



## Rapid and inexpensive purification of adenovirus vectors using an optimised aqueous two-phase technology

Saqlain Suleman<sup>a</sup>, Kuteiba Schrubaji<sup>a</sup>, Chrysovalanto Filippou<sup>a</sup>, Svetlana Ignatova<sup>b</sup>, Peter Hewitson<sup>b</sup>, Jonathan Huddleston<sup>b</sup>, Rajvinder Karda<sup>c</sup>, Simon N. Waddington<sup>c,d</sup>, Michael Themis<sup>a,e,\*</sup>

<sup>a</sup> Division of Biosciences, Department of Life Sciences, College of Health, Medicine & Life Sciences, Brunel University London, Uxbridge, Middlesex, UK

<sup>b</sup> Department of Chemical Engineering, College of Engineering, Design & Physical Sciences, Brunel University London, Uxbridge, Middlesex, UB8 3PH, UK

<sup>c</sup> Gene Transfer Technology, EGA Institute for Women's Health, University College London, London, WC1E 6HX, UK

<sup>d</sup> MRC Antiviral Gene Therapy Research Unit, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

<sup>e</sup> Division of Ecology and Evolution, Department of Life Sciences, Imperial College London, London, SW7 2AZ, UK

### ARTICLE INFO

#### Keywords:

Adenovirus type 5  
Aqueous two-phase partitioning system  
Caesium chloride  
Vaccine  
Viral vector purification

### ABSTRACT

Adenoviruses (AdVs) are used as gene therapy vectors to treat human diseases and as vaccines against COVID-19. AdVs are produced by transfecting human embryonic kidney 239 (HEK293) or PER.C6 virus producer cells with AdV plasmid vectors or infecting these cells with cell lysates containing replication-defective AdV. Cell lysates can be purified further by caesium chloride or chromatographic protocols to research virus seed stocks (RVSS) for characterisation to high quality master virus seed stocks (MVSS) and working virus seed stocks (WVSS) before downstream production of pure, high titre AdV. Lysates are poorly infectious, block filtration columns and have limited storage capability. Aqueous two-phase systems (ATPS) are an alternative method for AdV purification that rapidly generates cleaner RVSS for characterisation to MVSS. After testing multiple ATPS formulations, an aqueous mixture of 20 % PEG 600 and 20 % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (w/w) was found most effective for AdV partitioning, producing up to 97±3% yield of high-titre virus that was devoid of aggregates both effective *in vitro* and *in vivo* with no observable cytotoxicity. Importantly, AdV preparations stored at -20 °C or 4 °C show negligible loss of titre and are suitable for downstream processing to clinical grade to support the need for AdV vaccines.

### 1. Introduction

Adenoviruses (AdVs) are highly suited to effective gene transfer and commonly used in vaccine production to target both dividing and non-dividing cells and their biology is well understood (Cortin, Thibault et al. 2004; Ura, Okuda et al. 2014). The vectors replicate and are assembled within the nucleus of infected cells before undergoing lysis for the progeny virus to infect adjacent cells. AdV particles have a diameter of 90–110 nm containing capsid proteins and fibres making up their structure surrounding a linear double-stranded DNA genome (Russell, 2009). Seven AdV species (A–G) are known, which have been subdivided into 57 serotypes. Ad serotype 5 (AdV5) is the most commonly used for gene therapy with fibre proteins that bind coxsackievirus and adenovirus (CAR) receptors on permissive cells (Bergelson, Cunningham

et al. 1997; Roelvink, Lizonova et al. 1998; Kirby, Lord et al. 2001.) and an RGD motif that binds to the cell surface integrins for cell entry (Huang, Kamata et al. 1996; Davison, Diaz et al. 1997). AdV5 usually elicits a significant host immune response due to recognition of virus proteins by T lymphocytes and neutralising antibodies (Gilgenkrantz, Duboc et al. 1995; Yang, Su et al. 1996).

AdV vectors are designed to carry a large transgene payload and particles are usually produced at high titre from human embryonic kidney 293 (HEK293) or PER.C6 producer cells (Xie, Pilbrough et al. 2002). For therapeutic applications, replication-defective vectors, originally based on AdV5, have been generated. First generation vectors have early genes E1 and/or E3 regions removed allowing ~8 kb of transgenic sequences to be carried. These early genes that are necessary for virus replication have been inserted into the producer cells to

**Abbreviations:** AdV, Adenoviruses; ATPS, Aqueous two-phase systems; HEK293, human embryonic kidney 293.

\* Corresponding author at: Institute of Environment, Health & Societies, Division of Biosciences, Department of Life Sciences, College of Health, Medicine and Life Sciences, Brunel University London, Kingston Lane, Uxbridge, Middlesex, UB8 3PH, UK.

E-mail addresses: [michael.themis@brunel.ac.uk](mailto:michael.themis@brunel.ac.uk), [m.themis@imperial.ac.uk](mailto:m.themis@imperial.ac.uk) (M. Themis).

<https://doi.org/10.1016/j.jviromet.2021.114305>

Received 27 October 2020; Received in revised form 24 August 2021; Accepted 27 September 2021

Available online 6 October 2021

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complement transfection or infection of defective vector genomes into these cells to enable the generation of attenuated progeny virus particles (Fallaux, Kranenburg et al. 1996; Graham, Smiley et al. 1977; Fallaux, Bout et al. 1998). Due to the immune response caused by 1st generation AdV5, second generation vectors have been developed with further deletions of E2A, E2B and E4 genes with larger transgene capacity (Bischoff, Kirn et al. 1996; Fallaux, Kranenburg et al. 1996). Third generation vectors have all internal components removed, except replication origins and packaging signals leaving the 3' and 5' inverted terminal repeats (ITR) remaining and therefore require complementation with helper viruses. AdVs have also been engineered as conditionally replicative carrying mutated E genes that only allow replication in cells not expressing P53 or Rb proteins and so are suited to cancer gene therapy (Bischoff, Kirn et al. 1996).

Human and chimpanzee AdV based vaccines of various sorts have entered human trials against HIV, influenza, Ebola, tuberculosis, and malaria diseases. Natural wild type infection by AdV is common in humans and thus hAdV5 vaccines are recognised by pre-existing anti-hAdV5 antibodies in humans (Xiang, Gao et al. 2003; Liebowitz, Lindbloom et al. 2015). As a result of this, alternative serotypes have been used or AdV from alternative species such as the chimpanzee and gorilla (Cheng, Wang et al. 2015; Baden, Liu et al. 2015; Bos, Rutten et al. 2020; Capone, Raggioli et al. 2020; Folegatti, Ewer et al. 2020; Zhu, Li et al. 2020). Currently, these AdVs are in clinical trials to combat the COVID-19 pandemic.

To obtain large-scale good manufacture practice-certified (GMP) vector preparations at high titre, fed-batch cultures of anchorage independent cells are grown in serum-free suspension bioreactors with improved culture conditions for optimal growth density (Lesch, Heikkilä et al. 2015).

AdV production to clinical grade by adherent or suspension culture producer cells requires both up and downstream manufacturing processes that are time consuming, costly and difficult to scale up, thereby limiting AdV manufacture. AdV production requires producer cell plasmid transfection or infection with crude cell lysates containing AdV particles. This produces research virus seed stocks (RVSS) that are characterised to become master and working virus seed stocks (MVSS and WVSS). After infection and cell lysis, AdV particles can be subjected to CsCl density separation to remove producer cell genomes and proteins to generate pure AdV. Both the use of cell lysates and CsCl purification have limitations. Cell lysates have reduced infectivity and can block purification columns used in downstream processing, thereby reducing particle yield. CsCl Ad production avoids this but requires two rounds of ultracentrifugation, which not only significantly contributes to the lengthy time required for AdV preparation but also results in loss of infectious particles during the extraction process. Although automated continuous flow centrifugation can be easily scaled up (~2 kl) for laboratory use, the need for dialysis to remove this toxic salt adds further cost, time (10 h) and reduces yield. It can also result in particle aggregation that reduces viable infectious titre (Boychyn, Yim et al. 2001; Farid, 2007; McNally, Darling et al. 2014). The use of chromatography to purify lysed cell samples over packed columns also limits supernatant volumes that can be used, and columns can become clogged resulting in a low AdV yield (McNally, Darling et al. 2014). Importantly, robust, high-quality, characterised MVSS and WVSS are required for downstream processing to clinical grade virus. At the upstream stage, a method that could cost effectively improve the purity of the Ad starting material from crude cell lysates and match that of CsCl purification rapidly whilst avoiding early stage chromatography to provide RVSS would be highly beneficial to Ad production.

Aqueous two-phase systems (ATPS) have been shown to be successful in purifying rapidly several types of biomolecules, including proteins, using immiscible liquids as phases for separation (Albertsson, Andersson et al. 1982; Chao, Shum, 2020). ATPS offers a simple, rapid and promising alternative methodology for the rapid scale-up of vector preparations, whilst retaining particle infectivity and titre. This can

streamline the production of RVSS further for downstream applications. Polyethylene glycol (PEG) is especially useful in separating biomolecules and its hydrophobicity is directly correlated to its molecular weight. When PEG is combined with a kosmotropic salt, such as in the PEG300-phosphate ATPS, AdV can be purified in the upper aqueous phase, whilst remaining highly infectious (Negrete, Ling et al. 2007a, b). Dextran and methylcellulose/polyethylene glycol polymers can also be used to generate viable virus particles that are concentrated (up to 1000-fold) using low speed short centrifugation times. ATPS purification has also been applied successfully to adeno-associated virus (AAV) vector production using 10 % PEG8000/13.2 % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solutions that gently remove impurities and avoids dialysis to a level acceptable for *in vivo* gene transfer (Guo, Xiao et al. 2013; Rogers, Bond et al. 1996). Importantly, ATPS is cost effective and can be easily performed in any laboratory without the need for expensive equipment (Molino, Lopes et al. 2018). These demonstrate advantages of the ATPS for purification of vectors compared to CsCl or chromatographic protocols.

This work applies ATPS technology to generate RVSS of AdV vectors at reduced cost and time with high particle yield, purity and infectivity. Several immiscible liquid compound formulations will be screened for partitioning AdV particles from infected HEK293 T producer cell lysates, their infectivity and potential toxicity *in vitro*. The ATPS formulation that shows the highest vector recovery will also be applied *in vivo* to compare CsCl and ATPS purified AdV to ensure comparable levels of gene transfer and potential toxicity *in vivo*. Because ATPS technology is amenable to scale-up, we propose this technology ideal for the rapid manufacture of large amounts of RVSS for characterisation to MVSS under GMP conditions to support clinical grade therapeutic AdV and AdV-based vaccines.

## 2. Material and methods

### 2.1. Cell culture

Phosphate buffered saline (PBS) solution (Fisher Scientific, Loughborough) was prepared in 500 mL batches with sterile dH<sub>2</sub>O and autoclaved. Cells were cultured in complete medium prepared using Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX™ supplemented with 1% penicillin streptomycin and 10 % fetal bovine serum (all reagents are purchased Fisher Scientific). 0.05 % trypsin (Fisher Scientific) diluted in PBS was used to detach cells from their substratum. All reagents used showed low levels of endotoxins present and are cGMP compatible. IMRS2, HEK293 T and HepG2 cells were obtained from ATCC (Middlesex, UK). These were thawed and centrifuged at 1500 rpm for 5 min to pellet cells that were resuspended in 10 mL complete medium. Cells were incubated at 37 °C in the presence of 5% CO<sub>2</sub> and the medium changed every 2 days. Cells were passaged when reaching 70 %–80 % confluency.

### 2.2. Generation of adenovirus vectors

For this study, 1st generation human AdVRSVβgal or AdVCMVLuc (E1 and E3 regions deleted), which drive the β-galactosidase or luciferase genes by the RSV and CMV promoters respectively, were generated using HEK293 T cells. 1.5 × 10<sup>7</sup> cells grown in T175 culture flasks were infected with AdV (MOI of 10) for 72 h. Cells were mechanically detached from the flasks and pelleted at 1500 rpm for 10 min, followed by resuspension in 10 mL complete medium. Cells were subjected to 3 freeze thaw cycles between 37 °C and –80 °C and cell debris pelleted by centrifugation at 1500 rpm for 15 min. AdV was purified using high-speed CsCl density gradient ultracentrifugation and AdV particle concentration determined via OD<sub>260nm</sub> equivalent to AdV titre, for vector genomes and plaque assays on HEK293 T cells respectively, as previously described (Fallaux, Bout et al. 1998). AdV was also recovered via ATPS partitioning from freeze thawed HEK293 T cell batches (see below). AdRSVβgal and AdCMVLuc recovered by CsCl partitioning were

tested on HeLa cells for infectivity via Xgal staining for  $\beta$ -galactosidase or luciferase gene expression via *n*-luciferin light emission, respectively.

### 2.3. Analysis of AdV vectors for infectivity and replication incompetence

To investigate for the presence or absence of replication competent adenovirus (RCA), infected HeLa cells were cultured for a further 2 weeks after which time conditioned supernatants were filtered and then applied to fresh HeLa and HEK293 T cells. These cells were stained using X-gal for  $\beta$ -galactosidase gene expression or tested for luciferase gene expression after 48 h to indicate the presence of positively or negatively infected cells, as previously described (Clifton, 2012). No cells were stained positive (i.e. blue or emitting light) indicating replication incompetence of AdV.

### 2.4. Generation of ATPS phases and analysis of yield

Polymers and salts were weighed and dissolved in deionised water at 37 °C, 1.0M $\Omega$ cm<sup>-1</sup> (Puritec, Thames). Each system was vortexed, then left to separate at room temperature for 15 min. (Table 1). ATPS created were: 14 % - 14 % w/w PEG 1000 - K<sub>2</sub>HPO<sub>4</sub>, 14 % - 14 % w/w (C<sub>6</sub>H<sub>10</sub>O<sub>6</sub>)<sub>n</sub> - PEG 8000, 14 % - 14 % w/w PEG8000 - HNa<sub>2</sub>O<sub>4</sub>P, 14 % - 14 % w/w PEG 8000 - K<sub>2</sub>HPO<sub>4</sub>, 14 % - 14 % w/w PEG 600 - (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 % - 20 % w/w PEG 600 - (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 14 % - 14 % w/w UCON 3900 - Na<sub>2</sub>HPO<sub>4</sub>, 14 % - 14 % w/w UCON 970 - Na<sub>2</sub>HPO<sub>4</sub>, 14 % - 14 % w/w UCON 970 - C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>•2H<sub>2</sub>O (Fisher Scientific & Sigma- Aldrich). ATPS consisting of 14 % - 14 % w/w PEG8000 - HNa<sub>2</sub>O<sub>4</sub>P, 14 % - 14 % w/w PEG 600 - (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 14 % - 14 % w/w UCON 3900 - Na<sub>2</sub>HPO<sub>4</sub>, 14 % - 14 % w/w UCON 970 - Na<sub>2</sub>HPO<sub>4</sub> were not used due to the formation of crystal salts.

To assess the yield of vector recovery after ATPS, 10  $\mu$ L containing  $3.5 \times 10^{12}$  IU of AdVRSV $\beta$ gal purified using CsCl gradient centrifugation was added to a 1:1 ratio of mixed upper and lower ATPS phases (2 mL total). After gentle mixing, the phases were centrifuged at 1500 rpm for partitioning for 15 min. Each layer was then quantified for AdVRSV $\beta$ gal concentration by spectrophotometry at an optical density reading of 260 nm for vector genomes (vg) equating to vector particles per ml (vp/mL) to calculate recovery yield using NanoDrop™2000C (Mittereder, March et al. 1996). Recovery was also measured by infection assays on HepG2 cells.

AdV batches were also screened for aggregation as previously described (Lefevre, van Bekkum 2003). Briefly, spectrophotometric ratios from 320/260 and 340/260 readings were assessed and no ratios above 0.3 were found indicating no virus aggregation.

### 2.5. Infection and cytotoxicity assays of HepG2 and IMRS2 cell lines

HepG2 (liver) and IMRS2 (neuronal) cells were cultured in 12 well

**Table 1**

ATPS solutions tested for the purification of AdVRSV $\beta$ gal particles. Successful (+) and unsuccessful (-) ATPS solutions for AdV purification are shown for each ATPS tested. All solutions are at 14 % w/w unless otherwise stated. Initial Ad input for purification was  $3.5 \times 10^{12}$  particles/mL. To detect for the presence or absence of viral nucleic acids and proteins, Abs<sub>260nm</sub> readings of the upper phase of each ATPS were measured after purification using NanoDrop™2000C. Negligible concentrations of AdV was present in the lower phases (n = 3).

ATPS	Positive/Negative
PEG 1000/K <sub>2</sub> HPO <sub>4</sub>	+
(C <sub>6</sub> H <sub>10</sub> O <sub>6</sub> ) <sub>n</sub> /PEG 8000	+
PEG 8000/HNa <sub>2</sub> O <sub>4</sub> P	-
PEG 8000/K <sub>2</sub> HPO <sub>4</sub>	+
PEG 600/(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	-
20 % - 20 % w/w PEG 600/(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	+
UCON 3900/Na <sub>2</sub> HPO <sub>4</sub>	-
UCON 970/Na <sub>2</sub> HPO <sub>4</sub>	-
UCON 970/C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> •2H <sub>2</sub> O	+

dishes at  $2 \times 10^5$  cells/well and incubated at 37 °C with 5% CO<sub>2</sub> for 24 h. HepG2 cells have been shown to be highly permissive to AdV infection (Pan, Wu et al. 2012). 10  $\mu$ L of the upper and lower phases of each phase system containing purified AdVRSV $\beta$ gal were then added to each well for 24 h before replacing medium. 48 h post infection, cells were stained for LacZ expression to determine the most effective ATPS for AdV purification, as previously described (Clifton, 2012). Cell viability was analysed via trypan blue exclusion 48 h post AdVRSV $\beta$ gal infection, where live cells exclude dye as opposed to dead cells that do not.

### 2.6. Animal procedures

Outbred male and female CD1 mice were used in this study (Charles River, Oxford). All animal experiments conducted within this study were in agreement with the United Kingdom Home Office guidelines, approved by the ethical review committee and following the institutional guidelines at University College London. To test hAdV gene transfer *in vivo*, AdVCMVLuc vector that drives luciferase gene expression from the CMV promoter was generated by infection of HEK293 T cells followed by purification via 20 % - 20 % w/w PEG 600 - (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS. Purified vector from supernatant was titrated at  $2.9 \times 10^{13}$  vector genomes/mL via spectrophotometry. Healthy CD1 mice were anaesthetised with isoflurane with 100 % oxygen (Abbott Laboratories, London, UK) in a heated chamber at 37 °C. 100  $\mu$ L containing  $1 \times 10^9$  vector genomes/mL AdVCMVLuc particles was administered to adult mice via tail vein intravenous injection. After the injections, mice were allowed to recover fully before being returned to their cages.

### 2.7. Whole-body bioluminescence imaging

Mice were anaesthetised with isoflurane with 100 % oxygen and given an intraperitoneal injection of 15 mg/mL of *n*-luciferin (Gold Biotechnologies, ST Louis, USA). Mice were imaged after 5 min using a cooled charged-coupled device camera, (IVIS machine, Perkin Elmer, Coventry, UK) for between 1 s and 5 min. The regions of interest (ROI) were measured using Living Image Software (Perkin Elmer, Beaconsfield) and expressed as photons per second per centimetre squared per steradian (photons/second/cm<sup>2</sup>/sr).

### 2.8. Statistical analysis

Significant differences between data were derived using a Man Whitney U statistical test, with a P value cut off of 0.05 for all data sets.

## 3. Results

### 3.1. hAdV genome recovery and infectivity following ATPS partitioning

Of the 9 ATPS screened, 5 formulations provided virus recovery, of which the 3 most optimal yields were  $75 \pm 8.55$  % for 14 % - 14 % w/w PEG 1000/K<sub>2</sub>HPO<sub>4</sub>,  $47 \pm 5.94$  % for 14 % - 14 % w/w PEG 8000/K<sub>2</sub>HPO<sub>4</sub> and  $83 \pm 5.99$  % for 20 % - 20 % w/w PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS. Vector partitioning was mainly restricted to upper phases. The

**Table 2**

AdVRSV $\beta$ gal genome recovery after separation by ATPS. An initial input of  $3.5 \times 10^{12}$  Ad vg/mL were separated in PEG1000/K<sub>2</sub>PO<sub>4</sub>, PEG8000/K<sub>2</sub>PO<sub>4</sub> and PEG600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Abs<sub>260nm</sub> readings were averaged and corrected over a range of dilutions. AdV genome titre (vg/mL) was calculated by multiplying the average Abs<sub>260nm</sub> value by 1.1 and the product by  $10^{12}$  (n = 3) (Fallaux, Bout et al. 1998).

ATPS	Titre (vg/mL)	Yield/%
PEG 1000 /K <sub>2</sub> PO <sub>4</sub>	$2.6 \times 10^{12}$	$75 \pm 8.55$
PEG 8000/K <sub>2</sub> PO <sub>4</sub>	$1.6 \times 10^{12}$	$47 \pm 5.94$
PEG 600/ (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	$2.9 \times 10^{12}$	$83 \pm 5.99$



vector titre reached for these ATPS ranged from  $1.6 \times 10^{12}$  to  $2.9 \times 10^{12}$  vg/mL (Table 2).

To examine the infectivity of AdVRSV $\beta$ gal partitioning to each phase, samples from both upper and lower phases were used to infect HepG2 cells, (Pan, Wu et al. 2012). Negligible HepG2 gene transfer was detected after exposing cells to any ATPS lower phases. Quantification of percentage infection by AdVRSV $\beta$ gal, recovered through upper phase, was  $55.2 \pm 2.7$  % for PEG1000/K<sub>2</sub>HPO<sub>4</sub>,  $59.9 \pm 9.8$  % for PEG8000/K<sub>2</sub>HPO<sub>4</sub> and  $97 \pm 3$  % with PEG600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS is shown in (Fig. 1b).

The IMRS2 neuronal cell line, which is of non-liver origin was also used as an alternative cell line to determine the efficacy of AdV infection. To measure IMRS2 permissiveness to AdV infectivity, cells were exposed to  $10^{11}$ – $10^6$  CsCl purified AdV (Fig. 2a and b). Cell infection was observed as  $100 \pm 0$  % ( $10^{11}$ ),  $80 \pm 3.84$  % ( $10^{10}$ ),  $60 \pm 1.15$  % ( $10^9$ ),  $46 \pm 2.19$  % ( $10^8$ ),  $15 \pm 1.76$  % ( $10^7$ ) and  $1 \pm 0.88$  % ( $10^6$ ) using serial dilutions of AdV. IMRS2 cells appeared highly permissive to AdVRSV $\beta$ gal at each concentration.

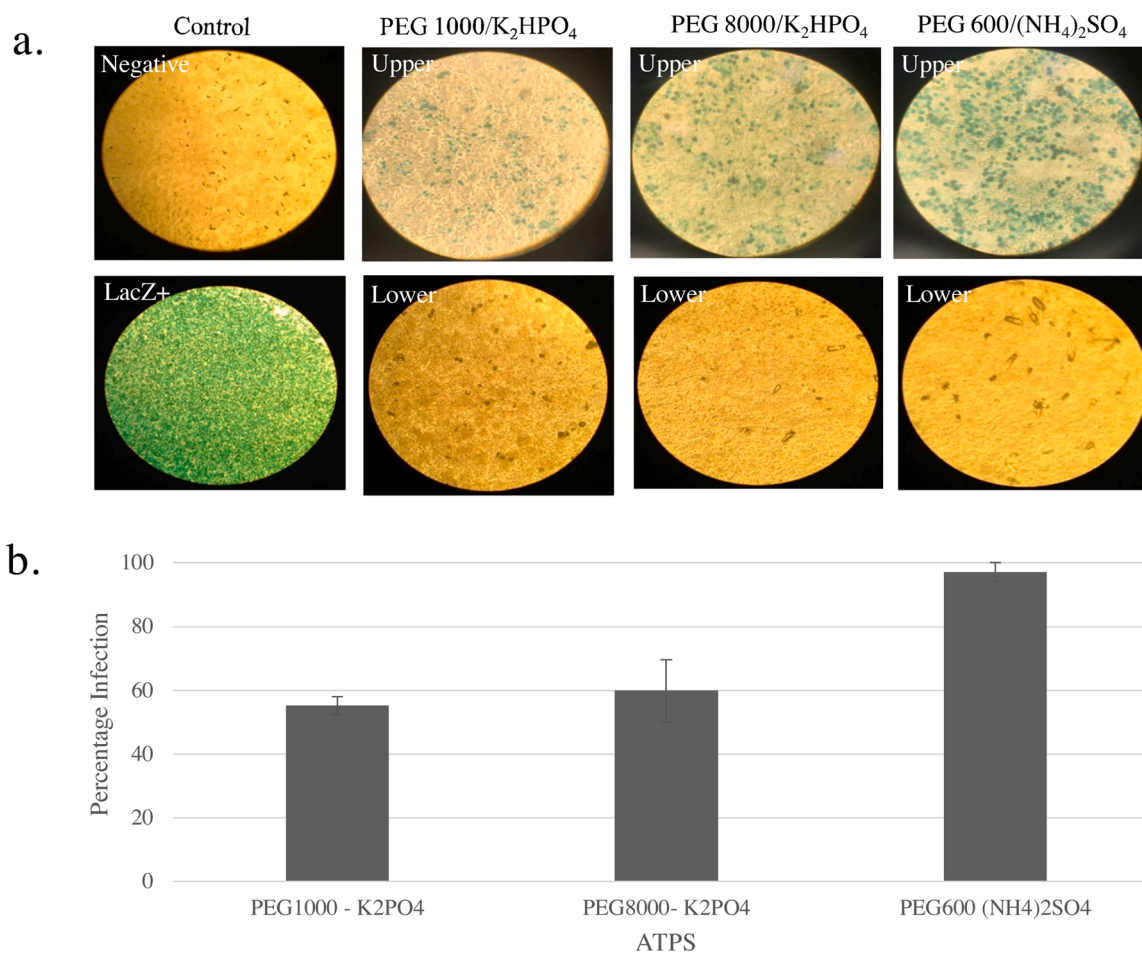
### 3.2. Isolation of Ad directly from 293 T producer cell lysate

The potential of ATPS to partition AdVRSV $\beta$ gal directly from infected HEK293 T cell lysates was assessed. HEK293 cells have previously been reported to produce high titre recombinant Ad virus (Wei, Fan et al. 2017; Chen, Wu et al. 2018). 293 T producer cells were infected with CsCl purified AdR $\beta$ gal seed stock at a MOI of 10. After producer cell infection, cells were isolated from culture flasks and subjected to

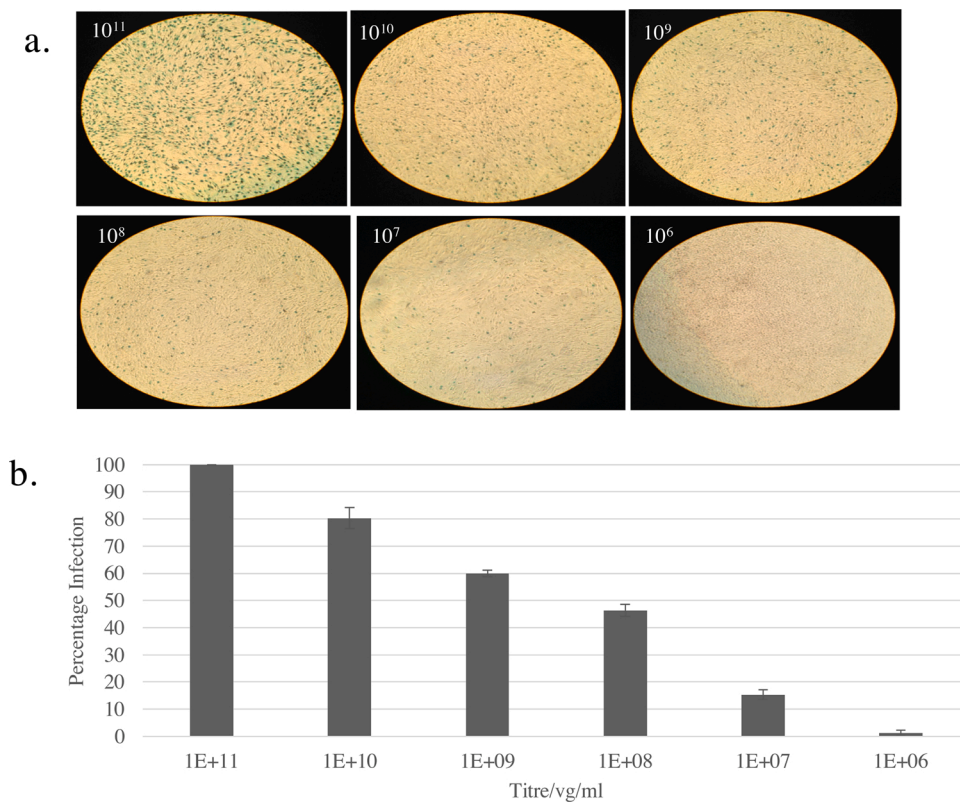
freeze thaw cycles for partitioning through each of the 3 ATPS. These products can be RVSS or stocks generated using cGMP grade reagents for characterisation to MVSS. HepG2 and IMRS2 cells were infected and cells positive for  $\beta$ -galactosidase gene expression were counted. HepG2 and IMRS2 infection was  $93.3 \pm 0.9$  % versus  $85.3 \pm 1.8$  % using PEG 1000/K<sub>2</sub>HPO<sub>4</sub>,  $91.7 \pm 2.4$  % versus  $30 \pm 1.2$  % using PEG 8000/K<sub>2</sub>HPO<sub>4</sub>.  $91.3 \pm 1.5$  % versus  $93.7 \pm 2.2$  % infection was observed with PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, closely matching that of percentage infection using CsCl purified AdVRSV $\beta$ gal partitioned through ATPS (Fig. 3a and b). Contaminating proteins can be detected via spectrophotometry (Glasel, 1995; Goldfarb, Saidel et al. 1951). All Abs<sub>260nm/280nm</sub> in ATPS purified AdV direct from cell lysates are above 0.6. Further HPLC analysis of ATPS purified AdV showed contaminating proteins present but to a lower extent that in crude lysates (data not shown). This demonstrates successful partitioning of AdV into the upper phase of ATPS, with the highest recovery using the PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> system.

### 3.3. Cell survival following infection by AdR $\beta$ gal recovered from ATPS formulations

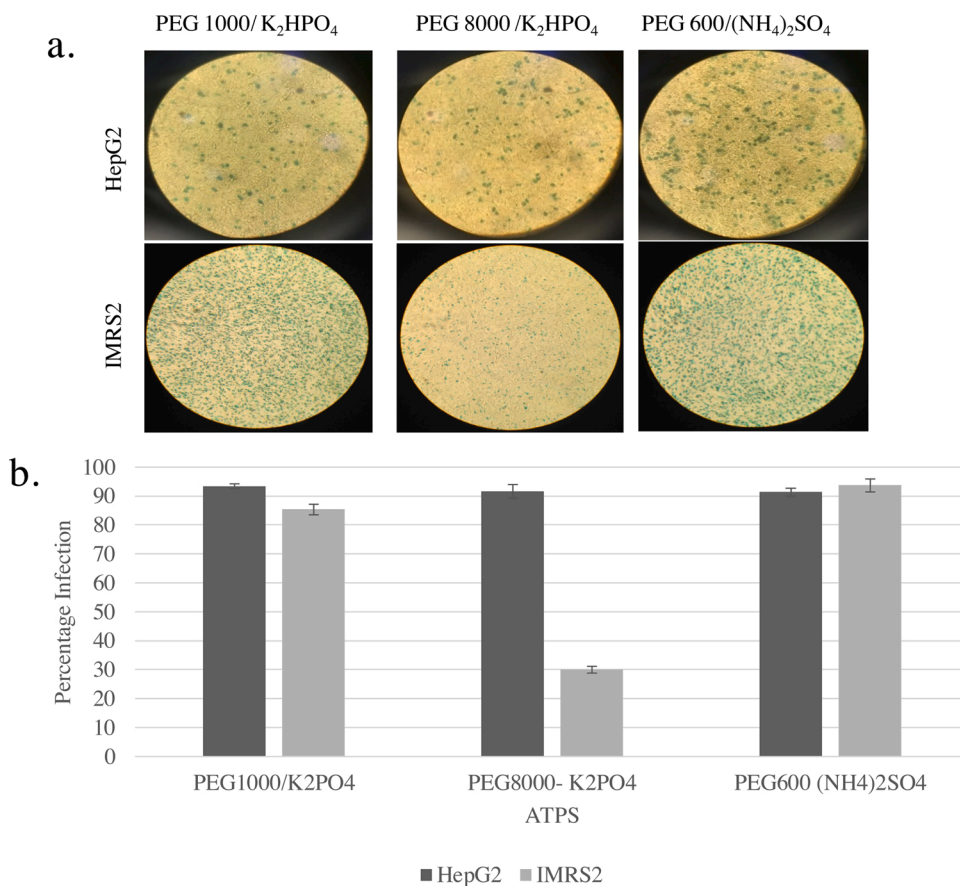
HepG2 cell survival was quantified using AdV after ATPS treatment and AdV infection (Fig. 3a). We found HepG2 viability after AdVRSV $\beta$ gal infection and gene transfer was similar between vector separated via ATPS from CsCl isolation or direct from HEK293 T cell lysates. Cells showed between  $77.33 \pm 5.06$  % and  $93.25 \pm 0.63$  % viability with PEG 1000/K<sub>2</sub>HPO<sub>4</sub> treatments. PEG 8000/K<sub>2</sub>HPO<sub>4</sub> treatments showed between  $87.25 \pm 4.40$  % and  $92.20 \pm 2.99$  % viability.



**Fig. 1.** Infection of HepG2 cells by AdVRSV $\beta$ gal. (a). HepG2 infection by CsCl prepared vector after partitioning by 3 ATPS formulations. Positive staining for  $\beta$ -galactosidase activity was identified in each ATPS upper phase, with highest infection by PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Uninfected HepG2 cells appear negative for enzyme activity. (b). Quantification of cell infection: PEG1000/K<sub>2</sub>HPO<sub>4</sub>;  $55.15 \pm 2.74$  %, PEG8000/K<sub>2</sub>HPO<sub>4</sub>;  $59.85 \pm 9.84$  %, PEG600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>;  $97.00 \pm 3.0$  %, (n = 5).



**Fig. 2.** Analysis of IMRS2 cell permissiveness to AdVRSVβgal infection. **(a).** Infected IMRS2 cells at various dilutions of AdV particles (vg/mL). Infection was detected by analysis of β-galactosidase activity demonstrating IMRS2 cells are highly permissive to infection. **(b).** Quantification of positive cells after exposure to serial dilutions of CsCl prepared AdVRSVβgal. IMRS2 infection was: 10<sup>11</sup>:100 ± 0%, 10<sup>10</sup>: 80 ± 3.84 %, 10<sup>9</sup>: 60 ± 1.15 %, 10<sup>8</sup>: 46 ± 2.19 %, 10<sup>7</sup>: 15 ± 1.76 % and 10<sup>6</sup>: 1 ± 0.88 % (n = 3).



**Fig. 3.** Analysis of AdV recovery direct from 293 T cell lysates **(a).** Infection of HepG2 and IMRS2 cells using AdVRSVβgal purified directly from 293 T cell lysate using 3 ATPS formulations. Infection of AdV was characterised by staining for β-galactosidase activity. **(b).** Quantification of HepG2 and IMRS2 cell infection by AdV purified directly from 293 T cell lysate. The upper phase presented the highest recovery of AdVRSVβgal particles and was used for infection. For HepG2 and IMRS2 cells, respectively, 93.3 ± 0.9 % and 85.3 ± 1.8 % cells were infected using PEG 1000/K<sub>2</sub>HPO<sub>4</sub>, 91.7 ± 2.4 % and 30.0 ± 1.2 % cells stained positive for AdV infection using PEG 8000/K<sub>2</sub>HPO<sub>4</sub>, 91.3 ± 1.5 % and 93.7 ± 2.2 % cells were infected using PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (n = 3).

PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> treatments showed a viability of between 83.75 ± 4.12 % and 93.50 ± 0.99 % (Fig. 4). No significant difference was found in comparison to untreated cells ( $P < 0.05$ ). This suggests no cytotoxicity due to ATPS treatment or AdV infection, including AdV purified direct from cell lysates.

### 3.4. In vivo application of AdCMVLuc

*In vivo* gene transfer was examined 3 days post injection of ATPS purified AdV (Fig. 5). None of the treated mice ( $n = 3$  each) displayed adverse reaction to vector injection, whilst displaying robust luciferase reporter expression. Levels of gene expression using ATPS purified AdV were comparable to CsCl purified AdV, indicating successful *in vivo* recovery.

## 4. Discussion

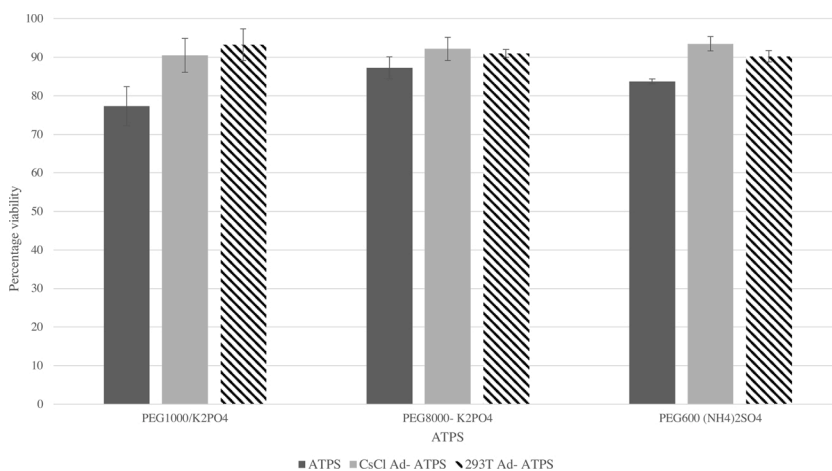
AdVs are important gene transfer vectors that are under development as licensed bioproducts for therapeutic applications including gene therapy of genetic diseases, vaccination or ‘virotherapy’. They are being developed for human diseases that are hereditary or acquired (Albertsson, Andersson et al. 1982; Oelmeier, Dismer et al. 2011, McNally; Darling et al. 2014) and preclinical studies have been initiated using these potentially therapeutic pharmaceuticals in a large number of laboratories (Rogers, Bond et al. 1996; Nemunaitis, Ganly et al. 2000; Oelmeier, Dismer et al. 2011; Clifton, 2012; Yan, Cao 2012; Guo, Xiao et al. 2013; Tan, Wang et al. 2015; Molino, Lopes et al. 2018). To service intensive clinical studies, manufacture requires cGMP production of large quantities of recombinant virus vectors, often in excess of 10<sup>15</sup> virus particles even for early phase clinical trials. Human AdV (hAdV) based vaccines have entered human trials against various pathogens but as hAd5 vaccines are recognised by pre-existing immune response in humans, novel AdV from different species have been developed (Baden, Liu et al. 2015; Cheng, Wang et al. 2015). hAdV5, hAdV26 and AdVs based on chimpanzee and gorilla adenoviruses are in clinical trials, including to combat the COVID-19 pandemic (Baden, Liu et al. 2015; Cheng, Wang et al. 2015; Bos, Rutten et al. 2020; Capone, Raggioli et al. 2020; Folegatti, Ewer et al. 2020; Zhu, Li et al. 2020). Such a variety of AdVs with differing complex surface topologies will require suitable manufacturing processes for their production at large scale. For example, the enormous challenge to meet the world-wide demand to vaccinate against COVID-19 is estimated to be 1.76 × 10<sup>19</sup> particles (Vellinga, Smith et al. 2014) and therefore rapid and cost effective alternatives to current manufacturing would be highly beneficial.

To generate high titre AdV on a small scale or from large-scale fed-batch cultures of anchorage independent producer cells, cells are transfected with plasmids carrying the cis and trans elements required

for particle production or infected with attenuated replication defective particles obtained directly from cell lysates or purified AdV. This production generates AdV RVSS for direct non-clinical use and usually is purified by CsCl buoyancy ultracentrifugation. AdV are then characterised and subsequently amplified to produce MVSS to be used in large scale downstream processing.

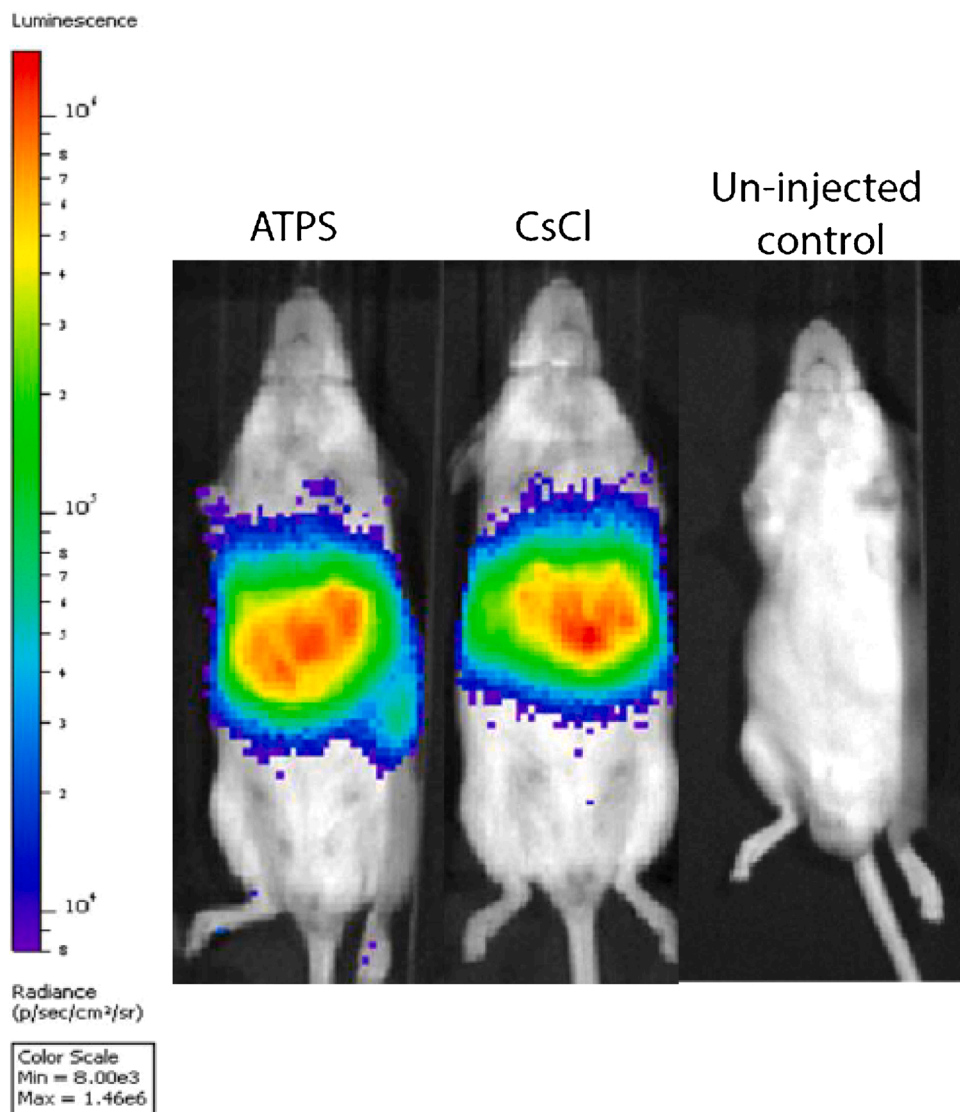
Before cell lysates can be used, they require filtration. These lysates are often contaminated with small cellular proteins or producer cell DNA. This can reduce producer cell infection and can result in the blockage of columns that are used in the filtration step prior to further purification. For downstream purification, although CsCl buoyancy ultracentrifugation is regarded to be an excellent method, this requires two rounds of ultracentrifugation and dialysis to remove this toxic salt, which is costly and time consuming. It also reduces particle yield and can result in particle aggregation thereby reducing viable infectious titre yield (Berkner, 1988; Baden, Liu et al. 2015; Cheng, Wang et al. 2015). Chromatographic methods over packed columns are also used to purify lysed cell samples, however these limit supernatant volumes and can also become clogged (Berkner, 1988). For clinical production of AdV, purification is achieved *via* ultrafiltration using tangential flow filtration followed by anion exchange and then further chromatographic steps.

Importantly, if the RVSS AdV particles required are relatively clear of cellular contaminants then downstream processing to MVSS would be more cost effective. To avoid the use of crude contaminated cell lysates or expensive and time consuming CsCl purification, we sought an alternative rapid, cost effective and simple method to generate RVSS AdV stocks for characterisation to MVSS. We also considered that this method should be amenable to provide AdV particles at a scale for downstream processing to high titre and infectious MVSS and WVSS AdV. ATPS technology has been shown successful to rapidly purify several types of biomolecules including proteins using immiscible liquids as phases for partitioning and permanent separation (Albertsson, Andersson et al. 1982). Its system provides an effective purification method, producing a relatively pure product with little to no need for dialysis. Moreover, it is an environmentally friendly, cost effective and easily scalable method that has been previously shown as an alternative methodology for rapid scale-up of AdV preparations, whilst retaining particle infectivity and titre. PEG has been found especially useful in separating biomolecules and its hydrophobicity is directly correlated to its molecular weight. When combined with a kosmotropic salt, such as PEG300-phosphate ATPS, highly infectious AdV partitioning occurs in the upper aqueous phase (Negrete, Ling et al. 2007a, b). Also, several polymers have been shown useful to generate concentrated viable virus particles using low-speed short centrifugation times. Sodium dextran sulphate is particularly useful because it allows salt removal from virus suspensions *via* precipitation, further concentrating virus stocks that are pure, viable are almost totally devoid of aggregates and dextran can also



**Fig. 4.** Analysis of cell survival after AdV/ATPS treatments. Percentage cell survival after treatment with upper phase ATPS +/- AdVRSVβgal. HepG2 cells + ATPS only (dark grey), + CsCl AdV (light grey) and + 293 T AdV (hash). PEG 1000/K<sub>2</sub>HPO<sub>4</sub> treatment showed viability of 77.33 ± 5.06 % (+ ATPS), 90.50(2.88) (+ CsCl AdV ATPS) and 93.25 ± 0.63 % (+293 T AdV). Cells treated with PEG 8000/K<sub>2</sub>HPO<sub>4</sub> upper phase showed a viability of 87.25 ± 4.40 % (+ATPS), 92.20 ± 2.99 % (+CsCl AdV) and 91.00 ± 1.83 % (+293 T AdV). PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> upper phase treatment showed a viability of 83.75 ± 4.12 % (+ATPS), 93.50 ± 0.99 % (+CsCl AdV) and 90.25 ± 1.49 % (+293 T AdV). No significant difference in viability was observed ( $P < 0.05$ ). This demonstrates the low toxicity of ATPS subsequent to AdV purification and recovery ( $n = \geq 4$ ).





**Fig. 5.** Application of PEG 600/  $(\text{NH}_4)_2\text{SO}_4$  ATPS purified AdVLuc *in vivo*. AdVLuc virus was purified using PEG 600/ $(\text{NH}_4)_2\text{SO}_4$  ATPS or CsCl ultracentrifugation.  $1 \times 10^9$  vg/mL AdVLuc particles recovered from the ATPS upper phase or CsCl were injected into CD-1 mice *via* their tail vein mice to determine *in vivo* gene transfer. Imaging for luciferase activity showed gene expression was comparable between the ATPS and CsCl purified vector thus demonstrating ATPS AdV can be used successfully *in vivo* without obvious side effects (a single representative mice of  $n = 3$  shown only).

be precipitated from virus preparations (Negrete, Ling et al. 2007a, b; Oelmeier, Dismar et al. 2012; Yan, Cao 2012; Tan, Wang et al. 2015). ATPS partitioning of adeno-associated vector (AAV) has also been shown using 10 % PEG8000/13.2 %  $(\text{NH}_4)_2\text{SO}_4$  solutions circumventing dialysis to generate particles pure enough for *in vivo* gene transfer (Rogers, Bond et al. 1996; Guo, Xiao et al. 2013).

To explore further the suitability of ATPS technology to generate effective AdV RVSS we tested several immiscible liquid compound formulations for their effectiveness in partitioning AdV particles from infected HEK293 T producer cells lysates. To test vector yield from 9 polymer/salt combinations, CsCl purified vector was firstly partitioned between these ATPS immiscible phases. Of these, 3 ATPS; 14 % - 14 % w/w PEG1000 -  $\text{K}_2\text{HPO}_4$ , and 14 % - 14 % w/w PEG8000 -  $\text{K}_2\text{HPO}_4$  and 20 % - 20 % w/w PEG 600 -  $(\text{NH}_4)_2\text{SO}_4$ , provided improved, high yield recovery above those previously described, of between 47–83 %. Particles appeared partitioned mainly in the upper, PEG rich phase and efficiently infected HepG2 cells with no observable cytotoxicity, with the most effective ATPS being PEG 600-  $(\text{NH}_4)_2\text{SO}_4$ . Using this optimal system, the quantities required for early phase clinical trials is estimated at  $1 \times 10^{15}$  virus AdV particles, which would require a volume of 350 mL. ATPS can be scalable up to 2 L batches, enough to easily achieve this estimate (Negrete, Ling et al. 2007b). Each ATPS was next used to directly partition AdV particles from 293 T cell lysates. While the T

antigen is undesirable in GMP manufacture of AdV vectors, this study shows improved proof of concept that these ATPS are useful for processing of high titre AdVs to RVSS. All three ATPS appeared to partition AdV particles, with the majority of high titre preparations in the upper aqueous phases. Infection of HepG2 and IMRS2 reached 93.3( $\pm 0.9$ )% and 85.3( $\pm 1.8$ )% using PEG 1000/ $\text{K}_2\text{HPO}_4$ , 91.7( $\pm 2.4$ )% and 30 ( $\pm 1.2$ )% using PEG 8000/ $\text{K}_2\text{HPO}_4$  and 91.3( $\pm 1.5$ )% *versus* 93.7( $\pm 2.2$ )% with PEG 600/ $(\text{NH}_4)_2\text{SO}_4$  for each cell line respectively, closely matching that of infection with CsCl purified vector partitioned through ATPS.

Vector purity is most important to avoid cytotoxicity, so to investigate whether any of the three ATPS or ATPS partitioned AdV cause cytotoxic side effects, each cell line was infected, and cell viability measured. Each ATPS alone or AdV particles purified by CsCl or direct from 293 T cell lysates when used to infect each cell line showed no toxicity, viabilities between approximately 90–94%, with PEG 600/ $(\text{NH}_4)_2\text{SO}_4$  partitioned vector being the highest. When using PEG 600/ $(\text{NH}_4)_2\text{SO}_4$  partitioned AdV carrying the luciferase gene to inject CD-1 mice *via* the tail vein, no adverse effects were observed when compared with animals infected by CsCl purified AdV.

PEG600 is commonly used in a clinical setting as an emulsifier, in ointments and toothpastes. The concentration of PEG600 used throughout this study remained within IGD limits and can be readily

cleared by the kidneys or oxidised to form acidic metabolites (D'souza and Shegokar, 2016).

We propose that ATPS methodology partitions AdV particles highly efficiently. It also offers a simple, scalable procure and long-term storage with low toxicity and high yield. Importantly, it avoids the need for expensive equipment and costly reagents for the production of RVSS Ad or stocks for characterisation to MVSS/WVSS and clinical grade AdV. AdV particles purified to high yields through the upper phase of PEG 600/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS can be used readily for *in vitro* and *in vivo* gene transfer. These products may be used to enter downstream processing for clinical production. ATPS is suitable to economies hoping to generate large amounts of AdV stocks for processing to generate Ad based vaccines against COVID-19.

## 5. Conclusion

The results of this study show that 20 % - 20 % w/w PEG 600 - (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> provided up to 97 ± 3% recovery yields of Ad particles by high-level partitioning of quality vector direct from producer cell lysates for *in vitro* and *in vivo* gene transfer. AdV can be produced in this way at high titre, devoid of aggregates and can be frozen for long-term storage without loss of viability. Viable particles are effective for gene transfer both *in vitro* and *in vivo* without obvious cytotoxic effects. As ATPS technology is readily scalable, without the need for expensive high-end equipment, it will be highly useful to generate RVSS of AdV for MVSS/WVSS characterisation and transport to centres throughout the world hoping to generate these particles as vaccines to counteract the coronavirus pandemic.

## Author contributions

**Saqlain Suleman:** Validation, Investigation, Writing- original draft, review and editing. **Kuteiba Schrubaji:** Validation, Investigation. **Chrysovalanto Filippou:** Validation, Investigation. **Svetlana Ignatova:** Conceptualisation, Methodology, Investigation, Supervision. **Peter Hewitson:** Conceptualisation, Investigation, Methodology, Supervision. **Jonathan Huddleston:** Conceptualisation, Validation, Methodology, Investigation. **Rajvinder Karda:** Validation, Formal analysis. **Simon Waddington:** Validation, Formal analysis, Supervision. **Michael Themis:** Conceptualisation, Methodology, Writing- Review and editing, Supervision.

## Funding source

This research was partly funded by a Brunel University London Brief award. No other public, commercial or not-for-profit sectors supported this work.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Acknowledgments

We would like to thank Generon, France for providing both AdRSVβgal and AdCMVLuc 1st generation vectors and their expertise in producing these vectors.

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