Linear Polyethylenimine-DNA Nanoconstruct for Corneal Gene Delivery

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Abstract

Purpose: This study investigated the efficiency and potential toxicity of a linear 22-kDa polyethylenimine (PEI)-DNA nanoconstruct for delivering genes to corneal cells and the effects of PEI nitrogen-to-DNA phosphate (N:P) ratio on gene transfer efficiency *in vitro* and *in vivo*.

Methods: A gel retardation assay, zeta potential measurement, bright-field microscopy, transfection with green fluorescent protein (GFP), immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) were used to characterize the physicochemical and biological properties and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH), and reactive oxygen species (ROS) assay for cytotoxicity of the linear PEI-DNA nanoconstruct using *in vitro* cultured primary human corneal fibroblast and *in vivo* mouse models.

Results: Of the several evaluated N:P ratios, the highest gene transfection efficiency achieved without any notable cytotoxicity was observed at an N:P ratio of 30:1 (N:P 30). *In vivo* gene transfer studies revealed substantial GFP gene delivery into the corneas of mice 3 days after a single 5-min topical application without any significant adverse ocular effects. Slit-lamp biomicroscope ophthalmic examination of the mouse exposed to the linear PEI-DNA nanoconstruct showed no evidence of hyperemia (redness), corneal edema, ocular inflammation, or epiphora (excessive tearing).

Conclusions: The 22-kDa linear PEI-DNA nanoconstruct is an efficient and well-tolerated vector for corneal gene therapy *in vitro* and *in vivo* and could be used as a platform for developing novel gene-based nanomedicine approaches for corneal diseases.

Keywords: linear PEI nanoparticles, cornea, gene delivery, N:P ratio, fibroblast

Introduction

THE SUCCESS OF GENE THERAPY relies on the development of a safe and efficacious nucleic acid transporter to shuttle therapeutic genes into cells of interest. The cornea is a very well-suited tissue for evaluating gene-based therapies because of its easily accessible and immune-privileged status.¹⁻⁴ Our laboratory has recently optimized techniques that allow for localized topical gene delivery into the cornea and we have successfully demonstrated highly efficient gene delivery into the corneas of mice and rabbits *in vivo*.⁵⁻⁹ The vast majority of the gene therapy studies that evaluated gene delivery into cornea have used viral vectors.⁵⁻¹¹ Although viral vectors offer the advantage of high transfection

efficiency, their generation is expensive and time consuming. Nonviral vectors offer an attractive alternative to viral vectors for corneal gene delivery. Nonviral vectors have the advantages of easy generation, an ability to carry large payloads including multiple genes, and offer more versatility for DNA conjugation. However, only a handful of nonviral vectors have been tested for gene delivery into the cornea.^{1-4,12,13} Further development of nonviral vectors for corneal gene delivery applications has been hampered by historically poor transfection efficiency. Therefore, there is a significant need to develop and evaluate more efficacious nonviral vectors for corneal gene delivery.

Cationic lipids and polymers have been widely tested for gene delivery.¹⁴⁻¹⁸ They interact with DNA forming nano-

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sized polyplexes that are readily taken up by the cells. Among cationic polymers, high transfection efficiency has made polyethylenimine (PEI) a highly attractive candidate for nonviral gene delivery.^{19–22} PEI is available in several isoforms. To date, high molecular weight branched PEI has shown the highest level of gene transfer in a wide variety of cells *in vitro* and in the kidney, lungs, brain, and liver *in vivo*.^{23–26} However, high cytotoxicity attributed to both apoptotic and necrotic cell death has hampered the further development of high molecular weight branched PEI.^{19–22,27,28} Therefore, we intended to search for a linear PEI with low toxicity and high gene transfer efficiency. This study was designed to test the gene transfer efficiency of 22-kDa linear PEI-DNA nanoparticles *in vitro* in human corneal fibroblast and *in vivo* in the corneas of mice. The published literature on branched PEI suggests that the relative ratio of PEI nitrogen-to-DNA phosphates (N:P ratio) in the PEI-DNA polyplex can modulate a number of physiochemical properties which, in turn, can affect the gene transfer ability and cytotoxicity.^{19–21,29} Thus, we also tested the effect of N:P ratio on the cytotoxicity and the gene transfer efficiency of linear PEI-DNA nanoparticles in an attempt to identify an N:P ratio for optimal *in vivo* corneal gene delivery with minimal or no toxicity.

Methods

Human corneal fibroblast culture

Primary human corneal fibroblast (HCF) cultures were generated from 12 donor human corneas procured from an eye bank (Saving Sight, Kansas City, MO) as reported previously.⁵ Corneal tissues were briefly washed with sterile cell culture medium, and the epithelium and endothelium were removed by gentle scraping with a no. 15 scalpel blade. The corneal stroma was cut into small pieces, placed onto a 100×200 mm culture dish (Thermo Fisher Scientific, Waltham, MA) containing minimum essential medium (MEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, and incubated in a humidified 5% CO₂ incubator at 37°C for 2 weeks or longer to obtain primary human corneal fibroblast, harvested by trypsin treatment. For further experiments, human corneal fibroblast up to passages 4 (P4) were used at ~80% confluence.

PEI-plasmid DNA nanoparticles preparation and characterization

The linearized PEI of 22-kDa size was prepared following the earlier reported method.³⁰ The PEI-DNA nanoparticles were prepared at various N:P ratios by adding appropriate amounts of 150 mM PEI in 100 µL of water dropwise with constant stirring to 2 µg plasmid DNA (pTRUF11 expressing GFP) in 100 µL of Diethyl dicarbonate (DEPC) water. A specific N:P ratio contains appropriate amount of PEI. To achieve appropriate amount of PEI, multiplied by 3 with amount of DNA taken (1 µg of nucleic acid contains 3 nmol of anionic phosphate) divided by 150 followed by multiplication with desired N:P ratio. The concentration of PEI was 150 mM. We tested N:P ratios of 2:1, 4:1, 8:1, 15:1, 30:1, and 60:1. The PEI-plasmid DNA complexation was confirmed with an agarose gel retardation assay by loading onto 1% agarose gel containing ethidium bromide, and subjecting to electrophoresis with a Tris–acetate–ethylenediaminetetraacetic acid run-

ning buffer. The zeta potential of the nanoparticles was measured using DelsaNano zeta potential analyzer (Beckman Coulter, Inc., Brea, CA).

Human corneal fibroblast transfection

The transfection solution was prepared by diluting the PEI-DNA nanoparticles with 2.5 mL Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The human corneal fibroblast cultures were incubated with the transfection solution for 6 h. After transfection solution incubation, the cultures were washed with phosphate-buffered saline (PBS) and allowed to grow in DMEM supplemented with 10% fetal bovine serum for 24 h.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The effects of PEI-DNA nanoconstruct on cellular viability were analyzed with a Cell Titer 96® Non-Radioactive Cell Proliferation Assay (MTT) following manufacturer's instructions (Promega, Madison, WI). Human corneal fibroblast cells were seeded in a 96-well plate at a density of 5×10^3 per well in 200 µL of MEM supplemented with 10% fetal bovine serum. After 24 h of incubation, PEI-DNA transfection solution at a different N:P ratio was applied to each well for 6 h, and thereafter 15 µL of Cell Titer 96® Non-Radioactive dye was added to each well for 4 h. Untransfected and plasmid-alone exposed cultures served as negative controls. Solubilization stop mix solution (100 µL/well) was then added to each well to solubilize the product. Sample absorbance was recorded at 570 nm with an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT).

Lactate dehydrogenase cytotoxicity assay

The HCF cultures grown in 96-well plates were challenged with PEI-DNA transfection solution of different N:P ratio for 6 h. Untransfected and plasmid-alone exposed cultures served as negative controls. Cytotoxicity was determined by lactate dehydrogenase (LDH) assay. The LDH assay was performed using Pierce LDH Cytotoxicity Assay Kit (Cat. no. 88953; Pierce) according to the manufacturer's instructions. The experiment was performed and LDH level was calculated with proper control like, cells treated with ultrapure water was spontaneous LDH activity control and, cells incubated with 10 µL of lysis buffer for 45 min, at 37°C in 5% CO₂ was treated as maximum LDH activity control.

Reactive oxygen species assay

The levels of intracellular reactive oxygen species (ROS) were analyzed using OxiSelect™ Intracellular ROS Assay Kit (STA-342; Cell Biolabs, Inc., San Diego, CA). Human corneal fibroblast cells were cultured in a 96-well culture plate for 24 h and washed with PBS before incubation in 100 µL of 1× cell-permeable fluorogenic probe 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 60 min. Cells were treated with PEI-DNA solution at different N:P ratio for 6 h. Untransfected and plasmid-alone exposed cultures served as negative controls. Fluorescence was analyzed with a fluorometric plate reader (BioTek) at 480/530 nm. Hydrogen peroxide, at 1,000 µM was used a positive control.

Detection and quantification of the delivered green fluorescent protein gene in mouse eye in vivo

To detect nanoparticle-mediated gene delivery, immunofluorescence and enzyme-linked immunosorbent assay (ELISA) for green fluorescent protein (GFP) were performed. HCFs were incubated for 6 h in transfection solution followed by washing with PBS and allowed to grow in DMEM +10% fetal bovine serum for 24 h. The cells were fixed in 4% paraformaldehyde solution for 30 min at room temperature, washed 3 times with PBS, mounted in vectashield containing 4'-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), viewed and photographed with a Leica fluorescent microscope (Leica DMI 4000B, Deerfield, IL) equipped with a digital camera (Spot-Cam RT KE, Sterling, MI). The percent transfection efficiency was calculated by counting the number of GFP-stained (GFP+) cells and the DAPI-stained nuclei at 200 $\times$ magnification in 12 randomly selected, nonoverlapping areas of each well of a 6-well cell culture plate. The ELISA for GFP was carried out following manufacturer's instructions using a commercially available kit (Cell Biolabs, Inc.).

Nanoparticle application to mouse cornea in vivo

Six- to eight-week-old female C57BL/6J mice weighing 18–21 g (JAX Stock no. 000664; The Jackson Laboratory, Bar Harbor, ME) were used for *in vivo* studies. All animals were treated in accordance with the tenets of the Association for Research in Vision and Research (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Harry S. Truman Memorial Veterans' Hospital in Columbia, Missouri. Twenty-four mice were divided into 3 groups ($n=8$ /group). The eyes of animals of group 1 received PEI-only, group 2 received DNA-only, and group 3 received PEI-DNA nanoconstruct. Only 1 eye of each animal was used for experiments. The contralateral eyes served as naive (no treatment) control. Before experimental manipulation, animals were anesthetized with an intraperitoneal injection of ketamine (130 mg/kg) and xylazine (8.8 mg/kg) and ophthalmic 0.5% proparacaine hydrochloride solution (Alcon, Ft. Worth, TX) was topically instilled into each eye for local anesthesia. Once fully anesthetized, the corneal epithelium of the mouse was removed by gentle scraping with a no. 64 Beaver blade (Becton-Dickinson, Franklin Lakes, NJ) using an operating microscope. After removing the corneal epithelium, eyes were washed with balanced salt solution (Alcon), wiped with a merocel sponge (Medtronic, Minneapolis, MN), and subjected to controlled cyclic air drying to dehydrate the corneal stroma as described previously.^{5,9} Immediately after drying, 2 μ L of PEI-DNA nanoparticle solution at an N:P ratio 30 (N:P 30:1) was topically applied to the mouse cornea for 5 min using a sterile custom-cloning cylinder as described previously.^{9,31–33}

Clinical eye examination and biomicroscopic imaging

Qualitative and ocular health examination of the anterior segment and adnexa of the mouse eyes (cornea, sclera, iris, ciliary body, lens, conjunctiva, and eyelids) were performed

at 12, 24, and 72 h after the nanoparticle application as described previously.^{34,35} In brief, ocular health was evaluated independently by at least 2 of the 3 examiners (J.T.R., R.T., and M.K.F.) at selected times using an established Draize and modified McDonald–Shadduck ocular scoring systems.³¹ With the Draize eye test, the severity of ocular lesions was scored in the following manner: in the cornea, by estimating the degree of opacity and area of involvement; in the iris, by examining pupillary light reflexes; and in the conjunctiva, by assessing the degree of redness, chemosis, and discharge. With the modified McDonald–Shadduck scoring system, ocular health scores were determined based on the cumulative average scores for corneal tissue (opacity, affected area, corneal neovascularization severity, and reepithelialization) and conjunctival tissue (congestion, chemosis, swelling, and discharge).³¹ The data on hyperemia, edema, and inflammation were collected. Biomicroscopic imaging of the mouse eyes was performed using a stereomicroscope (Leica, Wetzlar, Germany) equipped with a digital camera (SpotCam RT KE; Diagnostic Instruments, Inc., Sterling Heights, MI).

Tissue embedding and GFP immunohistochemistry

Mice were killed 3 days after the nanoparticle application. The eyes were then enucleated and embedded in liquid optimal cutting temperature compound (Sakura FineTek, Torrance, CA) within a 15 $\times$ 15 $\times$ 5-mm mold (Thermo Fisher Scientific) and snap frozen. The frozen tissue blocks were maintained at -80°C . Tissue sections (7 μ thick) were cut with a cryostat (HM 525M; Microm GmbH, Walldorf, Germany) and maintained frozen at -80°C until staining.

For GFP immunofluorescence, the corneal tissue sections were incubated with 5% bovine serum albumin for 30 min at room temperature, followed by chicken polyclonal anti-GFP antibody (1:200 dilution) (Millipore Cat. no. 06–896) for 90 min and Alexa 488 goat anti-chicken immunoglobulin G secondary antibody (1:500 dilution) (product no. A-21202; Thermo Fisher Scientific) for 1 h. The tissues were washed 3 times in PBS, mounted in vectashield containing DAPI, viewed and photographed with a Leica fluorescent microscope (Leica DM 4000B, Deerfield, IL) equipped with a digital camera (Spot-Cam RT KE).

Statistical analyses

Results were expressed as mean $\pm$ standard error of the mean. Statistical analysis was performed using a one-way analysis of variance and Tukey's test. The standard curve for ELISA and ROS data were subjected to regression analysis to permit the calculation of slope, intercept, and correlation coefficient. A value of $P<0.05$ was considered significant for all analyses.

Results

Characterization of PEI-DNA nanoparticles

The formation of a PEI-DNA complex was confirmed with a gel retardation assay. The agarose gel in Fig. 1 shows the electrophoretic migration of the plasmid DNA from the PEI-DNA nanoparticles at various N:P ratios. As is evident from Fig. 1A, the increase in N:P ratio caused a dose-dependent inhibition of DNA migration, thus confirming

FIG. 1. Plasmid DNA migration on agarose gel electrophoresis (A) and zeta potential (B) of PEI-DNA nanoparticles at various N:P ratios. PEI completely inhibited plasmid DNA migration at N:P ratios ≥ 15 (N:P 15:1), suggesting effective complex formation (A). Plasmid DNA (1 μ g) without PEI showed normal migration. “L” denotes 1 kb plus DNA ladder. Zeta potential indicates that the nanoparticles acquired progressively more positive surface charge with the increase in N:P ratio (B). PEI, polyethylenimine.

the successful complexation of PEI and plasmid DNA. At an N:P ratio ≥ 15 (N:P 15:1), a complete inhibition of DNA migration was observed and it also prevented the ethidium bromide dye from binding to the DNA resulting in no staining of the retained DNA band in the wells. The control plasmid DNA alone showed normal electrophoretic migration in gel.

Zeta potential is an indicator of the surface charge of PEI-DNA nanoparticle complexes. Figure 1B shows the zeta potential of the linear PEI-DNA nanoparticles at various N:P ratios. At the lowest tested N:P ratio of 4 (N:P 4:1), the zeta potential of linear PEI-DNA nanoparticle complexes was -2.6 , which indicates a net negative potential because of molar excess of the negatively charged plasmid DNA. With an increase in N:P ratio, the zeta potential became progressively positive because of the cumulative amounts of positively charged PEI and the values plateaued ~ 25 mV at N:P ratios of 30 (N:P 30:1) and 60 (N:P 60:1). The positive zeta potential of the nanoparticles tentatively helps them bind to the negatively charged cellular membrane, thereby facilitating their entry into the cells by endocytosis.

Effect on cell viability and morphology

Light microscopy images were evaluated to observe any morphological changes in human corneal fibroblast cultures exposed to linear PEI-DNA nanoparticles at various N:P ratios of DNA-nanoparticle solution. As is evident from Fig. 2A, no observable changes in morphology were noted in the human corneal fibroblast exposed to linear PEI-DNA nanoparticles at an N:P ratio ≤ 30 :1 (N:P 30:1). Both the untransfected and plasmid-alone control cell cultures and the cultures exposed to N:P ratios of N:P 2:1, N:P 4:1, N:P 8:1, N:P 15:1, and N:P 30:1 continued to demonstrate a healthy spindle-shaped morphology characteristic of corneal fibroblast. However, notable signs of cellular toxicity including nonadherent cells, and a loss of overall cell density and confluence was observed at the high N:P ratio 60 (N:P 60:1). Therefore, we intended to explore viability of fibroblast at a different N:P ratio. MTT assay confirmed that HCFs viability was not affected by increase in N:P ratio as $>95\%$ cells remained viable up to N:P ratio 30:1. HCF viability was reduced significantly (75 ; $P < 0.001$) at N:P ratio 60 (N:P 60:1) (Fig. 2B).

Effect on cytotoxicity and ROS levels

Figure 3 provides the cytotoxicity of different N:P ratios of DNA-nanoparticle solution to HCF measured with LDH (Fig. 3A) and ROS (Fig. 3B) assays. The various N:P ratios ≤ 30 (N:P 30:1) were noncytotoxic as they did not cause any significant leakage as evident from LDH assay. Nevertheless, the dose of N:P 60:1 caused leakage up to $23.7\% \pm 2.3\%$ in HCFs compared with untransfected or plasmid-exposed negative control. A similar pattern in ROS was observed in HCFs exposed to N:P ratio ≤ 30 (N:P 30:1). The ROS levels remained $<12.4\% \pm 3.3\%$ up to N:P 8:1, then gradually increased up to $30.2\% \pm 2.3\%$ up to N:P 30:1 followed by a significantly high ROS levels at N:P 60:1 ($P < 0.001$).

In vitro gene delivery to human corneal fibroblast

To test whether the N:P ratio affected gene delivery, we exposed the human corneal fibroblast cultures to linear PEI-DNA (pTRUF11-GFP plasmid) nanoparticles at various N:P ratios. Figure 4A provides the level and the localization of delivered GFP gene in human corneal fibroblast. As is evident from Fig. 4A, an increase in the number of GFP expressing corneal fibroblast was associated with an increase in the N:P ratio of the nanoparticles. The gene delivery efficiency of nanoparticles at various N:P ratios was quantified by calculating the percent transfection efficiency as shown in the graph (Fig. 4B). At N:P ratios of 2 (N:P 2:1) and 4 (N:P 4), a transfection efficiency of $<5\%$ was noted. At N:P ratios of 8 (N:P 8:1) and 15 (N:P 15), a significant increase in the transfection efficiency was observed to be $15\% \pm 2.3\%$ and $17\% \pm 3.7\%$, respectively ($P < 0.01$). Finally, a peak transfection efficiency of $24\% \pm 5.5\%$ ($P < 0.001$) was observed at an N:P ratio of 30 (N:P 30:1). The transfection efficiency then started to decline at an N:P ratio of 60 (N:P 60) largely because of significant cellular toxicity (data not given).

The transfection efficiency of various N:P ratios of linear PEI-DNA nanoparticles was further confirmed by quantifying the GFP protein with an ELISA. Figure 4C provides the amounts of GFP in the cellular lysates of human corneal fibroblast at various N:P ratios. At N:P 2 (N:P 2:1) and N:P 4 (N:P 4:1), very low levels of GFP protein were detected in the cell lysates of human corneal fibroblast. At N:P 8

A

FIG. 2. (A) Representative light microscopy images showing the effects of PEI-DNA (pTRUF11-GFP plasmid) nanoparticle application on the morphology of human corneal fibroblast. The nanoparticles did not cause any notable change in cellular morphology at N:P ratio ≤ 30 . Notable cellular toxicity was only observed at a high N:P ratio of 60:1 (N:P 60). Scale bar = 100 μ m. (B) Histogram shows HCF viability determined by the MTT assay. PEI-DNA nanoconstruct did not alter cellular viability up to N:P ratio 30 (N:P 30:1). However, a significant decrease in cellular viability was observed at N:P ratio 60 (N:P 60:1) (* $P < 0.001$). GFP, green fluorescent protein; HCF, primary human corneal fibroblast; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

(N:P 8:1) and N:P 15 (N:P 15:1), a significant increase ($P < 0.001$) in GFP protein levels was observed. The cellular lysates exposed to N:P 30 (N:P 30:1) showed the highest amount ($P < 0.001$) of GFP protein. However, with further increases in N:P ratio, the GFP protein levels started to decline (data not given).

In vivo gene delivery to mouse cornea

The promising *in vitro* gene transfer efficiency without significant toxicity at an N:P ratio 30 (N:P 30:1) prompted us to test the potential of linear PEI-DNA nanoparticles for *in vivo* gene delivery into the mouse cornea. The levels of gene transfer detected in the entire mouse corneas with

stereomicroscope and in serial corneal sections with immunohistochemistry 3 days after a single 5-min topical application of PEI-DNA solution are given in Fig. 5. Representative stereomicroscopy images of mouse eye in Fig. 5A and B illustrate that topical PEI-DNA solution was safe and did not cause any clinically appreciable hyperemia, edema, or inflammatory infiltrate in the mouse eye *in vivo*. The fluorescence stereomicroscopy was used to detect expression of GFP in the mouse eye *in vivo*. PEI-DNA nanoconstruct-exposed mouse corneas showed significant GFP gene delivery into corneal stroma (Fig. 5D) compared with the DNA-alone applied eyes (Fig. 5C). The dotted circles in Fig. 5C and D show the area where DNA alone or PEI-DNA was topically applied onto the mouse

FIG. 3. (A) Histogram showing cytotoxicity of PEI-DNA nanoconstruct to HCF quantified by LDH assay. PEI-DNA nanoconstruct N:P ratio ≤ 30 was noncytotoxic to HCFs. The N:P 60 (N:P 60:1) dose showed significant cytotoxicity to HCFs (* $P < 0.001$). (B) Levels of ROS were not affected by the tested PEI-DNA N:P ratio up to 30, whereas N:P 60 showed significantly elevated ROS compared with untransfected or plasmid-alone treatment (* $P < 0.001$). LDH, lactate dehydrogenase; ROS, reactive oxygen species.

FIG. 4. (A) Fluorescence microscopy images showing GFP expression in human corneal fibroblast exposed to PEI-DNA (pTRUF11-GFP plasmid) nanoparticles at various N:P ratios. The cultures were exposed to PEI-DNA nanoparticles for 6 h and then imaged for GFP expression 24 h later. Nuclei are stained blue with DAPI. Scale bar = 100 μ m. (B) Graph showing gene transfer efficiency of PEI-DNA (pTRUF11-GFP plasmid) nanoparticles at various N:P ratios. The gene transfer efficiency percentage was calculated by counting the number of GFP expressing cells (GFP+) and the total number of DAPI-stained nuclei at 200 $\times$ magnification in 12 randomly selected and nonoverlapping areas in each well of the 6-well cell culture plate. * P < 0.01; ΨP < 0.001 compared with N:P 2. (C) Graph showing ELISA quantification of GFP protein in the cell lysates of human corneal fibroblast cultures exposed to PEI-DNA nanoparticles at various N:P ratios. * P < 0.001 compared with N:P 2. DAPI, 4'-6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay.

cornea *in vivo*. The immunohistochemistry to characterize GFP levels in serial corneal sections of the DNA-alone and PEI-DNA-applied eyes are given in Fig. 5E and F, respectively. The PEI-DNA nanoconstruct efficiently delivered GFP gene into keratocytes of the anterior and midstroma of the mouse cornea *in vivo* (Fig. 5F). As expected, DNA-alone (Fig. 5E) or PEI-alone (Fig. 5A) treatment did not show any gene delivery into mouse keratocytes *in vivo*. The autofluorescence in the hair/eyelid was noted in animals that received DNA alone (Fig. 5C) or PEI-DNA (Fig. 5D) during stereo-biomicroscopy because of circular shape and small size of the mouse eye. This inference was drawn as immunohistochemistry and polymerase chain reaction analyses of eyelid/hair tissues showed gene delivery in these tissues.

Discussion

A wide variety of cationic lipids and polymers have been tested as gene delivery vectors. Among these cationic vectors, PEI has emerged as one of the most promising gene

delivery vectors.^{22–26} At a physiologic pH, PEI efficiently forms nanoparticulate complexes with the plasmid DNA and these nanoparticles readily enter the cells by endocytosis.^{36,37} After their efficient cellular uptake, PEI's proton sponge effect enables DNA release from endosomes because of proton and chloride influx, thus leading to endosome rupture by osmotic swelling.^{19–21} Branched and linear PEIs have been tested for gene transfer.^{22,38} The attempts to decrease the cytotoxicity by using low molecular weight branched PEI have resulted in a considerable loss of gene transfer efficiency.^{39,40} Recent studies have shown that the cytotoxic effect of PEI can be reduced by decreasing the branching that led to the development of linear forms of PEI. Linear PEI shows low toxicity while still retaining the capacity to offer high gene transfer efficiency.^{30,38,41–44}

In this study, we demonstrated that low molecular weight (22 kDa) linear PEI is a highly efficient vector for delivering genes into corneal cells *in vitro* and *in vivo* without compromising cellular morphology. The relative N:P ratio in these nanoparticles can influence a number of their physicochemical properties which, in turn, can affect their

FIG. 5. Representative biomicroscopy images demonstrate gene-transfer efficacy and safety of the PEI-DNA N:P 30, PEI alone and DNA alone to mouse cornea *in vivo*. The ophthalmic examinations revealed no hyperemia, edema, or inflammatory infiltrate in PEI-alone-treated (A) or PEI-DNA-treated N:P 30 (B) mouse eyes. Fluorescence stereomicroscopic evaluations in live animals detected no GFP gene delivery into eyes treated with DNA alone (C), whereas significant GFP gene transfer was observed in cornea of the eyes that were treated with PEI-DNA nanoconstruct (D). Dotted circles in (C, D) show areas where vector was applied. Immunofluorescence staining performed in corneal sections revealed significant GFP gene delivery into keratocytes of the anterior and midstroma (arrows) of mouse eyes that received PEI-DNA N:P 30 (F). No gene transfer was noted in DNA-alone-treated eyes. The fluorescent hair/eyelid in the DNA-alone (C) and PEI-DNA (D) eyes is because of autofluorescence. Scale bar = 100 μ M.

biological activity.^{19–21,29} Therefore, we prepared linear PEI-DNA nanoparticles at various N:P ratios and tested the hypothesis of whether the N:P ratio affects the plasmid DNA complexation, surface charge, transfection efficiency, and cytotoxicity of these nanoparticles. Indeed, the gel retardation assay revealed a stronger DNA complexation associated with the increase in N:P ratio. Zeta potential quantification showed that the nanoparticles acquired an increasingly positive surface charge with increasing N:P ratio. Multiple studies have reported that a positive surface charge on the vector promotes electrostatic interaction with the negatively charged cell membrane, leading to an enhanced cellular uptake and gene delivery through adsorptive endocytosis.^{18–21} As the nanoparticles acquired positive surface charge with the increase in N:P ratio, this led us to postulate that it will affect the gene transfer efficiency of linear PEI-DNA nanoparticles. Indeed, the direct correlation between the N:P ratio, surface potential, and the level of GFP gene transfer identified in this study confirms our hypothesis. The results of this study also suggest that linear PEI is well tolerated and safe because no alterations in cellular morphology was observed in the human corneal fibroblast exposed to PEI up to a ratio of N:P 30. Only at a high N:P ratio of N:P 60 (Fig. 2A) was derangement of fibroblast morphology observed, which is likely because of

the cellular toxicity caused by the interaction of high amounts of PEI with polyanionic cellular components.

Based on the encouraging gene transfer efficiency data and the low potential for toxicity observed *in vitro*, we decided to explore the potential for 22 kDa linear PEI to deliver genes to keratocytes *in vivo* using an optimized mouse model. For *in vivo* nanoparticle delivery, the corneal epithelium was scraped and partially dehydrated as optimized in the previous studies from our laboratory to achieve tissue-selective gene delivery to keratocytes within the corneal stroma.^{5,9} Surgical debridement (removal) of the corneal epithelium is frequently used clinically to treat epithelial defects and the epithelium typically regenerates completely in 48–72 h in a process called reepithelialization. The topical route of delivery was chosen for *in vivo* application to the cornea because it represents the most common and clinically relevant scenario, is the most convenient way of delivering drugs to the eye by ophthalmic drops, and avoids the pharmacokinetic challenges associated with systemically administered treatments. Our results show that topically applied linear PEI-DNA nanoparticles can selectively deliver therapeutically relevant levels of transgene into the cornea *in vivo*. Biomicroscopic examinations of the mouse corneas revealed no evidence of hyperemia, edema, or inflammatory infiltrate after topical application of linear PEI

for 5 min at an N:P ratio of 30 suggesting that linear PEI-DNA nanoparticles are safe for *in vivo* application.

In conclusion, the results of this study demonstrate that linear PEI-DNA nanoparticles are safe and efficient vectors for gene delivery into the cornea *in vivo*. Gene therapy holds promise to treat several acquired pathologies and genetic diseases affecting the cornea including corneal dystrophies for which there is currently no effective treatment available. This study shows a “proof of concept” that PEI-DNA nanoconstruct can be used for gene therapy *in vivo*. In addition, studies evaluating safety, efficiency, and toxicity will help to define the clinical utility of this technique.

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Author Disclosure Statement

No competing financial interests exist.

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