Published in final edited form as:

Microsc Res Tech. 2010 September ; 73(9): 866–877. doi:10.1002/jemt.20839.

Structural and Functional Consequences of Poly(ethylene glycol) Inclusion on DNA Condensation For Gene Delivery

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Abstract

Polycationic polymers have been used to condense therapeutic DNA into sub-micron particles, offering protection from shear-induced or enzymatic degradation. However, the spontaneous nature of this self-assembly process gives rise to the formation of multimolecular aggregates, resulting in significant polyplex heterogeneity. Additionally, cytotoxicity issues and serum instability have limited the *in vivo* efficacy of such systems. One way these issues can be addressed is through the inclusion of poly(ethylene glycol) (PEG). PEG has known steric effects that inhibit polyplex self-aggregation. A variety of PEGylated gene delivery formulations have been previously pursued in an effort to take advantage of this material's benefits. Due to such interest, our aim was to further explore the consequences of PEG inclusion on the structure and activity of gene delivery vehicle formulations. We explored the complexation of plasmid DNA with varying ratios of a PEGylated tri-lysine peptide (PEG-K³) and 25 kDa polyethylenimine (PEI). Atomic force and scanning electron microscopy were utilized to assess the polyplex size and shape, and revealed that a critical threshold of PEG was necessary to promote the formation of homogeneous polyplexes. Flow cytometry and fluorescence microscopy analyses suggested that the presence of PEG inhibited transfection efficiency as a consequence of changes in intracellular trafficking, and promoted an increased reliance on energy-independent mechanisms of cellular uptake. These studies provide new information on the role of PEG in delivery vehicle design and lay the foundation for future work aimed at elucidating the details of the intracellular transport of PEGylated polyplexes.

Keywords

Poly(ethylene glycol); Polyplex Formulation Design; Gene Delivery; Cellular Uptake; Transfection Efficiency

INTRODUCTION

DNA condensation *via* polycation interactions is dependent upon the nature and concentration of the material used. High molecular weight (i.e. > 10 kDa) polymers made up of positively-charged subunits, such as poly(L-lysine) (PLL), polyhistidine, or

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polyethylenimine (PEI), are effective in condensing DNA. (Breunig et al., 2007; Kichler et al., 2001; Malek et al., 2008; Oh et al., 2002; Petersen et al., 2002; Putnam et al., 2003; Ramsay et al., 2000; Sharma et al., 2005; Shim and Kwon, 2008; Zaitsev et al., 2004) 25 kDa PEI in particular has been shown to be exceptionally effective for DNA condensation. (Breunig et al., 2007; Kichler et al., 2001; Oh et al., 2002; Sharma et al., 2005) However, like most high molecular weight cationic polymers, PEI exhibits significant cytotoxicity and produces large, heterogeneous particles when complexed with DNA. Such variability and toxicity prevent conventional polymeric formulations from being widely utilized in current *in vivo* gene delivery applications, despite their successes in inducing gene expression *in vitro*. One way investigators have attempted to address these concerns involves the inclusion of poly(ethylene) glycol (PEG) in the formulation process.

PEG has proven useful for non-viral gene delivery applications because of its non-toxic chemical nature and ability to both provide steric protection and resist non-specific protein adsorption when conjugated to gene delivery materials. (Auguste et al., 2006; Choi et al., 1998; Malek et al., 2008; Putnam et al., 2003; Rimann et al., 2008; Walsh et al., 2006) The most common PEG molecules used for non-viral gene delivery are linear polymers with molecular weights of 5 kDa or greater. These polymers are typically grafted onto the surface of DNA polyplexes or integrated into block copolymers that are subsequently used to complex DNA. (Bartlett and Davis, 2007; Blessing et al., 2001; Brus et al., 2004; Kircheis et al., 2001a; Kircheis et al., 2001b; Merdan et al., 2005; Mishra et al., 2004; Ogris et al., 1999; Ogris et al., 2001; Varga et al., 2000; Wagner et al., 1990) These PEGylated polyplexes exhibit improved cytotoxicity profiles, and in some cases, have transfection efficiencies that can rival or exceed those of similar systems that lack PEG. (Choi et al., 1998; Malek et al., 2008; Ogris et al., 2001; Putnam et al., 2003; Rimann et al., 2008; Walsh et al., 2006) Unfortunately, in many cases, PEG incorporation destabilizes polyplexes by reducing the number of free amines available for DNA condensation, (Choi et al., 1998; Kaminskas LM, 2008; Pun, 2002; Rimann, 2008; Walsh, 2006; Wolfert and Seymour, 1996) and results in decreased transfection and altered subcellular trafficking. This suggests that it is important to gain a better understanding of the structural and functional consequences of PEG inclusion in gene delivery formulations. (Mishra et al., 2004; Suh et al., 2007)

To accomplish this goal, recent work has focused on the characterization of block copolymers consisting of a polycation and PEG. Specifically, PEG-PLL diblock copolymers have received significant interest because of their low toxicity and biocompatibility. (Choi et al., 1998; Rimann, 2008; Walsh, 2006) For example, Walsh et al. demonstrated the ability of a linear PEG-PLL copolymer containing a 30 residue lysine chain and a 5 kDa PEG chain to successfully complex and deliver DNA *in vitro*. (Walsh et al., 2006) This conjugate was shown to form 50 nm toroidal structures and rod-like structures 150 nm long and 20 nm wide. Rimann et al. investigated branched PEG-PLL copolymer architectures with a varying degree of PEG grafting, which formed polyplexes with an approximate hydrodynamic diameter of 80 nm. (Rimann et al., 2008) When PLL blocks with different levels of PEG grafting were compared, PLL grafted with a small amount of PEG (~ 5%) and formulated at N:P (PLL amine:DNA phosphate) ratios of ~ 3-12 exhibited the highest levels of cell transfection and maintained cell viability. Additionally, the molecular weight of the grafted PEG was shown to play a more significant role in determining transfection efficiency than the molecular weight of the polycation. Such observations support the premise that the inclusion of PEG within gene delivery formulations can have a significant impact on the bioactivity of delivery vehicles.

Despite recent improvements in PLL-based formulations, these formulations have in general proved to be less efficient than PEI-based ones. Consequently, significant effort has been focused on developing PEI-based formulations that include a PEG component for improved

biocompatibility. For example, Petersen et al. investigated the effect of grafting different sized molecular weight PEG molecules onto PEI, demonstrating the importance of copolymer architecture on dictating polyplex formation. (Petersen et al., 2002) Copolymers with many short PEG chains (of 500 Da each) were found to form large, diffuse, unstable polyplexes, whereas copolymers with a few long PEG chains (of 5 or 20 kDa) produced compact structures. These studies revealed that all PEGylated architectures produced significant improvements in cell viability and had transfection efficiencies that were at least as high as those of PEI homopolymer polyplexes. In another example, Ogris et al. developed PEI/ligand block copolymer formulations and bound PEG chains onto the surface of these polyplexes, demonstrating improved stability, lower toxicity, and more effective gene transfer *in vivo*. (Ogris et al., 1999) However, in further probing the mechanism for these effects *in vitro*, PEG was found to inhibit cellular association and transfection. (Ogris et al., 2001) Thus, while PEG inclusion can improve PEI- and PLL-based transfection in some cases, the effects are not easily predictable and the mechanism underlying these effects needs to be elucidated.

In an effort to further explore this phenomenon, as well as to explore an alternate method for polyplex PEGylation that would not reduce the number of free amines on the complexing polycation, we have examined a hybrid polyplex formation strategy involving PLL-PEG block copolymers and free PEI. We have synthesized a PLL-PEG copolymer consisting of 3 lysine residues connected to a PEG chain (PEG-K³ peptide) (Fig. 1A). In this work, we show that this PEG-K³ peptide associates with plasmid DNA but does not significantly condense it. Additionally, our results suggest that this loose electrostatic interaction enables PEG inclusion in the formulation prior to DNA complexation by higher molecular weight polycations such as 25 kDa PEI. PrePEGylation is expected to enable the formation of more homogeneous particles by preventing particle aggregation (unpublished observations). In this work, we have formulated DNA with various mixtures of PEG-K³ and PEI at a constant charge ratio (an N:P of 10) (Fig. 1B), with the goal of exploring the role of PEG inclusion on polyplex formation, and the resulting effects on cell uptake, transfection, and cytotoxicity. An N:P of 10 was chosen because it has previously been shown to be the most effective for PEI-DNA transfection *in vitro*. (Boussif et al., 1995) By combining the benefits of PEGylation *via* the PEG-K³ peptide with the condensation capabilities of 25 kDa PEI, a synergistic effect was observed that promoted efficient cell uptake. However, efficient cell uptake did not correlate with high transfection for the PEGylated formulations, suggesting that PEG inclusion altered subcellular trafficking. These findings provide insight on the effects of PEG inclusion on DNA condensation, and on the mechanisms by which PEG inclusion influences gene delivery.

MATERIALS AND METHODS

Reagents and Materials

The GWiz green fluorescent protein (GFP)-expressing plasmid was purchased from Genlantis (San Diego, CA), amplified in *E. coli*, and purified with a Qiagen MegaPrep kit. Briefly, competent DH-5 α cells were transformed with the GWiz plasmid and cultured overnight in Lysogeny Broth. Cells were lysed and the amplified plasmid was purified and recovered in accordance with the manufacturer's protocols. 25 kDa branched PEI was purchased from Sigma (St. Louis, MO). The molecular weight of the polymer was validated by light scattering and gel permeation chromatography (GPC) by the manufacturer. Peptide Nucleic Acid (PNA) clamps (AlexaFluor488-TCTCTCTC-OOO-JTJTJTJT-CONH₂, (Zelphati et al., 1999) where O = 8-amino-3,6-dioxaoctanoic acid and J = pseudoisocytosine) with either an AlexaFluor488 or an AlexaFluor555 terminus were synthesized by solid-phase synthesis and high performance liquid chromatography (HPLC)-purified to >95% purity by Panagene (Daejeon, Korea). Rink Amide resin was acquired

from Anaspec (Fremont, CA). Fmoc-Lys(Boc)-OH and HBTU were purchased from Novabiochem (Darmstadt, Germany). Fmoc-NH-PEG₅₀₀₀-COOH was obtained from Iris Biotech GMBH (Marktredwitz, Germany). Diisopropylethylamine (DIPEA) and piperidine were purchased from Sigma Aldrich (St. Louis, MO). α -Methoxy ω -succinimidyl succinate PEG was purchased from Polymer Source (Montreal, Canada). All other chemicals were obtained from Fisher Scientific (Fairlawn, NJ) unless otherwise specified.

PEG-K³ Synthesis

NH₂-PEG-KKK-CONH₂ was synthesized by standard solid-phase Fmoc chemistry. The synthesis was completed manually in 5 mL syringes (Torviq, Niles, MI) on a shaker at 30°C. Rink amide resin was swelled in dichloromethane (DCM) and dimethylformamide (DMF). Fmoc-Lys(Boc)-OH (4-fold excess) and Fmoc-NH-PEG₅₀₀₀-COOH (1.5-fold excess) were activated *in situ* by O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU). Each addition was completed with a 2 h (Lys addition) or 4 h (PEG addition) mixing step in DIPEA and DMF. After each addition, the Fmoc protecting group was removed by successive 1 min- and 10 min-long treatments with 20% piperidine in DMF. The resin was washed five times with DMF after each coupling and deprotection step. A final wash step was completed in dichloromethane (DCM). The peptide was cleaved off the resin using a trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H₂O (95:2.5:2.5, v/v/v) mixture. Crude peptide was precipitated by adding cold diethyl ether (−20°C) and collected by centrifugation. The crude peptide was dissolved in 0.1% TFA in double-deionized H₂O (ddH₂O) and lyophilized. Purification was completed using preparative reversed-phase HPLC (RP-HPLC) with a 0.1% TFA in ddH₂O (solvent A)/0.1% TFA in acetonitrile (solvent B) gradient on a Shimadzu Prominence Ultra-fast LC (UFLC) system (Columbia, MD). A Restek C18 column (150×21.2 mm, 5 μ m particle diameter, Lancaster, PA) was used at a 5 mL/min flow rate. The elution profile was created by running a gradient of 5 to 55% solvent B over 40 min followed by detection at 210 nm and collection. The molecular weight of the final product was verified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Supplementary Figure 1). 0.9 μ L of a 1 mg/mL sample was dried on the target between two layers of 0.9 μ L α -Cyano-4-hydroxycinnamic acid (HCCA) matrix (16 mg HCCA, 250 μ L ddH₂O, 250 μ L acetonitrile). Measurements were made using a Bruker Daltonics Omnistar system (Billerica, MA).

Particle Formulation

Varying amounts of PEG-K³ peptide were added to 500 μ L of 20 mM Hepes buffer (pH 8.0). Similarly, 20 μ g of plasmid DNA were added to 500 μ L of 20 mM Hepes buffer (pH 6.0). The PEG-K³ solution was added to the DNA solution in a drop-wise fashion while vortexing, and the combined solutions were allowed to form polyplexes at room temperature for 10 minutes. Then, varying amounts of a 0.45 μ g/ μ L solution of PEI in 20 mM Hepes buffer (pH 6.0) were added directly to the sample, mixed thoroughly, and incubated at room temperature for an additional 10 minutes prior to sample analysis. In all studies, an N:P ratio of 10 was maintained. For the turbidity assays, PEI polyplexes and surface PEGylated PEI polyplexes were also formulated as per (Ogris et al., 1999). For surface PEGylation, 20 μ L of a 4 mM solution of α -methoxy ω -succinimidyl succinate PEG in 20 mM Hepes buffer (pH 6.0) was added to a 1 mL solution of the PEI-DNA complexes (containing 20 μ g of DNA), and the resulting solution was stirred for 1 hour at room temperature prior to analysis.

Ethidium Bromide Exclusion Assays and Nuclease Protection Assays

Ethidium bromide exclusion assays were performed by utilizing intrinsic fluorescence measurements. Polyplexes were formed as described, over a range of ratios of PEI/PEG-K³, while maintaining an overall N:P ratio of 10. 1 mL samples of polyplexes were then

analyzed by incubating the polyplexes with 17.3 μ M ethidium bromide for 5 minutes at room temperature, and then measuring the resulting fluorescence spectra (518 nm excitation, 605 nm emission). Nuclease protection assays were conducted by a modification of existing protocols. (Bartlett and Davis, 2007) Briefly, 10 μ L of 100% mouse serum (Sigma) were added to 10 μ L of polyplex suspension (containing 200 ng of DNA), and the mixture was incubated for 4 hours at 37°C. Subsequently, termination buffer ("STOP" buffer) containing 38 mM EDTA, 154 mM NaOH, and 38 mM NaCl was added to stop the serum degradation process, and the resulting mixture was incubated for 15 minutes at 4°C. The DNA was then displaced from the polyplexes by incubation with 1 mg/mL heparin (Sigma), and the DNA containing samples were analyzed by agarose gel electrophoresis.

Atomic Force Microscopy and Scanning Electron Microscopy

100 μ L of polyplex suspension containing 2 μ g of DNA was prepared as described and allowed to settle overnight on a 12 mm-diameter mica coverslip. Each sample was subsequently fixed with 2% glutaraldehyde, washed three times with ddH₂O to remove leftover buffer, and treated with 2% osmium tetroxide for one hour at room temperature. After three washes with ddH₂O, ethanol was added to the fixed sample in increasing amounts (25%, 50%, 75%, 95%, and 100%) at ten minute intervals, and then the samples were immersed in pure ethanol for critical point drying with an Autosamdri 815B (Tousimis, Rockville, MD).

Atomic force microscopy (AFM) was performed on a Nanoscope IIIa atomic force microscope (Digital Instruments, Santa Barbara, CA) used in conjunction with an RTESP cantilever tip (303-344 kHz frequency; 20-80 N/m nominal spring constant). Samples were prepared as described on mica coverslips. Tapping mode AFM images were collected under ambient conditions at approximately 25°C. Height, phase, and amplitude modes were used for imaging, with scan rates of ~1 Hz. Image processing was performed using the Nanoscope IIIa software package.

Scanning electron microscopy (SEM) was performed on a Hitachi S-4700 field emission scanning electron microscope (Pleasanton, CA). Samples were prepared as described on mica, silver-glued upon aluminum SEM mounts, and sputtered at ~ 30 kV with an Au/Pd grid in a Denton carbon coater (Moorestown, NJ) under 50 mTorr argon. The sputtered samples were fixed in the sample holder and placed in the vacuum chamber of the microscope under low vacuum (10^{-3} torr) for imaging in ultra-high resolution with an upper mode at 3.0 kV and 40 μ A.

Images were analyzed with the particle analysis algorithm in the ImageJ software package (NIH, Bethesda, MD). SEM and AFM height images were uploaded into the ImageJ software package and converted into 8 bit images. Images were prepared for analysis by setting lower and upper threshold values to segment the image into features of interest and background features. These threshold values were selected qualitatively such that the number of surface features identified by the algorithm best corresponded to the number of surface features that were actually observed. The "analyze particles" tool was then used to count and measure objects. This tool functions by scanning the image until the edge of an object is found, and proceeding until the other end of the object is identified. The objects are then outlined using the wand tool and measured, and the Feret's diameter is recorded. Any object with a Feret's diameter greater than 20 nm is considered a "particle". The number of observations and the particle diameters were recorded, and the mean, standard deviation, and coefficient of variation of the particle diameters were calculated.

Salt Stability Assay

The salt-induced aggregation of the polyplexes was assessed *via* turbidity measurements, tracking the change in absorbance at 600 nm. A Beckman DU600 spectrophotometer (Brea, CA) was used for all turbidity assays. 500 μ L samples of polyplexes were formed as described, and an initial absorbance measurement was performed immediately following polyplex formation. 55 μ L of 10x phosphate buffered saline (PBS) solution (Invitrogen, Carlsbad, CA) was then added to the 500 μ L samples, resulting in saline concentrations of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM KH₂PO₄. The extent of the aggregation of the polyplexes was monitored as a function of time following the addition of PBS by turbidity measurements.

Cell Culture

Chinese hamster ovary (CHO) cells (ATCC, Manassas, VA) were maintained in a humidified, 37°C atmosphere containing 5% CO₂. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 μ g/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen, Carlsbad, CA).

Transfection

200,000 CHO cells were seeded into each well of a 6-well dish, and the cells were cultured overnight. Immediately prior to transfection, the growth medium was removed and replaced with 2 mL of serum-free Opti-Mem medium (Invitrogen). 500 μ L of polyplex suspension (containing 10 μ g DNA) was formed and added to the cells in a drop-wise manner. The polyplexes were then allowed to incubate with the cells in serum-free medium for 3 hours. At that time, the serum-free medium was removed, the cells were washed in PBS, and growth medium was added. The cells were analyzed by flow cytometry and fluorescence microscopy 24 hours after transfection. For flow cytometry, the cells were trypsinized, washed in 0.1% BSA, and reconstituted in PBS for analysis with a BD Biosciences FACSCalibur cytometer (San Jose, CA). For fluorescence microscopy, live cells were imaged with a Leica microscope according to established protocols.

Cell Viability

For cell viability assays, an ethidium homodimer dye (Invitrogen) was used to stain the nuclei of dead cells. 4 μ L of a 2 mM dye solution was added to 2 mL of media in each well of the transfected cell cultures 24 hours after transfection, and the cells and dye were incubated for 10 minutes at room temperature. The cells were subsequently imaged by fluorescence microscopy.

Cellular Internalization Experiments

Prior to complexation, a 30 nM solution of plasmid DNA was stained with YOYO-1 dye at a ratio of 1:50 (dye molecules to nucleotides) in 20 mM Hepes buffer (pH 6.0) for 5 minutes at room temperature. Alternatively, a 30 nM solution of plasmid DNA was incubated overnight at 37°C with 2 μ M AlexaFluor488 labeled PNA (PNA-F) or 2 μ M AlexaFluor555 labeled PNA (PNA-R) in a 20 mM Hepes buffer (pH 7.4). For some experiments, polyplexes containing YOYO-1- or PNA-F-labeled DNA were formed and added to the cells as previously described. Following a 3 hour incubation at 37°C, the cells were trypsinized, washed in 0.1% BSA, and reconstituted in PBS for analysis with a BD Biosciences FACSCalibur cytometer (San Jose, CA). Alternatively, PNA-R-labeled plasmids were complexed and added to cells in serum-free media for 3 hours at 4°C. Cells were fixed with 4% paraformaldehyde and stained with fluorescein-phalloidin and Hoechst dye (for F-actin and nuclear DNA, respectively), according to standard protocols. Fluorescence microscopy images at various z slices were then taken.

RESULTS

Condensation and Protection

To explore the efficiency of plasmid DNA condensation by the addition of varying mixtures of PEGylated (low molecular weight) and nonPEGylated (high molecular weight) polycations, ethidium bromide exclusion assays and agarose gel electrophoresis experiments were performed. Fig. 2A illustrates the fluorescence intensity spectra of ethidium bromide-stained DNA complexes made with varying combinations of the PEG-K³ peptide and PEI. At a total N:P ratio of 10, the PEG-K³ peptide did not significantly condense plasmid DNA on its own, whereas PEI/PEG-K³ mixtures with at least 25% of their amines contributed by PEI (PEI N:P 2.5/PEG-K³ N:P 7.5) effectively condensed the plasmid. Gel electrophoresis was used to evaluate the nuclease resistance of the plasmid following complexation with varying mixtures of PEI and PEG-K³ (Fig. 2B). Consistent with the ethidium bromide exclusion assays, the PEG-K³ peptide alone was insufficient to protect the plasmid against enzymatic degradation (as evidenced by the disappearance of a plasmid DNA band), whereas all formulations containing PEI resisted nuclease degradation to some extent, as shown by the maintenance of an intense plasmid DNA band following incubation with serum nucleases. Formulations containing at least 75% PEI amines appeared to be fully protected, as evidenced by the full-intensity plasmid DNA bands for the polyplex samples containing 75% and 100% PEI amines.

Colloidal Stability

Turbidity assays were performed to assess the tendency of the DNA-containing particles to aggregate upon the introduction of 150 mM salt (Fig. 3). In addition to the particles made with various PEI/PEG-K³ mixtures, particles made with 100% PEI and then surface-PEGylated were examined because they were expected to demonstrate the stable response for the assay. As predicted, the surface PEGylated particles (PEI N:P 10/PEG-K³ N:P 0 with surface PEGylation) did not aggregate upon the addition of salt, whereas the unPEGylated particles formulated with 100% PEI (PEI N:P 10/PEG-K³ N:P 0) aggregated significantly, as evidenced by the rapid increase in absorbance. In contrast, particles containing PEI amine levels of 50 – 75% aggregated moderately (PEI N:P 5/PEG-K³ N:P 5 and PEI N:P 7.5/PEG-K³ N:P 2.5). The particles containing PEI amine levels of 25% (PEI N:P 2.5 PEG-K³ N:P 7.5) did not have any appreciable increase in absorbance, suggesting that like the surface PEGylated polyplexes, these polyplexes were stable under physiological salt conditions. The PEI-free sample (PEI N:P 0 PEG-K³ N:P 10) also did not have an increase in absorbance, likely due to the fact that PEG was loosely associated with the plasmid DNA.

Polyplex Characterization

As previously mentioned, the heterogeneity of PEI-DNA polyplexes is typical for these systems, and is a direct consequence of the multimolecular aggregation that occurs during the formulation process; the incorporation of PEG into polyplex formulations has been proposed as a means of reducing this heterogeneity. To further examine these phenomena, AFM and SEM were utilized to characterize the polyplexes formulated with different mixtures of PEI and PEG-K³ (Fig. 4). Micrographs of these polyplexes suggested that the inclusion of PEG during formulation only resulted in a less heterogeneous population under certain conditions. Polyplexes formulated at ratios of PEI N:P 7.5 PEG-K³ N:P 2.5 (Fig. 4, B and F) and PEI N:P 5 PEG-K³ N:P 5 (Fig. 4, C and G) displayed greater heterogeneity relative to the PEI N:P 10 PEG-K³ N:P 0 polyplexes (Fig. 4, A and E). However, significant reductions in particle size and heterogeneity were observed for polyplexes formulated at PEI N:P 2.5 PEG-K³ N:P 7.5 (Fig. 4D)¹. The absence of distinguishable features in the SEM image for the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes (Fig. 4H) is a consequence of the limit of SEM resolution (features less than 10 nm from the surface are indistinguishable).

(Castle, 1997) No visible surface features were observed in either the AFM or SEM images for the PEI N:P 0 PEG-K³ N:P 10 sample (Supplementary Fig. 4), confirming the absence of complexed DNA. To quantify the observations on particle size and heterogeneity, statistical analyses on the particle size distributions of the polyplexes were performed, and are summarized in Table I. Consistent with the qualitative observations, the inclusion of PEG at the ratio of PEI N:P 2.5 PEG-K³ N:P 7.5, resulted in a reduction in polyplex diameter and heterogeneity relative to PEI N:P 10 PEG-K³ N:P 0 (48.3 $\pm$ 25.9 nm vs. 62.2 $\pm$ 58 nm), as determined *via* AFM (the analysis of SEM data were consistent with the analyses of AFM data). Additionally, the coefficient of variation was reduced from 0.93 to 0.54. Such observations confirm that the physical consequence of PEG inclusion in these formulations was a reduction in multimolecular aggregation above a critical threshold level of PEGylation. Below this threshold, average polyplex diameters increased to higher levels.

Transfection Efficiency and Cell Viability

To determine if PEG inclusion and the resulting alterations in polyplex size influenced cellular transfection, polyplexes made with various PEI/PEG-K³ mixtures were used to transfect CHO cells. After 24 hours, a PEI- and PEG-dependent trend in the transfection efficiency was observed *via* both flow cytometry and fluorescence microscopy, with no transfection observed for polyplexes formulated at a ratio of PEI N:P 2.5 PEG-K³ N:P 7.5 (Fig. 5C), and increasing levels of transfection observed as the levels of PEI increased (Fig. 5, D – F). A similar trend in polyplex cytotoxicity was observed, demonstrating the role of PEI in contributing to cell death (Fig. 6).

Cellular Internalization

The levels of polyplex internalization into CHO cells were investigated with polyplexes formulated with various PEI/PEG-K³ mixtures and fluorescently-labeled DNA. When YOYO-1-labeled plasmid DNA was incorporated into the polyplexes, flow cytometry revealed a nearly three-fold higher level of uptake for the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes relative to the PEI N:P 10 PEG-K³ N:P 0 polyplexes (Fig. 7A). The levels of PEI N:P 2.5 PEG-K³ N:P 7.5 polyplex internalization were also significantly higher than the levels of PEI N:P 5 PEG-K³ N:P 5 polyplex or PEI N:P 7.5 PEG-K³ N:P 2.5 polyplex internalization.

Because YOYO-1 fluorescence becomes quenched upon complexation and unquenched upon decomplexation, it was desirable to confirm if the strong intracellular fluorescence intensity of the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes was related exclusively to particle internalization, or if there were also a contribution from DNA decomplexation. Thus, PNA-F and PNA-R labeled plasmids were formulated into polyplexes and polyplex internalization was explored in CHO cells. Uptake was again observed to be three-fold greater with the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes relative to the PEI N:P 10 PEG-K³ N:P 0 polyplexes (Fig. 7B), suggesting that the differences in intracellular fluorescence were indeed related to differences in cellular uptake of the polyplexes. Further confirmation of this observation was obtained *via* fluorescence microscopy (Fig. 7, C and D). These experiments demonstrated the higher levels of PEI N:P 2.5 PEG-K³ N:P 7.5 polyplex internalization, and also displayed differences in non-cell associated polyplex accumulation (Fig. 7D). This non-cell

¹Although the image in Fig. 4D appears to contain somewhat less material than the images of the other polyplex formulations (Fig. 4, A – C), dilution studies with this particular formulation have suggested that the number concentration of polyplexes increases at this ratio of PEI to PEG-K³, consistent with the reduction in the average size of the polyplexes at this ratio. The increase in number concentration produces significant drying effects (data not shown), as verified by AFM/SEM on the formulations prepared at different dilutions. In an effort to uniformly assess the consequence of PEG inclusion in each mixture, the AFM/SEM images shown in Figure 4 were prepared and analyzed under identical conditions

associated polyplex accumulation was attributed to large polyplex aggregates in PEI N:P 10 PEG-K³ N:P 0 samples.

To explore the possibility that a non-energy-dependent mechanism might be contributing to the differences in polyplex uptake, cellular internalization experiments were performed at 4°C with PNA-R-labeled plasmids. Fig. 8 illustrates z-series images of cells transfected with either the PEI N:P 10 PEG-K³ N:P 0 polyplexes or with the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes. Under these low temperature conditions, low levels of surface-associated PEI N:P 10 PEG-K³ N:P 0 polyplexes and small amounts of internalized PEI N:P 10 PEG-K³ N:P 0 polyplexes were detected (Figure 8A, white arrows). In contrast, while low levels of surface-associated PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes were also observed under these conditions, significant levels of internalized PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes were detected. Identical uptake experiments performed at 37°C revealed negligible surface-associated fluorescence for both formulations (Supplementary Figure 3). These observations suggested that the internalization mechanism for the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes involved both energy-dependent and energy-independent pathways, whereas the internalization mechanism for the PEI N:P 10 PEG-K³ N:P 0 polyplexes was primarily energy-independent.

DISCUSSION

Studies on the effects of including PEG within gene delivery vehicle formulations have revealed a tendency for PEGylation to destabilize the polyplexes, as well as conflicting evidence on the pros and cons of PEGylation on gene delivery efficiency. (Banerjee et al., 2006; Choi et al., 1998; Kaminskas LM, 2008; Petersen et al., 2002; Pun, 2002; Rimann, 2008; Walsh, 2006; Wolfert and Seymour, 1996) To address these issues, a low molecular weight PEGylated polycation (PEG-K³ peptide) was synthesized and incorporated into a PEI-based DNA formulation, with the goals of establishing a less invasive means of polyplex PEGylation and further exploring the role of PEGylation on gene delivery.

Because it was expected that polyplex PEGylation by the PEG-K³ peptide would enable PEG inclusion without compromising PEI-based DNA compaction, ethidium bromide exclusion assays and nuclease protection assays were employed to explore DNA condensation with the PEI/PEG-K³ mixtures. As expected, the PEG-K³ polycation failed to efficiently condense plasmid DNA on its own, as shown in Fig. 2, A and B. Such a result was not surprising considering the small number of positively charged residues on this condensing reagent relative to 25 kDa PEI (where every third amino nitrogen can be protonated). (Boussif et al., 1995) When the PEG-K³ peptide was combined with PEI, a formulation of PEI N:P 2.5 PEG-K³ N:P 7.5 was found to effectively condense the plasmid and protect it against enzymatic degradation (Fig. 2, A and B). On a molar basis, this formulation consisted of approximately 0.19 moles of PEG for every mole of amine; by comparison, stable formulations of surface PEGylated PEI-DNA polyplexes were found to require 0.13 moles of PEG per mole of amine in order to promote colloidal stability (data not shown). Previous studies have suggested that the optimal level of PEGylation for *in vivo* use consists of 0.09 moles of PEG per mole of amine. (Ogris et al., 1999; Ogris et al., 2001) However, these studies utilized a low molecular weight PEI (i.e. 800 Da PEI) whereas our experiments employed a high molecular weight PEI as the primary condensing reagent. 800 Da PEI has 34% more primary amines than 25kDa PEI, resulting in more accessible binding sites for succinimide functionalized PEG. A precise determination of the reaction stoichiometry in such systems remains to be conducted however differences in PEG binding efficiency could play a key role. Overall, the PEG-K³-based PEGylation strategy appears to be comparable to existing methods for PEGylation. These observations suggest that the

amount and nature of the condensing polycation are critical parameters in determining the level of PEGylation needed to achieve stabilization.

Turbidity assays were used to determine the salt stability of the PEI/PEG-K³ formulations, in an effort to determine whether PEG-K³-based PEGylation resulted in steric stabilization (Fig. 3). The PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes did not reveal an appreciable increase in signal upon salt addition, indicating that no aggregation was occurring, whereas formulations containing lower levels of PEG aggregated upon salt addition. Comparing the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes with stable surface PEGylated PEI-DNA polyplexes demonstrates that the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes consisted of comparable levels of PEGylation.

Given that prePEGylation by the PEG-K³-based strategy was expected to reduce the heterogeneity of the polyplex formulations, the physical characteristics of the PEI/PEG-K³-based polyplexes were investigated using AFM and SEM. These assays indicated that the PEI N:P 2.5 PEG-K³ N:P 7.5 formulations produced smaller, more homogeneous polyplexes than the other formulations that were explored, including the formulation containing PEI in the absence of PEG-K³. Previous work has shown that DNA polyplexes containing a single plasmid are approximately 30 nm in diameter. (Dauty et al., 2002) The PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes were approximately 48 nm in diameter (as observed *via* AFM) and had size distributions with a smaller standard deviation and coefficient of variation than conventional PEI polyplexes. This suggests that this formulation consists of polyplexes containing 1 – 2 plasmids, and indicates that the prePEGylation strategy results in a reduction in polyplex heterogeneity as a consequence of the PEG-induced steric stabilization. This same formulation was also identified as the minimum composition of PEG to promote condensation and retain colloidal stability in response to physiological salt conditions.

The biological activity of the PEI/PEG-K³ polyplexes was assessed in CHO cells to determine the influence of PEG-K³-based PEGylation on transfection, cytotoxicity, and subcellular trafficking. When the N:P ratio was held constant, transfection and cytotoxicity both increased with increasing amounts of PEI and decreasing amounts of PEG-K³ up to a value of 75% PEI-derived amines (the PEI N:P 7.5 PEG-K³ N:P 2.5 formulation). This result was consistent with the literature, which has shown that the presence of free PEI in solution contributes to cell death, and that minimizing the amount of high molecular weight polycation helps to improve cell viability. (Boeckle et al., 2004) Also in agreement with the literature, (Nguyen et al., 2000; Prata et al., 2008; Tang et al., 2003) there is often a tradeoff between delivery efficiency and cytotoxicity. Reductions in delivery efficiency with increased PEGylation have in part been attributed to the tendency for PEG to reduce complexation efficiency. This does not appear to be the case under the present circumstances, given that the PEG-K³-based PEGylation method maintains polyplex compaction. Since the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes were smaller in size and may have a different surface charge, it is possible that physicochemical differences could influence transfection. The effects of particle size and charge on gene delivery have been thoroughly reviewed elsewhere (Millili et al., *Cancer Gene Therapy*, IN-PRESS). However, with these PEG-K³-based formulations, it is difficult to decouple the size and charge effects from the polymer-specific effects involved in delivery. Given the known effects of PEI on endosomal buffering (Sonawane et al., 2003), and other literature suggesting that polyplex PEGylation affects intracellular trafficking (Mishra et al., 2004), the intracellular fate of the polyplexes was of interest.

To further explore these hypotheses, the possibility of inefficient endosomal buffering and escape was investigated. This hypothesis was based on the fact that the PEG-K³ peptide was

the dominant material in the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes. Failure to effectively escape endosomal compartments during internalization could account for the poor transfection efficiencies observed for the formulations containing lower levels of PEI. Thus, the cells were treated with chloroquine (an endosome destabilizing reagent) at a variety of different concentrations and transfected with the polyplexes (data not shown). No improvements in transfection efficiency were observed, suggesting that endosomal escape is not a bottleneck for these delivery vehicles.

Since inefficient endosomal escape did not explain the trend in cell transfection, the cell internalization efficiencies of the PEI/PEG-K³ polyplexes were assessed by polyplex labeling and fluorescence microscopy. Surprisingly, the level of intracellular YOYO-1-labeled PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes was three-fold higher than the level of intracellular YOYO-1-labeled PEI N:P 10 PEG-K³ N:P 0 polyplexes. Although this effect might be attributed to higher levels of PEI N:P 2.5 PEG-K³ N:P 7.5 polyplex unpackaging, given that YOYO-1 fluorescence is unquenched upon DNA decomplexation, the same effects were observed when polyplexes were labeled *via* PNA-F-containing plasmids. One might expect that higher levels of cellular uptake would correlate directly with increased levels of cellular transfection, but studies have shown that these phenomena can be uncoupled. (Mishra et al., 2004; Ogris et al., 1999) In these examples, PEGylation appeared to alter the subcellular trafficking pathways employed by the differently-formulated polyplexes.

To further explore the cellular uptake pathways employed by the PEI/PEG-K³ polyplexes, cell uptake studies were conducted at 4°C. At low temperatures, energy-dependent internalization mechanisms, as well as intracellular transport, unpackaging, and cytosolic release, are inhibited. The PEI N:P 10 PEG-K³ N:P 0 polyplexes appeared to be surface-associated under these conditions, but the PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes appeared to contain both surface-associated and internalized polyplexes. (Fig. 8) This observation suggests that the inclusion of PEG in this formulation may have consequences for temperature-independent cellular processes. In particular, cellular internalization and trafficking could be altered, resulting in negligible gene expression despite strong uptake. Previous studies have identified five major uptake mechanisms for delivery vehicles: phagocytosis (Reddy et al., 2006), macropinocytosis (Patel et al., 2007; Swanson and Watts, 1995), clathrin-mediated endocytosis (Maxfield and McGraw, 2004; Takei and Haucke, 2001), caveolae-mediated endocytosis (Pelkmans and Helenius, 2002), and clathrin/caveolae independent internalization (Perret et al., 2005). PEI-based polyplexes are known to follow clathrin- and caveolae-dependent pathways (van der Aa et al., 2007); consequently future studies will explore the impact of PEG on the selection of these pathways. We will also investigate the role of non-energy dependent uptake for these formulations.

In conclusion, PEG is capable of inhibiting multimolecular aggregation, promoting colloidal stability, and assisting in the formulation of vehicles that are extremely effective at cellular uptake. However, the functional consequence of these structural improvements appears to be an alteration in intracellular navigation which is manifested in a reduction in transfection efficiency. Through improved understanding of the intracellular pathways taken by PEGylated vehicles, bottlenecks limiting gene expression effectiveness can be overcome, resulting in significant improvements in formulation design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

M.O.S. thanks the NSF (CBET-0707583), the University of Delaware Research Foundation, the ARO DURIP, and the NIH/NCRR INBRE (2 P20 RR016472-07) for financial support for this project. The authors acknowledge technical assistance from the Delaware Biotechnology Institute Bioimaging Center (AFM and SEM) and from John Larsen (Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF)).

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Fig. 1.

(A) The chemical structure of the PEG-K³ peptide. The PEG-K³ peptide was synthesized via solid phase peptide synthesis, and incorporates three lysine residues connected to a 5 kDa linear PEG chain. (B) The PEI polyplex formulation process. (C) The PEG-K³ polyplex formulation process. (D) The PEI/PEG-K³ polyplex formulation strategy. Stepwise addition of PEG-K³ (reaction step 1) and 25 kDa PEI (reaction step 2) facilitate the formation of efficiently complexed, PEGylated particles with low polydispersity.

Fig. 2. Complexation and protection of plasmid DNA with PEI/PEG-K³ mixtures. (A) Ethidium bromide exclusion assays. The fluorescence intensity spectrum (518 nm excitation, 605 nm emission) for each sample was normalized to the peak fluorescence intensity of the uncomplexed DNA. (B) Nuclease stability assay. The indicated samples were sequentially incubated with buffers containing (+) mouse serum, STOP buffer, and heparin, or with buffers absent (-) these reagents. The samples were subsequently analyzed by agarose gel electrophoresis.

Fig. 3. Turbidity measurements of salt stability. Sample absorbance was measured at 600 nm. PEI N:P 0/PEG-K³ N:P 10 (grey triangles), PEI N:P 2.5/PEG-K³ N:P 7.5 (white boxes), PEI N:P 5/PEG-K³ N:P 5 (grey Xs), PEI N:P 7.5/PEG-K³ N:P 2.5 (black circles), PEI N:P 10/PEG-K³ N:P 0 (black diamonds), and PEI N:P 10/PEG-K³ N:P 0 with surface PEGylation (black boxes). Error bars represent one standard deviation from the mean absorbance of three separate samples.

Fig. 4. AFM images of DNA polyplexes. (A) PEI N:P 10/PEG-K³ N:P 0, (B) PEI N:P 7.5/PEG-K³ N:P 2.5, (C) PEI N:P 5/PEG-K³ N:P 5, and (D) PEI N:P 2.5/PEG-K³ N:P 7.5. Each image is a representative image of multiple images collected from two separate samples. SEM images of DNA polyplexes. (E) PEI N:P 10/PEG-K³ N:P 0, (F) PEI N:P 7.5/PEG-K³ N:P 2.5, (G) PEI N:P 5/PEG-K³ N:P 5, and (H) PEI N:P 2.5/PEG-K³ N:P 7.5. The scale bars = 100 nm. Each image is a representative image of multiple images collected from three separate samples.

Fig. 5. Transfection efficiencies of polyplexes made with various PEI/PEG-K³ mixtures. Transfection was assessed in CHO cells 24 h post-transfection by fluorescence microscopy (A) - (F) and flow cytometry (G) to detect GFP fluorescence. The scale bar (shown in [A]) = 250 μ m. The data in (B) represent the mean values obtained from three transfection experiments, and the error bars represent one standard deviation from the mean.

Fig. 6. The cytotoxicity of polyplexes made with various PEI/PEG-K³ mixtures. The red color is associated with ethidium homodimer staining. Each experiment was performed three separate times, with representative images shown. The scale bar = 250 μ m.

Fig. 7. Polyplex internalization into CHO cells. Flow cytometry was used to assess YOYO-1-labeled (A) or PNA-F-labeled (B) polyplex internalization following three hours of incubation with cells at 37°C. The reported values represent the mean of measurements made on three separate cell samples; error bars represent one standard deviation from the mean. PNA-R labeled plasmids were formulated into PEI N:P 10 PEG-K³ N:P 0 polyplexes (C) or PEI N:P 2.5 PEG-K³ N:P 7.5 polyplexes (D) and incubated with cells for 3 hours at 37°C. The cells were then fixed and stained for F-actin (green) and nuclear DNA (blue). The images are representative images from 2 separate experiments. The scale bar (shown in [C]) = 25 μm.

Fig. 8. Polyplex internalization into CHO cells at 4°C. Z-series images were taken of F-actin (green), nuclear DNA (blue), and the fluorescently labeled polyplexes (red) following a 3 hour incubation of the PEI N:P 10 PEG-K³ 0 polyplexes (A) or the PEI N:P 2.5 PEG-K³ 7.5 polyplexes (B) with cells. White arrows point to surface associated polyplexes. The images are representative images from 2 separate experiments. The scale bar (shown in [A] and [B]) = 10 μm.

Table I
Statistical analyses of the particle size distributions obtained by analyzing AFM and SEM images of the polyplexes. The analyses were completed on a set of images representative of 3 SEM and 2 AFM replicates

	PEI N:P 2.5 PEG-K ³ N:P 7.5		PEI N:P 5 PEG-K ³ N:P 5	
	AFM	SEM	AFM	SEM
Standard Deviation (nm)	25.9	N/A	159.6	70.8
Average (nm)	48.3	N/A	96.2	86.7
Coefficient of Variation	0.54	N/A	1.66	0.82
Number of Observations	672	N/A	893	2713

	PEI N:P 7.5 PEG-K ³ N:P 2.5		PEI N:P 10 PEG-K ³ N:P 0	
	AFM	SEM	AFM	SEM
Standard Deviation (nm)	123.1	67.8	58.0	57.1
Average (nm)	83.6	61.2	62.2	63.5
Coefficient of Variation	1.47	1.11	0.93	0.90
Number of Observations	1027	2015	1487	2223