Screening amino acid additives as aerosolization modifiers for spray dried inhalable viral-vectored vaccines

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Abstract

Adenoviral vectored vaccines are a promising platform for immunization against respiratory diseases. Despite the advantages offered by dry powder over parenteral administration, like thermal stability and possible immunization at the site of infection, aerosolization of such dry powders is challenging. In this study, eight amino acids were investigated to improve the aerosolization of spray-dried human adenovirus type 5 (AdHu5) in a mannitol/dextran matrix. All samples were characterized for relative yield from spray drying, particle size and morphology, thermal properties, residual moisture content, aerosol performance, and bioactivity of the encapsulated AdHu5. All particles were in the respirable size range (except glycine and tryptophan); however, most other amino acid-containing powders did not improve or detract from the aerosolization properties of the spray dried powders compared to the control binary blend comprised of mannitol/dextran only. All powders (except glycine) showed glass transition temperatures (T_g) considered adequate to retain thermal stability of the viral vectors in powder when stored at room temperatures. Among the samples screened, amino acid-containing samples showed reduced viral activity than control. To investigate the reduction in viral activity of AdHu5 upon addition of amino acids, further studies are required.

1. Introduction

Dry powder vaccines have drawn interest, especially for efficient global vaccine distribution with reduced wastage, especially in resource-poor areas.[208] Dry powder vaccines offer several advantages over liquid vaccines, such as thermal stability, reducing the need for stringent cold chain protocols and offering extended shelf life, thus facilitating stockpiling as needed for pandemics.[11,172,209] Additionally, their inhalation allows non-invasive and easy administration of vaccines with less need for trained personnel.[17,210] Dry powder inhalation is an attractive method for pulmonary immunization, facilitating both mucosal and systemic immunity against respiratory diseases such as tuberculosis, influenza, and severe acute respiratory syndrome coronavirus (SARS-CoV-2) by neutralizing pathogens at the site of infection.[173]

Ideal particles for pulmonary delivery have aerodynamic sizes less than 10 μm to reach the lungs (above which the particles will deposit in the proximal airways mainly at the mouth, throat and the larynx), between 2-5 μm for deposition on the central airways, and 0.5-2 μm for peripheral airways.[17,100,211–213] Therefore, powders should be well aerosolized after inhalation with a high fine particle fraction (i.e., the fraction of particles below 5 μm) for enhanced immunogenic efficiency of the vaccine. Furthermore, low interparticle cohesive forces are essential to ensure proper flowability, de-agglomeration, and aerosolization of the dried powder.[163] These interparticle interactions are governed by electrostatic, hydrophobic, and capillary forces and can complicate dispersion of the vaccine due to particle aggregation in the inhaler.[214] Some of the commonly used approaches to enhance powder aerosolization include the addition of specific antistatic agents, co-delivery with carrier particles, or modification of the surface morphology (e.g. corrugated or textured).[186,214,215] However, use

of large carrier particles has the negative effect of diluting the active (bio)pharmaceutical ingredient (API), thereby reducing the potency of the inhaled powder.[29] In fact, corrugated particles are preferred for inhalable drugs since their decreased contact area caused by surface wrinkling lowers interparticle interactions.[186–188] These particles usually have a core-shell structure with a solid core composed of excipients preferred for interaction with the API and surface active agents for forming an outer shell.[192,216,217] Fortunately, particle properties, such as size, morphology, and moisture content can be controlled by optimizing various parameters in the spray drying process.[15,216,218]

Due to their surface active properties, amino acids are often considered shell forming excipients to enhance the aerosol properties of dry powder vaccines. Various amino acids, especially leucine, have been previously tested to improve pulmonary delivery of therapeutic molecules such as respiratory drugs, antibiotics, and antibodies.[22,131,132,180,183,189,211,219] In addition, formulations containing leucine as one of their components exhibit the desired corrugated particles as well as improved storage stability and protection from humidity.[188] There are no studies, however, considering how these amino acids affect the function and performance of dry powder vaccines based on viral vectors.

Biologics, like viruses, are sensitive to their environment, including several types of spray drying associated stresses like shear stress, thermophysical stresses, interfacial stresses, and dehydration.[26] Adenoviruses are non-enveloped viruses and considered more susceptible to such stresses with resulting losses of infectivity and efficacy than enveloped viruses. In addition to process stresses, certain excipients in formulated vaccines can alter the bioavailability and stability of the API.[220] In the case of amino acids, certain species have been noted to interact detrimentally with viral vectors either by chemical interactions[221] or

electrostatic interactions,[222,223] meaning that their positive contributions to particle flow may also have a negative impact on the vaccine efficacy. The viral vector used in the present study is a human serotype 5 adenovirus (AdHu5) which is a commonly selected vaccine platform for immunization against mucosal pathogens, including tuberculosis and COVID-19.[9,224] Understanding how amino acids in spray drying formulations affect AdHu5 activity as well as aerosolization properties of the powder produced is thus crucial for vaccine development.

The intent of the work was to determine whether the aerosolization of mannitol/dextran powders can be improved for inhalation by addition of shell forming amino acids. Amino acids have been established as enhancing aerosolization characteristics in spray dried powders containing small molecules like drugs, proteins, enzymes, and antibodies, but not always with consideration for their interactions with these ingredients. Research on interactions with adenoviruses (and other viral vectors) is critical for the emerging field of inhalable dry powder vaccines and yet such guidance is currently missing from the literature. It is natural to assume a viral vector will display sensitivity to amino acids (based on their molecular weight, polarity, and charge) and therefore analyzing the critically important viral activity compared to aerosolization performance is imperative. Therefore, the first step towards developing well aerosolized spray dried viral-vectored vaccines was to screen the well known aerosolization enhancers. This study evaluates the selection of amino acids as excipients for their effect on aerosolization and bioactivity of spray dried viral vectored vaccines. As such, a variety of amino acids were investigated from both polar and non-polar species, varying in their hydrophobic properties (e.g. leucine and tryptophan) versus hydrophilic properties (e.g. glycine and glutamine), as well as presence of positively charged (e.g. lysine and histidine) or negatively charged (e.g. glutamic acid) functionalities. The amino

acid-containing spray dried powders were characterized for particle size, morphology, thermal properties, aerosol performance, and viral vector activity.

2. Materials and Methods

2.1. Materials and Adenoviral Vector

D-mannitol, dextran (40 kDa), L-leucine, L-tryptophan, L-glutamine, L-glutamic acid, L-histidine, L-lysine, L-glycine, and tricine were purchased as USP grades from Sigma-Aldrich (Toronto, Canada). Ultrapure water was obtained from a Barnstead GenPure Pro water purification system (Thermo Fisher Scientific; Waltham, MA) with a resistivity of 18.2 MΩ cm. Recombinant replication-deficient human serotype 5 adenovirus expressing green fluorescent protein (AdHu5-GFP, referred to as AdHu5 from hereafter for consistency) was produced in the Vector Facility of the McMaster Immunology Research Centre (MIRC), as described previously.[72] The stock viral vector suspension was stored in a phosphate-buffered saline solution (PBS) with 10% (v/v) glycerol.

Culture media for lung epithelial (A549) cells was prepared from Alpha Minimum Essential Medium Eagle (α-MEM) in house according to the protocol by the supplier, Life Technologies (Toronto, Canada), with 1% streptomycin/penicillin (Invitrogen; Burlington, Canada) and 10% fetal bovine serum. PBS and trypsin were also prepared in house.

2.1.1. Excipient Selection and Formulation Composition

Nine formulations were spray-dried and assessed for their powder properties including particle size, morphology, thermal stability, aerosol performance, and viral activity. The control formulation consisting of mannitol and dextran in a 3:1 weight ratio was chosen due to its well studied ability to retain high viral activity by efficient encapsulation of AdHu5

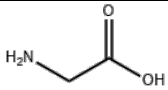
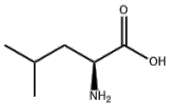
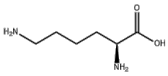
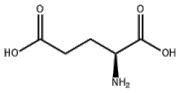
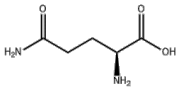
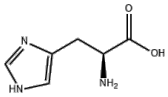
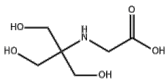
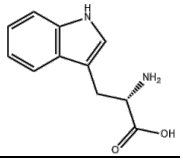
during spray drying.[9,11,152,160] The formulation affords exceptional thermostability of AdHu5 at room temperature over a prolonged period of storage and is intended for inhalation delivery. The fractions of stabilizing binary blend (mannitol/dextran), and the antigen, were kept the same throughout the study for high encapsulation efficiency.

One formulation, as control, consisted of only the blend and AdHu5, while the other eight formulations were prepared by supplementing with amino acids. Previous studies using amino acids in their formulations as a dispersibility enhancer considered a concentration range between 10-20% (w/w) and found an improvement in the aerosolization of spray dried powders.[163,225] Using such studies as guidance for the current work, a concentration of 13% (w/w) amino acids was used, after preliminary study with L-leucine testing 5-20% (w/w) and its effects on particle wrinkling. All formulation and processing details are described in Table 1. L-configuration amino acids investigated in this study include leucine, glutamine, tryptophan, lysine, histidine, glutamic acid, and glycine, but for conciseness the "L" is not explicitly mentioned henceforth. Tricine, a zwitterionic buffer and a derivative of glycine, was also chosen for study due to its buffering capacity. Properties of the selected amino acid excipients are listed in Table 2.

Table 1. Composition of spray drying formulations (both control and amino acid/tricine-containing formulations) and spray drying parameters used.

Composition of spray drying formulations		**Spray Drying Parameters**	
Total solid concentration (mg/mL)	11.5	Inlet temperature (°C)	120
Mannitol fraction (wt.%)	65	Spray gas flow (L/h)	439.11
Dextran fraction (wt.%)	22	Feed flow rate (mL/h)	217.5
Amino acid/tricine fraction (wt.%)	13	Outlet temperature (°C)	60 – 65
		Aspirator (m^3/h)	35

Table 2. Properties of the amino acids/tricine (in order of increasing molecular weight) used in formulations with AdHu5 for spray drying.

Amino acid (all "L" enantiomer)/ Tricine	**Molecular weight (Da)**	**Amino acid side chain polarity and charge**[*226]	**Hydrophobicity (at pH 7)**[227,228]	**Isoelectric point (pI)**[229] **at 25 °C**	**Structure**
Glycine	75	Non-polar	Hydrophobic	6.0	
Leucine	131	Non-polar	Very Hydrophobic	6.0	
Lysine	146	Polar, basic (positively charged)	Hydrophilic	9.7	
Glutamic Acid	146	Polar, acidic (negatively charged)	Very Hydrophilic	3.2	
Glutamine	147	Polar, (uncharged)	Neutral	5.7	
Histidine	155	Polar, basic (positively charged)	Neutral	7.6	
Tricine	179	-	-	Buffering pH range (7.4-8.8)	
Tryptophan	204	Non-polar	Very Hydrophobic	5.9	

[*] Charge of the amino acid side chain at pH=7.1, which is approximately the pH of spray drying formulations and cell media. The hydrophobicity is considered neutral for amino acids at pH 7 based on its hydropathy index when it does not lie exclusively in the hydrophobic or hydrophilic category.
[#]Tricine was also listed based on the molecular weight, however, it is not a naturally occurring amino acid.

2.2. Preparation of Spray-Dried Powders

A 10 mL aqueous solution consisting of 1 wt% mannitol/dextran (ratio 3:1; abbreviated as MD) was used as the control. A 10 µL dosage of high viral potency stock of AdHu5 at 10^{10} pfu (plaque forming units) was added for *in vitro* activity testing, making a final viral titer of 10^{7} pfu in a 10 mL feedstock solution for spray drying. The composition for the aqueous solutions of the eight other formulations is described in Table 1, where a portion of the binary blend was substituted with the amino acids. The prepared formulations were spray-dried using a B-290 mini spray dryer (Büchi, Switzerland) equipped with a high-performance cyclone and 0.7 mm nozzle, following the spray drying parameters described in Table 1.

Spray drying parameters were chosen based on a previous study in order to minimize AdHu5 losses during spray drying.[230] To allow meaningful comparisons between spray-dried samples, a single set of parameters without optimization were used for spray drying, resulting in higher activity losses for the adenovirus than normally acceptable.

The pH of spray drying formulations in this study was not measured for all the formulations, but the control formulation pH was 7. The overall charge on an amino acid in an aqueous solution is dependent on the physiological pH and the isoelectric point (pI) of amino acids (pH at which the amino acid has a net neutral charge and exists as a “zwitterion”). At pH > pI, amino acids acquire an overall negative charge, whereas at a pH < pI, amino acids acquire an overall positive charge.

2.3. Powder Characterization

Previously it has been demonstrated that powders spray dried with or without AdHu5

are morphologically identical by SEM and in particle properties due to the very small fraction (< 1/10,000th by liquid volume) of viral vector present in the vaccine formulation.[11] As such, the majority of particle characterization was carried out on powders spray dried without AdHu5 to avoid biosafety constraints. For all viral activity testing, powders with AdHu5 were used.

2.3.1. Geometric Particle Size Distribution

The geometric particle size of spray-dried samples was measured with laser diffraction analysis using a HELOS sensor with R2 lens (0.25/0.45 – 87.5 µm) (Sympatec GmbH, Clausthal-Zellerfeld, Germany). The powder (10 ± 1 mg) was dispersed through the Sympatec at a pressure that equals a 4 kPa pressure drop across the selected dry powder inhalation (DPI) device, ICOone® (a commercial single-dose dry powder inhaler, generously donated by Iconovo, Sweden). ICOone® is a single-dose, medium resistance ultralow-cost inhaler molded in one piece,[231] with the formulation intended to be protected by an Al-foil (though no foil was used in the study). These features make ICOone® suitable for the administration of inhaled vaccines. Laser diffraction data were based on the Fraunhofer theory. Geometric particle size experiments were analyzed in triplicates. Particle size span value was calculated using Equation 1, where D_{90} represents the diameter greater than 90% of measured particles, D_{50} represents the median diameter, and D_{10} represents the diameter greater than 10% of measured particles.

$$Span = \frac{D_{90} - D_{10}}{D_{50}} \qquad (1)$$

2.3.2. Particle Morphology (Scanning Electron Microscopy)

Particle morphology (surface topology, particle shape, and size) was analyzed using a Tescan Vega II LSU scanning electron microscope (Tescan USA, PA). Powder samples were

mounted on double-sided tape attached to an aluminum stub and dusted for excess powder. These samples were coated with a 24 nm layer of gold using a Polaron E5100 sputter coater (Polaron Equipment Ltd., Watford, Hertfordshire). Images were taken at an electron accelerating voltage of 5 kV and working distances between 11 and 17 mm.

2.3.3. Particle Aggregation and Surface Roughness

Due to the absence of direct physical characterization techniques for roughness of spherical shapes in the size range of 1 μm, an arbitrary scale was developed for aggregation and surface roughness from the SEM images. The arbitrary scale for aggregation ranged from 1 to 4 (1- dispersed, 2-slightly aggregated, 3-aggregated, and 4-very aggregated), and scores were assigned to spray-dried samples based on the number and size of aggregates seen in the images. For surface corrugation, a similarly arbitrary scale ranging from 1 to 4 (1-smooth, 2-slightly rough, 3-rough, and 4-very rough) was used but in this case, aided by surface roughness values obtained from image analysis in FIJI (ImageJ 2.0)[232] using the surface roughness plug-in.[233] Two images of each sample were used to assign the arbitrary scores to the samples.

2.4. Thermal Properties of the Powder (Differential Scanning Calorimetry)

Modulated differential scanning calorimetry (DSC) was performed, and thermograms were obtained for all the samples using a Q200 differential calorimeter (TA instruments; New Castle, DE). Spray-dried samples of 5-12 mg were weighed and hermetically sealed in Tzero aluminum pans for analysis. The processing history of samples was removed by a first ramp cycle starting from 20 °C until 120 °C at 10 °C/min to remove residual water from the samples and then cooled. Subsequently, samples underwent modulated heating up to 200 °C at a ramp

of 5 °C/min and oscillation of ±0.60 ºC/40 s under a purge of nitrogen gas flowing at 50 mL/min. The glass transition temperature for spray-dried powders was determined from the reversible heat flow curve using Universal Analysis software (TA Instruments, New Castle, USA).[234]

2.5. Thermogravimetric Analysis (TGA) for Moisture Content Analysis

The residual moisture content of the samples was determined using TGA/DSC 3 + instrument (Mettler Toledo; Columbus, OH). Spray dried samples without viral vector were heated in alumina crucibles at a rate of 5 °C per minute up to 150 °C under air. Stable mass loss up to 150 °C was monitored, and residual moisture content was calculated using the Star^{e} software (Mettler Toledo; Columbus, OH).

2.6. *In vitro* Aerosol Performance

Aerosol properties of the spray-dried samples were analyzed *in vitro* using a Next Generation Impactor (NGI) (MSP Corporation Model 170, Copley Scientific; Nottingham, UK) with a standard right-angled, USP metal inlet. The inhaler, ICOone®, was donated by ICONOVA AB (Lund, Sweden) to deliver the powder; the inhaler was not optimized for the formulation and was used as supplied. ICOone® was connected to the inlet via a custom-made silicone adapter, and the inhalation flow rate was controlled by a TSI (Model 4043A) flow meter connected to a vacuum pump. The impactor stages were fitted with disc filters (cut to fit the stages of the NGI), which were weighed before the experiment. Five ICOone® inhalers were weighed, and each inhaler was loaded with 10 ± 3 mg of spray-dried powder. The 5 inhalers were actuated in succession in order to obtain sufficient mass on the stages (filters) for gravimetric analysis. Actuations were performed at an inspiratory flow rate of 60 Lpm over 4 s to achieve 4 L of air withdrawal (USP <601>).[235] After actuation, the inhalers and all

the filters (on each stage) were weighed using a microbalance.

Emitted dose (ED) was determined gravimetrically as the percent of total loaded mass exiting the ICOone® inhaler post actuation. The total mass of powder deposited on the impactor stages (filters) from the 5 actuations was representative of an anticipated dose to the upper and lower respiratory tracts. The effective cut-off diameter of each stage was determined for sampling at 60 Lpm, and the mass median aerodynamic diameter (MMAD) of the powder was derived as the particle size that represented 50% of the cumulative fraction read from the plot of stage cut-off diameters vs. the mass fraction of powder measured on each stage. The Fine particle fraction, representative of the fraction of the powder particles with aerodynamic diameters <5 μm that could deposit on airways below the carina, was obtained by interpolation from the plot of stage cut-off diameters vs. the cumulative fraction of powder on the stages. The fine particle fraction was expressed relative to the emitted dose (FPF/ED) referred to as the FPF henceforth. FPF and MMAD were expressed as the mean of triplicate impactor runs (n=3).

2.6. *In vitro* Viral Activity Testing

2.6.1. Culturing A549 Cells

A549 (lung epithelial cells) were revived from liquid nitrogen storage and cultured in T150 culture flasks using Minimum Essential Medium Eagle (α-MEM). Cells were cultured at controlled environmental conditions at 37.0 °C and 5% CO_2 in a water jacketed CO_2 incubator (Forma Series II, Thermo Scientific Corporation; Waltham, MA). Cells were split and plated in a 96-well plate for *in vitro* testing.

2.6.2. Viral Activity of Spray-Dried Formulations

Samples containing viral vectors were stored on ice before spray drying to minimize

the potential activity loss due to storage at room temperature and were tested on the same day as they were spray dried. The spray-dried samples were reconstituted in α-MEM to achieve matching solids concentrations to the feed solutions used for spray drying, namely 1 % (w/w) for control and 1.15% (w/w) for amino acid/tricine-containing samples. A549 cells seeded at a concentration of 4 x 10^4 cells per well in a flat-bottom 96-well plate and transfected with 100 µL of reconstituted spray-dried powders after 24 h. After keeping the transfected cells overnight, the cells were prepared for flow cytometry as described previously.[26] The prepared cells were processed for flow cytometry and run on a MACSQuant Analyzer 10 (Miltenyi Biotec; Bergisch Gladbach, Germany). The data from flow cytometry were processed in FlowJo software (Tree Star; Ashland, OR) as described previously.[26] GFP expressing cells were obtained and compared against a standard curve for the modality of infection (0.1 to 100) vs. percentage GFP expressing cells. The titer of the spray-dried viral vector was thus calculated to assess titer log loss before and after spray drying by Equation 2.[26]

$$\text{Viral activity loss} = \text{Log}_{10}(\text{initial titer}) - \text{Log}_{10}(\text{final titer}) \qquad (2)$$

2.7. Data Analysis

Experimental data for aerosol behavior (MMAD and FPF) and viral activity were analyzed with a single-factor analysis of variance (ANOVA) using Microsoft Excel followed by a two-tailed t-test. Data with probability values less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Powder Characteristics

3.1.1. Powder Yield

Relative yield provides insight into the processability of formulations containing

different amino acids with low values representing poor drying characteristics. It is defined as the ratio of collected yield after spray drying a given formulation relative to the original yield of the control powder (original yield of mannitol/dextran without amino acids compared to the initial solids concentration: 47 ± 3.8 %); the relative yield varied from 10% to 110% depending on the choice of amino acid (Table 3). In most cases, the addition of amino acid reduced the yield significantly, except for leucine, where the yield exceeded the control. It is not uncommon for amino acids to cause powder to adhere to the walls of the drying chamber and cyclone of a spray dryer, resulting in lowered yields. This adhesion has been attributed to the temperature of the chamber walls, which approaches the glass transition temperature of the powder.[230] Among the tested amino acids, glycine led to the lowest relative yield (10%) from powder lost on the spray drying chamber due to it having the lowest T_g (Table 4); this amino acid has also been shown to reduce the relative yield in spray dried powders of glycine-aztreonam.[24]

3.3.1.2 Particle Morphology (Aggregation and Surface Roughness)

The particle morphology of the spray-dried powders was investigated by SEM (Figure 1). Based on the images, samples were assigned an arbitrary score for the degree of aggregation and surface roughness observed, summarized in Figure 2. The control mannitol/dextran (MD) particles appeared smooth, spherical and without significant aggregation, giving them the lowest scores in both categories among all samples. Leucine-containing particles showed considerable roughness and some deep cracks but with the least aggregation (Figure 1B). Overall, the particles containing leucine were the closest in appearance to the control. Glycine-containing particles (Figure 1C) had distinct spherical shapes despite aggregation and exhibited rough surfaces. The particles containing tryptophan

(Figure 1D) appeared rough (arbitrary score of 3) and larger than the others due to the highly fused aggregates seen in the SEM images. The particles fused together were not considered aggregates.

Samples containing polar amino acids (glutamine, lysine, histidine, and glutamic acid) (Figure 1E, F, G, and H, respectively) showed less aggregation than the powders containing non-polar amino acids, with particles generally close in size to the control. With the polar amino acids, particles were very rough, though with different morphologies. For example, the lysine-containing particles appeared slightly wrinkled, whereas the histidine-containing particles were pitted in appearance. Due to the high surface activity[236] and low molecular weight, leucine tends to be enriched at the air-particle interface resulting in hydrophobic surfaces for the leucine-containing particles;[237] this appears to reduce the interparticle interactions between particles. Reduced aggregation of leucine-containing powder is aided by the reduced contact area between wrinkled particles.[187,214]

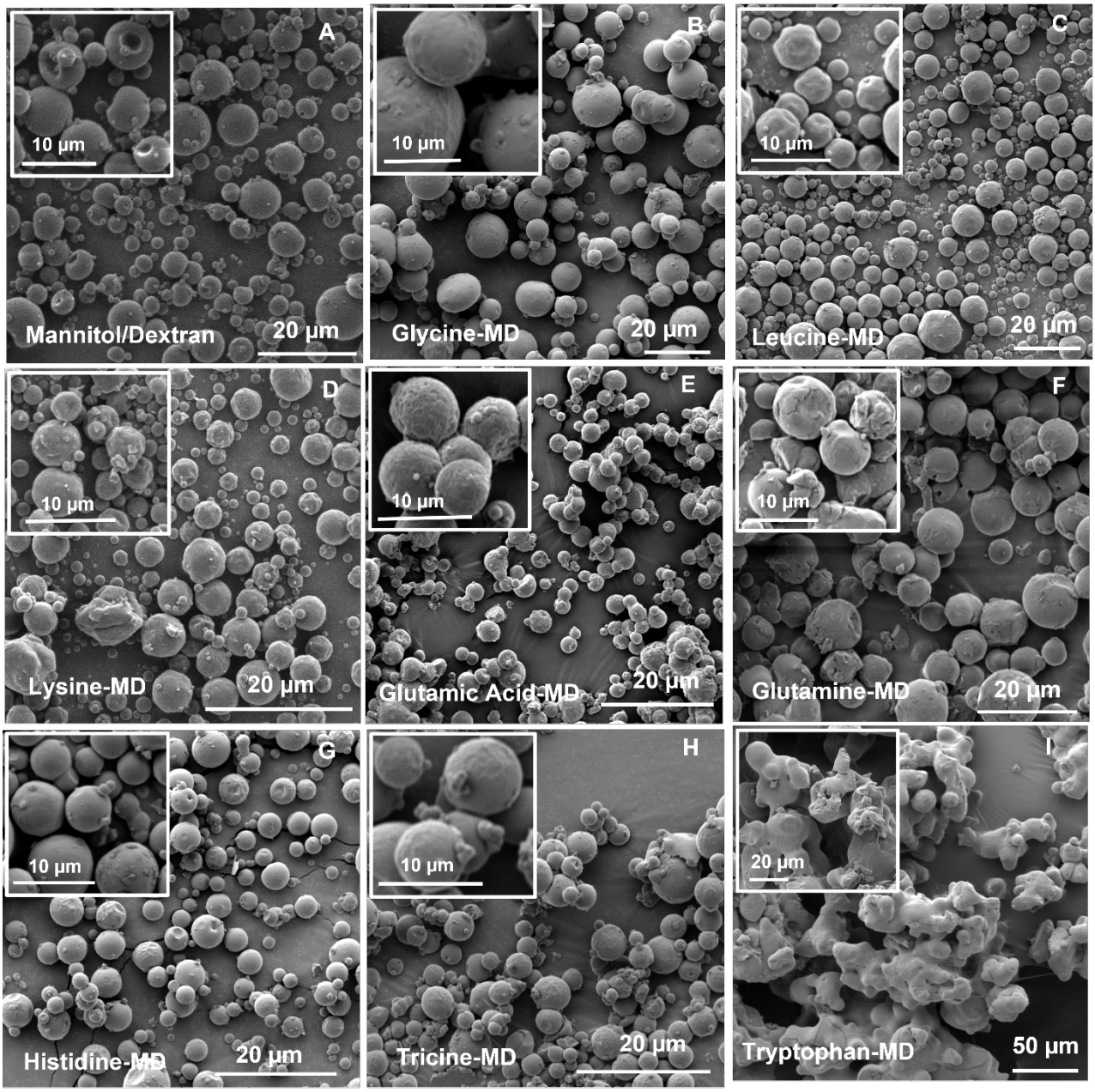


Figure 1. Scanning electron microscopy images for various spray dried MD-amino acid samples. [A. Mannitol/dextran (control) particles, B. glycine-containing particles, C. leucine-containing particles, D. lysine-containing particles, E. glutamic acid -containing particles, F. glutamine-containing particles, G. histidine-containing particles, H. tricine-containing particles, and I. tryptophan-containing particles.] All images are at magnifications between 700x and 3000x with a scale bar of 20 micrometers. The square boxes on top left of each sample shows double magnified images.

The wrinkled morphology of particles is governed by the presence of the shell forming

excipients in the formulation. Molecules (like amino acids) with fast crystallization and high initial saturation precipitate into a separate phase and accumulate at the surface to form a shell. Such shell formation occurs earlier for smaller droplets due to their smaller precipitation window.[192] Assuming that amino acids are present in the shell on the particle surface, smaller particles are more wrinkled than larger particles. Based on the assigned scores, the particle surface roughness of the spray dried samples (Figure 2) was found to be higher for polar uncharged samples compared to polar charged and non-polar (except leucine) amino acids containing particles. No clear trend was observed for aggregation of particles. Overall, spray dried powders showing the least aggregation and high surface roughness (i.e., leucine-containing particles, Figure 1B and Figure 2) are the preferred choice for inhalation.

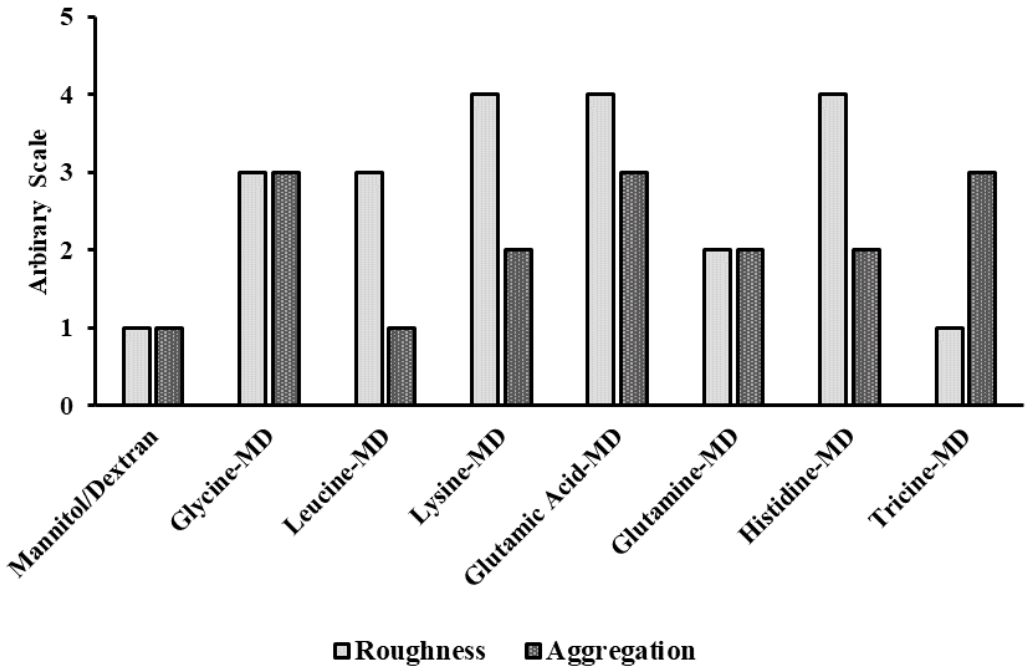


Figure 2. Particle wrinkling and aggregation from SEM images. Scale assigned for roughness: 1-smooth, 2-slightly rough, 3-rough, 4-very rough. Scale assigned for aggregation: 1-dispersed, 2-slightly aggregated, 3-aggregated, 4-very aggregated.

3.1.3. Geometric Particle Size and Size Distribution

Particle size reflects how effectively the powder samples should deposit in the lungs, and therefore it is an important factor for demonstrating clinical effectiveness of an inhaled product.[60,238] The geometric particle size distributions of the spray-dried powders were measured using laser diffraction. The mean geometric particle sizes (D_{50}) ranged from 5.7 µm to 29 µm (Table 3). The distribution breadth of samples is conveyed in Table 3 by the diameters corresponding to the 10% and 90% cumulative volume fractions (D_{10} and D_{90}, respectively) and the calculated span based on the three diameter parameters. The span of the control was 1.6, indicating a narrow distribution and consistent aerosolization behavior.[60,238]

Table 3. Powder characteristics (relative yield and geometric particle size distribution) of spray-dried MD-amino acid samples.

Sample name	Relative yield (%)	D_{50} (in µm)	D_{90} (in µm)	D_{10} (in µm)	Span value
Mannitol/Dextran	100 ± 11	5.9 ± 0.2	12 ± 1	2.4 ± 0.4	1.6 ± 0.1
Glycine-MD	10 ± 6	14 ± 3	44 ± 21	4.4 ± 0.7	2.8 ± 0.8
Leucine-MD	110 ± 13	5.7 ± 0.6	15 ± 3	3 ± 2	1.6 ± 0.1
Lysine -MD	66 ± 24	6.6 ± 2	11 ± 0.5	2.3 ± 0.3	1.7 ± 0.1
Glutamic Acid-MD	38 ± 10	8 ± 1	14 ± 4	2.6 ± 0.6	1.8 ± 0.1
Glutamine-MD	40 ± 16	8.6 ± 2	16 ± 3	2.9 ± 0.6	1.9 ± 0.5
Histidine-MD	32 ± 11	9.5 ± 2	20 ± 7	3.9 ± 0.9	1.7 ± 0.2
Tricine-MD	57 ± 12	7 ± 1	20 ± 9	3 ± 0.7	1.6 ± 0.1
Tryptophan-MD	49 ± 11	29 ± 5	68 ± 14	9 ± 1	2.1 ± 0.2

Glycine and tryptophan led to larger particles than the other amino acids, far too big to generally be considered suitable for inhalation. The D_{50} of the powder produced with glycine was not substantially above the respirable range, but the large span (3.0) indicated that a lot more particles were well above this range compared to some of the other amino acids.

Previously, glycine also produced large (144.5 μm) particles in a spray dried aztreonam blend.[24] The largest particles were found with tryptophan, with evidence by microscopy of fused primary particles formed either during drying or in the collection vial. Particles with tryptophan also showed a very high span suggesting that the majority of powder produced would show poor aerosolization and drug deposition when inhaled.[215] Overall, formulations with the other amino acids (except glycine and tryptophan) produced particles with geometrical size in the respirable range (1-10 μm), although only the control mannitol/dextran and leucine-containing particles were in the ideal size range (~5 μm) for delivery to the upper respiratory airways.

3.2. Thermal Properties

According to vitrification theory, degradation of biological activity requires some level of molecular mobility of the biologics. An encapsulating matrix in its "glassy state" will offer limited molecular mobility of a biologic preventing it from oxidative, humidity or thermal stresses thereby extending the potential for an extended shelf life. Thus, the glass transition temperature (T_g) is considered a suitable parameter in determining vaccine thermal stability. A T_g greater than 75 ^oC is preferred for vaccine storage at ambient temperature[162,239] though preferred formulations exhibited a T_g above 100 °C to allow for some plasticization by moisture to occur over time while still keeping the virus immobile. The T_g of spray-dried mannitol/dextran samples with and without amino acids are listed in Table 4, with the control particles exhibiting the highest value at 121 °C, consistent with values reported previously.[11,152,160] The measurement of T_g for the control mannitol/dextran was measured in duplicate and the standard error (± 2) is reported in Table 4. This range of standard error in the measurement of glass transition temperature has also been reported in other related

studies.[19,23] Based on this and the error ranges (5 to 10 °C) from previous studies in our group,[152,160,240] it was speculated the standard error in the measurements of glass transition temperature for amino acid-containing samples to be ≤10 °C. The T_g of mannitol/dextran blend has been reported to maintain high viral activity in the dried particles when exposed to storage temperatures as high as 55 °C.[11,152]

Table 4. Glass transition temperature of spray dried MD-amino acids and MD control samples.

Sample name	**Glass transition temperature (T_g) (°C)**
Mannitol/Dextran	123 ± 2
Glycine-MD	80 ± 10
Leucine-MD	111 ± 10
Lysine -MD	102 ± 10
Glutamic Acid-MD	105 ± 10
Glutamine-MD	108 ± 10
Histidine-MD	98 ± 10
Tricine-MD	101 ± 10
Tryptophan-MD	114 ± 10

Recognizing that dextran will not show any crystalline peaks,[241] the crystalline peaks of mannitol were expected to resemble the α-polymorphic form based on previous crystallinity studies.[11,242] No separate T_g was observed for the amino acids in the DSC traces of these spray dried formulations. Due to the high crystallinity of amino acids, a thermal transition in the smaller amorphous content will be difficult to detect by DSC especially if chain mobility is restricted in that phase by the surrounding crystals. As a result, the glass transition temperature was often difficult to identify from the baseline heat flow during the test. The glass transition temperatures of all samples containing amino acids in the table were above 75 ºC (except

glycine) and therefore treated as acceptable candidates in preparing a thermally stable vaccine. The lowest T_g and sample yield with glycine meant this amino acid was already ranked lowest as a viable candidate for our vaccine even before considering its flow behavior and viral activity. The lowest T_g and sample yield with glycine meant this amino acid was already ranked lowest as a viable candidate for our vaccine even before considering its flow behavior and viral activity.

3.3. *In vitro* Aerosol Performance

In vitro aerosolization and deposition properties of the spray-dried powders were analyzed using a Next Generation Impactor (NGI) to simulate human lung delivery. The chosen inhaler was found highly effective for dispensing the powders, with emitted dose (ED) values in Table 5 for all formulations ranging from 79-93% of the total dose. Low emitted doses (below 90%) were obtained for samples containing glutamic acid, tricine, and glutamine, which was attributed to their cohesiveness reflected in SEM and size analysis where otherwise well-formed primary particles were being held together as aggregates. In addition to particle size, this observation highlighted that powder emission from an inhaler is affected by particle morphology as well as surface roughness.

The flowability of powders can be impacted by residual moisture content in particles, moisture gained upon exposure to ambient conditions, and static charge if the moisture content in powders is very low. The residual moisture content in the spray dried powders was measured using the thermogravimetric analysis and was found to be in the range of 3% - 6%, Table 5. It is to be noted that the residual moisture content for spray dried mannitol/dextran and other formulations was found to be low (≤ 6%, Table 5); a previous study[240] found a similar moisture content (5%) for the same mannitol/dextran formulation being used as the

control in the current study. No clear trends were seen between the aerosolization properties (MMAD, FPF, and ED) of the samples and residual moisture content. The effects of residual moisture content, if any, with respect to amino acid content were expected to manifest in the particle morphology analysis. Therefore, all samples in this study were collected from the dryer and analyzed using NGI under identical conditions to maintain a baseline for comparison.

ED reflects the effectiveness of the inhaler to disperse the powder, but deposition of powder particles in the respiratory tract is better indicated by the aerosol particle size and the fine fraction of the emitted dose.[24] The emitted dose (ED) for all the samples was found to be comparable to each other and were found to be statistically similar. The aerosol particle size (MMAD) is accepted in the field of aerosolization as determining the site of deposition and distribution of inhaled dry powder aerosols.[60] While the geometric particle size distribution (GPSD) does not consider the particle characteristics, such as particle density and shape factor, the MMAD represents the size of complex shaped particles under an air flow. However, for our samples, all particles were spherical in shape, and therefore the MMAD and GPSD trends are expected to be very similar. The samples containing tryptophan and glycine showed very high MMAD (38 μm and 16 μm) due to their particle morphology and size and were not considered suitable for inhalation, consistent with the geometric particle size analysis. The MMAD for most other samples ranged from 4.4 μm to 6.5 μm, adequate for deposition in the lung.

Table 5. Aerosol performance (represented by MMAD, FPF, and ED and activity loss of spray dried powders from ICOone® inhaler using NGI operated at 60 Lpm.

Sample name	MMAD (µm)	% Fine particle fraction (FPF)	% Emitted dose (ED)	Residual moisture content (%)	Viral Activity Log Loss
Mannitol/Dextran	5.7 ± 0.6	24 ± 2	91 ± 1	4	1.1 ± 0.07
Glycine-MD	16 ± 0.9	8.2 ± 0.7	93 ± 2	5	1.7 ± 0.3
Leucine-MD	4.4 ± 0.8	29 ± 4	92 ± 2	4	1.5 ± 0.02
Lysine -MD	4.8 ± 0.6	22 ± 2	90 ± 2	3	2.0 ± 0.1
Glutamic Acid-MD	6.5 ± 0.2	20 ± 2	87 ± 3	3	2.2 ± 0.07
Glutamine-MD	6.1 ± 0.6	20 ± 6	79 ± 15	4	1.5 ± 0.2
Histidine-MD	6.2 ± 0.7	20 ± 2	90 ± 2	6	2.2 ±0.004
Tricine-MD	6.1 ± 0.03	22 ± 2	87 ± 1	4	1.1 ± 0.3
Tryptophan-MD	38 ± 3	6.6 ± 2	90 ± 3	2	2.2 ± 0.5

The fine particle fraction of the emitted dose was calculated based on the percentage of powder that was collected on stages 1 to 7 of the impactor (cut-off diameter ranging from 8.06 µm to 0.34 µm, respectively) relative to the emitted dose. All spray-dried samples demonstrated aerosolization (based on normalized FPF) ranging from 6.6% to 29% (Table 5). These values correlated well with other aerosolization studies and demonstrated similar range of values for fine particle fractions of spray dried powders. For example, Saluja et al. prepared a spray dried influenza subunit vaccine with a 37% FPF;[25] Sou et al. studied a mannitol/trehalose/leucine-containing spray dried formulation that showed 26% FPF;[163] and Gomez et al. found the FPF (presented as lung dose) of trehalose-based spray dried powders in the range of 18% to 33%.[225,243] As a result, range of FPF for our control and amino acids-containing powders (except glycine and tryptophan) is believed to be within the acceptable

range for inhalation of a powder vaccine, though these results are for a non-optimized formulation (to allow comparison between formulations without inclusion of process effects), and the FPF can be further improved with optimization.

Most of the amino acid-containing samples exhibited a similar degree of aerosolization as the control powder, with 24% of the emitted dose consisting of fine particles that reached as far as stage 6 of the NGI, which indicates aerosolization capable of reaching the bronchial airways. Glycine and tryptophan powders showed the poorest aerosolization, with 90% of the total powder being deposited on stages 1 and 2 of the NGI, depicting deposition in the oropharyngeal region and trachea only. Therefore, the glycine and tryptophan spray-dried powders were deemed unsuitable for inhalation which was corroborated by their particle size and morphology data.

Overall due to overlap in the MMAD and FPF values for the samples, all spray dried samples showed similar aerosol performance except glycine and tryptophan reduced the FPF significantly compared to the control and other amino acids. It was interesting though none of the amino acids resulted in better aerosolization performance despite their rougher surfaces compared to the relatively smooth features of the control. It is likely that there exists an optimal concentration of amino acids (as aerosolization enhancers) and optimized spray drying conditions to form a hydrophobic and crystalline shell capable of improving aerosolization of vaccine powders by reducing interparticle forces.

3.4. Viral activity of AdHu5 in Spray-Dried Powders

The bioactivity of dry powder viral vectored vaccines was determined by their ability to transfect cells viably.[9] *In vitro* viral activity of AdHu5 for spray dried samples with different amino acids and the control is given in Figure 3. The control (mannitol/dextran) showed a 1.1

± 0.07 log loss in activity, which was higher than losses reported previously for the same system when optimized spray drying conditions are used;[230] however, it was important to use identical spray drying conditions that worked for all of the formulations, and as such, the activities are not optimized but were deemed suitable for relative comparison. The sample means were found to be significantly different as per one-way ANOVA analysis ($p = 0.044$). All amino acid-containing powders showed significantly reduced viral activity compared to the control. Based on the overlap of error bars for viral activity values between the samples, all amino acid containing samples showed comparable activity loss. The adverse effects of amino acids on the integrity and biological function of non-viral LPD (lipid/polycation/pDNA) vectors (which are structurally similar to non-enveloped viruses like adenovirus) have been reported previously.[189,219] However, in our case, the reduction in viral activity is not attributed to innate toxicity of the amino acids themselves affecting cell viability (based on counting live cells after direct application of amino acid solutions, percentage of viable cells for all samples was found to be>97%).

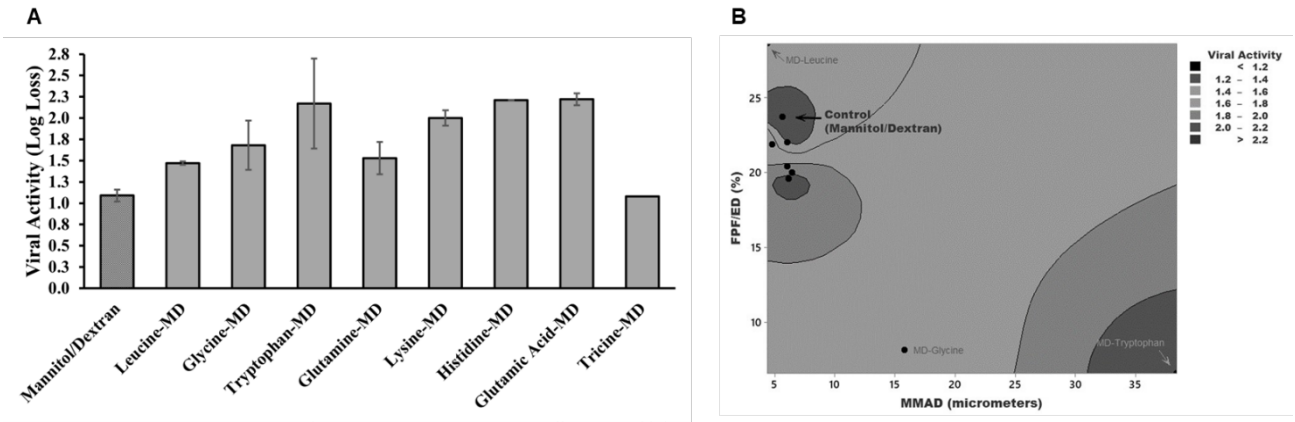


Figure 3. A. Log loss of viral activity of AdHu5 after transfecting A549 cells with spray-dried MD-amino acid samples. B. Contour plot for viral activity vs. aerosol performance.

The interaction between amino acids and viral vectors has been shown to be a function of molecular weight, side chain length, and the ratio of the number of OH groups and carbon

atoms.[244] The activity losses observed for these amino acid-containing formulations can be attributed partially to the aggregation of adenoviral vector in presence of amino acids in liquid formulations. Charged amino acid species (like histidine and glutamic acid) have been shown to be more detrimental to non-enveloped viruses like AdHu5 compared to enveloped viruses.[189,245] The charge can disturb stability of the viruses, thereby causing potential structural damages; such damages can lead to leakage of the genetic material and lower the activity of the viral vector.[160] However, finding the root cause of the reduction in AdHu5 activity upon addition of amino acids needs to be further investigated.

4. Conclusions

Based on the characterization and side-by-side screening, this study shows amino acids will be less valuable as flow aids to the emerging area of inhalable dry powder vaccines in contrast to their reported effectiveness in other aerosolized products. The absence of significant aerosolization enhancement may be attributed to the good performance of the control blend (mannitol/dextran) for inhalation already but the negative effect of these amino acids on bioactivity of the adenoviral vector is the stronger motivation for their inclusion in the study. Only leucine was felt by the authors to need further evaluation, if only to understand how amino acids were affecting the viral vector. The results further emphasize that spray drying sensitive biologics (viral vectored vaccines, in our case) requires careful selection of excipients such that the potency is preserved. It was believe that this study, although a bit on the negative side, provides new insights about the detrimental effects of well-known aerosolization enhancers on adenoviruses.

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