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Modified Poly(lactic-co-glycolic acid) Nanoparticles for Enhanced Cellular Uptake and Gene Editing in the Lung

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Abstract

Modified nanoparticles composed of a blend between poly(β -amino ester) (PBAE) and poly(lactic-co-glycolic acid) (PLGA), surface coated with cell-penetrating peptides (CPPs), have previously shown promise in gene delivery. In this study, we sought to evaluate pulmonary cellular uptake of these modified nanoparticles, and demonstrate their potential as vectors in pulmonary gene therapy. This work shows that surface modified PLGA/PBAE/CPP nanoparticles achieve higher cellular association than traditional nanoparticles composed of PLGA or PLGA/PBAE blends alone.

Keywords

nanoparticles; gene editing; pulmonary delivery; cellular uptake; cell-penetrating peptide

Polymeric nanoparticles (NPs) hold great potential in pulmonary drug delivery. While administration of freely soluble drug is effective in the treatment of several pulmonary conditions, there are often issues associated with drug dosing or drug stability.^[1] In the case of asthma, for example, administration of freely soluble steroids has proven effective, though the benefits are relatively short lived and require repeated dosing.^[1] In the case of genetic disorders, delivery of nucleic acids is often difficult due to nucleic acid stability, mucosal barriers, or repulsive charge interactions with the negatively charged cell membrane.^[1, 2] Polymeric NPs, however, offer many benefits to overcome some of these barriers, notably their ability to encapsulate and slowly release drug content, protect nucleic acid degradation, and shield nucleic acid charge.^[1, 3] Polymeric NPs can also be

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Supporting Information

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functionalized, allowing for enhanced or targeted delivery through NP surface modifications.^[4, 5] Moreover, the inhalation of NPs provides for a minimally invasive delivery route, resulting in high local concentrations of drug throughout the lung.^[3, 6]

In several studies, NP formulations have already been successful in delivering therapeutics to the lung.^[3, 6, 7, 8] Several methods have been employed to better understand lung deposition and particle uptake by cells. Single-photon emission computed tomography (SPECT) has been used to determine regional deposition and biodistribution of radiolabeled polyethylenimine(PEI)/siRNA constructs after intratracheal administration.^[8] Although the constructs were detected in the kidney, liver, and trachea, the strongest signals were detected in the lung.^[8] Regional deposition of aerosolized plasmid DNA (pDNA)/PEI complexes was likewise studied through the use of fluorescent microscopy.^[6] While pDNA/PEI constructs were detected throughout the lung, radiolabeled pDNA/PEI was detected at a slightly higher dose in the stomach.^[6] Most recently, lung deposition and cellular uptake of polyanhydride nanoparticles was investigated.^[9] Ex-vivo fluorescent imaging of lung tissue revealed the prolonged presence of encapsulated F1-V recombinant antigen at 2 hr and 48 hr time points relative to soluble antigen alone. Encapsulated F1-V antigen was further detected in specific cell populations of the lung (dendritic cells, macrophages, and alveolar epithelial cells) by flow cytometry (FACS).^[9]

In the case of genetic disorders, such as cystic fibrosis (CF), cell-specific uptake is of particular interest.^[10, 11] CF is characterized by defects in the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent chloride channel.^[12] Although CF affects epithelial surfaces across several organs, abnormal pulmonary pathology is the greatest cause of morbidity.^[12] Absence of functional CFTR in the lung airway epithelium results in viscid mucus, leading to increased respiratory difficulty, and greater entrapment of bacteria, particularly *Pseudomonas aeruginosa*.^[13] Recent evidence has also shown that CFTR is expressed by alveolar macrophages, and may contribute to the hyperinflammatory immune response observed in CF patients.^[10, 14] Bruscia and colleagues, for example, determined that a lack of functioning CFTR in CF macrophages resulted in increased toll-like receptor 4 (TLR4) cell-surface expression and abnormal TLR4 cellular localization, leading to hyperinflammatory signaling.^[11] Members of the same group later demonstrated that heme-oxygenase (HO-1) failed to compartmentalize normally in CF macrophages, due to decreased CAV-1 expression, resulting in a further failure to downregulate TLR4 signaling.^[13] While CF remains a prime candidate for gene therapy, delivery of biological agents to the diseased cells of the lung has proven difficult.

Previously, our group has demonstrated that NPs composed of a blend between poly(β -amino ester) (PBAE)^[15] and poly(lactic-co-glycolic acid) (PLGA) improve cellular uptake and plasmid transfection of CF bronchial epithelial cells (CFBE) relative to PLGA NPs alone.^[16] This formulation was further improved through surface modification with MPG, a cell-penetrating peptide (CPP).^[16] In this work, we sought to advance our understanding of these NP formulations by investigating the effects of these modifications *in vivo*. More specifically, we aimed to i) locally deliver NPs of varying material composition to the lung, ii) resolve population-specific uptake, iii) and provide proof of principle that these formulations could be used to treat pulmonary genetic diseases through gene editing of

target cell populations.^[17, 18] This is the first report of polymer NPs that are capable of gene-editing by triplex-forming oligonucleotides after delivery to the mucosal surface of the lung.

Use of fluorescently labeled probes is an effective approach for resolving cellular localization, because it allows for visualization using fluorescence or confocal microscopy of lungs slices. To accomplish our first two aims, NPs were loaded with a fluorescent dye, Coumarin-6 (C6), and administered intranasally (IN) to mice. Particle uptake was assessed by fluorescent microscopy and quantified by FACS. To investigate whether our NP formulations could be used in gene editing of relevant cell populations, NPs were loaded with triplex-forming PNAs and donor DNA oligonucleotides, capable of mediating site-specific homologous recombination with genomic DNA.^[17–19]

C6-loaded NPs were prepared using PLGA (NP-0), PLGA blended with PBAE at a ratio of 85:15 (wt:wt) (NP-15), or NP-15 with a DSPE-PEG-MPG surface coating (NP-15-MPG) (Figure 1A). This surface coating method has proven effective in presenting peptides on the surface of particles^[16, 20, 21] Here, we use this strategy to prepare NPs with the CPP, MPG, a synthetic peptide derived from the hydrophobic domain of the HIV gp41 fusion sequence, and a hydrophilic domain from the nuclear localization sequence (NLS) of the SV40 T-antigen.^[22] MPG has been shown to improve intracellular nucleic acid delivery through several mechanisms, including improved membrane permeability, and potentially improved nuclear trafficking.^[23] NPs encapsulating C6 were shown to be morphologically similar with diameters on the order of 150 nm as determined by SEM, and hydrodynamic diameters of approximately 300 nm as determined by DLS (Figure 1 B, C). Previously, we reported that these particle formulations released negligible amounts of C6 when incubated in PBS over a 24 hr period^[16], and that NPs containing C6 can be used as tracers in both cell and tissue samples.^[24]

To understand the localization of NPs within the lung, NPs loaded with C6 were administered IN to mice. After harvesting, and slicing, fluorescent imaging of the lung revealed that NPs delivered IN were co-localized with ciliated epithelial cells (CECs) as well as macrophages (Figure 2). Although co-localization was observed in mice treated with all three NP formulations, the lungs of mice treated with NP-15-MPG appeared to have a significantly greater concentration of particles distributed throughout the section. No colocalization was observed for type II alveolar epithelial cells (Figure S1). Fluorescent imaging of a 10-micron thick lung lobe further revealed that NP-15-MPG NPs were widely distributed throughout the proximal and distal airways of the lung (Figure S2).

The cellular localization of NPs in the lung was quantified using FACS. After treatments, as described above, lung tissue samples were harvested, and prepared for FACS analysis. NP fluorescence associated with lung cell types was quantified (Figure 3 A, B). Within the lungs of mice receiving the NP-15-MPG treatment, we observed that approximately 30% of all lung cells (excluding red blood cells which were removed by Ficoll separation) associated with, or internalized particles compared with 5% for NP-15 and 0.4% for NP-0 treated mice. To determine if different cell types preferentially took up the NPs, macrophages and type I alveolar epithelial cells (AEC Is) were stained, and the number of

these cell types that were C6+ was quantified (Figure 3 B, C). The NP-15-MPG NPs were associated with 80% of macrophages and AEC Is, while NP-15 was associated with 8% of macrophages and 2% of AEC Is. NP-0, however, was associated with less than 1% of each. These trends support our previous *in vitro* observations that NP-15 particles have greater cellular association than the NP-0 particles. Likewise, the addition of a DSPE-PEG-CPP surface coating to NP-15 improved cellular association considerably.^[16]

Although a high level of cellular association is necessary for successful PNA/DNA delivery, it is not sufficient for recombination due to the numerous intracellular barriers, even after successful cellular internalization. We have previously shown that PLGA NPs loaded with triplex-forming PNA oligonucleotides and donor DNA fragments can mediate site-specific gene editing in cells *ex vivo*^[17] and *in vivo*.^[18] For successful gene editing, the NPs must enter cells, and release their cargo, which is only active in the nucleus. To investigate whether our NP formulations could have an effect on cells in the lung, gene editing was explored in an EGFP654 transgenic mouse containing an aberrantly expressed intron within the EGFP gene.^[25] Correction of this splice site mutation results in EGFP expression (Figure 4 A). Blank NP-15 particles, as well as NP-0, NP-15 and NP-15-MPG particles loaded with a PNA and DNA for EGFP654 correction were delivered IN to EGFP654 mice four times over the course of two weeks. Five days after the last treatment, EGFP expression in the lung was quantified in all lung cells, then specifically in macrophages and AEC Is. The extent of gene modification increased with the addition of PBAE to the NPs (NP-0 to NP-15), and increased further with the addition of the MPG surface modification (NP-15 to NP-15-MPG) (Figure 4 B). In line with our previously reported *in vitro* results^[16], this increasing trend in gene correction correlated with increased transfection of NPs in the following order: NP-0 < NP-15 < NP-15-MPG (Figure 2).

The average modification efficiency due to NP-15-MPG particles in alveolar macrophages was double that observed in AEC Is (0.6% vs. 0.3% respectively) even though the NP-15-MPG NPs showed cellular association with approximately 80% of both cell types (Figure 3 C, D). The exact mechanism underlying the increased gene correction observed in macrophages as compared to AEC Is, despite similar levels of cellular association in both cell types, is not yet understood.

In vivo gene correction experiments were repeated for the NP-15 blank and NP-15-MPG treated groups (red symbols) to increase the number of replicates (Figure 4). For all experiments combined, gene correction efficiency is significantly enhanced with NP-15-MPG particles in the total lung cells, macrophage cells, and AEC I cells.

The efficiency of particle uptake and gene correction frequency *in vivo* depends on multiple factors. Once particles are administered, and deposited in the airway, there are a host of mucus and mucociliary clearance mechanisms that make cellular delivery of NPs a challenge. In the case of NP-15-MPG, we qualitatively observed a higher amount of NPs in lungs slices stained for macrophages and CECs (Figure 2). When designing our surface coated NPs, we chose PEG as a linker due to its low mucoadhesive properties and potential to enhance NP penetration through mucus.^[4, 26] Therefore, the PEG surface coating present on the NP-15-MPG NPs may have facilitated diffusion through the pulmonary mucus,

resulting in an elevated NP concentration at the epithelial surface and, thereby, increasing cellular uptake. Additional particle uptake of NP-15-MPG, may have resulted from the presence of the cell-penetrating peptide (MPG), which has shown successful cellular delivery of nucleic acids as a complex.^[22] The improved cellular association and gene correction of NP-15-MPG and NP-15 treatments, relative to NP-0 treatments, may be further due to the presence of PBAE, which has been previously shown to condense nucleic acids, and facilitate endosomal escape.^[27] Although the same trends in cell association and transfection were observed among our NP formulations *in vitro*^[16] and *in vivo*, future work should aim to study the diffusivity of these NP formulations in pulmonary mucus as it relates to material properties and NP surface charge.

The work presented here confirms that our NP formulations can be used for pulmonary delivery, resulting in successful association with macrophages, CECs, and AECs (types I and II). Knowledge of this population-specific uptake should enhance the development of therapeutics relevant to diseases that affect these cell types. While our proof of principle focused on the delivery of triplex-forming oligonucleotides for gene editing in airway cells, the NP formulations presented here can be further engineered to encapsulate a variety of therapeutic agents for cellular delivery.

Experimental Section

Materials

Poly (*DL* lactic-co-glycolic acid), 50:50 with inherent viscosity 0.95–1.20 dL/g, was purchased from DURECT Corporation (Birmingham, AL). Poly(beta amino ester) (PBAE) was synthesized by a Michael addition reaction of 1,4-butanediol diacrylate (Alfa Aesar Organics, Ward Hill, MA) and 4,4'- trimethylenedipiperidine (Sigma, Milwaukee, WI) as previously reported.^[15] DSPE-PEG(2000)-maleimide was purchased from Avanti Polar Lipids (Alabaster, AL). MPG peptide (GALFLGFLGAAGSTMGAWSQPKKKRKV) was purchased from the W.M. Keck Facility (Yale University). The donor DNA oligos were purchased from Keck (Yale University). Donor DNA was 5' and 3' end protected by three phosphorothioate internucleoside linkages. PNA monomers were purchased from ASM research chemicals and PNA was synthesized by standard Boc based protocols.^[28] In the PNA sequence, 'O' represents the 8-amino-2,6-dioxaoctanoic acid linker and 'J' stands for pseudoisocytosine, a replacement for cytosine that allows pH-independent triplex formation. Donor DNA was purchased from Midland Certified Reagent Company (Midland, TX, USA). The tcPNA sequence is: N terminus-KKK-JTTTJTTTJTTJ-OOO-TCTCTTTCTTTTCAGGGCA-KKK-C terminus. The Donor DNA sequence is: 5'-AAAGAATAACAGTGATAATTTCTGGGTAAAGGCAATAGCAATATCTCTGCATATAAATAT-3'

Synthesis of DSPE-PEG-Peptide Conjugates

MPG was covalently linked to DSPE-PEG-maleimide as previously reported.^[20] Briefly, cysteine-flanked (at the N-terminus) MPG was dissolved in 50µl of diH₂O. A reaction mixture consisting of 50µl TCEP bond breaker (ThermoScientific), 400µl of 100mM HEPES and 10mM EDTA reaction buffer at pH 7.0–7.4, and 50µl of the peptide solution

was allowed to react at room temperature for 1 hr. The reduced peptide solution was then added to 3X molar excess of DSPE-PEG-maleimide in reaction buffer and incubated at room temperature on a rotisserie rotator overnight. The next day the solution was dialyzed in 1X PBS to remove by-products from the reaction and stored at 4°C until use. As in previous work, we verified the conjugation of DSPE and MPG by matrix assisted laser desorption/ionization (MALDI) and SDS-PAGE using a Novex 16% Tricine Gel System (Life Technologies) and Coomassie Blue staining, per the manufacturer's protocol.^[16]

Nanoparticle Synthesis and Formulation

NPs were formulated as previously described.^[16] Briefly, PLGA or PLGA blended with PBAE at a wt/wt ratio of 15% was dissolved in dichloromethane (DCM). Coumarin-6 (C6) was added to the polymer solution at a 0.2% wt:wt C6:polymer ratio. Luciferase coding plasmid DNA (pGL4.13, Promega) or PNA/DNA complexes in 1X Tris EDTA buffer was added dropwise under vortex to the solvent-polymer blend solution. The solution was then sonicated on ice using a probe sonicator (Tekmar Company, Cincinnati, OH) to form the first water-in-oil emulsion. The first emulsion was rapidly added to a 5.0% aqueous solution of poly(vinyl alcohol) (PVA) under vortex to form the second emulsion and sonicated again. The emulsion was then added to a stirring 0.3% PVA stabilizer solution and stirred overnight to allow for residual solvent evaporation. Nanoparticles were centrifuged (3X, 9500 rpm, 15min) and washed in diH₂O to remove excess PVA prior to lyophilization (72hrs). Dried nanoparticles were stored at -20°C until use. To make surface-modified particles, DSPE-PEG-MPG was added to the 5.0% PVA solution during the second emulsion at a ratio of 5 nmol DSPE-PEG-MPG /mg polymer.

Scanning Electron Microscopy (SEM)

For SEM analysis, nanoparticles were first coated in gold. Particle morphology was analyzed using an XL-30 scanning electron microscope (FEI, Hillsboro, Oregon). ImageJ software analysis was used to determine particle diameter.

Dynamic Light Scattering

Hydrodynamic diameters of particles were analyzed with a Zetasizer Nano ZS (Malvern).

In-vivo Nanoparticle Administration

Female Balb/c mice and EGFP654 transgenic mice (obtained from Ryszard Kole and Rudolph Juliano at the University of North Carolina, and bred at Yale University) were used for this study. Animal use was conducted in accordance with the Yale University Institutional Animal Care and Utilization Committee (IACUC). Prior to treatment, mice were anesthetized with isofluorane. 4mg of NPs were dissolved in 50ul of PBS, sonicated in a water bath, and administered intranasally.

For lung distribution studies, mice were sacrificed four hours post-NP administration and perfused with heparinized PBS. Lungs were resected for further experiments as described below.

For delivery of PNA/DNA loaded NPs mice were treated twice a week for two weeks with an IN dose of 2mg NP/treatment. Mice were then sacrificed 5 days after the last treatment and lung samples were resected and prepared for flow cytometry analysis as described below. Nasal epithelia of these mice were also removed and mounted for microscopy analysis

Frozen section/staining

Lung samples were fixed in 4% paraformaldehyde overnight at 4°C then transferred to a 50mM sucrose solution for an additional 24 hrs. at 4°C. Tissues were then snap frozen in OCT media, sliced to 10µM sections using a cryostat, mounted on lysine coated microscopy slides and stored at -20°C.

Randomly selected slides were chosen for immunohistochemical analysis. Briefly, slides were rehydrated in PBS then incubated in a blocking solution (1%BSA and NGS) for 1hr followed by incubation with primary antibody (anti-beta-tubulin, ciliated epithelial cells; anti-prosurfactant C, type II alveolar epithelial cells; anti-CD68, macrophages) overnight at 4°C. Slides were washed 3 times in PBS and incubated for 1 hour 30 minutes with secondary antibody (goat anti-mouse-Texas Red), washed again three times and mounted with Vectashield containing Dapi.

Flow Cytometry

Lung samples were manually homogenized in a cell strainer with 5ml RPMI media. Ficoll separation was performed to remove remaining red blood cells and debris. Cells were aliquotted and stained for specific cell types (anti-F480, macrophage: anti-P2X7, alveolar epithelial cells) for 30 minutes, and washed three times before being fixed in 4% paraformaldehyde. Flow cytometry analysis was performed.

Statistical Analysis

Data for *in vivo* C6 NP uptake was collected in triplicate (n=3) and reported as mean +/- standard deviation. Unpaired, two-tailed t-test with 95% confidence interval (p<.05) was performed to determine significance of different particle formulations in uptake studies.

Data for gene correction experiments was collected in triplicate (n=3). Additional data points (in red) were collected from a second, separate experiment. Statistical significance comparing NP-15-MPG to NP-15 Blank treatment was calculated using an unpaired, two-tailed t-test with 95% confidence interval (p<.05), with the addition of Welch's correction to account for the wide variance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Schematic representation of NPs. NPs were formulated with a polymer blend of PLGA and PBAE. Particles encapsulated plasmid DNA (not shown in schematic) or PNA/DNA cargo and were surface modified with a DSPE-PEG-MPG moiety. B) SEM image of NPs. C) Size characterization of NPs when suspended in aqueous solution (hydrodynamic diameter) or after lyophilization (dry powder diameter).

Figure 2.

Lung tissue from mice treated with, NP-0, NP-15, and NP-15-MPG particles. Lung tissue was harvested 4 hours post administration. The nucleus (Dapi) is shown in blue.

Macrophages and CECs are shown in red, while NPs are shown in green. Insets depict co-localization of NPs with macrophages or CECs. All images were taken at 20X magnification. White scale bar is equal to 58 μm .

Figure 3.

FACS analysis of mice lungs treated with C6 loaded NPs four hours post IN administration.

A) FL1/FSC channel of all lung cells. B) Percentage of C6+ lung cells. C) Percentage of C6+ Macrophages. D) Percentage of C6+ Alveolar Epithelial Cells (Type I). *Indicates statistical significance in particle cellular association of NP-15-MPG relative to untreated and unmodified particle formulations; $p < 0.05$.

Figure 4.

Expression of EGFP after correction of EGFP gene splice site with PNA/DNA oligos. A) Schematic of PNA mediated homologous site-specific recombination of donor DNA to correct EGFP gene and restore EGFP expression. B) Percentage of GFP+ lung cells after treatment with different NP formulations. C) Percentage of GFP+ macrophage cells. D) Percentage of GFP+ type I alveolar epithelial cells. Experiments were repeated for NP-15-MPG and NP-15 Blank treated groups to increase the number of replicates (red symbols). *Indicates statistical significance in gene correction for the of NP-15-MPG treated groups above control (NP-15 Blank); $p < 0.05$.