



Crosslinked linear polyethylenimine enhances delivery of DNA to the cytoplasm

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ABSTRACT

Crosslinked polyethylenimines (PEIs) have been frequently examined over the past decade since they can maintain the transfection efficiency of commercially available, 25 k branched PEI, but exhibit less cytotoxicity. The argument is often made that the degradability of such polymers, generally synthesized with either disulfide or hydrolytically degradable crosslinkers, is critical to the high efficiency and low toxicity of the system. In this work, we present a crosslinked linear PEI (xLPEI) system in which either disulfide-responsive or non-degradable linkages are incorporated. As with previous systems, strong transfection efficiency in comparison with commercial standards was achieved with low cytotoxicity. However, these properties were shown to be present when either the degradable or non-degradable crosslinker was used. Uncomplexed polymer was demonstrated to be the critical factor determining transfection efficiency for these polymers, mediating efficient endosomal escape without signs of cell membrane damage. While several crosslinked PEI systems in the literature have demonstrated the effect of the disulfide moiety, this work demonstrates that disulfide-mediated unpackaging may not be as important as conventionally thought for some PEI systems.

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1. Introduction

Clinical translation of gene therapy requires safe, efficient vectors. While viral vectors are efficient, concerns regarding safety and immunogenicity have created a need for efficient synthetic vectors. One of the most studied polymers for DNA delivery is polyethylenimine (PEI), a polycation with high charge density capable of pH buffering in physiologically beneficial ranges [1]. PEI, available in either linear (IPEI) or branched (bPEI) forms, is not only one of the most efficient commercially available polymers, but also one of the most toxic [2]. It causes an acute cytotoxicity due to cell membrane disruption followed by induction of apoptosis by destabilization of mitochondrial membranes [3,4]. Though PEI has frequently been used in both animals and humans for gene delivery, it has also been shown to aggregate red blood cells, bind complement, and cannot easily be broken down and excreted [5,6].

Crosslinking low molecular weight PEI has emerged as a strategy for overcoming cytotoxicity while maintaining relatively high transfection efficiency [7,8]. Of particular interest are crosslinkers that are bioresponsive and can specifically degrade within the cytoplasm of the target cell [9–12]. Disulfide reduction and hydrolytic degradation are the strategies primarily employed to trigger intracellular release of DNA from crosslinked PEI. PEI containing hydrolytically degradable

crosslinks has been shown to have efficiency equal or greater to 25 k bPEI, one of the most common PEI products used for gene delivery, as well as reduced toxicity [13–17]. Disulfide crosslinked polymers have been designed to use the intracellular reducing environment as a stimulus for DNA release in the cytoplasm [10]. Breunig et al. have shown transfection efficiencies of over 60% combined with over 90% cell viability in several cell lines transfected with disulfide crosslinked IPEI [18,19]. Several other disulfide crosslinked PEI studies have reported efficiencies near or exceeding that of commercially available standards with reduced cytotoxicity [20–22]. While intracellular disulfide reduction is frequently cited as being an important feature for excellent transfection and low toxicity, very few studies directly compare disulfide crosslinked PEI with analogous non-degradable crosslinked PEI [23]. Some studies have modulated the intracellular reducing potential with either buthionine sulfoximine (BSO) or glutathione monomethyl ester (GSHMEE) [24–26], however these methods perturb normal cell physiology. In this work, we synthesize a series of crosslinked IPEI polymers containing either disulfide or non-degradable crosslinkers and evaluate the mechanism for high transfection efficiency and low cytotoxicity.

2. Materials and methods

2.1. Materials

Linear polyethylenimine (IPEI, $M_w = 2.5$ k, 25 k) was purchased from Polysciences (Warrington, PA). Branched polyethylenimine

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(bPEI, $M_w=25$ k) and other chemical reagents were purchased from Sigma Aldrich (St. Louis, MO). KB cells were obtained from ATCC (Manassas, VA). pCMV-EGFP-N1 plasmid DNA was obtained from Aldevron (Fargo, ND). All other cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

2.2. Polymer synthesis

One hundred (100) mg of 2.5 k IPEI was dissolved in a 2:1 mixture of methanol:dimethylsulfoxide (MeOH:DMSO) at a concentration of 0.33 g/mL. Succinic acid (SA) or dithiodipropionic acid (DTDP) was dissolved in 2:1 MeOH:DMSO at 0.3 g/mL and added to achieve crosslinker ratios of 0.02–0.1 mol crosslinker/mol IPEI monomer ($M_w=43$ g/mol) (see Fig. 1). N-hydroxysuccinimide (NHS) was dissolved in 2:1 MeOH:DMSO at 0.4 g/mL and 1.4 equivalents per carboxylic acid added. After all components were well mixed, 1.4 equivalents of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) dissolved in 2:1 MeOH:DMSO was added while the mixture was stirred vigorously. This reaction proceeded overnight and was then dialyzed against Milli-Q water for 2 days using a 12 k–14 k MWCO SpectraPDR membrane. Non-degradable, SA crosslinked polymers are labeled xL-Y, where Y is the mol % crosslinker (relative to IPEI monomer). Degradable, DTDP crosslinked polymers are labeled xL-SS-Y. xL-Y $^1\text{H-NMR}$ (400 MHz, D_2O) $\delta=2.6$ (t, $-\text{NCO}-\text{CH}_2-\text{CH}_2-\text{CON}-$), 3.0 (m, $-\text{CH}_2-\text{NH}-\text{CH}_2-$), 3.45 (t, $-\text{CH}_2-\text{NCO}-\text{CH}_2-$). xLSS-Y $^1\text{H-NMR}$ (400 MHz, H_2O) $\delta=2.6$ (t, $-\text{NCO}-\text{CH}_2-\text{CH}_2-\text{S}-\text{S}-$), $\delta=2.95$ (t, $-\text{NCO}-\text{CH}_2-\text{CH}_2-\text{S}-\text{S}-$), 3.0 (m, $-\text{CH}_2-\text{NH}-\text{CH}_2-$), 3.45 (t, $-\text{CH}_2-\text{NCO}-\text{CH}_2-$). See Supporting information for FTIR and GPC analysis.

2.3. Cell culture

KB cells were cultured in folate-free RPMI 1640 basal media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified 37 °C atmosphere at 5% CO_2 .

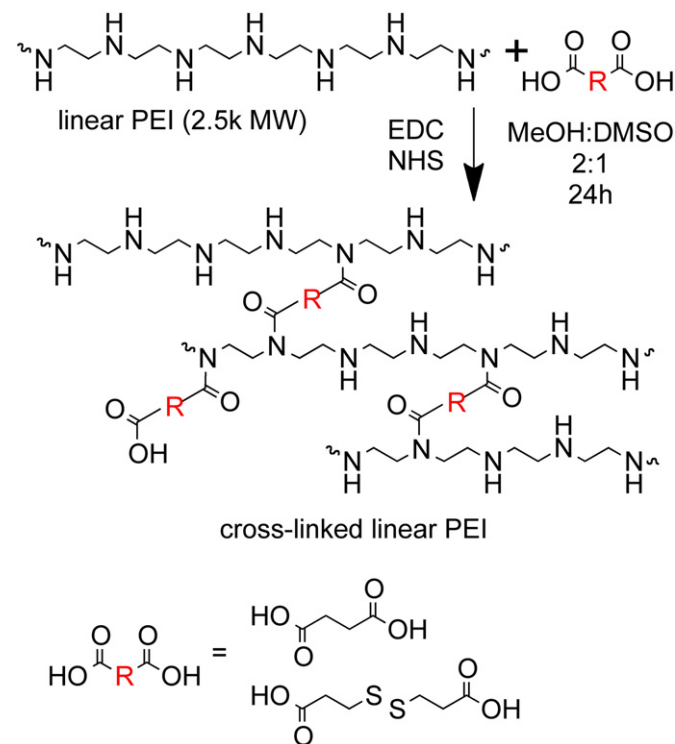


Fig. 1. Synthesis of crosslinked linear polyethylenimine using NHS/EDC chemistry with a diacid. For degradable crosslinks that break down by disulfide oxidation R contains a disulfide group.

2.4. Polyplex formation

Fifty (50) μL of polymer was aliquoted into wells of a 96-well plate at concentrations ranging from 0.333 mg/mL to 0.0033 mg/mL. Twenty-five (25) μL of plasmid DNA at 0.05 mg/mL in pH 7.4 phosphate buffered saline (PBS) was added to each well and mixed well via pipette. Complexes were allowed to stand for 10 min. One hundred (100) μL of a 1:200 (v/v) solution of Quant-It Picogreen reagent in PBS was added to the wells of an opaque 96-well plate. Twenty (20) μL of polyplexes was added to the Picogreen plate and mixed well. After 5 min, fluorescence measurements were made (ex. 490/em. 525) on a Tecan Infinite 200 Pro plate reader. Measurements were normalized to that of free DNA (100% uncomplexed DNA) and background Picogreen (0% uncomplexed DNA).

2.5. Transfection

Cells were trypsinized prior to transfection and seeded in 96-well plates at 4000 cells/well. Cells were allowed to grow overnight in a humidified 37 °C atmosphere at 5% CO_2 to be 60% confluent at the time of treatment. Polymers were diluted in PBS at 0.1 mg/mL initially and then further diluted into 25 μL of the same buffer in a separate 96-well plate at varying concentrations. Twenty-five (25) μL of pDNA at 0.05 mg/mL in PBS was then added to each well and mixed by gentle pipetting. After 10 min to ensure complex formation, 30 μL of the complexes was added to 200 μL of Opti-MEM. After further mixing, 150 μL of the complexes in Opti-MEM was added to the cells, from which growth media had been removed. Additional polymer was added either immediately or after 4 h. After 8 h, the complexes in Opti-MEM were removed and growth media was added. Cells were assayed at 48 h for GFP production using flow cytometry. 4000–10,000 live cells per sample were analyzed.

2.6. Flow cytometry

Flow cytometry was performed in U-bottom 96-well plates using an LSR II HTS Flow cytometer (Becton-Dickinson, Mountain View, CA). To prepare samples, media was removed from cells and replaced with 25 μL trypsin for 5 min. One hundred (100) μL of PBS supplemented with 2% FBS and 0.005 mg/mL propidium iodide was then added to each well, mixed, and the entire 125 μL cell suspension was transferred into a U-bottom 96-well plate.

2.7. Polyplex uptake

Cells were trypsinized prior to treatment and seeded in 96-well plates at 8000 cells/well. Cells were allowed to grow overnight in a humidified 37 °C atmosphere at 5% CO_2 to be 80% confluent at the time of treatment. Plasmid DNA was labeled with Rhodamine-CX using a Label-IT kit from Promega (Madison, WI). Polyplexes were formed and cells treated as described in the Transfection section, with labeled DNA mixed 1:1 (w/w) with unlabeled DNA. At the time indicated, cells were washed twice with PBS and analyzed using flow cytometry. No propidium iodide was used due to spectral overlap with Rhodamine-CX.

2.8. Endosomal escape

Cells were trypsinized prior to treatment and seeded in opaque, black-sided, clear-bottom 96-well plates at 8000 cells/well. Cells were allowed to grow overnight in a humidified 37 °C atmosphere at 5% CO_2 to be 80% confluent at the time of treatment. Cells were treated with 100 μL of Opti-MEM containing 0.15 mg/mL calcein and varying concentrations of polymer. After 2 h, the Opti-MEM was removed and cells were washed once with Opti-MEM and then incubated in Opti-MEM containing 0.1% (v/v) Hoechst 33342 for

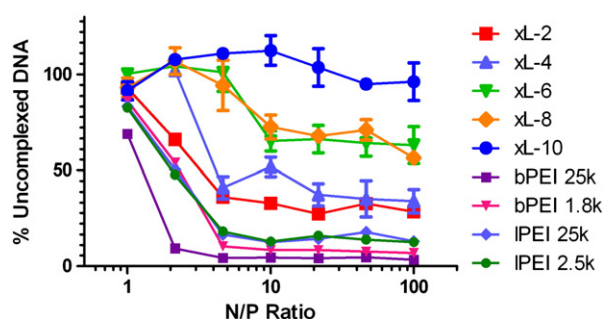


Fig. 2. Complexation of plasmid DNA by nondegradable crosslinked IPEI as a function of the percentage of crosslinker incorporated (e.g. xL-2 indicates 2% crosslinker).

20 min. After the cells were washed twice, 150 μ L of phenol-free Opti-MEM was added to each well before the plate was covered with an opaque sticker, foiled, and analyzed. Imaging was done using a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher, Waltham, MA) and analysis was done using the included software [27]. 300–3000 live cells per sample were analyzed.

2.9. Lactate dehydrogenase (LDH) release

The protocol used was adapted from the manufacturer's instructions for the Cyto-Tox ONE assay kit. Cells were trypsinized prior to treatment and seeded in 96-well plates at 8000 cells/well. Cells were grown overnight in a humidified 37 $^{\circ}$ C atmosphere at 5% CO₂ to be 80% confluent at the time of treatment. Cells were treated with 100 μ L of Opti-MEM containing varying concentrations of polymer. After 2 h, lysis buffer was added to untreated cells as a positive control. After 4 h, 50 μ L of supernatant was removed and added to an opaque 96-well plate. 50 μ L of Cyto-Tox ONE reagent was added and mixed with gentle pipetting. After 10 min, 25 μ L STOP solution was added and fluorescence was read on a Tecan Infinite 200 Pro plate reader (ex. 560 nm/em. 590 nm).

2.10. Cell viability

Cells were trypsinized prior to treatment and seeded in 96-well plates at 4000 cells/well. Cells were grown overnight in a humidified 37 $^{\circ}$ C atmosphere at 5% CO₂ to be 60% confluent at the time of treatment. Cells were treated with 100 μ L of Opti-MEM containing varying concentrations of polymer. After 4 h, polymers were aspirated and wells replenished with growth media. After 48 h, 10 μ L of a 5 mg/mL solution of MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added. After 4 h, media was aspirated and replaced with DMF to dissolve formazan crystals. Absorbance at 570 nm was read on a Tecan Infinite 200 Pro plate reader.

3. Results

3.1. Synthesis

Low molecular weight IPEI was crosslinked using EDC and NHS to form higher molecular weight structures more suitable for gene transfection (Fig. 1). Methanol/DMSO was chosen as a solvent system to enable high concentrations of reactants that could not be achieved in DMSO, methanol or water alone. NMR spectra showed that the incorporation of the crosslinker was nearly quantitative, however GPC analysis did not reveal a correlation between crosslinker ratio and molecular weight. Molecular weights (M_w) generally ranged from 30,000 to 50,000 g/mol. The lack of increase in molecular weight with greater crosslinker feed ratios was likely due to reaction between the activated carboxylic acid groups and methanol to form methyl esters on one of the two functional groups of the crosslinker.

These methyl esters subsequently hydrolyzed to form carboxylic acid groups during dialysis, thus decreasing interchain crosslinking while still incorporating a large amount of crosslinker. The presence of carboxylic acid groups in the crosslinked IPEI (xLPEI) polymers with high crosslinker ratios was confirmed by FTIR (see Supporting information). The ratio of the peak intensity of the carboxylic acid group to the tertiary amide group was much smaller for the 4% crosslinked polymers compared to the 8% crosslinked polymers.

3.2. Polyplex formation

Complexation of plasmid DNA was measured by exclusion of Picogreen (Fig. 2). Polyplexes were completely complexed at an N/P ratio between 5 and 10 for xLPEIs, commercial IPEIs, and 1.8 k bPEI. 25 k bPEI completely complexed DNA at an N/P ratio of 3, consistent with reports from Wu et al. [28]. The total amount of protection of DNA decreased with increasing crosslinker ratio. This is likely due to the introduction of carboxylic acids that neutralized the charge of the amines at high crosslinker feed ratios as mentioned above. When completely complexed, DNA was still more available to Picogreen in the xLPEI polyplexes (33%) than IPEI polyplexes (10%) and 25 k bPEI polyplexes (<5%). Polyplexes formed by xLPEIs in PBS had zeta potentials that were on average between +20 and +40 mV, with average diameters of approximately 350 to 500 nm (see Supporting information).

3.3. Transfection

Transfection of KB cells demonstrated that efficiency of non-degradable xLPEI was optimal at a crosslinker ratio of 4% (Fig. 3). At an N/P ratio of 40:1, 40% efficiency was achieved with over 90% viability, results consistent with those reported for similar materials [18]. This efficiency was slightly higher than that of 25 k bPEI, 25 k IPEI, and nearly as strong as Lipofectamine 2000. As expected, cell viability was much higher for the xLPEIs, though not for xL-2, which contained the lowest feed ratio of crosslinker. Cell viability increased monotonically with crosslinker ratio rather than achieving a maximum, as seen with transfection efficiency. xLPEIs required relatively high N/P ratios in

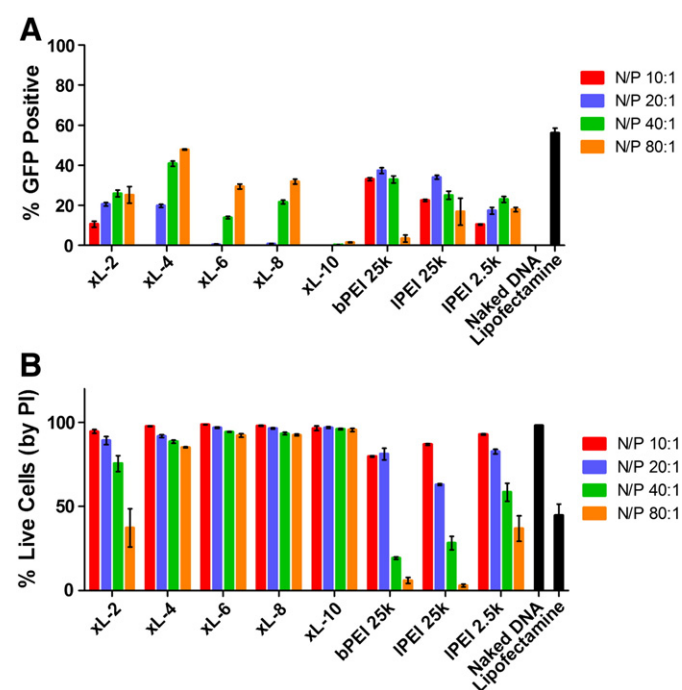


Fig. 3. (A) Transfection efficiency of cells transfected with nondegradable crosslinked IPEI as a function of crosslinking percentage and N/P ratio. (B) Relative viability of KB cells transfected in (A).

comparison to 25 k bPEI and IPEI, though xL-2 did show some transfection at N/P 10. Both transfection efficiency and cell viability were unchanged by the incorporation of a disulfide crosslinker (Fig. 4), suggesting that reduction-mediated xLPEI fragmentation may not be the primary means of DNA release from xL-SS polyplexes. Given the relatively weak DNA binding of the xLPEIs, release could be mediated electrostatically by exposure of the polyplex to the high concentrations of polyionic species in the intracellular environment and consequent shielding of the polyplex amine groups. The disulfide crosslinked xLPEI systems were readily degraded by DTT (see Supporting information), thus the similarity in release characteristics was not due to a lack of disulfide degradation as a result of our particular synthetic scheme.

3.4. Free polymer effects

To better understand the mechanisms by which xLPEI was able to transfect efficiently with low cytotoxicity, the importance of unbound polymer was investigated. Maximal transfection efficiency was observed at relatively high N/P ratios for xLPEIs (Fig. 3A). This finding suggests that a relatively large amount of free polymer is present in solution during transfection, which has been previously shown to increase transfection efficiency [28,29]. Previously, Wu et al. examined the effect of free versus polyplex-bound polymer by forming polyplexes at N/P ratios lower than typically used for optimal transfection, but sufficiently high to effectively condense DNA [28,30,31]. Here, this technique was used to decouple the effects of the free and polyplex-bound xLPEI. Cell uptake, transfection, and cytotoxicity were measured in two sets of experiments. First, the condensing, or polyplex-bound, polymer was kept constant (non-degradable 25 k bPEI) while the free polymer was varied (Fig. 5). Subsequently, the

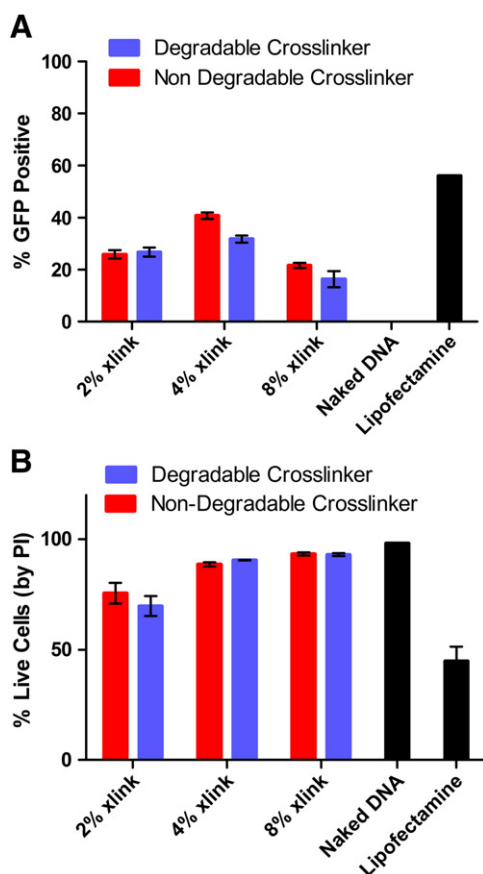


Fig. 4. (A) Transfection efficiency of cells transfected with crosslinked IPEI as a function of crosslinker degradability. Polyplexes were formed at an N/P ratio of 40:1. (B) Relative viability of KB cells transfected in (A).

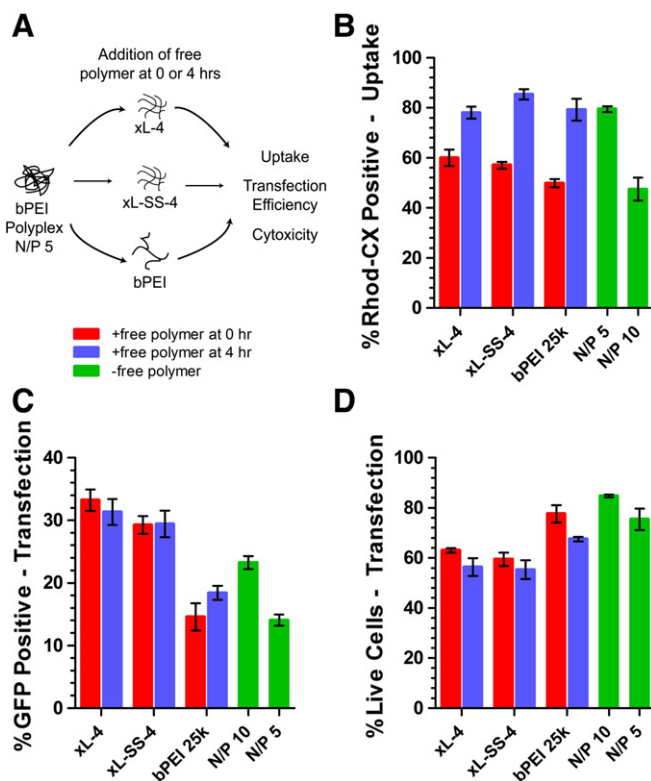


Fig. 5. (A) Experimental schematic. KB cells were treated with bPEI polyplexes formed at an N/P ratio of 5 and supplemented with free polymer at either 0 or 4 h. Cells treated with polyplexes formed at N/P 10 or N/P 5 and unsupplemented with free polymer were present as controls. Free xLPEI (xL-4 – succinic acid crosslinked, non-degradable, xL-SS-4 – disulfide linked, degradable) was added to form a total N/P ratio of 40. Free bPEI was added to form a total N/P ratio of 10. (B) Percentage of cells taking up Rhodamine-CX labeled DNA polyplexes as measured by flow cytometry 6 h after initial treatment. (C) Percentage of GFP positive cells 48 h after initial treatment. (D) Percentage of live cells 48 h after live treatment as measured by propidium iodide staining.

free polymer was held constant (xL-4) while the condensing polymer was varied (Fig. 6). In both cases, polyplexes were formed at N/P 10 (for xLPEI) or N/P 5 (for bPEI). These N/P ratios represent the lowest N/P ratio at which the maximum degree of complexation was reached for each system based on Fig. 2. No method of separation was used to remove free polymer as that would result in complexes that were thermodynamically unstable in media at physiological salt concentrations [29]. The remaining fraction of free polymer was added either immediately following transfection or 4 h later, after which the majority of the polyplex uptake is expected to have taken place.

Fig. 5 summarizes the effects of varying the free polymer while 25 k bPEI was held constant as the condensing polymer. Fig. 5B shows that cell uptake was high (greater than 50%) for bPEI, with or without added polymer; in fact the immediate addition of free polymer, regardless of composition, had a negative effect on bPEI polyplex uptake, agreeing with data from Boeckle et al. [29], who hypothesized that this was a result of competition by free polymer. When free polymer was added 4 h later, the original high cell uptake was recovered, supporting this explanation. Most importantly, there was no difference in uptake of bPEI polyplexes when the type of free polymer was varied. Thus uptake effects cannot explain the superior transfection of free xLPEI/bPEI polyplexes over free bPEI/bPEI polyplexes (Fig. 5C). This indicates that the superior efficiency of xLPEI is likely due to downstream events such as endosomal escape.

The addition of free xLPEI led to similar ~30% transfection efficiency using either degradable or non-degradable xLPEI (Fig. 5C). In fact, under the majority of conditions studied, there was no significant difference between degradable and non-degradable xLPEIs. Additionally, since

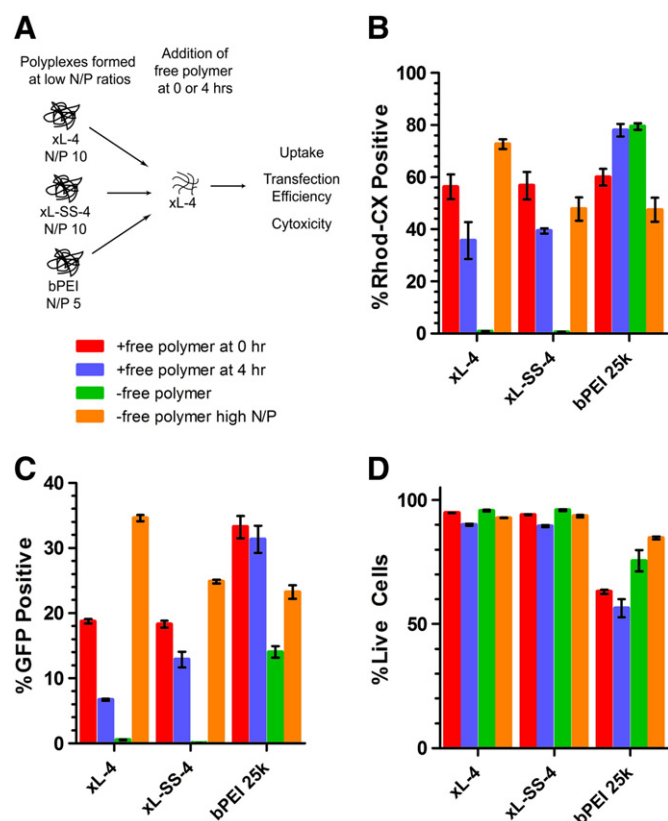


Fig. 6. (A) Experimental schematic. KB cells were treated with polyplexes formed at an N/P ratio of 5 (bPEI) or 10 (xLPEI) and either supplemented with free xL-4 polymer at 0 or 4 h, or not supplemented at all. Cells treated with polyplexes formed at optimal high N/P ratios (xLPEI N/P = 40, bPEI – N/P = 10) and unsupplemented with free polymer were also present as controls. Free xL-4 was added to form a total N/P ratio of 40. (B) Percentage of cells taking up Rhodamine-CX labeled DNA polyplexes as measured by flow cytometry 6 h after initial treatment. (C) Percentage of GFP positive cells 48 h after initial treatment. (D) Percentage of live cells 48 h after live treatment as measured by propidium iodide staining.

bPEI polyplexes must unpackage without the assistance of degradable linkers, the high efficiency achieved using free xLPEI supports the hypothesis that reduction-mediated unpackaging does not contribute to the high efficiency of xLPEI. Cytotoxicity (Fig. 5D) actually increased when xLPEIs were used as free polymer instead of 25 k bPEI, opposite of what might be expected based on standard transfection with homogeneous polyplexes (Fig. 3B). This may be due to xLPEI-mediated increased cytosolic delivery of bPEI, increasing the opportunity for bPEI to interact with mitochondria and cause apoptosis.

Next, the free polymer was held constant (xL-4) to compare the behavior of xLPEIs as condensing polymers (Fig. 6). xLPEI samples xL-4 and xL-SS-4 performed similarly, despite differences in degradability, as noted above. For the xLPEIs, the addition of free polymer was necessary to observe any uptake at all by cells (Fig. 6B). In the presence of free xLPEI added immediately, there was a significant boost in cell uptake (60%). However, when free xLPEI was added after 4 h, the delay led to a decreased uptake of 30–35% (Fig. 6B). Decreased uptake when xLPEI polyplexes were incubated in the cell culture media for extended periods could be due to gradual aggregation or the destabilization of the complexes in culture conditions over time. Cellular uptake of xLPEI complexes thus appears to depend on the presence of additional polymer; this additional polymer may interact with the cell membrane or further condense particles to facilitate entry of the polyplexes to the cell. However, the size of polyplexes formed in Opti-MEM was not significantly reduced when free polymer is added (see Supporting information). While further complexation by free polymer may play some role in increasing uptake for the loosely complexing xLPEI as Fig. 6B suggests, Fig. 5B and

C shows that xLPEI was clearly influencing the transfection process downstream of uptake as well, likely by enhancing endosomal escape.

In Fig. 6C, it can be seen that efficiency was generally higher using bPEI instead of xLPEI as the condensing polymer. Additionally, bPEI polyplexes did not suffer a drop in transfection efficiency when free polymer was added at 4 h rather than immediately (Fig. 6C). More generally, relative transfection efficiency followed the same trend as relative uptake (Fig. 6B). This implies that downstream events such as endosomal escape were achieved with similar efficiency, possibly as a result of the free polymer consistent across treatment groups. A notable exception was the bPEI complexes without free polymer added, which were taken up efficiently but did not transfect well, further indicating a role for xLPEI in facilitating endosomal escape. As was seen in earlier transfections, xLPEI was nearly non-toxic, while bPEI showed significant toxicity when used as the condensing polymer (Fig. 6D).

3.5. Endosomal escape

To determine the efficiency of free polymer in facilitating endosomal escape, a high-throughput calcein assay was used as previously described [27]; results of the assay are shown in Fig. 7A for each of several

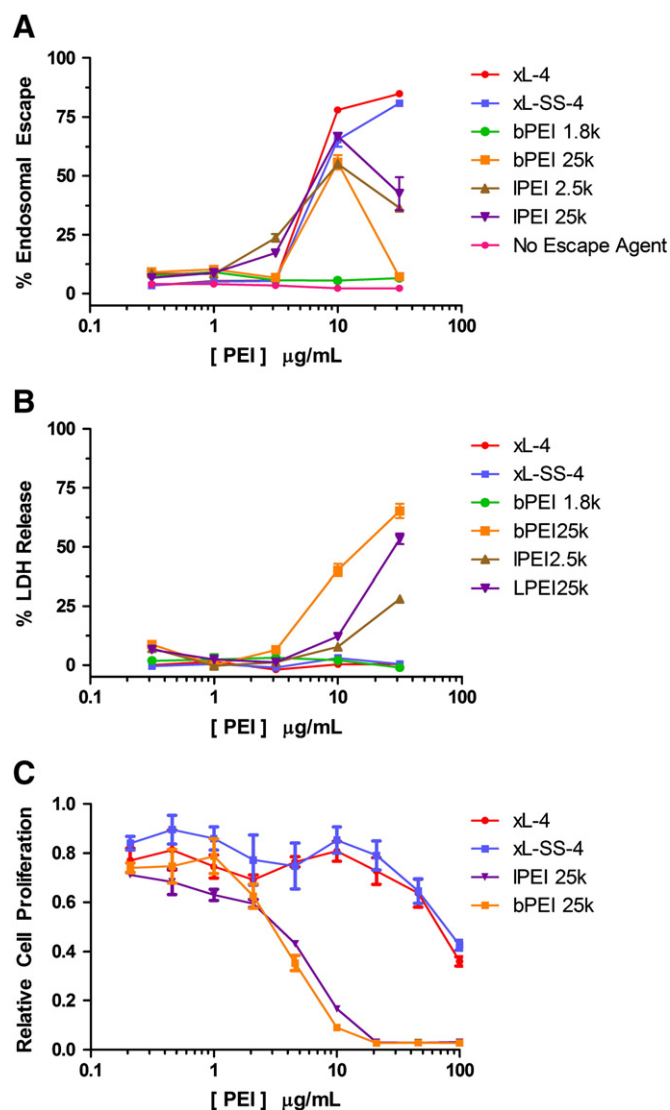


Fig. 7. (A) Activity of lactate dehydrogenase (LDH) in the supernatant of cells treated with crosslinked and commercial PEIs. (B) Percentage of cells exhibiting endosomal escape in high-throughput calcein assay (see methods) when treated with crosslinked and commercial PEIs. (C) Relative viability of cells as measured by an MTT assay.

PEI polymer systems. xLPEIs caused significant endosomal escape above a critical concentration, between 3 and 10 $\mu\text{g/mL}$, with a steep increase in efficiency to over 75%. In contrast, commercial lPEI showed a more gradual increase in escape, facilitating calcein escape in over 50% of cells at 10 $\mu\text{g/mL}$. 25 k bPEI was also able to cause calcein release at 10 $\mu\text{g/mL}$, while bPEI 1.8 k was not able to effect any release up to 30 $\mu\text{g/mL}$. The commercial PEIs showed a decrease in escape with concentrations above 10 $\mu\text{g/mL}$, possibly due to calcein leaking out of the cells entirely as a result of membrane damage. In order to determine the extent of membrane destabilization, LDH leakage was measured as a function of free polymer concentration (Fig. 7B). 25 k bPEI and lPEI showed significant membrane damage above 3 $\mu\text{g/mL}$, as did lPEI 2.5 k. Both xLPEI species did not show any membrane damage at concentrations up to 30 $\mu\text{g/mL}$, despite facilitating escape in over 75% of cells at this concentration. xLPEIs also demonstrated a ~ 1.5 log increase in LD50, with no toxicity effects at 10 $\mu\text{g/mL}$, the concentration at which escape becomes highly efficient (Fig. 7C).

4. Discussion

As mentioned in the [Introduction](#), different PEI crosslinking techniques have been reported, generally utilizing either a bifunctional crosslinker or derivitizing primary amines into thiols for subsequent disulfide formation. We opted to use EDC coupling, as it requires only dicarboxylic acid functionality from the crosslinker. The bifunctional crosslinker can be designed to be responsive to various stimuli, and crosslinking can be achieved in a one-pot approach in various solvents, depending on the solubility of the crosslinker. The choice of polycation may also be varied, as only primary or secondary amines are required for crosslinking — here lPEI was chosen due to favorable efficiency and toxicity reports from previous work [18]. This synthetic approach is potentially amenable to a combinatorial development with a library of polycations and linkers responsive to different stimuli. For this study, a reducible and non-reducible crosslinkers were chosen for incorporation. While crosslinker functionality was the critical feature for this design, the lengths were slightly different and thus a future study on the impact of crosslinker length could be of interest. The approach taken here resulted in crosslinked polymers with nearly quantitative integration of crosslinker. However, as noted in the [Results](#) section, the presence of methanol in the solvent system resulted in free carboxylic acid formation at higher crosslinker ratios, decreasing the positive charge and limiting the molecular weight. Wu et al. demonstrates the effect of controlled addition and an oxygen-free environment on the crosslinking of bPEI [32], however we desired to keep the synthesis simple and scalable for future combinatorial implementation.

xLPEI was able to form polyplexes, though xLPEI polyplexes were looser than those formed with commercial 25 k bPEI. DNA was more accessible, even at very high N/P ratios, than the 25 k bPEI and lPEI standards, likely a result of reduced net charge and thus reduced efficacy of DNA condensation. Gosselin et al. demonstrated how different crosslinker chemistry could be used to avoid this charge neutralization [7]. Transfection efficiencies achieved by xLPEIs agree well with those previously reported for KB cells [18]. The decrease in efficiency at higher crosslinking ratios is likely explained by the high amount of charge neutralization and poor complexation efficiency. Cell viability increases monotonically with crosslinker ratio, and hence charges neutralization, which may be the primary mechanism for decreased toxicity at the higher crosslink densities. At high N/P ratios, toxicity is likely dominated by free polymer effects, thus changes in polyplex size or charge should not have an impact on toxicity. Of great interest was the fact that both transfection efficiency and cell viability varied independent of crosslinker choice. This indicates that disulfide degradation may not be critical for either transfection or reduced toxicity in this xLPEI system.

The high transfection efficiencies shown in Fig. 3 were achieved at high N/P ratios, indicating that free polymer likely plays an important

role in transfection. Wu et al. found that in a typical N/P 10 formulation of 25 k bPEI, approximately 70% of the bPEI is free in solution. This agreed well with results from Wagner in which size exclusion chromatography was used to separate free polymer from polyplexes [29]. In both studies, it was found that transfection efficiency in the absence of free polymer was two orders of magnitude less efficient, but could be restored by the addition of free polymer, either immediately or several hours after initial polyplex addition. To investigate whether the high transfection efficiency was due to superior properties of xLPEI as free polymer, as a DNA condensation agent, or some combination of the two, a series of experiments was carried out in which 25 k bPEI was substituted for xLPEI for the above functions. The increase in transfection efficiency when xLPEI is used as free polymer along with 25 k bPEI polyplexes indicates that free xLPEI plays an important role in the increased transfection efficiency. To determine if the free polymer was functioning primarily to stimulate uptake of polyplexes, polyplex uptake was measured using labeled DNA. When 25 k bPEI was used to form polyplexes, uptake was relatively independent of free polymer addition, indicating that the primary role of free xLPEI was downstream of uptake. Cell viability was dependent on both the choice of condensation polymer and free polymer, as systems with 25 k bPEI included in either capacity experienced significant cytotoxicity. Differences in either efficiency or viability between degradable and non-degradable xLPEIs were absent in most cases. This is consistent with xLPEI free polymer being the dominant factor in generating high transfection levels, as cytosolic polyplex unpackaging, a property improved by disulfide degradation, would not depend on free polymer.

In experiments in which xLPEI was kept constant as the free polymer, uptake was greater when 25 k bPEI was used as the condensing polymer versus xLPEI. xLPEI was shown to be inefficient at both uptake and transfection in the absence of free polymer, suggesting that free polymer may be necessary for additional complexation prior to uptake. Additional polyplex condensation by free polymer was not seen in sizing experiments (see Supporting information), but Fig. 6 still suggests some role for this process. The role of free xLPEI in this system thus depends on the identity of the polyplex used; for loose polyplexes it serves to additionally complex polyplexes after addition and for all polyplexes it enhances transfection processes downstream of uptake, such as endosomal escape.

Endosomal escape was probed rapidly using a high-throughput implementation of a calcein release assay reported previously by our group [27,33]. xLPEIs were able to mediate escape in over 60% of cells at 10 $\mu\text{g/mL}$, the approximate concentration of free polymer in a 40:1 N/P formulation. In comparison, 25 k bPEI caused escape in less than 10% of cells at 3 $\mu\text{g/mL}$, approximately the concentration at which free polymer exists at the optimized 25 k bPEI formulation of N/P 10:1 [28]. 25 k bPEI was effective in causing escape at 10 $\mu\text{g/mL}$, but at slightly lower efficiencies than xLPEI, and causing significant membrane damage. This membrane damage also manifested itself as a loss of calcein at 30 $\mu\text{g/mL}$. However, xLPEI showed no evidence of membrane damage at 30 $\mu\text{g/mL}$, a concentration at which escape was seen in over 75% of cells. Overall cell viability was also shown to be substantially better for xLPEI and no loss of viability was seen at 10 $\mu\text{g/mL}$, the concentration that achieved escape in over 60% of cells. Taken together, this shows that free xLPEI is able to effectively mediate endosomal escape without the membrane toxicity observed in commercially available PEIs.

In summary, we have shown that polycations crosslinked with a disulfide linkage are not necessarily highly efficient and less toxic as a result of the disulfide linkage itself. In the case of the xLPEIs synthesized in this work, free polymer was shown to be primarily responsible for the high efficiency and decreased toxicity of this system. Free xLPEI was shown to be effective in mediating endosomal escape with no observed membrane toxicity. More investigation into the role of free polymer in delivery of DNA will increase the understanding of how polymer structure and delivery function are related. Further study of the *in vivo* implications of transfection mediated by free polymer could aid the design of

polymer gene delivery systems. Systems that do not require an excess of polymer to remain complexed in vivo will afford greater flexibility for formulation, as they are not limited to a specific minimum dose of free polymer. Consideration of polyplexes and free polymer as independent populations may also encourage evaluation of hybrid systems, in which one polymer is used to form polyplexes and another to potentiate downstream events such as endosomal escape. Here we find that xLPEI, with either degradable or nondegradable crosslinks, is a potentially viable system to use as an endosomal escape mediator. Focused in vivo experiments determining the biodistribution, toxicity, and efficacy of systems containing free polymer will clarify future design goals for these polymer gene delivery systems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2012.09.004>.

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