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 nanoporous 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 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 nanoporous 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 glycans 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 nonadhesive 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.

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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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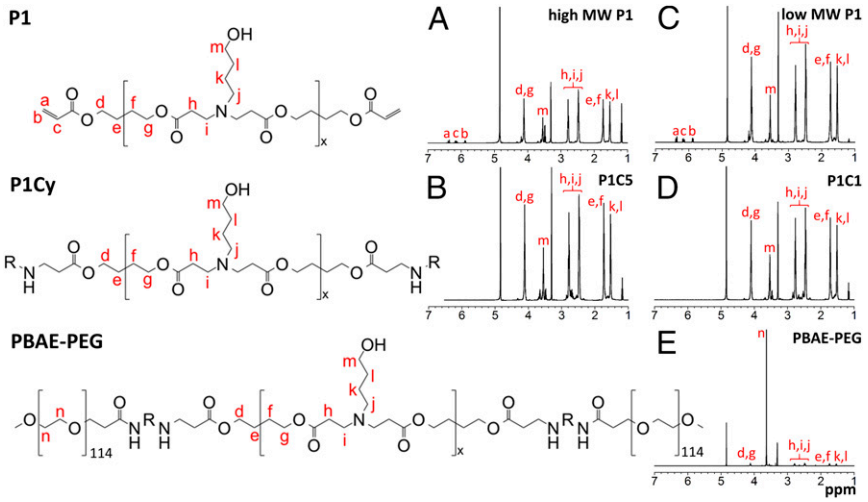

Fig. 1. Structures and ^{1}H NMR spectra of respective PBAE polymers and intermediates. NMR spectra of (*A*) high-MW (6.0–6.5 kDa) P1 PBAE polymer synthesized by a Michael addition reaction of 1,4-butanediol diacrylate and 4-amino-1-butanol (P1; x, 19–20), (*B*) high-MW P1 PBAE capped by 2-(3-aminopropylamino)ethanol (C5) (P1C5), (*C*) low-MW (3.8–4.2 kDa) P1 PBAE (x, 13–14), (*D*) low-MW P1 PBAE capped by 1,3-diaminopropane (C1) (P1C1), and (*E*) PEGylated P1C1 PBAE (PBAE-PEG) at a 2:1 PEG-to-PBAE molar ratio. End capping is confirmed by the loss of diacrylate peaks (a–c).

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) serotypes 1, 2, and 5 (23, 25), and a clinically tested polymeric nonviral gene vector ($CK_{30}PEG_{10k}$ DNA-NP) (26, 27) are unable to efficiently penetrate the mucus gel layer, thereby limiting their success in clinical trials (20, 22, 28). In addition, conventional gene vectors formulated with cationic polymers, such as polyethylenimine (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 the airway epithelium in vivo, inhaled gene vectors must be small enough to diffuse through the mucus mesh while possessing a muco-inert surface to avoid adhesion to mucus constituents (20, 22, 31–33). We have previously demonstrated that highly dense surface coatings of hydrophilic and neutrally charged polyethylene glycol (PEG) render therapeutic NPs resistant to mucoadhesion, thereby enabling rapid diffusion in airway mucus (20, 22, 31–33). Of note, conventional (lower-density) PEG surface coatings do not provide muco-inert surfaces, resulting in immobilization of particles in CF mucus (26, 27, 33).

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. 1*A*) with the end diacrylate groups capped by 2-(3-aminopropylamino ethanol) (C5) (Fig. 1*B*); 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. 1*C*) 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. 2*A* 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:PBAE-PEG) compacted plasmid DNA more tightly (~50 nm in diameter) and provided a near-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. 2*B*). We also found that PBAE-MPPs retained their physicochemical properties in bronchoalveolar lavage fluid (BALF) at 37 °C for 24 h with only a minor increase in particle diameter, whereas PBAE-CPs rapidly aggregated (Fig. 2*C*). 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 $CK_{30}PEG_{10k}$ DNA-NP (39)]. The physicochemical properties of PEI-CPs and PLL-CPs 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. S3*A*). Likewise, PBAE-MPPs exhibited significantly improved resistance against the adsorption of proteins present in human CF mucus compared with all other DNA-NP formulations (Fig. S3*B*). This is in accordance with the excellent colloidal stability of PBAE-MPPs in mouse BALF, which was not achieved with PBAE-CPs (Fig. 2*C*). 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).

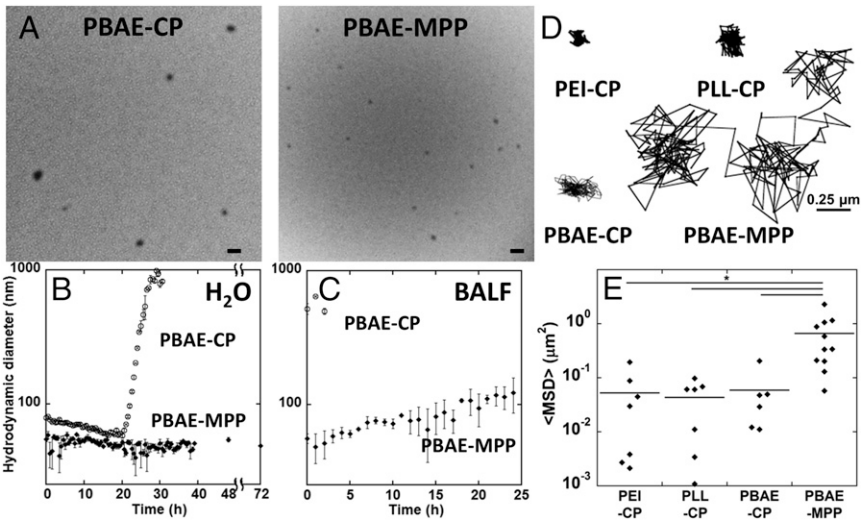

Fig. 2. In vitro and ex vivo characterization of DNA-NPs. (*A*) Transmission electron micrographs of PBAE-based DNA-NPs. (Scale bars, 200 nm.) (*B* and *C*) Hydrodynamic diameter of PBAE-based DNA-NPs over time in (*B*) H_2O at room temperature and (*C*) BALF at 37 °C. (*D*) Representative trajectories of various DNA-NPs in freshly expectorated CF mucus. (*E*) Dot plot of the ensemble-averaged geometric means of MSD (<MSD>) of DNA-NPs at a timescale of 1 s in individual mucus samples. Data represent the mean ± SEM. The asterisk denotes a statistically significant difference ($P < 0.05$).

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 with 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. 2*D*), 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. 2*D*) and diffused significantly faster ($P < 0.05$) than the conventional DNA-NPs (Fig. 2*E*). 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.

To test whether the enhanced colloidal stability and mucus-penetrating property of PBAE-MPPs provide improved particle distribution in the mouse lung in vivo, we intratracheally dosed Balb/C mice with Cy3-labeled DNA-NPs of various types via a microsprayer. PBAE-MPPs exhibited widespread distribution throughout the lung airways, whereas other conventional DNA-NPs (PBAE-CPs, PEI-CPs, and PLL-CPs) were all sparsely distributed (Fig. 3*A*). The airway coverage of PBAE-MPPs was ~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. 3 *B* and *C*). Similar results were observed in the lung parenchyma (Fig. 3 *D*–*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. 4*A*). 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 nonimmune 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

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

DNA-NP type	Storage*	Hydrodynamic diameter ± SEM, nm†	PDI†	ζ-Potential ± SEM, mV‡
PBAE-CPs	Fresh	84 ± 2.8	0.1	31.0 ± 1.3
	24 h at RT	262 ± 3.6	0.2	0.7 ± 1.2
	Lyophilized	178 ± 9.4	0.4	0.6 ± 0.6
PBAE-MPPs	Fresh	50 ± 1.1	0.1	0.5 ± 0.2
	24 h at RT	54 ± 1.9	0.1	1.6 ± 0.1
	Lyophilized	73 ± 2.3	0.2	2 ± 0.1

*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 ($n \geq 3$).

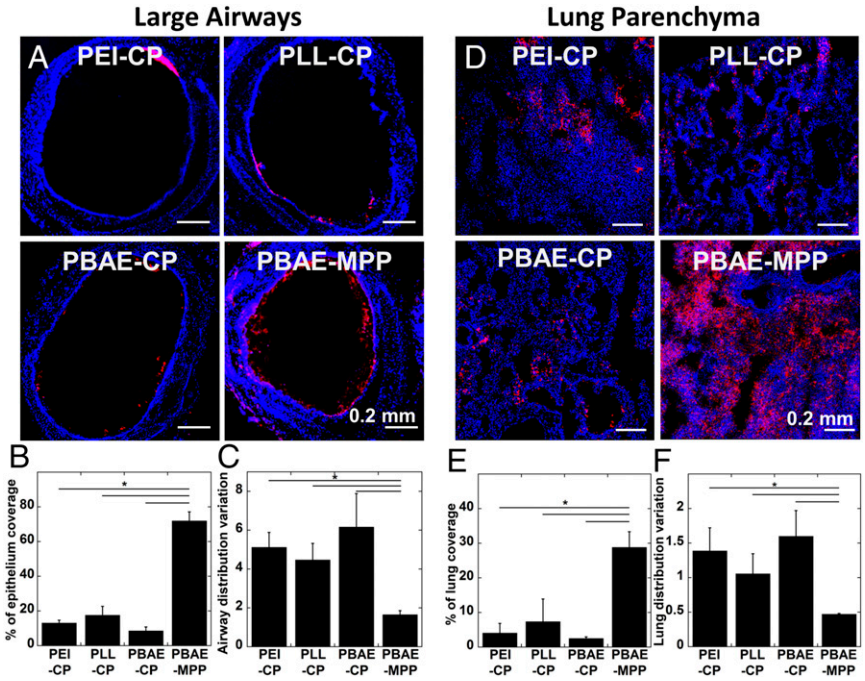

Fig. 3. In vivo distribution of DNA-NPs following intratracheal administration. (*A*) Representative images of DNA-NP distribution in large airways following intratracheal administration of the respective DNA-NPs (red). Cell nuclei are stained with DAPI (blue). (*B* and *C*) Image-based quantification of (*B*) coverage and (*C*) distribution variation of DNA-NPs in large airways. (*D*) Representative images of DNA-NP distribution in the lung parenchyma. (*E* and *F*) Image-based quantification of (*E*) coverage and (*F*) distribution variation of DNA-NPs in the lung parenchyma. Tissues were harvested 30 min after intratracheal administration of DNA-NPs. Data represent the mean ± SEM. The asterisks denote a statistically significant difference ($P < 0.05$).

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. 4*B*). 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. 4*B*). 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 efficacies 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

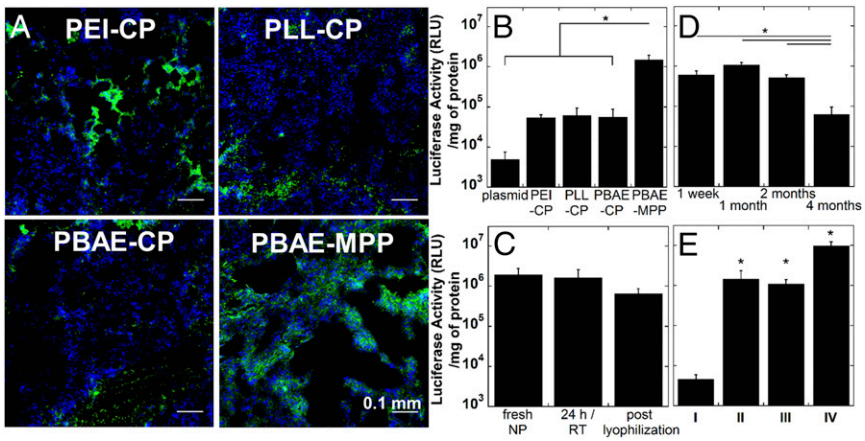

Fig. 4. In vivo transgene expression in mouse lungs following intratracheal administration of DNA-NPs. (*A*) Representative images of GFP expression (green) ($n = 3$). Cell nuclei are stained with DAPI (blue). (*B–D*) Luciferase expression ($n = 5$) mediated by (*B*) various DNA-NPs 1 wk after a single administration, (*C*) PBAE-MPPs stored at different conditions, and (*D*) PBAE-MPPs at different time points after a single administration. RT, room temperature. (*E*) Effect of repeated administrations on the transfection efficiency of PBAE-MPPs ($n = 4–5$). Mice were dosed once or twice with PBAE-MPPs carrying pBACH with a 2-wk interval and, 2 wk after the final pBACH dose, PBAE-MPPs carrying pBAL were administered. Luciferase expression was quantified 1 wk after the final administration. Groups I and II represent mice treated with a single dose of pBACH (negative control) and pBAL (positive control), respectively. Mice in groups III and IV were exposed to a single dose or double dose of pBACH, respectively, and subsequently dosed with pBAL. The two plasmids are identical except for the reporter coding sequences. Data represent the mean ± SEM. The asterisks denote a statistically significant difference from group I ($P < 0.05$).

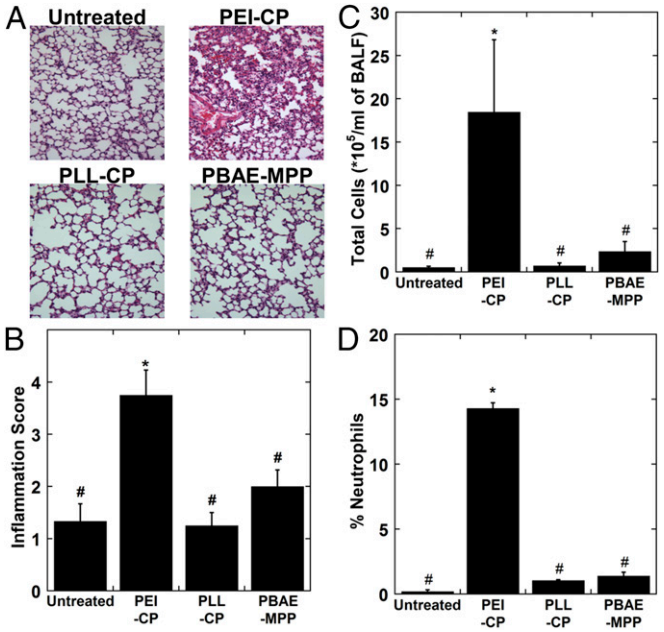

Fig. 5. In vivo safety profile of DNA-NPs. (*A*) Representative images of lung parenchyma 24 h after administration of DNA-NPs. (*B*) Histopathological scoring of lung inflammation. (*C* and *D*) Total cell counts (*C*) and % neutrophils (*D*) in bronchoalveolar lavage fluid following a single administration of DNA-NPs. Data represent the mean ± SD (n = 3–5). The differences are statistically significant ($P < 0.05$) compared with untreated control (*) or mice dosed with PEI-CPs (#).

greater transgene expression in the mouse lung compared with PEI-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 mediated 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. 4*C*), 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. 4*D*). 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. 4*B*). 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 problem (64). Here, we found that multiple administrations did not reduce the transfection efficiency of PBAE-MPPs (Fig. 4*E*).

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 PEI-CPs 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) (39). PEI-CPs induced a significant increase in total cell counts and percentage of neutrophils in BALF. 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 colloidal 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 unlabeled 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 MSD 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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