Physiochemical Properties of Low and High Molecular Weight Poly(ethylene glycol)-Grafted Poly(ethylene imine) Copolymers and Their Complexes with Oligonucleotides

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Inefficient delivery of antisense oligonucleotides (AOs) to target cell nuclei remains as the foremost limitation to their usefulness. Copolymers of cationic poly(ethylene imine) (PEI) and poly(ethylene glycol) (PEG) have been well-studied for delivery of plasmids. However, the properties of PEG−PEI−AO polyplexes have not been comprehensively investigated. Therefore, we synthesized a series of PEG−PEI copolymers and evaluated their physiochemical properties alone and when complexed with AO. The M_w of PEG was found to be the main determinant of polyplex size, via its influence on particle aggregation. DLS measurements showed that when PEG5000 was grafted to PEI2K and PEI25K, polyplex diameters were extremely small (range 10−90 nm) with minimal aggregation. In contrast, when PEG550 was grafted to PEI2K and PEI25K, polyplexes appeared as much larger aggregates (∼250 nm). As expected, the surface charge (ζ potential) was higher for polyplexes containing PEI25K than those containing PEI2K, but decreased with increased levels of PEG grafting. Surprisingly, within the physiological range (pH 7.5−5), the buffering capacity of all copolymers was nearly equivalent to that of unsubstituted PEI2K or PEI25K, and was barely influenced by PEGylation. The stability of polyplexes was evaluated using a heparin polyanion competition assay. Unexpectedly, polyplexes containing PEI2K showed stability equal to or greater than that of PEI25K polyplexes. The level of PEG grafting also had a dramatic effect on polyplex stability. The relationships established between molecular formulations and polyplex size, aggregation, surface charge, and stability should provide a useful guide for future studies aimed at optimizing polymer-mediated AO delivery in cell and animal studies. A summary of the relationships between polyplex structures and recent studies of their transfection capacity is provided.

Introduction

Small oligonucleotides such as antisense oligoribonucleotides (AOs) and interference RNA (siRNA) have seen a remarkable recent surge in popularity for basic and applied research. Over the past decade, a host of chemical modifications to the basic structure of AOs has greatly expanded their specificity, functionality, and resistance to degradation and thus markedly improved their potential usefulness.[1−7] In particular, chemically modified AOs are now seen as valuable agents for targeted down regulation of transcript expression, modulation of alternative splicing, and exon skipping.[4,6,8−10] Together, AOs and siRNA represent powerful agents that can now be used to leverage information gained from genomic and proteomic studies into knowledge of protein function, identification of drug targets, and therapeutic intervention.[4,6] Although the main application for new classes of AOs, and indeed siRNA, may be drug discovery, they also present an increasingly valuable basic research tool for studying protein function, signal transduction, and gene regulation.

The primary limitation that has most impeded our ability to utilize oligonucleotides to greater advantage is the lack of effective delivery systems. Alone, small oligonucleotides have very low transfection efficiency and are rapidly degraded by nucleases, necessitating the use of carrier molecules. Recently, several excellent reviews have defined the barriers that must be overcome for successful delivery of oligonucleotides to target cell nuclei, and have outlined progress in carrier-mediated delivery to overcome those barriers.[1,7,11−15] For carrier-mediated transfection of both AO and siRNA, the majority of studies have relied on cationic lipoplexes,[16] some of which are commercially available. Although significant progress has been made in improving lipoplexes for oligonucleotide delivery,[12,17] cytotoxicity and serum reactivity continue to hinder their usefulness especially for use in vivo.[18]

The cationic polymer poly(ethylene imine) (PEI) is well-known as an efficient nucleotide carrier due to formation of PEI−nucleotide complexes that show high stability, controllable size, adjustable unpackaging properties in cells, and flexibility for addition of moieties that target specific entities on cell membranes and intracellular structures.[19−24] PEI forms polyplexes with anionic nucleotides by charge coupling. In the absence of a targeting moiety, cellular uptake of the polyplexes appears to occur by nonspecific adsorptive endocytosis, although this process is not well understood. Escape from the endosomes is facilitated by protonation of amines on PEI, the so-called "proton sponge" effect.[24−26] Once released from endosomes, the nucleotides may enter the nucleus by a process that likely requires dissociation of the nucleotide from its polymer carrier.

The functionality of PEI as a nucleotide carrier is significantly improved by incorporating hydrophilic poly(ethylene glycol) (PEG) into PEG−PEI copolymers.[21,23] Complexation of PEG−PEI copolymers with nucleotides produces particles with a core−shell structure, whereby PEI−nucleotide is sequestered toward the particle core and the PEG chains form a brushlike

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corona, although the precise nature of this arrangement is still an open question. In general, PEG provides polyplexes with improved solubility, lower surface charge, diminished aggregation, lower cytotoxicity, and possibly improved stealthiness in the bloodstream. However, these desirable properties may come at a cost of lower transfection efficiency due to both reduced membrane interaction and less efficient endosomal escape. The precise function of PEGylation may depend on many factors including the PEG M_w, PEI M_w, stoichiometry of PEG grafting, type of nucleotide, and environment.

Although PEG−PEI copolymers have been primarily used for plasmid delivery, other studies have provided strong evidence that PEGylated PEI can be adapted to function as an effective carrier for cellular delivery of small oligonucleotides. Several recent reports have characterized the properties and transfection capacity of PEGylated PEI25K complexed with oligonucleotides.[27−32] Overall, these studies have provided valuable insights into the influence of the PEG chain length, extent of PEG grafting, and method of PEGylation on the polyplex structural properties, nuclease protection, complement activation, serum stability, transfection efficiency, and in vivo distribution. To a lesser extent, similar types of studies using much smaller molecular weight PEI2K have also described the physiochemical properties and transfection potential of PEG−PEI−AO polyplexes.[33,34] In addition, improvements in the transfection efficiency of PEG−PEI−oligonucleotide polyplexes have been attained by covalent attachment of cell-targeting ligands to PEG.[29,34]

Despite the aforementioned studies, we still have only a limited understanding of the physiochemical properties of PEG−PEI copolymers and the complexes formed upon interaction with oligonucleotides. To date, there has not been a rigorous comparison of the physiochemical properties of polyplexes containing high and low molecular weight PEI in terms of the influence of PEG shielding, the tendency to form aggregates, or stability. While some important principles that govern these processes can be understood from plasmid-based studies, the extreme differences in molecular weight and other physical attributes between small oligonucleotides and plasmids will result in significantly different properties of their respective copolymer−nucleotide complexes. In addition, all previous studies of PEG−PEI as an oligonucleotide carrier have focused only on polyplex properties, and have failed to report the properties of copolymers alone. Because small oligonucleotides are in the same molecular weight range as copolymers, the copolymer properties may contribute a much greater influence on the physiochemical characteristics of polyplexes than for plasmid-based polyplexes. Therefore, measuring the copolymer properties alone is a prerequisite to understanding the interactions of copolymers and oligonucleotides in polyplexes.

In this study we synthesized a series of PEG−PEI copolymers from branched PEI (M_w = 2000 and 25000) and PEG (M_w = 550 and 5000). We show that variation of PEG−PEI−oligonucleotide formulations produces polyplexes with a wide range of physiochemical properties that should significantly influence their adaptability for cellular delivery of oligonucleotides in differing environments. By utilizing copolymers that covered a wide range of PEI M_w, PEG M_w, and PEG grafting, we revealed a dynamic range of polyplex size, aggregation status, surface charge, and stability. The relationships established between polyplex formulations and physiochemical properties provides a foundation for optimizing polymer-mediated oligonucleotide delivery in cell and animal studies.

Table 1. Compositions and Molecular Weights of Investigated PEG−PEI Copolymers

copolymer	PEI M_w[a]	PEG M_w	PEG:PEI	copolymer M_w
PEI25000(PEG5000)$_{10}$	25000	5000	10	75000
PEI25000(PEG550)$_{25}$	25000	550	25	38750
PEI25000(PEG5000)$_{25}$	25000	5000	25	150000
PEI25000(PEG5000)$_{50}$	25000	5000	50	275000
PEI2000(PEG550)$_{10}$	2000	550	10	7500
PEI2000(PEG5000)$_{10}$	2000	5000	10	52000

[a] The M_w values for PEI refer to the values provided by the supplier, and the corresponding M_w values listed for copolymers are based on these values. Recently, the M_w of commercially available branched PEI25000 has been measured using SEC−MALLS and found to be substantially higher than indicated by the suppliers.[21,35] Therefore, the PEG:PEI ratios listed for the high M_w PEI25000-based copolymers may underestimate the actual ratio.

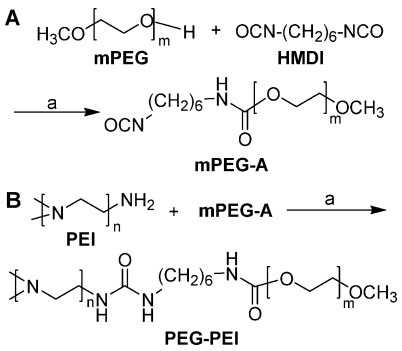

Figure 1. Synthesis of PEG−PEI copolymers. (A) (a) $CHCl_3$, N_2, reflux, 16 h; (**B**) (a) $CHCl_3$, N_2, reflux, 16 h.

Experimental Section

Chemicals and Instruments. Methoxypoly(ethylene glycol)-550 (mPEG550), mPEG5000, poly(ethylene imine)-2000 (PEI2000), PEI25000, and hexane-1,6-diisocyanate (HMDI) were all from Sigma-Aldrich (St. Louis, MO). Et_2O and hexanes (Acros-Organic, Fisher Scientific, Hampton, NH) were used as received. $CHCl_3$ (Acros) was distilled from CaH_2 prior to use. Unless otherwise indicated, we used an arbitrary single-stranded 20-mer deoxyoligonucleotide (ODN) as a model antisense oligonucleotide with the sequence 5′-GGCCAAAC-CTCGGCTTACCT-3′ (phosphodiester, crude lyophilized Na^+ salt, Trilink Biotechnologies, San Diego, CA). In some cases (where indicated) we instead used an AO with the sequence 5′- UCCAUUCG-GCUCCAAACCGG-3′ that was modified by 2-O-methylation and phosphorthioation (2OMeAO) and contained a 6-FAM label at the 5′ end (Proligo, Boulder, CO). Because of the large amounts of oligonucleotide involved, we used the ODN rather than the 6-FAM-2OMeAO for all experiments except the polyanion competition assay.

^1H (300 MHz) and ^{13}C (75.4 MHz) NMR spectra were recorded with a Mercury VX 300 MHz spectrometer (Varian, Palo Alto, CA) with tetramethylsilane (TMS) as an internal standard. Relative molecular weights were determined using a GPC instrument equipped with a Rainin data station, a Pl Gel mixed column (5 μm, Polymer Labs, Amherst, MA), and THF as the eluent at 0.85 mL/min. Detection was by refractive index (Knauer differential refractometer, Knauer, Berlin, Germany). Relative molecular weights (M_w from 580 to 1290000) were calculated using calibration plots constructed from polystyrene standards. FT-infrared spectra were recorded as KBr pellets on a Perkin-Elmer 1610 spectrometer (Perkin-Elmer, Wellesley, MA).

Polymer Synthesis. A series of PEG−PEI copolymers (Table 1) were prepared using a two-step procedure (Figure 1; also see the Supporting Information for details). Both mPEG5000 and mPEG550 were activated with HMDI, on the basis of a previously reported procedure.[21] Solutions containing mPEG and HMDI (10× molar excess) were refluxed in dry $CHCl_3$ under nitrogen for 16 h and then

concentrated. Excessive HMDI (bp 110 °C, 0.1 mmHg) was removed via vacuum distillation. The residue (mPEG−HMDI) was dissolved in a minimal amount of $CHCl_3$ and precipitated into hexanes (−30 °C for mPEG550) two or three times. For each reaction, it was ensured via ^{1}H NMR and FT-IR spectroscopy that no excessive HMDI was present in the product, and the purity was further confirmed with GPC.

PEI and activated mPEG, at a given molar ratio (Table 1), were dissolved in a small amount of $CHCl_3$ and refluxed for 16 h under N_2 (Figure 1). Copolymers containing PEI25000 were precipitated into Et_2O, whereas copolymers containing PEI2000 were obtained by evaporation of the solvent, followed by drying the products under high vacuum until weight constancy. Completeness of reactions was verified with ^{1}H NMR and FT-IR. All copolymers were stored at −20 °C in sealed vials under N_2.

The M_w values for PEI are the values provided by the supplier. The M_w of commercially available branched PEI25000 has been measured (SEC−MALLS) and reported to be substantially higher than indicated by the suppliers.[21,35] Therefore, the PEG:PEI ratios listed for the high M_w PEI25000-based copolymers may underestimate the actual ratio.

Polymer Buffering Capacity. The buffering capacity of all polymers was measured with pH titration assays. Polymers were dissolved in 150 mM NaCl and sonicated briefly. All polymer solutions were prepared to contain the same number of PEI amine groups (24 mM amine), which allowed for a direct comparison of the buffering capacity of each polymer per mole of amine. The pH of the polymer solutions was measured during slow addition of 0.01 N HCl (in 150 mM NaCl) under vigorous stirring. All experiments were done in duplicate.

Formation of Polyplexes. Polyplexes were prepared over a range of *N*:*P* values (1−15), where *N* represents moles of amine on PEI and *P* represents moles of phosphate on oligonucleotide. Copolymer solutions (in diH_2O) were added slowly to oligonucleotide solutions (in 1× PBS) to reach the required *N*:*P* ratio and vortexed briefly. Pilot DLS experiments using polyplexes of PEI25000(PEG5000)$_{10}$−AO (*N*:*P* = 5) and PEI2000(PEG550)$_{10}$−AO (*N*:*P* = 5) showed that the order in which the components were added did not influence the particle size distribution (data not shown). Thus, the mixing order described here was used for all experiments.

Dynamic Light Scattering. The hydrodynamic diameters of polymers and polyplexes in aqueous solutions were measured using DLS at an angle of 90° with a Brookhaven Instruments detector (BI-APD, Brookhaven Instruments, Holtzville, NY), equipped with a Melles Griot 25-LHP-928-246 laser (35 mW, maximum at 632.8 nm, Melles Griot, Carlsbad, CA) and goniometer (BI-200SM, Brookhaven Instruments). Copolymers alone were analyzed at 1 mg/mL in 3 mL of PBS. Polyplexes contained 60 μg of ODN (with the appropriate amount of polymer for the desired *N*:*P*) in a total 3 mL volume (PBS). All samples were filtered three times through a 450 nm dust filter. Filters, syringes, tubes, and other equipment were washed repeatedly with filtered diH_2O. For data analysis, the viscosity and refractive index of water at 25 °C (0.89 mPa·s and 1.333, respectively) were used. Polystyrene nanospheres (220 ± 6 nm, Duke Scientific Corp., Palo Alto, CA) were used to check the performance of the instrument.

All DLS results were based on four measurements, from two separately prepared samples. For each sample, data were acquired until the "total count number" was at least 3.0×10^6. Data were collected and analyzed with BIC dynamic light scattering software (version 3.37, Brookhaven Instruments) using the NNLS algorithm. Values smaller than 5 nm were considered to be below the reliable detection limit of the apparatus and were not included in the analysis.

ζ Potential. The ζ potential (surface charge) of polymers and polyplexes was determined at 25 °C using a Zeta Plus apparatus equipped with a 658 nm laser (Brookhaven Instruments). Samples were prepared in 1× PBS and then diluted 1:8 with diH_2O to ensure measurements were made under conditions of low ionic strength (conductance <4000 μS), where the surface charge of particles can be accurately measured. The final concentration of the polymer was 1 mg/mL. The surface charge of the polymers and polyplexes was analyzed by Doppler electrophoresis using default program settings (ζ potential analyzer, version 3.30). All data refer to 15 measurements from one sample.

Polyplex Stability: Polyanion Competition Assay. The relative stability of polyplexes was measured by measuring AO released from polyplexes in the presence of a competing polyanion. For these experiments the 6-FAM-labeled 2OMeAO was used. Polyplex solutions were incubated (10 min) in the presence of heparin (American Pharmaceutical Partners, Inc., Schaumburg, IL) over a range of concentrations. All polyplexes were formed at *N*:*P* = 5, and samples contained 0.8 μg of AO. Samples were run on agarose gels (1.5% in TAE buffer, 100 V, 25 min), and the intensity of the "free AO" band was quantified by densitometry using Alphaimager 3400 (Imgen Technologies, New City, NY). At a given heparin concentration, the percentage of AO dissociated from the polyplex was obtained by normalizing against the AO band measured from a sample containing AO only on the same gel. All experiments were performed in duplicate.

Results and Discussion

Synthesis of PEG−PEI Copolymers. A series of PEG−PEI copolymers were synthesized (Table 1) using a modification of previously described methods.[21] The synthesis of PEG−PEI copolymers involved the activation of mPEG and coupling of the activated mPEG (mPEG-A) with PEI (Figure 1). The purity and structural authenticity were monitored and confirmed with ^{1}H NMR and FT-IR spectroscopy. mPEG was activated by its reaction with a 10× molar excess of HMDI to minimize symmetric disubstitution of the diisocyanate. Thorough removal of excess HMDI was a key step, as it could otherwise act as a cross-linker during subsequent addition of PEI. Such cross-linking gives rise to a network copolymer of much higher molecular weight and decreased solubility. In particular, it was ensured that the ^{1}H NMR peaks of the methylene groups adjacent to urethane ($-CH_2$NHCO−, δ = 3.15; −NHCOOCH_2−, δ = 4.20) had about the same intensity as the CH_2 group adjacent to unreacted isocyanate ($-CH_2$N═C═O, δ = 3.30). The final coupling of mPEG-A with PEI was monitored with IR and ^{1}H NMR. The absence of the characteristic N═C═O absorption band ($\nu \approx 2270$ cm^{-1} in IR) and of the ^{1}H NMR peak at δ = 3.30 indicated the completion of the reaction. Details of the synthesis and structural analysis of all copolymers are described in the Supporting Information.

Hydrodynamic Diameter and Surface Charge of Polymers. To better understand the behavior of polyplexes, we first investigated the polymers alone. The hydrodynamic diameter of polymers was determined with DLS, using the NNLS algorithm, which is appropriate for polydisperse samples with potentially discrete (multimodal) size distributions.[36] Data were converted to "relative by number" plots, from which the mean hydrodynamic diameter was derived.

All four PEGylated PEI25000 copolymers exhibited very small hydrodynamic diameters of ∼10−30 nm, which were similar to the diameter of unsubstituted PEI25000 (11 nm, Figure 2). These data suggest that copolymers of PEI25000 grafted with PEG5000 do not exhibit extensive aggregation, and probably exist in solution as small oligomers (monomers, dimers, or trimers). For example, although the molecular weight of PEI25000(PEG5000)$_{50}$ (M_w = 275000) was 11 times greater than that of unsubstituted PEI25000 (11 nm), the hydrodynamic diameter (27 nm) increased by only a factor of ∼2.5.

In contrast, the diameters of PEG−PEI copolymers with low M_w PEI2000 were heavily influenced by the M_w of PEG (Figure 2). When high molecular weight PEG5000 was grafted to PEI2000, the copolymers were very small (15 ± 0.4 nm), with

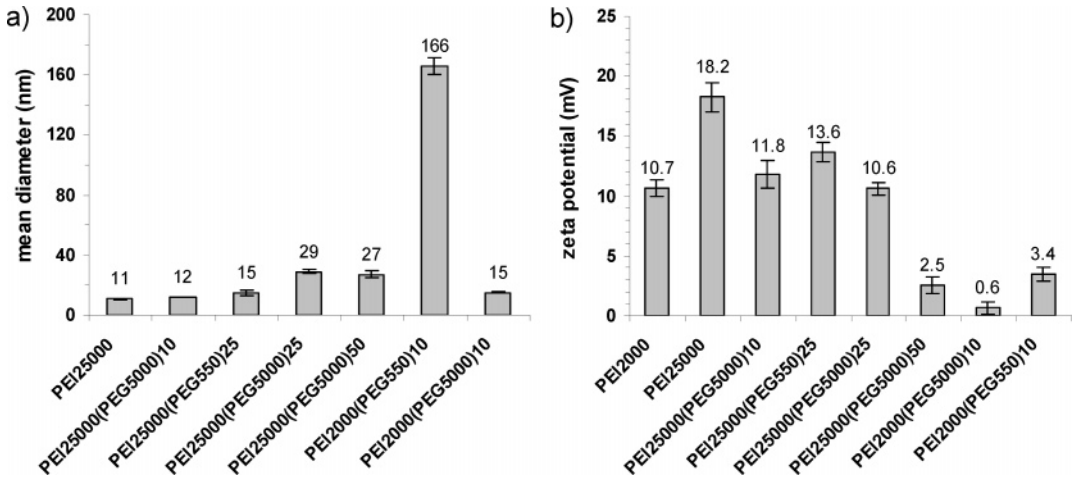

Figure 2. Mean diameter and ζ potential of PEG−PEI copolymers and PEI homopolymers in solution. (a) The mean hydrodynamic diameter was determined by dynamic light scattering and analyzed using the NNLS algorithm. Shown are the mean values (±SEM) from four measurements each from two separately prepared samples. (**b**) ζ potentials are shown as the mean (±SEM) from 15 measurements of a single sample.

minimal aggregation. However, grafting of low molecular weight PEG550 to PEI2000 produced copolymer particles about 10-fold larger (166 ± 5.7 nm), suggesting the presence of extensive aggregation.

Figure 2b shows the corresponding ζ potentials for the polymers in solution. As expected, increased levels of PEG grafting led to decreased surface charge. To effectively shield the charge of PEI25000 (18.2 ± 1.3 mV), PEG grafting of up to 50 chains per PEI was required. Whereas PEI25000-$(PEG5000)_{10}$ (11.8 ± 1.2 mV), PEG25000$(PEG550)_{25}$ (13.6 ± 0.8 mV), and PEI25000$(PEG5000)_{25}$ (10.6 ± 0.5 mV) each had a relatively high ζ potential, PEI25000$(PEG5000)_{50}$ (2.5 ± 0.7 mV) was well shielded.

The aggregation of PEG−PEI copolymers and their polyplexes in aqueous solution is determined by factors similar to those of colloids. Nonaggregating particles are formed only if there is enough repulsion (electrostatic or steric) between the individual particles. Our results indicate that, for PEI2000, grafting with high molecular weight PEG5000 was required to provide enough steric repulsion to deter polymer aggregation (Figure 2a), as seen in the comparison between PEI2000-$(PEG550)_{10}$ (166 ± 5.7 nm) and PEI2000$(PEG5000)_{10}$ (15 ± 0.4 nm). On the other hand, the greater electrostatic repulsive forces within PEI25000 sufficiently deterred particle aggregation even when grafted with low molecular weight PEG550 (i.e., PEI25000$(PEG550)_{25}$ = 15 ± 1.6 nm).

Hydrodynamic Diameter and Surface Charge of PEG−PEI−ODN Polyplexes. The diameter and surface charge of polyplexes formed from the six different copolymers was determined with DLS and ζ potential measurements over a range of *N*:*P* values (Figure 3). Overall, the DLS data suggest that the two copolymers with low molecular weight PEG550 formed polyplexes that appeared as aggregates at all *N*:*P* values, while all copolymers with PEG5000 formed much smaller polyplexes with minimal or no aggregation. The tendency of copolymers to form aggregated or nonaggregated polyplexes can be explained by a combination of the charge density of the PEI chains (electrostatic repulsion, which depends also on *N*:*P*) and size of the PEG chains (steric repulsion) that govern the stability of polyplex particles.

The copolymers with PEG5000 grafted to PEI25000 at 10, 25, and 50 PEGs per PEI each formed polyplexes with minimal aggregation, although in the case of PEI25000$(PEG5000)_{10}$ aggregation was sharply dependent on the *N*:*P* ratio. For this copolymer, an *N*:*P* ratio of 1 or 2 led to aggregated particles of ~150 nm and visible cloudiness in the solution. Addition of more copolymer to such solutions, until the *N*:*P* ratio reached 5 or higher, rapidly decreased the hydrodynamic diameter to approximately the same size as found for solutions of the polymer alone (~10−20 nm). The surface charge of these polyplexes was close to neutral at an *N*:*P* of 1 but increased steeply at *N*:*P* > 5. Therefore, at or near electrical neutrality these complexes had the tendency to aggregate, while the higher ζ potentials at *N*:*P* > 5 provided enough electrostatic repulsion to minimize aggregation. This *N*:*P* effect is in agreement with previous data reported for similar complexes. Kunath et al.[27] reported a diameter of 80 nm for complexes of PEI25000-$(PEG5000)_6$ and a 20-mer oligonucleotide at *N*:*P* = 7, and observed larger aggregates (~150 nm) of this polyplex at *N*:*P* = 3. Interestingly, this same group[28] more recently reported only a 10 nm diameter for complexes formed between PEI25000-$(PEG5000)_6$ and a 40-mer oligonucleotide (*N*:*P* = 7). Apparently, slight differences in the oligonucleotide chain length or chemistry impacted the aggregation behavior of these polyplexes. Aggregation at lower overall charge (i.e., lower *N*:*P* ratio) was also observed for PEI−DNA polyplexes.[37]

Interestingly, when the substitution of high molecular weight PEG5000 was increased, as in PEI25000$(PEG5000)_{25}$ and PEI25000$(PEG5000)_{50}$, the polyplex diameters increased only to about 50−90 nm. The small increases in diameter were expected on the basis of the increased molecular weight of the highly substituted copolymers and suggest that only minimal aggregation (perhaps dimers or trimers) occurred. Apparently, the formation of larger aggregates was prevented by steric repulsion from the high molecular weight PEG chains, despite the fact that electrostatic repulsion in these complexes was quite low (i.e., ζ potentials between 2.6 and 6.3 mV). These findings are in agreement with a previous report of 90 nm particles with a ζ potential of +5 mV for polyplexes of PEI25000$(PEG3400)_{40}$−AO.[29]

In contrast, when PEI25000 was grafted with low molecular weight PEG chains (PEG550), the polyplexes containing PEI25000$(PEG550)_{25}$ exhibited large aggregate particles (range 200−270 nm) even at high *N*:*P* ratios. Apparently, the low molecular weight PEG550 did not supply enough steric repulsion to deter aggregation. While this effect was not strong enough to cause aggregation of this copolymer in solution alone, partial neutralization of the cations by the addition of ODN

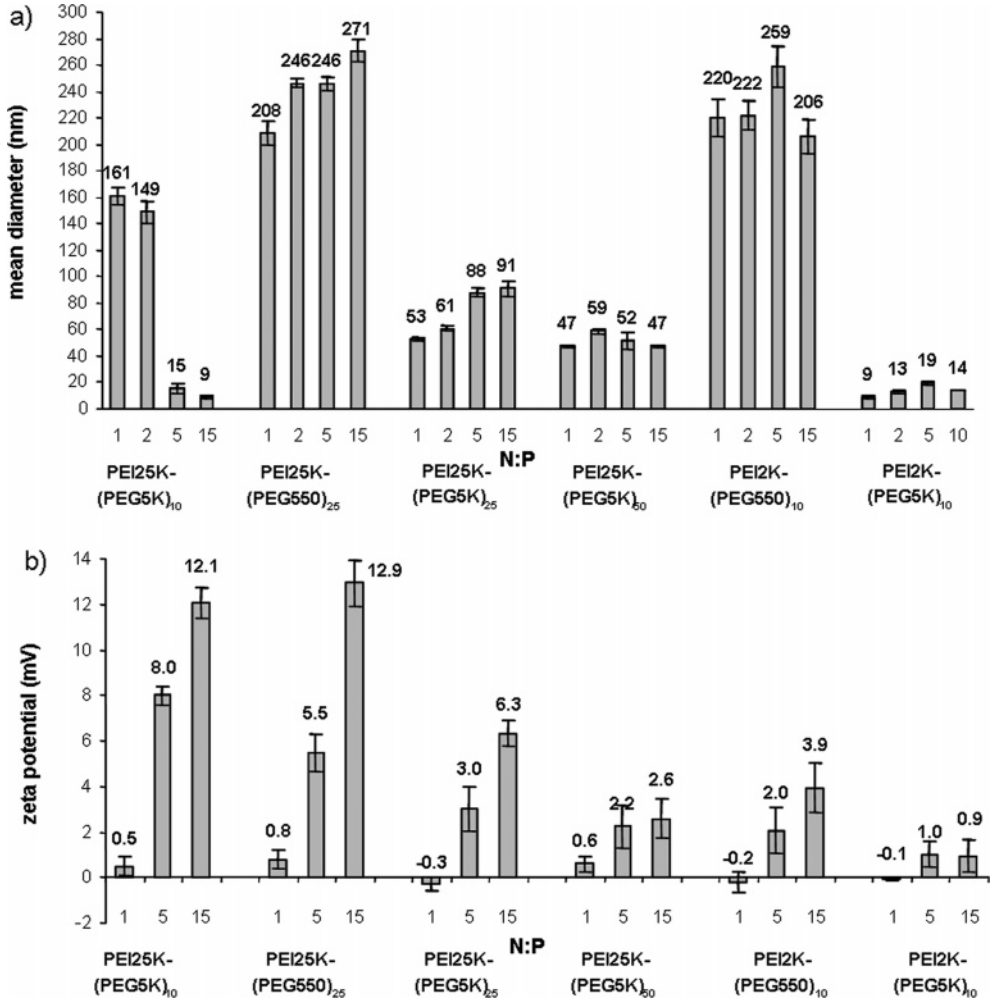

Figure 3. Mean diameter and ζ potential of PEG−PEI−ODN polyplexes over a range of *N*:*P* values. (**a**) The mean hydrodynamic diameter was determined by dynamic light scattering and analyzed using the NNLS algorithm. Shown are the mean values (±SEM) based on four measurements from two separately prepared samples. (b) ζ potentials are shown as the mean (±SEM) based on 15 measurements from a single sample.

appears to shift the equilibrium toward aggregation. In agreement with these results, Kunath et al.[27] found diameters of more than 300 nm for PEI25000(PEG550)$_{50}$ complexed with a 20-mer AO at *N*:*P* = 3.5 and 6.0. In contrast, Brus et al.[28] reported much smaller sizes of only 30−50 nm for PEI25000(PEG550)$_{18}$ and PEI25000(PEG550)$_{50}$ complexed with a 40-mer AO at an *N*:*P* of 6.7. Possibly, differences in the oligonucleotide chain length or chemistry may be the cause of this apparent discrepancy.

Analysis of polyplexes formed from low molecular weight PEI2000 further confirmed that the PEG chain length was a main determinant of polyplex aggregation. Polyplexes containing PEI2000(PEG5000)$_{10}$ exhibited small particles (10−15 nm) at both low and high *N*:*P* ratios. The surface charge of these polyplexes was very low (1 mV), even at an *N*:*P* of 5. Thus, the steric repulsion provided by PEG5000 was apparently sufficient to overcome the lack of electrostatic repulsion and avoid aggregation. This is in agreement with similar diameters reported for complexes based on PEI2000(PEG8000)$_{1.7}$.[33]

In marked contrast, polyplexes formed from PEI2000-(PEG550)$_{10}$ were highly aggregated (200−250 nm, Figure 3a) at all *N*:*P* ratios. Because these copolymers alone already formed aggregates in solution (166 nm), it was not surprising that the polyplexes were also aggregate particles. In this case the steric repulsion provided by PEG550 was not able to overcome the low level of electrostatic repulsion, as ζ potentials for the polyplexes were only about +1 mV, even at an *N*:*P* of 15.

In preliminary experiments we also measured the hydrodynamic diameter with DLS of a limited number of polyplexes comprised of AO rather than ODN. Overall, for polyplexes containing PEI2000(PEG550)$_{10}$ and PEI25000(PEG5000)$_{10}$ we found no detectable difference between the diameters of polyplexes formed from AO and ODN (data not shown). However, more rigorous analysis will be required to assess the extent to which differing chemistries of oligonucleotides influence polyplex structural properties.

Buffering Capacity of Polymers. The buffering capacity of all PEG−PEI copolymers and unsubstituted PEI was determined by a potentiometric titration assay (Figure 4). Titrations of PEGylated PEIs have rarely been reported, and included only a limited set of copolymers.[33,38] Therefore, we investigated the buffering capacities of all PEG−PEI copolymers and compared them with those of their PEI homopolymers. Because of the extensive amount of oligonucleotide that would have been required for accurate pH measurements of polyplexes, this assay was only performed on polymer solutions. The numbers of protonatable amine groups on PEI25000 and PEI2000 are estimated to be 580 and 45, respectively. Therefore, to directly compare the buffering capacities of the polymers, all measurements were made at an equivalent molar concentration of amine groups (i.e., 24 mM N).

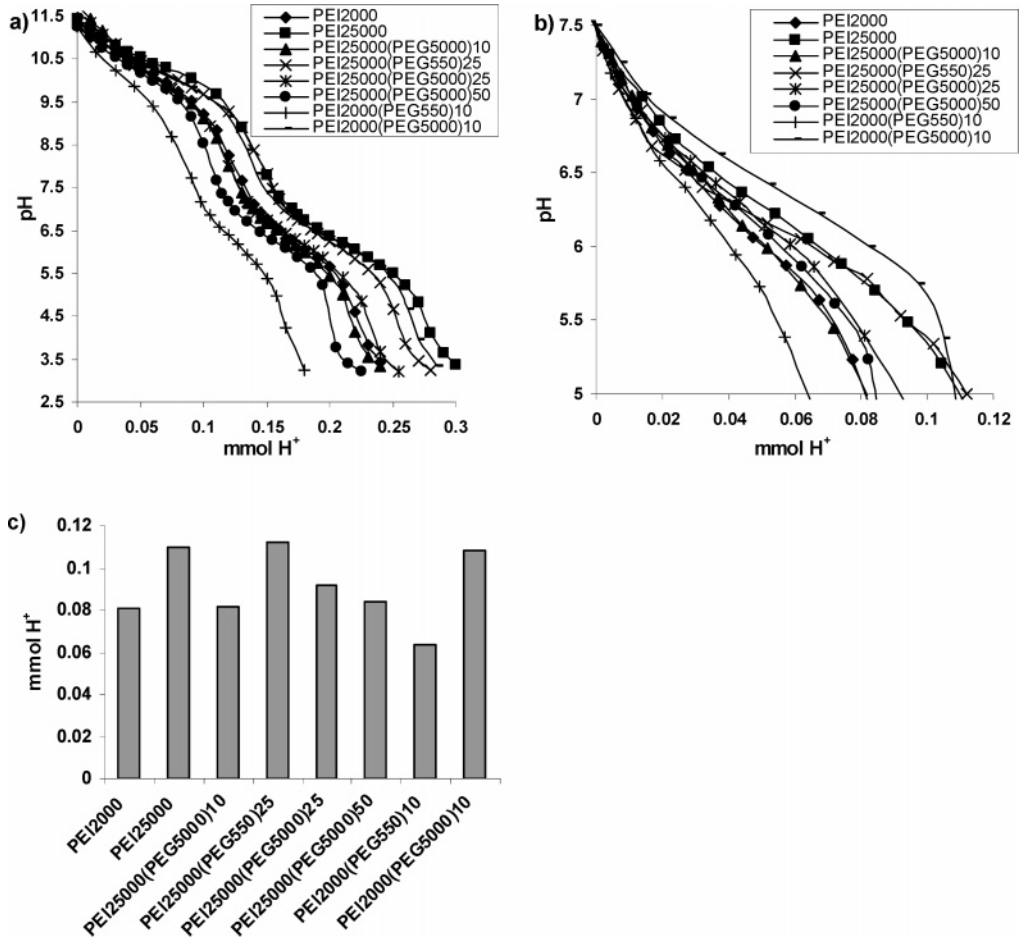

Figure 4. Buffering capacity of PEG−PEI copolymers and PEI homopolymers in solution. (a) Potentiometric titration curves over the pH range from 11.3 to 3.5. Each curve was determined as the average values from duplicate experiments. (b) Same data as in panel a, expanded to highlight the buffering capacity within the physiological pH range over which the copolymers exist during cellular transfection. To allow a better comparison between the polymers, the initial [H^+] was normalized to 0 at pH 7.5. (c) Total [H^+] required to lower the pH from 7.5 to 5 (i.e., the physiological range) taken from the plots in panel b.

Figure 4a shows the titration plots for all polymers over a pH range from 11.3 to 3.5. In general, all polymers behaved very similarly and exhibited two regions of higher buffering capacity (flatter curves, pH 11−9.5 and pH 7−5.5), neighbored by regions of lower buffering capacity (steeper curves). These two areas of higher buffering capacity can be assigned to the protonation of primary and secondary amino groups.[33]

Figure 4b shows the same data expanded to highlight the buffering capacity within the physiological pH range over which the copolymers exist during cellular transfection from the extracellular space to the lower pH (pH $\approx$ 5−6) endosomes. Importantly, for all polymers one of the two regions of higher buffering capacity falls within this physiological range. This is also demonstrated by the similarity in the total amount of protons taken up by the copolymers when titrated from pH 7.5 to pH 5 (Figure 4c). This finding is in agreement with data reported by Von Harpe et al.,[39] who found no significant difference in buffering capacity between PEI600000 and PEI800.

Overall, these data suggest that low and high molecular weight PEI-based copolymers should act as equally strong proton buffers during transit through endosomal compartments and facilitate escape from endosomes via the proton sponge mechanism.

Polyplex Stability in the Presence of Competing Polyanions. We chose heparin, a polysaccharide bearing sulfonate groups, as a model compound to evaluate polyplex stability in a polyanion competition assay. Heparin is a glycosaminoglycan (GAG), which is a linear, negatively charged polysaccharide. GAGs are the major components in the extracellular matrix of many tissues (for example, vascular walls and connective tissues), but are also found inside cells and on the cell surface.[40]

To evaluate polyplex stability, polyplexes were incubated with varying amounts of heparin (10 min) prior to separation on agarose gels (Figure 5). All polyplexes contained the same amount of 6-FAM-2OMeAO (0.8 μg) and were prepared at the same *N*:*P* ratio (*N*:*P* = 5). The intensity of the free AO band in heparin-incubated and zero-heparin polyplex samples was normalized against the intensity of the AO band from "AO only" samples. In some cases bands were observed between the sample wells and the AO only position. These bands were identified as complexes of polymer, AO, and heparin (data not shown), and were not counted as free AO in the quantification. For all polyplexes, when no heparin was added, AO was not released from the polyplexes and was visible in the sample well.[41,42] This indicates that all polyplexes were sufficiently stable in the absence of polyanions to deter electrophoretic separation of the copolymer and AO.

In Figure 6 the percentage of AO released is shown as a function of heparin concentration. A comparison of the concentration of heparin needed to release 50% of the AO (IC_{50}) was used to rank the relative stability of the polyplexes (Figure 6b). For the polyplexes based on PEGylated PEI25000, increas-

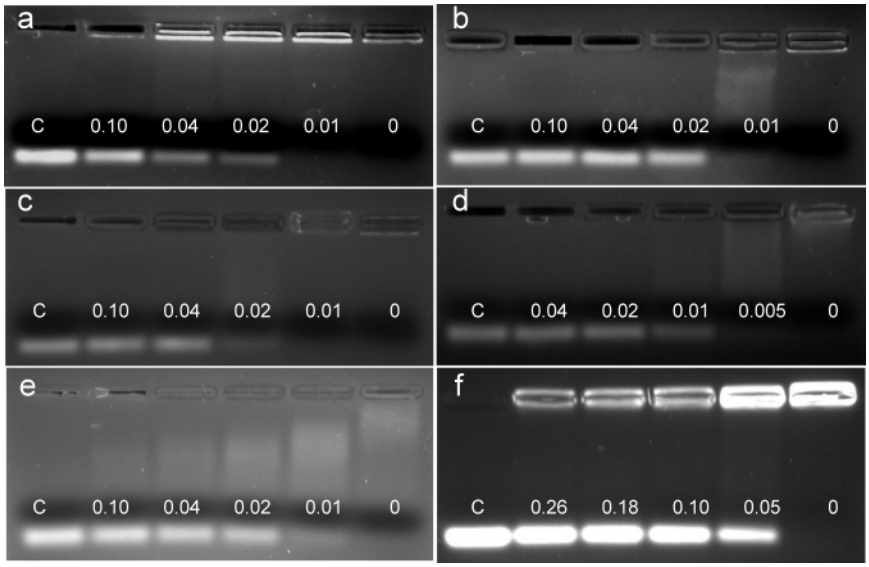

Figure 5. Stability of polyplexes in the presence of competing polyanions. Polyplexes containing PEG−PEI complexed with 6-FAM-AO were incubated with varying amounts of heparin (polyanion) prior to separation on agarose gels. All polyplexes contained 0.8 μg of 6-FAM-AO, and were prepared at the same *N*:*P* ratio (*N*:*P* = 5). Each panel shows a representative gel for a different polyplex, and includes the full range of heparin concentrations examined (μg/μL, as indicated in each lane). Control lanes (indicated as C) contained 0.8 μg of AO only. Gel identifications are as follows: (a) PEI25000(PEG5000)$_{10}$, (b) PEI25000(PEG550)$_{25}$, (c) PEI25000(PEG5000)$_{25}$, (d) PEI25000(PEG5000)$_{50}$, (e) PEI2000-(PEG550)$_{10}$, and (f) PEI2000(PEG5000)$_{10}$.

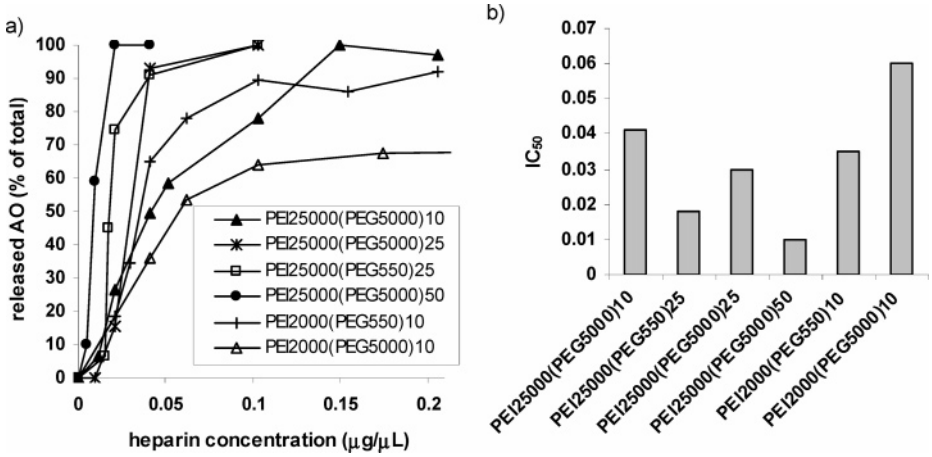

Figure 6. Polyplex stability evaluated with a polyanion competition assay. (a) Destabilization of PEG−PEI−AO polyplexes, each of *N*:*P* = 5, with 0.8 μg of 6-FAM-AO, by exposure to increasing amounts of heparin. The incubated samples were run on agarose gels, and the amount of released AO was quantified with densitometry by normalizing against the intensity of the band from a sample containing AO only in the same gel. All data are based on duplicate runs of gels as shown in Figure 5. (b) Heparin concentration required to release 50% of the AO from each polyplex (IC_{50} value).

ing the level of PEG grafting from 10 to 25 and 50 PEG chains per PEI dramatically reduced the polyplex stability. The IC_{50} value for PEI25000(PEG5000)$_{10}$ was 36%, 127%, and 310% higher than for PEI25000(PEG5000)$_{25}$, PEI25000(PEG550)$_{25}$, and PEI25000(PEG5000)$_{50}$, respectively. This behavior is explained by an effect of PEG shielding, where PEG partly deters electrostatic interactions between polycationic PEI and polyanionic AO. For the highly PEGylated copolymers, AO is most likely only loosely bound to the copolymer and may even be located partly within the PEG shell rather than in the PEI core. Conversely, for polyplexes of PEI25000(PEG5000)$_{10}$, the AO is apparently sequestered more toward the PEI core of these core−shell particles.

Surprisingly, the stability of PEI2000-based polyplexes was equal to or greater than that of the most stable PEI25000-based polyplex. The IC_{50} values for PEI2000(PEG5000)$_{10}$ and PEI2000-(PEG550)$_{10}$ were 46% higher and 17% lower, respectively, than for PEI25000(PEG5000)$_{10}$ (Figure 6b). Even at the highest heparin concentration tested, PEI2000(PEG5000)$_{10}$ polyplexes released just less than 70% of their AO. The mechanism behind the relatively high stability against polyanions of PEI2000-(PEG5000)$_{10}$ polyplexes is not clear. A possible explanation is that PEI2000(PEG5000)$_{10}$ polyplexes carry far fewer positive charges per particle than PEI25000-based polyplexes, and the attractive electrostatic interactions with heparin are relatively weak compared to the interactions with small AO. In the case of PEI2000(PEG550)$_{10}$ polyplexes, aggregation may partially diminish this effect. Regardless of the mechanism, the heightened stability of PEI2000-based polyplexes has important implications for their effectiveness as AO carriers.

In preliminary experiments we also measured the stability of a limited number of polyplexes comprised of ODN rather than AO. Because the ODN was not fluorescently labeled, free ODN in this assay was detected with ethidium bromide. Overall, polyplexes of polymer−ODN were less stable than those of polymer−AO (data not shown). These findings underscore the

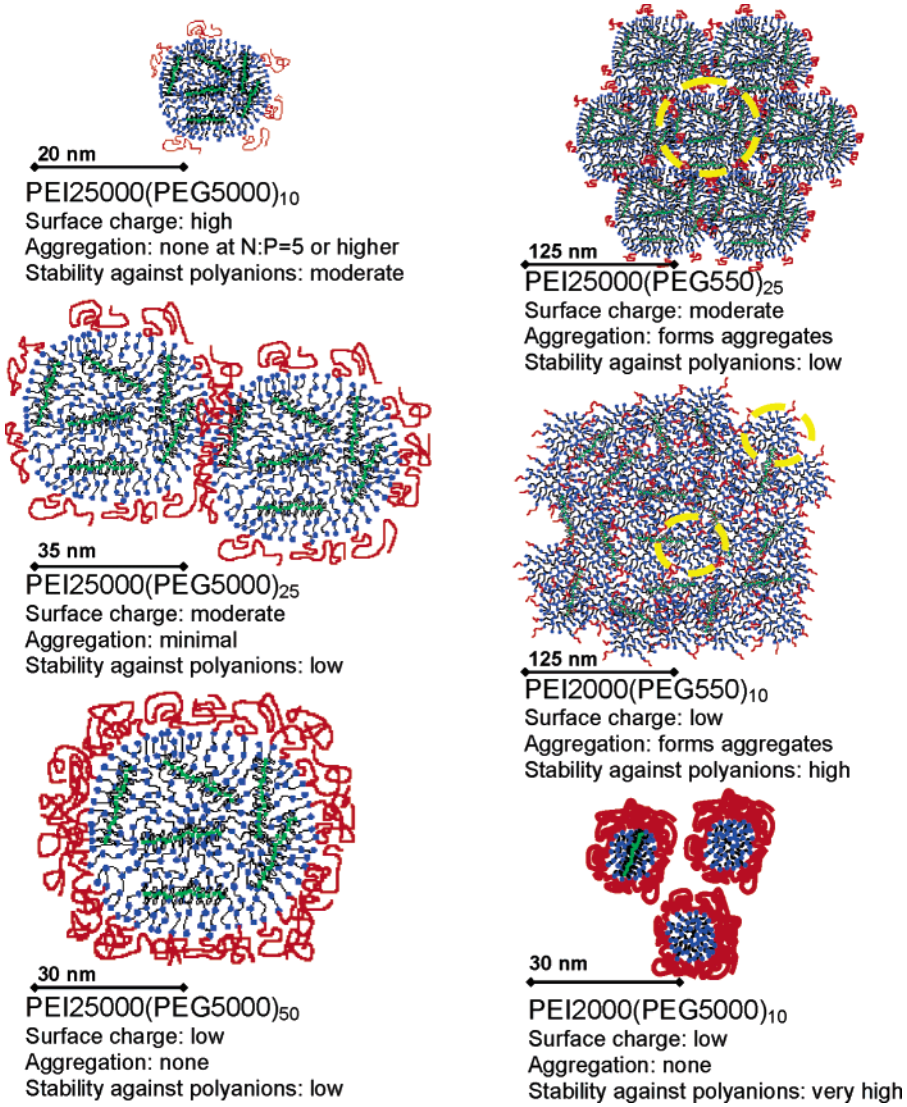

Figure 7. Schematic representations of various PEI−PEI−AO polyplexes (N:P = 5) and their physiochemical properties. PEI amine groups (blue) are connected through ethylene groups (black); attached PEG chains (red) exhibit a random coil conformation. Complexed AO (green) is embedded into PEI or interconnects several copolymers. For the PEI25000(PEG550)$_{25}$ and PEI2000(PEG550)$_{10}$ polyplexes, individual polyplexes within the aggregate particles are indicated by yellow circles. Although the structures are drawn assuming a "core−shell" polyplex structure,[33] as suggested by Petersen et al.,[21] low M_w PEG molecules might penetrate into the PEI molecules during synthesis, so a homogeneous copolymer is formed rather than the core−shell structure.

need to verify the stability of polyplexes depending on the particular oligonucleotide used in transfections, as oligonucleotide chemistry may be a key determinant of polyplex structural properties.

Summary of Polyplex Structures and Their Relationship to Transfection Capacity. In this paper we described PEG−PEI−oligonucleotide polyplexes with a wide range of properties including variability in size, aggregation, surface charge, and stability against competing polyanions. These properties are summarized in Figure 7 along with schematic representations of the polyplex structures. Overall, the PEG molecular weight was the main determinant of polyplex size, through its influence on aggregation. When grafted with PEG5000, both low and high molecular weight PEI-based copolymers formed small polyplexes, with minimal aggregation. Alternatively, when grafted with PEG550, both low and high molecular weight PEI-based copolymers formed complexes with oligonucleotides that appeared as much larger aggregates. The stability of polyplexes in a heparin-based polyanion competition assay showed that the level of PEG grafting and the molecular weight of PEI were the primary determinants of stability (Figure 6). Increasing the level of PEG grafting on PEI25000 resulted in extremely unstable polyplexes that readily dissociated into AO and copolymer. Low molecular weight PEI2000-based copolymers formed extremely stable polyplexes that did not dissociate even at the highest polyanion concentrations.

The combined physiochemical data reported here provide sufficient information to construct theoretical models of the polyplex structures (Figure 7), although the exact details remain uncertain. The model polyplex structures (all at N:P = 5) are based mainly on the DLS data and the molecular weights of the copolymers. At an N:P ratio of 5, one PEI25000-based copolymer with ~580 N groups can theoretically complex with ~6 AO molecules (20 P groups per molecule).

Due to the smaller number of amine groups within PEI2000 (~46 N groups per molecule), one AO molecule may be surrounded by ~2−3 copolymers. When PEG-mediated repulsion is weak, the AOs may be allowed to interconnect several copolymers and form aggregate structures, as observed for PEI2000(PEG550)$_{10}$ polyplexes. Alternatively, when PEG-mediated repulsion is strong, a single AO could localize within the core of one PEI2000 copolymer, leaving other copolymers

free in solution, such as illustrated for PEI2000(PEG5000)$_{10}$. It should be further mentioned that whether any PEGylated PEI-based polyplexes exhibit core−shell or homogeneous structures is still unresolved and requires more detailed scrutiny.[21]

To date, only limited transfection data are available for this family of polyplexes. The transfection capacity of PEI25000-(PEG5000)$_{10}$−AO polyplexes was evaluated in vitro in mature muscle cells isolated from *mdx* mice using quantitative confocal microscopy of fluorescently tagged polyplexes.[43] It was shown that the PEI25000(PEG5000)$_{10}$ copolymers functioned as effective, nontoxic carriers that facilitated delivery of AO to myonuclei. Although the copolymer and AO were colocalized within membrane-associated structures, the AO was efficiently translocated into myonuclei, while the copolymer was almost entirely excluded from entering the nuclei. Overall, AO uptake into myonuclei after transfection with PEI25000(PEG5000)$_{10}$−AO was about 5.5-fold higher than that after transfections with AO alone. Although the transfection capacities of other polyplexes have not been systematically investigated with this in vitro system, pilot studies indicated that the transfection efficiency was substantially lower for polyplexes with 25 and 50 PEGs per PEI25000 (unpublished observations). We speculate that the poor transfection capacity was primarily due to the poor polyplex stability (Figure 6), although reduced surface charge may also weaken polyplex−membrane electrostatic interactions and diminish the transfection capacity in this in vitro system.

The transfection capacity of three other polyplexes shown in Figure 7 has also been evaluated in vivo following intramuscular injections into skeletal muscle of *mdx* mice, which lack functional dystrophin (Williams et al. *Mol. Ther.*, accepted). The transfection capacity in this system was quantified by AO-mediated expression of dystrophin-positive muscle fibers. Overall, injection of polyplexes containing low M_w PEI2000-(PEG550)$_{10}$ resulted in about 3-fold more dystrophin-positive fibers than injections with high M_w PEI25000(PEG5000)$_{25}$ and PEI25000(PEG5000)$_{50}$.

Presently, we showed polyplexes containing PEI2000-(PEG550)$_{10}$ formed large aggregates bearing a low surface charge (Figure 3). Although polyplex aggregation has typically been considered to be an undesirable feature, the relatively high transfection capacity of the low M_w polyplexes (Williams et al. *Mol. Ther.*, accepted) suggests that aggregation may provide favorable characteristics for AO delivery. One possibility is that the aggregate polyplexes may provide a greater payload of AO with each endocytotic event. We are currently evaluating the transfection capacity of low M_w PEI-based polyplexes that do not show aggregation, to determine whether aggregation itself is a desirable feature in polyplex design. As shown in Figure 6, polyplexes containing low M_w PEI2000(PEG550)$_{10}$ were substantially more stable against competing polyanions than polyplexes containing high M_w PEI25000(PEG5000)$_{25}$ and PEI25000-(PEG5000)$_{50}$. Thus, the most likely explanation for the reduced transfection efficiency of the high M_w PEI-based polyplexes in vivo (Williams et al. *Mol. Ther.*, accepted) was their extreme lack of stability. In addition, pilot studies suggested that PEI25000(PEG5000)$_{10}$ copolymers that were extremely effective for in vitro transfection[43] were ineffective after intramuscular injection (unpublished observations). In this case, the excessive surface charge appeared to prevent distribution of the polyplexes throughout the musculature.

In summary, significant variability exists in the properties of a family of PEG−PEI−AO polyplexes that may have a significant impact on their capacity for cellular transfection. The wide range of properties suggests that this family of polyplexes may be adapted for use in divergent biological applications.

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Supporting Information Available. Details about the copolymer synthesis and their spectral characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Shi, F.; Hoekstra, D. Effective intracellular delivery of oligonucleotides in order to make sense of antisense. *J. Controlled Release* **2004**, *97* (2), 189−209.
(2) Kurreck, J. Antisense technologies. Improvement through novel chemical modifications. *Eur. J. Biochem.* **2003**, *270* (8), 1628−1644.
(3) Marcusson, E. G.; Yacyshyn, B. R.; Shanahan, W. R., Jr.; Dean, N. M. Preclinical and clinical pharmacology of antisense oligonucleotides. *Mol. Biotechnol.* **1999**, *12* (1), 1−11.
(4) Ravichandran, L. V.; Dean, N. M.; Marcusson, E. G. Use of antisense oligonucleotides in functional genomics and target validation. *Oligonucleotides* **2004**, *14* (1), 49−64.
(5) Opalinska, J. B.; Gewirtz, A. M. Nucleic-acid therapeutics: basic principles and recent applications. *Nat. Rev. Drug Discovery* **2002**, *1* (7), 503−514.
(6) Goodchild, J. Oligonucleotide therapeutics: 25 years agrowing. *Curr. Opin. Mol. Ther.* **2004**, *6* (2), 120−128.
(7) Dias, N.; Stein, C. A. Antisense oligonucleotides: basic concepts and mechanisms. *Mol. Cancer Ther.* **2002**, *1* (5), 347−355.
(8) Sazani, P.; Kole, R. Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. *J. Clin. Invest.* **2003**, *112* (4), 481−486.
(9) van Deutekom, J. C. Gene Therapy: The 'pro-sense' approach to Duchenne muscular dystrophy. *Eur. J. Hum. Genet.* **2005**, *13* (5), 518−519.
(10) Bremmer-Bout, M.; Aartsma-Rus, A.; de Meijer, E. J.; Kaman, W. E.; Janson, A. A.; Vossen, R. H.; van Ommen, G. J.; den Dunnen, J. T.; van Deutekom, J. C. Targeted exon skipping in transgenic hDMD mice: A model for direct preclinical screening of human-specific antisense oligonucleotides. *Mol. Ther.* **2004**, *10* (2), 232−240.
(11) Wiethoff, C. M.; Middaugh, C. R. Barriers to nonviral gene delivery. *J. Pharm. Sci.* **2003**, *92* (2), 203−217.
(12) Hughes, M. D.; Hussain, M.; Nawaz, Q.; Sayyed, P.; Akhtar, S. The cellular delivery of antisense oligonucleotides and ribozymes. *Drug Discovery Today* **2001**, *6* (6), 303−315.
(13) Merdan, T.; Kopecek, J.; Kissel, T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv. Drug Delivery Rev.* **2002**, *54* (5), 715−758.
(14) Roth, C. M.; Sundaram, S. Engineering synthetic vectors for improved DNA delivery: insights from intracellular pathways. *Annu. Rev. Biomed. Eng.* **2004**, *6*, 397−426.
(15) Dass, C. R. Vehicles for oligonucleotide delivery to tumours. *J. Pharm. Pharmacol.* **2002**, *54* (1), 3−27.
(16) Brazas, R. M.; Hagstrom, J. E. Delivery of small interfering RNA to mammalian cells in culture by using cationic lipid/polymer-based transfection reagents. *Methods Enzymol.* **2005**, *392*, 112−124.
(17) Ulrich, A. S. Biophysical aspects of using liposomes as delivery vehicles. *Biosci. Rep.* **2002**, *22* (2), 129−150.
(18) Semple, S. C.; Harasym, T. O.; Clow, K. A.; Ansell, S. M.; Klimuk, S. K.; Hope, M. J. Immunogenicity and rapid blood clearance of liposomes containing polyethylene glycol-lipid conjugates and nucleic Acid. *J. Pharmacol. Exp. Ther.* **2005**, *312* (3), 1020−1026.
(19) Bieber, T.; Meissner, W.; Kostin, S.; Niemann, A.; Elsasser, H. P. Intracellular route and transcriptional competence of polyethylenimine-DNA complexes. *J. Controlled Release* **2002**, *82* (2−3), 441−454.

(20) Suh, J.; Wirtz, D.; Hanes, J. Efficient active transport of gene nanocarriers to the cell nucleus. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (7), 3878−3882.
(21) Petersen, H.; Fechner, P. M.; Fischer, D.; Kissel, T. Synthesis, Characterization, and Biocompatibility of Polyethylenimine-*graft*-poly(ethylene glycol) Block Copolymers. *Macromolecules* **2002**, *35*, 6867−6874.
(22) Petersen, H.; Fechner, P. M.; Martin, A. L.; Kunath, K.; Stolnik, S.; Roberts, C. J.; Fischer, D.; Davies, M. C.; Kissel, T. Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjugate Chem.* **2002**, *13* (4), 845−854.
(23) Kichler, A. Gene transfer with modified polyethylenimines. *J. Gene Med.* **2004**, *6* (Suppl. 1), S3−10.
(24) Thomas, M.; Klibanov, A. M. Non-viral gene therapy: polycation-mediated DNA delivery. *Appl. Microbiol. Biotechnol.* **2003**, *62* (1), 27−34.
(25) Sonawane, N. D.; Szoka, F. C., Jr.; Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem.* **2003**, *278* (45), 44826−44831.
(26) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* **2005**, *7* (5), 657−663.
(27) Kunath, K.; von, H. A.; Petersen, H.; Fischer, D.; Voigt, K.; Kissel, T.; Bickel, U. The structure of PEG-modified poly(ethylene imines) influences biodistribution and pharmacokinetics of their complexes with NF-kappaB decoy in mice. *Pharm. Res.* **2002**, *19* (6), 810−817.
(28) Brus, C.; Petersen, H.; Aigner, A.; Czubayko, F.; Kissel, T. Physicochemical and biological characterization of polyethylenimine-graft-poly(ethylene glycol) block copolymers as a delivery system for oligonucleotides and ribozymes. *Bioconjugate Chem.* **2004**, *15* (4), 677−684.
(29) Schiffelers, R. M.; Ansari, A.; Xu, J.; Zhou, Q.; Tang, Q.; Storm, G.; Molema, G.; Lu, P. Y.; Scaria, P. V.; Woodle, M. C. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* **2004**, *32* (19), e149.
(30) Fischer, D.; Osburg, B.; Petersen, H.; Kissel, T.; Bickel, U. Effect of poly(ethylene imine) molecular weight and pegylation on organ distribution and pharmacokinetics of polyplexes with oligodeoxynucleotides in mice. *Drug Metab. Dispos.* **2004**, *32* (9), 983−992.
(31) Vinogradov, S. V.; Batrakova, E. V.; Kabanov, A. V. Nanogels for oligonucleotide delivery to the brain. *Bioconjugate Chem.* **2004**, *15* (1), 50−60.
(32) Jeong, J. H.; Kim, S. W.; Park, T. G. A new antisense oligonucleotide delivery system based on self-assembled ODN−PEG hybrid conjugate micelles. *J. Controlled Release* **2003**, *93* (2), 183−191.
(33) Vinogradov, S. V.; Bronich, T. K.; Kabanov, A. V. Self-assembly of polyamine-poly(ethylene glycol) copolymers with phosphorothioate oligonucleotides. *Bioconjugate Chem.* **1998**, *9* (6), 805−812.
(34) Vinogradov, S.; Batrakova, E.; Li, S.; Kabanov, A. Polyion complex micelles with protein-modified corona for receptor-mediated delivery of oligonucleotides into cells. *Bioconjugate Chem.* **1999**, *10* (5), 851−860.
(35) Kunath, K.; von Harpe, A.; Fischer, D.; Petersen, H.; Bickel, U.; Voigt, K.; Kissel, T. 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. Controlled Release* **2003**, *89* (1), 113−125.
(36) Morrison, I. D.; Grabowski, E. F.; Herb, C. A. Improved Techniques for Particle Size Determination by Quasi-Elastic Light Scattering. *Langmuir* **1985**, *1*, 496−501.
(37) Nguyen, H. K.; Lemieux, P.; Vinogradov, S. V.; Gebhart, C. L.; Guerin, N.; Paradis, G.; Bronich, T. K.; Alakhov, V. Y.; Kabanov, A. V. Evaluation of polyether-polyethyleneimine graft copolymers as gene transfer agents. *Gene Ther.* **2000**, *7* (2), 126−138.
(38) Benns, J. M.; Mahato, R. I.; Kim, S. W. Optimization of factors influencing the transfection efficiency of folate-PEG-folate-graft-polyethylenimine. *J. Controlled Release* **2002**, *79* (1−3), 255−269.
(39) Von Harpe, A.; Petersen, H.; Li, Y.; Kissel, T. Characterization of commercially available and synthesized polyethylenimines for gene delivery. *J. Controlled Release* **2000**, *69* (2), 309−322.
(40) Ruponen, M.; Ronkko, S.; Honkakoski, P.; Pelkonen, J.; Tammi, M.; Urtti, A. Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. *J. Biol. Chem.* **2001**, *276* (36), 33875−33880.
(41) Van Rompaey, E.; Engelborghs, Y.; Sanders, N.; De Smedt, S. C.; Demeester, J. Interactions between oligonucleotides and cationic polymers investigated by fluorescence correlation spectroscopy. *Pharm. Res.* **2001**, *18* (7), 928−936.
(42) Jones, N. A.; Hill, I. R.; Stolnik, S.; Bignotti, F.; Davis, S. S.; Garnett, M. C. Polymer chemical structure is a key determinant of physicochemical and colloidal properties of polymer-DNA complexes for gene delivery. *Biochim. Biophys. Acta* **2000**, *1517* (1), 1−18.
(43) Sirsi, S. R.; Williams, J.; Lutz, G. J. Poly(ethylene imine)-Polyethylene Glycol Copolymers Facilitate Efficient Delivery of Antisense Oligonucleotides to Nuclei of Mature Muscle Cells of *mdx* Mice. *Hum. Gene Ther.* **2005**, *16* (11), 1307−1317.

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