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Measurement of Adenovirus-Based Vector Heterogeneity

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Abstract

Adenovirus vectors have become an important class of vaccines with the recent approval of Ebola and COVID-19 products. In-process quality attribute data collected during Adenovirus vector manufacturing has focused on particle concentration and infectivity ratios (based on viral genome: cell-based infectivity), and data suggest only a fraction of viral particles present in the final vaccine product are efficacious. To better understand this product heterogeneity, lab-scale preparations of two Adenovirus viral vectors, (Chimpanzee adenovirus (ChAdOx1) and Human adenovirus Type 5 (Ad5), were studied using transmission electron microscopy (TEM). Different adenovirus morphologies were characterized, and the proportion of empty and full viral particles were quantified. These proportions showed a qualitative correlation with the sample s infectivity values. Liquid chromatography-mass spectrometry (LC-MS) peptide mapping was used to identify key adenovirus proteins involved in viral maturation. Using peptide abundance analysis, a ~5-fold change in L1 52/55k abundance was observed between low- (empty) and high-density (full) fractions taken from CsCl ultracentrifugation preparations of ChAdOx1 virus. The L1 52/55k viral protein is associated with DNA packaging and is cleaved during viral maturation, so it may be a marker for infective particles. TEM and LC-MS peptide mapping are promising higher-resolution analytical characterization tools to help differentiate between relative proportions of empty, non-infectious, and infectious viral particles as part of Adenovirus vector in-process monitoring, and these results are an encouraging initial step to better differentiate between the different product-related impurities.

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Abbreviations

LDF:	Low Density Fraction
HDF:	High Density Fraction
LC-MS:	Liquid Chromatography Mass Spectrometry
DTT:	Dithiothreitol
IAM:	Iodoacetamide
Ad5:	Human Adenovirus serotype 5
ChAdOx1:	Chimpanzee Adenovirus Oxford serotype Y25
CID:	Collision Induced Dissociation
CSH:	Charged Surface Hybrid
TEM:	Transmission Electron Microscopy
TFF:	Tangential Flow Filtration
AEX:	Anion Exchange Chromatography
P:I Ratio:	Ratio of the Number of Viral Particles to the Number of Infectious Viral Particles

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Introduction

Adenoviral vectors have many therapeutic and prophylactic uses[1] including commercial gene therapy products and vaccines [2]. They are a significant class of viral vector because they can deliver large nucleic acid payloads (8-36 kbp)[3], therefore can offer treatment that require delivery of a large transposon payload into a target genome. Adenoviruses were the first DNA virus to enter rigorous therapeutic development, because of its well-defined biology, its genetic stability, its high gene transduction efficiency and its relative ease of large-scale production. [4] They are appealing vaccine candidates due to their long shelf life , which can be prolonged further with different additives and formulation strategies [5,6-,7].

There are many assays and techniques available to characterize different structural attributes of adenoviruses in accordance with regulatory guidelines [8-10]. Current quality control assays for viral vector-based vaccines comprise of a suite of: particle count by PCR, particle to infectivity ratio (determined by cell-based infectivity assays), residual host cell DNA and residual host cell protein[9]. In this work, we evaluated the potential utility of higher resolution structural analysis, including liquid chromatography mass spectrometry (LC-MS) and transmission electron microscopy (TEM) as analytical characterization tools for use during process development of adenovirus-based vaccines. These analytical tools provide a greater degree of complementary product understanding, with TEM providing evidence of viral particle morphology and LC-MS allowing measurement of the viral proteome.

Chimpanzee Adenovirus (ChAdOx1) and Human Adenovirus Type 5 (Ad5), two vectors typically used in commercial manufacturing, were characterized in this study. Both viral vectors were isolated *via* caesium chloride density gradient ultracentrifugation and additionally for Ad5, anion exchange membrane chromatography, to evaluate the heterogeneity of the purified Adenoviruses. One of the issues with manufacturing of adenoviruses is the presence of product related impurities such as empty and immature (non-infectious particles). [11] Whilst the assays currently employed characterize their presence and ratio, they do not provide root cause information that could be used to elucidate cause(s) of these impurities, so that they may be rationally designed out of the manufacturing process.

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There is currently ongoing research in the adeno-associated virus (AAV) field to increase understanding on which features of the AAV correlate to potency [12]. Recently experiments by high-resolution native mass spectrometry investigated the composition of several AAV serotypes and highly heterogenous populations of capsids with variable composition were found [13].

Product related impurities in Adenoviruses can arise from the different stages of the virus maturation cycle of the virus particle, which undergoes a series of structural changes (Figure 1). These structural changes are governed by key adenovirus proteins which are involved in capsid assembly, DNA packaging and maturation of the particle. The key adenovirus proteins and their functions are listed in Table 1 and as part of the maturation process, many of these adenovirus proteins undergo proteolytic cleavage [11] as indicated. In addition, product related impurities may arise through mis-packaging and proteolytic cleavage; empty viral particles may have been harvested before maturation or may have been the result of errors in the packaging process that arrested maturation. The temporal sequence of events for maturation has many unanswered questions. [11]

Adenovirus shell proteins IIIa, VI and VIII, and core proteins VII, μ and TP are synthesized as precursors, and processed by the adenovirus protease (AVP) during assembly [11]. A quantitative proteomics study gave an indication of the AVP copy number, with only seven AVP molecules per viral particle [14]. Approximately, 2000 cleavages have to happen in each virion, leading to ~40 to ~300 cleavages per AVP copy [11]. These cleavages must take place internally in the highly crowded environment of the viral core as they interact with the viral DNA. The packaging scaffold L1 52/55k protein had been predicted to undergo cleavage by AVP [15]. Immature particles contain ~50 copies of full length L1 52/55k, and this protein is absent from mature virions [11 15].

Therefore, these viral proteins are potentially indicative markers of adenovirus maturation and may be observed via LC-MS to monitor the structure and ratios of various Ad5 particles. As some of the surface viral proteins have low copy numbers, they may be difficult to identify by LC-MS [11]. Additionally, the structural changes the particles undergo may cause morphological changes that can be observed using TEM[16]. Ultimately, these two methods could potentially be used to detect Ad5 product impurities and

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heterogeneity for process development, and the identified marker proteins could potentially be the basis for ELISA-based assays for use in quality control, GMP settings.

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Material and Methods

Cell lines and virus lab production

The human embryonic kidney cell line (HEK293) was obtained from the American Type Culture Collection (ATCC, USA) and grown to 80% confluency and passaged in Dulbecco s modified Eagle s medium (DMEM, with GlutaMAX, 4.5g/L glucose and pyruvate) containing 10% fetal bovine serum (FBS; Hyclone, USA), 100 U/mL of Penicillin G, and 100 µg/ml of Streptomycin (Thermo Fisher Scientific). Cells were cultured in a 5% CO_2 atmosphere at 37°C. Human adenovirus serotype 5 (Ad5) was prepared by seeding HEK293 cells at 2.1 x10^6 cells/mL in T-175 flasks and incubating for 24 hrs, after which the cells were infected with Ad5 virus stock (5.2×10^9 PFU/mL) at a multiplicity of infection (MOI) of 10. The cells were harvested 48 hrs post-infection when approximately 80% cytopathic effect was observed.

ChAdOx1 Vector CsCl and AEX purification

ChAdOx1-GFP and ChAdOx1 nCoV-19 vectors were prepared using the lab-scale production method described previously [17] and were purified by CsCl density-gradient ultracentrifugation by the Jenner Institute Viral Vector Core facility. Large-scale ChAdOx1 nCoV-19 vector was prepared using an AEX process based on methods described previously [9].

Ad5 CsCl purification

Infected cells (150 mL) were spun at 300 x g for 20 min, the supernatant was removed, and the cell pellet was resuspended in 13 mL of lysis buffer (50 mM Tris (pH 9.0), 0.1% Triton X-100 and 2 mM $MgCl_2$, with 25 µL nuclease added just before use) and transferred to a 50 mL centrifuge tube. The suspension was incubated at RT for 30 mins and 6 mL 5M NaCl was added. The sample was spun at 1900 x g for 5 mins and the supernatant recovered. The clarified supernatant (~18 mL) was transferred to the top of CsCl step gradient (1.25 g/cm and 1.35 g/cm CsCl solution) in 38.5 ml Beckman ultra-clear ultracentrifuge tubes. The ultracentrifugation tubes were filled to the top with HEPES buffer and

centrifuged in a Beckman SW20Ti rotor at 100,000 x g in a Beckman ultracentrifuge at 4°C for 2 hrs. The virus band containing the enriched complete particles was extracted and mixed with 1.35 g/mL CsCl solution and subjected to another round of ultracentrifugation at 100,000 x g for 18 hrs. The virus band was collected in 5 mL volume and dialyzed in Float-A-Lyzer® 100 K dialysis device (Repligen) against 1L of 50 mM Tris pH 8.0 buffer for 2 hrs. The virus samples were aliquoted and stored at -80°C.

Ad5 AEX purification

The AEX purification was performed according to the procedures described previously [17]. Briefly, the infected cells were lysed by incubating with lysis buffer (10% v/v Polysorbate 20, 50% w/v Sucrose, 20 mM $MgCl_2$, 500 mM Tris pH 8.0) with benzonase (50 unit/mL) for 2 hrs at 37°C with shaking at 110 rpm. The lysate was then clarified using a 23cm^2 HC pro depth filter (Millipore) followed by TFF step to concentrate and buffer exchange the lysate for the capture step. Using an Akta Pure (GE) a 0.18 mL bed volume Mustang Q-XT Acrodisc column (Pall) was loaded with diafiltered material at 5mL/min. Subsequently, column was washed with 5 column volumes of loading buffer (100 mM NaCl, 1 mM MgCl2, 0.1% v/v Polysorbate 20, 5% w/v Sucrose, 50 mM Bis-Tris, pH 6.5) and bound virus was eluted by a linear gradient of NaCl in elution buffer (1 M NaCl, 1 mM $MgCl_2$, 0.1% v/v Polysorbate 20, 5% w/v Sucrose, 50 mM Tris-HCl, pH 8.0). The purified virus was buffer exchanged against 1L of 50 mM Tris pH 8.0 buffer for 2 hrs, aliquoted and stored at -80°C.

Infectivity Assay

To measure the titer of infective Ad5 particles, an anti-Hexon immunostaining assay kit (Cell Biolabs Inc) was used [18]. A total of 2.5×10^5 HEK293 cells were cultured per well in 24-well plates 24 hrs before the infection. Serially diluted Ad5 virus was added to each well and cells were incubated for 48 hrs at 37°C in 5% CO_2. Briefly, cells were fixed by adding 100% cold methanol to each well and incubated at -20°C for 10 min. The cells were washed with three times and then blocked with 1% bovine

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serum albumin (BSA) in phosphate buffered saline (PBS). The anti-Hexon polyclonal antibody followed by secondary anti mouse horseradish peroxidase (HRP) conjugated antibody (Cell Biolabs Inc.) were added sequentially to each well according to the manufacturing instructions. Staining was developed by addition of 3,3 diaminobenzidine (DAB) substrate, and positive brown/black stained cells were counted by light microscopy and infectious titers (IFU/mL) were calculated for each well using the instruction on the kit.

Real-time quantitative PCR

All primers, probes and controls were from Adeno-X™ qPCR Titration Kit (Takara, Japan). Viral DNA was extracted from purified Ad5 using the NucleoSpin Virus kit (MN, Germany) according to the manufacturer s instructions and eluted in 30 µL of DNase and RNase-free water and stored at -20°C until use. Serial dilutions of the viral DNA sample were used as a template for qPCR to determine the threshold cycle (Ct) for each dilution. Real-time PCR assays were carried out in triplicate in a 25 µL final volume that included 2 µL of sample dilution, 12 µL of TB Green Advantage qPCR Premix (2X), 0.5 µL 50 × ROX Reference Dye, 0.5 µL of each primer (10 µM), and 9 µL of nuclease-free water on a CFX Connect (Biorad, USA) Real-Time PCR detection system under the following conditions: Denaturation at 95°C for 30 sec, then qPCR for 40 cycles at 95°C for 5 sec and 60°C for 30 sec and finally for dissociation curve, 15 sec at 95°C, 30 sec at 60°C and 15 sec at 95°C. The DNA copy number was then determined from a standard curve generated from a standard control with known genome copy number.

Reversed-Phase HPLC Analysis

ChAdOx1-GFP or Ad5 fractions were injected neat or were concentrated 10X using 100 kDa MWCO centrifugal filters (Millipore Sigma) prior to reversed-phase analysis. Adenovirus samples were injected into a 1220 LC system (Agilent Technologies) containing a Hypersil GOLD™ C4 column (4.6 x 250 mm, 5 µm, ThermoFisher Scientific). The LC gradient consisted of 20-65% B (A: 0.1% trifluoroacetic acid in water, B: 0.1% trifluoroacetic acid in acetonitrile) over 90 min at a flow rate of 0.2

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mL/min. Elution of each protein was monitored using the absorbance signal at 214 nm (UV214). For identification, peaks were collected manually and dried overnight at 30°C using a vacufuge (Eppendorf). The following day, a digestion solution (50 mM ammonium bicarbonate pH 7.7, 10 mM DTT, 10% acetonitrile) was added and the samples were incubated overnight at 37°C in the presence of 5 µg of trypsin or chymotrypsin. The samples were then subjected to LC-MS peptide mapping as described below.

LC-MS Peptide Mapping

ChAdOx1-GFP, ChAdOx1 nCoV-19, or Ad5 fractions were buffer exchanged into 50 mM Tris, 1 mM EDTA pH 8.0 using 100 kDa MWCO centrifugal filters (Millipore Sigma). The samples were then reduced using 10 mM DTT (5 min at 90°C), alkylated using 20 mM IAM (30 min at ambient temperature), and digested using 10 µg trypsin or chymotrypsin (overnight at 37°C). LC-MS peptide mapping was performed using a 1290 LC system (Agilent Technologies) connected in-line to a 6545XT quadrupole time-of-flight mass spectrometer (Agilent Technologies). Peptides were desalted and separated using a CSH C18 column (2.1 x 150 mm, 1.7 µm, Waters Corporation) held at 60°C. The LC gradient consisted of 0-40% B (A: water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid) over 60 min at a flow rate of 0.2 mL/min. The electrospray ionization parameters consisted of: 275°C gas temperature, 4,000V Vcap, and 175V fragmentor. Mass spectra were collected from 275-1700 m/z at 1 spectra/sec. The threshold for MS/MS analysis was 10,000 counts and the two most abundant ions were selected for CID fragmentation per cycle.

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MS Data Processing

Mass spectra were processed using MassHunter Bioconfirm v10.0 software (Agilent Technologies). The Ad5 (UniProt ID UP000004992) or ChAdOx (UniProt ID UP000110857) proteomes were used for the database search. Variable modifications included: Cys alkylation, Met oxidation, and Asn deamidation. The ion abundance ratio of confirmed peptides was then used to compare the relative

protein abundance between CsCl fractions. The number of confirmed peptides compared between CsCl fractions ranged from 5 (L1 52/55k) to 140 (Protein II) for the ChAdOx proteins.

Following data processing using MassHunter, the presence of host cell proteins in each viral sample was investigated using Proteome Discoverer v1.4 software (ThermoFisher Scientific) and the human proteome (UniProt ID UP000005640). The criteria used to confirm the presence of an HCP was 2 unique peptides per identified protein.

LC-MS Peptide Data Analysis

The relative abundances of various peptides within the virus proteome were determined using a method adapted from Silva *et al*. who demonstrated that, with an internal standard, the relative abundance and therefore quantity of proteins in a protein mix can be determined by comparison of the abundance of their constituent peptides identified from peptide mapping studies. [19] We adapted the procedure without the use of an internal standard to look at the relative abundance of peptides between two Adenovirus samples, and thus identified trends in the differences observed. We compared the ratios of all common peptides observed between the two Adenovirus samples being compared, rather than the top 3 most abundant as previously described [19], and then used box-whisker plots so that all the data were evaluated. For each of the ChAdOx1-GFP samples analyzed, the peptides identified from mass spectrometry peptide mapping (following trypsin and chymotrypsin digestion of the virus sample) were compared pair wise with the 2nd HDF and the relative proportion of the peptide abundance was determined for each viral protein identified. For ease of comparison, the relative abundance ratios determined for each sample were normalized against a viral protein that is known to not be modified during adenovirus assembly and maturation and that had greater than 3 common peptides identified. For relative comparisons of the different ChAdOx1-GFP CsCl fractions, the ratios were normalized against the Protein IX median ratio to be consistent with the HPLC peak area analysis in Figure 4a. For relative comparisons of the ChAdOx1 nCoV-19 samples from a large-scale AEX preparation and two lab-scale CsCl preparations, the ratios

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were normalized against the Protein II median ratio, as too few common protein IX peptides were identified.

Transmission Electron microscopy

Copper 200 mesh grids coated with continuous carbon (Agar Scientific Ltd. Essex, UK) were glow discharged for 60 sec just before use. 5 µL of sample were incubated on the grid for 1 min, blotted and then incubated with freshly filtered 5 µL 2% uranyl acetate (UA) (Merck Life Science UK Ltd. Dorset, UK) for 1 min, blotted, dried and then imaged.

Micrographs were collected on a Tecnai T12 G2 Twin microscope equipped with a LaB6 electron source and TVIPS F216 CCD camera. All micrographs were collected with a defocus value between 3 µm and 10 µm depending on the magnification.

Virus morphology analysis

Two datasets were collected for each purified Ad5 and ChAdOx1-GFP sample fraction. The cryo EM automated particle picking software SHPIRE-crYOLO was used to train two separate models based on the main morphological classes of virus particle observed in the micrographs[20]. The micrographs from the first dataset were used exclusively for training models by manually picking full or empty capsids. A particle was deemed “complete” if the contrast across the particle diameter was uniform, suggesting no stain infiltration. “incomplete” was characterized as having darker contrast in the capsid core and a lightly contrasting periphery (Supplemental Figure 1).

Models were built separately for Ad5-full, Ad5-empty, ChAdOx1-GFP-full and ChAdOx1-GFP-empty and then used for automated picking of the second dataset for each purification condition in crYOLO. The box manager GUI within crYOLO was used to inspect the picking quality manually.

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Adenovirus Protein (Variants)	Location	Function	Undergoes cleavage
L1 52/55k	Inside the empty capsid (non-structural protein)	x Assembly of infectious particle[11] x Encapsidation of viral DNA	9
Mu, X	Inside the capsid (core protein)	x Condenses Adenovirus genome x Alters accumulation of E2 proteins x Is involved in increasing DNA transfection efficiency[30]	9
Protease, AVP	Inside the empty capsid (core protein)	x Essential for virus maturation x Production of infectious progeny x Cleavage of precursors IIIa, VI, VIII, Mu, TP, L1 52/55k[30]	
Protein II, Hexon	Capsid	x Major coat protein x Hexon coat proteins are synthesised during late infection and form homo-trimers	
Protein III, Fiber Protein, Penton Base	Capsid	x Major capsid protein that self-associates to form penton base pentamers x Involved in virus secondary attachment to host cell after initial attachment by the fiber protein, and in endocytosis of virions x As the virus enters the host cell, penton proteins are shed.	
Protein IIIa	Minor Capsid	x Interacts with L1 52/55K to help with the encapsidation of the viral DNA	9
Protein V	Core protein	x Associates with the viral genome and bridges the core and the capsid proteins x Appears to be essential for virus replication in primary cells[30]	
Protein VI (VI(1), VI(2), VI(3))	Minor Capsid	x It functions as a cofactor for the adenovirus protease (AVP) x As a chaperone for nuclear transport x Essential for virus assembly and endosome lysis	9
Protein VII (VII(1), VII(2), VII(3))	Core protein	x Nuclear transport of the viral genome acts as cellular histone[30]	9

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Protein VIII (VIII(1), VIII(2), VIII(3))	Minor Capsid	x Structural component of the virion that acts as a cement protein on the capsid interior and which connect the peripentonal hexons and group-of-nine hexons together	9
Protein IX	Minor Capsid	x Most flexible molecule among the cement proteins. x Acts as a transcriptional activator of Ad genes x Reorganize host cell nuclear domains.	

Table 1. Key adenoviral structural proteins and their functional characteristics.[30]

Sample	**TEM analysis**			**Virus Particle Analysis**		
	Complete Particles	Incomplete Particles	Relative Abundance of Complete particle (%)	Virus particle concentration (VP/mL) – qPCR	Infective particle concentration (VP/mL)	Infectivity Ratio (P:I)
Ad5 CsCl	4185	240	95	4.3×10^{11}	1.2×10^{11}	4
Ad5 AEX	752	238	76	1.2×10^{11}	8.6×10^{09}	14

Table 2. TEM analysis of transmission electron microscopy images of Ad5 samples purified by CsCl ultracentrifugation or anion exchange chromatography.

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Sample	**TEM analysis**			**Mass Spectrometry Analysis**		**Virus Particle Analysis**		
	Complete Particles	Incomplete Particles	Complete particle percent (%)	L1 52/55k peak analysis (RA)	L1 52/55k peptide analysis (RA)	Virus particle Conc. (VP/mL) - qPCR	Infective Particle Conc. (VP/mL)	Infectivity Ratio (P:I)

ChAdOx 1st LDF	2291	1400	62	82	5.5	1.0×10^{11}	8.9×10^{08}	113
ChAdOx 2nd LDF	684	843	45	66	5.6	5.6×10^{10}	7.3×10^{08}	77
ChAdOx 1st HDF	3339	266	93	8	2.0	8.5×10^{11}	1.1×10^{10}	79
ChAdOx 2nd HDF	5791	50	99	10	1	3.4×10^{11}	3.4×10^{11}	56

Table 3. Analytical comparison of TEM; LC-MS and virus particle data for ChAdOx1 fractions taken from CsCl ultracentrifugation purification. relative peak abundance determined from RP-UHPLC. median relative abundance of L1 52/55k determined from peptide analysis.

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