Evaluation of Excipients for Enhanced Thermal Stabilization of a Human Type 5 Adenoviral Vector through Spray Drying

Daniel A. LeClair[1], Emily D. Cranston[1], Zhou Xing[2], Michael R. Thompson[1,*]

[1] Department of Chemical Engineering, McMaster University

Hamilton, Ontario, Canada L8S 4L7

[2] McMaster Immunology Research Centre & Department of Pathology and Molecular Medicine, McMaster University

Submitted to: International Journal of Pharmaceutics

January 2016

[*] Author to whom correspondence should be addressed.

Email: mthomps@mcmaster.ca

Tel: (905) 525-9140 x 23213

Evaluation of Excipients for Enhanced Thermal Stabilization of a Human Type 5 Adenoviral Vector through Spray Drying

Daniel A. LeClair[1], Emily D. Cranston[1], Zhou Xing[2], Michael R. Thompson[1,*]

ABSTRACT

We have produced a thermally stable recombinant human type 5 adenoviral vector (AdHu5) through spray drying with three excipient formulations (L-leucine, lactose/trehalose and mannitol/dextran). Spray drying leads to immobilization of the viral vector which is believed to prevent viral protein unfolding, aggregation and inactivation. The spray dried powders were characterized by scanning electron microscopy, differential scanning calorimetry, Karl Fischer titrations, and X-ray diffraction to identify the effects of temperature and atmospheric moisture on the immobilizing matrix. Thermal stability of the viral vector was confirmed *in vitro* by infection of A549 lung epithelial cells. Mannitol/dextran powders showed the greatest improvement in thermal stability with almost no viral activity loss after storage at 20°C for 90 days (0.7 ± 0.3 log $TCID_{50}$) which is a significant improvement over the current -80ºC storage protocol. Furthermore, viral activity was retained over short term exposure (72 hours) to temperatures as high as 55°C. Conversely, all powders exhibited activity loss when subjected to moisture due to amplified molecular motion of the matrix. Overall, a straightforward method ideal for the production of thermally stable vaccines has been demonstrated through spray drying AdHu5 with a blend of mannitol and dextran and storing the powder under low humidity conditions.

KEYWORDS: adenovirus, viral vector, vaccine, spray drying, thermal stability, moisture uptake

Introduction

Adenovirus-based gene transfer vectors have been increasingly developed as vaccine platforms against both old and newly emerging infections (Lasaro and Ertl, 2009; Majhen et al., 2014; Zhu et al., 2015). However, the real world application of adenoviral vectors, in particular in the developing countries, is limited by their instability when stored or transported at even mild temperatures. Alteration of genetic data within viral genomes for vaccine vector applications results in an increased instability in maintaining infectious function (Amalfitano et al., 1998; Havenga et al., 2006). Storage of these vectors within synthetic vials furthermore accelerates denaturing of proteins and loss of viral infectivity through aggregation. Thus, to maintain function, adenoviral vectors suspended in an aqueous medium require storage at temperatures close to -80°C to maintain 'cold chain' protocols (Nyberg-Hoffman and Aguilar-Cordova, 1999). This condition is critical for inhibiting molecular movements of the stored adenoviruses, hindering their aggregation else resulting in vector inactivation (Kumru et al., 2014; Rexroad et al., 2006, 2003). Immobilization of viral vectors within cold storage conditions are uneconomical, and potentially infeasible in areas around the globe requiring vaccination the most.

A major goal for both the World Health Organization and Bill & Melinda Gates Foundation is to alleviate cold chain requirements for vaccine storage and distribution (World Health Organization, 2011). Hence, thermal stability, as used in reference to new classes of vaccines, refers to the ability of a viral vector to be stored at elevated temperatures (above -80ºC) for prolonged duration without significant loss of activity. A promising approach capable of increasing thermal stability of labile vectors is through their dispersion within the amorphous phase of a solid matrix, termed as vitrification (Crowe et al., 1997; Rexroad et al., 2003).

Vitrification of viral vectors within sugars, polymers, amino acids, surfactants, and other materials has maintained viral activity at storage temperatures above typical cold chain temperatures (Alcock et al., 2010; Amorij et al., 2008; Maa et al., 2004).

Previous studies have dictated the importance of matrix physical and chemical properties on thermal stability.(Yu, 2001) The production of a solid matrix is known to greatly hinder the molecular movements of an entrapped adenoviral vector, thus preventing unfolding and aggregation (Ihnat et al., 2005). Selection of a purely amorphous matrix may result in a solid with high moisture sensitivity (Hancock and Zografi, 1993) which will reduce stabilization of any dispersed labile biological materials (Ahlneck and Zografi, 1990). Conversely, crystalline structures are moisture-resistant but not optimal for stabilizing dispersed biological materials due to poor incorporation within the matrix. Binary excipient mixtures are a novel consideration for stabilizing viral vectors since they can be used to balance the physical characeteristics of a formulation (Couchman, 1978; Penning and St. John Manley, 1996), though no current examples are systematically evaluated within the literature. The work presented here demonstrates the potential viability for semicrystalline powders as stabilizing matrices. As pharmaceutical excipients require regulatory approval for use, this work highlights that it is not necessary to be even more restrictive in excipient selection by not considering crystalline and semicrystalline materials. Furthermore, crystallinity may offer material advantages, as previous publications have demonstrated that crystalline regions can act as physical barriers for molecular movements and water sorption (Bronlund and Paterson, 2004; Mihranyan et al., 2004; Mizuno et al., 1998). The present work evaluates two binary sugars and one amino acid formulation to observe the effects of crystallinity and excipient glass transition temperature (T_g) on adenovirus stabilization.

Several drying processes such as spray drying, freeze drying and foam drying have been employed in recent years for producing dry powder forms of solid viral vector dispersions (Jin et al., 2010; Ohtake et al., 2010; Wong et al., 2007). Spray drying is increasingly preferred since its simple requirements facilitate product scalability (Ré, 1998) and favorable economics. During spray drying, a pressurized gas is used to disperse a liquid feed into small droplets within a drying chamber. Evaporation of heated aqueous droplets results in precipitation of the dissolved solutes and suspended materials. Current research aimed at improving thermal stability for labile biological materials has shown great success with spray drying vaccines ranging from attenuated pathogens to antigen-based formulations (Garmise et al., 2007; Jin et al., 2010; Ohtake et al., 2010; Saluja et al., 2010; Wong et al., 2007). The degree of thermal stabilization varies significantly depending on the dispersed biological material. For example, a spray dried bacillus Calmette-Guérin vaccine formulation with L-leucine demonstrated a minimal activity loss of approximately 2.0 log after 120 days at 25°C under high moisture protection (Wong et al., 2007). Alternatively, an antigen-based influenza subunit vaccine stabilized in inulin retained considerable immunogenicity for up to three years of storage at 20°C (Saluja et al., 2010). The variance in stability among spray dried biological materials emphasizes the need for specific evaluation of each vaccine backbone and excipient combination.

Human adenovirus type 5 (AdHu5) has been shown to be an effective vaccine vector for prevention of infectious diseases and has been developed in both liquid buffer and lyophilized forms (Frahm et al., 2012; Smaill et al., 2013). Current limitations to AdHu5 use stem from pre-existing AdHu5 immunity and the lack of a thermally stabilized form. It is estimated that 30 - 100% of the population, depending on geographical location, have been exposed to AdHu5 and therefore elicit an AdHu5-specific response upon infection (Appaiahgari and Vrati, 2014). The

anti-AdHu5 immunity pre-existing in most of the human population poses a potential limitation to the application of AdHu5-vectored vaccines. However, the results from our recent clinical vaccine trial suggest that the potency of AdHu5 vector system is able to diminish the negative effect of a pre-existing immunity (Smaill et al., 2013). Furthermore, AdHu5 vector is particularly amenable to vaccination via the respiratory mucosal route against lung infectious diseases and the human respiratory tract has been found to have minimal pre-existing anti-AdHu5 immunity (Richardson et al., 2011). Thus, it is expected for AdHu5-based vaccine to be even more effective when given via the respiratory mucosal route versus a parenteral route. In terms of thermal stability, AdHu5 has yet to be developed into a well-stabilized spray dried form. This work extends the possible applications of AdHu5 as a vaccine by producing a more thermally stable vector through spray drying with well-accepted excipients. More specifically, we have evaluated binary sugar and amino acid formulations consisting of semicrystalline and entirely crystalline excipient matrices to observe the effects of crystallinity and T_g on AdHu5 stability. The effects of storage time, temperature and humidity were systematically examined on spray dried vector infectivity for AdHu5, which to the best of our knowledge, has not been reported previously. The purpose of this work is to demonstrate a thermally stable spray dried AdHu5 vector and highlight the physical properties necessary for the best stabilization, which can be used to further the field of dry powder vector development. Future developments with these spray dried powders will focus on their use for inhalation and optimizing excipient ratios for better thermal stability of the labile material. The future use of these spray dried powders in inhalable applications is dependent on a suitable safety assessment, as the effects of administration of the studied excipients within this work to the lungs has not been fully established.

Materials and Methods

Chemicals and Adenoviral Vectors

Anhydrous lactose, D-(+)-trehalose dihydrate, D-mannitol, dextran (M_r 40000 kDa) and L-leucine were all purchased as USP grades from Sigma-Aldrich (Ontario, Canada). Culture media was produced from α-minimum essential medium (prepared in the lab according to protocol by Life Technologies (Ontario, Canada)) with 10% fetal bovine serum and 1% streptomycin/penicillin (Invitrogen; Ontario, Canada). X-Gal stock solution was purchased from EMD Millipore (Ontario, Canada). A recombinant replication-defective human type 5 adenovirus expressing *Escherichia coli* β-galactosidase (AdHu5LacZ) was produced in the vector facility of McMaster Immunology Research Centre as described previously (Xing et al., 1996).

Spray Drying

Spray dried powders were produced using a Büchi Mini Spray Dryer B-290 (Büchi; Switzerland) with 0.7 mm spray nozzle and high performance cyclone. The setup is shown schematically in Figure 1, consisting of 1) the spray drying nozzle, 2) the drying chamber, 3) the separating cyclone and 4) the collection chamber. The atomizing air was dried using an in-line silica gel desiccant air dryer (McMaster-Carr; Elmhurst, IL) and cleaned using an Aervent® 0.2 μm filter (EMD Millipore; Billerica, MA). Three excipient formulations were produced: (1) L-leucine, (2) 90% lactose and 10% trehalose and (3) 67% mannitol and 33% dextran (all compositions are quoted based on percent by weight). Excipient formulations were dissolved in Milli-Q® water. The AdHu5 vector was stored in a PBS buffer; however, its addition to the

excipient solution was negligible, being less than 1/10000th of the spray dried volume. The input concentration of viral vector for each formulation was 7.56 x 10^6 $TCID_{50}$/mg. The pH of the solution was 6.5. The formulations used within this work were selected from a number of excipients that have been employed within the pharmaceutical and spray drying industry (Amorij et al., 2008; Ohtake et al., 2010; Vehring, 2008; Wu et al., 2014; Yu, 2001) and can be found in the Inactive Ingredient Database for Approved Drug Products (FDA). The reported formulations (Table 1, in terms of excipients used and excipient ratio) were derived from preliminary spray drying experiments (results not included) that looked to achieve a high yield of non-agglomerating particles with matrices of significant amorphous content and high glass transition temperatures. The specific spray dryer conditions used for each formulation were based on those preliminary experiments attempting to minimize the adenoviral vector activity loss through processing. Recovery was calculated as a percentage of the mass of powder in the collection vessel compared to the input amount. All spray drying processes and powder collection were performed in a custom biosafety cabinet (Design Filtration; Ontario, Canada).

Table 1. Spray drying process parameters and powder recovery for each formulation.

Parameter	L-leucine	90% Lactose/10% Trehalose	67% Mannitol/33% Dextran
Solid Concentration (mg/mL)	3[a]	10	10
Spray Gas Flow (L/h)	439.11	666.93[b]	439.11
Inlet Temperature (°C)	90	90	120[c]
Outlet Temperature (°C)	54	48	65
Feed Flow Rate (mL/h)	145.0	145.0	217.5[d]
Recovery (%)	81.4	83.1	84.5

a: Formation of crumpled L-leucine particles after spray drying prevented concentrations of 10 mg/mL.
b: Increased spray gas flow for lactose/trehalose formulation increased powder production without any observed decrease in viral titre post-spray drying.
c,d: Greater inlet temperature and feed flow rate increased post-spray drying viral titre.

Sample Storage

Powder samples were stored for different durations at relative humidities (RH) of <10%, 45% and >90%. Storage humidity was produced with gel desiccants and saturated salt solutions

(potassium carbonate, 45% RH; potassium nitrate, >90% RH) within desiccators. The relative humidity within each compartment was confirmed before sample observation through direct measurement with a Fisher Scientific™ Traceable™ Jumbo Thermo-Humidity Meter (Fisher Scientific; Ontario, Canada), and found to vary ±3%. The liquid control containing only buffer and AdHu5LacZ was stored in a tightly sealed vial under ambient conditions due to the dehydrating effects of desiccants and saturated salts, though it is assumed that humidity would have no effect on the liquid sample. All aging trials examining the influence of humidity were performed at 20°C. Samples and storage conditions are listed in Table 2. Sealed samples were stored in closed 2 mL Nalgene General Long-Term Storage Cryogenic Tubes (Nalgene; Ontario, Canada).

Table 2. Storage conditions and corresponding sample formulations.

Storage Condition			Samples Stored
Temperature	Relative Humidity	Storage Time	
20°C	<10% RH	Up to 90 days (evaluated at 0, 1, 10, 45 and 90 days)	L-leucine Lactose/Trehalose Mannitol/Dextran
20°C	45% RH	Up to 90 days	L-leucine Lactose/Trehalose Mannitol/Dextran
20°C	>90% RH	15 days	L-leucine Lactose/Trehalose Mannitol/Dextran
20°C	Sealed, ambient humidity	Up to 90 days	Liquid Control
37°C	Sealed, ambient humidity	Up to 72 hours	Liquid Control Mannitol/Dextran
45°C	Sealed, ambient humidity	Up to 72 hours	Liquid Control Mannitol/Dextran
55°C	Sealed, ambient humidity	Up to 72 hours	Liquid Control Mannitol/Dextran

Particle Size and Morphology

Spray dried particle size and morphology was examined using a JEOL JSM-7000F scanning electron microscope (SEM) (JEOL Ltd.; Japan). Samples containing adenovirus vector

were inactivated through exposure to UV light for 30 minutes prior to imaging. All samples were applied to double-sided tape and coated with a 5.0 nm layer of platinum. Micrograph images were collected at several magnifications at a working distance ranging from 10.0-10.3 mm and an electron accelerating voltage ranging from 3.0-5.0 kV. All electron microscopy was conducted at pressures less than 5.0 x 10^{-4} Pa. SEM images were analyzed using ImageJ (Abràmoff et al., 2004) to measure the dimensions of nodular masses found on particle surfaces.

A Malvern Mastersizer 2000G (Malvern Instruments; United Kingdom) equipped with a He-Ne laser was used to measure particle size and size distribution. Powder formulations were dispersed in anhydrous ethanol at concentrations of 0.3 mg/mL, 1.0 mg/mL and 1.0 mg/mL for L-leucine, lactose/trehalose and mannitol/dextran formulations, respectively. Mean powder particle size was determined as the average diameter (n=3), and represented as Feret diameters as powders are not entirely spherical. Powder size span was calculated using Equation 1, where D(0.9) represents the diameter greater than 90% of measured particles, D(0.1) represents the diameter greater than 10% of measured particles and D(0.5) represents the median diameter.

$$Span = \frac{[D(0.9) - D(0.1)]}{D(0.5)} \quad (1)$$

X-ray Photoelectron Spectroscopy

Surface elemental analysis was performed using a Physical Electronics (PHI) Quantera II X-ray photo electron spectrometer (Physical Electronics; Chanhassen, MI). Samples containing adenoviral vectors were inactivated through exposure to UV light for 30 minutes. X-rays were generated using an Al source and focused with a quartz crystal monochromator. A monochromatic Al K-α X-ray (1486.7 eV) source was operated at 50W 15kV with a spot size of 200 μm and at pressures less than 2.67 x 10^{-6} Pa. Each recorded measurement was a survey

spectra average of five sample scans. Data manipulation and analysis was performed using PHI MultiPak software (Physical Electronics; Chanhassen, MI).

Argon etching was accomplished using a 500V beam of Ar to bombard the surface of each specimen. Approximately 100 nm was removed from each sample using etch rates based off of computer simulated Monte Carlo calculations (Williams et al., 2003).

Moisture Uptake of Spray Dried Particles

For analysis of moisture content, aged spray dried powders without the adenoviral vector were dissolved in formamide and injected into a Mettler Toledo DL39 Coulometric Karl Fischer Titrator (Mettler Toledo; Columbus, OH). The corresponding water content was determined using Equation 2:

$$Water\ content\ [ppm] = x \cdot \frac{f2 + f3}{f3} - \frac{f1 \cdot f2}{f3} \quad (2)$$

where *x* was the measured water content of the sample after Karl Fischer titration (ppm), and *f1* was the water content of the solvent used (ppm). Water content of the formamide solvent was measured by adding formamide without dissolved solids to the Karl Fischer titrator. *f2* was the measured mass of solvent (g) and *f3* represents the mass of dissolved sample (g).

Thermal Properties of the Spray Dried Particles

Thermograms for the three excipient formulations (without adenoviral vector) were measured using a differential scanning calorimeter (DSC). Samples of 3-10 mg were weighed into hermetically sealed aluminum pans for analysis in a Q200 Differential Scanning Calorimeter (TA Instruments; New Castle, DE). The procedure for measurement involved first equilibrating the sample at 4°C. Sample was heated to 300°C at a ramp rate of 10°C/min under a nitrogen

purge gas flowing at 50 mL/min. The heating rate was established from a previous study characterizing lactose and trehalose samples (Mazzobre et al., 2001). Thermal events were recorded from a single heating ramp to avoid dehydration of the sample.

Crystallinity of Spray Dried Particles

The excipient formulations (without adenoviral vector) were characterized by powder X-ray diffraction (XRD) on a Bruker D8 Discover with DAVINCI.DESIGN difractometer (Bruker; Billerica, MA) using a Cobalt Sealed Tube Source (λ_{avg} = 1.79026 Å, 2Θ = 5-70°). Small sample quantities were mounted on a silica base for examination. A blank silica sample signal was subtracted from each measured data set using GADDS software (Bruker; Billerica, MA), and the resulting signal intensity was integrated into a crystallographic figure through the use of DIFFRAC.EVA software (Bruker; Billerica, MA). Crystalline content was determined using TOPAS software (Bruker; Billerica, MA).

***In Vitro* Testing of Spray Dried Particles**

Culturing of A549 Cells

A549 lung epithelial cells were thawed from liquid nitrogen and cultured in T150 culture flasks using Alpha Minimum Essential Medium Eagle (α-MEM). All cell culturing was completed in a humidified Forma Series II Water Jacketed CO_2 Incubator (Thermo Scientific Corporation; Waltham, MA) at 37.0°C and 5.0% CO_2. When cells were 80-90% confluent, they were split to a new T150 flask and/or plated in a 96-well plate for *in vitro* testing.

Effect of Excipient Formulations on *In Vitro* Experiments

Approximately 10 mg of each spray dried formulation (without adenoviral vector) was dissolved within 100 μL of culture media and added to A549 epithelial cells plated within a 96-well plate through the use of a Gilson micropipette (Gilson; Middleton, WI). The concentration of 10 mg/100 µL was chosen because it is three times more than the concentration used for *in vitro* infection (3 mg/100 µL). Control samples contained only 100 μL of culture media. Cells were left to incubate for 24 hours. After incubation, A549 cells were trypsinized and tested for viability through use of a trypan blue solution (Life Technologies; Ontario, Canada). Cell suspensions were mounted on a Bright-Line™ hemacytometer (Reichert; Buffalo, NY) and the number of viable and nonviable cells were counted. Results are reported as percentage of viable cells measured and error bars represent the standard deviation (n=3).

Spray Dried Formulation Viral Infectivity

The retained viral activity or infectivity of AdHu5LacZ vector after spray drying and storage was determined by infecting plated A549 cells with approximately 3 mg of spray dried powder (input concentration of 7.56×10^6 $TCID_{50}$/mg) reconstituted in culture media right before testing. Eight-fold serial volume dilutions were created from each reconstituted sample, ranging from a dilution of 10^{-1} to a dilution of 10^{-8} or ranging from a dilution of 10^{0} to a dilution of 10^{-7}. Cells were incubated with AdHu5LacZ for 24 hour, and then fixed using a 0.2% glutaraldehyde (Sigma Aldrich; Ontario, Canada)/0.8% formaldehyde (Sigma Aldrich; Ontario, Canada) solution in phosphate buffered saline (%v/v) for less than five minutes. After removal of fixative, viral infection was detected as X-gal color reaction indicative of the cells transduced by infectious AdHu5LacZ viral particles present in the dried powder using the substrate 5-bromo-4-

chloro-3-indoyl β-(D)-galactoside (X-gal). The number of cells positive for color reaction was determined using an Axiovert 25 inverted light microscope (Zeiss; Germany). Median tissue culture infectious dose ($TCID_{50}$) was then calculated using the Reed-Muench method as detailed within the literature (Reed and Muench, 1938). Results are reported as loss of viral activity (log $TCID_{50}$/mg) and error bars are calculated as the standard deviation (n=3).

Data Analysis

Where applicable, results were statistically analyzed using the statistical package R (R Foundation for Statistical Computing; Austria). Results were considered statistically significant for $p \leq 0.05$.

Results and Discussion

Characterization of Spray Dried Powders without Adenoviral Vector

Size and Morphology

The spray dried powder formulations were imaged by SEM at varying magnifications to evaluate size and morphology, as shown in Figure 2. L-leucine particles were generally less than 10 μm in diameter (Figures 2a and 2b), with an average of 8.80 μm (Table 3). The size distribution was broadest for L-leucine compared to the other formulations and had a noted skewness in the Mastersizer favoring smaller particles. These particles had a "collapsed sphere" morphology as a result of the hydrophobic isobutyl side chain on L-leucine which enhanced its surface activity (Gliński et al., 2000), causing reduced diffusion within the drying droplet. This results in an early onset of particle precipitation and the formation of hollow spheres that are prone to collapse, as seen with the L-leucine formulation. Both lactose/trehalose (Figures 2c and 2d) and mannitol/dextran (Figures 2e and 2f) formulations showed spherical morphologies when

spray dried. These latter cases are indicative of systems with a lower Peclet number due to their enhanced solubility and reduced surface activity (Elversson and Millqvist-Fureby, 2005). Average particle diameter for the lactose/trehalose and mannitol/dextran powders were 32.2 μm and 7.92 μm, respectively (Table 3). The larger average particle size for the lactose/trehalose formulation can be attributed to a greater amount of agglomeration between developed particles (notable by the bridging outlined in Figures 2c and 2d). The span in the size distribution for these two formulations were smaller, as indicated in Table 3, compared to L-leucine and both showed a more normal distribution of sizes according to the Mastersizer around their means.

Table 3. Average spray dried particle size and span (calculated by equation 1), measured by Mastersizer, for the three formulations tested.

Formulation	D_{50} (μm)	Span
L-leucine	8.80	2.05
Lactose/Trehalose	32.2	1.73
Mannitol/Dextran	7.92	1.61

Moisture Uptake

The measured water content after storage under different humidity conditions is plotted in Figure 3 for the three spray dried formulations. For the 15 day evaluation, the least hygroscopic spray dried powder was produced with the L-leucine formulation. Total moisture content for L-leucine (measured as percent weight of the total) was 0.98%, 1.98% and 6.76% under controlled relative humidity conditions of <10, 45 and >90% RH, respectively. In comparison, the lactose/trehalose formulation absorbed significant amounts of water, measured as 2.54%, 4.21% and 17.08% for <10, 45, and >90% RH, respectively. A similar amount of moisture uptake was determined for the mannitol/dextran formulation; at <10, 45, and >90% RH, the respective 15 day measurements were 1.72%, 5.89% and 15.05%.

Many pharmaceutically relevant excipients have hydrogen bonding potential, allowing for the binding of water from their surrounding environment (Newman et al., 2008). The absorption of water within solid dispersions is generally deleterious, destabilizing their physical structure by depressing the T_g and inducing changes within the crystalline structure. As a result, minimal moisture uptake is optimal for the dispersed active ingredient to remain immobilized for as long as possible (Ahlneck and Zografi, 1990; Hancock and Zografi, 1994). The low water sorption capacity of L-leucine particles is due to the high crystalline content Both lactose and trehalose are considered to be highly hygroscopic materials and hence, it was not unexpected that the spray dried particles from these ingredients similarly showed high moisture uptake in the experiments. Mannitol is typically crystalline and non-hygroscopic (Naini et al., 1998), yet the inclusion of dextran produced spray dried particles with high moisture sensitivity.

All spray dried powders showed no significant change in moisture content after day 1 when stored at 45% RH or less. In comparison, when stored at >90% RH the powders continued to uptake water and showed no evidence of approaching an equilibrium condition within the tested 15 day period. The spray dried powders stored at >90% RH proved too difficult to preserve in their current state for the detailed characterizations that follow that they will not be reported on further. These powders agglomerated into large masses and were very sticky, proving to be difficult to work with and unsuitable for AdHu5 stabilization.

Crystallinity

The three spray dried powders were analyzed by X-ray diffraction as shown in Figure 4. The crystal structure differences were attributed to the different chemical composition of each formulation. Measured crystalline content is shown in Table 4 for each formulation immediately

after spray drying. The crystallinity for L-leucine was very high, as the particle was mostly crystallized. Both lactose/trehalose and mannitol/dextran formulations were measured to be semicrystalline.

Table 4. Measured crystallinity for all formulations immediately after spray drying.

Formulation	Measured Crystallinity (%)
L-leucine	>97%
Lactose/Trehalose	56%
Mannitol/Dextran	44%

Figure 4a displays diffraction patterns for the crystalline structure of spray dried L-leucine. L-leucine has a propensity to crystallize (Banno et al., 2004), and the sharp peaks in the diffractogram indicate a highly regular crystalline structure was immediately present after spray drying as well as after two weeks of storage at 20°C and <10% RH. Under conditions of 20°C and 45% RH, a small amount of peak broadening was observed for L-leucine. This broadening corresponds with a marginal increase in water content at these conditions, suggesting absorbed water may have partially dissolved and/or disrupted the L-leucine crystal structure.

The diffraction patterns in Figure 4b for the lactose/trehalose formulation displayed crystalline α-lactose monohydrate peaks as well as a large amorphous band, when powders were tested immediately after spray drying. The absence of diffraction peaks for α,α-trehalose dehydrate was unexpected due to its high crystallizing nature and from the fact that its crystalline form has been reported after freeze drying (Sundaramurthi and Suryanarayanan, 2010) but in this case its low concentration likely prevented detection by XRD. The significant amorphous content shown was expected having been previously reported for spray dried lactose (Lerk, 1993) and furthermore, blends of lactose and trehalose are known to inhibit crystallization in the complementary component (Miao and Roos, 2005). It has been previously reported that the presence of trehalose in a lactose system delayed and prevented lactose crystallization through

interference of growth of the crystal lattice (Mazzobre et al., 2001). After storage at 20°C for two weeks, the crystalline regions were less apparent by XRD, even at low humidity. The work of other authors has demonstrated that the onset of crystallization for lactose and trehalose blends occur at 65.6% RH (Miao and Roos, 2005), thus storage of these powders at 45% RH and <10% RH does not allow sufficient moisture for a thermodynamically equilibrated crystal structure to emerge. The broadening of diffraction peaks at these conditions coincided with a decrease in crystallinity similar to what happens when water is lost from a crystalline trehalose structure, resulting in a mostly amorphous material (Ding et al., 1996). This effect of dehydration on crystal structures has been previously reported under mild conditions for both raffinose- and trehalose-based systems (Saleki-Gerhardt et al., 1995; Willart et al., 2002). This process should be anticipated more so in spray dried systems, where the particle is trapped in an unfavourable state as a result of fast drying (Vehring et al., 2007). The broad peaks in Figure 4b (ii, iii) were attributed to the small-sized crystal domains detected by DSC (Rani et al., 2006; Willart et al., 2002).

Figure 4c shows the diffraction patterns for the mannitol/dextran formulation. As expected based on other studies (Taylor et al., 1959), dextran exhibited no crystalline peaks in XRD. The crystalline peaks of mannitol were shifted in the presence of dextran, though most closely resembled the α-polymorphic form (Hulse et al., 2009). After two week storage at 20°C and <10% RH, no change in crystallinity was observed. When stored at 20ºC and 45% RH, a single broad mixed-mode peak was found as a result of the significant water uptake shown in Figure 3. Both α- and β-polymorphic forms of pure mannitol are reportedly structurally stable in the presence of high RH for several weeks, though under those condition, crystallinity is gradually decreased (Hulse et al., 2009). The relatively hygroscopic nature of dextran leading to

greater water uptake, and the previous reports of mannitol crystal disruption from cosolutes such as dextran may have caused this (Kim et al., 1998).

Overall, the presence of moisture is detrimental to the structural stability of the matrix as seen by changes in crystal structure for all three excipient formulations. These changes in crystal structure are indicative of movements on the molecular scale, which correspond to activity loss in spray dried particles containing adenoviral vectors, as discussed further below.

Thermal Properties

The thermal transitions of spray dried particles from each of the three formulations were analyzed by DSC. The glass transition temperature and peak fusion/sublimation temperature (T_m) are given in Table 5. Spray dried L-leucine particles had sufficiently high crystallinity that the glass transition temperature could not be detected. An endothermic peak at 247°C corresponded to the sublimation of L-leucine (Martins et al., 2006). Both lactose/trehalose and mannitol/dextran formulations exhibited relatively high T_g values immediately after spray drying, measured at 115°C and 130°C, respectively. The T_m of 214°C for lactose/trehalose indicated a depressed melting point that was 8°C below the α-form of crystalline lactose (Listiohadi et al., 2009). The T_m of mannitol/dextran was 162°C, lower than the 170°C reported for pure mannitol (Hulse et al., 2009).

After storage for two weeks at 20°C and under dry conditions (<10% RH), the lactose/trehalose and mannitol/dextran powder T_g did not change noticeably, as shown in Table 4. However, after storage for two weeks at 20°C and intermediate humidity conditions (45% RH), the T_g for lactose/trehalose and mannitol/dextran decreased significantly to 15°C and 45°C

respectively. The decrease in measured T_g is caused by the plasticizing effect of absorbed water (Hancock and Zografi, 1994).

The T_m and especially T_g were key parameters in determining matrix stability in terms of immobility. Simply stated, higher T_g and T_m were preferred due to the higher ambient temperatures necessary to induce destabilizing molecular movement

Table 5. Measured glass transition temperature (T_g) and fusion/sublimation temperature (T_m) for three formulations.

Formulation	Day 0		Day 15: 20°C/0% RH		Day 15: 20°C/45% RH	
	T_g	T_m	T_g	T_m	T_g	T_m
L-leucine	-[a]	246.8°C	-[a]	252.5°C	-[a]	247.0°C
Lactose/Trehalose	114.8°C	213.8°C	113.8°C	213.7°C	17.6°C	208.1°C
Mannitol/Dextran	127.3°C	162.4°C	127.3°C	162.8°C	45.9°C	153.5°C

[a]: *L-leucine particles exhibited no detectable T_g.*

Effect of Excipient Formulations on *In Vitro* Experiments

To ensure accurate viral infection measurements, the base spray dried formulations without adenoviral vector were tested for their relative toxicity to the plated A549 cells. The powders were tested at a concentration level that was three times higher than that for later *in vitro* testing. Cell viability corresponding to each formulation is shown in Table 6. No cytotoxicity was observed for the three formulations based on the absence of any significant differences in cell viability between the formulations and the control (α-MEM).

Table 6. Measured A549 cell viability (%) after 24 hour incubation with each formulation (mean ± SD, n=3).

Formulation	Measured Viable Cells (%)
L-leucine	98 ± 3
Lactose/Trehalose	97 ± 1
Mannitol/Dextran	98 ± 2
Control	96 ± 2

Evaluation of Spray Dried Particles Containing Adenoviral Vector

Retained Viral Activity After Spray Drying

Spray dried powders containing human type 5 adenoviral vector expressing *Escherichia coli* β-galactosidase (AdHu5LacZ) were prepared and were indistinguishable in initial appearance from the powders without the viral vector. The high temperatures and shear rates experienced during the spray drying process could presumably lead to some loss in viral infectivity and as such, the vector activity for each formulation was tested immediately after spray drying. As shown in Figure 5, while the L-leucine formulation resulted in a relatively large loss in activity (2.6 ± 0.5 log), the lactose/trehalose and mannitol/dextran formulations exhibited excellent retention of adenoviral vector infectivity with less than 1.0 log loss. The activity loss for spray dried mannitol/dextran with AdHu5 particles was the smallest within this report, being only 0.3 ± 0.1 log. For all three formulations, the collected powder recovery after spray drying was greater than 80% (Table 1) which was important because it implied an efficient processing method where there was no significant loss of valuable biological material, such as the AdHu5LacZ vector tested here.

SEM micrographs of spray dried particles containing the adenoviral vector are shown in Figure 6, demonstrating differences in the extent to which the virus was incorporated within each matrix. The large AdHu5 activity loss observed for L-leucine particles may be due to phase separation of the excipient and the adenoviral vector. Nodules were observed at the powder surface (highlighted by black arrows in Figure 6a) which may imply that some AdHu5 is not fully encapsulated. This is in contrast to the L-leucine particles spray dried without AdHu5 shown in Figure 2a and 2b. These nodules were measured to be 99 ± 8 nm in diameter which is similar to the reported AdHu5 vector diameter of 70-100 nm (Kennedy and Parks, 2009). Separation could occur due to the high Peclet conditions of L-leucine during spray drying and

the “expelling” nature of forming crystals. Due to the low solubility of L-leucine molecules, supersaturation at the droplet surface is thought to occur early in the drying process (Vehring et al., 2007). Furthermore, a crystalline material is unlikely to form stabilizing bonds with any other material, as it is instead more favourable to continue the crystal structure without faults (Jackson, 1984). The coupling of high Peclet conditions and poor labile material stabilization in a crystalline product explains the greater loss of AdHu5 activity after spray drying with L-leucine. In Figure 6b, the addition of the adenoviral vector to the spray dried lactose/trehalose formulation also resulted in nodules on the particle surface, although these nodules were 330 ± 90 nm in diameter, much larger than the AdHu5 vector. These nodules, which were not seen in Figure 2 for particles without AdHu5LacZ, could indicate surface localization of the adenoviral vector, now better encapsulated within a layer of excipient than found with L-leucine. This interpretation of the morphology appears consistent with the infectivity data since the viral vector would have been better isolated from the environment than within L-leucine particles yet not quite so well shielded as in mannitol/dextran. No nodules were visible on the mannitol/dextran particles containing AdHu5 (Figure 6c) indicating complete incorporation.

To improve confidence as to the source of the nodule morphology, the surface characterization technique XPS was used to detect nitrogen as a marker for AdHu5LacZ because this element is not present in lactose, trehalose, mannitol or dextran. Testing by this technique was not done with L-leucine since no element could be identified as a unique marker in this case. The adenoviral vector was unlikely to be solely localized in the nodules but the presence of the nodules suggested closer proximity to the particle surface if they did in fact contain AdHu5LacZ. Nitrogen was detected once out of three tests at the surface of the lactose/trehalose particles and once again out of five tests after argon etching away a surface layer of 100 nm in thickness. The

same number of tests were conducted with mannitol/dextran particles, with nitrogen never being detected. The surface elemental composition for a selected test of the AdHu5LacZ control and both lactose/trehalose and mannitol/dextran powders is shown in Table 7. Due to the detection threshold of the XPS instrument, a concentration of the AdHu5 vector along the tested surface is necessary for nitrogen to be shown in the result. As this is not always the case, it is at times not possible to detect nitrogen. We propose that this method of surface detection for AdHu5 is not entirely concrete, however it offers a valuable insight into potentially observing viral vector locations on formed particles. More confident results with higher resolution could be attained through more rigorous testing, but this would require significant time and resources and is outside the scope of this project. However, the description of the nodules as containing AdHu5LacZ seems compelling based on these SEM and XPS results, and provides a reasonable explanation, in part, for the differences in thermal stability noted between these three excipient formulations.

Table 7. Elemental composition by XPS for lactose/trehalose and mannitol/dextran samples (n=8) before and after argon etching 100 nm into the sample.

Formulation	% Composition Pre-etch			% Composition Post-etch		
	C	O	N	C	O	N
AdHu5LacZ Control[a]	46.4	32.3	9.8	-	-	-
Lactose/Trehalose	51.8	48.2	0.0	52.0	47.8	0.7[b]
Mannitol/Dextran	57.6	42.4	0.0	58.9	41.1	0.0

[a]*Only relevant elements are listed for AdHu5LacZ Control. Salt ions from PBS are not listed.*
[b]*Nitrogen content was present in two lactose/trehalose samples of eight.*

To highlight the fact that the presence of nodules indicating poorly incorporated AdHu5 can only be part of the explanation for the differences in thermal stability seen in the tests, the measured viral infectivity loss of only 0.7 ± 0.1 log for the lactose/trehalose formulation needed to be reiterated. The greater activity retained with this formulation compared to L-leucine is

attributed to the exceptional stabilizing profile of trehalose with other bioactive compounds (Mazzobre et al., 1997). The total encapsulation of AdHu5LacZ in mannitol/dextran powders is correlated to the low activity loss after spray drying. As described previously, particle formation is affected by diffusion of the excipient components in aqueous solution during spray drying. Dextran diffusion is heavily restricted compared to lactose and trehalose, which are much smaller sugars (Uedaira and Uedaira, 1985). Thus precipitation at the droplet surface likely begins with dextran, providing less opportunity for adenovirus segregation to the outer particle surface. These results highlight that the chemical nature of the excipients plays a role in their ability to trap and stabilize AdHu5 vector and that morphological inspection of spray dried particles offers further insight into the ability of some formulations to maintain viral infectivity better than others.

Viral Infectivity after Storage at 20°C and Differing Humidity

Figure 7 shows the resulting adenoviral vector titre loss through storage at 20ºC at <10% RH for each spray dried formulation up to 90 days. For the liquid control (AdHu5LacZ in buffer), significant AdHu5 vector infectivity was lost after 42 days at 20°C and there was no measurable activity after 90 days. This relatively rapid loss of adenoviral vector activity corresponds to previously reported data (Alcock et al., 2010) and further highlights the need for vector stabilization at temperatures above the normal cold chain storage conditions of -80°C. All excipient formulations outperformed the liquid control. However, the mannitol/dextran formulation was able to retain higher viral activity than the other formulations at day 90 for the low humidity condition ($p<0.01$). The measured AdHu5 titre loss at day 90 was 0.7 ± 0.3 log with mannitol/dextran-formulated vector, only slightly higher than the 0.3 ± 0.1 log measured

directly after spray drying. Adenoviral vectors stabilized within lactose/trehalose did not maintain the same degree of function as those stabilized within mannitol/dextran. After 90 days, the measured loss of infectivity for these samples was measured at 3.1 ± 0.3 log. Similarly, L-leucine exhibited poor excipient stability as the measured activity loss on day 90 was 4.0 ± 0.2 log. For all formulations, the vector activity loss was greatest within the first two weeks. This was likely due to the greater amount of molecular movements within the particle as it transitioned to an equilibrated state post-spray drying.

Figure 8 shows the loss of AdHu5LacZ infectivity for each spray dried formulation during storage for up to 90 days at 20°C under moderate moisture conditions (45% RH). By day 90, all formulations were considered to be inactive. This represents a significantly greater viral activity loss at 45% RH compared to <10% RH (noted above) which is attributed to the uptake of water by the spray dried powders within the enclosed storage system. Hygroscopic sugars, and even non-hygroscopic L-leucine particles, will take up detectable quantities of water from the humid air (as demonstrated in Figure 3). As measured by XRD, the crystallographic profiles for both lactose/trehalose and mannitol/dextran powders showed increasing disorder with water uptake over time. XRD peaks from L-leucine particles exhibited only slight broadening indicating disruption of the initially crystalline spray dried structure. Matrix destabilization was further emphasized by the decrease in T_g observed for lactose/trehalose and mannitol/dextran samples (Table 5). This significant decrease in T_g indicated a greater molecular mobility within the stored samples. Overall, the increase in moisture resulted in an accelerated rate of viral titre loss. Whereas in drier conditions the rate of titre loss leveled off after approximately ten days (referring to Figure 7), the same is not observed at 45% RH. This emphasizes the need to store potential AdHu5 vaccines in low humidity environments, however this is more easily maintained

than -80°C temperatures and can be done, for example, using blister packs which are common practice in the pharmaceutical industry.

Overall, the best-performing powder throughout the storage tests was mannitol/dextran which also encapsulated the adenoviral vector without producing nodules after spray drying (as measured by SEM and XPS). The high T_g for lactose/trehalose particles implied a great thermal stability should have been expected but this was not observed. This could best be explained through the excipient matrix instability as observed through XRD (Figure 4b). The glass-like state is known to be thermodynamically unfavourable as the polymer glass is being held with a higher volume and entropy than its equilibrium state (Farhoodi et al., 2012; Struik, 1977). Physical aging of amorphous and semicrystalline materials can potentially cause a slow relaxation to a more thermodynamically favourable state, even at temperatures below the glass transition (Struik, 1977). This change occurs more significantly in lactose/trehalose particles than mannitol/dextran particles. These molecular movements result in a greater loss of adenoviral vector function throughout storage. Although similar instability is not observed for L-leucine through XRD, a failure to properly incorporate the AdHu5LacZ vector within the matrix renders it a poor excipient formulation for long term storage.

Viral Infectivity at Elevated Temperatures

Generally, the mannitol/dextran formulation outperformed the other excipients in its ability to retain adenoviral vector activity under mild storage conditions. This formulation was thus subsequently used to test the thermal stability of AdHu5LacZ at more extreme temperatures of 37°C, 45°C and 55°C. As shown in Figure 9, spray drying adenoviral vector with the mannitol/dextran formulation was found to thermally stabilize the virus significantly more than

the liquid control after 72 hours of storage at increased temperatures ($p<0.05$). For the liquid control, there was no measurable AdHu5LacZ activity after 30 minutes at 45°C, or for any harsher storage conditions. In contrast, the mannitol/dextran formulation exhibited good activity after 72 hours at both 37°C and 45°C. Only at a storage temperature of 55°C was there a significant loss of viral infectivity after 72 hours for the mannitol/dextran particles. This was due to the increase in energy within the system, which promotes viral protein unfolding (Pace and Hermans, 1975; Pace and Vanderburg, 1979). This effect is more pronounced in liquid control samples because there is more molecular movement possible. It is presumed from these results that the mannitol/dextran matrix sufficiently restricts the molecular-scale movements of AdHu5LacZ through immobilization within the vitrified particle, as seen previously for dried influenza viral vaccines (Amorij et al., 2008).

Conclusions

In conclusion, this work has demonstrated that spray drying is able to produce a high yield of powders capable of incorporating AdHu5 vectors in amino acid or sugar matrices. These matrices allow for an increase in viral vector infectivity, compared to the liquid control, even when the powders are stored at high temperatures. Thermal stability is influenced by particle morphology; amorphous structures can form secondary bonds with the viral vector leading to immobilization which prevents protein unfolding and aggregation. While some degree of matrix crystallinity can aid in avoiding moisture uptake, which in turn restricts both molecular rearrangements and decreasing T_g, highly crystalline excipients exhibited viral nodules on the spray dried particle surface and a subsequent decrease in viral activity. The significant loss of

viral vector activity in highly crystalline matrices can be avoided through incorporation of a semicrystalline structure, where amorphous content is able to stabilize the AdHu5 vector.

Improved thermal stability is most pronounced with mannitol/dextran formulations, where the viral vector was fully encapsulated by the matrix and the semicrystalline morphology remained relatively constant over mild storage conditions. More specifically, particles containing AdHu5 and mannitol/dextran are considered almost fully active after storage for 90 days at 20°C with less than 10% RH, as well as after short term storage at extreme temperatures (stable for 72 hours at 45°C and 24 hours at 55°C). This represents a substantial improvement in thermal stability compared to AdHu5 stored in a liquid buffer matrix.

AdHu5-based vectors have been exploited to develop novel vaccines for a number of important infectious diseases (Lasaro and Ertl, 2009; Majhen et al., 2014; Zhu et al., 2015). For instance, we have found a intramuscular dose of 10^8 pfu AdHu5-based TB vaccine to be safe and effective in a phase 1 clinical trial (Smaill et al., 2013). The effective dose of future inhalational applications in humans is expected to be even smaller than the intramuscular dose. Such doses are within the range of production capacity by using our best currently developed spray drying technology. Furthermore, an increased initial concentration of AdHu5 in each formulation can be explored through future studies. Overall a processing method and matrix formulation were developed to allow AdHu5-based vaccines to be stored above typical cold chain temperatures, making their deployment more straightforward and at a fraction of the cost compared to current viral storage methods. However, relative humidities above 45% were detrimental to maintaining viral activity due to moisture uptake and increased mobility of the matrix. As a result, spray dried particles with adenoviral vectors should be stored in low humidity conditions using dry packaging examples already existent in the industry (Rubio et al., 2008, 2006). Although a

semicrystalline matrix was not able to perform exceptionally well at elevated relative humidities, the potential for a semicrystalline stabilizing matrix is apparent at low relative humidities. Extending on this, it may be beneficial to further examine binary mixtures in the future for AdHu5 thermal stability. This is a significant step towards long term storage of AdHu5 vectors with increased thermal stability.

Acknowledgements

Authors like to thank Dr. Pelton for equipment use, Xuan Yang for SEM assistance, Danielle Covelli for XPS assistance, Victoria Jarvis for XRD assistance and Anna Zganiacz and Xueya Feng for cell culture training and general assistance. The authors would also like to thank both the Canadian Centre for Electron Microscopy and the Biointerfaces Institute for use of their facilities. This study is supported by funds from the Canadian Institutes of Health Research and Natural Sciences and Engineering Research Council of Canada.

References

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

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

Alcock, R., Cottingham, M.G., Rollier, C.S., Furze, J., De Costa, S.D., 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. Sci. Transl. Med. 2, 19ra12. doi:10.1126/scitranslmed.3000490

Amalfitano, A., Hauser, M.A., Hu, H., Serra, D., Begy, C.R., Chamberlain, J.S., 1998. Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. J. Virol. 72, 926–933.

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, 1256–73. doi:10.1007/s11095-008-9559-6

Appaiahgari, M.B., Vrati, S., 2014. Adenoviruses as gene/vaccine delivery vectors: promises and pitfalls. Expert Opin. Biol. Ther. 2598, 1–15. doi:10.1517/14712598.2015.993374

Banno, N., Nakanishi, T., Matsunaga, M., Asahi, T., Osaka, T., 2004. Enantioselective Crystal Growth of Leucine on a Self-Assembled Monolayer with Covalently Attached Leucine Molecules 428–429.

Bronlund, J., Paterson, T., 2004. Moisture sorption isotherms for crystalline, amorphous and predominantly crystalline lactose powders. Int. Dairy J. 14, 247–254. doi:10.1016/S0958-6946(03)00176-6

Couchman, P.R., 1978. Compositional variation of glass transition temperatures. 2. Application of the thermodynamic theory to compatible polymer blends. Macromolecules 11, 1156–1161. doi:10.1021/ma60066a018

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. doi:10.1006/cryo.1997.2020

Ding, S.-P., Fan, J., Green, J.L., Lu, Q., Sanchez, E., Angell, C. a., 1996. Vitrification of trehalose by water loss from its crystalline dihydrate. J. Therm. Anal. 47, 1391–1405. doi:10.1007/BF01992835

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

Farhoodi, M., Mousavi, S.M., Oromiehie, A., Mansour, H., 2012. A Study on Physical Aging of Semicrystalline Terephthalate below the Glass Transition Point Polyethylene. J. Appl. Res. Technol. 10, 698–702.

Frahm, N., DeCamp, A.C., Friedrich, D.P., Carter, D.K., Defawe, O.D., Kublin, J.G., Casimiro, D.R., Duerr, A., Robertson, M.N., Buchbinder, S.P., Huang, Y., Spies, G. a., De Rosa, S.C., McElrath, M.J., 2012. Human adenovirus-specific T cells modulate HIV-specific T cell responses to an Ad5-vectored HIV-1 vaccine. J. Clin. Invest. 122, 359–367. doi:10.1172/JCI60202

Garmise, R.J., Staats, H.F., Hickey, A.J., 2007. Novel dry powder preparations of whole inactivated influenza virus for nasal vaccination. AAPS PharmSciTech 8, E81. doi:10.1208/pt0804081

Gliński, J., Chavepeyer, G., Platten, J.K., 2000. Surface properties of aqueous solutions of L-leucine. Biophys. Chem. 84, 99–103.

Hancock, B.C., Zografi, G., 1994. The Relationship Between the Glass Transition Temperature and the Water Content of Amorphous Pharmaceutical Solids. Pharm. Res. 11, 471–477.

Hancock, B.C., Zografi, G., 1993. The use of solution theories for predicting water vapor absorption by amorphous pharmaceutical solids: A test of the Flory-Huggins and Vrentas models. Pharm. Res. doi:10.1023/A:1018901325842

Havenga, M., Vogels, R., Zuijdgeest, D., Radosevic, K., Mueller, S., Sieuwerts, M., Weichold, F., Damen, I., Kaspers, J., Lemckert, a., van Meerendonk, M., van der Vlugt, R., Holterman, L., Hone, D., Skeiky, Y., Mintardjo, R., Gillissen, G., Barouch, D., Sadoff, J., Goudsmit, J., 2006. Novel replication-incompetent adenoviral B-group vectors: High vector stability and yield in PER.C6 cells. J. Gen. Virol. 87, 2135–2143. doi:10.1099/vir.0.81956-0

Hulse, W.L., Forbes, R.T., Bonner, M.C., Getrost, M., 2009. The characterization and comparison of spray-dried mannitol samples. Drug Dev. Ind. Pharm. 35, 712–718. doi:10.1080/03639040802516491

Ihnat, P.M., Vellekamp, G., Obenauer-Kutner, L.J., Duan, J., Han, M. a, Witchey-Lakshmanan, L.C., Grace, M.J., 2005. Comparative thermal stabilities of recombinant adenoviruses and hexon protein. Biochim. Biophys. Acta 1726, 138–51. doi:10.1016/j.bbagen.2005.06.006

Jackson, K.A., 1984. Crystal growth kinetics. Mater. Sci. Eng. 65, 7–13. doi:10.1016/0025-5416(84)90194-0

Jin, T.H., Tsao, E., Goudsmit, J., Dheenadhayalan, V., Sadoff, J., 2010. Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). Vaccine 28, 4369–4375. doi:10.1016/j.vaccine.2010.04.059

Kennedy, M.A., Parks, R.J., 2009. Adenovirus virion stability and the viral genome: size matters. Mol. Ther. 17, 1664–1666. doi:10.1038/mt.2009.202

Kim, A.I., Akers, M.J., Nail, S.L., 1998. The physical state of mannitol after freeze-drying: Effects of mannitol concentration, freezing rate, and a noncrystallizing cosolute. J. Pharm. Sci. 87, 931–935. doi:10.1021/js980001d

Kumru, O.S., Joshi, S.B., Smith, D.E., Middaugh, C.R., Prusik, T., Volkin, D.B., 2014. Vaccine instability in the cold chain: Mechanisms, analysis and formulation strategies. Biologicals 42, 237–259. doi:10.1016/j.biologicals.2014.05.007

Lasaro, M.O., Ertl, H.C.J., 2009. New Insights on Adenovirus as Vaccine Vectors. Mol. Ther. 17, 1333–1339. doi:10.1038/mt.2009.130

Lerk, C.F., 1993. Consoloidation and Compaction of Lactose. Drug Dev. Ind. Pharm. 19, 2359–2398.

Listiohadi, Y., Hourigan, J.A., Sleigh, R.W., Steele, R.J., 2009. Thermal analysis of amorphous lactose and α-lactose monohydrate. Dairy Sci. Technol. 89, 43–67. doi:10.1051/dst:2008027

Maa, Y.F., Ameri, M., Shu, C., Payne, L.G., Chen, D., 2004. Influenza vaccine powder formulation development: Spray-freeze-drying and stability evaluation. J. Pharm. Sci. 93, 1912–1923. doi:10.1002/jps.20104

Majhen, D., Calderon, H., Chandra, N., Fajardo, C.A., Rajan, A., Alemany, R., Custers, J., 2014. Adenovirus-based vaccines for fighting infectious diseases and cancer: progress in the field. Hum. Gene Ther. 25, 301–17. doi:10.1089/hum.2013.235

Martins, T.S., Matos, J.R., Vicentini, G., Isolani, P.C., 2006. Synthesis, characterization, spectroscopy and thermal analysis of rare earth picrate complexes with L-leucine. J. Therm. Anal. Calorim. 86, 351–357. doi:10.1007/s10973-005-7309-0

Mazzobre, M.F., del Pilar Buera, M., Chirife, J., 1997. Protective Role of Trehalose on Thermal Stability of Lactase in Relation to its Glass and Crystal Forming Properties and Effect of Delaying Crystallization. LWT - Food Sci. Technol. 30, 324–329. doi:10.1006/fstl.1996.0231

Mazzobre, M.F., Soto, G., Aguilera, J.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. doi:10.1016/S0963-9969(01)00115-6

Miao, S., Roos, Y., 2005. Crystallization Kinetics and X□ray Diffraction of Crystals Formed in Amorphous Lactose, Trehalose, and Lactose/Trehalose Mixtures. J. Food Sci. 70, 350–358. doi:10.1111/j.1365-2621.2005.tb09976.x

Mihranyan, A., Llagostera, A.P., Karmhag, R., Strømme, M., Ek, R., 2004. Moisture sorption by cellulose powders of varying crystallinity. Int. J. Pharm. 269, 433–442. doi:10.1016/j.ijpharm.2003.09.030

Mizuno, A., Mitsuiki, M., Motoki, M., 1998. Effect of Crystallinity on the Glass Transition Temperature of Starch. J. Agric. Food Chem. 46, 98–103. doi:10.1021/jf970612b

Naini, V., Byron, P.R., Phillips, E.M., 1998. Physicochemical stability of crystalline sugars and their spray-dried forms: dependence upon relative humidity and suitability for use in powder inhalers. Drug Dev. Ind. Pharm. 24, 895–909. doi:10.3109/03639049809097269

Newman, A.W., Reutzel-Edens, S.M., Zografi, G., 2008. Characterization of the "hygroscopic" properties of active pharmaceutical ingredients. J. Pharm. Sci. doi:10.1002/jps.21033

Nyberg-Hoffman, C., Aguilar-Cordova, E., 1999. Instability of adenoviral vectors during transport and its implication for clinical studies 955–957.

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, 1275–84. doi:10.1016/j.vaccine.2009.11.024

Pace, C.N., Hermans, J., 1975. The stability of globular proteins. Crit. Rev. Biochem. Mol. Biol. 3, 1–43.

Pace, C.N., Vanderburg, K.E., 1979. Determining globular protein stability: guanidine hydrochloride denaturation of myoglobin. Biochemistry 18, 288–292. doi:10.1021/bi00569a008

Penning, J.P., St. John Manley, R., 1996. Miscible Blends of Two Crystalline Polymers. 2. Crystallization Kinetics and Morphology in Blends of Poly(vinylidene fluoride) and Poly(1,4-butylene adipate). Macromolecules 29, 84–90. doi:10.1021/ma950652l

Rani, M., Govindarajan, R., Surana, R., Suryanarayanan, R., 2006. Structure in dehydrated trehalose dihydrate--evaluation of the concept of partial crystallinity. Pharm. Res. 23, 2356–67. doi:10.1007/s11095-006-9058-6

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

Reed, L., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J.

Epidemiol. 27, 493–497.

Rexroad, J., Martin, T.T., McNeilly, D., Godwin, S., Middaugh, C.R., 2006. Thermal Stability of Adenovirus Type 2 as a Function of pH. J. Pharm. Sci. 95, 1469–1479. doi:10.1002/jps

Rexroad, J., Wiethoff, C.M., Green, A.P., Kierstead, T.D., Scott, M.O., Middaugh, C.R., 2003. Structural Stability of Adenovirus Type 5 92, 665–678.

Richardson, J.S., Abou, M.C., Tran, K.N., Kumar, A., Sahai, B.M., Kobinger, G.P., 2011. Impact of systemic or mucosal immunity to adenovirus on Ad-based Ebola virus vaccine efficacy in guinea pigs. J. Infect. Dis. 204 Suppl , S1032–42. doi:10.1093/infdis/jir332

Rubio, B., Martínez, B., García-Cachán, M.D., Rovira, J., Jaime, I., 2008. Effect of the packaging method and the storage time on lipid oxidation and colour stability on dry fermented sausage salchichón manufactured with raw material with a high level of mono and polyunsaturated fatty acids. Meat Sci. 80, 1182–1187. doi:10.1016/j.meatsci.2008.05.012

Rubio, B., Martínez, B., González-Fernández, C., García-Cachán, M.D., Rovira, J., Jaime, I., 2006. Influence of storage period and packaging method on sliced dry cured beef "Cecina de Leon": Effects on microbiological, physicochemical and sensory quality. Meat Sci. 74, 710–717. doi:10.1016/j.meatsci.2006.06.002

Saleki-Gerhardt, A., Stowell, J.G., Byrn, S.R., Zografi, G., 1995. Hydration and dehydration of crystalline and amorphous forms of raffinose. J. Pharm. Sci. 84, 318–323. doi:10.1002/jps.2600840311

Saluja, V., Amorij, J.P., Kapteyn, J.C., de Boer, a. H., Frijlink, H.W., Hinrichs, W.L.J., 2010. A comparison between spray drying and spray freeze drying to produce an influenza subunit vaccine powder for inhalation. J. Control. Release 144, 127–133. doi:10.1016/j.jconrel.2010.02.025

Smaill, F., Jeyanathan, M., Smieja, M., Medina, M.F., Thanthrige-Don, N., Zganiacz, A., Yin, C., Heriazon, A., Damjanovic, D., Puri, L., Hamid, J., Xie, F., Foley, R., Bramson, J., Gauldie, J., Xing, Z., 2013. A human type 5 adenovirus-based tuberculosis vaccine induces robust T cell responses in humans despite preexisting anti-adenovirus immunity. Sci. Transl. Med. 5, 205ra134. doi:10.1126/scitranslmed.3006843

Struik, L.C.E., 1977. Physical aging in plastics and other glassy materials. Polym. Eng. Sci. 17, 165–173.

Sundaramurthi, P., Suryanarayanan, R., 2010. Trehalose Crystallization During Freeze-Drying: Implications On Lyoprotection. J. Phys. Chem. Lett. 1, 510–514. doi:10.1021/jz900338m

Taylor, N.W., Zobel, H.F., Hellman, N.N., Senti, F.R., 1959. Effect of Structure and Crystallinity on Water Sorption of Dextran. J. Phys. Chem 63, 599–603. doi:doi:10.1021/j150574a036

Uedaira, H., Uedaira, H., 1985. Sugar-Water Interaction from Diffusion Measurements. J. Solution Chem. 14, 27–34.

Vehring, R., 2008. Pharmaceutical particle engineering via spray drying. Pharm. Res. 25, 999–1022. doi:10.1007/s11095-007-9475-1

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

Willart, J.F., De Gusseme, a., Hemon, S., Descamps, M., Leveiller, F., Rameau, a., 2002. Vitrification and polymorphism of trehalose induced by dehydration of trehalose dihydrate. J. Phys. Chem. B 106, 3365–3370. doi:10.1021/jp012836+

Williams, K.R., Gupta, K., Wasilik, M., 2003. Etch rates for micromachining processing - Part II. J. Microelectromechanical Syst. 12, 761–778. doi:10.1109/JMEMS.2003.820936

Wong, Y.-L., Sampson, S., Germishuizen, W.A., Goonesekera, S., Caponetti, G., Sadoff, J., Bloom, B.R., Edwards, D., 2007. Drying a tuberculosis vaccine without freezing. Proc. Natl. Acad. Sci. U. S. A. 104, 2591–5. doi:10.1073/pnas.0611430104

World Health Organization, 2011. Global Vaccine Action Plan 2011-2020.

Wu, L., Miao, X., Shan, Z., Huang, Y., Li, L., Pan, X., Yao, Q., Li, G., Wu, C., 2014. Studies on the spray dried lactose as carrier for dry powder inhalation. Asian J. Pharm. Sci. 9, 336–341. doi:10.1016/j.ajps.2014.07.006

Xing, Z., Ohkawara, Y., Jordana, M., Graham, F.L., Gauldie, J., 1996. Transfer of Granulocyte-Macrophage Colony-stimulating Factor Gene to Rat Lung Induces Eosinophilia, Monocytosis, and Fibrotic Reactions. J. Clin. Invest. 97, 1102 – 1110.

Yu, L., 2001. Amorphous pharmaceutical solids: Preparation, characterization and stabilization. Adv. Drug Deliv. Rev. 48, 27–42. doi:10.1016/S0169-409X(01)00098-9

Zhu, F., Hou, L., Li, J., Wu, S., Liu, P., Zhang, G., Hu, Y., Meng, F., Xu, J., Tang, R., Zhang, J., 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 6736, 1–8. doi:10.1016/S0140-6736(15)60553-0

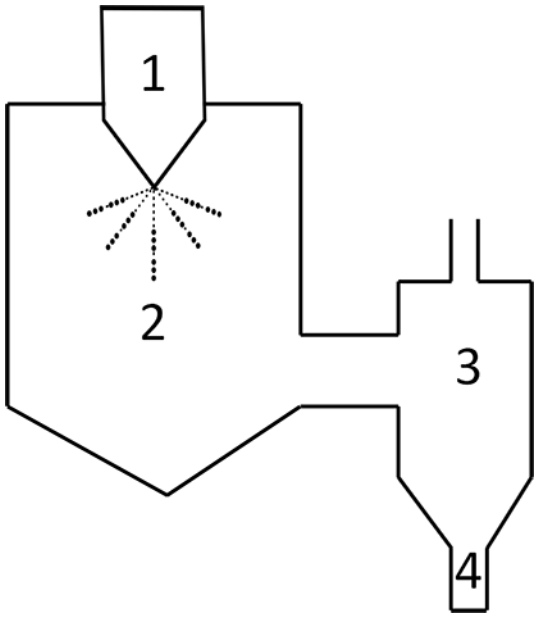

Figure 1. Schematic diagram of the spray drying process. Labeled components are 1) the spray dryer nozzle, 2) the spray drying chamber, 3) the separating cyclone and 4) the collection chamber.

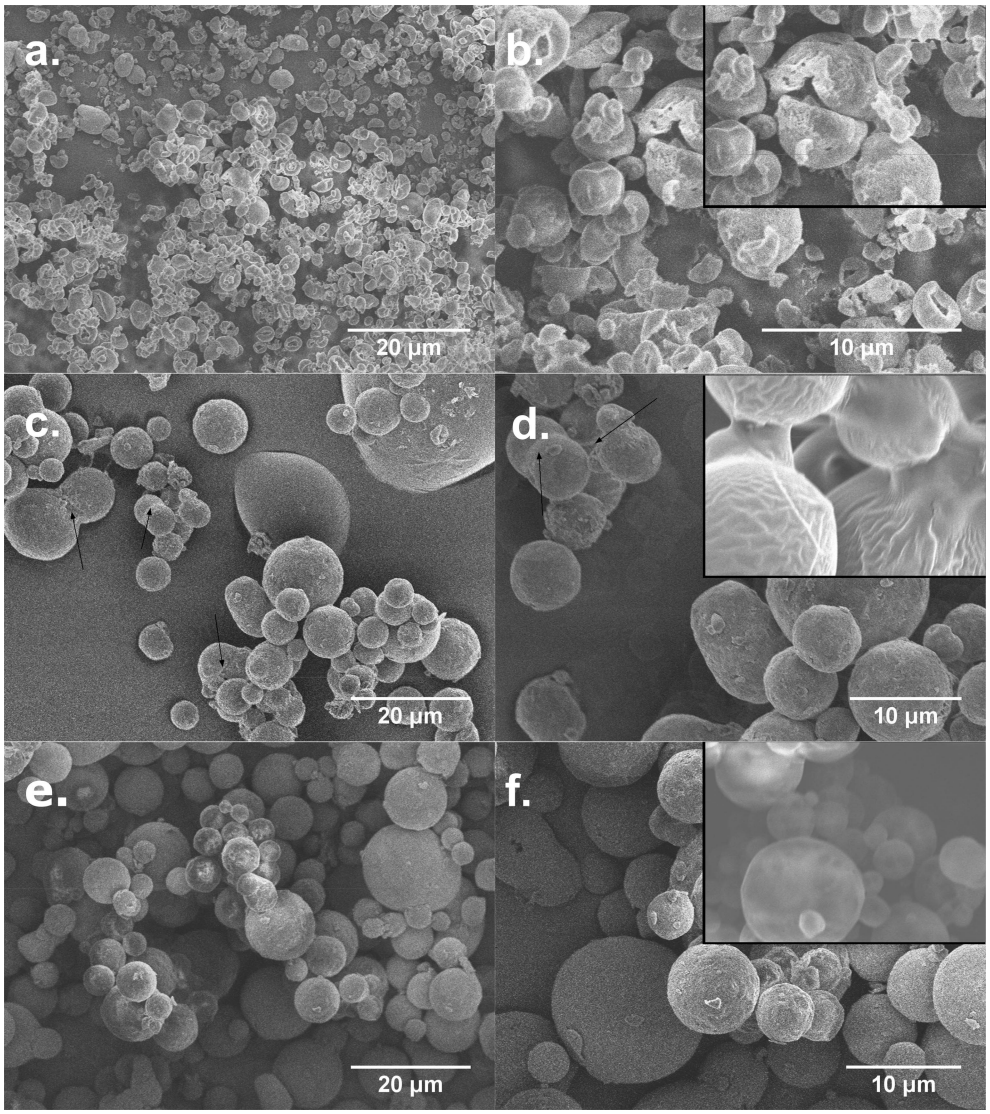

Figure 2: Spray dried particles imaged by scanning electron microscopy composed of L-leucine (a,b), lactose/trehalose (c,d) and mannitol/dextran (e,f). Insets shown detail particle bridging when present at electron microscope magnifications of 5000x (panel b) and 10000x (panels d, f). These spray dried formulations do not contain adenoviral vector.

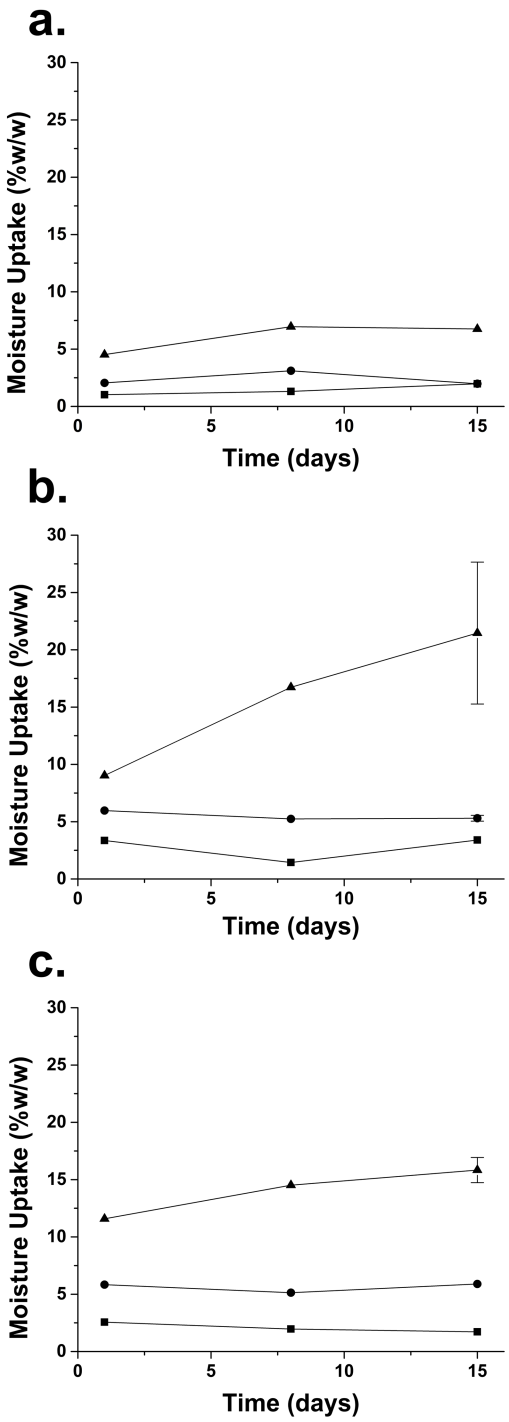

Figure 3: Moisture uptake (wt. %) of L-leucine (a), lactose/trehalose (b) and mannitol/dextran (c) formulations after storage for up to two weeks at 20°C and relative humidities of <10% (□), 45% (□) and >90% (□). Data is shown as mean (± SD).

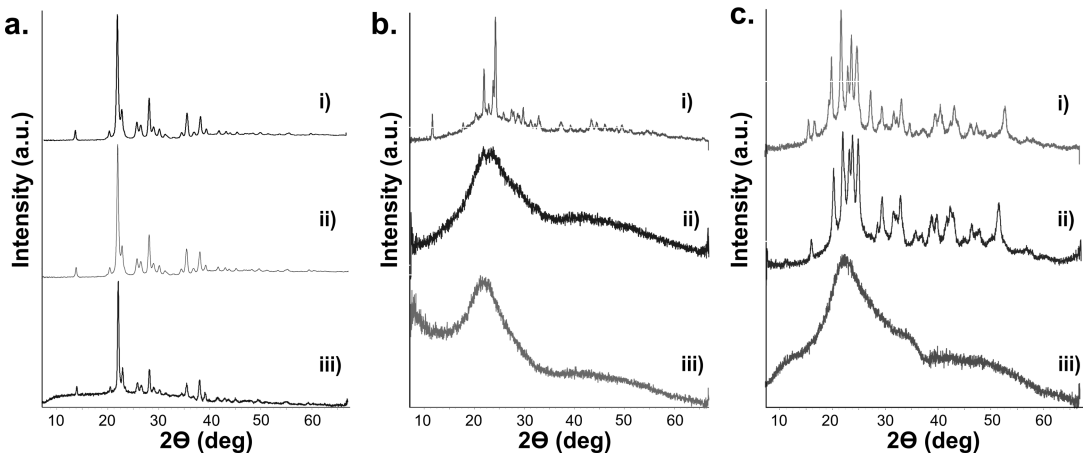

Figure 4: X-ray diffraction peak crystal intensities (arbitrary units) measured across a range of x-ray incidence angles for L-leucine (a), lactose/trehalose (b) and mannitol/dextran (c) formulations. Storage conditions are immediately post spray drying (i), two weeks storage at 20°C and <10% RH (ii) and two weeks storage at 20°C and 45% RH (iii).

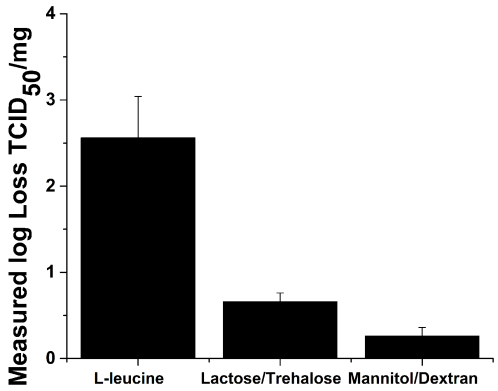

Figure 5: Measured loss of AdHu5 infectivity for L-leucine, lactose/trehalose and mannitol/dextran, formulations after spray drying (i.e. process loss at t=0 days). Data is shown as mean ± SD (n = 3).

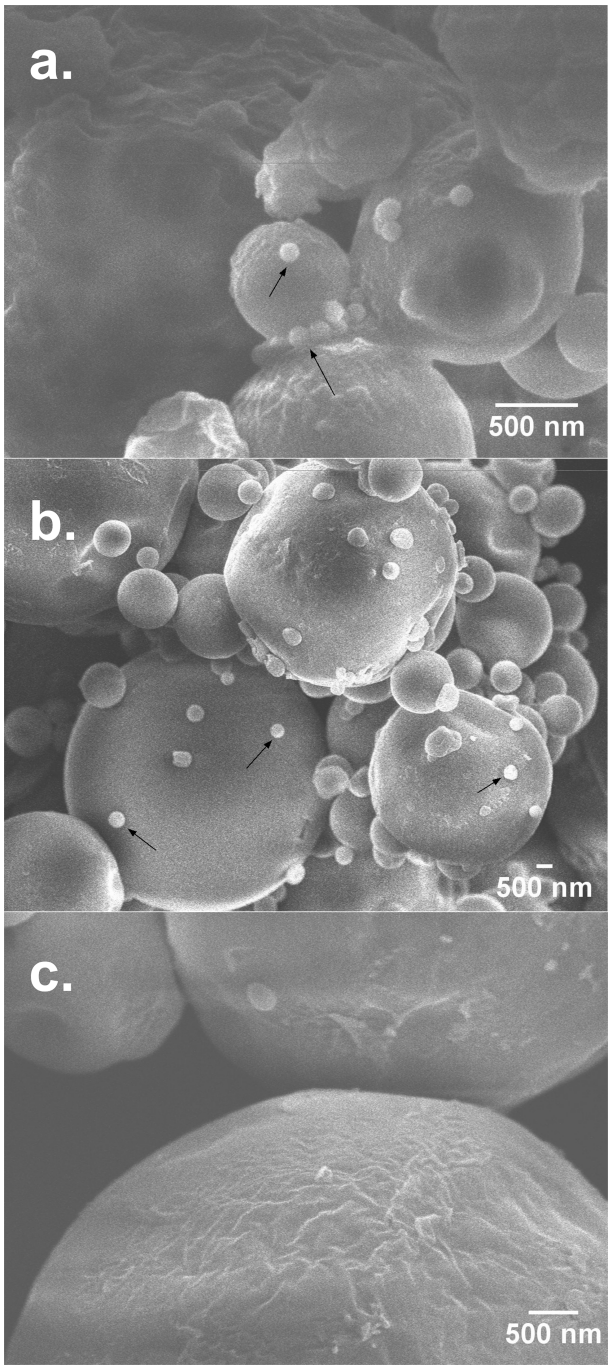

Figure 6: SEM micrographs of formulations spray dried with adenoviral vector: L-leucine (a), lactose/trehalose (b) and mannitol/dextran (c). Proposed AdHu5 nodules present on the particle surface are indicated by black arrows.

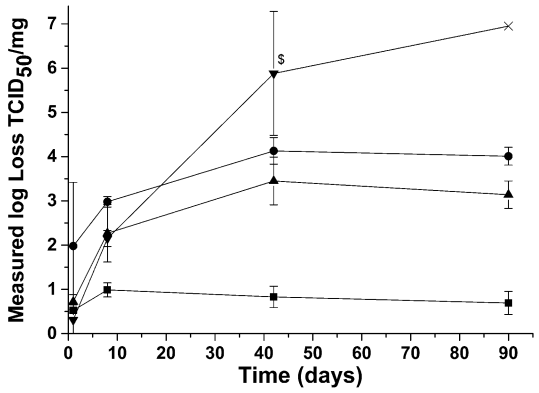

Figure 7: Measured log loss of AdHu5 vector infectivity after storage up to 90 days at 20°C and <10% RH for liquid control (□), L-leucine (□), lactose/trehalose (□) and mannitol/dextran (□),formulations. Initial time point is at t=1 day. Three repeat samples were stored for each formulation and $ denotes viral activity below the detection limit for one repeat and '×' denotes viral activity below the detection limit for all repeats. Data is represented as mean ± SD for three repeat samples and mean ± range for two repeat samples.

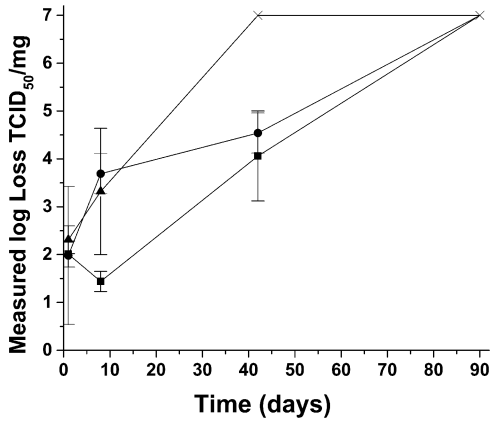

Figure 8: Measured log loss of AdHu5 vector infectivity after storage up to 90 days at 20°C and 45% RH for L-leucine (□), lactose/trehalose (□) and mannitol/dextran (□)formulations. Initial time point is at t=1 day. Three repeat samples were stored for each formulation and '×' denotes viral activity below the detection limit for all repeats. Data is represented as mean ± SD for three repeat samples.

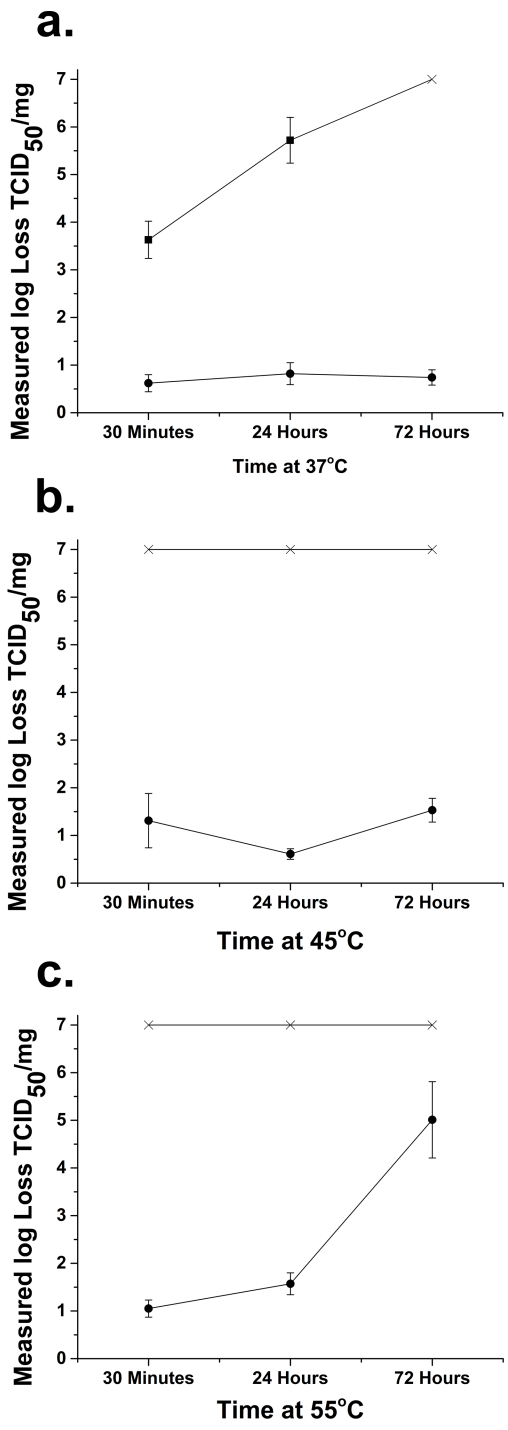

Figure 9: Measured AdHu5 infectivity loss for both control (□) and mannitol/dextran formulation (□) at a storage of 37°C (a), 45°C (b) and 55°C (c) for up to three days. Three repeat samples were stored for each formulation and '×' denotes viral activity below the detection limit for all repeats. Data is represented as mean +/- SD for three repeat samples and mean +/- range for two repeat samples.