an approximately spherical, well compacted shape. In contrast, the linear construct appears with both complexing agents as worm-like strands, i.e., an apparently differential tertiary structure.

Discussion

To advance clinical application of gene transfection/delivery, progress in two key technical aspects, namely improved nanomaterials-based vectors and optimal DNA cargo structure needs to be achieved. For transfection vectors, nanomaterials are seen as a promising route to the required optimal balance between nucleic acid binding and release at the target site. In addition, such vectors combine stealth properties (protecting the cargo
from premature scavenging by the immune system) with targeting capabilities to the target organ and target cell type. Optimally structured nucleic acid cargo is not less important, as this study shows that the topology of the expression vector has a substantial impact on expression success in vitro. In this study we performed comparative analysis of the transfection efficiency and cytotoxicity of linear 25 kDa PEI with the commercially available non-viral vector Lipofectamine in HeLa cells. Our study supports that a circular plasmid offers advantages in terms of average expression intensity and expression homogeneity among cancer cells, while the linearized version led to expression in a limited number of cells. The findings were consistent for both the fluorescence microscopy as well as flow cytometry. The results showed that the transfection efficiency of linear 25 kDa PEI is dependent on the DNA concentration and N/P ratio. The highest GFP expression could be detected at N/P ratio 40 as already seen by others (16). It is known that the N/P ratio plays a crucial role for maximization of the transfection efficiency by influencing the size and the charge of the PEI/DNA complexes (17). Therefore, higher N/P ratios increase DNA condensation and endocytic uptake by the cells (18). Although as seen in numerous studies, high transfection efficiency with PEI is mostly combined with high cytotoxicity whereas our study revealed very low toxicity values. The high amount
of free PEI at higher N/P ratios could be a key factor for the disruption of the endosomal membrane leading to higher expression efficiency from the PEI-transfected DNA.

Lipofectamine is a lipid-based transfection agent, which is known to be highly efficient for the transfection of a variety of cells. Our study revealed that transfection efficiency of Lipofectamine was up to two times higher than PEI. Although Lipofectamine shows better transfection efficiency, the MFI of PEI was higher than for Lipofectamine and increased with increasing DNA concentration. Moreover, we also investigated the transfection efficiency of circular and linearized plasmid DNA. Our results showed a reduced percentage of GFP expression and MFI from linearized plasmid DNA after 48 h for both Lipofectamine and PEI compared with circular DNA.

In principle, differential expression may be due to different composition of the vectors, e.g., different promoters etc. Differential expression may be a consequence of differential vector-cell binding and uptake as we believe that in non-receptor targeted transfection used in this work, positive charge, i.e., a high N/P ratio facilitates non-specific binding and uptake. Differential expression may be a consequence of intracellular processes like degradation by nucleases, of nuclear translocation or even integration of a vector into chromosomal DNA. Not all these factors could be assessed in this study, but future work will focus on an in-depth understanding of the differential impact of these factors.

From the electron microscopy data, we found a notable difference in the shape of the complexes: circular DNA had random coil appearance in well compacted, roughly spherical shape, while linearized DNA appeared as worm-like strands, both, when complexed with Lipofectamine or with polyethyleneimine. While the intrinsic persistence length of a double stranded DNA coil is known to be approximately 50 nm, defining the dimension of a DNA random coil, the binding of additional molecules to a DNA strand has the potential to alter its properties: polyethyleneimine, as a polycation, will bind through electrostatic interaction at multiple sites on a DNA strand, has an intrinsic persistence length below 1 nm in suited buffer and behaves, if stretched to a different shape, like an elastic rubber. At the scale of an individual polyethyleneimine molecule, a compacting molecule may therefore impart an additional compacting force on the DNA. Charge of the complex may also significantly influence the overall shape: if complexation imparts an overall charge on the complex, electrostatic forces tend to stretch a segment within the Debye radius (i.e., the electrostatic screening distance by the aqueous electrolyte). The N/P ratio was â« in our experiments because transfection rates were low at N/P below 10. Interestingly, at N/P ratios of 1.0, an impact on circular plasmid tertiary structure has been reported using a different polycationic polymer (19). Particle size and shape are important variables in the cell biology of endocytosis and phagocytosis (20). This suggests that the shape of the transfection particle may be an important factor for successful gene transfer.

Thus, for successful transfection of DNA into eukaryotic cells, and in particular for clinical in vivo applications (21), we hypothesize that control of shape of the DNA/cationic vector is an important design variable to be understood and controlled.

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References


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Xueya Wang studied molecular biology at the University of Basel from where she received her diploma in 2004 and compiled her PhD thesis entitled “Antigen – presentation by vascular endothelium: its role for CTL – mediated vascular injury” under the guidance of Prof. Dr. Barbara Biedermann in 2008. Afterwards Xueya joined the group of Prof. Patrick Hunziker as a senior scientist. Her research interests are nanoscale polymer delivery systems for the safe and specific delivery of therapeutic molecules and intelligent materials to target tissues and cells in humans focusing on atherosclerosis and cancer therapy.
Patrick Hunziker has studied Medicine at the University of Zurich, Switzerland. He received a doctoral degree based on thesis work in experimental immunology from the University of Zurich and did further research in experimental hematology at University Hospital in Zurich, Switzerland. He earned specialist degrees in Internal Medicine, Cardiology and Intensive Care Medicine. As a fellow of the Massachusetts General Hospital, Harvard Medical School, he worked on cardiac imaging in a joint project with the Massachusetts Institute of Technology, Cambridge. His professional activities in Europe, the US, Africa and China gave him a broad insight into the needs for the medicine of the future in a variety of settings. Hunziker became involved in medical applications of Nanoscience in the late 1990s and has been the pioneer physician in Nanomedicine in Switzerland since then. With improved prevention, diagnosis and cure of cardiovascular disease as his main research topic, he worked in the nanoscience fields of atomic force microscopy, nano-optics, micro/nanofluidics, nanomechanical sensors and polymer nanocarriers for targeting. He is the founding president of the European Society of Nanomedicine, cofounder of the European Foundation for Clinical Nanomedicine and coinitiator of the European Conference for Clinical Nanomedicine and is clinically active as deputy head of the Clinic for Intensive Care Medicine at the University Hospital Basel, Switzerland. In November 2008 Patrick Hunziker became professor for Cardiology and Intensive Care Medicine at the University of Basel.