

Thermal stability of spray dried vaccine powders encapsulating enveloped and non-enveloped viral vectors

By: Steven Toniolo
McMaster University, toniolsp@mcmaster.ca

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Lay Abstract

Most vaccines lose their activity when stored at room temperature and therefore are required to be stored at temperatures between 4 °C and -80°C to maintain vaccine potency. The refrigeration required to meet these temperatures is costly and limits the distribution of vaccines to resource poor areas in the world where refrigeration technology is uncommon. Spray drying, a process that rapidly dries a solution to form a dry powder, was used to trap vaccines within a sugar matrix to protect the vaccine from heat and structural changes that would otherwise deactivate it. The spray dried powders demonstrated higher thermal stability than liquid samples, which decreases the need for refrigeration during transportation and storage. Thermal stability trends for spray dried powders produced with various sugars and categories of viruses are presented.

Abstract

This thesis work aims to improve the thermal stability of vesicular stomatitis virus (VSV) through spray drying and investigates differences in thermal stability in a matrix between enveloped and non-enveloped viral vectors. The spray drying process was used to dry and encapsulate the VSV vector within the glassy amorphous phase of a matrix of carbohydrate excipients, which imparted increased thermal stability. Viral activity was maintained when the powders were stored for 30 days at 37 °C, in contrast to the liquid control that lost all activity after 15 days. The best excipients for enhancing thermal stability of VSV were trehalose and a 3:1 blend of trehalose and dextran. Immunogenic response from the spray dried trehalose-VSV particles was detected through an *in vivo* study with Female BALB/c mice after storing the vaccine for 15 days at 37 °C.

Two enveloped viral vectors, VSV and influenza, and a non-enveloped viral vector, human type 5 adenoviral vector (AdHu5) were spray dried with the same formulations to observe how excipients enhance thermal stability and encapsulate the different groups of viruses. The thermal stability of both enveloped viral vectors was enhanced the most when spray dried with trehalose or a 3:1 trehalose/dextran blend, and exhibited the greatest activity loss when spray dried with a mannitol/dextran blend, determined by *in vitro* TCID₅₀ assays measuring GFP expression. Conversely, the best performing excipient formulation for the non-enveloped viral vector was a mannitol/dextran blend. This led to the hypothesis that the encapsulation mechanism differs between the two groups of viruses. The glass transition temperature (T_g) of the spray dried formulations (without virus) stored at 37 °C for 10 days was measured to infer the potential molecular mobility of the viral vector within the primarily amorphous matrix. Formulations containing dextran exhibited the smallest depression in T_g after storage, indicating minimal increase in molecular mobility over time. RNA leakage from aged spray dried powders containing VSV was quantified to investigate the encapsulation mechanism of enveloped viral vectors and followed a similar trend to the *in vitro* activity tests. VSV with poor performing excipients yielded the least detectable RNA/pfu after three days of storage at 45°C, suggesting that the lipid envelopes ruptured and released viral RNA which denatured during storage. This work demonstrates that the VSV vector can be thermally stabilized through spray drying but highlights that different carbohydrates interact differently with enveloped versus non-enveloped viral vectors, providing a guideline for future work with the advent of new vaccines.

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Nomenclature

AdHu5	human type 5 adenovirus
CTC	controlled temperature chain
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
FDA	US Food and Drug Administration
GFP	green fluorescent protein
MEM	minimum essential medium
PBS	phosphate buffered saline
RNA	ribonucleic acid
SEM	scanning electron microscopy
T_g	glass transition temperature
T_m	phase transition temperature
VSV	vesicular stomatitis virus
WHO	World Health Organization

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 and Dr. Emily D. Cranston.

Chapter 1: Introduction

1.1 Vitrification of Vesicular Stomatitis Virus Vaccines through Spray Drying and Thermal Stability Comparison Between Enveloped and Non-Enveloped Viral Vectors

The field of virology faces challenges around the development and distribution of vaccines globally, due to limitations surrounding the storage and transportation of vaccines. Cold chain requirements make it necessary to keep vaccines stored and transported at a specified temperature in order to maintain the activity and stability of the vaccine vector; this storage temperature can be as low as $-80\text{ }^{\circ}\text{C}$ or as high as $8\text{ }^{\circ}\text{C}$ depending on the antigen [1]. This requirement impedes the bulk stockpiling of vaccines, particularly in underdeveloped countries that lack the technology and resources to maintain these cold temperatures for vaccine stability. Recognizing this limitation and the associated large energy costs of refrigeration, the World Health Organization has actively promoted the development of thermally stable vaccines which would minimize the need for cold chain storage [2].

Recent research to alleviate the demand for cold chain requirements explores the production of thermally stable biologics through vitrification via rapid drying processes. While lyophilisation is commonly used within industry, the limitations on throughput and batch time [3] have resulted in a shift towards spray drying of vaccines as a tunable, low cost and easily scalable process [4]. Numerous vaccines have been spray dried resulting in enhanced thermal stability including vaccines for measles [5], tuberculosis [6] and influenza [7]; however, this is the first work that investigates the spray drying of a formulation with vesicular stomatitis virus (VSV). Moreover, there is a fundamental absence of published work examining the differences in thermal stability of spray dried enveloped and non-enveloped viral vectors.

1.2 Research Objectives

The goal of this thesis is to enhance the thermal stability of spray dried VSV with a variety of sugar-based excipients and characterize differences in thermal stability between spray dried enveloped and non-enveloped viral vectors using the same sugar formulations. These differences were highlighted using three viral vectors: two enveloped viruses: VSV expressing green fluorescent protein (VSVGFP), and influenza mNeon, and a non-enveloped virus: human type 5 adenovirus expressing green fluorescent protein (AdHu5GFP), which all elicit a response that can be measured *in vitro* by fluorescence microscopy with a cell imaging system. The work is separated into two studies:

Thermal Stabilization of VSV:

This study aims to develop a formulation to thermally stabilize VSV vectors through spray drying and evaluate the immunogenicity of spray dried excipients with a VSV vector *in vivo*. We demonstrate that a spray dried powder of VSV in trehalose and trehalose/dextran will retain activity for 30 days when stored at 37 °C and exceeded the liquid control during the *in vitro* study.

Stabilization of Enveloped versus Non-Enveloped Viral Vectors:

This study aims to distinguish trends in activity losses over time between two sets of spray dried enveloped viruses compared to a non-enveloped virus spray dried with the same formulations. The work examined the role of selected sugars in stabilizing the lipid membrane of the enveloped viral vector to prevent leakage of viral genetic material and the significance of the glass transition temperature (T_g) in the thermal stabilization of all types of viruses, to ultimately provide a guideline for selecting excipients to spray dry thermally stable enveloped or non-enveloped viral vectors.

1.3 Thesis Outline

This thesis is divided into five chapters, including this chapter. Chapter 2 is a literature review of published work on the underlying mechanisms of vitrification, stabilization of lipid membranes, differences in virus structure between enveloped and non-enveloped viruses, and the process of spray drying and its use to enhance the thermal stability of viruses. In Chapter 3, the thermal stability of spray dried VSV with various excipients and its immunogenicity *in vivo* is studied. Chapter 4 examines the differences in thermal stability of enveloped and non-enveloped viruses spray dried with various excipients and highlights property parameters to consider for improved thermal stability. Lastly, Chapter 5 presents the conclusions of the thesis and future routes to further examine the findings discussed in the thesis.

Chapter 2: Literature Review

2.1 Vitrification

Vitrification is a promising approach for the long-term protection and storage of biologics that leads to minimal loss of biological activity. The principle of vitrification is based on using a primarily amorphous or glassy matrix to entrap biologics on the molecular level offering protection from structural changes due to denaturation or aggregation over time [8]. The current standard for preservation of biologic materials involves freezing an aqueous suspension at temperatures close to $-80\text{ }^{\circ}\text{C}$ and guaranteeing constant maintenance of similar storage conditions during transportation to minimize activity loss [9]. However, maintaining the storage and transportation requirements of vaccines (known as cold chain requirements) can be costly and difficult, especially in underdeveloped countries, which have led researchers to explore more thermally stable vitrification methods such as lyophilisation and spray drying [4].

In nature, numerous examples of vitrification occur that exploit the presence of sugars to illicit a state of anhydrobiosis, where an organism reaches a state of near dehydration and maintains a reduced metabolic function [10]. Upon rehydration, these anhydrobiotic organisms will resume an active metabolism and avoid irreversible damage that would normally occur upon dehydration. Anhydrobiotic organisms such as brine shrimps cysts [11] and several species of nematodes [12] have been found to contain high concentrations (up to 20% dry weight) of trehalose. The role of trehalose and sugar molecules in stabilizing dry membranes and phospholipid bilayers in nature has been extensively studied [13] and the understanding of these principles have led to the wide spread use of sugar molecules to artificially thermally stabilize biologics through rapid drying to form a primarily amorphous matrix.

The amorphous phase of the matrix that is formed during vitrification is randomly ordered allowing for many stable structural conformations that entrap the biologic preventing protein unfolding, aggregation and structural changes [14]. The formation of the solid amorphous phase can hinder the molecular movements of entrapped biologics, leading to a reduction in activity loss during room temperature storage, especially with materials exhibiting high glass transition temperatures [15]. The glass transition temperature of a material is often used as a governing property in vitrification and is described as the temperature at which a glassy material will undergo a transition into a rubbery state when heated [16]. When a material is supercooled from a temperature above the glass transition temperature, the material will undergo vitrification and form a primarily amorphous matrix [16]. At temperatures below the glass transition temperature, materials will still experience translational molecular movements, but the movements occur less frequently and decreases in frequency with decreasing temperature [14]. Physical stability of the biologic is attained when the storage temperature of the entrapped biologic is below the Kauzmann Temperature, which is approximately 50 K below the glass transition temperature [17]. At the Kauzmann temperature, the energy in the system is low enough that the molecular mobility of an amorphous matrix is negligible [14], [18]. While vitrification is favoured to stabilize biologics in the dry state, a direct interaction via hydrogen bonding with the biologic is additionally required to maintain function upon dehydration and subsequent rehydration. Thus, a

primarily amorphous material that is able to form direct interactions with the biologic and possesses a high T_g is ideal to retain biological activity through vitrification.

2.2 Stabilization of Lipid Membranes

Lipid membranes are found throughout living organisms and are integral in cellular metabolic function. Due to the miniscule size of the lipid cell membrane (7.5 – 10 nm thick), direct visual observation of the phospholipid membrane is difficult [19]. However, experimental evidence through labelling, x-ray diffraction and calorimetry led to the widely accepted fluid mosaic model to describe the behaviour and structural properties of the phospholipid cell membranes [19].

The fluid mosaic model defines the lipid membrane as a thin bilayer composed of amphiphilic fatty acids arranged in sheet-like structures with the hydrophilic heads facing outwards and the hydrophobic tails directed inwards as illustrated in Figure 1.

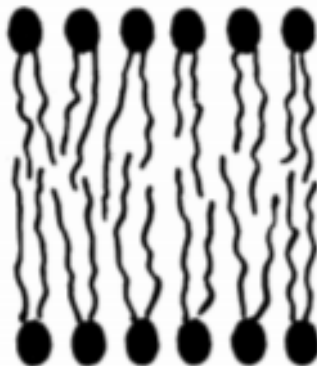


Figure 1: A phospholipid bilayer: schematic cross-sectional view. The filled circles represent the ionic and polar head groups of the phospholipid molecules, which make contact with water; the wavy lines represent the fatty acid chains. Reproduced from [19].

Embedded within the lipid membrane are integral proteins that can either transverse the lipid bilayer or peripheral proteins that extend exclusively from the inner or outer surfaces of the membrane [19]. These proteins are critical to maintain cell homeostasis through ion and molecule exchange across the cell membrane and act as cell signaling receptors to the extracellular matrix [19]. The phospholipids that comprise the lipid membrane are constantly moving past one another leading to the lipid membrane often being characterized as fluid-like [19]. This fluid-like behaviour allows for the free movement of proteins along the surfaces of the lipid membranes as well as permits the absorption of larger biomolecules into the cell via endocytosis. The fluidity of the lipid membrane is entropically driven by the presence of water

surrounding the polar phosphate heads of the membrane phospholipids [20]. There are typically 10-12 water molecules that are hydrogen-bonded around each phosphate head in a phosphatidyl choline based lipid membranes [21]. However, upon dehydration, the removal of water causes an increased van der Waals attraction between the hydrocarbon tails of the phospholipids which results in fusion of the bilayer and increased rigidity (Figure 2) [19, 21].

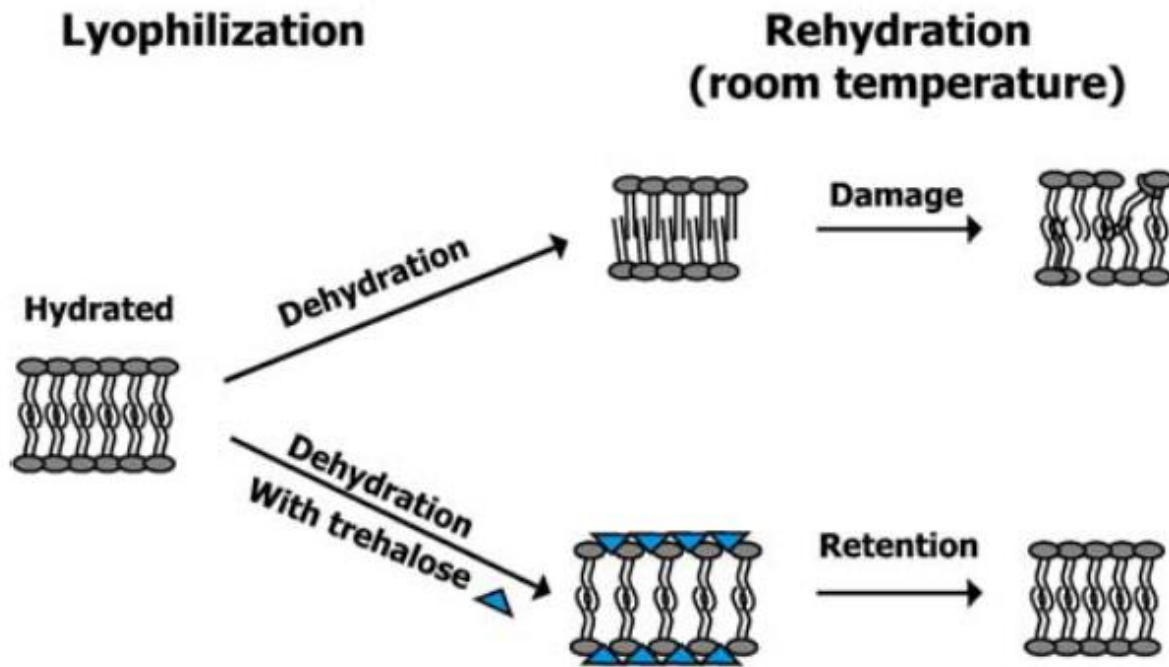


Figure 2: Water replacement hypothesis for lipid bilayers. The diagram shows how trehalose is thought to stabilize dry lipid bilayers. Reproduced from [22].

The loss of fluidity in the lipid bilayer leads to leakage of the internal cellular components and reduced function upon rehydration as the bilayer locally transitions from a gel state back to liquid-crystalline [21]. Alternatively, the lipid membrane will reach a gel state if the temperature of the lipid membrane is decreased below its phase transition temperature (T_m), the temperature at which the bilayer will transition from a fluid state to a gel state [20]. It has been observed that the dehydration of lipid membranes increases the phase transition temperature, leading to the transformation of the lipid membrane from a liquid-crystalline state to a gel state at physiological temperatures. As such, the T_m is often used to identify alterations in the behaviour of a lipid membrane in the dry state.

To mitigate the increase in phase transition temperature and the rigidity observed during dehydration, sugar molecules have been used to stabilize the lipid and protein surface by replacing the hydrogen bonds formed between the water and phospholipid surface [13]. The

water replacement hypothesis states that the stabilization of phospholipid bilayers is due to the interactions between -OH groups on polyhydroxy sugars and the phosphate head of the membrane [21],[23]. For example, the T_m for POPC (1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocoline) in the hydrated state is $-3\text{ }^{\circ}\text{C}$ and when dried without the presence of sugar the T_m of the lipid increases to $61\text{ }^{\circ}\text{C}$ [24]. When the POPC was dried with the addition of sugar, the phase transition temperature did not exceed $6\text{ }^{\circ}\text{C}$ [24]. It was also discovered by Crowe et al. that liposomes dried with trehalose and rehydrated, retained as much as 100% of their original contents, whereas those dried without a stabilizing sugar leaked their contents upon drying and rehydration [21]. Thus, water replacement with -OH based sugar molecules must be considered in conjunction with vitrification to successfully achieve thermal stabilization of biologics.

2.3 Virus Structure and Stability of Adenoviral, VSV and Influenza

Vectors

Generally, viral vectors can be structurally characterized into two categories: enveloped and non-enveloped. The genetic material of enveloped viruses is encased in a protective protein capsid, covered with an lipid bilayer envelope containing viral proteins that assist the virus in entering a host cell [25], whereas non-enveloped viruses lack a lipid bilayer as shown in Figure 3.

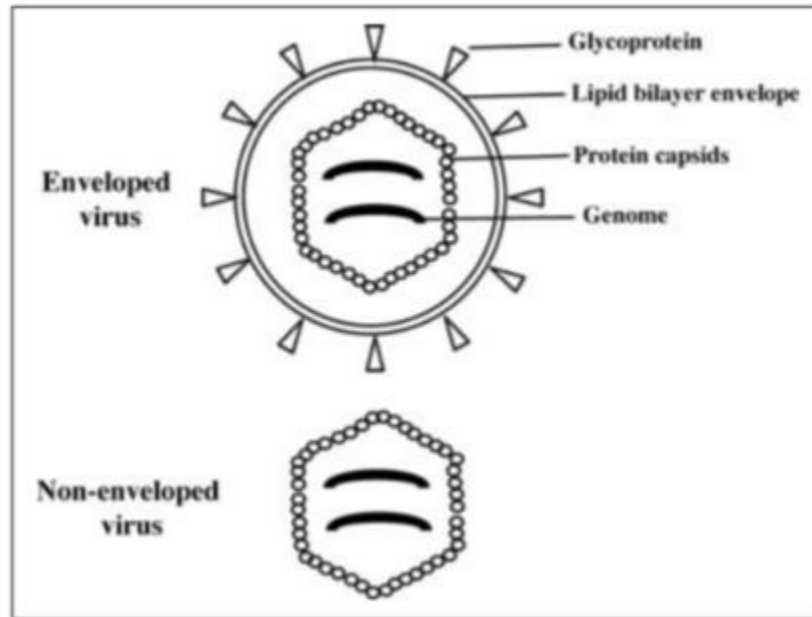


Figure 3: Graphic structures of a typical enveloped virus and non-enveloped virus. Reproduced from [26].

The lipid envelope is sensitive to desiccation and heat, resulting in the enveloped virus being more susceptible to damage outside of a host cell [27]. Protruding from the lipid envelope

are glycoproteins that assist the virus to identify and bind to receptor sites of the host membrane [27][28]. Once the virus attaches to the host cell's membranes, the lipid bilayer of the virus fuses with the lipid membrane of the host cell allowing the capsid and viral genome to enter and infect the host as shown in Figure 4 [29]. At which point, the viral genetic material is incorporated within the host cell's DNA and begins the process of replicating the viral genetic material within the host.

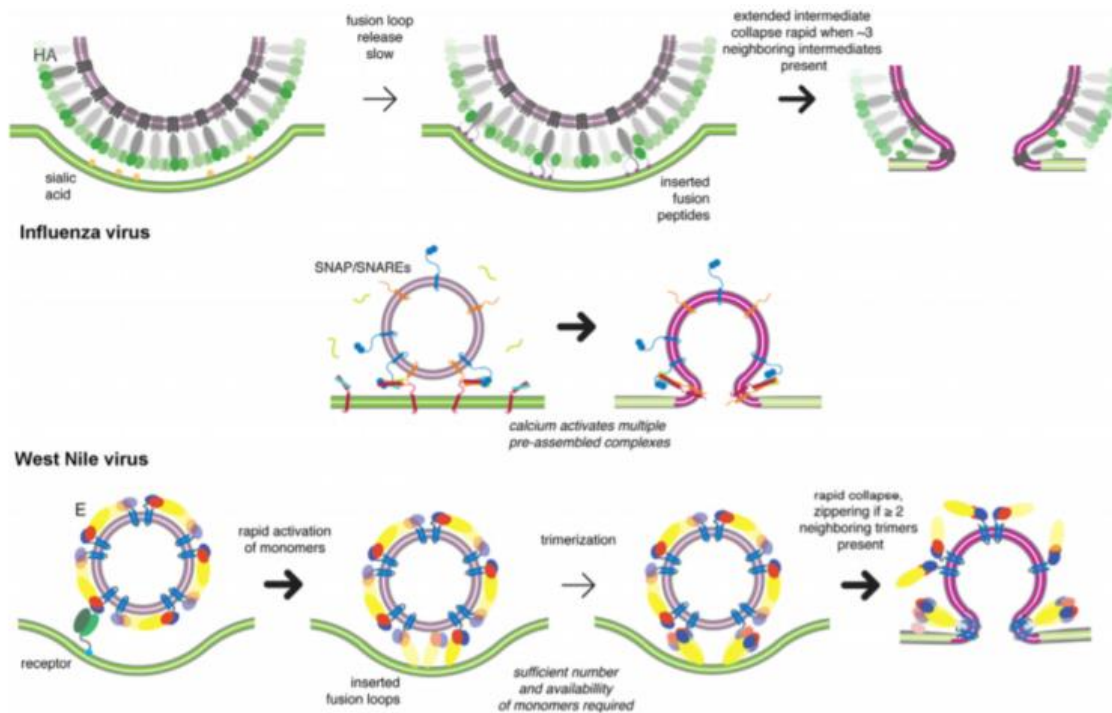


Figure 4: Comparison of fusion as catalyzed by influenza virus HA, SNAREs, flavivirus E protein. Reproduced from [29].

Non-enveloped viruses such as adenovirus, lack a lipid bilayer surrounding the protein capsid, and as such the virus is more stable and robust but follows a different infection pathway than enveloped viruses [27]. Initially, non-enveloped viruses follow a similar infection pathway relying on viral attachment to the host cell through viral fibres. However, due to the lack of a lipid envelope surrounding the protein capsid, the virus does not fuse with the host cell but rather undergoes endocytosis and is engulfed into the cell [30]. Once the viral particle is taken inside the host cell, the capsid shell is broken down by the cellular body and the genetic material either is released into the cell where it will be incorporated with the host cell's DNA [30] or the virus genome is transcribed and replicated by the host cell [31].

Along the infection pathway, many factors could lead to an unsuccessful infection and ultimately a loss of viral activity. If the infection pathway is disrupted, a vaccine is unable to elicit a host response and the vaccination process is ineffective. Viruses require intact viral fibres to bind to the host cells, and when exposed to hydrophobic surfaces or high temperatures

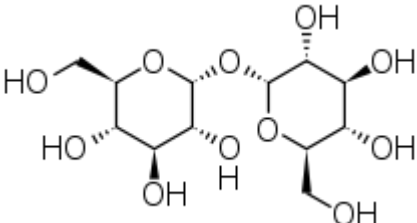
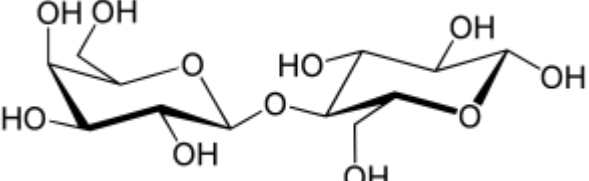
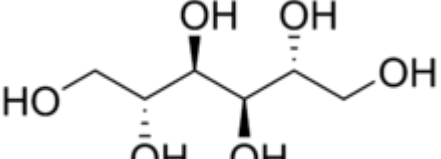
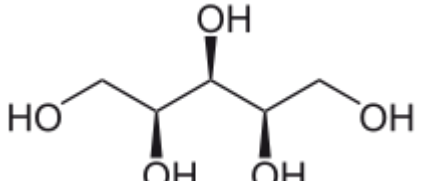
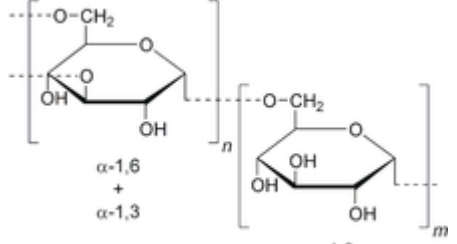
can lead to denaturing of the proteins as well as changes in the fibre structure leading to reduced infectivity [32]. Additionally, to ensure fusion with the host cell, the lipid bilayer of the enveloped virus must remain flexible and fluid-like [29]. Dehydration of the virus lipid bilayer can lead to fusion of the individual phospholipid heads of the lipid bilayer, creating a rigid viral lipid membrane [21]. The increased rigidity of the viral lipid envelope prevents fusion with the membrane of the host cell, inhibiting entry of the virus. With the increase in rigidity of the lipid membrane, leakage of the internal genetic material will occur, leading to inactivity of the virus and an overall lower infectivity [20].

2.4 Excipient Selection

The addition of excipients to stabilize biologics for prolonged storage in the dry or liquid state must adhere to a set of criteria to ensure that the material is safe for human administration while maintaining the desired function of improved stability. The goal of developing these formulations is to ultimately administer the products to humans, and as such the safety profiles and cytotoxicity of the materials must be thoroughly understood and approved by the US Food and Drug Administration (FDA) and Health Canada. Due to cytotoxicity standards, materials that are derived from animal-based molecules or materials that could disrupt normal biological function such as surfactants are undesirable [33].

Selected excipients must also improve upon the thermal stability of the biologic when in the dry state. Considering the *water replacement hypothesis* mentioned earlier, selected excipients should possess the ability to adequately replace the hydrogen bonds between water and the biologic to prevent unfolding of the protein components. From previous work [34], sugar-based molecules containing numerous hydroxyl groups (-OH) have been identified as exceptional candidates in replacing the hydrogen bonds formed between the displaced water molecules and the protein components of biologics. Amino acids, are another type of excipient that are able to form hydrogen bonds through their amine functional groups. The main classes of sugars are: reducing sugars, non-reducing sugars, sugar alcohols, polysaccharides and complex carbohydrates. Examples of sugars pertinent to this work are shown in Table 1.

Table 1: Chemical Structures of Various Sugar-Based Excipients

Excipient	Chemical Structure
Trehalose (Non-reducing Sugar)	
Lactose (Reducing Sugar)	
Mannitol (Sugar Alcohol)	
Xylitol (Sugar Alcohol)	
Dextran (Polysaccharide)	

These carbohydrates have been utilized significantly as stabilizers in the food industry [35] and can thus be deemed as appropriate stabilizing excipients for pharmaceutical applications depending on the target site for administration [34, 35].

2.5 Spray Drying

The process of spray drying utilizes high temperatures and pressurized gas to aerosolize a liquid feed into mini droplets which rapidly evaporate into a dry powder (Figure 5).

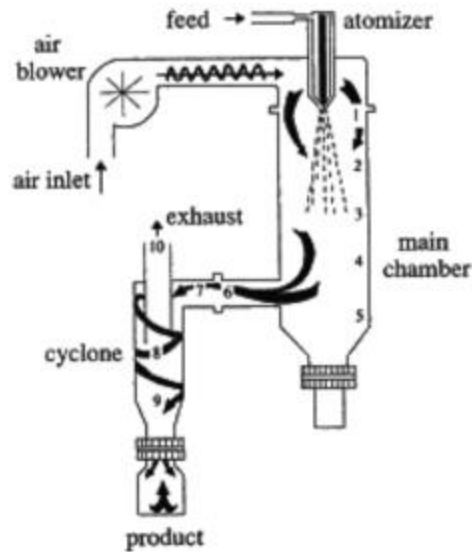


Figure 5: Diagram of a typical bench-top spray dryer. Reproduced from [38].

The latent heat of vaporization that occurs during the evaporation process, maintains a low overall temperature of the drying particle compared to the surrounding hot air, reducing thermal stress on the particle [4]. Lyophilisation as a drying method is well established within industry, however the batch process can be time consuming require days to complete [3]. Lyophilisation can also result in decreases of biologic activity during the freezing and drying process, and further processing via milling is required to break down the dried material into granules [4]. The unique ability to tune the production of fine dry particles affords spray drying a significant advantage over conventional drying methods such as lyophilisation and vacuum drying. The spray drying process, unlike lyophilisation and milling, can control the morphology of the produced particles which allows for the possibility to produce inhalable vaccines [34]. By adjusting the spray drying temperature, pressurized gas flow rate, the solute concentration within the liquid feed and the liquid feed rate into the nozzle; the particle size, degree of crystallinity and density can be controlled. The Peclet number (Pe_i) is often used to characterize the drying behaviour of the particle and describes the relationship between the evaporation rate (κ) and the diffusivity of the solutes within the drying droplet (D_i) [39].

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

A low Peclet number (< 1) indicates the diffusion of the components within the droplet is much faster relative to the evaporation rate, which will produce solid uniform particles. A high Peclet number (> 1) will lead to a greater probability of producing a hollow particle with high surface enrichment of the drying component (E_i). The Peclet number can also be used to approximate the surface enrichment or the concentration of a component ($c_{s,i}$) relative to the mean droplet concentration ($c_{m,i}$).

$$E_i = \frac{c_{s,i}}{c_{m,i}} = 1 + \frac{Pe_i}{5} + \frac{Pe_i^2}{100} - \frac{Pe_i^3}{4000}$$

As the water is evaporated, the solute concentration increases until the solute precipitates into a dry particle, often designated as the saturation time (τ_{sat}) [40]. The remaining time between saturation and complete drying of the droplet is characterized as the precipitation time (τ_p) or the time available for the drying component to crystallize [40]. Components which crystallize during the precipitation window will experience a significant increase in the Peclet number and form a crystalline shell with a hollow core.

In addition to tunability, the spray drying method is easily scalable for commercial applications with large production demands. As such, spray drying has been utilized as a reliable tool for numerous decades in the food industry to encapsulate and retain food flavours in the dry powder form [41]. It is because of this success, that spray drying has recently been explored as a viable method to preserve pharmaceuticals and vaccines through the vitrification and stabilization of biologics [42].

2.5.1 Thermally Stable Dry Powder Vaccines Prepared by Spray Drying

Spray drying of vaccines to achieve thermal stabilization is a relatively new concept, yet a few literature examples exist that have demonstrated the ability of spray dried vaccines to outperform liquid controls when stored at higher temperature. Ohtake et al. generated a heat-stable measles vaccine via spray drying using a mixture of trehalose, sucrose, L-arginine, human serum albumin and glycerol in potassium phosphate buffer that resulted in a titre log loss of 1 log after 8 weeks stored at 37 °C [43]. A hepatitis B vaccine with improved thermal stability has also been demonstrated through spray drying with a mixture of trehalose and sodium hydrogen phosphate [44]. A comparison between spray drying and freeze drying of an Influenza vaccine yielded a titre log loss rate per week of 0.72 and 0.66 respectively when stored at 37 °C for 8 weeks. Furthermore, the use of sugars other than trehalose have been shown to stabilize a recombinant human type 5 adenoviral vector as LeClair et al. were able to achieve a log loss of <1 log after 90 days of storage at 20 °C when spray drying the adenoviral vector with a mannitol/dextran formulation [34]. While some examples of successful thermal stabilization of vaccines via spray drying exist within academia, the advent of new vaccines will require further exploration and testing.

Chapter 3: Accelerated Storage Study Investigating the Thermal Stability of Spray Dried VSV-Vectored Vaccines and Immunogenicity *In Vivo*

3.1 Introduction

Vesicular stomatitis virus (VSV) is a single stranded RNA enveloped virus that has been explored as an oncolytic viral platform to target cancer cells [45], a potential vaccine for tuberculosis [46], and more recently has been developed into an experimental vaccine for protection against the latest Ebola virus outbreak [47]. The VSV platform was found to be advantageous for use during an outbreak due to the shorter time required to induce the full production of protective antibodies through humoral immunity [48], as well as the relatively low innate immunity in humans [47].

VSV vectors stored in aqueous media, like all vaccines, must be refrigerated to maintain their efficacy over time [1]. Due to this requirement, cold chain protocols are in place to maintain refrigeration during transportation and storage for liquid vaccines, which reduces the possibility to stockpile large quantities of vaccines and also limits distribution to resource-poor areas during an outbreak, such as Southeast Asia and Africa [49]. The World Health Organization (WHO) has advocated for a controlled temperature chain (CTC) which permits vaccines to be exposed for short periods of time outside of the cold chain temperature, exceeding 2-8 °C [2]. In order to obtain CTC approval, vaccines must endure temperatures in excess of 40 °C for a minimum of 3 days [2]. To mitigate the need for cold chain storage of liquid vaccines, research has been directed to develop thermally stable vaccines through the vitrification of excipients into glassy powders [50]. Efforts to vitrify adenoviral-based and influenza viral vectors with sugar-based molecules have been successful and thermal stability for prolonged periods while stored in the dry state have been demonstrated [7], [34]. This work represents the first study of stabilizing VSV following similar methods.

Within industry, lyophilisation has often been selected as the preferred method of drying biologics [51]. However, lyophilisation has its deficiencies, as many biologics are susceptible to the stresses that occur during the freezing and drying processes [4], and the batch process can take days or weeks to complete, limiting throughput [3]. A promising approach that has already demonstrated the ability to produce dry vaccines, while maintain efficacy, is spray drying [1]. Briefly, spray drying uses pressurized gas to aerosolize a liquid feed into a chamber containing heated air. The mini liquid droplets rapidly evaporate, to form a dry powder where it is collected in a cyclone chamber. The ability to tune characteristics of the dry particles such as the particle size, degree of crystallinity and density, as well as the scalability of the process and short batch time have made spray drying an attractive alternative to lyophilization. The spray drying of dry

powder vaccines against measles [5], tuberculosis [6], hepatitis B [44] and influenza [7] have been explored and established with thermal stability being achieved in some cases [34].

While there has been some notable success in achieving thermal stability of influenza and adenoviral-based vectors, it cannot be assumed that all vaccines will behave similarly under vitrification. There is an inherent need to address the underlying phenomenon that not all excipients encapsulate the various families of viruses similarly. Thus, the objective of our study was to investigate the effects of various excipients on the thermal stability of a spray dried VSV viral vector *in vitro* and how its immunogenicity translates *in vivo*. Through spray drying of the VSV vector expressing green fluorescent protein (VSVGFP) we have identified which of the explored excipients minimize the initial viral activity loss that occurs due to the spray drying process. Additionally, the spray dried powders encapsulating VSV were subjected to accelerated storage conditions to investigate which excipients afforded the best thermal stability to the viral vector. To address any possible defects to the viral structure that may hinder *in vivo* immunogenicity, a spray dried VSVAg85A vector was similarly aged and tested on mice. The goal of this work was to highlight which excipients perform best with the VSV vector (without optimization), such that they minimize activity loss during the spray drying process and maximize thermal stability over long-term storage, reducing the dependency on cold chain storage.

3.2 Materials and Methods

3.2.1 Chemicals and VSV Vectors

D-(+)-trehalose dihydrate, D-Mannitol, Dextran (M_r 40,000 kDa), anhydrous Lactose, Xylitol were purchased from Sigma-Aldrich (Ontario, Canada). Culture media was prepared from Alpha Minimum Essential Medium Eagle (α -MEM) (in house according to protocol by the supplier, Life Technologies; Ontario, Canada) with 10% fetal bovine serum and 1% streptomycin/penicillin (Invitrogen; Ontario, Canada). Recombinant replication-deficient Vesicular Stomatitis Virus-vectored vaccines (VSVAg85A and VSVGFP) were produced in the vector facility of McMaster Immunology Research Centre as described previously [46].

3.2.2 Spray Drying of VSV-Vectored Vaccines

Spray dried powder vaccines were produced by spray drying using a Mini Spray Dryer B-290 (Büchi; Switzerland) with 0.7 mm spray nozzle and high performance cyclone attachments as previously described [34]. For all spray dried excipients, the spray dryer was operated at a spray gas flow rate of 439 L/h, feed solution of 234 mL/h and a nozzle inlet temperature of 110 °C. The spray dried formulations were selected through preliminary testing not shown and previous work [34].

3.2.3 Vaccine Sample Storage

Powder samples were stored individually in closed 2 mL Nalgene General Long-Term Storage Cryogenic Tubes (Nalgene; Ontario, Canada) sealed with Parafilm Wax (Bemis NA; Wisconsin, US). The tubes were placed in a resealable plastic bag and stored within a glass jar

filled with gel desiccant and sealed with Parafilm Wax to ensure a low humidity environment (<10% RH). The glass jars were sealed within another resealable plastic bag and placed in a water bath for temperature control. The liquid control containing only PBS buffer and VSV were stored in a plastic resealable bag within a tightly sealed glass jar without gel desiccant.

3.2.4 *In Vitro* Activity Testing of Spray Dried VSV

3.2.4.1 Culturing of Vero Cells

Vero cells isolated from kidney epithelial cells were thawed from liquid nitrogen and cultured with Alpha Minimum Essential Medium Eagle (α -MEM) in T150 culture flasks. Cell culturing was completed in a humidified Forma Series II Water Jacketed CO₂ Incubator (Thermo Scientific Corporation; Waltham, MA) at 37°C and 5.0% CO₂. When cells appeared to be 80-90% confluent, they were split into a new T150 culture flask and plated in a 96-well plate for *in vitro* testing.

3.2.4.2 Spray Dried Viral Infectivity

The viral activity after the spray drying process and subsequent storage was determined by an endpoint dilution, infecting plated Vero cells with 4 mg of powder (initial concentration of 1.3×10^9 pfu/g) reconstituted in culture media. After the overnight incubation of plated Vero cells, the cell media was removed and replaced with an eight-fold serial dilution from each reconstituted sample at a volume of 100 μ L per well and a total of 4 wells per sample. After overnight incubation, viral infectivity was detected by the presence of Green Fluorescent Protein (GFP) expression indicated by a bright green hue to cells, observed under an EVOS FL Cell Imaging System with a GFP filter (Thermo Scientific Corporation; Waltham, MA). A positive GFP response of a single cell within a well constituted a positive infection response with respect to the endpoint dilution. If greater than 50% of the wells in the row were non-expressive, the dilution was determined to have reached its endpoint and the median tissue culture infections dose (TCID₅₀) was calculated using the Reed-Muench method [52]. Using the Poisson distribution, the results were multiplied by a factor of 0.69 to convert to an approximated pfu value and reported as a viral activity loss of pfu/g of material with error bars calculated as the standard deviation (n =3).

3.2.5 *In Vivo* Evaluation of Immunogenicity of Spray Dried Vaccines

Female BALB/c mice, 6-8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a specific pathogen-free level B facility at McMaster University. All experiments were completed in accordance with the guidelines from the Animal Research and Ethics Board at McMaster University. Immunization of the mice was carried out via the intramuscular route through delivery of the vaccine into the hind legs of the animal. Tested vaccine samples were either fresh liquid control (1×10^7 pfu), stored liquid control (initial concentration of 1×10^7 pfu stored for 7 or 15 days at 37 °C, or reconstituted spray dried powders (viral vector concentration was adjusted to 1×10^7 pfu to account for spray dry process loss, as determined by *in vitro* testing) stored for 0, 7 or 15 days at 37 °C. Powder samples of the spray dried vaccine were reconstituted in 100 μ L of sterile PBS solution. Control samples of the

vaccines were completed in 100 μ L of sterile PBS solution. The basis of the 10^7 pfu per mouse dose was chosen based on our previous published work using the same dose of a recombinant human type 5 adenoviral vector (AdHu5Ag85A) [53].

3.2.5.1 Spleen and lung mononuclear cell isolation

Mice were sacrificed by cervical dislocation. The recovered lungs were digested with collagenase type 1 (Sigma-Aldrich, St Louis, MO) at 37 °C in an agitating incubator. Spleen mononuclear cells were isolated as described previously [54]. The digested tissue was crushed through a 100 μ m basket filter to obtain a single-cell suspension. All cells that were isolated were re-suspended in a complete RPMI 2640 (RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine).

3.2.5.2 Tetramer immunostaining and flow cytometry

Mononuclear cells from the spleen and lungs were processed, immunostained and analyzed as previously described [53], [54]. Cells were plated into U-bottom 96-well plates at a concentration of 20 million cells per mL. The cells were then washed and blocked with CD16/CD32 in 0.5% bovine serum albumin/phosphate-buffered saline for 15 min on ice and then stained with the respective fluorochrome-labelled monoclonal antibodies. Cells were then processed according to the manufacturer's instructions (BD Biosciences, San Jose, CA). The monoclonal antibodies used included CD4-allophycocyanin-Cy7, CD8a-phycoerythrin-Cy7, and CD3-CyChrome. For immunostaining of tetramer, a tetramer for the immunodominant CD8 T-cell peptide (MPVGGQSSF) of Ag85A bound to the BALB/c major histocompatibility complex class I allele H-2L (NIH Tetramer Core, Atlanta, GA) was used. Immunostained cells were run on an LSR II flow cytometer (BD Biosciences, San Jose, CA) and 250,000 events per sample were collected and analyzed on the FlowJo software (version 10; Tree star, Ashland, OR).

3.3 Results

3.3.1 Initial Activity Loss of Spray Dried VSV and after One Day of Storage at 45 °C

Spray dried powders containing the VSV vector expressing GFP were prepared with various excipients. All spray dried powders were produced under similar spray drying conditions, and were not individually optimized. The storage conditions selected were in accordance with the requirements for CTC stated by the WHO of demonstrated activity after storage in excess of 40 °C for a minimum of 3 days [2]. The activity log loss for selected single and blends of excipients after the spray drying process (Day 0) and after 1 day stored dry at 45 °C (Day 1) in pfu/g along with their respective excipient ratios by mass is shown in Table 1.

Table 2: Activity log loss (pfu/g) of VSVGFP spray dried with various excipients immediately after spray drying (Day 0) and after 1 day of storage at 45 °C

Excipient	Day 0 Activity Log Loss (pfu/g)	Day 1 Activity Log Loss (pfu/g)
Trehalose	0.8	3.8
Lactose	1.3	6.6
Mannitol	4.8	6.1
Trehalose/Dextran (3:1)	1.1	4.0
Trehalose/Dextran (2:2)	1.4	4.0
Trehalose/Dextran (1:3)	1.1	5.2
Trehalose/Lactose (2:2)	0.6	4.0
Mannitol/Dextran (2:1)	2.3	6.3
Mannitol/Dextran (3:1)	2.6	8.3
Xylitol/Dextran (1:3)	1.1	4.8

It was observed that blends of excipients generally outperformed single excipients both in initial activity loss as well as after storage for 1 day at 45 °C. The exception was trehalose, which demonstrated low initial activity loss after spray drying of VSVGFP as well as the lowest activity loss after 1 day of storage. This data did not include statistical triplicates and was primarily used to select the excipients and blend ratios for further accelerated storage studies.

3.3.2 *In Vitro* Testing of Spray Dried VSV after Accelerated Storage

To evaluate the ability of sugar-based excipients to thermally stabilize the VSV vector over prolonged storage (up to 30 days), a select group of single and blended excipients were spray dried with the VSVGFP vector and stored at low relative humidity conditions above 37 °C. Additionally, the higher than room temperature storage temperatures allowed for a rapid determination of excipient performance, as the biological activity loss is proportional to increased temperature[50]. After storage, the spray dried samples were reconstituted, and Vero cells were infected, and the measured activity log loss is displayed in Figure 6.

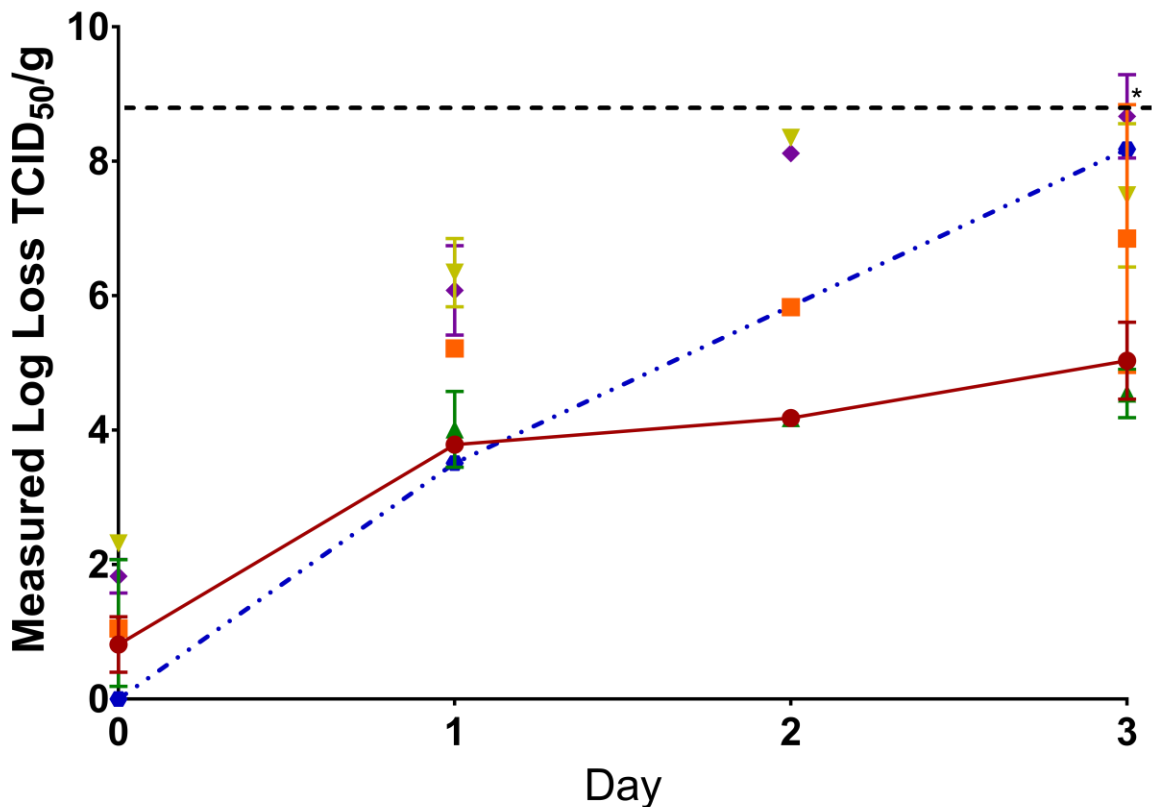


Figure 6: Measured log loss of VSVGFP vector activity after storage at 45°C and <10% RH for liquid control(◆), trehalose (●), dextran (◆), mannitol/dextran 2:1 (▼), trehalose/dextran 3:1(▲), trehalose/dextran 1:3 (■). Process loss is at $t = 0$ day and the first stored time point is at $t = 1$ day. Data are represented as mean \pm SD for three repeat samples. Symbols marked with (*) indicate 1 of 3 samples was below the detectable limit. The solid line and dashed-dotted line are used to highlight the best performing excipient and positive control, respectively. The horizontal dashed line represents the lower limit for viral activity detection and all data points above this line are considered inactive samples relative to the starting titre.

After 1 day of accelerated storage at 45°C the positive liquid control demonstrated less activity loss than the spray dried powders. However, after 3 days of storage the positive control exhibited an activity loss of 8 log, whereas the best performing excipients of trehalose and trehalose/dextran (3:1) demonstrated an activity loss of 5.04 ± 0.57 log and 4.54 ± 0.36 log respectively. The same excipients spray dried with VSVGFP were then stored at 37 °C for up to 30 days and infected in a similar manner to better quantify the activity loss profile of the vector. The spray dried samples were again compared to the positive control but for longer times and at a slightly lower temperatures (Figure 7).

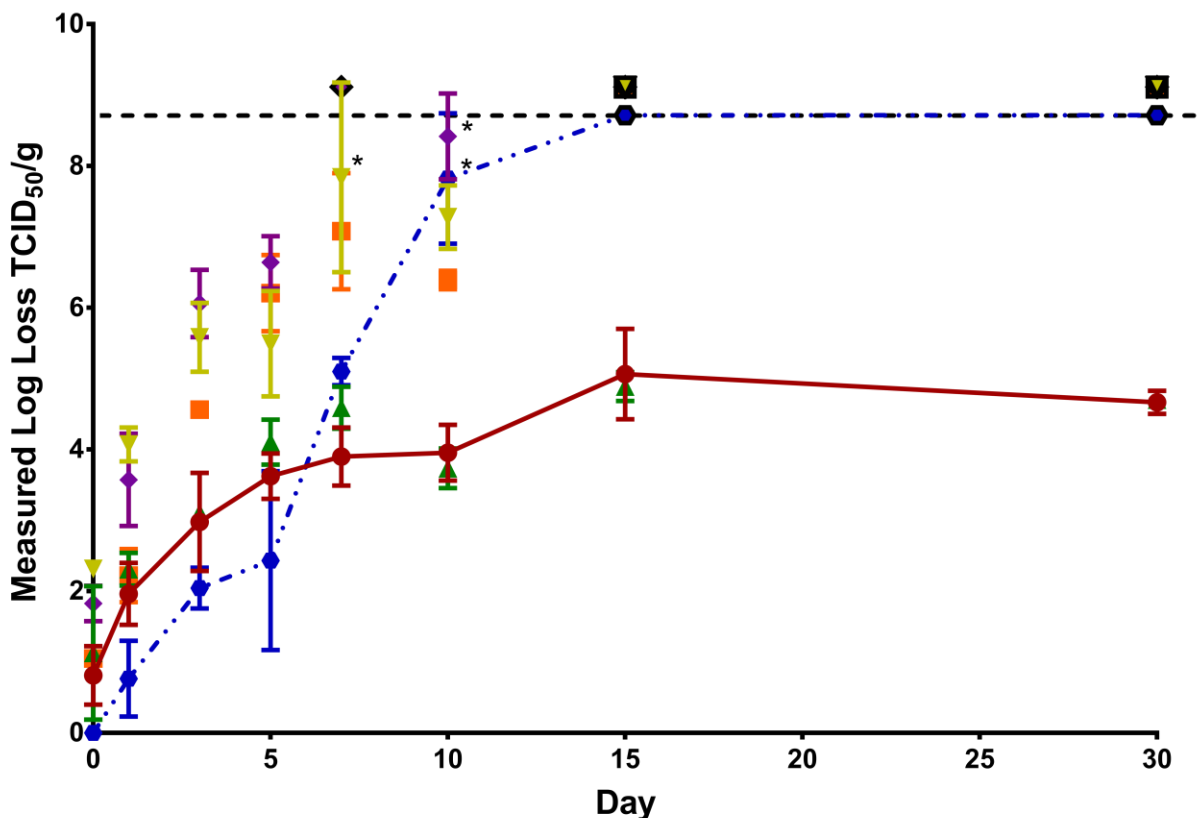


Figure 7: Measured log loss of VSVGFP vector infectivity after storage at 37°C and <10% RH for liquid control(●), trehalose (●), dextran (◆), mannitol/dextran 2:1 (▼), trehalose/dextran 3:1(▲), trehalose/dextran 1:3 (■). Process loss is at $t = 0$ day and the first stored time point is at $t = 1$ day. Data are represented as mean \pm SD for three repeat samples. Hollow symbols indicate all 3 samples were below the detectable limit. Symbols marked with (*) and (\$) indicated 1 or 2 samples of 3 were below the detectable limit, respectively. The solid line and dashed-dotted line are used to highlight the best performing excipient and positive control, respectively. The horizontal dashed line represents the lower limit for viral activity detection and all data points above this line are considered inactive samples relative to the starting titre.

Similar to the 45 °C storage data, trehalose and trehalose/dextran (3:1) were the best performing excipients and minimal activity loss of the VSVGFP vector was observed. After 7 days of storage at 37 °C, the activity loss of the positive control was greater than trehalose and trehalose/dextran samples and all activity of the positive control was lost by Day 15. After 10 days of storage, the activity loss of trehalose and trehalose/dextran (3:1) appeared to reach a plateau. The mannitol/dextran blend demonstrated the most activity loss initially and over time and was deemed to be the worst performing excipient blend.

3.3.3 Evaluation of Immunogenicity *In Vivo*

Based on our promising *in vitro* data showing the ability of trehalose to thermally stabilize VSV after spray drying, we furthermore assessed the immunogenicity of our spray dried

vaccine in a mouse animal model. While the GFP cell assay can provide insight on the activity of the VSV virus in a spray dried particle after storage, it is imperative to know the *in vivo* efficacy to determine if the thermal stability translates to the immunization process. Both methods target epithelial cells, but the *in vivo* study examines the T cell response of the host, from the infected epithelial cells. Trehalose was chosen for the *in vivo* study because it was the best performing excipient during the storage study at 37 °C. As such, trehalose was spray dried with VSVAg85A and compared against the liquid control of VSVAg85A in PBS buffer. The samples were stored in a similar manner to the *in vitro* experiments for 0, 7 and 15 days at 37 °C and low relative humidity. Mice were then immunized with a liquid control or a spray dried equivalent of VSVAg85A. The mice were processed 2 weeks post immunization at the peak of the immune response during the effector phase. The frequency of Ag85A tetramer-specific CD8⁺ (CD8⁺Tet⁺) T cells harvested from the spleen were determined using tetramer staining to examine the immune response and are reported in Figure 8.

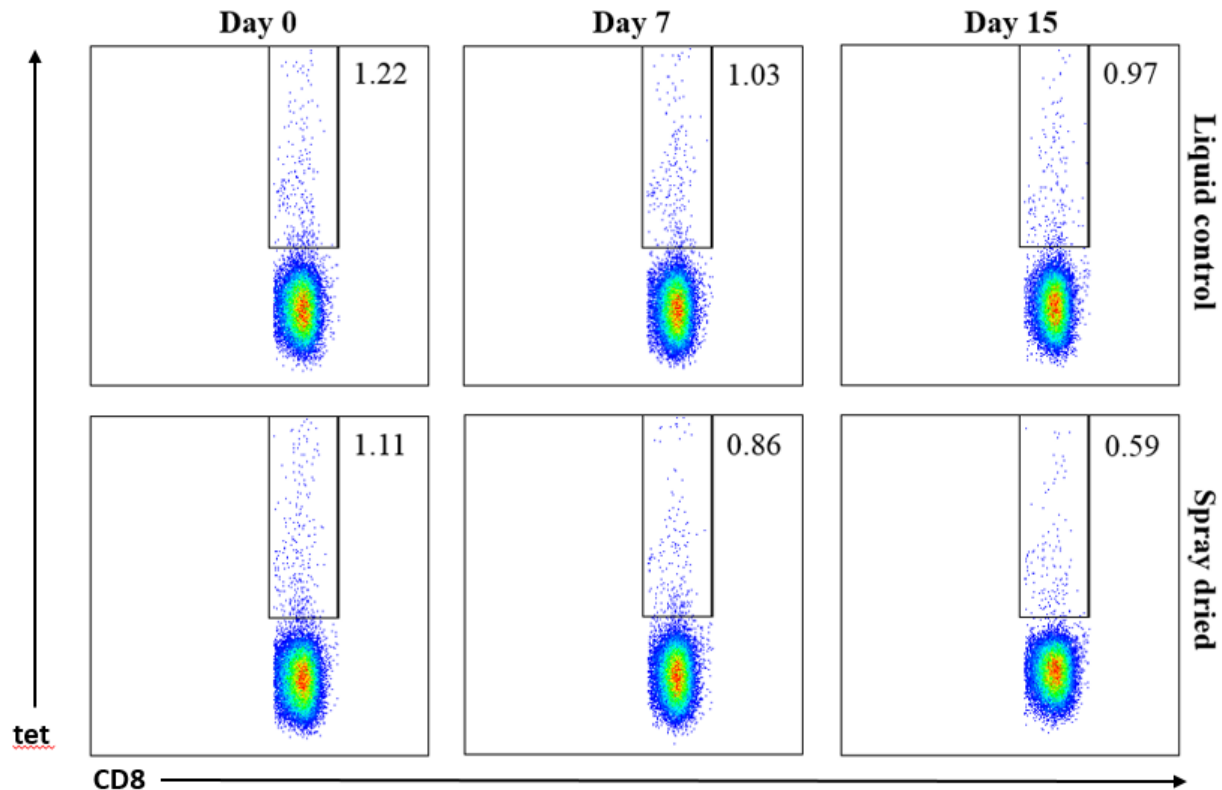


Figure 8: Intramuscular immunization with 1×10^7 pfu of spray dried VSVAg85A with trehalose or an equivalent liquid vaccine control that had been stored for 7 or 15 days at 37 °C and low relative humidity or with the same amount of fresh cryopreserved liquid vaccine control. Animals were sacrificed 2 weeks post-immunization. Frequencies (%) of Ag85A tetramer-specific CD8⁺ (CD8⁺Tet⁺) T cells in spleen were determined. Data are expressed as representative dotplots (frequencies) of T cells.

Immunogenicity of the spray dried vector post spray drying was determined to be similar to the liquid control indicating that the spray dried sample did not lose activity *in vivo* as a result of the processing method. However, the Day 7 and Day 15 liquid control does not correlate with

the *in vitro* data as the frequencies of the liquid control remain higher than the spray dried samples at similar time points. This may be a result of the low starting titer (and the measurement precision) with the VSV samples compared to our past work with AdHu-based vaccines; experiments to investigate further are underway.

3.4 Discussion

The purpose of the study was to demonstrate that VSV-vectorized vaccines can be spray dried with suitable excipients to retain thermal stability in a powder form and maintain activity in both *in vitro* and *in vivo* tests after storage. Previous work has established that spray drying is a viable method to thermally stabilize influenza [1] and adenoviral vectors [34], but the VSV vector has not been examined before. Our work highlights that trehalose and a blend of trehalose/dextran at a 3:1 weight ratio, spray dried from a 4 wt% solution, were both able to thermally stabilize the VSVGFP vector and retain its activity, even after dry storage of 30 days at 37 °C (Figure 7) – this is a significant improvement compared to the generally prescribed cold chain storage requirements for vaccines.

The role of trehalose in the improved stabilization of the VSV vector is likely based on its ability to replace the water molecules typically surrounding the lipid membrane envelope of the virus. Upon dehydration without added excipients, the lipid envelope becomes rigid as the hydrogen bonds of the water are removed and an increase in van der Waals forces leads to the hydrophobic tails of the lipid bilayers aggregating and becoming transiently leaky [20]. When rehydrated, the lipid membrane experiences localized rigidity leading to the leakage of inner viral material through gaps between the membrane [20]. To effectively stabilize lipid membranes in the dry state, the hydrogen bonds that exist between water molecules and the phosphate heads must be substituted. The *water replacement hypothesis* [55] suggests that the OH groups of carbohydrates can replace the main hydration shell surrounding the polar phosphate head of the lipid membrane of the enveloped virus [56].

Trehalose has shown to be an excellent carbohydrate in stabilizing lipid membranes of biologics in a dehydrated state [21] and has been used to stabilize enveloped viruses, such as influenza vectors, in the dry state [57]. The -OH bonds of the trehalose molecules form direct hydrogen bonds with the phosphate heads of the lipid bilayer, effectively replacing the removed water molecules [16]. Additionally, trehalose is able to form a primarily amorphous matrix to encapsulate and protect the viral vectors from external stresses and fusion of the lipid membranes which would lead to leakage [56]. Due to the branched nature of dextran it tends to be fairly amorphous, but the poor performance observed for the spray dried dextran-only sample is likely due to steric hindrance as the larger molecule is unable to replace the hydrogen bonds formed between the water molecules and individual phosphate heads of the lipid membrane [16]. The blend of trehalose/dextran (3:1), was successful due to the relatively large amount of trehalose that could be used to replace the hydrogen bonds. Furthermore, the spray dried mannitol/dextran blend, which demonstrated exceptional thermal stability with adenoviral vectors [34], was the worst performing excipient blend when stabilizing the VSV vector likely due to the high tendency of mannitol to crystallize, which will lead to membrane rupture and leakage of inner viral material [58].

The *in vivo* data did not correlate with the *in vitro* trends as the liquid positive control exhibited a larger immunogenic response after 15 days of storage at 37 °C than the spray dried trehalose sample. The overall immunogenic response of the liquid control of VSV at Day 0 was deemed to be lower than previous work with other viral vectors [59] and all samples were considered to be a low frequency response. Since, VSV is typically used as a booster, it is not as effective in eliciting an immune response as a primer during immunization [60]. For future studies, VSV should be used as a heterologous booster after an initial priming with an adenoviral vector as per previous experiments [59] to achieve a stronger immune response.

While trehalose, the best performing excipient, experienced a loss greater than 4 log over 30 days at 37 °C, it is imperative to note that the spray drying parameters for all excipient blends were not optimized. Parameters such as initial excipient concentration, inlet spray dry temperature and spray gas rate can be adjusted to achieve optimal conditions for minimal activity loss. Thus, there exists a possibility that further reduction in activity loss may be achieved in future studies to stabilize the virus when stored for a minimum of 3 days at temperatures exceeding 40 °C to meet the WHO requirement for CTC.

3.5 Conclusion

Our data demonstrates that we are the first to successfully enhance the thermal stability of the VSV viral vector in a dry state via spray drying. Spray dried VSV with trehalose outperformed all spray dried samples and the liquid control, and was able to retain viral activity after 30 days of storage at 37 °C. The immune response of spray dried VSV *in vivo* was lower than the liquid control after storage, likely due to low inherent immunogenicity of the VSV vector. A more detailed conclusion can be found in the conclusion section in Chapter 5.

Chapter 4: Thermal Stability of Enveloped versus Non-Enveloped Viral Vectored Vaccines

4.1 Introduction

The stabilization of vaccines through cryopreservation is the current best practice to ensure that stored vaccines maintain their efficacy when administered to patients [1]. This often requires vaccines in aqueous media to be kept below 2-8 °C [1], resulting in the necessity of a cold chain management system for the transportation and storage of the vaccine. However, the implementation of a cold chain system in the developing world is not feasible, due to limited infrastructure and as such the World Health Organization (WHO) has advocated for alternative storage methods to alleviate the dependency on cold chain systems [2]. This has led to the development of thermally stable vaccines to preserve the virus in the dry state based on vitrification [50].

Broadly, viral vectors can be structurally classified into two groups, enveloped and non-enveloped viral vectors. Enveloped viral vectors, such as vesicular stomatitis virus (VSV) and Influenza, have their genetic material confined within a protein capsid that is surrounded by a lipid membrane [28]. The genetic material of non-enveloped viral vectors, like Adenoviral vectors, is enclosed within a rigid protein capsid and lacks a lipid membrane [61]. There are two proposed mechanisms that work in conjunction towards the preservation of biologics in the dry state, eliminating the dependency for cold chain storage; *water replacement hypothesis* and vitrification theory [20].

Water replacement hypothesis examines the stabilization of the virus with sugars based on chemical interactions. The hypothesis states that upon dehydration, the hydroxyl groups of the sugar, form hydrogen bonds with the protein capsid to maintain the native protein structure and prevent denaturation [62]. With respect to lipid membranes, upon dehydration, the loss of the hydrogen bonds acting to stabilize the phosphate heads of the lipid membrane, will lead to an increase in van der Waals attraction between the hydrocarbon tails of the lipid bilayer. This attraction results in the fusion and increased rigidity of the lipid membrane bilayer, as the membrane transitions into a gel state [20]. The temperature at which this transition occurs is known as the phase transition temperature (T_m), below which a membrane is in the gel state. The (T_m) increases when the hydrogen bonds between water and the phosphate heads are not replaced [21]. As the gel state lipid membrane is rehydrated, it locally transitions back to the fluid-like liquid crystalline phase, resulting in nonuniform fluidity and leakage of the internal components. The hydroxyl groups of sugar molecules are able to replace the hydrogen bonds formed between water and the phosphate heads of the dehydrated lipid membrane, stabilizing the phase transition temperature and preserving membrane fluidity and viral function [22].

Vitrification theory is centered around the concept that proteins and other biologics remain active through encapsulation by a rigid, amorphous sugar glass matrix [8]. Amorphous

structures are ideal for vitrification as the numerous conformations allow for improved encapsulation of the biologics compared to a crystalline material [57]. The encapsulation of the biologic reduces protein denaturation that would occur during aggregation of the biologics or external thermal stresses. The molecular mobility of the glassy amorphous phase of the matrix will govern how well a biologic is encapsulated over time and can be characterized by the difference between the storage temperature of a material and its glass transition temperature (T_g) [14]. As the storage temperature approaches the material T_g , the molecular mobility of the material increases which will lead to reduced encapsulation and ultimately protein denaturation [14]. When the storage temperature of a material is 50 °C below the T_g , known as the Kauzmann temperature, the molecular mobility of the amorphous phase is deemed to be insignificant and is ideal for long-term vitrification of biologics [40]. The presence of water through high humidity has demonstrated the ability to depress the T_g of many matrix materials resulting in rapid activity loss [34], as well as degradation of encapsulated biologics over time [14]. Additionally, low T_g materials have a greater tendency to recrystallize at ambient temperatures due to the higher molecular mobility of the material, which is not desired for vitrification [14].

Vitrification can be achieved through the heating and super cooling of a material to produce an amorphous solid. Lyophilisation is the preferred method for rapid drying of biologics in industry, but the process is time consuming, is limited by its batch throughput [3] and the biologics can be disturbed by stresses endured during the freezing and drying process [4]. An alternative to lyophilisation that has garnered attention in academic research recently, is the use of spray dryers to produce dry vaccines [1]. The process uses pressurized gas to aerosolize a liquid feed containing the vaccine and excipient formulations, into a heated chamber, where it is rapidly dried into dry particles which can be collected in a cyclone chamber [63]. The spray drying process is considered as an alternative to lyophilisation due to its tunability, scalability and relatively short batch time, and has been utilized to encapsulate and protect vaccines [1].

Recent efforts by our group to improve thermal stability of viral vectors through the process of spray drying, yielded encouraging results [33], however there is a fundamental lack of knowledge on how different viral vectors behave when spray dried with various excipients. This study examines the differences in thermal stabilization of spray dried viral vectors that are either enveloped (influenza and VSV) or non-enveloped (adenoviral human type 5; AdHu5). Our aim is to elucidate the dominant mechanisms that are involved in stabilizing enveloped vs. non-enveloped viral vectors to ultimately reduce the dependency on cold chain systems that are needed to maintain vaccine potency during transportation and storage.

4.2 Materials and Methods

4.2.1 Chemicals and Viral Vectors

D-(+)-Trehalose dihydrate, D-Mannitol, Dextran (M_r 40,000 kDa), anhydrous Lactose were purchased from Sigma-Aldrich (Ontario, Canada). Culture media was prepared from Alpha Minimum Essential Medium Eagle (α -MEM) (in house according to protocol by the supplier,

Life Technologies; Ontario, Canada) with 10% fetal bovine serum and 1% streptomycin/penicillin (Invitrogen; Ontario, Canada). Phosphate Buffered Saline (PBS) was prepared in house. Recombinant replication-deficient human type 5 adenovirus expressing green fluorescent protein (AdHu5GFP) and vesicular stomatitis virus-vectored vaccines expressing green fluorescent protein (VSVGFP) were produced in the vector facility of McMaster Immunology Research Centre as described previously [46]. The A/Puerto Rice/8/1934 H1N1 mNeon-2A-HA virus (Influenza mNeon) was gifted from Dr. Heaton of Duke University. These viruses will be referenced from this point on as AdHu5, VSV and influenza for simplicity.

4.2.2 Spray Drying of Viral Vectors

Spray dried powder vaccines were produced by spray drying using a Mini Spray Dryer B-290 (Büchi; Switzerland) with 0.7 mm spray nozzle and high performance cyclone attachments as previously described [34]. For all spray dried excipients, the spray dryer was operated at a spray gas flow rate of 439 L/h, feed solution of 234 mL/h and a nozzle inlet temperature of 110 °C. The concentration of all feed solutions is 4 wt% solute.

4.2.3 Storage of Powder Vaccine

Powder samples were stored individually in closed 2 mL Nalgene General Long-Term Storage Cryogenic Tubes (Nalgene; Ontario, Canada) sealed with Parafilm Wax (Bemis NA; Wisconsin, US). The tubes were placed in a resealable plastic bag and stored within a glass jar filled with gel desiccant and sealed with Parafilm Wax to ensure a low humidity environment (<10% RH). The glass jars were sealed within another resealable plastic bag and placed in a water bath for temperature control at 37 °C. The liquid control containing only PBS buffer and VSV, Influenza or AdHu5 were stored in a plastic resealable bag within a tightly sealed glass jar without gel desiccant.

4.2.4 Differential Scanning Calorimetry (DSC)

Thermograms for the spray dried powders (without viral vector) were measured by differential scanning calorimetry (DSC). Powder samples were weighed out between 4-8 mg and hermetically sealed in zero hermitically sealed pans. The samples were analyzed with a Q200 Differential Scanning Calorimeter (TA Instruments; New Castle, DE). Samples were tested using a modulated DSC protocol from 20 °C to 200 °C using a rate of 1 °C/min under a nitrogen purge gas flow rate of 50 mL/min. The glass transition temperature (T_g) of the sample was determined using TA Universal Analysis software (TA Instruments; New Castle, DE).

4.2.5 RNA Staining

Spray dried samples of VSV were stored as described above for 3 days at 37 °C. The samples were reconstituted with nuclease free water. A liquid control of VSV in PBS was dried and stored under the same conditions. The RNA staining was conducted in an opaque flat bottom 96 well plate with the QuantiFluor RNA System (Promega; Madison, WI) in accordance with the Promega QuantiFluor RNA System Protocol [64]. Fluorescence was measured using a Spectramax i3 (Molecular Devices; San Diego, CA). To mitigate the effects of the different

excipients on the fluorescence readout, standard curves were prepared for each excipient or blend of excipients at the same concentration of the reconstituted spray dried samples.

4.2.6 *In Vitro* Testing of Viral Vectors

4.2.6.1 Cell Culturing

Vero cells isolated from kidney epithelial cells and A549 lung epithelial cells were thawed from liquid nitrogen and cultured with Alpha Minimum Essential Medium Eagle (α -MEM) in T150 culture flasks. Cell culturing was completed in a humidified Forma Series II Water Jacketed CO₂ Incubator (Thermo Scientific Corporation; Waltham, MA) at 37°C and 5.0% CO₂. When cells appeared to be 80-90% confluent, they were split into a new T150 culture flask and/or plated in a 96-well plate for *in vitro* testing.

4.2.6.2 GFP Infection and Endpoint TCID Calculation

The viral activity after the spray drying process and subsequent storage was determined by an endpoint dilution in a 96 flat bottom well plate. For the VSVGFP vector, plated Vero cells were infected with 4 mg of powder (initial concentration of 1.30×10^9 pfu/g) reconstituted in culture media. For Influenza mNeon, plated Vero cells were infected with 4 mg of powder (initial concentration of 2.51×10^5 pfu/g) reconstituted in culture media. For AdHu5GFP, plated A549 cells were infected with 4 mg of powder (initial concentration of 2.71×10^8 pfu/g). After the overnight incubation of plated the respective cells, the cell media was removed and replaced with an eight-fold serial dilution from each reconstituted sample at a volume of 100 μ L per well and a total of 4 wells per sample. After overnight incubation, viral infectivity was detected by the presence of a GFP expression indicated by a bright green hue to cells, observed under an EVOS FL Cell Imaging System with a GFP filter (Thermo Scientific Corporation; Waltham, MA). A positive GFP response of a single cell within a well constituted a positive infection response with respect to the endpoint dilution. If greater than 50% of the wells in the row were non-expressive, the dilution was determined to have reached its endpoint and the median tissue culture infections dose (TCID₅₀) was calculated using the Reed-Muench method [52]. Using the Poisson distribution, the results were multiplied by a factor of 0.69 to convert to an approximated pfu value and reported as a viral activity loss of pfu/g of material with error bars calculated as the standard deviation (n =3).

4.3 Results

AdHu5 and VSV viral vectors were subjected to an initial accelerated storage study at 45 °C for up to 3 days. The activity data collected guided a more extensive study of the thermal stability of AdHu5, VSV and Influenza at 37 °C for up to 30 days.

4.3.1 Thermal Stability of Viral Vectors after Spray Drying

Various excipients were selected to examine initial activity loss after spray drying and activity loss after 1 day of storage at 45 °C for VSV or AdHu5; activity log loss values in pfu/g are reported in Table 2.

Table 3: Activity log loss of VSV and AdHu5 vectors spray dried with various excipients after spray drying (Day 0) and 1 day of storage at 45 °C in a low relative humidity environment.

Excipient (ratio)	VSV		AdHu5	
	Activity Log Loss after 0 Days Storage (pfu/g)	Activity Log Loss after 1 Day Storage (pfu/g)	Activity Log Loss after 0 Days Storage (pfu/g)	Activity Log Loss after 1 Day Storage (pfu/g)
Trehalose	1.4	3.8	3.6	3.9
Dextran	3.1	6.1	5.5	5.8
Mannitol	3.3	6.1	3.3	5.7
Lactose	2.4	6.6	4.00	4.3
Trehalose/Dextran (1:3)	2.4	5.2	3.5	5.6
Trehalose/Dextran (3:1)	1.2	4.0	2.7	4.1
Mannitol/Dextran (2:1)	3.4	6.3	0.9	3.2

Notable differences were observed in activity log loss between AdHu5 and VSV when spray dried with the same excipient or blends of excipients. Trehalose and trehalose/dextran (3:1 ratio) demonstrated the lowest initial loss of VSV activity after spray drying at 1.38 ± 0.41 log and 1.23 ± 0.07 log respectively and retained the most activity after 1 day of storage, with losses measuring 3.78 ± 0.05 log and 4.01 ± 0.57 log, respectively. Single excipients of dextran, mannitol and lactose when spray dried with VSV had high initial losses and all excipients exceeded 6 log loss after 1 day of storage. When VSV was spray dried with the mannitol/dextran blend it experienced the highest initial loss out of all selected excipients of 3.41 ± 0.07 log loss and had a high activity loss of 6.34 ± 0.51 log after 1 day of storage.

Conversely, when AdHu5 was spray dried with the mannitol/dextran blend, the lowest activity loss was observed, with losses at Day 0 and Day 1 reported to be 0.92 ± 0.70 log and 3.19 ± 0.36 log, respectively. Trehalose/dextran (3:1 ratio) was the next best performing excipient when minimizing activity loss of AdHu5 with Day 0 and Day 1 activity loss of 2.65 ± 0.66 log and 4.08 ± 1.31 . All other single and blends of excipients when spray dried with AdHu5 exhibited high initial and storage loss. The best performing excipients for each virus type was later evaluated at a lower temperature to examine the thermal stability trends over a prolonged period of time.

4.3.2 Thermal Properties of Sugars After Storage

Spray dried excipients (with no virus included) were stored in a low relative humidity environment at 37 °C to investigate the changes in glass transition temperature over time. The T_g decreases because of the plasticization effect of water on carbohydrates which leads to increased molecular mobility of the matrix. The glass transition temperatures of the samples were measured using DSC (Table 3).

Table 4: Glass transition temperatures of excipients after spray drying (Day 0), and after storage for 10 days at 37 °C in low relative humidity.

Sample	T_g (Day 0, 37 °C)	T_g (Day 10, 37 °C)
Trehalose	110	72
Trehalose/Dextran (1/3)	88	86
Trehalose/Dextran (3/1)	88	67
Mannitol/Dextran (2/1)	113	101
Dextran	125	113

Both the dextran and mannitol/dextran particles exhibited the highest T_g values after spray drying, measured at 125 °C and 113 °C respectively. Trehalose spray dried samples had a similarly high T_g of 110 °C. The trehalose/dextran ratios of 1:3 and 3:1 had identical T_g values after spray drying at 88 °C and were the lowest of the excipients tested.

After 10 days of storage at 37 °C, trehalose and 3:1 trehalose/dextran exhibited a significant drop in T_g , and became the lowest T_g matrices at 72 °C and 67 °C respectively. While low, these T_g values are still reasonable to meet the Kauzmann temperature during storage at room temperature (but not at 37 °C). The T_g of mannitol/dextran and dextran samples both dropped by 12 °C after 10 days of storage and were measured to be 101 °C and 113 °C. The 1:3 trehalose/dextran yielded the smallest change in T_g after 10 days of storage barely decreasing from 88 °C to 86 °C.

4.3.3 30 Day Accelerated Storage of Viral Vectors

To evaluate the thermal stability of enveloped and non-enveloped viral vectors long term, selected excipients (the same as in Table 3) were spray dried with AdHu5, VSV and influenza vectors and subjected to storage at 37 °C in low relative humidity for up to 30 days. The measured *in vitro* activity log loss of AdHu5 is displayed in Figure 9, VSV in Figure 10 and influenza in Figure 11.

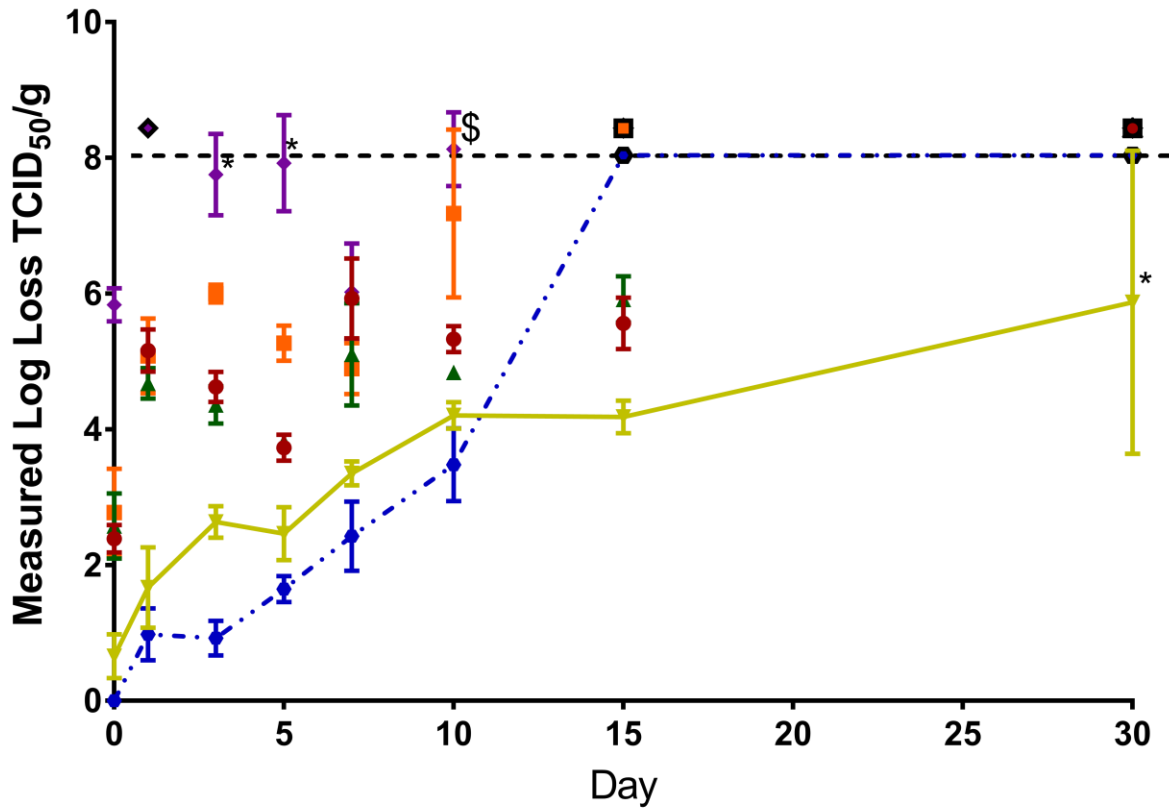


Figure 9: Measured log loss of AdHu5 vector infectivity after storage at 37°C and <10% RH for liquid control(●), trehalose (●), dextran (◆), mannitol/dextran 2:1 (▼), trehalose/dextran 3:1(▲), trehalose/dextran 1:3 (■). Process loss is at $t = 0$ day and the first stored time point is at $t = 1$ day. Data are represented as mean \pm SD for three repeat samples. Hollow symbols indicate all 3 samples were below the detectable limit. Symbols marked with (*) and (\$) indicated 1 or 2 samples of 3 were below the detectable limit, respectively. The solid line and dashed-dotted line are used to highlight the best performing excipient and the positive control respectively. The horizontal dotted line represents the lower limit for viral activity detection and all data points above the line indicate an inactive sample relative to the starting titre.

Of all the spray dried samples with AdHu5, mannitol/dextran exhibited the lowest activity losses and was the only excipient to retain activity after 30 days of storage at 37 °C, with 1 sample of the set of triplicates yielding no activity. Dextran was the worst excipient at stabilizing the AdHu5 vector as spray dried samples lost most of their activity after only 1 day of storage. By Day 30, all samples except for mannitol/dextran were determined to have lost all activity. After 15 days of storage the AdHu5 liquid control was deemed to be inactive as no response was observed in infected cells.

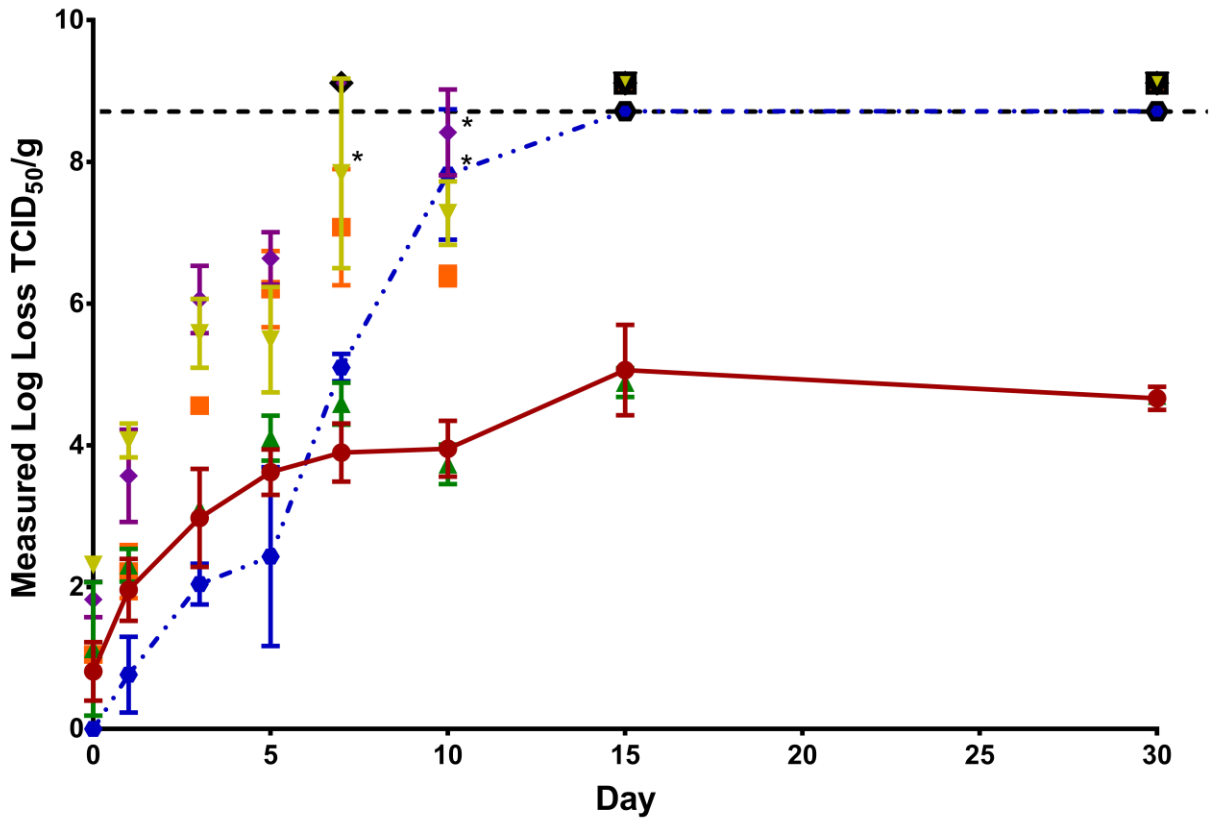


Figure 10: Measured log loss of VSV vector infectivity after storage at 37°C and <10% RH for liquid control(●), trehalose (●), dextran (◆), mannitol/dextran 2:1 (▼), trehalose/dextran 3:1(▲), trehalose/dextran 1:3 (■). Process loss is at $t = 0$ day and the first stored time point is at $t = 1$ day. Data are represented as mean \pm SD for three repeat samples. Hollow symbols indicate all 3 samples were below the detectable limit. Symbols marked with (*) and (\$) indicated 1 or 2 samples of 3 were below the detectable limit, respectively. The solid line and dashed-dotted line are used to highlight the best performing excipient and the positive control respectively. The horizontal dotted line represents the lower limit for viral activity detection and all data points above the line indicate an inactive sample relative to the starting titre.

As discussed in Chapter 3, the best performing excipients with VSV were trehalose and trehalose/dextran (3:1 ratio) that were able to retain activity after 30 days of storage contrary to the other spray dried samples which lost all activity after 15 days of storage. The activity loss of the liquid control exceeded the trehalose and trehalose/dextran samples after 7 days of storage at 37 °C. All activity of the liquid control was lost after 15 days of storage. The activity loss of trehalose and trehalose/dextran (3:1) appeared to reach a plateau by Day 10. Dextran was the worst performing formulation and the mannitol/dextran blend experienced the greatest loss in activity due to the spray drying process.

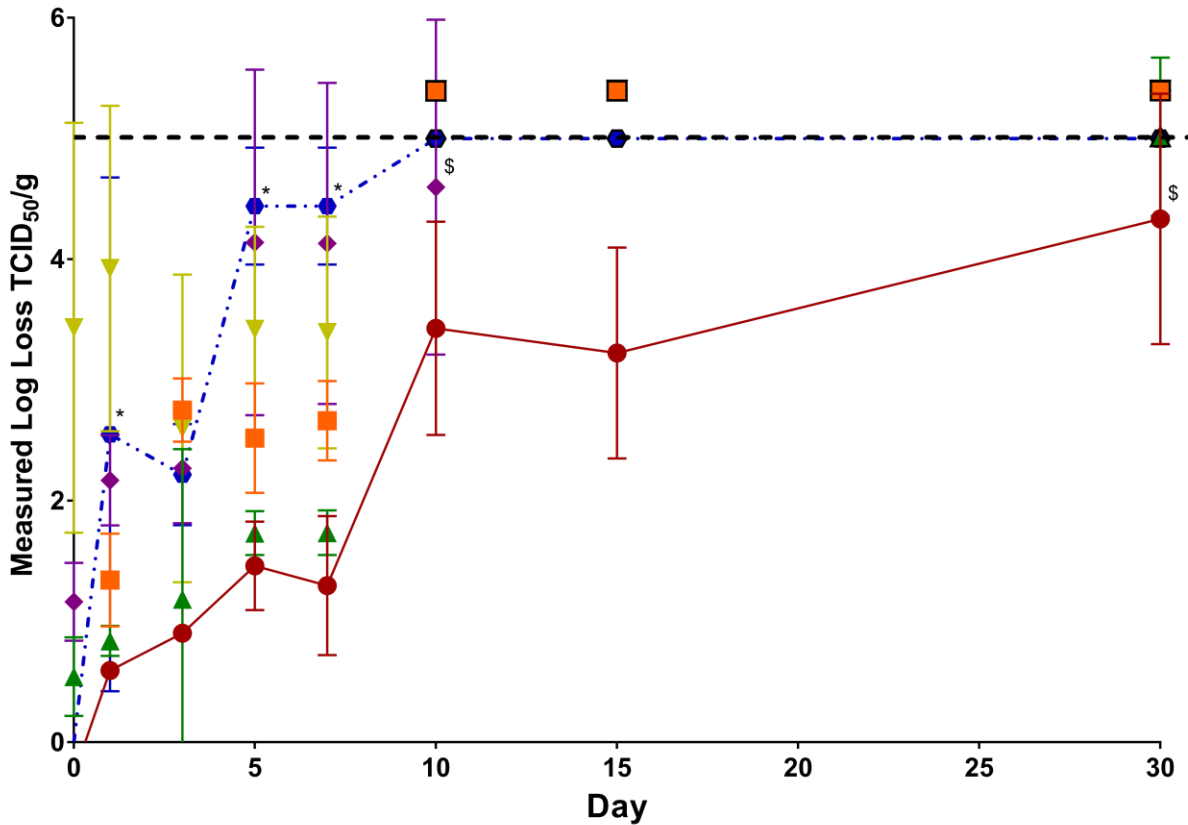


Figure 11: Measured log loss of Influenza vector infectivity after storage at 37°C and <10% RH for liquid control(●), trehalose (●), dextran (◆), mannitol/dextran 2:1 (▼), trehalose/dextran 3:1(▲), trehalose/dextran 1:3 (■). Process loss is at t = 0 day and the first stored time point is at t = 1 day. Data are represented as mean ± SD for three repeat samples. Hollow symbols indicate all 3 samples were below the detectable limit. Symbols marked with (*) and (\$) indicated 1 or 2 samples of 3 were below the detectable limit, respectively. The solid line and dashed-dotted line are used to highlight the best performing excipient and the positive control respectively. The horizontal dotted line represents the lower limit for viral activity detection and all data points above the line indicate an inactive sample relative to the starting titre.

The best excipient for stabilizing the influenza vector was trehalose, followed by trehalose/dextran (3:1 ratio) – similar to the results with VSV. By Day 15, the trehalose formulation was the only sample that retained activity as all other formulations were above the lower limit for viral activity detection. After 30 days, only 1 out of 3 samples of the trehalose formulation was able to elicit a positive infection response. The liquid control lost all activity after 10 days of storage and the mannitol/dextran blend yielded the highest initial activity loss. The initial starting titre of the influenza vector was approximately 3 log lower than both the AdHu5 and VSV, and as such activity losses appeared to be relatively greater.

4.3.4 Quantification of Viable RNA and Denaturation during Storage of VSV Powders

To quantify the amount of VSV RNA that was protected during storage, the viable RNA from the enveloped VSV virus after spray drying was measured using a fluorescence RNA binding dye. Samples were tested immediately after spray drying and after 3 days storage at 45 °C at low RH and compared to VSV dried from buffer solution and aged following the same protocol. The amount of RNA in nanograms per pfu was calculated and is shown in Figure 12.

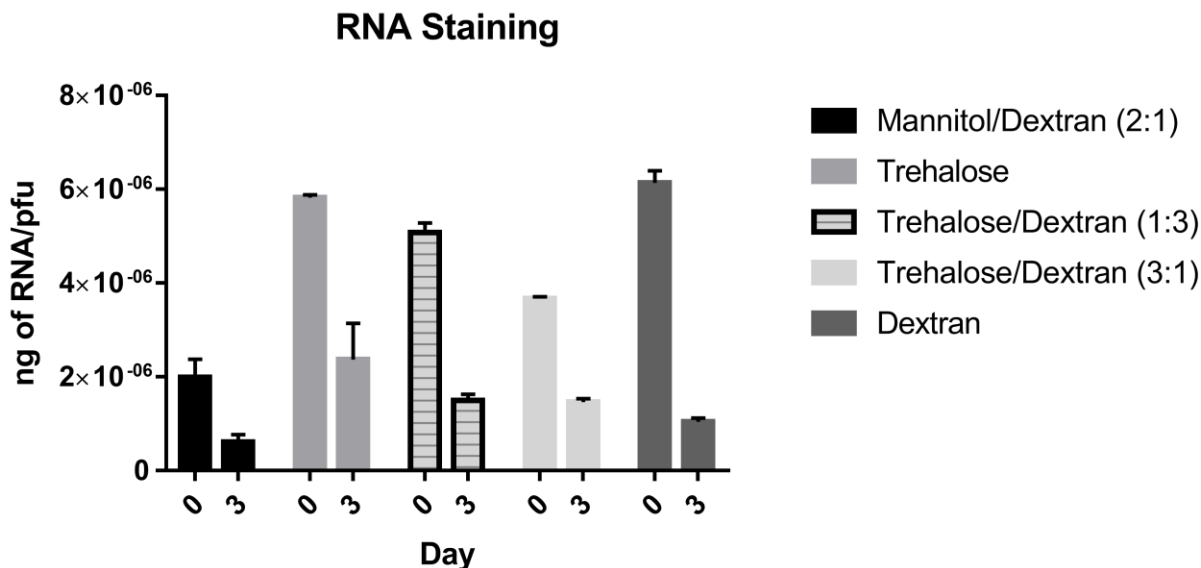


Figure 12: Concentration of viable RNA in ng/pfu of spray dried VSV samples after spray drying (Day 0) and after 3 days of storage at 45 °C in low relative humidity. RNA concentration was detected using a fluorescent RNA binding dye.

Large RNA values in Figure 12 indicate well-protected RNA (and correlate with activity testing, i.e. Fig. 10, with the exception of dextran) and imply minimal leakage of RNA during the spray drying/reconstitution/ageing steps because exposed RNA quickly denatured and became undetectable by the fluorescent dye. This interpretation is supported by a control experiment whereby stock RNA at 50 ng of RNA/μL was dried and tested; after 3 days at 45 °C the detectable RNA decreased 65% to 17 ± 2 ng of RNA/μL. This test confirms that unprotected RNA exposed to high temperatures in the dry state denatures and cannot be measured with the current fluorescent assay.

The VSV-mannitol/dextran powder had the lowest concentration of detectable RNA at $2.0 \pm 0.4 \times 10^{-6}$ ng/pfu after spray drying and also had the lowest detectable RNA after 3 days of storage ($0.6 \pm 0.2 \times 10^{-6}$ ng/pfu) indicating a poor excipient choice for VSV. The VSV-trehalose sample had a high reading at Day 0 and experienced the smallest decrease in detectable RNA over the 3 days of storage, showing that most RNA was protected. The VSV-dextran powder also exhibited a high RNA reading on Day 0 but then had the most significant decrease

(from $6.1 \pm 0.3 \times 10^{-6}$ ng/pfu to $1.04 \pm 0.08 \times 10^{-6}$ ng/pfu after 3 days) which suggests that the matrix could not prevent denaturation of RNA during storage. A dried VSV-only control (no excipient) yielded the lowest detectable RNA concentration of all samples – after 3 days of storage only $0.37 \pm 0.01 \times 10^{-6}$ ng/pfu were measured.

4.4 Discussion

It is evident through our storage data that the same formulations of excipients do not thermally stabilize enveloped and non-enveloped viral vectors equivalently when spray dried. The best performing excipients to be using in the spray drying of VSV and influenza viral vectors, were trehalose and trehalose/dextran (3:1 ratio), showing similarities between the two the enveloped viral vectors. The best performing spray dried formulation for AdHu5, the non-enveloped vector, was mannitol/dextran, which experienced the greatest activity loss when spray dried with VSV and influenza.

These differences in stabilization can be attributed to the vitrifying properties of the sugar excipients and the chemical interactions or lack thereof between the sugars and the outer surfaces of the viral vectors. The use of dextran in blends would appear to be preferred due to the high T_g of dextran and its ability to minimize T_g depression over time as indicated in Table 3. Since dextran is a large molecule, it has low molecular mobility and as such, movement of the virus within the matrix should be limited and ensure that the virus remains encapsulated and does not aggregate, which would result in a loss of activity [14]. However, spray dried samples with dextran demonstrated poor activity retention for both enveloped and non-enveloped viruses (Figure 9, 10, 11). The use of dextran as the only excipient was determined to be non-ideal, as its large size and branching makes it unable to closely encapsulate the vaccine, replace water hydrogen bonds and prevent aggregation, resulting in reduced activity as shown previously for encapsulated protein systems [65]. When dextran was used in conjunction with smaller molecules, such as mannitol and trehalose, it can limit molecular mobility of the matrix, while the smaller molecules directly interact with the vaccine and minimize activity loss [65].

Furthermore, polysaccharide glucans, such as dextran are unable to stabilize the phospholipid layer of membranes due to steric hinderance [66] which prevents the hydroxyl groups of dextran from replacing the water molecules surrounding the phosphate heads. Wolkers et al. demonstrated that while dextran and a protein analogue were able to interact through hydrogen bonding, dextran and a lipid micelle had minimal interaction [16]. Thus, while dextran is an attractive excipient for thermal stabilization of vaccines due to its high T_g , its inability to form physical/chemical bonds with lipid membranes makes it a poor primary material for stabilizing enveloped viruses and as such should be used as a secondary excipient in blends at low relative concentrations.

Trehalose has been shown to effectively replace the water molecules surrounding the phosphate heads of the lipid membrane upon dehydration, satisfying the *water replacement hypothesis*. It is able to maintain the T_m of a lipid membrane upon dehydration, which is necessary to stabilize enveloped viral vectors [67]. As a disaccharide, trehalose possesses a relatively high T_g , and forms a primarily amorphous glass via spray drying, vitrifying the viral

vector. The depression of the T_g of trehalose during storage at elevated temperatures, suggests that the matrix experienced molecular movements resulting in the lower T_g [14]. This is apparent in the activity data of spray dried VSV (Figure 10), as the trehalose samples experienced increased activity loss over the first 10 days of storage before reaching a plateau. The material approaches the Kauzmann temperature leading to increased molecular mobility of the matrix which correlates with a decrease in activity up to Day 10. This depression could be due to residual moisture after spray drying as trehalose is known to be a hygroscopic material [68], however residual moisture testing was outside of the scope of this project. The plateaus observed in activity loss of VSV after Day 10 at 37 °C (Figure 10) with spray dried trehalose and trehalose/dextran (3:1 ratio) suggest that the molecular mobility of the matrix had reached equilibrium [14] and it is possible that further depression in T_g would not occur.

While trehalose was able to retain activity of the AdHu5 viral vector and outperform the liquid control, the mannitol/dextran blend was able to retain activity through all 30 days of storage at 37°C as shown in Figure 9. The mannitol/dextran blend was the best at minimizing activity loss of AdHu5 over time due to the high T_g of the blend and the ability of mannitol to form hydrogen bonds with the protein capsid of the non-enveloped virus [69].

As a component in blends for enveloped vectors, mannitol is not ideal, as the material readily crystallizes which could lead to crystal nucleation in the dry state and pierce the lipid membrane of enveloped viruses [58]. Polyols like mannitol can alter the phase of a lipid membrane from a bilayer to a hexagonal II phase, resulting in leakage [70]. Once the lipid membrane is perturbed, the genetic material of the virus will leak out and lower the infectivity of the viral vector. If the sugar is unable to stabilize the lipid membrane, the inner RNA will leak into the dry environment. The exposure of RNA to elevated temperatures in a dry state will lead to denaturation of the RNA as indicated by the diminished fluorescence response of our stored RNA control. The decrease in detectable RNA from Day 0 to Day 3 for all excipients (Figure 12) is a direct indicator of unprotected RNA, likely outside of the virus. A smaller decrease over time, however, suggests less exposed RNA as a result of better encapsulation. The VSV-mannitol/dextran particles had the lowest concentrations of detectable RNA both at Day 0 and Day 3 indicating significant denaturation. Trehalose and trehalose/dextran (3:1 ratio) maintained high concentrations of intact RNA after 3 days of storage, which correlates with the high activities observed *in vitro*.

After analyzing the trends and differences between the spray dried formulations with VSV, influenza and AdHu5 a set of general criteria can be identified for the production of thermally stabilize enveloped or non-enveloped viral vectors. When stabilizing non-enveloped viruses, a glassy matrix with high T_g is preferred that has minimal molecular mobility and encapsulates the virus. However, over time the amorphous phase of the matrix will shift, so materials that maintain T_g with time are ideal. Stabilization of enveloped viruses also require a glassy matrix with high T_g , but the formulation must contain sugars that have demonstrated the ability to form hydrogen bonds with the phosphate heads of lipid membranes to maintain fluidity of the lipid bilayer and prevent a phase transition to the gel state. Thus, when selecting excipients for

stabilization of enveloped viruses in the dry state both the T_g of the material and the T_m of the lipid must be considered.

4.5 Conclusion

It is apparent through our data that there are differences in the thermal stabilization of enveloped and non-enveloped when spray dried with the same formulations. Trehalose and trehalose/dextran (3:1 ratio) spray dried samples retained the most activity of enveloped viruses (VSV and influenza) and mannitol/dextran samples retained the most activity for the non-enveloped virus (AdHu). Conversely, mannitol/dextran was the least successful at stabilizing the VSV and influenza vectors. The RNA staining results demonstrated that poor performing excipient-VSV blends were unable to retain and protect viral RNA when stored in a dry state at high temperatures. A more comprehensive conclusion can be found in Chapter 5.

Chapter 5: Conclusions and Outlook

Thermal stabilization of vaccines will alleviate the demand for cold chain systems, improving access to resource poor areas of the world, and allow for stockpiling of vaccines for emergency use in the event of a severe disease outbreak. The spray drying of vaccines with carbohydrate-based formulations has shown potential in producing thermally stable dry powder vaccines and preliminary thermal stability with a variety of viral vectors has been achieved. For this thesis, we presented a spray dry formulation that excelled in thermally stabilizing VSV and investigated the stability differences between enveloped and non-enveloped viral vectors when spray dried with the same set of excipients.

In the VSV stabilization section (Chapter 3), we demonstrated the ability to thermally stabilize the VSV viral vector in a dry state through the process of spray drying. The trehalose and trehalose/dextran (3:1 ratio) formulation were the most successful at retaining viral activity at high storage temperatures. While the trehalose sample experienced an *in vitro* activity loss of 4 log after 30 days of storage at 37°C, it greatly outperformed other spray dried excipients and the liquid control which lost all activity after 15 days of storage. The immunogenicity of spray dried VSV was determined to be lower than the liquid control after storage, and the overall immune response of the fresh liquid control was deemed to be low and thus the *in vivo* experiments will need to be revisited in future studies.

In the enveloped versus non-enveloped section (Chapter 4), we presented differences in thermal stability between enveloped and non-enveloped viruses with various excipients. The trehalose and trehalose/dextran (3:1 ratio) spray dried samples outperformed the liquid control when stabilizing the enveloped viral vectors of VSV and influenza and were able to retain activity after 30 days of storage at 37 °C. The mannitol/dextran formulation was the worst at stabilizing the enveloped viral vectors but was the best at thermally stabilizing the non-enveloped viral vector, AdHu5. The RNA staining results of spray dried and stored VSV-excipient combinations highlighted the denaturation of RNA with poor performing excipients, whereby unprotected viral RNA became undetectable in dry, high temperature conditions.

5.1 Future Work

Future work should look at optimizing trehalose and blends with trehalose for thermal stabilization of VSV through adjustment of the various spray drying parameters as well as the concentration of components in the feed. For future *in vivo* studies, higher initial titres of VSVAg85A will be required to elicit a stronger immune response within the animal to better distinguish the effects of storage on the immunogenic response of a liquid control versus the spray dried VSV-trehalose powder. Additionally, characterization of the hydrogen bonding interactions between blends of sugars and lipids (used as analogues of the viral lipid bilayer envelope) would be beneficial to further understand the threshold concentration at which dextran begins to hinder the hydrogen bonding of other excipients in the blend and further support the *water replacement hypothesis* as the dominant stabilization mechanism with enveloped viruses. Finally, an in-depth study of the depression of the glass transition temperature over time as well as the relaxation enthalpy of various blends of excipients and how they relate to retained viral

activity would aid in the selection of excipients for vitrification without necessarily requiring extensive activity testing, this could significantly reduce testing costs in industry.

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