Pharmaceutical Research, Vol. 22, No. 3, March 2005 (© 2005)
DOI: 10.1007/s11095-004-1874-y

Research Paper

Cross-linked Small Polyethylenimines: While Still Nontoxic, Deliver DNA Efficiently to Mammalian Cells *in Vitro* and *in Vivo*

Mini Thomas,[1] Qing Ge,[2] James J. Lu,[2] Jianzhu Chen,[2] and Alexander M. Klibanov[1,3]

Received August 6, 2004; accepted December 3, 2004

Purpose. Polyethylenimine (PEI) is among the most efficient nonviral gene delivery vectors. Its efficiency and cytotoxicity depend on molecular weight, with the 25-kDa PEI being most efficient but cytotoxic. Smaller PEIs are noncytotoxic but less efficient. Enhancement in gene delivery efficiency with minimal cytotoxicity by cross-linking of small PEIs via potentially biodegradable linkages was explored herein. The hypothesis was that cross-linking would raise the polycation's effective molecular weight and hence the transfection efficiency, while biodegradable linkages would undergo the intracellular breakdown after DNA delivery and hence not lead to cytotoxicity. Toward this goal, we carried out cross-linking of branched 2-kDa PEI and its 1:1 (w/w) mixture with a linear 423-Da PEI via ester- and/or amide-bearing linkages; the *in vitro* and *in vivo* gene delivery efficiency, as well as toxicity to mammalian cells, of the resultant cross-linked polycations were investigated.
Methods. The efficiency of the cross-linked PEIs in delivering *in vitro* a plasmid containing β-galactosidase gene and their cytotoxicity were investigated in monkey kidney cells (COS-7). Dynamic light scattering was used to compare the relative DNA condensation efficiency of the unmodified and cross-linked PEIs. *In vivo* gene delivery efficiency was evaluated by intratracheal delivery in mice of the complexes of a luciferase-encoding plasmid and the PEIs and estimating the luciferase expression in the lungs.
Results. Cross-linking boosted the gene delivery efficiency of the small PEIs by 40- to 550-fold *in vitro*; the efficiency of the most potent conjugates even exceeded by an order of magnitude that of the branched 25-kDa PEI. Effective condensation of DNA was evident from the fact that the mean diameter of the complexes of the cross-linked PEIs was some 300 nm with a narrow size distribution, while the complexes of the unmodified small PEIs exhibited a mean size of >700 nm with a very broad size distribution. At concentrations where the 25-kDa PEI resulted in >95% cell death, the conjugates afforded nearly full cell viability. The cross-linked PEIs were 17 to 80 times more efficient than the unmodified ones *in vivo*; furthermore, their efficiencies were up to twice that of the 25-kDa PEI.
Conclusions. Cross-linking of small PEIs with judiciously designed amide- and ester-bearing linkers boosts their gene delivery efficiency both *in vitro* and *in vivo* without increasing the cytotoxicity. The high efficiency is dependent on the nature of the linkages and the PEIs used.

KEY WORDS: biodegradability; COS-7 cells; cross-linking; cytotoxicity; *in vitro* gene delivery; *in vivo* gene delivery; plasmid DNA; polyethylenimine.

INTRODUCTION

Gene therapy, the treatment or prevention of inherited (1–3) or acquired (4–6) diseases by the delivery of DNA, has attracted much attention as a promising therapeutic strategy during the past two decades (7). However, after more than a thousand clinical trials, no gene therapy protocol has yet been approved by the U.S. Food and Drug Administration. The negative charge and biodegradability of nucleic acids pose a major challenge to their delivery, and the primary hurdle to the clinical success of gene therapy has been the absence of carriers (vectors) that can deliver DNA and RNA into target cells safely and efficiently (8–10). Viral vectors, used in most clinical trials, are plagued by such problems as immunogenicity (11), insertionals mutagenesis (12), and even germ cell line alterations (13). Cationic liposomes (7) and polycations (8–10) are the two major classes of nonviral vectors investigated for gene delivery. Polycations, such as polylysine, are yet to reach clinical trials due to their inferior efficiency compared to viral vectors and liposomal formulations (14,15). However, the discovery that polyethylenimine (PEI) is able to deliver DNA on par with the best cationic liposomes (15) has led to intense research both in terms of understanding (16–19) and improving (17,20–22) gene delivery by PEI, as well as designing its mimics (23–26).

The relatively high transfection efficiency of PEI was

[1] Department of Chemistry and Division of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.
[2] Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.
[3] To whom correspondence should be addressed. (e-mail: klibanov@mit.edu)

proposed to be due to its "proton-sponge" effect, and much evidence supports this hypothesis (17,19). According to the latter, the partially protonated polycation absorbs protons brought into the endocytic vesicles by the ATPase proton pump, leading to the accompanying influx of chloride ions and ultimately to osmotic swelling and rupture of the endocytic vesicles. The gene delivery efficiency and cytotoxicity of PEI vary with its molecular weight. For example, the branched 2-kDa PEI is some two orders of magnitude less efficient than the branched 25-kDa polycation at the same concentration (17), but the former is not cytotoxic whereas the latter is. Increasing the hydrophobicity of small PEIs enhances their transfection efficiency substantially (17,27–29), as does increasing the effective molecular weight (30–33). Previously, we found that conjugation of the 2-kDa PEI to gold nanoparticles boosted the optimal transfection efficiency of this PEI *in vitro* 12 times (thus making it some 6 times higher than that of the 25-kDa PEI) (33). However, this conjugation also raised toxicity of the 2-kDa PEI, thus limiting the applicability of the conjugates *in vivo,* particularly as relatively high concentrations were required for optimal efficiency.

The foregoing polycation toxicity issue must be resolved, especially as repeated administrations would be required in most clinical applications, unlike in the case of many viral vectors that integrate into the host genome and thus guarantee long-term expression. We hypothesized that enhancement in gene delivery efficiency with minimal cytotoxicity may be achieved by cross-linking of small PEIs with linkages that are potentially biodegradable. Such linkages comprise esters, amides, orthoesters, acetals, glycosides, and disulfides; polymers containing these bonds are increasingly common in biomedical and pharmaceutical applications (34), including gene delivery (23–24,31,35–40). Gene delivery using soluble biodegradable polycations has had only limited success with a few exceptions (23–24,40). In many instances, where low cytotoxicity was achieved, transfection efficiency was far inferior to the 25-kDa PEI (36–39); in others, low or moderate cytotoxicity with moderate efficiency has been reported (31,35). Even for systems with relatively high *in vitro* efficiency (23–24,40), their potency in the presence of serum or *in vivo* performance are not known.

Herein we report that with certain combinations of cross-linkers and PEIs, the gene delivery efficiency of small PEIs can be enhanced up to 550-fold *in vitro* and 80-fold *in vivo*. Furthermore, the efficiency of such conjugates *in vitro* is an order of magnitude higher, and *in vivo* is up to twice as high, than that of the 25-kDa PEI. Importantly, at a concentration of the latter polycation that resulted in >95% cell death, the cross-linked PEIs maintained 95% cell viability.

MATERIALS AND METHODS

Chemicals

All reagents and solvents used were of the highest grades available from commercial sources. Branched 25- and 2-kDa PEIs and the linear 423-Da PEI were obtained from Sigma-Aldrich (St. Louis, MO, USA). EGS (ethylene glycol bis[succinimidylsuccinate]) and DSS (disuccinimidyl suberate) were purchased from Pierce Biotechnology Inc. (Rockford, IL, USA).

Synthesis of Cross-linked PEIs

One-half gram (11.6 mmol in PEI; monomer M_W = 43) of 2-kDa PEI or its 1:1 (w/w) mixture with the linear 423-Da PEI were dissolved in 4 ml of 50% (v/v) aqueous dimethyl sulfoxide (DMSO). One-quarter mmol of DSS (92 mg) or EGS (114 mg) dissolved in 1 ml of DMSO was added to the above-referenced solutions of PEI at room temperature with stirring. The resulting reaction mixture was incubated at 37°C in an incubator shaker for 12 h. The total volume of the reaction mixture was then made up to 10 ml with DMSO in each case, the incubation was continued for another 36 h, and the cross-linked products were then stored at 4°C. The products were purified by dialysis against water (MW cutoff of 500 Da), lyophilized, and confirmed by NMR (in D_2O, 300 MHz). The extent of functionalization of PEI, estimated from the ratio of the -CH_2-CH_2-NH(H)- signals (2.5–2.8 ppm, multiplet) to -CH_2-CH_2-N(H)CO- signals (3.3 ppm, broad singlet), was consistent with the molar ratio of PEI/cross-linker used for reactions in each case.

Capillary Viscometry

PEI solutions were prepared in 500 mM NaCl, and the time required for the solution ($t_{solution}$) and the solvent ($t_{solvent}$) to flow through the capillary of an Ostwald capillary viscometer under atmospheric pressure was recorded. These values were used to calculate the inherent viscocity (η_{inh}) = ln η_{rel}/c, where $\eta_{rel} = t_{solution}/t_{solvent}$ and c is the concentration of PEI. The Mark-Houwink parameters K and *a* (40,41) were determined using PEI standards of known molecular weights.

Plasmid

gWiz Beta-Gal (8278 bp) encoding the β-galactosidase gene was purchased from Aldevron (Fargo, ND, USA). This plasmid, containing the β-galactosidase gene under the control of a modified promoter from the cytomegalovirus (CMV) immediate early gene, was obtained in a ready-to-use form as a 5.0 mg/ml stock solution in water.

Cell Culture and Transfection

COS-7 cells (SV40-transformed kidney cells of an African green monkey) were kindly provided by Kris Holley (MIT, Cambridge, MA, USA) and were cultured in DMEM (American Type Culture Collection, ATCC) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-Invitogen Corp., Greenland, NY, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Sigma-Aldrich). Cells were grown at 37°C in a humidified air atmosphere containing 5% CO_2 and passaged every 3–4 days. The cells (1.5×10^5/well) were plated on Costar 6-well tissue culture clusters 24 h before transfection.

The plasmid DNA (gWiz Beta-Gal) in the amount of 1.25 μg per well was used for transfection. Quantities of plasmid and PEIs given below correspond to experiments done in triplicate. The ratios of PEI and DNA in the polyplexes (PEI/DNA complexes) are expressed as N/P, defined as the ratio of PEI's nitrogens/DNA's nucleotide phosphate. The polyplexes were prepared by adding appropriate amounts of the polycation in 75 μl of water to 3.75 μg of plasmid DNA in 75 μl of

20 mM phosphate buffered saline (PBS), followed by pipette mixing. The resulting solutions were incubated at room temperature for 20 min and then diluted to 3 ml with DMEM containing 10% fetal bovine serum (FBS) immediately prior to the addition of the polyplexes into the cells. The medium was removed from each well, and 1.0 ml/well of the aforementioned transfection medium was added, followed by incubation at 37°C in a humidified air (5% CO_2) atmosphere for 6 h. The transfection medium was then removed, and the cells were further incubated under the same conditions in a complete medium (1.5 ml/well) for 18 h. Thereafter, the medium was removed from each well, and the cells were washed twice with Dulbecco's PBS without $CaCl_2$ and $MgCl_2$ (Sigma-Aldrich). The cells in each well were lysed with 560 μl of Reporter Lysis Buffer (Promega, Baltimore, MD,USA) following the manufacturer's protocol, and the lysates were assayed in a 96-well format for β-galactosidase activity spectrophotometrically by monitoring the absorbance of *o*-nitrophenolate at 420 nm (17,33). The results were expressed as relative β-galactosidase activity/mg protein. Total protein was estimated from the BCA (bicinchoninic acid; Sigma-Aldrich) assay (17,33). The results are expressed as a mean ± SD, n = 3. Small PEIs and the corresponding cross-linked PEIs were compared at each N/P ratio for the calculation of the p values using the program Sigma Plot (Systat Software, Point Richmond, CA, USA).

Cytotoxicity Measurements

Cells were cultured as outlined above. Solutions of 75 μl containing appropriate amount of PEI in water were diluted to 150 μl with 20 mM PBS and then to 3 ml with DMEM containing 10% FBS. The medium was removed from wells, and the above-referenced PEI solutions were added at 1 ml/well. Control cells were treated the same way except that PEI was omitted. After incubation at 37°C in a humidified air atmosphere (5% CO_2) for 6 h, PEI-containing medium was replaced with complete medium. Cytotoxicities were evaluated by measuring the metabolic activity of the cells 18 h later using the MTT assay (17,33). Briefly, the culture medium was removed, and the cells were treated with 1.0 ml of DMEM containing 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich]. After incubation at 37°C in a humidified air atmosphere (5% CO_2) for 4 h, the medium was removed and 560 μl of DMSO was added to each well to dissolve the formazan crystals produced from the reduction of MTT by viable cells. After an overnight incubation, the absorbance of the DMSO solution from each well was measured at 570 nm in a 96-well format following appropriate dilutions. The results were expressed as percentages relative to control cells (mean ± SD, n = 3).

Particle Size Measurements

Polyplex sizes were measured by dynamic light scattering using a Brookhaven ZetaPALS system equipped with a particle sizer (Brookhaven Instruments Corporation, Holtsville, NY, USA). Correlation functions were collected at a scattering angle of 90° (50 mW laser, incident beam = 676 nm), and polyplex sizes were calculated using the MAS option of the manufacturer's particle sizing software (version 2.30). Viscocity and refractive index of pure water at 25°C were used for the analysis. Polyplex sizes are expressed as effective diameters (based on intensity weighted analysis) assuming a log-normal distribution. Complexes were prepared as in transfection experiments, except that the amounts of PEI and DNA were scaled up to a total volume of 1.0 ml of polyplexes from 150 μl. Specifically, appropriate amounts of PEI (for each N/P ratio) in 500 μl of water were added to 25 μg of DNA in 500 μl of 20 mM PBS. This procedure was followed by pipette mixing and incubation at room temperature for 20 min, and the polyplexes were then subjected to light scattering experiments. The results were expressed as a mean ± SD, n = 3. Polyplexes of the parent small PEIs and the corresponding cross-linked PEIs were compared at each N/P ratio for the calculation of the p values.

Gene Delivery in Mice

pCMV-luc DNA (Promega) in water, mixed with the PEIs in water at indicated N/P ratios, were incubated at room temperature for 15 min. Fifty microliters of the mixture containing 30 μg of DNA were administered into the lungs of 8-week-old male C57BL/6 mice (Taconic Farms, Germantown, NY, USA) using an Intrapulmonary Aerosolizer (Penn-Century, Philadelphia, PA, USA). Twenty-four hours after pCMV-luc DNA administration, lungs were harvested and homogenized in 300 μl of Cell Lysis Buffer (Marker Gene Technologies, Eugene, OR, USA). All animal research adhered to the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985). Twenty microliters of the lysates were mixed with 100 μl of the Luciferase Assay System (Promega), and the luminescence was measured using an Optocomp I luminometer (MGM Instruments, Hamden, CT, USA). Protein concentrations were determined using the BCA assay. The results were expressed as a mean ± SD, n = 3. Small PEIs and the corresponding cross-linked PEIs were compared for the calculation of the p values.

RESULTS AND DISCUSSION

Low efficiency compared to viral vectors, as well as toxicity (8,9,20,21,42), are among the major hurdles to the clinical utility of polycationic gene delivery vectors. Their toxicity arises at both the cellular and organ levels. A plausible reason for cellular toxicity is the interaction of polycations with anionic macromolecules in the cell, such as RNA, genomic DNA, and even anionic proteins, thus impeding the latter's normal cellular functions. In fact, the mechanism of unpacking of polycation-DNA complexes is obscure and may involve a polyion exchange reaction with these polyanions. Such reactions would be particularly deleterious in the case of synthetic polymers, such as PEI, as there are no known pathways for their metabolism. At the organ level, the kidney can successfully eliminate molecules only if their molecular weight is below 30 kDa. (36).

Based on the foregoing knowledge, cross-linking of nontoxic small PEIs using potentially degradable linkages was conceived by us as a way of increasing their molecular weight while maintaining the proton sponge capacity and nontoxic nature. An increase in the molecular weight was expected to raise the polycation's DNA-condensation ability, resulting in the formation of more compact complexes with narrow size distribution. This effect, in turn, should facilitate their cellular uptake and afford higher transfection efficiencies, while po-

tentially degradable linkages were expected to maintain low toxicity.

DSS and EGS that result in amide and amide/ester linkages upon reaction with PEI were chosen as the cross-linking agents (Fig. 1). Cross-linking reactions were carried out in aqueous-organic media to ensure that the products are water-soluble. Reaction conditions, such as solvent and temperature, nature of cross-linking agents, cross-linker/PEI molar ratio, and dilution, were expected to affect the molecular weight of the products and hence their biological performance. It is worth noting that poly(ethylene glycol)–cross-linked small PEIs synthesized in organic solvents were reported to have low water solubility and only a modest, some 3-fold, enhancement in efficiency compared to the parent (37).

Capillary viscometry was used to ascertain increase in average molecular weights upon cross-linking. The molecular weights of 2-kDa PEI and its 1:1 (w/w) mixtures with linear 423-Da PEI cross-linked with EGS and DSS were determined to be 8.77, 2.44, 7.94, and 2.52 kDa, respectively. The cross-linked mixtures were expectedly weighted toward lower values due to a 4.7-fold molar excess of the 423-Da PEI over the 2-kDa one.

The cross-linked PEIs were first tested for their *in vitro* transfection efficiency and found to be some 40 to 550 times superior to the respective parent PEIs at the same concentrations (Fig. 2). The optimal N/P ratios, defined as those exhibiting the highest transfection efficiency, were consistent with the molecular weights of the cross-linked PEIs and the nature of the linkages. For example, DSS–cross-linked 2-kDa PEI, with its amide linkage, was expected to be more stable to hydrolytic degradation under physiologic conditions than that cross-linked with EGS. Note that hydrolytic degradation of poly(amino esters) under physiologic conditions has been reported previously (36,39–40). The optimal N/P ratios of the polyplexes of the 2-kDa PEI cross-linked with DSS and EGS were 10 and 30, respectively. Higher biodegradability implies that a portion of the polycation might get degraded in the extra-/intra- cellular environment, thus necessitating a higher concentration of the polycation to achieve optimal efficiency. Interestingly, the optimal efficiency of 2-kDa PEI cross-linked with DSS was some 1/30 of that cross-linked with EGS, probably because higher hydrolytic degradability of the EGS-based conjugate facilitates the release of DNA (vector unpacking) and hence enhances the transcriptional availability of DNA (43). Similarly, in the case of the smaller, hybrid cross-linked PEIs, the polycation with a higher hydrolytic stability (DSS-based) exhibited a lower optimal N/P ratio than the more labile (EGS-based) one: 30 vs. 50. The optimal efficiency of the two cross-linked PEIs in this case differed only by 3-fold.

Light scattering experiments were used to gain insights into the difference in transfection efficiency between the cross-linked and parent PEIs, as well as between the different cross-linked PEIs. Consistent with the increase in transfection efficiency, the polyplexes of all of the cross-linked PEIs were smaller than their parents at the N/P ratios of both 10 and 30 (Table I). Also, at N/P = 10, 2-kDa PEI cross-linked with DSS and EGS exhibited lower mean diameters (527 and 495 nm, respectively) than the two cross-linked hybrid PEIs (675 and 683 nm, respectively), consistent with the former's higher molecular weight. Moreover, increasing the N/P ratio to 30 resulted in a 100–200 nm reduction in the mean diameter of all the PEIs (Table I). The mean sizes of the parent small PEIs were still high, above 700 nm, and these complexes were also characterized by a wide distribution of particle sizes (Table I and Fig. 3). In contrast, DNA complexes of the DSS–cross-linked hybrid and EGS–cross-linked 2-kDa PEI, the two polycations that afforded the highest transfection efficiencies, had a roughly 300-nm mean particle diameter and a narrow size distribution (Table I and Fig. 3). All the particles were under 400 nm in diameter in both instances. The EGS–cross-linked hybrid had a higher mean diameter (502 nm) at N/P = 30, presumably explaining the requirement of higher N/P (= 50) for optimal efficiency in this case. The DSS–cross-linked 2-kDa PEI, whose optimal N/P ratio was 10, also exhibited much smaller mean particle diameter and narrow size distribution at N/P = 30 compared to 10. The inferior efficiency of the smaller particles in this case is likely due to the low release of DNA from the tightly compacted complexes (less facile vector unpackaging). Thus, particle size measurements provided an independent explanation for the results of *in vitro* transfection experiments.

It is noteworthy that the cross-linked PEIs described herein, although larger than their parents, still have relatively low molecular weights. Nevertheless, despite their relatively low molecular weight (e.g., compared to the 25-kDa), they afford high transfection efficiency. The lower limit for

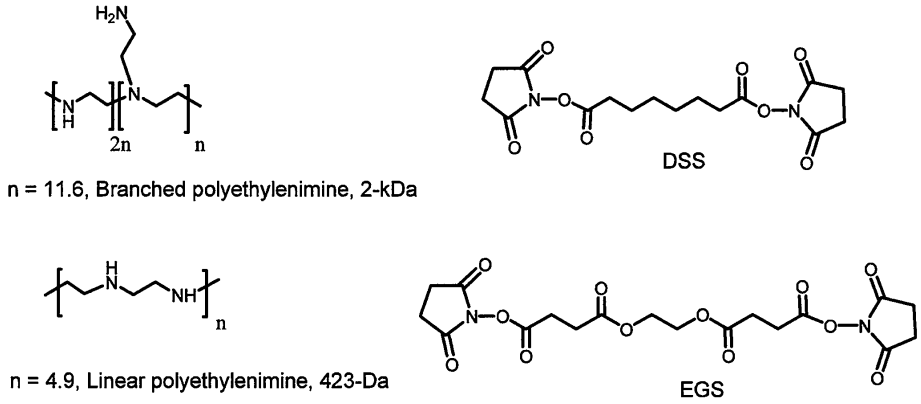

Fig. 1. Chemical structures of the small polyethylenimines (PEIs) and of the cross-linking agents used in this study.

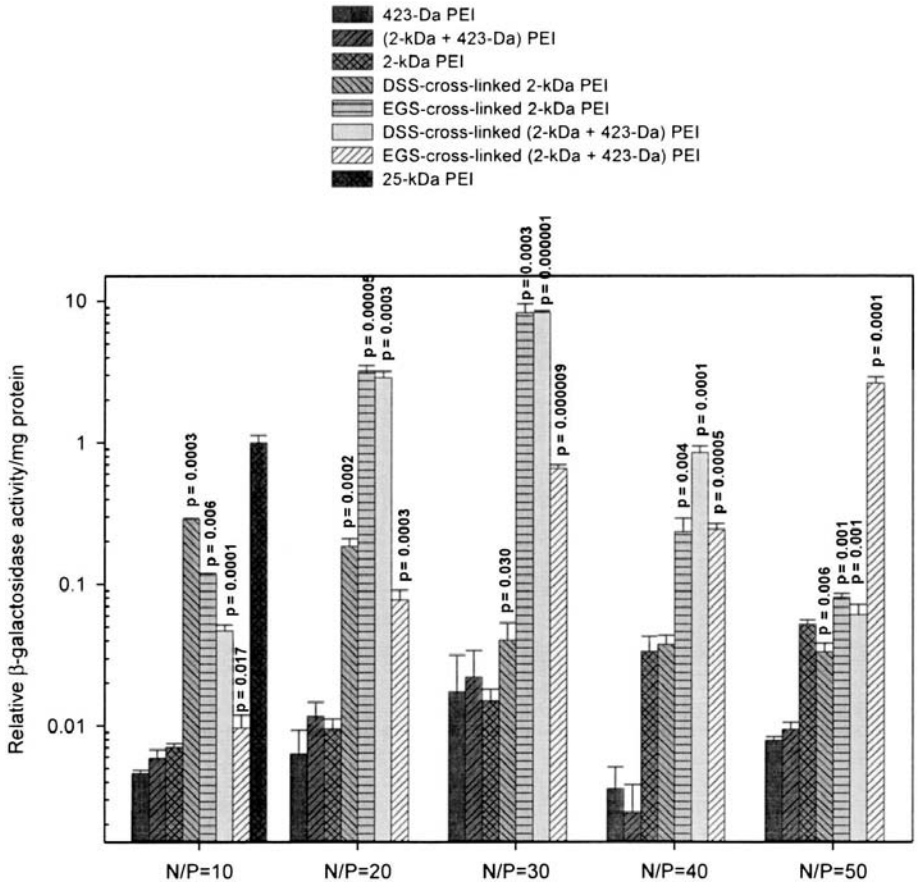

Fig. 2. Expression in COS-7 cell culture of β-galactosidase gene mediated in the presence of serum by the unmodified small PEIs, the branched 25-kDa PEI, and the cross-linked small PEIs. The error bars represent SD, n = 3.

the latter in terms of molecular weight and the concentration required for optimal efficiency (defined by N/P) may be expected to vary for different classes of polycations. For example, dodecylated 2-kDa PEI synthesized by us previously was some 400-fold more efficient than the parent even though the average molecular weight had increased by only about 800 Da and the optimal N/P in this case was 20. For the unmodified 2-kDa PEI, the optimal N/P is around 150 (33). These results, in combination with those of the current experiments, suggest that the efficiency and the N/P ratio required for optimal efficiency are decided by a combination of molecular weight, hydrophobicity, and degradability of the polycations. In general, lower optimal N/P ratios are indicative of the greater DNA condensation ability of the polycations and thus are suitable candidates for *in vivo* gene delivery.

The results of the cytotoxicity experiments involving the

Table I. Comparison of the Effective Diameter and Size Distribution[a] of the Polyplexes of the Unmodified Small PEIs, the Branched 25-kDa PEI, and the Cross-linked Small PEIs

PEI	Diameter (nm) (N/P = 10)	Half-width	Diameter (nm) (N/P = 30)	Half-width
25-kDa	472 ± 32	33 ± 2	207 ± 5	31 ± 28
2-kDa + 423-Da	899 ± 44	283 ± 82	745 ± 22	305 ± 12
2-kDa	810 ± 91	430 ± 105	714 ± 47	291 ± 30
EGS–cross-linked (2-kDa + 423-Da)	675 ± 7 (p = 0.001)	222 ± 12	502 ± 22 (p = 0.0002)	154 ± 105
DSS–cross-linked (2-kDa + 423-Da)	683 ± 46 (p = 0.0005)	172 ± 47	352 ± 6 (p = 0.00001)	25 ± 0.4
EGS–cross-linked 2-kDa	495 ± 28 (p = 0.005)	76 ± 67	299 ± 8 (p = 0.0001)	53 ± 55
DSS–cross-linked 2-kDa	527 ± 21 (p = 0.007)	205 ± 59	174 ± 3 (p = 0.00004)	24 ± 19

PEI, polyethylenimine; EGS, (ethylene glycol bis[succinimidylsuccinate]); DSS, disuccinimidyl suberate.
[a] Defined by the half-width of the size distribution plots, see Fig. 3.
The results are represented as mean ± SD, n = 3.

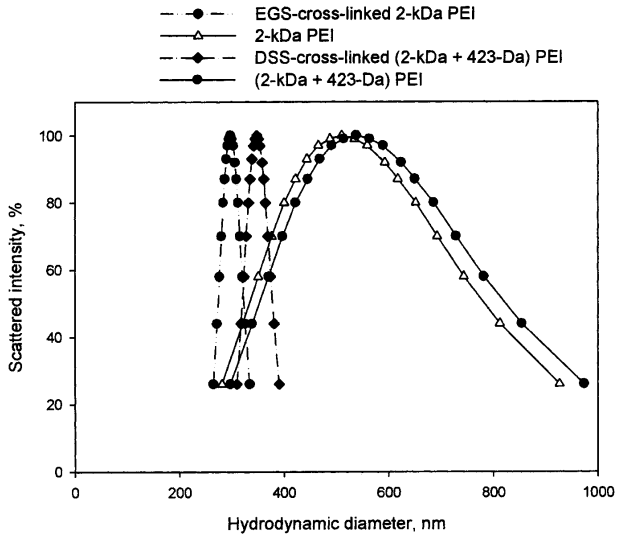

Fig. 3. Comparison of the effective diameter and of the size distributions of the polyplexes of the most efficient cross-linked PEIs and those of the respective parents. N/P = 30 in all the cases. Representation plots are presented, see Table I for SD.

PEIs are presented in Fig. 4. The parents, small PEIs, were nontoxic even at the highest concentrations used. In contrast, 25-kDa PEI was highly toxic, with cell viability dropping more than half at concentrations above 20 μg/ml and to under 5% above 35 μg/ml PEI. By comparison, all the cross-linked PEIs were far more benign. In fact, the EGS–cross-linked PEIs were as nontoxic as the parent small PEIs. Of the two DSS–cross-linked PEIs, the 2-kDa one was more toxic than the hybrid. As in transfection experiments, the toxicity profiles of the cross-linked PEIs were consistent with their relative degradability and molecular weights. Importantly, the most potent cross-linked PEIs, which are an order of magnitude more efficient than the 25-kDa polycation, exhibited 100% or 95%

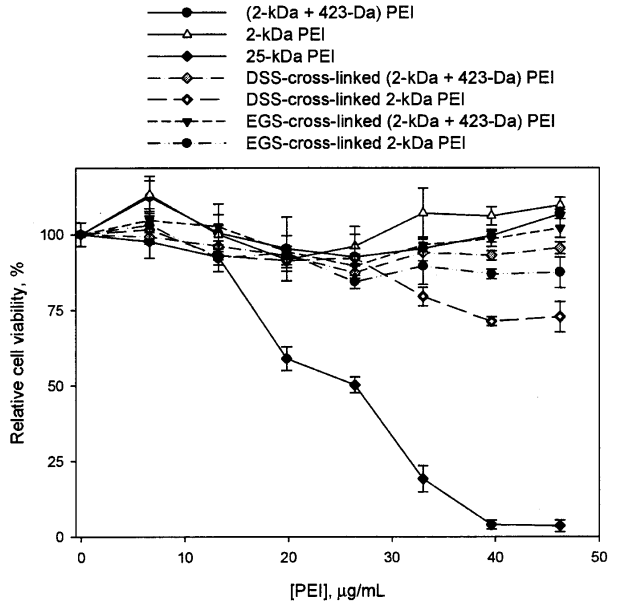

Fig. 4. Comparison of the cytotoxicities induced by the unmodified small PEIs, the branched 25-kDa PEI, and the cross-linked small PEIs to COS-7 cells measured by the MTT assay. The error bars represent SD, n = 3.

cell viability at concentrations where 25-kDa resulted in >95% cell death.

The most potent cross-linked PEIs were next tested for their efficiency in gene delivery into mouse lung through intratracheal administration. Lung is the affected organ in the case of many serious and potentially fatal diseases, such as cystic fibrosis, lung cancer, influenza, and severe acute respiratory syndrome, and therefore serves as the target for their treatment by gene delivery. The first two diseases, e.g., may be treated by delivering plasmid DNA containing the cystic fibrosis transmembrane conductance gene (CFTR gene) and the tumor suppressor p53 gene, respectively (1,44,45). The last two diseases may be treated by the delivery of siRNA against specific genes of the viruses (6,46). Intratracheal administration has the advantage over the systemic one in that the delivered nucleic acid is primarily expressed in the lungs, thus avoiding unwanted expression in other organs. However, several barriers to nonviral gene delivery to the airway exist (47). For example, mucins (high-molecular-weight glycosylated proteins) and lung sufactants impede cationic liposome–mediated transfection (47), possibly by disrupting the DNA/lipid complex.

The most potent polycations created by us, EGS–cross-linked 2-kDa PEI and DSS–cross-linked small PEI hybrid, were initially tested at N/P = 20 to 40 for their efficiency to deliver DNA. Complexes formed at N/P of 20 and 30 were found to be most effective (Fig. 5): 17 and 80 times above those of the parent PEIs, the 2-kDa and its equal mixture with the 423-Da polycation, respectively. Furthermore, EGS–cross-linked 2-kDa PEI was as effective as 25-kDa PEI *in vivo,* and the DSS–cross-linked hybrid was 2.5-fold more potent.

In conclusion, by employing a properly selected combination of PEIs and potentially biodegradable cross-linking agents, both the *in vitro* and *in vivo* gene delivery by small PEIs was enhanced by up to 550- and 80-fold, respectively, without increasing cytotoxicity. The methodology described herein may easily be adapted to high-throughput synthesis

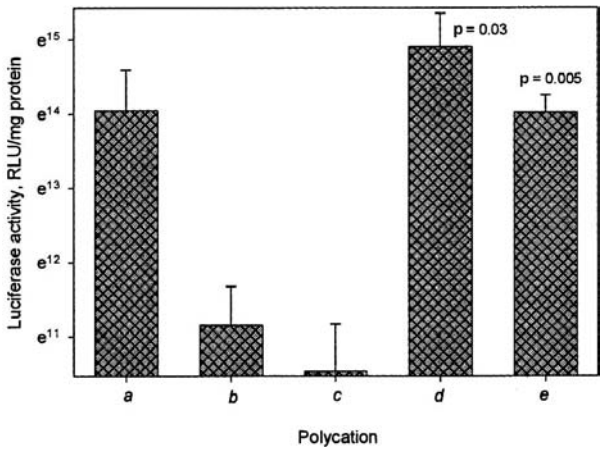

Fig. 5. Comparison of the *in vivo* gene delivery efficiency of a luciferase-encoding plasmid mediated by the 25-kDa PEI, unmodified small PEIs, and the cross-linked small PEIs. (a) 25-kDa, N/P = 10; (b) 2-kDa, N/P = 20; (c) (2-kDa + 423-Da), N/P = 30; (d) DSS–cross-linked (2-kDa + 423-Da), N/P = 30; (e) EGS–cross-linked 2-kDa, N/P = 20. Polyplexes were introduced intratracheally into mice using an intrapulmonary aerosolizer. The error bars represent SD, n = 3.

and screening of cross-linked small PEI libraries to identify polycations with even more superior *in vitro,* and eventually *in vivo,* performance.

ACKNOWLEDGMENTS

This work was financially supported by NIH grant GM26698 (to A. M. K.) and by NSF though the Biotechnology Process Engineering Center at MIT, as well as by NIH grants AI50631 and AI56267 (to J. C.). J. J. L. thanks the National Science and Engineering Research Council of Canada (NSERC) for a Postdoctoral Fellowship.

REFERENCES

1. L. M. Schwiebert. Cystic fibrosis, gene therapy, and lung inflammation: for better or worse? *Am. J. Physiol.* **286**:L715–L716 (2004).
2. H. S. Kingdon and R. L. Lundblad. An adventure in biotechnology: the development of haemophilia A therapeutics - from whole-blood transfusion to recombinant DNA to gene therapy. *Biotechnol. Appl. Biochem.* **35**:141–148 (2002).
3. C. J. Kuo, F. Farnebo, E. Y. Yu, R. Christofferson, R. A. Swearingen, R. Carter, H. A. von Recum, J. Yuan, J. Kamihara, E. Flynn, R. D'Amato, J. Folkman, and R. C. Mulligan. Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. *Proc. Natl. Acad. Sci. USA* **98**: 4605–4610 (2001).
4. C.-H. Lecellier and O. Voinnet. RNA silencing: no mercy for viruses? *Immunol. Revs.* **198**:285–303 (2004).
5. A. J. Frater, S. J. Fidler, and M. O. McClure. Gene therapy for AIDS and other infectious diseases. *Gene Ther.* **9**:189–213 (2002).
6. Q. Ge, L. Filip, A. Bai, N. Tam, H. N. Eisen, and J. Chen. Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl. Acad. Sci. USA* **101**:8676–8681 (2004).
7. Gene therapy clinical trials worldwide provided by the Journal of Gene Medicine. http://www.wiley.co.uk/genmed/clinical/.
8. M. E. Davis. Non-viral gene delivery systems. *Curr. Opin. Biotechnol.* **13**:128–131 (2002).
9. M. Thomas and A. M. Klibanov. Non-viral gene therapy: polycation-mediated DNA delivery. *Appl. Microbiol. Biotechnol.* **62**: 27–34 (2003).
10. C. M. Wiethoff and R. C. Middaugh. Barriers to nonviral gene delivery. *J. Pharm. Sci.* **92**:203–217 (2003).
11. E. Check. Gene therapy: a tragic setback. *Nature* **420**:116–118 (2002).
12. S. Hacein-Bey-Abina, C. Von Kalle, M. Schmidt, M. P. McCormack, N. Wulffraat, P. Leboulch, A. Lim, C. S. Osborne, R. Pawliuk, E. Morillon, R. Sorensen, A. Forster, P. Fraser, J.I. Cohen, G. de Saint Basile, I. Alexander, U. Wintergerst, T. Frebourg, A. Aurias, D. Stoppa-Lyonnet, S. Romana, I. Radford-Weiss, F. Gross, F. Valensi, E. Delabesse, E. Macintyre, F. Siqaux, J. Soulier, L. E. Leiva, M. Wissler, C. Prinz, T. H. Rabbitts, F. Le Deist, A. Fischer, and M. Cavazzana-Calvo. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**:415–419 (2003).
13. N. Boyce. Trial halted after gene shows up in semen. *Nature* **414**:677–678 (2001).
14. G. Y. Wu and C. H. Wu. Receptor-mediated gene delivery and expression *in vivo*. *J. Biol. Chem.* **263**:14621–14624 (1988).
15. O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, and 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**:7297–7301 (1995).
16. W. T. Godbey, K. K. Wu, and A. G. Mikos. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc. Natl. Acad. Sci. USA* **96**:5177–5181 (1999).
17. M. Thomas and A. M. Klibanov. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**:14640–14645 (2002).
18. J. Suh, D. Wirtz, and J. Hanes. Efficient active transport of gene nanocarriers to the cell nucleus. *Proc. Natl. Acad. Sci. USA* **100**: 3878–3882 (2003).
19. N. D. Sonawane, F. C. Szoka Jr., and A. S. Verkman. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem.* **278**:44826–44831 (2003).
20. E. Wagner. Strategies to improve DNA polyplexes for *in vivo* gene transfer: will "artificial viruses" be the answer? *Pharm. Res.* **21**:8–14 (2004).
21. A. Kirchler. Gene transfer with modified polyethylenimines. *J. Gene Med.* **6**:S3–S10 (2004).
22. M. L. Forrest, G. E. Meister, J. T. Koerber, and D. W. Pack. Partial acetylation of polyethylenimine enhances *in vitro* gene delivery. *Pharm. Res.* **21**:365–371 (2004).
23. Y.-B. Lim, S.-M. Kim, H. Suh, and J.-S. Park. Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and non-toxic gene delivery carrier. *Bioconjug. Chem.* **13**:952–957 (2002).
24. A. Akinc, D. M. Lynn, D. G. Anderson, and R. Langer. Parallel synthesis and characterization of a degradable polymer library for gene delivery. *J. Am. Chem. Soc.* **125**:5316–5323 (2003).
25. A. Kichler, C. Leborgne, J. Marz, O. Danos, and B. Bechinger. Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proc. Natl. Acad. Sci. USA* **100**:1564–1568 (2003).
26. Y. Liu, L. Wenning, M. Linch, and T. M. Reineke. New poly(D-glucaramidoamine)s induce DNA nanoparticle formation and efficient gene delivery into mammalian cells. *J. Am. Chem. Soc.* **126**:7422–7423 (2004).
27. S.-O. Han, R. I. Mahato, and S. W. Kim. Water-soluble lipopolymer for gene delivery. *Bioconjug. Chem.* **12**:337–345 (2001).
28. S. Kim, J. S. Choi, H. S. Jang, H. Suh, and J. Park. Hydrophobic modification of polyethyleneimine for gene transfectants. *Bull. Korean Chem. Soc* **22**:1069–1075 (2001).
29. C. L. Gebhart, S. Sriadibhatla, S. Vinogradov, P. Lemieux, V. Alakhov, and A. V. Kabanov. Design and formulation of polyplexes based on pluronic-polyethylenimine conjugates for gene transfer. *Bioconjug. Chem.* **13**:937–944 (2002).
30. N. Oku, Y. Yamazaki, M. Matsuura, M. Sugiyama, M. Hasegawa, and M. Nango. A novel non-viral gene transfer system, polycation liposomes. *Adv. Drug Deliv. Revs.* **52**:209–218 (2001).
31. M. A. Gosselin, W. Guo, and R. J. Lee. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjug. Chem.* **12**:989–994 (2001).
32. H. Petersen, K. Kunath, A. L. Martin, S. Stolnik, C. J. Roberts, M. C. Davies, and T. Kissel. Star-shaped poly(ethylene glycol)-block-polyethylenimine copolymers enhance DNA condensation of low molecular weight polyethylenimines. *Biomacromolecules* **3**:926–936 (2002).
33. M. Thomas and A. M. Klibanov. Conjugation to gold nanoparticles enhances polyethylenimine's transfer of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. USA* **100**:9138–9143 (2003).
34. J. Panyam and V. Labhasetwar. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv. Drug Deliv. Rev.* **55**:329–347 (2003).
35. J. Wang, H.-Q. Mao, and K. W. Leong. A novel biodegradable gene carrier based on polyphosphoester. *J. Am. Chem. Soc.* **123**: 9480–9481 (2001).
36. H. Peterson, T. Merdan, K. Kunath, D. Fisher, and T. Kissel. Poly(ethylenimine-co-L-lactamide-co-succinimide): a biodegradable polyethylenimine derivative with an advantageous pH-dependent hydrolytic degradation for gene delivery. *Bioconjug. Chem.* **13**:812–821 (2002).
37. C.-H. Ahn, S. Y. Chae, Y. H. Bae, and S. W. Kim. Biodegradable poly(ethylenimine) for plasmid DNA delivery. *J. Control. Rel.* **80**:273–282 (2002).
38. Y.-B. Lim, S.-O. Han, H.-U. Kong, Y. Lee, J.-S. Park, B. Jeong, and S. W. Kim. Biodegradable polyester, poly[(α-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. *Pharm. Res.* **17**:811–816 (2000).
39. Y.-B. Lim, Y. H. Choi, and J.-S. Park. A self-destroying polycationic polymer: biodegradable poly(4-hydroxy-L-proline ester). *J. Am. Chem. Soc.* **121**:5633–5639 (1999).
40. M. L. Forrest, J. T. Koerber, and D. W. Pack. A degradable

polyethylenimine derivative with low toxicity for highly efficient gene delivery. *Bioconjug. Chem.* **14**:934–940 (2003).
41. A. von Harpe, H. Petersen, Y. Li, and T. Kissel. Characterization of commercially available and synthesized polyethylenimines for gene delivery. *J. Control. Rel.* **69**:309–322 (2000).
42. Y. Tan and L. Huang. Overcoming the inflammatory toxicity of cationic gene vectors. *J. Drug Target* **10**:153–160 (2002).
43. D. V. Schaffer, N. A. Fidelman, N. Dan, and D. A. Lauffenburger. Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnol. Bioeng.* **67**:598–606 (2000).
44. C. Moon, Y. Oh, and J. A. Roth. Current status of gene therapy for lung cancer and head and neck cancer. *Clin. Cancer Res.* **9**:5055–5067 (2003).
45. A. C. Willis and X. Chen. The promise and obstacle of p53 as a cancer therapeutic agent. *Curr. Mol. Med.* **2**:329–345 (2002).
46. Y. Zhang, T. Li, L. Fu, C. Yu, Y. Li, X. Xu, Y. Wang, H. Ning, S. Zhang, W. Chen, L. A. Babiuk, and Z. Chang. Silencing SARS-CoV spike protein expression in cultured cells by RNA interference. *FEBS Lett.* **560**:141–146 (2004).
47. D. J. Weiss. Delivery of gene transfer vectors to lung: obstacles and the role of adjunct techniques for airway administration. *Mol. Ther.* **6**:148–152 (2002).