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Formulation of a Dry Powder Influenza Vaccine for Nasal Delivery

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ABSTRACT

The purpose of this research was to prepare a dry powder vaccine formulation containing whole inactivated influenza virus (WIIV) and a mucoadhesive compound suitable for nasal delivery. Powders containing WIIV and either lactose or trehalose were produced by lyophilization. A micro-ball mill was used to reduce the lyophilized cake to sizes suitable for nasal delivery. Chitosan flakes were reduced in size using a cryo-milling technique. Milled powders were sieved between 45 and 125 μm aggregate sizes and characterized for particle size and distribution, morphology, and flow properties. Powders were blended in the micro-ball mill without the ball. Lyophilization followed by milling produced irregularly shaped, polydisperse particles with a median primary particle diameter of ~21 μm and a yield of ~37% of particles in the 45 to 125 μm particle size range. Flow properties of lactose and trehalose powders after lyophilization followed by milling and sieving were similar. Cryo-milling produced a small yield of particles in the desired size range (<10%). Lyophilization followed by milling and sieving produced particles suitable for nasal delivery with different physicochemical properties as a function of processing conditions and components of the formulation. Further optimization of particle size and morphology is required for these powders to be suitable for clinical evaluation.

KEYWORDS: Intranasal delivery, dry powder, influenza vaccine, mucoadhesive, lyophilization.

INTRODUCTION

Influenza is a highly contagious disease that causes high morbidity and mortality worldwide each year. Most deaths currently associated with influenza in industrialized countries occur among the elderly—those over 65 years of age.[1]

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Vaccination has a major impact on preventing the epidemic spread of the disease. The World Health Organization recommends that elderly persons, and persons of any age who are considered to be at high risk because of underlying health conditions, be vaccinated. Influenza vaccination can reduce both health care costs and productivity losses associated with influenza illness.[2] Current efforts to control influenza are based on the use of annual inactivated intramuscular (IM) or live attenuated, cold-adapted trivalent intranasal (IN) liquid vaccines.

Recently, contamination of trivalent influenza suspensions for IM injection resulted in a vaccine shortage. The discovery of contaminated injectable doses resulted in a 48% shortfall in the number of doses that the US federal health agencies had planned to distribute in 2004–2005.[3] This led to the rationing of influenza vaccines, with priority given to elderly persons, immunocompromised persons, and professional health care workers. Steps must be taken to ensure that a vaccine shortage will not happen in the future.

Delivery of vaccines and therapeutic drugs to the nasal and pulmonary systems has been studied extensively.[4-8] These approaches offer noninvasive alternatives to needle-based delivery. IN delivery of vaccines has been shown to elicit both mucosal and systemic immune responses.[9-12] However, in most cases liquid formulations have been used. The development of heat-stable powder vaccine formulations and delivery technologies may help overcome the low-temperature storage and distribution requirements associated with current vaccine delivery methods. Dry powder formulations are potentially superior to liquid formulations in their sterility and stability, facilitating mass vaccination and eliminating the need for the cold chain.[13-17] Few dry powder vaccine formulations have been prepared, and the ones that have been reported were not intended for nasal delivery.[15,16,18,19] The reasons for the limited interest in powders are difficult to enumerate. However, the production of powders with reproducible particle size and distribution, stability, and performance characteristics may be a perceived barrier to development when compared with the production of solution formulations for parenteral or nasal administration.

Vaccination against influenza via the IN route has drawn considerable attention. The majority of infectious agents, including influenza, invade the human body through mucosal routes, making IN administration an attractive vaccination method. The ability to elicit both mucosal and systemic immunity and the ease and safety of administration are advantages that IN delivery has over standard parenteral delivery. FluMist (Medimmune Vaccines, Inc, Gaithersburg, MD) elicits mucosal immunity and was shown to be safe and well-tolerated in healthy pediatric and adult populations.[2,20,21] However, this live attenuated vaccine is not indicated for children under age 5, elderly persons, and immunocompromised persons.[22] As has always been the case for IM influenza vaccines, FluMist must be stored as a frozen liquid and thawed immediately before use to maintain potency. In the developing world, in particular, there is an urgent need to overcome such cold chain requirements and to provide single-use, nonrefillable delivery technologies that require minimal training. While dry powder vaccines have the potential to meet requirements, there have been very few reported studies investigating IN delivery of dry powder vaccines. Furthermore, the powders used in prior studies were not fully characterized, and there was no description of a device suitable for reproducible IN powder delivery in humans.[13,14,17]

In our hypothesis, we proposed that the residence time of the dry powder vaccine on the nasal mucosa would influence the efficacy of the vaccine. Elements of this hypothesis were addressed through the following steps: developing a dry powder antigen preparation; processing the powder to achieve appropriate particle size characteristics (10-50 μm); and incorporating a mucoadhesive compound to potentially enhance residence time on the nasal mucosa,[23] thus amplifying the immune response elicited. The latter biological effects are not described in this article.

A previously published study using the formulation of antigen-excipient-mucoadhesive described in this article focused on the ability of a powder vaccine to elicit a significant serum antibody response in rats equivalent to that of a liquid vaccine administered via IN delivery or IM injection.[24] Chitosan was employed in the formulation to potentially increase the residence time on the nasal mucosa. When dosed in animals, the powder vaccine described in this article generated strong nasal mucosal and systemic immune responses using the combination of an IN delivery device and a formulation of influenza vaccine. In the previously published studies, the powder vaccine was shown to be more stable than liquid vaccine at various temperatures and relative humidities. IN powder with mucoadhesive generated serum antibody titers >10 times that of IM injection, IN liquid, or IN powder without mucoadhesive. Comparable nasal immunoglobulin A (IgA) titers were generated in the groups receiving IN doses containing antigen. No nasal IgA response was seen in animals receiving IM doses or in animals receiving IN doses with excipients alone.

The present article is the first detailed description of the preparation and characterization of the antigen powders used in the subsequent in vivo studies.[24]

Materials and Methods

Non-spray-dried crystalline lactose monohydrate (Mallinckrodt Baker, Paris, KY, referred to as lactose), D-(+)-trehalose dihydrate (referred to as trehalose), and chitosan (MW 50 000 to 190 000 Da, minimum 85% deacetylated) were purchased from Sigma Chemical Co (St Louis, MO). Whole inactivated influenza virus (WIIV) of the H1N1 strain, A/PR/8/34, purchased from Charles River SPAFAS (North Franklin, CT; Lot4PRI000901, 2 mg/mL), was used as a liquid preparation as provided by the vendor or formulated as a powder. WIIV was propagated in the allantoic fluid of chicken eggs, purified from sucrose gradient; inactivated by beta-propiolactone; and resuspended in HEPES saline.

Powder Preparation of Vaccine

Lyophilization

The final formulation contained 100 μg of influenza vaccine blended in 10 mg of lactose or trehalose. A solution with 5 mg/mL of total solids was to be prepared. For vaccine antigen samples, 1 mL of vaccine suspension was thawed and added to the disaccharide solution, stirred, and placed in vials for freeze-drying (Kinetics Flexi-Dry, Kinetics Thermal Systems, Stone Ridge, NY). The vials were frozen at –20°C and placed on the manifold at a temperature of –55°C and chamber pressure of 5 x 10^{-3}mm Hg. A drying time of 48 hours was adopted following preliminary experiments with trehalose. To minimize humidity effects, subsequent work was completed in a dry box (<15% relative humidity).

Particle Size Reduction

A micro-ball mill (SPEX Certiprep #3117, Metuchen, NJ) was used to reduce particle size for small quantities of powder (<300 mg). The mill consists of a stainless steel vessel containing several ¼-inch steel balls. The micro-ball mill was sealed and mounted on an oscillating motor (Variable Speed Jig Saw, Black and Decker Inc, Towson, MD) to provide agitation. Run parameters are shown in Tables 1 and 2.

Sieving

Milled powders were sieved (model no SS-5 sieve shaker, Gilson Co, Worthington, OH) with 300, 125, and 45 μm sieves. The powders were sieved for 2 hours in tap mode, and particles in the 45 to 125 μm size range were collected.

Table 1. Parameters and Particle Size and Distribution for Steel Ball Milling of Lactose Experiments (n = 3)*

Run	Milling Load, mg	Milling Speed, rpm	Milling Time	D50, μm, mean (SD)	Span, mean (SD)
1	240	200	5 minutes	—	—
2	200	200	10 minutes	—	—
3†	200	200	10 minutes	—	—
4	200	600	5 minutes	—	—
5	200	600	15 minutes	—	—
6	100	600	20 minutes	—	—
7	100	200	15 minutes	30.5 (5.1)	5.20 (2.87)
8	100	200	30 minutes	22.1 (7.0)	3.43 (0.77)
9	100	200	1 hour	12.4 (1.0)	3.70 (0.48)
10	100	200	2 hours	7.5 (1.7)	3.66 (0.35)
11	150	200	30 minutes	18.3 (2.2)	2.98 (0.10)

*Particle sizing was not completed on runs with milling speeds of 600 rpm or milling loads ≥ 200 mg. SD indicates standard deviation.
†One steel ball was used as the milling media, with the exception of Run 3, in which 2 balls were used.

Mucoadhesive Compound

Chitosan was supplied as solid flakes roughly 2 to 4 mm in size. The flakes are not brittle at room temperature; their flexibility reduces their susceptibility to size reduction by a simple milling technique. Consequently, a cryo-milling technique was used. Sixty milligrams of chitosan was placed in a vial with a single steel ball. The vial was immersed in liquid nitrogen, left to cool for 5 minutes, and then placed on the jigsaw for milling at 1600 rpm for 20 minutes. This process was repeated 3 times, and the resulting powder was then sieved as above.

Physicochemical Characterization

Particle Size Analysis

Each powder was sized by laser diffraction (Malvern 2600 Series, Worcestershire, UK) with a small liquid dispersion sample cell. The sample cell has an integral stir bar and a volume of ~5 mL. The cuvette was filled with 1% wt/wt Span 80 (Sigma) in light, white mineral oil (Sigma). Powder samples of ~10 mg were dispersed in 2 mL of 1% wt/wt Span 80 in the light, white mineral oil, sonicated for 5 minutes, and then placed into the sample cell one drop at a time until a laser obscuration between 2% and 30% was achieved (n = 3). Results were reported as the median diameter (D50) and the span ([D90-D10]/D50). This technique was previously validated using light microscopy (data not shown).

Powder Morphology

The powders were adhered to double-sided adhesive carbon tabs (Ted Pella Inc, Redding, CA) on aluminum stubs (Ernest F. Fullam, Inc, Latham, NY) scanning electron microscopy (SEM). A Polaron sputter coater (model E-5200, Electron Beam Services, Agawan, MA) was used to coat the samples with gold-palladium. A scanning electron microscope (JEOL 6300, JEOL Corp, Peabody, MA) was used with an electron beam at an acceleration voltage of 15 kV and a working distance of ~25 mm. For examination of the freeze-dried cakes, samples were dusted onto a double-stick carbon tape, then coated with platinum to ~4 nm. A scanning electron microscope (LEO 1450 VP, Leo Microscopy, Inc, Thornwood, NY) was used with an electron beam at an acceleration voltage of 20 kV. Representative areas of the stub were photographed.

Flow Properties

Bulk and Tapped Densities. The bulk and tapped densities were determined for bulk powders, 45 to 125 μm size fraction of unmilled powders and 45 to 125 μm size fraction of milled powders (n = 3). Approximately 10 mL of powder was measured.[25] Dividing the difference between the tapped and bulk densities by the tapped density determines the Carr's Compressibility Index (CCI).[26]

Table 2. Parameters and Particle Size and Distribution for Steel Ball Milling of Trehalose Experiments (n = 3)*

Run	Milling Load, mg	Milling Speed, rpm	Milling Time	D50, μm, mean (SD)	Span, mean (SD)
12	100	200	15 minutes	13.2 (0.8)	2.19 (0.08)
13	100	200	30 minutes	11.3 (2.6)	2.27 (0.24)
14	100	200	1 hour	30.1 (8.0)	4.03 (1.28)
15	100	200	2 hours	12.5 (3.5)	2.34 (0.21)
16	150	200	15 minutes	23.2 (0.2)	2.04 (0.09)
17	150	200	30 minutes	21.4 (0.4)	2.14 (0.04)

*SD indicates standard deviation.

Static Angle of Repose. Measurements of the static angle of repose were conducted for bulk powders, 45 to 125 μm sieved fraction of bulk powders and 45 to 125 μm sieved fraction of milled powders (n = 3). Approximately 10 mL of powder was measured using a funnel and a flat collection surface.[27]

Statistical Analysis

The experimental data were analyzed by a 1-way analysis of variance.

Results and Discussion

Powder Preparation of Vaccine

Lyophilization

The freeze-dried material formed white, porous cakes showing slight shrinkage, but no large-scale collapse was observed. The morphology of lyophilized material was observed using SEM (Figure 1).

Particle Size Reduction

Very little particle size reduction occurred for the larger load (200 mg) milling runs or for runs shorter than 15 minutes (Runs 1-5). When milled at the higher velocity (600 rpm, Run 6), the powder was recovered in large flakes, suggesting melting and recrystallization of the lactose under the higher energy conditions. In general, trehalose powder was reduced in size more rapidly by milling than lactose was. Milling was most effective for a smaller load of powder.

Ball milling of powders is a common particle size reduction method in which a powder formulation is fractured by the repetitive collision of stainless-steel or ceramic balls or cylinders (milling media). Total milling time, speed, powder mass, size of mill, and number of milling media elements are all important variables for milling and must be determined separately for each formulation.[28] The effect of milling speed on production of 45 to 125 μm particles may be explained by the degree of aggregation. Faster milling speed corresponds to faster particle size reduction. Typically, the degree of milling reaches a plateau and then decreases because of the creation of aggregates.[28] For the effect of ball loading, milling occurs more slowly for a higher percentage of filling since the milling media has reduced freedom of motion. The combined effect of milling time and powder load also may be explained by speed of milling since a longer mill time is required to mill a larger load of powder. Milling methods generally result in nonspherical solid particles with a wider size distribution than in spray-drying. In addition, frictional heating of the milling must be controlled, as it can reduce the activity of the thermolabile materials such as vaccines. A slow milling speed was used to minimize the potential for milling to reduce vaccine potency.

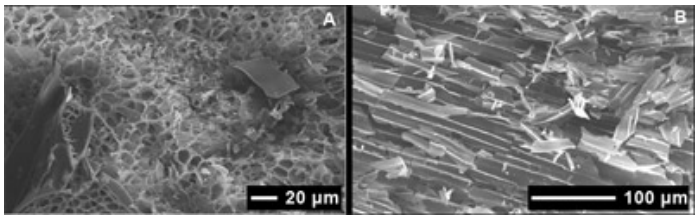

Figure 1. Scanning electron micrographs of (a) top surface (500×) and (b) cross-section (500×) of the lyophilized cake.

[Should a "Sieving" head and section appear here, as at this point in the Materials and Methods section?]

Mucoadhesive Compound

Particle sizing by sieving of chitosan after cryo-milling indicated that a very small percentage (<10%) of powder was produced in the desired size range of 45 to 125 μm. This sieved powder fraction was recovered for use as a blended mucoadhesive in the vaccine-containing formulation.[24]

Physicochemical Characterization

Particle Size Analysis

Particle size and distribution are important factors in the development of a dry powder nasal formulation. Particles <10 μm have the potential to be deposited in the lower respiratory tract if inhaled.[29] This can lead to issues with potential toxicities as well as reduce the dose fraction reaching the intended site of delivery. Particles that are too large and/or in too high of a mass may not dissolve efficiently on the nasal mucosa.[27]

Particle sizing results are shown in Tables 1 and 2. Particle size analysis was not completed on Runs 1 to 6, as stated above.

Lactose. One- and 2-hour mill times resulted in a large proportion of particles that were potentially respirable (<10 μm) and unsuitable for nasal delivery. The 15-minute mill time resulted in ~35% of particles exhibiting volume diameters between 45 and 125 μm, as did the 30-minute mill time (Figure 2). In the case of the 15-minute mill time, a significant proportion of particles were >125 μm, while for the 30-minute mill time a significant proportion of particles were <10 μm and no particles were >125 μm. Therefore, a mill time between 15 and 30 minutes would maximize the number of particles in the 45 to 125 μm size range. Alternatively, a slightly larger mill loading would slow down the milling rate so that particles in the desired size range could be generated without a large percentage of respirable

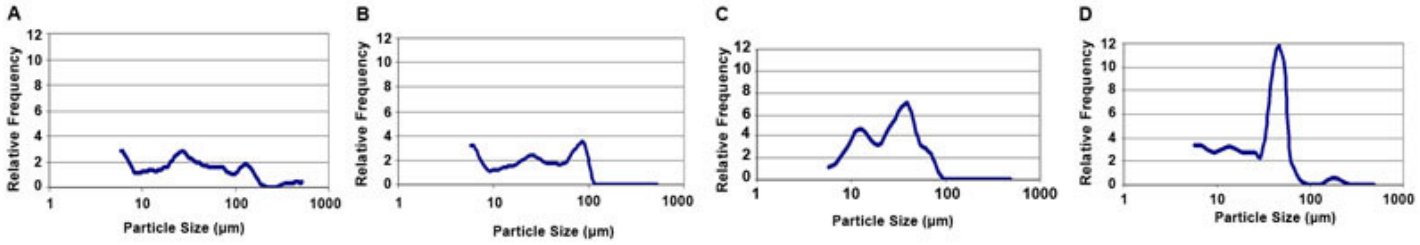

Figure 2. Particle sizing results for (a) lactose, 100 mg, 15-minute mill time (D10 = 9.9 μm, D50 = 28.0 μm, and D90 = 128.0 μm); (b) lactose, 100 mg, 30-minute mill time (D10 = 4.7 μm, D50 = 22.2 μm, and D90 = 85.3 μm); (c) trehalose, 150 mg, 30-minute mill time (D10 = 6.1 μm, D50 = 21.0 μm, and D90 = 50.6 μm); and (d) trehalose, 150 mg, 30-minute mill time, 45 to 125 μm sieved fraction (D10 = 8.3 μm, D50 = 28.8 μm, and D90 = 128.0 μm).

particles. Consequently, powder loads of 150 mg were used for subsequent lactose formulations.

Trehalose. Trehalose milled at 60 or 120 minutes tended to form aggregates, increasing the median diameter of the resultant powder. Increasing the powder load within the mill from 100 to 150 mg increased the fraction of particles in the 45 to 125 μm particle size range from 5% and 7% to 21% and 37%, for 15 and 30 minutes, respectively. These conditions (150 mg, 200 rpm, 1 steel ball, 30-minute mill time) maximized the production of appropriately sized particles and were chosen as the production conditions.

The span is a measure of the polydispersity of the powder. Milling of freeze-dried cakes effectively produced particles with sizes appropriate for nasal delivery. However, the span of the lyophilization followed by milling (LFM) powders indicates that a large number of fine particles are present. It should be noted that the particle sizing of powders dispersed in light mineral oil with surfactant determines the primary particle size of the powders. This includes fine particles that are aggregated with larger particles. Further characterization of particle size by an aerodynamic method will be required to adequately describe the impact of these fine particles on deposition into the lower respiratory tract.

As with other processing methods, excipient choice is crucial for the optimization of the formulation. Disaccharide sugars are commonly used in lyophilized formulations because they are effective cryoprotectants.[30] Lactose and trehalose have been used in a variety of formulations for nasal delivery. However, lactose is a reducing sugar that has been shown to degrade proteins when stored as a dried solid at room temperature.[31] Trehalose, on the other hand, is not a reducing sugar and does not cause proteins to lose activity. Because of this, initial milling optimization was completed with lactose, but the final formulation was optimized by using trehalose.

Powder Morphology

Scanning electron micrographs of trehalose powder after milling are shown in Figure 3. LFM produced polydisperse, irregularly shaped particles.

When delivered in vivo, the nasal IgA responses elicited by IN powder delivery were more variable compared with those induced by the liquid vaccine.[24] This variability may be due, in part, to the differences in particle size distribution and morphology observed for these powders, leading to less reproducibility in emitted dose. Additional work to optimize the formulation is underway to address this issue. Evaluation of the lyophilized cakes revealed the presence of pores 10 to 40 μm in diameter (Figure 1). These pores were produced from long channels formed by the crystallization and removal of water. When these cakes are milled, the particles produced are needlelike and are difficult to blend uniformly with other excipients. Further evaluation of the

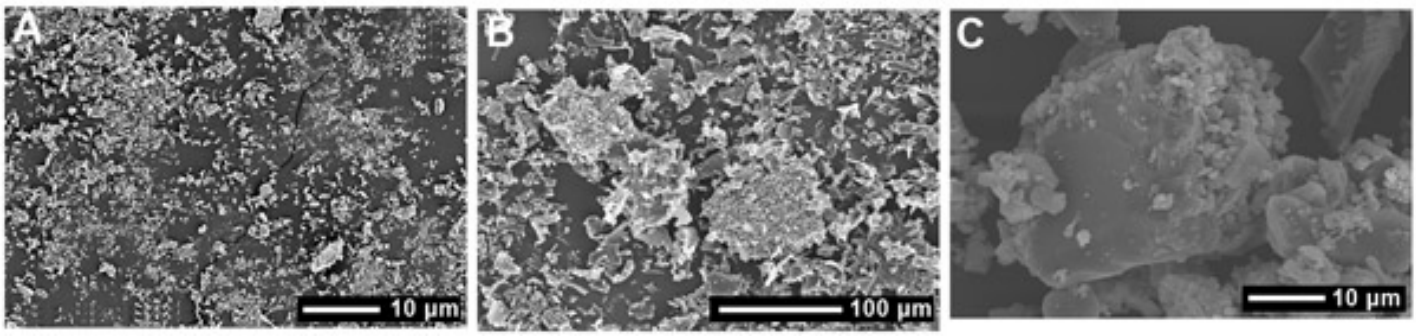

Figure 3. Scanning electron micrographs of trehalose powder, 150 mg, 30-minute mill time.

Table 3. Particle Size Data and Flow Properties of Lactose and Trehalose (n = 3)*

	Powder	Median Diameter, D50 (μm), mean (SD)	Span, mean (SD)	Carr's Compressibility Index, mean (SD)	Static Angle of Repose (°), mean (SD)
Lactose	Bulk	25.8 (0.7)	2.64 (0.05)	29.70 (1.69)	49.0 (0.5)
	Sieved bulk	18.3 (0.7)	2.59 (0.06)	33.33 (0.95)	49.5 (3.7)
	Milled, sieved	15.3 (2.2)	2.90 (0.06)	29.50 (1.33)	37.3 (1.6)
Trehalose	Bulk	37.6 (0.8)	2.14 (0.07)	23.80 (1.47)	30.3 (3.6)
	Sieved bulk	34.1 (0.4)	2.12 (0.04)	22.97 (1.34)	21.6 (1.5)
	Milled, sieved	37.1 (4.5)	4.47 (3.36)	33.94 (3.74)	37.4 (1.7)

*SD indicates standard deviation.

freeze-drying process will be required to produce a more uniform distribution of particles by creation of a lyophilized cake with smaller pores. In addition, alternative methods of manufacture, such as spray-freeze-drying, might solve the problem.

Flow Properties

The flow properties measure the cohesive forces of a powder.[25,27] In these experiments, manufacturing conditions were optimized for producing particles in the desired size range (45-125 μm), not for flow properties. However, the flow properties do affect the performance of the final product. The ability of a powder to flow is one of the factors that affect the mixing of different materials to form a powder blend.[25] This may be important for the blending of the virus and excipient with the mucoadhesive compound. Also, because nasal delivery requires a fluidization of the powder bed, it is conceivable that flow properties of powders could affect the emitted dose from the device and deposition in the nasal cavity.

Bulk and Tapped Densities. CCI values are reported in Table 3. The bulk lactose, sieved bulk lactose, and both lyophilized followed by milling and sieving (LFMS) powders had similar CCI values ($P < .05$). The LFMS powders had bulk densities of ~0.07 g/mL as compared with the bulk and sieved bulk powder, which had bulk densities of 0.4 to 0.6 g/mL. Bulk trehalose and sieved bulk trehalose had CCI values that were significantly lower than those of any of the other powders ($P < .05$).

The more compressible a powder is (ie, the higher the CCI), the greater the cohesive forces of a powder, making it flow poorly. A free-flowing powder has a CCI less than ~20% to 21%.[26] Bulk trehalose and sieved bulk trehalose had CCI values of ~23. However, the LFMS powder containing trehalose had a CCI value of ~34. Generally, compared with powders with larger particle sizes, powders with smaller particle sizes pack better, increasing the bulk density of the powders. However, this is not the case with trehalose. This difference can be attributed to the irregular, needlelike shape of the particles, which leads to larger voids in the powder. LFMS trehalose and lactose powders exhibit needlelike morphology; bulk lactose is an elongated crystal and therefore has a similarly high CCI value.

Static Angle of Repose. Static angles of repose of the powders are reported in Table 3. Both the bulk lactose and the sieved bulk lactose had static angles of repose of ~49°. The sieved bulk trehalose had the smallest angle of repose. LFMS produced powders with similar static angles of repose in each material ($P > .05$).

The greater the static angle of repose, the greater the cohesive force of a powder. Powders with static angles of repose less than 40° are considered to be free-flowing powders; with angles greater than 50°, the powders flow poorly or not at all.[26,28] Lactose and trehalose powders have similar flow properties after manufacture by LFMS. The smaller particle size of the LFMS trehalose powder, when compared with the sieved bulk trehalose powder, indicates a significant decrease in the powder's potential ability to flow. In the case of lactose, the LFMS powder has particle size characteristics similar to those of the sieved bulk powder, but the static angle of repose indicates that the LFMS powder exhibits a flow that is better than the sieved bulk powder's.

CONCLUSIONS

Powders of different composition (lactose, trehalose, and chitosan) prepared under a variety of processing conditions (milling, powder quantity, and number of steel balls) exhibited a range of physicochemical properties (particle size and distribution, morphology, bulk/tapped density, and angle of repose). A powder vaccine formulation suitable for nasal delivery was obtained by lyophilization, followed by milling and sieving under optimized conditions. Further optimization in conjunction with the development of a delivery device is required for these observations to be suitable for clinical studies.

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