Acoustic levitation as a screening method for excipient selection in the development of dry powder vaccines

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Abstract

Spray drying is emerging as a promising technique to produce thermally stable powder vaccines containing viral vectors. One of the most important factors in developing dry powder vaccines is the selection of the excipient carrier, however this process is time intensive, and uses large amounts of costly viral material. In this work, an acoustic levitator modified with a hot air delivery system was evaluated for its ability to mimic spray drying and acts as a screening method for excipient selection. The ability of three binary excipient blends to stabilize a human type 5 adenovirus was evaluated through an *in vitro* activity assay based on the expression of green fluorescent protein. Dried particle size and shape, glass transition temperature, moisture content, and crystallinity of powders produced by levitation and spray drying were compared. The particles created in the acoustic levitator under moderate heat can be considered representative of the powders that would be produced via spray drying. Viral vector titre losses differ between the methods, however, the trends with respect to excipient performance remain the same. Key material characteristics such as particle morphology and thermal properties are conserved when using the levitator. The acoustic levitator is a good starting point for dry powder vaccine development, and can be used to identify promising excipients while consuming minimal amounts of the viral vector.

Keywords

Acoustic levitation, spray drying, screening method, vaccine, excipient, droplet drying

1. Introduction

One of the most commonly reported techniques for creating thermally stable vaccines is vitrification, where a biologic is encapsulated within the amorphous phase of a solid excipient (Crowe et al., 1997). Carbohydrates, amino acids, and proteins have all been reported in the literature as successful excipients to encapsulate and retain biologic potency at higher temperatures than current refrigeration conditions necessary for cold chain compliancy (Alcock et al., 2010; Amorij et al., 2008; LeClair et al., 2016a; Ohtake et al., 2010). Freeze drying is the method traditionally used for vitrification in the pharmaceutical industry; however, spray drying is becoming more wide-spread and increasingly preferable due to its low cost, scalability, high throughput processing, and easy customization (Amorij et al., 2008; I Ré, 1998; Langford et al., 2018).

Previous work has shown spray drying to be an effective method for preparing a thermally stable viral-vectored vaccine, with retained activity demonstrated even after storage at elevated temperatures as high as 55^{o}C (LeClair et al., 2016a, 2016b). However, the selection of an excipient or blend of excipients that will successfully stabilize a viral vector is often done by trial and error, which can consume large amounts of expensive viral vectors, and even after selection must be optimized to reach its full potential as a stabilizer (Kristensen and Chen, 2010; LeClair et al., 2016b). In order to avoid the pitfalls associated with excipient selection, an effective screening method is required that preserves key characteristics of spray dried powders while reducing either the time or the costs involved in successful development of the vaccine platform.

The literature does not contain any examples of screening methods used to select excipients for spray dried pharmaceutical products (of any type, not just limited to vaccines),

although several single droplet analysis techniques have been reported as models for the spray drying process itself (Schutyser et al., 2012; Vehring et al., 2007). The main premise of any screening method is that it must match the trends seen in activity loss after spray drying. That is, one would conclude that the same excipient is ideal from both spray drying and the screening method, and additionally that the screening method would preserve certain key powder properties that have been identified as crucial for thermal stabilization, such as particle morphology and glass transition temperature (LeClair et al., 2016a, 2016b).

Acoustic levitation is one method suggested in the literature for evaluating single droplet drying kinetics, as well as modelling particles produced in a spray dryer (Sadek et al., 2015; Sloth et al., 2006). In this method, a high amplitude standing wave is created using a reflector plate to reflect an ultrasonic acoustic wave. When a small object or droplet is placed in this standing wave, the upward pressure from the wave counteracts the downward force of gravity and the droplet is suspended in place (Yarin et al., 1999, 1998). In order to avoid oscillations in position, the distance between the reflector plate and the wave generator can be adjusted until stable levitation is achieved.

The extended and motionless droplet drying in the levitator compared to a spray dryer allows for *in situ* measurements to be made as particles dry. Studies have been done using X-ray scattering to track crystallization of materials in the levitator as a function of time, and other contactless characterization techniques such as Raman scattering have also been employed (Klimakow et al., 2010; Leiterer et al., 2008). Mondragon et al. (2011) investigated the drying kinetics of multiphase droplets using an acoustic levitator modified to produce high-temperature conditions equivalent to those found in a spray dryer. They reported similar changes to drying behaviour with changing temperature, volume, and concentration, as those reported for spray

drying (LeClair et al., 2016b; Mondragon et al., 2011). Schiffter and Lee (2006a) noted that the Sherwood and Reynolds dimensionless numbers for levitated droplets were within the same range as those produced by spray drying, asserting that acoustic levitation followed an equivalent droplet drying mechanism. Additionally, they found that they could use the acoustic levitator to produce particles of mannitol, trehalose, and catalase that showed similar morphologies to spray dried powders (Schiffter and Lee, 2006). Acoustic levitation has been considered as 'slow motion' spray drying, to determine the deactivation kinetics of an enzyme by removing droplets at various timepoints during the drying process (Lorenzen and Lee, 2012). We infer that particle morphology as a function of drying time could also be investigated using the slower drying process that occurs during levitation.

In our current work, we seek to confirm that important particle morphology characteristics are conserved between the screening method and the production method, and that importantly, when a viral vector is included in the formulation, that activity loss from both methods is consistent. Initially, our concerns with acoustic levitation as a screening method were the fact that the particles produced are much larger, there is no shear, and the drying time is much longer than in spray drying. Conversely, acoustic levitation provides a unique opportunity to separate the effects of processing conditions on the drying of materials since the droplet can be suspended indefinitely.

Herein we demonstrate the use of single-droplet drying via acoustic levitation as an excipient selection screening method for the spray drying of sugar-encapsulated viral vectors. Specifically, a human type 5 adenovirus expressing green fluorescent protein (AdHu5GFP) was tested with mannitol/dextran, lactose/trehalose, and xylitol/dextran binary excipient blends. Although the viral activity losses from spray drying and levitation were not the same, the trends

in activity loss with excipient blends were comparable. X-ray diffraction (XRD), scanning electron microscopy (SEM), differential scanning calorimetry (DSC), and thermogravimetric analysis (TGA) were used to characterize powder crystallinity, size and shape, thermal properties, and water content, respectively. Spray-dried and levitated particles were directly compared and powder property trends were correlated with activity losses to identify promising excipient blends. Acoustic levitation offers a less costly method than spray drying to select and optimize excipients. This study also identified the key powder properties that need to be matched to develop the best possible screening method for spray drying. To the best of our knowledge, this is the first report of acoustic levitation as a means of producing vaccine or virus-containing particles.

2. Materials and Methods

2.1 Chemicals and adenoviral vector

Excipients chosen for the study included anhydrous lactose, D-mannitol, D-(+)-trehalose dihydrate, dextran (M_r 40,000 kDa), and xylitol, all purchased as USP grades from Millipore-Sigma (Ontario, Canada). Cell media was prepared in-house using Life Technologies protocol from α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin (Invitrogen, Ontario, Canada). A recombinant replication deficient human serotype 5 adenovirus expressing green fluorescent protein (AdHu5GFP) was produced in-house at the vector facility of the McMaster University Immunology Research Center as described previously (Roediger et al., 2008; Wang et al., 2004).

2.2 Spray drying, and acoustic levitation

Three excipient blend formulations were evaluated: (1) 90% lactose and 10% trehalose, (2) 75% mannitol and 25% dextran, and (3) 25% xylitol and 75% dextran (all compositions are based on percent by weight). Mannitol/dextran and xylitol/dextran formulations were dissolved into purified water to make a 4 wt.% solution, while lactose/trehalose was dissolved into purified water to give a 1 wt.% solution. All water was purified using a Barnstead GenPure Pro water purification system from ThermoFisher Scientific (Waltham, MA) and had a resistivity of 18.2 MΩ cm. For all powders containing viral vector, 10 µL of viral vector stock solution was added to the dissolved excipient solution. The stock viral vector solution consists of phosphate buffered saline (PBS) with 10% by volume glycerol. Each milliliter of viral stock contains 2.35×10^{12} viral particles, and 3.8×10^{10} plaque forming units (pfu).

Spray dried powders were produced using a B-290 Mini Spray Dryer (Büchi, Switzerland) equipped with a 0.7 mm spray nozzle and a high performance cyclone following a protocol described previously (LeClair et al., 2016a, 2016b). All spray drying experiments were completed without optimization per formulation, using a constant nozzle temperature of 110°C, an outlet-measured air temperature of 55°C, a spray gas flow rate of 439.11 L/h, and a feed solution rate of 188.5 mL/h (corresponding to a pump setting of 13%). For all characterization techniques that did not require activity testing, AdHu5GFP was not included in the formulation due to biosafety requirements. Previous results from our group have found that powders spray dried both with and without viral vector are virtually indistinguishable by SEM due to the small amount of viral vector added to the solutions (less than 1/10,000th of the liquid volume) (LeClair et al., 2016a). All powder characterization was performed on the same day as the powders were produced to avoid any effects from aging.

Levitated particles, from the same excipient-virus solutions prepared for spray drying, were produced using a 13D10 ultrasonic levitator with a single-walled process chamber (tec5USA; Plainview, NY). A 10 µL glass syringe (Hamilton; Reno, NV) with a 26G needle (Becton Dickinson; Ontario, Canada) was used to deposit liquid droplets and collect dried particles from the acoustic levitator. A custom-made hot air delivery system was used to heat a suspended droplet. The relative humidity within the levitator corresponded to ambient room conditions (35-43% RH), considerably lower than in a spray dryer (near 100% RH). The system was monitored by a K-type thermocouple (Omega, Canada) and a USB microscope (AVEN; Ann Arbor, MI). MicroViewer 1.3M software (AVEN; Ann Arbor, MI) was used to record video and images of the droplet drying process and ImageJ (National Institute of Health) was used to measure the dimensions of the dried particles (Abràmoff et al., 2004). Spray drying yields were in the range of 20 – 100 mg, and levitation yields were in the range of 0.2 – 1.5 mg. The lactose/trehalose blend had lower yields than mannitol/dextran and xylitol/dextran due to the lower concentration of solute in solution. There were several nodes in the levitator available for multiple droplets to simultaneously dry; however, particles were dried one at a time in the study to avoid difficulties in retrieving one without disrupting others.

2.3 Particle imaging

Particle size and shape were investigated using a JEOL JSM-7000 F scanning electron microscope (SEM) (JEOL Ltd., Japan). Particles were attached to aluminum stubs using double-sided tape and sputter-coated with a 5.0 nm layer of gold. Images were collected at working distances between 5.4 and 13.1 mm and electron accelerating voltages between 3.0 and 10.0 kV. Image analysis was done using ImageJ software (Abràmoff et al., 2004).

2.4 Differential scanning calorimetry (DSC)

A Q200 differential scanning calorimeter (TA Instruments; New Castle, DE) was used to measure the glass transition temperatures of powders (without viral vector). Powders were weighed into hermetically sealed aluminum pans and heated from 20°C to 300°C at a rate of 10°C per minute under a nitrogen purge gas flowing at 50 mL/min. A modulation period of 60 seconds and a modulation amplitude of 1°C was used. Thermal events were recorded from a single heating ramp to avoid dehydration of the powder and compare data between preparation methods.

2.5 Thermogravimetric analysis (TGA)

Spray dried and levitated powders without viral vector were heated in alumina crucibles at a rate of 5°C per minute up to 150°C under argon gas using a TGA/DSC 3+ instrument (Mettler Toledo; Columbus, OH). Star^e software (Mettler Toledo; Columbus, OH) was used to monitor the mass and determine the stable mass loss at 100°C.

2.6 Powder crystallinity

The powders produced from different excipient formulations (without viral vector) were characterized by powder X-ray diffraction (XRD) on a Bruker D8 Discover with DAVINCI.DESIGN diffractometer (Bruker; Billerica, MA) using a cobalt sealed tube source ($\lambda_{avg} = 1.79026$ Å, $2\theta = 5 - 70°$). Powders were mounted on a silica base, and a blank silica signal was subtracted from the data using GADDS software (Bruker; Billerica, MA). The signal intensity was integrated into a crystallographic figure using DIFFRAC.EVA software (Bruker; Billerica, MA) and crystalline content determined using TOPAS software (Bruker; Billerica, MA).

2.7 Excipient solution viscosity

Dynamic viscosities of the three excipient blend solutions were measured using a Discovery Hybrid Rheometer (TA Instruments; New Castle, DE) with a Peltier plate. A 40 mm cone geometry was used. Experiments were done at a controlled temperature of 25°C, and a flow sweep was conducted from a shear rate of 0.1 s^{-1} to 5000 s^{-1} using approximately 0.5 mL of solution. All excipient solutions exhibited shear thickening behaviour beginning at a shear rate of 400 s^{-1}. At a shear rate of 100 s^{-1} and a temperature of 25°C, the lactose/trehalose blend had a viscosity of $2.4 \cdot 10^{-4}\ Pa \cdot s$, mannitol/dextran had a viscosity of $3.4 \cdot 10^{-4}\ Pa \cdot s$, and xylitol/dextran had a viscosity of $4.1 \cdot 10^{-4}\ Pa \cdot s$. As the shear rate was increased, the differences in solution viscosities diminished.

2.8 *In vitro* activity testing

A549 lung epithelial cells were cultured in T150 culture flasks with prepared α-MEM culture media and incubated in a humidified Forma Series II water jacketed CO_2 incubator (Thermo Scientific Corporation; Waltham, MA) at 37°C and 5.0% CO_2. At 80-90% confluency, cells were either split into a new T150 flask or plated into a 96-well plate for activity testing.

On the same day that they were produced, spray dried and levitated powders containing AdHu5GFP were reconstituted in 1 mL of culture media and eight-fold serial volume dilutions were created ranging from 10^0 to 10^{-7} of the reconstituted powder. A549 cells were then incubated with the dilutions for 24 hours, after which they were imaged for GFP fluorescence using an EVOS FL cell imaging system (ThermoFisher Scientific; Waltham, MA). The median tissue culture infectious dose ($TCID_{50}$) was calculated using the Reed-Muench method (Reed and Muench, 1938) and then compared to the initial titre to determine titre loss incurred from spray drying/drying in the acoustic levitator.

3. Results and Discussion

The viral vector candidate selected for the study, human type 5 adenovirus, is a promising vaccine platform for immunization against various mucosal pathogens, while a dry powder form of the vaccine is considered an ideal deliverable form for inhaled administration (Afkhami et al., 2016; Zhu et al., 2015). This viral vector must be stored at -80^{o}C in aqueous media in order to retain its activity, meaning it is susceptible to damage at any of the temperature conditions used in this study. A high viral potency in the stock solution of 10^{10} pfu was used and when added to the excipient solution resulted in a dilution to 10^{7} pfu in 10 mL. This high concentration of viral vector minimized the number of repeated levitated particle drying experiments needed (approximately 7 particles which takes 7 runs) to produce a strong detectable response by *in vitro* analysis. Each particle dried in the levitator takes approximately 15 min and as such, a starting titer lower than 10^{10} pfu would require more particles and considerably more time to obtain reproducible *in vitro* data. The three binary excipient blends selected as candidates in this work resulted from a preliminary study examining twenty different excipients (and excipients blends) and were chosen for their acceptability in the pharmaceutical industry. The blends were also deliberately chosen with the understanding that they would lead to a range of retained viral activity after drying: lactose-trehalose was expected to show poor stability in contrast to xylitol-dextran which was shown in preliminary studies to produce excellent thermal stability with AdHu5GFP.

A single set of operational parameters was used in the spray dryer, without optimization, to compare the excipients under comparable drying conditions. This allowed for meaningful comparisons between spray dried powders as well as between levitated and spray dried powders. Lack of optimization meant viral activity losses were higher than normally acceptable but for

screening excipients, the trend in activity between formulations was the primary consideration. Our group has previously shown that the optimization of spray drying conditions plays an important role in minimizing viral activity losses caused by processing (LeClair et al., 2016b).

3.1 Comparing viral vector activity between spray dried and levitated powders

3.1.1 Room-temperature acoustic levitation

Acoustic levitation offers a unique opportunity to decouple contributing physical parameters affecting droplet drying, specifically for their effect on the retained activity of the viral vector. The most notable feature of the acoustic levitator is the ability to prepare particles at any ambient temperature where the solvent exhibits a vapour pressure, whereas the constrained space in a spray dryer requires conditions closer to the solvent boiling point that may harm the activity of the virus. Studying droplet drying at room temperature, which cannot be accomplished in a spray dryer, minimizes thermal contributions to deactivation while allowing researchers to investigate activity losses caused physically by drying phenomena, and chemically by associative effects as the excipients replace water surrounding the viral vector. Fig. 1 shows viral vector activity loss with the three binary excipient blends and compares spray dried powders to particles dried in the levitator at room temperature.

[Fig. 1]

Results in Fig. 1 imply that drying through levitation at room temperature does not suitably mimic spray drying; both the activity loss values and trends in activity are different for the two methods. Drying through levitation showed less loss of the original viral titer (i.e., higher activity) compared to spray drying in two out of three cases. In general, the spray dried powders had large titer losses (4.6 log loss for lactose/trehalose and 2.7 log loss for mannitol/dextran).

Xylitol/dextran showed excellent retention of activity with a low titer loss for both levitated (1.1 log) and spray dried (0.06 log) powders. A log loss of 1.1 is likely a realistic upper limit to continue testing/optimizing a given screened candidate for product development. Despite the lack of agreement between the two methods, this set of experiments demonstrates that acoustic levitation can be used to produce dried particles that retain viral activity, which has not been shown previously, and which alleviated concerns about physical distortions under the acoustic wave possibly causing damage to the viral vector.

The difference in activity losses between the two methods provides insight into how drying time, temperature and shear forces may affect viral activity during drying. The lower activity losses seen in levitated particles imply that longer drying times did not adversely affect the viral vector despite prolonged exposure to temperatures above -80^{o}C (where the excipient and viral vector have high mobility). The greater losses seen in spray dried samples are attributed to the higher drying temperature and/or exposure of the viral vector to high shear in the nozzle, which have both been shown to negatively impact spray dried biologics (Grasmeijer, 2016; LeClair et al., 2016b). The effects of drying temperature were subsequently further explored.

3.1.2 Acoustic levitation at elevated temperatures

To more closely mimic the surrounding air conditions in the spray dryer, as well as to investigate the effect of air drying temperature on particle formation, excipient blends containing AdHu5GFP were dried at 30°C, 40°C, 55°C, and 62°C within the acoustic levitation chamber. For reference, air temperature in the drying chamber and cyclone unit of the spray dryer was measured to be 55^{o}C. In the acoustic levitator a temperature of 62°C was selected as the uppermost limit that would not cause damage to the equipment. Fig. 2 shows the activity losses

for levitated particles dried with increasing air temperature for the three excipient blends (compared to spray drying data from Fig. 1).

[Fig. 2]

Fig. 2 shows that as the air temperature was increased in the acoustic levitator, all three formulations showed a decrease in titer loss going from room temperature up to 40°C. Above 40°C, titer losses for all three formulations increased once again but never exceeded the results at room temperature. Evaporative cooling as the droplet dried partially shielded the viral vector from thermal degradation but as the surrounding air temperature rose, this benefit was increasingly diminished. All temperatures tested for mannitol/dextran and lactose/trehalose blends indicated better viral stability than from the spray dryer; however, for xylitol/dextran the spray dryer remained the better performing drying method. From this we can infer that slightly higher air temperature does not necessarily infer more damage occurring to the viral vector, as it is partially protected from the heat by the evaporating liquid phase and benefits from a more rapid drying rate. With faster drying there will be less opportunity for the virus to migrate to the liquid-air interface. However, above a certain temperature, viral damage becomes the dominant outcome in spray drying encapsulated vaccine particles (LeClair et al., 2016b).

Excipient solutions evaporated in a Petri dish at 55°C were found to have higher activity losses (3.0 log loss for mannitol/dextran, 2.8 log loss for lactose/trehalose; no measurable activity was found for xylitol/dextran) than either spray drying or levitation. This data suggests that the extended drying time necessary for basic evaporation is non-representative of spray drying powders. The acoustic levitator also avoids the air-water-solid interface present in more basic evaporation experiments. This air-water-solid interface has been shown to cause increased inactivation of bacteriophage, making removal of the solid phase during drying beneficial to

mimicking spray drying (Thompson and Yates, 1999). Several studies have also been published on the effect of acoustic streaming on droplet drying kinetics, which would change how the particles dry compared to the spray dryer (Bänsch and Götz, 2018). However, the disruption in air flow caused by the hot air in the levitator is likely greater than the acoustic streaming effect, and comparisons between the evaporated controls, levitation, and spray drying show that acoustic streaming is not detrimental to mimicking the spray dryer.

Variability in the test data was assessed with the mannitol/dextran formulation by running trials in true triplicate. The main source of error and variability in the results was found to come from the endpoint dilution *in vitro* assay for measuring viral activity, determined by testing control samples of the stock viral vector for activity as well; the control showed a standard deviation of 0.204 log for the three trials. The overall conclusion drawn from Fig. 2 was that moderate heat (30-55°C) enabled acoustic levitation to produce similar trends in activity between the excipient blends when compared to spray drying, even though titer losses remained significantly lower.

3.1.3 Effect of particle size on activity loss

The major physical difference between acoustic levitation and spray drying is the size of the particles produced. Particles produced using the spray dryer have diameters in the range of 10 – 50 μm (LeClair et al., 2016a), whereas particles created via acoustic levitation have diameters approximately one order of magnitude larger, in the range of 300 – 800 μm. In the acoustic levitator, particles are necessarily large since they need to be collected and manipulated manually by an operator, and it is desirable that individually they contain as much virus as possible so that only a few particles (approximately 7) are needed for a detectable response in *in vitro* (or *in vivo*)

testing. To determine the effect that droplet size had on viral activity post drying, three different droplet sizes were placed at 40°C in the levitator without changing the mannitol/dextran concentration in solution. The smallest particles were created by levitating 0.5 μL of solution, the smallest amount that was easily detachable from the needle tip, and produced particles with an average Feret diameter of 360 ± 30 μm. The largest particles that could be levitated in a stable manner used 2.8 μL of solution and gave particles with an average Feret diameter of 780 ± 60 μm. The average Feret diameter for the intermediate size was 510 ± 50 μm. Fig. 3 reports the average particle sizes and resulting activity losses for mannitol/dextran levitated particles.

Activity testing shows that there is no significant difference in the titer loss of viral vector activity caused by changing the particle size; the variation in log loss data has already been attributed to the endpoint dilution method. All tested particle sizes show titer losses of 1.5 log or less, which indicate overall good performance, as expected for the mannitol/dextran formulation with AdHu5 (LeClair et al., 2016a). While particle size intuitively may be linked to activity loss (based on a higher interfacial area per unit volume at which viruses may be deactivated with smaller particles), the size range investigated here (360 – 777 μm) did not show this trend. Importantly, Fig. 3 shows that the difference in particle size between the acoustic levitator and spray dryer does not invalidate the former as a screening method.

[Fig. 3]

3.2 Size and shape of spray dried and levitated powders

Particle size and shape of the dry powder vaccine is considered to impact its long term storage stability, where surface cracking/distortions and proximity of the virus to the surface influence the rate of declining activity. We have previously reported that spray dried particles

showing surface nodules exhibited higher activity losses though the source of the nodules could not be conclusively identified as the virus (LeClair et al., 2016a). Particles created using both the spray dryer and the acoustic levitator at 40°C, imaged using SEM, are compared side-by-side in Fig. 4.

[Fig. 4]

As shown in Fig. 4, the shape and surface morphology of dried particles are consistent between acoustic levitation and spray drying. Both lactose/trehalose and mannitol/dextran show spherical particles, indicating a low Peclet number caused by high solubility and reduced surface activity (Elversson and Millqvist-Fureby, 2005). Mannitol/dextran particles display a rougher, more wrinkled surface than the smooth lactose/trehalose particles. Xylitol/dextran particles display a more dimpled and slightly collapsed shape, indicating a higher Peclet number which would cause the formation of hollow spheres that may collapse. This may be caused by the higher fraction of dextran present in these particles (75% vs. 25%), as the high molecular weight of dextran would hinder diffusion and lead to earlier precipitation at the droplet surface. The most notable difference between the methods is that acoustically levitated particles appear more oblong rather than spherical for all excipient blends due to the pressure exerted on the levitating droplet by the acoustic wave. The acoustic wave may also be the cause of small striations that are seen on the surface of some levitated particles (Fig. 4d) but not on the spray dried particles.

3.3 Powder glass transition temperature, water content, and crystallinity

There are several factors present in spray drying that are not easily replicated in the acoustic levitator and that may cause differences in titer losses between particles created by the two methods, as highlighted in Fig. 1 and Fig. 2. One of these is the shear stress experienced by

the liquid feed solution through the spray dryer nozzle, causing atomization. Previous work has demonstrated that increased shear can cause denaturation of proteins, and damage to the biologic may be even more pronounced when combined with the increase in air-water interface caused by atomization (Ghandi et al., 2012; LeClair et al., 2016b; Maa and Hsu, 1996, 1997). However, by using relatively low solute concentrations (1% and 4%) in the trials, viscosity is low (and differences between the three excipient blends are minimal; see values in Materials and Methods section 2.7) which suggests that shear was not a dominant cause for the differences in titer losses between formulations in spray drying specifically. Future studies may clarify the importance of shear on final activity losses.

Particle glass transition temperatures and moisture contents for all three excipient blends produced by both methods were analyzed to explain the differences in viral activity (Table 1). All acoustically levitated particles denoted in Table 1 were made at 40°C (i.e., the center point in the range of acceptable temperatures). Particles with the xylitol/dextran and mannitol/dextran blends displayed similar moisture contents between spray dried and levitated powders, with T_g values varying due to the slight differences in the amount of water present. Levitated lactose/trehalose had over five times the moisture content of the spray dried powder, depressing the blend T_g significantly to 91°C from 116°C; water is a known plasticizer of carbohydrates (Hancock and Zografi, 1994). The results show that minor differences in moisture content can produce large differences in T_g. Viewing this information in combination with the activity loss results in Fig. 2, the implications of moisture content can be seen: powders with a higher T_g and hence lower moisture content generally experienced a lower titre loss (or higher T_g leads to higher viral activity in dried particles). The relevance of T_g on viral stability for thermally stable vaccines was previously reported by LeClair (2016a). The increased mobility of the viral vector

in the encapsulating excipient opens it up to degradation mechanisms that are dependent on movement such as aggregation, and therefore a higher glass transition temperature is desirable because higher external temperatures are required to cause destabilization of the viral vector (Bhandari and Howes, 1999; Duddu and Dal Monte, 1997; Hancock et al., 1995). Several groups have published results linking moisture content of dried materials to enzyme and viral activity loss (Perdana et al., 2012; Savage et al., 1998).

[Table 1]

Levitated particles were found to have similar or higher moisture content to spray dried powders of the same formulation. This was expected due to the higher temperatures found in the spray dryer that are not replicated in this case by the levitator, causing more water to evaporate from spray dried powders even though the residence time is much shorter than in the levitator. The differing moisture content can also be related to differences in the crystallinity of the dried particles between the two methods. XRD analysis was done to determine whether the powders were crystalline or amorphous, and to examine the extent of crystallization that was occurring during the different drying processes.

X-ray diffractograms for the three excipient blends are presented in Fig. 5. All three formulations displayed similar XRD spectra between spray dried and levitated powders. Lactose/trehalose and xylitol/dextran displayed amorphous diffraction patterns with a single broad peak for both methods. Lactose and trehalose are known to inhibit crystallization in each other by interfering with crystal lattice growth, causing the amorphous peak seen here (Mazzobre et al., 2001; Miao and Roos, 2005). The amorphous peak of the xylitol/dextran blend is similar to the peak for amorphous xylitol reported using wide-angle X-ray scatterings (WAXS) by Palomäki, and dextran was not expected to show any crystalline peaks in XRD based on previous

reports (Palomäki et al., 2016; Taylor et al., 1959). Mannitol/dextran displayed a crystalline spectrum that was similar to that published by us for spray dried mannitol/dextran (LeClair et al., 2016a). Levitated mannitol/dextran was found to have 62% crystalline content, higher than the 40% crystalline content measured for spray dried mannitol/dextran. This is likely due to the extended drying time in the levitator, which prolongs the period available for the excipients to crystallize (Hoe et al., 2014). This may account for the lower moisture content seen in the levitated mannitol/dextran powder as well, as the higher crystallinity makes it less likely to uptake moisture (Ahlneck and Zografi, 1990). Overall, these results show that the acoustic levitator can create either crystalline or amorphous particles that are representative of the powders produced through spray drying.

[Fig. 5]

4. Conclusion

These results represent a library of new data comparing powder vaccines comprised of different excipient blends with AdHu5GFP and produced using spray drying and acoustic levitation (under similar conditions). The viral activity trends discussed here are all based on non-optimized spray drying conditions, and fairly large variances seen in the *in vitro* assay employed may add increased uncertainty to the results. However, after thorough characterization of viral activity and physical properties of dried powders, we believe that the acoustic levitator can act as a screening method to narrow down potential excipients to the most promising options based on activity testing, T_g values, and moisture content. More specifically, ideal formulations should lead to particles with moderate water content (ca. 10%) and T_g values over 100°C, which can easily be assessed on particles created by acoustic levitation. Heating the acoustic levitator chamber in the 30-55°C range was the key to obtaining similar trends in viral activity between

the two drying methods and the larger size of particles produced by acoustic levitation was not a decisive factor in predicting viral activity. The downside to using the acoustic levitator is the large amount of time necessary to produce enough mass for activity testing and characterization (approximately three hours to generate a milligram of powder), however, the microlitre amounts of liquid sample needed means that this method does not use large amounts of potentially costly viral vector.

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References

Abràmoff, M.D., Magalhães, P.J., Ram, S.J., 2004. Image processing with imageJ. Biophotonics Int. 11, 36–41. https://doi.org/10.1117/1.3589100

Afkhami, S., Yao, Y., Xing, Z., 2016. Methods and clinical development of adenovirus-vectored vaccines against mucosal pathogens. Mol. Ther. - Methods Clin. Dev. 3, 16030. https://doi.org/10.1038/mtm.2016.30

Ahlneck, C., Zografi, G., 1990. The molecular basis of moisture effects on the physical and chemical stability of drugs in the solid state. Int. J. Pharm. 62, 87–95.

https://doi.org/10.1016/0378-5173(90)90221-O

Alcock, R., Cottingham, M.G., Rollier, C.S., Furze, J., Costa, S.D. De, Hanlon, M., Spencer, A.J., Honeycutt, J.D., Wyllie, D.H., Gilbert, S.C., Bregu, M., Hill, A.V.S., 2010. Long-Term Thermostabilization of Live Poxviral and Adenoviral Vaccine Vectors at Supraphysiological Temperatures in Carbohydrate Glass 2, 1–9.

Amorij, J.P., Huckriede, A., Wilschut, J., Frijlink, H.W., Hinrichs, W.L.J., 2008. Development of stable influenza vaccine powder formulations: Challenges and possibilities. Pharm. Res. 25, 1256–1273. https://doi.org/10.1007/s11095-008-9559-6

Bänsch, E., Götz, M., 2018. Numerical study of droplet evaporation in an acoustic levitator. Phys. Fluids 30. https://doi.org/10.1063/1.5017936

Bhandari, B.R., Howes, T., 1999. Implication of glass transition for the drying and stability of dried foods. J. Food Eng. 40, 71–79. https://doi.org/10.1016/S0260-8774(99)00039-4

Crowe, J.H., Oliver, A.E., Hoekstra, F.A., Crowe, L.M., 1997. Stabilization of Dry Membranes by Mixtures of Hydroxyethyl Starch and Glucose: The Role of Vitrification. Cryobiology 35, 20–30. https://doi.org/10.1006/cryo.1997.2020

Duddu, S., Dal Monte, P., 1997. Effect of glass transition temperature on the stability of lyophilized formulations containing a chimeric therapeutic monoclonal antibody. Pharm. Res. 14, 591–595.

Elversson, J., Millqvist-Fureby, A., 2005. Particle size and density in spray drying - Effects of carbohydrate properties. J. Pharm. Sci. 94, 2049–2060. https://doi.org/10.1002/jps.20418

Ghandi, A., Powell, I.B., Howes, T., Chen, X.D., Adhikari, B., 2012. Effect of shear rate and

oxygen stresses on the survival of Lactococcus lactis during the atomization and drying stages of spray drying: A laboratory and pilot scale study. J. Food Eng. 113, 194–200. https://doi.org/10.1016/j.jfoodeng.2012.06.005

Grasmeijer, N., 2016. Improving protein stabilization by spray drying: formulation and process development. University of Groningen.

Hancock, B.C., Shamblin, S.L., Zografi, G., 1995. Molecular Mobility of Amorphous Pharmaceutical Solids Below Their Glass Transition Temperatures. Pharm. Res. An Off. J. Am. Assoc. Pharm. Sci. https://doi.org/10.1023/A:1016292416526

Hancock, B.C., Zografi, G., 1994. The Relationship Between the Glass Transition Temperature and the Water Content of Amorphous Pharmaceutical Solids. Pharm. Res. An Off. J. Am. Assoc. Pharm. Sci. https://doi.org/10.1023/A:1018941810744

Hoe, S., Ivey, J.W., Boraey, M.A., Shamsaddini-shahrbabak, A., Javaheri, E., Matinkhoo, S., Finlay, W.H., Vehring, R., 2014. Use of a Fundamental Approach to Spray-Drying Formulation Design to Facilitate the Development of Multi-Component Dry Powder Aerosols for Respiratory Drug Delivery. Pharm. Res. 449–465. https://doi.org/10.1007/s11095-013-1174-5

I Ré, M., 1998. Microencapsulation By Spray Drying. Dry. Technol. 16, 1195–1236. https://doi.org/10.1080/07373939808917460

Klimakow, M., Leiterer, J., Kneipp, J., Rossler, E., Panne, U., Rademann, K., Emmerling, F., 2010. Combined Synchrotron XRD / Raman Measurements : In Situ Identification of Polymorphic Transitions during Crystallization Processes. Langmuir 26, 11233–11237. https://doi.org/10.1021/la100540q

Kristensen, D., Chen, D., 2010. Stabilization of vaccines: Lessons learned. Hum. Vaccin. 6, 227–231. https://doi.org/10.4161/hv.6.3.11618

Langford, A., Bhatnagar, B., Walters, R., Tchessalov, S., Ohtake, S., 2018. Drying technologies for biopharmaceutical applications: Recent developments and future direction. Dry. Technol. 36, 677–684. https://doi.org/10.1080/07373937.2017.1355318

LeClair, D.A., Cranston, E.D., Xing, Z., Thompson, M.R., 2016a. Evaluation of excipients for enhanced thermal stabilization of a human type 5 adenoviral vector through spray drying. Int. J. Pharm. 506, 289–301. https://doi.org/10.1016/j.ijpharm.2016.04.067

LeClair, D.A., Cranston, E.D., Xing, Z., Thompson, M.R., 2016b. Optimization of Spray Drying Conditions for Yield, Particle Size and Biological Activity of Thermally Stable Viral Vectors. Pharm. Res. 33, 2763–2776. https://doi.org/10.1007/s11095-016-2003-4

Leiterer, J., Delißen, F., Emmerling, F., 2008. Structure analysis using acoustically levitated droplets. Anal. Bioanal. Chem. 391, 1221–1228. https://doi.org/10.1007/s00216-008-2011-2

Lorenzen, E., Lee, G., 2012. Slow motion picture of protein inactivation during single-droplet drying: a study of inactivation kinetics of L-glutamate dehydrogenase dried in an acoustic levitator. J. Pharm. Sci. 101, 2239–2249. https://doi.org/10.1002/jps

Maa, Y.-F., Hsu, C.C., 1996. Effect of high shear on proteins. Biotechnol. Bioeng. 51, 458–465. https://doi.org/10.1002/(SICI)1097-0290(19960820)51:4<458::AID-BIT9>3.0.CO;2-H

Maa, Y.F., Hsu, C.C., 1997. Protein denaturation by combined effect of shear and air-liquid interface. Biotechnol. Bioeng. 54, 503–12. https://doi.org/10.1002/(SICI)1097-0290(19970620)54:6<503::AID-BIT1>3.0.CO;2-N

Mazzobre, F., Soto, G., Aguilera, José M.Buera, M.P., 2001. Crystallization kinetics of lactose in sytems co-lyofilized with trehalose . Analysis by differential scanning calorimetry. Food Res. Int. 34, 903–911.

Miao, S., Roos, Y.H., 2005. Crystallization Kinetics and X-ray Diffraction of Crystals Formed in Amorphous Lactose , Trehalose , and Lactose/Trehalose Mixtures. J. Food Sci. 70, 350–358.

Mondragon, R., Hernandez, L., Enrique Julia, J., Carlos Jarque, J., Chiva, S., Zaitone, B., Tropea, C., 2011. Study of the drying behavior of high load multiphase droplets in an acoustic levitator at high temperature conditions. Chem. Eng. Sci. 66, 2734–2744. https://doi.org/10.1016/j.ces.2011.03.033

Ohtake, S., Martin, R.A., Yee, L., Chen, D., Kristensen, D.D., Lechuga-Ballesteros, D., Truong-Le, V., 2010. Heat-stable measles vaccine produced by spray drying. Vaccine 28, 1275–1284. https://doi.org/10.1016/j.vaccine.2009.11.024

Palomäki, E., Ahvenainen, P., Ehlers, H., Svedström, K., Huotari, S., 2016. Monitoring the recrystallisation of amorphous xylitol using Raman spectroscopy and wide-angle X-ray scattering. Int. J. Pharm. 508, 71–82. https://doi.org/10.1016/j.ijpharm.2016.04.074

Perdana, J., Fox, M.B., Schutyser, M.A.I., Boom, R.M., 2012. Enzyme inactivation kinetics: Coupled effects of temperature and moisture content. Food Chem. 133, 116–123. https://doi.org/10.1016/j.foodchem.2011.12.080

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.

Roediger, E.K., Kugathasan, K., Zhang, X.Z., Lichty, B.D., Xing, Z., 2008. Heterologous boosting of recombinant adenoviral prime immunization with a novel vesicular stomatitis virus-vectored tuberculosis vaccine. Mol. Ther. 16, 1161–1169. https://doi.org/10.1038/mt.2008.59

Sadek, C., Schuck, P., Fallourd, Y., Pradeau, N., Le Floch-Fouéré, C., Jeantet, R., 2015. Drying of a single droplet to investigate process–structure–function relationships: a review. Dairy Sci. Technol. 95, 771–794. https://doi.org/10.1007/s13594-014-0186-1

Savage, M., Torres, J., Franks, L., Masecar, B., Hotta, J.A., 1998. Determination of adequate moisture content for efficient dry-heat viral inactivation in lyophilized factor VIII by loss on drying and by near infrared spectroscopy. Biologicals 26, 119–124. https://doi.org/10.1006/biol.1998.0140

Schiffter, H., Lee, G., 2006. Single-droplet evaporation kinetics and particle formation in an acoustic levitator. Part 2: Drying kinetics and particle formation from microdroplets of aqueous mannitol, trehalose, or catalase. J. Pharm. Sci. 96, 2284–2295. https://doi.org/10.1002/jps

Schutyser, M.A.I., Perdana, J., Boom, R.M., 2012. Single droplet drying for optimal spray drying of enzymes and probiotics. Trends Food Sci. Technol. 27, 73–82. https://doi.org/10.1016/j.tifs.2012.05.006

Sloth, J., Kiil, S., Jensen, A.D., Andersen, S.K., Jørgensen, K., Schiffter, H., Lee, G., 2006. Model based analysis of the drying of a single solution droplet in an ultrasonic levitator. Chem. Eng. Sci. 61, 2701–2709. https://doi.org/10.1016/j.ces.2005.11.051

Taylor, B.N.W., Zobel, H.F., Hellman, N.N., 1959. Effect of structure and crystallinity on water

sorption of dextrans. J. Phys. Chem. 617, 1955–1959. https://doi.org/10.1021/j150574a036

Thompson, S.S., Yates, M. V, 1999. Bacteriophage Inactivation at the Air-Water-Solid Interface in Dynamic Batch Systems. Appl. Environ. Microbiol. 65, 1186–1190.

Vehring, R., Foss, W.R., Lechuga-Ballesteros, D., 2007. Particle formation in spray drying. J. Aerosol Sci. 38, 728–746. https://doi.org/10.1016/j.jaerosci.2007.04.005

Wang, J., Thorson, L., Stokes, R.W., Santosuosso, M., Huygen, K., Zganiacz, A., Hitt, M., Xing, Z., 2004. Single Mucosal, but Not Parenteral, Immunization with Recombinant Adenoviral-Based Vaccine Provides Potent Protection from Pulmonary Tuberculosis. J. Immunol. 173, 6357–6365. https://doi.org/10.4049/jimmunol.173.10.6357

Yarin, A.L., Brenn, G., Kastner, O., Rensink, D., Tropea, C., 1999. Evaporation of acoustically levitated droplets 399, 151–204.

Yarin, A.L., Pfaffenlehner, M., Tropea, C., 1998. On the acoustic levitation of droplets. J. Fluid Mech. 356, 65–91. https://doi.org/10.1017/S0022112097007829

Zhu, F.C., Hou, L.H., Li, J.X., Wu, S.P., Liu, P., Zhang, G.R., Hu, Y.M., Meng, F.Y., Xu, J.J., Tang, R., Zhang, J.L., Wang, W.J., Duan, L., Chu, K., Liang, Q., Hu, J.L., Luo, L., Zhu, T., Wang, J.Z., Chen, W., 2015. Safety and immunogenicity of a novel recombinant adenovirus type-5 vector-based Ebola vaccine in healthy adults in China: Preliminary report of a randomised, double-blind, placebo-controlled, phase 1 trial. Lancet 385, 2272–2279. https://doi.org/10.1016/S0140-6736(15)60553-0

Table 1. Glass transition temperatures measured by DSC, and moisture content measured by TGA for three binary excipient formulations, spray dried and acoustically levitated at 40°C.

Formulation	Glass Transition Temperature (°C)		Moisture Content	
	Spray Dried	Levitated	Spray Dried	Levitated
Lactose/Trehalose	116	91	4.4%	23.0%
Mannitol/Dextran	117	130	10.7%	7.0%
Xylitol/Dextran	142	118	8.4%	10.7%

Fig. 1

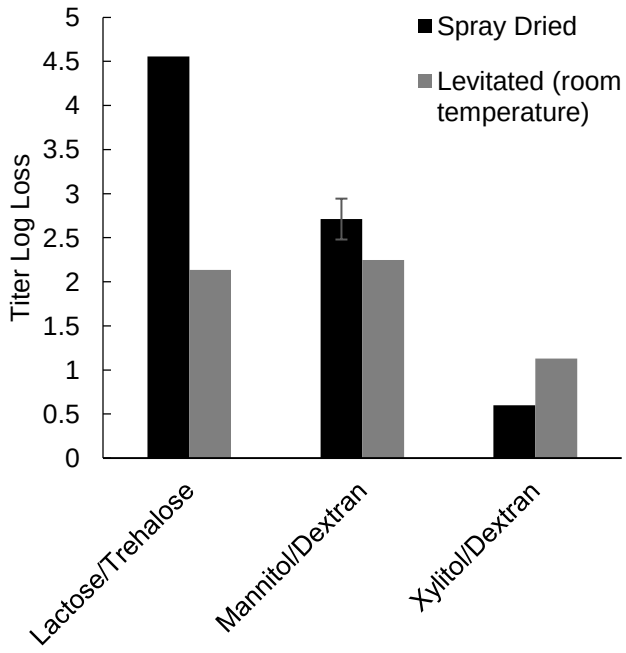

Titer Log Loss
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Spray Dried
Levitated (room temperature)
Lactose/Trehalose
Mannitol/Dextran
Xylitol/Dextran

Fig. 2

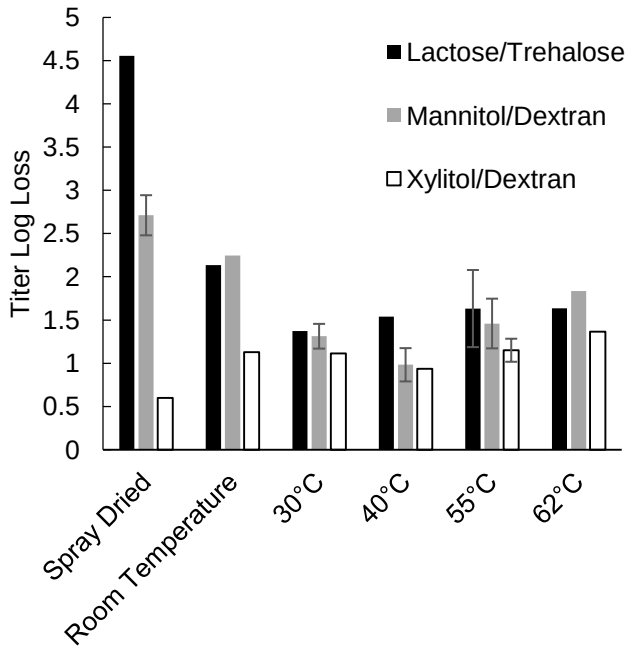

Titer Log Loss
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Lactose/Trehalose
Mannitol/Dextran
Xylitol/Dextran
Spray Dried
Room Temperature
30°C
40°C
55°C
62°C

Fig. 3

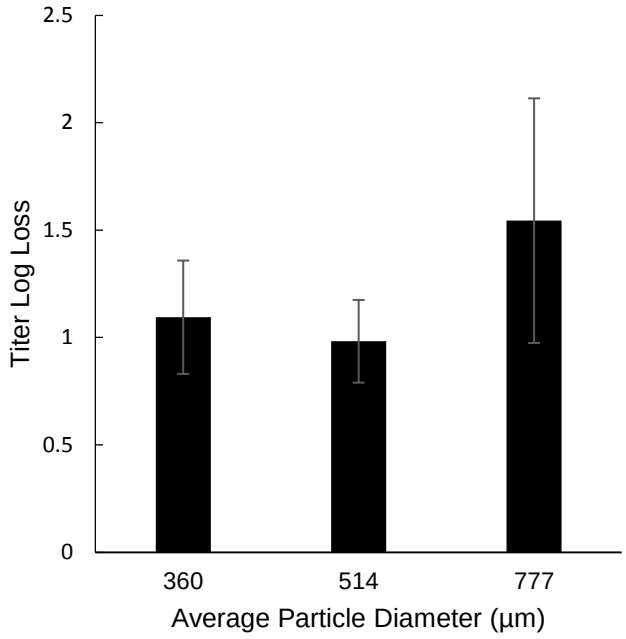

2.5
2
1.5
1
0.5
0
Titer Log Loss
360
514
777
Average Particle Diameter (µm)

Fig. 4

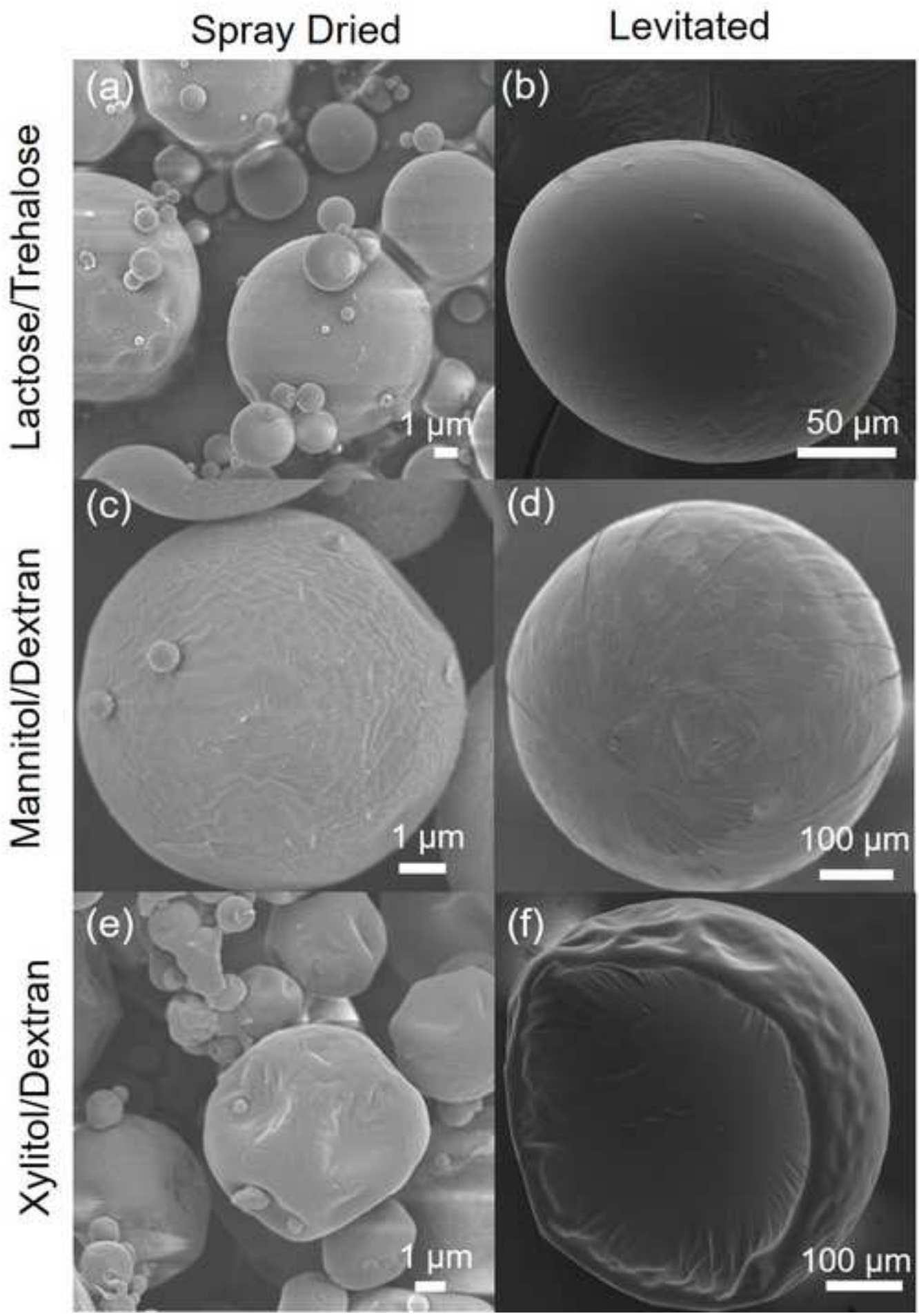
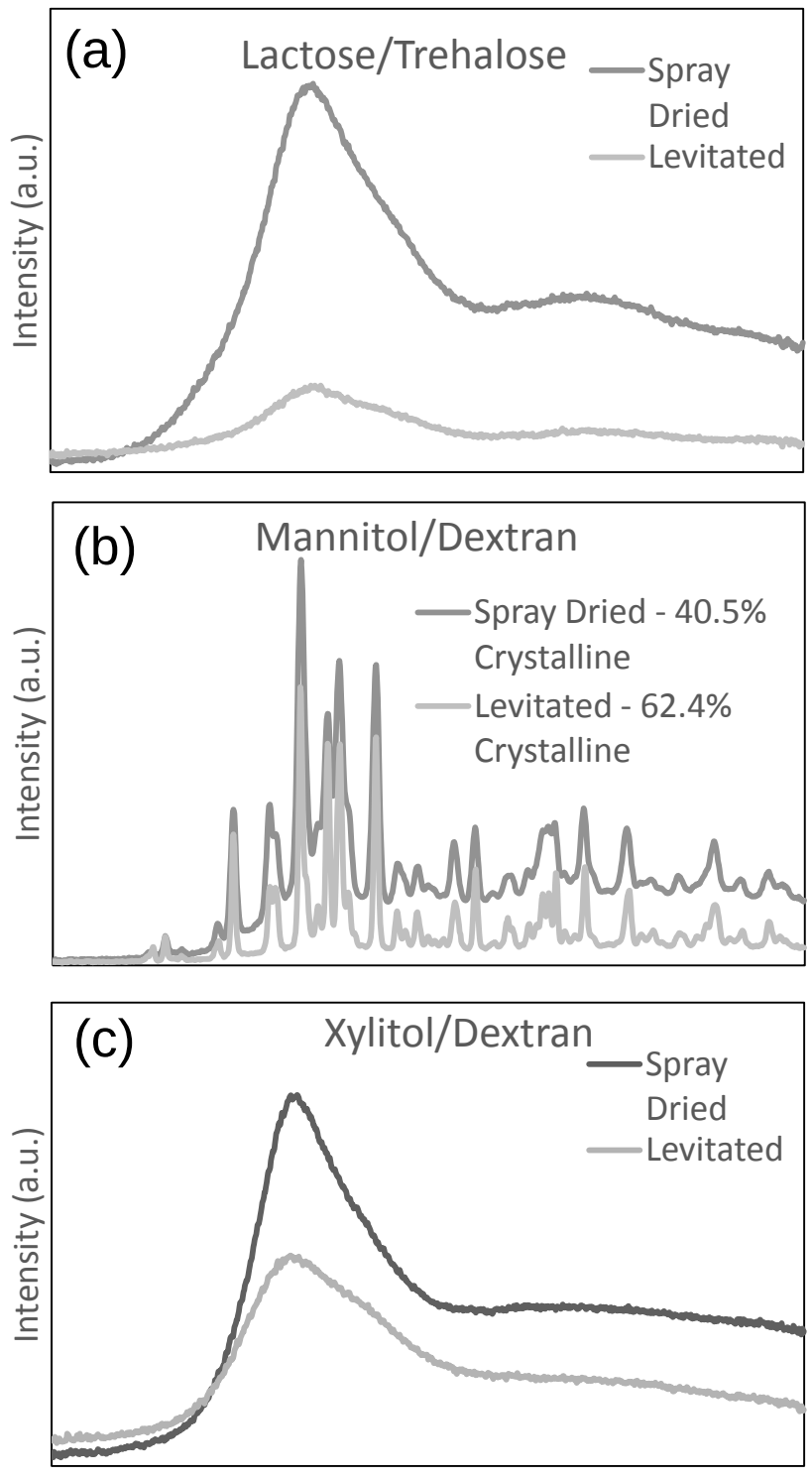

Spray Dried
Levitated
Lactose/Trehalose
Mannitol/Dextran
Xylitol/Dextran
(a)
(b)
(c)
(d)
(e)
(f)
1 μm
50 μm
1 μm
100 μm
1 μm
100 μm

Fig. 5


(a)
Lactose/Trehalose
Spray Dried
Levitated
Intensity (a.u.)
(b)
Mannitol/Dextran
Spray Dried - 40.5% Crystalline
Levitated - 62.4% Crystalline
Intensity (a.u.)
(c)
Xylitol/Dextran
Spray Dried
Levitated
Intensity (a.u.)