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Specific effects of PEGylation on gene delivery efficacy of polyethylenimine: Interplay between PEG substitution and N/P ratio

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ABSTRACT

While an effective non-viral gene carrier, 25 kDa branched polyethylenimine (PEI) is cytotoxic, and decreasing its toxicity while maintaining its functionality is vital. Conjugation of carriers with polyethylene glycol (PEG) is a common approach to decreasing toxicity and improving biodistribution; however, the effect of PEGylation on PEI transfection efficacy is contradictory at present. The aim of this work was to reveal the details of this dependence. Polymers were synthesized by grafting 2 kDa PEG to 25 kDa PEI at multiple ratios. Unlike typical investigations, parallel studies based on either total polymer weight or PEI-backbone weight were employed at the same time for accurate investigation into the specific effects of PEGylation. Polymers were assessed for toxicity and plasmid DNA (pDNA) binding, while polyplexes were formed at various polymer/pDNA weight ratios and monitored by dynamic light scattering (DLS) in the presence of serum. The efficacy of the polyplexes for pDNA delivery and transgene expression in HEK293 cells was assessed by flow cytometry. This approach unexpectedly revealed that increased PEG substitution caused lower toxicity and pDNA-binding on a per total polymer weight basis, but not on a per PEI-backbone weight basis. DLS indicated that high PEGylation prevents an increase in polyplex size in the presence of serum. Plasmid uptake and transgene expression were found to have a complex relationship with PEG substitution, dependent on the polymer/plasmid-DNA weight ratio. PEGylation generally decreased the transfection efficacy of PEI, but under ideal conditions of PEG substitution and polymer/pDNA ratio, PEGylation provided more effective carrier formulations than the native PEI itself.

1. Introduction

Polyethylenimine (PEI) was one of the first polycations to act as a successful transfection reagent [1]. Its capacity to complex nucleic acid is attributed to formation of electrostatic interactions between the anionic phosphate backbone of nucleic acids and its high density of charged amines. Upon condensation, the positively charged polyplexes enter cells by various uptake mechanisms, depending on both cell type and particle size [2–5]. The gradual decrease in pH during endocytosis is thought to cause a proton sponge effect by which increased protonation of PEI causes an influx of counterions and an inward osmotic gradient, hence rupturing the vesicular membrane and releasing entrapped polyplexes [6,7]. The ability of PEI to effectively deliver nucleic acid intracellularly has led to extensive investigation into the effectiveness of various forms of the polymer in terms of molecular weight (MW) and degrees of branching [8,9]. Despite optimization of the PEI by modifying the architecture of the polycation, the main detriment of PEI remains its toxicity. The cytotoxic effect of PEI has been attributed to both necrotic and apoptotic mechanisms resulting from membrane damage and can be reduced by decreasing branching and MW [10–12]. One solution to the toxicity problem is incorporation of biodegradable linkages in PEI-based polymers to facilitate their breakdown to less toxic smaller PEI [13–15]. This modification, however, conceivably has no effect on the toxicity of the polymer in its intact high MW state. Additionally, it fails to address other problematic issues concerning the biodistribution of PEI polyplexes when used for in vivo applications [16].

An alternative solution to PEI's shortcomings is to covalently attach polyethylene glycol (PEG) to PEI polymers. The highly hydrophilic nature of PEG produces a hydration shell around its conjugated partner, hence reducing intermolecular interactions and resultantly decreasing toxicity. PEG incorporation is an established method to improve the biodistribution of nanocarrier systems, including a variety of polyplexes and PEI-based systems

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[17–20]. PEI synthesis with bifunctionalized PEG to form alternating block copolymers has been performed with a variety of linkage chemistries [21–23]. Similarly, simply grafting PEG to PEI has been performed using a variety of PEG MW and degrees of substitution [24–28]. In all studies where the toxicity is assessed, covalent modification of PEI with PEG appears to reduce the toxicity of the polymer, but its specific effect regarding nucleic acid delivery was conflicting and/or poorly defined.

In terms of transfection efficacy, some studies have suggested that incorporation of PEG into PEI-based polymers has either little effect or possibly a beneficial effect, depending on PEG MW and ratio of polymer to nucleic acid [23–25]. Others demonstrated that PEGylation generally decreases the effectiveness of PEI [28–30], but this inhibitory effect may depend on the payload molecule itself (pDNA or siRNA) and whether nuclear delivery is required or not. This contention is further complicated by the fact that the majority of studies assessing transfection are pursued by normalizing transfection to protein concentrations (i.e., luciferase assays) rather than by individual cell analysis using flow cytometry. The latter is more precise, especially in situations where toxicity is involved and typical normalization methods (e.g., per protein or DNA unit) conceal the investigated effect. Finally, reported studies frequently do not account for the contribution of PEG to total polymer weight when comparing polymers on a per weight basis. This leads to comparisons of different concentrations of PEI backbone and does not accurately assess the effect of PEGylation on PEI. Considering that PEI is a leading non-viral alternative for gene therapy and that PEGylation is a firmly established method of improving biocompatibility, addressing this controversy and clarifying the effect of PEG on PEI-facilitated gene delivery is imperative.

Both the ratio of polymer/nucleic acid and the degree of PEG substitution are critical factors for transgene expression. The two variables may be responsible for both increasing and decreasing the effectiveness of PEGylated PEI in an interdependent manner [27,31]; however, this relationship is poorly defined at present. For this reason, the present work reports a focused examination of these two variables and their relationship to physical properties and transfection efficiencies of PEI–PEG polyplexes using a range of polymer/pDNA ratios and PEG substitutions. These studies were approached in a manner that addresses the effects of PEGylation on a per-polymer weight basis (i.e., using total polymer weight as the independent variable) and on a per-PEI backbone weight basis (i.e., using PEI backbone weight as the independent variable) in order to accurately discern the specific effect of PEGylation on PEI. This parallel approach was not previously utilized and permitted a novel examination into which effects of PEGylation previously described without PEG weight correction fail to persist when analyzed more rigorously on a per-backbone basis.

2. Materials and methods

2.1. Materials

Both 25 kDa branched PEI and trinitrobenzene sulfonate (TNBS) were purchased from Sigma–Aldrich (St Louis, MO), and 2 kDa mPEG-NHS (succinimidyl carboxy methyl ester of PEG) was purchased from Creative PEG Works (Winston-Salem, NC). The sources of all plasmids and tissue culture reagents were described previously [32]. SYBR Green I was purchased from Invitrogen (Carlsbad, CA). Cy3 labelling kit was purchased from Mirus Bio (Madison, WI).

2.2. PEI–PEG synthesis and characterization

PEG grafting was accomplished by conjugating mPEG-NHS to PEI amines (Fig. 1). PEI–PEG polymers were synthesized by combining 4 mg ml^{-1} branched 25 kDa PEI dissolved in phosphate buffer (0.1 M pH 7.2) with an equal volume of 75% DMSO containing mPEG-NHS at 0.5, 2.5 and 7.5 mM. After reacting for 4 h at room temperature, conjugates were extensively dialyzed against double-distilled dH_2O and lyophilized. Polymers were analyzed by ^{1}H-NMR in D_2O and the degree of PEG substitution was determined by comparing the peaks of CH_2CH_2O (~3.6 ppm) with CH_2CH_2N (~2.5–3.1 ppm). Differences in primary amines between each polymer were also detected via a colorimetric assay, in which 20 μl of aqueous polymer was combined in a 96-well plate with 180 μl TNBS solution (0.34 mM TNBS in 50 mM borate buffer, pH 9.0). Following 0.5 h incubation at 37 °C, absorbance of each sample was assessed at 405 nm with a microplate reader. The slope (m) of each polymer (Δ absorbance/Δ total polymer concentration) was compared with the slope of unmodified PEI to determine the percentage modification of primary amines: $100\% \times \{[m(\text{unmodified PEI}) - m(\text{polymer of interest})] \div [m(\text{unmodified PEI})]\}$.

2.3. Assessment of polymer toxicity

The details of cell culture and passage were described in Ref. [32]. Polymers were added to HEK293 cells in 24-well plates 24 h after cell seeding. In one study, the amount of polymer added was based on the total polymer weight and, in another, only the weight of the PEI backbone was accounted to correct for PEG contribution to the total polymer weight. Both studies were performed in triplicate. After 24 h, 100 μl of 5 mg ml^{-1} methylthiazolydiphenyl-tetrazolium bromide (MTT) was added to each well and incubated for 2 h, after which medium was replaced with 0.5 ml DMSO. Then 100 μl of each sample was transferred to a 96-well plate and the absorbance read at 570 nm. Relative cell viability (%) was determined by normalizing with untreated cells.

2.4. Assessment of polymer–DNA binding affinity

The complexes of polymers and plasmid DNA (pDNA) were formed with polymer/DNA weight ratios from 0 to 8. Two distinct studies were performed using polymer concentrations based on total polymer weight and PEI component alone. Two microliters of 0.025 μg μl^{-1} gWIZ-GFP was vortexed with 118 μl polymer of various concentrations (in 150 mM NaCl). After 30 min, 200 μl of SYBR Green I (1X) was added and gently vortexed, and 200 μl of complexes were transferred to a 96-well plate for analysis (λ_{ex} = 485 nm, λ_{em} = 527 nm). Polyplexes were assessed for percentage complexation by comparing the fluorescence (F) of samples with the reference which contained pDNA but no polymer, and the background which contained neither: $100\% \times \{[F(\text{DNA only}) - F(\text{polymer/DNA weight ratio})] \div [F(\text{DNA only}) - F(\text{background})]\}$. Three parameter sigmoidal curves for each polymer (SigmaPlot11) and bc_{50} values (polymer/DNA ratio with 50% binding) were determined. Two independent quintuplicate experiments were performed for each experimental group. For native PEI, the nitrogen/phosphorus (N/P) ratio for PEI/pDNA complexes was assumed to be 7.74 for a PEI:pDNA weight ratio of 1:1. For PEG-modified PEI, the N/P ratio in the complexes was calculated by considering the weight fraction of PEI alone in the complexes. This was followed by correction for the amines consumed by PEG grafting from the integrated ^{1}H-NMR.

2.5. Dynamic light scattering

Polyplexes were formed by combining polymers (2 mg ml^{-1} in 150 mM NaCl) with 4 μg gWIZ-GFP pDNA made up to a total volume of 100 μl with 150 mM NaCl, and immediately vortexing. Polymer/pDNA weight ratios were 2, 5 and 10 (determined with and without PEG weight correction). Following 30 min incubation,

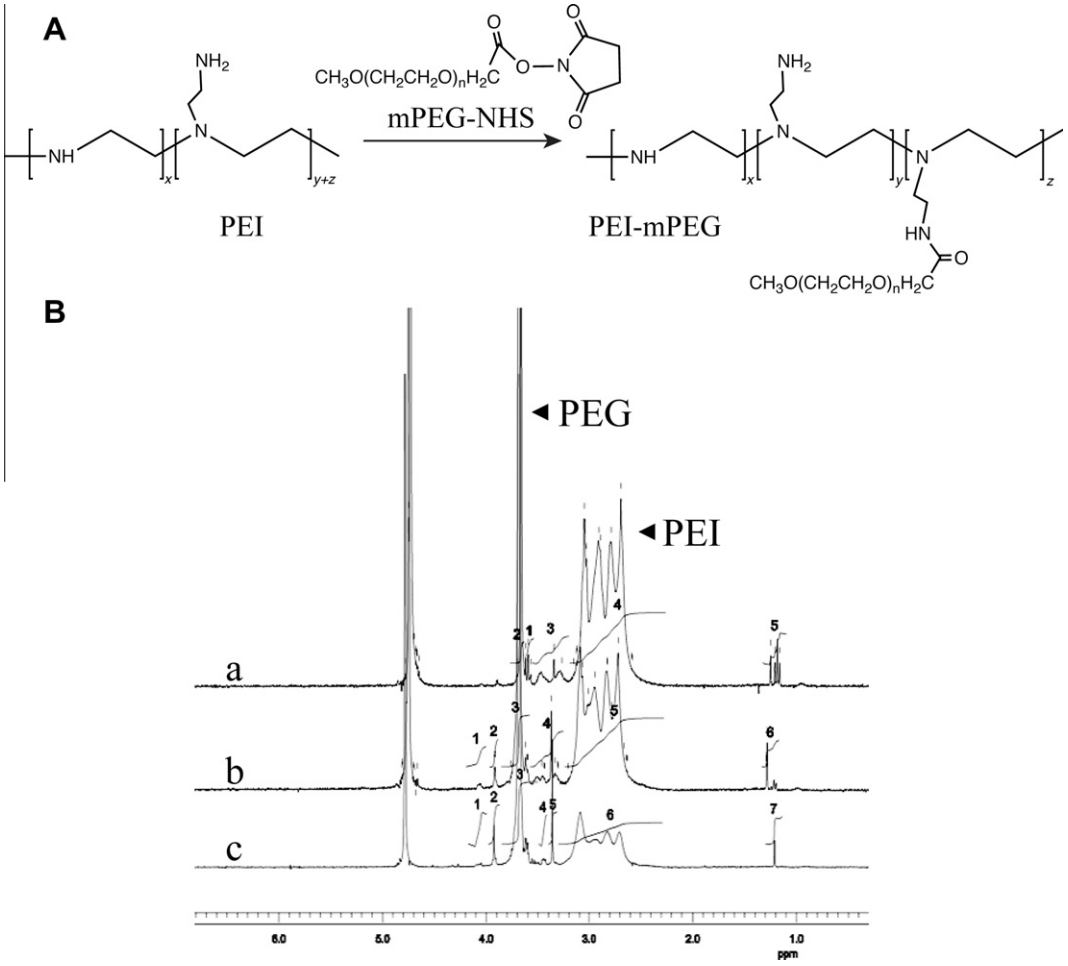

Fig. 1. (A) Reaction scheme for PEGylation of PEI via NHS moiety of functionalized methoxy-PEG. (B) ^{1}H-NMR spectra of polymer products. Conjugates were synthesized by reacting 25 kDa branched PEI with varying concentrations of 2 kDa mPEG-NHS: (a) 0.25 mM; (b) 1.25 mM: (c) 3.75 mM. By comparing the peaks of CH_2CH_2O (~3.6 ppm) with CH_2CH_2N (~2.5–3.1 ppm), the PEG substitutions of the three conjugates were determined as 2.7, 13.6 and 37.2 PEG chains/PEI backbone.

complexes were added to 0.6 ml medium with 10% serum. DLS was performed immediately after adding to medium and 1 h later, using a Brookhaven ZetaPlus. Five consecutive measurements (20 s) were made for each sample (25 °C, 660 nm, real = 1.333 imag. = 0). Two independent samples were prepared per experimental group.

2.6. DNA uptake studies

gWIZ was labelled using a Mirus Cy3 Label IT kit, and the extent of labelling was determined using A_{260} and A_{550} values, as per the manufacturer's instructions. The reacted plasmid was found to have ~24 labels per plasmid and was diluted with unlabelled plasmid such that there was an average of five labels per plasmid, and the final gWIZ concentration was 0.4 mg ml^{-1}. HEK293 cells were seeded 24 h prior to transfection in 48-well plates (in triplicate). Complexes were formed as for DLS measurements with either labelled or unlabelled gWIZ (control) and added to cells 30 min after vortexing (0.5 μg DNA well^{-1}). After 5 h, the medium was replaced, cells were prepared for flow cytometry 24 h post-transfection and analyzed for Cy3 fluorescence (FL2 channel [32]).

2.7. Assessment of transgene expression

Complexes were formed as for DLS measurements with either gWIZ-GFP or gWIZ (blank control) and added to HEK293 cells in 24-well plates 30 min after vortexing (1 μg DNA well^{-1}). After 5 h, the medium was replaced, and cells were processed for flow cytometry and analyzed for GFP fluorescence (FL1 channel) after 48 h, as described in [32]. Two independent experiments were performed in triplicate. Gating of positive cells was performed by setting a control of untreated cells to exhibit 1–1.5% positive events. Since cells transfected with gWIZ blank plasmid gave autofluorescence values identical to that of untreated cells, these results were not shown for uptake and transfection studies.

2.8. Statistical analysis

The results shown are summarized as mean ± SD of the indicated number of experiments (see legends). Where indicated, the coefficient of correlation (r^2) was calculated using Pearson's correlation method. Statistical differences were investigated by ANOVA ($p < 0.05$) and Tukey post hoc tests.

3. Results and discussion

3.1. Polymer characterization

PEGylation of PEI was confirmed by ^{1}H-NMR (Fig. 1). ^{1}H-NMR spectra integration indicated PEG substitutions of the three conjugates to be 2.7, 13.6 and 37.2 PEG chains/PEI backbone, which result in polymers consisting of 17.7 wt.%, 52.1 wt.% and 74.8 wt.%

PEG (Table 1). The colorimetric TNBS assay for the PEI primary amines demonstrated (Fig. 2a) that grafting reactions with a higher mPEG-NHS concentration produced polymers with a relatively decreased slope in the TNBS assay (m: Δabsorbance/Δtotal polymer concentration). From these slopes, the extent of primary amine modifications for the three conjugates were calculated to be 52.9%, 79.4% and 99.9%. The absolute accuracy in the latter case is expected to be limited by the sensitivity of TNBS assay. Based on the ^{1}H-NMR and TNBS data, wt.% PEG composition and% primary amine modification corroborated each other with a significant Pearson correlation (Fig. 2b; $r^2 = 0.9063$, $p = 0.0485$). Hence, direct modification of the PEI backbone with PEG was apparent, as opposed to simply a mixture of separate unconjugated reagents in the product. This assortment of polymers with a range of confirmed substitutions permitted a comprehensive survey of the effects of PEGylation on PEI polyplexes in regard to their toxicity, effectiveness and physical properties.

3.2. Effect of PEG modification on polymer toxicity

The MTT assay indicated that PEGylation decreased the toxicity of PEI (Fig. 3a). However, this could be as a result of decreasing amount of PEI added to the cells as the PEG contribution to total polymer weight was increased. When the amount of each polymer added to the cells was corrected so that an equal amount of PEI backbone was added per well, PEGylation appeared to have no effect on toxicity (Fig. 3b). Toxicity data were then re-plotted to explore correlations between the wt.% PEG and the observed viability. This analysis revealed no significance for any concentration when an equal amount of backbone was applied to the cells (Fig. 4b) whereas, without this PEG weight correction, significant correlations were found (Fig. 4a). For polymer concentrations 5, 10 and 15 μg ml^{-1}, the correlation coefficients (r^2) were 0.968, 0.964 and 0.988, respectively ($p = 0.0162$, $p = 0.0185$, $p = 0.0062$). Hence, it is concluded that PEGylation was effective in decreasing toxicity on a per-polymer basis, but not on a per-PEI backbone basis.

The observation that incorporation of PEG decreases the toxicity of PEI on a per-polymer basis is well supported by previous studies [23,27,31]. This was expected, as these studies conducted toxicity assays by varying the weight of polymer added to cells and the toxic component, PEI backbone, decreases in proportion to total polymer weight with PEG substitution. All these studies cannot definitively conclude that PEG decreases the toxicity of PEI on a per-backbone basis. Studies that form polyplexes at identical N/P ratios with polymers with varying amounts of PEG composition address this question more accurately. Cytotoxicity assays previously performed in this manner generally indicated that the increased PEG composition decreased toxicity [24,28,30]. This is contradictory to the findings here that, on a per-PEI backbone basis, PEGylation had no effect on toxicity. The major difference between the reported studies and this work is that they assessed the toxicity of their polymer library by forming complexes, whereas the present study used free polymer. Considering that PEI-based polymers are more toxic in free form than formulated as a complex [28], it is possible that the differences in toxicity were not detected as a result of free polymers having similar (and greater) toxicities, whereas with complexed polymers, differences in toxicity due to PEG composition may be more apparent. This is logical, considering that differences in PEG substitution would most likely produce greater hydration differences at polymer–environment interfaces in a smaller volume (a polyplex) than in a larger volume (a free polymer) where there is more exposed PEI backbone and hence more opportunity for PEI-environment interaction uninhibited by PEG. The differences between the parallel per-polymer and per-backbone studies may have particularly important implications for in vitro polyplex incubations over extended periods of time, as well as for in vivo administration.

3.3. pDNA binding by PEGylated PEI

The effect of PEGylation on pDNA binding is summarized in Fig. 5, where the binding was independently assessed as a function of total polymer concentration (Fig. 5a) or PEI backbone concentration alone (Fig. 5b). Binding curves with polymer/pDNA (w/w) ratios determined using the total polymer weight shifted to the right with increasing PEGylation, whereas no shifts were seen if the polymers were added such that an equal amount of PEI backbone was present in each solution. This indicated that PEGylation only affected binding as a result of its contribution to the weight of the total polymer. Upon combining the data from both studies by converting the w/w ratios to N/P values, the same pattern was seen: PEGylation only shifted the binding curves rightward if unequal PEI backbone was being added (Fig. 6a). A correlation between the bc_{50} values and the percentage of PEG composition was then pursued based on the binding curves obtained. The results (Fig. 6b) showed increasing bc_{50} with increasing % PEG content in each polymer ($r^2 = 0.922$, $p = 0.0404$), indicating a detrimental effect of the presence of PEG in polymer formulation. However, when equal PEI backbone was present, there was no correlation between the bc_{50} values and the % PEG content in each polymer. Hence, on a per-polymer basis, PEGylation affected DNA binding, but not on a per-PEI backbone basis.

Decreased DNA binding due to increased PEG composition has been noted before [24,27,28,31]. These studies monitored DNA binding by adjusting the N/P ratio, and all but one noted a decreased binding affinity specifically with 25 kDa PEI conjugated to 2 kDa PEG (the same MW PEG used in this work). They are in disagreement, however, with the finding that, when equal backbone was present for each polymer, no differences in binding were noted as a function of N/P ratio or weight ratio. The observations reported by other groups were in agreement with the present binding curves that were based on the total polymer weight. Binding differences noted between studies with and without PEG weight correction using the parallel approach implies that, despite PEGylation, much of the PEI backbone remained accessible for DNA binding. This may suggest that steric effects are negligible for the range of substitutions tested, or a conformational change in polymers permits efficient binding to DNA. The binding curves in Fig. 6, however, are not completely overlapping, and it is possible that (i) subtle differences among PEGylated samples and (ii) experimental errors might have prevented complete overlap among the binding curves.

3.4. Sizes of PEI–PEG polyplexes

DLS measurements indicated that most polyplexes were between 200 and 300 nm (Fig. 7). Particle size generally increased after 60 min incubation in medium with 10% serum compared with initial sizes, and this increase appeared more prominent with increasing polymer/DNA and PEI backbone/DNA ratios. PEGylation appeared to prevent the size increase, especially at the highest PEG substitution. In general, no remarkable differences in size were noted between particles formed with either equal polymer or equal PEI backbone. Particle sizes at both time points also tended to increase with increasing polymer/DNA and PEI backbone/DNA ratios. The only exception was the most PEG substituted polymer; for the study based on PEI backbone/DNA ratios, all ratios produced similar sized polyplexes, while for the study based on polymer/DNA ratios, the size decreased from a ratio of 2 to 5 and then slightly increased from 5 to 10. The polyplex with the most striking difference in size after 60 min incubation in 10% serum was formed

Table 1
Degree of substitution of branched PEI (25 kDa) with PEG (2 kDa) determined using the ^{1}H-NMR spectra by comparing the peaks of CH_2CH_2O (~3.6 ppm) with CH_2CH_2N (~2.5–3.1 ppm).

[PEG]/[PEI] ratio in synthesis	PEG substitution (PEG chains/PEI)	Total polymer MW (kDa)	Wt.% PEG (PEG/total polymer)	% amines modified (from NMR)	Weight correction factor
0/0.08 mM	0	25.0	0.0	0.0	1
0.25 mM/0.08 mM	2.7	30.4	17.7	0.5	1.214
1.25 mM/0.08 mM	13.6	52.2	52.1	2.3	2.086
3.75 mM/0.08 mM	37.2	99.3	74.8	6.4	3.974

The degree of substitution was used to calculate the proportion of PEG weight to PEI weight and amine modification for each polymer; the weight correction factor was also calculated in order to determine the weight of each polymer required to have the same weight of backbone as unmodified PEI.

using the moderately substituted polymer (13.6 PEG/PEI) at a PEI backbone/DNA ratio of 10. Given that only one preparation exhibited this notable increase, this may be indicative that the aggregation of PEI–PEG polyplexes was the result of a multivariable dependence, which presumably works in a non-linear fashion over some ranges of PEG substitution, PEI/DNA weight ratio and incubation time.

The sizes in this study were generally larger than previous DLS measurements of PEI–PEG polyplexes. In some cases, this lower size range (~100 nm at 0 min) may be attributed to the use of low ionic buffers, such as 5 mM HEPES or 10 mM NaCl [23,30]. Larger size ranges (150–250 nm) closer to the range observed here were noted, using solutions with ionic strengths more similar to the cell medium, such as PBS or 150 mM NaCl [27,29]. Presumably, the higher ionic strength allowed better shielding of polyplexes, facilitating their interactions and formation of aggregates in solution. The presence of 10% serum, which was avoided by most studies, may explain the greater size of polyplexes observed in this work. In one study, where the size measurements of PEI polyplexes were reported in 1% serum, the observed sizes (210–663 nm) were in line with the current study at similar DNA and polymer concentrations [32].

The previous studies were in agreement with the present authors' observations that PEGylation generally decreased polyplex size, particularly after extended incubation. This is most likely the result of the hydration shell produced by PEG providing steric protection of polyplexes. While the present studies noted a general increase in polyplex size with increasing polymer/DNA ratio, both Zhong et al., who used PEG–PEI–PEG triblock copolymers, and Zhang et al., who used PEI–PEG, observed a decrease in size with increasing polymer/DNA ratio [23,27]. The presence of 10% serum in this study, and the use of 5 mM HEPES and PBS in the other studies may be the cause of this difference. The use of serum was considered an absolute necessity to mimic better the culture conditions in which the polyplexes function and to facilitate the direct comparison between size measurements and transfection studies undertaken in complete medium.

Zeta-potential measurements were not included in this study, since it was not possible to measure them in cell culture buffers. Nonetheless, it is posited that, if polymers with higher PEG substitution demonstrate a reduced zeta-potential as polymer/DNA and backbone/DNA weight ratio increases, this trend would probably be more severe for assays performed on a per-polymer weight basis. This could be attributed to both PEI backbone composition and PEI–DNA electrostatic interactions reducing concomitantly with increasing PEG substitution, owing to the lack of PEG weight correction.

3.5. Plasmid delivery to cells by PEI–PEG polyplexes

Plasmid uptake was assessed using Cy3-labeled gWIZ to measure the relative delivery efficiency mediated by polymers. Two general trends were evident (Fig. 8). First, plasmid delivery was found to increase with increasing polymer/DNA ratio. This was the case whether the complexes were prepared with equal polymer or PEI backbone amount. The only exception to this was with the most PEGylated polyplexes prepared without PEG weight correction; complexes containing 37.2 PEG/PEI had no uptake at any ratio (Fig. 8a), possibly as a result of too little PEI backbone. Secondly, while PEG-modified polymers gave lower plasmid delivery on an equal polymer weight basis, correcting for the PEI backbone in complexes resulted in equivalent delivery in the case of low-modified PEI. To reveal better the effect of PEGylation, a surface plot of plasmid delivery as a function of both PEG-content and N/P ratio was plotted (Fig. 8c). At low PEG composition (<20%), a general trend of increasing cell delivery with increasing N/P ratio was evident, whereas in the higher PEG range, an uptake peak in the range 40–60% PEG weight at N/P = 40–60 was apparent.

It was previously demonstrated that, using a range of substitutions and MW, PEG modification generally decreased the uptake of pDNA [28]. The likely reason for this is PEG creating a steric barrier between the polyplex surface and cell surface proteoglycans, inhibiting interaction and decreasing uptake. The data here suggested a more complex relationship, where an optimal cellular delivery was evident at select PEG amounts and N/P ratios. The increase in uptake with increasing N/P ratio at low PEG composition is easily explained by the increasing cationic nature of polyplexes, which is more conducive to cell interactions. The cause for the peak in the ideal range of N/P ratio and PEG composition is less obvious, seeing that PEG should decrease any ionic interaction between charged amines and the immediate environment of a polyplex. One possibility may be that polyplexes in this range have sufficient amines to initiate binding, despite PEGylation, and this particular composition of PEG chains causes minor destabilization of the polyplex upon binding. Such destabilization would reveal more amines than typically available in a less PEGylated polyplex and, as a result, improve binding.

3.6. Transfection properties of PEI–PEG polyplexes

Transfection efficiency of PEG-modified PEI polymers is shown in Fig. 9 (a,b: mean GFP fluorescence of cell population; c,d: % GFP-positive cells). When complexes were formed with ratios based on total polymer weight (Fig. 9a and c), complexes at the low ratio (=2) were relatively ineffective for transgene expression, as seen with a significant decrease in % transfection compared with unmodified PEI for polymers with 13.6 and 37.2 PEG/PEI ($p = 0.01$ and $p = 0.008$). However, effectiveness was greatly improved with increasing weight ratio. A higher weight ratio was needed to increase effectiveness for polymers with higher PEG substitution compared with polymers with lower substitution. Such a transfection pattern has been noted previously by independent groups for N/P ratio <10 with 2 kDa PEG grafted PEI [29] and for N/P ratio <~20, in which the pattern was more severe for polymers with a higher PEG composition due to greater PEG substitution and/or PEG weight [30].

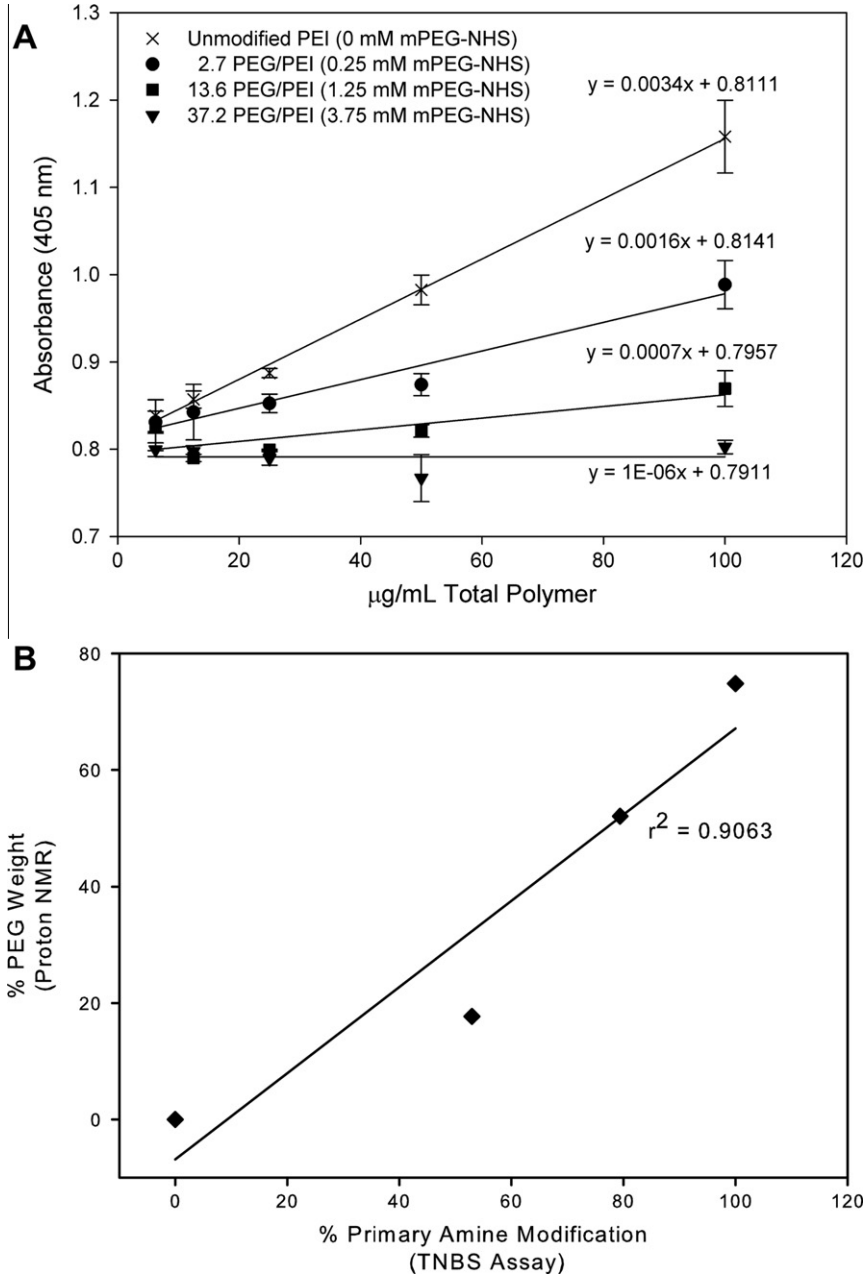

Fig. 2. (A) Changes in PEI amine groups after PEG substitution, as measured by the TNBS assay. The slope for each polymer was compared with the slope of unmodified PEI to determine the percentage of primary amine modification: 52.9%, 79.4% and 99.9% modification was obtained for increasing concentration of NHS–PEG. (B) Correlation between the % modification (from TNBS assay) and wt.% PEG (determined by ^{1}H-NMR) of the conjugates was analyzed by Pearson correlation ($r^2 = 0.9063$).

When the same transfection study was conducted again using PEG weight correction, such that equal PEI backbone was used for all complexes, the effects of PEG grafting became more apparent (Fig. 9b and d). In general, mean GFP fluorescence and % transfection were relatively lower for polymers with higher PEG substitution. Mean fluorescence was significantly decreased compared with unmodified PEI at a high weight ratio (=10) for polymers with 13.6 and 37.2 PEG/PEI ($p = 0.011$ and $p = 0.003$). With % transfection, the general trend of decreasing transfection with increasing PEGylation appeared only past a certain substitution threshold, which is well demonstrated by a decrease in effectiveness for 37.2 PEG/PEI polymer, but not 13.6 and 2.7 PEG/PEI polymers. The presence of a substitution threshold has been observed previously with PEI conjugated with 2 kDa and 5 kDa PEG [27,31]. Zhang et al. observed a decrease in transfection at N/P ratios 10, 20 and 30 (compared with unmodified) for 2 kDa PEG-conjugated PEI at 2.9 and 10.5 PEG/PEI, but not 1.89 PEG/PEI. For the 5 kDa PEG-conjugated PEI, the same effect was seen at 1.8 PEG/PEI and 7.2 PEG/PEI, but not at 0.66 PEG/PEI substitution. Tang et al. similarly noted a decrease for 2 kDa PEG-conjugated PEI at 2.5, 5.0 and 14.5 PEG/PEI, but not at 1.0 or 1.3 PEG/PEI [31]. Such a threshold effect has also been noted for grafted PEG chains of lower MW. Grafting with PEG chains of 0.35 kDa, 0.75 kDa and 1.9 kDa decreased transfection (albeit only at specific N/P ratios), and the substitution threshold resulting in decreased effectiveness appeared lower for polymers grafted with a higher PEG MW [30]. Given that the specific substitution threshold appears dependent on the PEG MW, the grafting threshold for each PEGylated polymer must be determined empirically. The grafting threshold of 2 kDa PEG-conjugated PEI, for example, appears to fall in the range

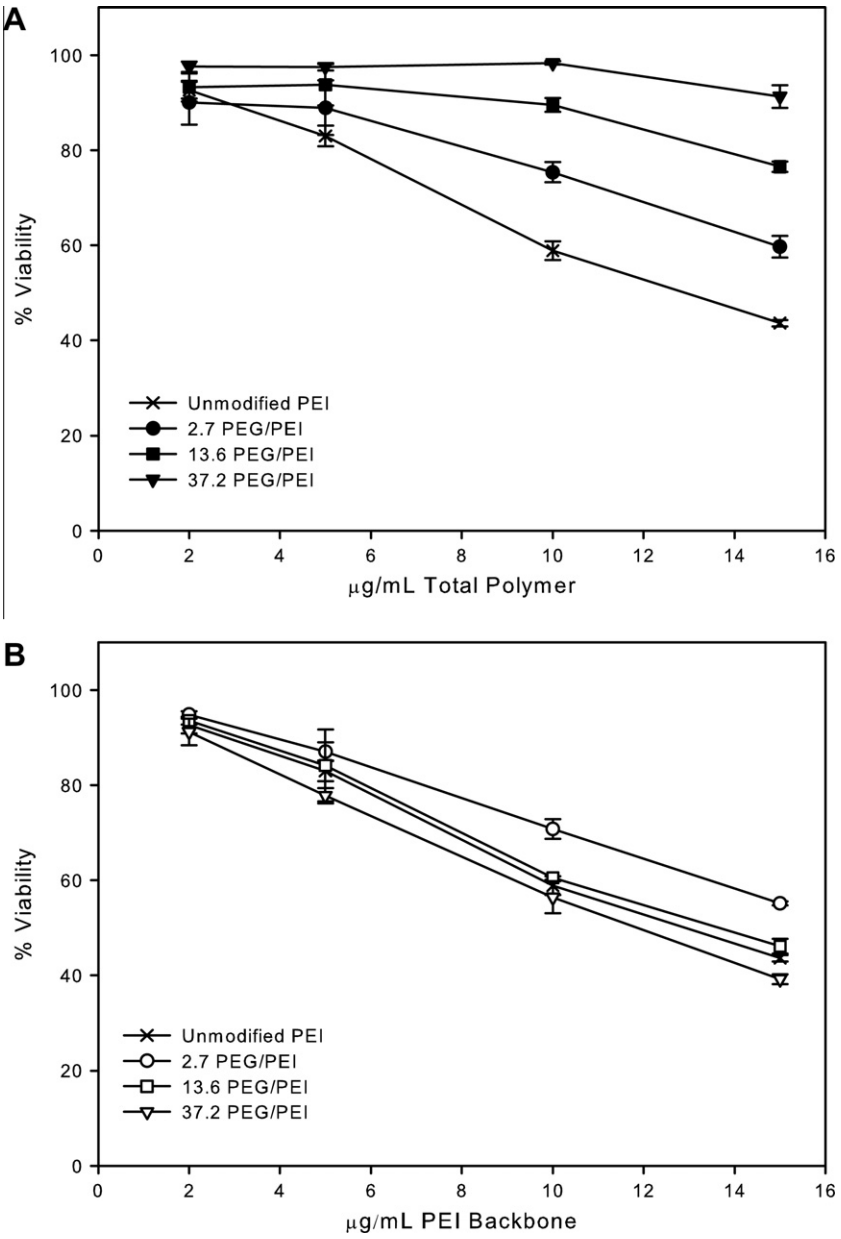

Fig. 3. Toxicity of unmodified and PEGylated PEI, as determined by MTT assay. The results were averaged from triplicate toxicity experiments. The observed viability values were summarized for distinct studies performed using either (A) total polymer concentration (includes PEG component) or (B) the PEI backbone (excludes PEG component). Significant differences in polymer toxicity were evident when one considered the total polymer concentration, but little difference in toxicity was evident when the cell viability was expressed as a function of PEI concentration alone.

1.3–2.5 PEG per 25 kDa PEI, based on the conservative estimate of Tang et al. or 2.7–13.6 PEG per 25 kDa PEI in this study. Ultimately, knowing this threshold assists in deciding the optimal degree of substitution in combination with knowing the effect of PEG grafting on toxicity and the severity to which exceeding this threshold affects transfection.

Unlike the uncorrected study, however, increasing the polymer/pDNA ratio did not generally increase transfection when PEG contribution to total polymer weight was corrected. This observation may have been the result of an increasing proportion of free to DNA-bound polymer as the ratio of PEI backbone/DNA increased and PEG grafting became detrimental to binding. Free polymers are likely to compete in binding to cellular components such as the proteoglycans of cell membranes and endosomal membranes. Increased PEG grafting of this uncomplexed polymer may interrupt interactions directly or indirectly by presenting steric hindrance between the particle and its binding partner. Alternatively, PEGylation could decrease complex stability as demonstrated by increased sensitivity of PEGylated PEI to heparin-induced DNA displacement [25,28]; this decrease in effectiveness at high N/P ratio and high PEGylation may be a result of premature unpacking in cytosol (possibly in endosomes) before nuclear association. The polyplexes within this range of N/P ratio and PEGylation may have a density of hydrophilic PEG chains exceeding an instability threshold not experienced by polyplexes, which either have a lower N/P ratio or lower % PEG composition.

It must be noted that there was an increase in transfection efficiency from a weight ratio of 2 to 5 followed by a decrease from a ratio of 5 to 10. This observed peak was the highest of all preparations including unmodified PEI, and suggests PEGylation

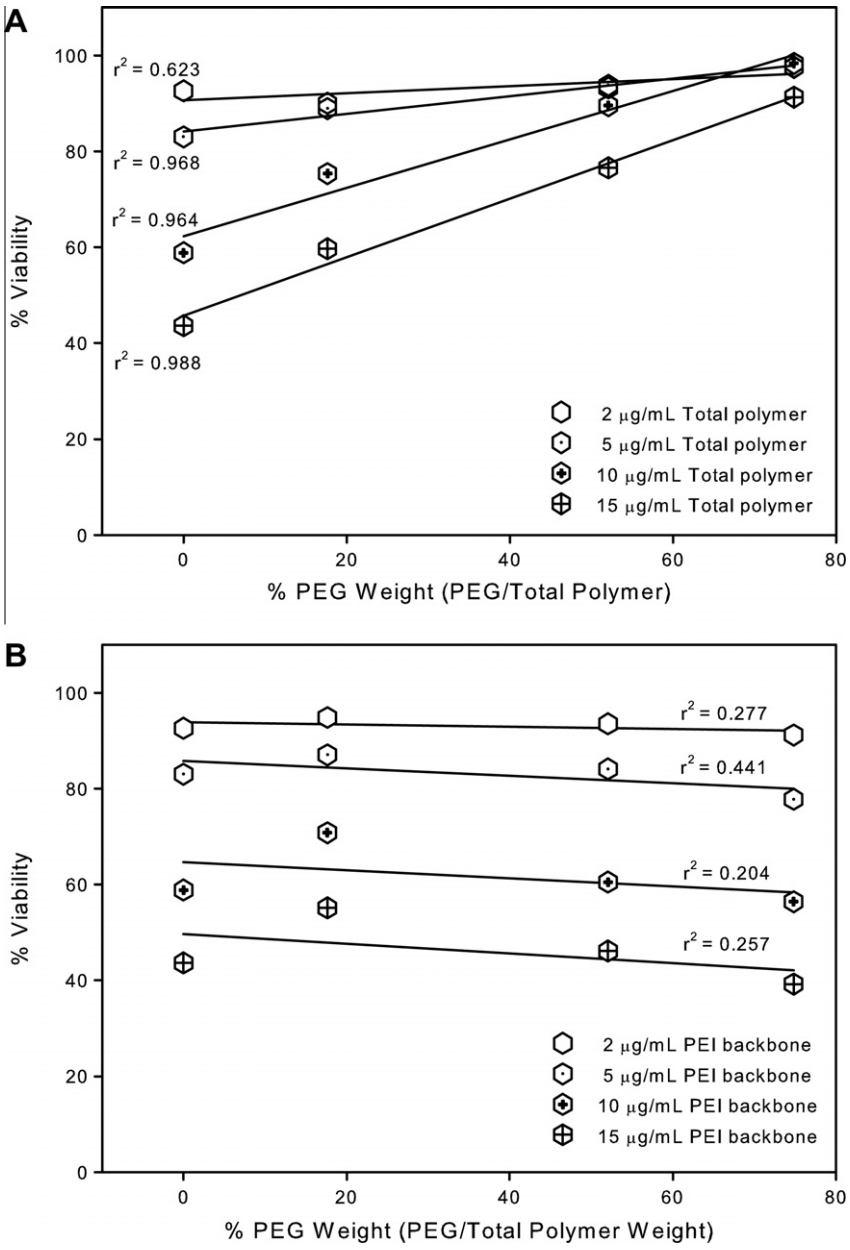

Fig. 4. Correlation between cell viabilities and PEG composition. The association between wt.% PEG and cell viability for distinct studies (A) without and (B) with PEG weight correction. Note the lack of correlations when cell viabilities were plotted as a function of PEI content (by excluding PEG fraction) at PEI concentrations of 2, 5, 10 and 15 μg ml^{-1}.

to be beneficial for transfection if the parameters of PEG substitution and weight ratio are amenable. Such an optimal effect of PEGylation has been noted previously [27,31]; Zhang et al. observed an increase in transfection compared with unmodified PEI using 2 kDa PEG-conjugated PEI with 1.9 PEG/PEI at a N/P = 30, as well as a smaller increase using 5 kDa PEG-conjugated PEI with 0.7 PEG/PEI at the same N/P ratio. Tang et al. observed optimal transfection exceeding that of unmodified PEI using 2 kDa PEG with 1.0 PEG/PEI in the N/P range 5–20, as well as a similar result using a substitution of 1.3 PEG/PEI. While Petersen et al. found that all their PEGylated polymers had similar or improved transfection compared with unmodified PEI, the optimal polymer had 35 PEG/PEI of 0.55 kDa PEG used at a N/P = 50, followed by a polymer having 2 PEG/PEI of 5 kDa PEG used in the N/P range 20–50 [24]. Hence, based on the present work and previous observations, it appears that, when the grafted PEG chains are in the 2–5 kDa range, a low substitution (0.7–2.7) at N/P = 20–50 is more effective than unmodified PEI. Also, as demonstrated by Petersen et al., conjugating PEG chains of lower weight at a higher substitution may exceed the effectiveness of unmodified PEI, suggesting that this phenomenon may be partly the result of the proportion of PEG and PEI within the polymer and not specifically the substitution ratio or the PEG chain length [24].

The reason for higher efficiency of PEG-substituted PEI may lie in differences in intracellular trafficking. Previous studies suggest that siRNA delivery is less inhibited by PEI PEGylation than plasmid delivery [25,28], and lower stability of PEGylated complexes was attributed to greater cargo release intracellularly. By extension, it is possible that higher cytosolic unpacking may be the same reason for a high degree of PEGylation decreasing plasmid transfection; premature unpacking of polyplexes in the cytosol decreases the likelihood of the plasmid bypassing the nuclear envelope, owing

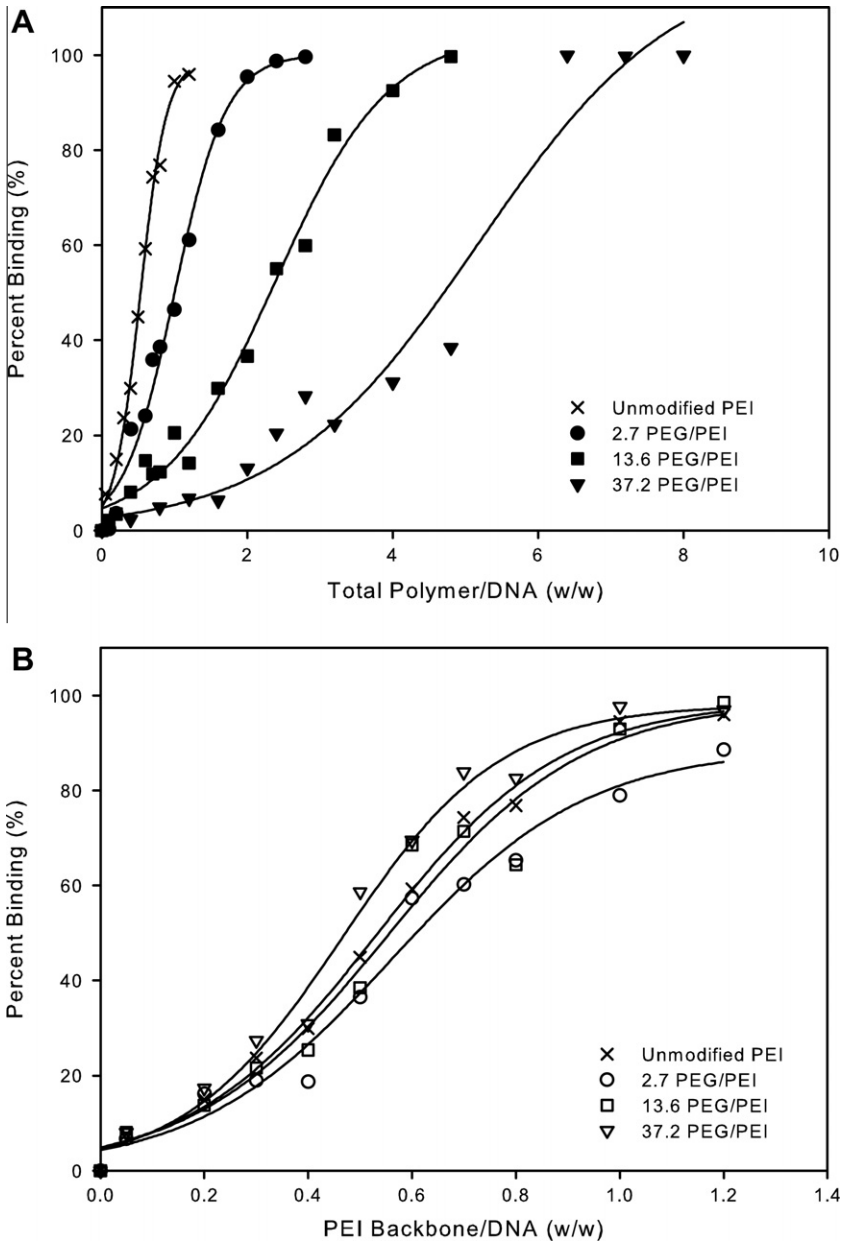

Fig. 5. Polymer binding to pDNA. The complexation of gWIZ-GFP plasmid with increasing concentrations of polymers was monitored using SYBR Green I dye. The results are summarized from distinct studies which assessed binding either as a function of (A) total polymer concentration or (B) PEI alone. Note the differences in binding pattern for the two types of concentrations employed.

to its larger size and negative charge [33]. The question remains then, how does a low degree of PEG substitution increase the transfection efficiency of PEI, compared with unmodified PEI? One possibility is that, while a polyplex with a high PEG composition will prematurely unpack in the cytosol, a polyplex with a low PEG composition would remain intact in the cytosol and, upon entrance to the nucleus, would exhibit improved unpacking by the competing chromatin compared with unmodified PEI. Such a mechanism is supported by the observations that branched PEI remained complexed with pDNA 24 h after transfection, and colocalization of the two components was noted in the nucleus, which suggests that intact polyplexes can undergo nuclear uptake [34,35]. Perhaps a small amount of instability induced by low PEG modification, which is not significant in cytosolic events, may have a significant impact in nuclear events. The increase in transfection efficiency noted with low PEG substitution, whether it is due to modulating the location of unpacking or not, serves as a rationale for PEI PEGylation independent of its effect on polymer toxicity.

In order to reveal better the effect of N/P ratio on transfection, the transfection results from different weight ratios are combined in Fig. 10. With increasing N/P ratio, all polymers followed a similar trend at low N/P ratios, followed by divergence with increasing N/P ratios for each polymer. In the low N/P range, where transfection efficiency starkly increased with N/P ratio, the degree of PEGylation appeared to have little influence on effectiveness, as seen with the seemingly indiscriminate order of data points of each polymer. The N/P ratio appeared to be the predominant variable, in that the data in this range are linear and superimposed, especially for % transfection. At higher N/P ratios, in contrast, different effectiveness ceilings seemed to exist for each polymer. For N/P > 20, polymers with moderate and high PEG substitution (13.6 and 37.2 PEG/PEI) had decreased effectiveness compared with unmodified PEI,

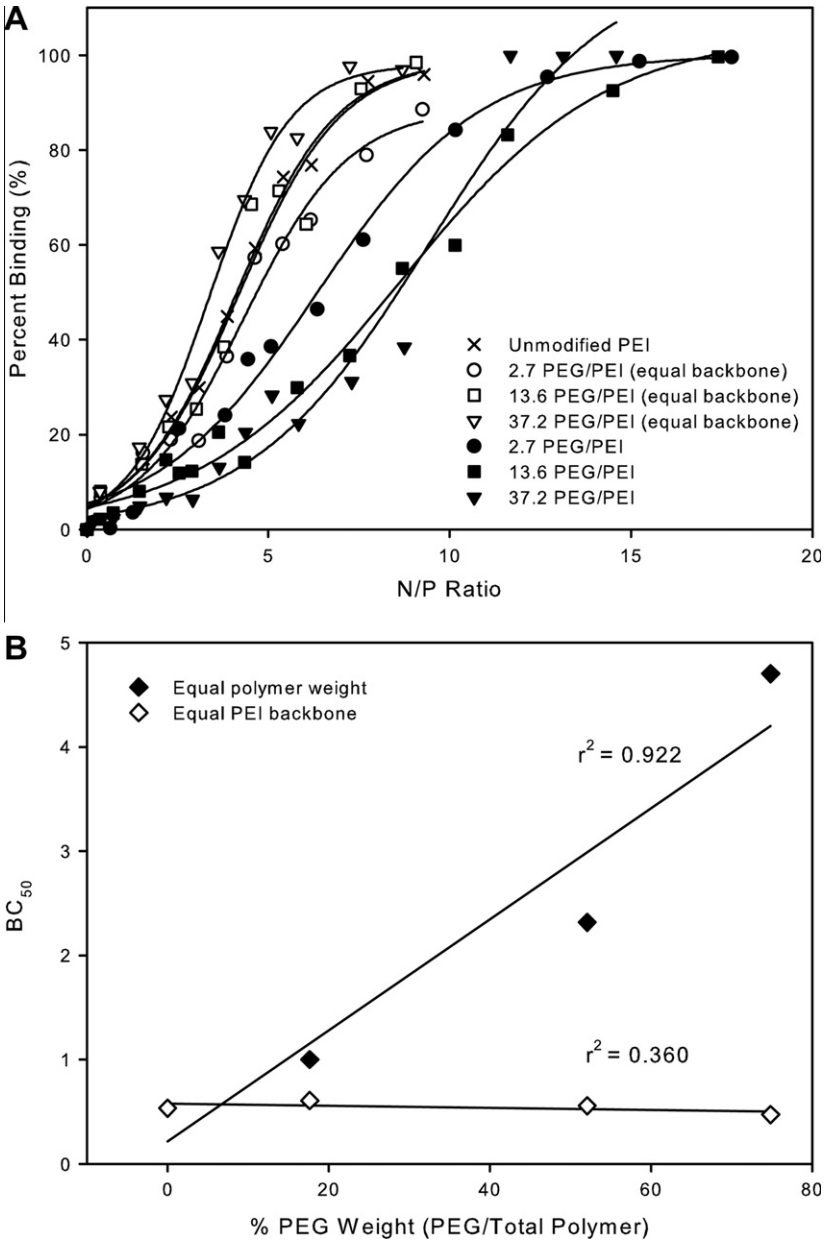

Fig. 6. (A) Polymer binding to pDNA as a function of N/P ratio and (B) correlation to PEG composition. Results from Fig. 5 were combined by plotting the curves as a function of N/P ratio. Pearson correlation between the %PEG weight and the bc_{50} of each polymer (determined from Fig. 5) was calculated for both studies. While an increasing bc_{50} was obtained with increasing wt.% PEG in polymers for the study without PEG weight correction, no effect of wt.% PEG was noted for the study with PEG weight correction.

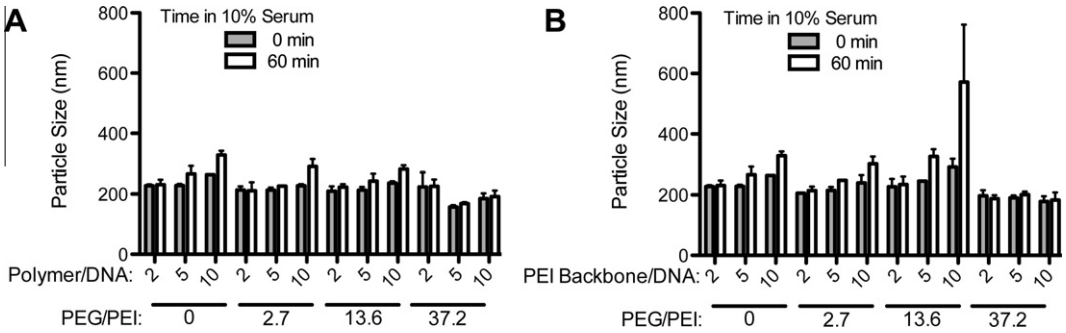

Fig. 7. Polyplex sizes. Polyplexes were formed at polymer:pDNA weight ratios (w/w) of 2, 5 and 10, based on (a) total polymer amount or (b) the PEI backbone amount. Two independent samples were prepared for each type of complex, and average sizes obtained from these studies were combined.

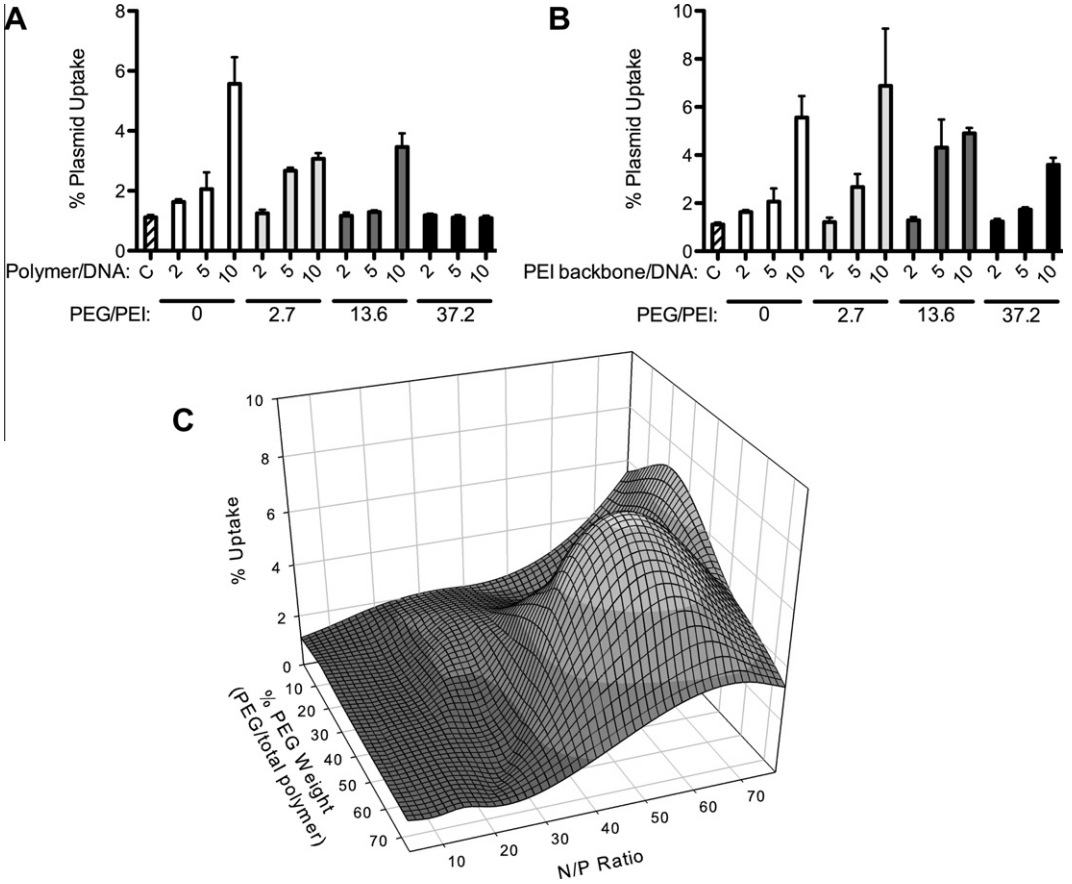

Fig. 8. Plasmid delivery by native and PEG-modified PEI. Polyplexes were formed with Cy3-labeled gWIZ using polymer:pDNA weight ratios (w/w) of 2, 5 and 10 based on either (A) total polymer amount or (B) PEI backbone amount. Data are mean (±SD) of triplicate experiments. Note the generally increasing plasmid delivery at increasing polymer:pDNA weight ratios. Surface plot of plasmid delivery as a function of N/P ratio and wt.% PEG is shown in (C).

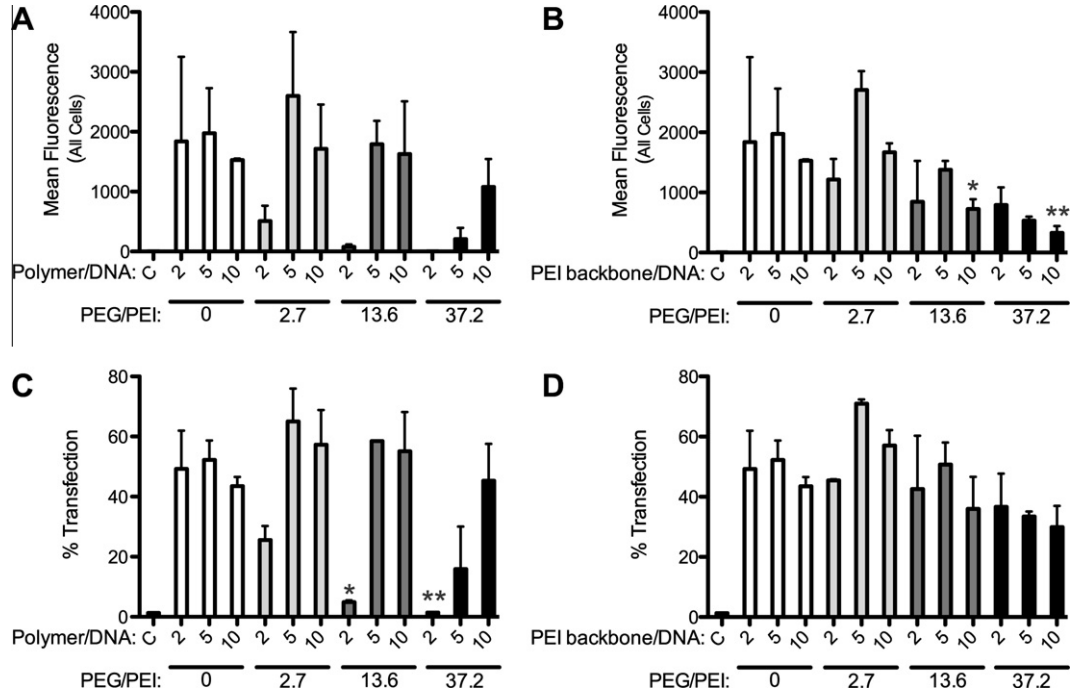

Fig. 9. Transfection efficacy of native and PEG-modified PEI. (A, B) The mean fluorescence and (C, D) % transfection of HEK293 cells was determined by flow cytometry after transfection with gWIZ-GFP. Distinct studies were performed with polyplexes at weight ratios (w/w) 2, 5 and 10 based on (A, C) total polymer or (B, D) PEI backbone amounts. Data are mean (±SD) of two independent experiments performed in triplicate. Asterisks indicate significant differences compared with unmodified PEI at the same weight ratio: $^{*}p < 0.05$; $^{**}p < 0.01$.

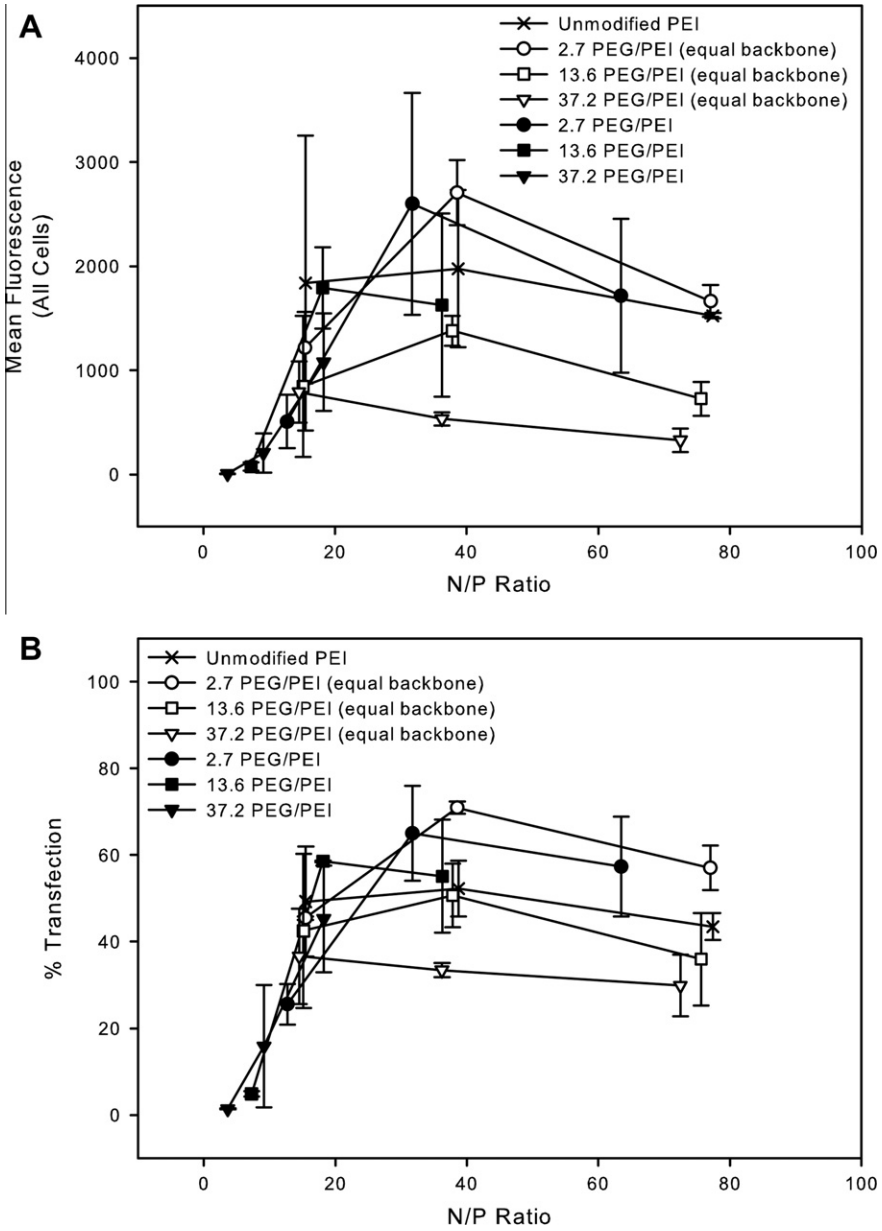

Fig. 10. Transfection efficacy as a function of N/P ratio. (A) The mean fluorescence and (B) per cent transfection of HEK293 cells treated with polyplexes formed with gWIZ-GFP at different polymer:pDNA weight ratios. All results shown in Fig. 9 were used to present the transfection efficiency as a function of N/P ratio.

while minor PEG substitution (2.7 PEG/PEI) resulted in similar or increased effectiveness. This transition of "superimposed linearity" to differing "effectiveness ceilings" may represent the transition from the need for an adequate number of positive charges to form condensed particles to a mechanism revealing the specific effect of PEG grafting when the adequately functioning polyplexes (i.e., sufficient cationic charge) were formed. In this regard, (1) for complexes with limited PEI content, PEG seems to have little effect on transfection, and (2) in the presence of sufficient PEI (i.e., adequate assembly), increasing PEG substitution generally resulted in detrimental effects on transfection efficiency, as evidenced by decreasing effectiveness ceilings with higher PEGylation.

While specifically analyzing the role of PEG composition and N/P ratio independently is invaluable, the two variables ultimately work in concert. Consequently, three-dimensional surfaces exploring the effect of both variables on mean GFP fluorescence and % cells transfected are displayed in Fig. 11. It is evident that there is an optimal combination of the two variables, as well as ineffective combinations highlighted by the valleys and troughs of the surfaces. Both surfaces clearly illustrate that the relationship of effectiveness with PEG substitution is complex and is inextricably related to N/P ratio. Ultimately, the resulting surfaces are expected to be the result of the physicochemical and intracellular trafficking differences among the polyplexes examined. All polyplexes in this study were in the range 200–300 nm (with the exception of one formulation increasing to ~600 nm after incubation in 10% serum). For polyplexes with sizes <500 nm, uptake relies on both clathrin-mediated and caveolin-mediated endocytosis (with the preferred mechanism dependent on cell type), while polyplexes >500 nm use fluid phase endocytosis [3,4,36]. The polyplexes are expected to be internalized in a similar manner; observed differences in transfection should not be attributed to polyplex size. Note that this analysis assumes no specific changes in uptake mechanisms due to the presence of PEG, and that particle size dictates the

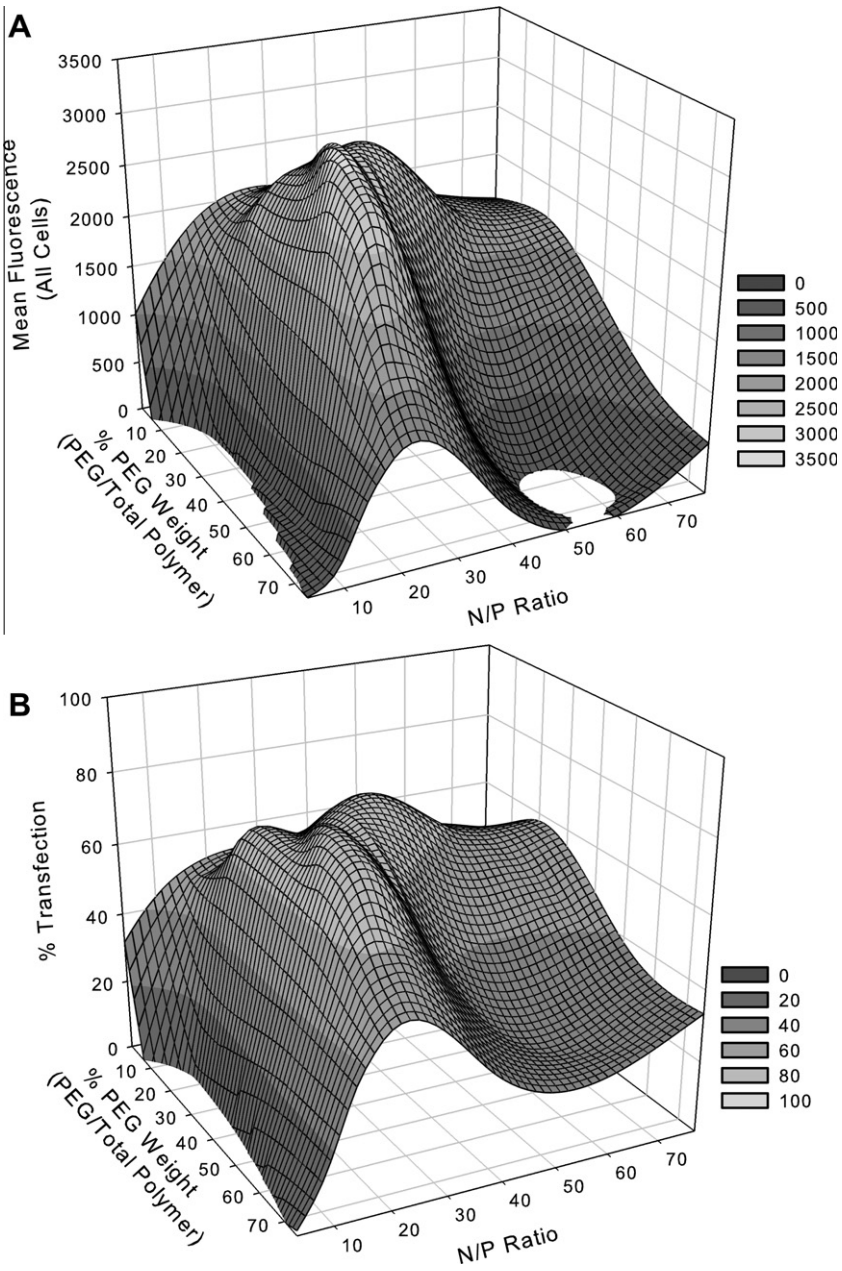

Fig. 11. Surface plots of (A) mean fluorescence and (B) per cent transfection. The mean fluorescence and % transfected cells as a result of treatment with gWIZ-GFP polyplexes, as determined by flow cytometry. The transfection efficiency was plotted as a dependent function of N/P ratio and wt.% PEG.

internalization mechanism. It was also interesting to note that the uptake peak of Fig. 8c (40–65% PEG; N/P = 40–70) was in the same region with low transfection (>45% PEG; N/P > 45; Fig. 11), and not in the region with high transfection (<55% PEG; N/P = 20–35). This lack of overlap suggests that uptake and transfection are not directly related.

4. Concluding remarks

The complex relationship between PEG substitution and N/P ratio explains why there are disagreements in the literature regarding the effect of PEGylation on PEI effectiveness. The fact that some studies report a beneficial effect, whereas others report an inhibitory effect may be the result of various studies only observing a limited portion of the effectiveness surface, such that some studies may be in a trough, while others limit their observations to a peak. Given the various effects of PEG length on transfection, the shape of such surfaces will most likely depend not only on the N/P ratio and PEG substitution, but also on the MW of grafted PEG. While this makes direct comparisons between studies difficult, it also suggests that the screening of any PEI–PEG library requires an in-depth study using a wide range of substitutions and N/P ratios. While many studies have noted the importance of these variables, the relevance of the two variables acting in concert has only been considered via a transfection surface recently: Ulasov et al. [33] reported the transfection surfaces (PEG chains/PEI vs N/P vs %transfection) of numerous cell types, all having similar peaks, but with the elevation dependent on cell type. The present study investigated a broader range of substitutions and N/P ratios in comparison, using 0–37.2 PEG/PEI with a maximum N/P = ~70, whereas Ulasov et al. used 0–8 PEG/PEI with a

maximum N/P = ~40. However, the surfaces they observed are remarkably similar to the surfaces reported here within the same corresponding region. Most notably, Ulasov et al. noted a peak at PEG/PEI = 1.2 in the N/P range 30–40, while the present work revealed a similar peak at the low substitution of PEG/PEI = 2.7 in the N/P range 31.7–38.5. This provides support for the accuracy of both observations and suggests HEK293 cells transfect in a similar manner to the cell types that Ulasov et al. tested.

It is important to recognize both the limits and transferable predictions of these surfaces. While these observations are useful for in vitro purposes, ex vivo gene therapy approaches and possibly some localized in vivo therapies, these data may not accurately predict the efficacy of injected polyplexes for all locally or systemically delivered gene therapies. It has been demonstrated with mice injections that blood levels remained higher for polyplexes with many short PEG chains compared with fewer longer PEG chains [20]. This is presumably due to differences in opsonization, and this study highlights the importance of optimization in the most appropriate and similar conditions that the polyplexes will be applied in. An in vivo investigation adopting the approaches established here, with a focus on the multivariable relationship between polymer/pDNA ratio and PEG substitution, may prove an illuminative guide for systemic gene delivery. Finally, knowing the optimal PEG substitution and N/P ratios may be useful in designing novel carriers other than 25 kDa PEI and/or help to better design carriers that combine other PEG moieties (e.g., with different MW and architectures) and assisting biomimetic peptides. The incorporation of endosomal escape peptides and nuclear localization sequences into PEI–PEG polyplexes formed using the optimal PEG substitution and N/P ratio outlined in the present studies may produce an additive effect on transfection efficiency. The complex relationship noted between N/P ratio and PEG substitution in determining transgene expression may be applicable to other conjugated ligands. This would suggest that detailed studies, similar to those described in this work regarding PEG grafting, should be conducted for each ligand to develop the best possible nanocarriers for gene delivery. As such, given the distinct conclusions generated using a parallel approach, future studies may also benefit from independently examining delivery systems on a per-polymer and per-backbone basis to determine precisely the effects of a particular ligand or copolymer.

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References

[1] Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA 1995;92:7297–301. http://dx.doi.org/10.1073/pnas.92.16.7297.

[2] Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 2004;377:159–69. http://dx.doi.org/10.1042/BJ20031253.

[3] Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther 2005;12:468–74. http://dx.doi.org/10.1016/j.ymthe.2005.03.038.

[4] Vongersdorff K, Sanders NN, Vandenbroucke R, Desmedt SC, Wagner E, Ogris M. The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. Mol Ther 2006;14:745–53. http://dx.doi.org/10.1016/j.ymthe.2006.07.006.

[5] Ogris M, Steinlein P, Carotta S, Brunner S, Wagner E. DNA/polyethylenimine transfection particles: influence of ligands, polymer size, and PEGylation on internalization and gene expression. AAPS Pharm Sci 2001;3. http://dx.doi.org/10.1208/ps030321 [article 21].

[6] Behr JP. The proton sponge: a trick to enter cells the viruses did not exploit. Chimia 1997;51:34–6.

[7] Sonawane ND, Szoka FC, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine–DNA polyplexes. J Biol Chem 2003;278:44826–31. http://dx.doi.org/10.1074/jbc.M308643200.

[8] Godbey WT, Wu KK, Mikos AG. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res 1999;45:268–75. http://dx.doi.org/10.1002/(SICI)1097-4636(19990605)45:3<268::AID-JBM15>3.0.CO;2-Q.

[9] Wightman L, Kircheis R, Rossler V, Carotta S, Ruzicka R, Kursa M, et al. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. J Gene Med 2001;3:362–72. http://dx.doi.org/10.1002/jgm.187.

[10] Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. Mol Ther 2005;11:990–5. http://dx.doi.org/10.1016/j.ymthe.2005.02.010.

[11] Kafil V, Omidi Y. Cytotoxic impacts of linear and branched polyethylenimine nanostructures in A431 cells. BioImpacts 2011;1:23–30. http://dx.doi.org/10.5681/bi.2011.004.

[12] Kunath K, von Harpe A, Fischer D, Petersen H, Bickel U, Voigt K, et al. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. J Control Release 2003;89:113–25. http://dx.doi.org/10.1016/S0168-3659(03)00076-2.

[13] Jiang HL, Arote R, Jere D, Kim YK, Cho MH, Cho CS. Degradable polyethylenimines as gene carriers. Mater Sci Technol 2008;24:1118–26. http://dx.doi.org/10.1179/174328408X341807.

[14] Bahadur KCR, Uludag H. A comparative evaluation of disulfide-linked and hydrophobically-modified PEI for plasmid delivery. J Biomater Sci 2011;22:873–92. http://dx.doi.org/10.1163/092050610X496297.

[15] Koo H, Jin GW, Kang H, Lee Y, Nam K. Zhe Bai C, Park JS. Biodegradable branched poly(ethylenimine sulfide) for gene delivery. Biomaterials 2010;31:988–97. http://dx.doi.org/10.1016/j.biomaterials.2009.10.0 04.

[16] Alexis F, Pridgen E, Molnar LK, Farokhzad OC. Factors affecting the clearance and biodistribution of polymeric nanoparticles. Mol Pharm 2008;5:505–15. http://dx.doi.org/10.1021/mp800051m.

[17] Owens DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm 2006;307:93–102. http://dx.doi.org/10.1016/j.ijpharm.2005.10.010.

[18] Vachutinsky Y, Kataoka K. PEG-based polyplex design for gene and nucleotide delivery. Isr J Chem 2010;50:175–84. http://dx.doi.org/10.1002/ijch.201000018.

[19] Ogris M, Brunner S, Schuller 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;6:595–605. http://dx.doi.org/10.1038/sj.gt.3300900.

[20] Kunath K, von Harpe A, Petersen H, Fischer D, Voigt K, Kissel T, et al. The structure of PEG-modified poly(ethylene imines) influences biodistribution and pharmacokinetics of their complexes with NF-κB decoy in mice. Pharm Res 2002;19:810–7. http://dx.doi.org/10.1023/A:1016152831963.

[21] Lungwitz U, Breunig M, Blunk T, Gopferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 2005;60:247–66. http://dx.doi.org/10.1016/j.ejpb.2004.11.011.

[22] Ahn CH, Chae SY, Bae YH, Kim SW. Biodegradable poly(ethylenimine) for plasmid DNA delivery. J Control Release 2002;80:273–82. http://dx.doi.org/10.1016/S0168-3659(01)00547-8.

[23] Zhong Z, Feijen J, Lok MC, Hennink WE, Christensen LV, Yockman JW, et al. Low molecular weight linear polyethylenimine-*b*-poly(ethylene glycol)-*b*-polyethylenimine triblock copolymers: synthesis, characterization, and in vitro gene transfer properties. Biomacromolecules 2005;6:3440–8. http://dx.doi.org/10.1021/bm050505n.

[24] Petersen H, Fechner PM, Martin AL, Kunath K, Stolnik S, Roberts CJ, et al. Polyethylenimine-*graft*-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. Bioconjug Chem 2002;13:845–54. http://dx.doi.org/10.1021/bc025529v.

[25] Mao S, Neu M, Germershaus O, Merkel O, Sitterberg J, Bakowsky U, et al. Influence of polyethylene glycol chain length on the physicochemical and biological properties of poly(ethylene imine)-*graft*-poly(ethylene glycol) block copolymer/siRNA polyplexes. Bioconjug Chem 2006;17:1209–18. http://dx.doi.org/10.1021/bc060129j.

[26] Remaut K, Lucas B, Raemdonck K, Braeckmans K, Demeester J, De Smedt SC. Protection of oligonucleotides against enzymatic degradation by pegylated and nonpegylated branched polyethyleneimine. Biomacromolecules 2007;8:1333–40. http://dx.doi.org/10.1021/bm0611578.

[27] Zhang X, Pan SR, Hu HM, Wu GF, Feng M, Zhang W, et al. Poly(ethylene glycol)-block-polyethylenimine copolymers as carriers for gene delivery: effects of PEG molecular weight and PEGylation degree. J Biomed Mater Res A 2008;84:795–804. http://dx.doi.org/10.1002/jbm.a.31343.

[28] Malek A, Czubayko F, Aigner A. PEG grafting of polyethylenimine (PEI) exerts different effects on DNA transfection and siRNA-induced gene targeting efficacy. J Drug Target 2008;16:124–39. http://dx.doi.org/10.1080/10611860701849058.

[29] Germershaus O, Merdan T, Bakowsky U, Behe M, Kissel T. Trastuzumab–polyethylenimine–polyethylene glycol conjugates for targeting Her2-expressing tumors. Bioconjug Chem 2006;17:1190–9. http://dx.doi.org/10.1021/bc0601119.

[30] Sung SJ, Min SH, Cho KY, Lee S, Min YJ, Yeom YI, et al. Effect of polyethylene glycol on gene delivery of polyethylenimine. Biol Pharm Bull 2003;26:492–500. http://dx.doi.org/10.1248/bpb.26.492.

[31] Tang GP, Zeng JM, Gao SJ, Ma YX, Shi L, Li Y, et al. Polyethylene glycol modified polyethylenimine for improved CNS gene transfer: effects of PEGylation extent. Biomaterials 2003;24:2351–62. http://dx.doi.org/10.1016/S0142-9612(03), 00029-2.

[32] Hsu CY, Hendzel M, Uludag H. Improved transfection efficiency of an aliphatic lipid substituted 2 kDa polyethylenimine is attributed to enhanced nuclear association and uptake in rat bone marrow stromal cell. J Gene Med 2011;13:46–59. http://dx.doi.org/10.1002/jgm.1526.

[33] Ulasov AV, Khramtsov YV, Trusov GA, Rosenkranz AA, Sverdlov ED, Sobolev AS. Properties of PEI-based polyplex nanoparticles that correlate with their transfection efficacy. Mol Ther 2011;19:103–12. http://dx.doi.org/10.1038/mt.2010.233.

[34] Itaka K, Harada A, Yamasaki Y, Nakamura K, Kawaguchi H, Kataoka K. In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine. J Gene Med 2004;6:76–84. http://dx.doi.org/10.1002/jgm.470.

[35] Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci USA 1999;96:5177–81. http://dx.doi.org/10.1073/pnas.96.9.5177.

[36] Hufnagel H, Hakim P, Lima A, Hollfelder F. Fluid phase endocytosis contributes to transfection of DNA by PEI-25. Mol Ther 2009;17:1411–7. http://dx.doi.org/10.1038/mt.2009.121.