Published in final edited form as:
Pharm Res. 2012 February ; 29(2): 500–510. doi:10.1007/s11095-011-0576-5.

The Effects of PVP(Fe(III)) Catalyst on Polymer Molecular Weight and Gene Delivery via Biodegradable Cross-Linked Polyethylenimine

Victor W.T. Shum[1], **Nathan P. Gabrielson**[1], **M. Laird Forrest**[2], and **Daniel W. Pack**[1,*]

[1]Department of Chemical and Biomolecular Engineering, University of Illinois, Box C-3, 600 South Mathews Avenue, Urbana, Illinois, 61801

[2]Department of Pharmaceutical Chemistry, University of Kansas, 2095 Constant Avenue, Lawrence, Kansas 66047

Abstract

Purpose—Crosslinked, degradable derivatives of low-molecular-weight polyethylenimine (PEI) are relatively efficient and non-cytotoxic gene delivery agents. To further investigate these promising materials, a new synthetic approach was developed using a poly(4-vinylpyridine)-supported Fe(III) catalyst (PVP(Fe(III))) that provides more facile synthesis and enhanced control of polymer molecular weight.

Methods—Biodegradable polymers (D.PEI) comprising 800-Da PEI crosslinked with 1,6-hexanediol diacrylate and exhibiting molecular weights of 1.2, 6.2, and 48 kDa were synthesized utilizing the PVP(Fe(III)) catalyst. D.PEI/DNA polyplexes were characterized using gel retardation, ethidium bromide exclusion, heparan sulfate displacement, and dynamic light scattering. *In vitro* transfection, cellular uptake, and cytotoxicity of the polyplexes were tested in human cervical cancer cells (HeLa) and human breast cancer cells (MDA-MB-231).

Results—D.PEIs tightly complexed plasmid DNA and formed 320- to 440-nm diameter polyplexes, similar to those comprising non-degradable, 25-kDa, branched PEI. D.PEI polyplexes mediated 2- to 5-fold increased gene delivery efficacy compared to 25-kDa PEI and exhibited 20% lower cytotoxicity in HeLa and no toxicity in MDA-MB-231. In addition, 2- to 7-fold improved cellular uptake of DNA was achieved with D.PEI polyplexes.

Conclusions—PVP(Fe(III)) catalyst provided a more controlled synthesis of D.PEIs, and these materials demonstrated improved *in vitro* transfection efficacy and reduced cytotoxicity.

Keywords

polyethylenimine; non-viral gene delivery; biodegradable polymer; polymer supported ferric chloride

Introduction

Gene therapy is a promising technique for treating diseases that are caused by genetic mutations, which can lead to malfunction or deficiency of proteins. For example, intractable diseases such as hemophilia, cystic fibrosis, and severe combined immunodeficiency (SCID) are related to specific aberrant genetic mutations (1–2). Currently, the most challenging aspect of gene therapy is delivery of therapeutic genes into target cells and/or tissues

*To whom correspondence should be addressed: Tel.: +1 217 244 2816, Fax: +1 217 333 5052, dpack@illinois.edu.

efficiently and without significant side effects. Most current gene delivery methods suffer critical drawbacks that have prevented gene therapies from getting past the clinical trial development phase (3–4). New materials providing safe and effective gene delivery are required to advance gene therapy into the clinic (5–7).

Polyethylenimine (PEI) is one of the most studied cationic polymers in gene delivery due to its commercial availability and relatively high *in vitro* transfection efficiency. PEI exhibits high primary, secondary, and tertiary amine densities, allowing easy ligand functionalization to enhance cell targeting and uptake (8–9). The 25-kDa, branched form of PEI has been shown to provide high transfection efficiency *in vitro*, but its high cytotoxicity prevents it from being used clinically. As a result, branched PEI has been relegated to use as a model material and as a standard to compare transfection efficacy of gene delivery materials. Much research has been done to modify or functionalize branched PEI to reduce polymer toxicity while improving gene delivery efficacy and cellular uptake.

In general, transfection efficiency is directly related to the molecular weight of PEI, while toxicity is inversely related. Gosselin et al. demonstrated that 25-kDa PEI displays gene delivery efficiency 50-fold higher than 800-Da PEI but reduces cell viability by more than half (10). Since toxicity should be one of the major considerations in any gene delivery vector's design, researchers have explored various alternatives to temporarily increase the molecular weight of low molecular weight cationic polymers. The resulting polymers can condense DNA and possess gene delivery efficiency similar to their high molecular weight counterparts and degrade into a less toxic low molecular weight version of the polymers inside the cells. One way to accomplish this goal is to cross-link low molecular weight PEI, which is relatively non-toxic, with a degradable cross-linker. Many synthetic approaches have been investigated using different cross-linker-polymer combinations, resulting in biodegradable polymers possessing reduced cytotoxicity and improved transfection efficiency. Examples of successful cross-linkers include an acid-labile imine linker between 1.8-kDa PEI and glutaraldehyde, degradable chitosan-linked PEI copolymer, and disulfide cross-linked PEI (11–13). These results indicate that the cross-linking design can be crucial for synthesizing degradable synthetic gene delivery vectors that show both low toxicity and high efficiency.

One of the strategies used in synthesizing degradable polycations is the Michael addition of amines to acrylate groups. Lynn et al. first screened a library of 140 diacrylate cross-linked amine polymers, showing some specific combinations of diacrylates and amine monomers with promising transgene expression (14). Forrest et al. later described the synthesis of biodegradable PEI derivatives by cross-linking 800-Da, branched PEI with 1,3-butanediol diacrylate or 1,6-hexanediol diacrylate. The resulting degradable PEI derivatives showed two- to 16-fold increase in transfection efficiency compared to 25-kDa, branched PEI and were also essentially non-toxic (15). However, the synthesis procedure was reported to be inconsistent and difficult to control.

In the present study, we report an alternative facile synthesis of biodegradable PEI (D.PEI) derivatives by Michael addition utilizing poly(4-vinylpyridine) (PVP)-supported ferric chloride (PVP(Fe(III))) heterogenous catalyst. Fe(III) has the ability to catalyze the diacrylate cross-linking reaction, but separating it from PEI post-synthesis is difficult due to PEI's metal chelating potential (16). By incorporating a polymer support, the PVP(Fe(III)) provides a low-cost, heterogeneous catalyst that allows easy catalyst recovery (17). As a result, this alternative synthesis scheme provides a more controllable PEI cross-linking method and D.PEIs with wider range of molecular weights, as well as enhanced transfection properties.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Aldrich (St Louis, MO) unless otherwise specified.

Cells and Plasmids

The MDA-MB-231 human breast carcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). The HeLa human cervical carcinoma cell line was a gift from Dr. Sandra McMasters (University of Illinois, Urbana, IL). Cells were cultured according to their ATCC protocols at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM). The growth medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The 5.3-kilobase expression vector pGL3 (Promega, Madison, WI), coding for firefly luciferase gene driven by the SV40 promoter and enhancer, was produced by Elim Biopharm (Hayward, CA) and used without further purification.

PVP(Fe(III)) Catalyst and Biodegradable PEI Syntheses

Polymer supported ferric chloride was synthesized by suspending 1 g of poly(4-vinylpyridine) (PVP) in 10 mL of 0.1 M $FeCl_3$ solution in double-distilled water in a scintillation vial. An additional 1 mmol of $FeCl_3$ was added into the mixture and stirred at room temperature for 30 minutes. The PVP-immobilized Fe(III) solid was filtered under vacuum, washed with double-distilled water, and dried at 60°C overnight.

D.PEI was synthesized by cross-linking 800-Da branched PEI with 1,6-hexanediol diacrylate cross-linker. Briefly, 1 g of 800-Da branched PEI was dissolved in 10 mL of methanol at room temperature in a scintillation vial. Various molar equivalents of PVP(Fe(III)) catalyst to mole of PEI, between 0.05 to 0.15, in addition to an equimolar amount of cross-linker to PEI, were added into the mixture to form a suspension. The mixture was sealed and stirred for 24 h, and centrifuged at 4500 rpm for 10 min to remove the catalyst. Unreacted materials were extracted using petroleum ether. The methanol was removed by using a rotating evaporator at 67 °C and 150 rpm for 25 min until only viscous D.PEI sample was left. The final polymer sample was used without further purification and stored at −80 °C.

Polymer Characterizations

^{1}H NMR (Variant Unity 400) was used to examine the structure and cross-linking density of D.PEI in D_2O. The extent of cross-linking was determined by peak integration at: δ=1.25–1.40 ppm (br m, -$COOCH_2CH_2CH_2$-, 2 H, ester linker), δ=1.40–1.60 ppm (br m, -$COOCH_2CH_2$-, 2 H, ester linker), δ=2.33–2.47 ppm (br m, $CH_2CH_2NHCH_2CH_2COOCH_2$, 2 H, ester linker), δ=2.47–3.3 ppm (br m, CH_2CH_2N, PEI ethylenes), δ=3.5–3.6 ppm (t, ^{3}J=6.6MHz, -HOCH_2-, 2 H, hydrolyzed ester linker), and δ=4.1 ppm (m, -COOCH_2-, 2H, ester linker). For the cross-linked PEI degradation study, each sample was dissolved in D_2O and incubated at 37 °C for 0 h, 24 h, 72 h, and 192 h, and the ^{1}H-NMR spectrum was acquired using the same apparatus. The fraction of remaining cross-linked PEI was determined by integrating and comparing the ester peaks at δ=4.1 ppm (m, -COOCH_2-, 2H, ester linker) and δ=3.5–3.6 ppm (t, ^{3}J=6.6MHz, -HOCH_2-, 2 H, hydrolyzed ester linker).

Molecular weight of D.PEI was determined using capillary viscometry (18). Briefly, polymers were dissolved in 0.5 M $NaNO_3$ to achieve three different solutions with concentrations between 1 to 5 g/L, and the viscosities were measured using a kinematic viscometer (Cannon, State College, PA) at 25 °C. Based on the measurements, reduced, specific, inherent, and limiting viscosities of the polymer solution were calculated. By

plotting the Staudinger-Mark-Houwink relationship with known molecular weight PEI standards, the molecular weight of each D.PEI sample was calculated based on the sample's viscosity.

Polyplex Formation

For polyplex characterization, 1 μg of DNA was diluted in PIPES buffer (120 mM NaCl, 16 mM PIPES at pH 7.2) and mixed with various amounts of polymer, dissolved in double distilled water, to form the desired polymer and DNA weight–to-weight ratio polyplexes. For polyplexes used for *in vitro* transfection, 0.5 μg of DNA was used to form polyplexes instead. Each polyplex sample was incubated at room temperature for 15 min after formation before any additional experiments were performed.

Polyplex Gel Retardation

After the 15 min incubation at room temperature, DNA sample buffer (Bio-Rad, Hercules, CA) was added into the polyplexes. The samples were electrophoresed on a 1% agarose gel for 30 min at 120 V. The gel was visualized with ethidium bromide (Bio-Rad, Hercules, CA) staining.

Ethidium Bromide Exclusion Assay

Ethidium bromide (0.5 μg) was added into each polyplex sample in a 96-well microplate and incubated at room temperature for an additional 10 min. Fluorescence was excited at 510 nm and emission detected at 595 nm in a Synergy 2 Multi-Mode Fluorescence Microplate Reader (Bio Tek, Winooski, VT). The sample fluorescence value was normalized by values for wells containing DNA only, after subtracting background fluorescence measured from wells containing ethidium bromide only. Each measurement was performed in triplicate.

Heparan Sulfate Competitive Displacement Assay

Various amounts of heparan sulfate, dissolved in double-distilled water, were added into each polyplex sample to achieve desired weight-to-weight ratios between heparan sulfate and DNA. The mixtures were incubated at room temperature for another 15 min after which 3 μL of DNA sample buffer was added into each mixture. The final mixtures were loaded onto a 1% agarose gel and electrophoresed for 30 min at 120 V. The gel was visualized with ethidium bromide. For the PEI degradation study, each polymer was incubated at 37 °C for 0 h, 24 h, 72 h, and 192 h. Polyplexes and heparan sulfate displacement assay were prepared as described above.

Dynamic Light Scattering

Based on the optimal transfection ratio, the corresponding amount of DNA and polymer were diluted into 200 μL PIPES buffer to form the desired polymer and DNA weight–to-weight ratio polyplexes. The polyplexes were incubated at room temperature for 15 min, and double distilled water was added to reach a final sample volume of 2 mL. Polyplex size was measured by Brookhaven Instruments Corporation 90 Plus Particle Size Analyzer (Holtsville, NY) before and after 4 h of incubation at 37 °C. Each measurement was repeated five times.

In Vitro Transfection

Cells (MDA-MB-231 or HeLa) were seeded in a 24-well plate at 8×10^4 cells/well in growth medium with serum 24 h before transfection. On the day of transfection, growth medium was replaced with fresh serum-free DMEM. Before transfection, 50 μL polyplexes in PIPES buffer were formed with 0.5 μg pGL3 and various amount of polymer to achieve desired

polymer/DNA ratios. The polyplexes were incubated at room temperature for 15 min and added into each well. Four hours post-transfection, polyplexes were removed and replaced with fresh growth medium with serum. Twenty-four hours post-transfection, luciferase expression was determined using the Promega Luciferase Assay System (Promega), which reports expression levels in relative light units (RLU) as measured on a Lumat L9507 luminometer (Berthold, GmbH, Germany). Each experiment was performed in quadruplicate. Luciferase expression (RLUs) was normalized by total cell protein using BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Cytotoxicity Assay

The cytotoxicity of polymer on the MDA-MB-231 and HeLa cells was characterized using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) (Promega) according to manufacturer's protocol. Briefly, 8×10^3 cells/well were seeded in 96-well microplate in growth medium containing 10% FBS. Twenty-four hours after seeding, the medium was replaced with serum-free DMEM, and polymer was added to the cells to achieve a final concentration between 0 and 50 μg/mL. Following a 4 h incubation period, the medium was replaced with fresh serum-containing medium and incubated for another 24 h, after which 15 μL of the Dye Solution was added into each well, and cells were incubated for another 4 h. After incubation, 100 μL of the Solubilization Solution/Stop Mix was added into each well and incubated for another 1 h. The absorbance at 570 nm, with reference wavelength of 650 nm, was measured using Tecan Safire 2 Fluorescence Reader (Tecan, Mannedorf, Switzerland). The absorbance of medium was subtracted from the viable cell absorbance and normalized to cells with no polymer. Each polymer and polymer concentration were tested six times in each experiment.

Polyplex Uptake via Flow Cytometry

In a 24-well plate, 8×10^4 cells/well were seeded in growth medium with serum 24 h before transfection. On the day of the transfection, growth medium was replaced with fresh serum-free DMEM. The intercalating dye YOYO-1 (Invitrogen, Carlsbad, CA) was mixed with pGL3 according to the ratio 25 nL YOYO-1/μg DNA. Fifty microliters of polyplexes in PIPES buffer were formed with 0.5 μg pGL3/YOYO-1 and various amount of polymer to achieve desired polymer/DNA ratios. Two hours post-transfection, the cells were trypsinized and analyzed on a BD LSR II Flow Cytometer System (BD, Franklin Lakes, NJ). Median peak fluorescence was recorded and normalized to cells transfected with pGL3/YOYO-1 in the absence of polymer. Each measurement was performed in triplicate.

Results

Synthesis and characterization of biodegradable PEI

Three D.PEI samples were synthesized as described above by cross-linking equimolar amounts of 800-Da, branched PEI and 1,6-hexanediol diacrylate, and catalyzed with 0.1, 0.15, and 0.2 molar equivalents of PVP(Fe(III)) catalyst per mole of PEI (Fig. 1). The molecular weights of the resulting D.PEIs were 48 kDa, 6.2 kDa, and 1.2 kDa (termed D.PEI-48, D.PEI-6.2, and D.PEI-1.2, respectively), determined using capillary viscometry (Supplementary Table 1) (18). Compared to the 14 kDa and 30 kDa D.PEI generated by the non-catalyzed reaction reported by Forrest et al. (15), the alternative synthesis reported here provides a wider range of D.PEI molecular weights for characterization and gene delivery studies.

The structure and degradation of polymeric gene delivery vectors are important parameters for safe, efficient gene transfer. Non-degradable PEI may accumulate inside the cell, increasing toxicity, and leading to reduced transfection efficiency (19). To investigate the

structure and degradation of D.PEI samples, we incubated samples at 37 °C in D_2O for various amounts of time and obtained their ^1H NMR spectra (Supplementary Figure 1). To calculate the extent of D.PEI degradation, the relative number of protons from the methylene group and the hydroxyl group adjacent to the carboxyl group was determined by integrating the corresponding peaks in the spectra (20). The degradation rates of D.PEIs were approximately the same (Fig. 2). D.PEI-1.2 degraded the most but all three D.PEIs have ~50% ester bond remaining after 24 h incubation.

Another D.PEI characteristic of interest is the degree of cross-linking. Highly cross-linked, low molecular weight PEI is expected to behave similarly to its high molecular weight non-cross-linked counterpart, including improved DNA binding and transfection efficiency, due to similar molecular weights. By utilizing elemental analysis, we approximated the cross-linking density of each D.PEI sample through mass balances (Table 1). As expected, the higher molecular weight D.PEI possessed higher degree of cross-linking, and vice versa. D.PEI-48, D.PEI-6.2, and D.PEI-1.2 exhibited cross-linking densities, defined as mole percent of nitrogen atoms in PEI attached to 1,6-hexanediol diacrylate, of 16.9%, 15.1%, and 14.1%, respectively. In addition to cross-linking density, it is also important to understand the cross-linking structure of the D.PEIs. To determine whether the samples have linear or branched structure, we calculated the number of PEI chains cross-linked with 1,6-hexanediol diacrylate (Table 1) (21). The results show that D.PEI samples with higher cross-link density have more diacrylates attached to a PEI chain. More importantly, all three D.PEIs show that, on average, each sample has more than two diacrylates linked with a single PEI chain. This finding implies that the D.PEI samples are branched. In particular, the D.PEI-48 has more than three diacrylates attached to a PEI chain, which indicates a high level of branching. Due to the loss of flexibility and steric hindrance as a result of this branching, the D.PEIs tend not to condense DNA as efficiently as their unmodified 25 kDa counterpart.

Characterization of D.PEI/DNA Polyplexes

One of the important characteristics of non-viral gene delivery vehicles is the ability to condense DNA efficiently in order to be taken up by the cells. To determine the capability of the polymers to bind DNA, we electrophoresed polyplexes formed at various polymer/DNA ratios in agarose gel to observe the necessary amount of polymer needed to completely inhibit DNA migration (Fig. 3). Unmodified 25-kDa, branched PEI required 0.2 μg polymer/μg DNA to completely retard the DNA (Fig. 3A). The D.PEI samples retarded DNA migration at 0.5 μg polymer/μg DNA (Fig 3B-3D). Upon closer examination, D.PEI-48 prevented migration of most DNA at 0.3 μg D.PEI/μg DNA (Fig. 3B), while 0.4 μg D.PEI/μg DNA was needed for D.PEI-6.2 and D.PEI-1.2 (Fig. 3C and Fig. 3D). These results indicate unmodified 25 kDa PEI bound DNA much more efficiently than D.PEI. This observation may be attributed to a decrease in the number of protonated amines due to attachment to the cross-linker, steric hindrance, or reduced flexibility due to the branched D.PEI structure.

To be endocytosed, it is critical that the cationic polymer is able to form nanoscale complexes with plasmid DNA (22). Dynamic light scattering was used to determine the size of the polyplexes immediately after polyplex formation at each polymers' optimal transfection ratio, and again after 4 h incubation at 37 °C (Fig. 4). For all polymers, polyplex size increased after incubation, likely due to aggregation. Unmodified 25 kDa PEI polyplexes, at polymer/DNA ratio of 2:1 (w:w), exhibited the smallest effective diameter both before and after incubation, despite these polyplexes containing 7.5-fold to 12.5-fold less polymer by mass than the three D.PEI polyplexes, at polymer/DNA ratio of 15–25 (w:w). This result shows that unmodified 25 kDa PEI condensed plasmid DNA better than the D.PEIs, which is consistent with the presence of higher cationic surface charges. For the

D.PEIs, the polyplexes' diameters were inversely related to the molecular weights of the polymers before incubation. D.PEI-1.2 polyplexes showed the largest effective diameter, even though there was ~1.6-fold more polymer present, confirming that higher molecular weight PEI can condense DNA better than its lower molecular weight counterpart. The trend is not as clear after 4 h of incubation, but the inverse relationship between molecular weight and polyplex size among the D.PEI samples was still observed.

Polymeric gene delivery vectors should exhibit tight binding to plasmid DNA to protect and avoid early release of DNA. To determine the "tightness" of DNA binding, we investigated the polymer's ability to exclude ethidium bromide, an intercalating dye that fluoresces much more strongly when bound to double-stranded DNA. To quantify the degree of condensation between D.PEIs and DNA, ethidium bromide fluorescence was measured in the presence of polyplexes at various polymer/DNA ratios (Fig. 5). Unmodified 25 kDa PEI condensed DNA tightly at polymer/DNA ratio of 0.5:1 (w:w) showing only 1% normalized fluorescence. The D.PEI samples required more polymer, 0.5 μg, 1 μg, and 1 μg polymer/μg DNA for D.PEI-48, D.PEI-6.2, and D.PEI-1.2, respectively, to achieve minimum normalized fluorescence and similar DNA binding "tightness" as unmodified 25 kDa PEI. This indicates that although all PEI samples are able to tightly condense DNA, D.PEIs appear to form "looser" polyplexes than unmodified PEI.

Upon entering the cells, the DNA inside the polyplexes must be released in order for transcription to occur. One way to measure the strength of the polymer/DNA binding is to displace the DNA with a polyanion like heparan sulfate (HS). The amount of HS needed to competitively displace DNA from polyplexes indicates the strength of binding (23). To determine the relative DNA binding strength of the D.PEIs before and after degradation (0 to 192 hours incubation at 37 °C), along with unmodified 25 kDa and 800 Da PEIs, various amounts of HS were added to polyplexes prepared at 2:1 (w:w) polymer/DNA ratio and electrophoresed in agarose gel (Fig. 6). Twenty-five kDa and 800 Da PEI required ~18 μg HS/μg DNA and 12 μg HS/μg DNA to displace the DNA, respectively (Fig. 6A and Fig. 6B). All the D.PEI samples required less HS compared to unmodified 25 kDa PEI, ~12 μg HS/μg DNA, to dissociate the DNA (Fig. 6C–6N). This suggests that it is easier to unpackage DNA from the D.PEIs than the unmodified PEI. Approximately the same amount of HS was required for all D.PEI samples and for the 800 Da PEI, indicating that molecular weight of the D.PEI is not the only factor that controls DNA binding. Due to steric effects and loss of flexibility discussed previously, DNA can bind more strongly to the higher overall positive charge of 25 kDa PEI than even the highest molecular weight of cross-linked D.PEI. This explains why even the D.PEI-48 sample needs less HS to displace DNA compared to the 25 kDa control.

In the tested incubation period, there is no significant change in the amount of HS needed to release DNA from the polyplexes for all D.PEI samples. This is expected since D.PEIs and 800 Da PEI released DNA with the same amount of HS present. This could be due to the relatively low cross-linking density of the D.PEIs, where the DNA binding of the unmodified 800 Da PEI, the starting material of the D.PEI, might overwhelm the effect of the reduced DNA binding strength from cross-link degradation.

In Vitro Transfection of D.PEI/DNA Polyplexes

Transfection efficacies of polyplexes formed with unmodified and cross-linked PEIs at various polymer/DNA ratios were studied in HeLa and MDA-MB-231 cell lines (Fig. 7). In HeLa cells, at the optimal polymer/DNA ratio (10:1 w:w), both D.PEI-48 and D.PEI-6.2 provided ~5–fold more transgene expression than unmodified, 25-kDa, branched PEI (PEI/DNA = 2:1 w:w), while transgene expression with D.PEI-1.2 (D.PEI/DNA = 25:1 w:w) was ~3-fold greater than with unmodified PEI (Fig. 7A). In MDA-MB-231 cells, D.PEIs also

mediated greater transgene expression than 25 kDa PEI, but not as significantly as in HeLa cells (Fig. 7B). At their optimal polymer/DNA ratios, transgene expression mediated by D.PEI-48 and D.PEI-6.2 (D.PEI/DNA = 15:1 w:w) was ~1.6–fold greater than unmodified PEI (PEI/DNA = 2:1 w:w), while D.PEI-1.2 (D.PEI/DNA = 20:1 w:w) provided similar transfection as unmodified PEI. The optimal polymer/DNA ratio was 2:1 for unmodified 25 kDa PEI in both HeLa and MDA-MB-231 cells. However, optimal polymer/DNA ratios for D.PEIs were significantly higher (10–25:1 w:w), likely due to the weaker DNA condensation and “looser” DNA binding compared to unmodified PEI.

Cytotoxicity of D.PEI

In order for the D.PEIs to be considered as potential vectors for gene delivery, they need to exhibit low or no toxicity. To determine polymer toxicity, viability of HeLa and MDA-MB-231 cells upon exposure to various amounts of polymers was measured using a MTT assay (Fig. 8). Unmodified 25 kDa PEI reduced cell viability to 10% at polymer concentrations greater than 15 μg/mL and 30 μg/mL in HeLa (Fig. 8A) and MDA-MB-231 (Fig. 8B) cells, respectively. All D.PEIs were significantly less cytotoxic in both cell lines compared to unmodified PEI. D.PEI-48 was the most toxic of the three D.PEI samples; at 50 μg/mL (the maximum concentration investigated), it yielded 40% and 80% cell viability in HeLa and MDA-MB-231 cells, respectively. D.PEI-1.2 was the least toxic polymer, causing essentially no decrease in cell viability in both HeLa and MDA-MB-231 cells at 50 μg/mL. At the same concentration, D.PEI-6.2 displayed 50% and 100% viability in the two cell lines. All polymers were more cytotoxic to HeLa cells than MDA-MB-231 cells.

Cellular Uptake of D.PEI/DNA Polyplexes

The first step in the gene delivery process is cellular internalization of polyplexes, typically by an endocytic mechanism. We measured uptake of fluorescently labeled polyplexes by fluorescence-activated cell sorting (FACS) (Fig. 9). Overall, MDA-MB-231 cells endocytosed 2- to 3-fold more polyplexes than HeLa cells for all polyplexes at the polymer/DNA ratios tested. For unmodified 25 kDa PEI, approximately the same amount was endocytosed at all polymer/DNA ratios in both HeLa and MDA-MB-231 cells. HeLa cells internalized similar amounts of D.PEI and unmodified PEI polyplexes, except at polymer/DNA = 25:1 (w:w), where the cells endocytosed slightly more D.PEI-48 polyplexes than unmodified PEI polyplexes (Fig. 9A). Unlike HeLa cells, MDA-MB-231 cells endocytosed more D.PEI polyplexes than unmodified PEI polyplexes, except at polymer/DNA = 2:1 (w:w) (Fig. 9B). At polymer/DNA ratios that resulted in the maximal uptake, D.PEI-48, D.PEI-6.2, and D.PEI-1.2 polyplexes show 3-, 7-, and 4-fold higher cellular uptake than unmodified PEI polyplexes, respectively.

Discussion

Safety and efficacy are arguably the most important factors in gene therapy. Polymers and lipids have been optimized for gene delivery by chemically modifying their structures or by conjugating ligands onto their surfaces (24). Specifically, the addition of degradable cross-linker in branched PEI has been extensively investigated in order to reduce the cytotoxicity and increase the transfection efficiency of branched PEI (11). We have further characterized and optimized the branched PEI derivatives first reported by Forrest et al. due to their relatively simple chemistry and efficient gene transfer (15).

Utilizing a heterogeneous PVP-supported Fe(III) catalyst, we synthesized a set of three D.PEIs with a wide range of molecular weights (1.2–48 kDa) through Michael addition of 800-Da PEI to 1,6-hexanediol diacrylate, resulting in D.PEIs with ~15% cross-linking density (Table 1). NMR confirmed the presence of ester linkages in each D.PEI and the

degradation of the D.PEI when incubated at 37 °C (Fig. 2). Both gel retardation (Fig. 3) and ethidium bromide exclusion (Fig. 5) studies indicated that all the D.PEIs were able to bind to DNA as tightly as unmodified 25 kDa PEI (Fig. 3 and Fig. 5). At their optimal transfection ratios, the D.PEI polyplexes were ~1.4-fold larger than the unmodified 25 kDa PEI polyplexes, most likely due to the fact that the optimal D.PEI/DNA ratios for transfection were 7- to 12-fold higher than that of 25 kDa PEI (Fig. 4). However, after a 4 h incubation, which simulated the 4 h exposure of polyplexes to DMEM during transfection, aggregation of D.PEI and 25 kDa PEI polyplexes resulted in similarly sized particles, making it unlikely that polyplex size plays a major factor in the greater transfection efficiency of the D.PEIs.

All three D.PEIs mediated ~2- to 5-fold better transfection efficacy compared to unmodified 25 kDa PEI, in both HeLa and MDA-MB-231 cell lines, at their respective optimized transfection ratios (Fig. 7). In particular, D.PEI-48 and D.PEI-6.2 consistently transfected better than D.PEI-1.2 in both cell lines, which is consistent with findings that suggest improved transfection efficiency with increasing PEI molecular weight (10). However, the fact that D.PEI-48 required ~5- to 7-fold more polymer than unmodified 25 kDa PEI to achieve optimal transfection indicates that, in addition to molecular weight, other factors including cytotoxicity, cellular uptake and polyplex unpackaging may contribute to the D.PEIs' improved transfection efficiency.

Additionally, we demonstrated that the D.PEIs are less cytotoxic than unmodified 25 kDa PEI. D.PEIs exhibited almost no toxicity in MDA-MB-231 cells; D.PEI-48 and D.PEI-6.2 reduced viability to ~50%, while D.PEI-1.2 was not toxic in HeLa cells at 50 μg/mL (Fig. 8). The optimal transfection ratio for 25 kDa PEI is 2:1 (w:w), which is approximately equal to a PEI concentration of 2 μg/mL. This concentration corresponds to about 60% and 80% viability for HeLa and MDA-MB-231 cell lines, respectively. Unlike unmodified PEI, our D.PEIs show above 80% viability in HeLa cells and almost no toxicity in MDA-MB-231 cells at their respective optimal transfection ratios. This reduced toxicity partially explains why transfection efficiency improved for D.PEI but decreased for unmodified PEI at increasing polymer/DNA ratios above 2:1 (w:w).

As discussed previously, cellular uptake and DNA unpackaging are two important aspects of gene delivery. HeLa cells internalized approximately the same amounts of unmodified PEI and D.PEIs, except for D.PEI-48, while MDA-MB-231 cells internalized 2- to 7-fold more D.PEI-containing polyplexes than unmodified PEI (Fig. 9). The increased cellular uptake of D.PEIs over unmodified PEI in MDA-MB-231 cells is in good agreement with the gene delivery activity of the polymers. However, this is not the case for HeLa cells, where there is no significant difference in uptake to correlate with the increase in transfection. To explain this discrepancy, we investigated the polymer's ability in unpackaging DNA through heparan sulfate competitive displacement assay. At the same polymer/DNA ratio, D.PEIs more readily dissociated from DNA than unmodified 25 kDa PEI in the presence of heparan sulfate (Fig. 6), which likely contributes to the increase in transfection efficiency for D.PEIs in both HeLa and MDA-MB-231 cells. These results demonstrate that one must take multiple intracellular barriers into account when designing potential non-viral gene delivery vectors.

Compared to the biodegradable PEI first reported by Forrest et al. (15), this present work has further characterized the D.PEI and investigated the effects of molecular weight on D.PEI-mediated gene delivery. Forrest et al. synthesized 14 kDa and 30 kDa biodegradable PEI by cross-linking 800 Da PEI with 1,3-butanediol diacrylate and 1,6-hexanediol diacrylate, respectively, via Michael addition without catalyst and under different reaction conditions than that reported here. Forrest et al. showed that their D.PEIs were capable of condensing DNA, exhibited reduced toxicity, and improved transfection efficiency in several cell lines,

but were unable to conclusively determine why the D.PEI were more effective than the 25 kDa control or the effect of D.PEI molecular weight on gene transfer activity. However, we synthesized D.PEIs with a wide range of molecular weights, which has allowed us to correlate D.PEI's molecular weight with various gene delivery properties of cross-linked PEI. Specifically, we have shown that the improved transfection efficiency of D.PEIs compared to unmodified PEI is due to a combination of molecular weight, cytotoxicity, polyplex internalization, and DNA unpackaging ability.

Several groups have reported various diacrylate cross-linked PEIs and shown at least 50-fold improvement in transfection efficiency and reduced cytotoxicity compared to the controls (25–26). However, it is difficult to draw a direct comparison between those polymers and the polymers reported here, mainly due to the use of different cell lines and controls. In addition, many of those reports only studied the effects of size, degradability, and cytotoxicity of the cross-linked PEI polyplexes on transfection, but neglected the effects of intracellular barriers including cellular uptake and polyplex unpackaging on gene delivery. Without such characterization, it is not possible to fully understand or compare cross-linked PEIs as polymeric gene delivery vectors and to explain relative enhancement in gene transfer ability.

Conclusions

We reported a controllable synthesis of biodegradable PEI by cross-linking 800 Da PEI and 1,6-hexanediol diacrylate by Michael addition via a polymer-supported heterogeneous catalyst. The resulting polymers have a wider range of molecular weights and have demonstrated 2- to 5-fold more efficient gene transfer compared to commercially available, 25 kDa PEI. The improvement in gene delivery of these D.PEIs is likely due to a combination of their low cytotoxicity, easy DNA unpackaging, and high cellular uptake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (GM085222). Flow cytometry was performed at the Flow Cytometry Facility of the Roy J. Carver Biotechnology Center at the University of Illinois.

References

1. Drumm ML, Pope HA, Cliff WH, Rommens JM, Marvin SA, Tsui LC, et al. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. Cell. 1990; 62(6):1227–1233. [PubMed: 1698126]
2. Kay MA, Rothenberg S, Landen CN, Bellinger DA, Leland F, Toman C, et al. In vivo gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. Science. 1993; 262(5130): 117–119. [PubMed: 8211118]
3. Check E. Gene therapy put on hold as third child develops cancer. Nature. 2005; 433:561.
4. Marshall E. Gene Therapy Death Prompts Review of Adenovirus Vector. Science. 1999; 286(5448): 2244–2245. [PubMed: 10636774]
5. Luo D, Saltzman M. Synthetic DNA delivery systems. Nat Biotechnol. 2000; 18:33–37. [PubMed: 10625387]
6. Park TG, Jeong HJ, Kim SW. Current status of polymeric gene delivery systems. Adv Drug Deliv Rev. 2006; 58(4):467–486. [PubMed: 16781003]
7. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discovery. 2005; 4:581–593.

8. Suk JS, Suh J, Choy K, Lai SK, Fu J, Hanes J. Gene delivery to differentiated neurotypic cells with RGD and HIV Tat peptide functionalized polymeric nanoparticles. Biomaterials. 2006; 27(29): 5143–5150. [PubMed: 16769110]
9. Ogris M, Brunner S, Schüller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 1999; 6(4):595–605. [PubMed: 10476219]
10. Gosselin MA, Guo W, Lee RJ. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. Bioconjugate Chem. 2001; 12(6):989–994.
11. Kim YH, Park JH, Lee M, Kim YH, Park TG, Kim SW. Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. J Control Release. 2005; 103(1):209–219. [PubMed: 15710512]
12. McKenzie DL, Smiley E, Kwok KY, Rice KG. Low molecular weight disulfide cross-linking peptides as nonviral gene delivery carriers. Bioconjugate Chem. 2000; 11(6):901–909.
13. Wong K, Sun G, Zhang X, Dai H, Liu Y, He C, et al. PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver adminstration in vivo. Bioconjugate Chem. 2006; 17(1):152–158.
14. Lynn DM, Anderson DG, Putnam D, Langer R. Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. J Am Chem Soc. 2001; 123(33):8155–8156. [PubMed: 11506588]
15. Forrest ML, Koerber JT, Pack DW. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. Bioconjugate Chem. 2003; 14(5):934–940.
16. Kim EM, Jeong HJ, Park IK, Cho CS, Bom HS, Kim CG. Monitoring the effect of PEGylation on polyethylenimine *in vivo* using nuclear imaging technique. Nucl Med Biol. 2004; 31(6):781–784. [PubMed: 15246369]
17. Chari MA, Syamasundar K. Polymer-supported ferric chloride as a heterogeneous catalyst for chemoselective deprotection of acetonides. Synthesis. 2005; 5:708–710.
18. von Harpe A, Petersen H, Li Y, Kissel T. Characterization of commercially available and synthesized polyethylenimines for gene delivery. J Control Release. 2000; 69(2):309–322. [PubMed: 11064137]
19. Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A. A two-stage poly(ethylenimine)-mediated cytoxicity: implications for gene transfer/therapy. Mol Ther. 2005; 11(6):990–995. [PubMed: 15922971]
20. Zhong Z, Song Y, Engbersen JFJ, Lok MC, Hennink WE, Feijen J. A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s. J Control Release. 2005; 109(1–3):317–329. [PubMed: 16081184]
21. Kichler, A.; Behr, JP.; Erbacher, P. Polyethylenimines: A Family of Potent Polymers for Nucleic Acid Delivery. In: Huang, L.; Hung, MC.; Wagner, E., editors. Nonviral Vectors for Gene Therapy. 1. San Diego: Academic Press, Inc; 1999. p. 191-204.
22. Ziello JE, Huang Y, Jovin IS. Cellular endocytosis and gene delivery. Mol Med. 2010; 16(5–6): 222–229. [PubMed: 20454523]
23. Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. PNAS. 1996; 93(22):12349–12354. [PubMed: 8901584]
24. Merdan T, Kunath K, Petersen H, Bakowsky U, Voigt KH, Kopecek J, et al. PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice. Bioconjugate Chem. 2005; 16(4): 785–792.
25. Thomas M, Ge Q, Lu JJ, Chen J, Klibanov AM. Cross-linked small polyethylenimines: while still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo. Pharm Res. 2005; 22(3):373–380. [PubMed: 15835742]
26. Dong W, Li S, Jin G, Sun Q, Ma D, Hua Z. Efficient gene transfection into mammalian cells mediated by cross-linked polyethylenimine. Int J Mol Sci. 2007; 8(2):81–102.

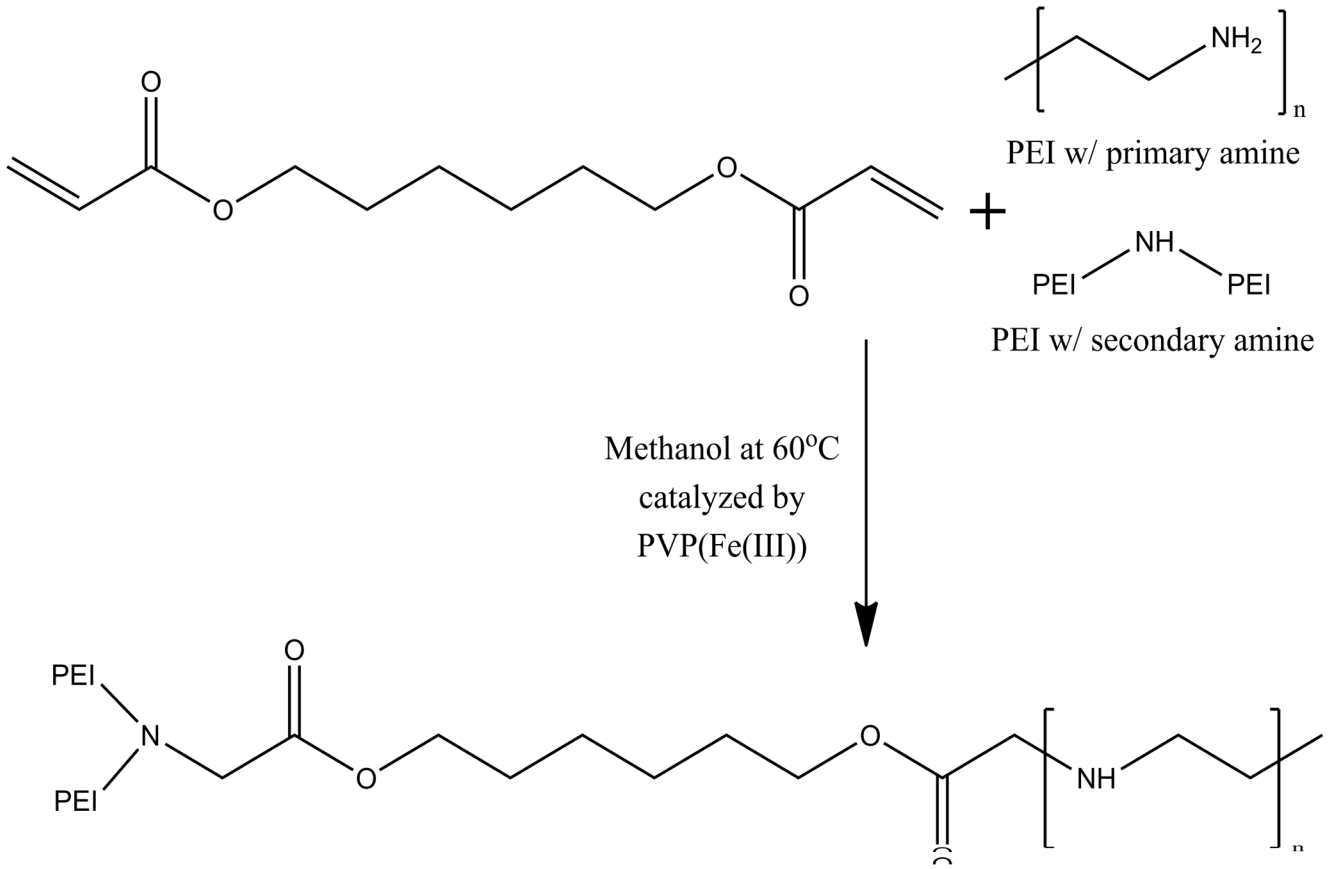

Figure 1.
Synthesis of biodegradable PEI derivatives. 800 Da PEI is cross-linked with 1,6-hexanediol diacrylate in methanol overnight at 60°C, catalyzed by PVP(Fe(III)) heterogenous catalyst. The diacrylate groups can react with primary and secondary amines of the PEI.

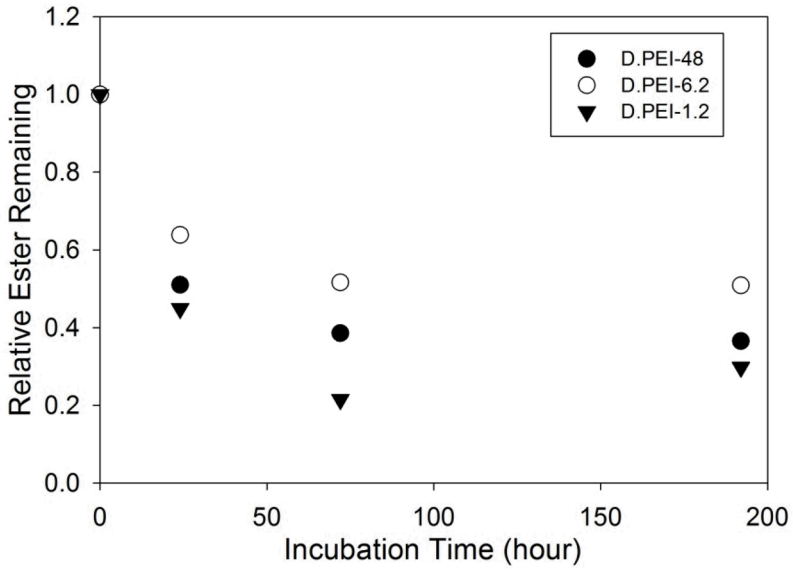

Figure 2.
Degradation of D.PEIs in D_2O at 37 °C for various incbuation periods. Degradation was calculated based on the integrals of the ester linker and hydrolyzed ester linker peaks determined by ^{1}H-NMR.

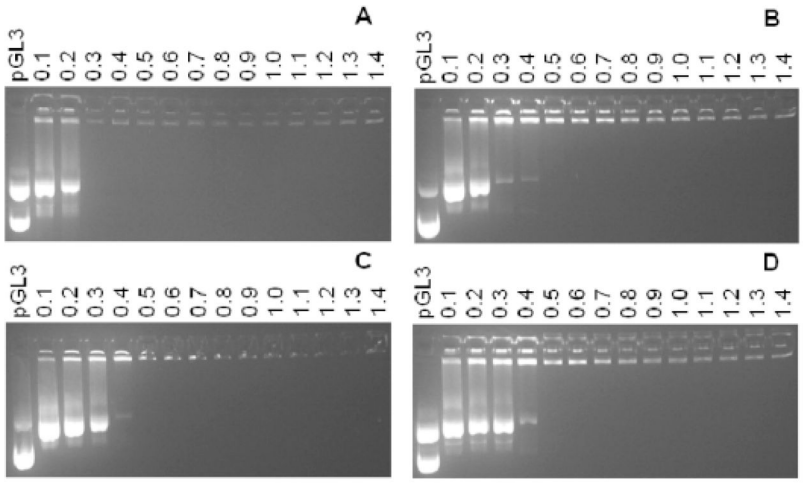

Figure 3.

Gel retardation of polymer/DNA polyplexes. (A) Unmodified 25 kDa PEI, (B) D.PEI-48, (C) D.PEI-6.2, (D) D.PEI-1.2. The polymer to DNA weight-to-weight ratio for each polyplex is listed above the corresponding lane.

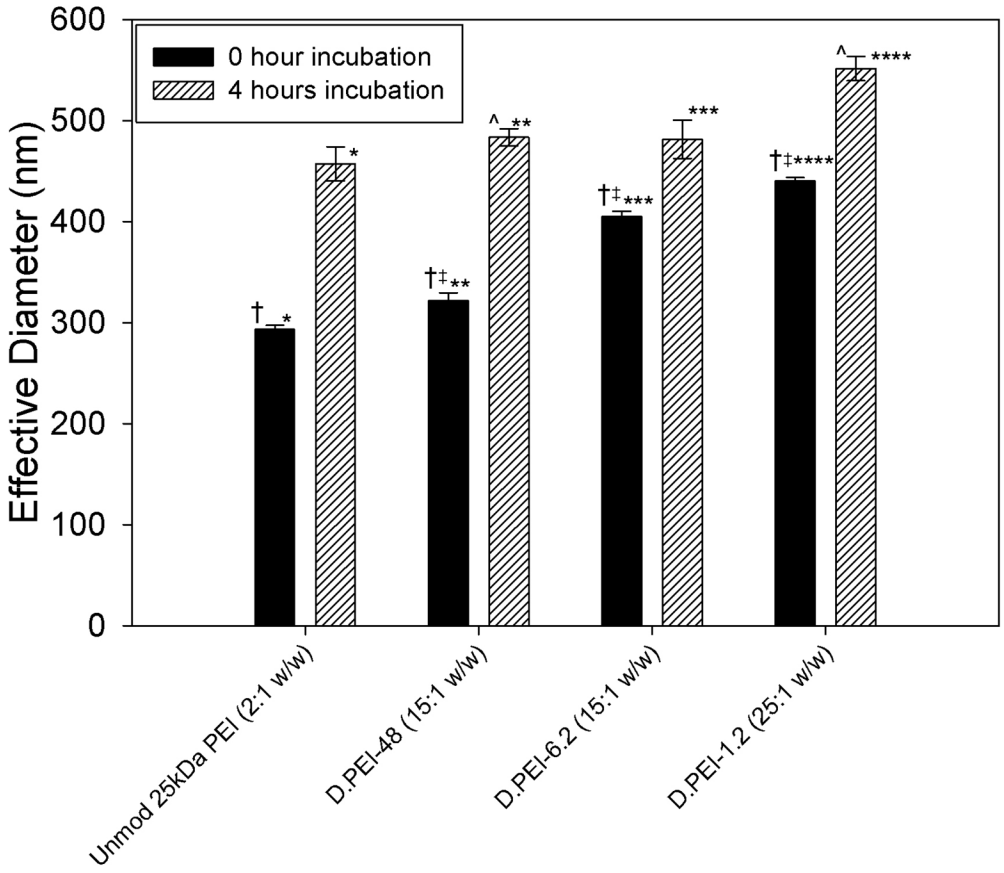

Figure 4.

The effective diameters of unmodified 25 kDa PEI and three D.PEIs' polyplexes before and after 4 hours incubation at 37°C, at their respective optimal transfection ratio (N=3, error bars represent standard deviation). The symbols (*, **, ***, ****, †, ‡, ^) indicate pairwise comparisons by t-test with $p < 0.01$.

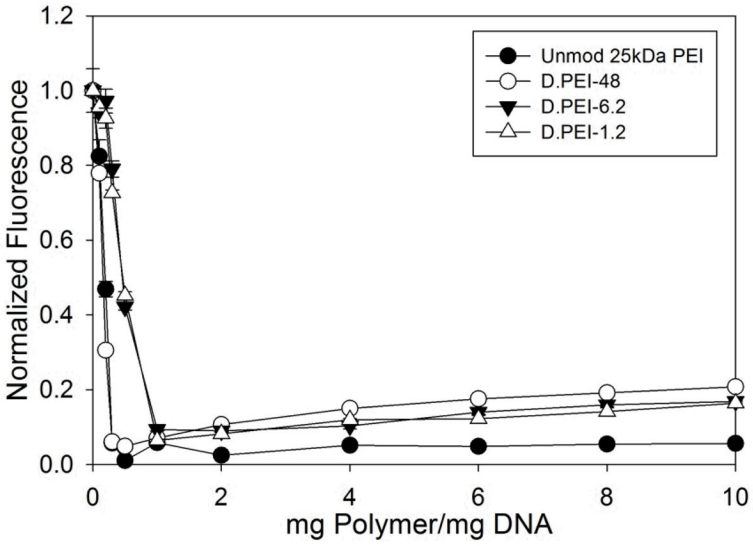

Figure 5.

Ethidium bromide exclusion assay. Polyplexes were formed with unmodified 25 kDa PEI or D.PEIs with DNA at various ratios in the presence of ethidium bromide. Normalized fluorescence was calculated by $(F-F_0)/(F_{DNA}-F_0)$. F, fluorescence of polyplexes and ethidium bromide; F_0, fluorescence of ethidium bromide only; F_{DNA}, fluorescence of DNA and ethidium bromide (N=3, error bars represent standard deviation).

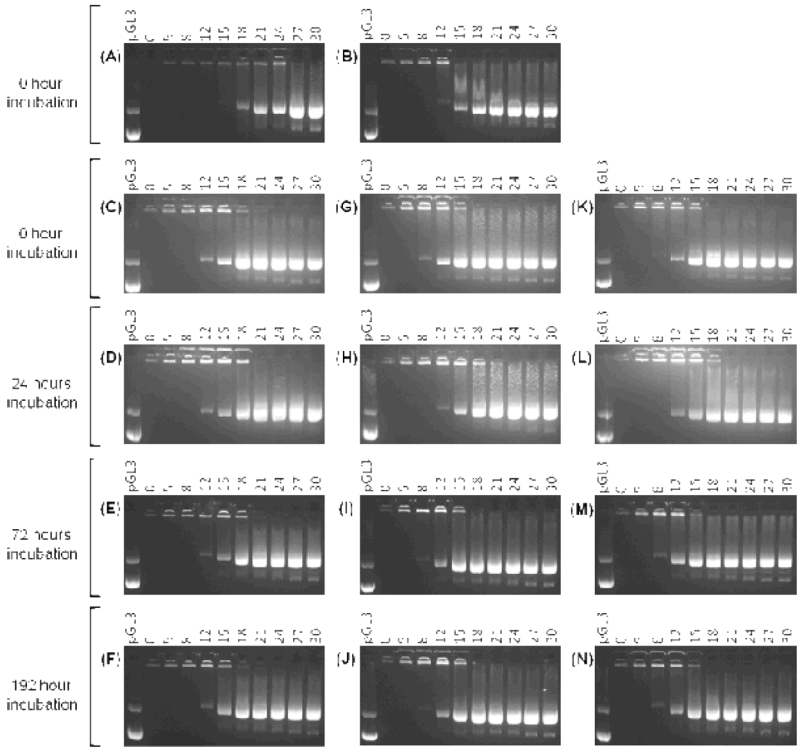

Figure 6.

Heparan sulfate displacement of polymer/DNA polyplexes at 2:1 (w:w) ratio. D.PEIs were incubated at 37 °C for 0, 24, 72, and 192 hours before complexation with DNA. (A) unmodified 25 kDa PEI, (B) unmodified 800 Da PEI, (C–F) D.PEI-48, (G–J) D.PEI-6.2, (K–N) D.PEI-1.2. The heparan sulfate/DNA ratio (w:w) for each polyplex is given above the corresponding lane.

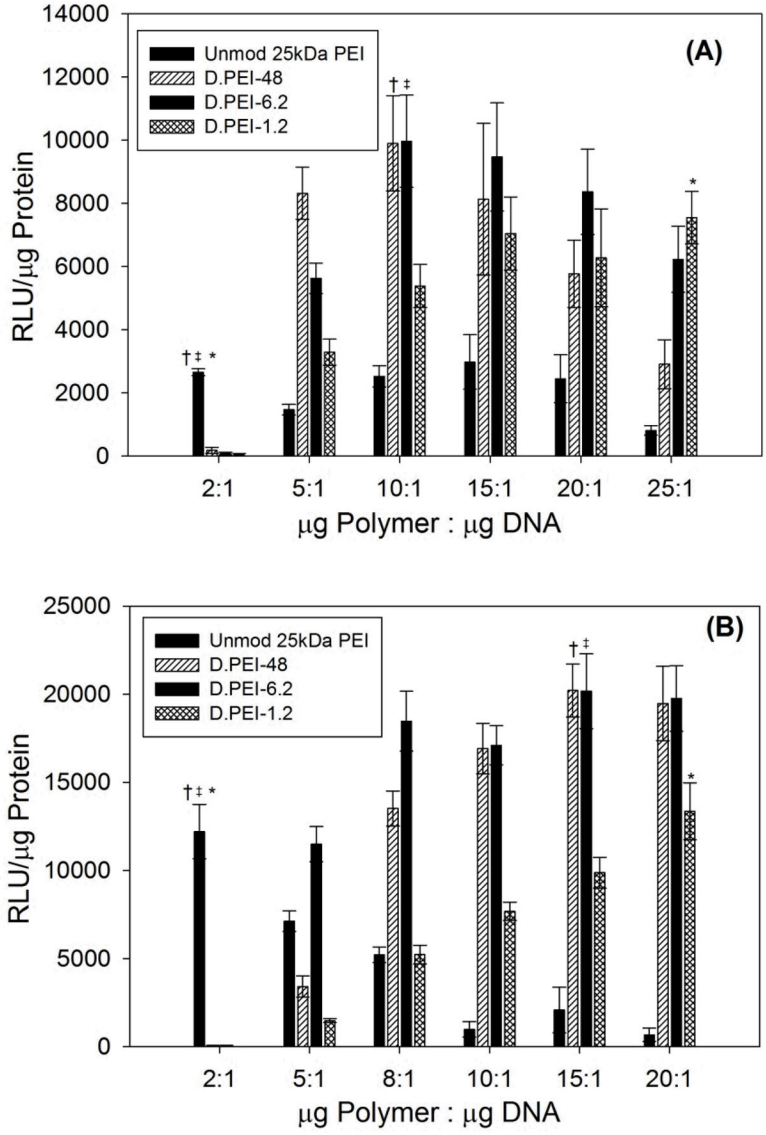

Figure 7.

In vitro transfection of (A) HeLa cells and (B) MDA-MB-231 cells with polyplexes formed with pGL3 and unmodified 25 kDa PEI or D.PEIs at various polymer/DNA ratios (t-test: †, $p < 0.01$; ‡, $p < 0.01$; *, $p < 0.01$). Luciferase expression in the cell lysates is reported as relative light unit (RLU) normalized by the total amount of protein the cell lysates (N=4, error bars represent standard deviation).

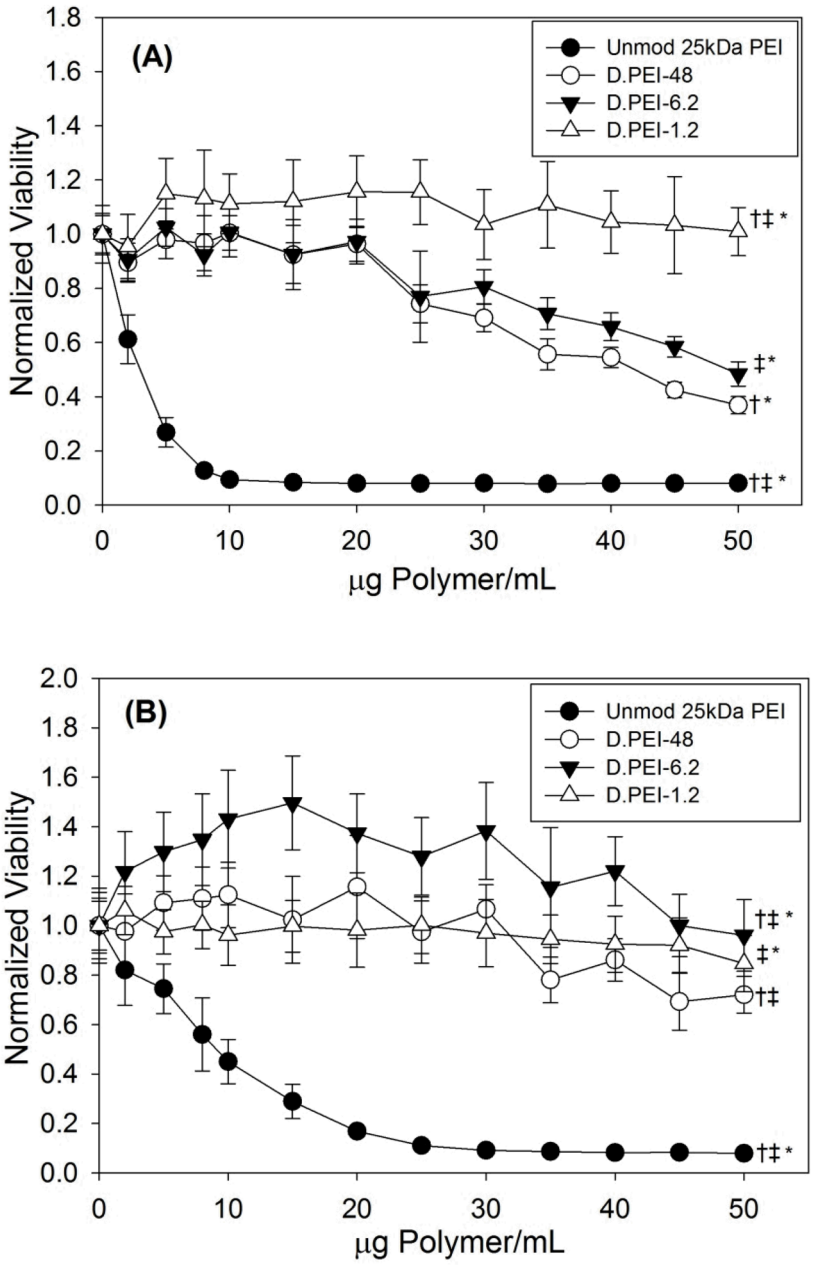

Figure 8.

Cytotoxicity of biodegradable PEI derivatives reported as normalized metabolic activity in (A) HeLa cells and (B) MDA-MB-231 cells in the presence of varying amounts of unmodified 25 kDa PEI and D.PEIs (ANOVA: †, D.PEI-48, $p < 0.05$; ‡, D.PEI-6.2, $p < 0.05$; *, D.PEI-1.2, $p < 0.05$). Metabolic activity was normalized to control with no polymer present (N=6, error bars represent standard deviation).

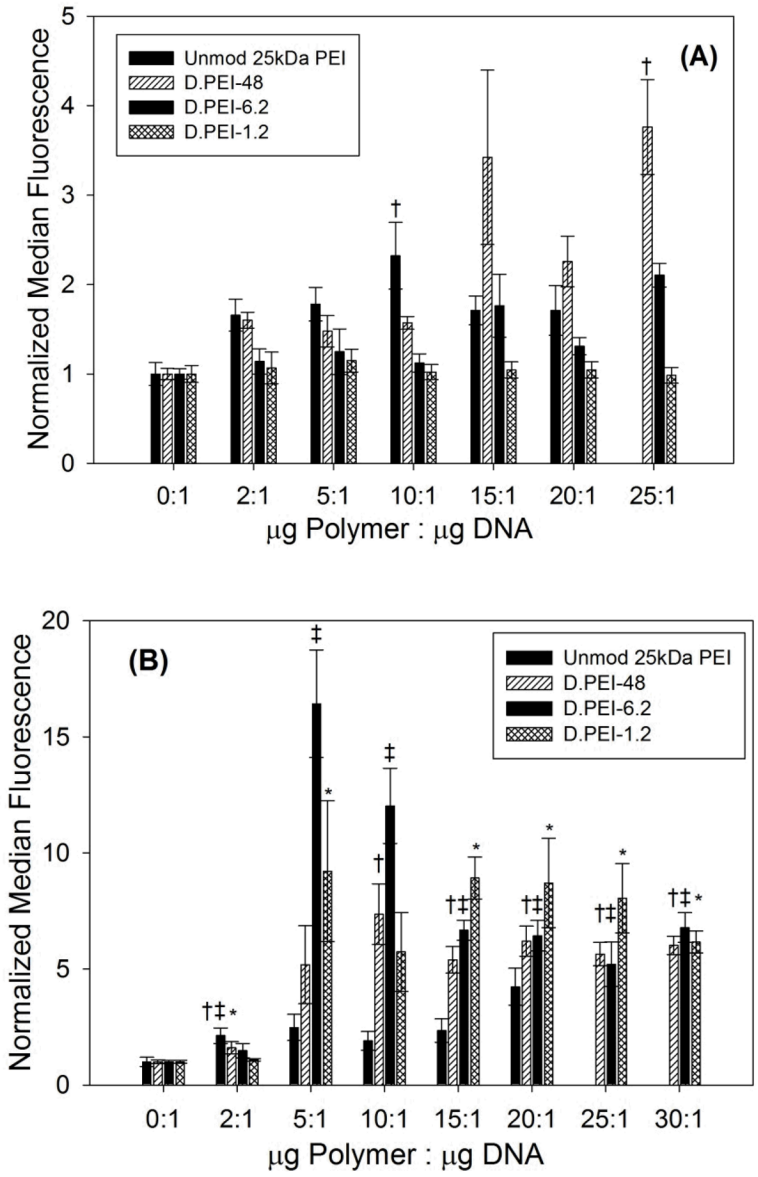

Figure 9.

Cellular uptake of polyplexes containing YOYO-1 intercalated pGL3 in (A) HeLa cells and (B) MDA-MB-231 cells (t-test: †, $p < 0.01$; ‡, $p < 0.01$; *, $p < 0.01$). Results are reported as median fluorescence normalized by control with no polymer present using FACS (N=3, error bars represent standard deviation).

Table 1

Elemental analysis, calculated % cross-linking and # diacrylate cross-linked with a PEI chain for each D.PEI sample

D.PEI Samples	Weight % Carbon	Weight % Nitrogen	Weight % Hydrogen	% Cross-linking[a]	# Cross-linker attached to a PEI chain
D.PEI-48	46.85	19.96	10.46	16.9	3.14
D.PEI-6.2	49.07	20.26	10.19	15.1	2.81
D.PEI-1.2	50.65	20.46	10.50	14.1	2.62

[a]Mole percent of nitrogen atoms in PEI attached to 1,6-hexanediol diacrylate