Cryoprotective agents influence viral dosage and thermal stability of inhalable dry powder vaccines

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Abstract

Increasing viral dosage within dry powder vaccines reduces the powder mass required to elicit an immune response through pulmonary delivery. This work analyzes how cryoprotective agents affect viral activity, particle properties and thermal stability of a spray dried, inhalable vaccine vector under high viral loading. Stock suspensions of a human serotype 5 adenovirus (AdHu5) vector in either neat phosphate buffered saline (PBS), 10% glycerol in PBS, or 5% trehalose in PBS were added to a mannitol-dextran formulation prior to spray drying. At high viral loading, spray dried powder containing glycerol had a viral titre log loss of 2.8 compared to 0.7 log loss using neat PBS. Powders containing glycerol had a lower glass transition temperature (T_g) compared to all other formulations, permitting greater viral mobility and exposure to heat damage. Inclusion of glycerol also promoted particle cohesion during spray drying and lower yields. Using 5% trehalose as a cryogenic alternative, viral powders had a viral log loss of 1.5 and the highest displayed thermal stability over time. Additionally, trehalose-containing powders had smaller particles with lower water moisture content and higher powder yield compared to glycerol-containing powders. These findings demonstrate the importance of cryoprotective agent selection when developing thermostable vaccine powders.

Key Words

adenovirus, spray drying, inhalation, glycerol, thermal stability, cryoprotective agent

1. Introduction

One of the greatest challenges in securing global vaccine access is the need for a reliable and economical temperature control infrastructure. Traditional vaccine storage and transport requires that temperatures be kept between 2 ºC to 8 ºC at all times, a protocol commonly referred to as the cold-chain (Nyberg-Hoffman and Aguilar-Cordova, 1999, Kroger, A Bahta, L Hunter, 2020). Depending on the biologic, ultra-low temperature storage down to -80 ºC may also be required to ensure vaccine stability (Emmer and Ertl, 2019). Failure to meet these rigorous protocols can result in vaccine wastage or risk of healthcare workers administering vaccines with decreased potency (Ashok et al., 2017). This requirement is particularly limiting for developing nations in tropical or semiarid climates that deal with unreliable power supply and frequent equipment failure (Bogale et al., 2019, Comes et al., 2018). These global regions are also disproportionately more impacted by the spread of infectious diseases such as tuberculosis (TB). The World Health Organization estimates that over 95% of all TB cases and resulting deaths occur in developing countries (World Health Organization, 2020). To reduce both disease prevalence and infrastructure limitations, scientific efforts are now focusing on developing vaccines that can maintain efficacy under elevated temperature conditions (Alcock et al., 2010, Ohtake et al., 2010). Thermostable vaccines will reduce global cold-chain dependency, offering a solution to the supply challenges in regions with limited resources.

One of the new TB vaccine strategies has focused on adenoviral vectors as effective modes for gene transfer and antigen delivery (Radošević et al., 2007, Ronan et al., 2009, Smaill et al., 2013, Afkhami et al., 2017). Although adenoviral vectors are highly unstable at and above room temperature, our group has previously shown that spray drying technology can effectively

encapsulate adenovirus and maintain thermal stability in powder form by using a mannitol-dextran excipient blend (Leclair et al., 2016a, Thompson et al., 2017, Toniolo et al., 2019). Immobilization of viral vectors or proteins within a glassy particle matrix prevents aggregation and denaturation to preserve biologic activity *in vitro (*Jin et al., 2010, Toniolo et al., 2019, Leclair et al., 2016a) and *in vivo* (Tyne et al., 2013, Afkhami et al., 2017). Excipient formulations used in spray dried micro-encapsulations must exhibit strong compatibility with the entrapped biologic to retain its activity, while also displaying appropriate particle morphology and thermal stability (Morgan et al., 2019, Toniolo et al., 2019).

Formulation selection in the context of spray drying typically refers to the excipients used for viral vector encapsulation, but the stock adenovirus suspension also has specific formulation requirements. Adenoviruses are highly susceptible to deactivation, therefore long-term storage protocols recommend the use of cryoprotective agents to prevent damage induced by freezing and thawing (Evans et al., 2004). These cryoprotective agents are often selected based on adenovirus purification procedures (Croyle et al., 1998), with many protocols specifying 10% glycerol in PBS due to the historical use of glycerol for cryopreservation of viruses and proteins (Kanegae et al., 1994, Altaras et al., 2005, Hubálek, 2003). Sucrose is another commonly used cryoprotective agent, known to achieve long term AdHu5 stability in liquid formulations stored between 2-8 ºC (Sene, 1998, Evans et al., 2004). Although sucrose is effective at maintaining adenovirus infectivity under refrigeration conditions, relatively high concentrations of the excipient are required to achieve stability at elevated temperatures (Sene, 1998, Rexroad et al., 2003, Pelliccia et al., 2016). As a non-reducing sugar, trehalose has interesting potential as a cryogenic alternative that can stabilize viral vectors under frozen conditions but has not been as extensively studied to date (Croyle et al., 2001). Adenovirus stability has been well documented in the context of

cryopreservation and liquid vaccine formulation, but the impacts of stock viral suspension within spray dried powders has not been previously reported in literature.

Administering spray dried powders as inhalable vaccines is particularly advantageous for respiratory diseases like TB as they can reach the deep lung for a targeted immune response (Saluja et al., 2010). For inhalable solid dosages, it is crucial that the powder has correct particle size for deep lung penetration and contains sufficient viral potency to elicit an immune response *in vivo*. Viral losses will occur during the spray drying process and within the lungs during pulmonary delivery, so initial viral loading (i.e., the volume of adenovirus stock suspension added to an excipient formulation) must account for anticipated losses as well as the necessary dose to achieve efficacy. However, increasing viral potency of a spray dried vaccine powder for stronger immunogenic response *in vivo* requires that adenoviral stock suspension be carefully considered for its impact on created particles. Initial trials by the authors to increase potency by 3-10 times, simply through increasing the quantity of added adenoviral stock, produced vaccine powders with very low yields and poor adenovirus activity, contrary to the intent of increasing viral dosage. This study explores the influence of cryoprotective agents within adenovirus stock suspension on the spray drying of a human serotype 5 adenovirus vector expressing GFP (AdHu5-GFP) encapsulated in a mannitol-dextran excipient blend. The goal of this work was to create a vaccine powder of higher viral potency, while also maintaining thermal stability and inhalable powder properties at higher viral loading.

2. Materials and Methods

2.1 Chemicals and Biologics

Dextran (M_r 40000 Da) and D-mannitol were sourced as USP grades from Millipore-Sigma (Ontario, Canada). D-(+)-trehalose dihydrate and glycerol were also purchased from Millipore-Sigma (Ontario, Canada). Water was purified in house utilizing a Barnstead GenPure Pro purification system operating at a resistivity of 18.2 MΩ-cm from ThermoFisher Scientific (Waltham, MA, USA). Cell culturing was performed using A-549 epithelial lung tumor cells grown in α-minimum essential media (α-MEM) combined with 10% fetal bovine serum and 1% streptomycin/penicillin, as prepared based on Life Technologies protocols from Invitrogen (ON, Canada). A recombinant, replication-deficient human serotype 5 adenovirus vector was prepared locally in the vector facility within the McMaster University Immunology Research Centre and modified to express Green Fluorescent Protein (GFP), referred to as AdHu5-GFP. Stock suspensions of the adenoviral vector were prepared in parallel using either phosphate buffered saline (PBS), 10% glycerol in PBS or 5% trehalose in PBS. Initial viral titres were determined via plaque forming assay and were found to be $5.7 \times 10^8\ pfu/mL$ (PBS buffer), $1.4 \times 10^{10}\ pfu/mL$ (10% glycerol in 1X PBS) and $4.4 \times 10^8\ pfu/mL$ (5% trehalose in 1X PBS).

2.2 Sample Preparation

Excipient solution was prepared at a concentration of 1% solids by weight containing a mixture of mannitol and dextran (3:1 ratio by weight respectively) dissolved in purified water. This ratio of excipients was selected based on previous work reporting successful viral encapsulation and thermal stability of the AdHu5-GFP vector (Leclair et al., 2016a). All physical characterizations were conducted on placebo powders, in which a 60 µL solution of cryoprotective

agent in PBS (referred to as the placebo storage solution) was added to 10 mL of excipient feed solution and subsequently spray dried. The placebo powders used for particle characterization did not contain AdHu5-GFP vector. Placebo storage solutions were prepared by adding cryoprotective agents to autoclave sterilized PBS at the concentrations outlined in Table 1; the table includes calculated relative weight contributions of cryoprotective agents and salt residuals within the spray dried powders. Calculated compositions do not account for any residual moisture that may be retained in the powder after spray drying. The calculated dry composition values were determined based on spray drying a 10 mL excipient batch size loaded with 60 µL of placebo storage solution.

Powders containing AdHu5-GFP were prepared by spray drying a 10 mL excipient feed solution containing 10 µL, 30 µL, 60 µL or 90 µL of the adenoviral stock suspensions described in Section 2.1. Spray drying small batch volumes in these trials was intended to conserve usage of the viral vector.

Table 1. Placebo storage solutions used to prepare spray dried mannitol-dextran placebo powder and the calculated relative contributions of cryoprotective agents and salt residuals within the final dry powder.

Placebo Storage Solution	**Cryoprotective Agent Composition**	**Composition in Dry Powder (wt.%)**	
	% Glycerol in 1X PBS (by weight)	***Glycerol***	***Salts***
1	0	0.0	1.2
2	2	1.5	1.1
3	5	3.6	1.1
4	10	7.0	1.0
5	15	10.1	0.9
	% Trehalose in 1X PBS (by weight)	***Trehalose***	***Salts***
6	5	2.9	1.1
7	10	5.6	0.5

2.3 Spray Drying Parameters

All spray dried powders were produced using a B-290 Mini Spray Dryer manufactured by Büchi (Switzerland) using a 0.7 mm nozzle. To ensure processing consistency, all spray dryer settings were the same for each batch. A feed flow rate of 234 mL/h (pump setting 13%) was used with a spray gas flow rate of 357 L/h (rotameter reading 30 mm), an aspirator flow rate of 35 m^3/h (100% aspiration) and inlet temperature of 120 ℃. These conditions were selected to optimize yield, viral activity and particle size (LeClair et al., 2016b). Outlet temperature was maintained between 56-62 ℃. Upon completion, powders were transferred from the collection tube of the spray dryer into microcentrifuge tubes within containment of the biosafety cabinet.

2.4 Collected Yield and Handling of Dry Powder

Collected yield was calculated as the percentage of removable powder mass recovered from the collection vial compared to the total mass of mannitol-dextran excipient in the feed solution prior to atomization. Low collected yields indicate that powder residual was stuck to the walls of both the cyclone and collection vial, limiting powder removal. Following powder collection, all powders were stored in the presence of Drierite® anhydrous indicating desiccant (W.A Hammond Drierite Company Ltd.) in sealed tubes within a benchtop desiccator at room temperature. To avoid exposure to ambient humidity during transport between workspaces, samples were stored in a vacuum sealed container containing additional desiccant.

2.5 A549 Cell Culturing

A batch suspension of low passage A549 cells were thawed from cryo-storage in liquid nitrogen. Cells were added to α-MEM that was pre-warmed to 37 ºC and immediately spun down in a centrifuge at 1400 rpm for 5 min. After discarding the supernatant, the cell pellet was

resuspended in media to form a single cell suspension before plating in T150 flask. The flask was incubated overnight in a CO_2 water jacketed incubator from Forma Scientific Inc. (Marietta, OH, USA) at 37 ºC and 5% CO_2. Cell media was changed the next day to remove dead cells and residual dimethyl sulfoxide (DMSO) from the media used in cryo-storage. Cells revived from cryo-storage were passaged twice before *in vitro* testing was conducted. Upon reaching 80-90% confluency, cells were plated in a 96-well plate for viral activity testing and passaged into a fresh T150 culture flask.

2.6 Viral Activity Testing via Flowcytometry

Viral activity was tested in vitro by infecting 40,000 confluent A549 cells with spray dried vaccine powders, as described previously (Morgan et al., 2020). Each spray dried sample was reconstituted in α-MEM to a 1% solids concentration. Cell infection took place within one hour of spray drying to neglect the influence of varying storage conditions in our analyses. Following infection, culture plates were incubated at 37 ℃ for approximately 24 h. Media was then aspirated from each well and cells were washed with 1X PBS prior to trypsinization. Each sample was transferred as a single cell suspension to a 4 mL tube and spun for 5 min at 1400 rpm in a centrifuge. After decanting, 1 mL of 1X PBS/ 2 mM EDTA (prepared in house) was added to each sample before centrifuging again at 1400 rpm for 5 min and decanting. To facilitate cell fixation, 1 mL of 1% paraformaldehyde (PFA) prepared in house was added to each sample. This was followed by a 10-min incubation period at room temperature. A third and final spin at 1800 rpm was performed and remaining liquid was decanted before adding 200 µL of flow cytometry staining buffer (FACS buffer, prepared in house) to each sample.

Flowcytometry was performed using a CytoFlex LX flow cytometer from Beckman Coulter Life Sciences (Indianapolis, IN, USA) with CytExpert software used for data acquisition.

A minimum of 10,000 events were analyzed per sample, representing at least 25% of cells plated. GFP expression was determined using FlowJo® software from BD (New Jersey, USA). Auto-gating of the data was used to identify the live cell population and remove artifacts of cellular debris and doublet formation that could skew the results. The overall percentage of GFP expression was compared to a negative control. A calibration curve was then used to correlate the percentage of GFP to the viral multiplicity of infection (MOI) which represents the ratio of plaque forming units to the number of cells tested. A viral titre was calculated in terms of pfu/mL, followed by pfu/mg to normalize based on collected powder weight. Results were discussed in terms of viral titre log loss to compare final titres with the initial amount of virus loaded in each sample.

2.7 Aging and Thermal Stability of Viral Vector Powder

The impact of each stock viral suspension on spray dried powder thermal stability was assessed using accelerated aging conditions. Each vaccine powder was incubated at 45 ºC in a water bath (ThermoFisher Scientific, Waltham, MA, USA) for 72 hours prior to testing viral activity via flowcytometry. To prevent any moisture uptake during the aging process, samples were stored in microcentrifuge tubes sealed with Parafilm®, which were placed within a plastic bag containing desiccant (Toniolo et al., 2019). The bag was stored in a glass jar sealed with Parafilm® containing additional desiccant and finally placed into a larger plastic bag before placing in the water bath. After the 72-hour incubation period, samples were removed from the water bath and diluted in α-MEM for subsequent cell infection. Viral activity testing via flowcytometry was conducted the following day. Replicate testing was conducted for all sample points, with a minimum of two true replicates per cryogenic agent tested.

2.8 Particle Characterization

2.8.1 Thermal Properties of Spray Dried Powder

Thermograms for each spray dried placebo powder were generated using a Q200 Differential Scanning Calorimeter (DSC) from TA Instruments (New Castle, DE, USA). Between 6-12 mg of sample stored on desiccant was weighed into an aluminum pan that was hermetically sealed. The best technique found to highlight the glass transition temperature (T_g) of the simple and complex carbohydrates in the formulation involved two normal heat-cool cycles, first between 20-120 ºC and then 20-160 ºC with a ramp rate of 25 ºC/min. This was followed by a third pass slowly heating the sample up to 200 ºC at 2 ºC/min under modulation with an amplitude of $\pm$1 ºC and a period of 60 s. The T_g was quantified using Universal Analysis 2000 software from TA Instruments (New Castle, DE, USA). All placebo powders were tested in duplicate as true replicates.

2.8.2 Moisture Content of Spray Dried Powder

Retained moisture content of a spray dried powder was determined using a C10SX coulometric Karl Fisher titrator (Mettler Toledo, Columbus, OH, USA). The placebo powder was weighed in a glove box configured with a dry nitrogen flush to maintain a relative humidity of <10%. Samples were dissolved in 2 mL of anhydrous formamide prior to titration. A volume of 0.1 mL sample solution was injected in the titrator for measurement. After applying blank corrections, the moisture content was determined within each sample.

2.8.3 Particle Sizing

Placebo powder was dispersed from an ICOone® inhaler that was generously provided by Iconovo (Lund, Sweden). Particle size distribution was detected and analyzed using a Helos R-series laser diffraction sensor from Sympatec GmbH (Pulverhaus, Germany) outfitted with a R2

lens with a focal length of 50 mm. Reliable detection for the R2 lens ranges from 0.45 – 87.5 µm. Cumulative distributions of particle sizes were generated for powder samples of weighed mass between 8-11 mg. Stated particle size was based on the median particle diameter of the distribution (D50), representing the 50th percentile of the distribution. The 10th (D10), 50th (D50) and 90th (D90) percentiles of the particle size distribution were used to calculate span, shown in Eq.1.

$$Span = \frac{D90 - D10}{D50} \quad (1)$$

2.8.4 Imaging of Spray Dried Particles

To visualize particle morphology and relative aggregation, spray dried placebo powders were imaged using a Tescan Vega II LSU scanning electron microscope from Tescan Orsay Holding, a.s. (Brno, Czech Republic). Prior to imaging, samples were placed on double-sided carbon tape and sputter-coated with a layer of 24 nm of gold using a Polaron E5100 sputter coater from Quorum Technologies Limited (Laughton, UK). All images were captured using an electron accelerating voltage of 5 kV with a working distance ranging from 17.03-17.33 mm and a magnification of 4000.

2.9 Freeze-Thaw Stability of AdHu5 Vector Stock Suspensions

Freeze-thaw stability of AdHu5-GFP stock suspension was tested by thawing a single vial of AdHu5-GFP viral vector suspended in PBS. It should be noted that this vial had been thawed once before commencing freeze-thaw studies. Prior to storage at -85 ºC, the vial of AdHu5-GFP vector had an initial viral titre of 5.7x10^{8} pfu/mL. Once thawed, 10 µL aliquots of viral stock suspension were diluted in PBS by a factor of 100 and each respective cryoprotective agent was added to achieve the following concentrations by weight: 0% glycerol (PBS only), 5% glycerol, 10% glycerol, 5% trehalose and 10% trehalose. From this viral buffer dilution, 100 µL was

removed for viral titre determination via flow cytometry using the protocol outlined in Section 2.6. Remaining samples were frozen at -85 ºC for 7 days before thawing at room temperature and repeating the activity test. For each subsequent freeze-thaw cycle, 100 µL was removed from each respective storage buffer. This process was repeated four times. Sample dilution was accounted for in the calculation of viral titre at each freeze-thaw cycle.

Results and Discussion

3.1 Impacts of Buffer Composition on Viral Activity

Preparing a vaccine powder for inhalable delivery requires that the adenovirus is active and is delivered at sufficient concentration to elicit an immune response. Our previous work focused on stabilizing the viral vector but only studied *in vivo* immunogenicity of reconstituted powders prepared with 10 µL adenoviral stock suspension per 100 mg excipient, resulting in a viral titre of ~10^6 pfu/mg in the spray dried powder (Afkhami et al., 2017). Although this viral dosage was sufficient for intramuscular delivery in mice, evaluation of this powder through pulmonary delivery requires an increased dosage up to 9-fold to achieve a detectable response. Following conventional purification and cryo-storage protocols for AdHu5, the adenoviral stock suspension contained 10% glycerol in PBS in our initial trials to increase dosage. It was not initially anticipated that increasing the volume of stock adenoviral suspension in the excipient solution would have a negative impact on viral activity in the spray dried product. However, Figure 1 shows that increased loading of the adenoviral stock suspension containing 10% glycerol led to substantially increased viral titre log losses upon spray drying up to 3.3 log loss at a high viral loading of 90 µL added to 100 mg of excipient.

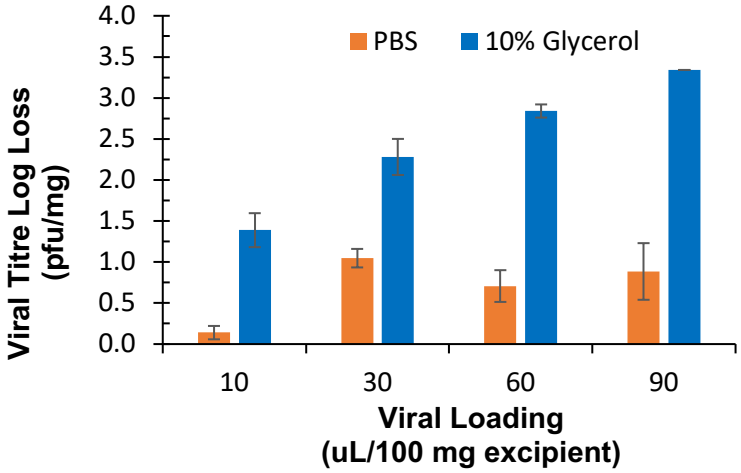

Figure 1. AdHu5-GFP viral activity log loss in spray dried powders as a function of viral loading with either neat PBS (no glycerol) or 10% glycerol in PBS as the adenoviral stock suspension. Error bars represent the standard error between averaged duplicates.

In contrast, when the PBS adenovirus stock suspension was added to the formulation, spray dried powders showed more acceptable changes in viral titre log loss when viral loading was increased from 10 to 30 µL/100 mg excipient. Viral activity then remained relatively constant at 1 log loss up to the highest viral loading of 90 µL/100 mg. For both stock adenoviral suspensions used, an increased viral loading corresponded to an increase in the amount of buffer salt within the spray dried matrix composition. Viral activity was essentially preserved during spray drying with increased PBS stock adenoviral suspension, suggesting that the buffer salts are much less detrimental to viral stability in the powder product compared to the cryoprotective agent glycerol. Ohtake et al. similarly found that viral titre process losses did not increase significantly by adding a potassium phosphate buffer in concentrations between 25-50 mM to an excipient solution prior to spray drying measles virus (Ohtake et al., 2010). For comparison, all tested adenoviral stock suspensions in the present study contained a low concentration of potassium phosphate of 1.76 mM in addition to 134 mM sodium chloride and 100 mM disodium phosphate salts. The slight

increase in AdHu5-GFP titre log loss observed for PBS loading of 30 µL/100 mg and higher could indicate the need for buffer salt optimization in the adenoviral stock suspension, but this was beyond the focus of the present study.

3.2 Impact of Increasing Stock Viral Suspension on Collected Yield

Using the adenoviral stock suspensions with and without glycerol, Figure 2 reveals that the cryoprotective agent presented significant challenges in collecting adequate process yields. We observed that increasing the volume of glycerol in the feed solution resulted in a strong negative correlation with the yield collected. Qualitatively, the higher glycerol content caused the powder to become increasingly adhesive on the glassware of the spray dryer. Much of these yield losses occurred due to particle adhesion on the walls of the spray dryer's cyclone and drying chamber which limited the amount of powder that could be collected. In comparison, by removing glycerol from the adenoviral stock suspension, the collected yields remained constant across all loadings of PBS adenoviral stock suspension. Similar findings have been reported by another group who experienced decreased yield when adding glycerol to a zein-based spray dried encapsulation of an antimicrobial peptide (Xiao and Zhong, 2011). With batch yields often used as an indicator of process efficiency, this data further highlights the importance of investigating cryoprotective agents for their impact on spray drying viral encapsulations effectively. The adenovirus was assumed to be evenly distributed within the excipient feed solution, meaning that increased yield losses proportionally reduced the amount of adenovirus recovered in the collection vial. It should be noted that small volume batches were prepared for this study which accounts for the low yields overall, but we still consider the trend meaningful. Process yields from a laboratory spray dryer are expected to increase as batch volumes are scaled up (Nekkanti et al., 2009).

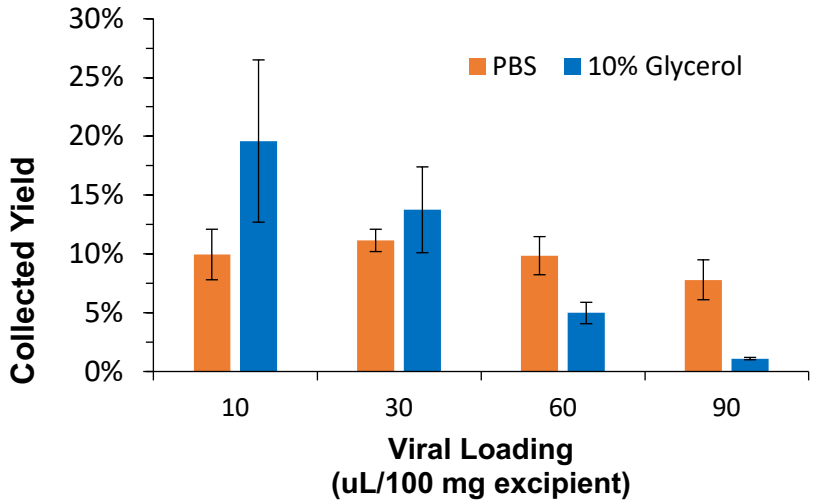

Figure 2. Percent yield of spray dried powder recovered from the spray dryer collection chamber based on a 10 mL batch volume with 1% solids concentration. Increased loadings of AdHu5-GFP (suspended in either PBS or 10% glycerol) were added to the feed solution prior to spray drying. Error bars represent the standard error between averaged duplicate samples.

It should be noted that there was no significant difference in average viscosity of the feed solutions containing placebo storage solutions with and without glycerol in Figures 1 and 2 (tested using a U-tube viscometer, data not shown). Wan et al. similarly reported no significant change in viscosity of a 1% *w/v* hydroxypropyl methylcellulose solution with 30% glycerol added prior to spray drying (Wan et al., 1992). Since our feed solution viscosity did not significantly change with the addition of glycerol, self-diffusion of the primary mannitol-dextran excipients and virus as well as the initial droplet drying dynamics are all expected to be consistent between formulations.

3.3 Influence of Cryoprotective Agents of Powder Properties

To further understand the direct impact of glycerol on powder properties, the amount of added glycerol in the spray dried powder was varied while keeping the concentration of excipients, virus, and salt constant, according to Table 1. Figure 3 shows that as glycerol content in the dry powder increased from 0 wt.% to 10 wt.%, there was a corresponding increase in viral titre log

loss of 0.7. For reference, the dry powders previously discussed in Figures 1 and 2 contained 10 wt.% glycerol at a loading of 90 µL/100 mg excipient. Discrete ranges of error did not overlap between the 0 wt.% glycerol and 10 wt.% glycerol dry powder formulations, thus indicating a statistical difference between these datasets. Since viral load and salt concentration were consistent for all preparations in Figure 3, glycerol content was the fundamental difference between particle composition in this case. Although glycerol represented a minor component of the overall particle matrix, it hindered the ability to produce a viral powder of sufficient AdHu5-GFP activity.

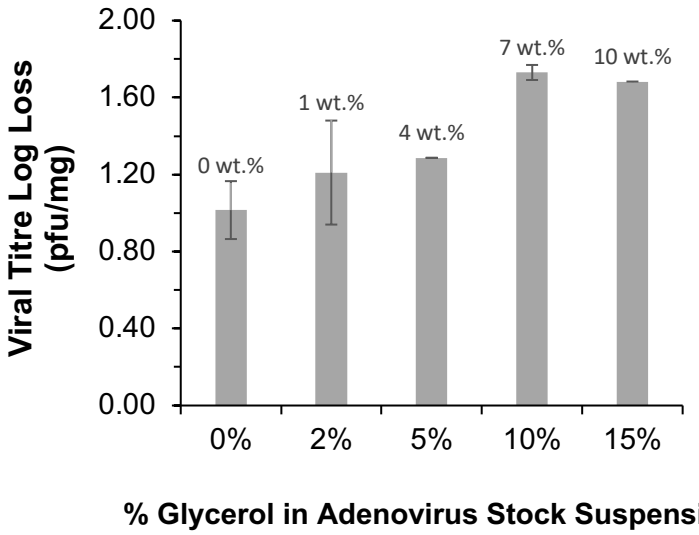

Figure 3. Log loss (pfu/mg) of AdHu5-GFP viral titre in spray dried powder as a function of increasing glycerol concentration within the adenovirus stock suspension. Data labels refer to the wt.% of glycerol incorporated within the spray dried particle matrix. All samples contain the same viral loading of 60 µL/100 mg excipient and equivalent salt content. Error bars represent the standard error between averaged duplicate samples.

Placebo powders were produced with either glycerol or trehalose as the cryoprotective agent and the resulting powder properties were characterized (Table 2). Trehalose was selected as an alternative to glycerol, based on its ability to participate in thermal stabilization of the powder

(Leclair et al., 2016a) and prevention of adenovirus deactivation due to freeze-thaw damage (Hubálek, 2003). In terms of thermal properties, the spray dried powders containing glycerol were found to have decreased T_g values compared to those containing trehalose or PBS storage solution alone. For example, incorporating the 10% glycerol placebo storage solution led to a T_g that was 20 ºC lower than the preparation using PBS, whereas the powder prepared with a 5% trehalose placebo storage solution resulted in a T_g of 120 ºC (the highest of all samples tested). A plasticization effect was observed with the addition of glycerol that caused a decrease in particle T_g (Carrigy and Vehring, 2019). Considering the Kauzmann temperature is 50 ºC below the T_g, formulations that have a T_g of 100 ºC or below are more likely to experience virus mobility and are less desirable for long term room temperature storage (Toniolo et al., 2019). Since the spray dryer outlet temperature typically ranged from 55-65 ºC, particles containing 5% and 10% glycerol stock suspension could experience more viral deactivation while sitting in the collection tube during spray drying. Virus located at the particle surface seems especially vulnerable to heat damage and deactivation during spray drying (LeClair et al., 2016b). AdHu5 is particularly susceptible to capsid rupture and can experience a 3 log loss in viral titre after only one minute of exposure to 56 ºC if freely mobile (Pereira et al., 1967). Using 5% trehalose as an alternative cryogenic storage solution maintained the Tg high enough with respect to the Kauzmann temperature, impeding viral mobility in the dry powder and effectively preserving adenovirus functionality. Plasticization was not detectable in the trehalose formulation as compared to the formulations containing glycerol. As both a cryogenic agent and a non-reducing sugar, trehalose promotes adenovirus stability by helping to prevent stresses caused by dehydration during the drying process (Preston and Randolph, 2021). Since trehalose is able to form hydrogen bonds, it

replaces the role of water during the drying process to offer adenovirus stability as the glassy matrix forms (Ohtake et al., 2010, Toniolo et al., 2019).

Table 2. Summary of spray dried mannitol-dextran particle properties based on the addition of placebo storage solution added at a loading of 60 µL/100 mg excipient. Placebo storage solutions refer to the percentage of each cryogenic component in PBS before addition to the excipient formulation.

Placebo Storage Solution	Cryogenic Composition in Dry Powder	Glass Transition Temperature	Water Content	Median Particle Diameter, D50 [µm]	Span [µm]
5% Trehalose in PBS	3 wt.%	120 ± 10 ºC	3.76%	4.3	1.8
PBS Buffer	0 wt.%	118 ± 5 ºC	3.73%	5.6	1.9
5% Glycerol in PBS	4 wt.%	103 ± 4 ºC	4.89%	6.6	2.1
10% Glycerol in PBS	7 wt.%	98 ± 4 ºC	5.39%	6.6	2.0

Particle size and residual water content are important characteristics to be considered for spray dried powders intended for inhalation. Ideally, particles should be 5 µm or smaller to reach the deep lung region (Darquenne, 2012). All tested powders had particle sizes near or under the target median diameter and the span was relatively constant (Table 2). Particles containing 5% trehalose displayed an ideal median diameter of 4.3 µm which is highly suitable for pulmonary delivery. On the other hand, glycerol-containing particles were 1-2 µm larger than particles with trehalose or PBS alone. This trend was supported by SEM (Figure 4) where powders containing 5% and 10% glycerol placebo storage solutions both appeared to contain slightly larger particles than powders with PBS and 5% trehalose.

One of the contributing factors for the larger particle size was water content. Powders containing 5% trehalose or PBS storage solutions had low water content, around 3.75%, while the

powders containing glycerol had higher levels of absorbed water after being stored at comparable conditions. Water retention increased by approximately 1.5% for the glycerol containing particles, compared to preparations with trehalose or PBS alone. Glycerol is highly hygroscopic and has been noted to cause high moisture absorption within polymeric-protein microstructures (Chen et al., 2005). Due to this affinity for water, glycerol-containing powder experienced moisture swelling which was indicated by larger particles. Inhalable powders containing glycerol could have limited functionality in an inhaler due to moisture-related particle cohesion.

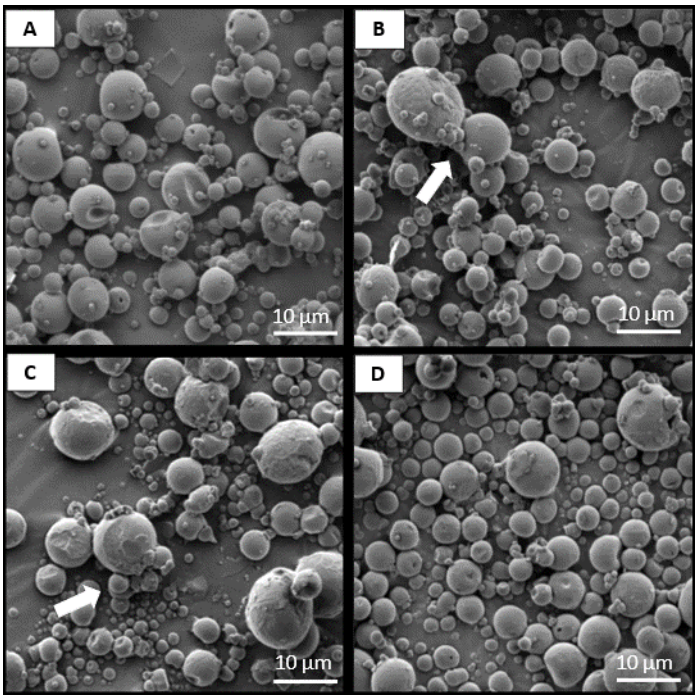

Figure 4. Scanning electron microscope (SEM) images of mannitol-dextran spray dried powders showing particle morphology for placebo powder samples with 60 µL/100mg excipient of the following storage solutions: (A) 0% glycerol, (B) 5% glycerol, (C) 10% glycerol, (D) 5% trehalose. Arrows indicate areas of particle bridging caused by cohesion. All images were captured at 4000X magnification with a scale bar representing 10 µm in length.

SEM micrographs suggested the particles were more cohesive for those powders containing either 5% or 10% glycerol storage solution, due to clumping shown in Figure 4. In

contrast, powders that contained only PBS or the 5% trehalose placebo solution did not display notable cohesive behaviour. Work conducted by Wan et al. has shown that spray drying plasticizers, such as glycerol, along with polymers for drug microencapsulation led to similar particle cohesion and agglomeration (Wan et al., 1992). The observed result of cohesion within powders containing glycerol directly affected the process yield from the spray dryer, as reported in Figure 2. During atomization, glycerol likely formed liquid capillary bridges with contact points on neighbouring particle droplets or the glass walls of the spray dryer to minimize interfacial energy (Herminghaus et al., 2019). In the initial stages of drying, particles experience high viscosity and surface tension around the glass transition region which can result in an increased bridge formation and higher cohesive strength or "stickiness" if the particles come into contact with the glass surfaces of the spray dryer (Wan et al., 1992, Foster et al., 2006, O'Callaghan and Hogan, 2013). Foster et al. have demonstrated that in amorphous sugars the rate of cohesion is proportional to the value of $T - T_{g,}$ such that liquid bridge formation is faster at temperatures exceeding the T_g (Foster et al., 2006). Since the outlet temperature and residence time in the spray dryer were constant for each batch of powder, the lower T_g associated with glycerol-containing powders led to an increase in the T-T_g value and a higher rate of cohesion.

In addition to glassware adhesion and yield loss during spray drying, there was also concern that particle cohesion will reduce the respirable fraction dispersed from an inhaler based on prior reported experiences for dry powders with higher water content (Maggi et al., 1999). To achieve efficacious delivery *in vivo*, residual moisture and subsequent cohesion must therefore be minimized. Based on these results, using 5% trehalose or neat PBS as a cryoprotective stock prevented particle cohesion appropriately within the spray dried powder.

3.4 Viral Buffer Freeze-Thaw Stability

Removing glycerol entirely from the stock viral suspension led to stronger viral activity, better powder properties and improved spray drying yield. However, the reason for including a cryoprotective agent within an adenoviral stock suspension must also be addressed to prevent issues during powder processing. A viral suspension often undergoes repeated freeze-thaw cycling prior to batch powder preparation, making cryoprotective agents crucial for maintaining adenovirus integrity. Figure 5 compares the viral titre of adenoviral stock suspensions containing either PBS, glycerol (5% and 10%) or trehalose (5% and 10%) during repeated freeze-thaw cycles. Each adenoviral stock suspension tested appeared to offer nearly identical cryoprotective behaviour as there were no significant differences at any of the sampling timepoints. However, it should be noted that these buffers were prepared by adding each cryoprotective agent to a thawed aliquot of virus originally suspended in pure PBS. This aliquot had an initial titre of $5.7\times 10^{8} pfu/mL$ but underwent one freeze-thaw cycle prior to commencing the freeze-thaw study. The drop in PBS viral titre to $3.3\times 10^{7} pfu/mL$ by the second freeze-thaw cycle was attributed to the lack of added cryoprotective agent. Adding a cryoprotective agent after an initial freeze-thaw did not appear to substantially alter the progression of additional viral damage. Without a cryoprotective agent (using only PBS), the viral titre decreased by 0.7 log between the second and fifth freeze-thaw cycle.

As an alternative to glycerol, trehalose has previously been used to protect viruses against damage due to freeze-thaw cycling (Hubálek, 2003). Based on identical performance between glycerol and trehalose as cryoprotective agents in this work, trehalose was considered a robust alternative to glycerol for cryo-storage of adenovirus intended for dry powder vaccine preparation. Moving forward, a concentration of 5% trehalose was preferred because it minimized the amount of cryoprotective agent added, while also maintaining cryogenic effectiveness compared to 10%

trehalose which did not appear to offer additional freeze-thaw stability. Incorporating trehalose as a cryoprotective agent at high concentrations will impact the excipient formulation intended to immobilize the virus within the spray dried powder. Although trehalose has robust stabilization behavior with most viral vectors upon spray drying, lower cryoprotective agent content will allow for more optimization opportunities. As noted above, spray dried powders formulated with 5% trehalose placebo storage solution offered ideal particle sizing for inhalation, a high T_g and low moisture content.

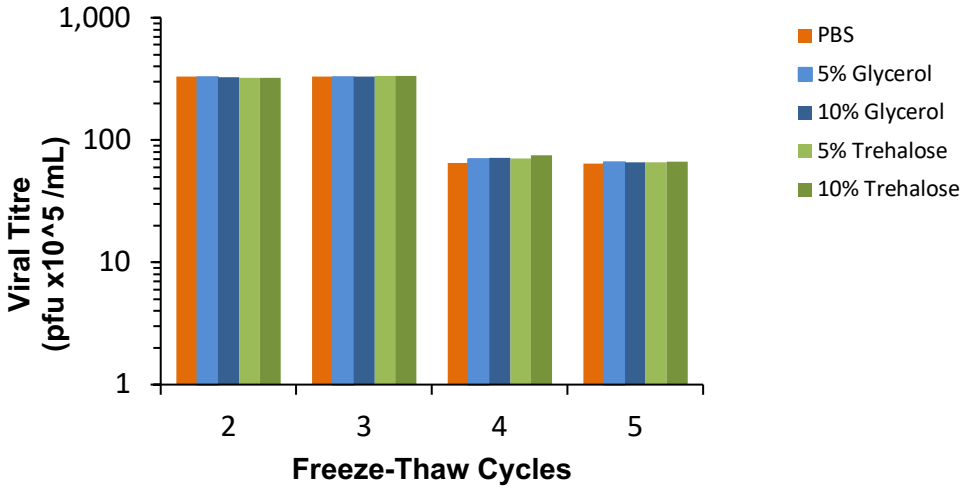

Figure 5. Viral titre of AdHu5 following progressive freeze-thaw cycling of adenoviral stock solutions containing PBS only, 5% glycerol, 10% glycerol, 5% trehalose and 10% trehalose, respectively.

3.5 Thermal Stability of Spray Dried Powder

To test the impacts of selected cryoprotective agents on thermal stability of the spray dried powder, viral activity was assessed after 72-hour exposure to 45 ºC (Table 3). These accelerated aging conditions are robust and comparable to the degradation tests set forth by the World Health

Organization to test viral stability of the oral poliomyelitis vaccine (Galazka et al., 1998). For accurate comparison, a high viral loading of 60 μL/100 mg excipient was used for each of the formulations tested. Viral losses related to the spray drying process were captured by the viral activity log loss of each 'fresh' sample. Losses related to the aging process and subsequent thermal stability of a formulation were indicated by the differences between viral log loss in fresh and aged samples. In all tested cases, the range of error for fresh and aged activity values did not overlap and therefore indicated significant statistical difference. Larger standard error was observed with larger sample sizes and was associated with natural variability between environmental conditions and cell culture.

Table 3. Viral titre log loss observed in mannitol-dextran spray dried powder with a viral loading of 60 μL/100 mg excipient of AdHu5-GFP stored in 5% trehalose in PBS, neat PBS and 10% glycerol in PBS adenoviral stock suspension. Samples were tested immediately after spray drying (fresh) and after exposure to accelerated aging conditions (aged).

Adenoviral Stock Suspension	**Collected Yield (%)**	**Sample Type**	**Viral Titre Log Loss (pfu/mg)**	**Sample Size (n)**
5% Trehalose in PBS	13 ± 2	Fresh	1.5 ± 0.3	n = 5
		Aged	2.0 ± 0.2	n = 2
PBS	10 ± 2	Fresh	0.7 ± 0.2	n = 3
		Aged	1.98 ± 0.03	n = 2
10% Glycerol in PBS	5 ± 1	Fresh	2.84 ± 0.08	n = 2
		Aged	3.4 ± 0.0	n = 2

Addition of a 5% trehalose viral stock suspension allowed for relatively low process losses (1.5 log loss) and excellent thermal stability with an additional 0.5 log loss due to high temperature aging. In combination with higher collected yields, 5% trehalose stock viral suspension helped to maintain the intended thermal stability and preserved activity under high viral dosage. Without a cryoprotective agent, the formulation containing only PBS showed the lowest thermal stability,

with an additional 1.3 log loss of viral titre during the aging process. Despite having lower initial process losses, removing the cryoprotective agent appeared to hinder adenovirus stability during prolonged exposure to high temperatures. In comparison, the addition of a 10% glycerol stock viral suspension only resulted in an additional viral titre log loss of 0.6 following aging but was also associated with low collected yields and high process losses. The order of decreasing thermal stability coincided with the decrease in respective T_g presented in Table 2, reiterating that the formulation with 5% trehalose adenoviral stock suspension experienced less molecular mobility and less viral damage under thermal stress. Targeted losses in adenovirus vaccine processing were around 0.5 log loss (Evans et al., 2004), which indicated the need for further processing improvements to reach pharmaceutically acceptable limits for this formulation. It is understood that with additional process optimization (LeClair et al., 2016b), viral losses occurring during spray drying can be further reduced.

4. Conclusions

Despite the necessity of cryoprotective agents for long-term freeze-thaw viral stability, the consequences of their addition within thermally stable vaccine preparations cannot be overlooked. It has been shown that introducing small volumes of glycerol into an excipient blend had a plasticizing effect on the excipient carrier in a spray dried powder. This impacted viral immobilization, consequently causing a reduction of adenovirus activity under high viral load. Although this work only focuses on the encapsulation of an AdHu5 vector, it is expected that the plasticizing effect of glycerol could also impact the yield and activity of other spray dried vectors. Interactions between glycerol and a particle matrix are applicable to most excipient sugars, suggesting that processing efficiency of other spray dried formulations may be hindered by the

inclusion of glycerol. Removing the cryoprotective agent entirely from the adenoviral stock suspension improved the viral activity of spray dried powder, but this method also negatively impacted freeze-thaw stability and decreased thermal stability. As a cryoprotective alternative to glycerol, it was demonstrated that a 5% trehalose adenoviral stock suspension did not depress the T_g of a mannitol-dextran spray dried powder and retained viral activity and thermal stability. Sucrose is another cryogenic alternative that could be further explored for this purpose. Though not considered in the present study, our initial testing showed that 5% and 10% sucrose could provide freeze-thaw stability similar to 5% trehalose (data not shown). However, the impact of incorporating sucrose within a mannitol-dextran spray dried encapsulation remains unknown. Cryoprotective agents may account for less than 10 wt.% of the overall spray dried particle composition, but they are shown in this study to play significant roles as matrix components and influence viral encapsulation, particularly at high viral loading. Improving viral load in spray dried vaccines to optimize *in vivo* delivery and immunogenic response must therefore consider the impacts of all ingredients, even those in the stock viral suspension.

Acknowledgements

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Cryoprotective agents influence viral dosage and thermal stability of inhalable dry powder vaccines: Supplementary Material

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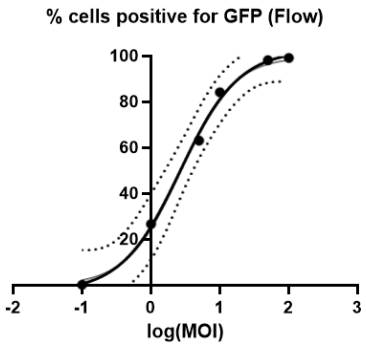


Figure S1. Standard curve used to convert the percentage of cells expressing green fluorescent protein (GFP), determined via flowcytometry, to a corresponding modality of infection (MOI). Calibration was determined based on measuring GFP expression of samples with known MOI of 0.1, 1, 5, 10, 50, 100.

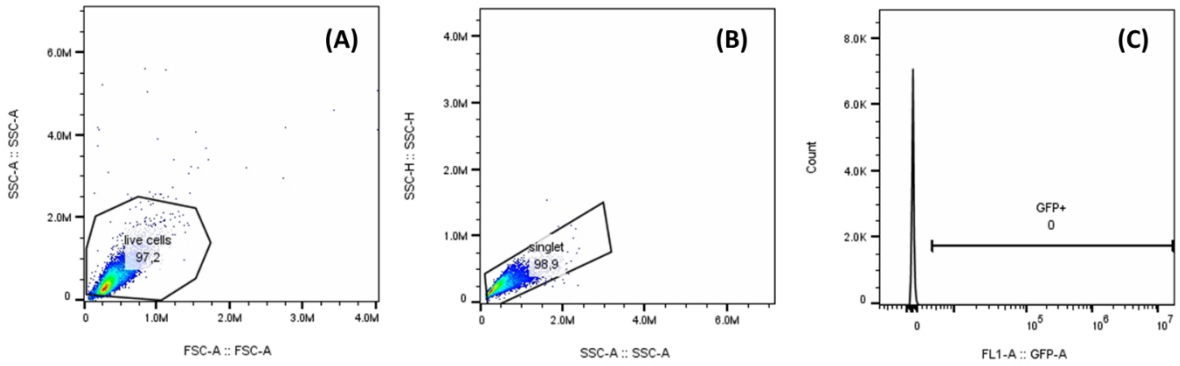
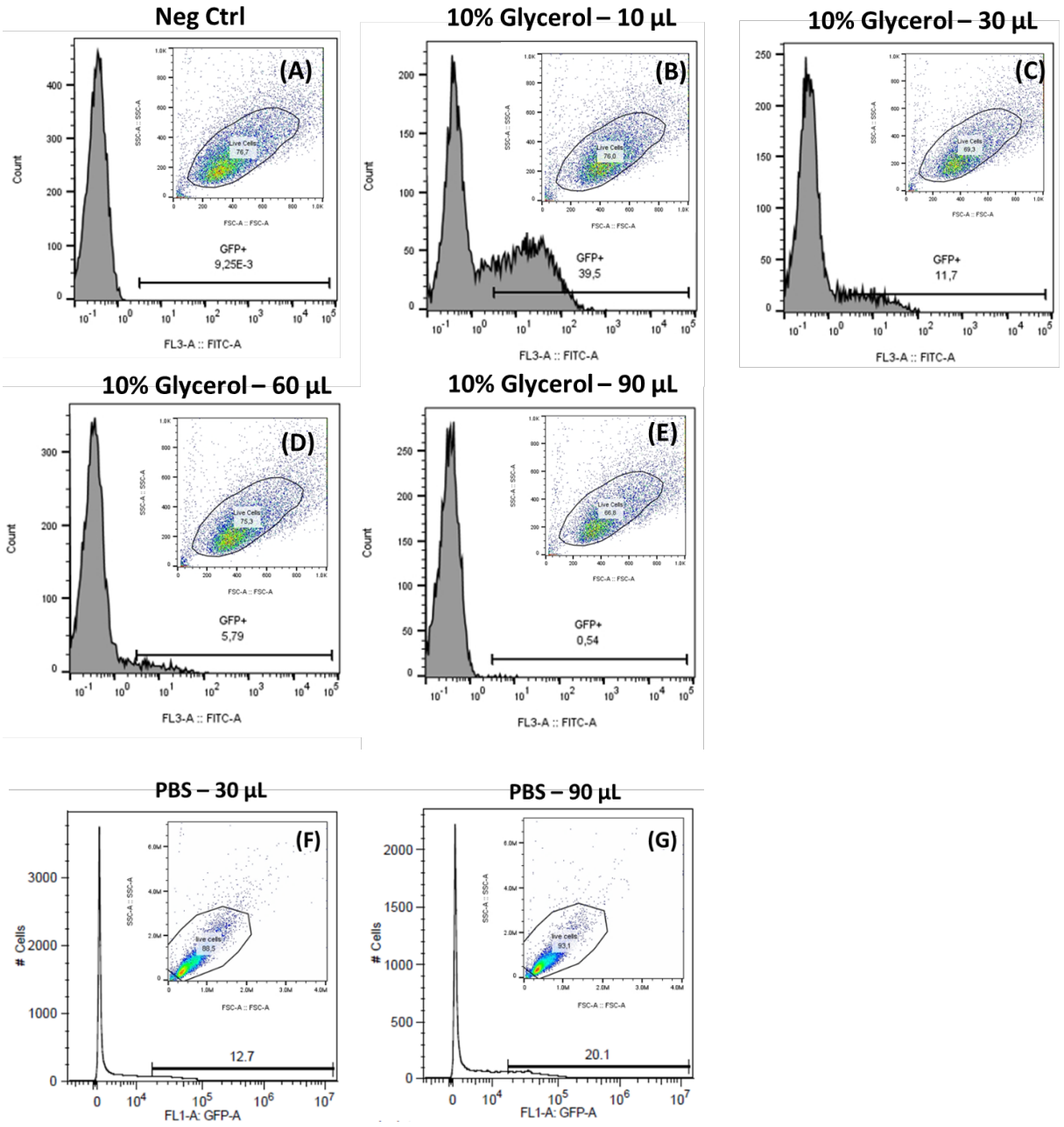


Figure S2. Cell population gating process of an uninfected negative control sample using FlowJo software. The autogating tool was used to select the live cell population from the scatterplot (A), followed by a user-defined gate to seperate live cell singlet events from non-singlet events (B) and a final user-defined gate to determine positive GFP expression in live cells as compared to the negative control population (C). For infected cell populations, viral titre is normalized based on mass of reconstituted powder as well as volume and intial titre of stock suspension.



710 **Figure S3.** Histogram counts of cells expressing GFP and corresponding event scatterplot (inset) for each sample analyzed via flowcytometry. Panel (A) represents an uninfected cell populaiton as a negative control. Samples shown were infected with reconstituted spray dried powder prepared with either a 10% glycerol AdHu5 stock suspension added at volumes of (B) 10 µL, (C) 30 µL, (D) 60 µL and (E) 90 µL or a PBS-only AdHu5 stock suspension added at (F) 30 µL and (G) 90 µL.

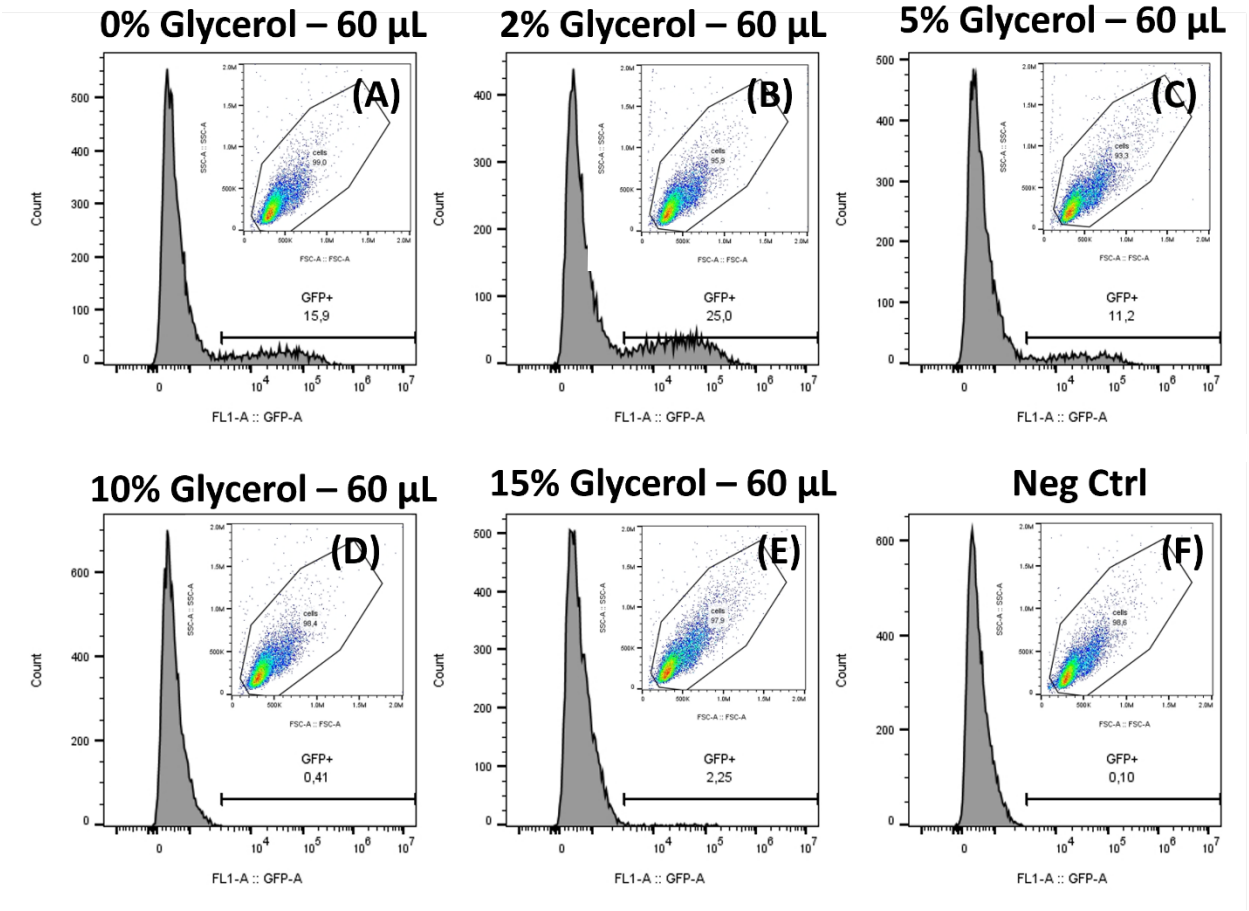


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Figure S4. Histogram counts of cell GFP expression and corresponding event scatterplot (inset) in samples analyzed via flowcytometry where cells were infected with reconsititued powder spray dried with 60 µL of AdHu5 stock suspensions containing (A) 0% glycerol, (B) 2% glycerol, (C) 5% glycerol, (D) 10% glycerol
720 and (E) 15% glycerol. Panel (F) represents an uninfected cell population as the negative control.

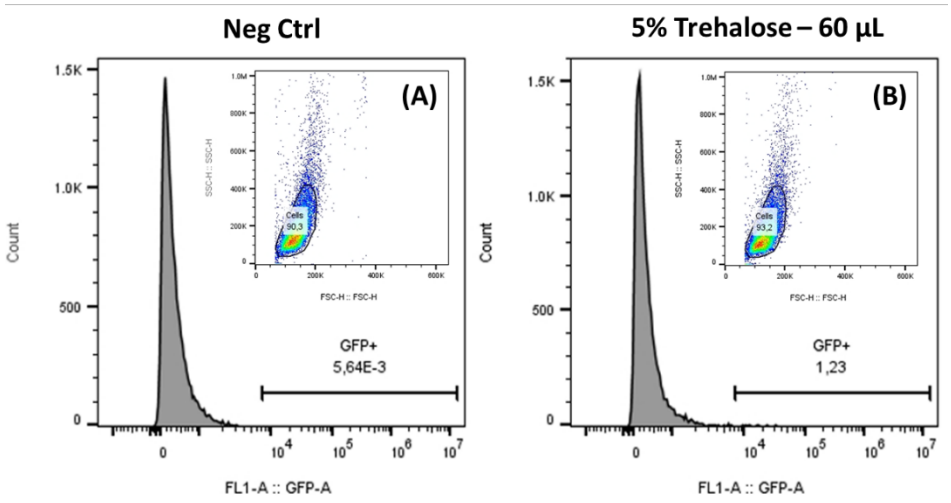

Figure S5. Histogram counts of positive GFP expression and corresponding event scatterplot (inset) analyzed via flowcytometry where cells were either uninfected (A) or infected with reconsititued powder spray dried containing 60 µL of 5% trehalose AdHu5 stock suspensions.