Gene Delivery Properties of End-Modified Poly(β-amino ester)s


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Here, we present the synthesis of a library of end-modified poly(β-amino ester)s and assess their utility as gene delivery vehicles. Polymers were synthesized using a rapid, two-step approach that involves initial preparation of an acrylate-terminated polymer followed by a postpolymerization amine-capping step to generate end-functionalized polymers. Using a highly efficient poly(β-amino ester), C32, we show that the terminal amine can greatly affect and improve polymer properties relevant to gene delivery. Specifically, the in vitro transfection levels can be increased by 30% and the optimal polymer:DNA ratio lowered 5-fold by conjugation of the appropriate end group. The most effective modifications were made by grafting primary diamine molecules to the chain termini. The added charge and hydrophobicity of some derivatives enhanced DNA binding and resulted in the formation of polymer–DNA complexes less than 100 nm in diameter. In addition, cellular uptake was improved 5-fold over unmodified C32. The end-modified poly(β-amino ester)s presented here are some of the most effective gene-delivery polycations, superior to polyethylenimine and previously reported poly(β-amino ester)s. These results show that the end-modification of poly(β-amino ester)s is a general strategy to alter functionality and improve the delivery performance of these materials.

INTRODUCTION

Incorporation of new genetic information into cells is a promising strategy for the treatment of many inherited and acquired genetic disorders. In order for gene-based therapeutics to be clinically applicable, a safe and efficient delivery system for DNA needs to be developed. Modified viruses are the most effective delivery vectors (6–8). Despite their widespread use, both PLL and PEI have significant disadvantages that may ultimately limit their clinical utility. In particular, both polymers are known to be very cytotoxic and have relatively low transfection efficiencies compared to viruses, especially in nondividing cells (9–11).

Poly(β-amino ester)s are an alternative class of cationic polymers that have been recently developed and explored as gene delivery vectors (12). These polymers are degradable by hydrolysis of backbone ester bonds and contain tertiary amines to facilitate DNA binding (12). They are synthesized by Michael addition of bifunctional amines to diacrylates, and can be prepared without solvents, catalysts, or complex protecting-group strategies (12, 13). The polymer molecular weight and chain end groups can be easily controlled by adjusting the amine/diacrylate monomer ratio (14, 15). Ease of synthesis, along with the commercial availability of many amine and diacrylate monomers, has allowed for the generation of large, structurally diverse libraries of poly(β-amino ester)s (15, 16). High-throughput transfection screens have identified many polymers that are capable of transfecting cells with much higher efficiencies than PEI, while also demonstrating less toxicity both in vitro and in vivo (15, 17). The polymer libraries have also been useful to elucidate structure–function relationships. These studies have shown that high molecular weight, amine-terminated poly(β-amino ester)s with hydroxyl-functionalized side chains are highly efficient polymers for gene delivery (15). The most effective polymer discovered, C32, has also been used in vivo for the gene-based treatment of prostate cancer (17).

The continued development of poly(β-amino ester)s for gene therapy and other biomedical applications requires an effective method to chemically modify these materials. As with other polycations, it is necessary to incorporate additional levels of functionality such as serum stability and cell targeting to improve the gene delivery properties of these polymers. The ideal approach to poly(β-amino ester) modification would involve a chemistry that is simple, versatile, and adaptable to a high-throughput format. It is important to generate many polymeric derivatives, since any modification has a nontrivial effect on gene delivery. Previous studies have shown that even single carbon or functional group differences between polymer repeat units can drastically affect their transfection efficiencies (15, 16).
We have recently shown that end-modification of poly(β-amino ester)s is a useful approach to improve both the in vitro and in vivo polymer transfection efficiency (18). In this study, we present a rapid, parallel approach to synthesize end-modified poly(β-amino ester)s and explore the effects of end-group structure on polymer gene delivery properties. End-modified polymers were synthesized following a two-step procedure in which an acrylate-terminated base polymer is first prepared by polymerization using excess diacylate over amine monomer. In a second stage, the base polymer was reacted with various amine reagents to generate amine-capped polymer chains. Following this approach, we have generated a library of end-modified C32 polymers and demonstrate that the terminal amine has a large effect on C32 transfection. Similar to polymer repeat units, end segments differing in a single additional carbon or functional group can drastically affect the polymer delivery properties. In addition, the terminal amine structure has a large influence on cytotoxicity, physical properties, and cellular uptake of polymer–DNA complexes. These results indicate that end-modification is a useful strategy to functionalize poly(β-amino ester)s and improve their gene delivery performance.

EXPERIMENTAL SECTION

Materials. Polyethyleneimine (water-free, 

\[ M_w \approx 25 \text{ kDa}, \quad M_n \approx 10 \text{ kDa} \] ) was purchased from Sigma-Aldrich (St. Louis, MO). A 25 mM sodium acetate buffer solution pH 5.2 (NaAc buffer) was prepared by diluting a 3 M stock (Sigma-Aldrich). 1,4-Butanediol diacrylate (99%) and 5-amino-1-pentanol (97%) were from Alfa Aesar (Ward Hill, MA). Amine capping reagents were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics/Fisher Scientific (Pittsburgh, PA), TCI America (Portland, OR), Molecular Biosciences (Boulder, CO), and Toronto Research Chemicals (Ontario, Canada). All chemicals were used as received without any further purification. PicoGreen and RediPlate 96 PicoGreen dsDNA Quantitation Kit were purchased from Molecular Probes (Eugene, OR). pCMV-Luc plasmid DNA stock solution (1 mg/mL in water) was obtained from Elim Biopharmaceuticals (Hayward, CA). gWIZ-β-gal plasmid DNA stock solution (5 mg/mL) was obtained from Aldevron (Fargo, ND). The MTT Cell Proliferation Assay and Bright Glo Luciferase Assay Kits were purchased from Promega Corporation (Madison, WI). White and black polystyrene tissue culture plates were purchased from Invitrogen Corporation (Carlsbad, CA) unless otherwise noted. COS-7 cells were obtained from ATCC (Manassas, VA) and maintained in phenol red free DMEM supplemented with 10% fetal bovine serum and 100 units/mL of penicillin/streptomycin. All cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA) unless otherwise noted. COS-7 were grown at 37 °C in a 5% CO₂ atmosphere.

Methods. ¹H NMR was conducted on a Varian Inova 500 MHz Spectrometer.

Synthesis of Acrylate-Terminated C32 Poly(β-amino ester). Acrylate-terminated C32 polymer was dissolved in DMSO at 31.13 wt%/wt. Amine capping reagents were dissolved in DMSO at 0.25 M. End-chain capping reactions were performed by mixing 321 mg of polymer/DMSO solution with 800 μL of amine solution. Reactions were performed in Eppendorf tubes with constant agitation for 24 h. Polymers were stored at −20 °C until use for each experiment. ¹H NMR of C32-Ac capped with 5-amino-1-pentanol (i.e., C32–32) (δ (ppm) 1.2–1.4 (m, -NCH₂(CH₂)₂CH₂OH), 1.6 (bs, -N(CH₂)₂COOCH₂CH₂), 2.2–2.4 (m, -COOCH₂CH₂N- and -NCH₂(CH₂)₂OH), 2.4–2.7 (m, -COOCH₂CH₂N-), 3.3 (t, J = 6.6 Hz, -N(CH₂)₂CH₂OH), 4.0 (bs, -N(CH₂)₂COOCH₂CH₂-).

Polymers were synthesized following a two-step procedure in which a diacrylate-terminated base polymer is first prepared by polymerization using excess diacylate over amine monomer. In a second stage, the base polymer was reacted with various amine reagents to generate amine-capped polymer chains. Following this approach, we have generated a library of end-modified C32 polymers and demonstrate that the terminal amine has a large effect on C32 transfection. Similar to polymer repeat units, end segments differing in a single additional carbon or functional group can drastically affect the polymer delivery properties. In addition, the terminal amine structure has a large influence on cytotoxicity, physical properties, and cellular uptake of polymer–DNA complexes. These results indicate that end-modification is a useful strategy to functionalize poly(β-amino ester)s and improve their gene delivery performance.

Polymers at 100 mg/mL in DMSO were diluted accordingly into NaAc buffer to concentrations that yield the different polymer:DNA weight ratios. One hundred microliters of diluted polymer solution was mixed vigorously with 100 μL of DNA (60 μg/mL in NaAc buffer) in a 96-well polystyrene plate. The solutions were left undisturbed for 5 min, after which time 120 μL of each was added to 800 μL of cell culture media in a deep-welled polycrylonite plate. The media over the cells was then removed with a 12-channel aspirator wand followed by the addition of 150 μL/well of polymer–DNA complex solution. Complexes were incubated over the cells for 1 h after which time they were aspirated off and replaced with 105 μL/well of fresh cell culture media. Cells were allowed to grow for three days at 37 °C, 5% CO₂, and then analyzed for luciferase protein expression.

Luciferase expression was analyzed using Bright-Glo assays kits. Briefly, 100 μL/well of Bright-Glo solution was added to the cell plates. The plates were gently agitated to promote mixing for 2 min Luminescence was then measured on a Perkin Elmer Victor 3 plate luminometer using a 1% neutral density filter and 1 s/well counting time.

Measurements of Polymer Cytotoxicity. Cytotoxicity measurements of polymer–DNA complexes were performed essentially as described for the transfection experiments except that cellular metabolic activity was measured instead of Luciferase protein expression. One day after the transfection, MTT reagent was added to the cell plates at 10 μL/well. The plates were incubated at 37 °C for 2 h. Detergent reagent was then added at 100 μL/well, and the cell plates were left in the dark at room temperature for 4 h. Optical absorbance was measured at 570 nm using a Molecular Devices SPECTRAmax PLUS384 absorbance plate reader and converted to % cell viability relative to untreated cells.

Polymer–DNA Binding Assay with PicoGreen. Polymer solutions at 100 mg/mL in DMSO were diluted into NaAc buffer to a final concentration of 6 mg/mL. In a half-area 96-well plate, 50 μL well of diluted polymer was added to 50 μL/well of DNA (60 μg/mL in NaAc buffer). The solutions were mixed vigorously and allowed to sit undisturbed for 5 min to allow for polymer–DNA complexation. After this time, 100 μL/well of PicoGreen solution was added. PicoGreen working solution was prepared by diluting 80 μL of the purchased stock into 15.2 mL NaAc buffer. After 5 min, 30 μL/well of polymer–DNA–PicoGreen solution was added to 200 μL/well of DMEM media in black 96-well polystyrene plates. The plate fluorescence was then measured on a Perkin Elmer Victor 3 plate reader using an FITC filter set (excitation 485 nm, emission 535 nm). The relative fluorescence (RF) was calculated using the following relationship

\[ RF = \frac{(F_{\text{sample}} - F_{\text{blank}})}{(F_{\text{DNA}} - F_{\text{blank}})} \]

where \( F_{\text{sample}} \) is the fluorescence of the polymer–DNA–PicoGreen sample, \( F_{\text{blank}} \) is the fluorescence of a sample with no
polymer or DNA (only PicoGreen), and $F_{\text{DNA}}$ is the fluorescence of DNA–PicoGreen (no polymer).

**Polymer–DNA Complex Size.** Polymer solutions at 100 mg/mL in DMSO were diluted into NaAc buffer at the appropriate concentration. Concentrations were adjusted for each polymer so that the final polymer:DNA ratio was the same as the ratio that produced the highest transfection. To prepare polymer:DNA complexes, 100 µL of diluted polymer was added to 100 µL of DNA (60 µg/mL in NaAc) and pipetted vigorously. Complexation was allowed to proceed undisturbed for 5 min, after which time 150 µL of the sample was diluted into 1.8 mL of DMEM media. Polymer:DNA complex size was measured on a Zeta-PALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY; 15 mW laser; 676 nm incident beam, 90° scattering angle). Effective particle diameters were calculated from the autocorrelation function using the MAS option of the BIC particle-sizing software assuming a log normal distribution. The solution viscosity and refractive index were assumed equal to those of pure water at 25 °C.

**Cellular Uptake Assay.** Uptake measurements of polymer–DNA complexes were performed essentially as described for the transfection experiments but using the β-galactosidase (β-gal) plasmid. Instead of quantifying protein expression levels after three days, total cellular DNA was isolated 4 h post-transfection using a DNeasy 96 Tissue Kit (Qiagen; Valencia, CA). DNA isolation was performed according to the supplied instructions and included two PBS washes of the cells before lysis to ensure that surface-bound complexes were removed (19). Total DNA was quantified using a Redi-Plate 96 PicoGreen dsDNA Quantification Kit following the supplied instructions.

The amount of β-gal DNA delivered was quantified using RT-PCR with a custom Taqman primer and probe set specific for the β-gal plasmid (Applied Biosystems; Foster City, CA). The primers and probe spanned an 80 bp sequence in the β-gal coding region of the plasmid and had the following sequences: forward primer 5′-TTA CAG GGC GGC TTC GTC T-3′, reverse primer 5′-TAA GCC GAC CAC GGG TTG-3′, and the Taqman probe 5′-6FAM-CTG GGT GGA TCA GTC GCT GAT TAA ATA TGA TG-TAMRA-3′. The primers and probes were used at concentrations of 400 nM and 25 nM, respectively. After activating the Taq enzyme at 95 °C for 10 min, 40 cycles of amplification were performed, with each cycle consisting of 95 °C for 15 s, 60 °C for 1 min, followed by a fluorescent plate read using a Chromo4 Continuous Fluorescence Detector (MJ Research; Waltham, MA). Plasmid copy numbers were determined by comparing the RT-PCR cycle threshold values to a plasmid standard curve and analyzed using the Opticon Monitor 3 software package (MJ Research).

**RESULTS AND DISCUSSION**

**Polymer Synthesis.** We developed a two-step approach to synthesize end-modified C32 poly(β-amino ester)s, as illustrated in Figure 1A,B (18). In the first step, acrylate-terminated C32 polymer was prepared by mixing the corresponding diacrylate and amine monomers in a 1:2 molar ratio, as shown previously (Figure 1A) (14). This ratio was selected since C32 and many other top-performing, amine-terminated polymers are made at an inverse ratio of 1:1.2 acrylate/amine (15). We hypothesized then that the exact opposite ratio may be optimal so that the
relative number of interior to terminal units is approximately preserved, with the end-capping step causing a relatively small change to this balance. Therefore, the diacrylate/amine ratio selected directly controls the final molecular weight. For many poly(β-amino ester)s, molecular weights greater than 10 kDa are usually most effective and can be achieved using a 1.2:1 molar monomer ratio (15). For the C32-acrylate polymer (C32-Ac), the weight-average molecular weight is approximately 8800 Da, relative to polystyrene standards, with a 1.9 polydispersity index.

In the second step, acrylate-terminated C32 polymer is reacted with various amine molecules to generate amine-capped polymer chains (Figure 1B). In this way, the chain ends contain amine functionalities different than those present in the interior of the polymer. Most chain-capping reactions are performed using a primary amine molecule, which results in an amine-capped polymer containing secondary amines at the chain end points. Secondary amine molecules are also used but result in tertiary amine groups at the polymer ends. The 37 different amine molecules used for this secondary capping step are shown in Figure 1C. These compounds were selected on the basis of their DMSO solubility, biocompatibility, and ability to assess structure–function relationships. In addition, many of these molecules have proven useful in the synthesis of poly(β-amino ester)s with high transfection efficiencies (15).

The end-capping reaction occurs via an amine-acrylate Michael addition, identical to that used in the polymerization. Since acrylates have no detectable reactivity toward hydroxyls, ethers, tertiary amines, amides, aromatics, and some types of heterocycles, all of these functionalities can be incorporated at the chain ends using the appropriate amine reagents (16). The reaction is carried out by mixing a concentrated polymer solution with an excess of amine in DMSO at room temperature. The conditions have been optimized with excess amine to fully end-cap all chains without causing any detectable cross-linking or aminolysis of backbone ester bonds, as determined by 1H NMR and GPC analysis (data not shown). Presumably, aminolysis is avoided due to the higher reactivity of acrylates compared to esters and the mild end-capping reaction conditions used. The resulting end-modified polymers can be directly tested for transfection efficiency without prior purification, since the DMSO and excess amine were determined to be nontoxic (data not shown). Therefore, this chemistry permits many polymers with structurally diverse end functionalities to be synthesized and screened in parallel. We show here that such a synthetic method is useful to assess end amine structure–function relationships and improve the gene delivery properties of poly(β-amino ester)s.

For the analysis presented here, we use C32 as a base polymer to examine the effects of the end amine structure on polymer transfection and also explore structure–function relationships. This polymer was identified from previous studies to be the most efficient poly(β-amino ester) for gene delivery (15). In principle, other base polymers, diacrylate/amine ratios, and amine-capping agents may be used and could generate more effective polymers for gene delivery.

**Cellular Transfections.** The DNA delivery efficiency of end-modified poly(β-amino ester)s was evaluated using a high-throughput assay (20). Concentrated polymer solutions in DMSO were diluted in sodium acetate buffer and complexed with plasmid DNA to form polymer–DNA nanoparticles. A range of polymer:DNA weight ratios was tested for each polymer, since this parameter is known to have a critical effect on polycation-mediated transfection (15). Nanoparticles were then diluted into cell culture media and incubated on COS-7 cells. The diluting media contained 10% serum to account for the effect of serum proteins on polymer transfection.

The transfection efficiencies of amine-terminated C32 polymers are shown in Figure 2. The average luciferase expression levels, measured in relative light units (RLUs), are given for...
each polymer at five different polymer:DNA ratios. We have previously shown the transfection data for each end-modified polymer at its optimal ratio and present here the complete data set containing all polymer:DNA ratios (18). Also included are the transfection data for 25 kDa branched PEI, one of the most efficient commercially available polycations, and C32, one of the best performing poly(β-amino ester)s synthesized to date. The unmodified, acrylate-terminated C32 polymer is also shown on the far right and demonstrates weak activity that is similar to other acrylate-terminated poly(β-amino ester)s (14). While amine-capping reactions of this polymer were verified by 1H NMR analysis, the data in Figure 2 provide functional confirmation by the large increase in transfection between acrylate- and amine-terminated polymers. An overall inspection of the data reveals that the structure of the terminal amine has a dramatic effect on the C32 transfection efficiency. In general, polymers capped with hydrophilic amine end groups containing hydroxyls or additional amines proved most effective. In contrast, chain termination with more hydrophobic amines containing alkyl chains or aromatic rings led to much lower transfection activity.

Perhaps the most important result is that very subtle structural differences in just the terminal amine can have a large effect on polymer transfection efficiency. This is most evident by comparing the C32–36 and C32–52 polymers. The C32–52 polymer, which contains a six-carbon alkyl chain extending from the terminal secondary amine, has a maximum transfection only twice that of naked DNA. In contrast, the C32–36 polymer is 34-fold more effective than C32–52, but only differs in a single hydroxyl group on carbon-6 at the chain end. In fact, the C32–36 polymer is half as effective as C32, demonstrating that a single functional group, in this case a terminal hydroxyl, can significantly alter the polymer delivery properties. A similar effect can be seen between the C32–95 and C32–110 polymers, which consist of terminal decylamines containing either a hydroxyl group or primary amine on carbon-10, respectively. In this case, substituting the terminal hydroxyl for an amine improves the transfection performance by over 1 order of magnitude. This same substitution pattern also changes the optimal polymer:DNA ratio. Comparing two highly efficient polymers, C32–122 and C32–124, the former displays very high RLU output at a 20:1 ratio, whereas the latter requires 5-fold more polymer (i.e., a 100:1 ratio) to achieve the same effect. A similar trend is also seen between the C32–36 and C32–106 polymers. Therefore, amine-capping molecules with hydroxyls and primary amines are most effective, with the latter being optimal at 5-fold lower polymer:DNA ratios in general.

Polymers terminated with primary diamine molecules had the highest transfection efficiency, as determined by both RLU output and lowest optimal polymer:DNA ratio. Specifically, the C32–102 polymer had a very similar transfection profile to that of C32, with a maximum occurring at the highest polymer:DNA ratio of 100:1, but had an overall 30% higher RLU output. This demonstrates that modification at the chain ends can significantly improve the delivery performance. Primary diamine capping also lowered the optimal polymer:DNA ratio substantially in many cases. Seven primary amine-terminated polymers had optimal polymer:DNA ratios of 20:1, while one polymer, C32–110, had a maximum RLU at a 10:1 ratio. The transfection profile at the 20:1 ratio for diamine-capped polymers, C32–102 through C32–111, appears to be a skewed bell-shaped curve with a maximum occurring at the C32–108 polymer. This indicates that larger alkyl chains bridging the diamine functionalities are generally more effective than their short-chain counterparts, with an optimum of eight carbons. The C32–108 polymer had an optimal transfection at a 20:1 ratio that is almost as high as that for C32, which requires a 100:1 ratio. Such a significant reduction in the amount of polymer needed to mediate high levels of transfection has important implications for in vivo delivery, where the amount of polymer injected needs to be limited to minimize toxic side effects (18).

**Cytotoxicity.** Many polycations have been shown to elicit considerable cell toxicity that may limit their utility as gene delivery vectors (10, 11). The biocompatibility of cationic polymers is determined by a number of factors that include molecular weight, charge density, type of amines, polymer structure (linear, branched, dentritic), and chain flexibility (21–27). In general, high molecular weight polymers with a high density of primary and/or secondary amines usually result in substantial cytotoxicity (28).

The cytotoxicity of end-modified poly(β-amino ester)s was evaluated using the MTT assay. This colorimetric test is based on the ability of mitochondrial reductase enzymes in viable cells to reduce 3-(4,5-dimethylythiazol-2-yl)-2,5-diphenyl tetrazolium bromide to a purple formazan. Toxicity of end-modified polymers was assessed by performing the same transfection experiment, but assaying for metabolic activity instead of luciferase expression. All polymers were tested at the highest 100:1 polymer:DNA weight ratio, which corresponds to an approximate 400 µg/mL concentration of polymer on the cells. Toxicity analysis at such a high polymer concentration may explain the differences in polymer transfection at high polymer:DNA ratios and simultaneously assess polymer biocompatibility under very aggressive conditions that may be important for their future use and development.

The cytotoxicity levels of end-modified C32 polymers are shown in Figure 3. The percentage of viable cells is displayed as a function of the amine-terminated polymer. Positive and negative control conditions are shown to the far right for PEI and naked DNA. At such high polymer concentrations, PEI is very cytotoxic, as reflected by the low 3% cell viability. In contrast, both C32 and the acrylate-terminated C32 polymer (C32-Ac) show no significant affects on the growth and metabolism of COS-7 cells.

The majority of end-modified C32 polymers show good biocompatibility. This is especially true of all polymers capped with primary monoamine reagents, regardless of the functional groups extending from the amine. Aromatic, alkyl, hydroxyl, secondary and tertiary amines, and imidazole functionalities at the chain end points do not appear to induce any significant adverse effects. Therefore, elevated cytotoxic effects do not sufficiently explain the low transfection ability of polymers terminated with the more hydrophobic amines. In contrast to the polymers capped with monoamines, polymers terminated with primary diamine molecules compromise cell viability to varying extents. While the increased charge is a determining factor, the overall toxicity is also strongly dependent on the hydrophobicity of the end group. In general, increasing the size of the alkyl chain bridging the amine groups increases the toxicity, as is evident by comparing the C32–102 through C32–109 polymers. Furthermore, C32–121, a polymer containing a terminal poly(ethylene glycol) amine with an eight-atom spacer between amine groups, is much less toxic than the corresponding alkyl derivative, C32–108. This indicates that both the spacing between amines and the degree of hydrophobicity in the terminal amine spacer are important determinants of end-amine toxicity.

These significant cytotoxic effects, in large part, explain the decreasing transfection ability of most primary diamine-capped polymers at the higher polymer:DNA ratios. The additional charge, in conjunction with increased hydrophobicity, may be particularly damaging to the cell membranes, since both properties are known to disrupt lipid bilayers (26).

**DNA Binding.** An important requirement for cationic transfection agents is the ability to bind and condense plasmid DNA
for cell entry (29). In general, higher molecular weight polymers with increased cationic charge density display stronger DNA binding at low polymer:DNA ratios (21, 30, 31). While strong electrostatic interactions are important to effectively condense and deliver the DNA, the polymer must possess a mechanism to unbind from the DNA once inside the nucleus (32). For this reason, the poly(β-amino ester)s may be particularly advantageous, since they undergo hydrolysis with short half-lives (12), which may aid in DNA unpackaging.

The binding and condensation of DNA by polycations is often monitored using an agarose gel electrophoresis assay (33). This assay can be used to adequately determine the minimum polymer:DNA ratio required for plasmid condensation but does not provide any information on the strength of this interaction. In contrast, dye-binding assays provide a quantitative measure of the polymer–DNA binding event at all polymer:DNA ratios (34–36). As a result, we utilized a PicoGreen dye penetration assay to determine the degree of plasmid condensation by the end-modified poly(β-amino ester)s. In this assay, polymer–DNA complexes are formed in a manner similar to their preparation for transfection experiments. The complexes are then mixed with a PicoGreen dye solution, diluted into cell culture media, and the solution fluorescence is measured. The dye exhibits fluorescence only when it intercalates between the DNA base pairs. High fluorescence is typically seen with free plasmid, but significant reductions can occur for polymer–DNA complexes in which the DNA is partially shielded from dye penetration. The magnitude of this fluorescence reduction relative to free DNA correlates to the strength of the polymer–DNA interaction (14, 37).

The DNA binding levels for each end-modified polymer are shown in Figure 4. Fluorescence measurements relative to free DNA are given at the optimal transfecting polymer:DNA ratio for each polymer. With the exception of monoamine PEG-terminated polymers, all end-modified materials displayed some level of DNA binding. In general, increased cationic charge at the end groups increased the polymer–DNA binding affinity. This effect is most noticeable by comparing the results of the
PEG amine-capped polymers. The monoamine-capped polymers, C32–123 and C32–124, displayed no measurable binding. However, the substitution of a single primary amine for a hydroxyl at the chain ends (C32–121 and C32–122) leads to increased polymer–DNA binding and less dye penetration. This result indicates that a single functional group only at the very end point of the polymer can bring about large changes in polymer function. Similar conclusions were reached when assessing the overall transfection ability of the polymer but now are seen at just one part of the delivery process.

Perhaps the most noticeable trend in the data is that polymers terminated with primary diamine molecules are most effective at condensing and binding DNA. Additional secondary or tertiary amines at the chain ends were not as effective at increasing the DNA binding ability of the polymer, possibly due to pKₐ differences or a more sterically crowded environment that may prevent their electrostatic interaction with DNA (38, 39). Similar to the cytotoxicity data, more effect is seen with increased terminal hydrophobicity in addition to the added positive charge. In general, smaller relative fluorescence is seen as the alkyl chain length is increased between terminal amine groups, as is evident by comparing polymers C32–102 through C32–110. These results are supported by lower DNA binding affinity of polymers terminated with the more hydrophilic primary ethylene glycol amine polymers (C32–121 and C32–122).

**Polylex Sizing.** Simple electrostatic interactions between the polycation and the negatively charged DNA can often lead to their spontaneous self-assembly into cationic polymer–DNA nanoparticles (40, 41). The physical properties of these complexes are particularly important for their subsequent uptake into cells. Complexes with a positive surface charge and a diameter less than 200 nm are usually sufficient for cellular endocytosis (42). These properties are dependent upon a number of polymer characteristics and the polymer-DNA mixing technique (43–47). Since the terminal amine has demonstrated significant effects on the DNA binding ability of polymers, it should also affect the physical properties of the polymer–DNA complexes.

The effective diameter of complexes formed between end-modified poly(β-amino esters) and plasmid DNA were measured using dynamic light scattering. Polymer–DNA complexes were formed at the optimal transfecting polymer:DNA ratio for each polycation and then diluted into cell culture media prior to each measurement. Concentrations, solution compositions, and polymer–DNA complexing procedures in each step were identical to those used in the transfection assay. In this way, the nanoparticle physical properties measured in this experiment reflect the actual particle properties in the transfection screen.

Average diameters of the polymer–DNA complexes are presented in Figure 5 for each end-modified C32 polymer. The average diameter varied between 85 to 220 nm, demonstrating the crucial effects of terminal amine structure on the physical properties of polymer–DNA complexes. Also shown on the far right is the average diameter of C32 complexes, which is determined to be 152 nm. In a previous study, C32 complexes were diluted into HEPES buffer and subsequently measured to be 79 nm in diameter (15). This difference in particle size illustrates the large effect of serum proteins to disrupt or interact with cationic polymer–DNA complexes. Increases in polymer–DNA complex size in the presence of serum have been seen in our previous studies and with other polycations such as PEI, and have a well-known effect on polymer–DNA properties (48, 49). Importantly, the C32 complex diameter is still below the threshold for endocytosis and maintains high transfection levels.

All end-modified polymers formed complexes with effective diameters in a suitable range for cellular uptake. Only two polymers, C32–101 and C32–121, formed complexes with diameters slightly above 200 nm. The former material consists of highly charged chain end groups, whereas the latter contains a short PEG diamine at the chain end points. In general, the PEG-terminated polymers (C32–121 to C32–124) formed larger complexes with diameters between 150 and 220 nm. Despite the large size and weak DNA binding of these polycations, they can still deliver DNA with relatively high efficiencies. This effect is also true of most polymers terminated with monoprimar amine molecules. These polymers, shown on the left side of Figure 5, mostly result in complexes with diameters greater than 150 nm and lower DNA binding ability than primary diamine-capped polymers. Although a general conclusion cannot be made, it is interesting to note that some polymers with very low transfection efficiencies (e.g., C32–117, −52, −101) also form relatively large complexes, suggesting that their physical properties may not be conducive to uptake.

Similar to the DNA binding data, particle sizing appears to be more favorable for polymers capped with primary diamine molecules. For almost all of these polymers, their complexes with DNA have diameters between 85 and 130 nm. The more hydrophilic PEG diamines, C32–121 and C32–122, are the exception, illustrating the importance of a hydrophobic alkyl chain space between amines at the terminus. Although the trend is not as pronounced as that for the DNA binding, it appears that the sizing is somewhat improved by increasing the alkyl chain length. This is especially true at the long chain lengths where C32–109 and C32–110 form the smallest complexes that have diameters less than 100 nm. These polymers assemble into smaller complexes with DNA compared to those terminated with

![Figure 5](image-url)
addition of secondary and/or tertiary amines, again illustrating the benefits of primary amines at the chain ends over these other amine functionalities. Consequently, it appears that polymers terminated with alkyl primary diamine molecules have the highest DNA binding affinity and assemble into the smallest polymer–DNA complexes.

DNA Uptake. Differences in the physical properties of polymer–DNA complexes can lead to differences in the rates and levels at which they are endocytosed into cells. Previous studies with poly(β-amino ester)s have shown that smaller complexes with high cationic surface charge are more favorable for cellular uptake (42). In addition, amine termination has been shown to promote higher cellular internalization over the corresponding acrylate-terminated polymer (44). In light of these findings and the terminal amine effects on polymer–DNA properties, we measured the uptake levels of end-modified C32 polymers. Although previous studies with poly(β-amino ester)s used a novel fluorescence-based technique (19), we choose to use a DNA extraction and real-time PCR (RT-PCR) amplification protocol to quantify the amount of endocytosed DNA (9). This method provides (1) high sensitivity due to the PCR amplification, (2) linearity over several orders-of-magnitude, (3) the ability to quantify DNA uptake without prelabeling the plasmid, and (4) a high-throughput, 96-well plate format to simultaneously and rapidly analyze all polymers. For this experiment, transfections were performed following the standard protocol using a DNA extraction and real-time PCR (RT-PCR) amplification protocol to quantify the amount of endocytosed DNA (9). The results show that this single substitution at the terminal hydroxyl, can have a large effect on cell interactions and endocytosis. Extending the comparison further, C32–106 mediating the highest plasmid internalization that is 30-fold greater than free DNA. The most obvious trend in the data is the improved uptake that occurs for polymers with additional terminal amines. This is evident for polymers containing extra secondary and tertiary end amines (C32–60 through C32–87) and mostly for those with an additional primary end amine (C32–102 through C32–122). These results suggest that conjugation of targeting ligands to the chain ends may be a promising strategy to achieve cell-specific delivery.

The differences in uptake between each polymer also explain some important differences in their transfection efficiencies. First, many polymers that are poor transfection agents also displayed very low uptake (e.g., C32–17, C32–93, C32–95). This indicates that the extra charge alone at the chain end points (compared to C32-Ac) is not sufficient to promote C32-DNA endocytosis. Specific functional groups at the chain ends, such as hydroxyls and amines, have an enhanced capacity to interact with cell surfaces and increase uptake as compared to more hydrophobic terminal segments. For example, the transfection differences between C32–36 and C32–52 are largely related to their differences in uptake. This comparison demonstrates that a single functional group in the polymer chain, in this case, a terminal hydroxyl, can have a large effect on cell interactions and endocytosis. Extending the comparison further, C32–106 differs from these two materials by a terminal primary amine. The results show that this single substitution at the terminal amine carbon-6 can increase uptake by over 20-fold. This effect is even more surprising considering that the polymer:DNA ratio used for C32–106 is 20:1, 5-fold less than that used for C32–36 and C32–52. In general, the increased uptake by the polymers capped with primary diamines largely explains their increased effectiveness at reduced polymer:DNA ratios. The overall transfection levels may not be substantially improved over C32 and other nonprimary amine polymers because the terminal

**Figure 6.** Polymer–DNA uptake into COS-7 cells. DNA uptake levels are shown in the number of plasmids per cell for each end-modified C32 polymer (C32-X).
functionalities may have important effects on other downstream gene delivery barriers such as endosomal escape, cytosolic trafficking, or nuclear import.

CONCLUSIONS

In this work, we present a fast and simple method to generate end-modified polymer derivatives. Variations in the terminal amine structure are shown to affect many properties that are important for effective polymeric gene delivery. Surprisingly, single carbon and functional group differences only at the chain ends have large effects on the physical properties of polymer–DNA complexes, their cellular uptake, and subsequent transfection. The best results occurred with primary diamine end modifications, which improved transfection of the C32 poly(β-amino ester) and lowered the optimal polymer:DNA ratio by 5-fold. These modifications effectively decreased the polymer–DNA nanocomplex size and increased cellular uptake. This suggests that ligand attachments to the polymer ends may be a promising strategy to achieve cell-specific targeting and improve the in vivo biodistribution of polymer–DNA complexes. To our knowledge, the modified poly(β-amino esters) presented here are some of the most effective and efficient degradable polyplexes to date, superior to PEI and previously optimized poly(β-amino ester)s. We are currently evaluating the in vitro transfection potential of these polymers for the systemic treatment of cancer.

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LITERATURE CITED


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