Highly compacted biodegradable DNA nanoparticles capable of overcoming the mucus barrier for inhaled lung gene therapy

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Gene therapy has emerged as an alternative for the treatment of diseases refractory to conventional therapeutics. Synthetic nanoparticle-based gene delivery systems offer highly tunable platforms for the delivery of therapeutic genes. However, the inability to achieve sustained, high-level transgene expression in vivo presents a significant hurdle. The respiratory system, although readily accessible, remains a challenging target, as effective gene therapy mandates colloidal stability in physiological fluids and the ability to overcome biological barriers found in the lung. We formulated highly stable DNA nanoparticles based on state-of-the-art biodegradable polymers, poly(β-amino esters) (PBAEs), possessing a dense corona of polyethylene glycol. We found that these nanoparticles efficiently penetrated the nonporous and highly adhesive human mucus gel layer that constitutes a primary barrier to reaching the underlying epithelium. We also discovered that these PBAE-based mucus-penetrating DNA nanoparticles (PBAE-MPPs) provided uniform and high-level transgene expression throughout the mouse lungs, superior to several gold standard gene delivery systems. PBAE-MPPs achieved robust transgene expression over at least 4 mo following a single administration, and their transfection efficiency was not attenuated by repeated administrations, underscoring their clinical relevance. Importantly, PBAE-MPPs demonstrated a favorable safety profile with no signs of toxicity following intratracheal administration.

lung gene therapy | mucus-penetrating particles | nanotechnology | biodegradable polymer | nonviral gene delivery

Gene therapy holds promise for a wide range of hard-to-treat inherited and acquired diseases. Most gene therapy clinical trials have investigated vectors derived from viruses due to their intrinsic capacity to infect cells. However, viral vectors possess several shortcomings, including low packaging capacity, technical difficulties in scale-up, high cost of production, and/or risk of mutagenesis (1). Furthermore, vector-inactivating immune responses are frequently observed with repeated administrations or prior exposures (2, 3). Nonviral gene vectors do not have these limitations, and have demonstrated promise in vitro and in preclinical settings (4). Among several promising polymers, poly(β-amino esters) (PBAEs) provide a library of nontoxic, biodegradable polymers for the compaction of nucleic acids. Numerous high-throughput in vitro screening studies of PBAE-based gene vector libraries have shown efficient gene transfer in a cell-specific manner (5–13). However, in vitro behavior of gene vectors does not usually predict in vivo performance, largely due to harsh physiological environments and a variety of extracellular barriers (14–16). Specifically, the hydrolytic nature and relatively low positive charge density of PBAE may reduce colloidal and DNA compaction stabilities in physiological conditions, thereby limiting their use in vivo and, thus, the potential for clinical applications. Here we sought to develop highly stable PBAE-based DNA nanoparticles (DNA-NPs) and test their ability to provide gene transfer in the airways in vivo.

The lung airway is accessible via inhalation and, thus, it is one of the most straightforward targets for localized gene delivery (17). However, the mucus layer lining the airways forms a nonporous adhesive barrier that thwarts gene vectors from reaching the underlying epithelium (18). The airway mucus gel is composed of a dense mesh of mucin fibers containing a high density of negatively charged glycan interspersed with periodic hydrophobic regions (19). In the airways of patients with respiratory diseases, including cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), elevated levels of bacterial and endogenous DNA, as well as actin filaments from degraded neutrophils, reinforce the barrier properties of airway mucus (20, 21). Based on the diffusion rates of nonadsorptive nanoparticles (NPs) of various sizes, we previously estimated the average pore size of spontaneously expectorated mucus from CF

Significance

Therapeutically relevant lung gene therapy is yet to be achieved. We introduce a highly translatable gene delivery platform for inhaled gene therapy based on state-of-the-art biodegradable polymers, poly(β-amino esters). The newly designed system is capable of overcoming challenging biological barriers, thereby providing robust transgene expression throughout the entire luminal surface of mouse lungs. Moreover, it provides markedly greater overall transgene expression in vivo compared with gold standard platforms, including a clinically tested system. The clinical relevance is further underscored by the excellent safety profile as well as long-term and consistent transgene expression achieved following a single and repeated administrations, respectively.


Conflict of interest statement: The MPP technology described in this publication was developed at the Johns Hopkins University and is licensed to Kala Pharmaceuticals, J.H. is a founder of Kala Pharmaceuticals and currently serves as a consultant. J.H. and Johns Hopkins University own company stock; J.H.’s relationship with Kala Pharmaceuticals is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

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Significance

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patients to be 140 ± 50 nm (range 60–300 nm) (22). As a consequence of the elevated adhesivity and tighter mesh size of CF mucus, adenovirus (23, 24), adeno-associated virus (AAV) sequences of the elevated adhesivity and tighter mesh size of CF success in clinical trials (20, 22, 28). In addition, conventional gene vectors formulated with cationic polymers, such as polyethyleneimine (PEI) and poly-l-lysine (PLL), are immobilized in airway mucus regardless of their size due to the positively charged particle surface that interacts with the negatively charged mucus constituents (18). Gene vectors trapped in airway mucus are rapidly cleared from the lung by mucociliary clearance (MCC) or cough-driven clearance (29, 30). Thus, for efficient gene transfer to efficiently penetrate the mucus gel layer, thereby limiting their success in clinical trials (20, 22, 28).

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We synthesized high-DNA (33). Using a two-step Michael addition reaction (6, 13, 34), a mixture of non-PEGylated and PEGylated polymers to condense plasmid DNA with non-PEGylated PBAE only, displayed a depletion of all reactions was confirmed by NMR (Fig. 1). The MWs estimated by NMR (Fig. 1) were similar to those determined by gel permeation chromatography (GPC) (Fig. S2).

Results and Discussion

To formulate mucus-penetrating DNA-NPs based on PBAEs, we used a previously established blending strategy in which we used a mixture of non-PEGylated and PEGylated polymers to condense DNA (33). Using a two-step Michael addition reaction (6, 13, 34), we synthesized high-molecular-weight (MW) non-PEGylated PBAE polymers (Fig. 1A) with the end diacrylate groups capped by 2-(3-aminopropylamino) ethanol (C5) (Fig. 1B); the capping group was selected from our PBAE library (Fig. S1). To produce PEGylated PBAE polymers (PBAE-PEG), low-MW PBAE polymers were first synthesized in a similar fashion (Fig. 1C) but with the end diacrylate groups capped by 1,3-diaminopropane (C1). Subsequently, methoxy-PEG-succinimidyl succinates were reacted with the terminal primary amine groups at both ends of the C1-capped PBAE polymer chain (Fig. 1 C–E). The completion of all reactions was confirmed by NMR (Fig. 1). The MWs estimated by NMR (Fig. 1) were similar to those determined by gel permeation chromatography (GPC) (Fig. S2).

Using these polymers, we formulated PBAE-based DNA-NPs. Conventional DNA-NPs (PBAE-CPS), formulated by compacting plasmid DNA with non-PEGylated PBAE only, displayed a particle hydrodynamic diameter of ~85 nm and a positive surface charge (ζ-potential ~30 mV) (Fig. 2A and Table 1). A blend of PBAE and PEGylated PBAE at an optimized ratio based on PBAE mass (wt/wt ratio of 2:3 PBAE:PEG) compacted plasmid DNA more tightly (~50 nm in diameter) and provided a neutral surface charge (~2 mV), suggesting that the particle surface was densely shielded with PEG chains (mucus-penetrating PBAE/DNA; PBAE-MPPs). PBAE-MPPs exhibited improved colloidal stability in water compared with PBAE-CPS (Fig. 2B). We also found that PBAE-MPPs retained their physicochemical properties in bronchoalveolar lavage fluid (BALF) at 37 °C for 24 h with a minor increase in particle diameter, whereas PBAE-CPS rapidly aggregated (Fig. 2C). The increase in NP size beyond the average mucus mesh spacing size, as observed in PBAE-CPS, hinders the diffusion of PBAE-CPS in the airway mucus, leading to rapid clearance by MCC before they reach the airway epithelium. Furthermore, particle aggregates deposited in the airspace are likely engulfed by alveolar macrophages and subsequently cleared from the lung (35–37).

This formulation strategy can be generalized to formulate PBAE-MPPs with various leading core PBAE polymers (Fig. S1 and Table S1) (7–13), resulting in DNA-NPs with similar physicochemical properties.

To test whether PBAE-MPPs possess dense PEG surface coatings, we assessed the ability of these DNA-NPs to resist adsorption of pulmonary macromolecules compared with leading nonviral gene vectors, including PBAE-CPS, non-PEGylated PEI/DNA [PEI-CPS; considered a gold standard (38)], and conventionally PEGylated PLL/DNA [PLL-CPS; a replicate of the CKPEG10k DNA-NP (39)]. The physicochemical properties of PEI-CPSs and PLL-CPSs are shown in Table S2. Following incubation of DNA-NPs in mouse lung protein lysates, we found that protein adsorption on the surface of PBAE-MPPs was negligible, whereas 79%, 56%, and 66% of protein quantity was found associated with PEI-CPs, PLL-CPs, and PBAE-CPs, respectively (Fig. S3B). This is in accordance with the excellent colloidal stability of PBAE-MPPs in mouse BALF, which was not achieved with PBAE-CPS (Fig. 2C). Of note, the conventional PEG coating of PLL-CPS did not significantly reduce protein adsorption compared with non-PEGylated DNA-NPs, suggesting that a dense surface coverage of PEG is required to preclude adsorption of pulmonary macromolecules onto NPs.

We next compared the in vitro transfection efficiency of PBAE-MPPs in BEAS-2B human bronchial epithelial cells with that achieved by PBAE-CPS, PEI-CPS, and PLL-CPS (Fig. S4).
PBAE-CPs exhibited the highest transfection efficiency, presumably attributable to the biodegradable nature of PBAEs that facilitates intracellular release of the packaged plasmid DNA (8, 40, 41). In accordance with previous reports that PEGylation may reduce in vitro transfection (14, 42), PBAE-MPPs exhibited significantly lower in vitro transfection efficiency compared to uncoated PBAE-CPs. However, PBAE-MPPs transfected BEAS-2B cells as efficiently as non-PEGylated PEI-CPs and significantly better than PLL-CPs. We hypothesized that the ability of PBAE-MPPs to retain high in vitro transfection efficiency and colloidal stability in physiological conditions would enhance its overall gene transfer efficacy in vivo.

Using multiple particle tracking (MPT), we next investigated the diffusion of various DNA-NPs in mucus freshly expectorated by CF patients. MPT allows simultaneous tracking of thousands of individual fluorescently labeled particles in biological media at high spatiotemporal resolution and provides various transport parameters such as mean square displacement (MSD), a measure of the distance traveled by particles at a given time interval (i.e., timescale) (43, 44). PLL-CPs, PEI-CPs, and PBAE-CPs were unable to efficiently penetrate CF mucus (Fig. 2D), in accordance with our previous finding that non-PEGylated or conventionally PEGylated nonviral gene vectors are largely hindered or immobilized in CF mucus (33). In contrast, PBAE-MPPs exhibited highly diffusive trajectories in CF mucus (Fig. 2D) and diffused significantly faster ($P < 0.05$) than the conventional DNA-NPs (Fig. 2E). Overall, PBAE-MPPs diffused in CF mucus at an average rate only 50-fold lower than their theoretical diffusion rate in water, whereas PEI-CPs, PLL-CPs, and PBAE-CPs were slowed on average by 350-, 630-, and 700-fold, respectively. The airway coverage of PBAE-MPPs was $\sim 70%$, with minimal variation in airway distribution (i.e., highly uniform distribution), in sharp contrast to 20% coverage at best with large variations observed for all other DNA-NPs (Fig. 3B and C). Similar results were observed in the lung parenchyma (Fig. 3D–F), likely due to the dense surface PEG coatings that reduce particle aggregation and phagocytosis by alveolar macrophages (35–37).

We next investigated whether the improved distribution of PBAE-MPPs also improved transgene expression in the mouse lung; various DNA-NPs carried plasmid DNA encoding for green fluorescent protein (GFP) driven by a cytomegalovirus (CMV) promoter and were administered intratracheally. We found that GFP transgene expression was sporadic in the lungs of mice treated with PBAE-CPs, PEI-CPs, and PLL-CPs, whereas PBAE-MPPs provided uniform transgene expression throughout the lungs (Fig. 4A). Flow cytometric analysis revealed that PBAE-MPPs mediated transgene expression in more than 15% of the total lung cell population in vivo following a single intratracheal administration (Fig. S5). In contrast, PBAE-CPs transfected only 3% of total cells, similar to previous findings with other leading nonviral (45–47) and viral (48, 49) vectors. PBAE-MPPs demonstrated transgene expression in 12% of the CD45-negative immune cells in the mouse lung, in contrast to the predominant transduction of immune cells achieved by viral vectors (50). Of note, the nonimmune cells in the lung lumen include airway epithelial cells, which comprise less than 1% of the total respiratory epithelial surfaces (51), as well as alveolar epithelial cells types I and II, which constitute 8% and 15% of the total peripheral lung cells, respectively (52, 53). Our findings suggest that a single intratracheal administration of PBAE-MPPs mediated efficient transgene expression in mouse lung epithelial cells. The widespread transgene expression achieved uniquely with PBAE-MPPs may have important implications for the success of lung gene therapy in the clinic.

Table 1. Physicochemical properties of PBAE-based DNA-NPs

<table>
<thead>
<tr>
<th>DNA-NP type</th>
<th>Storage</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Hydrodynamic diameter ± SEM, nm</th>
<th>PDI ± SEM</th>
<th>ζ-Potential ± SEM, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAE-CPs</td>
<td>Fresh</td>
<td>84 ± 2.8</td>
<td>84 ± 2.8 ± 0.1</td>
<td>0.1</td>
<td>31.0 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>24 h at RT</td>
<td>262 ± 3.6</td>
<td>262 ± 3.6 ± 0.2</td>
<td>0.2</td>
<td>0.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>178 ± 9.4</td>
<td>178 ± 9.4 ± 0.6</td>
<td>0.4</td>
<td>0.6 ± 0.6</td>
</tr>
<tr>
<td>PBAE-MPPs</td>
<td>Fresh</td>
<td>50 ± 1.1</td>
<td>50 ± 1.1 ± 0.5</td>
<td>0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>24 h at RT</td>
<td>54 ± 1.9</td>
<td>54 ± 1.9 ± 0.6</td>
<td>0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Lyophilized</td>
<td>73 ± 2.3</td>
<td>73 ± 2.3 ± 0.2</td>
<td>0.2</td>
<td>2 ± 0.1</td>
</tr>
</tbody>
</table>

*PBAE-based DNA-NPs were characterized directly after formulation, following a 24-h storage at room temperature, or following lyophilization and subsequent rehydration.

†Hydrodynamic diameter and polydispersity index (PDI) were measured by dynamic light scattering (DLS) in water (pH 7.0). Data represent the mean ± SEM ($n \geq 3$).

‡ζ-Potential was measured by laser Doppler anemometry in 10 mM NaCl (pH 7.0). Data represent the mean ± SEM ($n \geq 3$).
clinic, specifically when broad therapeutic effect throughout the lung is required. For example, CF patients with certain cystic fibrosis transmembrane conductance regulator (CFTR) mutations, which retain ∼10% of functional expression per cell, are generally not afflicted by CF lung diseases (54). This suggests that even modest levels of CFTR gene transfer throughout the airways, rather than a high level of spatially confined transgene expression, may be required to restore the defects in CF lungs. Moreover, thanks to the identification of genetic targets, the ability to achieve widespread transgene expression may provide therapeutic breakthroughs for diseases such as COPD and asthma that affect both airways and lung parenchyma (55, 56).

We subsequently investigated whether the ability of PBAE-MPPs to penetrate the mucus layer and reach the underlying epithelium enhances the overall gene transfer efficacy following intratracheal administration. The various DNA-NPs were formulated with a luciferase plasmid DNA driven by a human β-actin promoter. All conventional DNA-NPs (PBAE-CPs, PEI-CPs, and PLL-CPs) provided significantly enhanced transgene expression levels compared with naked plasmid DNA control (Fig. 4B). Among the conventional DNA-NPs, PLL-CPs have demonstrated a gene transfer level on par with AAV serotype 2 in a clinical trial (39, 57), and PEI-CPs have provided in vivo transgene expression comparable to the only gene delivery system being tested currently in a clinical trial for CF gene therapy (GL67A) (58, 59). PBAE-MPPs mediated a statistically significant ∼25-fold higher overall transgene expression level compared with these gold standard nonviral gene vectors (Fig. 4B).

The significantly enhanced in vivo gene transfer by PBAE-MPPs compared with PBAE-CPs underscores that the ability of PBAE-MPPs to remain stable in physiological conditions and to overcome the mucus barrier more than offsets the inferior gene delivery capacity often observed with PEGylated gene vectors in vitro (Fig. S4). We next compared the in vivo gene transfer efficiencies of PBAE-MPPs and nonbiodegradable PEI-based DNA-NPs that we previously developed and confirmed to achieve efficient mucus penetration and thus airway gene transfer in vivo (PEI-MPPs) (33). We found that PBAE-MPPs provided significantly
greater transgene expression in the mouse lung compared with PBAE-MPPs (Fig. S6). This is likely attributable to the well-established ability of PBAEs to provide superior intracellular gene transfer compared with PEI (60). Overall, both MPP formulations mediate significantly higher absolute levels of transgene expression compared with conventional DNA-NPs (PBAE-Cps, PEI-Cps, and PLL-Cps). It should also be noted that both storage in aqueous solution for 24 h at room temperature and reconstitution after lyophilization did not reduce the in vivo gene transfer efficacy of PBAE-MPPs (Fig. 4C), suggesting that PBAE-MPPs may be stored long-term as a stable pharmaceutical product.

The lack of sustained gene expression remains a limiting factor for effective nonviral gene therapy of chronic diseases (61). We demonstrated that a combination of PBAE-MPPs and plasmid DNA driven by an unmethylated human β-actin promoter provides sustained levels of transgene expression for at least 2 mo (Fig. 4D). Although the level decreased at 4 mo postadministration, it remained comparable to the levels achieved by leading conventional DNA-NPs at 1 wk postadministration (Fig. 4B). It should be noted that transgene expression will eventually fade due to the episomal nature of the delivered plasmid DNA and the turnover of transfected cells; the turnover of mouse airway epithelial cells has been shown to occur every 6 and 17 mo in trachea and bronchioles, respectively (62). Thus, it is imperative to achieve high-level transgene expression each time following repeated administration, especially for the treatment of chronic disorders (63). The inactivation of viral vectors via neutralizing antibodies and other host immune responses generated by prior exposure is a major obstacle (64). Here, we found that multiple administrations did not reduce the transfection efficiency of PBAE-MPPs (Fig. 5E).

We next sought to test the safety of the various gene vectors following inhalation. We discovered by histology that PBAE-MPPs and PLL-Cps did not cause acute inflammation, whereas the lungs of mice treated with PBAE-MPPs showed evidence of widespread immune cell infiltration (Fig. 5 A and B). PLL-Cps were well-tolerated by patients with CF in a phase I/IIa clinical trial, likely because they contain PEG (albeit less PEG than PBAE-MPPs) (59). PEI-Cps induced a significant increase in total cell counts and percentage of neutrophils in Balb/C. On the other hand, cell counts were not significantly different between the lungs of untreated controls and mice that inhaled PLL-Cps or PBAE-MPPs (Fig. 5 C and D).

In summary, biodegradable PBAE-MPPs provide colloidial stability in physiological conditions and capacity to efficiently penetrate airway mucus, leading to widespread transgene expression in the lungs in vivo. PBAE-MPPs demonstrate significantly enhanced overall gene transfer efficacy compared with leading nonviral gene vectors (and potentially viral vectors) as well as safety comparable to a nonviral gene delivery system found safe in human trials (PLL-Cps). The PBAE-MPP formulation strategy is simple and generalizable to a variety of biodegradable cationic polymers.

**Materials and Methods**

We established a library of PBAE polymers that consists of the polymer variants previously shown to provide efficient in vitro gene transfer (Fig. S1) (7–13). PBAE polymers were synthesized by a two-step Michael addition, as previously reported (Fig. 1) (6–13, 34). Methoxy-PEG-succinimidyl succinates (5 kDa; JenKem Technology) were reacted with the terminal primary amine groups at both ends of the end-capped linear PBAE polymer for the synthesis of PBAE-PEG polymers. The intermediates and final polymer products were characterized by NMR (Fig. 1) and GPC (Fig. S2). DNA-NPs were formulated by the dropwise addition of unlabelled or fluorescently labeled plasmid DNA solution to a polymer solution under optimized conditions (33) and characterized by transmission electron microscopy, dynamic light scattering, laser Doppler anemometry, and in vitro protein binding assay. MPT was used to measure the MSF of fluorescently labeled DNA-NPs in freshly expectorated mucus from CF patients (32, 33, 65). For the in vivo assessment of DNA-NP distribution and transgene expression in the lung airways and airspace, overall transfection efficiency, and in vivo safety profile, a 50-μL solution of DNA-NPs at a 0.5 mg/mL plasmid DNA concentration was intratracheally administered to Balb/C mice using a microsprayer (MicroSprayer Aerosolizer Model IA-1C; Penn-Century). All animals were handled in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee. In vivo distribution and transfection of DNA-NPs were characterized using confocal microscopy (66), flow cytometry (49), and luminometry (33). Detailed experimental procedures are provided in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Reagents. Reagents were purchased from different companies as follows: 1,4-butendiol diacrylate, 5-amino-1-pentanol, 4-amino-1-butanol, and 1-(3-aminopropyl)-4-methylpiperazine (C4) from Alfa Aesar; 1,3-diaminopropane (C1) and 2,2-dimethyl-1,3-propanediamine (C2) from Sigma-Aldrich; N-(3-aminopropyl)pyrrolidine (C3) from Acros Organics; 2-(3-aminopropylamino) ethanol (C5) from Oakwood Chemical; N-(3-aminopropyl) diethanolamine (C6) and 1,11-diamino-3,6,9-trioxadecane (C7) from Tokyo Chemical Industry; and methoxy-polyethylene glycol (PEG)-succinimidyl succinate from JenKem Technology. Solvents were purchased as follows: ethyl ether anhydrous from Fisher Scientific; and dimethyl sulfoxide anhydrous (DMSO) and tetrahydrofuran (THF) from Sigma-Aldrich.

Polymer Synthesis. Following an in-depth literature search (1–7), we established a library of leading poly(β-amino ester) (PBAE) polymers previously shown to provide efficient in vitro gene transfer (Fig. S1). PBAE polymers were synthesized by a two-step Michael addition reaction, as previously reported (Fig. 1) (1–9). First, 1,4-butendiol diacrylate and 4-amino-1-butanol or 5-amino-1-pentanol were reacted at a 1:1.1 mole ratio at 90 °C for 24 h to synthesize high–molecular-weight (MW) uncapped P1 or P2 PBAE polymers (6.0–6.6 kDa), respectively. Polymers were then precipitated in cold ether three times to remove any unreacted species, dried under vacuum, and lyophilized. The molecular mass of 6.0–6.6 kDa was estimated using NMR, assuming two end acrylate groups per polymer. The number of repeating units was estimated to be 19–20 (Fig. 1A, peaks j–m). The MW was also characterized by gel permeation chromatography (GPC), yielding a number-averaged MW (Mn) of 6,050 and weight-averaged MW (Mw) of 9,900, with a polymer dispersity index (Dp) of 1.63 (Fig. S2A). For capping of the end acrylate groups, P1 or P2 PBAE polymers were dissolved in THF at 100 mg/mL and 30 molar equivalents of one of C1–C7 capping groups (Fig. S1) was added (to ensure end capping rather than further propagation). The reactions occurred while stirring at room temperature for 4–5 h. The end-capped PBAE polymers were retrieved by precipitation in cold ether to remove unreacted groups, dried under vacuum, and lyophilized. Purity and complete end capping were confirmed by the absence of the acrylate proton peaks (a–c) in the NMR spectrum (Fig. 1B).

For the synthesis of PEGylated PBAE polymers (PBAE-PEGs), a three-step reaction scheme was used. First, a P1 PBAE polymer possessing lower MW was synthesized by reacting 1,4-butendiol diacrylates and 4-amino-1-butanol at a 1.2:1 molar ratio. Based on NMR analysis, the molecular mass was estimated to be 3.8–4.2 kDa, assuming two end acrylate groups per polymer. The number of repeating units was estimated to be 13–14 (Fig. 1C, peaks j–m). The MW was also characterized by GPC, yielding a number-averaged Mn of 4,600 and Mw of 5,800 with a Dp of 1.25 (Fig. S2B). The low-MW P1 PBAE polymers were then capped with C1 as described earlier, and the end capping was confirmed by NMR (Fig. 1D). Subsequently, methoxy-PEG-succinimidyl succinates were reacted with the two terminal primary amine groups at both ends of the C1-capped PBAE polymer chain. To do this, we transferred the resulting P1C1 PBAE polymers and 2.05 molar equivalents of 5 kDa methoxy-PEG-succinimidyl succinate to a glass vial, vacuumed, and purged with nitrogen. The mixture of reactants was dissolved in THF and reacted while stirring at room temperature overnight. The final PBAE-PEG polymer product was precipitated and washed with cold ether three times, dried under vacuum, and lyophilized. PEG conjugation was confirmed with NMR (Fig. 1E, peak n). Of note, the lower-MW P1 PBAE polymer was used for the synthesis of PBAE-PEG (at a PEG-to-PBAE molar ratio of 2:1) to increase the PEG-to-PBAE weight-to-weight (wt/wt) ratio of the individual PBAE-PEG polymers and, thus, achieve a higher PEG content when particles are formulated at a fixed amount of PBAE polymer. All polymers were dissolved in DMSO at 100 mg/mL and stored at −20 °C for further use.

The P1C5 PBAE polymer was selected as a core polymer for the formulation of PBAE-based DNA nanoparticles (DNA-NPs) to be tested throughout the study. The rest of the PBAE polymers in the library were synthesized to show that our DNA-NP formulation strategy is highly generalizable, regardless of the chemical variations of the PBAE polymers. Mucus-penetrating PBAE/PEG nanoparticle (PBAE-MPP) formulations based on various core PBAE polymers can potentially be used to screen for enhanced gene transfer, including the potential for improved gene delivery to specific cell types, while retaining their mucus-penetrating property.

1H NMR Characterization. 1H NMR spectra of the intermediates and the final polymer products dissolved in deuterated methanol (MeOH-d4; Cambridge Isotope Laboratories) were recorded on a Bruker spectrometer (500 MHz). 1H chemical shifts are reported in ppm (δ) and the MeOH peak was used as an internal standard. Data were processed using MestRe-C software (Mestrelab Research) (Fig. 1).

Gel Permeation Chromatography. Polymer MW was analyzed by GPC using a Waters Breeze System and three Styragel columns (7.8 × 300 mm) in series: HR 1, HR 3, and HR 4 (Waters). A solution of 95% (vol/vol) THF/5% (vol/vol) DMSO/0.1 M piperidine was used as a mobile phase, and the samples eluted at a flow rate of 1 mL/min.

DNA-NP Formulation. The luciferase-expressing plasmid driven by a human β-actin promoter (pBAL) and the mCherry-expressing plasmid driven by a human β-actin promoter (pBACH) were produced by Copernicus Therapeutics, and a green fluorescent protein (GFP)-encoding plasmid driven by a cytomegalovirus (CMV) promoter (pEGFP) was purchased from Clontech Laboratories. A Mirus Label IT Tracker Intracellular Nucleic Acid Localization Kit (Mirus Bio) was used to fluorescently label plasmid DNA with a Cy3 fluorophore. PBAE-MPPs were formulated by the dropwise addition of five volumes of labeled or unlabeled plasmid DNA (0.1 mg/mL) to one volume of a swirling polymer solution at a PBAE-to-plasmid DNA wt/wt ratio of 60:1. Both solutions were preadjusted to pH 6.0 using a 0.1 M hydrochloric acid solution. The polymer solution consisted of a mixture of PBAE and PBAE-PEG at a wt/wt ratio of 2:3 based on PBAE mass. For comparison, leading DNA-NPs, including conventional non-PEGylated polyethyleneimine-based DNA-NPs (PEI-CPs) (10), conventionally PEGylated poly-l-lysine–based DNA-NPs (PLL-CPs) (10–12), and conventional non-PEGylated PBAE-based DNA-NPs (PBAE-CPS) (1, 3, 7, 13, 14), as well as densely PEGylated PEI-based mucus-penetrating particles (PEI-MPPs) (10), were formulated as previously described. DNA-NPs were washed with three volumes of ultrapure distilled water and reconstituted to 0.5–1 mg/mL using Amicon Ultra Centrifugal Filters [100,000 molecular-weight cutoff (MWCO); Millipore]. To calculate the DNA concentration, we included 5% fluorescently...
labeled DNA and measured fluorescence intensity using a Synergy Mx Multi-Mode Microplate Reader (BioTek Instruments).

We characterized the hydrodynamic diameter and polydispersity index (PDI) of DNA-NPs in water by dynamic light scattering (DLS), and the ζ-potential was measured in 10 mM NaCl at pH 7.0 by laser Doppler anemometry using a Zetasizer Nano ZS90 (Malvern Instruments). DNA-NPs were imaged using transmission electron microscopy (H7600; Hitachi High Technologies America) to determine their morphology and dimensions. Stability of DNA-NPs in ultrapure water was measured by DLS for 40 h every 15 min and following that every 24 h or until PDI >0.5. Similarly, stability of DNA-NPs in bronchoalveolar lavage fluid (BALF) at 37 °C was monitored every 30 min for 25 h or until PDI >0.5.

**Protein Binding Assay.** To determine the extent of protein adsorption onto the surface of DNA-NPs, we incubated DNA-NPs in a protein lysate solution of either mouse lungs or human cystic fibrosis (CF) mucus. Mucus samples were freshly collected from CF patients visiting the Johns Hopkins Adult Cystic Fibrosis Clinic via spontaneous expectoration, under informed consent on a protocol approved by the Johns Hopkins Medicine Institutional Review Board, as previously described (10, 15, 16). The protein lysate solutions were prepared by homogenizing whole lungs from mice or human CF mucus in 1 mM tissue protein extraction reagent (T-PER; Thermo Scientific) using a Bullet Blender Storm 24 homogenizer (Next Advance). Following a 30-min centrifugation at 1,000 × g at 4 °C, we quantified the protein concentrations in the supernatants using a Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce). DNA-NPs (plasmid concentration of 30 μg/mL) were incubated in a 200-μL protein lysate solution (protein concentration of 1.3 mg/mL) for 5 min, and the bound proteins were isolated via Amicon Ultra Centrifugal Filters (100,000 MWCO). The respective protein lysate solutions at the same protein concentration without DNA-NP incubation were used as a control. The amount of unbound protein was measured by Western blot-based quantification of a housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Cell Culture.** Human bronchial epithelial (BEAS-2B) cells (ATCC) were cultured in DMEM/F12 (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. When cells were 70% confluent, they were harvested and homogenized by incubating in 1 mg/mL collagenase A (Boehringer Ingelheim) for 30 min at 37 °C (17). The resulting homogenates were serially titrated using 10- and 40-μm cell strainers (Fisher Scientific). The isolated cells were pelleted, incubated in a cold red cell lysis buffer (Quality Biological), and washed with PBS. A 100-μL solution of 109 cells was fixed using 0.5% formaldehyde for 30 min and permeabilized using 1% Triton X-100 (Fisher Scientific) for 10 min. Each procedure was accompanied by extensive PBS washing. Subsequently, cells were incubated with 1 μg of Alexa Fluor 488-conjugated anti-GFP antibody (BioLegend) (18) and 1 μg of Cyanine 5.5-conjugated anti-CD45 antibody (Molecular Probes) (19) in 0.01% Triton X-100 for 30 min. The cells were washed twice and analyzed using an Accuri C6 cell analyzer (BD Biosciences). Data were analyzed using BD Accuri C6 software and FCS Express 5 Plus (De Novo Software).

**Animal Studies.** Female BALB/c mice, 6–8 wk old, were anesthetized with an i.p. injection of 2,2,2-tribromoethanol (Sigma-Aldrich) working solution prepared in normal saline. All animals were treated in accordance with the policies and guidelines of the Johns Hopkins University Animal Care and Use Committee.

To study the distribution of DNA-NPs and transgene expression in large airways and lung parenchyma, a 50-μL solution of DNA-NPs carrying Cy3-labeled plasmid DNA or pEGFP, respectively, at a 0.5 mg/mL plasmid DNA concentration was administered intratracheally using a microsprayer (MicroSprayer Aerosolizer Model IA-1C; Penn-Century) (n = 3 per group). Animals were killed 30 min or 48 h after the administration to determine the distribution of DNA-NPs or GFP transgene expression, respectively. Lungs were harvested, flash-frozen in OCT, and cryosectioned using a CM1950 cryostat (Leica Biosystems). To study the distribution of Cy3-labeled DNA-NPs, slides were stained with DAPI (Molecular Probes) and imaged using a confocal LSM 710 microscope (Carl Zeiss) under 20× magnification. For distribution of GFP expression, slides were immunologically stained using a GFP (B-2):sc-9996 monoclonal antibody (Santa Cruz Biotechnology) and Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes) as well as DAPI and imaged using a confocal LSM 710 microscope under 20× magnification. Untreated mice were used as controls to determine the microscope settings that did not introduce any background fluorescence. Image-based quantification of distribution as well as distribution variation of DNA-NPs in the large airways and lung parenchyma was performed using the histogram function of the image processing and analysis software ImageJ (NIH).

For flow cytometry analysis of in vivo gene transfer, we also treated mice with a 50-μL solution of DNA-NPs carrying pEGFP at a 0.5 mg/mL plasmid DNA concentration using a microsprayer (n = 3–5 per group). At 48 h postadministration, the lungs were harvested and homogenized by incubating in 1 mg/mL collagenase A (Boehringer Ingelheim) for 30 min at 37 °C (17). The resulting homogenates were serially titrated using 10- and 5-μL serological pipettes, followed by passage through 70- and 40-μm cell strainers (Fisher Scientific). The isolated cells were pelleted, incubated in a cold red cell lysis buffer (Quality Biological), and washed with PBS. A 100-μL solution of 106 cells was fixed using 0.5% formaldehyde for 30 min and permeabilized using 1% Triton X-100 (Fisher Scientific) for 10 min. Each procedure was accompanied by extensive PBS washing. Subsequently, cells were incubated with 1 μg of Alexa Fluor 488-conjugated anti-GFP antibody (BioLegend) (18) and 1 μg of Cyanine 5.5-conjugated anti-CD45 antibody (Molecular Probes) (19) in 0.01% Triton X-100 for 30 min. The cells were washed twice and analyzed using an Accuri C6 cell analyzer (BD Biosciences). Data were analyzed using BD Accuri C6 software and FCS Express 5 Plus (De Novo Software).
To assess the overall level of in vivo transgene expression, a 50-μL solution of DNA-NPs carrying pBACH at 0.5 mg/mL plasmid DNA concentration was administered intratracheally \((n = 5 \text{ per group})\) using a microsprayer. Animals were killed 1 wk after a single intratracheal administration, and luciferase activity on lung tissue homogenates was measured as previously described \((10)\) using a standard luciferase assay kit and a 20/20n luminometer. The RLU was normalized by total protein concentration measured by BCA protein assay. The effect of different storage conditions on in vivo gene transfer efficacy was assessed using the identical method \((n = 5 \text{ per group})\); PBAE-MPPs were administered following 24 h of storage in an aqueous solution at room temperature or following lyophilization and rehydration using 2% sucrose for cryopreservation and compared with freshly prepared PBAE-MPPs. To assess long-term transgene expression, luciferase activity was measured 1 wk, 1 mo, 2 mo, and 4 mo after administration of a single dose of PBAE-MPPs \((n = 5 \text{ per group})\). Finally, to assess the effect of repeated dosing on transfection efficacy, a multidose study was performed as previously described \((10)\). Briefly, mice were dosed once or twice with PBAE-MPPs carrying pBACH with a 2-wk interval between doses. Two weeks after the final pBACH dose, PBAE-MPPs carrying pBAL were administered. Luciferase expression was quantified 1 wk after the only or final administration. Groups I and II in Fig. 4E represent mice treated with a single dose of pBACH (negative control) or pBAL (positive control), respectively. Mice in groups III and IV were exposed to one or two doses of pBACH, respectively, and subsequently dosed with pBAL. The two plasmids are identical except for the reporter coding sequences.

To assess in vivo safety, mice were intratracheally treated with DNA-NPs at the identical dose used for in vivo gene transfer studies. Animals were killed 24 h postadministration; lungs were harvested and fixed in paraffin, or BALF was collected for total and differential cell counting. Slices were cut, subjected to hematoxylin and cosin staining, and scored for inflammation, as previously described \((20)\).

**Statistical Analysis.** Statistical significance was analyzed with a two-tailed Student’s \(t\) test assuming unequal variances. Multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by a post hoc test using SPSS 18.0 software.

Fig. S1. PBAE polymer library design. Uncapped PBAE polymers were synthesized by a Michael addition reaction of diacrylates (1,4-butanediol diacrylate) and amine alcohols (4-amino-1-butanol or 5-amino-1-pentanol) to yield P1 and P2. Subsequently, uncapped PBAE polymers were capped with one of the C1–C7 capping groups.

Fig. S2. Characterization of P1 PBAE polymer by GPC. Chromatogram of (A) representative high-MW P1 used for the synthesis of non-PEGylated PBAE polymers (Mn 6,050; Mw 9,900; Ð 1.63) and (B) representative low-MW P1 used for the synthesis of PBAE-PEG polymers (Mn 4,600; Mw 5,800; Ð 1.25).
Fig. S3. Adsorption of pulmonary proteins onto DNA-NPs. Western blot-based quantification of unbound protein levels following incubation of different DNA-NPs with (A) mouse lung lysate (n = 8) and (B) human CF mucus lysate (n = 4) at a protein concentration of 1.3 mg/mL. Data represent the mean ± SEM. Differences are statistically significant as indicated (*P < 0.05, **P < 0.01, ***P < 0.001).

Fig. S4. In vitro transfection of DNA-NPs. Luciferase activity secondary to in vitro transfection of human bronchial airway epithelial cells (BEAS-2B). Data represent the mean ± SEM. The asterisk denotes a statistically significant difference (P < 0.05); # denotes statistically significant differences (P < 0.05) compared with PLL-CPs and plasmid DNA control.
Fig. S5. Flow cytometric analysis of in vivo transgene expression in mouse lungs following intratracheal administration of PBAE-based DNA-NPs. (A) Gating of the total cell population. (B) FL1-H (GFP) signal of the total lung cell population in untreated control mice. (C) Composite representation of differentiation between nonimmune cells (CD45−) and immune cells (CD45+) in the mouse lung. (D–I) Composite representation of FL1-H (GFP) signal in (D and G) the total lung cell population, (E and H) nonimmune cells, and (F and I) immune cells following intratracheal administration of (D–F) PBAE-MPPs and (G–I) PBAE-CPs carrying GFP-expressing plasmid DNA (n = 3–5). FSC, forward scatter; SSC, side scatter.

Fig. S6. In vivo transgene expression in mouse lungs following intratracheal administration of different MPP formulations. Luciferase expression (n = 5–7) mediated by PEI-MPPs and PBAE-MPPs. Data represent the mean ± SEM. The asterisk denotes a statistically significant difference (P < 0.05).
Table S1. Physicochemical properties of PBAE-MPPs formulated with various core polymers from the PBAE polymer library

<table>
<thead>
<tr>
<th>PBAE polymer*</th>
<th>Hydrodynamic diameter ± SEM, nm†</th>
<th>PDI†</th>
<th>ζ-Potential ± SEM, mV‡</th>
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<tbody>
<tr>
<td>P1C1</td>
<td>50 ± 0.9</td>
<td>0.1</td>
<td>1.1 ± 0.6</td>
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<tr>
<td>P1C2</td>
<td>47 ± 0.6</td>
<td>0.2</td>
<td>1.9 ± 1.1</td>
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<td>P1C3</td>
<td>59 ± 1.0</td>
<td>0.1</td>
<td>−0.9 ± 0.3</td>
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<tr>
<td>P1C4</td>
<td>57 ± 4.0</td>
<td>0.1</td>
<td>2.3 ± 1.2</td>
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<tr>
<td>P1C5</td>
<td>51 ± 0.3</td>
<td>0.1</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>P1C6</td>
<td>58 ± 2.0</td>
<td>0.1</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>P1C7</td>
<td>57 ± 1.0</td>
<td>0.1</td>
<td>1.2 ± 1.9</td>
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<tr>
<td>P2C1</td>
<td>46 ± 1.9</td>
<td>0.1</td>
<td>−0.3 ± 0.6</td>
</tr>
<tr>
<td>P2C2</td>
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<td>0.3</td>
<td>14 ± 0.7</td>
</tr>
<tr>
<td>P2C3</td>
<td>57 ± 10.6</td>
<td>0.2</td>
<td>6.9 ± 0.6</td>
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<tr>
<td>P2C4</td>
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<tr>
<td>P2C5</td>
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<tr>
<td>P2C6</td>
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<td>0.3</td>
<td>−0.7 ± 10.2</td>
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<tr>
<td>P2C7</td>
<td>44 ± 0.3</td>
<td>0.1</td>
<td>0.3 ± 0.4</td>
</tr>
</tbody>
</table>

*PBAE core polymers from the PBAE polymer library (Fig. S1).
†Hydrodynamic diameter and PDI were measured by dynamic light scattering in water (pH 7.0). Data represent the mean ± SEM (n ≥ 3).
‡ζ-Potential was measured by laser Doppler anemometry in 10 mM NaCl (pH 7.0). Data represent the mean ± SEM (n ≥ 3).

Table S2. Physicochemical properties of PEI-CPs and PLL-CPs

<table>
<thead>
<tr>
<th>DNA-NP type</th>
<th>Hydrodynamic diameter ± SEM, nm*</th>
<th>PDI*</th>
<th>ζ-Potential ± SEM, mV†</th>
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</thead>
<tbody>
<tr>
<td>PEI-CPs</td>
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<td>27 ± 1.4</td>
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<tr>
<td>PLL-CPs</td>
<td>47 ± 3.4</td>
<td>0.3</td>
<td>0.9 ± 1.6</td>
</tr>
</tbody>
</table>

*Hydrodynamic diameter and PDI were measured by dynamic light scattering in water (pH 7.0). Data represent the mean ± SEM (n ≥ 3).
†ζ-Potential was measured by laser Doppler anemometry in 10 mM NaCl (pH 7.0). Data represent the mean ± SEM (n ≥ 3).