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Poly(D,L-lactide-co-glycolide) Nanoparticle Agglomerates as Carriers in Dry Powder Aerosol Formulation of Proteins

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Abstract

A dry powder aerosol drug delivery system was designed with both nano- and microstructure to maximize the protein loading via surface adsorption and to facilitate delivery to the deep lung, respectively. Ovalbumin was employed as a model protein to adsorb to and controllably flocculate DOTAP-coated PLG nanoparticles into “nanoclusters” possessing low density microstructure. The mechanism of nanoparticle flocculation was probed by evaluating the effects of ionic strength, shear force, and protein concentration on the geometric and aerodynamic diameters of the nanoclusters as well as the protein adsorption efficiency. Salt ions were found to compete with ovalbumin adsorption to nanoparticles and facilitate flocculation; therefore, formulation of nanoclusters for inhaled drug delivery may require the lowest possible ionic strength to maximize protein adsorption. Additional factors, such as shear force and total protein–particle concentration can be altered to optimize nanocluster size, suggesting the possibility of regional lung delivery. Immediate release of ovalbumin was observed, and native protein structure upon release was confirmed by circular dichroism and fluorescence spectroscopy studies. Controlled flocculation of nanoparticles may provide a useful alternative to spray drying when formulating dry powders for pulmonary or nasal administration of protein therapeutics or antigens.

1. Introduction

Inhaled aerosols are an effective means to treat diseases infecting the lung, including asthma and cystic fibrosis, which require treatment locally in the bronchial or alveolar region.^{1,2} Alternatively, the large surface area of the pulmonary system (~140 m²), attributable to the alveolar epithelium in the deep lung region, and ready access to the circulation offer the potential for noninvasive systemic delivery of macromolecular therapeutics including peptides, proteins, DNA, etc.^{3–8} Efficiently administering aerosols to the deep lung remains an encumbrance when locally treating lung tissue or when utilizing the lung as a gateway for systemic drug delivery.⁹

Deposition of inhaled particles in the deep lung requires significant control of the particle size, a major deterrent to utilizing the lung for drug delivery.^{9–11} Particles possessing an aerodynamic diameter greater than 15 μm are subject to deposition in the mouth and throat, while submicron particles are commonly exhaled.^{12–14} Studies have consistently verified that ~1–3 μm solid particles deposit most efficiently in the deep lung, that is the bronchiole and alveolar region.^{12–14} In light of this, low density particles are being developed in several

laboratories as a means to deliver drugs to the deep lung.^{9,15–17} These particles possess large geometric diameters (d_p), but because of their low mass densities (ρ) exhibit much smaller aerodynamic diameters (d_{aero}) as described by eq 1,¹⁵

$$d_{aero} = d_p [(\rho/\rho_{ref})/\gamma]^{0.5} \quad (1)$$

where ρ_{ref} is a reference mass density (e.g., 1 g/cm³) and γ is a dynamic shape factor (typically 1 for a sphere).

In addition to improvements in aerosol microstructure, other studies have focused on developing nanoparticles as drug delivery vehicles to the pulmonary system.^{18,19} Nanoparticles may not be desirable as aerosols since submicron particles are often exhaled following administration.^{12–14} Particles of this size also suffer from a propensity to agglomerate uncontrollably,^{20,21} thus reducing their utility as aerosols. An advantage that nanoparticles offer to pulmonary drug delivery is the increased surface area (per total particle mass) available for drug adsorption.²² Moreover, macromolecules adsorbed to the surface of nanoparticles may offer the advantage of improved stability and activity as compared to encapsulated proteins.²³

Poly(D,L-lactide-co-glycolide) (PLG) is an attractive polymer for fabricating nano- and microparticles for drug delivery because of its biodegradability and biocompatibility;²⁴ however, despite its approved use in parenteral formulations, the use of PLG in inhaled drug formulations remains to be accepted. Concerns surrounding the slow degradation rate of PLG and the potential for accumulation of the polymer in the lungs are obstacles in the development of such pulmonary delivery vehicles that remain to be evaluated;^{25–27} however, recent work by Kissell and others demonstrate the safety of PLG nanoparticles compared to nondegradable nanoparticles (polystyrene) in rats.²⁷

We have designed an inhalable dry powder formulation possessing both nano- and microstructure to increase adsorption of a macromolecular drug and improve delivery to the deep lung, respectively. Surface charge interactions were exploited for controlled flocculation of nanoparticles using ovalbumin as a model protein drug, and various properties of the nanoparticles were evaluated for their effect on protein adsorption and flocculate properties. Factors such as surface charge of the nanoparticles, ionic strength, shear force, protein–particle ratio, and total protein–particle concentration were found to influence the formation, size, and density of the flocculated nanoparticles, or “nanoclusters.” Ovalbumin was immediately released from the lyophilized nanoclusters into 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl (PBS) and maintained native protein structure as determined by fluorescence and circular dichroism (CD) measurements. This formulation approach leverages agglomeration of nanoparticles in solution as protein carriers and may serve as an alternative to spray drying when formulating dry powder aerosols. Ultimately, we believe that the nanocluster delivery system may enhance the transport of macromolecules to the deep lung for both local and systemic delivery with the potential for delivering synergistic, combination therapeutics.

2. Materials and Methods

2.1. Materials

PLG (50:50, 0.41 dl/g) was purchased from Lactel (Pelham, AL). Poly(vinyl alcohol) (88 mol% hydrolyzed; PVA) was purchased from Polysciences, Inc. (Warrington, PA). 1,2-Dioleoyl-3-trimethylammonio propane, chloride salt (DOTAP) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids, Inc.

(Alabaster, AL). Albumin from chicken egg white (ovalbumin, grade V, minimum 98%) was purchased from Sigma (St. Louis, MO). All other reagents were purchased in high purity from Fisher Scientific (Pittsburgh, PA) and used without further purification.

Aqueous solutions of each lipid were prepared by first drying and then redissolving the lipid in water. Briefly, lipid in chloroform was added to a volumetric flask. A stream of nitrogen was used to dry the lipid in the bottom of the flask while gradually rotating the flask to create a film. Distilled, deionized water (ddH₂O) was added to the flask containing the dry lipid film, and the flask was swirled and sonicated to mix and dissolve the lipid.

2.2. Fabrication of Charged Nanoparticles

A modified emulsion solvent evaporation method^{28,29} was employed to create cationic, anionic and neutral nanoparticles. Briefly, nanoparticles were fabricated by sonicating (50% amplitude) or homogenizing (30 000 rpm) 3 mL of 1.67% (w/v) PLG dissolved in an acetone/methanol (5/1) solvent mixture into 25 mL aqueous solution containing one of the following: DOTAP (cationic), dioctyl sulfosuccinate (DSS) (anionic), cetyltrimethylammonium bromide (CTAB) (cationic), or PVA (neutral). DOTAP and CTAB nanoparticles were prepared using sonication, while all others were prepared using homogenization. The suspension was stirred for 4 h to evaporate the solvent. The precipitated nanoparticles were then washed with ddH₂O and sonicated in a water bath to break up any loosely associated agglomerates. The nanosuspension was then filtered through a KimWipe laboratory tissue to remove large, particulate matter (i.e., agglomerates, film-like residue, etc.) and characterized.

2.3. Characterization of Nanoparticles

The geometric diameters and polydispersities of the nanosuspensions were evaluated using dynamic light scattering (Brookhaven Instruments Zeta Potential Analyzer, Holtsville, NY). Nanoparticles were diluted in ddH₂O (~100×), and three 1 min measurements were obtained at 25 °C for each nanosuspension. The same instrument was employed to quantify the zeta potential, an indicator of surface charge, of the nanoparticles in 1 mM sodium nitrate solution. Three runs of 15 cycles were acquired, and the mean zeta potential (ζ) was recorded.

2.4. Adsorption of Ovalbumin to Charged Nanoparticles

Ovalbumin was employed as a model macromolecular drug for these studies. Working solutions of ovalbumin in PBS were prepared, and the exact concentration of each was determined by UV absorbance spectroscopy (Agilent 8453) using the absorbance at 280 nm and an extinction coefficient of 0.696 mL/mg · cm. Aliquots (100 μ L) of ovalbumin working solutions were added to a constant mass of cationic, anionic, and neutral nanoparticles (1 mL) in 2 mL microcentrifuge tubes (final salt concentration was 14 mM). Samples were prepared in triplicate and tumbled end-over-end for 1 h at 4 °C to facilitate adsorption. Centrifugation (14 000g) was used to pellet the particles, and the supernatant was assayed for unbound ovalbumin using UV absorbance spectroscopy.

Total surface area was estimated using the following process:³⁰ (1) the mean diameter measured by dynamic light scattering was used to calculate the surface area of a single nanoparticle, (2) the mass of a single nanoparticle was estimated using the volume of a single nanoparticle and the absolute density of PLGA, measured previously in our laboratory using a helium pycnometer,³¹ (3) the concentration of nanoparticles (determined by lyophilizing a known volume of the nanosuspension in a preweighed tube and then weighing the mass of particles postlyophilization) and the mass of a single particle was used to calculate the total number of particles, and (4) the surface area of a single nanoparticle was

multiplied by the total number of particles to arrive at the total surface area. Although the calculations do not provide an absolute value for total adsorptive surface area, we do believe that this approach yields a close approximation.

2.5. Flocculation of DOTAP Nanoparticles with Ovalbumin

2.5.1. Effects of Ovalbumin Concentration, Ionic Strength, and Shear Force during Tumbling on Flocculation

—The mechanism of flocculation of the DOTAP nanoparticles in the presence of ovalbumin was probed by comparing flocculates prepared in ddH₂O and PBS to evaluate effects of ionic strength. Additionally, the effect of headspace during tumbling (i.e., shear force) was evaluated by comparing the size of the resulting flocculates prepared in 2 mL microcentrifuge tubes and 15 mL centrifuge tubes. To each tube, ovalbumin working solution (408 μ L) in either ddH₂O or PBS was added to 400 μ L of DOTAP-coated nanoparticles (1.16 mg) to yield a weight ratio of ovalbumin to nanoparticles on the order of approximately 0, 0.01, 0.10, 0.30, and 0.60. The concentrations of ovalbumin working solutions were 0, 0.028, 0.28, 0.85, 1.7 mg/mL, respectively. Blanks (without ovalbumin) were prepared to evaluate the role of the buffer salts in flocculation. The final salt concentration in all samples prepared using PBS was ~81 mM. The samples were tumbled end-over-end at 4 °C for 1 h, and the geometric diameters of the resulting flocculates were measured using a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). Additionally, the effect of protein adsorption on the surface charge was evaluated using a zeta potential analyzer. Forty microliters of ovalbumin-adsorbed nanoparticles were added to 2 mL of 1 mM sodium nitrate solution. The zeta potential was quantified as described above. Furthermore, the remaining sample was centrifuged to pellet the particles, and the supernatant was assayed for unbound protein concentration using UV absorbance spectroscopy.

2.5.2. Effects of Total Protein-Particle Concentration on Flocculation—A weight ratio of 0.66 ovalbumin to nanoparticles and a total mass of 1.74 mg of ovalbumin and nanoparticles were maintained for all samples. Aliquots of the DOTAP-coated nanoparticle suspension (400 μ L) were added to 15 mL centrifuge tubes and diluted by adding 500 μ L, 2, 5, or 10 mL of PBS or ddH₂O. A 408 μ L aliquot of the appropriate 2.81 mg/mL ovalbumin working solution (PBS or ddH₂O) was then added to each tube to yield a final total concentration of 1.3, 0.62, 0.33, and 0.16 mg/mL, respectively. The samples were treated as described above to facilitate flocculation and analyzed using a Multisizer 3 Coulter Counter to measure the geometric diameters of the flocculates.

2.6. Dry Powder Nanoclusters

Flocculates (808 μ L) were prepared in triplicate in 2 mL microcentrifuge tubes containing a weight ratio of ovalbumin to nanoparticles of ~0.01, 0.10, 0.30, and 0.60 in either ddH₂O or PBS as described above. The final ionic strength of the nanosuspensions prepared using PBS was ~81 mM. To maximize sample recovery, the flocculates from each of the three 2 mL tubes were combined into a 15 mL centrifuge tube, frozen at –80 °C, and lyophilized using a Labconco benchtop lyophilizer (Kansas City, MO).

2.6.1. Characterization of Nanocluster Particle Morphology—The size and morphology of the dry powder nanoclusters were evaluated using a LEO 1550 field emission scanning electron microscope (SEM) with secondary electron detection. The flocculates were coated on a platform and sputtered with gold prior to imaging at 10 000 $\times$ magnification.

2.6.2. Characterization of Nanocluster Aerosol Properties—The aerodynamic diameters of the dry powder nanoclusters were evaluated using an Aerosizer LD (Amherst

Process Instruments Inc.). Data were collected over ~70 s using a 300 μm nozzle and low shear force.

2.6.3. Tap Test Method To Determine Bulk Powder Density—The bulk density of the dry powder was estimated using a microtap test approach. Briefly, dry powder nanoclusters were added to preweighed microcentrifuge tubes, and the tubes were weighed back to determine the mass of powder. The tubes were then tapped on the benchtop 30 times to compress the dry powder. The volume of the powder was approximated by comparing the compressed powder level to the level of a known volume of water. The density was calculated by dividing the mass of powder by the approximated volume. Samples were analyzed in triplicate in most cases, but because of a sample shortage, the powder at a protein–particle ratio of 0.28 prepared in ddH₂O was only evaluated in duplicate. The bulk powder densities of the nanoclusters prepared in ddH₂O at protein–particle ratios of 0.02 and 0.10 were not quantified because of lack of sample.

2.7. Release of Ovalbumin from Nanoclusters

Studies were conducted to evaluate the release kinetics of ovalbumin from the dry powder nanoclusters. Release was monitored in PBS and in an aqueous suspension of DPPC vesicles (13.25 mg/mL). Nanoclusters (78.1 and 35.6 mg) were suspended in 15 mL of PBS and 10 mL of DPPC, respectively. Aliquots (500 μL) were transferred to 2 mL microcentrifuge tubes and incubated at 37 °C while shaking at 175 rpm. Duplicate samples (time = 0) were immediately centrifuged at 13 000g for 30 min to pellet the particles. The supernatant was assayed for protein concentration by UV absorbance spectroscopy (supernatant from DPPC samples were diluted 2 $\times$ to minimize interference with absorbance near 280 nm). After removal of the supernatant, the remaining pellet was dissolved in 40 μL of dimethyl sulfoxide (DMSO), and the polymer was then precipitated by addition of 350 μL of PBS. The samples were centrifuged at 13 000g for 10 min to pellet the polymer, and the supernatant was again assayed for unreleased protein concentration by UV absorbance spectroscopy.

2.7.1. Stability of Released Ovalbumin—After flocculate formation and prior to lyophilization, the flocculates were washed with ddH₂O to remove excess, unbound ovalbumin. To wash the flocculates, the samples were centrifuged at 500 rpm for 5 min. The supernatant was discarded, and the flocculates were resuspended in ddH₂O. This was repeated, and the washed flocculates were resuspended in ~2 mL of ddH₂O, frozen at –80 °C, and lyophilized. Since immediate release of ovalbumin from the nanoclusters was observed (see Results), the lyophilized nanoclusters were resuspended in 1 mL of PBS to release ovalbumin and centrifuged at 14 000g for 10 min to pellet the particles. The supernatant was isolated and analyzed using UV absorbance, fluorescence (PTI QuantMaster, Lawrenceville, NJ), and circular dichroism (CD; Jasco J-810, Easton, MD) spectroscopy to evaluate the conformational stability of the released ovalbumin. UV absorbance was employed to determine the concentration of ovalbumin in the samples. Fluorescence studies utilized the tryptophan (Trp) residues of ovalbumin as an intrinsic fluorescence probe sensitive to the polarity of their environment to determine whether the tertiary structure of ovalbumin was altered during flocculation. Fluorescence spectra of the released ovalbumin and a control containing ovalbumin in PBS were collected from 305–400 nm using an excitation wavelength of 295 nm (>95% Trp emission). CD was employed to evaluate whether the secondary structure of ovalbumin was altered during flocculation. CD spectra of the sample and control were acquired in three accumulations from 260–195 nm with a scanning speed of 50 nm/min and 1.0 nm resolution.

3. Results

3.1. Characterization of Charged Nanoparticles

The sizes and surface charges of PLG nanoparticles were modified using four different charged coating materials (Table 1). Particles of approximately 200–300 nm were created with relatively low polydispersity values (<0.16). Polydispersity is the relative standard deviation of a sample size; therefore, the low polydispersity value suggests a narrow size distribution of particles, or minimal variance of particle sizes. In some cases, the size of the nanoparticles changed with concentration of coating material (data not shown). Furthermore, the particle surface charge was modified by including various charged compounds in the aqueous phase during particle synthesis. The resulting particles were strongly positively charged ($+42.6 \pm 3.2$ mV) when using DOTAP as the surfactant and less positively charged ($+23.0 \pm 1.7$ mV) when using CTAB. Neutral particles (-2.6 ± 3.2 mV) were achieved using PVA, and particles with a strong negative charge (-36.4 ± 2.1 mV) were generated using DSS as the coating material.

While the surface charge of most particles remained constant for different preparations of the same type of particle, the particles prepared with CTAB showed drastic variation between batches prepared using the exact same technique. To investigate this, the zeta potential was measured after each wash step. These measurements revealed a progressively declining surface charge of the CTAB-coated nanoparticles after as many as five subsequent washes (data not shown). This suggests that CTAB was gradually washed off the surface of the nanoparticles. CTAB was not pursued in further flocculation studies. Wischke et al. also recently reported a finding similar to ours using the double emulsion procedure.³² They observed agglomeration of the nanoparticles during washing and also attributed this to desorption of the CTAB.³²

3.2. Adsorption of Ovalbumin to Charged Nanoparticles

The isoelectric point (pI) of the model macromolecular drug, ovalbumin, is 4.6;³³ therefore, a net negative charge on the protein is expected at neutral pH. The increased adsorptive capacity observed for binding of ovalbumin to the cationic nanoparticles (DOTAP and CTAB) suggests that electrostatic forces may be at least partially responsible for adsorption (Figure 1). Interestingly, ovalbumin also adsorbed to the anionic nanoparticles, albeit at much lower levels (approximately 2-fold less), suggesting that other forces (e.g., van der Waals, hydrogen bonding, apolar interactions, etc.) must also play a role. The least amount of binding was observed for the PVA-coated nanoparticles. This may be attributed to the neutral, hydrophilic nature of the particles possessing no attractive forces for binding of ovalbumin.

3.3. Flocculation Studies

During the adsorption studies, flocculation was induced during tumbling in the presence of ovalbumin for the samples containing DOTAP nanoparticles; therefore, a series of experiments were conducted to evaluate the properties and characterize the mechanism of flocculation of these materials.

3.3.1. Effects of Ovalbumin Concentration and Ionic Strength—Comparison of the geometric sizes and size distributions of the flocculates revealed that the presence of salt enhanced the ability of the nanoparticles to form flocculates (Figure 2), as indicated by the presence of a peak measured by the Multisizer 3. In the absence of salt (Figure 2A), the particles did not readily flocculate except when the ratio of ovalbumin to particles was 0.12 (w/w). When considering these findings, it should also be considered that Multisizer 3 measurements are conducted in ionic diluent (Isoton II). This influence of ionic strength on

formation of the flocculates confirms a charge–charge interaction, not an agglomeration or aggregation event. At weight ratios of 0 and 0.01 in the absence of salt in the 2 mL tubes, some larger flocculates ($\sim 11\text{--}12\ \mu\text{m}$) were detected, yet significant nanoparticle structure having the same particle diameter as the nascent nanosuspension remained present as detected by dynamic light scattering. A similar result was observed for flocculates at a weight ratio of ovalbumin to nanoparticles of 0.33 prepared in a 15 mL tube, where shear during tumbling would be greater than in the 2 mL tube; however, the nanoparticle size was slightly larger than the nascent nanoparticles (i.e., $399.2 \pm 13.4\ \text{nm}$ vs $298.6 \pm 3.9\ \text{nm}$). Furthermore, at these concentrations of ovalbumin, the distributions showed increased noise (i.e., standard deviations) compared to the 0.12 weight ratio. At lower concentrations of ovalbumin, this noise may be attributed to equilibrium between flocculated and free nanoparticles resulting from an excess of nanoparticles or a balance between nanocluster assembly and shear occurring at the measurement orifice on the Multisizer 3. Ovalbumin begins to saturate the particle surface and eventually tethers nanoparticles together, forming flocculates at a weight ratio of 0.12, and the nanocluster structure stabilized, resulting in a higher signal-to-noise ratio. At higher ovalbumin content, however, the protein saturates the nanoparticle surface, neutralizing the nanoparticle's surface charge. This results in a smaller sized flocculate and eventually inhibits flocculate formation as more ovalbumin is added.

A similar result was observed at low ovalbumin–particle ratios in the presence of salt (Figure 2B). Generally, salt led to enhanced flocculate formation (i.e., signal-to-noise) at all weight ratios of ovalbumin to nanoparticles when compared to flocculates formed in water alone. At protein–particle ratios of 0 and 0.01, the size distributions remained relatively noisy and revealed flocculate sizes larger than at higher ovalbumin content. In this case, the salt ions may have bound to the particles, thereby shielding some of the positive charge on the nanoparticle surface and facilitating flocculation, even in the absence of ovalbumin. Similar to flocculate formation in the absence of salt, there seemed to be an optimal protein–particle ratio of ~ 0.13 (w/w) in the presence of salt. At this concentration, the size distribution was less noisy than at lower ovalbumin concentrations, although the difference is not as distinct as in the absence of salt (Figure 2). Contrary to the data without salt, flocculate formation occurred completely at higher ratios of ovalbumin to nanoparticles. The ability to form flocculates at higher ovalbumin concentrations in PBS was probably due to charge neutralization of the particles and protein by the salt ions. Further evidence of this is the reduced ovalbumin content adsorbed to the surface of the nanoparticles in the presence of salt (Table 2). Additionally, the data in the presence of salt displayed a general decrease in flocculate size with increasing ovalbumin concentration (Figure 2B). This may be attributed to the greater charge shielding on the nanoparticles as more ovalbumin is adsorbed.

The roles of ovalbumin and salts in facilitating flocculation also have important implications on the amount of drug that can be loaded. In the absence of salt, only ovalbumin was involved in flocculation; therefore, a greater amount of ovalbumin can be incorporated into the flocculates (Table 2). At ovalbumin–particle ratios of ~ 0.12 , approximately 3–4 times more ovalbumin is incorporated into the flocculates in ddH₂O than in PBS. At other concentrations of ovalbumin, the relative effect was the same; however, at a weight ratio of ~ 0.66 , the amounts of ovalbumin incorporated into the flocculates were relatively equal in both ddH₂O and PBS. The reduced ovalbumin adsorption in the presence of PBS suggests competitive binding between the salt ions and ovalbumin. While this finding reveals interesting mechanistic properties of flocculate formation, it may have an even greater impact on guiding formulation development of these inhalable drug delivery systems.

To further characterize adsorption of ovalbumin to the cationic DOTAP-coated nanoparticles, zeta potential measurements were acquired as a function of ovalbumin

concentration (Figure 3). As more ovalbumin was adsorbed, the surface charge was reduced, neutralized, and became slightly anionic upon saturation of the nanoparticle surface. The negative charge of the ovalbumin-saturated nanoparticle is not surprising given the acidic pI of the protein. In the absence of salt, binding of ovalbumin became saturated at approximately 70 μg ovalbumin ($\sim 3.5 \mu\text{g}$ ovalbumin adsorbed per total surface area). Conversely, saturation of ovalbumin was not achieved within the range of concentrations evaluated in the presence of salt. In fact, the binding curve in the presence of salt seemed to be relatively linear beyond $\sim 2.5 \mu\text{g}$ ovalbumin added per total surface area (Figure 3, black squares). These findings again argue that the binding of ovalbumin is affected by the charge shielding of the salt ions and suggests a binding equilibrium strongly dependent on the unbound protein concentration.

The plots of zeta potential vs ovalbumin concentration provide some helpful insight into the mechanism of flocculation (Figure 3, gray symbols). A protein–particle ratio of 0.12 (w/w) corresponds to 2.0 μg ovalbumin per total surface area. At this point, the nanoparticle surface was nearly saturated, and the nanoparticle surface charge was approaching zero. This argues that saturation of the nanoparticle surface led to enhanced flocculation, which is not surprising given that electrostatic attractive forces between particles would be the greatest when the particle surface is saturated.

3.3.2. Effects of Headspace during Tumbling—The studies evaluating the effects of salt and ovalbumin concentration on flocculation were performed using 2 and 15 mL tubes using a constant volume to evaluate the effect of agitation and shear during tumbling on the flocculate size. In most cases, preparation of the flocculates in the 15 mL tube resulted in flocculates $\sim 2 \mu\text{m}$ smaller than those prepared in the 2 mL tube (Figure 2, black vs gray). An increase in shear was expected to disrupt flocculate formation and reduce flocculate size. This suggested that shear force may be another variable that could be tuned to optimize the size of nanoclusters to achieve $\sim 100\%$ fine particle fraction ($< 5 \mu\text{m}$).

At weight ratios of 0 and 0.01 ovalbumin to nanoparticles in ddH₂O, large flocculates (~ 11 – $12 \mu\text{m}$) were formed in the 2 mL tubes that were not observed in the 15 mL tubes (Figure 2A). This could be attributed to the increased shear in the larger tubes during tumbling, leading to disintegration of any flocculates formed. In the case of the flocculates formed in the absence of ovalbumin, the result was surprising given that the ionic strength was zero and the only materials in solution were ddH₂O and the nanoparticles. Since the properties of the nascent nanoparticles were retained for these samples as determined by dynamic light scattering measurements, it is possible that the process of measuring the geometric diameter using the Multisizer 3 Coulter Counter may have induced flocculate formation. This technique involves suspension of the particles in an isotonic saline solution prior to measurement; therefore, the brief exposure (~ 30 s) of samples to this solution may have been enough to facilitate a small degree of flocculation. This explanation, however, was not supported by the data for flocculates prepared in the 15 mL tube since no flocculates were detected.

3.3.3. Effects of Total Protein-Particle Concentration on Flocculate Size—Since the incidence of encountering another particle would be reduced at more dilute concentrations of protein and nanoparticles, a constant ratio of ovalbumin to nanoparticles was diluted to four different concentrations. A protein–particle ratio of 0.66 (w/w) was randomly chosen for these studies. At this ratio, no flocculates were formed in ddH₂O (see results described above). The same was observed for all dilutions in ddH₂O (Figure 4A). In PBS, however, the flocculate size achieved at a weight ratio of 0.66 ovalbumin to nanoparticles was affected by the overall protein–particle concentration (Figure 4B). As the system was diluted from 1.33 to 0.62 to 0.30 mg/mL, a decrease in flocculate size was

observed. This can be attributed to a reduced incidence of encountering another protein molecule, nanoparticle, or flocculate. At 0.16 mg/mL, the flocculate size was increased slightly from 0.33 mg/mL, a likely result of the decreased shear during tumbling since the headspace was reduced to only ~4 mL in this sample (compared to ~9 mL for 0.33 mg/mL, ~12 mL for 0.62 mg/mL, and ~13.5 mL for 1.3 mg/mL samples). Additionally, this may be due to a more pronounced influence of salt in the flocculate formation since the concentrations of ovalbumin and salt were changing inversely to one another, and thus the incidence of encounter between protein and particle is decreasing as that of salt and particle is increasing. This explanation is further supported by the larger flocculate size observed in the absence of ovalbumin when 10 mL of PBS was used to dilute the nanosuspension (Figure 4B).

3.4. Characterization of Dry Powder Nanoclusters

After lyophilization of the flocculates, the aerosol properties of the dry powder nanoclusters were evaluated. For these studies, no additional excipients were added, and the dry powder was analyzed as recovered after freeze-drying without additional processing. In general, nanocluster formulations possessed an aerodynamic diameter desirable for delivery to the deep lung (i.e., ~1–3 μm) (Table 3).¹³ Given the large aperture of the Aerosizer nozzle (i.e., 300 μm), it is expected that larger particles would not be excluded from the measurement, suggesting accurate aerodynamic diameters. Interestingly, the mean aerodynamic diameter of nanoclusters prepared in PBS was usually slightly smaller than for those prepared in ddH₂O, and the standard deviations were also smaller. This was also evident in the narrower size distribution observed for the nanoclusters prepared in PBS (data not shown). Furthermore, the effect of ovalbumin concentration on the aerosol properties of the flocculates was not significant (Table 3), suggesting that factors such as salt concentration and shear force may be more important for controlling the particle size while the protein concentration need only be maximized according to the dose. Additionally, negligible sticking of the nanoclusters to the chamber walls was observed, suggesting that nanocluster powders may mitigate problems associated with deposition inside the inhaler common for some dry powder aerosol formulations.

Comparing the geometric and aerodynamic diameters of the nanoclusters (Figure 2 and Table 3, respectively), the density of the material was expected to be rather low, despite large standard deviations for both measurements due to broad particle size distributions resulting from the irregular shapes of nanoclusters. This was confirmed by microscopy studies and quantified using a microtap test. SEM images revealed that the nanoclusters are highly porous and irregularly shaped (Figure 5), a desirable characteristic for enhancing dry powder aerosolization and flowability, respectively.^{31,34} The bulk density of the powders ranged from 0.15–0.26 g/mL (Table 3). For nanoclusters prepared in PBS, the powder density seemed to decrease as the protein–particle ratio was increased (Table 3). This could be attributed to an increasing excess of free protein in the powder. Since the lyophilized protein is less dense than the nanoparticles, an excess of protein would be expected to yield a reduction in powder density. A similar trend was observed for the nanoclusters prepared in ddH₂O (Table 3). The density of the nanocluster powder prepared in ddH₂O at a protein–particle ratio of 0.28 was greater than that prepared in PBS at the same ratio. This supports the conclusion that free ovalbumin in the powder leads to a decreased powder density since more protein was adsorbed to the particles in ddH₂O than in PBS.

3.5. Release Studies and Stability of Released Ovalbumin

Release of ovalbumin from the nanoclusters was evaluated in PBS. Measuring the concentration of ovalbumin released from the nanoclusters into PBS revealed that ~100% of the ovalbumin was released immediately. The amount of ovalbumin remaining in the pellet

(i.e., ovalbumin not released) was also determined. There was no ovalbumin detected in the pellet, further confirming that ~100% of the ovalbumin was released immediately into PBS. The release of ovalbumin was also investigated using a lung surfactant model (DPPC); however, no differences were observed in the rate of release. This indicates that nanoclusters may be useful for delivery of a “rapid release” drug or a synergistic combination of drugs in which the adsorbed drug works cooperatively with a controlled-release drug encapsulated in the nanoparticles.

Spectroscopic techniques were employed to evaluate the stability of ovalbumin upon release. Intrinsic Trp fluorescence spectra of ovalbumin in PBS and after release from the nanoclusters showed peak maxima at approximately 330 nm (Figure 6A). CD spectra of the released ovalbumin and the protein dissolved in PBS were also very similar and displayed double minima at 208 and 222 nm, characteristics representative of significant α -helix character (Figure 6B). These findings suggest that the native tertiary and secondary structures of ovalbumin are retained upon release from the nanoclusters.

4. Discussion

The large surface area available for drug absorption in the lung makes the pulmonary route very attractive for delivering drugs systemically.¹⁵ Additionally, dry powder inhalers (DPIs) are noninvasive, easy to use and improve bioavailability of macromolecular drugs by avoiding unfavorable environments (e.g., gastrointestinal tract in the case of oral delivery).^{7,35} Unfortunately, a major obstacle of efficiently administering dry powder aerosols to the lungs exists.⁹ Control of particle size has been said to be “the single most important design variable of a DPI formulation”.³⁵ Particles must be ~1–3 μm to become successfully deposited with high efficiency in the deep lung.^{12,13} Exhalation of smaller particles and impaction in the cheek and throat of larger particles greatly reduces the delivery efficiency of suboptimal sized particles.^{12,13}

Despite the historical focus of drugs for pulmonary delivery being on small molecules, recent efforts have been made to develop inhalable protein formulations for human insulin (diabetes), interleukin-1 receptor (asthma therapy), heparin (blood clotting), α -1 antitrypsin (emphysema and cystic fibrosis), interferons (multiple sclerosis and hepatitis B and C), and calcitonin and other peptides (osteoporosis).⁵ Although the first FDA-approved inhalable insulin (Exubera) was pulled from the market in October 2007 because of low demand resulting from high costs to the consumers, acceptance of this product serves as motivation for the development of other inhalable macromolecular drug formulations. For delivery of adsorbed macromolecules, the large particle size needed for pulmonary delivery greatly diminishes the amount of drug that can be delivered for a given mass of powder. The increased surface area available for adsorption to nanoparticles is much more attractive for delivering large amounts of an adsorbed drug. The novel nanocluster system described here provides a formulation strategy to combine both nanostructure for maximum drug loading and microstructure for efficient delivery to the deep lung.

Optimization of adsorption properties is a critical step in the development of an inhalable formulation of nanoclusters since the protein must be adsorbed to carrier particles. For comparison, a 3 mg Exubera blister dose contains 5.1 mg of powder (insulin plus excipients), of which only 1.0 mg of insulin is successfully delivered to the deep lung³⁶ (the remainder is retained in the blister pack or becomes caught in the mouth and throat upon inhalation). This calculates to 0.20 mg of insulin delivered per mg of material (i.e., 1.0 mg insulin/5.1 mg total powder). For the nanoclusters reported here, the calculated ratio is much lower, suggesting that more material would be needed for delivery of the same dose of drug. It is possible to further refine nanocluster formulations for maximum protein adsorption by

selecting an alternative coating material for nanoparticles (of opposite charge relative to the pI of the protein), by optimizing ionic strength and pH of the solution to maximize charge interactions, by varying the shear force during flocculation and by adjusting the relative concentrations of protein and nanoparticles. Alternatively, it may be desirable to chemically cross-link a positively or negatively charged tag (e.g., histidine tags used in protein purification) to the therapeutic protein to facilitate charge–charge interactions. This approach may be useful for the delivery of proteins possessing a pI ~ 7.

The difficulties of simulating drug release/dissolution in the lung remain a substantial barrier to formulation testing. DPPC is the major component of lung surfactant;^{37,38} however, a solution of DPPC at the natural concentration is above the critical micelle concentration. Thus there exists a suspension of DPPC vesicles in an aqueous medium at that concentration. Release studies frequently utilize a suspension of particles immersed in the selected release medium, which does not accurately represent the environment upon deposition in the lung. Instead, when a drug is delivered to the deep lung, the drug sticks to the surface of the lung surfactant and is not actually immersed, but this remains very difficult to model in vitro.

Only a few techniques are practiced for producing DPI products: spray drying, spray freeze-drying, milling, and precipitation methods.^{39–41} While these techniques can be useful for micronizing small molecule drug compounds, the processes can often be too harsh for biomacromolecules.³⁹ Milling often causes degradation of biomacromolecules resulting from the extreme energy required for breaking apart coarse particles. This technique also provides minimal control over particle properties, a necessity for efficient pulmonary drug delivery.⁴¹ Spray drying has had limited success for micronizing biopharmaceuticals but is desirable since particles can typically be generated in a onestep process. Meanwhile, particle size remains difficult to control, and degradation of the biopharmaceutical product due to the high shear rates, elevated temperature, or inclusion of volatile cosolvents is a frequent cause for failure.^{1,41,42} In contrast, a spray-dried insulin formulation, Exubera, was approved by the FDA showing that this technique holds promise for inhalation therapies although large excipient-to-active ratios may be required. Spray freeze-drying often results in degradation of biomacromolecules due to stress of the protein during freezing and drying;⁴¹ however, the nanocluster method must also endure drying or freeze-drying. Moreover, spray freeze-drying is undesirable because of the time required and the high cost of the equipment.⁴¹ Supercritical fluids have recently been pursued to rapidly precipitate particles³⁹ and may be suitable for macromolecules.⁴³ This approach can be more tedious and expensive than alternative techniques and thus is not yet extensively utilized.³⁹

A couple of key particle engineering approaches that focus on affecting the aerodynamic properties of drug or excipient particles have emerged in recent years. Although nanoparticle agglomeration (e.g., liposomes, drug nanoparticles, etc.) has been reported previously, these efforts differ from the approach reported here and may not be ideal for macromolecular formulations. Recent studies have focused on the fabrication of large, porous particles.^{9,16} These particles possess good aerodynamic properties for delivery to the lung.⁴⁴ To date, such particles have been fabricated using a spray drying or spray freeze-drying process; thus, similar disadvantages may exist as mentioned above for spray drying.

For typical DPI designs, a micronized drug is blended with larger carrier particles and must be released or desorbed before it is bioavailable.³⁹ In contrast, nanoclusters allow the protein drug to be immediately released in its native form, making this delivery system especially attractive for treatment of local diseases of the lungs (e.g., asthma, chronic obstructive pulmonary disease, etc.) or other ailments in which rapid bioavailability of the drug is desired with easy, quick administration of the drug.³⁵ Upon dissolution or desorption of the

protein from the nanoclusters, the microstructure becomes disrupted; therefore, formulation of nanoclusters may also be considered an excipient approach to delivering nanoparticles to the deep lung. Nanoparticles have also been proposed as a mechanism to further extend the bioavailability of an encapsulated drug by evading clearance by phagocytic alveolar macrophages and by reducing mucociliary clearance.^{15,16,27,41,45–50} One should be cautioned that the slow degradation rate of PLG and other polymers may lead to accumulation of the polymer in the lungs, which could cause adverse reactions;^{17,25,26} therefore, careful exploration of these systems must be completed, although recent reports by Kissell and others suggest that PLG nanoparticles are benign for short-term exposure.²⁷

5. Conclusions

A dry powder formulation based on nanoparticles assembled in solution (“nanoclusters”) possessed characteristics favorable for formulation of dry powder aerosols for delivery to the deep lung. The microstructure of the nanoclusters provided aerodynamic diameters necessary for delivery to the deep lung, while the nanostructure provided a large surface area for adsorption of protein. Ionic strength was found to be a critical variable that may need to be balanced to facilitate optimal adsorption of a macromolecular drug and control of flocculation to produce nanoclusters. Competitive binding to the nanoparticle surface between salt ions and protein was found to greatly reduce the amount of protein adsorbed. The trends observed regarding shear force and total protein–particle concentration indicated that these variables may be modified to optimize the characteristics of the nanoclusters. The immediate release of ovalbumin from the nanoclusters suggests that this delivery system may be useful for the administration of a “rapid release” formulation to treat local pulmonary infections or disease or for systemic delivery of therapeutics. Furthermore, nanoclusters may ultimately be extended to deliver combination therapies by adsorbing a drug and encapsulating another within the nanoparticles. By customizing the surface charge of PLG nanoparticles to the pI of the macromolecular therapeutic, we expect that this nanocluster system may be useful for the formulation of many types of protein drugs for pulmonary delivery.

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Figure 1.

Adsorption isotherms for binding of ovalbumin (pI, 4.6) to charged nanoparticles (gray diamonds: DOTAP, ζ +42.6 mV; black circles: CTAB, ζ +23.0 mV; gray triangles: DSS, ζ -36.4 mV; black squares: PVA, ζ -2.6 mV). The data are normalized for total surface area of the nanosuspension. Error bars representing standard deviations ($N=3$) are present for each point; however, because of their small magnitude, they are not visible in most cases.

Figure 2. Geometric size distributions of nanoclusters prepared using various ratios (w/w) of ovalbumin to nanoparticles (0, 0.01, ~0.12, 0.33, ~0.67) as a function of ionic strength (ddH₂O-A; PBS-B) and tube size (2 mL, gray; 15 mL, black). The error bars represent standard deviations ($N=3$ for 2 mL, $N=2$ for 15 mL).

Figure 3.

Adsorption of ovalbumin to DOTAP nanoparticles in ddH₂O (triangle) and PBS (square). The gray data points correspond to the *y*-axis on the right and represent changes in the nanoparticle surface charge upon adsorption of ovalbumin. The black data points represent changes in the amount of ovalbumin adsorbed to 1.16 mg DOTAP nanoparticles and correspond to the left *y*-axis. Error bars representing standard deviations ($N=3$) are present for each point; however, because of their small magnitude, they are not visible in most cases.

Figure 4.

Geometric size distributions of nanoclusters prepared using ddH₂O (A) and PBS (B) as a function of total protein-particle concentration. The volume given in parentheses within the legend corresponds to the volume of PBS or ddH₂O used to dilute the nanosuspension prior to addition of ovalbumin. In the case of PBS (B), the ionic strength of the solution was increased to 111, 137, 149, and 154 mM as the total protein-particle concentration was reduced; therefore, variations in ionic strength may also have an influence on the size differences observed. For ease of interpretation, only a representative plot is shown for each concentration; the results were reproducible ($N = 2$).

Figure 5. Scanning electron micrograph depicting the porosity of a nanocluster imaged at 10 000 $\times$ magnification. The nanocluster contains nanoparticles cross-linked with protein, which results in a webbed appearance. A similar image of agglomerated PLGA nanoparticles was recently published by the authors in *Langmuir*.⁵¹

Figure 6.

Evaluation of the stability of ovalbumin upon release from the nanoclusters. Retention of ovalbumin's native secondary and tertiary structure was confirmed upon release using fluorescence (A) and circular dichroism (B) spectroscopy, respectively. The spectra of the released ovalbumin (black line) were compared to a control of ovalbumin in PBS (gray line). Molar ellipticity is presented in the standard units, [deg cm²/dmol].

Table 1Characteristics of Nanoparticles ($N=3$)

coating material, concentration	size ^a (nm)	zeta potential (ζ , mV)
DOTAP, 50 μ M	271.5 $\pm$ 9.1	42.6 $\pm$ 3.2
CTAB, 0.5% (w/v)	296.2 $\pm$ 4.1	23.0 $\pm$ 1.7
PVA, 0.5% (w/v)	182.3 $\pm$ 2.1	-2.6 $\pm$ 3.2
DSS, 0.1% (w/v)	313.0 $\pm$ 18.9	-36.4 $\pm$ 2.1

^aThe polydispersity values were less than 0.16 in all cases.

Table 2

Extent of Ovalbumin Incorporation into Nanoclusters As a Function of Ionic Strength (i.e., ddH₂O vs PBS) and Shear Force during Tumbling (i.e., tube size) ($N = 3$)

ratio of ovalbumin to nanoparticles (w/w) ^a		% ovalbumin adsorbed					
		ddH ₂ O			PBS		
ddH ₂ O	PBS	tube vol, 2 mL	tube vol, 15 mL	tube vol, 2 mL	tube vol, 2 mL	tube vol, 15 mL	
0	0	N/A	N/A	N/A	N/A	N/A	
0.01	0.01	100.0 ± 0.0	100.0 ± 0.0	49.4 ± 4.4	89.0 ± N/A ^b		
0.12	0.13	42.4 ± 7.4	58.3 ± 0.2	12.0 ± 0.6	21.4 ± 0.2		
0.33	0.33	24.5 ± 2.4	28.7 ± 4.4	8.6 ± 0.1	14.0 ± 2.1		
0.67	0.66	8.3 ± 0.7	9.1 ± 0.1	5.9 ± 0.1	9.3 ± 0.3		

^aStandard deviations are on the order of ± 0.00–0.01.

^bStandard deviation is not available because of a technical error during data acquisition.

Table 3

Aerodynamic Properties and Bulk Powder Densities of the Lyophilized Nanoclusters as a Function of Ionic Strength (i.e., ddH₂O vs PBS) and Ovalbumin Content ($N = 3$)

ratio of ovalbumin to nanoparticles (w/w) ^a		mean aerodynamic diameter (μm) ^b		mode aerodynamic diameter (μm) ^c		Bulk powder density (g/mL)	
ddH ₂ O	PBS	ddH ₂ O	PBS	ddH ₂ O	PBS	ddH ₂ O	PBS
0.02	0.01	2.8 ± 1.8	3.1 ± 1.7	2.24	2.45	n/a	0.26 ± 0.03
0.10	0.11	3.2 ± 1.9	2.6 ± 1.6	2.45	2.04	n/a	0.23 ± 0.03
0.28	0.29	3.3 ± 1.9	2.9 ± 1.8	2.57	2.20	0.20 ± 0.01	0.15 ± 0.03
0.55	0.56	3.1 ± 1.9	2.9 ± 1.7	2.86	2.24	0.15 ± 0.02	0.15 ± 0.03

^a Standard deviations are on the order of ± 0.00–0.01.

^b Standard deviations reported for size distributions. SD between replicates was on the order of ± 1%.

^c As given in the data report obtained from the Aerosizer.