THERMALLY STABLE HUMAN TYPE 5 ADENOVIRUS THROUGH SPRAY DRYING: STORAGE EFFICACY AND PROCESS OPTIMIZATION

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TITLE: Thermally Stable Human Type 5 Adenovirus through Spray Drying: Storage Efficacy and Process Optimization

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Lay Abstract

Many vaccines and their base components inherently deteriorate in function at moderate temperatures. Storage by refrigeration at temperatures ranging between 4°C and -80°C is the norm. Such refrigeration is costly for long term storage and significantly limits where vaccines can be sent. This reduces the availability of vaccines in locations around the world where these storage conditions are infeasible but vaccines are needed most. Spray drying, a process which forms dry powders from solution, was used; the solution contained sugars or amino acids to surround and protect the sensitive vaccine component. The produced powders from this work exhibited enhanced thermal stability compared to the control, reducing the need for refrigeration during storage and transport. The spray drying process was further optimized for industrial use by maximizing the amount of powder recovered and ensuring the particle size was appropriate for inhalable use, but most importantly, minimizing losses in therapeutic effectiveness during processing. This production of a thermally stable vaccine is advantageous because is allows for better world-wide accessibility and reduces overall production and delivery costs.

Abstract

This thesis investigates enhancing the thermal stabilization of a human type 5 adenoviral vector (AdHu5) through spray drying. The spray drying process was used to dry and effectively immobilize the AdHu5 within a mixture of carbohydrate or amino acid excipients into a powder form, resulting in significantly increased thermal stabilization of the viral vector. Spray dried powders were characterized by scanning electron microscopy, differential scanning calorimetry, Karl Fischer titrations, X-ray diffraction (XRD), and X-ray photoelectron spectroscopy (XPS) to identify the effects of temperature and atmospheric moisture on the immobilizing matrix. The best performing spray dried powder in terms of thermal stability consisted of an excipient blend of mannitol and dextran. Response surface methodology was employed to optimize production of these mannitol/dextran powders; measured responses were those relevant to industrial processing of a therapeutic material, namely powder yield for maximizing quantity, particle size for maximizing production of inhalation-deliverable powders, and adenoviral vector response for minimizing the loss of therapeutic activity. The spray drying process parameters of inlet temperature, spray gas flow rate, liquid feed rate and solute concentration in the feed were optimized resulting in a powder yield of 90%, percentage of ideally-sized particles of 50% and a near-zero viral vector titre loss of 0.25 log loss median tissue culture infectious dose ($TCID_{50}$). The spray dried mannitol/dextran powders proved to have exceptional thermal stability during long term storage as minimal viral vector activity loss was observed when stored at 20°C for 90 days at low relative humidity (0.7 ± 0.3 log $TCID_{50}$) in comparison to the liquid control which exhibited complete activity loss under the same storage conditions. Furthermore, viral activity of

mannitol/dextran powders was retained over short term exposure (72 hours) to temperatures as high as 55°C whereas the liquid control expectedly lost all AdHu5 activity after 30 minutes. Overall, this work provides a guideline for the production of thermally stable powders and active biopharmaceuticals, such as AdHu5 vectors for vaccine applications, using the spray drying process.

Acknowledgements

First, I would like to start by thank my supervisors, Dr. Michael R. Thompson, Dr. Emily D. Cranston and Dr. Zhou Xing. Their guidance has made everything possible, and I am truly lucky to have been supported by them over the years. I find their drive and passion for research to be a major inspiration, and I can't begin to fully express how valuable it has been to have these opportunities with them.

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Nomenclature

Ad	adenoviral
AdHu5	human type 5 adenovirus
AdCh68	chimpanzee type 68 adenovirus
ANOVA	analysis of variance
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
FE	spray dryer liquid feed rate
$F_{1\text{-}5\mu m}$	percentage of ideally sized particles
LacZ	β-galactosidase
MEM	minimum essential medium
M.tb	*mycobacterium tuberculosis*
PBS	phosphate buffered saline
RSM	response surface methodology
S	solute concentration in the liquid feed
SEM	scanning electron microscopy
SG	spray gas flow rate
T	viral vector titre loss
TE	inlet temperature of spray dryer
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XPS	X-ray photoelectron spectroscopy
XRD	X-ray powder diffraction
Y	powder yield

Declaration of Achievement

I declare that the research presented here is original work completed and originally drafted by me, with editorial assistance from my supervisors Dr. Michael R. Thompson, Dr. Emily D. Cranston and Dr. Zhou Xing.

Chapter 1: Introduction

1.1. Vitrification of Adenoviral Vector Vaccine Platforms through Spray Drying

Adenoviruses have gained interest from researchers for their use as viral vectors in vaccines (Appaiahgari and Vrati, 2014). Adenoviral vectors have proven to be advantageous due to their efficient transduction of genetic material, high immunogenicity and favourable safety profile compared to other viral vector platforms, such as retroviral vectors (Appaiahgari and Vrati, 2014; Bauer et al., 2008). Combining efficient introduction of foreign DNA into host cells and induction of host immune response makes adenoviral vectors especially useful as vaccine tools for combating infectious diseases.

In particular, Xing and colleagues have developed a recombinant replication-deficient human type 5 adenoviral-based vaccine expressing the immunogenic *Mycobacterium tuberculosis (M. tb)* antigen 85A (AdHu5Ag85A)(Smaill et al., 2013). Current work has shown this platform to be effective in protecting against infectious pulmonary tuberculosis, especially when delivered via inhalable mucosal routes (Wang et al., 2004). Respiratory administration of the vaccine provides better retention of immune cells within the lung tissue and airways.

A major pitfall for adenoviral vectors is their poor thermal stability which leads to loss of bioactivity at even moderate temperatures (Alcock et al., 2010). Generally, storage at temperatures above -80°C gives adenoviral vectors a limited shelf life (Croyle et al., 1998; Evans et al., 2004). This is particularly disadvantageous because infectious disease protection is most needed in third world countries, such as Sub-Saharan Africa, where

ambient temperatures are high and refrigeration during transportation and storage is often infeasible. This challenge has been outlined by the Bill & Melinda Gates Foundation, a grant-making foundation that contributes significantly to global health (McCoy et al., 2009). Thus, an objective of the Bill & Melinda Gates Foundation is to produce more thermally stable vaccine platforms.

Current approaches to produce more thermally stable bioactive molecules range from storage in liquid buffer formulations to various solid forms (Croyle et al., 2001; Evans et al., 2004; Saluja et al., 2010; Wong et al., 2007). Some liquid formulations have seen moderate success leading to enhanced stability at temperatures as high as 4°C; however, the use of potentially cytotoxic compounds, such as ionic surfactants, require extensive dilutions to reduce the toxicity of the medium prior to administration (Croyle et al., 1998). Drying methods to produce stabilized solid bioactive compounds, most commonly freeze drying and spray drying, have also shown enhanced stability at elevated temperatures (Maa et al., 2004; Ohtake et al., 2010; Wong et al., 2007). Extensive research has investigated freeze drying of labile materials for producing thermally stable forms; however, the process is lengthy, costly, and offers little to no control over the produced particle characteristics (Franks, 1998). In contrast, spray drying can be operated as a continuous process, is low cost compared to other drying processes, and offers a significant degree of control over the final particle properties (Vehring et al., 2007).

Spray drying of labile materials for enhanced thermal stability has been established within the literature primarily focusing on protein- and bacteria-based therapeutics(Vehring, 2008). However, the full potential for spray drying labile materials

has not been met due to a lack of process and formulation knowledge (Ameri and Maa, 2006). As the interactions between formulation components and labile materials are extremely material specific, there is a need for formulation testing with each labile material, including adenoviral vectors such as AdHu5. Furthermore, there is a lack of published work addressing how spray drying processes should be adjusted for final products containing these labile materials, looking at factors such as powder yield, particle size and the activity of labile material post-processing. This is the first work that investigates the spray drying of a formulation for AdHu5 and provides in a systematic manner, knowledge that allows for smarter choice and investigation for future labile materials.

1.2. Research Objectives

The overall goal of this thesis is to study the spray drying process as a method to prepare thermally stable labile materials, specifically AdHu5 vectors, which may lead to improved vaccines. This work is accomplished using AdHu5LacZ, a model AdHu5 vector that elicits a response that can be measured *in vitro*. The work is divided into two projects:

Thermal stabilization of AdHu5:

This project aims to develop a thermally stabilizing formulation for use in spray drying with AdHu5 vectors. In doing so, we (1) demonstrate a spray dried powder of AdHu5 in mannitol/dextran that retains its bioactivity for up to three months when stored at 20°C and (2) systematically evaluate the influence of the spray dried particle

crystallinity, moisture content and glass transition temperature, on the AdHu5 thermal stability.

Effect of spray drying process parameters on powders containing labile materials:

This project aims to correlate spray drying process parameters with the production of thermally-stabilizing powders. We link spray drying process parameters, including inlet temperature, gas flow rate, liquid feed rate, and excipient concentration to (i) powder yield, (ii) particle size and (iii) bioactivity of the labile material. While the exact optimized process parameters will depend on the chosen excipient and labile material, the overall trends and governing interactions elucidated here provide a valuable "guide map" for future investigations.

1.3. Thesis Outline

This thesis is divided into five chapters, including this chapter. A literature review discussing the published work on viral vector stability and methods used to enhance thermal stability, including freeze drying and spray drying, is provided in Chapter 2. Chapter 3 details a study examining formulations, and relating particle properties such as glass transition temperature and crystallinity to the enhanced stability of a human type 5 adenoviral vector (AdHu5LacZ). In Chapter 4, work is presented which studies the effect of the spray drying process parameters on retaining AdHu5LacZ activity post-processing, as well as the powder yield and yield of particles within an inhalable size range. Finally, Chapter 5 presents conclusions and future directions for this thesis work.

Chapter 2: Literature Review

2.1. Stability of Adenoviral-Based Vectors

Adenovirus-based vectors have demonstrated great effectiveness as vaccine tools due to their efficient transduction of foreign DNA into host cells and the innate induction of an immune response from the adenoviral backbone (Appaiahgari and Vrati, 2014). Studies by Wang et al. have shown that the transduction of genetic data for immunogenic antigens, such as the antigen 85A secreted by *M. tb*, confers protective immunity against subsequent infection (Wang et al., 2004). Furthermore, the high immunogenicity of adenoviral vectors reduces the need for adjuvant delivery alongside vaccine platforms. This prevents the use of compounds that contain their own stability and safety profiles, and overall allows for a more effective and safe vaccine (Aucouturier et al., 2001).

Adenoviral infectivity is dictated by its viral structure, shown in Figure 2.1 (Tatsis and Ertl, 2004). Although infection is an increasingly complex process, it is very briefly summarized here. Simply, the virus is a collection of genetic data encoded within DNA encased in a proteinous structure. Rux et al. have shown that the proteinous encasement consists of an icosahedral capsid shell with protruding fibres (Rux and Burnett, 2004). These viral fibres are necessary for host cell attachment (Martin et al., 2003). Infection proceeds following viral attachment through endocytosis by the host cell, where engulfment of the viral particle allows for it to be taken inside (Meier and Greber, 2004). Intake of the viral particle into the cellular body initiates breakdown of the capsid shell, and releases the genetic data. Work by Meier et al. and Medina-Kauwa have shown that dismantling of the adenoviral capsid proceeds through a carefully coordinated and complex process (Medina-Kauwe, 2003; Meier and Greber, 2004). Overall, the critical

point within these works is that successful adenoviral infection, and consequently viral vector activity, depends completely on functioning proteins and fibre components within the capsid shell.

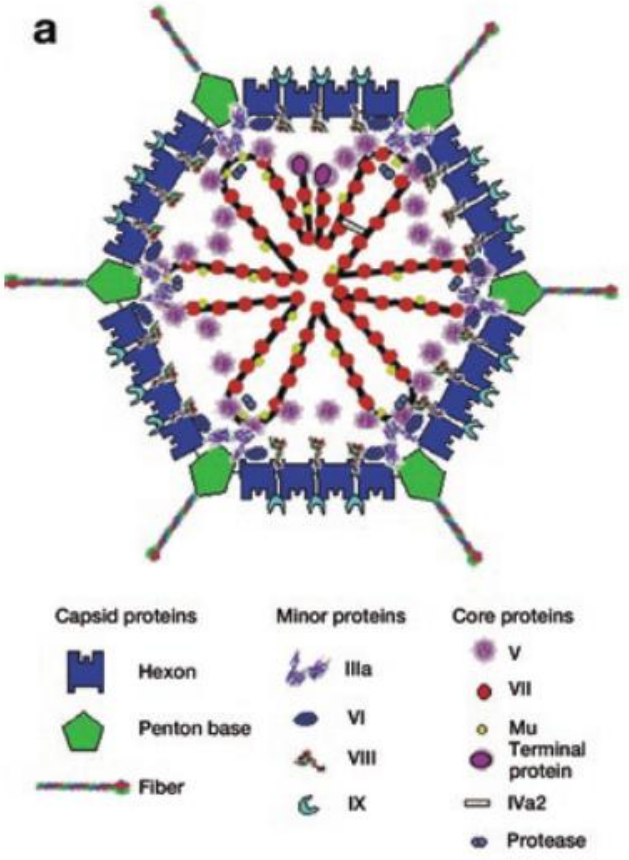

Figure 2.1. 2D slice showing adenoviral structure consisting primarily of proteins. Shown is capsid, comprised of hexon units, penton bases, and infection fibers. Genetic data is stored inside. Image reproduced from (Kennedy and Parks, 2009)**.**

Protein function has been readily established within the literature as dependent upon protein structure (Newton, 1995). Protein structure consists of primary, secondary, tertiary and quaternary levels, and thus alteration at one of these levels is detrimental to protein performance (Eisenhaber et al., 1995). Poor thermal stability exhibited by

adenoviral vector platforms is a result of changes in protein structure, also termed denaturation. Commonly, protein structures contain hydrophobic amino acid residues that reside within the interior of the three-dimensional protein structure. Exposure of these proteins to hydrophobic surfaces, such as air-water interfaces or the walls of synthetic vials, can result in denaturing as hydrophobic moieties are "released" from protein interiors due to unfolding. These denaturing events reduce the activity of viral vector platforms significantly. Furthermore, the exposure of hydrophobic residues amongst viral particles can lead to irreversible protein aggregation. Overall, to prevent loss of activity of adenoviral-based vectors, the denaturing of proteins must be avoided. The differences in protein structure are shown in Figure 2.2, comparing a folded protein to a completely unfolded and linear structure.

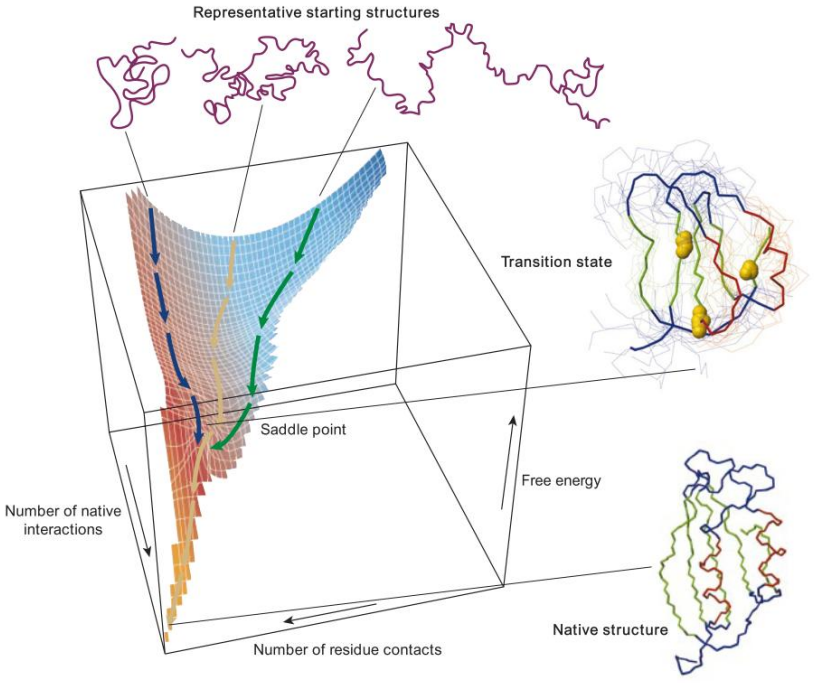

Figure 2.2. Two states of protein structure, folded and functional (left) and linear and denatured (right). Image reproduced from (Dobson, 2003)**.**

2.2. Enhanced Thermal Stability through Vitrification

As denaturing of viral proteins results in activity loss, entrapment on the molecular level is necessary to prevent changes in protein structure. One established strategy is vitrification, whereby the labile material, in this case the viral vector, is entrapped within a solid glass.

Today, the gold standard for vitrification is storage within frozen water-based stock solutions. Cruz et al. have explored the storage of adenoviral storage in frozen buffer at -80°C (Cruz et al., 2006). Further advancements have been made towards developing better frozen buffer solutions, as Croyle et al. published viral solutions maintaining stability at -20°C for extended periods of time (Croyle et al., 2001). Croyle et al. noted changes in stability of these viral solutions through addition of various excipients, such as glycerol, sucrose and trehalose. It was determined that excipients in the storage buffer affect processing and storage of vitrified samples.

This occurs as added excipients may interact with the labile material during freezing and provide stabilizing bonds that prevent disruption of the native protein structure (Honadel and Killian, 1988). Furthermore, the addition of excipients disturbs the formation of ice crystals, which prevents the destruction of labile materials by mechanical forces and entrapment between crystal faces. Back et al. demonstrated stronger stabilizing interactions between the viral protein hydrophobic groups and their environment in sucrose or glycerol-containing solutions compared with water (Back et al., 1979). Thus, the three dimensional structure of proteins is better maintained within solutions containing these excipients. Overall, the work within this field has illustrated the importance of excipient-labile material interactions, as well as the molecular entrapment

of the material of interest (Arakawa et al., 1993; Prestrelski et al., 1993). The choice of excipient(s) used in these storage buffers continues to be investigated, but one critical disadvantage of cryopreservation is the requirement of a completely frozen aqueous system. Molecular entrapment of any stored labile material is lost at elevated temperatures when aqueous systems are in the liquid state (Croyle et al., 1998; Cruz et al., 2006).

2.3. Selection of Excipients for Stabilization

Stabilization benefits from excipients make them a valuable addition to any formulation. However, strict selection criteria is necessary to make them viable within the pharmaceutical industry. As these products are intended for human use, the perspective of the US Food and Drug Administration (FDA) must be considered before an excipient can be included. Generally, this means the toxicity and safety profiles of each material should be properly characterized and understood (Osterberg and See, 2003). These constraints prevent the use of some potentially useful excipients due to toxicity concerns, such as surfactants and animal-based molecules (Srinivasan et al., 2010).

The major guidelines for an appropriate excipient selection are then i) exceptional stability profile and ii) low toxicity. As stability is imparted from excipient to labile biomolecule generally through hydrogen bonding, the excipient of choice should have high potential for this. These two criteria outlined are generally fulfilled through the use of sugar-based molecules, such as glucose, lactose, sucrose, etc. Carbohydrates are effectively able to hydrogen bond and maintain protein structure, and have been widely

used within the food industry demonstrating acceptable safety profiles (Jeffrey and Lewis, 1978; McDonald and Thornton, 1994). Thus their use naturally lends to being a stabilizing excipient for pharmaceutical applications (Takano et al., 2002).

2.4. Vitrification through Drying

Solid state excipients can maintain viral activity at higher temperatures through the removal of water. Vitrification of viral vectors within excipients consisting solely of sugars, amino acids, polymers and/or surfactants has been established to protect activity at temperatures greater than the melting point of water (Alcock et al., 2010). Jahanshahi-Anbuhi et al. developed tablets of labile biomolecules encapsulated within pullulan which exhibited retained activity for up to 60 days at room temperature (Jahanshahi-Anbuhi et al., 2014). Additionally, Alcock et al. entrapped viral vectors through vitrification within a trehalose-sucrose glass (Figure 2.3) which maintained activity for up to six months at temperatures as high as 25°C (Alcock et al., 2010). However, this method of preservation requires desiccation over periods of time to produce these sugar membranes, making it difficult to produce industrially-relevant quantities. Thus, drying methods, such as freeze drying and spray drying, have been investigated for quick removal of water and the production of a solid powder.

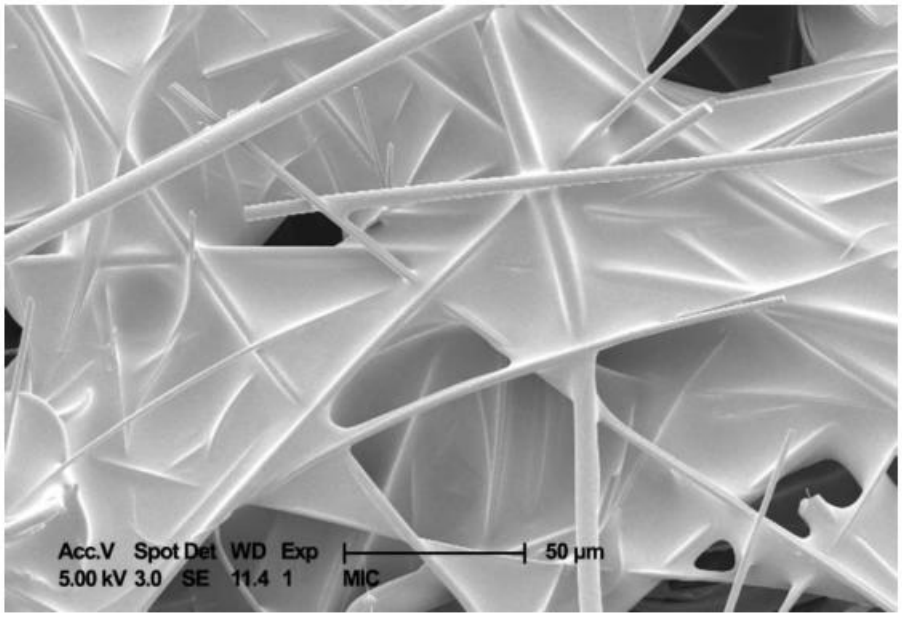

Figure 2.3. Vitrification of viral vectors within a trehalose-sucrose film. Image reproduced from (Alcock et al., 2010)**.**

All drying methods lead to precipitation of the solubilized materials and can induce dehydration stresses on the viral vector (Ohtake et al., 2010). Reduction of these stresses, and effective long term viral vector storage, is accomplished through appropriate excipient selection. For example, Crowe et al. examined trehalose as an excipient for freeze drying due to its advantageous stabilizing properties (Crowe et al., 1996) which include 1) direct interaction between the trehalose molecule and labile material, primarily through hydrogen bonding and 2) the high glass transition temperature (T_g; the temperature point at which a substance transitions from a hard, immobile glassy state to a soft, mobile state) of amorphous trehalose glasses. These findings echo the discussion amongst cryopreservation, where both high interactivity between excipient and labile material and high molecular entrapment are beneficial for stability.

Freeze drying is a two stage process (Boss et al., 2004). Freezing, the first stage, is accomplished by depressing the temperature of the sample below the freezing point of the solvent. In most biological cases, this solvent is water. During this stage, ice crystal growth is initiated and will alter the porous nature of the end product. The second stage, drying, subjects the sample to vacuum conditions (and sometimes the addition of heat) to cause sublimation of the solvent. The solvent sublimates to the vapour phase and is removed from the sample, resulting in a dry product.

Freeze drying has been well documented for the stabilization of many labile materials. Zhai et al. achieved minimal process loss of a Herpes simplex virus through freeze drying with the excipients trehalose, sucrose and Tris-HCl buffer (Zhai et al., 2004). The freeze dried product is shown in Figure 2.4. Talsma et al. demonstrated good adenoviral-vector stability through freeze-drying with sucrose, observing approximately 1 log loss in viral vector titre after 24 hours storage at 20°C (Talsma et al., 1997). Pikal et al. were able to successfully store human growth hormone for up to six months at 25°C after freeze drying with the excipients mannitol and glycine (Pikal et al., 1991). The previous work within this field demonstrates the specificity of formulation for each thermally stabilized labile material; unfortunately, formulations showing high success with one labile material may have much less success with another. Although the freeze drying method is promising as a thermally stabilizing technique, two main issues remain: 1) freeze drying is costly and difficult to scale industrially due to the requirement of refrigeration and vacuum conditions and 2) there is a lack of current formulation knowledge needed to choose the best excipient for a given labile material and achieve

thermal stabilization goals. Spray drying thus attempts to address these two issues because it is a more industrially relevant process and it allows for systematic evaluation of process conditions and formulation properties necessary for thermal stabilization of labile materials.

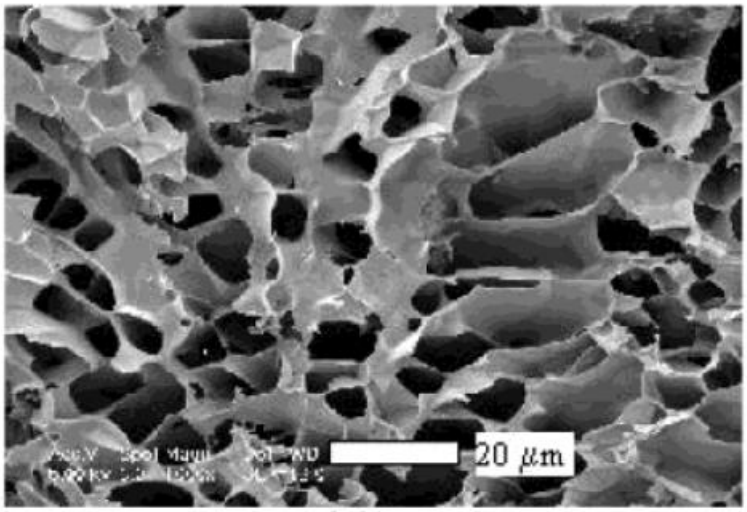

Figure 2.4. Freeze dried sample of Herpes simplex virus-2 vitrified in sucrose and dextran. Image reproduced from (Zhai et al., 2004)**.**

2.5. Spray Drying

Spray drying has been used for decades, but primarily within the food industry as a method to microencapsulate and protect ingredients and flavours from degradation (Gharsallaoui et al., 2007; Nisha et al., 2009). The use of this processing technique in commercial applications that demand large quantities, such as in the food industry, lends credibility to its scalability. This feature is very useful for the production of pharmaceuticals, for which spray drying has been employed within the past (Marchiori et al., 2012; Najafabadi et al., 2004).

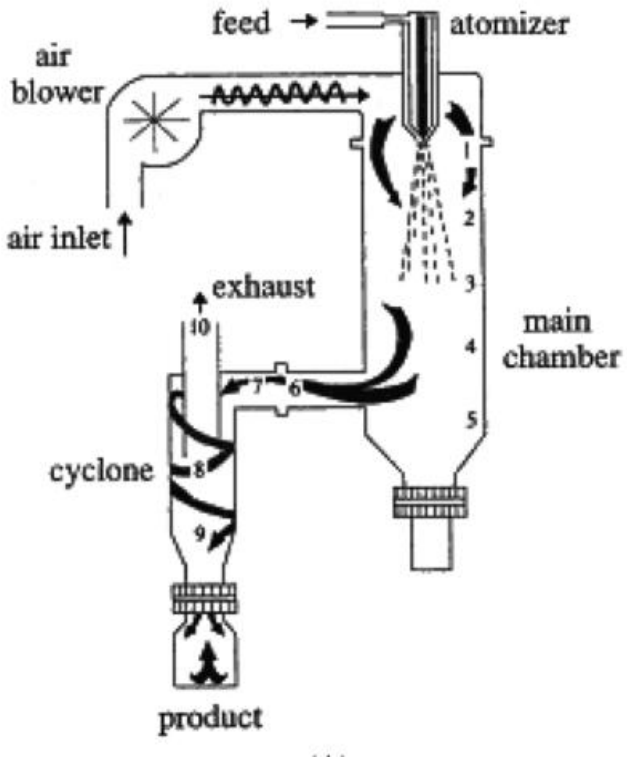

Figure 2.5. Schematic of the spray drying process. Liquid feed is atomized into the main chamber where is it dried. Powder is then collected as the product from the cyclone. Image reproduced from (Ameri and Maa, 2006)**.**

In comparison to freeze drying, spray drying requires high temperatures and pressurized gas to produce a dry powder; a typical spray drying setup is shown in Figure 2.5. A liquid feed is distributed to the spray dryer nozzle, where the pressurized gas atomizes it into tiny droplets (Parti and Paláncz, 1974). Breakup of the liquid feed is determined by three properties within the system: liquid feed inertia, surface tension and spray gas aerodynamic forces (Lin and Reitz, 1998). The types of liquid breakup are shown in Figure 2.6, where the most desirable for spray drying would be feed atomization (Figure 2.6d). High temperatures evaporate water from the dispersed droplets by

convection due to the large increase in surface area. Dehydration concentrates the solutes within each droplet, leading to precipitation and formation of a dry particle.

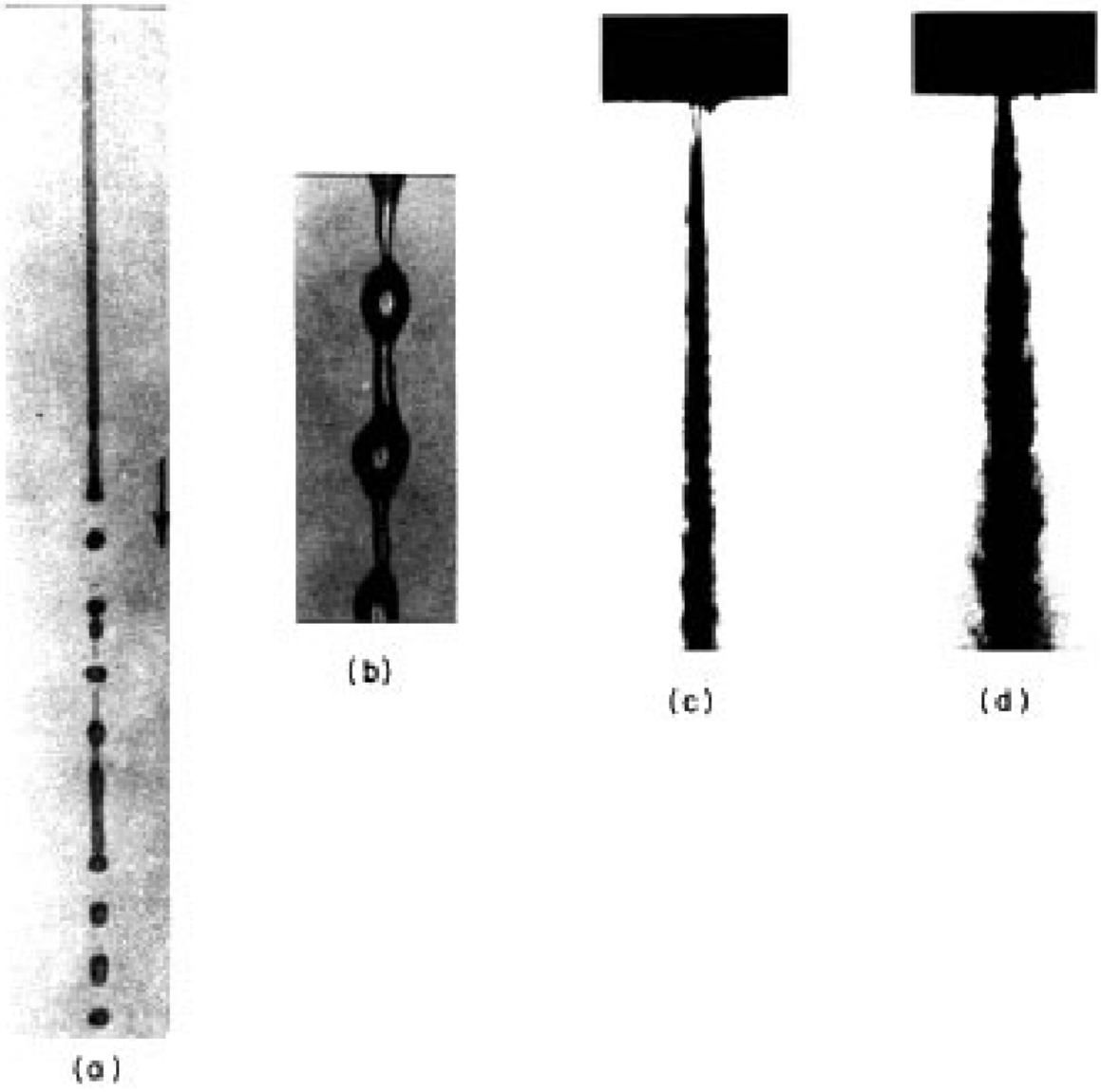

Figure 2.6. Breakup of regimes of a liquid feed using a pressurized gas: a) Rayleigh breakup, occurs downstream with drop diameters larger than nozzle diameter; b) First wind-induced regime.where droplets formed are similar in size to nozzle diameter; c) Second wind-induced regime where breakup occurs downstream with droplets smaller than the nozzle diameter; d) Atomization regime where breakup occurs immediately with droplets much smaller than nozzle diameter. Image reproduced from (Lin and Reitz, 1998)**.**

Spray drying is a controllable process through adjustment of several parameters. The spray drying temperature, pressurized gas flow rate, liquid feed entering the nozzle and solute concentration within the liquid feed are all controllable. This provides flexibility in determining the spray dried particle size and morphology, as well as the specific stresses applied during the spray drying process.

Encapsulation and vitrification of labile materials within spray dried particles offer protection from thermal deactivation. Just as in freeze drying, the immobility of the solid excipient phase, characterized by the T_g for amorphous products, prevents protein denaturing and aggregation. Again, it is most beneficial to spray dry using excipients capable of providing stabilizing interactions with the labile material, such as hydrogen bonding, which can increase the unfolding temperatures of the proteinous materials (Amorij et al., 2008). Furthermore, the addition of specific excipients can change the aerosol performance of the resulting powder, such as the addition of L-leucine, as demonstrated by Aquino (Aquino et al., 2012).

Although spray drying is a relatively new process within the biopharmaceutical industry, a few examples exist within the literature. In 2007, Wong et al. spray dried a model *Mycobacterium* with L-leucine, observing approximately a 3 log loss in titre over a 300 day period (Wong et al., 2007). Similarly, Ohtake et al. demonstrated a thermally stable attenuated measles vaccine through spray drying with the excipients trehalose, sucrose, L-arginine, glycerol, human serum albumin, among others (Ohtake et al., 2010). Corbanie et al. stabilized a live attenuated Newcastle disease virus within trehalose, polyvinylpyrrolidone and bovine serum albumin through spray drying (Corbanie et al.,

2007). As shown by these recent examples, the excipient composition of each formulation varies depending on the specific labile material requiring thermal stabilization. Thus, as outlined previously, this thesis hopes to address this issue. Specifically, this work uses spray drying and aims to develop a thermally stabilizing formulation for one of the most common viral vectors in use, human type 5 adenovirus (AdHu5) (Magnusson et al., 2007; Tatsis and Ertl, 2004; Zhu et al., 2015); to the best of our knowledge, this is the first report of adenoviral vectors processed by spray drying.

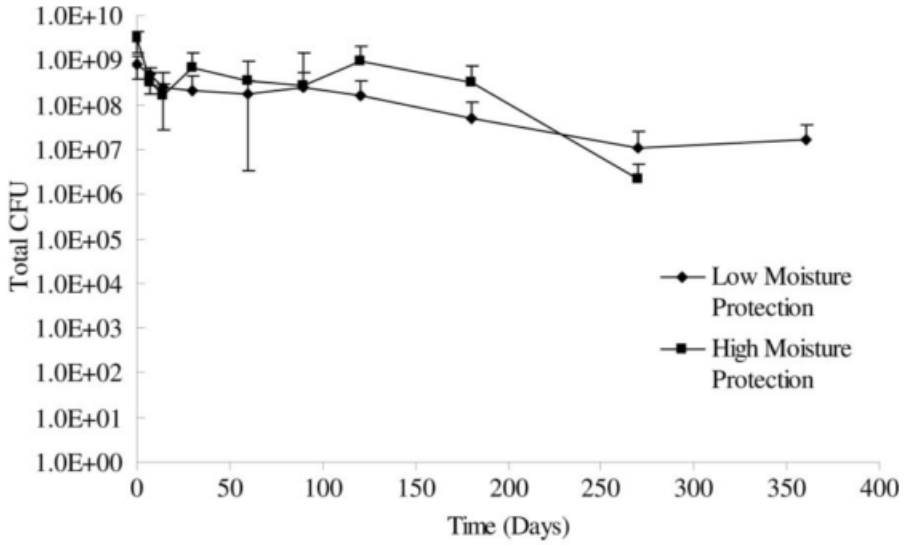

Figure 2.7. Vitrification of model *Mycobacterium* using L-leucine. Particles display approximately 2 log loss in titre after 300 days. Image reproduced from (Wong et al., 2007)**.**

2.5.1. Particle Formation from Spray Drying

A major advantage of spray drying is the ability to control particle characteristics through processing. Adjustment of the process parameters such as the drying temperature and spray gas flow rate can alter the particle size and morphology, as well as the distribution of excipients within the particle. Drying is often described using the Peclet

number (Pe, Equation 1), which is the ratio between the drying rate (κ) and the diffusivity of solutes (D_i) (Vehring et al., 2007).

$$Pe = \frac{\kappa}{8D_i}$$

The Peclet number is critical to spray drying as it describes which event is faster: evaporation of the water solvent, or diffusion of the solutes. As particle formation begins with solute precipitation, high drying rates correspond to a lack of solute diffusion and early precipitation. These conditions, more simply described as high Peclet number drying ($\kappa > D_i$), often result in larger, hollow particles. This is observed because evaporation of the water occurs more quickly than diffusion of the solutes within the drying droplet. Thus, solute concentration quickly increases at the interface or droplet surface. In contrast, low Peclet drying conditions more readily produce solid particles. The appearance of spray dried particles prepared under low to high Peclet drying conditions is shown in Figure 2.8: the spray dryer inlet temperature, and thus, drying rate (κ) is increased from top panel to bottom panel.

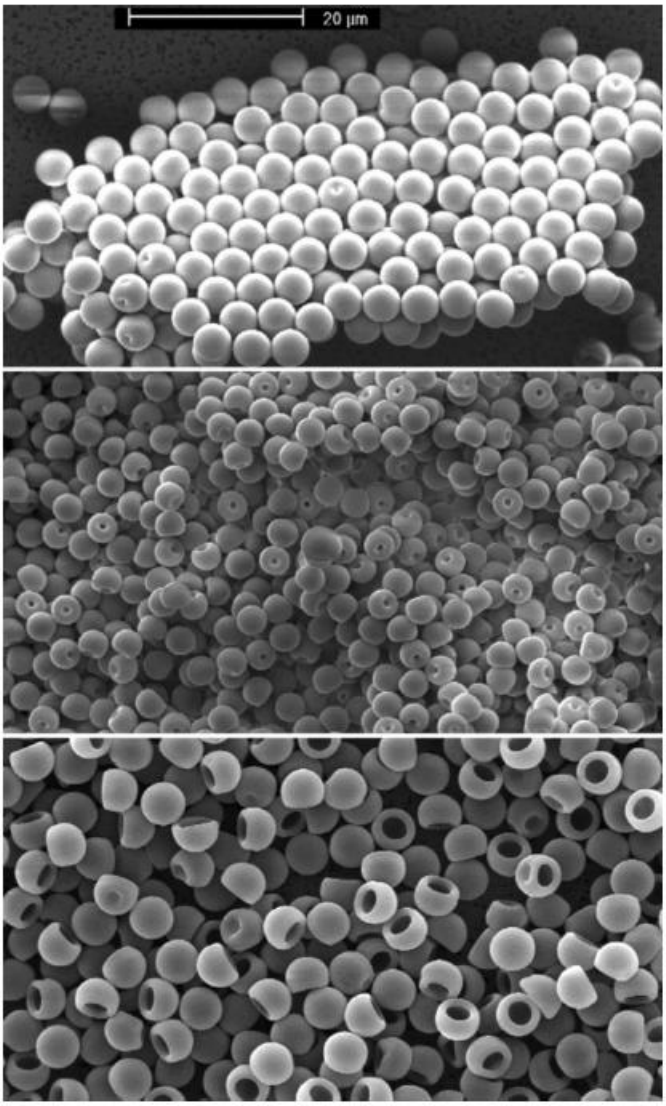

Figure 2.8. Scanning electron microscopy images of particles formed at different Peclet numbers. Drying temperatures are (top) 25°C – low Peclet drying, (middle) 50°C, and (bottom) 125°C – high Peclet drying. Image reproduced from (Vehring et al., 2007)**.**

The implication of Equation 1 is that different solutes will concentrate at different rates. Thus, there exists a potential for surface enrichment of specific excipients when spray drying. As the droplet evaporates within the spray dryer and its surface recedes, diffusion of each excipient occurs due to the concentration gradient between the

concentrated droplet surface and more dilute droplet interiors. As different molecules diffuse at different rates, the distributions of excipients within the drying product are not equal. This can be both beneficial or detrimental; for example, poor diffusion of the labile material will lead to additional activity loss due to high concentrations at the droplet surface (where exposed surface biomolecules are prone to deactivation). As a positive example, use of a surface active compound, such as L-leucine, will lead to surface enrichment of the excipient and better encapsulation of the labile materials (Vehring, 2008). This is shown in Figure 2.9, as the addition of L-leucine evidently modifies the surface of spray dried immunoglobulin particles. The particle surface transitions from smooth with only immunoglobulin within the spray dried system to rough and crumpled when a mixture of L-leucine and immunoglobulin is used.

The use of spray drying offers better adjustment over particle size, morphology and distribution of excipients compared to other drying processes, such as freeze drying. The benefit in control over excipient distribution is clear due to retained activity and better encapsulation of the active labile material, which for pharmaceutical purposes is the more costly ingredient. Both size and morphology are interesting parameters within the pharmaceutical industry because, for example, adjustment allows for the creation of inhalable particles.

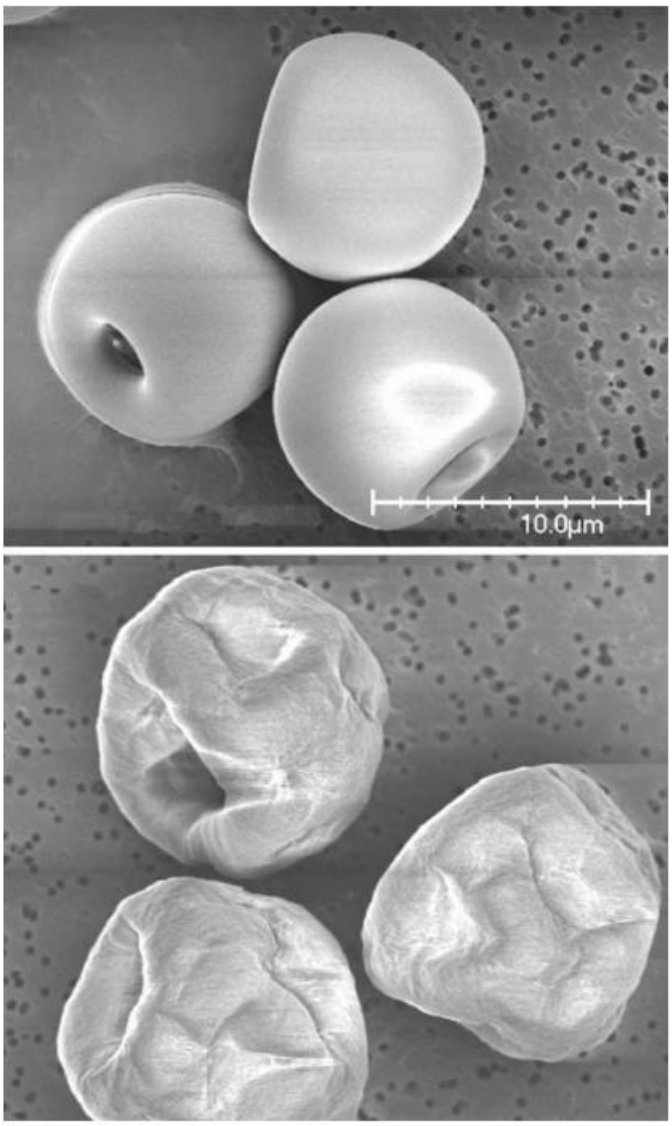

Figure 2.9. Scanning electron microscopy images of particles produced by spray drying. Shown is solely immunoglobulin (above) and immunoglobulin with L-leucine (below). Image reproduced from (Vehring, 2008)**.**

2.5.2. Spray Drying of Inhalable Particles

As outlined previously, the production of inhalable particles is of interest due to the better therapeutic effect of a more localized treatment (Wang et al., 2004) which is particularly relevant for the development of tuberculosis vaccines. Particles suitable for

inhalation should be within the size range of 1 - 5 µm (Menache et al., 1995). Previously, Elversson et al. produced inhalable lactose particles through spray drying (Elversson et al., 2003). In their work, the authors showed that adjustment of spray gas flow rate and solids concentration within the feed allowed for production of particles there were within a suitable size range for inhalation. The percentage of particles suitable for inhalation are labeled as particles within the ideal size range, or $F_{1\text{-}5\mu m}$.

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Chapter 3: Enhanced Thermal Stabilization for Human Type 5 Adenoviral Vector through Spray Drying

In chapter 3, all experiments and work were conducted by myself. The paper was drafted by myself, and thoroughly revised to the final version by Dr. Michael R. Thompson, Dr. Emily D. Cranston and Dr. Zhou Xing. This chapter has been submitted to *International Journal of Pharmaceutics*.

Enhanced Thermal Stabilization for Human Type 5 Adenoviral Vector through Spray Drying

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Enhanced Thermal Stabilization for Human Type 5 Adenoviral Vector through Spray Drying

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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 -80°C 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

3.1. 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 -80^{o}C) 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 non-cytotoxic 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.

3.2. Materials and Methods

3.2.1. 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 *Escheria coli* β-galactosidase (AdHu5LacZ) was produced in the vector facility of McMaster Immunology Research Centre as described previously (Xing et al., 1996).

3.2.2. 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 3.1, 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 $1/10000^{th}$ of the spray dried volume. The pH of the solution was 6.5. The composition and spray drying process parameters (Table 3.1) were optimized on preliminary experiments looking to achieve a high yield of non-agglomerating particles with matrices of significant amorphous content and high glass transition temperature (T_g), and most importantly, minimal adenoviral vector activity loss. Yield 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 3.1. 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]
Yield (%)	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.

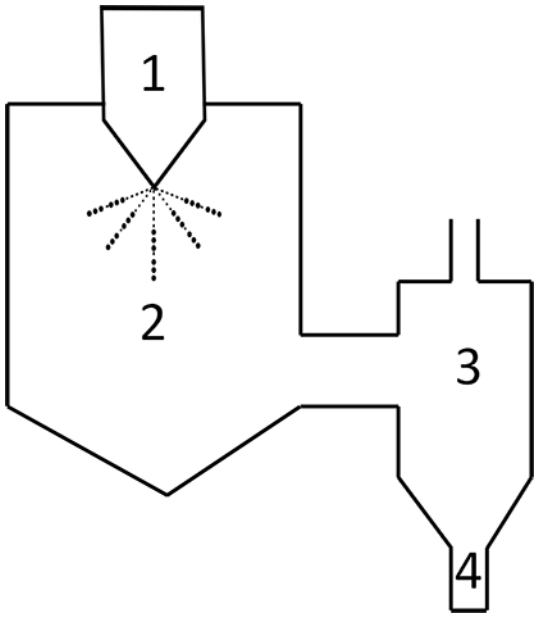

Figure 3.1. Schematic diagram of the spray drying process. Labeled components are 1) the spray dryer nozzle, 2) the spray drying chamber, 3) the separating cyclone and 4) the collection chamber.

3.2.3. 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%. Liquid control samples containing only buffer and AdHu5LacZ were stored in tightly sealed vials under ambient conditions and were not stored with desiccants or saturated salts. All aging trials examining the influence of humidity were performed at 20°C. Samples and storage conditions are listed in Table 3.2. Sealed samples were stored in closed 2 mL Nalgene General Long-Term Storage Cryogenic Tubes (Nalgene; Ontario, Canada).

Table 3.2. 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

3.2.4. 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 5.0×10^{-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.

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

3.2.5. 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).

3.2.6. 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).

3.2.7. 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.

3.2.8. 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).

3.2.9. *In Vitro* Testing of Spray Dried Particles

3.2.9.1. Culturing of A549 Cells

A549 lung epithelial cells were thawed from liquid nitrogen and cultured in T150 culture flasks using culture media. 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.

3.2.9.2. Excipient Toxicity

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). 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).

3.2.9.3. 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. The dosages were small in volume, as up to 100 mg of powder could be contained within a 2 mL Nalgene General Long-Term Storage Cryogenic Tube. Furthermore, each 3 mg dosage could be reconstituted in as little as 60 µL of PBS. These low powder dosage amounts indicated low space constraints for storage and transport. 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).

3.2.10. 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 $p \leq 0.05$.

3.3. Results and Discussion

3.3.1. Characterization of Spray Dried Powders without Adenoviral Vector

3.3.1.1. Size and Morphology

The spray dried powder formulations were imaged by SEM at varying magnifications to evaluate size and morphology, as shown in Figure 3.2. L-leucine particles were generally less than 10 µm in Feret diameter (Figures 3.2a and 3.2b), with an average of 8.80 µm (Table 3.3). These particles had a "collapsed sphere" morphology as a result of the hydrophobic isobutyl side chain on L-leucine which enhanced its surface activity (Glińiski et al., 2000), causing reduced diffusion within the drying droplet. Particle precipitation that is greatly limited by excipient diffusion throughout the drying process has a high Peclet number. In these cases, evaporation occurs more quickly than diffusion, and thus diffusion of the excipient is the limiting factor (Vehring, 2008). 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 3.2c and 3.2d) and mannitol/dextran (Figures 3.2e and 3.2f) 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 Feret diameter for the lactose/trehalose and mannitol/dextran powders were 32.2 μm and 7.92 μm, respectively (Table 3.3). 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 3.2c and 3.2d). The span listed in Table 3.3, is indicating a wide particle size distribution for all spray dried formulations. Overall, particle sizes were larger than might be expected from the initial solution concentrations. This is a result of the spray drying process, as completely condensed particles are unlikely to form, as shown in Figure 3.2b.

Table 3.3. 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

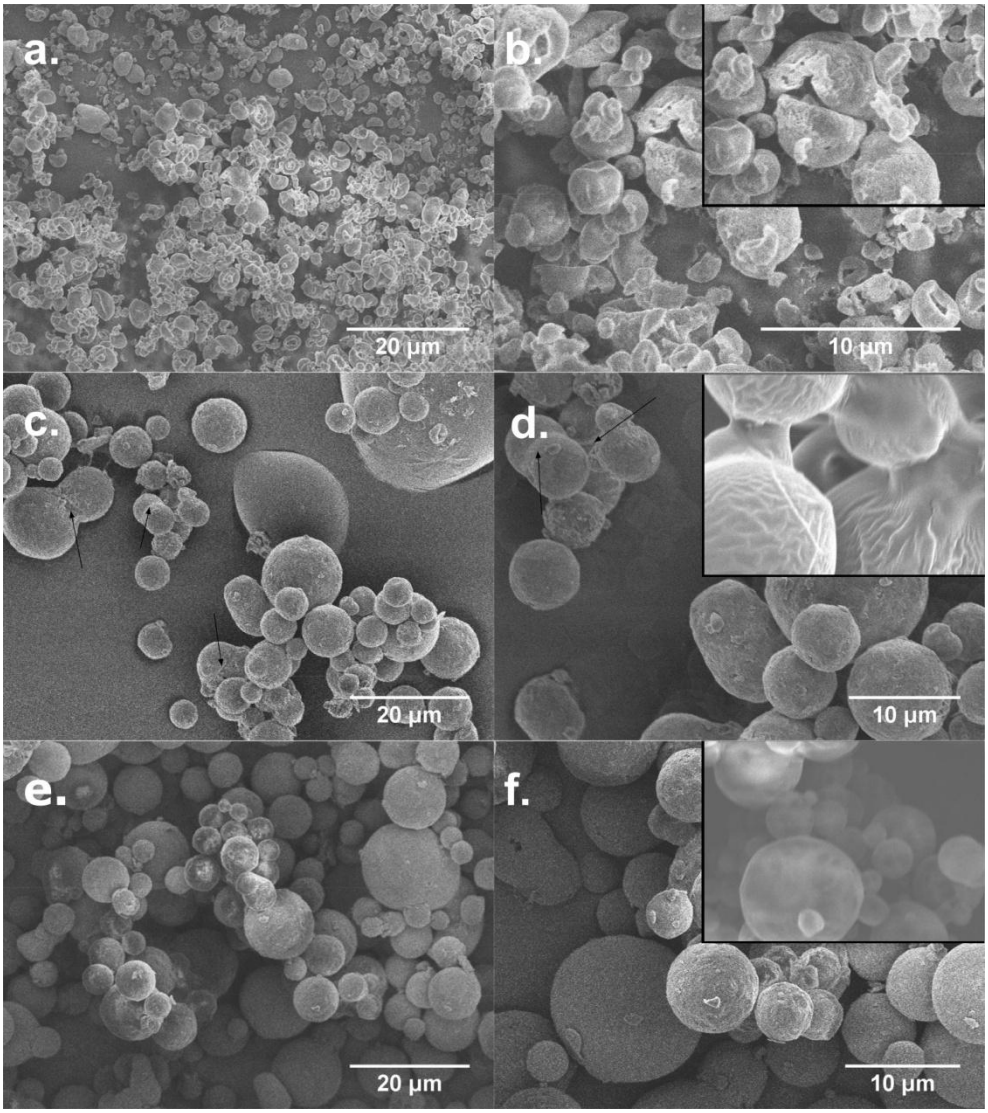

Figure 3.2. Spray dried particles imaged by scanning electron microscopy composed of L-leucine (a,b), lactose/trehalose (c,d) and mannitol/dextran (e,f). Insets shown detail particle bridging when present at electron microscope magnifications of 5000x (panel b) and 10000x (panels d, f). These spray dried formulations do not contain adenoviral vector.

3.3.1.2. Moisture Uptake

The measured water content after storage under different humidity conditions is plotted in Figure 3.3 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. Furthermore, the spray drying process orients the hydrophobic isobutyl groups towards the air phase and hydrophilic amide groups towards the water phase during evaporation (Raula et al., 2008, 2007). 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.

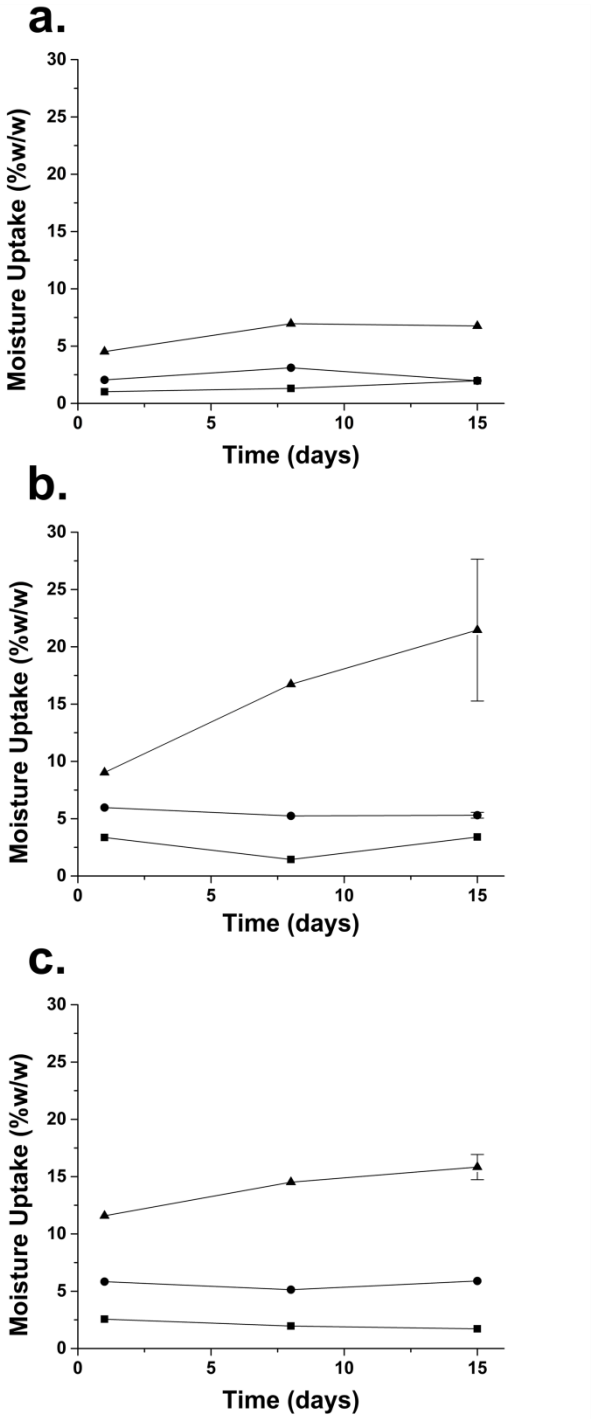

Figure 3.3: Moisture uptake (wt. %) of L-leucine (a), lactose/trehalose (b) and mannitol/dextran (c) formulations after storage for up to two weeks at 20°C and relative humidities of <10% (■), 45% (●) and >90% (▲). Data is shown as mean (± SD).

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.

3.3.1.3. Crystallinity

The three spray dried powders were analyzed by X-ray diffraction as shown in Figure 3.4; crystal structure differences were attributed to the different chemical composition of each formulation. Measured crystalline content is shown in Table 3.4 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. Crystallinity of each formulation was determined using x-ray diffractograms, as it has been previously published that measuring crystallinity of specific materials from differential scanning calorimetry thermograms can lead to erroneous results (Lehto et al., 2006).

Table 3.4. Measured crystallinity for all formulations immediately after spray drying.

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

Figure 3.4a 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 3.4b for the lactose/trehalose formulation displayed crystalline α-lactose monohydrate peaks as well as a large amorphous peak, 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). 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 3.4b (ii, iii) were attributed to the small-sized crystal domains detected by DSC and notably identified by other authors as a crystal structure is reverted to a mostly vitrified glass (Rani et al., 2006; Willart et al., 2002).

Figure 3.4c 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 20°C and 45% RH, a single broad mixed-mode peak was found as a result of the significant water uptake shown in Figure 3.3. 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).

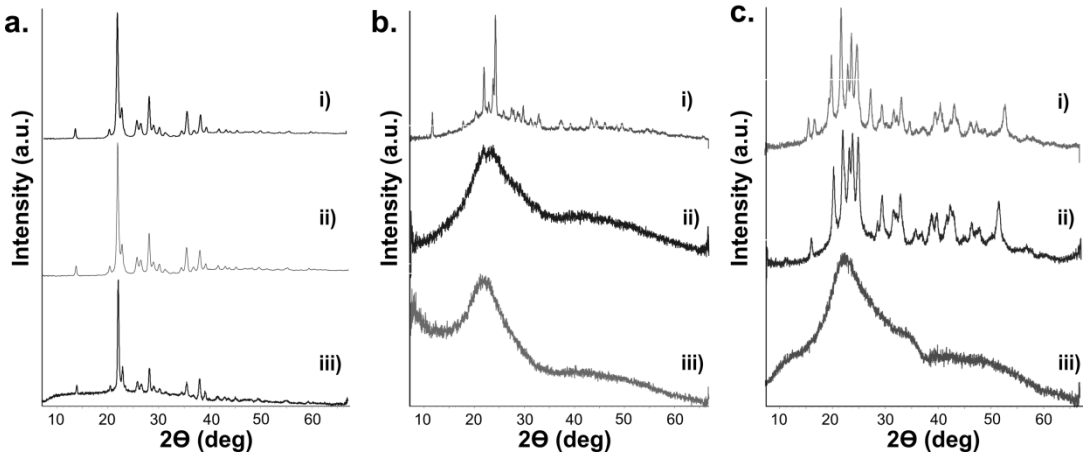

Figure 3.4: X-ray diffraction peak crystal intensities (arbitrary units) measured across a range of x-ray incidence angles for L-leucine (a), lactose/trehalose (b) and mannitol/dextran (c) formulations. Storage conditions are immediately post spray drying (i), two weeks storage at 20°C and <10% RH (ii) and two weeks storage at 20°C and 45% RH (iii).

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.

3.3.1.4. 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 3.5. 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 3.5. 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 now necessary to induce destabilizing molecular movement. However, if T_g and T_m alone were used to predict viral vector stability then L-leucine would appear to be best, followed by lactose/trehalose and mannitol/dextran which are similar in thermal properties, however, this is not the trend observed for viral activity emphasizing the importance of water uptake, degree of crystallinity, particle morphology and *in vitro* assays to properly predict excipient performance.

Table 3.5. 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.*

3.3.1.5. Excipient Cytotoxicity

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 at a dosage level that was three times higher than used for the particles containing adenovirus. Cell viability corresponding to each formulation is shown in Table 3.6. 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 (cell culture media).

Table 3.6. 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

3.3.2. Evaluation of Spray Dried Particles Containing Adenoviral Vector

3.3.2.1. Retained Viral Activity After Spray Drying

Spray dried powders containing human type 5 adenoviral vector expressing *Escheria coli* β-galactosidase (AdHu5LacZ) were prepared and were indistinguishable in

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 3.5, 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. Within the literature, activity loss post-spray drying can be reportedly greater than 3.0 log, though this is highly variable depending on the labile material used and the method of measuring activity (Jin et al., 2010; Ohtake et al., 2010). 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 3.1) 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 3.6, 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 3.6a) 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 3.2a and 3.2b. 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). The diffusion coefficient for this phase-separated supersaturated domain is magnitudes smaller than that of lactose, trehalose, mannitol or dextran. The result is a Peclet number much greater than 1.

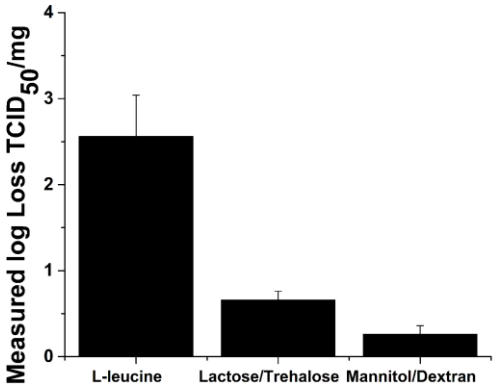

Figure 3.5. Measured loss of AdHu5 infectivity for L-leucine, lactose/trehalose and mannitol/dextran, formulations after spray drying (i.e. process loss at t=0 days). Data is shown as mean ± SD (n = 3).

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 3.6b, 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 3.2 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 3.6c) 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 and hence XPS should have an increased probability detecting the nitrogen should this hypothesis prove valid. 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.

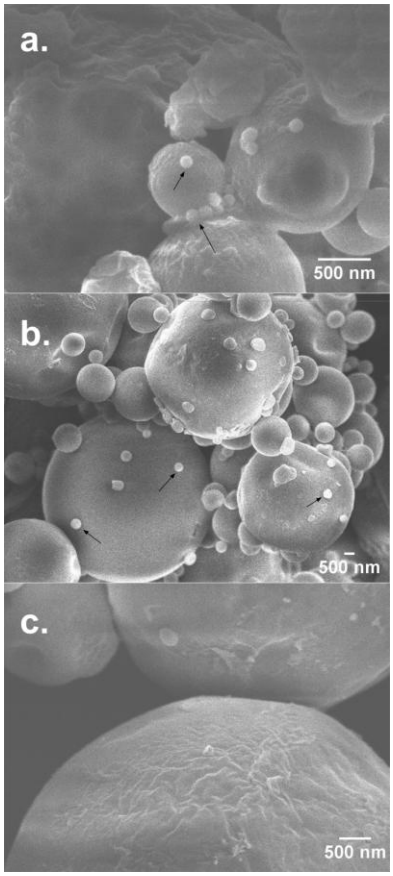

Figure 3.6. SEM micrographs of formulations spray dried with adenoviral vector: L-leucine (a), lactose/trehalose (b) and mannitol/dextran (c). Proposed AdHu5 nodules present on the particle surface are indicated by black arrows.

The same number of tests were conducted with mannitol/dextran particles, with nitrogen never being detected. The surface elemental composition for selected tests of both lactose/trehalose and mannitol/dextran powders is shown in Table 3.7. As an approximate calculation, AdHu5LacZ is estimated to be composed of 20% nitrogen by weight (Green and Pina, 1963; Pina and Green, 1965), and thus for XPS with a detection threshold of 0.5 wt.%, the surface area coverage of AdHu5LacZ to excipient must be over 2.5% for a signal that is readily distinguishable from instrumental noise. This level of surface coverage is possible but the distribution of components is not expected to be

perfectly uniform over different areas sampled by the XPS beam. Thus in some XPS spectra, we were able to detect nitrogen, whereas in others it was absent. We propose that this method of surface detection for AdHu5 is not entirely concrete, however it offers a valuable insight into 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 3.7. 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
Lactose/Trehalose	51.8	48.2	0.0	52.0	47.8	0.7[a]
Mannitol/Dextran	57.6	42.4	0.0	58.9	41.1	0.0

[a]*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). Trehalose has previously been proposed to be a good "water replacing" molecule throughout the drying process as it is able to replace stabilizing hydrogen bonds between water molecules and viral protein

structures (Crowe et al., 1996). Thus, although some segregation between adenoviral vector and formulation excipients may be occurring, trehalose provides good stabilization throughout the spray drying process. In addition, total vector loss from segregation between the adenoviral vector and excipient matrix was not necessarily properly observed through SEM. It is highly probable that some AdHu5 that failed to be incorporated within the matrix would become detached from the particle at some point. 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.

3.3.2.2. Viral Infectivity after Storage at 20°C and Differing Humidity

Figure 3.7 shows the resulting adenoviral vector titre loss through storage at 20^{o}C 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.

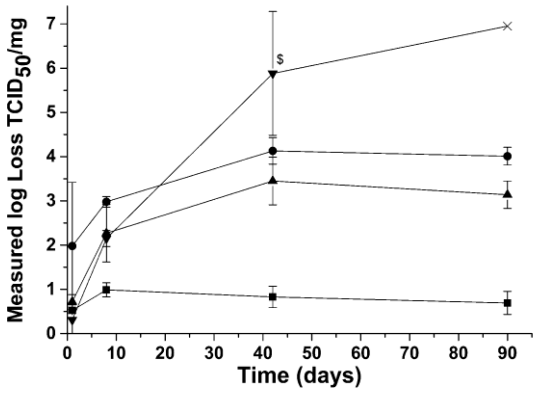

Figure 3.7. Measured log loss of AdHu5 vector infectivity after storage up to 90 days at 20°C and <10% RH for liquid control (▼), L-leucine (●), lactose/trehalose (▲) and mannitol/dextran (■),formulations. Initial time point is at t=1 day. Three repeat samples were stored for each formulation and $ denotes 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 3.8 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 3.3). 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. The increase in excipient molecular motion caused by moisture uptake is clearly linked to the destabilization of the adenoviral vector.

Matrix destabilization was further emphasized by the decrease in T_g observed for lactose/trehalose and mannitol/dextran samples (Table 3.5). 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 3.7), 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.

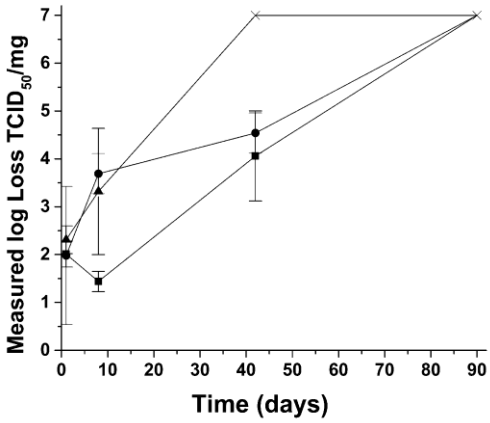

Figure 3.8. Measured log loss of AdHu5 vector infectivity after storage up to 90 days at 20°C and 45% RH for L-leucine (●), lactose/trehalose (▲) and mannitol/dextran (■)formulations. Initial time point is at t=1 day. Three repeat samples were stored for each formulation and '×' denotes viral activity below the detection limit for all repeats. Data is represented as mean ± SD for three repeat samples.

Overall, the best-performing powder throughout the storage tests, based on retained viral activity, 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 3.4b). 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 XPS, a failure to properly incorporate the AdHu5LacZ vector within the matrix renders it a poor excipient formulation for long term storage.

3.3.2.3. 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 (<10% RH in all cases). As shown in Figure 3.9, 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 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).

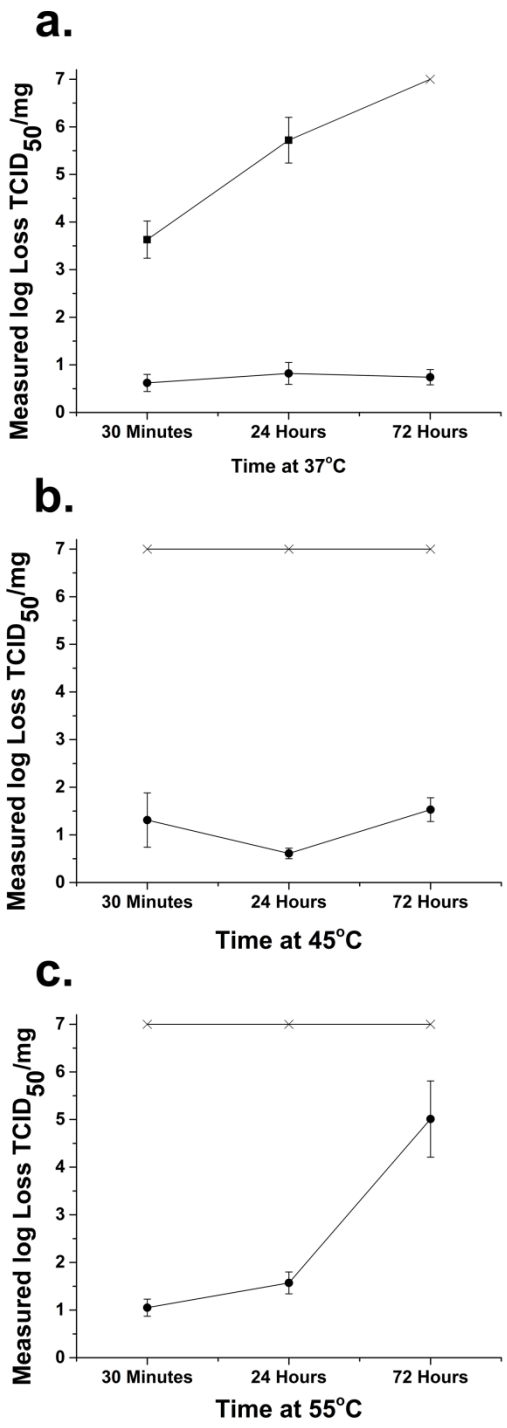

Figure 3.9. Measured AdHu5 infectivity loss for both control (■) and mannitol/dextran formulation (●) at a storage of 37°C (a), 45°C (b) and 55°C (c) for up to three days. Three repeat samples were stored for each formulation and '×' denotes viral activity below the detection limit for all repeats. Data is represented as mean +/- SD for three repeat samples and mean +/- range for two repeat samples.

3.4. 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 aids 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.

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.

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Chapter 4: Optimization of Spray Drying Conditions for Yield, Particle Size and Biological Activity of Thermally Stable Vaccines

In chapter 4, all experiments and work were conducted by myself. The paper was drafted by myself, and thoroughly revised to the final version by Dr. Michael R. Thompson, Dr. Emily D. Cranston and Dr. Zhou Xing. This chapter has been submitted to *International Journal of Pharmaceutics*.

Optimization of Spray Drying Conditions for Yield, Particle Size and Biological Activity of Thermally Stable Vaccines

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ABSTRACT

Recent developments in thermally stable vaccine powders involve using the spray drying process. Optimizing such processes is challenging since the active biopharmaceutical ingredient is often determined too costly to use until the final selection of operating conditions. This paper considers the effects of spray drying parameters on i) yield, ii) particle size and iii) viral vector activity of a mannitol/dextran encapsulated recombinant human type 5 adenoviral vector vaccine, to demonstrate that the biopharmaceutical must be included earlier in the optimization. Response surface methodology (RSM) was used to optimize parameters including inlet temperature, spray gas flow rate, liquid feed rate and solute concentration in the feed. Generally, good conditions for maintaining viral activity led to reduced yield and fewer particles of the desired size. Within the range of parameters tested, the yield varied from 50%–90%, the percentage of ideally size particles was 10%–50%, and the viral vector titre loss was 0.25-4.0 log loss. RSM indicates that the most significant spray drying parameters are the inlet temperature and spray gas flow rate. It was not possible to optimize all three output variables with one set of parameters. Overall, this work provides a guideline for the spray drying of active biopharmaceuticals.

KEYWORDS: spray dry, adenovirus, viral vector, particle size, titre, yield, process parameters, surface response methodology, optimization

4.1. Introduction

Thermal sensitivity in biopharmaceutics ranging from antibiotics to viral vectors remains a major problem today (Brandau et al., 2003; Craig et al., 1999; Sovizi, 2010). Diminished pharmacological activity notably occurs at moderate temperatures through chemical degradation and protein denaturation for these drug candidates (Abdul-Fattah et al., 2007; Ahlneck and Zografi, 1990; Craig et al., 1999). Maintaining efficacy demands adherence to *cold chain* storage and handling protocols which increase the cost of the drug to patients and complicates widespread distribution (Bishara, 2006). As a result, it has become a global challenge to increase the thermal stability of many biopharmaceuticals to ease logistic concerns. The vector platform used in this paper is a human type 5 adenovirus (AdHu5) encoding LacZ for *in-vitro* detection of activity. It is a known thermally unstable biopharmaceutical shown to lose activity even at moderately low temperatures (Ihnat et al., 2005; Rexroad et al., 2006). Normal storage protocols for AdHu5 vectors require temperatures of -80°C, thus it is a suitable biologic to model processing aspects of developing a thermally stable vaccine.

Spray drying is favoured as a robust, scalable, continuous process for producing particulate-form pharmaceutics and recently has become an important method for increasing the thermal stability of labile vaccine platforms (Abdul-Fattah et al., 2007; LeClair et al., 2016; Vehring, 2008). The process dries biologics within glass-forming

ingredients to create particles whereby these therapeutic actives are immobilized in stabilizing matrices. These stabilizing matrices can consist of sugars, sugar alcohols, amino acids, and surfactants, as they effectively replace the water-stabilizing bonds of isolated biomolecules as moisture is removed from the system throughout the drying process (Crowe et al., 1996). The high glass transition temperatures of these matrix species (often $T_g > 100^oC$) restrict molecular movement to the degree that aggregation of an active biopharmaceutical ingredient, such as a vaccine platform, is not possible (Bhandari and Howes, 1999). The entrapped biologic within a stabilizing matrix is prevented from degrading and as a result, retains a higher activity after storage in moderate temperature environments.

Spray drying is already popular within the pharmaceutical industry, used for the manufacture of numerous solid oral dosage forms due to its continuous nature, short residence time, excellent particle size control, and of course, low cost (Broadhead et al., 1992; Gibbs et al., 1999; Vehring, 2008). The use of heat and pressurized sterile gas to produce a dry powder is inexpensive. Furthermore, spray drying has been shown to provide reasonable scalability from the lab to industrial-scale processes (Thybo et al., 2008). This method of manufacture has been an easy choice for active pharmaceutical ingredients (API) that exhibit good-to-excellent stability at high temperatures. However, this method is often overlooked for labile ingredients in the face of more expensive approaches such as lyophilisation for drying biopharmaceuticals, never properly considering the short residence time and thermal environment within sprayed droplets. As a result, little process understanding exists to meet the stated global challenges (World

Health Organization, 2011) of producing thermally stable vaccines by spray drying methods. Particularly of concern to this paper, the effect of spray drying parameters on particle properties involving therapeutics are largely unexplored within the literature.

This paper examines the main operational variables to spray drying a thermally stable vector platform, including: inlet temperature, gas flow rate, liquid feed rate, and the solute concentration in the feed liquid. Its intent is to highlight how the effects of these parameters differ on the measured response of viral activity versus more traditional spray drying variables such as powder yield and particle size. Earlier studies related to the present work have optimized the yield of similar powder materials though without inclusion of a biologic (Elversson and Millqvist-Fureby, 2005; Maury et al., 2005). Further work valuable in the present study has modelled the steps to particle formation throughout the spray drying process (Vehring et al., 2007). The work demonstrates that the setup of a spray drying process intended to produce these new thermally stable vaccines is challenged by the requirement of maintaining therapeutic activity and that a true optimal operating condition can only be determined with the viral vector present. The results of this process study are considered timely in the face of the global urgency to develop thermally stable vaccines and in recognition that little knowledge is available linking spray drying process parameters to viral activity (as the primary consideration) and powder yield and particle size as secondary parameters. While the exact optimized spray drying conditions will depend on the specific labile material and excipient chosen, the overall trends and governing interactions elucidated here for AdHu5LacZ in a

mannitol/dextran matrix provide valuable "process rules" for spray drying other biopharmaceuticals.

4.2. Materials and Methods

4.2.1. Chemicals and Adenoviral Vectors

D-mannitol and dextran (M_r 40,000 kDa) were 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 the supplier, 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 *Escheria coli* β-galactosidase (AdHu5LacZ) was produced in the vector facility of McMaster Immunology Research Centre as described previously (Xing et al., 1996).

4.2.2. Spray Drying, Process Parameters and Response Criteria

Powders were produced from spray drying with a Mini Spray Dryer B-290 (Büchi; Switzerland). The spray dryer was equipped with a 0.7 mm spray nozzle and high performance cyclone. The pressurized spray gas was dried and filtered using an in-line silica gel desiccant air dryer (McMaster-Carr; Elmhurst, IL) and Aervent® 0.2 μm filter (EMD Millipore; Billerica, MA). The feed solution was composed of mannitol and dextran (at a weight ratio of 67% mannitol and 33% dextran) along with the AdHu5LacZ

vector (1.5 x 10^6 pfu). The T_g for this formulation has previously been measured to be approximately 130°C (LeClair et al., 2016). The aspirator rate was fixed at 35m^3/h for each case.

Adjusted process parameters in the study were inlet temperature (TE), gas flow rate (SG), liquid feed rate (F), and the solute concentration in the feed liquid (S). Although the actual chamber temperature is important to the drying process, it is a correlated variable with many of the parameters tested and hence was not suitable to study. The parameter values used throughout the optimization study are shown in Table 4.1, with each individual run listed; each parameter was studied at three levels to consider non-linear effects on the process. Optimization was performed based on the response criteria of (i) increasing the cumulative size fraction of spray dried particles in the range of 1-5 µm which are suitable for dry powder inhalation ($F_{1\text{-}5\mu m}$), (ii) maximizing the overall powder yield (Y) and (iii) minimizing AdHu5LacZ titre log loss (lost bioactivity) as a result of spray drying (T); the rank of importance given to each response variable will be discussed later.

Table 4.1. Design of Experimental Runs Examining Spray Drying Parameters.

Run	Inlet Temperature (°C)	Gas Flow Rate (L/h)	Liquid Feed Rate (mL/h)	Solute Concentration (% w/w)
1	120	357	217.5	0.50
2	90	468	145.0	0.75
3	120	357	217.5	0.50
4	90	468	145.0	0.25
5	150	468	145.0	0.25
6	90	468	290.0	0.75
7	150	246	145.0	0.25
8	150	468	290.0	0.75
9	120	357	217.5	0.50
10	90	246	145.0	0.25
11	150	468	145.0	0.75
12	90	246	290.0	0.25

13	90	468	290.0	0.25
14	150	468	290.0	0.25
15	150	246	290.0	0.25
16	150	246	290.0	0.75
17	90	246	145.0	0.75
18	120	357	217.5	0.50
19	90	246	290.0	0.75
20	150	246	145.0	0.75
21	90	357	217.5	0.50
22	150	357	217.5	0.50
23	120	246	217.5	0.50
24	120	468	217.5	0.50
25	120	357	145.0	0.50
26	120	357	290.0	0.50
27	120	357	217.5	0.25
28	120	357	217.5	0.75

4.2.3. Imaging of Spray Dried Powders

The spray dried particles without viral vector were examined using a JEOL JSM-7000F scanning electron microscopy (SEM, JEOL Ltd., Japan). Previous examination has shown the powders to retain the same size and morphology with viral vector included (LeClair et al., 2016). Powder samples were mounted with double-sided tape and sputter-coated with approximately 5 nm of gold. Micrograph images were collected at a working distance of 10.0 mm to 10.3 mm and an electron accelerating voltage of 5.0 kV. Electron microscopy was performed at pressures below 5.0×10^{-4} Pa. Resulting images were analyzed using ImageJ software (Abràmoff et al., 2004).

4.2.4. Sizing and Yield of Spray Dried Powders

Particle size was determined using a Malvern Mastersizer 2000 (Malvern Instruments, United Kingdom) equipped with a He-Ne laser. The powders were suspended in anhydrous ethanol at concentrations of 1.0 mg/mL; the tested powder could

not include the viral vector in this test out of concerns for contaminating the instrument but a comparison of particle sizes with and without AdHu5LacZ by SEM found no significant difference (LeClair et al., 2016). A representation of ideal particle size with a Feret diameter between 1-5 μm was based on the cumulative weight fraction, $F_{1\text{-}5\mu m}$, in the desired size range quantified as a percentage. This size range represents the suitability of the powder sample for intranasal pharmaceutical delivery as this size offers the best penetration into the lungs (Elversson et al., 2003). The first moment (i.e. d_{50}) of the size distribution was not considered as an appropriate optimization parameter in this study as some spray drying conditions produced such coarse particles that a bimodal distribution was found which inappropriately skews the average (Dixon, 1950). Choosing an interval size and accounting for the percentage of particles within that range was considered a more accurate manner to optimize the process and the size range chosen is identified as suitable for pulmonary delivery.

Powder yield was calculated simply as the mass of the powder out of the spray dryer divided by the mass of excipients originally spray dried. This amount is converted to percentage through multiply by 100.

4.2.5. *In Vitro* Testing of Spray Dried Particles

4.2.5.1. Culturing of A549 Cells

A549 lung epithelial cells stored in liquid nitrogen were thawed. Cells were cultured with culture media in T150 flasks. The cell culture flasks were incubated in a Forma Series II Water Jacketed CO_2 Incubator (Thermo Scientific Corporation; Waltham,

MA) at 37.0°C and 5.0% CO_2. When cells reached 80-90% confluency, they were either split to a new culture flask or plated in a 96-well plate for *in vitro* testing.

4.2.5.2. Spray Dried Formulation Viral Infectivity (Titre Log Loss)

Spray dried powders containing AdHu5LacZ were dissolved in culture media and then added to 96-well plates containing A549 lung epithelial cells for *in vitro* testing. Eight-fold serial volume dilutions were created at dilutions of 10^0 to 10^{-7} of the reconstituted sample. The A549 lung epithelial cells were incubated with the AdHu5LacZ dilutions for 24 hours. Afterward, the cells were fixed with a 0.2% glutaraldehyde (Sigma Aldrich)/0.8% fomaldehyde (Sigma Aldrich) solution in phosphate buffered saline (%v/v) for less than five minutes. The fixative was removed, and viral activity was detected using the X-gal color reaction with 5-bromo-4-chloro-3-indoyl β-(D)-galactoside. Cells positive for LacZ transfection were observed with an Axiobert 25 inverted light microscope (Zeiss, Germany). The resulting median tissue culture infectious dose ($TCID_{50}$) was calculated using the Reed-Muench method (Reed and Muench, 1938). This end titre was compared with the initial titre to determine total titre loss from spray drying.

4.2.6. Data Analysis

The optimization study was produced using a Box-Wilson Central Composite Design response surface methodology within Design-Expert 7.0 (Stat-Ease, Minneapolis, MN). The experimental design was set up with the four process parameters at three levels, as discussed in Section 2.2. Three replicates were included for the center point to evaluate

uncertainty. The initial factorial trials were performed in a random order to minimize bias. Data was analyzed and all statistical plots were created using the statistical package R (R Foundation for Statistical Computing, Austria). Results were determined to be statistically significant at $p \leq 0.05$.

4.3. Results

To represent the significance of each process variable to the responses of the weight fraction of particles with the desirable size, powder yield and titre log loss, a linear regression model was constructed. An ideal optimized set of process conditions from the standpoint of being industrially relevant, would see vaccine powders produced with $F_{1\text{-}5\mu m}$ > 95%, >85% powder yield and less than 1 log loss; the goal of the program was to ultimately develop a thermally stable tuberculosis vaccine intended to be administer by dry powder inhaler. Each response was defined in Section 2. The three response surfaces corresponding to the selected process variables are given in Table 4.2.

Table 4.2. Response Surfaces for the Spray Drying of Mannitol/Dextran Particles Containing AdHu5LacZ.

Response variable	Expression*	Eqn.
$F_{1-5\mu m}$ (%)	$F_{1-5\mu m} = 25.89 - 3.94TE + 8.68SG - 2.60F - 8.87S - 1.02TE \cdot F + 5.64SG \cdot S - 6.90TE \cdot TE + 9.60F \cdot F$	(1)
Powder Yield (%)	$Y = 82.34 - 6.80TE - 2.93F - 3.54S - 6.02TE \cdot F + 17.81TE \cdot S + 21.44F \cdot S - 14.08F \cdot F$	(2)
Titre Loss (log)	$T = 0.99 + 0.67TE + 0.81SG - 0.011F - 0.92S + 0.79F \cdot S + 1.76SG \cdot SG$	(3)

* Spray drying process parameters: inlet temperature (TE), gas flow rate (SG), liquid feed rate (F) and solute concentration (S).

4.3.1. Percent of Ideally Sized Particles ($F_{1\text{-}5\mu m}$)

In examining the effect of spray drying parameters on $F_{1\text{-}5\mu m}$, all four parameters were found to be significant terms of the surface response model (Equation 1 in Table 4.2) along with the second order terms of TE•TE and F•F, and the two-way interactions of SG•S and TE•F. The analysis of variance (ANOVA) results for $F_{1\text{-}5\mu m}$ in regards to spray drying parameters are shown in Table 4.3.

Table 4.3. ANOVA results for $F_{1\text{-}5\mu m}$ (%)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value
Model	2548.90	8	318.61	146.59	<0.0001
TE	279.27	1	279.27	128.49	<0.0001
SG	1353.47	1	1353.47	622.71	<0.0001
F	121.68	1	121.68	55.98	<0.0001
S	372.65	1	372.65	171.45	<0.0001
TE•F	16.81	1	16.81	7.73	0.0119
SG•S	133.98	1	133.98	61.64	<0.0001
TE•TE	160.60	1	160.60	73.89	<0.0001
F•F	311.59	1	311.59	143.36	<0.0001
Residual: Lack of Fit	37.74	16	2.36	1.99	0.3145

The effect of each parameter on $F_{1\text{-}5\mu m}$ is shown in the given surface plots in Figure 4.1. $F_{1\text{-}5\mu m}$ was found to be as low as 10% and as high at 45% in the study but never approached the ideal case (i.e. >95%). The second-order effect of inlet temperature meant that a maxima occurred for $F_{1\text{-}5\mu m}$ at moderate temperatures. Conversely, the second-order effect of liquid feed rate meant that a minima occurred for $F_{1\text{-}5\mu m}$ within the range of tested rates. Increasing the solute concentration reduced $F_{1\text{-}5\mu m}$ while increasing the gas flow increased $F_{1\text{-}5\mu m}$.

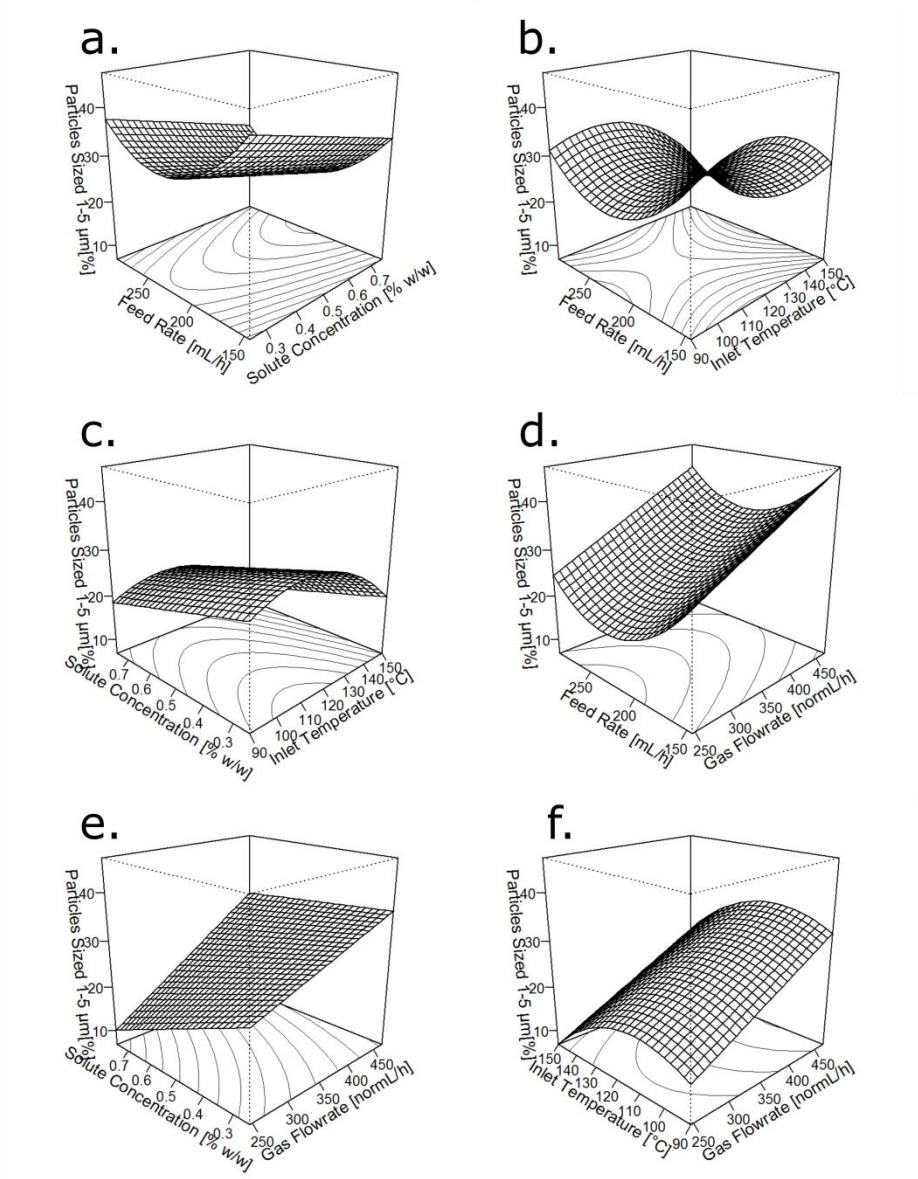

Figure 4.1. Surface plots for statistical model (Equation 1, Table 4.2) of percentage of particles sized 1-5 µm (%) against all process parameters.

4.3.2. Powder Yield

ANOVA results regarding the regression model for powder yield are given in Table 4.4. The significant main model terms were TE and S, whereas the significant second order term was F•F, and two-way interactions were TE•S, F•S, and TE•F. The

model of surface response for powder yield is given as Equation 2, in Table 4.2. Powder yield ranged from > 90% to 50% within this study.

Table 4.4. ANOVA results for model parameters of powder yield (%).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value
Model	6347.31	7	906.76	8.89	<0.0001
TE	829.74	1	829.74	8.13	0.0099
F	154.48	1	154.48	1.51	0.2328
S	59.41	1	59.41	0.58	0.4544
TE•F	579.61	1	579.61	5.68	0.0272
TE•S	1334.08	1	1334.08	13.07	0.0017
F•S	1933.80	1	1933.80	18.95	0.0003
F•F	1274.44	1	1274.44	12.49	0.0021
Residual: Lack of Fit	1691.14	17	99.48	0.85	0.6503

Surface plots in Figure 4.2 shows the influence of each spray drying process parameter on powder yield. Inlet temperature exhibited a complex influence on powder yield by being correlated with solute concentration and feed rate, often producing a maxima in the range of 120^{o}C. A lower yield was produced using a higher solute concentration for the matrix ingredients dissolved in the supplied liquid. The solute concentration and feed flow rate interacted positively on yield; the same was true for the interaction between inlet temperature and solute concentration.

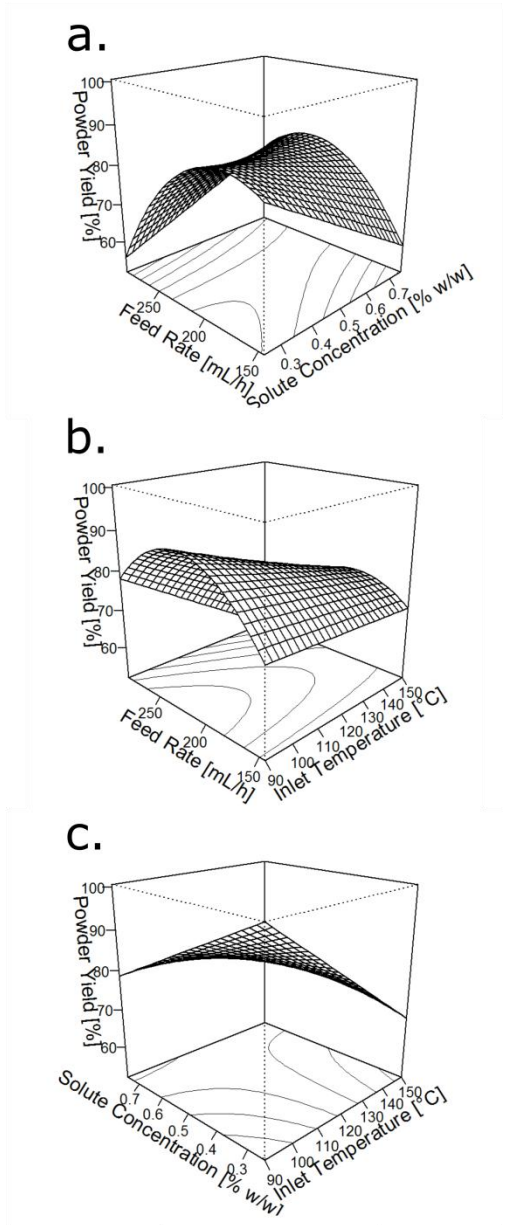

Figure 4.2. Surface plots for statistical model (Equation 2, Table 4.2) of powder yield (%) against all process parameters.

4.3.3. Titre Loss

The ANOVA results for the model relating viral vector titre loss to spray drying parameters are shown in Table 4.5; the computed response surface for titre loss is given

by Equation 3, Table 4.2. The main model terms determined to be significant ($p \leq 0.05$) were TE, SG, S, while the significant second-order effect was SG•SG and the two-way interaction was F•S. Their adjustment over the range of levels tested significantly altered the resulting total titre loss.

Table 4.5. Analysis of variance (ANOVA) for AdHu5LacZ total titre loss (log).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value
Model	46.39	6	7.73	17.59	<0.0001
TE	7.99	1	7.99	18.18	0.0003
SG	11.76	1	11.76	26.75	<0.0001
F	2.154×10^{-3}	1	2.154×10^{-3}	4.901×10^{-3}	0.9448
S	4.02	1	4.02	9.14	0.0065
F•S	2.60	1	2.60	5.92	0.0240
SG•SG	20.01	1	20.01	45.52	<0.0001
Residual: Lack of Fit	7.63	18	0.42	0.80	0.6805

Surface plots modeling the effects of process parameters on total titre loss are shown in Figure 4.3; the lowest titre loss was sought in this study (corresponding to the highest retained bioactivity). The titre loss varied from less than 0.5 log loss to 4.0 log loss compared to the original viral vector stock (control). This implied that there is a wide range of spray drying parameters that can lead to viable biopharmaceuticals where less than 1 log loss is considered good but greater than 2 log loss is poor. The chosen surfaces in Figure 4.3 demonstrate the influence of the significant processing parameters on the resulting viral vector titre in the collected powders. The total titre loss for the adenovirus-containing powder was reduced by increasing the solute concentration within the liquid feedstock but was increased at high gas flow rate and high inlet temperatures. The feed rate generally did not influence the resulting viral vector titre loss except when correlated

with changes in the solute concentration. In these cases, a higher feed rate minimized the negative outcome of lowering the solute concentration.

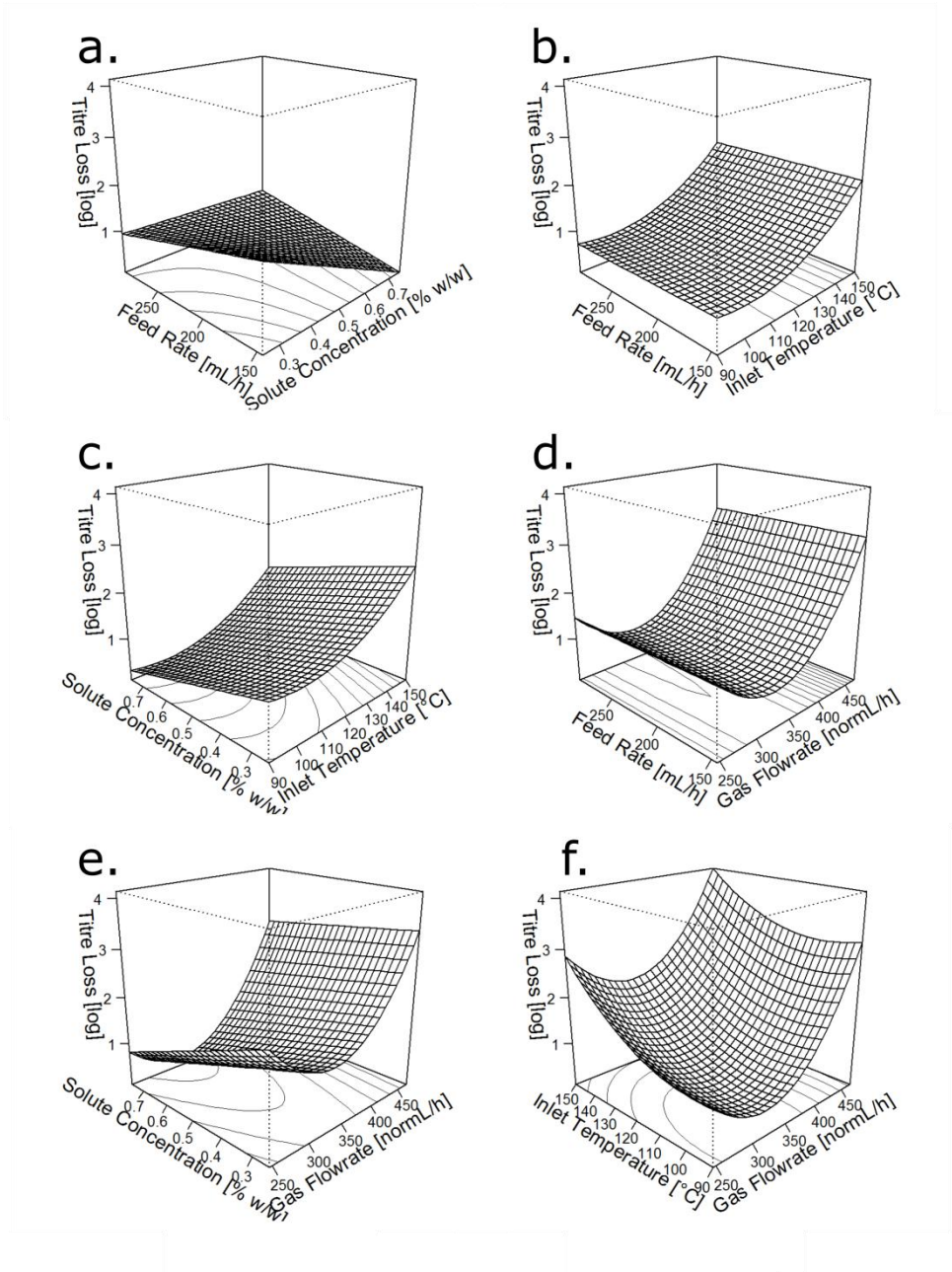

Figure 4.3. Surface plots for statistical model (Equation 3, Table 4.2) of AdHu5LacZ total titre loss (log) against all process parameter combinations.

4.4. Discussion

4.4.1. Process Parameter Effects on Particle Size ($F_{1\text{-}5\mu m}$)

Typically, the low-end tail of the particle size distribution extends into the 1-5 µm range ($F_{1\text{-}5\mu m}$); the dominant proportion of particles are much larger than the identified ideal size range sought in this study. Examples of two distinctly different size distributions arising in the study are given in Figure 4.4 with the fraction of particles contributing to $F_{1\text{-}5\mu m}$ highlighted.

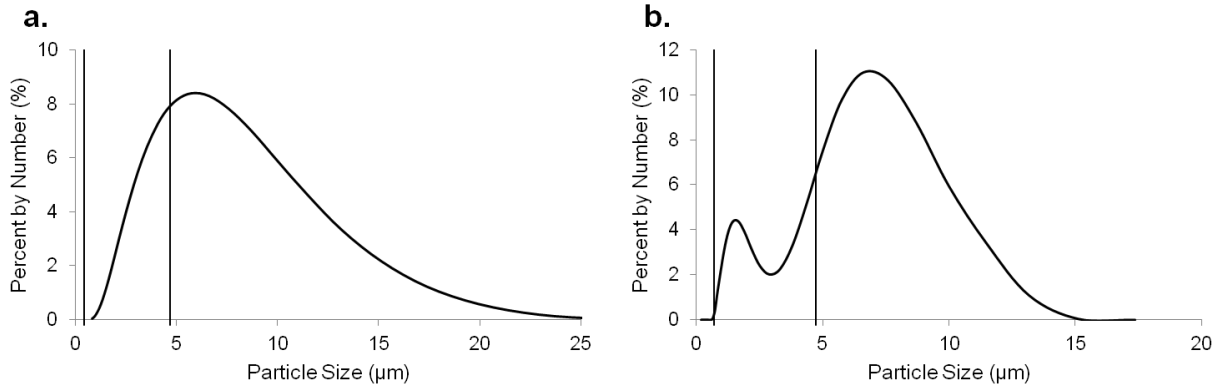

Figure 4.4. Particle size distributions of two trials are shown with the ideal size range ($F_{1\text{-}5\mu m}$) outlined by the vertical black lines. Distribution (a) presents a typical size distribution formed from non-collapsing particles (Run 2) whereas (b) shows a size distribution formed from collapsing and breaking particles (Run 6).

Inlet temperature of the spray dryer exhibits a second-order relationship with $F_{1\text{-}5\mu m}$ (Figures 4b, c, f) where the highest and lowest temperatures tested produce a lower amount of particles in the desired size range of 1-5 µm. At the higher end of the tested temperature range, particle temperature approaches the glass transition temperature of the matrix species and coalescence based on bridge formation between particles becomes possible, as observed in Figure 4.5 (Roos, 2002). Furthermore, the high Peclet (*Pe*) number corresponding to these drying conditions leads to a higher likelihood of larger

sized (yet hollow) individual particles since surface solidification occurs much faster than solute diffusion within the droplet (Vehring et al., 2007). The *Pe* number given in Equation 4 represents the balance of evaporation rate (κ, Equation 5) versus solute diffusion, D_i in a process (Winterton, 1999):

$$Pe = \frac{\kappa}{8D_i} \tag{4}$$

$$\kappa = hA(T - T_\infty) \tag{5}$$

where the evaporation rate is increased by a larger temperature gradient between the drying droplet (T) and the air in the spray drying chamber (T_∞). Thus, increasing the inlet temperature of the spray dryer increases drying, which is characterized by a greater Peclet number. The formation of hollow particles often occurs using matrix solutes that poorly diffuse from the droplet surface, such as L-leucine, as shown in other studies (Aquino et al., 2012; Najafabadi et al., 2004). However, it has also been observed that even highly soluble molecules can form hollow particles at high evaporation rates (Elversson et al., 2003). The sunken depressions observed on the surfaces of formed mannitol/dextran particles by SEM in Figure 4.5 are characteristic of such hollow structures, and thus larger in size than particles with a compact core. Conversely, low inlet temperatures facilitate slower drying, which is more likely to produce denser particles, but being in the presence of high moisture for too long can lead to agglomeration. Water is a plasticizer to the matrix ingredients used here, depressing the glass transition temperature and promoting particles to bind together should they collide (Hancock and Zografi, 1994).

The overall effect is the production of aggregated particles. Some of the large aggregates that are formed from low temperature drying are shown in Figure 4.6.

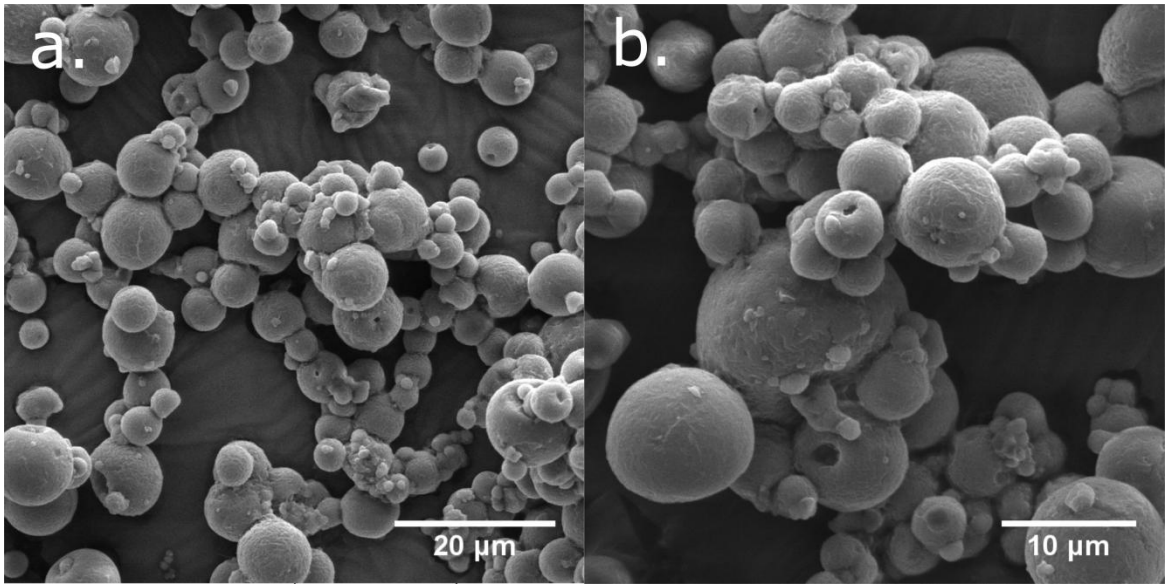

Figure 4.5. Spray dried particles without AdHu5LacZ formed at an inlet temperature of 150°C as imaged by SEM at (a) 1000x and (b) 3000x magnification. Significant bridging between particles is observed.

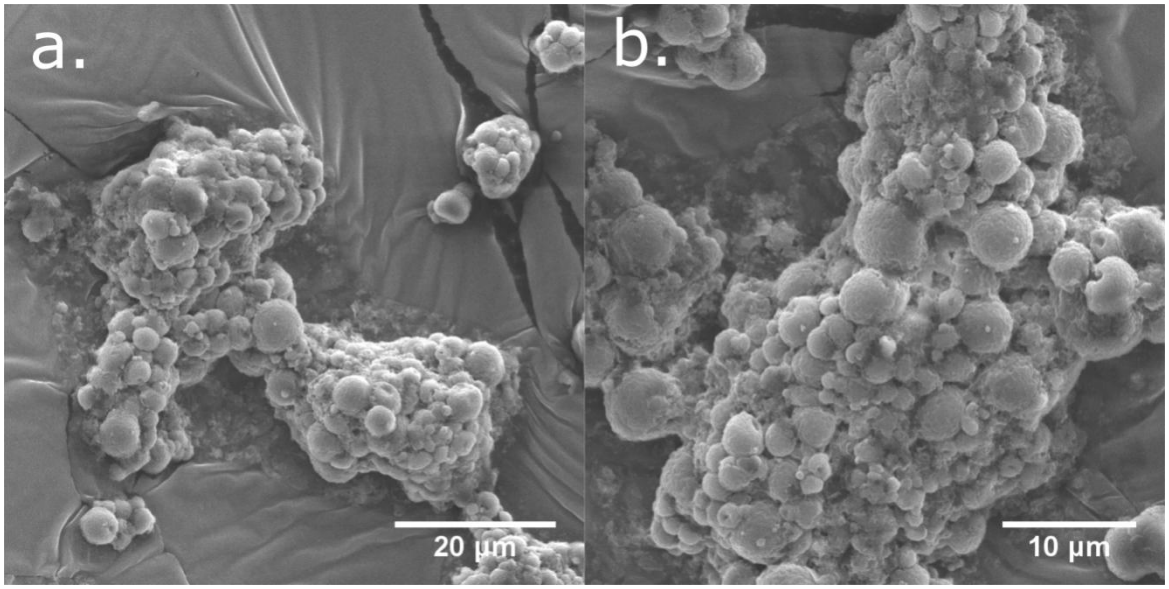

Figure 4.6. Spray dried particles without AdHu5LacZ formed at an inlet temperature of 90°C as imaged by SEM at (a) 1000x and (b) 3000x magnification Particles adhere to each other due to incomplete powder drying.

Our results indicate that gas flow is linearly related to $F_{1\text{-}5\mu m}$. Hede et al. have demonstrated this relationship through the Weber number (*We*) where larger Weber numbers result in smaller sized particles (Hede et al., 2008). The increased inertial terms of the Weber number caused by higher gas velocity is more likely to overcome the surface energy of the fluid to produce smaller droplets and hence smaller dry particles. Increasing the Weber number, which infers a dominance of kinetic forces over interfacial forces, is accomplished by increasing the spray gas velocity (U_g) or reducing the surface tension of the solution (σ). This is shown in Equation 6, where increasing the spray gas velocity greatly increases the Weber number, reducing the dry particle size.

$$We = \frac{\rho {U_g}^2 l}{\sigma} \tag{6}$$

A second-order relationship was also found between $F_{1\text{-}5\mu m}$ and the liquid feed rate (Figures 4a, b, d) with a minimum determined within the range of tested conditions. At low feed rates, a smaller amount of liquid is dispersed to form smaller droplets (Lin and Reitz, 1998). The trend resembles previous work modeling an exponential decrease in formed droplet size from an atomizer as the liquid feed to the system is reduced (Hede et al., 2008). Increasing feed rate based on this theory should form large droplets; however, another phenomenon which begins to dominate causes this trend to gradually reverse. At a constant solute concentration, the larger particles are more hollow with a thinner shell than particles formed from smaller droplets (Elversson et al., 2003). The result is particles

more prone to collapsing and breaking, which produces smaller-sized particles. These particles are shown in Figure 4.7, where the appearance of broken particles presumably leads to the creation of finer-sized debris. A single collapse/break can form many particles of ideal size, and thus the percentage of particles between 1 and 5 microns increases at high feed rates. Particle size data shown in Figure 4.4b indicates that such breakage can lead to bimodal distribution. It is known that a higher feed rate also corresponds to a lower outlet temperature within the spray dryer (Maa et al., 1997), which would indicate lower Peclet number drying. It was generally measured, and observed in Figure 4.7, that the effect of a high feed rate was more significant in producing hollow particles than the lower Peclet number drying was in preventing them. However, the mentioned influence of the feed rate on the drying temperature seems present in the interaction parameter TE•F and so was a significant contributing effect overall (Table 4.2). The interaction parameter explains the small minima present when adjusting the feed rate or increasing inlet temperatures for fixed feed rate. This reduction in $F_{1\text{-}5\mu m}$ is presumably due to less collapsed particles on account of the change in Peclet drying induced by the change in the feed flow rate. These changes are most prevalent in cases where Peclet drying is most extreme, i.e. at high inlet temperatures.

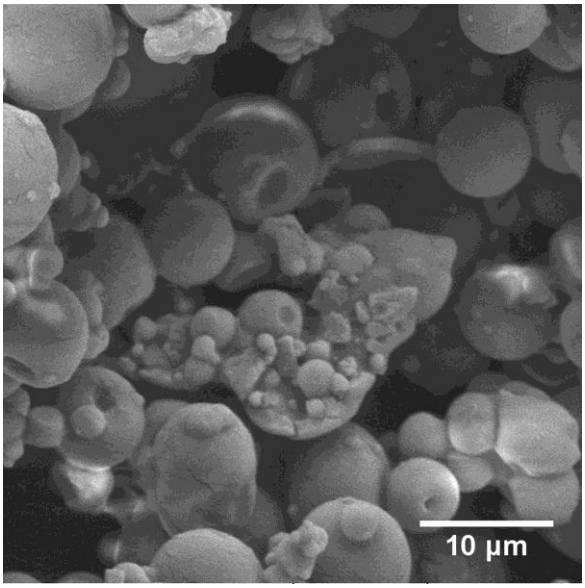

Figure 4.7. Spray dried particles without AdHu5LacZ formed at the high liquid feed rate imaged by SEM.

The solute concentration has a simple linear effect on $F_{1\text{-}5\mu m}$, like gas flow rate, with fewer particles of ideal size occurring due to preferential growth as the solute concentration was increased. This is related to how particles form during the spray drying process. As water evaporates at the air-droplet interface, the surface localized solute concentration increases. This creates a gradient in solute concentration radially within the droplet. The net result is the movement of solute material to the centre as the droplet shrinks through evaporation (Huang, 2011; Vehring et al., 2007). Eventually, a threshold is reached where the concentration of solute within the drying water droplet is high enough for precipitation of the matrix to begin. As precipitation occurs the particle is formed. When starting with a higher matrix solute concentration, the onset of precipitation occurs earlier during the evaporation process – resulting in a larger particle in the end.

4.4.3. Powder Yield

The response surface with respect to powder yield shows similar trends to $F_{1\text{-}5\mu m}$ for the most significant processing parameters. Yield displays a simple linear dependency on inlet temperature, decreasing yield as TE increases, despite the non-negligible interaction terms for inlet temperature with solute concentration and feed rate (Equation 2). The increased likelihood of particle bridging with increased inlet temperature mentioned in Section 4.2 (and seen in Figure 4.5) will similarly result in more particles adhering to the spray chamber wall. By sticking to the wall, less powder was collected from the separating cyclone and hence yield decreased.

A second-order relationship exists for feed rate with a displayed maximum in the range of tested conditions. Yields decrease at higher and lower feed rates due to particle adherence to the spray dryer glassware. At low feed rates, there is less liquid for evaporation within the drying chamber, thus raising temperatures within the spray drying glassware (Maa et al., 1997). Higher drying temperatures results in more free-flowing and adhering particles due to the particle T_g. Higher feed rates lower the temperature within the spray dryer, but also increases the moisture content of the particles (Joo and Yong, 2007; Tonon et al., 2008). Greater particle water content reduces the matrix T_g, thus although the temperature within the spray dryer is lower, the temperature at which the particles become sticky and free flowing is also lower. Sticky and free flowing particles readily adhere to the spray dryer chamber walls, reducing powder yield. These effects are evident from the interaction between inlet temperature and feed rate (TE•F, Figure 4.2b). At the maximum for each of these conditions, both spray dryer temperature and particle

moisture are elevated. The reduced glass transition temperature of these particles at high temperatures causes the greatest particle adherence, and loss of yield.

Interaction between the feed rate and solid matrix concentration (F•S) is shown in Figure 4.2a. The yield exhibits a parabolic dependency on the feed rate, as discussed previously. Increasing the solute concentration increases the feed rate at which the maximum yield occurs, meaning optimal powder yields occur at higher feed rates for higher matrix solid concentrations, and at lower feed rates for lower matrix solid concentrations. This is not a major shift, as the optimal powder yield feed rate at the lowest and greatest matrix solid concentrations is approximately 190 mL/h and 230 mL/h, respectively, indicating only a modest change in feed rate for varying matrix solid concentrations. This small variation is likely a result of the thermal property changes at these conditions. As described, major powder losses occur due to powder adhering to the wall of the spray drying chambers, which occurs at temperatures approaching or above the particle T_g. Increasing matrix solid concentration reduces the amount of remaining moisture in a formed particle by increasing the ratio of precipitating solute to water per drying droplet. This change in moisture content increases the particle T_g and thus shifts the feed rate necessary for optimal powder yield, as observed in Figure 4.2a.

4.4.4. Titre Loss

To understand the influence of spray drying parameters on the observed loss in viral activity it is important to understand the many routes, both physical and chemical, that can lead to adenovirus deactivation. To retain activity, the virus must maintain its

structure and stay intact, which requires that the viral proteins preserve their primary, secondary, tertiary and quaternary structure (Klein et al., 1979; Norrby, 1969). Protein unfolding and denaturation can occur chemically through protein interactions with hydrophobic surfaces (including the air-water interface) leading to changes in secondary and tertiary structure (Dill and Shortle, 1991; Tanford, 1962) or through chemical reactions, such as peptide cleavage, which change the primary structure (Jacobson and Schaffer, 1973). Protein aggregation and protein unfolding due to elevated temperature are other common routes to deactivation where protein function is lost by being in the denatured state (Baldwin, 1986; Day et al., 2002; Ihnat et al., 2005; Monahan et al., 1995). Losses of viral activity can also occur from physical forces, such as high shear, which can mechanically disrupt/damage the virus particles (D'Souza et al., 2009; Maa and Hsu, 1997, 1996). Thus minimal AdHu5LacZ titre loss is achieved by preventing chemical, thermal and physical inactivation.

The adjustment of inlet temperature within the spray dryer controls the total energy in the drying system. Thus, the inlet temperature, as set by the temperature of the spray dryer nozzle, is a very important parameter, and determined to be the only factor that was significant for all three responses (Tables 4.3 - 5). Increasing this temperature increases the drying rate of the particles but also the free energy for deformation/protein denaturation of a labile material such as the viral vector. This is seen experimentally as an increase in the total titre loss of AdHu5LacZ with increasing inlet temperature, which agrees with previous results which studied protein denaturation as a function of temperature (Dill and Shortle, 1991; Dill, 1990). While high inlet temperatures are not

optimal for powder yield or particles of ideal size either, there is a limit to which inlet temperature can be lowered because particle development (i.e. formation of dry particles, overall powder yield, etc.) will also be negatively affected.

Increasing gas flow rate during spray drying increases the total titre loss (Figures 4.3d, e, and f). A larger gas flow rate increases the shear applied to the liquid feed, breaking the liquid into smaller-sized droplets and correspondingly, increases the total air-water interface. The amount of shear applied to the fluid in a two-fluid nozzle atomizer is estimated through Equation 7, where U_{av} refers to the average velocity of the spray gas and liquid feed, U_l refers to the velocity of the liquid feed and D_L refers to the diameter of the spray nozzle(Hede et al., 2008).

$$\dot{\gamma} \approx \frac{2(U_{av} - U_l)}{D_L} \tag{7}$$

Previous work has shown that protein denaturation can be related to shear forces on biological compounds (Ghandi et al., 2012; Maa and Hsu, 1997). The trend observed in those studies showed that biological activity decreased exponentially for increasing shear rates. Our study lacks a sufficient number of different gas flow rates to model an exponential trend but we do find a second-order dependency as denoted by the significant model terms being SG, SG•SG in Equation 3 and displayed in Figures 4.3d, e, and f. The increase in air-water interfacial area resulting from smaller-droplet sizes at high shear rates is further detrimental to native protein structure. Air-water interfaces act as hydrophobic “surfaces” and enhance the rate of protein denaturing (Maa and Hsu, 1997;

Tanford, 1962). Thus the rate of denaturing of viral vector proteins is increased along the surface of each formed droplet as the spray gas rate increases (Graham and Phillips, 1979).

Increasing the feed rate should increase shear forces while the liquid is passing through the nozzle, but as indicated by Equation 7, increasing feed rate will actually decrease the apparent shear rate on droplets formed at the end of the nozzle at a given gas flow rate. Due to the loss in viral vector activity by increased shear from spray gas flow rates, the expectation is a reduction in titre loss at high flow rates. However, feed rate is not a significant term in Equation 3 which indicates that its effect on shear rate was small relative to our observations with gas flow rate. This is due to the differences in magnitude between the spray gas flow rate and liquid feed rate, thus subtraction of U_l from U_{av} is negligible when U_{av} is much greater than U_l. Overall, there was no observed effect on the total titre loss by adjusting the feed flow rate to the nozzle, indicating minimal influence of this variable relative to the other contributing parameters of inlet temperature and gas flow rate (Table 4.5).

In this work, mannitol and dextran is chosen as a good excipient matrix for viral vector stabilization (LeClair et al., 2016) and so changing the matrix solute concentration in the feed will primarily affect the ability for the matrix to encapsulate the bioactive agent. Stabilization of other viruses has also been demonstrated through spray drying with sugar matrices (Ré, 1998; Saluja et al., 2010). As predicted, the total titre loss is lowered by increasing the matrix solute concentration in the liquid feed (Table 4.5). The natural state for AdHu5 is within an aqueous environment where hydrogen bonding from water

molecules maintains proper protein structure (Ippolito et al., 1990; Tarek and Tobias, 2002). Thus viral vector protein denaturation can occur throughout drying due to the loss of water molecules (Prestrelski et al., 1993). Matrix solutes are able to replace these stabilizing interactions and prevent a loss of protein structure during drying (Crowe et al., 1996; Liao et al., 2002). This explains the preference of hydrogen-bonding molecules, such as carbohydrates, for stabilization of biological materials through vitrification (Amorij et al., 2008). Thus the greatest viral vector stabilization is achieved at the greatest feed matrix concentration, as this corresponds to the greatest potential for water hydrogen-bonding replacement. At high liquid feed rates, the solute concentration has a negligible effect on the AdHu5 titre loss (Figure 4.3a). This is represented by the interaction parameter for liquid feed rate and solute concentration (F•S) in Table 4.5. High feed rates correspond to higher retention of water within spray dried particles. The requirement for water replacing bonds is lower in particles that retain water.

4.4.5. Significance of Findings

A summary of our findings is presented in Figure 4.8 to highlight the complex nature of the dependent parameters. All three responses cannot be maximized within a given set of processing conditions which then require the outputs to be prioritized. Clearly, the incorporation of a labile material requires that titre loss be minimized – most users will agree that this is the primary constraint given the high cost of producing, for example, viral vectors. Process yield and particle size can then be optimized as secondary parameters depending on matrix material and processing costs and intended method of

delivery of the biopharmaceutical. Figure 4.8 shows that careful optimization of one parameter will result in suboptimal values for the other responses. For example, increasing the solute concentration decreases the loss of the viral vector through spray drying, but also decreases the powder yield and amount of particles in the desired size range. A good compromise for all three responses could be to spray dry with medium-low inlet temperature, medium spray gas flow rate, medium feed rate, and high solute concentration noting that inlet temperature and gas flow are the most influential parameters.

While the exact responses will differ for different formulations and spray dryer setups, the trends and interactions outlined and discussed here can serve as a guide for future investigations. The results are intended to emphasize why optimization studies are needed and that focusing only on a specific response, such as particle size, can be detrimental towards the overall purpose (i.e., optimizing particle size in this system would lead to a reduction in powder yield and viral vector activity).

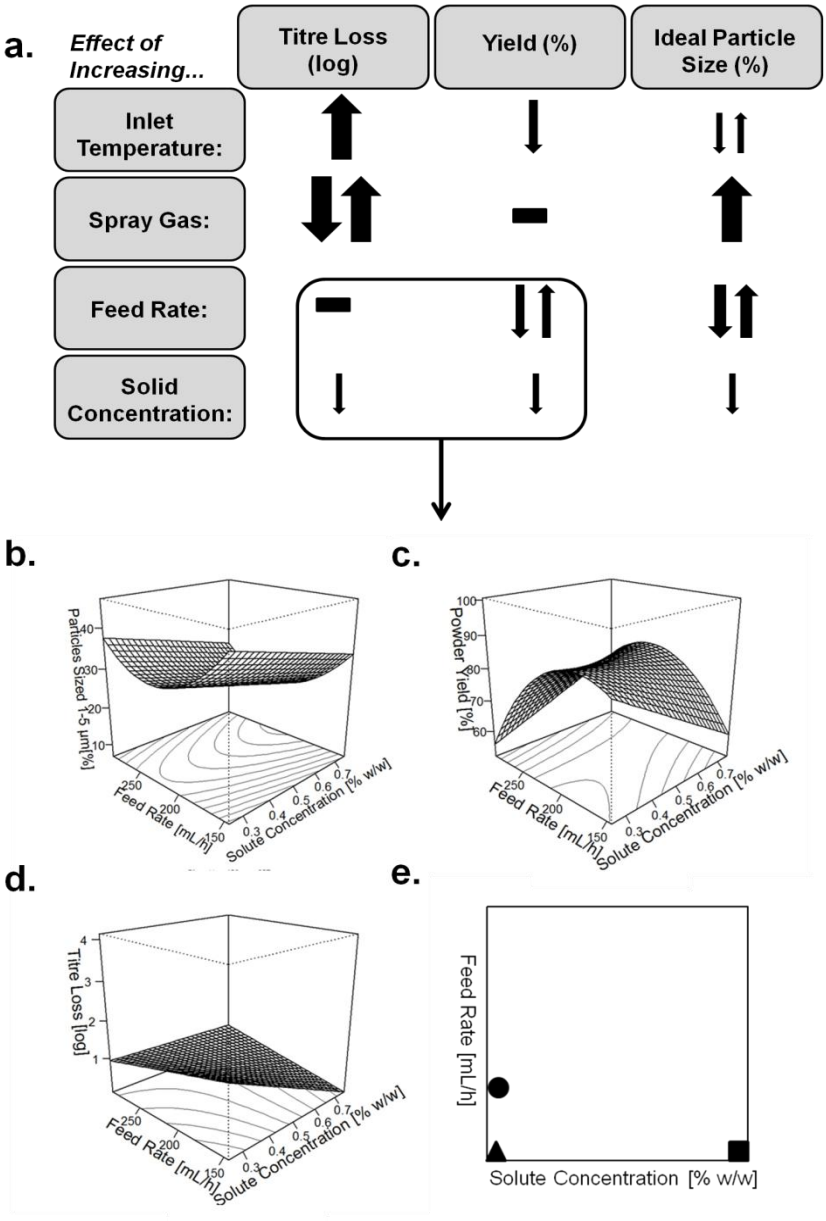

Figure 4.8. a) Effect of input parameters (inlet temperature, spray gas flow, feed rate, matrix solid concentration) on the viral vector titre loss (log), powder yield (%) and the percentage of ideally sized particles (%). The magnitude of the effect is demonstrated by the thickness of the arrow. Below are plots demonstrating differences between b) particles sized 1-5 µm, c) powder yield and d) titre loss. e) This is highlighted in the two dimensional plot, showing optima for particles sized 1-5 µm (▲), powder yield (●) and titre loss (■).

4.5. Conclusions

The aim of this work is to provide “process rules” for processes attempting to spray dry with labile materials. The work presented here models the influence of spray drying parameters on viral vector activity, powder yield and particle size. Overall, this work highlights the importance of these spray drying parameters and allows for navigation within the design space of each model in order to better optimize current and future processes. The statistical models constructed suggest that the spray drying process can be adjusted to account for control over each of the three measured responses, however it also emphasizes the competing nature in optimizing for any particular response. Optimal output of one specific response can lead to significant loss in the other responses. Overall, the results presented can be applied to current spray drying processes, both laboratory- and industry-scale, to achieve desirable particle properties for all three responses. While new processes may use different excipients with a different labile material than what was used here, the trends and interactions remain constant, proving to be a valuable asset for future studies.

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Chapter 5: Concluding Remarks and Recommendations

The thermal stabilization of labile biomolecules, such as AdHu5, is an active area of research due to global need. Proper thermal stabilization will offer enhanced long term storage, and improve the ability to ship vaccines to remote areas of the world. Spray drying is particularly advantageous in producing thermally stable vaccines over other methods due to its low processing costs and high process tunability for altering the properties of a final product. For this thesis, we have presented a spray dried formulation offering a high degree of thermal stability for AdHu5 LacZ. This formulation was optimized for spray dryer processing in terms of powder yield, particle size, and retained viral vector activity.

In the formulation development section (Chapter 3), we have presented a thermally stable spray dried formulation for AdHu5 based on a mixture of sugars or on an amino acid. All of the selected stabilizing excipients showed no significant cytotoxicity with regards to AdHu5. The mannitol/dextran formulation was the most successful based on its ability to retain good viral vector activity (<1 log loss) for up to 90 days at 20°C. Furthermore, the formulation outperformed the liquid control at all stages of testing, particularly at elevated temperatures of 37°C, 45°C and 55°C. It was notable that with an ambient moisture above a relative humidity of 10% that the mannitol/dextran formulation performed almost as poorly in terms of thermal stabilization as the liquid control. However, overall the findings pointed to the potential for exceptional thermal stabilization with this sugar mixture assuming dry conditions.

In the optimization section (Chapter 4), we presented our findings on the effects of several spray drying process parameters on the viral vector activity, powder yield, and

production of particles of desirable size with the mannitol/dextran formulation. Response surface measurements based on adjusting the inlet temperature, spray gas flow rate, liquid feed flow rate, and the solute concentration in the feed, showed the possibilities of powder yields of ~90%, a percentage of ideal sized particles at ~50%, and a minimal viral vector titre loss of approximately 0.25 log loss. Coupled with the work in Chapter 3, the combined findings allow for the production in high yields of particles with a desired size and maintained activity of AdHu5 at elevated temperatures.

Future work within this field of research should aim to produce particles that are better stabilized when ambient moisture is present as the particles presented here are very moisture-sensitive. A potential solution to this challenge is the development of core-shell particles, where the core consists of a stabilizing environment for the viral vector, and the shell consists of a material resistant to environmental factors. Additional work useful within this field would be developing a spray dried particle to co-deliver pharmaceutical drugs with the labile biomolecule. Often times pharmaceutical drugs can offer benefits to the active ingredient, such as the use of adjuvants with non-immunogenic vaccines. The combination of both within a spray dried particle would make for simpler processing and administration of these systems in conjunction with the benefits for thermal stabilization observed here. Additionally, it would be advantageous to further test the current formulation within *in vivo* studies, to ensure the benefits observed translate over for animal models. Lastly, changing the biomolecule stabilized within the excipient matrix would be worth testing. Stabilization of other adenovirus types, such as AdCh68 (chimpanzee adenovirus type 68), are potentially more efficacious as viral vectors due to

the prevalence of neutralizing antibodies against human type adenovirus within the population.