Mucoadhesive particles (MAP) have been widely explored for pulmonary drug delivery because of their perceived benefits in improving particle residence in the lungs. However, retention of particles adhesively trapped in airway mucus may be limited by physiologic mucus clearance mechanisms. In contrast, particles that avoid mucoadhesion and have diameters smaller than mucus mesh spacings rapidly penetrate mucus layers (mucus-penetrating particles [MPP]), which we hypothesized would provide prolonged lung retention compared to MAP. We compared in vivo behaviors of variously sized, polystyrene-based MAP and MPP in the lungs following inhalation. MAP, regardless of particle size, were aggregated and poorly distributed throughout the airways, leading to rapid clearance from the lungs. Conversely, MPP as large as 300 nm exhibited uniform distribution and markedly enhanced retention compared to size-matched MAP. On the basis of these findings, we formulated biodegradable MPP (b-MPP) with an average diameter of <300 nm and examined their behavior following inhalation relative to similarly sized biodegradable MAP (b-MAP). Although b-MPP diffused rapidly through human airway mucus ex vivo, b-MAP did not. Rapid b-MPP movements in mucus ex vivo correlated to a more uniform distribution within the airways and enhanced lung retention time as compared to b-MAP. Furthermore, inhalation of b-MPP loaded with dexamethasone sodium phosphate (DP) significantly reduced inflammation in a mouse model of acute lung inflammation compared to both carrier-free DP and DP-loaded MAP. These studies provide a careful head-to-head comparison of MAP versus MPP following inhalation and challenge a long-standing dogma that favored the use of MAP for pulmonary drug delivery.

INTRODUCTION

Inhalation of drug-loaded nanoparticles (NP) is a promising approach for the treatment of diseases that affect the lungs, including asthma, chronic obstructive pulmonary disease, cystic fibrosis (CF), and lung cancer (1–4). Biodegradable NP may be delivered into the lungs via nebulization or as a dry powder to achieve high airway deposition, where they may then provide (i) protection of drug payloads against enzymatic and/or hydrolytic degradation or inactivation (5–7), (ii) controlled release of drugs over prolonged periods of time locally (8, 9), (iii) reduced local and systemic side effects by lowering total dose, dose frequency, and systemic exposure (10, 11), and (iv) potential to overcome a variety of extracellular and intracellular barriers due to tailorability and geometric size (12–16).

In an attempt to enhance drug residence time in the lungs following inhalation, drugs are typically packaged into biodegradable NP that adhere to mucus, namely, mucoadhesive particles (MAP) (17–20). MAP have been typically engineered to possess cationic (21, 22) or thiolated (23, 24) surfaces that interact with negatively charged glycosylated or cysteine-rich hydrophobic domains of airway mucus fibers, respectively. However, those highly cationic or thiol-decorated NP have been formulated with hydrophobic core polymers (21, 24), and thus, the mucoadhesion may have been attributed to hydrophobic interactions as well (25, 26). The dogma is that MAP may be retained for longer duration in the lungs by decreasing mucociliary clearance (MCC) rates (27), through rheological changes of mucus via multivalent mucus-particle interactions (28–31). However, by definition, MAP adhere to the most superficial mucus gel layer and, thus, are not expected to reach the underlying periciliary layer (PCL) that is cleared less rapidly (32). Moreover, we hypothesized that MAP would not spread uniformly within the mucus gel layer or PCL, potentially leading to uneven delivery of inhaled drugs within the airways that may reduce drug efficacy.

We and others have demonstrated that airway mucus forms an adhesive and steric barrier to conventional NP (that is, MAP) regardless of particle diameter (25, 33–36). Using multiple-particle tracking (MPT), we found that particles as large as 200 nm can rapidly penetrate freshly collected human respiratory mucus, obtained from healthy donors (26) and CF patients (25) ex vivo, but only when the NP surface was passivated with dense coatings of poly(ethylene glycol) [PEG; that is, mucus-penetrating particles (MPP)]. However, a recent publication argued that although MPP are diffusive over short distances and time scales in airway mucus (as confirmed via MPT ex vivo), transport across the mucus gel layer in the airways in vivo, with a thickness of up to a few tens of micrometers, is not feasible (37). Thus, we sought to directly compare the in vivo behavior of MPP to MAP of various sizes...
following inhalation. Additionally, we compared the efficacy of a corticosteroid, dexamethasone sodium phosphate (DP), delivered either via a biodegradable MPP formulation (b-MPP), a biodegradable MAP formulation (b-MAP), or the carrier-free soluble drug in a mouse model of lipopolysaccharide (LPS)–induced acute lung inflammation.

RESULTS
Characterization of NP
To test whether the more rapid diffusion of MPP compared to MAP in mucus ex vivo would correlate to a more uniform airway distribution and/or a prolonged airway retention, we first produced model polystyrene (PS) NP with dense surface coatings of PEG (PS-PEG MPP) by conjugating 5-kDa PEG chains to carboxyl groups on the particle surfaces, as previously described (34, 38, 39). Because the average mucus mesh spacing for mouse respiratory mucus is unknown, we investigated a broad range of NP sizes (60 to 1000 nm in diameter). In agreement with our previous findings (34), dense PEG coverage of NP resulted in marginal increases in particle diameters and significantly increased (to near neutral) regardless of particle size (Table 1). In comparison, PS NP without PEG coatings (PS MAP) exhibited negative ζ-potentials, ranging from −34 to −83 mV, reflecting the presence of unconjugated carboxyl groups on the NP surface.

We also synthesized b-MAP and b-MPP in a size range of 100 to 200 nm, because well-PEGylated NP with particle diameters <200 nm are known to penetrate human respiratory mucus and CF sputum (25, 26). First, we fabricated b-MPP using a diblock copolymer composed of poly(lactic-glycolic acid) (PLGA) and PEG (PLGA-PEG), namely, PLGA-PEG MPP, for mechanistic in vivo studies of lung distribution and retention. Uncoated PLGA NP were used as a MAP control (PLGA MAP). Both formulations exhibited hydrodynamic diameters of 130 to 140 nm, but whereas the average ζ-potential of PLGA MAP was strongly negative (−73 mV), that of PLGA-PEG MPP was near neutral (−6 mV) due to the dense PEO coating (Table 2). Second, we synthesized b-MPP from all generally-regarded-as-safe (GRAS) components, using PLGA as a core and Pluronic F127 (PLGA/F127 MPP) to provide a noncovalent muco-inert surface coating. Pluronics are a class of triblock copolymers composed of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO-PPO-PEO), where PPO adsorbs onto hydrophobic surfaces, whereas PEO (also known as PEG) imparts nonadhesive surface coatings (40). We have previously shown that dense F127 coatings lead to rapid penetration of PLGA/F127 MPP through human cervicovaginal mucus (41). Pluronic F68 coating was used as a MAP control (PLGA/F68 MAP), because it does not provide sufficient shielding of the surface to prevent mucus-NP adhesive interactions (41). The hydrodynamic diameters of PLGA/F127 MPP and PLGA/F68 MAP were comparable (180 to 200 nm) (Table 2). As a control, we formulated another uncoated, PLGA NP free of Pluronics (PLGA/PF MAP) having a similar diameter (190 nm). Similar to PLGA-PEG MPP, the ζ-potential of PLGA/F127 MPP was near neutral (~8 mV). In contrast, ζ-potentials of PLGA/F68 MAP and PLGA/PF MAP were −23 and −64 mV, respectively, underscoring insufficient and absent surface shielding.

It is imperative that NP retain their physicochemical properties under relevant physiological conditions. We thus investigated whether dense PEG coatings on NP promote their colloidal stability in mouse bronchoalveolar lavage fluid (BALF) (Fig. 1, A and B). Unlike uncoated PLGA/PF MAP, particle diameters and polydispersity indices were well preserved for both PLGA/F127 MPP and PLGA/F68 MAP following 24 hours of incubation in BALF. We next assessed the in vivo safety of these GRAS NP following inhalation on the basis of histological analysis (fig. S1) and immune cell profiling in BALF (fig. S2). Whereas lungs of mice dosed with PLGA/F127 MPP were virtually indistinguishable compared to saline-treated controls, mice treated with either PLGA/PF MAP or PLGA/F68 MAP displayed signs of acute inflammation

We also formulated PLGA/F127 MPP and PLGA/F68 MAP loaded with a corticosteroid. To increase the drug loading level, we adapted a previously reported method of coordinate complexation of the water-soluble corticosteroid DP with zinc, followed by encapsulation into NP (42, 43). The physicochemical properties of DP loaded into PLGA/F127 MPP (DP/PLGA/F127 MPP) and PLGA/F68 MAP (DP/PLGA/F68 MAP) are shown in Table 2. DP/PLGA/F127 MPP exhibited near-neutral ζ-potential (−4 mV), whereas DP/PLGA/F68 MAP possessed a highly negative surface charge (−23 mV). Morphological examination via transmission electron microscopy revealed spherical NP geometry with no evidence of drug precipitation (Fig. 1C). Both DP/PLGA/F127 MPP and DP/PLGA/F68 MAP exhibited DP loading levels of ~8.5% w/w and sustained in vitro DP release over 10 days (Fig. 1D).

### Table 1. Physicochemical characterization of PS NP

<table>
<thead>
<tr>
<th>Nominal size (nm)</th>
<th>Particle type</th>
<th>Diameter (nm)</th>
<th>PDI</th>
<th>ζ-Potential (mV)</th>
<th>Mucus penetration</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>60-nm PS</td>
<td>58 ± 1</td>
<td>0.12</td>
<td>−34 ± 2</td>
<td>X</td>
</tr>
<tr>
<td>60</td>
<td>60-nm PS-PEG</td>
<td>66 ± 3</td>
<td>0.18</td>
<td>−12 ± 1</td>
<td>O</td>
</tr>
<tr>
<td>100</td>
<td>100-nm PS</td>
<td>90 ± 5</td>
<td>0.06</td>
<td>−44 ± 3</td>
<td>X</td>
</tr>
<tr>
<td>100</td>
<td>100-nm PS-PEG</td>
<td>107 ± 3</td>
<td>0.03</td>
<td>−5 ± 1</td>
<td>O</td>
</tr>
<tr>
<td>300</td>
<td>300-nm PS</td>
<td>282 ± 2</td>
<td>0.04</td>
<td>−50 ± 2</td>
<td>X</td>
</tr>
<tr>
<td>300</td>
<td>300-nm PS-PEG</td>
<td>307 ± 9</td>
<td>0.03</td>
<td>−3 ± 1</td>
<td>O</td>
</tr>
<tr>
<td>1000</td>
<td>1000-nm PS</td>
<td>1025 ± 13</td>
<td>0.13</td>
<td>−83 ± 4</td>
<td>X</td>
</tr>
<tr>
<td>1000</td>
<td>1000-nm PS-PEG</td>
<td>1045 ± 20</td>
<td>0.11</td>
<td>−5 ± 1</td>
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</tbody>
</table>
Ex vivo diffusion of biodegradable MPP and MAP in human CF sputum

To ensure that the b-MPP formulations used in this study, specifically PLGA-PEG MPP and PLGA/F127 MPP, efficiently penetrated human mucus ex vivo, their transport rates were measured in CF sputum freshly expectorated by CF patients. The greater diffusion of PLGA-PEG MPP and PLGA/F127 MPP compared to PLGA MAP and PLGA/F68 MAP in airway mucus was first qualitatively confirmed (movies S1 to S4) and then quantified using MPT. PLGA MAP and PLGA/F68 MAP both exhibited confined particle trajectories, whereas PLGA-PEG MPP and PLGA/F127 MPP exhibited Brownian-like particle trajectories (Fig. 2A). The median mean-squared displacement (MSD) of particles at a time scale of 1 s was nearly 800-fold greater for PLGA-PEG MPP compared to PLGA MAP and 25-fold greater for PLGA/F127 MPP compared to PLGA/F68 MAP (Fig. 2B).

We have previously shown via MPT that PS-PEG MPP with diameters as large as 200 nm are capable of diffusing rapidly in human airway mucus, whereas 500-nm PS NP are unable to do so regardless of dense surface PEG coatings, suggesting that they are too large to fit through the mucus pores (4, 7, 25, 26). However, we had not investigated the diffusion behaviors of PS NP having an intermediate diameter of 300 nm. Thus, we have confirmed here that 300-nm PS-PEG MPP exhibit significantly greater median MSD compared to non-PEGylated counterparts (that is, 300 PS MAP) in CF sputum (fig. S3).

Lung distribution and retention of variously sized polystyrene MPP and MAP

To examine whether rapid mucus penetration over short time scales ex vivo correlates with particle transport across thick mucus layers in vivo, we investigated the distribution of variously sized PS MAP and PS-PEG MPP in the mouse conducting airways following intranasal administration. We found that PS-PEG MPP with diameters of 60, 100, and 300 nm uniformly distributed throughout the mucus layer in the airways, showing little to no aggregation and penetration into folds found in the airway epithelium (Fig. 3, A to C). In contrast, similarly sized PS MAP were usually found in large, clumped aggregates positioned away from the epithelium (Fig. 3, A to C). The findings correlated well with the diffusion behavior of NP on the lumen of freshly

Table 2. Physicochemical characterization of biodegradable NP.

Hydrodynamic diameter and PDI were measured by DLS. ζ-potential was measured at pH 7.4 in 10 mM NaCl. Mucus penetration indicates the ability of the NP to penetrate CF sputum, as determined by MPT in Fig. 2.

<table>
<thead>
<tr>
<th>Particle type</th>
<th>Diameter (nm)</th>
<th>PDI</th>
<th>ζ-Potential (mV)</th>
<th>Mucus penetration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>141 ± 15</td>
<td>0.14</td>
<td>−73 ± 4</td>
<td>X</td>
</tr>
<tr>
<td>PLGA-PEG</td>
<td>128 ± 6</td>
<td>0.09</td>
<td>−6 ± 1</td>
<td>O</td>
</tr>
<tr>
<td>PLGA/PF</td>
<td>190 ± 7</td>
<td>0.09</td>
<td>−64 ± 2</td>
<td>X</td>
</tr>
<tr>
<td>PLGA/F68</td>
<td>180 ± 17</td>
<td>0.09</td>
<td>−23 ± 4</td>
<td>X</td>
</tr>
<tr>
<td>PLGA/F127</td>
<td>200 ± 8</td>
<td>0.14</td>
<td>−8 ± 2</td>
<td>O</td>
</tr>
<tr>
<td>DP/PLGA/F68</td>
<td>150 ± 10</td>
<td>0.08</td>
<td>−23 ± 1</td>
<td>O</td>
</tr>
<tr>
<td>DP/PLGA/F127</td>
<td>260 ± 21</td>
<td>0.10</td>
<td>−4 ± 2</td>
<td>O</td>
</tr>
</tbody>
</table>

Fig. 1. Characterization of GRAS-based biodegradable NP. Ex vivo stability of NP in BALF at room temperature for 2 or 24 hours, as measured by (A) hydrodynamic diameter and (B) PDI. (C) Transmission electron micrographs of DP/PLGA/F127 and PLGA/F68 NP. Scale bars, 100 nm. (D) Release kinetics of DP from DP/PLGA/F127 and DP/PLGA/F68 NP. Data represent means ± SD. *P < 0.05, **P < 0.01.
excised mouse tracheas (movies S5 and S6), where PS-PEG MPP 60 to 300 nm in diameter diffused rapidly in mouse tracheal mucus, whereas the movement of PS MAP of all sizes was strongly hindered or immobile. In contrast to smaller NP (≤300 nm), there was no difference in the airway distributions of 1000-nm PS MAP and PS-PEG MPP (Fig. 3D); 1000-nm MAP and MPP were both found poorly distributed and away from the epithelial surface, similar to the observations with smaller PS MAP, suggesting that 1000-nm particles are too large to fit through the mucus mesh in the airways regardless of surface coating.

Next, we measured the time course retention of MPP versus MAP in the lumen of mouse lungs by quantifying the amount of NP in the BALF. The retention of 60-nm PS-PEG MPP was enhanced compared to that of 60-nm PS MAP at each time point studied (Fig. 3E). After 2 hours, more than 85% of the initially deposited 60-nm PS-PEG MPP were retained in the lung lumen, whereas only ~45% of PS MAP remained (P < 0.01). Similarly, 100- and 300-nm PS-PEG MPP showed enhanced retention compared to similarly sized PS MAP at each time point studied. At least 70% of 100- and 300-nm PS-PEG MPP were retained at 2 hours after administration, but only ~30% of 100- and 300-nm PS MAP remained in the lung at the same time point (P < 0.01; Fig. 3, F and G). In contrast, the retention of 1000-nm PS MAP and PS-PEG MPP was not statistically different at any time point studied (Fig. 3H), with 2-hour retention values of ~45 and ~50%, respectively.

**Lung distribution and retention of biodegradable MPP and MAP**

On the basis of the findings with model PS NP, we investigated the distribution of b-MPP and b-MAP in the mouse conducting airways.
following inhaled administration. Similar to the model PS-PEG MPP as large as 300 nm (Fig. 3, A to C), PLGA-PEG MPP and PLGA/F127 MPP each displayed uniform distribution throughout the bronchial surfaces in close proximity to the airway epithelium (Fig. 4, A and B). In contrast, μ-MAP formulations (that is, PLGA MAP and PLGA/F68 MAP) were again found in large clumps away from the airway epithelium (Fig. 4, A and B). We observed that PLGA-PEG MPP penetrated deep (approximately 200 μm from the epithelial surface) into a submucosal gland found in the mouse trachea (Fig. 4, C and D, yellow arrow). PLGA MAP were exclusively located in large aggregates in the luminal mucus layer (LML) of the trachea (Fig. 4C, white arrow), similar to that found with MAP in the bronchi.

We next studied the retention of PLGA-PEG MPP and PLGA MAP via the same lavage method used for the model NP study. PLGA-PEG MPP were retained longer in the lung lumen compared to PLGA MAP at each time point studied (Fig. 4E). PLGA-PEG MPP showed a similar behavior to PS-PEG MPP of similar size, with more than 80% retained in the lung at 2 hours after administration. PLGA MAP were rapidly cleared from the lung, similar to PS MAP having similar particle diameter, with only ~25% retained in the lung after 2 hours (P < 0.01). To measure the retention of PLGA-PEG MPP and PLGA MAP in the entire lung (including NP that cannot be collected via lavage), we measured the fluorescence of NP retained in excised whole-lung tissues at various time points after inhalation using an in vivo imaging system (Fig. 4F). The retention of PLGA-PEG MPP was significantly greater at each time point up to 24 hours compared to that of PLGA MAP. At 6 hours, approximately 75% of PLGA-PEG MPP were retained, whereas only ~50% of PLGA MAP were retained at the same time point (P < 0.01).

**In vivo efficacy comparison of DP-loaded biodegradable MPP and MAP**

To determine whether the improved particle uniformity and enhanced retention of μ-MPP yield improved efficacy of DP in a model of acute lung inflammation, we intranasally dosed LPS-challenged mice with DP/PLGA/F127 MPP, DP/PLGA/F68 MAP, or carrier-free soluble DP (Fig. 5). At a dose of 1 mg/kg, soluble DP had little anti-inflammatory effect (Fig. 5, A and B). In contrast, treatment with the identical DP dose of DP/PLGA/F127 MPP significantly reduced the recruitment of inflammatory cells to the lung, as evidenced by the decrease in total BALF cell count compared to treatment with soluble DP or saline (P < 0.01) (Fig. 5A). Treatment with DP/PLGA/F68 MAP also moderately reduced the inflammatory cell infiltration compared to saline-treated controls (P < 0.05); however, the effect was not statistically significant compared to soluble DP. In addition, only the DP/PLGA/F127 MPP provided significant reduction of a proinflammatory cytokine, tumor necrosis factor-α (TNF-α) (P < 0.01) (Fig. 5B).

**DISCUSSION**

MAP have been widely used to improve drug delivery to the lung airways (17–21), including demonstration of improved sustained drug levels in the lungs compared to carrier-free drug formulations (17, 18, 20). However, we found that inhaled NP capable of efficiently penetrating human airway mucus ex vivo (that is, MPP ≤300 nm in diameter) exhibited greatly improved particle distribution and retention in the lungs in vivo compared to MAP of similar size, and, further, that a drug loaded into MPP was more effective than the same drug loaded into MAP. The improved retention of these MPP is most likely attributed to their ability to overcome MCC by rapidly diffusing deep into the mucus layer, as evidenced by the presence of MPP in epithelial folds as well as a submucosal gland. MPP exhibited enhanced retention in the lung airways compared to MAP, suggesting that a direct correlation
exists between mucus penetration and improved retention. The rapid clearance of MAP from the lung in the retention experiments reported here agrees well with the time scale reported for MCC (44, 45) and was faster than would be expected for clearance mediated via macrophages [24 to 48 hours for airway macrophages and weeks to months for alveolar macrophages (46)]. Notably, we have demonstrated similar beneficial effects of MMP in the vagina (47, 48) and gastrointestinal tract (49) in vivo. These findings altogether suggest that MMP are a promising alternative to mucoadhesive formulations for therapeutic delivery to the lung airways.

A recent study suggested that MMP, despite the rapid diffusion over short time and length scales, are not able to penetrate physiologically thick respiratory mucus layers (~10 to 100 μm) (37). They found that PEG-coated magnetic dextran/iron oxide NP were unable to penetrate horse respiratory mucus under the constant force of a magnet. These results are in stark contrast to the wide distribution of MMP observed in the present study, where MMP were well distributed throughout the conducting airways, which may result in larger areas with a more permeable PCL. Such large permeable areas might account the presence of nonciliated cells, including secretory cells, which are often found in the airway epithelium (67). The presence of these cells may create permeable gaps in PCL where secreted mucins diffuse out to reach the LML. There are fewer ciliated cells in the lower conducting airways, which may result in larger areas with a more permeable PCL.

directional magnetic force, such that NP that entered into small mucus pockets would be unlikely to escape. Under physiological conditions, muco-inert NP would be able to diffuse back and forth in the pores, escape from them, and continue their journey in the mucus.

The clinical implications of this work are significant, because improved lung distribution and retention achieved by MMP may enhance the efficacy of other drugs that could treat a large number of lung diseases. Mucus penetration of NP will also enhance the probability that therapeutic NP may reach the underlying epithelial cells protected by the mucus layer. Inhaled corticosteroids suffer from poor lung pharmacokinetics and short duration of action because of their rapid clearance by MCC as well as absorption into the blood (57). Repeated dosing is often required to maintain the local drug concentration in the therapeutic window, which may transiently expose patients to elevated local and/or systemic drug concentrations, leading to toxicity/side effects. Delivery of corticosteroids in MMP formulations may resolve these shortcomings by providing long-term therapeutic drug concentrations in the lung without the need for frequent dosing. We found that b-MPP loaded with DP improved the efficacy in reducing inflammation in a mouse model of LPS-induced acute lung inflammation. Likewise, Nafee and co-workers recently demonstrated that inhaled ultrasmall lipid NP capable of penetrating mucus greatly enhanced the antivirulence efficacy of an anti-infective, quorum-sensing inhibitor (36). In the case of lung cancer, tumor cells are susceptible to chemotherapy only at distinct phases of the cell cycle (58). The sustained therapeutic drug concentrations enabled by MMP may augment the chance of targeting tumor cells at the appropriate phases of the cell cycle in the lung airways. Prolonged retention of MMP may also be helpful in CF gene therapy, which has been unsuccessful, to date, in clinical trials (59, 60). We have recently reported that newly engineered mucus-penetrating DNA NP significantly enhanced pulmonary gene transfer compared to conventional gene vectors (61), including one that has shown efficacy comparable to a leading viral gene vector widely tested in clinical trials (that is, adeno-associated virus serotype 2) (59, 62).

MMP that rapidly penetrate the outer LML into the deeper PCL may be retained significantly longer, because this layer is believed to be nearly stationary (63, 64). This mechanism has been hypothesized to explain the enhanced retention of human serum albumin compared to sulfur colloids in the canine lungs (32), as well as the ability of certain viral particles to infect mucosal surfaces (65). However, this mechanism has recently been challenged by the seminal findings of Button and co-workers (66). In vitro experiments that used an air-liquid interface culture of primary human bronchial epithelium revealed that dextran probes larger than 40 nm were excluded from the PCL, whereas smaller dextran molecules partitioned into the layer closer to the epithelium (66). On the basis of this observation, they postulated that the PCL may behave as a fine mesh rather than a watery layer in which inhaled objects can freely diffuse. However, because the observations were made in vitro only, it is unclear whether this exact behavior would be replicated in vivo and, if so, whether it is observed throughout the conducting airways regardless of branching generation. Furthermore, this model does not take into account the presence of nonciliated cells, including secretory cells, which are often found in the airway epithelium (67). The presence of these cells may create permeable gaps in PCL where secreted mucins diffuse out to reach the LML. There are fewer ciliated cells in the lower conducting airways, which may result in larger areas with a more permeable PCL.

**Fig. 5. In vivo anti-inflammatory effects of GRAS-based biodegradable NP carrying DP in the lungs of mice challenged with Pseudomonas aeruginosa LPS.** Mice were challenged twice with LPS at 0 and 6 hours. At t = 24 hours, LPS-treated mice received DP/PLGA/F127 or DP/PLGA/F68 NP at a dose of 1 mg/kg. Control LPS-treated mice received either carrier-free DP or saline. Mice were sacrificed at 48 hours for BALF analysis. (A) Total inflammatory cell counts. (B) Concentration of TNF-α. Data represent means ± SD. *P < 0.05, **P < 0.01.
We found in this study that MAP, regardless of particle diameter, were rapidly removed from the lumen of the lung in vivo. This suggests that previously reported favorable outcomes achieved with a drug in MAP compared to carrier-free soluble drug formulations may be partly attributed to the benefits intrinsic to NP-based drug delivery systems rather than NP mucadenhesion per se. In contrast, MPP were uniformly distributed throughout the airway mucus layer and exhibited improved retention, resulting in improved therapeutic efficacy compared to carrier-free drug and drug delivered by a MAP formulation. These findings suggest that MPP, at least those up to 300 nm in diameter, provide an attractive alternative to the use of MAP to enhance pulmonary delivery of therapeutics.

**MATERIALS AND METHODS**

**Formulation of model MPP**

Fluorescently labeled carboxyl-modified PS NP (Invitrogen) were PEPEGylated via carbodiimide cross-linking chemistry, as previously described, to generate MPP (39). Details are provided in the Supplementary Materials.

**Characterization of NP**

Hydrodynamic diameters and \( \zeta \)-potentials of all NP were measured by DLS and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS (Malvern Instruments) in 10 mM NaCl (pH 7.4) at 25°C, as per the manufacturer’s instructions.

**Fluorescent labeling of biodegradable polymers**

PLGA2A (15 kDa; Lakeshore Biomaterials) and methoxy-PEG5k-PLGA45k (Jinan Daigang Biomaterial Co.) were labeled with a carboxyl-reactive Alexa Fluor dye (AF488, AF555, or AF647), as previously described (68). Reaction details are provided in the Supplementary Materials.

**Preparation of biodegradable NP**

PLGA and PLGA-PEG NP were formulated by an emulsification solvent evaporation method, as previously described (68). Briefly, 40 mg of polymer, including 20 mg each of fluorescently labeled and unlabeled polymer, was dissolved in dichloromethane (DCM) (0.2 and 0.8 ml for MAP and MPP, respectively. The organic DCM phase was then added to an aqueous phase of 5 ml of 0.5% cholic acid sodium salt (CHA), followed by probe sonication for 2 min (30% amplitude). The emulsion was then added to a stirring solution of 35 ml of 0.5% CHA and allowed to harden for 3 hours under magnetic stirring at 700 rpm. To ensure complete removal of DCM, the beaker was transferred to a vacuum chamber for 30 min. Biodegradable NP, either b-MPP or b-MAP, were collected by centrifugation (10,000g, 30 min) and washed twice in 35 ml of ultrapure water. NP were characterized as described above.

GRAS-based NP, including PLGA/PA, PLGA/PEG, and PLGA/F127 NP, were prepared by a solvent diffusion method, as previously described (41). Ten milligrams of polymer was dissolved in 1 ml of tetrahydrofuran (THF) and added dropwise to 40 ml of ultrapure water and 5% F68 (BASF) and 5% F127 (BASF) aqueous solution for PLGA/PA, PLGA/PEG, and PLGA/F127, respectively, under magnetic stirring at 700 rpm. After rotary evaporation for 30 min to remove the THF, the NP were collected by centrifuging at 10,000g for 25 min, washed twice with respective aqueous solutions, and re-suspended in 0.4 ml of ultrapure water. NP were characterized as described above.

**Preparation of DP-loaded biodegradable NP**

DP was encapsulated into Pluronic-coated PLGA NP following a modified solvent diffusion method, as previously described (42, 43). Briefly, a DP-zinc complex was formed by adding 1 ml of 0.5 M zinc acetate aqueous solution to 0.5 ml of an aqueous solution containing 10 mg of DP (Sigma-Aldrich). After centrifuging at 20,000g for 5 min, the precipitated DP-zinc complex and 50 mg of PLGA1A (3.2 kDa; Lakeshore Biomaterials) were dissolved in 2.5 ml of THF, followed by the addition of 20 ml of triethanolamine. The mixture was added dropwise into 100 ml of an aqueous solution containing either 5% F68 or 5% F127 while stirring to form DP-loaded b-MAP or b-MPP, respectively. After rotor evaporation for 30 min to remove the THF, 1 ml of 0.5 M EDTA aqueous solution (pH 7.5) was added to the NP suspension to chelate excess zinc and solubilize any unencapsulated DP-zinc complexes. The NP were then separated from unencapsulated DP, zinc, and EDTA by centrifuging at 10,000g for 25 min, washed twice with 5% F68 or 5% F127, and resuspended in 0.4 ml of ultrapure water. The hydrodynamic diameters and \( \zeta \)-potentials of NP were characterized as described above. NP morphology was visualized using a Hitachi H-7600 transmission electron microscope (Hitachi Co. Ltd.).

**Animals**

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins Medical Institutions. Female CF-1 or Balb/c mice (6 to 8 weeks; Harlan) were allowed to acclimate for 1 to 2 weeks before handling, with access to food and water ad libitum throughout the experiments.

**Ex vivo stability of biodegradable NP in BALF**

Mice were challenged twice with 100 μg of *P. aeruginosa* LPS (serotype 10; Sigma-Aldrich) at 0 and 6 hours by intranasal instillation. After 48 hours, animals were sacrificed, and the lungs were harvested. BALF was collected by sequentially lavaging the lungs three times with 1 ml of PBS, and the recovered fluids were pooled for each animal. Subsequently, BALF samples collected from four animals were pooled, cells were separated by centrifugation, and supernatant was collected and syringe-filtered (0.2 μm). The protein content of the BALF supernatant was measured using the BCA (bicinchoninic acid) Protein Assay Kit (Pierce). Twenty microliters of stock NP solution (20 mg/ml) was added to 0.4 ml of BALF (a protein concentration of 0.6 mg/ml) and incubated at room temperature. At 2 and 24 hours after incubation, hydrodynamic diameters of NP were measured by DLS, as described above.

**DP loading and release kinetics**

To measure the DP content of Pluronic-coated biodegradable NP, the NP were freeze-dried, weighed, and dissolved in 0.5 ml of acetonitrile. Subsequently, 1 ml of 50 mM EDTA was added to chelate zinc and solubilize encapsulated DP, and the DP concentration was measured by reverse-phase high-performance liquid chromatography (HPLC). Briefly, isocratic separation was performed on a Shimadzu Prominence LC system equipped with a Pursuit 5 C18 column (Varian Inc.). The mobile phase was composed of acetonitrile/water (35/65 v/v) containing 0.1% trifluoroacetic acid (flow rate, 1 ml/min). Column effluent was monitored by ultraviolet detection at 241 nm. The drug loading and encapsulation efficiency were calculated according to the following equations

\[
\text{Drug loading} \% = \left( \frac{\text{amount of DP in NP/weight of NP}}{100} \right)
\]
Encapsulation efficiency (%) = \frac{\text{measured loadings}}{\text{theoretical loadings}} \times 100 \quad (2)

To measure the in vitro release of DP, 200 \mu l of the NP suspension (approximately 25 mg/ml) was sealed in a Spectra/Por 6 dialysis membrane (molecular weight cutoff, 10 kDa; Spectrum Laboratories Inc.). The sealed dialysis membrane was placed into a 50-ml conical tube containing 12 ml of PBS and incubated at 37°C on a platform shaker (140 rpm). The entire release medium was collected at predetermined intervals and replaced with 12 ml of fresh PBS. The concentration of DP in the release medium was measured by HPLC, as described above.

**Collection of CF sputum**

Sputum samples spontaneously expectorated by male and female CF patients aged 23 to 40 were collected at the Johns Hopkins Adult Cystic Fibrosis Program. The procedures conformed to ethical standards, and the sputum collection was performed under informed consent on a protocol approved by the Johns Hopkins Medicine Institutional Review Board. Two to three samples were acquired from the weekly CF outpatient clinic, placed on ice upon collection and during transport, pooled to minimize patient-to-patient variation, and studied the same day. The total number of individual samples used for the present studies was 9.

**MPT of biodegradable NP in CF mucus**

Diffusion of fluorescently labeled (AF555) biodegradable NP in human CF sputum was studied by MPT, as we have previously described (26). Details are provided in the Supplementary Materials.

**Ex situ tracking of model NP in freshly excised mouse trachea**

Fluorescently labeled PS-based MAP (excitation/emission maxima of 505/515 nm; Invitrogen) and MPP (excitation/emission maxima of 660/680 nm; Invitrogen) were mixed together at a concentration of 2 mg/ml in sterile, hypotonic PBS (that is, diluted PBS). Female CF-1 mice were anesthetized under continuous flow of 2% isoflurane (Baxter Healthcare Corp), and 50 \mu l of the NP suspension was administered via intranasal instillation. Mice were sacrificed 30 min after administration, and the tracheas were carefully resected and cut longitudinally to generate “half-pipes” that were laid face-up on microscope slides. The trachea was instantaneously flattened and sealed with a coverslip, and movies of NP were captured at a temporal resolution of 66.7 ms for 20 s with a Zeiss Axio Observer D1 inverted epifluorescence microscope (Zeiss) equipped with a Photometrics Evolve 512 camera (Photometrics) and MetaMorph software (Molecular Devices) after incubation for 1 hour. Areas where all NP were immobilized were avoided to ensure that NP were located in regions with intact tracheal mucus [some studies suggest that the mucus gel layer is discontinuous (69–72)], and care was taken to avoid areas with convection. Fluorescently labeled MAP and MPP were imaged in the exact same field of view to allow direct comparison of tracking results.

**Distribution of NP in lung airways**

Fluorescently labeled PS-based or PLGA-based MAP and MPP were mixed together at a concentration of 2 or 15 mg/ml, respectively, in sterile, hypotonic PBS. Female CF-1 mice were anesthetized under a continuous flow of 2% isoflurane, and 50 \mu l of the NP suspension was administered via intranasal instillation. Mice were sacrificed after 30 min, and the lungs were carefully resected, added to plastic cryomolds filled with optimum cutting temperature (OCT) compound (Sakura), and frozen in liquid nitrogen. Lungs were sectioned, mounted, stained with DAPI, and imaged on an epifluorescence microscope (Zeiss). Details are provided in the Supplementary Materials.

**Retention of NP in the lung**

Retention of fluorescently labeled NP in the lungs was measured by two complementary methods: the lavage method, which only measures NP in the lung lumen, and the whole-lung method, which measures NP in entire lung tissue. The whole-lung method accounts for particles deposited in the conducting airways as well as the alveolar regions that are not accessible via the lavage method.

For the lavage method, female CF-1 mice (6 to 8 weeks) were anesthetized under a continuous flow of 2% isoflurane, and 50 \mu l of the NP suspension was administered via intranasal instillation. Mice were sacrificed at various time points via cervical dislocation, and the lungs were resected, rinsed briefly in PBS, and lavaged three times with 1 ml of PBS to collect BALF. Fluorescently labeled NP in the BALF was measured on a 96-well fluorescent plate reader (BioTek Synergy 2, BioTek).

For the whole-lung method, similarly treated female CF-1 mice were sacrificed at various time points via cervical dislocation, and the lungs were resected, rinsed briefly in PBS, laid on a labeled petri dish, and stored at −20°C until imaging. Imaging was performed with Xenogen IVIS Spectrum live animal imaging system (Caliper Life Sciences). Fluorescence was monitored at excitation/emission wavelengths of 640/680 nm with an exposure time of 1 s. Quantification of fluorescence was performed with Living Image 2.5 software (PerkinElmer). Details are provided in the Supplementary Materials.

**In vivo anti-inflammatory efficacy of DP-loaded NP**

Mice were challenged twice with 100 \mu g of LPS at 0 and 6 hours by intranasal instillation. After 24 hours, carrier-free DP or DP-loaded NP were administered in saline by oropharyngeal aspiration at a DP dose of 1 mg/kg (n = 8). Mice in a control group received saline (n = 8). After 48 hours, animals were sacrificed, and lungs were harvested. BALF was collected and analyzed for total and differential cell counts and concentration of TNF-α as follows. The lungs were sequentially perfused three times with 1 ml of PBS, and the recovered BALF was pooled for each animal. Cells were collected by centrifugation, and the supernatant was stored at −80°C for enzyme-linked immunosorbent assay (ELISA). Red blood cells were lysed with ammonium-chloride-potassium (ACK) lysing buffer (Quality Biological Inc.). Total cell counts were determined using a Vi-CELL XR automated cell viability analyzer (Beckman Coulter Inc.), and a cytokine, TNF-α, was assayed using a Quantikine ELISA kit (R&D Systems Inc.). Cytokine concentrations in BALF were adjusted for dilution by the urea method (73) and expressed as a concentration of epithelial lining fluid (pg/ml).

**In vivo safety profile of biodegradable NP**

GRAS-based biodegradable NP were administered in saline by oropharyngeal aspiration at a dose of 1 mg per mouse (50 mg/kg) (n = 9). Control mice were treated with saline (n = 9). After 24 hours, animals were sacrificed, and lungs were harvested for either BALF (n = 7) or tissue histology (n = 2). BALF was collected and analyzed for total cell counts and TNF-α content, as described above. Differential cell counts were performed via cytospin with a Shandon Cytospin 3 centrifuge (Shandon Scientific) after staining with hematoxylin and eosin.
SUPPLEMENTARY MATERIALS

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/4/e1601556/DC1

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA), followed by Tukey post hoc test or Games-Howell test using SPSS 18.0 software (SPSS Inc.). Differences were considered to be statistically significant at a level of P < 0.05.

REFERENCES AND NOTES


Nanoparticles that do not adhere to mucus provide uniform and long-lasting drug delivery to airways following inhalation
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