Small-Molecule End-Groups of Linear Polymer Determine Cell-Type Gene-Delivery Efficacy

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Gene delivery has the potential to treat a range of inherited and acquired diseases. Research has primarily focused on the use of viral vectors for this purpose, due to efficient infection of cells with viruses as well as long-term gene expression. However, the use of viral vectors for gene therapy is limited by safety concerns, production/manufacturing challenges, and limited nucleic acid-carrying capacity.[1,2] Increased attention has been focused on biomaterials including cationic polymers as gene-transfection agents[3–5] due to their electrostatic interactions with plasmid DNA to form cationic nanoparticles. Polymers, including polyethylenimine (PEI), are useful in a variety of gene-therapy applications.[6–10] One promising class of polymers, poly(β-amino ester)s (PBAEs), are degradable and have enhanced delivery compared to PEI.[11–13] Recent studies show that the amine-terminated polymers are generally more effective at promoting cellular uptake and DNA delivery than their acrylate-capped counterparts.[14,15]

We hypothesized that small molecules conjugated to the ends of the linear polymers could modify the gene-delivery efficacy of these polymers in a cell-type-specific manner. Initially, we screened a large library of PBAEs for gene delivery.[16] The base polymer with the highest gene-transfer efficiency, C32, is synthesized by mixing 1,4-butanediol diacrylate (C) with 5-amino-1-pentanol (32) at a 1.2:1.0 amine to diacrylate molar ratio. The C32 polymer has excellent biocompatibility in vivo, effectiveness as a gene-delivery vector for the treatment of prostate cancer via intratumoral injection in mice, and is promising as a delivery system for pancreatic cancer.[17,18]

Using a combinatorial approach, an initial library of end-modified C32 polymers was synthesized and tested.[19–21] Beyond increasing efficacy in an easy-to-transfect cell line, we wanted to explore whether small-molecule end-groups could change the gene-delivery efficacy differentially among varying cell types and in difficult-to-transfect conditions such as 100% serum. Therefore, we synthesized a new library of end-modified C32 polymers using structural information gained from previous studies, where we noted that diamine end-groups that contain a three-carbon spacer between amine functionalities were most effective.[13] We chose flow cytometry (and green fluorescent protein (GFP) expression) to quantify transfection efficacy so that we could analyze cell populations, which is not possible with luminescence assays. Previously, we have seen good correlation between these two methods.[16] The synthesis, biophysical characterization, and delivery efficacy of the nanoparticles formed with these new polymers is presented here.

A library of polymers was synthesized from a base polymerization of C and 32 to form acrylate-terminated C32 (Fig. 1a) and then this polymer was end-capped (Fig. 1b) with small molecules that contain amine groups (Fig. 1c). Polymers were characterized by ^{1}H-NMR spectroscopy and gel permeation chromatography (GPC) (see Experimental Sec. and Fig. S1 and S8). The effect of modifying the ends of the same base polymer with different amine end-groups is dramatic. Figures 2 and 3 demonstrate that the end-group modulates gene delivery from very low (<1% positive) to very high (>90% positive) and does so in a cell-specific manner.

Through electrostatic interactions, these polymers bind DNA and form nanoparticles. We examined the particle size and zeta potential of these particles by dynamic light scattering at the same concentrations and media conditions as used during transfection, as has been described previously.[22] We found that for most polymers, 30 and 50 weight polymer:weight DNA (wt/wt) ratio formulations formed particles of similar size. However, changes to the polymer end-group could considerably vary the particle size from no particle formation to larger than 400-nm particles (Fig. 2a). End modification had minimal effects on the particle zeta potential (Fig. 2b) and both 30 wt/wt and 50 wt/wt formulations had the same, neutral zeta potential. Most polymers formed nanoparticles that were ~200 nm in size and had a neutral zeta potential.

We initially screened our library of end-modified C32 polymers in COS-7 monkey kidney fibroblasts in 10% serum with a 600 ng DNA dose per well (Fig. 2c). At a 30 wt/wt ratio, five new polymers (206, 213, 216, 227, and 228) achieved a nominally higher transfection rate than the previous best polymer, C32-117. At a 50 wt/wt ratio, 11 new polymers achieved a nominally higher transfection rate than the previous best polymer. Almost all of the new polymers showed normal viability at the concentrations and wt/wt ratio tested (Fig. 2d), although a few of the best polymers showed significant toxicity (206, 208, 210, and 253). The four cytotoxic polymers may be useful at lower polymer doses but are less suitable for transfection than the other high-performing

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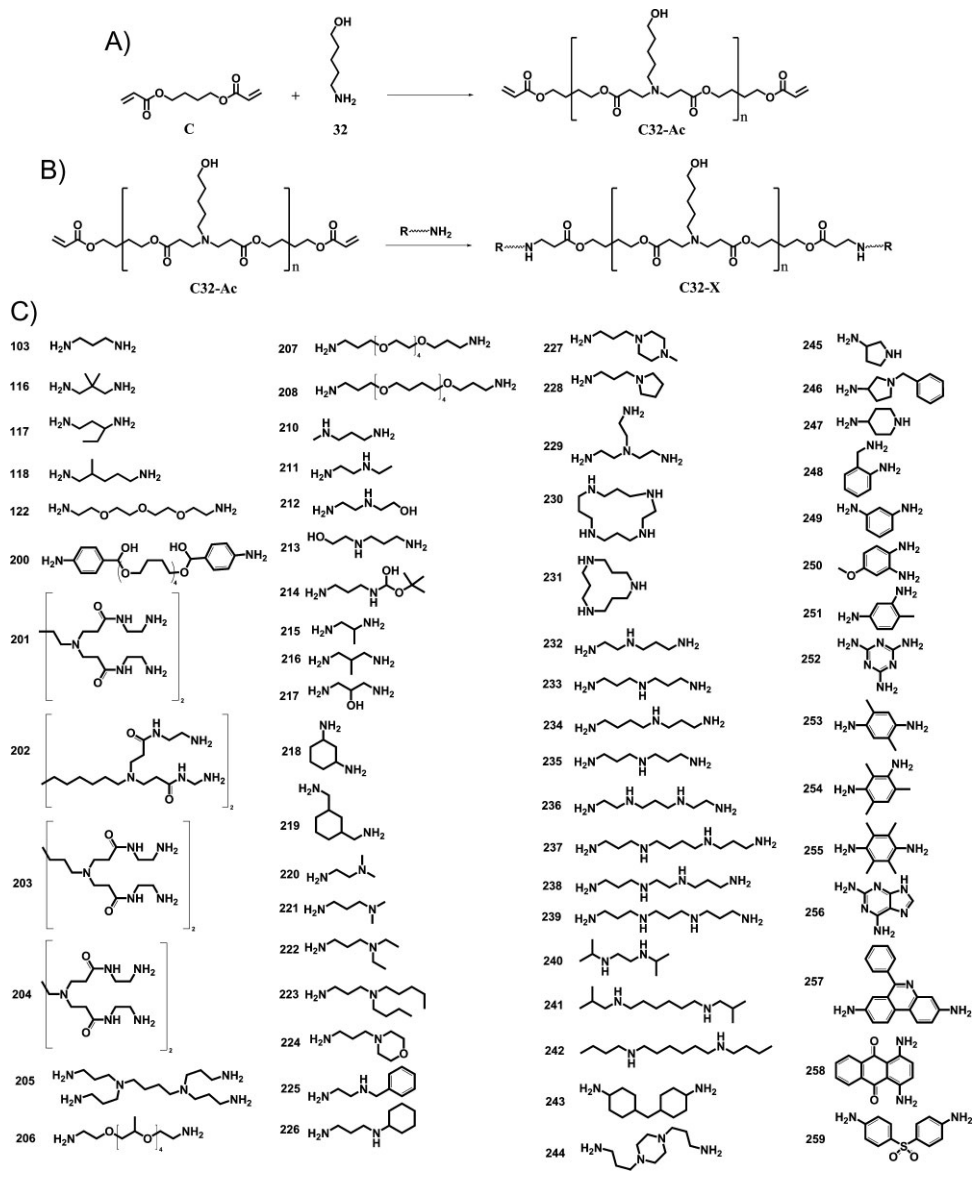

Figure 1. A) Synthesis of acrylate-terminated C32 polymer (C32-Ac). B) Synthesis of end-modified C32 polymers (C32-X). C) Amine capping molecules.

polymers that also have high viability (such as for example 213, 216, 227, 228, 245, and 254). Interestingly, biophysical properties of the nanoparticles (Fig. 2) or molecular weight (Supporting Information, Fig. S9) did not significantly correlate to transfection efficacy. This suggests that the functionality of the small-molecule polymer end-groups does not come from tuning the physical properties of the nanoparticle, but instead from changing the interaction between the complex and the cell, perhaps by playing a role in cellular uptake.

To investigate the biological effects of end-modification, we compared the gene delivery of the nanoparticles across additional cell lines including HeLa, a human cervical cancer-cell line (Fig. S2), HepG2, a human hepatocellular carcinoma (Fig. S3), HUVEC, human primary endothelial cells (Fig. S4), DC 2.4, murine dendritic cells (Fig. S5), and hMSC, human mesenchymal stem cells (Fig. S6). All experiments were conducted in standard cell-growth media that includes 10–12% serum proteins. Several of the polymers (206, 208, 210, 213, 221, 227, and 228) were effective at transfecting HeLa cells and were comparable to the commercially available reagent, Lipofectamine 2000. In HepG2 cells, a different set of lead polymers (210, 218, 221, 227, and 228) was most effective. In HUVECs, a hard-to-transfect human primary cell type, several polymers including 117, 210, and 213 were highly effective, delivering exogenous genes to over twice as many cells as Lipofectamine 2000 (Fig. S5). Polymer C32-210 is particularly interesting, as it is both more efficient relative to the other similarly structured PBAEs and more effective, meaning that less than half the amount of polymer 210 (30 wt/wt) is needed to achieve similarly high transfection (~75% positive) compared to polymer 213

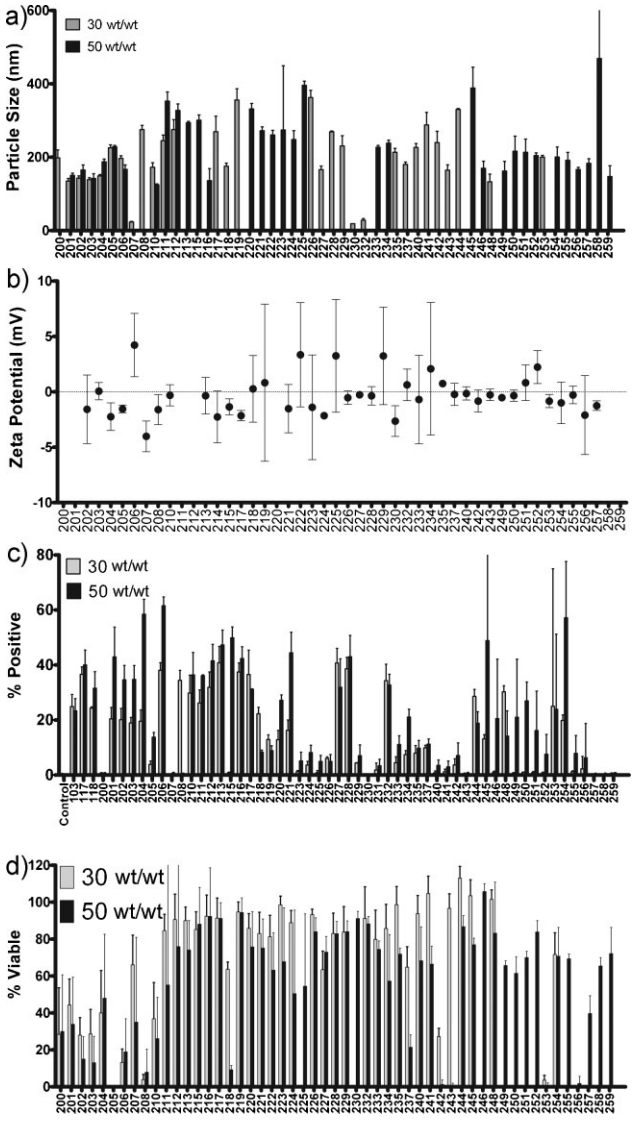

Figure 2. Biophysical and gene delivery characterization of small-molecule-modified polymers. a) Particle size, b) zeta potential, c) gene-delivery efficacy in COS-7, and d) viability in COS-7. Biophysical characterization shown for each polymer at its optimal formulation (mean ± standard deviation).

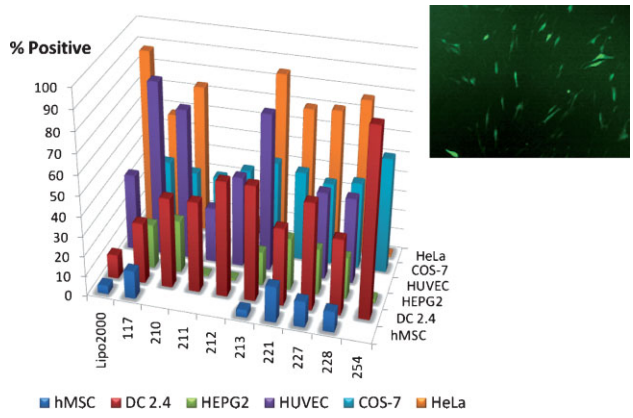

Figure 3. Gene-delivery efficacy of lead small-molecule-end-capped polymers across six cell types. Each bar represents the optimal formulation for each polymer and ranges from 30–75 (wt/wt). Inset: Fluorescence microscopy image showing the gene-delivery efficacy of 221 to human mesenchymal stem cells (~18% positive cells).

(75 wt/wt). Many other polymers were unable to achieve this high level of efficacy at any formulation. The fact that such small changes to polymer structure can make the polymer more relatively efficient (or, in the case of C32-206 or 211, relatively inefficient) is surprising (Fig. S4). End-modified polymers were found to be particularly effective for gene delivery to DC 2.4 cells (Fig. S5). Lipofectamine 2000, a commercially available non-viral transfection reagent, transfects 10% of these antigen-presenting immune cells. Polymer C32-254, on the other hand, is able to transfect 90% of these cells. This result suggests the potential utility of these biodegradable particles as efficient genetic vaccines. A final difficult-to-transfect cell type that we are interested in is hMSC. Here, polymer C32-221 is the lead biomaterial and, while transfection is not as high as in other cell types, it remains significantly higher than levels from Lipofectamine 2000 (Fig. S6a, b and e). The polymeric nanoparticles are also shown to be minimally cytotoxic to these fragile cells (Fig. S6c–d), similar to the COS-7 findings. To further increase gene delivery to hMSCs, we increased the DNA dose, which enhanced gene expression for some polymers up to 5-fold, while not having any adverse effects on cell viability. Some of these formulations also retain effectiveness in the presence of 100% serum during transfection, with C32-206 and C32-204 best for COS-7s and HUVECs, respectively (Fig. S7). This suggests that some of these materials may retain their effectiveness following intravenous administration. As compared to Lipofectamine 2000, these polymers require a higher dose of biomaterial (wt/wt polymer) to form optimized gene-delivery particles.

A few polymers have high transfection across most cell types and point to some general features of highly effective polymeric gene-delivery particles. Polymers C32-213, C32-227, and C32-228 are particularly effective and structurally very similar. They, like all polymers in this library, contain the same polymer backbone and hydroxyl-terminated side chains. In addition, they each contain an end-group that is composed of two amines separated by exactly three carbons and then terminated in a slightly differing arrangement of a few atoms. Unlike many end-groups in the library (such as 103-208 and 232-239), these three polymers are not terminated by primary amines. Polymers C32-213, C32-227, and C32-228 have molecular weights between 8 and 16.7 kDa, form nanoparticles that are 170–290 nm in size (slightly larger than average), and have a zeta potential of ~0 mV (most neutral) in the presence of cell media.

While some polymers are strong transfection agents overall, none of the polymers is optimal for all cell types simultaneously (Fig. 3). Rather, for each cell type there is a particular polymer that is most effective (COS-7: C32-206, HeLa: C32-213, HepG2: C32-210, HUVEC: C32-117, DC2.4: C32-254, hMSC: C32-221). Some polymers are also highly effective for delivery to one cell type, while simultaneously being poor for delivery to another (for example, C32-254 is especially effective for delivery to DC2.4s rather than HepG2s or HeLas, whereas C32-228 transfects 80% of

HeLas, but only 10–40% in the other five cell types tested). When a double DNA dose is used and multiple cell types are transfected side by side in the same plate, the same cell-specificity trends are observed, although the transfection of HeLas increases overall (Fig. S10). Future work is needed to elucidate the mechanisms for this cell specificity, which may be due to changes to particle-uptake kinetics and/or polymer/DNA binding interactions that regulate the DNA release. Combined with other modalities for cell targeting, such as targeting ligands[23,24] or transcriptional targeting,[17] proper biomaterial selection may further improve the safety/efficacy window for nanomedicine.

In conclusion, our approach has revealed materials that are promising for the nonviral delivery of genes to cancer cells, immune cells, and human stem cells and points to polymer end-groups as a regulators for cell-type specificity.

Experimental

Polymer Synthesis: Acrylate-terminated poly(β-amino ester) C32-Ac was synthesized by mixing 3.532 g of 1,4-butanediol diacrylate (17.8 mmol) with 1.533 g of 5-amino-1-pentanol (14.8 mmol) for 24 h at 90 °C in the dark. End-chain-capping reactions were performed by mixing 160 μL of C32-Ac/DMSO solution (500 mg mL^{-1} polymer in DMSO) with 640 μL of 0.25 M amine solution. Excess amine is used to fully end-modify the polymer without causing detectable crosslinking or aminolysis. Reactions were performed in 1.5 mL Eppendorf tubes with constant agitation for 24 h at room temperature. Following initial screening, lead polymers 117, 210, 211, 212, 213, 221, 227, 228, and 254 were scaled up by adding 9.1 g of THF to 5 g of C32-Ac, vortexing, and then transferring to a 100 mL flask with a stir bar. 40 mL of 0.25 M end-capping amine solution was then added and the mixture was left stirring at room temperature in the dark for 24 h. End-modified polymers were precipitated by the addition of 10 times the volume of diethyl ether and centrifugation at 2500 rpm for 2 min. Polymers were washed twice with diethyl ether and dried in a vacuum desiccator.

Polymer Characterization: Polymers were characterized on a Varian mercury spectrometer by ^{1}H-NMR spectroscopy (300 MHz, $CDCl_3$, δ): C32-Ac: 1.3–1.6 (m, –NCH$_2$(CH_2)$_3$CH$_2$OH), 1.7 (bs, –N(CH$_2$)$_2$COOCH$_2$CH_2– and CH$_2$CHCOOCH$_2$CH_2–), 2.3–2.5 (m, –COOCH_2CH$_2$N– and –NCH_2(CH$_2$)$_4$OH), 2.7–2.8 (m, –COOCH$_2$CH_2N–), 3.6 (bs, –N(CH$_2$)$_4$CH_2OH), 4.1 (bs, –N(CH$_2$)$_2$COOCH_2CH$_2$–), 4.2 (m, CH$_2$CHCOOCH_2CH$_2$–), 4.4 (bs, –N(CH$_2$)$_5$O*H*), 5.9 (m, CH_2CHCOOCH$_2$CH$_2$–), 6.1–6.2 (m, CH$_2$C*H*COOCH$_2$CH$_2$–), 6.4 (m, CH_2CHCOOCH$_2$CH$_2$–). End-modification of polymers was confirmed by the disappearance of the acrylate peaks at 5.9, 6.1–6.2, and 6.4 ppm. Organic phase GPC was performed using a solution of 0.1 M piperidine in 95% THF/5% DMSO (v/v) as the eluent at a flow rate of 1.0 mL min^{-1} in a Waters GPC system equipped with an autosampler (Waters Corporation, Milford, MA). A Phenogel (Phenomenex, Torrance, CA) MXL column (5 μm, 300 mm × 7.8 mm) and a Waters Styragel HR4 column were used in series and the molecular weights of the polymers are reported relative to monodisperse poly(2-vinylpyridine) standards. Unmodified C32-Ac and most end-modified polymers synthesized generally had a molecular weight M_w of ~8500 Da. However, 4 polymers synthesized in larger scale, C32-221, -227, -228, and -254, had higher M_w of ~14–20 kDa, approximately twice as large as the others, indicating low levels of crosslinking of the C32 oligomers by these diamines (Fig. S8). The small variance in molecular weight among the polymers does not appear to be correlated to particle size, zeta potential, or transfection efficacy (Fig. 2, S8, and S9) among the cell types tested. Polymers were stored at −20 °C with desiccant.

Particle-Size and Zeta-Potential Measurements: Measurements were carried out using a ZetaPALS dynamic light scattering detector as previously described [24].

Cell Culture: COS-7 (ATCC, Manassas, VA), HeLa (ATCC), HepG2 (ATCC), and HUVECs (Lonza, Walkswille, MD) were grown in accordance with manufacturer instructions. DC2.4s were generously given to us by Herman Eisen (MIT) and cultured according to published procedures [25]. Bone marrow-derived hMSCs (Lonza, Walkswille, MD) were grown in MSC growth medium consisting of Dulbeccos-modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 mM sodium pyruvate, and 100 units of penicillin and streptomycin. Cells were subcultured upon confluence until passage 5 before use.

Cell Transfections: Cells were plated in 96-well plates at 15000 cells per well and allowed to adhere overnight. EGFP-1 DNA (Elim Biopharmaceuticals, Hayward, CA) was diluted in 25 mM sodium acetate (NaAc, pH = 5) to 0.04 mg mL^{-1}. Polymers at 100 mg mL^{-1} in DMSO were diluted into NaAc buffer to yield the different polymer to DNA weight ratios. 100 μL of diluted polymer solution was mixed vigorously with 100 μL of DNA solution in 500 μL Eppendorf tubes. After 10 min wait time, 180 μL of each was added to 720 μL of media in a deep-well polypropylene plate. Subsequently, the media over the cells was removed with a 12-channel aspirator wand and followed by the addition of 150 μL well^{-1} of polymer–DNA-complex solution in media. Complexes were incubated over the cells for 4 h, then removed by aspiration, and replaced with 100 μL well^{-1} of fresh media. Cells were allowed to grow for 2 days at 37 °C and 5% CO_2 and then analyzed by fluorescence-activated cell sorting (FACS) for GFP expression. For transfections in 100% serum, we prepared the particles in 25 mM NaAc buffer, similar to all other formulations. These particles were then added to 100% serum instead of regular complete cell medium and the diluted particle mixtures were subsequently added to freshly aspirated cells. While the particle diluent was initially 100% serum rather than cell medium, the final concentration of serum is 80% serum during the transfection due to the presence of residual buffer. No cell-culture medium or any other additives were present during transfection. For hMSC experiments, all volumes were scaled up 5-fold and performed in 24-well plates. 75000 cells were seeded 24 h prior to transfection and particle doses of 3 μg and 6 μg DNA were added to the cells for 4 h, after which they were removed by aspiration and replaced with 500 μL well^{-1} of fresh media.

FACS: GFP expression was measured using FACS on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) or BD LSR II. Cells were aspirated, washed, aspirated, trypsinized, and then suspended in FACS buffer (98% phosphate buffered saline (PBS), 2% FBS, 1:200 propidium iodide) while in 96-well plates. Propidium iodide staining was used to exclude dead cells from the analysis and at least 5000 live cells per sample were acquired. 2D gating was used to separate increased auto-fluorescence signals from increased GFP signals to more accurately count positively expressing cells. Gating and analysis was performed using FlowJo 6.3 software (TreeStar, Ashland, OR, USA).

CellTiter Assay for Cell Viability: 96-well plates were seeded with COS-7 cells in DMEM (10% FBS, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) to 15000 cells per well and allowed to attach overnight. Transfection was performed as described previously. The media and complexes were removed by aspiration 4 h post-transfection and replaced with fresh media. 24 h post-transfection, 20 μL of CellTiter 96 AQ$_{ueous}$ One Solution Reagent was added to each well. Absorbance measurements were taken following manufacturer instructions. Viability is reported as normalized cell-metabolic activity compared to untreated controls. hMSC viability measurements were performed similarly.

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