Title: **Ultra scale-down approaches to study the centrifugal harvest for viral vaccine production.**

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Short running title: **Centrifugation ultra scale-down for viral vaccines**

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Abstract

Large scale continuous cell-line cultures promise greater reproducibility and efficacy for the production of influenza vaccines, and adenovirus for gene therapy. This paper seeks to use an existing validated ultra scale-down tool, which is designed to mimic the commercial scale process environment using only millilitres of material, to provide some initial insight into the performance of the harvest step for these processes. The performance of industrial scale centrifugation and subsequent downstream process units is significantly affected by shear. The properties of these cells, in particular their shear sensitivity, may be changed considerably by production of a viral product, but literature on this is limited to date. In addition, the scale-down tool used here has not previously been applied to the clarification of virus production processes. The results indicate that virus infected cells do not actually show any increase in sensitivity to shear, and may indeed become less shear sensitive, in a similar manner to that previously observed in old or dead cell cultures. Clarification may be most significantly dependent on the virus release mechanism, with the budding influenza virus producing a much greater decrease in clarification than the lytic, non-enveloped adenovirus. A good match was also demonstrated to the industrial scale performance in terms of clarification, protein release and impurity profile.

Key words: Scale-down, centrifugation, viral vaccine, shear, gene-therapy

Introduction

Viral products have been successfully used as both prophylactic and therapeutic vaccines, yet have unique processing challenges depending on the choice of enveloped or non-enveloped virus utilised. This paper uses ultra scale-down studies to explore the harvest by continuous centrifugation of a non-enveloped adenovirus and an enveloped influenza virus, both of which are increasingly important viral products. Adenovirus is one of the leading candidates currently being studied for use as a vector in both prophylactic vaccine design and in gene therapy (Silva et al. (2010), Waehler et al. (2007), Zhang and Godbey (2006)), with particular potential in oncology, and therapeutic vaccines (Kotterman et al., 2015). 526 reported trials are currently ongoing (Edelstein, 2016) and licenced products include Gendicine in China for head and neck cancer (Kotterman et al. (2015), Kasala et al. (2016)). Meanwhile, in addition to seasonal influenza, the continuing potential for pandemic influenza also remains a high profile, public health priority (Kamps et al. (2006),Osterhaus et al. (2011)). The most successful and widely used strategy for combating influenza has been prevention via vaccination (Hedlund et al. (2010), Salomon and Webster (2009), Muennig and Khan (2001)). Scale up of production is set to pose a major challenge in the future for both these products. Adenovirus production is shifting from traditional adherent culture to larger scale bioreactor based systems, potentially introducing complexities, in particular the challenges associated with factors such as shear (Pettitt et al., 2016), which is of particular significance in downstream processing, and needs to be understood. For influenza vaccine production too, there is an urgent need for more efficient and cost-effective processes than the current egg-based system with its numerous shortcomings (Feng et al., 2011), to meet the requirement for billions of doses in stock piles, and rapid response to emergence of new pandemic strains, alongside the large annual seasonal influenza vaccine production (Chua and Chen (2010) ,Osterhaus et al. (2011)). The demand is such that there are several continuous cell-line based candidates and processes in development, supported by both commercial and public funded ventures (Genzel and Reichl, 2009). These continuous cell-line candidates and processes must be well characterised.

Boychyn et al. (2004) have developed an ultra scale-down technique which accounts for the impact of shear in large scale continuous centrifugation on the harvest from mammalian cell based bioreactor cultures. Ultra scale-down uses millilitres of material to imitate the effects of industrial processes, with the aim of facilitating rapid scale-up of new products (Wolff and Reichl (2011),Titchener-Hooker et al. (2008)), troubleshooting and broadening product and process understanding; all with lower cost and reduced biosafety risks through minimised volume requirements (Rathore and Sofer, 2005). The centrifuge ultra scale-down model has previously been used successfully to predict the performance of various centrifuges for mammalian cell cultures (Hutchinson et al. (2006), Zaman et al. (2009)), protein precipitates (Boychyn et al., 2004) and flocculants (Berrill et al., 2008), including comparison to large scale of clarification (Boychyn et al., 2004), solids remaining, fragment antibody recovery and process impurity concentrations (Lau et al., 2013), and impact on subsequent filtration performance (Zaman et al., 2009). No literature has been published, however, on the use of the ultra scale-down mimic in predicting the performance on virus infected cell harvests. Nor are the authors aware of any literature on the effects of shear on virus infected cells. This paper therefore aims to address this and gain insights into the performance of these important processes using this well characterised and already successfully demonstrated laboratory mimic.

Theoretical Considerations

The cell and the virus life cycle

In this paper, two contrasting viruses are considered. Adenovirus is a non-enveloped, icosahedral virus with a double strand of DNA at its core, of approximately 70-100nm in size (Waehler et al., 2007), with 52 identified serotypes (Harrison, 2010), of which we concentrate here on serotype 5, and made up of approximately 13 structural proteins (Russell, 2009). Influenza, on the other hand, is a pleomorphic enveloped virus, varying in size between 80 and 120nm (The Rapid Reference to Influenza Resource Center Team. Adapted from Wilschut, 2006), with segmented negative-sense,

single RNA strands (Chang et al., 2015), and 9 viral proteins (Shaw et al., 2008), of which HA and NA are deemed to be the main antigens in the viral vaccine (Osterhaus et al., 2011).

There are a number of mechanisms by which viruses may initiate release of mature virions from the host cell, which will vary chiefly as a function of the virus type, with non-enveloped icosahedral viruses being released by a different mechanism than enveloped viruses, which only becomes infective once it acquires its lipid envelope from the cell membrane upon egress from the cell (Garoff et al., 1998). It may be anticipated that the mechanism by which the virus initiates release from the host cell will have significant implications for the subsequent clarification. Whilst influenza buds from the plasma cell membrane (Roberts et al., 2015), adenovirus accelerates the death and lysis of the cell and ultimately the disruption of the cell membrane (Murali et al., 2014). The membrane penetration of non-enveloped viruses is also distinctly different from that of enveloped viruses (Wiethoff et al., 2005). There are a large number of permeations with respect to the modifications which viruses may induce in cell structure and physiology, both during propagation and at virus release. The following concentrates therefore on the broad release mechanism for these two virus types, as it is currently understood, and its impact on process clarification.

Influenza viruses modify the plasma cell membrane of infected cells by insertion of the membrane proteins and destruction of the receptors for haemagglutinin prior to budding (Skehel and Wiley, 1995). The capsid and core are assembled at the membrane at the point of budding, at lipid rafts where cholesterol/lipid compositions differ from that of the standard cell membrane and viral membrane proteins are concentrated, and which may remain dispersed over the cell surface or coalesce to form a large lipid raft zone from which multiple viruses bud. Other viruses which bud in this fashion include HIV-1 and Ebola virus. In other enveloped viruses the assembly of the core occurs in the cell cytoplasm before transport to the membrane. To form an envelope around the virus the membrane must deform, and eventually separate from the host cell membrane, to achieve which the local chemical and physical membrane properties must be modified (Perlmutter and Hagan (2015), Rossman and Lamb (2011)).

Adenoviruses by contrast are transcribed, replicated and assembled in the infected cell nucleus and released by cell lysis (McLean et al., 2008). According to Hulo et al. (2011) cell membrane disruption is achieved via addition of virus encoded viroporins to the cell membrane. According to Gros and Guedan (2010) adenovirus release is generally inefficient with large amounts of the virus retained inside the cell. It is for this reason that a cell lysis step is not uncommon with adenovirus processing. Properties of viruses themselves may vary depending on the cell type which produces them, particularly enveloped viruses (Shaw et al. (2008), Schaap et al. (2012), Shigematsu et al. (2014)), but the quality of the virus itself is not considered in this study.

Many viruses display a degree of tropism, meaning that they propagate better in some cells than others. Although numerous continuous cell lines are available for the propagation of both influenza and adenovirus the work presented here uses EB66 (Valneva), a relatively new duck cell line, and HEK, a well-established human cell line.

Although it is not widely discussed it has been stated in the literature that infected cells are more 'fragile' and thus more susceptible to damage by shear stress (Silva et al. (2010)). This seems intuitively a logical assumption, and this paper therefore seeks to test this as a starting hypothesis. It should be noted that virus behaviour and cell destruction mechanisms may vary not just between viruses, but between the cell types they infect (Lynch et al. (2011), Murali et al. (2014)). Hence the interest in examining side-by-side two such different cells and viruses. Equally, however, it would not be wise to draw too general a set of conclusions from the outcomes of this work alone.

Ultra Scale-Down of continuous centrifugation

This system has been extensively used and validated over the last 15 years, and is discussed extensively in the literature. In summary scaling of solids settling between large scale and small scale laboratory centrifuges is discussed by Maybury et al. (2000) who follows on from work by Mannweiler and Hoare (1992). Maybury et al. (2000) also demonstrated the importance of shear for clarification at large scale. CFD has been used by Boychyn et al. (2001) in discussion of the difference between high and low shear centrifuges, to locate the shear in the feedzone of the

centrifuge and to demonstrate comparability in shear levels between this feedzone shear and that at the disc tip in the rotating shear device. The authors went on to use a combination of pre-shearing in the rotating shear device and laboratory scale centrifugation to replicate the clarification of protein precipitates in industrial and pilot scale continuous centrifuges of various designs.

Materials and Methods

The overall experimental flow scheme is summarised in Figure 1.

Cell culture

HEK293 cells (85120602-1VL SIGMA293 Cell Line, European Cell Bank) were grown as adherent cell cultures in T-175 flasks (660160, Greiner Bio-one, UK), using defined DMEM media (21969-035, Gibco, Life technologies, UK) with 10%FBS (10270-106, Gibco, Life technologies, UK) and 5.5ml L-glutamine (25030-024, Gibco, Life technologies, UK), and in a Sanyo incubator at 37°C and 5%CO_2. Cell culture and virus replication conditions are summarised in Table 1. For non-virus infected runs the cell culture conditions were similar in range to those seen in virus infected runs at the time of infection. In order to remove the samples from the T-flasks and prepare them for shearing the following steps were undertaken: (i) free liquid was removed from the T-flask, (ii) any remaining attached cells were washed with PBS, then trypsin added for a maximum of 3 minutes at 37°C, the trypsin neutralised by addition of fresh media and the cells spun down, and the pellet re-suspended in the free liquid removed in step (i).

EB66 cells (Valneva, France) were grown and infected by GSK in a 50-litre single use bioreactor. The cells were grown to a high cell density before dilution in fresh media and other constituents for viral propagation, and infected. Sample volumes were taken at 4 and 5 days after infection, and subjected to the shear conditions of interest (within 2 hours), and then clarified, and OD measured (within the same day). For the non-virus infected culture a small volume was taken from the main bioreactor and diluted in the same material as for infection, except without the addition of the virus seed. As with the virus infected samples the non-virus infected sample volume was subjected to a

range of shear conditions within 2 hours of being extracted from the bioreactor / diluted, and then clarified and OD measured within the same day.

Virus seed

The Adenovirus kit used was supplied by Clinical BioManufacturing Facility (Oxford, UK). It was an ad5 control stock (D419, p53).

The influenza virus runs used three separate influenza strains: A/Shanghai/2/2013 (H7N9), A/Gyrfalcon/Washington (H5N8) from the CDC and A/Duck/Bangladesh/19097/2013(H5N1) from St Jude Children's Research Hospital.

Rotating Disc Device

The rotating disc shear device consists of an aluminium alloy disc of 0.1 cm thickness and 4 cm diameter, contained within a chamber of 1 cm height and 5 cm diameter. This rotating disc, which is detailed elsewhere (Boychyn et al., 2001, Boychyn et al., 2004, Hutchinson et al., 2006, Levy et al., 1999, Zaman et al., 2009, Boychyn et al., 2000), was designed and constructed at UCL.

The disc was rotated for a fixed period at a fixed speed via a shaft and motor. To fill the rotating shear device chamber requires approximately 15-20ml of sample, such that all air bubbles are cleared from the chamber. Samples were subjected in quadruplicate to the following six conditions: sheared at one of the following speeds, 0, 6, 8, 10 and 12krpm, for 20 seconds, or at a speed of 18krpm for 1 minute, in that order. These speeds are equivalent to a maximum energy dissipation rate of 0, 27, 90, 210 and 420kW/kg respectively. It is anticipated that a low shear continuous centrifuge may be well represented by a speed of 6krpm and the feed zone shear of a high shear continuous centrifuge by a rotating shear device speed of 12krpm.

Centrifugation

The influenza process harvest was conducted using a PSC-5 disc stack centrifuge (GEA, Milton Keynes, UK), at a speed of 8.5krpm and a flowrate of 200l/h.

Clarification

For uninfected HEK cells, uninfected EB66 cells, adenovirus infected HEK cells and influenza infected EB66 cells the cells were first subjected to shear then clarified in an Eppendorf centrifuge using the following conditions: 1ml, 1.5ml and 2ml spun down for 5 minutes at 3000rpm and the same volumes spun down for 5 minutes at 6000rpm. For well spun supernatant 2ml was spun down for 30 minutes at 6000rpm. For HEK cells (adenovirus infected, and uninfected) the Eppendorf 5430 centrifuge was used with rotor FA-45-30-11 (Eppendorf, UK), whilst for EB66 (influenza infected, and uninfected) cells the 5427R Eppendorf centrifuge with rotor FA-45-48-11 (Eppendorf, UK), using only the upper row. In the Eppendorf 5430 centrifuge the centrifuge conditions listed are equivalent to a $V/t\Sigma$ of 9.0, 13.2, 17.1, 2.2, 3.3 and 4.3 $x10^{-8}$m/s respectively, and in the 5427R Eppendorf centrifuge to 5.3, 7.7, 9.8, 1.3, 1.9 and 2.45 $x10^{-8}$m/s respectively.

For HEK cells (adenovirus infected, and uninfected) the absorbance (OD) of the supernatant, feed and well spun supernatant was measured at 600nm, as being close enough to the desired OD of 650nm, by transferring 200µl of the supernatant to a translucent 96 well plate (82.1581, Sarstedt, UK), which was kept agitated until measurement, and measured on an InfiniTe 200 plate reader (Tecan, UK), using a rotationally symmetric layout of replicate samples. By comparing the absorbance of the supernatant to that seen in the feed stock, and in the supernatant of a well spun sample, the clarification was derived, as outlined elsewhere by Maybury et al. (2000). Maybury et al. (2000) also describe how to match the performance at pilot and industrial scale to that at laboratory scale, based on sigma theory.

For EB66 cells (influenza infected and uninfected) sheared cells were also clarified using a larger laboratory scale centrifuge, Allegra X-15R, rotor SX4750A (Beckmann), using 14ml falcon tubes. The conditions used were as follows: 7ml and 14ml spun down for 7minutes at 3000rpm, and the same volumes spun down for 10minutes at 4000rpm, as well as 13, 10, 7, 5 and 2ml spun down for 5minutes at 4000rpm. These centrifuge conditions are equivalent to a $V/t\Sigma$ of 6.7, 1.5, 2.8, 5.7, 10.5, 7.8, 5.3, 3.7 and 1.4 $x10^{-8}$m/s respectively. The absorbance (OD) was measured at 650nm by using an UltraSpec 2000 (Pharmacia Biotech, Uppsala, Sweden). For biosafety reasons influenza infected

samples were pipetted into the cuvettes under a flow hood and stoppered with a cap. The samples were then agitated just before measurement, by inverting several times, to ensure that particle settling did not affect the accuracy of measurements.

The conditions listed above for the larger laboratory scale centrifuge are designed to match the Q/Σ conditions in the PSC-5 centrifuge (6.3×10^{-8} m/s). However, it should be noted that the g-forces will also differ between this centrifuge, the smaller laboratory centrifuge and the PSC-5. It has been shown elsewhere that for certain cell types, specifically algal, the g-forces may also be of interest with respect to the clarification and settling (Xu et al., 2015b). The g-forces for the various centrifuges are therefore summarised as follows: the small Eppendorf centrifuge has a g-force ranging from 1,600 to 14,000xg, the larger laboratory centrifuge has a g-force ranging from 5,900 to 41,000xg and the PSC-5 has a g-force ranging from 7,000 to 20,000xg, over the operating ranges used.

For EB66 (influenza infected, and uninfected) the supernatant samples used for western blots and protein assays were produced by spinning down 7ml of the sheared sample at 3000rpm for 7minutes, conditions which should roughly match the Q/Σ of the PSC-5 centrifuge at the run conditions used in this process.

Cell count

The HEK cells (adenovirus infected, and uninfected) were counted using a haemocytometer. Trypan blue was used to confirm the relative viability of HEK cells infected with adenovirus 3 and 6 days post infection for runs 7, 8, 9 and 10 as listed in Table 1.

The EB66 cells (influenza infected, and uninfected) were counted using a ViCell XR Series (Beckmann Coulter, UK). The pre-prepared cell type EB66 and EB66PI were selected, for which the parameters are a minimum diameter of 6 and 9um, a cell brightness of 82 and 85%, a cell sharpness of 85 and 100, a viable spot brightness of 66 and 70%, and a viable cell spot area of 5 and 8% respectively, and a maximum diameter of 35um. The analysis software version was 2.03.

Western Blot

The presence of adenovirus for HEK cell cultures was confirmed by western blot against a rabbit adeno coat proteins primary antibody (ab6982, Abcam, Cambridge, UK), and a goat anti-rabbit HRP secondary (ab6721, Abcam, Cambridge, UK), and also, on another blot, against a mouse hexon protein primary antibody (10R-8460, Fitzgerald, Acton, USA) and a rabbit anti-mouse HRP secondary (ab6728, Abcam, Cambridge, UK). The samples were prepared by dilution 1:1 in NP40 buffer, then dilution 1:1 of the diluted sample in STB. Equal quantities of protein were added to each well. The gel was a BOLT 4-12% bis-tris 10 well mini gel (Invitrogen, Thermo Fisher scientific, UK) using a mini gel tank (Invitrogen, Thermo Fisher scientific, UK) run at 200V, transferred to a PVDF membrane (IB24001, Invitrogen, Thermo Fisher scientific, UK) using the iBlot 2 Dry transfer (Thermo Fisher scientific, UK). Molecular weight markers were SeeBlue pre-stained protein standard (LC5625, Thermo Fisher scientific, UK). The chemiluminesence substrate used was Supersignal West Pico (34080, Thermo Fisher scientific, UK). Imaging was completed using a ChemiDoc MP (Bio-rad, UK). Image Lab version 5.2.1. (Bio-Rad, UK) was used for band position and strength identification.
For the virus infected EB66 cells the concentration of EB66 host proteins in the supernatant was quantified using a rabbit primary antibody against EB66 host cell proteins developed by GSK and a rabbit anti-sheep IgG HRP secondary (P0448, Dako). For both the western blot and SDS-PAGE equal sample volumes were added per well. The gel was a Criterion XT precast 4-12% bis-tris 18 well comb gel (bio-rad, Ca) using a bio-rad criterion cell gel tankrun at 200V, transferred to a nitrocellulose membrane (162-0145, Bio-Rad, UK) using the wet blotting method, with a criterion blotter unit (Bio-Rad, UK). Molecular weight markers were Precision Plus Protein WesternC Blotting Standards (161 0376, Bio-Rad, UK), against Precision Protein StepTactin-HRP Conjugate (161 0380, Bio-Rad, UK). A gel was run, using the same samples, and stained with Sypro Ruby protein stain (S21900, Invitrogen). Molecular weight markers for the gel were Precision Plus Protein unstained Protein Standards, step-tagged recombinant (161 0363, Bio-Rad, UK). The chemiluminesence substrate used was Supersignal West Pico (34080, Thermo Fisher scientific, UK) which was added drop-wise across the membrane surface directly before imaging. Imaging was completed using an Image Quant LAS4000

(GE). GelAnalyzer version 2010a (GelAnalyzer.com, Lazar and Lazar) was used for band position and strength identification.

Particle Size Distribution Analysis

The particle count and size distribution was measured for the supernatant following shear and clarification in a laboratory centrifuge of 2ml, at a speed of 6000rpm for 5minutes (as detailed above, under clarification). This supernatant was diluted 10x in PBS before being measured using a Nanosight tracker LM10 (Nanosight Ltd, UK), the software version 3.1 and the camera type CCD.

Virus identification

In addition to western blot (described above) TEM and β-Galactosidase Reporter Gene Staining Kit (GALS, Sigma Aldrich, Saint Louis, USA) were used to confirm identity and presence of adenovirus in HEK cultures.

Influenza virus was identified by GSK by testing against HA antibodies. Western blots were also performed against HA anti-bodies, demonstrating the presence of influenza virus.

Protein Concentration

For HEK cells (infected with adenovirus, and uninfected) the protein concentration was measured for the purposes of the Western blot. The protein concentration was measured using the modified Lowry method (reagents 500-0114, 500-0113 and 500-0115, Bio-rad, UK) in acrylic semi-micro cuvettes (67.740, Sarstedt, UK), with the OD at 750nm measured on a biomate3s spectrophotometer (thermo scientific, UK). For the EB66 cells (infected with influenza, and uninfected) the sample was diluted to three different dilutions, and chemicals added to denature and precipitate the protein. It was then spun down, and the supernatant removed, leaving the protein pellet. This procedure was followed for biosafety reasons. The protein concentration was then measured in duplicates, using a Lowry reaction, with a maximum variance accepted across the three different dilutions..

Infectivity Assay

Infective adenovirus in the supernatant was quantified using the blue forming units (BFU) assay, which involved the identification of cells which had been transfected with the LACz gene (Mittereder

et al. (1996), Cell_Biolabs_Inc (2004)), using the Beta-galactosidase staining kit (GALS, Sigma Aldrich, Saint Louis, USA), and their quantification.

A 96 well plate was coated with Poly L Lysine (PLL) (P4832, Sigma) by incubating with 30ul per well for 5 minutes, removing the PLL and leaving for a further 2 hours to dry, all at room temperature. 100ul of HEK293T cells at a concentration of 10^5cells/ml were added per well, and left overnight in an incubator.

The frozen sample was thawed on ice, and dilutions, in cell media, prepared with dilution factors of $2x10^3$, $2.5x10^4$, $5.1x10^4$, $7.2x10^4$, $2.1x10^5$, $4.3x10^5$, $8.5x10^5$ and $1.7x10^6$. The media in each well was removed and replaced with 100ul of diluted sample. Each dilution was prepared in triplicate, with fresh media added to 3 of the columns, acting as a blank. The plate with infected sample was left in the incubator for 1 hour, then the adenovirus containing media was removed and replaced with 100ul of fresh media, and the plate incubated overnight.

Wells were washed twice with 30ul of PBS, left in 30ul 0.5% gluteraldehyde fixation solution for 5minutes, washed twice again with 30ul of PBS, and left in the incubator overnight in 30ul of staining solution.

The total number of cells which stain blue was counted for each well at a magnification of 5x and, where this number was between 50 and 200, corrected for dilution and averaged, to give the BFU/100ul.

Results & Discussion

Clarification of HEK cells infected with Adenovirus serotype 5

HEK cells grown in an adherent culture were sheared either a number of days following infection, or without being infected with adenovirus, and clarification measured at lab scale. It was anticipated that the cells might show increased sensitivity to shear following infection that could lead to the generation of fine debris which might impact on clarification (Silva et al., 2010), but as can be seen in Figure 2 and Figure 3 this was not the case. No significant decrease in clarification was seen in the first 3 days following infection, and following longer infection periods the clarification improved

significantly. This is in agreement with the cell count data, which shows no difference in cell destruction relative to initial cell numbers for the first 3 days following infection, and no impact of shear on cell count following a longer period of infection, i.e. a significant decrease in shear sensitivity. Trypan blue was used to demonstrate that this change is likely to be due to cell death which occurs somewhere between the 3rd and 4th day of infection. It has been shown elsewhere (Tait et al., 2009) that older or dead cells in non-virus infected cultures may be less shear sensitive, possibly due to the flaccid and weakened cell structure of the dead cell in the shear velocity gradients.

Within the first 3 days following infection there is evidence that shear may cause release of virus from cells. Immunoblots (Figure 4) were conducted showing that the viral protein concentrations in the supernatant increase with increasing shear, as shown in Figure 5. Although the proteins selected for plotting are nominally identified, it should be noted that these identifications are designed for illustrative purposes only and may not be completely unambiguous, this has no bearing on the conclusions drawn. Overall measures of protein concentration in the supernatant show an increase with increasing shear, with each lane on the blots run at equal protein concentration. From which it appears that viruses or viral proteins are released at a greater rate than host cell proteins. This is supported by an increase of more than a log in infective units in the supernatant, based on a duplicate measurement using a blue forming units assay for quantification of virus by counting transfected cells. This has implications for the best approach to harvesting of adenovirus at large scale where, as standard, because so much of the virus is inside the cell, a detergent lysis process or homogenisation is conducted to release the adenovirus from the cell mass. Standard small scale processes, which rely on low shear pelleting followed by freeze-thaw to lyse cells and release the virus product will not accurately reflect what happens in such large scale processes, and therefore will give limited insight into the challenges and opportunities. In particular, the addition of a higher-shear disc stack centrifugation step prior to lysis will clearly have implications for the product location and yield, and potentially for the relative importance placed on the processing of the

supernatant. This is further complicated by the uncertainty respecting the relative quality of viral particles in the supernatant and in the cells which would also need to be taken into account (Altaras et al., 2005).

Taken together, the evidence for virus release, whilst the infected cells appear no more likely to be destroyed by shear than uninfected cells probably simply implies that the virus production processes are efficient, with cells producing more protein as virus production factories than they do for normal metabolic activities. Indeed this agrees well with evidence that adenovirus production is highly metabolically demanding (Silva et al., 2010), and with the high burst volumes shown for adenovirus in HEK (Xu et al., 2015a), and also with evidence that adenoviruses may induce autophagy (Balvers et al., 2011). It may also be noted that the evidence gathered to date does not preclude the possibility that the additional virus release is due to detachment of virus which was bound to the external cell membrane, as oppose to destruction of virus infected cells. Further work is required to verify the source of the additional virus in the supernatant following shear.

Interestingly the cell lysis by this virus does not appear to significantly decrease the clarification. Visually the cells appear to remain largely whole and intact, with the population increasing substantially over the post-infection period. The debris count, as measured in the size range ~60 to 600nm, does not appear to increase substantially, reinforcing the impression given by visual inspection. The virus induced lysis takes the form of an increased permeable cell membrane, as oppose to the absolute destruction of the cell, under these culture conditions.

Although these results would need to be confirm for suspension cultures they potentially provide some valuable insights for the numerous large scale production processes being developed, or coming on-line for adenovirus, particularly with respect to harvest windows and decisions regarding harvesting product from the supernatant, cell mass, or both.

Clarification of EB66 cells infected with Influenza

EB66 cells infected with influenza show a distinctly different clarification to uninfected EB66 cells. In contrast to the above result, with HEK cells infected with adenovirus, Figure 6 (A-D) shows that the

clarification efficiency for influenza virus infected cells is significantly below that of non-virus infected cells based on a 95% confidence interval (n=3). It also appears to show an increase in the impact of shear. As shear increases, the clarification efficiency decreases and is worsened with increased viral infection duration.

Again, as for HEK cells and adenovirus, the cell count was measured for different shear levels, for both influenza virus infected and uninfected EB66 cells. Figure 7, however, by contrast with the clarification data discussed above, shows a similar pattern to that seen in adenovirus infected HEK: the number of cells destroyed by shear decreases following infection, particularly for the highest shear level. Application of the same explanation, of a greater number of dead cells, is supported by the decrease in viability from 96+/-4.5% (95% CI, student t-test, n=3), to 73+/-9.2% and 77+/-10.8% (95% CI, student t-tests, n=2) for uninfected EB66 cells, and 4 and 5 days post infection respectively. The viability of sheared cells is marginally but consistently greater than for unsheared cells in the virus infected cells, whilst in the non-virus infected cells it is less. For cells subjected to shear in the rotating shear device at a speed of 12krpm, for example, the viabilities of the uninfected EB66 cells was 92%, and cell viabilities of influenza infected cell at 4 and 5 days post infection were 78% and 81% respectively. As a percentage of the original number in the sample therefore the non-viable cell count decreased more than the viable cell count. This may imply that cells deemed to be non-viable are marginally more likely to be destroyed by shear, possibly including infected cells. This could not be confirmed by infectivity assays, as with adenovirus, because influenza virus released by shear would not be enveloped, and therefore not mature, viable or infective.

Taken together the decrease in clarification between uninfected and virus infected cells, and the increasing shear insensibility indicated by the cell count, implies that clarification is being impacted by the debris formed by virus induced cell lysis during or following budding; the Vi-Cell images certainly show a noisier background after a number of days of infection which may be membrane components which form during virus egress from the cell. It may be noted that whilst the slope of the curves in Figure 6 (A-D) increase with virus infection, the distance between the slopes,

representing different shear levels, only shows a clear visible increase after 5 days of infection. This may represent a combined effect of the impact of virus infection on the cells and the subsequent increasing sensitivity of the clarification to shear.

The influenza virus was cultured at pilot scale (50litres), and clarified using a disc stack centrifuge (PSC-5). Prior to these influenza based studies this disc stack centrifuge was run with both HEK and CHO cells at the same flow rate and rotational speed as used for the clarification of the influenza harvest. The clarification of these HEK and CHO cell cultures were measured both for the disc stack centrifuge and using the ultra scale-down mimic (consisting of rotating disc and laboratory centrifuge), which has been used extensively for the prediction of clarification in mammalian cell cultures. This work confirmed that the centrifuge in question is a low shear centrifuge, with rotating shear device speeds of the order of 4.6 and 6 krpm (HEK and CHO respectively) giving a good prediction of clarification and protein release levels (Melinek et al., 2017). As this scale-down mimic has not yet been validated for use with virus infected continuous cell line harvest it was deemed important to include validation at scale, in discussing the implications of the presented results. Figure 6E therefore includes the clarification as measured for the disc stack supernatant. The equivalent rotating shear device speed required to accurately match the disc stack centrifuge clarification, overall protein release and relative concentration for a number of host cell proteins (Figure 8 and Figure 9) is approximately 4.5krpm. This result is therefore consistent with expectations.

It was noted that there was an unexpected discrepancy between the small and large lab scale centrifuges (compare Figure 6D and Figure 6E). Clarification from the larger lab scale centrifuge was consistently below that of the smaller lab scale centrifuge. Given the costs and industrial setting of this work it was not possible to complete sufficient repeats to verify the significance of this difference. However, it was noted that this effect was less visible in the non-infected EB66 cells. It has been hypothesised that this difference may be due to differences in the g-forces or head present in these different systems, but again it was not possible to fully verify this hypothesis. It may also be

observed that the infected EB66 cells give a less consistent measure of clarification. This may be due to (i) differences in the way the debris or cells pellet, or (ii) difference in the supernatant make up, such that there is a tendency to form solid impurities, which manifest as a floating 'scum-film layer' following virus infection, or (iii) differences in methodology, as noted in the materials and methods, clarification section.

Conclusions

Studies were conducted on the impact of shear on HEK and EB66 cells before and after infection with adenovirus and influenza respectively. The interest of these studies was in the implications for the harvest and subsequent processing of adenovirus and influenza for therapeutic and prophylactic viral vaccines. These are both areas of major importance, in which continuous cell line production of a viral product is involved and where scale may play a major role in cost and availability of sufficient product. Although it cannot be said definitively from the data that virus infected cells are not more sensitive to shear at some point in the infection process, the evidence strongly suggests that cells used to propagate enveloped influenza virus in cell culture may be more shear sensitive and more difficult to clarify in comparison to non-enveloped adenovirus. The clarification may be worse for cells where the virus produces a significant amount of lysis debris, and unchanged, or even improved for cells where the virus leaves the cell structure largely intact. Further work is required before any definite patterns can be drawn for clarification and shear sensitivity for different cell types, virus types and culture conditions. Further it has been demonstrated that the ultra scale-down laboratory mimic previously developed for use with mammalian cells gives a good prediction on a range of parameters for the harvest by continuous centrifugation of EB66 cells producing influenza virus. Particularly with respect to viral vectors, where substantial expense, expertise and biosafety issues may also play a role, this represents demonstration of a highly promising tool for timely and robust process development.

Acknowledgments

The authors wish to thank the influenza research and development team at GSK for their help and support, in particular Jessica Jonker, Helge Abrecht, Michael Klomp, Pierre-Yves Vitry and Laurane Selmeus.

Conflict of interest

Sandrine Dessoy is an employee of the GSK group of companies and owns shares in GSK.

This work was sponsored by University College of London and was financially supported by GlaxoSmithKline Biologicals S.A. and Engineering and Physical Science Research Council (EPSRC).

Author contributions:

Melinek, Dessoy, Mukhopadhyay and Bracewell were involved in the conception and design of the study. Melinek acquired the data. Melinek analyzed and interpreted the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

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Table 1. Summary of culture conditions for adenovirus / HEK cell runs

Figure 1. Experimental Flowsheet

Figure 2. Normalised clarification of (A) uninfected HEK cells, (B), infected HEK cells with adenovirus for less than 3 days, and (C) infected HEK cells with Adenovirus for between 4 and 6 days. Cells have been subjected to disruption by a rotating shear device at varying shear rates prior to clarification in a laboratory centrifuge, with rotating shear device speeds of 0krpm (squares), 6krpm (triangles), 8krpm (crosses), 10krpm (circles) and 12krpm (diamonds) respectively. Error bars represent 95% confidence intervals using student t-test (n=6, 3 and 5 for 8 and 10krpm, and n=6, 4 and 6 for 0, 6 and 12krpm, for figures A, B and C respectively).

Figure 3. Normalised cell counts for HEK cells, not infected with adenovirus (diamonds), infected with adenovirus for less than 3 days (squares) and infected with adenovirus for between 4 and 6 days (crosses). Cells have been subjected to disruption by a rotating shear device at varying shear rates, and then counted using a haemocytometer. Error bars represent 95% confidence intervals using student t-test (n=4).

Figure 4. Sample immunoblot, tagged with antibodies against Adenovirus type 5 coat proteins. Lanes are, in order, molecular weight markers, adenovirus type 5 kit virus, lysate of HEK cells infected with adenovirus and subjected to rotating shear device speeds of 0, 18 and 12krpm, a reference of adenovirus infected HEK cells, lysate of uninfected HEK cells, and supernatant of HEK cells infected with adenovirus and subjected to rotating shear device speeds of 0, 18 and 12krpm. N.B. Equal mass of protein in each lane.

Figure 5. Graphs show relative concentrations for selected viral proteins (identified on the immunoblot in Figure 4). Graphs on the top are of lysate concentrations, and on the bottom are of concentration in the supernatant. Striped is unsheared, dotted has been subjected to an intermediate level of shear (12krpm in the rotating shear device) and filled has been subjected to a high level of shear (18krpm in the rotating shear device).

Figure 6. Normalised clarification of EB66 cells, (A) uninfected with influenza virus, (B,C) infected with influenza virus for 4 days and (D) infected with influenza virus for 5 days. Cells have been subjected to disruption by a rotating shear device at varying shear rates prior to clarification in a laboratory centrifuge, with rotating shear device speeds of 0krpm (squares), 6krpm (triangles), 8krpm (crosses), 10krpm (circles) and 12krpm (diamonds) respectively. Measurements shown are for a single representative run (as all influenza runs used different viral strains), with the exception of (A), where triplicate results are shown. Normalised clarification of EB66 cells, infected with flu virus for 5 days, where cells have been subjected to disruption by a rotating shear device at varying shear rates prior to clarification in a Beckmann laboratory centrifuge, using 15ml falcon tubes, with rotating shear device speeds of 0krpm (squares), 6krpm (triangles), 8krpm (crosses), 10krpm (circles) and 12krpm (diamonds) respectively (E). Measurements shown in E are for two separate runs, each using a different viral strain of influenza. In addition red circles give the clarification measured on the PSC-5 centrifuge.

Figure 7. Cell counts for EB66 cells, not infected with influenza virus (triangles), infected with influenza virus for 4 days (squares) and infected with influenza virus for 5 days (diamonds). Cell have been subjected to disruption by a rotating shear device at varying shear rates, and then counted using a Vi-Cell. In (A) measurements shown are for three separate runs each using a different influenza A viral strain (A/Gyrafalcon/Washington H5N8, A/Shanghai/2/2013 H7N9 and A/Duck/Bangladesh/19097/2013(H5N1)). In (B) average normalised measurements are shown for pre-infection and the two post-infection time points.

Figure 8. Sample Immunoblot, tagged with antibodies against EB66 host cell proteins. Lanes are, in order, molecular weight markers, supernatant of influenza virus infected EB66 cells subjected to rotating shear device speeds of 6 and 12krpm respectively, supernatant of EB66 cells not infected with virus subjected to a rotating shear device speed of 6krpm, supernatant of influenza virus infected EB66 cells subjected to rotating shear device speeds of 6, 12, 6, 0 and 0krpm, the supernatant from PSC-5 and the same virus run as in the other lanes, lysate from influenza virus infected EB66 cells subjected to rotating shear device speeds of 0, 6 and 12krpm and a reference virus infected supernatant sample (the supernatant from the PSC-5 on the first virus run). N.B. Equal volume of sample was loaded in each lane.

Figure 9. Graphs show relative concentrations for selected host cell proteins (identified on the immunoblot in Figure 8). Graphs show average concentrations for two separate run (with different viral strains) and from the day before harvest for the second run. Cells have been subjected to disruption by a rotating shear device at varying shear rates prior to clarification in a Beckmann laboratory centrifuge, using 15ml falcon tubes. The protein concentrations for each run have been normalised such that the concentration for the PSC-5 centrifuge supernatant for that run is 100. In the bar charts striped is unsheared, dotted has been subjected to a low level of shear (6krpm in the rotating shear device) and filled has been subjected to a high level of shear (12krpm in the rotating shear device). Error bars are standard deviation of duplicate measurement (n.b. 'run 1 – 1 day' has duplicates for the mid shear level only).