Development of a High Recovery Adenovirus Purification Process Using Anion Exchange Nanofibers

A thesis submitted to University College London for the degree of

DOCTOR OF ENGINEERING

by

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Declaration

I hereby declare that the work presented in this thesis is solely my own work and that to the best of my knowledge the work is original, except where otherwise indicated by reference to other authors.

Jordan P Turnbull

10 October 2018
Abstract

The manufacture of high quality virus at an industrial scale remains a challenge. In the present study, the ability of nanofiber adsorbent functionalised with quaternary (Q) amine ligands to purify adenovirus 5 (Ad5) from crude feeds was investigated. The hypothesis for this study is that nanofibres modified with Q amine ligands will enhance purification of Ad5, enabling recovery of the virus at high, infectious yields from crude cell lysate feeds. The nanofiber adsorbent technology are non-woven regenerated cellulose nanofibers that present an open pore structure 0.2-2.0µm with large inter-fiber space and shallow bed height. These are features previously reported to be advantageous for separation of viral vectors.

The upstream process for propagation of Ad5 stocks involves use of a HEK293 cell line that can allow proliferation of the virus as it contains the E1 gene deleted from Ad5. Optimal culture conditions for reproducible production of infective Ad5 from HEK293 are poorly characterised. Therefore, we performed investigations of the influence of cell passage (P: P2, P5, P10), metabolic activity (mitochondrial activity: MTT assay), and rate of proliferation on generation of infective Ad5. HEK293 passage influenced the yield of Ad5 produced. Cells of a low passage (P2) generated lower amounts of virus than those of higher passage (P5, P10). This was due to lower growth rates of P2 cells than P5 and P10 cells. The intermediate passage cells (P10) presented with the highest growth rates that were significantly greater ($P \leq 0.05$) than that of P2 cells. The mitochondrial activity of P2 cells were lower than that of P10 cells but P5 cells presented with greater mitochondrial activity than P10 cells. HEK293 cells were used at passages 10-15 to propagate Ad5 for the nanofibre optimisation and screening phases of the study.

The Q amine modified nanofibres are novel and therefore preliminary investigations of their function for separation of proteins and viruses are necessary. Conditions for the use of nanofibre were optimised initially by assessing the ability of those matrices to purify bovine serum albumin (BSA) and thyroglobulin. High recovery separations of BSA and thyroglobulin were achieved with nanofibres compared to Q-
Sepharose and POROS chromatography materials. A number of gradient elution methods were applied to purify Ad5 from a clarified bulk feed lysate. Ad5 was purified from a clarified bulk feed and protein VIII, a protein marker for infective mature capsids was identified (via mass spectrometry) in a high salt elution fraction. Our study is the first to describe evidence for identification of a cleaved mature capsid protein in a preparative chromatographic purification step. Evaluations of the effects of process variables that may induce stress on Ad5 during separation procedures showed that high salt concentrations in elution buffers and increased flow rates did not affect Ad5 recovery and infective yield. Lowering the Ad5 feed volume from 50mL to 20 mL lead to improvements in resolution of eluted virus peaks and increases in infective Ad5 yields from 69% to 78%.

Further optimisation of nanofiber-mediated purification of Ad5 and screening of nanofibers functionalised with Q amine ligands at low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) densities was performed. Clarified crude and filtered (500 kDa hollow fiber tangential flow filtration system) Ad5 feeds were purified using nanofibers at low, medium and high Q amine ligand densities. Results showed that by maintaining short process times, infective Ad5 recoveries of over 90% were achieved and those are the highest infective recoveries of Ad5 achieved to date. Prolonged adsorption durations on Q amine nanofibers showed significant losses in Ad5 product quality on medium and high ligand densities over extended binding durations of up to 24 min. Each ligand density produced several Ad5 populations over a single run with unique infective ratios (1-16.04 virus particle/infectious virus particle).

Increasing Q amine ligand density improved resolution and separation of intact Ad5 capsids from host cell protein impurities and product-related impurities including free hexon (a major capsid coat protein) and replication-defective Ad5 capsids that contained DNA. Using 0.125mL adsorbent, flow rates in excess of 70 mL/min could be applied to nanofiber adsorbents for separation of Ad5, indicating efficiency in the
purification workflow. Nanofiber nanofibers exhibited high dynamic binding capacity for Ad5 vectors in excess of $2.39 \times 10^{10}$ virus particles.

This study demonstrates the utility of Q amine functionalised nanofibre nanofibers for high recovery purification of infective Ad5. The bioprocess workflow devised for separation of Ad5 from crude cell lysate feed generated from an optimised upstream process can be scaled up for industrial manufacture of therapeutic Ad5 containing genetic payloads. Quaternary amine functionalised nanofibre nanofibers present features that clearly indicate their potential as next generation bioprocessing tools.
Adenovirus is being developed as a vaccine and oncolytic vector therapy. Manufacture of viral vectors used for vaccination and gene therapy remains costly and challenging. The primary bottleneck in viral vector processes is the downstream purification. Implemented here is the use of a novel scalable purification platform developed by the UCL spin out, and PhD sponsor, company Puridify Ltd for high yield purifications of adenovirus vector. This methodology can now be readily implemented into a range of industrial viral vector processes. The presentation of thesis data as part of Puridify business engagement, regular interaction with The Clinical BioManufacturing Facility and an oral presentation at a conference, and a submitted abstract for a conference in November, will aid wider academic and industrial engagement for the purification of adenovirus using nanofiber technology.

Moreover, the research presented in this these has demonstrated the commercial value of nanofiber purification of adenovirus. This has direct value for Puridify Ltd, as it demonstrates high product quality is achieved after purification. Conference proceedings resulting from this thesis have provided further exposure for Puridify within the bioprocess community aiding future commercial and academic partnerships.

The implementation of the methodology and technology developed in this thesis by viral vector manufacturers will enable high quality product purifications. The ability to purify high quality adenovirus with minimal loses to infective recoveries over the processing step could increase therapeutic efficacy per dose, reducing dose volume. This would have significant implications for bioprocess economics reducing the overall volume of product that requires stringent storage conditions to maintain stability from process to patient.

Loss of vector infectivity during bioprocess is still poorly understood with a greater understanding of adenovirus virology required to inform quality by design process development. Here adenovirus lifecycle was analysed to determine the presence of
therapeutically viable vector purified over the nanofiber chromatography step, identifying a protein marker for maturity by implementing a novel suite of analytics. This was then used to develop a novel high resolution separation of different adenovirus populations demonstrating the value of such an approach with high infective recoveries.

In summary, the knowledge, methodologies, insight and expertise developed as a result of this research that will beneficially impact academic understanding of viral vector downstream processing but commercial development of the sponsor company Puridify Ltd., advisory organisation The Clinical BioManufacturing Facility and the wider bioprocessing community.
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Finally thank you to all my friends and colleagues at UCL.
Dedicated to my grandfather

Terry Rowland
(1926-2016)

A man of great industry
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno associated virus</td>
</tr>
<tr>
<td>Ad5</td>
<td>Adenovirus serotype 5</td>
</tr>
<tr>
<td>AdV</td>
<td>Adenovirus</td>
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<tr>
<td>AVP</td>
<td>Adenovirus maturation protein</td>
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</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CQA</td>
<td>Critical quality attribute</td>
</tr>
<tr>
<td>DBC</td>
<td>Dynamic binding capacity</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DF</td>
<td>Diafiltration</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DoE</td>
<td>Design of experiments</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>DSP</td>
<td>Downstream processing</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European medicines agency</td>
</tr>
<tr>
<td>Empty</td>
<td>Capsid containing no genetic material</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GON</td>
<td>Group of nine</td>
</tr>
<tr>
<td>GOS</td>
<td>Group of six</td>
</tr>
<tr>
<td>HCP</td>
<td>Host cell protein</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>HEP-C</td>
<td>Hepatitis-C</td>
</tr>
<tr>
<td>HIC</td>
<td>Hydrophobic interaction</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICH</td>
<td>International Council for Harmonisation</td>
</tr>
<tr>
<td>IEX</td>
<td>Ion exchange</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IND</td>
<td>Investigational new drug</td>
</tr>
<tr>
<td>IVP</td>
<td>Infective virus particle</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MMC</td>
<td>Mixed mode</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem Mass spectrometry</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre ($10^{-9}$ m)</td>
</tr>
<tr>
<td>NVP</td>
<td>Non-infective virus particle</td>
</tr>
<tr>
<td>PAC</td>
<td>Prespotted anchorchip</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Q</td>
<td>Quaterniary amine</td>
</tr>
<tr>
<td>QbD</td>
<td>Quality by design</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SHIV</td>
<td>Simian/human immunodeficiency virus</td>
</tr>
<tr>
<td>SME</td>
<td>Small and medium-sized enterprises</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TFF</td>
<td>Tangential flow filtration</td>
</tr>
<tr>
<td>TG</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>TMP</td>
<td>Transmembrane pressure</td>
</tr>
<tr>
<td>ToF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>UF</td>
<td>Ultrafiltration</td>
</tr>
<tr>
<td>USP</td>
<td>Upstream processing</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>VP</td>
<td>Virus particle</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
</tbody>
</table>
w/w   Weight/weight
β-Gal   β -Galactosidase
Chapter 1  Introduction

1.1  The Application of Adenovirus 5 as a Gene Delivery Vector

Adenovirus 5 particles are 90-100 nm, non-enveloped, icosahedral capsids that carry a linear, 26-45 kb of unsegmented, double-stranded DNA genome (San Martin, 2012). Adenovirus 5 is an attractive gene delivery vector due to structural stability, ability to carry large transgene payloads and broad tissue tropism (Crystal, 2014). Adenovirus 5 vectors are an advantageous therapeutic platform as they are well-characterised (Puig et al., 2014). Since 2017, adenovirus vectors have been used in 20% of all gene therapy trials (Lee et al., 2017). Twenty current clinical trials are described in Table 1. The Ad5 vector fulfils three broad roles in these gene therapy trials, 1.) in an oncolytic capacity for treatment of cancers, 2.) as a vaccine whereby the vector expresses a foreign antigenic protein, or 3.) for gene therapy that involves the vector expressing a non-mutant protein to correct for a genetic mutation. Early clinical trials for gene correction using Ad5 were limited due to the majority of patients expressing antibodies against common Ad5 serotypes, and nonspecific binding of Ad5 to blood components that lead to viral inactivation (Keeler et al., 2017).

Modification of Ad5 vectors, to generate chimeric vectors and chemical alterations to the Ad5 capsid have prevented some of the early issues regarding cell specificity and host immunity (Capasso et al., 2014). These modifications were performed using knowledge about the structures of different adenovirus serotypes (Wold and Toth, 2013). Adenovirus 5 was subject to a strenuous safety review after a patient’s death was reported in 1999, but this viral vector has subsequently been confirmed as clinically safe (Kotterman et al., 2015). Adenovirus 5 is one of several promising viral vectors candidates along with adeno associated virus (AAV), lenti virus, herpes simplex virus, vaccinia virus, retrovirus, and vesicular stomatitis virus (Finer and Glorioso, 2017).
As an oncolytic therapy, the robust immune response to the Ad5 vector is advantageous to successful therapeutic outcomes (Keeler et al., 2017). Oncolytic viruses are a promising anti-tumour therapy as they selectively replicate in tumour cells. The viral progeny generated in the tumour cells spread to neighbouring tumour cells increasing the dose efficacy and renewing the vector therapy in situ (Niemann and Kühnel, 2017). Oncolytic viruses activate toll-like receptor signalling pathways leading to acute local tumour inflammation (Wongthida et al., 2011). Inflammatory cytokines released from activated immune cells induce tumour cell cytotoxicity leading to a further influx of immune cells into the tumour (Di Paolo et al., 2014).

Tumour associated microvasculature can be disrupted by oncolytic viruses, priming the adaptive immune cells through a disruption of intratumoral immune homeostasis and promotion of the innate immune response (Niemann and Kühnel, 2017). The efficacy of Ad5 delivered therapy is increased by this induction of the innate and adaptive immune responses (Sobol et al., 2011). The simplicity of the viral vector genome allows replication machinery reprogramming so that the virus is able to specifically respond to tumour-specific molecular alterations by initiating replication and subsequent cell lysis (Russell et al., 2012). A number of promising Ad5 clinical trials for different cancers including prostate, ovarian, bladder and refractory solid tumours are currently being performed (Hemminki et al., 2015, Freytag et al., 2014, Kim et al., 2013, Burke et al., 2012, Pesonen et al., 2012).

Adenovirus 5 vectors can be modified to carry a genetic payload coding for transient, antigenic protein expression. Replication defective Ad5 vectors have been extensively employed as vaccines because they are able to induce a strong humoral and T cell response to the vector encoded antigen, mediating long lasting immune responses (Lasaro and Ertl, 2009). As such, adenoviruses are efficacious vaccine vectors against diseases that have typically been difficult to vaccinate against, including mucosal pathogens such as influenza, tuberculosis (TB) and human immunodeficiency virus (HIV) (Afkhami et al., 2016). Chimpanzee-derived replication-defective Ad5 encoding ebolavirus glycoprotein was shown to generate
protection against acute lethal Zaire ebolavirus challenge in macaques demonstrating potential to provide rapid immunity in an acute human outbreak setting (Stanley et al., 2014). Advances in adenoviral vectorology, viral packaging cell lines, immunity and vaccine immunology have led the development of adenoviral-vectored vaccines against a number of pathogens for which there does not exist an effective vaccination strategy (Afkhami et al., 2016).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Conditions</th>
<th>Interventions</th>
<th>URL Reff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safety Study of Adenovirus Vector Engineered to Express hIL-12 in Combination With Activator Ligand to Treat Melanoma</td>
<td>Melanoma</td>
<td>Biological: INXN-2001 Drug: INXN-1001</td>
<td><a href="https://ClinicalTrials.gov/show/NCT01397708">https://ClinicalTrials.gov/show/NCT01397708</a></td>
</tr>
<tr>
<td>Safety, Tolerability, and Immunogenicity of the Ebola Chimpanzee Adenovirus Vector Vaccine (cAd3-EBO), VRC-EBOADC069-00-VP, in Healthy Adults</td>
<td>Healthy Adult Immune Responses to Vaccine</td>
<td>Biological: VRC-EBOADC069-00-VP Biological: VRC-EBOADC076-00-VP</td>
<td><a href="https://ClinicalTrials.gov/show/NCT02231866">https://ClinicalTrials.gov/show/NCT02231866</a></td>
</tr>
<tr>
<td>Safety of and Immune Response to a DNA HIV Vaccine Followed By an Adenoviral Vector HIV Vaccine in Healthy Adults</td>
<td>HIV Infections</td>
<td>Biological: VRC-HIVDNA016-00-VP Biological: VRC-HIVADV014-00-VP Biological: VRC-HIVDNA016-00-VP placebo Biological: VRC-HIVADV014-00-VP placebo</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00125970">https://ClinicalTrials.gov/show/NCT00125970</a></td>
</tr>
<tr>
<td>A Phase I Clinical Trial to Evaluate the Ebola Adenovirus Vector Vaccine (Ad5-EBOV) in Healthy Adults.</td>
<td>Ebola Virus Disease</td>
<td>Biological: Low dose Ebola Zaire vaccine (Ad5-EBOV) Biological: High dose Ebola Zaire vaccine (Ad5-EBOV) Biological: placebo (one dose) Biological: placebo (two doses)</td>
<td><a href="https://ClinicalTrials.gov/show/NCT02326194">https://ClinicalTrials.gov/show/NCT02326194</a></td>
</tr>
<tr>
<td>Study Title</td>
<td>Disease/Treatment</td>
<td>Biological/Dose</td>
<td>Details</td>
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<tr>
<td>Phase 1 Placebo-controlled, Randomized Trial of an Adenoviral-vector Based Norovirus Vaccine</td>
<td>Norovirus Gastroenteritis</td>
<td>Drug: VXA-G1.1-NN Oral Vaccine Drug: VXA Placebo Tablets</td>
<td><a href="https://ClinicalTrials.gov/show/NCT02868073">https://ClinicalTrials.gov/show/NCT02868073</a></td>
</tr>
<tr>
<td>Safety of and Immune Response to a DNA HIV Vaccine Followed By Boosting With One of Two Serotypes of Adenoviral Vector HIV Vaccine in Healthy Adults</td>
<td>HIV Infections</td>
<td>Biological: VRC-HIVADV027-00-VP Biological: VRC-HIVADV038-00-VP Biological: VRC-HIVDNA044-00-VP</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00472719">https://ClinicalTrials.gov/show/NCT00472719</a></td>
</tr>
<tr>
<td>Safety of and Immune Response to Two Different HIV Vaccines, Each Followed by an Adenoviral Vaccine Boost, in HIV Uninfected Adults</td>
<td>HIV Infections</td>
<td>Biological: VRC-HIVADV014-00-VP Biological: VRC-HIVDNA009-00-VP Biological: FFB Biological: PBS</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00270218">https://ClinicalTrials.gov/show/NCT00270218</a></td>
</tr>
<tr>
<td>Safety of and Immune Response to a DNA HIV Vaccine Followed by an Adenoviral Vaccine Boost Given Three Different Ways to HIV Uninfected Adults</td>
<td>HIV Infections</td>
<td>Biological: VRC-HIVDNA009-00-VP Biological: VRC-HIVADV014-00-VP</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00384787">https://ClinicalTrials.gov/show/NCT00384787</a></td>
</tr>
<tr>
<td>Phase I Pilot Study of Ad5-CB-CFTR, an Adenovirus Vector Containing the Cystic Fibrosis Transmembrane Conductance Regulator Gene, in Patients With Cystic Fibrosis</td>
<td>Cystic Fibrosis</td>
<td>Genetic: Ad5-CB-CFTR</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00004779">https://ClinicalTrials.gov/show/NCT00004779</a></td>
</tr>
<tr>
<td>A Booster Dose of Ad5-EBOV in Healthy Adults After Primary Immunization</td>
<td>Ebola Virus Disease</td>
<td>Biological: 4Å—10^10vp/1ml Ebola Zaire vaccine (Ad5-EBOV) Biological: 1.6Å—10^11vp/2ml Ebola Zaire vaccine (Ad5-EBOV)</td>
<td><a href="https://ClinicalTrials.gov/show/NCT02533791">https://ClinicalTrials.gov/show/NCT02533791</a></td>
</tr>
<tr>
<td>Study Title</td>
<td>Condition</td>
<td>Biological: placebo</td>
<td>URL</td>
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<td>---------------------------------------------------------------------------</td>
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<tr>
<td>A Clinical Trial on the Candidate Vaccine cAd3-EBOZ in Healthy Adults in Switzerland</td>
<td>Ebola Vaccines</td>
<td>Biological: cAd3-EBOZ vaccine Biological: Placebo (for cAd3-EBOZ vaccine)</td>
<td><a href="https://ClinicalTrials.gov/show/NCT02289027">https://ClinicalTrials.gov/show/NCT02289027</a></td>
</tr>
<tr>
<td>A Phase I Trial to Evaluate Ad5-EBOV in Healthy Adult Africans in China.</td>
<td>Ebola Virus Disease</td>
<td>Biological: Ad5-EBOV</td>
<td><a href="https://ClinicalTrials.gov/show/NCT02401373">https://ClinicalTrials.gov/show/NCT02401373</a></td>
</tr>
<tr>
<td>Administration of AdVEGF-All6A+ to Myocardium of Individuals With Diffuse CAD Via Minimally Invasive Surgery</td>
<td>Coronary Artery Disease</td>
<td>Biological: AdVEGF-All6A+ Biological: AdNull</td>
<td><a href="https://ClinicalTrials.gov/show/NCT01757223">https://ClinicalTrials.gov/show/NCT01757223</a></td>
</tr>
<tr>
<td>Administration of Autologous Dendritic Cells (DCs) Infected With an Adenovirus Expressing Her-2</td>
<td>Breast Neoplasms</td>
<td>Biological: CD34+ derived DCs</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00197522">https://ClinicalTrials.gov/show/NCT00197522</a></td>
</tr>
<tr>
<td>Phase I Study of HIV Adenoviral Vector Vaccine in Healthy Subjects Using Needle or Biojector Injection</td>
<td>HIV Infections</td>
<td>Drug: VRC-HIVADV014-00-VP</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00709605">https://ClinicalTrials.gov/show/NCT00709605</a></td>
</tr>
<tr>
<td>Safety and Effectiveness of HIV-1 DNA Plasmid Vaccine and HIV-1 Recombinant Adenoviral Vector Vaccine in HIV-Uninfected, Circumcised Men and Male-to-Female (MTF) Transgender Persons Who Have Sex With Men</td>
<td>HIV Infections</td>
<td>Biological: DNA plasmid vaccine Biological: Recombinant adenoviral serotype 5 (rAD5) vector vaccine Biological: DNA vaccine placebo Biological: HIV-1 recombinant adenovirus vaccine placebo</td>
<td><a href="https://ClinicalTrials.gov/show/NCT00865566">https://ClinicalTrials.gov/show/NCT00865566</a></td>
</tr>
<tr>
<td>Phase 1 Trial of Ebola Vaccine in Mali</td>
<td>Ebola Virus Disease</td>
<td>Hemorrhagic Fever</td>
<td>Biological: Ebola Chimpanzee Adenovirus Vector Vaccine (cAd3-EBO Z Biological: Booster-MVA-BNA® Filo or saline placebo</td>
</tr>
<tr>
<td>Study Title</td>
<td>Disease(s)</td>
<td>Biological(s)</td>
<td>Drug(s)</td>
</tr>
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<tr>
<td>A Study of Ad-RTS-hIL-12 With Veledimex in Subjects With Glioblastoma or Malignant Glioma</td>
<td>Glioblastoma Multiforme</td>
<td>Anaplastic Oligoastrocytoma</td>
<td>Biological: INXN-2001 Drug: veledimex</td>
</tr>
</tbody>
</table>
Vaccination against disease prevents up to 3 million deaths per year (Ulmer et al., 2006), and is widely regarded as one of the most important medical achievements (Grimm and Ackerman, 2013). In 2010 the Bill and Melinda Gates Foundation, the World Health Organization, the National Institute of Allergy and Infectious Diseases, and the United Nations Children’s Fund launched the Decade of Vaccines (DoV). The aims of the DoV over the next 10 years, are to scale up research, development and delivery of vaccines to the world’s poorest countries, with a coverage of 90% (Keith et al., 2013). The conglomerate organization has projected the scheme could prevent the death of 7.6 million children under the age of five (Steinglass et al., 2011). A cost of around US $800 to US $1.8 billion is commonly cited for the development of a new drug (Waye et al., 2013). In 2009 alone, more than US $45 billion was spent globally on pharmaceutical research and development (Waye et al., 2013). Vaccines now fall into a category of biotech products that comprise more than 50% of the pharmaceutical product development pipeline (Milne and Kaitin, 2010).

The application of next-generation sequencing and proteomic techniques has allowed a paradigm shift in vaccine development research (Grimm and Ackerman, 2013). Researchers are now able to identify novel candidate immunogens by mining entire microbial genomes, proteomes and transcriptomes utilizing this reverse vaccinology approach has garnered considerable success including antigens for, *Streptococcus pneumonia*, pathogenic *Escherichia coli* and the tick *Haemaphysalis flava* (Moriel et al., 2010, Hiller et al., 2007, Liu et al., 2018). The impact of vaccine development is becoming increasingly apparent. This therapy will be crucially important to combat the emergence of antibiotic resistance that in conjunction with the abated development of novel antibiotics has resulted in pathogenic, multi-drug resistant microbial strains posing a genuine threat to public health (Grimm and Ackerman, 2013).

### 1.2 Adenovirus 5 as a Research Model Platform

Adenovirus 5 is a pathogen in humans and it is also present in most vertebrates, (Perez-Berna et al., 2012, Harrach et al., 2011). In humans, Ad5 commonly causes
mild gastrointestinal, respiratory and eye infections (Cheng et al., 2007). An Ad5 infection is typically subclinical but Ad5 causes pathology in immunocompromised patients and this accounts for a significant level of morbidity (Leen and Rooney, 2005). Utilization of Ad5 as an experimental system has allowed investigation of fundamental processes in eukaryotic cell life, e.g. splicing and apoptosis (Silva et al., 2010). Adenoviruses have shown potential as vectors for gene transfer into mammalian cells, vaccine delivery and oncolysis (Shiver et al., 2002, Lasaro and Ertl, 2009, Yamamoto and Curiel, 2010). Human species Ad5 is the most well understood adenovirus serotype and it is also the most frequently used serotype in clinical studies (Kalyuzhniy et al., 2008). The majority of current gene delivery vectors are genetically modified versions of Ad5 (Wold and Toth, 2013).

A thorough understanding of the structure of Ad5 is important for several reasons. The range of therapeutic applications for Ad5 vectors can be increased with comprehensive mapping of the Ad5 capsid structure. The pathogenic effects of Ad5 can be addressed with increased knowledge about the structure of the virus (Kinchington et al., 2005). The large size and structural intricacy of Ad5 highlights this virus as a suitable experimental model for complex virus assembly.

### 1.3 The Structure of Adenovirus 5

Adenovirus 5 is a large non-enveloped virus, with a dsDNA genome (Riske et al., 2013). Measuring at ~950 Å, the icosahedral capsid is assembled from around 11 different types of protein (Perez-Berna et al., 2009). The organization of the Ad5 capsid is remarkably complex with a triangulation number of pseudo $T = 25$ (Sutjipto et al., 2005). The theoretical framework for the structure of spherical viruses was used to predict that the Ad5 capsid would comprise $60 \times 25 = 1500$ structural subunits: 12 pentamers forming the vertices, plus 240 hexamers (Caspar and Klug, 1962). These early theoretical models predicted that the structural subunits would be chemically identical. A combination of chromatographic purification and electron microscopy revealed that the 6-fold and 5-fold coordinated capsomers were chemically distinct (Ginsberg et al., 1966).
As recently as 2009, only the three major components of the adenoviral capsid proteins, hexon, penton and fibre, were described in atomic detail. Valentine and Pereira produced the first images of the extended fibers which protrude from each vertex (Valentine et al., 2003). Since then, the location of the major coat proteins along with their structural and life cycle significance has been associated with the discovery and characterization of the minor coat proteins. A total of nine minor coat proteins have been identified with a combination of known crystal structures and increasingly detailed cryo-electron microscopy maps (Perez-Berna et al., 2012). The nomenclature for all adenovirus polypeptides follows roman numerals in order of decreasing molecular weight, determined by SDS-PAGE electrophoresis. Using this terminology, the alternative names for hexon (108 kDa), penton (63 kDa) and fibre (61.5 kDa) are polypeptide II, polypeptide III and polypeptide IV respectively (Table 2) (San Martin, 2012).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Uncleaved Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II (Hexon)</td>
<td>Major capsid protein that self assembles</td>
<td>108007</td>
</tr>
<tr>
<td>III (Penton)</td>
<td>Major capsid protein that forms penton base pentamers. Involved in in secondary attachment of virus to host cell</td>
<td>63293</td>
</tr>
<tr>
<td>pIIIa</td>
<td>Structural component of the virion mediating interactions between hexon proteins</td>
<td>65253</td>
</tr>
<tr>
<td>IV (Fiber)</td>
<td>Mediates initial attachment of virus to host cell. Forms spikes that extend from each vertex of the icosahedral capsid. Interacts with host coxsackievirus and adenovirus receptor CAR</td>
<td>61585</td>
</tr>
<tr>
<td>IVa2</td>
<td>Component of the packaging machinery which encapsidates the viral DNA into preformed capsids</td>
<td>50887</td>
</tr>
<tr>
<td>V</td>
<td>Promotes viral assembly through nucleoplasmic redistribution of nucleolar nucleophosmin</td>
<td>41000</td>
</tr>
<tr>
<td>pVI</td>
<td>During virus assembly, promotes hexon trimers nuclear import through nuclear pore complexes via an importin alpha/beta-dependent mechanism.</td>
<td>26996</td>
</tr>
<tr>
<td>pVII</td>
<td>Binds strongly to DNA suggesting it mediates wrapping and condensing of genome</td>
<td>21992</td>
</tr>
<tr>
<td>pVIII</td>
<td>Internal structural component of the virion that stabilises capsid interior</td>
<td>24687</td>
</tr>
<tr>
<td>IX</td>
<td>Structural component of the virion that acts as a cement protein on the capsid exterior</td>
<td>14458</td>
</tr>
<tr>
<td>pμ</td>
<td>Condenses viral genome upon maturation this action is relaxed to prime capsid genome ejection</td>
<td>8846</td>
</tr>
<tr>
<td>pTP</td>
<td>Protein covalently bound to the viral genome acting as a primer for viral genomic replication</td>
<td>76500</td>
</tr>
<tr>
<td>AVP</td>
<td>Cleaves viral precursor proteins (pTP, pIIIa, pVI, pVII, pVIII, and pX) giving rise to mature virions. Protease slides along the viral DNA to cleave viral precursor proteins.</td>
<td>23068</td>
</tr>
</tbody>
</table>
1.2.1 Hexon

Hexon is the primary building block of the Ad5 capsid with a copy number of 720 monomers and 240 trimers (San Martin, 2012). The intricate folding of the hexon trimer stabilises the Ad5 capsid structure (Rux et al., 2003, Rux and Burnett, 2000). The position of hexon and minor interactions with other coat proteins induce conformational changes in the N- and C- termini of the hexon monomer (Perez-Berna et al., 2009). Located at the innermost part of the hexon base, N- and C-termini establish interactions between hexons. A property of viral coat proteins is the mobility of their terminal regions in solution. Mobile terminal regions function as conformational switches that modulate the different quasi-equivalent interactions within the icosahedral capsid (San Martin, 2012).

Mobile regions are not confined to the innermost parts of the hexon, hexon towers display hyper variable mobile loops that are believed to be involved in defining the specific serological response of the virus (Liu et al., 2010). As the hexon structure is investigated further, this protein has been shown to play a crucial role in tropism determination as well as receptor binding and entry, functions previously believed to be performed exclusively by the penton proteins (Kalyuzhniy et al., 2008).

The geometric coat architecture is made up of systems of hexon and penton tiles. The first system is known as the Group of Nine (GON) hexons (Figure 1). These structures were identified using mild chemical dissociation conditions. A GON forms the central hexon of each facet, but it does not form the hexons surrounding the penton or peripentonal (Prage et al., 1970). Two different systems of tiles are used to describe the icosahedral architecture, a.) the central GON of each facet and b.) the five peripental hexon trimers (group of six: GOS) together with the penton base (Figure 1) (San Martin, 2012).
Figure 1. Schematic of the Ad5 hexon capsid conformation. (A) The hexon trimer is displayed as a hexagon with a triangle representing the positions of the three towers. The two different faces of the pseudo-hexagonal hexon base are labelled s (single monomer) and t (two monomer). (B) A representation of a penton and five adjacent facets. The red symbols represent the icosahedral symmetry axis. The four numbered hexon trimers represent an asymmetric unit (AU). The penton and hexons highlighted in green represent a Group of Six (GOS). Hexons in the Group of Nine (GON) are represented in white (San Martin, 2012).
The hexon structure was determined by X-ray diffraction that generated a 6 Å resolution crystallographic model and it was the first animal infecting virus protein to be crystallized (Pereira et al., 1968). Solving the structure of Ad5 hexon revealed that the trimeric capsomer presented a pseudo 6-fold hexagonal base. Negative staining revealed that the hexon trimer comprised three towers that were twisted with respect to the hexagonal base (Figure 2) (Burnett et al., 1985) This conformation allows for a close-packed protein shell for protection of the viral genome. It is achieved by repetition of a structural motif at the base of each hexon monomer forming an 8-stranded β-barrel with a “jellyroll” topology (Perez-Berna et al., 2012). Each capsid facet is formed by 12 trimers of hexon (Figure 1) (San Martin, 2012) A GON consists of nine trimers of the major coat protein, hexon, forming a central plate for each of the 25 faces of the structure (Rodriguez-Ortega et al., 2006).

The icosahedral asymmetric unit (AU) describes four hexon homotrimerers, positioned in four different environments. Because of the trimeric nature of hexons, the AU classification includes the penton. As such \( 4 \times 3 \) (hexon) + 1 (penton) = 13 independent polypeptides (Figure 1) (Perez-Berna et al., 2012). This is in contrast to the initial 25 different environments of the monomeric hexon AU of the \( T = 25 \) icosahedron predicted by the Casper and Klug quasi-equivalence theory; the Ad5 capsid is commonly described as \textit{pseudo} \( T = 25 \) (Perez-Berna et al., 2009).
Figure 2. The structure of Ad5 capsid and internal proteins. (A) Icosahedral model of Ad5 shell built from a low resolution cryoEM map. (B) Diagram of adenovirus proteins, highlighting the level of complexity within each virion (Mangel and San Martin, 2014, San Martin, 2012). Adenovirus proteins prefixed with a ‘p’ denote proteins that undergo proteolysis by adenovirus maturation protein (AVP) as part of a maturation which causes a disassociation of the adenovirus genome from the capsid and a capsid stiffening, priming the capsid for uncoating under endosomal acidification.
1.2.2 Penton Base and Fibre

The penton base (polypeptide III) and fibre (polypeptide IV) are involved in the initial stages of infection and form the vertex capsomers (San Martin, 2012). Specifically, the distal, C-terminal fibre nob binds to the cell surface protein coxsackie- and adenovirus receptor (CAR), this initial host cell attachment is true for most human serotypes (Bergelson et al., 1997). Each penton base monomer N-terminal arm (residues 37-51) extends away from the β-barrels (main body of the protein) towards the viral core. This anchors the penton within the GOS by interacting with the N-terminal domains of two IIIa monomers (Perez-Berna et al., 2012). Experimental disruption of this interaction causes virions to lose their pentons (San Martin et al., 2008).

1.2.3 Polypeptide IIIa

There is a total copy number of 60 polypeptide IIIa monomers distributed within the capsid, one per asymmetric unit (Saban et al., 2006). Five polypeptide IIIa monomers form a ring formation underneath each vertex on the inner capsid surface (San Martin et al., 2008). The N-terminal domain of polypeptide IIIa binds to two peripentonal hexons and the penton base - this knit maintains the GOS structure and is known as the “GOS-glue domain” (San Martin, 2012). Polypeptide IIIa also interacts with one of two independent copies of polypeptide VIII via a long helix known as the VIII-binding domain (San Martin et al., 2008). This is a key interaction that contributes to the architecture of Ad5 by tethering the GON to the GOS, through the central plate facet of the GON (San Martin, 2012).

The roles of polypeptide IIIa in the viral cycle may be to stabilize the vertex region and the packaged genome upon assembly, and to regulate vertex and genome release during virus uncoating (San Martin et al., 2008). Serotype specific interactions between the N-terminal domain of IIIa and the putative scaffold protein L1 52-55K are required to promote correct genome packaging, highlighting an even more complex structural role for polypeptide IIIa (Ma and Hearing, 2011).
1.2.4 Protein IVa2

The molecular mechanism for Ad5 genome packing into the capsid is reported to involve an ATP-hydrolysing molecular machine that drives DNA insertion through a portal structure into a preformed prohead, similar to that of bacteriophages and herpes viruses (Ostapchuk et al., 2011). Protein IVa2 may be the ATPase driving this packaging mechanism. In vitro studies have shown that IVa2 binds to ATP and Ad5 protein L4-22K, that subsequently binds to Ad5 genome sequences required for packaging, i.e. packaging domain between nucleotides 200 and 400 (Zhang and Imperiale, 2007).

The L1-52/55K protein also associates with the packaging domain in vivo (Perez-Romero et al., 2005). Adenovirus 5 mutants generated by mutating the genes encoding the IVa2, L4-22K, L1-52/55K proteins produce progeny virus with defects associated with packaging and therefore lack a DNA core (Ostapchuk et al., 2011). However, protein IVa2 seems to play a more complex role in Ad5 capsid packaging and assembly of the empty virus particle containing motifs associated with binding DNA as well as motifs for the binding and hydrolysis of ATP (Koonin et al., 1993). Ostapchuk et al. (2011) developed an Ad5 mutant that was not able to produce protein IVa2. The engineered virus particles did not contain a detectable DNA core suggesting that a major role of protein IVa2 is DNA packaging.

1.2.5 Protein V

Protein V is a minor core protein bridging the core and capsid proteins; a capsid contains approximately 157 copies of protein V (Vayda et al., 1983). Following Ad5 infection, a nucleoplasmic redistribution of nucleolar nucleophosmin 1 (NPM1) occurs. Protein V localises in nucleoli during Ad5 infection and as the infection progresses it translocates to the nucleoplasm where it is assimilated into infectious virus particles. Protein V promotes NPM1 translocation from the nucleoli to the nucleoplasm (Ugai et al., 2010). This translocation is correlated with Ad5 assembly and propagation. Interestingly, NPM1 is co-purified with empty capsids from
infected cells, and specifically interacts with empty Ad5 suggesting NPM1 plays a role in viral assembly, but is not present in infective virus particles. Protein V mediated NPM1 redistribution plays an essential role in capsid formation and replication (Ugai et al., 2012).

1.2.6 Polypeptide VI

Polypeptide VI serves multiple roles in the infection mechanism of Ad5 (San Martin, 2012). Upon entry, the polypeptide VI N-terminal amphipathic helix deforms the curved structure of the endosomal membrane, allowing the virus to escape into the cytosol (Wiethoff et al., 2005). Polypeptide VI also aids microtubular trafficking to the nucleus facilitated by a ubiquitin ligase interacting motif (PPxY) (Wodrich et al., 2010). The PPxY motif on polypeptide VI allows this protein to act as an activator of adenoviral gene expression (Schreiner et al., 2012). The virion forms within the nucleus of infected cells, allowing structural viral proteins to be imported into the nucleus (San Martin, 2012).

The C-terminus of polypeptide VI contains a nuclear localization signal and this facilitates an interaction with importin α/β, mediating importation of the major coat protein hexon (Wodrich et al., 2003). The C-terminal is a substrate and cofactor of the adenovirus proteinase (AVP). Activated AVP mediates maturation of Ad5 that leads to formation of infectious virions (Mangel et al., 1996). Polypeptide VI binds to a loop in the inner cavity of the hexon trimer and to dsDNA joining the core of the virus to the internal face of icosahedral shell (Wodrich et al., 2003). The copy number (360) of polypeptide VI raises questions about its distribution in the capsid core. At one copy for every two hexons its does not appear to follow the icosahedral symmetry presented by the other adenoviral proteins (Sutjipto et al., 2005).

1.2.7 Protein VII

The major core protein, protein VII represents 10% of the total mass of the Ad5 particle and functions via a similar mechanism as a histone-like core protein because it binds tightly to the Ad5 genome (Ostapchuk et al., 2017). Viral dsDNA trigger a
cellular DNA damage response cascade that severely inhibits viral replication if not impeded (Weitzman and Ornelles, 2005). Protein VII inhibits the early stages of this response, whilst binding to and regulating cellular chromatin and isolates immune danger signals to dampen immune signalling (Avgousti et al., 2016). During infection, the Ad5 fiber protein engages with CAR, the penton base binds to cellular αvβ3/5 integrins, and the tethered virus is internalised by endocytosis into clathrin-coated endosomes (Reddy and Nemerow, 2014). Adenovirus 5 capsids that do not contain protein VII are able to undergo endocytosis into the cell. However, those capsids are not able to eject their genetic material into host cells and this results in failure of the late stages of the infection cycle (Ostapchuk et al., 2017). Cleavage of protein VII induces an increase in the internal pressure of the capsid that is suggested to facilitate nuclear ejection (Ortega-Esteban et al., 2015).

1.2.8 Polypeptide VIII

The function of polypeptide VIII is not well characterised but this protein has been shown to confer a thermolabile phenotype when mutated (Liu et al., 1985). Crystallography and CryoEM analyses of the structure of Ad5 has shown that polypeptide VIII is located on the internal side of the capsid (Liu et al., 2010, Reddy et al., 2010). The asymmetric unit contains two independent monomers of polypeptide VIII. One monomer links each GOS to each five surrounding GONs, by binding to polypeptide IIIa and to the hexon bases at the periphery of the GOS (San Martin, 2012). The second monomer is located around the icosahedral 3-fold symmetry axis and stabilizes the GON by interaction with protein IX (San Martin, 2012).

As the viral capsid matures, polypeptide VIII undergoes proteolytic maturation. Initially synthesized as a precursor (pVIII, 277 residues), the protein undergoes cleavage by AVP at three potential residues (112, 131, 157). The cleavage site that is used and the fragments of polypeptide VIII that remain in the mature virion are not known (Diouri et al., 1996, San Martin, 2012). Mass spectrometry studies investigating the function of polypeptide VIII during Ad5 maturation have produced
conflicting results. A previous study indicated that only the N-terminal remains in the mature virion (Liu et al., 2003). Another study detected only the C-terminal fragment in the virion core (Takahashi et al., 2006). A report demonstrated findings suggesting that both N- and C-terminal fragments remain in the capsid core (Lehmberg et al., 1999, Chelius et al., 2002). A previous study showed that the precursor form of pVIII, and the two largest fragments pVIII_N and pVIII_C interact with the packaging protein IVa2 (Chelius et al., 2002).

1.2.9 Polypeptide IX

Polypeptide IX is located on the outermost part of the Ad5 capsid. It plays an integral structural role that involves binding together the hexons in each GON, whilst binding GONs to GONs (Perez-Berna et al., 2009). Polypeptide IX forms a web over the outer side of the Ad5 capsid (Perez-Berna et al., 2009). The polypeptide comprises 140 residues and is a 185 Å molecule (San Martin, 2012). Twelve monomers of polypeptide IX form four triskelions, a three armed protein trimer that forms round hexagonal proteins (Fotin et al., 2004). Each monomer joins through hydrophobic interactions whilst interacting with hexons via another β-strand augmentation. A C-terminal α-helix, the rope domain, joins with the C-terminal helices of three copies of polypeptide IX. Adenovirus 5 virions either contain this intricate incorporation of polypeptide IX to the capsid or in some cases they do not show the interactions of the core protein (Scheres et al., 2005).

Interestingly, previous studies have shown that polypeptide IX is not critical for capsid assembly and instead the protein plays an integral role in capsid stability with polypeptide IX-deleted mutants presenting a low thermostability (Perez-Berna et al., 2009). Polypeptide IX is present in empty or immature Ad5 virion assembly intermediates (San Martin, 2012). Of all the characterized Ad5 structural proteins, polypeptide IX is only present in Mastadenovirus (mammalian infecting species). Polypeptide IX is responsible for an immune response interference and modulates viral tropism (de Vrij et al., 2011). All structural studies to date have indicated that at least one domain of polypeptide IX is exposed on the capsid surface in an ideal
position for host interaction. The location of polypeptide IX on the capsid surface has identified this protein as a target for modification of Ad5 to create structural motifs for virus targeting, imaging or immunization (San Martin, 2012). Although polypeptide IX does not play a major role in capsid maturation it is vital for the final stages of viral entry. After escaping from the cell endosome, the partially disassembled virion travels along the microtubule network towards the nuclear pore before complete disassembly and transport of the viral genome into the nucleus (Perez-Berna et al., 2009). This mechanism is modulated by microtubule motor kinesin-1 and polypeptide IX is the principal viral component responsible for this interaction (San Martin, 2012).

1.2.10 Protein X

The function of the Ad5 capsid core protein X (protein μ) is poorly understood. Protein X highly basic and it is 19 amino acids in length. The core protein binds tightly to DNA and previous reports indicate that it may be involved in viral genome condensation (Anderson et al., 1989).

1.3 The Life Cycle of Adenovirus 5

Adenovirus 5 undergoes proteolytic processing during its maturation life cycle stage that primes the capsid for infection. Adenovirus 5 maturation involves the proteolytic cleavage of several proteins by AVP. Adenovirus proteinase utilises a peptide cleaved from one of the adenovirus protein substrates which it uses as a ‘molecular sled’ sliding along the Ad5 genome and cleaving proteins as it moves (Mangel and San Martin, 2014). XGG-X sequence motifs that are modulated by AVP that recognizes (M/I/L) XGX-G and (M/I/L), cleave minor capsid proteins, IIIa, VI and VIII and core proteins VII, μ and the terminal protein (TP) (Figure 2b). Maturation of Ad5 causes the capsid to become meta-stable, (providing it is not subject to environmental stress) priming it for a stepwise uncoating. AVP substrates, polypeptide V and polypeptide VI are cleaved and this decouples the condensed genome from the capsid, reducing the capsid stabilisation effect and aiding vertex
release (Perez-Berna et al., 2012). Polypeptide IIIa and polypeptide VIII act as a molecular stitch to link hexon trimer groups to form groups of six (GOS). As protein IIIa and protein VIII are cleaved, this stitch weakens and a brittle Ad5 capsid is formed (San Martin, 2012). The cleavage of protein VII is attributed to an increase in the internal pressure of the mature capsid which aids the ejection of the viral genome upon uncoating in the endosome (Ortega-Esteban et al., 2015). Maturation also results in capsid removal of proteins L1-52/55K and L4-22K (Ostapchuk et al., 2011).

The immature capsid containing the uncleaved precursor proteins is unable to infect host cells as it is unable to uncoat. The capsid core is more compact and stable than the mature capsid. The condensing action of uncleaved core proteins that pin the capsid to the genome core, stabilizes the capsid (Perez-Berna et al., 2009). A HAdV-2 thermosensitive mutant (tsl) was developed to investigate structural and functional aspects of Ad5 maturation (San Martin, 2012). When grown at 39°C tsl does not package AVP, and capsids that contain protein precursors are produced (Rancourt et al., 1995). The viral packaging remains unimpaired, whilst the cleavage mechanism is blocked. This model has been replicated in HAdV-5 (Imelli et al., 2009).

There are three major differences between the mature and immature Ad5 virions. First, in the inner capsid surface of tsl cryoEM studies have shown there are extra densities of protein located between the ring of peripental hexons and the hexons that make up the GON (Perez-Berna et al., 2009). This is known as the ‘molecular stitch’, it aids capsid formation by holding the GOS in place during assembly, but it must be removed to allow vertex release for uncoating (Perez-Berna et al., 2009). The ‘stitch’ comprises 216-225 residues in the polypeptide IIIa VIII-binding domain and 45 residues between the cleavage sites residues 110 and 159 in polypeptide VIII (Liu et al., 2010). The stich is a structure formed by the central peptides of uncleaved pVIII and IIIa.

CryoEM highlighted a second difference inside each tsl hexon cavity (Silvestry et al., 2009). A weak density observed in sub-nanometer resolution structural studies of
the mature particles has been attributed to polypeptide VI (Reddy et al., 2010). Therefore in tsI where the precursor form of protein VI, pVI, is present, the interaction with the hexon is different, resulting in a more uniform occupancy or ordering of the part of protein VI inserted within the internal hexon cavity (San Martin, 2012). The change in conformation relates to the change in protein VI during the viral cycle. Initially the strong bond between protein VI and hexon permits the transport mechanism during assembly and a weaker interaction allows effective release of protein VI from the capsid into the endosome (Perez-Berna et al., 2009).

Organization of the capsid core is the final major difference between immature and mature Ad5 particles. During maturation the core transforms from an ordered to disordered state (Silvestry et al., 2009). Disrupting tsI virions, releases very compact, spherical cores of DNA suggesting that protein VII and protein μ induce a stronger dsDNA condensing ability than their cleaved mature versions (San Martin, 2012). These small proteolytic changes to individual Ad5 capsid and core proteins collectively result in a remarkably different capsid, exhibiting differentiated physical properties and infectious/therapeutic capability. Those changes to the capsid during maturation provide a set of capsid properties that could be exploited for an immature and mature vector separation process design.

1.4 Purification of Adenovirus 5

The ability to generate large volumes of viral stocks through improved hightitre fermentations has placed an increasing burden on the viral downstream processing, particularly chromatography (Uyeki and Cox, 2013, van der Loo and Wright, 2016). The most common chromatography modes for vector purification are ion exchange (IEX), affinity, mixed mode (MMC), size exclusion (SEC) and hydrophobic interaction (HIC) (Nestola et al., 2015). However the majority of process step investigations have concentrated on ion exchange in bind and elute mode (Sharon and Kamen, 2018). The ultimate goal of viral vector downstream processing is to obtain a product of high potency separated from process related impurities (e.g. benzonase, extractables and leachables) and product related impurities (host cell
proteins: HCP, DNA, viral free protein, aggregates, empty capsids) (Nestola et al., 2015). The product should meet the stringent quality guidelines outlined by the regulatory authorities such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA) (ICH, 2007).

1.4.1 Ultracentrifugation

Downstream processing of viral vectors represents a significant bottleneck and a primary expense for their production (Vellinga et al., 2014). Typically, industry and academia have relied heavily on ultracentrifugation for downstream purification of viral vectors (Morenweiser, 2005). This technique separates large, dense viral vectors from other impurities based on the density of the viruses. The success of ultracentrifugation has highlighted this procedure as the gold standard processing step for purification of viral vectors and as a result it has not been optimised into a new process. Ultracentrifugation produces highly purified viral vector preparations (Ugai et al., 2005) despite major drawbacks including poor scalability and high operating costs (Vicente et al., 2011c). The major advantage of ultracentrifugation is that packed and empty Ad5 capsids are separated due to differences in density of the empty capsid and the genome containing (packed) virus particle in caesium chloride gradients of 1.29 to 1.3g/cm³ and 1.34 g/cm³ respectively (Sundquist et al., 1973). A caesium chloride density gradient is an appropriate tool for separation of empty and packed Ad5, but immature and mature Ad5 virions that both contain a genome are indistinguishable. Empty and immature Ad5 capsids that are product related impurities, cause low infectivity titres, poor yields, and provide a source of non-functional antigenic material. Despite limitations, ultracentrifugation remains the benchmark process for clinical grade Ad5 purification (Sharon and Kamen, 2018).

1.4.2 Resins

Ion-exchange chromatography is scalable and GMP compliant. It is the favoured method of purification for vaccines, virus like particles and viral vectors (Forcic et al., 2011, Puig et al., 2014, Urbas et al., 2011). Anion-exchange resins were
repurposed to construct scalable purification platforms for purification of proteins and low molecular weight biotherapeutics. The limitations of conventional resin-based chromatographic platforms became apparent as target biotherapeutics have increased in size and complexity (Huyghe et al., 1995).

Mass transfer of the mobile phase over and between resin particles is attributed to convective flow, and diffusion mass transfer dictates movement of the mobile phase into the inner functionalised surface of the resin (Lyddiatt, 2002). Resin chromatography media has been the workhorse for protein purification for many years (Hardick et al., 2013). The pore size of resin media used in protein purification is approximately 30nm and vector species size can range from 20-300nm (Jungbauer, 2005). Viral vector particles are unable to access the internal functionalised surface, resulting in 30-50 fold decreases in column capacity for macromolecules when compared to protein standards (Lyddiatt and O'Sullivan, 1998, Trilisky and Lenhoff, 2007).

To address this problem, the surface area available to bind molecules can be increased by decreasing the size of the beads or increasing their porosity. Those modifications can, however, cause increased back pressure (Palsson et al., 1999). In gigaporous material the large macromolecules diffuse slowly into the pores, becoming the rate limiting step (Lyddiatt, 2002). Huyghe et al. (1995) pioneered early work into ion-exchange resins for the purification of viral vectors achieving low yields of 49% from a 5 x 10^{10} virus particle (VP) challenge. Low recoveries and capacities of Ad5 purification are widely reported even for gigaporous columns such as Sepharose XL™ (Lucero et al., 2017). A tandem diethyl amino ethyl (DEAE) and PolyFlo® (Puresyn, Inc., Malvern, PA) resin process step was developed by Green et al. (2002). The tandem step with a 10^{14} VP input generated recoveries of 73% ± 4% for the DEAE column and 84% ±12% for the PolyFlo® columns step.

Smaller viral vectors, e.g. the 20nm capsid diameter adeno-associated virus (AAV) has been purified with resin matrices to high rates of recovery as the pore exclusion effect is easier to circumvent with the small AAV virus. Another study demonstrated
successful separation of empty and full AAV particles utilizing Q-Sepharose - a ratio of empty capsids to packed capsids was 0.2:1 (Qu et al., 2007). High pH buffers (pH 9.0) may be important to achieve empty capsid separations and high yields of infective vector (Urabe et al., 2006). The study concluded that the DNA packaged inside AAV particles results in significant, exploitable changes in particle interactions with ion-exchange chromatographic media. The inclusion of DNA is believed to either induce conformational changes in the capsid proteins of AAV or the presence of DNA results in a greater anionic property that influences the behaviour of the virus on ion exchange resins. Whilst resins operated in bind and elute mode for vector purification show low capacity, new resins types such as Capto™ Core 700 (GE Life Sciences) are being developed for vector polishing steps. The bead technology excludes virus from the functionalised inner surface by an inactive shell and has seen novel applications such as being directly added to viral slurry (James et al., 2016).

1.4.3 Monoliths

A number of new scalable chromatography adsorbents have been developed for purification of viruses to improve binding capacities and yields. Monoliths were introduced in the early 1990s as a new column technology for rapid separation of molecules. Monoliths offer substantial improvements on resins with respect to recovery, capacity and reduction in process time (Pfaunmiller et al., 2013). Monolith columns are based on polymethacrylate and are manufactured with a radial geometric design, a single block of solid matrix with highly interconnected macropores through which biomolecules are transported by convective flux (Nestola et al., 2015). Monoliths are viable chromatography platforms for Ad5 purification (Whitfield et al., 2009) as well as the separation of much larger enveloped virus species including Vaccinia viruses (250 x 350nm) (Vincent et al., 2017).

The key advantage of monoliths over resins is large pores that allow high flow rates at a low back pressure (Jandera et al., 2006). Preparation of a monolith is relatively simple. A homogenous polymerization mixture is produced that contains the
monomers (functional and cross-linked monomers), porogenic solvents and a radical initiator (Pfaunmiller et al., 2013). Monolith adsorbents can be synthesized in situ adapting to the shape of their moulds.

Figure 3. Comparison of the mass transfer properties of chromatographic matrices (Source: Puridify).

Monoliths rely primarily on convective mass transfer of the mobile phase due to large pore and channel architecture (Figure 3) (Pfaunmiller et al., 2013). Although the architecture of those materials can be indirectly altered, small, randomly produced channels cause column heterogeneity. Small pores in monolith structures increase their surface area but the pores also exhibit size exclusion properties that cause flow restrictions. These have been shown to cause clogging especially when large volumes of DNA are present in the bioreactor bulk (Nestola et al., 2015). Enlarging the pores permits greater flow rates but molecules are less likely to form contacts with ligands bound to the support wall of the monolith (Tennikov et al., 1998).
A quaternary amine (QA) monolithic column platform was developed by Whitfield et al. (2009) using the Bio-Monolith QA made by Agilent. The column step was able to operate with Ad5 loads of $3 \times 10^{10}$ to $9.6 \times 10^{11}$ VP/mL. Using Sartobind anion direct, Peixoto et al. (2008) achieved an 62% recovery of infectious Ad5 determined by cell fluorescence. The recovery of Ad5 loaded with recombinant gene therapy was improved from 28% using a Q-Sepharose™ XL column to 35% using a monolithic column (CIM™ QA-1), and Ad5 was purified at high flow rates of 5 mL min$^{-1}$ by Lucero et al. (2017). The CIM™ QA-1 was the preferred choice over the weak anion CIM™ DEAE-1. The final infective ratio of virus particle to infective units (VP:IU) was 13:1, a range documented as acceptable for potency by the food and drug association (FDA) (Kramberger et al., 2015). These studies highlight the high recoveries of Ad5 that can be achieved using scalable monolithic platforms.

A study utilizing a monolithic chromatography support carrying DEAE ligands in a polishing step for canine adenovirus serotype 2 (CAd2) was unable to separate empty and packed capsids (Segura et al., 2012). The primary focus of the study was to create a method for purification of CAd2 from a crude cell lysate. Microfiltration and ultra/diafiltration were used for clarification and concentration of the crude viral stocks. Hydrophobic interaction chromatography using Fractogel propyl tentacle resin was used to remove the bulk contaminating proteins, in a step that lead to the recovery of high CAV-2 yields (88%). Finally, anionic exchange chromatography was used achieving a CAV-2 recovery of 58-69%. The final purified material consisted of 9.9±2.2% empty capsids. Segura et al. (2012) concluded that protein aggregates disrupted the difference in the charge densities of the empty and packed CAV-2. Monoliths provide a promising alternative to packed bed resin vector purification demonstrating improved process efficiencies when switched to a convective media platform. Alternative convective media platforms have emerged as a cost effective strategy for large volume processing of viral feeds (Nestola et al., 2015).
1.4.4 Membranes

Membrane adsorbents have been utilised primarily in the biopharmaceutical industry for flowthrough mode monoclonal antibody purification (Weaver et al., 2013). Membranes consistently yield low pressure drops and minimal compression or channelling (Nestola et al., 2015). Compared to packed bed separations, membrane absorption devices are able to operate at high flow rates, with fast adsorption kinetics, whilst inducing minimal pressure drops (Figure 4) (Schneiderman et al., 2011). Rapid adsorption of molecules is achieved due to their ready access to ligands on the adsorbent surface and therefore minimal internal diffusion (Chang et al., 2011). Similar to monoliths, membranes are typically used as a disposable unit, eliminating lengthy cleaning and validation procedures (Nestola et al., 2015).

A number of membrane adsorbents are commercially available. Mustang® Q membrane units (supplier: PALL) are a popular unit platform due to high recoveries and operation flow rates. A previous study showed that an 80% Ad5 VP recovery was achieved with a $1.0 \times 10^{13}$ VP load, 65% IVP recovery was observed at 20 column volumes /min using a $3.7 \times 10^{11}$ IVP load, and a drop in recovery of 46% IVP was observed at higher flowrates of 40 cv/min using a 0.03 mL Acrodisc unit (Kutner et al., 2004). Vicente et al. (2011a) investigated the effect of ligand density on the recovery of a recombinant baculovirus using a membrane adsorbent. The study showed that a high ligand density did not lead to an increased binding capacity. The purity of the recovered virus was improved by reducing the ligand density and with the low ligand density membrane, a recovery of 85%, a 20% improvement on the higher ligand density membrane was achieved. The low ligand density membrane also allowed substantial clearance of process and product related impurities (Vicente et al., 2011a). McNally et al. (2014) achieved 50% infectious recovery of lentivirus using an anion exchange membrane adsorbent. The open pore structure of membranes (Figure 3) allows for rapid processing times and high recoveries, and the membrane surface can be functionalised to optimise purification of various viral vectors. Despite those advantages, membranes still suffer some drawbacks include low capacity and poor resolution (Nestola et al., 2015).
1.4.5 Nanofibers

Nanofibers are non-woven regenerated cellulose membranes that present an open pore structure with large inter-fiber space and shallow bed height, features previously reported to be advantageous for separation of viral vectors (Wickramasinghe et al., 2006, Hardick et al., 2011). Nanofibers (consisting of randomly overlaid fibers with diameters of <1000 nm) present an attractive alternative to conventional chromatographic materials because these operating conditions lead to a reduction in processing times and concomitant reduction in waste and solvent use (Schneiderman et al., 2011).

The electrospinning technique that was used to produce electrospun textile fibers was first reported in 1934 when Formhals patented this process (Formhals, 1934). To produce a membrane consisting of electrospun fibers, a cellulose polymer solvent is drawn through an electrically charged spinneret, connected to a (5-30 kV) power supply (Hardick et al., 2011). A syringe pump is used to eject the polymer solution through a needle tip at a constant rate, forming a droplet. The voltage applied to the droplet causes it to form a Taylor cone, i.e. charged liquid jet. Electrostatic repulsion is initiated at small bends inside the fiber, causing a whipping process that elongates the jet stretching it to nanometre-scale before it collects on a grounded collector plate (Hardick et al., 2011). The polymer solidifies, forming a fiber as the solvent evaporates (Figure 4) (Reneker and Yarin, 2008).
Diethyl amino ethyl (DEAE) derivatisation of cellulose nanofibers was described by Hardick et al. (2013). The study described the beneficial flow properties, binding capacities and fouling performance of the DEAE derivatised nanofibers, highlighting the potential of the adsorbent for protein purification. The processing times of nanofibers for purification of proteins are shorter than those of other packed bed materials (Figure 3). The binding capacities of commercially available chromatography materials are comparably lower (Schneiderman et al., 2011). Nanofibers present a high aspect ratio (low bed height to diameter) resulting in a complex flow distribution (Hardick et al., 2011). Electrospun carbon nanofibers functionalized with various surface ligand chemistries have been shown to adsorb 10 times as much protein as microfiber nanofibers (Schneiderman et al., 2011). The permeability of nanofiber mats is substantially higher than resin or monolith counterparts (Zhang et al., 2008, Schneiderman et al., 2011). Nanofibers demonstrate
a good reusability, with very low levels of fouling, but are often marketed as disposable (Hardick et al., 2013).

Table 3. Comparison of chromatography stationary phases (Orr et al., 2013).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Resin</th>
<th>Monolith</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Pressure Drop</td>
<td>High</td>
<td>Low-Moderate</td>
<td>Low</td>
</tr>
<tr>
<td>Dominant Transport</td>
<td>Diffusion</td>
<td>Convection</td>
<td>Convection</td>
</tr>
<tr>
<td>Binding Capacity</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
</tr>
<tr>
<td>Resolution</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>Hardware Cost</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Inexpensive</td>
</tr>
<tr>
<td>Hardware Footprint</td>
<td>Extensive</td>
<td>Extensive</td>
<td>Small</td>
</tr>
</tbody>
</table>

A critical factor that defines large molecule chromatography is the recovery of the bioactive form of the target (Orr et al., 2013). Nanofibers that comprise a high surface area and large interfiber space should be amenable to high recovery vector process steps, allowing for convective mass transfer of the large product (Table 3). Factors specific to the Ad5 capsid should be considered when designing capture and separation conditions of nanofiber chromatography. Nanofiber membranes present a customisable chromatographic adsorbent which can operate at high flow rates because of a low bed height and open interfiber adsorbent architecture. These convective mass transfer properties of the platform should be amenable to high yield recovery of the bioactive form of the Ad5 product.

1.5 Objectives and Experimental Aims

1.5.1 Hypothesis

The current generation of downstream processes for Ad5 vectors can be considerably improved by using novel nanofibre membranes for their purification. Those chromatography biomaterials will allow high efficiency separation and enhanced
scaling up of Ad5 and therefore significantly reduce losses of their infective yield. The physical architecture and chemical properties of nanofibre membranes will allow for greater resolution with an increased processing capacity for high throughput ionic separations of Ad5. Achievement of the following objectives and associated experimental aims will demonstrate the feasibility of nanofibre membranes for high yield purification of infective Ad5.

1.5.2 Objectives

1. Develop a methodology platform for efficient upstream production of Ad5 viral vector.

2. Develop a downstream process for Ad5 capture from a complex feed stream to generate high recovery yields of infective Ad5 product.

3. Perform a chromatographic screen of a range of different quaternary (Q) amine functionalised nanofibre membranes to assess their resolution for Ad5 from a complex feed stream.

4. Develop a process for optimal binding recovery yields of Ad5 using Q amine nanofibre membranes and further characterise those to refine binding capacities and resolution.

1.5.3 Experimental Aims

1. Determine cell culture conditions that contribute to production of infective Ad5 by assessing the metabolic activity and proliferation rates of HEK293 cells used for propagation of Ad5.
2. Investigate linear and/or step gradient chromatography procedures to develop methods for purification of Ad5 from an ultrafiltered cell lysate feed stream using nanofiber membranes functionalised with Q amine ligands.

3. Perform assays to determine the ability of purified Ad5 to infect cells and subsequently measure the recovery efficiency (perform a mass balance) at each stage of the purification process. Follow up infectivity assays with analytical assays to:
   a. Assess the presence of Ad5 genes using qPCR.
   b. Determine the function of purified Ad5 using an infectivity assay.
   c. Identify (using Western blotting and mass spectrometry) Ad5 capsid coat and core proteins levels to demonstrate the presence or absence of cleaved core proteins that will indicate mature, infective (packed), immature, non-infective (packed), and empty, non-infective Ad5 capsids.
   d. Examine the integrity of purified Ad5 capsid structure using transmission electron microscopy.

4. Apply the methodology developed for chromatographic purification of Ad5 from complex feed streams to screening Q amine nanofibre membranes with various (low, medium and high) ligand densities. Modify the protocol as required for individual ligand densities and select the protocol and that allows optimal recovery of Ad5 at high binding capacities and resolution.
Chapter 2 Materials and Methods

2.1 Materials

Replication defective (E1 gene removed) Ad5 (prepared in 2.5% (v/v) glycerol) that contained a β-galactosidase gene insert was kindly gifted from the Clinical BioManufacturing Facility (Oxford, UK). Dulbecco’s modified Eagle’s medium (DMEM) without phenol red, sodium pyruvate, penicillin/streptomycin, and L-glutamine were purchased from Life technologies, Thermo Fisher Scientific (Paisley, UK). Fetal bovine serum, Trypsin-EDTA, Tween20, sodium chloride, hydrochloric acid, ethanol, sodium thiosulphate, sodium acetate, sodium nitrate, sodium carbonate, formaldehyde, ethylenediaminetetraacetic acid, disodium dehydrate, sodium dodecyl sulphate, 2-mercaptoethanol, glycerol, bromothenol blue, sodium hydroxide, Tris hydrochloride, di-methyl formaldehyde, uranyl acetate, ammonium bicarbonate, acetonitrile, 1,1,1-Trichloro-2,2,2-trichloroethane, 1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethane, PCR-grade H₂O, HCCA (a-cyano-4-hydroxycinnamic acid), 0.1% trifluoroacetic acid (TFA), β-galactosidase Reporter Gene Staining Kit, poly-L-lysine, ammonium phosphate monobasic and 1X phosphate buffered saline were bought from Sigma-Aldrich (Gillingham, Dorset, UK). Peptide Calibration Standard II (#222570) was purchased from Bruker (Coventry, UK), Tris base was purchased from Santa Cruz Biotechnology Inc (Heidelberg, Germany) and molecular weight markers were from Invitrogen [distributed by Thermo Fisher] (Paisley, UK).

Rabbit polyclonal antibody to Ad5, goat polyclonal antibody to rabbit IgG (HRP-conjugated), and rabbit polyclonal antibody to mouse IgG (HRP-conjugated) were from Abcam (Cambridge, UK). The mouse monoclonal antibody to Ad5 Hexon was bought from Fitzgerald [distributed by 2BScientific Limited] (Upper Heyford, UK). Trypan Blue Solution, Vybrant™ MTT cell proliferation assay kit, super signal west pico chemiluminescent substrate, the iBlot 2 dry blotting system, NuPAGE™ Novex™ Precast 4-12%, 1.0 mm depth, 10-well, BisTris mini-gels, NuPAGE™ Novex™ Precast 10%, 1.0 mm depth, 10-well, BisTris mini-gels, 20X MES SDS gel...
running buffer, acetic acid, restore Western blot stripping buffer and methanol were from Thermo Fisher (Paisley, UK).

The modified Lowry protein assay kit and bovine serum albumin protein standard II were purchased from Biorad (Watford, UK). The HEK293 cell line used for the generation of adenoviral stocks and β-galactosidase infectivity titre were purchased from American Tissue Culture Collection (Manassas, VA, USA). T-175 Cellstar® culture flasks, and HYPERFlask M Straight neck, CellBIND (Corning, Wiesbaden, Germany). Nanofiber adsorbents were made to a range of quaternary amine ligand densities by Puridify (Stevenage, UK), in 0.125mL column volume formats. Sequence Grade Modified Trypsin was purchased from Promega (Southampton, UK). The host cell protein ELISA kit F650R was from Cygnus Technologies (Southport, NC, USA).

2.2 Methods

2.2.1 HEK293 Cell Culture

HEK293 cells were cultured in T-175 CellStar flasks or HYPERFlasks® in an incubator set at 37°C and 95% humidity with a 5% (v/v) CO₂ enriched atmosphere. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) penicillin/streptomycin, and 2mM L-glutamine (Biochrom, Cambridge, UK). Cells were passaged when they reached 80% confluency. Briefly, media was removed, the cell monolayer was carefully washed with 1X sterile Dulbecco’s phosphate buffered saline (PBS) to remove residual medium and cell debris. The cells were incubated with trypsin-EDTA (diluted 1:2 with 1X sterile PBS) for 1 min before they were detached by gently knocking the sides of the flask. Trypsin was neutralised by dilution with an equal volume of medium. Trypsinised cells were centrifuged at 1000 rpm for 5 min, the supernatant was discarded and the cell pellet was suspended in an appropriate volume of supplemented medium. The viability and numbers of cells were determined by treating cells with Trypan blue dye (Trypan blue exclusion assay) and
counting them using a manual haemocytometer. A 10 µL aliquot of the cell suspension was mixed with an equal volume of Trypan blue dye and 10 µL was removed and pipetted onto the haemocytometer grid under a coverslip. The dye will gain entry into dead cells with damaged plasma membranes so they will appear blue and the live cells with intact membranes will not be stained. To count cells, the haemocytometer was moved over the set of 16 corner squares and the 25 middle squares. The average cell count from each of the sets of 16 corner squares and the middle 25 squares was calculated. To determine the number of cells in 1 mL of medium, the average number of cells was multiplied by 10,000. The volume of a 16 corner squares or the middle 25 squares = W x H x D = 1 x 1 x 0.1 = 0.1 mm3 = 0.0001 mL. Therefore to calculate the number of cells in 1 mL, the average number of cells in 0.0001 mL is multiplied by 10,000. The solution from this calculation was multiplied by 2 to correct for the 1:2 dilution from the Trypan Blue addition and this solution was multiplied as required to correct for the volume of medium that the cells are suspended in, i.e. multiply by 5 if the cells are suspended in 5 mL medium. The percentage viability of the cell suspension was calculated by expressing the number of live cells as a percentage of the total number of cells (live and dead).

2.2.2 Preparation of Adenovirus 5 Stocks

Adenovirus 5 stocks were generated by adding 100 µL, 5.1 x 10^9 virus particles of Ad5 (in 2.5% (v/v) glycerol) to culture flasks containing HEK293 cells at 80% confluency. The cells were incubated for 48 h in an incubator set at 37°C and 95% humidity with 5% (v/v) CO₂ before they were stored at -80 °C.

2.2.3 Propagation of Adenovirus 5

Adenovirus 5 was propagated in HEK293 cells by adding a 10mL Ad5 stock (see section 2.2.2) containing 10^9 infectious units of Ad5 to 10 tier HYPERFlasks® containing HEK293 cells at 80% confluency. The cells were incubated at 37°C for 48 hours and 95% humidity with 5% (v/v) CO₂. The cell medium was transferred to 50 mL centrifuge tubes. The tubes were incubated on dry ice for 30 min and then
thawed at room temperature for 40 min. This cycle of freezing and thawing was performed three times to disrupt the cell membrane. The cell lysate was then centrifuged at 2000 rpm for 10 min and supernatant containing released Ad5 particles was retained.

2.2.4 Clarification of Crude Adenovirus 5 Preparations

To harvest propagated Ad5, cells were transferred from HYPERFlasks® to 50 mL centrifuge tubes. Cells were incubated on dry ice for 30 min and thawed at 37°C for 40 min. The cycle of freezing and thawing was performed three times to disrupt the cell membrane. The cell lysate was then centrifuged at 2000 rpm for 10 minutes, and the resulting supernatant was filtered using 33mm polyethersulfone (PES) membrane sterile syringe driven filters (0.45µm, Merck Millipore, Feltham, UK) before they were pooled.

2.2.5 Tangential Flow Filtration of Crude Adenovirus 5 Preparations

Tangential flow filtration of the clarified cell lysate was conducted on a KR2i system using a 500kDa molecular weight cut off (MWCO) D06-E500-05-N hollowfiber (length 65cm, surface area 370cm², Spectrum Labs, Breda, The Netherlands) at a flow rate of 20ml/min and transmembrane pressure of 2Psi (± 0.5). Ultrafiltration membranes are routinely used to concentrate biological products of interest with the ultrafiltration membrane selected to reject the product which is concentrated in the feed suspension (retentate). The cell lysate was concentrated 4X and dialysed in binding buffer (20mM Tris, pH 7.4). A 5X volume of retentate sample was then diluted 1 in 4 to original harvest volume.

2.2.6 Chromatography

Experiments were performed using an AKTA Avant (GE Healthcare Life Sciences, Buckinghamshire UK), with online measurements of pH, conductivity and UV absorbance (260 and 280 nm). The nanofiber membrane was equilibrated with 10 ml wash buffer (20 mM Tris, pH 7.4) at a flow rate of 10 ml/min. Adenovirus 5 feed
was loaded onto the column using a sample pump (S9H AKTA). A gradient elution (1M NaCl 20mM Tris, pH 7.4) was applied to nanofiber membranes at a flow rate of 10 ml/min to elute Ad5 bound to the nanofibre membrane. The column was washed with 2M NaCl 20mM Tris, pH 7.4. A step elution process schedule was then determined by peak analysis from gradient elution separations using UNICORN™ 6 control software (GE Healthcare Life Sciences, Buckinghamshire UK). Eluted purified Ad5 fractions were collected using a F9-R fraction collector (GE Healthcare Life Sciences, Buckinghamshire UK).

2.2.7 Measurement of Adenovirus 5 Infective Titre

The detection and quantification of Ad5 units that were able to deliver the β-galactosidase gene were analysed as a measure of sample infectivity using the β-galactosidase Reporter Gene Staining Kit (Sigma-Aldrich, Taufkirchen Germany). Reactions were conducted following manufacturer’s instructions but modified for a 96-well plate format. Briefly, plates were coated with 0.01% (v/v) poly-L-lysine for 10 min. HEK293 cells were plated in volumes of 100 µL at a density of 4 x 10⁵ cells/mL and cultured overnight to attach to the plate surface. Serial dilutions of Ad5 samples were prepared using supplemented DMEM and cell monolayers were treated with 100 µL diluted Ad5 - the plate was incubated for 1h at 37°C. Adenovirus 5 was then removed from wells, replaced with 100 µL of supplemented medium and the plate was incubated overnight at 37°C. To stain, media was removed from wells, cells were washed twice with PBS, fixed with 1X fixation buffer (20% formaldehyde, 2% glutaraldehyde in 10X PBS) and incubated for 10 min at room temperature. Fixed cells were washed two times with PBS and stained for 24 h at 37°C with 30 µL staining solution. The Ad5 vector contains a β-galactosidase gene insert, and this gene is transiently expressed in HEK293 cells infected with Ad5. β-galactosidase catalyses the hydrolysis of β-galactosidases. X-Gal generates an indigo-blue colour in cells which express the β-galactosidase gene. Blue stained cells were counted manually using a light microscope to determine the infective titre of Ad5 (EVOS FL Cell Imaging System, Thermo Fisher, East Grinstead, UK).
2.2.8 Identification of Empty and DNA-packed Adenovirus 5 Capsids using rtPCR Analysis

To assess total numbers of Ad5 capsids containing DNA, samples were analysed using the Adeno-X™ Rapid Titer Kit (Takara Bio Europe, Saint-Germain-en-Laye, France). Adenovirus 5 feed and purified fractions were pre-treated with DNAse to remove exogenous DNA, and then they were chemically lysed with protease. DNA was isolated using NucleoSpin® Virus Columns (Takara Bio Europe, Saint-Germain-en-Laye, France). Samples were added to the master reaction mix in a 96 well plate so that each well contained 2 µL of unknown sample or standard control DNA, 6.8 µL PCR-grade H₂O, 0.4 µL Adeno-X forward primer (10 µM), 0.4 µL Adeno-X reverse primer (10 µM), 0.4 µL ROX™ Reference Dye LMP, 10.0 µL SYBR® Advantage qPCR Premix. Reactions were performed using a CFX Connect™ Real-Time PCR Detection System (Applied Biosystems, CA, USA) using the following cycle conditions: stage 1 = 95°C for 30 seconds; stage 2 = 95°C for 5 seconds, followed by 60°C for 30 seconds (40 repetitions); stage 3 = dissociation curve of 95°C for 10 seconds, 65°C to 95°C increment 0.5°C every 5 seconds. To ensure that recoveries obtained from NucleoSpin® Virus Columns (Takara Bio Europe, Saint-Germain-en-Laye, France) were not affected by the range of salt conditions present in the elution samples, a range of samples containing standard control DNA containing 20mM Tris, and a range of salt concentrations from 0-0.5 M NaCl (all pH 7.4) were analysed. The data obtained from this demonstrated that buffer conditions of the elution fractions would not affect the column recovery from the columns or the subsequent reaction.

2.2.9 Separation of Adenovirus 5 Proteins using Gel Electrophoresis

Adenovirus 5 feed purified and feed fractions were concentrated using Vivaspin® Turbo 4 (Sartorius, Gottingen Germany). Total protein amounts were quantified using the modified Lowry protein assay. Protein samples were prepared for gel electrophoretic separation by treatment with Laemmli reducing sample treatment buffer (50 mM Tris-HCl, 4% (w/v) SDS (Sigma), 10% (v/v) β-mercaptoethanol (Sigma), 20% (v/v) glycerol (Sigma), a trace of Coomassie brilliant blue R (Sigma))
and they were heated at 95°C for 5 min. Proteins were separated using NuPAGE™ precast 4-12%, BisTris mini-gels (Thermo Fisher, East Grinstead, UK) with gels run at 100V (constant volts) per gel.

2.2.10 Silver Staining Analysis of Adenovirus 5 Proteins

Silver staining analysis of Ad5 proteins mobilised in 4-12% Bis-Tris gels was performed as this is a sensitive protein staining method that can allow visualisation of low amounts of proteins in gels. After the gel electrophoresis step, proteins were fixed (40% methanol, 10% acetic acid, 50% MilliQ water) inside the gel for 30 min at room temperature under gentle agitation. Fixed gels were washed in MilliQ water three times for 5 min. Proteins were sensitized by incubating the gel in sensitizing buffer: 0.2% (w/v) sodium thiosulphate, 33% (v/v) ethanol, 0.05M sodium acetate, at room temperature for 30 min. Sensitizing buffer was removed and the gels were washed three times for 10 min in MilliQ water. Proteins were coated in silver ions by incubating the gels in 12mM silver nitrate for 20 minutes at room temperature under agitation. To develop the gel, excess silver nitrate was removed by washing for four 30 s washes in MilliQ water, before the gel was submerged in developer solution (0.06M sodium carbonate, 0.0004% formaldehyde (v/v)). After adequate development, developer solution was removed and the gel was submerged in stop solution (0.0025M ethylenediaminetetraacetic acid disodium salt dehydrate) for 10 min. The developed gel was washed three times in MilliQ water for 5 min, before it was imaged using an Amersham Imager 600 (GE Healthcare Life Sciences, Buckinghamshire UK).

2.2.11 Identification of Adenovirus 5 Proteins using Western Blotting

Adenovirus 5 proteins separated in 10% Bis-Tris gels were transferred from gels to polyvinylidene fluoride (PVDF) membranes using an iBlot™ 2 gel transfer device following the manufacturer’s instructions. Non-specific binding sites on blots were blocked with 5% (w/v in 1X TBS-T) milk for 1 h at room temperature before the blots were incubated overnight at +4°C in anti-Ad5 polyclonal antibody diluted
1:4000 in 2% (w/v in 1X TBS-T) milk. Blots were washed three times in 1X Tris buffered saline-tween (TBS-T) for 5 min before they were incubated (with agitation) in the secondary antibody, a goat polyclonal antibody to rabbit IgG, (conjugated to horse radish peroxidase) diluted 1:8000 in 2% (w/v in 1X TBS-T) milk, for 2 h at room temperature. The blots were washed three times in 1X TBS-T for 5 min before they were incubated in enhanced chemiluminescent reagent for 1 min. Adenovirus 5 proteins were imaged using an Amersham Imager 600 (GE Healthcare Life Sciences, Buckinghamshire UK). The anti-Ad5 polyclonal antibody was stripped from blots to identify Ad5 Hexon on the same PVDF membrane. Membranes were washed with 1X TBS-T 3 times for 5 min before they were submerged into Restore Western blot stripping buffer (Thermo Fisher Scientific) for 30 min with agitation at room temperature. Stripped blots were washed with 1X TSB-T three times for 5 min and blocked in 5% (w/v in 1X TBS-T) milk for 1 h at room temperature. Blots were incubated overnight at +4°C in anti-Ad5 Hexon antibody at 1:5000 dilution in 2% (w/v in 1X TBS-T) milk. They were washed three times for 5 min and then incubated with the secondary antibody, rabbit polyclonal antibody to mouse IgG (HRP-conjugated): 1:8000 dilution in 2% (w/v) milk. The blots were washed three times in 1X TBS-T for 5 min before they were incubated in enhanced chemiluminescent reagent for 1 min. Adenovirus 5 hexon was imaged using an Amersham Imager 600 (GE Healthcare Life Sciences, Buckinghamshire UK).

2.2.12 Quantification of Host Cell Proteins in Adenovirus 5 Preparations

The presence and concentration of host cell protein (HCP) in Ad5 samples were analysed using the HEK293 HCP ELISA kit F650R (Cygnus Technologies, Southport, NC, USA) following manufacturer’s instructions. The assay is a two-site immunoenzymetric assay. The UV absorbance were read at 450 and 650 nm using the Safire2 plate reader (Tecan Group Ltd, Zürich, Switzerland). The reaction is performed in microtiter strips coated in capture antibody, binding to HEK293 cell proteins. A horse radish peroxidase-labelled anti-HEK293 antibody is reacted simultaneously, forming a complex of solid phase antibody-HEK293 HCP-enzyme labelled antibody. Strips are washed to remove unbound reactants and
tetramethylbenzidine is reacted, with the amount of hydrolysed substrate read on a microtiter plate.

2.2.13 Analysis of Adenovirus 5 Structure using Transmission Electron Microscopy

Transmission electron microscopy was used to visualise empty and packed Ad5 capsids. To perform the analysis, Ad5 particles were negatively stained by adding uranyl acetate to Ad5 feed and purified fractions in 20mM Tris pH 7.5, at a range of NaCl concentrations depending on elution conditions. The stained samples were dropped onto a carbon grid (400 mesh) and loaded onto JEOL 1010 Transmission Electron Microscope (JEOL, Peabody, MA USA) before they were imaged. Transmission electron microscopy allows for high resolution imaging of virus capsid structures. Empty adenovirus capsids lack a DNA genome and they adsorb the uranyl acetate stain into the capsid cavity resulting in a dark core distinguishing them from DNA containing capsids (Sutjipto et al., 2005).

2.2.14 Analysis of Adenovirus 5 Binding to Nanofibre Membranes using Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to image Ad5 particles bound to nanofibre membranes. Due to its open structure, the nanofibre bed did not require manipulation to be visualised using SEM. Nanofibers were mounted on aluminium stubs using adhesive carbon taps. Mounted samples were coated in a 2 nm layer of gold/palladium using a 681 Gatan ion beam coater (Roper Industries, Abingdon UK). Sputter coating for SEM is the process of applying gold/palladium onto a non-conducting or poorly conducting biological specimen. An ultra-thin (2nm in depth) coating of electrically-conducting metal (in this case gold/palladium) prevents charging of the specimen that in turn prevents charging of the specimen. This increases the amount of secondary electrons that can be detected on the surface of the specimen and enhances the signal to noise ratio (Golding et al., 2016). Samples were imaged using a JEOL 7401 FEG-SEM (JEOL, Peabody, MA US).
2.2.15 Peptide Digest of Adenovirus 5 for Mass Spectrometry Analysis

Adenovirus 5 feed and purified fraction proteins were separated on 4-12% Bis-Tris gels, silver stained, and protein bands at molecular weights that corresponded to Ad5 capsid coat (hexon) and core proteins (protein V, VI, VII, and VIII) were excised for trypptic digestion. Gel pieces were washed in 50mM ammonium bicarbonate:acetonitrile (1:1) for 15 min and then centrifuged at 1000 rpm for 1 min using a centrifuge 5430 (Eppendorf, Hamburg, Germany). The supernatant was discarded, gel pieces were washed in acetonitrile for 15 min and they were centrifuged for 1 min at 1000 rpm. Gel pieces were hydrated by submerging in 10mM DDT in 50mM ammonium bicarbonate for 30 min at 56°C before they were centrifuged for 1 min at 1000 rpm and the supernatant was discarded. The gel pieces were then washed in acetonitrile for 5 min and centrifuged for 1 min at 1000 rpm. The supernatant was discarded and the gel pieces were incubated in 55mM iodoacetamide in 50mM ammonium bicarbonate for 20 min at room temperature.

The gel pieces centrifuged for 1 min at 1000 rpm and the supernatant was discarded. They were then washed in 50mM ammonium bicarbonate for 15 min, centrifuged for 1 min at 1000 rpm and this step was repeated. The gel pieces were shrunk by submerging in acetonitrile for 5 min and they were centrifuged for 1 min at 1000 rpm. The supernatant was discarded and gel pieces were dried using a Savant™ SpeedVac™ High Capacity Concentrator vacuum centrifuge (ThermoFischer, East Grinstead, UK). Dried gel pieces were rehydrated in digestion buffer: 10mM ammonium bicarbonate, 10% acetonitrile, containing 10ng/µL of Sequence Grade Modified Trypsin (Ref: V5111, Promega, Wisconsin, USA) for 30 min on ice. The supernatant was removed and replaced with digestion buffer: 10mM ammonium bicarbonate, 10% acetonitrile. Samples were incubated with trypsin overnight at 37°C. Peptides were extracted by adding acetonitrile to gel pieces and sonicating for 15 min. Gel pieces were centrifuged for 1 min at 1000 rpm and the supernatant collected. A buffer containing 50% (v/v) acetonitrile and 5% (v/v) formic acid was added to gel pieces and the samples were sonicated for 15 min. The supernatant was collected and pooled.
Matrix assisted laser desorption/ionisation (MALDI) time of flight (ToF) tandem mass spectrometer (MS/MS) was used to identify Ad5 proteins from tryptic digests. The mass spectrometry analysis involved the use of an ultrafleXtreme™ (Bruker, Coventry, UK) in positive ion reflector mode and 50% laser power. The highest intensity peaks for each target spot were then selected for MS/MS analysis. Peptide masses with an ion score exceeding the threshold set for p < 0.05 were interrogated using the Mascot algorithm (matrix-science.com) to search all taxonomies in the SwissProt database. Instrument run setup options were: fixed modifications, carbidomethyl (C); variable modifications, oxidation (M); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: +/− 50 ppm; fragment mass tolerance +/− 0.5 Da using a standard 700–3500Da protein acquisition method. Adenovirus 5 tryptic digests (0.5µL) were loaded onto Prespotted AnchorChip (PAC) targets (Bruker) and they were dried at room temperature. Matrix solution (0.5µL): 0.7 mg/mL HCCA (a-cyano-4-hydroxycinnamic acid) was dissolved in a solvent mixture containing 85% acetonitrile, 15% water, 0.1% trifluoroacetic acid (TFA) and 1 mM ammonium phosphate monobasic, spotted onto the Ad5 sample on the PAC target and allowed to dry at room temperature. Peptide calibration matrix solution was prepared following manufacturer’s instructions: Peptide Calibration Standard II (#222570, Bruker, Coventry, UK) was dissolved in 125µL TA30 solvent (30:70 [v/v] acetonitrile: 0.1% TFA in water). Calibration solution was mixed 1 part with 200 parts matrix solution, and 0.5µL of this solution was deposited onto the calibrant anchor spots on the PAC. The PAC was loaded into the ultrafleXtreme™ and flex imaging software was used to conduct a 3-point teaching procedure to position targets within the instrument before samples were ionised with a laser. The matrix adsorbs the UV light which is vaporised together with the sample, charged ions are directed through a drift space and the time of flight the ion analysed by a detector plate. The time of flight through the drift space is a function the mass-to-charge ration (m/z) of the ion.
Chapter 3  Cell Line Characterisation of HEK293 to Ensure Consistent, High Yield Production of Infective Adenovirus 5 Particles

3.1 Introduction

Batch to batch reproducibility of viral vectors is a critical concern for their manufacture; cell density and health both result in variations in virus yields (Vellinga et al., 2014). The HEK293 cell line was developed by Graham et al. (1977) and it is the primary cell line for Ad5 production. The adenovirus 5 (Ad5) vector propagated in HEK293 cells in the present study does not contain the E1 region that codes for subunits E1A and E1B. E1A plays an important role in Ad5 replications, transactivating other early units whilst deregulating cell cycle controls. E1B inactivates apoptotic pathways (Dormond et al., 2009). As deletion of E1 impairs viral replication, Ad5 is propagated in the HEK293 cell line which provides the E1 sequence in \textit{trans}.

Viral replication induces major changes in cell physiology that are strongly linked to the cell metabolic state (Rodrigues et al., 2014). Viral component synthesis, capsid assembly, release and the severity of virus propagation in the host was shown to be directly linked to the metabolic efficiency of cells, and to the ability of cells to provide adequate amounts of metabolic precursors (Petiot et al., 2015). During Ad5 infection, HEK293 cells disfavour glutamine over other amino acids as carbon sources; asparagine is essential for virion formation and the cells respond by favourably consuming asparagine/aspartate (Nadeau et al., 2000). Shashkova et al. (2009) showed dramatic reductions in cell mitochondrial activity in virus-infected cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. They demonstrated a decrease in mitochondrial activity in cancerous cell lines five days after infecting those cells at a multiplicity of infection (MOI) greater than $10^2$ (MOI: the ratio of infective virus particles (IVP) to number of cells (Dormond et al., 2009)).
The passage/age of cells influences growth rates and yields of Ad5, with greater production of Ad5 in cell monolayers than cell suspensions, and with older generations of cells exhibiting a higher growth rate (Park et al., 2004). The Ad5 E1 gene integrated into the HEK293 cell line is an immortalised gene and is involved in the growth stimulatory process (Quinlan, 1993). The continued expression of E1A in later generation cells required for activation of the cell cycle, may result in cells with an altered growth pattern (Park et al., 2004). Whilst HEK293 is the platform of choice for production of Ad5, a major drawback is the production of small amounts of replication competent virus that can arise through homologous recombination of the E1 sequence (Hehir et al., 1996). However, this can be mitigated by reducing the number of Ad5 batches generated with previous harvests. An aliquoted stock of Ad5 can be produced and stored at -80°C, with batches being generated from this stock (Lochmüller et al., 1994). In the present results chapter, early and intermediate HEK293 passages were investigated to determine the effects of their metabolic activity, proliferation and passage on the yield and infectivity of Ad5 virus that they produce.

3.2 Objectives

a. To design a processing platform that will ensure reproducible production of high yields of infective Ad5 particles.

3.2.1 Aims

a. Define processing parameters for manufacture of high yields of infective Ad5.

b. Optimise process workflows to allow rapid generation of Ad5 product.

c. Determine processes that will ensure reproducible production of infective Ad5.
3.3 Methods Summary

3.3.1 Analysis of HEK293 Mitochondrial Activity using the MTT Assay

HEK293 cells at passage (P) 2, P5, and P10 were seeded at a range of densities (1X10³ to 1X10⁵ cells/mL) in 96-well tissue culture plates (Corning® Costar® TC-Treated 96 Well Plates, Sigma-Aldrich, Gillingham, UK) and they were cultured in supplemented DMEM for 24 h to adhere to the plate surface. Mitochondrial activity was analysed using the Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific, Paisley, UK). Briefly, media was removed from cell monolayers in the 96-well plate and replaced with 100 µL of fresh culture medium before 10 µL of 12 mM MTT stock solution was added to each well. Cells were incubated with the MTT reagent at 37°C for 4 h and after this labelling step, all but 25 µL medium+MTT solution was removed from the wells and this was replaced with 50 µL dimethyl sulfoxide. Cells were mixed thoroughly with the DMSO solution until they were solubilised, releasing the blue formazan product formed due to reduction of the tetrazolium salt by dehydrogenases and reductases in cells. The solution was incubated at 37°C for 10 min. Absorbance was read at 540 nm using a Safire2 (Tecan Group Ltd, Zürich, Switzerland).

3.3.2 Analysis of the Effect of HEK293 Cell Passage on their Proliferation

HEK293 cells at P2, P5, and P10, were seeded into 12-well tissue culture plates (Corning® Costar® TC-Treated 12 Well Plates, Sigma-Aldrich, Gillingham, UK) at a range of densities (1X10³ to 1X10⁵ cells/mL) for 24 h, 48 h and 96 h growth periods. After each culture period, cell colonies were imaged using an EVOS FL Cell Imaging System (Thermo Fisher Scientific, Paisley, UK), before they were trypsinised and counted using a haemocytometer. The viability of cells was determined using the Trypan blue exclusion assay. Briefly, trypsinised cells were centrifuged (at 1000 rpm for 5 min) and the resulting cell pellet was suspended in 100 µL medium. A 10 µL aliquot of the cell suspension was mixed with 10 µL Trypan blue dye, and 10 µL of this mixture was pipetted onto a haemocytometer to count live cells (unstained) and dead cells (stained blue). The results from
experiments performed to investigate the growth patterns (over 24h, 48 h, and 96 h) of HEK293 cells, will be used as the basis for experiments to analyse the growth dynamics (using a growth curve) of those cells. The passage and density that show the most efficient exponential cell growth over time will be chosen to construct the growth curve.

3.4 Results

3.4.1 Analysis of the Influence of HEK293 Cell Passage on their Proliferation

Reproducible upstream processes are essential for investigation of the capabilities of prototype chromatography columns for purification of complex biotherapeutics. Bioprocess workflows that result in minimal batch to batch variation of products are ideal for industrial manufacture of biotherapies. Batch to batch variability in the production of adenovirus vectors remains a challenge (Lusky, 2005). The Ad5 material utilised in the present study contained an E1 deletion; vectors containing this deletion fail to express late genes unless E1 is provided by the cell host. High passage HEK293 cells propagate Ad5 that contains the E1 gene due to a recombination event with the host genome (Lochmüller et al., 1994). Cell passage was reported to influence the expression of mammalian transgenes over time (Kantardjieff and Zhou, 2013). Therefore, clear processing guidelines for culture conditions of cell lines used to propagate Ad5 vectors are necessary.

The effects of HEK293 cell passage on their proliferation was examined to determine the growth dynamics of those cells at early (P2 and P5) and intermediate (P10) passages. Passage 10 HEK293 cells proliferated at a greater rate than P2 and P5 cells (Figure 5). Images of cell colonies (Figure 6) were consistent with the quantitative results of cell proliferation. Passage 10 cells initially seeded at $2.5 \times 10^4$ (per mL), proliferated to a significantly greater extent over 24 h, 48 h, and 96 h growth periods than P2 and P5 cells seeded at the same density. Over 96 h, the growth of P10 cells initially seeded at a lower density than $2.5 \times 10^4$ (per mL) ($2 \times 10^4$ per mL) was greater than that of P2 and P5 cells. At higher densities ($5 \times 10^4$ and $1 \times 10^5$), P10
cells grew exponentially during 24 h, 48 h, and 96 h and at those densities P10 cell growth was significantly greater than that of P2 and P5 cells (p value ≥0.05). The proliferation of HEK293 cells was not dramatic at low densities (1 x 10^3, 5 x 10^3, 1 x 10^4, and 1.5 x 10^4 per mL) – the difference in cell growth was minimal between passages and culture periods. These data indicate that P10 is the appropriate passage for HEK293 cells that can be used to propagate Ad5 because at this passage, cells will proliferate rapidly during a brief (3-4 days) culture period.
Figure 5. Seeding cells at P2, P5, and P10 at a range of densities showed that the growth rate of P10 cells was consistently greater than P2 and P5 (n=3). Error bars represent standard error of the mean.
Figure 6. Colonies of P2, P5 and P10 cells over 24h, 48h, and 96h growth periods. Scale bar represents 400µm (n=3).

To further refine culture conditions for HEK293 cells, the growth dynamics of P10 cells were investigated by constructing a growth curve (over a period of 10 days) using those cells at a density ($2.5 \times 10^4$) that is estimated (based on the previous growth profile experiments) to proliferate to 100% confluency (in a 12-well plate) during 10 days. P10 cell proliferation exhibited a 1-4 day lag phase after seeding at $2 \times 10^4$ cells/mL, before entering an exponential growth phase from day 4-10 (Figure 7). High levels of necrotic cells were observed from day 8. A previous study performed to propagate Ad5 in HEK293 cells recommends that cells should be
seeded at ~10^6 and allowing to grow for 2 to 3 days before infection (Petiot et al., 2015). Typically, HEK293 cells at a confluency of approximately 70% are infected with Ad5; this is a density of cells that is high enough to allow for cell growth whilst the virus propagates (Chillón and Bosch, 2013). Infection at high confluency results in HEK293 cell stress over the incubation period from overcrowding. This causes down regulation of the E1 gene (Petiot et al., 2015). The growth curve for P10 cells demonstrate proliferation over 1-4 days that is consistence with previous results (see Figure 5) as well as normal exponential cell growth.

![Figure 7](image-url)

Figure 7. Passage 10 HEK293 cell growth curves, cells were stained and counted using Trypan blue (n=3). Error bars represent standard error of the mean.

The mitochondrial activity of HEK293 cells was investigated because the metabolic efficiency of cells can influence Ad5 propagation (Petiot et al., 2015). Passage 2, P5 and P10 HEK293 cells were seeded into 96-well plates at the range of densities used
for investigations of growth dynamics of those cells (Figure 5) and the MTT assay was performed to measure mitochondrial activity. The MTT assay involves the conversion of water soluble MTT to insoluble formazan (Mosmann (1983). Endocytosis and reduction of MTT require ATP and NADH (Liu et al., 1997).

Passage 5 cells presented greater mitochondrial activity than P2 and P10 cells (Figure 8 and Figure 9). The low growth rate of passage 5 cells that was similar to that of P2 cells (Figure 5), indicates that the link between mitochondrial activity and proliferation of HEK293 cells may not be linear. For all cell passages, mitochondrial activity increased as cell densities were elevated. The low mitochondrial activity of P2 cells compared to those of P5 and P10 cells is consistent with the growth rate of P2 cells that was also lower than that of P5 and P10 cells (Figure 5). The reduction in mitochondrial activity is likely due to cell stress. Vander Heiden et al. (2009) have demonstrated that the metabolism of proliferating cells favours aerobic glycolysis enabling better acquisition and metabolism of nutrients required for proliferation. Together with the cell growth results, the mitochondrial activity data do not provide complete information regarding the health of HEK293 cells. To perform a thorough investigation of potential links between HEK293 cell proliferation and metabolism and passage cycles, a wide range of healthy and senescent cells should be screened.
Figure 8. The mitochondrial activity of P2, P5 and P10 HEK293 cells over a range of densities. The reduction of tetrazolium salts to formazan is observed by a colour change from yellow to blue/purple and the absorbance is measured at 540nm (n=3). Error bars represent standard error of the mean.
Figure 9. Images of HEK293 cells analysed using the MTT assay. Wells show the colour change of tetrazol reduction to formazan (n=3).
3.4.2 Effect of HEK293 Cell Passage on Propagation of Infective Ad5 Particles

E1 deleted Ad5 containing a β-galactosidase gene insert was purified using ultracentrifugation. The quality of the Ad5 stocks was analysed using transmission electron microscopy (Figure 10). Whilst damaged capsid structure are visible in Figure 10 packed Ad5 capsid preparations presented very little damage and the stock was also highly concentrated, indicating that a good quality Ad5 stock was used for the propagation process. Empty Ad5 capsids do not contain a genetic payload. As such, the capsids are not therapeutically functional but contain the same protein capsid structure as infective, packed Ad5 capsids. The capsid coat of empty Ad5 is mainly antigenic and they are an important product impurity (Takahashi et al., 2006). Caesium chloride ultracentrifugation removes these process impurities due to differences in the densities of packed and empty Ad5 capsids (Ugai et al., 2005).
Figure 10. Transmission electron microscopy imaging of Ad5 stock purified by caesium chloride ultracentrifugation. The images show highly concentrated Ad5 vectors and low amounts of empty and damaged vectors. Arrow shows damaged capsid. Empty capsids (highlighted with red circles) have a darkened capsid as more stain is adsorbed into the void usually filled with genomic material.
Crude harvests of Ad5 propagated in P2, P5 and P10 HEK293 cells were analysed using a β-galactosidase cell stain. β-galactosidase (β-gal) is used as a marker for successful transfection of the reporter gene, LacZ. Adenovirus 5 contains the LacZ gene insert and successful infection of HEK293 cells with Ad5 results in transient expression of β-gal in host cells. Expression of β-gal can then be detected in cells as an indigo-blue colour when stained with X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (Lawrence et al., 1999). HEK293 cells were cultured in HYPERFlasks for 72 h before they were infected with stock Ad5, with a freeze thaw harvest 48 h after infection. Adenovirus 5 propagated from P2, P5 and P10 cells were used to infect P10 cells for assessment of the yield of infective Ad5 from different HEK293 passages.

Passage 10 cells produced Ad5 of the highest infective yield (Figure 11 and Figure 12) despite presenting a lower mitochondrial activity than P5 (Figure 9). Passage 2 cells propagated the lowest amount of infective Ad5 due to low cell densities during the 72 h culture period before they were infected with stock Ad5. These data demonstrated that whilst the mitochondrial activity of P2 cells was comparable to that of P5 and P10 cells (see Figure 3), low growth rates limited the production of Ad5 in this early passage. It is hypothesised that due to the high growth rates of P10 at time of infection there is a higher cell count than in P5, resulting in higher total yield of Ad5 at time of harvest. The time pressures of process schedules for Ad5 propagation would render P2 unsuitable for use in process development for this viral vector. Propagation of high yields of infective Ad5 may be achieved in either P5 or P10 HEK293 cells. The differences in growth rate and mitochondrial activity between those cell passages may not significantly impact Ad5 production. The rapid growth rate of passage 10 cells indicates that this could be the preferred HEK293 passage for development of efficient workflows for Ad5 production.
Figure 11. Infective Ad5 propagation in P2, P5 and P10 HEK293 cells. Cells were seeded and cultured for 72hr before being incubated with stock Ad5 (n=3). Error bars represent standard error of the mean.
Figure 12. Infection of cells with Ad5 propagated from P2, P5 and P10 HEK293 cells. Images show blue cells that express β-galactosidase due to infection with LacZ containing Ad5 (n=3). Scale bar represents 400µm.

To confirm the presence of Ad5 in harvests from P2, P5 and P10 HEK293 cells, Western blotting analyses were performed to identify Ad5 capsid proteins (Figure 13). The major capsid coat protein, hexon was detected from P2, P5 and P10 harvests together with another coat protein, penton and the internal capsid core protein, protein V. The expression levels of those proteins was similar between P5 and P10 Ad5 harvests but the P2 Ad5 harvest contained lower amounts of capsid proteins than P5 and P10 Ad5 harvests. Adenovirus 5 samples were clarified (clarif) by filtration using a 0.45µm syringe filter and dilute (Dil) protein samples were concentrated (conc) using a 100 kDa molecular weight cut off (MWCO) centrifuge
Clarification and concentration of P10 Ad5 protein samples appeared to reduce expression of capsid proteins compared to P5 Ad5 protein samples, but this did not occur in some P10 samples. These data show that the lower yield of Ad5 produced by P2 cells than P5 and P10 cells is reflected in the expression of Ad5 capsid proteins from the virus produced by cells at those passages.

Figure 13. Identification of capsid proteins from Ad5 propagated in P2, P5 and P10 HEK293 cells by western blot. A. Adenovirus 5 samples were clarified (clarif) by filtration using a 0.45μm syringe filter and dilute (Dil) protein samples were concentrated (conc) using 100 kDa MWCO centrifuge filter. B. The blot was re-probed using an anti-Ad5 hexon antibody to detect hexon alone. (n=3).
To investigate the quality of Ad5 protein from harvests of this virus propagated in P2, P5 and P10 HEK293 cells, gel electrophoresis and silver staining analyses were performed (Figure 14). Adenovirus 5 samples were loaded onto 4-12% Bis-Tris gels at 1.5 µg total protein per well and proteins were fixed before they were stained with silver nitrate. The concentration step increased the intensity of protein bands from Ad5 harvests produced by P2 and P5 and P10 cells.
Figure 14. Silver staining analysis of Ad5 protein from virus harvests from P2, P5 and P10 HEK293 cells. Adenovirus 5 samples were clarified (clarif) by filtration using a 0.45μm syringe filter and dilute (Dil) protein samples were concentrated (conc) using 100 kDa MWCO centrifuge filter. Adenovirus 5 proteins were separated using 4-12% Bis-Tris gels at 1.5 µg total protein per well and proteins were fixed before they were stained with silver nitrate (n=3).
3.4.3 Results Summary

The results from this chapter describing investigations of strategies for cell line characterisation of HEK293 for optimised production of Ad5, demonstrate favourable conditions for production of high, infective yields of this virus. The objective to identify optimal processing conditions for Ad5 has not, however, been achieved. At P10, HEK293 cells presented a growth rate that was greater than that of P2 and P5, indicating that the higher passage would be most suitable for establishing rapid Ad5 processing workflows. The mitochondrial activity of P5 HEK293 cells that was higher than that of P2 and P10 cells did not correlate with the growth rates of those cells. Without an indication of the mitochondrial activity of healthy and senescent HEK293 cells, it is not possible to conclude that the higher mitochondrial activity of P5 cells shows that they are more or less healthy than P2 and P10 cells. The propagation of Ad5 in P2, P5 and P10 cells correlated with the growth rate of those cells, i.e. the greatest yield of infective Ad5 was produced by P10 cells that presented with the highest rate of proliferation. This is hypothesised to be because of a higher cell count at the time of harvest. Although P10 was chosen as the HEK293 passage for processing Ad5, P5 may also be considered as at this passage HEK293 cells propagate similar yields of Ad5 as P10 cells.

3.5 Discussion

The HEK293 cell line is the primary platform for production of Ad5 (Sharon and Kamen, 2018). HEK293 cells were generated by transforming adenoviral DNA into embryonic kidney cells resulting in constitutive expression of Ad5 E1 protein (Lin et al., 2014). Those cells can be adapted for use in suspension culture using serum free media and their scalability and consistency in culture have made them an attractive platform for production of viral vectors by regulatory agencies (Sharon and Kamen, 2018).

Comparisons of the growth of HEK293 cells at early (P2 and P5) and intermediate (P10) passages demonstrated that seeded at recommended densities, P10 cells
proliferated to a greater extent than earlier cell passages, P2 and P5. Under Ad5 processing conditions, a higher cell passage may be favourable for high yield propagation of the virus. A workflow that allows achievement of the highest cell densities in the shortest time periods are more desirable as this reduces the length of processing schedules whilst providing the highest level of substrate for Ad5 propagation (Kovesdi and Hedley, 2010). Research was performed to understand the growth rates and culture passage/age of caco-2 cell lines. Older generations of cells were shown to reach plateau phase sooner (Briske-Anderson et al., 1997). Increasing cell passage can induce negative effects on cell growth. In a culture, cells are under environmental pressures; most cell cultures will represent a heterogeneous mix of competing populations.

Cell media can be supplemented with growth factors, nucleic acids and salts, but these nutrients are a finite resource. Sub-populations of cells can outcompete others by growing faster through more efficient use of these resources by down regulating unnecessary genes. Checkpoint genes are typically removed in immortal cell lines and this exacerbates genomic instability (Yoshioka et al., 2015). The understanding of mitochondrial activity as a marker for immortalised cell metabolic health seems to be an area requiring further study (Vander Heiden et al., 2009). A reduction in the metabolic activity of P10 cells was observed in the present study but due to a higher growth rate than P2 or P5 cells, P10 cells yielded the highest Ad5 concentrations. More in-depth analyses of the link between cell proliferation and metabolic activity are clearly warranted in the current study. Those could include investigations of the cell cycle using bromodeoxyuridine staining protocols (Cecchini et al., 2012) and cell apoptosis by assessing caspase 3/7 and annexin V levels (Telford et al., 2002).

Typically Ad5 infection can be induced using approximately $10^5$ cells/mL and the virus is harvested when cells achieve approximately $1 \times 10^6$ cells/mL (Kamen and Henry, 2004). HEK293 P10 cells were shown to reach these cell densities during shorter culture periods than P2 or P5 cells. With cell numbers of more than $0.5 \times 10^6$, a reduction in specific virus production is observed (Kamen and Henry, 2004). This
decline has been attributed to nutrient deficiency and metabolite accumulation that hinders cell metabolism. Rates in cell consumption of amino acids and oxygen have been shown to increase dramatically after Ad5 infection, and at a high cell density it is suggested that the decrease in metabolism reduces the ability of cells to support Ad5 production (Dormond et al., 2009). A favourable metabolic state for the production of Ad5 has been shown to involve an increase in glycolytic and tricarboxylic acid fluxes as well as ATP production (Nadeau et al., 2000). This requirement for a higher metabolic state was shown to increase transfection rates in late passage cultures of HEK293 (Molinas et al. (2014). These findings support data described in the present study demonstrating that P10 and P5 cells presenting with higher mitochondrial activities than P2 cells, were able to propagate higher yields of Ad5 than the low passage (P2) cells. Although P2 cells appear to proliferate at a lower rate than P5 and P10 cells. Therefore, comparisons of growth curves for those passages (P2, P5, P10) should be performed and if those show similar growth rates over time, differences in their ability to propagate Ad5 may be due to other properties.

3.6 Conclusion

The results described in this chapter provide a number of upstream conditions that can be used in a strategy for optimal propagation of Ad5 from a HEK293 cell line. Although those conditions require further evaluations, we have defined a platform for upstream analysis of Ad5 vectors. This work is important because a well-characterised upstream process is essential for reproducible generation of Ad5 vector stocks. This not only has implications for upstream manufacture, in which reduction in culturing periods is desirable, but also cell based infectivity analyses of the Ad5 vector. The results generated here showing that P10 allows propagation of high yields of Ad5 will be applied to preceding chapters.
Chapter 4  Optimisation of Downstream Processes for Purification of Adenovirus 5 Particles

4.1  Introduction

Nanofiber membranes are non-woven regenerated cellulose membranes that present an open pore structure with large inter-fiber spaces and shallow bed height, features previously reported to be advantageous for separation of viral vectors (Wickramasinghe et al., 2006). They are one of a number of different adsorbent solutions in development to increase the process efficiency for manufacture of biotherapeutics (Vincent et al., 2017, Nestola et al., 2014c, Nestola et al., 2014b). Those novel adsorbent membranes present properties that allow higher recoveries than conventional resin-based chromatography systems. The binding capacity and process performance of resin beads are reduced significantly as product size increases and this is particularly severe when purifying macromolecular complexes, e.g. viral vectors and virus like particles (Wickramasinghe et al., 2006). These biotherapeutics cannot reach the internal functionalised surface because of pore exclusion due to their large size.

Empty adenovirus 5 (Ad5) capsids that do not contain DNA in their core are less dense than DNA packed capsids (Vellekamp et al., 2001). Empty Ad5 capsids can be depleted using caesium chloride ultracentrifugation (Tollefson et al., 1999). However, this process is time consuming, expensive and scales poorly (van der Loo and Wright, 2016). Over its life cycle, an Ad5 capsid will develop from an immature to a mature capsid, through a series of proteolytic events that prime the virion for ejection of genetic contents and subsequent propagation (Perez-Berna et al., 2012). The immature virion is therapeutically deficient and unable to release its genetic payload (Perez-Berna et al., 2009). The immature capsid contains a DNA core and exhibits the same density as the mature capsid, making it indistinguishable by ultracentrifugation. The empty and immature virions represent two important product impurities. Studies have not been performed to address the removal of immature Ad5 capsids as a product impurity. Only one research group (San Martin (2012)) that
studies virion biophysics is currently performing investigations into separation of packed Ad5 particles.

In addition to issues with separation of packed and empty Ad5 capsids, product impurities can adversely affect the therapeutic properties of Ad5 vectors. Removal of product impurities represents one of the greatest viral vector bioprocessing challenges, placing a burden on their industrial processing and therapeutic delivery (van der Loo and Wright, 2016). Improving the dose efficacy of gene therapies is important as the majority of adult patients exhibit immune responses (through proinflammatory cytokine release) caused by the gene carrying vector and transduced cells (Higginbotham et al., 2002). Immune response side effects would be significantly reduced if a dose of viral vector only contained Ad5 capsids that were packed with the gene therapy and did not include a proportion of impurities.

Shallow bed convective adsorbent matrices have previously been used for clearance of process impurities (Podgornik et al., 2013). The Ad5 feed will contain a heterogenous mix of empty, immature and mature vectors. Only mature vectors exhibit therapeutic efficacy. Minimising process steps will maintain product quality, by limiting processing damage to capsids. The nanofiber material used in the present study is highly porous and columns provided are packed in a short bed height format (Hardick et al., 2011) optimal for high resolution separations of vectors (Podgornik et al., 2013). We hypothesise that the difference in mature and immature Ad5 characteristics will allow for these two subtypes to be separated over the processing step. The presence of mature cleaved Ad5 proteins will be detected using mass spectrometry analysis.

4.1.1 Objectives

   a. To optimise a downstream process for purification of Ad5 from a clarified cell lysate using nanofiber membranes.
4.1.1.1 Aims

- Determine whether or not the architecture of nanofiber membranes is suitable for separation of macromolecules.
- Explore bioprocess step parameters for purification of Ad5 on nanofiber membranes.
- Investigate the utility of nanofiber membranes for separation of empty and packed Ad5 capsid subtypes using a range of analytical methods.
- Assess potential causes underlying the reduction of Ad5 yield during separation using nanofibre membranes.

4.2 Methods Summary

4.2.1 Anion Exchange Chromatographic Separation of Clarified Adenovirus 5 Feed Components

A HEK293 cell lysate containing propagated Ad5 was centrifuged at 2000 rpm for 10 min and filtered using a 0.45 µm syringe filter. Chromatographic separations were conducted using an AKTA Avant (GE Healthcare Life Sciences, Buckinghamshire UK), with online measurements of pH, conductivity and UV absorbance (260 and 280 nm). The Ad5 feed was bound to 0.125 mL nanofiber adsorbent functionalised with quaternary amine ligand chemistry at a ligand density of 820 µmol/g. The column was equilibrated in 20 mM Tris (pH 7.4), and a gradient elution of up to 20 mM Tris, 1 M NaCl (pH 7.4) was applied. Peaks were determined and a step gradient elution method was developed. Peak samples were collected in 2 mL fractions using a F9-R fraction collector (GE Healthcare Life Sciences, Buckinghamshire UK).

4.2.2 Silver Staining Analysis of Adenovirus 5 Proteins

Adenovirus 5 crude feed and purified fractions were concentrated using Vivaspin® Turbo 4 (Sartorius, Gottingen Germany). Protein was quantified using the modified Lowry protein assay and they were separated via SDS-PAGE using NuPAGE™
precast 4-12%, Bis-Tris mini-gels (Thermo Fisher, East Grinstead, UK) with gels run at 100V (constant volts) per gel.

Silver staining analysis of Ad5 proteins mobilised in Bis-Tris gels was performed as it is a sensitive protein staining method that can allow visualisation of low amounts of proteins in gels. After the gel electrophoresis step, Ad5 proteins were fixed in gels, then sensitized by incubating the gel in sensitizing buffer at room temperature for 30 min. Proteins were coated in silver ions by incubating the gels in 12mM silver nitrate for 20 minutes at room temperature under agitation. The gel was developed and silver stained protein bands were imaged using an Amersham Imager 600 (GE Healthcare Life Sciences, Buckinghamshire UK).

4.2.3 Identification of Adenovirus 5 Proteins using Western Blotting

Adenovirus 5 proteins were transferred from gels to polyvinylidene fluoride (PVDF) membranes using an iBlot™ 2 gel transfer device following the manufacturer’s instructions. Blots were blocked with 5% milk (w/v), incubated with anti-Ad5 polyclonal antibody diluted 1:4000 in 2% (w/v in 1X TBS-T) milk. Blots were washed three times in 1X Tris buffered saline-tween (TBS-T) for 5 min before they were incubated (with agitation) in the secondary antibody, a goat polyclonal antibody to rabbit IgG, (conjugated to horse radish peroxidase) diluted 1:8000 in 2% (w/v in 1X TBS-T) milk, for 2 h at room temperature. Blots were developed using the enhanced chemiluminescent reagent kit, SuperSignal™ West Pico PLUS Chemiluminescent Substrate. Adenovirus 5 proteins were imaged using an Amersham Imager 600 (GE Healthcare Life Sciences, Buckinghamshire UK). The anti-Ad5 polyclonal antibody was stripped from blots to identify Ad5 Hexon on the same PVDF membrane. Membranes were incubated with anti-Ad5 Hexon antibody at 1:5000 dilution in 2% (w/v in 1X TBS-T) milk. They were washed three times for 5 min and then incubated with the secondary antibody, rabbit polyclonal antibody to mouse IgG (HRP-conjugated): 1:8000 dilution in 2% (w/v) milk.
4.2.4 Mass Spectrometry Analysis of Adenovirus 5

The total amount of protein in the Ad5 feed and purified fractions was quantified using the modified Lowry protein assay before protein samples were prepared in Laemmli buffer. Adenovirus 5 samples (1.5 µg total protein per sample per lane) were separated on Bis-Tris gels and proteins were silver stained. Protein bands of Ad5 containing fractions were excised, protein was extracted from the gel pieces, and treated with trypsin. Tryptic digests were analysed using a matrix assisted laser desorption/ionisation (MALDI) time of flight (TOF) mass spectrometer (UltrafleXtreme™ (Bruker, Coventry, UK)) in positive ion reflector mode. Mass spectra were analysed and Ad5 proteins were identified.

4.2.5 Determination of Ad5 Features Using Transmission Electron Microscopy

Adenovirus 5 containing samples were analysed for damage or core darkening using a JEOL 1010 Transmission Electron Microscope (JEOL, Peabody, MA USA). Adenovirus 5 empty capsids do not contain DNA and exhibit a darkened core or an incomplete capsid.

4.2.6 Analysis of the Effects of Salt Stress on Adenovirus 5 Infectivity

Infective Ad5 yield was assessed under conditions involving the use of a high concentration salt elution buffer to determine whether or not this treatment can affect Ad5 recovery. Adenovirus-containing, clarified cell lysate feed was thoroughly mixed via a 20 s vortexing step using a Waverly V1 Series Vortex Mixer (Waverly Scientific, Waverly, IA, USA) and divided into 500µL aliquots into eppendorfs. The 500µL aliquots of Ad5-containing clarified cell lysate feed were then mixed 1:1 with binding buffer to a final concentration of 20mM Tris, pH 7.4 and one of a range of NaCl concentrations (0, 0.15, 0.25, 0.375, 0.5, 0.75, 1 M NaCl). Three technical repeats were performed and after a 24 h incubation period in the various salt buffers, sample infectivity was analysed.
4.2.7 Analysis of the Effects of Flow Rate on Adenovirus 5 Infectivity

To determine the effects of high flow rates on infective recoveries, a 5mL clarified Ad5 feed was injected over unmodified cellulose nanofiber adsorbent membrane. Using a sample pump at flow rates of 5, 10, 20, 30, 40, 50, 60, 70 mL/min Ad5 permeate was collected and analysed for infective recovery.

4.3 Results

4.3.1 Imaging Adenovirus 5 Binding to Nanofibre Membranes under Batch Conditions

To optimise the function of chromatographic matrices functionalised with chemical moieties, experiments must be designed to determine the mechanisms for virus binding to and interactions with those materials (Wickramasinghe et al., 2006). In particular, it is important to understand the manner that virus to virus repulsion affects their binding patterns to the adsorbent (Trilisky and Lenhoff, 2007) and consequently how smaller process impurities affect the spread of bound virus across the functionalised surface (Vicente et al., 2011b). Adenovirus 5 particles were ionically bound to anion exchange nanofibers under batch conditions by submerging nanofiber disks into binding buffer containing Ad5 and the disks were imaged using scanning electron microscopy (SEM) (Figure 15). The images presented here potentially indicate binding patterns of Ad5 onto the adsorbent surface during chromatography runs for purification of the virus.

Adenovirus 5 virions measure approximately 90nm in length and are clearly visible bound to the nanofiber adsorbent. Other host cell components are also clearly visible as a layer bound to the functionalised surface. To elute the bound virus, nanofiber membranes were submerged in binding buffer and suspended into a high salt (1M NaCl, 20 mM Tris, pH 7.4) elution buffer. Removal of Ad5 capsids and host cell impurities was clearly visible under high salt elution conditions (Figure 15). These images are possibly the first to demonstrate the binding of Ad5 particles to an
adsorbent surface. The open structure of nanofibers allows for SEM imaging with minimal sample preparation e.g. slicing cross sections into the adsorbent.

![SEM images of Ad5 bound to an ion exchange nanofiber membrane](image)

Figure 15. Scanning electron microscopy images of Ad5 bound to an ion exchange nanofiber membrane.

4.3.2 Analysis of the Binding Capacities of Large Globular Proteins to Nanofibre Membranes

The open pore structure of the nanofiber adsorbent (Figure 15) will allow convection mass transfer of the mobile phase and proteins within the mobile phase will have access to the entire functionalised surface. Therefore, the reduction in dynamic binding capacity observed with larger biotherapeutics as a result of pore exclusion on resins is not likely to occur with nanofibers. Anion exchange chromatography columns were compared using bovine serum albumin (BSA) (~3nm diameter) and thyroglobulin (~20nm diameter) to determine whether or not the larger protein was excluded from the functionalised surface of the nanofiber membrane. Exclusion of the protein would be observable as a reduction in binding capacity.
Table 4. The nanofiber adsorbent presents a high dynamic binding capacity for thyroglobulin (~20nm) and BSA (~3nm). Binding capacity was assessed to show pore diffusion limitations of current generation resin adsorbents for large proteins.

<table>
<thead>
<tr>
<th>Adsorbent Material</th>
<th>Ligand</th>
<th>Protein</th>
<th>Binding Capacity (g/L of adsorbent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanofiber</td>
<td>DEAE</td>
<td>Thyroglobulin</td>
<td>29.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>25.98</td>
</tr>
<tr>
<td>Nanofiber</td>
<td>Branched DEAE</td>
<td>Thyroglobulin</td>
<td>50.12</td>
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<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>44.16</td>
</tr>
<tr>
<td>Nanofiber</td>
<td>Glycidol DEAE</td>
<td>Thyroglobulin</td>
<td>20.05</td>
</tr>
<tr>
<td>Sepharose FF</td>
<td>Q</td>
<td>Thyroglobulin</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>28.92</td>
</tr>
<tr>
<td>POROS</td>
<td>Q</td>
<td>Thyroglobulin</td>
<td>13.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BSA</td>
<td>54.62</td>
</tr>
</tbody>
</table>

Resin binding capacity for thyroglobulin was considerably lower than that for BSA (Table 4). The nanofiber adsorbent displayed a higher dynamic binding capacity for thyroglobulin than for BSA. The open pore structure of the nanofiber membranes allow for convective mass transfer of proteins independent of their difference in size. The decrease in dynamic binding capacity for thyroglobulin exhibited by POROS and Sepharose was either due to pore exclusion from the inner functionalised surface resulting in lowered column efficiency, or entrapment within the material as a result of the larger protein not diffusing out of the adsorbent during the elution phase. Nanofibers with branched ligand architecture, also significantly increased binding capacity. Extending the charged ligand on an inert glycidol arm did not, however, increase binding capacity for thyroglobulin. These data demonstrate that nanofibers are a suitable platform technology for large biotherapeutic separations, with no observable reductions in dynamic binding capacity due to binding of thyroglobulin that is a large globular protein.
4.3.3 Investigation of the Effects of a High Volume Load of Crude Adenovirus 5 Feed on Anion Exchange Nanofiber Membranes

Figure 16. Adenovirus 5 loaded as a clarified cell lysate feed in media was bound and eluted from nanofiber membrane columns. An elution peak profile was achieved from a 50 ml load (400 column volumes) on a quaternary (Q) amine ligand modified nanofiber membrane, at a flow rate of 10ml/min (n=3). ‘FT’ denotes flow through peak, ‘P’ denotes elution peaks.
The dynamic binding capacities of protein models are useful as a guide for interactions of biotherapeutics with chromatographic matrices. However, they cannot be a substitute for performing individual binding studies using biotherapeutics of interest. The purification of infective Ad5 from a complex feed is a challenge. Although Ad5 is structurally stable (Perez-Berna et al., 2012), it is a complex labile product. Therefore, the design of a batch process should be optimally maintained by reducing steps in the workflow. Initial studies were conducted to purify a clarified cell lysate Ad5 feed using nanofiber membranes that are capable of separating complex feeds. A Q amine ligand was selected (based on a review of literature) for functionalisation of the adsorbent. A step elution profile was applied and three peaks produced (Figure 16). A prominent flow through was observed from the 50 mL load. The Q amine diethyl amino ethyl (DEAE) nanofiber membrane column was able to bind and elute feed components from a clarified crude cell lysate feed containing Ad5. These data indicate that Q amine DEAE membranes can be used to perform a simple and quick bind and elute process step following a single filtration step using a 0.45 µm syringe filter.

4.3.3.1 Analysis of Infectious Adenovirus 5 Capsid Recovery

The primary goal of downstream processing is to purify a biological product to a standard that maintains its normal function and structure. The ability of recovered Ad5 material to infect HEK293 cells was used as a marker of product quality. This infectivity assay is the simplest measure of product quality. β-galactosidase (β-gal) is used as a marker for successful transfection of the reporter gene, LacZ. Adenovirus 5 contains the LacZ gene insert so infection of HEK293 cells with Ad5 results in transient expression of β-gal in host cells. Expression of β-gal can then be detected in cells as an indigo-blue coloration when stained with X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside). Fractions (Figure 16) were collected and assayed to measure the population of infective Ad5 capsids (Figure 17). Elution fractions P2 and P3 contained infectious adenovirus material. P2 contained the highest amount of infective Ad5 and P1 contained no detectable infective material. These data shows that Q amine nanofiber membranes can reproducibly purify two separate populations.
of infective Ad5 from a crude feed. As P1 did not contain infective Ad5, this peak represents removal of process impurities.

Figure 17. Infective recoveries of Ad5 on Q amine nanofiber membranes from a lysate feed. Fractions were collected from a 20mL load of crude cell lysate (CCL) and analysed for total infective units (IFU) by staining for β-galactosidase. Adenovirus was collected in peaks 2 and 3 and breakthrough of Ad5 was observed in flow through (FT) fractions 1-5 (n=3). Error bars represent standard error of the mean.

A mass balance infective recovery of 69% was recorded. Column overloading was observed, with Ad5 material breaking through in the first fraction collected (FT1). The 0.125 mL nanofiber columns were challenged with a 50mL clarified cell lysate that represented a feed load of 400 column volumes (CV). The Ad5 feed is clarified via filtration with a 0.45μm syringe filter that removes large cellular debris, e.g. cell membrane fragments that can cause column fouling. The Ad5 feed is complex and
contains a mixture of host cell proteins, DNA and Ad5 derived impurities that
compete with Ad5 to bind to nanofibre membrane Q amine ligands. The feed
complexity reduces available Q amine ligands that leads to a decrease in Ad5
binding capacity and a lowering of column productivity. Possible solutions to this
problem include reducing load volume and increasing the number of purification
runs.

Figure 18. The expression of Ad5 coat (hexon and penton) and core (protein V and protein
VI) proteins in crude feed and purified fractions. Protein was prepared from a 50 mL Ad5
crude feed purified on Q amine nanofiber membranes, quantified, separated (5 µg total
protein per lane) on 10% Bis-Tris gels and transferred to polyvinylidene fluoride membranes
before identification of Ad5 proteins via Western blotting using an anti-Ad5 polyclonal
antibody (n=3).
The expression of Ad5 capsid coat and core proteins was investigated to determine the presence of mature Ad5 in the crude feed and purified fractions, and to examine how the presence or absence of Ad5 proteins correlated with the infectivity of Ad5 feed and purified fractions. A polyclonal antibody raised against Ad5 was used to identify capsid coat and core proteins using the Western blotting technique. Hexon, penton, and protein V were detected in feed, flow through and peak fractions, and protein VI was detected in peak 1 and peak 2 fractions (Figure 18). P1 did not contain infective Ad5 (Figure 17) but Ad5 proteins were detected on the blot (Figure 18). Empty Ad5 capsids do not contain DNA and therefore are not able to infect cells, but they comprise the same capsid protein components as packed Ad5 capsids (Sutjipto et al., 2005). These data suggest that although host cell proteins are depleted from the Ad5 feed during the chromatography run, purified Ad5 fractions contain virus derived impurities. Those may be removed by modifying the chromatography method to include a linear gradient step to resolve Ad5 capsids from product related impurities.
4.3.3.2 The Protein Profile of Purified Adenovirus 5

To visualise process impurities in Ad5 crude feed and purified fractions, protein preparations from those samples were investigated using silver staining analysis. Silver staining showed that elution peaks P1 and P2 are much less complex than the Ad5 feed. P1 and P2 present distinct protein banding patterns (Figure 19). P3 contained very low levels of protein that were not detected on the stained gel. P2 and P3 both contained infective Ad5 (Figure 17), but no infective material was recovered in P1. The flow through fractions from FT1-FT5 show that as the permeate volume increases during the loading phase the mobile low molecular weight proteins are bound to the column. As the column is saturated with Ad5, the functionalised surface of the nanofiber may become sterically inaccessible to Ad5 that is still present in the mobile phase. It is likely that host cell proteins and other impurities gain access to the Q amine ligands before the Ad5 particles. The depletion of those impurities may be observed in the protein profiles of FT1-FT3 that show many protein bands compared to those of FT4-FT5 that show a reduction in the number of protein bands.
Figure 19. Silver stained proteins from Ad5 crude feed (50 mL load) and purified fractions. Lanes on 4-12% Bis-Tris gels were loaded with 1.5 µg total protein that was quantified using the modified Lowry protein assay.

4.3.3.3 Characterisation of Adenovirus 5 Capsids to Identify Impurities

To further investigate the presence of Ad5 derived impurities in purified fractions, transmission electron microscopy (TEM) was used to visually assess virus preparations. Adenovirus 5 capsids can be distinguished from eukaryote cell endosomes or membrane fragments (approximately 100 nm length) using TEM. Purified fractions P2 and P3 (Figure 20) contain complete Ad5 capsids - the hexon group of nine (GON) structures that are part of the capsid are clearly visible. Interestingly, P2 (Figure 20, panel F) contains sheared capsids that may be a result of processing or more likely a result of exposure to the vacuum conditions required for TEM imaging. It is impossible to deduce the origin of these capsids with regard to their subtype using TEM imaging alone as they appear as capsid fragments.
Fraction P1 (Figure 20, panel B) contains particles of a similar size to Ad5 capsids. The structures did not display the regularity in morphology observed in Ad5 capsids but were abundant in the sample. Transmission electron micrographs of the reference Ad5 feed compared to those of purified Ad5 fractions shows that capsids are clearly visible in purified fractions and they are not visible in the feed. These data show that anion exchange nanofiber membranes can achieve separation of Ad5 from a crude feed.
Figure 20. Transmission electron microscopy analysis showed the presence of Ad5 particles in fractions P2 (E and F, damaged capsid highlighted with arrow) and P3 (G and H). A - low magnification image of lysate feed, B - high magnification image of feed showing Ad5 vector (circled) and an endosome (highlighted with arrow), C - Sample P1 showing unidentified structures of either empty Ad5 capsids or endosomes.
4.3.3.4 Identification of Mature and Immature Adenovirus 5 Capsids using Mass Spectrometry

Identification of mature and immature Ad5 capsid populations from purified Ad5 fractions (P2 and P3) was not achieved using the β-galactosidase infectivity assay together with qPCR, Western blotting and TEM analyses. Fractions P2 and P3 elute at different salt concentrations, with smaller populations eluting under high salt conditions. This difference in ionic charge could be the result of capsid damage or loss of capsid structures that alter the overall capsid charge, most likely due to loss of the delicate capsid fiber protein that is a critical quality attribute for Ad5 propagation through to host cell recognition (Perez-Berna et al., 2009).

As the Ad5 capsid matures, it stiffens and becomes brittle through proteolysis of capsid coat proteins pIIIa, pVI, pVIII and core proteins pVII, pµ, pTP, mediated by adenovirus proteinase (AVP) (Perez-Berna et al., 2012). Matrix assisted laser desorption/Ionisation (MALDI) – time of flight (TOF) tandem mass spectrometry (MS/MS) was applied to the detection of Ad5 core proteins that are cleaved as this virus matures. Purified Ad5 fractions and Ad5 crude feed were separated on 4-12% Bis-Tris gels and protein bands were stained with silver. Stained protein bands were cut from the gel and Ad5 protein was extracted via a tryptic digest before they were analysed using MALDI-TOF MS/MS (Figure 21).

Peptide fingerprints (MS/MS files) were converted to mzXML files and submitted to the Swiss-Prot database for identification of Ad5 proteins. A peptide sequence match was detected for protein VIII (120 total copies per virion). Adenovirus 5 protein VIII undergoes a major cleavage by AVP at amino acid site 111 and 157 (Mangel and San Martin, 2014). The sequence before this site forms a functional, mature cleaved VIII protein. Uncleaved protein VIII has a molecular weight of 25 kDa and the analysed protein band was removed at approximately 14 kDa. These data indicate that the identified peptide was cleaved protein VIII and mature, packed Ad5 capsids are present in purified fraction peak 3. Figure 22 shows the fragmentation patterns of protein VII in peaks 2 and 3, different fragmentation patterns were observed
suggesting differences in structural properties of protein VII analysed in peak 2 and 3. An explanation for this could be that immature capsids are present in peak 2 and have not yet undergone cleavage of protein VII, although further work is required.

Figure 21. Mature Ad5 protein VIII was detected in peak 3 (P3). Protein VII was detected in both peak 2 and peak 3 (P2 and P3). Red squares on the gel represent band sections extracted for MALDI-TOF-MS/MS, the blue square represents an extracted band where uncleaved pVIII should be present but was not detected.
Figure 22. MALDI mass spectrum of Ad5 proteins VII from peaks 2 and 3 show distinct fragmentation patterns.

4.3.4 Evaluation of Process Stress Parameters on Infective Adenovirus 5 Yield

Losses in Ad5 infective capacity and yield as well as structural damage to the virus may be sustained over the duration of a processing step. This is one of the fundamental challenges of vector bioprocessing. Enveloped viruses are more vulnerable to process derived damage than non-enveloped (capsid) viruses. The loss
in Ad5 infectivity after purification may be the result of a number of stressors. Loss in Ad5 infectivity following purification was broadly attributed to particle entrapment (Wickramasinghe et al., 2006). Shear stress has also been well-documented as a cause of loss of Ad5 infectivity, particularly in ultrafiltration diafiltration (UF/DF) modules (Subramanian et al., 2005, Nestola et al., 2014a). The void space in resins that constitutes 40% of total column volume has been implicated in the damage of labile molecules as it is linked to shear stresses that increase in direct proportion to flow rate (Kirkland et al., 2000). The laminar flow properties of monoliths and nanofiber membranes minimise eddy formation and shear stress (Hahn et al., 2002, Hardick et al., 2013). However, the effects of column-derived shear on Ad5 vectors is poorly understood.
Figure 23. Increasing flow rates from 5-70 mL/min on non-functionalised nanofiber membranes for separation of a 5 mL load of clarified Ad5 cell lysate feed (CV = 0.125 mL).

Nanofiber membranes are able to operate at exceptionally high flow rates that exceed those used for commercially available adsorbents (Hardick et al., 2011). This
property is due to their open pore structure. To assess whether or not flow rate induced shear affects the infectivity of an Ad5 feed, a 5mL load of the crude, clarified virus (from HEK293 cell lysate) was perfused through a non-functionalised nanofiber column. The 0.125mL nanofiber columns were able to operate at exceptionally high flow rates (Figure 23), whilst maintaining operable pre-column pressures of 1.25 MPa. There was no correlation between flow rate and infective recovery of Ad5 using a nanofiber column (Figure 24).

Figure 24. Infective recoveries of Ad5 did not show significant losses after flow rates of 70mL/min on a non-functionalised nanofiber column (n=3). The red data point at 0mL/min represents feed IFU. Error bars represent standard error of the mean.

These data show that the low recovery of infective Ad5 observed in the previous experiments is not likely due to flow rate as those exceeding 10mL/min (flow rate used in previous experiments) did not affect Ad5 infectivity. Loss in infective Ad5
recovery could be attributed to buffer conditions. An investigation of pH stress on Ad5 was conducted by Perez-Berna et al. (2012) and it showed that at pH <6 the mature, infective Ad5 capsid begins to deform and crack, ejecting the genetic material. Immature Ad5 capsids are more resistant to thermal and chemical stress. The early endosome, which facilitates intracellular transport of the vector during infection, presents a mildly acidic environment that strips away the virus coat, a process that is essential for successful viral propagation (Mangel and San Martin, 2014).

Buffer conditions were maintained at pH 7.4 for Ad5 processing. Therefore, loss of Ad5 infectivity cannot be due to acidity of the virus environment. In results section 4.3.3 the purified Ad5 fraction P3 was eluted under high salt conditions. This fraction was diluted immediately after it was collected to reduce salt stress on the vector. The purified fractions were then serially diluted over several steps to obtain appropriate concentrations for the β-galactosidase infectivity assay. To investigate the possibility that Ad5 infectivity is limited by high concentrations of salt in elution buffers, Ad5 was exposed to a high salt concentration for 24 h. Adenovirus 5 was very resistant to high levels of salt with no correlation between high salt concentrations and loss of infective capsids (Figure 25).
Figure 25. Adenovirus 5 infective recovery after salt stress showed no significant losses after 24 h of exposure. Red data point represents Ad5 maintained in media under the same conditions (n=3). Error bars represent standard error of the mean.

4.3.5 Improving Nanofiber Performance by Reduction of Adenovirus 5 Loading

Reducing the volume of Ad5 feed may decrease the burden on the nanofiber adsorbent. Previous results (see section 4.3.3) show that the purified Ad5 product is lost in the flow through. A reduction in Ad5 feed permeate is likely to increase chromatographic resolution by preventing column overloading (Avis and Wu, 1996). The low volume (20 mL) Ad5 feed improved peak resolution of purified virus compared to the 50mL load (400 column volumes) (see Figure 16). Purified Ad5 fraction P2 that contained two peaks was resolved from the 20mL feed (160 column
volumes) (Figure 26), via a step elution method. A modification to the UV ratio of P2 in the peak tail (Figure 26) suggests that further separation of this peak is achievable.
Figure 26. Improved separation of Ad5 was achieved by increasing the number of steps in the elution schedule. A 20ml clarified Ad5 feed was used with a Q amine nanofiber (CV = 0.125 mL) (n=3). ‘FT’ denotes flow through peak, ‘P’ denotes elution peaks.
4.3.5.1 Determining Adenovirus 5 Eluent Product Quality

Figure 27. Infectious Ad5 particles were detected in Peaks 2, 3 and 4 using a higher resolution step elution method and a lower loading volume (20 mL) of clarified cell lysate (n=3). Error bars represent standard error of the mean.

The function of Ad5 purified from the 20 mL feed was assessed by quantifying levels of infective virus particles from purified fractions (Figure 27). Fractions P3 and P4 contained similar ratios of infective Ad5 (Table 5). A greater separation was achieved by including an extra elution step, creating four elution peaks - infective Ad5 was detected in P2-P4. Overall recovery of infective Ad5 was 78% over the processing step. However, a large pool of infective Ad5 was detected in the flow through. This virus did not bind to the adsorbent possibly due to competition from impurities. High resolution separations of Ad5 are achievable using a clarified cell lysate feed. The early breakthrough of Ad5 suggests that further work is required to minimise competitive binding of feed impurities.
Figure 28. DNA containing Ad5 virus particles (VP) were detected in Peaks 2-4 using a higher resolution step elution method and a lower loading volume (20 mL) of clarified cell lysate (n=3). Error bars represent standard error of the mean.

DNA containing capsids were eluted in peak P2, P3 and P4 with the largest amount collected in P3 (Figure 28). A low mass balance of VP was observed at 48%. Whilst this could be explained by irreversible binding, it is more likely that the number of Ad5 capsids present in the feed may be overestimated. The qPCR assay relies on the removal of extra-virion DNA through a DNase step which precedes capsid lysis and Ad5 DNA analysis; insufficient DNA lysis in the complex feed may have resulted in an over estimate of the total number of VP in the feed. Increasing the concentration of DNase added as part of the sample preparation would facilitate complete extra-
virion DNA lysis. This explanation is supported by the infective recovery which did not exhibit the same drop in recovery.

Table 5. The recoveries of infective virus particles and Ad5 containing DNA compared as an infectivity coefficient - number of filled copies per infective unit.

<table>
<thead>
<tr>
<th>Ad5 containing sample</th>
<th>Infectious Virus Particle (IVP)</th>
<th>Virus Particle Number (VP)</th>
<th>Infectivity coefficient (VP/IVP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td>1.31E+09</td>
<td>1.14E+10</td>
<td>8.66</td>
</tr>
<tr>
<td>FT1</td>
<td>2.06E+08</td>
<td>1.03E+09</td>
<td>4.99</td>
</tr>
<tr>
<td>FT2</td>
<td>4.23E+08</td>
<td>2.06E+09</td>
<td>4.86</td>
</tr>
<tr>
<td>P1</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>P2</td>
<td>1.05E+07</td>
<td>5.06E+08</td>
<td>48.28</td>
</tr>
<tr>
<td>P3</td>
<td>3.28E+08</td>
<td>2.86E+09</td>
<td>8.71</td>
</tr>
<tr>
<td>P4</td>
<td>6.09E+07</td>
<td>6.03E+08</td>
<td>9.89</td>
</tr>
</tbody>
</table>

A comparison of the ratios of packed Ad5 capsids and infective units (or the infective coefficient) (Table 5) vary between samples. The infective coefficient is greatest for P2 that contained a large ratio of packed and non-infective Ad5. The total number of Ad5 particles in P2 is low relative to P3 and P4. The overall infectious recovery mass balance is high. It is comparable with Ad5 recovery data observed in a membrane ion exchange process (Lucero et al. (2017)).

4.3.5.2 Evaluation of the Quality of Purified Adenovirus 5

To evaluate the quality of Ad5 purified fractions, gel electrophoresis and silver staining analyses were performed to visualise purified Ad5 proteins. Silver staining analysis showed that P1 contained more protein bands than P2, P3 and P4 (Figure 29). The protein band pattern for P2, P3 and P4 were similar showing that low molecular weight proteins were not stained and high molecular weight bands were visualised. Protein bands at 20 – 50 kDa were stained in the P1 fraction but those were not stained in the P2, P3 and P4 fractions. The Ad5 feed, FT1 and FT2 fractions
were over stained because the P1, P2, P3 and P4 fractions that contained comparatively lower levels of protein required prolonged exposure to the silver stain developer to be visualised. The stained gel showed that more protein was removed in the flow through fractions than the peaks. Those may be host cell proteins and product impurities.

<table>
<thead>
<tr>
<th>MWM (kDa)</th>
<th>Feed</th>
<th>FT1</th>
<th>FT2</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
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<tr>
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</table>

Figure 29. SDS-PAGE and silver staining analysis shows distinct Ad5 protein profiles for purified fractions. Gels (4-12% Bis-Tris gels) are loaded with 0.5 µg total protein per lane.
The expression of Ad5 capsid coat and core proteins were determined in purified fractions (P1, P2, P3 and P4) to confirm results showing infectivity of purified Ad5. Ad5 proteins were identified using an anti-Ad5 polyclonal antibody and the blot was stripped before it was reprobed using an anti-Ad5 Hexon antibody. The capsid coat proteins hexon and penton were identified and the core protein, protein V was also detected (Figure 30). Protein V was only present in P3 and P4 and it was more abundant in P3 than P4 – those data are in line with the infectivity results showing the highest levels of infective Ad5 were present in P3 (see Figure 27 and Table 5).

Figure 30. Hexon, penton and protein V are expressed in Ad5 fractions purified from a 20 mL feed. Gels (10% Bis-Tris gels) are loaded with 5 µg total protein per lane.
4.3.5.3 Characterisation of Adenovirus 5 Capsids Purified from a Low Volume Feed

Empty Ad5 capsids are not detected using the β-galactosidase infectivity assay or qPCR analysis. Transmission electron microscopy (TEM) has previously been employed to detect empty vector capsids (Qu et al., 2007). Adenovirus 5 capsids could not be detected in purified fraction P1 using TEM (Figure 31, panel A). Fraction P2 contained a low number of Ad5 capsids and fraction P3 contained packed and empty Ad5 capsids (Figure 31). Fraction P4 also contained Ad5 capsids. Empty and packed Ad5 capsids were distinguished because empty capsids absorb the uranyl acetate TEM assay stain whereas packed capsids are not stained. The penton structure on the capsid surface was observed in Figure 31, panel H. Penton is often difficult to image and there is increased interest surrounding the apparent heterogeneity of fiber protein (involved in host cell recognition) between adenovirus serotypes (Hackenbrack et al., 2017).

The routine use of TEM analysis as part of downstream virus purification work flows may not be possible as it is labour intensive, but this technique has proven to be a vital tool for identification of Ad5 capsid subtypes (empty or packed). The depletion of empty capsids presents a challenge. Nanofiber membranes can remove a small population of mature, packed infective Ad5 from feeds that contain empty Ad5 species. Modifications to the nanofibre membrane surface chemistry together chromatography protocols may lead to development of a process that allows efficient separation of packed and empty Ad5 capsids.
Figure 31. Transmission electron micrographs show the absence of Ad5 capsids in purified fractions in P1 (A), and the presence of Ad5 capsids in P2 (B and C), P3 (D and E), and P4 (F, G and H).
4.3.6 Results Summary

The results described in this chapter demonstrate that nanofiber membranes can purify high molecular weight macromolecules, e.g. BSA and thyroglobulin. A stepwise elution method was developed for the purification of Ad5 from a crude feed with a range of analytical methods used to identify and quantify infective Ad5 particles from purified fractions. Preliminary investigations were performed to identify mature and immature Ad5 capsids from purified fractions using tandem mass spectrometry (MS/MS). Those data showed that mature, packed Ad5 capsids were present in fractions purified from a high volume (50 mL) crude feed of the virus. Evaluations of the effects of process variables that may induce stress on Ad5 during separation procedures showed that high salt concentrations in elution buffers and increased flow rates did not affect Ad5 recovery and infective yield. Lowering the feed volume (20 mL) of Ad5 lead to improvements in resolution of eluted virus peaks and increases in infective Ad5 yields.

4.3.7 Discussion

In the present results chapter, a clarified cell lysate feed was applied to a nanofiber adsorbent under a simplified downstream workflow. The open inter-fibre architecture of those chromatographic matrices relies on diffusive mass transfer that is able to operate using a relatively crude feed of clarified cell lysate (Hardick et al., 2013, Wickramasinghe et al., 2006). Typically downstream vector processes incorporate a UF/DF step to dialyse virus into binding buffer, this however can result in a loss in infective units (Nestola et al., 2014a). Removing process steps increases product therapeutic efficacy as labile viral vectors degrade over time (Sharon and Kamen, 2018). We have shown in the current study that minimal sample preparation is required for small scale purification of Ad5.

A viral vector purification platform technology should show good binding capacity for large molecules. We showed that significant losses in resin binding capacity was observed when comparing a BSA (~3nm) and thyroglobulin (~20nm) feed. Large
proteins, viral vectors or virus like particles are excluded from the inner functionalised surface of matrices because they are too large to diffuse into the pores (Wickramasinghe et al., 2006). As the majority of the functionalised surface on a resin is contained in these pore channels, this restricted access was observed as a decrease in the dynamic binding capacity of the adsorbent. The POROS™ column also exhibited a reduced binding capacity for the larger protein. Pore size for POROS™ is listed as 1,000-3,600 Angstrom (100-360 nm), suggesting that whilst the pores are several times larger than thyroglobulin, diffusion into the inner functionalised surface was still hindered, reducing the efficacy of the column for purification of this protein.

Wickramasinghe et al. (2006) showed that despite the high open structure of Sartobind® S membranes the dynamic binding capacity was far higher for BSA (7.9 mg/mL) than for thyroglobulin (4.9 mg/mL). The gradual equilibration of the flowthrough titre to the feed titre is known as the ‘car parking’ model (Talbot et al., 2000). Initial binding occurs randomly, steric hindrance or geometric blocking of unbound particles by adsorbed particles slows the rate of binding as saturation is approached. The surface coverage of particles on the adsorbent is always less than the total surface area of the chromatographic material. This shielding becomes greater as the size of virus particles increase if the solute species is large relative to the space between adsorbed species (Wickramasinghe et al., 2006). Nanofiber membranes present a higher surface area to volume ratio than membranes, with a lower fiber diameter (Hardick et al., 2011) and it is hypothesised that this increase in surface area and reduction in fiber diameter reduces steric hindrance of the larger thyroglobulin protein allowing for a higher dynamic binding capacity of large biotherapeutics than other convective media.

Model proteins are useful biological tools for evaluation of chromatography column performance but they provide limited information about the purification of viral vectors that are considerably more complex. In the present study, Ad5 purification on nanofibre membranes was optimised to develop a process workflow that allowed
efficient recovery of infective virus at reasonably high yields. High volumes of Ad5 were observed in the flow through fractions at a 50mL and 20mL load, this has been previously described in membrane experiments (Wickramasinghe et al. (2006). The published study described a rapid breakthrough for purification of virus particles. However, unlike membranes, nanofibers exhibit equal dynamic binding capacity for small and large proteins. In the current study, steric hinderance of Ad5 by bound Ad5 and other feed components resulted in rapid breakthrough of Ad5 from nanofibre membranes. Filtration strategies have been investigated as a means of improving vector purification steps to improve column efficiency and resolution by removing feed impurities and increasing available functionalised surface area (Nestola et al. (2014a).

Despite column overload with feed components the adsorbent still achieved a resolution capability expected of convective media (Podgornik et al., 2013), with at least two populations of Ad5 resolved from the nanofiber step. Charge differences exhibited by empty, immature and mature Ad5 capsids could explain the separation of these two populations. Mature Ad5 capsids are characterised by stiffening of the capsid through proteolytic cleavage of six core capsid proteins by the adenovirus proteinase (AVP) enzyme. Mature, packed Ad5 capsids can eject their genome into host cells allowing for virus propagation or transfer of genetic payload. Immature and empty capsids are product impurities that can represent a major proportion of therapeutic Ad5 vectors; this imbalance of Ad5 impurities to packed, mature virus can limit the efficacy of Ad5 mediated therapy.

A therapeutic load can present low VP to IVP ratios (a low number of therapeutically viable capsids when compared to the total population of capsids). High doses of Ad5 vectors with low VP:IVP ratios are required to overcome natural immunity to the delivery vector (Afkhami et al., 2016). Downstream processes can be developed to separate mature and immature Ad5 capsids by modifying chromatography procedures and the surface chemistry of nanofibre membranes. Analytical evaluation of purified Ad5 can involve methods for identification of empty, mature and
Matrix assisted laser desorption/ionisation (MALDI) time of flight (TOF) tandem mass spectrometry (MS/MS) that has been demonstrated previously as a viable technique for identification of viruses may resolve the issue of identifying mature and immature Ad5. Three distinct serotypes of polio virus were identified by Calderaro et al. (2014), and Lewis et al. (1998) demonstrated structural differences between wild type and mutant Tobacco Mosaic Virus using MALDI-TOF MS/MS. Demonstrating the potential of mass spectrometry to investigate the small differences in protein composition in closely related forms of a virus species, Jin et al. (2017) used this technique as a means of examining the post-translational modifications of adeno associated virus (AAV) capsid proteins which give rise to serotype heterogeneity.

In the present study, two populations of Ad5 isolated from the nanofiber column step, were analysed using MALDI-TOF MS/MS. This technique was applied to determine the presence of mature, infective Ad5 capsids in feed and purified fractions. The core protein that is cleaved upon maturation of Ad5, protein VIII, was identified. To our knowledge, our study is the first to demonstrate the presence of cleaved protein VIII in Ad5 purified from an ion exchange membrane column. Protein VII that is a substrate for AVP mediated proteolysis was also identified. Cleavage of protein VII causes disassociation of the genome from the capsid, reducing its capacity as a structural scaffold and increasing the internal pressure of the capsid (Perez-Berna et al., 2012). The purified Ad5 obtained here contained DNA and the fractions were infective. Western blot analyses showed expression of Ad5 hexon and penton as well as protein V from the core capsid in purified fractions. Further analysis using TEM demonstrated the presence of packed Ad5 capsids in purified fractions that were previously shown to be infective.

Alterations in the structure of Ad5 capsids during maturation may influence the mechanisms of virus binding to nanofibre membranes - a mature Ad5 capsid is
brittle. Protein IIIa and VIII form a molecular stitch holding hexon trimer groups (groups of six (GOS)) together. As these proteins are cleaved, this stitch weakens resulting in formation of a brittle capsid (San Martin, 2012). The precise stage of maturation at which Ad5 is able to infect host cells is not known. Purified Ad5 from the 50mL load study was eluted in three peaks that presented various abilities to infect cells. Peak 1 did not contain Ad5, peak 2 contained packed immature capsids and peak 3 contained packed, mature capsids. The elution conditions for the Ad5 containing peaks may indicate conditions for separation of mature and immature capsids. Peak 3 Ad5 was eluted at a higher salt concentration than peak 2 capsids, potentially due to differences in charges of mature and immature capsids. Modification of the nanofibre membrane surface may permit individual capture of mature and immature virus particles in a charge dependent manner.

Process parameters including elution buffer salt concentrations and flow rates will also impact the separation of Ad5 capsid subtypes as well as the percentage recovery of virus. Here, the effect of elution buffer salt concentration on Ad5 infectivity was assessed. The ability of Ad5 to infect cells was not limited after prolonged periods to high salt exposure. Perez-Berna et al. (2012) investigated thermal and acidic stress on Ad5 capsid integrity finding significant damage to the capsid was observed <pH 6 and >40°C. Rexroad et al. (2006) showed that Ad5 exhibited high stability with up to 1M NaCl over a wide pH range, corroborating the findings described in the current study. Our data can be applied as a basis for process design as high ionic strength buffers were required to elute all Ad5 samples.

We showed that flow rates of up to 70 mL/min did not diminish Ad5 infectivity. Shear forces are generated in the void space of resin columns have been suggested as one cause of structural damage to labile macromolecular structures (Gagnon, 2008). Convective media are able to operate at high flow rates and are typically not packed to minimise void space (Podgornik et al., 2013). Shear forces were assessed as a possible cause for the loss of Ad5 infectivity. Nanofiber membranes can typically operate at exceptionally high flow rates (70ml/min and above) allowing for rapid
processing times. No significant loss in infective recovery was observed at flow rates of 70mL/min. This may not be due to high tolerance of Ad5 to shear stress (Subramanian et al., 2005), but because of the open pore structure of the nanofibers reducing vortex formations due to minimal void space in the nanofiber layer (Hardick et al., 2011). The open pore structure allowed for the nanofibers to operate at these flow rates without the column failure previously shown by Hardick et al. (2013). However, feed quality here was significantly poorer. These data suggest that chromatography run times can be greatly shortened to increase process efficiencies and it will be possible to modify chromatography methods that require the use of various salt concentrations for elution of mature and immature Ad5 capsids.

In the current study, by utilising a lower feed volume from 50 mL to 20 mL the total infective Ad5 recovery increased from 69% to 78%. This may be a result of adverse effects of higher concentrations of virus eluting from the column at higher loading volumes. Aggregation of adenovirus has shown to be affected by concentration of capsids, pH and conductivity (Kahler et al., 2016). Aggregation of vector capsids can be minimised by using non-ionic detergents to attenuate hydrophobic interactions and sodium chloride to mediate electrostatic interactions (Konz et al., 2005). Irreversible binding of virus to the adsorbent has been reported in a number of viral processes as a potential contributor to loss in recovery (Grein et al., 2012, McNally et al., 2014). The reason for the observed increase in overall recovery at lower loading volumes is not yet clear and requires further investigation.

4.4 Conclusion

Taken together, the results described in the present chapter demonstrate optimisation of some features of the purification procedure for Ad5. We show that process optimisation is required to improve resolution and performance of the nanofiber membranes, particularly with regard to recovery of packed, mature Ad5 capsids and depletion of empty and immature virus particles. We have presented here, a simple process step for the rapid purification of functional (infective) Ad5 vectors from a clarified bulk cell lysate, with minimal processing steps. These data demonstrate the
ability of nanofiber membranes to process complex Ad5 feeds with application of a range of downstream analytical procedures that validate the function, yield, purity and identity of the Ad5 product.
Chapter 5  Investigation of Quaternary Amine Functionalised Nanofiber Membranes for Purification of Infective Adenovirus 5 at High Yield

5.1 Introduction

Adenovirus 5 (Ad5) vectors are complex macromolecular structures comprising of a non-enveloped protein capsid encasing a dsDNA core. Fiber pentons on the capsid surface are critical to the initial stages of Ad5 infection as they are involved in attachment of the virus to host cells (San Martin, 2012). They can be lost on adsorbent surfaces during binding and elution resulting in losses in infective Ad5 yield. Adenovirus 5 exhibits different physical characteristics during the virus life cycle, e.g. the mature virus is brittle (Perez-Berna et al., 2012), and this structural property may affect how Ad5 interacts with nanofiber membranes over the adsorption and desorption process.

The duration of the adsorption process may affect Ad5 infective yield. Reports show that increasing ligand densities on cation exchange resins enhances haemoglobin and ferritin binding, retention and resolution (Kopaciewicz et al., 1985) but this modification does not affect the dynamic binding capacity of monoclonal antibodies (Hardin et al., 2009). The binding behaviour of viral vectors will be more complex than that of proteins as their macromolecular structures are more intricate. A functionalised gel layer membrane adsorbent was shown to exclude large 100 nm length molecules (Tatárová et al., 2009). Increasing the ligand density of chromatographic matrices may be detrimental to vector purification, as excessive binding sites favour the adsorption of process impurities.

Vicente et al. (2011) showed that ion exchange matrices modified with diethylamine ligands at a low density not only decreased concentrations of host cell protein and dsDNA, but it also removed non-infective recombinant baculovirus (rBV) whilst increasing infective rBV yields by 20%. Those studies indicate that the addition of functional groups to nanofiber membranes may increase the capture of Ad5 whilst reducing host cell contaminants. The current results chapter will focus on
investigation of Ad5 purification using nanofibre membranes modified with quaternary amine (Q) ligands at low, medium and high densities. Quaternary amine ligands have been and continue to be used to functionalise Ad5 purification platforms. Lucero et al. (2017) found that genome containing Ad5 recoveries were improved from 28% to 34% by changing from a Q Sepharose column to CIM QA-1 monolith column. This shows a marked improvement in recovery when using convective media platform technology. Results described here show a > 90% recovery of genome containing Ad5, and > 90% recovery of infective Ad5.

5.1.1 Objectives

a. To investigate the ability of nanofibre membranes modified with Q amine ligands (low, medium and high density) to purify infective Ad5 at high yields.

5.1.2 Aims

a. Evaluate the effect of an ultrafiltration/diafiltration (UF/DF) step on Q amine nanofibre column resolution.

b. Determine the effects of Q amine functionalisation on nanofiber adsorbent performance, focussing on resolution and recovery of infective Ad5.

c. Assess the life cycle performance of Q amine functionalised nanofibre membranes.

d. Examine the binding capacity for Ad5 of Q amine functionalised nanofibre membranes.

5.2 Methods Summary

5.2.1 Clarification of Adenovirus 5 Crude Feed

Adenovirus 5 propagated in a HEK293 cell was transferred to 50 mL centrifuge tubes. The cell lysate was then placed on dry ice for 30 min and thawed at 37°C over a 40 min period. This cycle of freezing and thawing was performed 3 times to disrupt
the cell membrane. The cell lysate was then centrifuged at 2000 rpm for 10 min, and the supernatant containing Ad5 was filtered using a 0.45µm syringe filter. The clarified Ad5 containing solution was pooled. Tangential flow filtration (TFF) of the clarified Ad5 crude feed was conducted on a KR2i TFF system with a 500 kDa molecular weight cut off (MWCO) D06-E500-05-N hollowfiber (length 65cm, surface area 370cm²; Spectrum Labs, Breda, The Netherlands) at a flow rate of 20ml/min and transmembrane pressure of 2Psi (± 0.5). Adenovirus 5 crude feed was concentrated four-fold, before it was suspended into binding buffer (20mM Tris, pH 7.4) via a buffer exchange procedure.

5.2.2 Chromatography

5.2.2.1 Gradient Elution of Adenovirus 5
Adenovirus 5 containing feeds that were filtered using the TFF procedure, were purified using nanofibre membranes to determine whether or not incorporating the TFF step into the purification procedure would reduce process impurities and change Ad5 binding characteristics to the nanofiber adsorbent. Chromatography experiments were performed using an AKTA Avant (GE Healthcare Life Sciences, Buckinghamshire UK), with online measurements of pH, conductivity and UV absorbance (260 nm and 280 nm). The nanofiber was equilibrated with 10 mL wash buffer containing 20mM Tris, pH 7.4 at a rate of 10 ml/min and 5ml Ad5 feed at a concentration of ~10⁸ virus particles per mL (VP/ml) was loaded onto the nanofibre column. The column was washed with binding buffer until conductivity reached a constant reading. A 20ml gradient elution was applied to the absorbent up to 1M NaCl 20mM Tris, pH 7.4 at a rate of 10 ml/min. The column was washed with 2M NaCl 20mM Tris, pH 7.4.

To examine the effect of prolonged adsorption durations on the nanofibre membrane adsorbent, 5ml of clarified Ad5 feed was loaded onto the nanofibre column and wash steps containing 10, 40, 80 or 240ml of elution buffer were passed through the column at a flow rate of 10ml/min. This method was performed to reproduce scaled up processing conditions that would typically include a larger loading volume and
extended durations of Ad5 bound to the adsorbent. The low, 5mL volume of Ad5 load was used to reduce the disparity in binding durations of Ad5 at the front of the feed volume compared to Ad5 at the end of the feed volume.

To optimise the chromatography method for purification of Ad5 on nanofiber membranes, a gradient elution method was assayed. A 50ml Ad5 feed containing ~10⁸ filled virus particles per mL (VP/ml) was loaded onto 0.125ml nanofiber adsorbent. The column was washed with binding buffer until conductivity reached a constant reading. A 20ml gradient elution was applied to the adsorbent up to 1M NaCl 20mM Tris, pH 7.4. A constant flow rate of 10ml/min was maintained over the duration of the process step. Flow through fractions were collected using a F9-R fraction collector (GE Healthcare Life Sciences, Buckinghamshire UK).

5.2.2.2 Resolution of Eluted Adenovirus 5
The resolution of eluted Ad5 peaks was achieved using a step elution method. Adenovirus 5 peaks from 20ml gradient elutions were resolved. Finer peak resolution was achieved by extending the gradient elutions when multiple peaks with similar isoelectric points were identified. A constant flow rate of 10ml/min was maintained to minimise effects of prolonged adsorption durations on Ad5 infective recovery. Elution fractions were collected using a F9-R fraction collector (GE Healthcare Life Sciences, Buckinghamshire UK). All samples were diluted in 1X phosphate buffered saline to minimise the effects of high salt on recovery of infective Ad5.

5.3 Results

5.3.1 Comparison of Clarified and Buffer Dialysed Adenovirus 5 Feeds
The crude Ad5 feed propagated from HEK293 cells was clarified with 0.45µm filters and 50% of this sample was then ultrafiltered and diafiltered using a 500kDa TFF system to remove bulk host cell impurities before dialysis into binding buffer. Both UF/DF and non UF/DF Ad5 feeds were analysed using the β-galactosidase infectivity assay to characterise the effect of processing on Ad5 infective potency.
After filtration, the retentate presented an infective recovery of 89% compared to the crude cell lysate (CCL). A 5ml volume of diafiltered Ad5 feed was loaded onto anion exchange nanofibers at 10 ml/min (Figure 32), and a 20ml gradient elution of up to 1M NaCl was applied to the column. The elution profile was then compared to a 5ml load of unfiltered crude Ad5 feed under the same process conditions.

This process was repeated for membranes functionalised with low, medium and high densities of Q amine ligands. A large flowthrough peak was observed for all Q amine ligand density nanofibers with loading of a 5 mL crude, unfiltered Ad5 feed into the chromatography system. A lower total flowthrough peak area was recorded for the filtered Ad5 feed when compared to the crude, unfiltered Ad5 feed, suggesting removal of host cell impurities from the retentate during the filtration step. However, a loss of 11% of total infective units was also recorded. Elution profiles for CCL Ad5 feeds are distinct across all three fiber types with components binding more tightly and requiring higher ionic strength to elute as ligand density increases. There are more subtle differences seen for the TFF treated material, which are more noticeable at the highest charge density. An explanation could be that with minimal contaminants present in the TFF material interactions between Ad5, contaminant and the charge surface that allow discrimination for the CCL material are reduced. The distinct elution profile across the three fiber types, demonstrate different separation capabilities of nanofibers as the Q amine ligand density increases. This suggests that by tailoring the ligand functionalisation of the nanofibers it is possible to optimise Ad5 purification process for improved separations.
Figure 32. Elution profile comparison of Ad5 on low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) Q ligand density nanofibers (CV = 0.125mL). Ad5 was separated from a clarified cell lysate (CCL) and a tangential flow filtration (TFF) UF/DF 500 kDa retentate diafiltered into binding buffer (20mM Tris, pH 7.4). Loads (5 mL) of both Ad5 feeds containing a total load of $5.6 \times 10^8 \pm 5.6 \times 10^7$ IVP were used. Chromatograms were generated using a 20 mL gradient elution at 10 mL/min from 0 M NaCl, 20mM Tris pH 7.4, to 1M NaCl, 20mM Tris pH 7.4 (n=3).
5.3.2 Extended Adsorption Periods on Quaternary Amine Functionalised Nanofibre Membranes Reduce Adenovirus 5 Infectivity

Poor viral vector recoveries over an ion exchange chromatography step have been attributed to prolonged adsorption periods that cause degradation of capsid integrity and entrapment of virus particles inside complex internal adsorbent structures (Trilisky and Lenhoff, 2009). Hardick et al. (2013) showed that the large inter-fibre space and unique open presentation of the functionalised surface of nanofibers minimises diffusive mass transfer limitations, a property which has been shown to be detrimental to capacity and recovery of large biotherapeutic molecules (Wickramasinghe et al., 2006). The open fibre structure of nanofibre (Figure 15) may minimise entrapment events suggesting loss in infective units is a result of irreversible binding or capsid damage.

The effects of prolonged binding duration (Figure 33) on the recovery of infective Ad5 (Figure 34) was analysed. Clarified Ad5 feed (5 mL) was loaded onto nanofiber columns and adsorption durations were selected to approximately replicate binding durations of current industrial vector processes. Complete recovery of infective capsids was observed after the shortest binding duration (1 min) using low ligand density nanofibers. Extending binding durations from 4-24 min using low ligand density nanofibers did not cause a significant decrease in the infectivity of Ad5 eluate, with recoveries between 87-90%. At an extended adsorption duration of 24 min there was a dramatic loss of almost 50% in total infective capsids for medium and high ligand density nanofibers. Significant losses in Ad5 infective recoveries were also observed on high ligand density membranes after adsorption periods of 1-8 min and 8-24 min adsorption periods. The highest recovery of infective Ad5 at 97% was achieved with low ligand density membranes during the shortest adsorption period of 1 min.
Figure 33. Representative elution profile of four chromatography runs of clarified cell lysate Ad5 feed with varying wash durations (10, 40, 80, 240 mL or 1, 4, 8, 24 min), using medium (750 µg/mol) Q ligand density nanofibers. Each variable was repeated in triplicate for a total of twelve runs. Differences in peak shape were not observed between low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) Q ligand densities. Nanofibers were loaded with $6.22 \times 10^8$ IVP of Ad5 in a clarified cell lysate feed (CCL) feed (5 mL at a flow rate of 10 mL/min). Samples were eluted with a 1M NaCl, 20mM Tris pH 7.4 wash, followed by a column wash of 2M NaCl (n=3).

The substantial losses in Ad5 infectivity observed with use of the medium and high ligand density nanofibre membranes indicate capsid damage potentially. Capsid structural proteins may over the time period be impacted by multipoint binding leading to them being strongly retained on the column (McNally et al., 2014). Alternatively this may cause deformation of the capsid as it is ‘pulled’ onto the
functionalised surface over the adsorption duration damaging the capsid. These data suggest that although medium and high ligand density nanofibre membranes limit the recovery of infective Ad5 over extended adsorption periods, Ad5 product function can be maintained if a rapid processing is utilised with those membrane types.

Figure 34. Recovery of adenovirus 5 infectivity during adsorption to nanofiber based ion exchangers, measured by a cell based β-galactosidase reporter assay. Low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) Q ligand density nanofibers (CV = 0.125 mL) were loaded with $6.22 \times 10^8$ IVP of Ad5 in a clarified feed ($n=3$). Error bars represent standard error of the mean.
5.3.3 Comparison of the Effects of Low, Medium and High Quaternary Amine Ligand Density on Resolution of Adenovirus 5 Separation

The effects of increasing the density of Q amine ligands on the nanofibre membrane surface on Ad5 purification were assessed. A 5ml filtered (TFF) Ad5 feed was purified using a step elution process to maximise the resolution of Ad5 feed components whilst maintaining recovery of infective vector (Figure 35). Elution steps were determined by selecting for peaks identified from gradient elutions (see Figure 32). A 5 mL volume was chosen to minimise process time and maximise recovery of infective Ad5. The double peaks of MP3 and HP4 could be resolved further, but as they did not contain infective Ad5, the length of the chromatography run was not extended to achieve improved resolution. Instead, chromatography run durations were limited to short periods to maximise Ad5 product recovery. The highest peak resolution was achieved using high ligand density nanofibers.
Figure 35. The impact of increasing Q amine ligand density on the resolution of Ad5 feed components. Elution peak profiles of low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) Q amine ligand density nanofibers were recorded from a chromatography run of 5 mL (2.39 x 10⁸ VP, 5.6 x 10⁹ IVP) TFF feed loaded onto a 0.125 mL nanofiber column at a flow rate of 10 mL/min (n=3).
5.3.4 Reproducibility and Life Cycle Performance of Quaternary Amine Functionalised Nanofibre Membranes

High and reproducible performance of chromatography tools are paramount in bioprocessing (Rathore and Sofer, 2005). Therefore, the effects of multiple, sequential chromatography runs on nanofiber membrane performance were assessed by performing a step elution process nine consecutive times over three different batches of low, medium and high density Q amine ligand membranes (Figure 36 - batch one: blue, batch two: red, batch three: green). Peak elution steps were achieved with 1M NaCl and a 2M NaCl wash was included between runs to enable column recovery. There was no detectable loss in performance after nine runs across all three nanofiber ligand densities, suggesting a 2M NaCl wash was sufficient to remove all Ad5 feed components between runs. Batch to batch variability of nanofiber membranes was assessed for all three ligand densities. Minimal variability between the three individual batches of each membrane type (low, medium and high) suggests good manufacturing reproducibility.
Figure 36. Performance of low, medium and high ligand density nanofibre membranes after multiple uses. Performance was assessed by performing nine bind/elute runs with a 5 mL load of TFF Ad5 feed in succession and this was repeated for three individual batches of low, medium and high ligand density nanofibers (n=3).
5.3.5 Quaternary Amine Functionalised Nanofibre Membranes Achieve Efficient, High Yield Purification of Infectious Adenovirus 5 Particles

Loading volumes for high resolution separation of Ad5 (Figure 35) were maintained at a relatively low volume (5 mL) or 40 column volumes (CVs) to maximise resolution at this loading. This approach ensured that adsorption periods were short and Ad5 capsid recovery was high. Product was not detected in the flowthrough. To determine ligand density performance when challenged with a large volume load, a 50 mL (400 CVs) TFF Ad5 feed (2.39 x 10^{10} VP, 5.6 x 10^{9} IVP) was loaded onto membranes (Figure 37). Five 10 mL flowthrough fractions were collected and screened for the presence of infective Ad5 capsids. Infective Ad5 capsids were not present in the fractions collected. There was no difference between the ligand densities in performance for loads up to the maximum attempted (1.91 x 10^{11} VP/mL of nanofiber). Nestola et al. (2014c) previously reported a negative impact on DBC when increasing directly grafted ligand density which was not observed here, however this is likely because capacity was not reached. This effect was not observed here and this is likely because the binding capacity of nanofibre membranes was not achieved. Nanofiber membranes are able to bind high amounts of Ad5. In vivo therapeutic loads range from as low as 10^8 to 10^{12} virus particles (VP) per dose depending on the therapy and site of administration. In a vaccine for tuberculosis, Ad5 was administered at 10^9 VP/dose (Smail et al., 2013) and as an oncolytic vector for hepatocellular carcinoma, Ad5 was administered at 10^{11} VP/dose (Habib et al., 2001). Whilst further work to determine capacity is required, the observed experimental scale can produce enough material for ten 10^9 VP doses, with the potential to fulfil multiple doses of a higher concentration with only a small scale up to a 1mL column format. The results presented here demonstrate potential clinical utility of the nanofiber adsorbent due to high dynamic binding of Ad5 particles.
Figure 37. High loadings of adenovirus feed material to quaternary amine exchange nanofibers. A 50 mL (high volume) TFF Ad5 feed (2.39 x 10^{10} VP, 5.6 x 10^9 IVP) was separated using low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) Q amine ligand density nanofibers (CV = 0.125 mL) (n=3). ‘FT’ denotes flow through.
5.3.6 Recovery of Infectious Adenovirus 5 Particles from Quaternary Amine Functionalised Nanofibre Membranes

High infective product recovery is the primary challenge when purifying viral vector. It is necessary to assess both the total recovery of Ad5 capsids and their infective potency across each unit operation. In Table 6 this data is presented for the runs in Figure 35. Quantitative PCR analysis was used to determine the recovery of total Ad5 VP. One fraction (LP4) containing Ad5 (1.94 x 10⁹ VP) was separated and two fractions (LP3 and LP5) that had low amounts of Ad5 were purified on the low ligand density membranes. Medium ligand density membranes separated MP5 that contained the majority of packed Ad5 (2.25 x 10⁹ VP) with Ad5 containing fractions MP4 and MP6 that contained lower titres. The high ligand density membranes isolated peak HP6 that contained most of the packed Ad5 (1.99 x 10⁹ VP).

Ad5 particle infectivity was measured by β-galactosidase staining, particles containing collected from low ligand density nanofibers were infective (Table 6). LP4 contained a ratio of 4.59 VP/IVP. MP5 contained a ratio of 5.12 VP/IVP, and HP6 contained a ratio of 4.00 VP/IVP. These peaks showed the highest titre from each run. The highest proportion of packed, non-infective Ad5 capsids were purified using high ligand density membranes and they were isolated in HP7 with a ratio of 16.04 VP/IVP, suggesting clearance of a population of lower quality Ad5. VP/IVP ratios can be used to determine product quality, all ratios for the highest titre peaks (LP4, MP5 and HP6) presented here are well accepted ranges for therapeutically viable by the FDA (Kramberger et al., 2015). Analysis of total capsid recovery provides evidence of separations of distinct populations of Ad5, with varying resolution capabilities across all three fibre types. Purified fractions of Ad5 that exhibit reduced infectivity as a result of damage or because they have not yet matured, represent an important product related impurity. The removal of those impurities from the final Ad5 product is important.
Removal of host cell protein (HCP) impurities across the UF/DF and chromatography step was high with a >95% removal (compared to non-purified Ad5 feed) of HCPs (Table 6).

The mass balances of packed, infective Ad5 capsid recovery across all nanofiber membranes were high (Table 6) compared to other membrane adsorbers (Nestola et al., 2014c) and monoliths (Lucero et al., 2017). Despite the different ligand densities presenting unique elution profiles, the highest titre peaks (LP4, MP5 and HP6) showed a relatively consistent infective ratio, however increasing the ligand density increased salt concentration required for elution. The total recovery of packed, infective Ad5 particles were similar across low, medium and high ligand density nanofibers. Quaternary amine density also affected the profiles of eluted fractions that did not contain infectious Ad5 (Figure 38). Peaks LP1, LP2, MP1, MP2, MP3 and HP1, HP2, HP3, HP4, HP5 did not contain VP or IVP and they showed distinct protein banding patterns. The proteins that are present in those fractions may be virus derived impurities or host cell proteins. The fractions collected later in the chromatography runs (LP4, LP5, MP4, MP5, MP6, HP5, HP6, HP7) contained very low amounts of total protein that were below the limits of detection for silver staining analysis.
Table 6. The total recoveries of infective Ad5 units (IVP, analysed by β-Gal stain), DNA containing (VP, analysed by qPCR) Ad5 units and the ratio of these two populations within all Ad5 containing peaks separated on low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) Q ligand density nanofibers. No qPCR signal was detected for samples LP3 and MP4. Good amounts of host cell protein was shown to be removed from the Ad5 containing feed, when compared to crude cell lysate Ad5 harvest (1.3 mg/mL) (n=3).

<table>
<thead>
<tr>
<th>Ad5 containing sample</th>
<th>Sample Volume (mL)</th>
<th>Infectious particle number (IVP)</th>
<th>Standard Error of the Mean</th>
<th>Total IVP Recovery to IVP recovery from TFF</th>
<th>IVP Recovery Standard Error</th>
<th>Viral Particle Number</th>
<th>Standard Error of the Mean</th>
<th>Total VP Recovery to VP recovery from TFF</th>
<th>VP Recovery Standard Error</th>
<th>Infectivity coefficient (VP/IVP)</th>
<th>Eluted NaCl concentration (M)</th>
<th>HCP conc (µg/mL)</th>
<th>Percentage HCP removal from crude feed</th>
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<td>Total</td>
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<td>9.40E+06</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>1304</td>
<td>356</td>
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<td>LP3</td>
<td>8</td>
<td>1.40E+07</td>
<td>4.00E+06</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.29</td>
<td>38.2</td>
<td>97.1%</td>
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<td>4.22E+08</td>
<td>8.72E+06</td>
<td>1.94E+09</td>
<td>5.06E+07</td>
<td>4.59</td>
<td>4.09</td>
<td>40.0</td>
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<tr>
<td>Density</td>
<td>LP5</td>
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<td>8.73E+06</td>
<td>3.26E+08</td>
<td>3.03E+07</td>
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<td>90.2%</td>
<td>3.81%</td>
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<td>42.3</td>
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<td>2.69E+08</td>
<td>3.86E+07</td>
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<tr>
<td></td>
<td>Total</td>
<td>5.14E+08</td>
<td>91.4%</td>
<td>4.81%</td>
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<td>11.91%</td>
<td>4.93</td>
<td>53.3</td>
<td>95.9%</td>
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<td>HP6</td>
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<td>1.48E+07</td>
<td>1.99E+09</td>
<td>4.71E+07</td>
<td>4.00</td>
<td>0.61</td>
<td>33.2</td>
<td>97.5%</td>
<td></td>
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<tr>
<td></td>
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<td>HP7</td>
<td>6</td>
<td>2.55E+07</td>
<td>5.41E+06</td>
<td>4.09E+08</td>
<td>1.44E+07</td>
<td>16.04</td>
<td>1</td>
<td>54.5</td>
<td>95.8%</td>
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<td></td>
</tr>
<tr>
<td>Density</td>
<td>Total</td>
<td>5.23E+08</td>
<td>92.9%</td>
<td>3.59%</td>
<td>2.40E+09</td>
<td>99.6%</td>
<td>2.57%</td>
<td>4.59</td>
<td>43.9</td>
<td>96.6%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

153
Figure 38. Proteins from fractions purified using low (440 µg/mol), medium (750 µg/mol), high (1029 µg/mol) ligand density nanofibres were separated using 4-12% Bis-Tris gels and silver stained. Peaks containing packed, infective adenovirus capsids are highlighted in red (n=3).
5.3.7 Separation of Free Hexon Capsid Protein

Hexon is a key protein in the Ad5 capsid but can also be found in non-assembled forms (Klyushnichenko et al., 2001). It has been shown to be immunogenic and represents an important product related impurity (Bradley et al., 2012). A western blot (Figure 39) was used to show the distribution of hexon during the separations shown in Figure 35. Hexon was identified in the purified fractions, LP3, LP4, MP5, and HP6, demonstrated to contain packed and infective Ad5 capsids. Hexon is also found in MP3 and HP4 fractions that do not contain infective Ad5 particles and therefore is free hexon protein that is not incorporated into complete capsids. This suggests with medium and high ligand density nanofibres it was possible to isolate free hexon from capsid bound hexon. There is scope to scale up this process for industrial production of therapeutic payloads of viral vectors.
Figure 39. Western blot using a Hexon antibody with a secondary antibody (rabbit polyclonal antibody to mouse IgG (HRP-conjugated) showing Adenovirus 5 hexon expression in purified fractions from low (440 µg/mol), medium (750 µg/mol) and high (1029 µg/mol) Q ligand density nanofibers collected from step elution chromatograms (n=3). A molecular weight marker (MWM) and Ad5 from a clarified cell lysate (CCL) and a tangential flow filtration (TFF) UF/DF 500 kDa retentate diafiltered into binding buffer (20mM Tris, pH 7.4) was also loaded.
5.4 Results Summary

Two feed streams derived from the same Ad5 batch were purified using low, medium and high Q amine ligand density membranes. One feed stream was pre-processed with an ultrafiltration/diafiltration (UF/DF) step using tangential flow filtration (TFF) into binding buffer and another was clarified (clarified cell lysate: CCL) using a 0.45µm syringe filter to remove cell debris. The hypothesis for this work is that decreasing the complexity of the Ad5 feed by performing the UF/DF step will enhance the recovery of Ad5 from Q amine functionalised membranes. The premise for increasing ligand density is that this modification will regulate the strength of interactions between the virus and adsorbent, leading to improvements in the resolution and binding capacity nanofibers. Loss of infectious Ad5 as a result of extended on-column adsorption periods was analysed to determine the cause of typically poor Ad5 downstream recoveries.

Results showed that:

a.) Adenovirus 5 from clarified and filtered feeds was separated in distinct eluate profiles between the two virus preparations (filtered and unfiltered) and the different membrane types.

b.) Prolonged adsorption durations (24 min) of Ad5 on membranes caused a 50% reduction in recovery of infective Ad5.

c.) Low ligand density membranes allowed efficient recovery of infective Ad5 after long (24 min) adsorption periods.

d.) Increasing the density of Q amine ligands on nanofibre membranes enhanced the resolution of eluted Ad5.

e.) The Q amine modified nanofibres do not lose performance after multiple (nine), consecutive chromatography runs.

f.) High infective recoveries of up to 90% were achieved from low, medium and high Q amine ligand density membranes.
Adenovirus 5 hexon identified in purified from virus containing peaks Q amine functionalised nanofibers supported results showing clearance of product related impurity from high titre Ad5 peaks.

5.5 Discussion

Membrane ion exchange materials present with a number of advantages that make them viable platform technologies for macromolecular purification (Hardick et al., 2013, Wickramasinghe et al., 2006). Altering the surface chemistry of ion exchange membranes has shown promise in process optimisation (Nestola et al., 2014c). In the present study, ion exchange nanofiber membranes modified with a quaternary (Q) amine ligand at a range of densities (low, medium and high) were investigated to assess their suitability as a processing platform for Ad5 purification.

In the present study, the density of Q amine ligands on nanofibre membranes was demonstrated to enhance the recovery of packed, mature, infective Ad5 particles. Ligand density has been shown to influence both protein retention and mass transfer characteristics in sulphopropyl cation exchange chromatography (Fogle et al., 2012). The surface area of the Q amine functionalised nanofiber membrane is significantly higher than that of commercially available membrane adsorbents. This large surface area may increase contact events between target and adsorbent (Hardick et al., 2013). The mass balance of infective Ad5 capsid recovery showed that this was exceptionally high across low, medium and high ligand density nanofibre membranes. To our knowledge, the infective recoveries of >90% for Ad5 on Q amine functionalised membranes presented here are the highest published to date. Previous chromatography screens of Ad5 have shown little difference between ligand density and recovery of Ad5. Poor recoveries of Ad5 were observed with increases in the ligand density of a Q amine functionalised membrane - 60-70% of the virus load was recovered in the flowthrough by Nestola et al. (2014). The experiments were operated in a 96 well format (column volume: 23µL) using a step elution and the conclusion of the study was that the virus did not form contacts with the functionalised surface during its migration through the column.
Vicente et al. (2011) showed that there was no increase in dynamic binding capacity of recombinant baculovirus with increases in the density of a hydrogel based functionalisation. The hypothesis of this reported study was that the ligand hydrogel layer was only accessible to process impurities. The Q amine ligands used in the current study are ‘directly’ attached to the adsorbent surface creating a very different adsorbent landscape compared to the hydrogel modified matrix. Another study investigating functionalised chromatography matrices described a switch in the elution order of lysozyme and cytochrome c on range of ligand density cation exchange resins (Wu and Walters 1992). Cytochrome c eluted first on low ligand density and lysozyme eluted first on high ligand density resins. Large macromolecular targets, e.g. Ad5 are able to bind to an adsorbent through multiple binding sites. Yamamoto et al. (2009) analysed the binding properties of 30nm, 50nm and 100nm charged liposome particles and showed that a single particle of Ad5 could interact with the adsorbent using 25-30 binding sites. This strong attachment to the adsorbent has been utilised for separations of large biotherapeutics from smaller feed components, which do not bind as strongly. Short monolithic columns have been used to separate tomato mosaic virus (Kramberger et al., 2004), Ad5 (Whitfield et al., 2009), Ad3 virus-like dodecahedric particles (Urbas et al., 2011), and hepatitis B surface antigen VLP produced in yeast (Burden et al., 2012) from those types of heterogeneous feeds. In all of these examples, a sharp virus peak was observed due to convective flow of the mobile phase as well as short bed height (Podgornik et al., 2013).

In a study investigating the effects of increasing cation ligand density of resins for monoclonal antibody (mAb) dynamic binding capacity and impurity clearance, Fogle et al. (2012) operated columns in bind and elute mode using gradient elution. A mAb in a monomeric form, a triple light chain high molecular weight variant, and a basic charge variant were selected. Ligand density increased resolution of the monomer and the charge variant increased, but resolution of high molecular weight variant and basic charge variant decreased. This varying of the ligand density and resolution
capability of closely related variants derived from the same macromolecule has also been demonstrated in the current study with Q amine functionalised membranes.

The published data are partially consistent with molecular dynamic simulations performed by Dismer and Hubbuch (2010), calculating that the electrostatic energy for the interaction of lysozyme with a two dimensional surface grew larger as ligand spacing decreased resulting in different binding orientations of the lysozyme. The interpretation of selectivity is observed here as Ad5 subspecies exhibit different surface charge distributions, which may influence their binding if ligand spacing is varied. Analysis of total capsid recovery provides compelling evidence of separations of distinct populations of Ad5, with varying resolution capabilities across all three fibre types. Purified fractions of Ad5 that exhibit reduced infectivity as a result of damage or because they have not yet matured, represent an important product related impurity. The removal of those impurities from the final Ad5 product is important.

The Q amine modification of nanofibre membranes is also an advantage as it can allow removal of empty Ad5 capsids from a feed. This is currently achieved using a routine ultracentrifugation procedure, varying density between the empty capsids and virus particles (Sundquist et al., 1973). Separating immature virus particles from infective virus particles is not possible based on density separations. We have shown that nanofiber membranes may allow separation of these Ad5 subspecies (see Chapter 4). In the present results chapter, using medium and high ligand density nanofibers, it was possible to achieve a separation of product peaks from a hexon rich peak. Adenovirus 5 hexon forms the major building block of the virus capsid (>60%) (Perez-Berna et al., 2012) and non-assembled hexon represents major product impurity due to its antigenic properties.

Free floating hexon was removed from an adenovirus feed using a DEAE-Fractogel anion exchange step by Green et al. (2002), eluting, as shown here at low ionic strength. A two-step process could incorporate a high ligand density capture of HP6 to remove HCP and low quality vector in HP7. This would be followed by a low
ligand density polishing step optimised to capture highly infectious virus. The higher infective recovery titres observed for low ligand density fibers would make this a favourable selection over medium ligand density fibres, if longer contact time is required to polish larger volumes. In the current study, low product quality fractions were observed in high ligand density fraction HP7 (16 VP: IVP). Using qPCR it was not possible to detect VP in fractions LP3 and MP4. This suggests that either poor recovery was achieved for the DNA isolation step during sample preparation or that sample Ad5 DNA concentration was below the range of the standard curve. Whilst all VP to IVP ratios reported here are within an acceptable range (Kramberger et al., 2015), the ability to separate multiple distinct populations in a single run has not been shown before in a membrane ligand density screen (Nestola et al., 2014c, Fernandes et al., 2013).

5.6 Conclusion

The combined attributes of varying Q amine ligand density as well as a shallow bed height allowed for high resolution separations of Ad5 at least partially in line with previous findings (Fogle et al., 2012, Podgornik et al., 2013). We show that Q amine functionalised nanofibre materials allow very high recoveries (>90%) of infectious Ad5, that to our knowledge has not been published. Critical to Ad5 recovery is adsorption time, which when reduced from 24 min to 8 min improved from approximately 50% to >90%. Taken together, these data demonstrate enhanced purification capabilities of nanofibre membranes due to functionalisation with Q amine ligands of various densities. The significant recoveries of infective Ad5 particles achieved using Q amine modified membranes exceed those of alternative functionalised chromatographic matrices. The present study indicates that Q amine nanofibre membranes have the potential to be developed as a leading industrial ion exchange matrix.
Chapter 6  General Discussion

6.1 Discussion Summary

The results described here demonstrate novel nanofiber membrane chromatography matrices that can be customised with chemical moieties to produce a purification platform that allows high recovery of infective Ad5 particles. The inherent open pore structure of nanofiber membranes allows convective mass transfer of the mobile phase and therefore uninhibited access of biomolecules to the adsorbent surface. This feature, together with the shallow bed height of nanofibre membranes allows for reproducible and refined separations of Ad5 product and impurities. Nanofibres also perform separations of packed, mature (infective) and immature (non-infective) as well as empty Ad5 capsid subtypes.

6.1.1 Development of an Upstream Process Platform to Ensure Consistent, High Yield Production of Infective Adenovirus 5 Particles

Mammalian cell production of viral vectors at an industrial scale is an aspect of upstream processing that can be complex, specific to the cell types used and dependent on virus infection and propagation pathways that are poorly understood (Petiot et al., 2015). In the present study, the growth dynamics and metabolic activity of HEK293 cells that are used in the present study for propagation of Ad5 were investigated to determine optimal conditions for production of infective Ad5.

The propagation of Ad5 in P2, P5 and P10 HEK293 cells correlated with the growth rate of those cells, i.e. the greatest yield of infective Ad5 was produced by P10 cells that presented with the highest rate of proliferation. The mitochondrial activity of P5 HEK293 cells that was higher than that of P2 and P10 cells did not correlate with the growth rates of those cells. These data indicated that HEK293 cells at an intermediate passage (P10) may be the most suitable for Ad5 propagation, but P5 may also produce infective Ad5 at levels that are comparable to P10. The association between metabolic activity and health in HEK293 cells is not well-investigated. Therefore, further investigations are needed to draw clear conclusions about
differences between mitochondrial activities of various cell passages. Further investigations are also necessary to dissect the influence of cell health and activity on the yields and infectivity of Ad5 that are propagated. Experiments to determine the effects of cell culture age on Ad5 propagation can involve:

a. Screening HEK293 cells at a wide range of passages (P1 – P30) for propagation of Ad5 to examine the influence of culture age on Ad5 yield and infectivity.

b. Measuring the metabolic activity, proliferation and viability of HEK293 cells at the same passages used for Ad5 propagation to determine the manner that cell health correlates with the ability of the cells to produce virus.

c. Apply a number of different metrics for measuring cell health including:
   i. Live cell staining using EnduRen™ Live Cell Substrate (Promega).
   ii. Nuclear staining of live cells using Hoechst (ThermoFisher) to examine changes in nuclear morphology.
   iii. Cell apoptosis using CellEvent™ Caspase-3/7 Green Detection Reagent or annexin V staining kits (ThermoFisher).
   iv. Cell metabolic activity using alamarBlue, a resazurin-based solution that is reduced by living cells.
   v. 5-bromo-2’-deoxyuridine (BrdU) staining (ThermoFisher) to investigate cell proliferation. Cycling cells are labelled BrdU, a synthetic analogue of thymidine that incorporates into newly synthesized genomic DNA during the S-phase of mitosis.

6.1.2 Optimisation of Downstream Processes for Purification of Adenovirus 5 Particles

Downstream purification processes are usually optimised to design workflows that are complementary to the chromatography materials in use. The results described in this chapter demonstrated purification of Ad5 particles from a crude feed using a stepwise elution method. Tandem mass spectrometry (MS/MS) demonstrated the presence of mature Ad5 capsids in fractions purified from a 50 mL crude Ad5 feed.
Evaluations of the effects of process variables that may induce stress on Ad5 during separation procedures showed that high salt concentrations in elution buffers and high flow rates did not affect Ad5 recovery and infective yield. Lowering the feed volume (20 mL) of Ad5 lead to improvements in resolution of eluted virus peaks and increases in infective Ad5 yields.

The use of MS/MS has shown promise as a tool to characterise an increasing portfolio of therapeutic viral vectors. A clear understanding of these complex labile macromolecules is required to ensure sufficient process characterisation for their efficient purification (Chandran et al., 2017). Johnson et al. (2018) compared the protein composition of two lentivirus vectors produced by transient transfection and a stable producer cell system. A smaller number of envelope protein species were observed on the stably produced vectors. The novel use of MS/MS to identify mature (infective) and immature (non-infective) Ad5 showed that cleaved protein VIII, that is a marker of Ad5 separation, was present in purified fractions of this virus. To our knowledge, these data are the first line of evidence demonstrating the chromatographic separation of infective and non-infective Ad5 using mass spectrometry. The MS/MS analysis was variable, i.e. the results showing cleaved protein VIII were difficult to reproduce. A high throughput method, for example, Capturem™ Trypsin (Takara Bio, Saint-Germain-en-Laye, France) that uses a membrane immobilised trypsin for protein to peptide cleavage at room temperature in a spin column format, can reduce incubation times from hours to minutes. This method may allow more efficient extraction of Ad5 protein from gels for MS/MS analysis.

Tandem mass spectrometry is limited as it cannot indicate the presence of complete capsids, i.e. proteins derived from complete capsids or free virus protein are indistinguishable. Transmission electron microscopy is commonly used to characterise complete capsid recovery (Earley et al., 2017). The identification of Ad5 capsids using TEM may be complemented by characterisation and quantification of virus particles using the NanoSight (Malvern, Salisbury, UK) that characterises
nanoparticles from 10nm - 2000nm in solution, and that can be operated at a higher throughput than TEM with less sample preparation. NanoSight analysis showed that particles of 100 nm were lost during the chromatography step from Ad5 feeds. The potential of NanoSight platform to identify and quantify empty capsids warrants further optimisation. The NanoSight may also be used to identify sheared capsids that would be non-infective and would not show under qPCR analysis as the genome core would be exposed to DNAse digestion. Work in the field of nanoparticle-tracking analysis to improve virion selectivity is ongoing (Heider and Metzner, 2014).

An alternative method for quantifying product quality is to determine whether or not the UV spectra of Ad5 capsids matches that of a standardised Ad5 protein and DNA absorbance ratio. The assay was first described by Maizel et al. (1968) and is widely used to characterise Ad5 produced under GMP conditions. The theoretical composition of Ad5 is 87% protein and 13% DNA, producing a 260/280 ratio of 1.2-1.50 for good quality material. However, as the assay requires concentrations of approximately $10^{12}$ virus particles per mL, it is not likely to be high throughput and routinely used.

The use of MS/MS together with a number of different orthogonal analytical assays for characterise and quantify Ad5 may also be applied to investigate other gene delivery vectors including AAV and lentivirus. Increasing interest in viral vectors from the biotechnology industry could allow development of greater diversity in the use of viruses for clinical applications. Further experiments to generate purified Ad5 material for MALDI-TOF:

a. Optimise an ultracentrifugation purification step to remove host cell proteins in Ad5 elution fractions for MALDI-TOF.

b. Develop and optimise a high-throughput protein preparation step for protein digestion and extraction, so that larger sample sizes can be prepared for MALDI-TOF analysis.
c. Explore technology transfer of nanofiber anion exchange capture for the purification of enveloped viral vectors that exhibit significant losses to infective titre during processing.

6.1.3 Investigation of Quaternary Amine Functionalised Nanofiber Membranes for Purification of Infective Adenovirus 5 at High Yields

Modification of the nanofibre membrane surface with quaternary (Q) amine ligands of various densities (low, medium and high), significantly improved their purification performance. High recoveries of up to 90% infective (packed, mature) Ad5 were achieved from low, medium and high Q amine ligand density membranes. Furthermore, Q amine modified nanofibres do not lose binding capacity after multiple, consecutive chromatography runs. Increasing the density of Q amine ligands on nanofibre membranes enhanced the resolution of eluted Ad5 and low ligand density membranes allowed efficient recovery of infective Ad5 after long (24 min) adsorption periods.

The ligand species diethylaminoethyl (DEAE) is a common anionic material. Anionic ligands, e.g. DEAE, that are modified with branched ligands in a gel layer present properties that include exclusion of viral vectors into the gel layer (Nestola et al., 2014b, Vicente et al., 2011). Heldt et al. (2009) explored the effects of high versus low density peptide ligands augmented with an ethylene oxide spacer arm for capture of Porcine Porvovirus (PPV). At low ligand densities of 0.008 mmol/g a reduction in non-specific binding of competing proteins (bovine serum albumin: BSA) was reported. At high ligand densities of 0.1 mmol/g, a bound virus would shield further binding of more virus to unused affinity ligands promoting non-specific binding of BSA. Porcine Porvovirus, like Ad5 capsids, are comprised of a ‘rough’ surface of protein ‘spikes’ and ‘canyons’. A low ligand density adsorbent conformation containing affinity ligands attached to 35Å spacer arms was suggested to be optimal for selectivity. This study highlights the potential for production of finely tuned platform ligand conformations. The development of a high throughput
A comprehensive study of the effects of mechanical and pH stress on Ad5 was conducted by Perez-Berna et al. (2012). Mature Ad5 capsids were shown to be more prone to capsid damage as a result of mechanical stress and acidification. In the present study, Ad5 was resilient to salt and shear stress and the effects of extended periods bound to medium and high Q amine ligand nanofiber adsorbents had a negative impact on Ad5 recovery. Process design optimised for recovery of Ad5 may require a two-step process, first removing process impurities using a high Q amine ligand density exchanger and then a polishing step to collect a purified fraction of Ad5 that exhibits a low VP/IVP infective ratio. As process scale increases, loading volumes increase, requiring a scale up in column size to process the larger feed volume. Future optimisation should focus on minimising on column contact time between adsorbent and vector. Viral vector process intensification using simulated moving bed chromatography has been explored previously for a range of vectors with recoveries shown in Table 7.

<table>
<thead>
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<th>Virus</th>
<th>Recovery (%)</th>
<th>Purification Step</th>
<th>References</th>
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<td>Influenza</td>
<td>69</td>
<td>Capto Core 700</td>
<td>(Blom et al., 2014)</td>
</tr>
<tr>
<td>Canine Adenovirus</td>
<td>86</td>
<td>Steric-Exclusion Chromatography</td>
<td>(Fernandes et al., 2013)</td>
</tr>
<tr>
<td>Ad5</td>
<td>86</td>
<td>Size Exclusion Chromatography</td>
<td>(Nestola et al., 2014b)</td>
</tr>
<tr>
<td>Bacteriophage</td>
<td>90</td>
<td>Expanded-bed Adsorption</td>
<td>(Lee et al., 2012)</td>
</tr>
<tr>
<td>Ad5</td>
<td>65</td>
<td>Aqueous Two-Phase Separation</td>
<td>(Peixoto et al., 2006)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>90</td>
<td>Size Exclusion Chromatography</td>
<td>(Negrete et al., 2007)</td>
</tr>
<tr>
<td>AAV</td>
<td>97</td>
<td>Capto Core 700</td>
<td>(Guo et al., 2012)</td>
</tr>
<tr>
<td>VLP rotavirus</td>
<td>85</td>
<td>Expanded-bed Adsorption</td>
<td>(Benavides et al., 2006)</td>
</tr>
<tr>
<td>VLP B19</td>
<td>92</td>
<td>Aqueous Two-Phase Separation</td>
<td>(Luechau et al., 2011)</td>
</tr>
</tbody>
</table>

Hardick et al. (2015) have explored simulated moving bed (SMB) chromatography using ion exchange nanofibres showing high operation flow rates of 2,400 cm/h
could improve productivity 15-fold. As shown here previously, extended loading periods has a detrimental impact of infective recovery of Ad5. As processes scale larger columns will be implemented. In order to reach capacity of larger columns increased loading periods, and therefore increased binding durations, will be required. A nanofiber SMB phase operation could rapidly cycle a series of smaller columns. By loading the first column to capacity and then redirecting feed load onto a second column, the first column could have bound Ad5 rapidly eluted. This would minimise binding duration at higher feed volumes that would be required with a single larger column. As such SMB could potentially allow high infective recoveries whilst processing large volumes of feed at scale.

Future experiments to build further optimisation of Ad5 subtype separations using nanofiber adsorbents:

a. Develop high-throughput method for Ad5 elution fraction sample preparation and administration for cell based β-Galactosidase assay to increase sample analytical capacity using an automated liquid handling platform, e.g. Tecan (Tecan Group Ltd, Männedorf, Switzerland).

   i. Develop processes for Ad5 capsids that contain a green fluorescent protein insert for flow cytometry counting.

b. Develop a two-step purification protocol using two anion ligand densities and determine product related impurity clearance capability whilst maintaining the infectious titre of Ad5.

   i. Decrease chromatography run times by loading Ad5 in a high salt buffer to reduce adsorption duration further and prevent host cell proteins (HCP) and other product related impurities from binding to the nanofiber adsorbent

c. Assess anion exchange nanofiber dynamic capacity for Ad5 prepared with ultrafiltration/diafiltration steps.
i. Load Ad5 in a high salt buffer to prevent binding of HCP and other product related impurities to the nanofiber adsorbent and thereby increase dynamic binding capacity.

ii. Determine whether or not Q amine ligand density affects dynamic binding capacity of Ad5.

iii. Compare Q amine functionalised nanofibre membranes to commercially available adsorbents.

d. Scale up purification of Ad5 using larger nanofiber columns.

i. Explore implementation of continuous process optimised for short adsorption periods to maintain an infective Ad5 titre at higher loading volumes.

e. Begin to develop processes using a therapeutic Ad5 vector.

f. Explore prototype cation exchange nanofiber membranes for purification of Ad5 and compare their performance to the anion exchange membranes used and developed in the current study.

6.2 Conclusion

Nanofiber membranes are a promising platform for product related impurity separations of Ad5 vectors, which arguably represent one of the most heterogenous virus vector species. Efforts to increase clearance of product related impurities will likely increase as understanding of vectors grows. This is driven by larger investments in a growing gene therapy product range as well as a need to further optimise processes to ensure better product quality for a growing patient stream. This platform of purification and identification of high quality infective vectors can be transferred to other equally complex viral vectors in both analytical and scaled up industrial processes.


QUINLAN, M. 1993. Expression of antisense E1A in 293 cells results in altered cell morphologies and cessation of proliferation. Oncogene, 8, 257-265.


8.1 Introduction

Viral vectors and vaccines have become a major focus in R&D pipelines across not only the major pharmaceutical pipelines but also form a significant proportion of new patents filed by academic institutions leading to a rise in vaccine based small and medium-sized enterprises (SME). Gene therapy company valuation has seen a dramatic increase in recent years as of Jan 4th 2018 Voyager therapeutics market capitalisation (cap): $484.82 million; current share price: $17.99, Nighstar Therapeutics market cap: $405.63 million; current share price: $14.43, Tocagen market cap: $247.22 million; current share price: $12.48, Kadmon Holdings market cap: $290.98 million; current share price: $3.70 (https://investingnews.com/daily/life-science-investing/genetics-investing/4-gene-therapy-stocks-to-watch/). Adenovirus serotype (Ad5) is a promising viral vaccine vector; with several Ad5 vectored therapies at various stages in Phase trials (see Chapter 1). The Ad5 virion therapy has been developed to express human immunodeficiency virus (HIV) antigens Env, Gag and Pol, the study has shown significant reductions in per-exposure acquisition risks when challenged with simian/human immunodeficiency virus (SHIV) (Barouch et al., 2013). Alongside this, Ad5 vector therapies currently in phase trials include treatments for Ebola, Glioma, Malaria, Non-muscle invasive bladder cancer and Hepatitis-C (HEP-C) (https://clinicaltrials.gov/ct2/results?term=viral+vector&Search=Search).

Whilst there have been significant advances in the upstream technology to culture high titres of viral capsids, downstream processing has progressed more slowly. The separation of large biotherapeutics with ‘retro-fitted’ chromatographic materials such as monoliths and resins, have resulted in low recoveries and infective yields. This has meant that a significant number of viral vector processes still incorporate a caesium chloride ultracentrifugation downstream processing step, a process that is inefficient and scales poorly. Current analysis shows that viral vector manufacturing capacity must increase by 1-2 orders of magnitude in order to support the proposed
commercial supply requirements for many promising therapy candidates (van der Loo and Wright, 2016). Nanofibre technology offers a novel material architecture that is advantageous for the separation of large biotherapeutics (Hardick et al., 2013).

8.2 The Regulation of Leachables and Extractables

The promise of the rapid processing of viral vector material using nanofibres must also be backed up with scrupulous testing to ensure that the technology is market ready in order to capitalise on the current interest and industrial need to purify large volumes of viral vectors.

Single use technology has seen a rapid growth in interest and uptake in biopharmaceutical manufacturing, particularly in drug development startups where flexibility is a key priority, especially at the early stages (Shukla and Gottschalk, 2013). However amongst the primary concerns with disposable platforms is presence of leachables and extractables, and as such the Food and Drug Administration (FDA) and International Council for Harmonisation (ICH) expect the selection of qualified and safe materials in order to manage and minimise risk (Gao and Allison, 2016). The evaluation of leachables and extractables has become a major aspect of quality by design.

A disposable chromatography column may introduce process-related contaminants into the final product in the form of leachables from the contact materials of the column (Gao and Allison, 2016). Extractables are defined as components which are extracted from materials directly in contact with the product when exposed to solvents under the controlled and aggressive conditions of time and temperature. Extractables are normally determined under worst case conditions whilst leachables are compounds which can migrate into drug product under normal operating conditions (Bestwick and Colton, 2009).
A detailed risk assessment must be conducted to demonstrate that risks are controlled to an acceptable level. The risk assessment takes into account a number of factors such as the extractables and leachables profile, process operating conditions, the function and application of the single use contact material and finally the proximity of the process step to the final drug product (Gao and Allison, 2016). Leachables and extractables from upstream platforms may be removed to acceptable levels in the downstream processing step. The identification of extractables and leachables from chromatography resins, membranes and monoliths and the column components is a critical consideration of column quantification (Langer, 2014). Tests conducted on the resins include analysis of the chromatographic matrices, the functional groups, porosity of the material under the various conditions required in the process (Rathore et al., 2003). The material of the nanofiber column material should also be evaluated (such as tubing and casing) assessing how the materials behave under process operating conditions, including the effect of multiple uses and sanitization steps on the extractables profile (Gao and Allison, 2016).

8.3 Viral Vector Regulation

The manufacture of new drugs including complex biotherapeutics such as viral vectors need to comply with cGMP as required under section 501 (a)(2)(B) of the Federal Food, Drug and Cosmetic Act (FD&C Act) and as described in 21 Code of Federal Regulations (CFR) parts 210 and 211. Extensive characterisation and quality control (QC) testing is required for human clinical trials (van der Loo and Wright, 2016). This requires large investments to ensure each batch of investigational product meets pre-determined specifications regarding purity, potency, identity and safety to establish a fully compliant GMP manufacturing process. As such many SME’s developing vector products develop non-validated assays for product characterisation at the early stages and then satisfy regulation bodies by validating those assays and tighten the specifications during clinical development (van der Loo and Wright, 2016).
The FDA created ‘CGMP for Phase 1 Investigational Drugs’ to differentiate Phase 1 drugs and drugs that are in late Phase clinical trials or are already being manufactured commercially (Food and Administration, 2012). In this document it is outlined that validation of manufacturing processes that are characterised by the repeat commercial batch production are not suitable for the production and manufacture of most Phase 1 clinical trial drugs. Quality control testing for safety of a viral vector must be validating from the beginning of the clinical development process. Safety assessments include product contaminants and product related impurities (Wright, 2008). The level of quality control at various stages of the development process is shown in Figure 40. During clinical development, manufacturing process improvements and experience garnered over multiple batch productions allow for progressive tightening of specifications (van der Loo and Wright, 2016). The identification of critical quality attributes of Ad5 vectors are key to characterising and quantifying batch quality. An adenovirus viral vector therapy batch would be characterised by viral genome titre, infectious titre, product identity (presence of Ad5 capsid and genome) and therapeutic gene identity (Clément and Grieger, 2016). To commercialise the process these optimised assays must be made GMP compliant before being submitted to test for specificity, reproducibility, linearity, robustness and sensitivity. An independent quality assurance body evaluates manufacturing and QC processes for compliance with regulations and guidelines (Clément and Grieger, 2016). One of the challenges of characterisation and QC analysis of viral vectors is that they exhibit a high degree of complexity (van der Loo and Wright, 2016). Viral vectors also all exhibit unique biophysical characteristics, unique to the species and serotype, as such a unique analytical method must be developed and validated for each viral vector delivered gene therapy product and each serotype (van der Loo and Wright, 2016). Most gene therapy products exist at an early stage in development with very little advanced stage experience available by which to build best practices (Sharon and Kamen, 2018).
Figure 40. Progression of GMP compliance as a product develops from the pre-clinical stage working to authorisation for an Investigational New Drug (IND) application to Biological License Application (BLA) and Commercial Manufacturing (van der Loo and Wright, 2016)

Viral vector QC testing can be divided into those that are similar to existing analysis developed and validated for recombinant protein or vaccine products and those that are specific to vectors (van der Loo and Wright, 2016). Analysis for host cell protein and DNA can be transferred from recombinant processes, whilst new analytical methods and QC analysis must be developed to characterise and quantify the unique feature of product related impurities (Wright and Zelenaiia, 2011). Quantifying product quality loses of virions over the chromatography step is challenging and requires multiple assays each of which if used in isolation is insufficient for thorough characterisation. The lack of a standardised method for full Ad5 characterisation means that it is difficult to conduct comparative studies of recoveries of different Ad5 therapeutic vectors over different processes with regard to viral genome titre, infectious titre, product identity (presence of Ad5 capsid and genome) and therapeutic gene identity (Wright, 2008). Potency assays are designed to quantify functional activity of the product and its ability to deliver and express the
vector transgene (van der Loo and Wright, 2016). Potency is critical in demonstrating lot-to-lot consistency and stability of the vector during storage and residual DNA impurities packed within the vector (Dolgin, 2012). Viral vector-encapsidated nucleic acid impurities cannot be removed by Benzonase and may include DNA fragments from plasmids and from producer cells (Wright, 2014). This has proven challenging to address as the mechanism of viral packaging of host cell DNA is only beginning to be understood, with improved approaches to vector production plasmid design to mitigate risk, as well as purification that is able to exploit differences in physico-chemical properties between therapeutic vectors and vector related impurities (Sharon and Kamen, 2018). There is increasing interest in reducing the footprint of large complex analytical units and simplifying sample preparation to facilitate rapid characterisation of viral vectors, as shown with the Vironova’s MiniTEM transmission electron microscopy system (Turkki et al., 2017). Many of these methods are not yet validated to the standards required for licensed products, and further innovations in analytical method development is required (van der Loo and Wright, 2016).

European guidelines state that products must be fully GMP compliant regardless of the stage of clinical trial (Ghani, 2014). This can create a challenging environment for US academic manufactures of Phase 1 drugs, when they are audited by international pharmaceutical companies (van der Loo and Wright, 2016). Validated manufacturing systems for the manufacture, material and document control are too costly for small academic ventures, requiring venture capital to ensure compliance with FDA and European guidelines. As the field matures pharmaceutical and biotech industry are asking full compliance of their academic partners in order to minimise the risk as the product is put forward for Biological License Application as part of the commercialisation process (van der Loo and Wright, 2016).

Taking a broader view, gene editing is a hotbed of regulatory issues that rightly extends to ethical concerns. There are still gaps in understanding which are halting the commercialisation of these therapies. A primary example is the gene delivery
viral vector Adeno-associated virus (AAV), this has been shown to transduce multiple different cell types delivering sustained gene expression (Nathwani et al., 2014). In theory a nuclease could be delivered by the AAV vector that can transduce target and non-target cells, which could potentially create ongoing genotoxic DNA damage in target and non-target cell types (Kohn et al., 2016). Whilst the potential therapeutic significance of viral vectors warrants the diligence and effort to develop platforms in line with GMP standards, labile viral vectors exhibit a complexity that makes full characterisation for the purpose of quality control (QC) challenging.

8.4 Conclusion

Nanofibre technology is a promising next generation chromatography material for the purification of viral vectors. Puridify has been able to collaborate with SME’s developing these investigatory drugs at an early stage. Developed here in this thesis is a suite of analytics incorporating novel use of MALDI-ToF to identify a mature capsid protein marker for infectious Ad5. Whilst it is unlikely that this quality method could be incorporated into characterising batches in a production setting it will help understand and characterise process effects and purification capability of scalable processes.