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Research Paper

Identification of Novel Superior Polycationic Vectors for Gene Delivery by High-throughput Synthesis and Screening of a Combinatorial Library

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Purpose. Low efficiency and toxicity are two major drawbacks of current non-viral gene delivery vectors. Since DNA delivery to mammalian cells is a multi-step process, generating and searching combinatorial libraries of vectors employing high-throughput synthesis and screening methods is an attractive strategy for the development of new improved vectors because it increases the chance of identifying the most overall optimized vectors.

Materials and Methods. Based on the rationale that increasing the effective molecular weight of small PEIs, which are poor vectors compared to the higher molecular weight homologues but less toxic, raises their transfection efficiency due to better DNA binding, we synthesized a library of 144 biodegradable derivatives from two small PEIs and 24 bi- and oligo-acrylate esters. A 423-Da linear PEI and its 1:1 (w/w) mixture with a 1.8-kDa branched PEI were cross-linked with the acrylates at three molar ratios in DMSO. The resulting polymers were screened for their efficiency in delivering a β-galactosidase expressing plasmid to COS-7 monkey kidney cells. Selected most potent polymers from the initial screen were tested for toxicity in A549 human lung cancer cells, and *in vivo* in a systemic gene delivery model in mice employing a firefly luciferase expressing plasmid.

Results. Several polycations that exhibited high potency and low toxicity *in vitro* were identified from the library. The most potent derivative of the linear 423-Da PEI was that cross-linked with tricycle-[5.2.1.0]-decane-dimethanol diacrylate (diacrylate **14**), which exhibited an over 3,600-fold enhancement in efficiency over the parent. The most potent mixed PEI was that cross-linked with ethylene glycol diacrylate (diacrylate **4**) which was over 850-fold more efficient than the physically mixed parent PEIs. The relative efficiencies of these polymers were even up to over twice as high as that of the linear 22-kDa PEI, considered the "gold standard" for *in vitro* and systemic gene delivery. The potent cross-linked polycations identified were also less toxic than the 22-kDa PEI. The optimal vector *in vivo* was the mixed PEI cross-linked with propylene glycol glycerolate diacrylate (diacrylate **7**); it mediated the highest gene expression in the lungs, followed by the spleen, with the expression in the former being 53-fold higher compared to the latter. In contrast, the parent PEIs mediated no gene expression at all under similar conditions, and injection of the polyplexes of the 22-kDa PEI at its optimal N/P of 10 prepared under identical conditions killed half of the mice injected.

Conclusions. High-throughput synthesis and transfection assay of a cross-linked library of biodegradable PEIs was proven effective in identifying highly transfecting vectors. The identified vectors exhibited dramatically superior efficiency compared to their parents both *in vitro* and in an *in vivo* systemic gene delivery model. The majority of these vectors mediated preferential gene delivery to the lung, and their *in vivo* toxicity paralleled that *in vitro*.

KEY WORDS: acrylates; cross-linking; cytotoxicity; gene delivery; organ specificity; polyethylenimine; systemic delivery.

INTRODUCTION

Medical interventions based on nucleic acids promise revolutionary advances in the treatment of human diseases. Originally aimed to correct genetic disorders, and thus termed gene therapy, this pharmaceutical strategy has been much broadened in its scope as both DNA and RNA (e.g., siRNA) have emerged as potential therapeutic agents against many inherited and acquired diseases (1–4). However, after more than a thousand clinical trials, no gene therapy protocol has been approved yet by the U.S. Food and Drug Adminis-

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tration. The negative charge, large size, and sensitivity to hydrolytic degradation of nucleic acids pose a major challenge to their delivery; in fact, the primary hurdle to the clinical success of gene therapy has been the absence of carriers (vectors) that can deliver DNA into target tissues and organs safely and efficiently (5–9).

Viral vectors, employed in most clinical trials, are plagued by such problems as immunogenicity (10,11), insertional mutagenesis (12), and even germ cell line alterations (13,14). Therefore, polycations and cationic liposomes have been investigated as alternatives to viral vectors (5,7,15–19). These non-viral vectors offer significant potential advantages, including ease of production, high stability, and low immunogenicity. Unlike recombinant viral vectors, wherein the therapeutic nucleic acid is covalently inserted into the viral genome, non-viral ones interact non-covalently with DNA/siRNA to form nanometer-sized particles readily amenable to endocytosis by mammalian cells. These features allow much flexibility in terms of tailoring the vectors for desired biological effects. Unfortunately, however, low efficiency and cytotoxicity are major drawbacks of existing non-viral vectors (5–9).

Two complementary approaches have been explored to improve non-viral vectors. One is iterative, wherein selected chemical modifications are brought about to affect one or more steps involved in non-viral delivery. Although this approach has contributed to a better understanding and improvement of gene delivery (9,17,20,21), the latter is a multi-step process (22) and hence vector modifications enhancing one step might affect another adversely. As a result, little net gain in efficiency may be achieved in many cases (20), making the iterative process time-consuming. An alternative approach involves making a large number of vectors employing minimal synthetic and purification steps, followed by *in vitro* screening for transfection efficiency in a high-throughput fashion. This emerging combinatorial approach (23–27) has had some early successes, notably the development of β-amino esters which are effective in DNA delivery by direct intra-tumor injection (24,25). Herein we report novel, highly transfecting vectors *in vitro* discovered from a combinatorial library of biodegradable polyethylenimine (PEI) derivatives synthesized by cross-linking small PEIs using high-throughput methods, as well as their application in a systemic gene delivery model in mice.

MATERIALS AND METHODS

Chemicals

All reagents and solvents used were of the highest grades available from commercial sources. The linear 423-Da PEI and all acrylate esters were obtained from Sigma-Aldrich (St. Louis, MO); the branched 1.8-kDa PEI was from Polysciences (Warrington, PA). All acrylates were purchased from Sigma-Aldrich, except for acrylate **5** which was from Polysciences.

Synthesis of Cross-linked PEIs

PEI stock solutions (423-Da PEI or its 1:1 (w/w) mixture with 1.8-kDa PEI) were prepared in DMSO. Then 3.5-ml solutions containing 0.5 g PEI (11.6 mmol; monomer's $M_w = 43$) were aliquoted into glass vials. Acrylate cross-linkers (0.278, 0.556, or 1.11 mmol) dissolved in 2 ml of DMSO were added to the PEI solutions in glass vials at room temperature with stirring using magnetic pellets. The resulting reaction mixtures were sealed and shaken in the dark at 65°C for 48 h, and the cross-linked products were then stored at 4°C. NMR analyses performed on selected samples after precipitation with diethyl ether and subsequent drying (see below) confirmed the complete consumption of the cross-linking agents.

Processing of Cross-linked PEIs for *In Vitro* and *In Vivo* Experiments

For initial *in vitro* screening, the cross-linked PEI stock solutions in DMSO were serially diluted with 0.2 M acetate buffer (pH 5) in a 96-well format and used for complex formation with DNA at different N/P ratios, also in a 96-well format.

After the initial screening, selected promising cross-linked PEIs were purified as follows. One milliliter each of the stock solutions was mixed with 5 ml of diethyl ether. After a few minutes the ether layer was removed, and the residual gummy solids were washed with 4 ml each of diethyl ether twice, air dried, and then freeze-dried. Portions of the freeze-dried products were weighed into glass vials and dissolved in water. The pH values of the resultant solutions were adjusted to 4.5 using 1 M HCl. The concentrations of these stock solutions were 150 mM in PEI monomer units, and were stored at −20°C until use.

Plasmid

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

Cell Culture and Transfection

COS-7 cells (SV40-transformed kidney cells from an African green monkey) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (American Type Culture Collection, ATCC), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO-Invitogen, Greenland, NY) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, both from Sigma-Aldrich). The cells were grown at 37°C in a humidified-air atmosphere containing 5% CO_2 and passaged every 3–4 days; 3×10^4 cells were plated per well in Costar® 96-well tissue culture clusters 24 h before transfection.

gWiz™ Beta-Gal was used for transfections at 300 ng per well. The relative quantities of PEI and DNA in the complexes (polyplexes) were expressed as N/P ratios. All complexes were prepared in a 96-well plate format. The polyplexes were prepared by adding appropriate amounts of the polycation dissolved in 10 μl of acetate buffer (pH 5) or water (pH 4.5) to 300 ng of the plasmid DNA in 10 μl of a 150 mM aqueous NaCl, followed by mixing with a multichannel pipette. The resulting solutions were incubated at room temperature for 20 min and diluted to 200 μl with

DMEM containing 10% FBS immediately prior to their addition onto the cells. Culture medium was removed from each well, and 200-μl aliquots of the aforementioned transfection solutions were added per well, followed by incubation at 37°C in a humidified-air (5% CO_2) atmosphere for 24 h. Thereafter, the medium was removed from each well, and the cells were washed with Dulbecco's PBS without $CaCl_2$ and $MgCl_2$ (Sigma-Aldrich). The cells in each well were lysed with 200 μl of the Reporter Lysis Buffer (Promega, Baltimore, MD) following the manufacturer's protocol, and the lysates were assayed spectrophotometrically in a 96-well format for β-galactosidase activity by monitoring the absorbance of *o*-nitrophenolate at 420 nm (20). The results were expressed as a relative β-galactosidase activity per mg protein. Total protein was estimated by a BCA assay (Sigma-Aldrich; 20). The results were expressed as mean ± SD with $n = 4$.

Cytotoxicity Measurements

A549 cells were plated in 96-well plates 24 h before treating with PEIs. Solutions (20 μl) containing appropriate amounts of PEIs in 150 mM aqueous NaCl were diluted to 200 μl with DMEM containing 10% FBS. The culture medium was removed from the wells, and the PEI solutions were added at 200 μl/well followed by incubation at 37°C in a humidified-air atmosphere (5% CO_2) for 24 h. Control cells were treated the same way, except that PEI was omitted. Cytotoxicities were evaluated by measuring the metabolic activity of the cells using the MTT assay (20). Briefly, the culture medium was removed, and the cells were treated with 200 μl/well 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 200 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. The results were expressed as percentages relative to control cells (mean ± SD, $n = 4$).

Gene Delivery in Mice

All animal experiments adhered to the Principles of Laboratory Animal Care (NIH publication #85-23). To obtain the desired N/P ratios, appropriate volumes of PEI stock solutions were diluted to 500 μl with 5% aqueous glucose, added to equal volumes of the glucose solutions containing 450 μg of the plasmid DNA (gWiz™ Luc), and mixed. The resulting polyplexes were incubated at room temperature for 10 min. Then 6–8 week old Black Swiss male mice (Taconic Farms, Germantown, NY) were injected retroorbitally with 200 μl of the aforementioned polyplexes containing 90 μg of DNA/mouse under anesthesia. After 24 h, the mice were euthanized by CO_2 inhalation; their lungs, kidneys, livers, hearts, and spleens were collected, and suspended in 500 μl of lysis buffer prepared by mixing 4 ml of 5× Passive Lysis Buffer (Promega), 800 μl of 8.7 mg/ml PMSF (Sigma-Aldrich) in methanol, 400 μl of the protease inhibitor cocktail (Sigma-Aldrich), and 14.8 ml of water. The samples were freeze-thawed, homogenized by probe sonication for 20 s, and centrifuged. The 10-μl supernatants (either as such or after dilution with the lysis buffer) were mixed with 50 μl of the Luciferase Assay Reagent (Promega), and the luminescence was measured using an Optocomp I luminometer (MGM Instruments, Hamden, CT; 28). Protein concentrations were determined using the BCA assay. The results were expressed as a mean ± SD, $n = 4$.

RESULTS AND DISCUSSION

Ethylenimine polymers (PEIs) belong to one of the most efficient family of cationic compounds for delivery of plasmid DNA into mammalian cells (29–31). The transfection efficiency of PEIs partially relies on their ability to capture the protons which are transferred into the endosomes during their acidification (31–33). In particular higher molecular weight (≥22-kDa) PEIs are relatively more efficient vectors, but are also toxic (28,34–36). A plausible reason for their cellular toxicity is likely interactions of the polycations with anionic macromolecules in the cells. The molecular mechanism of dissociation of polymer-DNA complexes is poorly understood, and may involve an exchange reaction with these polyanions: it has been suggested that most PEI-DNA polyplexes de-condense in the cytoplasm, presumably because they interact with such polyanionic molecules such as mRNA, phosphatidylserine, or proteoglycans (37). These reactions would be particularly deleterious in the case of such synthetic polymers as PEIs because there are no known pathways for their metabolism. In addition, toxicity also arises as a result of the interaction of PEI with the plasma membrane (38) and also components of the blood (systemic delivery; 39,40). The polycation toxicity must be addressed, especially since high doses and repeated administration would be required in most clinical applications due to (1) a relatively low efficiency of non-viral vectors, and (2) the fact that non-viral gene delivery results in transient gene expression.

Both gene delivery efficiency and cytotoxicity of PEIs greatly vary with their molecular weights. We previously showed that increasing the effective molecular weight of small PEIs boosted their transfection efficiency. These modifications of short-chained PEIs included hydrophobic derivatization (20), conjugation to gold nanoparticles (41), and cross-linking with potentially biodegradable linkages (36). These approaches were conceived to increase the PEI's effective molecular weight, while still maintaining its proton sponge capacity and non-toxic nature. An increase in the molecular weight by cross-linking, for example, raised the polycation's DNA-condensing ability resulting in the formation of more compact complexes with narrower size distribution. This, in turn, afforded much higher transfection efficiencies, that are up to an order of magnitude greater than the 25-kDa branched PEI, while biodegradable linkages ensured low toxicity (36). The latter studies, and also similar studies by other groups employing different small PEIs and cross-linkers (34,42–44) have shown that the transfection efficiency of the cross-linked PEIs compared to their parents depended upon factors such as the molecular weight of the parental small PEIs, the nature of cross-linkers, and PEI/cross-linker ratio. Such an outcome is expected considering the complexity of non-viral gene deliv-

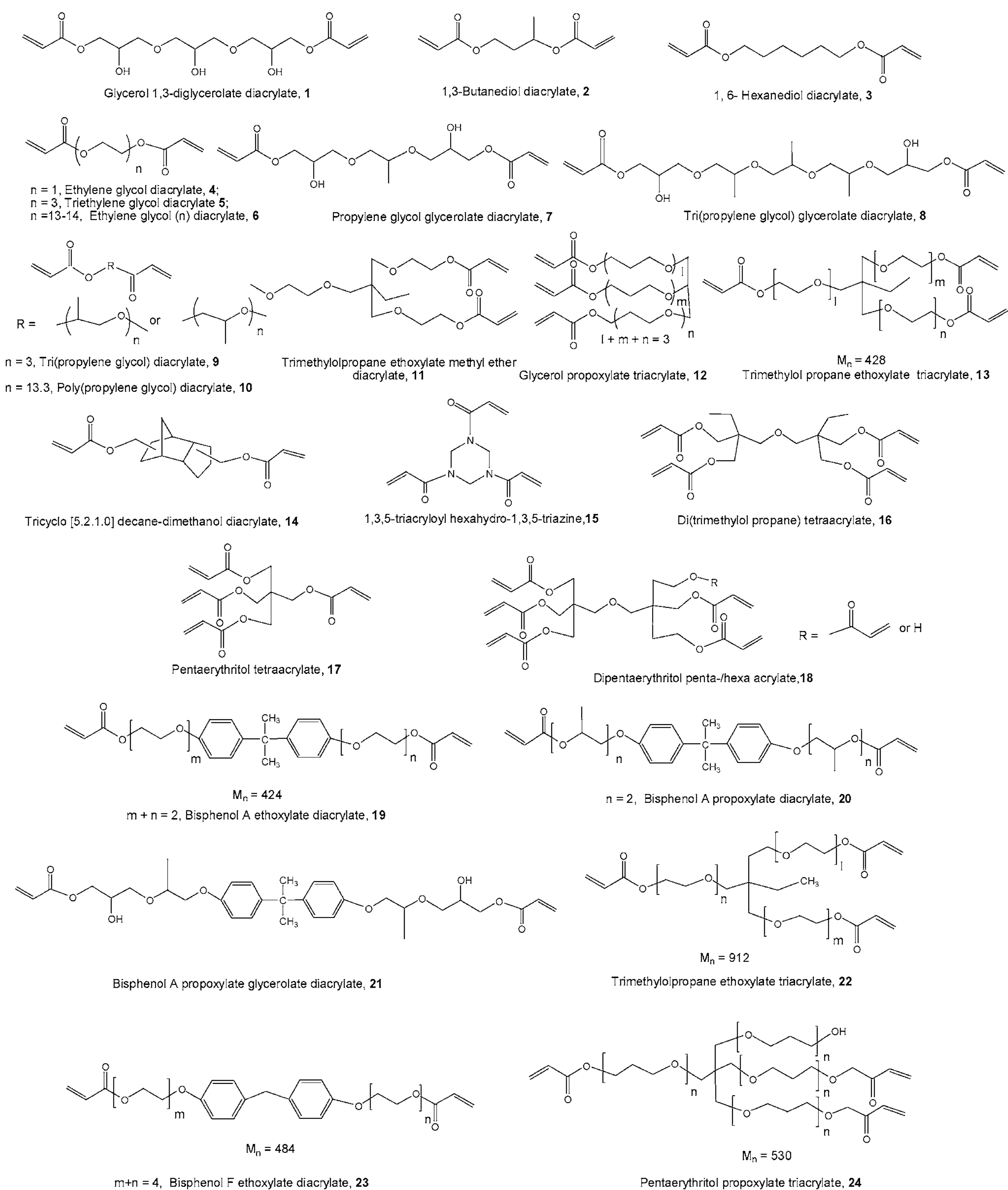

Fig. 1. Chemical structures of the bi- and oligo-functional acrylate cross-linking agents used in this study.

ery. DNA transfection is a multi-step process involving (1) DNA condensation, (2) cellular uptake, (3) endosomal escape, (4) vector unpackaging, and (5) nuclear localization (22,45). The overall efficiency of a vector is determined by how its physico-chemical properties affect these steps. In particular, such factors as hydrophobicity, the number of positive charges and of protonatable nitrogens, and topology of the polymer should influence the various steps of transfection and hence the ultimate efficiency of the vector. For example, tight binding with DNA is expected to favor its condensation, which is a desirable property; at the same time, excessively tight binding is likely to lead to poor vector dissociation due to

over-stabilization of the complexes, resulting in lower efficiency (21,36). Screening a rationally created library increases the likelihood of finding the most optimal vectors overall.

Based on the above rationale we expanded our studies to the creation and screening of a combinatorial library of cross-linked PEIs with the goal of identifying biodegradable (i.e., those containing carboxylic ester linkages that are amenable to hydrolysis under the physiological pH of 7.4 (44,46), and also by carboxyl esterases (47) vectors that are efficient and yet less toxic, *in vitro* and in systemic gene delivery, compared to the linear 22-kDa PEI, the 'gold standard' among polycationic vectors (48,49).

In the present work, we cross-linked two short-chained PEIs—a linear 423-Da PEI and a branched 1.8-kDa PEI—with 24 bi- and oligo-functional acrylates depicted in Fig. 1. Some of the cross-linked products formed gel-like insoluble mass in DMSO (the reaction solvent), and thus were unsuitable for gene delivery experiments. Furthermore, in many instances the gel-like precipitates formed soon after the addition of the cross-linking agents suggesting that the conjugate-addition reactions between the acrylate groups and the aliphatic amine groups of PEIs employed here are facile even at room temperature. Among the derivatives of the 423-Da PEI, those that precipitated were the ones cross-linked with the following diacrylates at the specified PEI: acrylate molar ratios given in parentheses: **4** (10.5:1), **10** (10.5:1), **12** (21:1 and 10.5:1), **13** (10.5:1), **15–17** (10.5:1), **18** (21:1 and 10.5:1), and **24** (10.5:1). Similarly, the derivatives of the mixed PEIs that precipitated were those cross-linked with **1** (10.5:1), **2** (10.5:1), **3–5** (21:1 and 10.5:1), **6** (10.5:1), **12–14** (21:1 and 10.5:1), **15** (10.5:1), **16–18** (all three ratios), **19** (10.5:1), **20** (all three ratios), **22** (10.5:1), **23** (10.5:1), and **24** (21:1 and 10.5:1). The formation of such precipitates depended upon the size of the parent PEIs and on the cross-linking ratio suggesting that higher molecular weight of the products and more extensive cross-linking both favored precipitation.

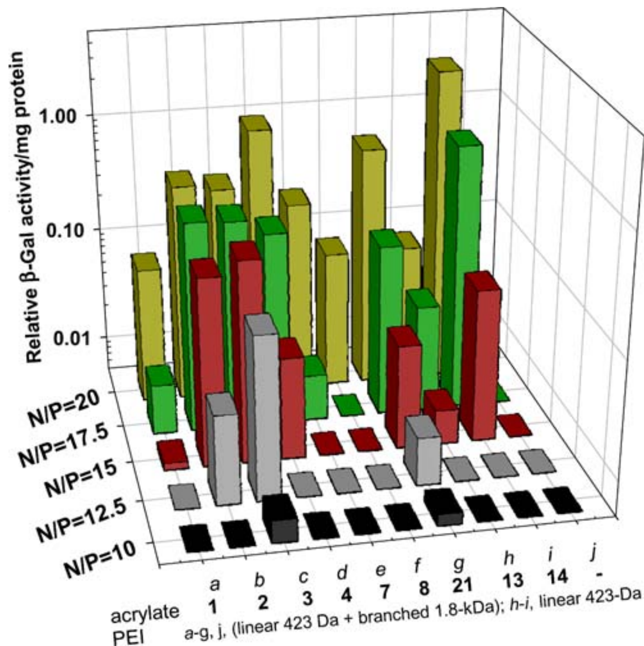

Fig. 2. Comparison of the β-galactosidase activity in COS-7 monkey kidney cells following transfection by selected most potent cross-linked small PEIs (*a–i*) and the physical mixture of the parent 1.8-kDa and 423 Da PEIs (*j*) at different N/P ratios from 10 to 20; $n = 4$. Efficiency of the linear 22-kDa PEI is taken as a unity. The linear 423-Da PEI alone did not mediate any enhancement in gene expression over naked DNA at the above N/P ratios (not shown). The PEI monomer to acrylate molar ratios for cross-linking were *a–d* and *f–g*, 42:1; *e* and *h*, 21:1; and *i*, 10.5:1.

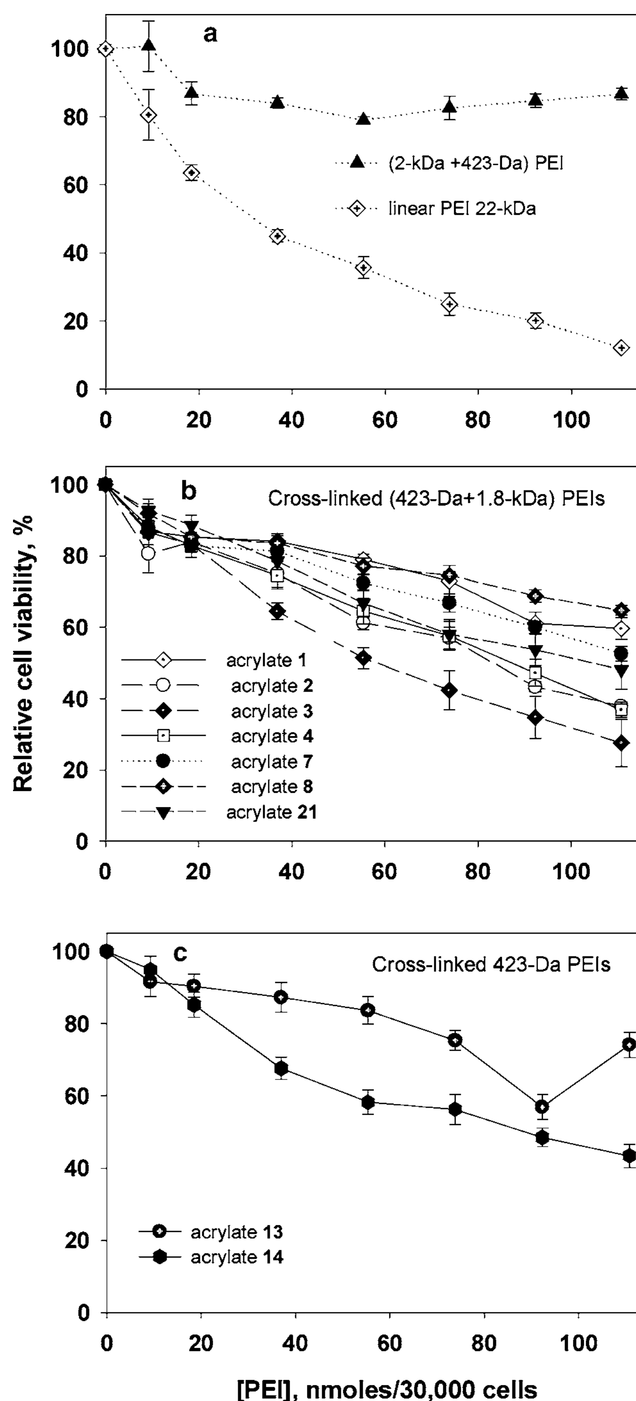

Fig. 3. Comparison of the cytotoxicities induced by the unmodified small PEIs (**a**), the linear 22-kDa PEI (**a**), and the cross-linked small mixed and individual PEIs (**b** and **c**) in A549 human lung cancer cells measured by the MTT assay; $n = 4$.

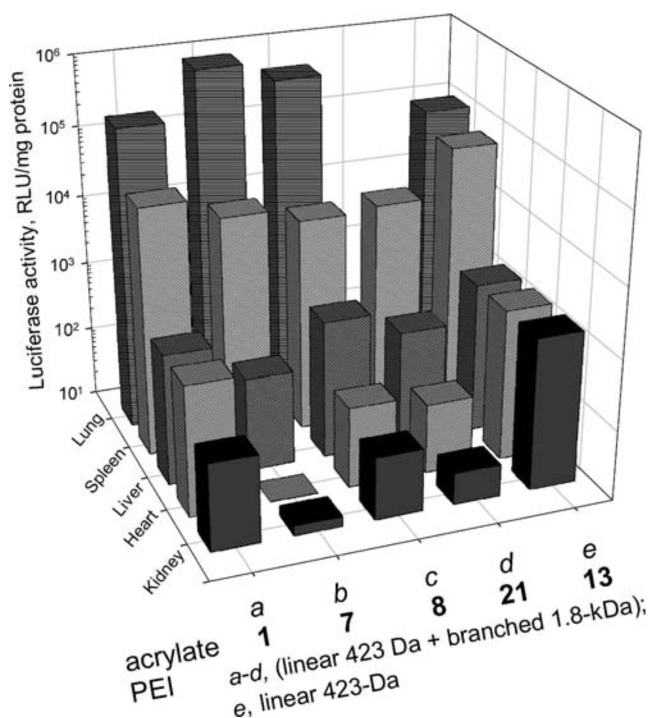

Fig. 4. Comparison of luciferase activities in different mouse organs following systemic delivery of a luciferase plasmid by selected cross-linked small PEIs at the optimal N/P ratios *in vivo*; $n=4$. The N/P ratios are, 15 (*a–b*), and 17.5 (*c–e*). See the legend to Fig. 2 for the respective PEI/acrylate ratios used for synthesizing these cross-linked PEIs. The parent PEIs mediated no gene expression. Administration of the polyplexes of the 22-kDa PEI prepared at N/P = 10 under identical conditions resulted in the death of half of the mice.

The remaining cross-linked PEIs were initially screened for their *in vitro* transfection efficiency in the COS-7 monkey kidney cells because of these cells' relatively high transfectability, which is conducive to a high-throughput screening that typically involves a low number of cells per well and small amount of DNA. The cross-linked PEIs were first tested for their efficiency at N/P ratios of 10, 20, and 40. None of the cross-linked polymers exhibited high efficiency at N/P = 10. Nine most potent polymers that exhibited optimal efficiency at N/P = 20 were selected, precipitated from DMSO with diethylether, and used for further experiments. Note that the processing of the potent PEIs employed herein was only intended to remove DMSO; hence the cross-linked PEIs obtained may contain some non-cross-linked parents. Although fractionation of cross-linked PEIs (higher molecular weight) may alter their *in vitro* and *in vivo* performance, this issue was outside of the scope of the present work.

Results of *in vitro* transfection experiments on COS-7 cells using the above-referenced PEIs are presented in Fig. 2. The most potent derivative of the linear 423-Da PEI was that cross-linked with tricyclo-[5.2.1.0]-decane-dimethanol diacrylate (diacrylate **14**) at a 10.5:1 PEI monomer/diacrylate molar ratio. This polymer exhibited a 3,670-fold enhancement in efficiency at N/P = 20 over the parental PEI at the same N/P. The most potent mixed PEI was that cross-linked with ethylene glycol diacrylate (diacrylate **4**) at a 42:1 PEI monomer/diacrylate molar ratio, which was 854 times more efficient than the parents at N/P = 20. Highest gene expression was observed at N/P = 20 with all the other cross-linked PEIs presented in Fig. 2 as well. At this N/P, the mixed PEIs cross-linked with acrylates **1**, **2**, **3**, **7**, **8** and **21** exhibited 63-, 320-, 281-, 186-, 64-, and 501-fold enhancement in efficiency over the physical mixture of the parental PEIs, respectively. The 423-Da PEI cross-linked with acrylate **13** exhibited a 113-fold enhancement in efficiency over the parent PEI. Further, the relative efficiency of the diacrylate **14** cross-linked PEI was 2.5 times greater ($p = 0.0002$), and that of the diacrylate **4** cross-linked PEI was similar ($p = 0.48$), to that of the 22-kDa linear PEI, the 'gold standard' among polycationic vectors.

The N/P ratios for optimal efficiency of PEIs vary inversely with their molecular weights (20,28,36,41). Given the higher optimal N/P of 20 for the cross-linked PEIs compared to that of 10 for the 22-kDa PEI, it is reasonable to assume that the molecular weights of the former are below 22-kDa.

The nine cross-linked PEIs whose transfection efficiency is presented in Fig. 2 and their parents were further tested for *in vitro* cytotoxicity in a A549 human lung cancer cell line. Even at the highest concentration used, treatment with the physical mixture of the 423-Da and 1.8-kDa PEIs retained some 90% cell viability (Fig. 3a). All the cross-linked PEIs were found to be less toxic than the linear 22-kDa PEI, although cross-linking did increase the toxicity of the small PEIs compared to the parents (Fig. 3b,c). The increase in toxicity in the case of the mixed PEIs (Fig. 3b) depended upon the type of linkages, with the lowest toxicity exhibited by those that were cross-linked with the acrylates **1**, **7**, **8**, and **21**. All of these bore hydrophylic hydroxyl substituents in the linker region. The remaining three cross-linked mixed PEIs that were relatively more toxic were the ones cross-linked with acrylates **2**, **3**, and **4**; all of these bore hydrophobic alkyl linkages. These results show that the nature of the linkages indeed affects toxicity.

Given these cross-linked PEIs' lower *in vitro* toxicity compared to linear PEI 22-kDa, they were next examined for systemic gene delivery in a mouse model using a Luciferase containing plasmid. Systemic delivery offers the opportunity to reach distal tissues and organs that are not directly accessible. Further, this model also provided an opportunity to test the organ specific delivery, if any, of the different cross-linked PEIs.

In pilot experiments, 45, 60, and 90 μg DNA/mouse were used. Luciferase activity (gene expression) was found to be the highest at 90 μg DNA/mouse; therefore, all further experiments were performed using this DNA dose unless specified otherwise. Complexes were prepared in 5% aqueous glucose, and N/P ratios in the 12.5–20 range were used. PEI stock solutions prepared at pH 4.5 were used for complex formation; use of neutral pH, buffers and presence of salts were found to result in substantial precipitation of the polyplexes, making them unsuitable for injections.

Diacrylate **1**-cross-linked mixed PEI was tested at N/P = 15 and 17.5. At the former ratio, the highest gene expression was observed in the lungs, followed by a 10 times lower expression in the spleen (Fig. 4, *a*). Increasing the N/P ratio to 17.5 resulted in the death of two of the four mice tested. Mixed PEI cross-linked with acrylates **2** and **3** were first tested at N/P = 12.5. Since all the mice injected with the polyplexes of these PEIs died, higher N/P ratios were not

tested. Similarly, all four mice injected with the polyplexes (N/P = 15) of mixed PEI cross-linked with acrylate **4** also died. Importantly, these three PEIs were the most toxic among cross-linked mixed PEIs identified in the *in vitro* experiments (Fig. 3b) suggesting a correlation between *in vitro* and *in vivo* toxicities for the cross-linked PEIs. Interestingly, a physical mixture of the parent small PEIs resulted in substantial precipitation upon complex formation with DNA at N/P = 15. Injection of the supernatants (200 μl/mouse) resulted in the death of two of the four mice injected. The two mice that survived exhibited no gene expression in any of the major organs. The above behavior is possibly due to the presence of a larger percentage of unbound PEI in solution, given the poor DNA binding efficiency of the small PEIs. Injection of equal amount of free PEI (i.e., without DNA) also resulted in the death of one mouse, and another mouse being sick, supporting this notion. It may be mentioned that an earlier study also has shown that presence of free PEI increases the toxicity of polyplexes (40).

Acrylate **7**-cross-linked mixed PEI was first tested at N/P = 15. The highest gene expression was observed in the lungs, followed by a 53-fold lower expression in the spleen (Fig. 4, *b*). Increasing the N/P to 17.5 and 20 cut the gene expression by half, implying that optimal expression was already achieved at N/P = 15. No mice died at any of these N/P ratios. The optimal N/P for mixed PEI cross-linked with acrylate **8** was 17.5, and the highest gene expression was observed in the lungs followed by spleen (Fig. 4, *c*).

Interestingly, unlike acrylate **1** and **7** cross-linked mixed PEIs, acrylate **21**-cross-linked mixed PEI exhibited the highest gene expression in the spleen, followed by liver at all N/P ratios tested (15, 17.5, and 20), with over 100 times lower expression in the lung compared to the spleen; no deaths of mice were observed at any of the above N/P ratios with this PEI. Highest gene expression was observed at N/P = 17.5 (Fig. 4, *d*). Acrylate **14**-cross-linked linear PEI was toxic at N/P = 15. Note that although most efficient *in vitro*, this PEI is also among the most toxic of the cross-linked PEIs *in vitro* (Fig. 3c). Similar to acrylate **1** and **7** cross-linked mixed PEIs, the acrylate **13**-cross-linked linear PEI also exhibited the highest gene expression in the lungs, although the difference in expression between lung and spleen is only moderate in this case (Fig. 4, *e*). It may be noted that the highest gene expression *in vivo* was mediated by acrylate **7**-cross-linked PEI, followed by the acrylate **8**-cross-linked PEI (Fig. 4, *b* and *c,* respectively). Highest expression is observed in the lungs with these PEIs. Earlier studies, including ours, have reported higher levels of gene expression in the lungs mediated by unmodified PEIs (i.e., with no ligand conjugated for targeting specific organs). Although the mechanism of this specificity is not fully understood, it may be due to association of PEI polyplexes with blood components leading to their aggregation and subsequent entrapment in the lung capillary bed (39,40). In support of this hypothesis, treating mice with anticoagulants prior to gene delivery has been shown to decrease the binding of blood cells to lung endothelium and to strongly reduce reporter gene expression (39). Further, complexes of even larger PEIs (22- or 25-kDa PEI) have been reported to contain unbound/loosely bound PEIs. Removal of unbound PEI (purification of the complexes) does reduce the toxicity *in vitro* and *in vivo*, but also results in a 200-fold reduction in the *in vivo* expression (lung) and in some 10-fold reduction in other organs (40). Addition of free PEI to the purified complexes restores the original levels of expression. Based on these observations it has been suggested that loosely bound/free PEI present in the complexes contributes to their aggregation by binding with blood components, and also contribute the endosomal escape of the complexes, thereby, in turn, contributing to the enhanced gene expression (40). From the present work, it appears that the nature of the cross-linker also influences the organ specificity of the polyplexes. Characteristics of the cross-linkers that determine the organ specificity of the polyplexes formed by these cross-linked PEIs is currently under investigation.

Importantly, two of the four mice injected with the linear 22-kDa PEI at its optimal N/P ratio of 10 died. Even when the N/P ratio was reduced to 7.5, two of the four mice injected with the polyplexes appeared close to death, and they exhibited severely damaged lungs. Toxicity to mice exhibited by the polyplexes of the 22-kDa PEI at the relatively low N/P ratios of 10 and 7.5 is in line with the greater *in vitro* toxicity of this PEI (Fig. 3a) compared to the cross-linked PEIs.

In closing, by employing a combinatorial library approach, we discovered several highly potent cross-linked PEIs that are at least as efficient and/or less toxic than the 22-kDa linear PEI *in vitro*. Two non-toxic PEIs derivatives that mediated high levels of luciferase expression in the lungs *in vivo* upon systemic delivery of the complexes were found. These PEIs could be further developed for gene therapy of lung diseases such as cystic fibrosis (50) and lung cancer (51). In addition, one non-toxic PEI derivative that exhibited preferential delivery to the spleen was also identified from the in vivo experiments.

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