Pulmonary Delivery of Butyrylcholinesterase as a Model Protein to the Lung

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Supporting Information

ABSTRACT: Pulmonary delivery has great potential for delivering biologics to the lung if the challenges of maintaining activity, stability, and ideal aerosol characteristics can be overcome. To study the interactions of a biologic in the lung, we chose butyrylcholinesterase (BuChE) as our model enzyme, which has application for use as a bioscavenger protecting against organophosphate exposure or for use with pseudocholinesterase deficient patients. In mice, orotracheal administration of free BuChE resulted in 72 h detection in the lungs and 48 h in the bronchoalveolar lavage fluid (BALF). Free BuChE administered to the lung of all mouse backgrounds (Nude, C57BL/6, and BALB/c) showed evidence of an acute cytokine (IL-6, TNF-α, MIP2, and KC) and cellular immune response that subsided within 48 h, indicating relatively safe administration of this non-native biologic. We then developed a formulation of BuChE using Particle Replication in Non-Wetting Templates (PRINT). Aerosol characterization demonstrated biologically active BuChE 1 μm cylindrical particles with a mass median aerodynamic diameter of 2.77 μm, indicative of promising airway deposition via dry powder inhalers (DPI). Furthermore, particulate BuChE delivered via dry powder insufflation showed residence time of 48 h in the lungs and BALF. The in vivo residence time, immune response, and safety of particulate BuChE delivered via a pulmonary route, along with the cascade impaction distribution of dry powder PRINT BuChE, showed promise in the ability to deliver active enzymes with ideal deposition characteristics. These findings provide evidence for the feasibility of optimizing the use of BuChE in the clinic; PRINT BuChE particles can be readily formulated for use in DPIs, providing a convenient and effective treatment option.

KEYWORDS: butyrylcholinesterase, pulmonary delivery, biologics, micro/nanoparticle

1. INTRODUCTION

Pulmonary delivery is an emerging alternative therapeutic approach to the common parenteral route of administration for delivery of biologics. The lung enables local delivery with a large...
surface area for absorption of proteins, as well as potential for noninvasive systemic delivery.\textsuperscript{1,2,3} Delivery of biologics, including active enzymes, to the lung is of particular interest for multiple diseases. For example, cystic fibrosis patients are currently treated with inhalation of rhDNase, an enzyme that aids in breaking down the sputum build up in the lungs.\textsuperscript{3} Patients with pulmonary oxidative stress benefit from antioxidant enzyme, such as superoxide dismutase and catalase, in order to prevent lung injury.\textsuperscript{4} Even systemic diseases have been shown to benefit from inhaled biologics; inhaled insulin has demonstrated optimal residence time of approximately 50 h in mice; however, this is not a convenient method due to the bulky nature of the device, the lack of universal enzyme stability, and the susceptibility to protein degradation. Recent progress in dry powder inhaler (DPI) development provides new opportunities for formulations with increased protein stability, overcoming many of the traditional challenges associated with nebulizers. Utilizing particles from a DPI device that could deliver enzymes such as DNase or antioxidant enzymes in a targeted method would advance current treatment options. It has been widely shown that aerosol size and shape effect residence time, rate of phagocytosis, and success of particulate treatments in the lung.\textsuperscript{5−8} However, the balance between protein stability, particle formulation, and aerosol characteristics remains a challenge in realizing the potential of DPI therapeutics, particularly for successful administration of an active enzyme or biologic to the lung with high efficiency.\textsuperscript{13}

To further explore the feasibility and properties of an enzyme delivered to the lung as a dry powder, we chose butyrylcholinesterase (BuChE) as a model enzyme. BuChE is an endogenous enzyme synthesized by the liver, which serves as a catalyzer for the hydrolysis of esters in choline. BuChE can be potentially used in a diverse array of applications, such as a future prophylactic measure against organophosphate (OP) exposure or for treatment of patients with pseudocholinesterase deficiency. OP poisoning remains a serious global health concern, with both accidental and deliberate exposure leading to thousands of deaths annually.\textsuperscript{14} As a prophylactic measure, BuChE scavenges OPs through a covalent bond at the active site, which leads to the inactivation of itself and the OPs.\textsuperscript{15} Human BuChE has been delivered systemically by intravenous (i.v.), intraperitoneal (i.p.), or intramuscular (i.m.) routes, with the latter two having a mean residence time of approximately 50 h in mice; however, this showed limited neutralization of inhaled OPs in guinea pigs.\textsuperscript{16,17} Rosenberg et al. introduced a new approach to address OP poisoning by creating a “pulmonary bioshield” using pulmonary delivery of BuChE to lungs with a polyethylene glycol (PEG)-modified recombinant macaque BuChE. Orotracheal administration prior to nerve gas exposure in mice showed a dose-dependent protection against toxicity, indicating the benefits of BuChE administration directly to the airways.\textsuperscript{18} In addition to protection and scavenging for OP exposure, inhaled BuChE could be used as a supplement to patients with pseudocholinesterase deficiency, an understudied condition where 1 in 3000 people have trouble breaking down certain anesthetics due to an abnormality in endogenous BuChE.\textsuperscript{19,20} These patients also suffer toxicity from common medicines, such as lidocaine and prilocaine.\textsuperscript{21,22}

To achieve efficient lung deposition of BuChE, we employed Particle Replication in Nonwetting Templates (PRINT) technology to fabricate BuChE particles for pulmonary delivery as a potential self-administered DPI. PRINT is a top-down particle fabrication technology that allows the engineering of precisely defined particles by exquisitely and independently controlling particle characteristics such as size, shape, chemical composition, and surface chemistry. Previous work by the lab has shown controlled deposition in the lung as a function of PRINT particle shape and size, as well as adaptability for use with several biologics.\textsuperscript{3,23,24} However, these biologics were not tested extensively in actual lung administration. The flexibility of the PRINT platform lends itself to further optimization of BuChE protein parameters to enhance particle deposition with accurate fabrication of uniform particles. We sought to provide a better understanding of the lung environment, retention, and activity of our model enzyme, BuChE. Furthermore, our studies investigated BuChE distribution in in vivo models postorotracheal administration, as well as the associated immune responses. Utilizing PRINT to meet the formulation challenge in pulmonary delivery, we also developed a novel 1 μm cylindrical BuChE particle to demonstrate a balanced formulation that maintains protein activity and has ideal aerosol characteristics.

2. MATERIALS AND METHODS

2.1. Animals. Male mice were housed in pathogen-free facilities at the University of North Carolina at Chapel Hill (UNC) and treated at 6 weeks of age. Standard guidelines for care and use of laboratory animals as approved by the Institution of Animal Care and Use Committee at UNC were followed. Foxn1nu/nu (Nude) mice were bred in-house; C57BL/6 and BALB/c mice were obtained from Jackson Laboratories.

2.2. Protein Source. Tetrameric equine butyrylcholinesterase (referred to as BuChE in this article) (ID C7512) at ≥10 U/mg was purchased from Sigma-Aldrich and used directly with no further purification. Specifically, lot SLBB2114V (50 U/mg solid as reported by Sigma) was used for instillation studies and lot SLB7404V (19.7 U/mg solid as reported by Sigma) was used in particles for dry powder insufflation studies. Lastly, a high purity eqBuChE stock (ID C4290), lot SLB1774V (331 U/mg solid as reported by Sigma), was used to demonstrate transformability of particle fabrication. Equine BuChE is 88% homologous with murine BuChE, 93% homologous to human BuChE, and is an accepted translational model for human BuChE. Furthermore, several researchers have shown that homologous systems do not result in anti-BuChE antibodies\textsuperscript{26,27} and have used non-homologous systems to establish proof of concept.\textsuperscript{17,26,28−30}

2.3. Orotracheal Administration and Residence of BuChE. Mice were dosed with BuChE orotracheally at a maximum volume of 50 μL at 80 mg/kg with n = 3 per study. BuChE activity was confirmed prior to dosing. Mice were anesthetized using isoflurane, placed on a 45° board by their upper incisors, the tongue was held away from the trachea and the dose was administered to the back of the mouth using a pipet. The nose was gently clamped so that the mouse could aspirate the liquid into the lung. At each time point, mice were anesthetized with ketamine, then blood was collected and centrifuged for plasma analysis. Bronchoalveolar lavage fluid (BALF) was collected from the lungs using 1× PBS (1 mL, Sigma-Aldrich)
at 24, 48, 72, 96, and 168 h with a single flush for protein analysis and two sequential flushes for cell collections. Cells were separated from BALF samples via centrifugation. Protocols were adapted from previous work.\textsuperscript{16,24,31}

For biodistribution analysis, a 3 \textit{wt} \% Dylight 680 tagged BuChE mixture (Fisher Scientific, USA) was administered orotracheally. Manufacturer’s instructions were followed for Dylight 680 tagging with an optimized 3-fold ratio of dye to protein (Figure S1) where a minimal activity loss was detected in the tagged BuChE. BuChE activity of the 3 \textit{wt} \% mixture was measured before administration to ensure proper dosing based on activity. BALF and plasma were collected as described above. Following a 10 mL PBS perfusion, organs were harvested, followed by detection via \textit{in vivo} imaging system (IVIS) Lumina optical imaging (emission filter Cy5.5 and excitation filter 675 nm). Radiant efficiency per gram was measured for all organs, plasma (100 \textmu L), and BALF (100 \textmu L). Saline treated mice were harvested at 0, 24, 96, and 168 h. BuChE treated mice were harvested at 24, 48, 72, 96, and 168 h.

Twenty micrograms of lipopolysaccharide (LPS) was administered as a positive control for inflammatory immune responses, 1X PBS was administered at 50 \mu L as a negative control, and an untreated group was also included in all studies.

2.4. Cytokine Measurements. The levels for IL-6, TNF-\alpha, KC, and MIP2 were measured in BALF and plasma samples using xMAP technology and Luminex Assays (RD Systems) with the assistance of the UNC Cytokine and Biomarker Analysis Facility on BALF and plasma samples. Select samples were tested using BD OptEIA kits (following manufacturer’s instructions) to confirm Lumexin Assay results (data not shown).

2.5. Flow Cytometry. Cells from BALF samples were blocked with anti-CD16/32 (FC block, eBioScience) and stained with CD11c-PE (BioLegend), CD45-PacificBlue (BioLegend), Ly6G Alexa700 (BioLegend), and SiglecF-APC (BDPharmin-gen). The gating scheme (Figure S2), briefly, consists of looking at CD45+ cells (leukocytes), then CD11c+ to differentiate alveolar macrophages, followed by Ly6g+ indicating Neutrophils, or SiglecF- indicating dendritic cells. CD11c-SiglecF+ indicated alveolar macrophages. Gating scheme based on work by Guilliams et al.\textsuperscript{12} Cells were fixed using 2\% PFA in PBS. All data were collected using the Beckman Coulter CyanAn ADP and analyzed using FlowJo Software (Tree Star).

2.6. Enzyme Activity Measurements. Total cholinesterase levels were measured, with an increase in levels attributed to dosed BuChE; therefore, BuChE activity was considered anything above the established baseline. VITROS CHE DT Slides for cholinesterase detection were used for all sample analysis with the VITROS Chemistry Products DT Specialty Calibrator kit per the standard operating protocol. The assay involves cholinesterase hydrolyzing butyrylthiocholine to thiocholine, which reduces the potassium ferriyanide to potassium ferrocyanide. Reference spectrophotometry was then used to monitor the rate of color loss. The UNC-Chapel Hill Animal Clinical Chemistry Core performed the assay.

Another assay used to measure activity is the Ellman assay, which utilizes the same substrate, butyrylthiocholine; however, it is known for interference due to hemoglobin and unstable reagents.\textsuperscript{33} Therefore, we evaluated a BuChE specific assay, DetectX (Arbor Assays, K1016-F1), which also used the same substrate. We found comparable findings to our VITROS assay; yet, the VITROS system provided results in a more accurate, consistent, and time/cost-effective manner. Assay consistency and BuChE stability was confirmed as shown in Figure S3.

2.7. Particle Fabrication. BuChE protein microparticles were fabricated via a PRINT method adapted from Xu et al.\textsuperscript{23} \textit{α}-D-Lactose and glycerol were purchased from Acros. Briefly, a 15\% (w/v) solution of 37.5\% BuChE, 37.5\% \textit{α}-D-lactose, and 25\% glycerol by weight was used to cast a film on a poly(ethylene terephthalate) (PET) sheet. For \textit{in vivo} studies, Dylight680-tagged BuChE at 10 \textit{wt} \% was used in the formulation. Following heat gun application, the transparent film was laminated onto PRINT mold with 1 \textmu m disc features (provided by Liquidia Technologies). The top-heated laminator was set to 73 °C with a laminating pressure of 100 psi. Particles were then removed from the filled mold by a second lamination step at the same settings onto a Plasdone covered PET sheet. The Plasdone film containing particles was then dissolved in isopropanol to obtain 1 \mu m of BuChE particles. The BuChE particles were washed with isopropanol multiple times to remove \textit{α}-D-lactose, glycerol, and residual Plasdone resulting in a primarily protein particle. The particles were further characterized via thermal gravimetric analysis (TGA) (TA Q5000) for concentration and scanning electron microscopy (using a 2 nm thick gold palladium alloy coating) (Hitachi model S-4700) for size and shape confirmation. Particle enzymatic cholinesterase activity was measured using a colorimetric butyrylthiocholine-based assay described earlier to confirm protein content and accurate dosing. The particle composition was determined via high performance liquid chromatography (HPLC), based on previously published protocols.\textsuperscript{23} Briefly, a known concentration of particles was dispersed in water, then BuChE was filtered out (Amicon Ultra, 0.5 mL, MWCO 30k). The filtrate (containing glycerol and lactose) was analyzed using HPLC, and the concentrate was measured to confirm BuChE concentration. A Hi-plex Ca column (Agilent, 300 × 7.7 mm, 8 \mu m) was used with a mobile phase of pure water over 35 min with a flow rate of 0.6 mL/min with an evaporative light scattering detector at 30 C. Area under the curve was measured for a Lactose peak at 9.3 min and a glycerol peak at 16.3 min to determine particle composition using a lactose and glycerol standard curve.

2.8. Cascade Impactor Lung Deposition. BuChE particles were lyophilized in tert-butanol, as previously described,\textsuperscript{8} to obtain a dry powder. The aerodynamic properties of the dry powder were characterized with an Andersen Cascade Impactor (ACI, ThermoScientific) in triplicate. Powder aerosols were dispersed via an insufflator device and a 3 mL syringe as the hand pump at 2 mg fill weights (Penn Century Inc., PA, USA). Standard ACI protocol was used as previously discussed.\textsuperscript{24} Briefly, collection plates were coated with poly(ethylene glycol) MW 300. The flow rate was 28.3 L/min for 8 s. Deposited particles were collected using 500 \mu L of water, followed by flash freezing and lyophilization. Lyophilized samples were then resuspended in a concentrated volume of water for quantification of deposition per stage based on BuChE activity. It was confirmed that this corresponds directly to the relative mass theoretically deposited on each plate based on a standard of BuChE activity as it correlates to mass of protein.

2.9. Dry Powder Insufflation. Mice were anesthetized using ketamine and placed on a 45° board by their upper incisors, and a laryngoscope was used to facilitate insertion of the PennCentury insufflator device. Male C57BL/6 mice (n = 3) were dosed with 1 \mu m of cylindrical BuChE protein particles (fabricated and lyophilized as described above) at 2 mg fill weights to demonstrate potential for dry powder administration. The PennCentury was applied following standard manufacturer procedures and previously published methods.\textsuperscript{34,35} Briefly, the
dose was administered at the tidal volume of mice (200 μL) with five actuations of the air pump. The actual dose delivered was determined by weighing the device before and after dose actuations. Following administration, the mice were placed on a heated blanket and given a reversal agent (Antisedane) before being placed for monitoring. At each time point, the mice were harvested as described above. Briefly, BALF was collected with 1 mL of 1x PBS and lungs were imaged via IVIS.

2.10. Statistical Analysis. GraphPad Prism v6.0 was used for statistical analyses as shown in figures and described in figure legends. Biodistribution of BuChE data was analyzed using two-way ANOVA with Tukey’s multiple comparisons test compared to saline at 24 h. Cytokine response analysis and cell population distributions included two-way ANOVA with Tukey’s multiple comparisons test compared to saline 24 h with LPS excluded from analysis. BuChE activity in particles was analyzed using two-way ANOVA with Tukey’s multiple comparisons test. Biodistribution of 1 μm dry powder particles was analyzed using two-way ANOVA with Tukey’s multiple comparisons test compared to negative control.

3. RESULTS

3.1. Biodistribution of Orotracheal Administered BuChE in Nude Mouse Model. Dylight680 tagged BuChE was utilized to monitor biodistribution of BuChE postorotracheal administration in male Nude mice, n = 3 at 80 mg/kg (Figure 1). Upon harvest, lungs were lavaged and imaged via IVIS (Figure 1A), showing detection of BuChE up to 96 h with significant difference over negative control up to 72 h (Figure 1B). BuChE in the BALF was detected via IVIS up to 48 h postinstillation with a significant difference over the negative control at 24 h (Figure 1C) and correlated with BuChE activity that was detected up to 48 h (Figure 1D). BALF BuChE concentrations were measured from the fluid layer lining the airways of the lung. Lung concentrations were measured after bronchoalveolar lavage was collected. Comparing detection of BuChE in the lungs versus BALF at the same time point suggests uptake of BuChE by lung tissue the longer BuChE remained in the lungs.

Given the increased retention of BuChE in the lungs after pulmonary instillation, other organs were analyzed for potential BuChE accumulation at each time point with no BuChE detected in the spleen, kidney, liver, or plasma compartments at any time points (Figure S4). No endogenous BuChE was detected in mice treated with saline orotracheally. Furthermore, no additional BuChE was detected systemically over baseline.

3.2. Immunological Assessment of Orotracheal Administration of BuChE in Different Murine Models. Lung biology is a complex field that leaves many questions unanswered. To our knowledge, information is lacking on the biological effects in the lungs after direct biologic administration. We sought to understand the effect of BuChE administration on clearance times and immunological response in order to ultimately utilize the lung as a therapeutic target. Furthermore, previous studies have demonstrated that the particular mouse model used can affect results due to genetic/immunological variation.36 For our initial studies, Nude mice were chosen due to their naturally low endogenous plasma BuChE levels (Table S1), which would allow for easier detection of our dosed BuChE due to higher signal-to-noise ratio. However, to better understand the potential immunological response, examining additional mouse models was important to compare variance among different lung environments and better understand protein clearance from the lungs. Martin and Frevert detail how diverse the immune environments and better understand protein clearance from the lungs.37 Therefore, we used athymic Nude mice to represent a T-cell deficient lung, C57BL/6 to represent a normal human lung, and BALB/c mice to represent an immunocompetent lung.

Figure 1. Biodistribution of BuChE. Dylight680 tagged BuChE detected postorotracheal administration at 80 mg/kg (n = 3) shown in (A) representative IVIS images of treated lungs over time (color bar scale: min = 3.13 × 10^−4, max = 6.61 × 10^−4). (B) Lung and (C) BALF compartments in male Nude mice with (D) activity in the BALF measured. Results are representative of two repeated studies, n = 3 each. saline mice were included as controls. *p < 0.05, ***p < 0.001, ****p < 0.0001, n.s. = not significant; two-way ANOVA with Tukey’s multiple comparisons test compared to saline.
represent a typical asthmatic lung model. BuChE was orotracheally administered at 80 mg/kg with saline negative control and LPS positive control treatments. BALF and plasma samples were collected at 6, 24, and 48 h. BuChE activity was confirmed throughout (Figure S5). We tested cytokine levels of TNF-α (Figure 2A), IL-6 (Figure 2B), KC (Figure S6A), and MIP-2 (Figure S6B) in each mouse model in both the BALF and plasma (Figure S7). Alveolar epithelium derived cytokines and macrophage-derived cytokines (TNF-α, IL-6, and IL-8) were tested. MIP-2 and KC are the murine homologues to human IL-8. Overall, the Nude mice exhibited a greater initial increase in all cytokine levels than the BALB/c or C57BL/6 mice, respectively; however, the cytokine response subsided between 24 and 48 h in all three strains.

Furthermore, to determine the effect of BuChE pulmonary administration on cell recruitment in the lung, flow cytometry was used to examine the relative cell populations of macrophages, granulocytes, and dendritic cells in the BALF (Figure 2C). Representative flow cytometric analysis of BALF cells is shown in Figure S2. A clear influx of granulocytes was seen at 6 h in all three strains, with the Nude mice having a greater influx than the BALB/c or C57BL/6 mice. However, the granulocyte levels decreased to near saline levels by 48 h in all three-mouse strains. It is important to note that the commercial source of administered BuChE was found to contain endotoxins (data not shown).

3.3. Fabrication and Utilization of PRINT BuChE Protein Particles to Demonstrate Pulmonary Cascade Impactor Deposition. To establish particles for therapeutic use, we fabricated cylindrical PRINT particles with a diameter of 1 μm and a height of 1 μm (Figure 3A). Importantly, protein activity, crucial for use in protection against OPs, was maintained throughout processing (Figure 3B). Following fabrication, the particle composition was determined via HPLC, establishing the majority of the particles were composed of BuChE, with lactose and glycerol removed during the postfabrication processing. Particle composition was 98% BuChE, 1.15% lactose, and 0.51% glycerol for particles used in cascade impaction studies and in vivo (Figure 3C). Higher activity BuChE particles were 93% BuChE, 6.22% lactose, and 0.73% glycerol (Figure 3D). BuChE particles were then lyophilized for dry powder deposition analysis. Aerosol sizing was performed from a PennCentury insufflator device with an Andersen Cascade Impactor (ACI), which is typically used to correlate to human lung deposition for particles (Figure 4A). PennCentury is routinely used for precise dry powder delivery to mice; therefore utilizing the PennCentury device allows us to compare in vitro data to in vivo studies.

PRINT 1 μm cylindrical particles fell within the respirable range with aerodynamic properties indicative of deposition in peripheral airways, as compared to larger particles. The average mass median aerodynamic diameter (MMAD) was determined to be 2.77 μm with an average geometric standard deviation (GSD) of 1.39 and a fine particle fraction (FPF) of 82.90% based on percent-emitted dose that was less than 5 μm (Figure 4B). Aerosols generated from these particles show potential for ideal deposition in the human lung with implications beyond the scope of current clinical standards.
3.4. Dry Powder Insufflation of PRINT BuChE Protein Particles in C57BL/6 Mouse Model. After assessment of dispersion properties, 1 μm of BuChE cylindrical dry powder particles tagged with Dylight-680 were administered to C57BL/6 male mice to demonstrate DPI administration utilizing a PennCentury insufflator. BuChE particle distribution was determined in the BALF and lungs. BuChE particles were detected in the lungs using IVIS imaging (Figure 5A) with detection up to 72 h and significant difference over the untreated baseline up to 48 h (Figure 5B). BuChE in the BALF was detected up to 48 h with significant difference from untreated controls (Figure 5C) and a corresponding detection of activity for 48 h (Figure 5D).

4. DISCUSSION

Utilizing biologics in pulmonary delivery that have both optimal aerosol characteristics and biological stability is a critical goal for the field. Diseases ranging from cystic fibrosis, pulmonary hypertension, asthma, and lung malignancies, not to mention patients with lung transplants rejection,18,19,20 could benefit from direct local treatment using dry powder particles, which can be easily administered in a targeted, noninvasive manner.41 Our studies utilize a model protein, BuChE, for its potential use in treatment for organophosphate poisoning or pseudocholinesterase deficiency. Furthermore, our work serves as a stepping-stone for biologic based particles for dry powder inhalation. For example, adapting our methods for human growth factor can provide the lacking balance of maintaining activity with good aerosol characteristics for treating growth deficiency.1,13 The study presented here examined pulmonary administration of BuChE in vivo to better understand local lung profiles and any resulting immunological implications. Importantly, fabricated particulate BuChE maintained enzymatic activity and optimal deposition characteristics and subsequently displayed ideal deposition profiles.

We first tested free BuChE via orotracheal instillations to create a baseline comparison for our dry powder particle studies. Without further chemical modifications, the current PRINT formulation was instantly dissolvable upon instillation, allowing us to directly relate our particle formulation to the free BuChE molecule studied in aqueous in vivo environments (Figure S8). In our initial studies, orotracheally administered BuChE was readily detectable in the BALF and lungs. BALF BuChE concentrations represented the amount of enzyme residing in the fluid layer lining the airways of the lung. Lung concentrations represented BuChE amounts remaining in the lung after bronchoalveolar lavage. To our knowledge, BuChE detection and retention in lungs has not been previously addressed, but its presence could be indicative of why previous studies have shown protection against OPs after pulmonary administration of BuChE.18 It is hypothesized that as BuChE deposits in lungs it adheres or is taken up by cells and tissue, as seen in the 48 h spike, followed by a gradual clearance from tissue after 72 h (Figure 1).
It is likely removed through multiple clearance mechanisms of the lung, including involvement of peptidases and lung absorption. Additionally, the main alveolar epithelium barrier restricts systemic exposure of larger proteins (>50 kDa) delivered to the lung, explaining the lack of significant BuChE levels in the bloodstream after lung deposition. Current data show no presence of BuChE in plasma, spleen, kidneys, or liver up to 168 h; however, future exploration of BuChE detection in blood after 168 h with dosing of higher BuChE activity may yet offer opportunities for systemic detection.

Cell distribution and cytokine levels in lungs are important to understanding immunological response to direct BuChE treatment and future translation of enzyme delivery to the lung. As we saw in Figure 1, there was no endogenous BuChE detected in the BALF; we were interested if administration of an enzyme non-native to the lung would have adverse responses. Another essential component to assessing immunological response is to mimic the diverse lung population of human patients. We sought to do this by comparing three mouse models representing varied immune responses: a T-cell deficient lung with athymic Nude mice, a normal human lung with C57BL/6 mice, and an asthmatic lung with BALB/c mice. Many therapeutics are not effective due to unintentional stimulation of the host immune response affecting residence time and potentially being harmful to the host; therefore, it is important to assess the implications of BuChE administration. Furthermore, the immunological consequence of delivering active particulate enzyme to the lung is largely understudied. Previously published work demonstrated minimal immune response with pulmonary delivery of non-biologically active PRINT formulations of different shapes and sizes. Utilizing saline- and LPS-treated mice as our negative and positive controls, respectively, we observed that BuChE-treated mice initially showed an influx of granulocytes, followed by a restoration of baseline cell populations at 48 h. This indicated an acute response to the administered protein, which was alleviated 48 h postinstillation. It is important to note that active BuChE is still detectable in the BALF and lungs at 48 h. Furthermore, in all mouse models, there was an initial influx in cytokine levels postadministration of BuChE in the lungs, which then decreased within 48 h to normal levels. All mouse models showed cell distribution and cytokine level recovery in this time period, with Nude mice having a slightly more severe initial influx, BALB/c with an intermediate influx, and C57BL/6 with the least influx. This suggests that the athymic Nude mice have a delay in immune protection, while normal mice react rapidly to control the immune response. The initial influx in cytokine levels in all strains within BALF can be attributed to the actual addition of protein to lungs but was more likely due to endotoxin associated with the unformulated commercially available protein. This protein is routinely used in research studies and contains <1 EU/μg of protein, which is the typical endotoxin content of most commercially available proteins but sufficient to potentially elicit an innate immune response. Further studies need to be conducted with endotoxin-free BuChE to shed light on the source of inflammation. However, these promising results show that current BuChE administered directly to the lungs will not cause a severe, long-term immune response. Therefore, it can be predicted that protein particles will be a safe treatment option despite the diverse human lung population.

After establishing how free BuChE acts in the lungs, as well as establishing the best mouse model with minimal immune responses, we used PRINT technology to develop a BuChE dry powder particle formulation to provide efficient delivery of BuChE to the lung. With the amenability of PRINT, we were able to formulate the particles with our choice of BuChE source, allowing us to vary particle dose based on the activity concentration of the BuChE utilized in fabrication while maintaining composition profiles of greater than 90% active BuChE content (Figure 3). The fabricated 1 μm BuChE particle aerosol showed promise for deep lung delivery into the alveolar
region due to a high fraction being in the respirable range (less than 5 μm). Based on accepted translation of ACI data to the respiratory tract, 2–3 μm of MMAD would have a theoretical deposition near the secondary bronchi. However, several studies have shown that this can be altered due to numerous variables, including flow rate and person to person variability, potentially resulting in deeper deposition near the terminal bronchi. Furthermore, a low GSD of 1.39 was determined, which indicated minimal aggregation and easy dispersion of this formulation (Figure 4). This supported the use of 1 μm of BuChE cylinders for pulmonary delivery via DPI. In comparison, our commercial source of BuChE does not have particle size properties amenable to dispersion in the insufflator, as it was too bulky for dispersion using this method. Thus, the PRINT BuChE particle formulation is an ideal candidate for controlled and efficient BuChE airway deposition.

Successful in vitro testing of PRINT BuChE particles demonstrated the ability for targeted deposition; therefore, confirming a residence time comparable to free BuChE was necessary. The dry powder particles were detectable in both the lungs and BALF for 48 h at a significant difference over baseline, with corresponding activity detection in the BALF (Figure 5). In comparison, the free BuChE resided in the lungs for 48 h with some detection at 72 h, while BuChE in the BALF lingered between 24 and 48 h (Figure 1). It is important to note the difference in delivery methods of instillation, direct administration to the lung, of free BuChE versus insufflation of the particulate BuChE. The direct instillation guaranteed the entire dose is administered. However, with the dry powder insufflation, powder can be exhaled while dosing, swallowed into the stomach, etc.; therefore, the entire reported dose delivered does not reflect the actual dose that entered the lungs. Due to this, it is important to assess trends and limitations in each individual study. Furthermore, PennCentury insufflation is the main way to administer dry powder particles, but it has limitations including small dose loading chamber and low dispersion of dose due to limited mouse lung capacity. Utilizing this device is further limited by the expertise of the dose administrator to place the device accurately in the trachea on the first attempt. Nevertheless, this method allowed us to demonstrate the ability of our particles to have comparable residence time as free BuChE. Future studies will incorporate different pulmonary administration techniques, such as a nose-cone chamber with larger animal models. Most importantly, we have demonstrated that the particles, in current formulation, can maintain activity in vivo as long as the free BuChE with the advantage of targeted deposition, as shown via cascade impaction. This is promising for future translational studies utilizing our BuChE dry powder particles.

Overall, we have established that PRINT BuChE particles can provide targeted deposition as well as comparable residence time as free BuChE. The aerodynamic properties presented here can be further tuned by altering the geometric properties of the particles, which is readily achieved through the PRINT process. Based on previous work in our lab on modifying the controlled degradation of protein particles, we are now exploring ways to chemically tune the degradation of BuChE particles for extended release in a single dose administration. This approach will provide higher residence times and efficacy for future treatments, as well as adequate lung deposition characteristics, with minimal immunologic consequences. Future studies will translate application to larger animal models and the use of other biologics, including human BuChE. This work demonstrates effective and safe delivery of BuChE as a DPI formulation, which offers potential for novel clinical treatments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00066.

Representative flow cytometry analysis of BALF cells, assay validation, additional biodistribution, and cytokine analysis, as well as activity confirmation and particle imaging (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): J.M.D. is a founder and maintains a financial interest in Liquidia Technologies.

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ABBREVIATIONS

OP, organophosphate; BuChE, butyrylcholinesterase; PRINT, particle replication in nonwetting templates; DPI, dry powder inhaler; AChE, acetylcholinesterase; ACh, acetylcholine; i.e.,...
intravenously; i.p., intraperitoneally; i.m., intramuscularly; BALF, bronchoalveolar lavage fluid; IVIS, in vivo imaging system; LPS, lipopolysaccharide; PET, poly(ethylene terephthalate); HPLC, high performance liquid chromatography; ACI, Andersen Cascade Impactor; MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation; FPF, fine particle fraction.

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