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

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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 % (NH4)2SO4 (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 (Ad5V) 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 Ad5V, 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.

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compliment transfection or infection of defective vector genomes into these cells to enable the generation of attenuated progeny virus particles (Fallaux, Kränenburg 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, Kränenburg 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 AdV5 vaccines are recognised by pre-existing anti-AdV5 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 manufacturing 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, Heikila 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 agglomeration that reduces viable infectious titre (Boychyn, Tim 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 using 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 % (NH4)2SO4 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 dH2O 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 % CO2 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 AdVRSVgal or AdVMVLuc (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 × 10⁷ 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 OD₂₆₀nm 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). AdVRSVgal and AdCMVLuc recovered by CsCl partitioning were
tested on HeLa cells for infectivity via Xgal staining for β-galactosidase or luciferase gene expression via β-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 β-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⊙m-1 (Purite, 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 - KPO4, 14 % - 14 % w/w (C6H12O6)n - PEG 8000, 14 % - 14 % w/w PEG8000 - HNa2O4P, 14 % - 14 % w/w PEG 8000 - K3HPO4, 14 % - 14 % w/w PEG 600 - (NH4)2SO4, 20 % - 20 % w/w PEG 600 - (NH4)2SO4, 14 % - 14 % w/w UCON 3900 - Na2HPO4, 14 % - 14 % w/w UCON 970 - Na2HPO4, 14 % - 14 % w/w UCON 970 - Na2HPO4, 20 % - 20 % w/w PEG 8000 - (NH4)2SO4, 14 % - 14 % w/w UCON 3900 - Na2HPO4, 14 % - 14 % w/w UCON 970 - Na2HPO4. ATPS consisting of 14 % - 14 % w/w PEG8000 - HNa2O4P, 14 % - 14 % w/w PEG 600 - (NH4)2SO4, 14 % - 14 % w/w UCON 3900 - Na2HPO4, 14 % - 14 % w/w UCON 970 - Na2HPO4 were not used due to the formation of crystal salts.

To assess the yield of vector recovery after ATPS, 10 μL containing 3.5 × 1012 IU of AdVRSV/β-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 20 min, the upper phase was collected and the vector concentration by spectrophotometry at an optical density range of dilutions. AdV genome titre (vg/mL) was calculated by multiplying the vector concentration by 1012 vector genomes/mL via spectrophotometry. Healthy CD1 mice were anaesthetised with isoflurane with 100 % oxygen and given an intraperitoneal injection of 15 mg/kg of β-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 analysed via Xgal staining for β-gal infection, and via −luciferin light emission, respectively. Significant differences between data were derived using a Mann-Whitney U test with a P value 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 ± 8.55 % for 14 % - 14 % w/w PEG 1000/K3HPO4, 47 ± 5.94 % for 14 % - 14 % w/w PEG 8000/ K3HPO4 and 83 ± 5.99 % for 20 % - 20 % w/w PEG 600/(NH4)2SO4 ATPS. Vector partitioning was mainly restricted to upper phases. The
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 AdVRSVβgal 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 AdVRSVβgal, recovered through upper phase, was 55.2 ± 2.7 % for PEG1000/K$_2$HPO$_4$, 59.9 ± 9.8 % for PEG8000/K$_2$HPO$_4$ and 97 ± 3 % with PEG600/(NH$_4$)$_2$SO$_4$ 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 ± 0% ($10^{11}$), 80 ± 3.84 % ($10^{10}$), 60 ± 1.15 % ($10^9$), 46 ± 2.19 % ($10^8$), 15 ± 1.76 % ($10^7$) and 1 ± 0.88 % ($10^6$) using serial dilutions of AdV. IMRS2 cells appeared highly permissive to AdVRSVβgal at each concentration.

3.2 Isolation of Ad directly from 293 T producer cell lysate

The potential of ATPS to partition AdVRSVβgal 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 AdRSVβgal 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 β-galactosidase gene expression were counted. HepG2 and IMRS2 infection was 93.3 ± 0.9 % versus 85.3 ± 1.8 % using PEG 1000/K$_2$HPO$_4$, 91.7 ± 2.4 % versus 30 ± 1.2 % using PEG 8000/K$_2$HPO$_4$, 91.3 ± 1.5 % versus 93.7 ± 2.2 % infection was observed with PEG 600/(NH$_4$)$_2$SO$_4$ closely matching that of percentage infection using CsCl purified AdVRSVβgal partitioned through ATPS (Fig. 3a and b).

Contaminating proteins can be detected via spectrophotometry (Glasel, 1995; Goldfarb, Saidel et al. 1951). All Abs$_{260nm}/$280nm in ATPS purified AdV direct from cell lysates are above 0.6. Further HPLC analysis of ATPS purified AdV showed contaminating proteins present but to a lower extent that in crude lysates (data not shown). This demonstrates successful partitioning of AdV into the upper phase of ATPS, with the highest recovery using the PEG 600/(NH$_4$)$_2$SO$_4$ system.

3.3 Cell survival following infection by AdRSVβgal recovered from ATPS formulations

HepG2 cell survival was quantified using AdV after ATPS treatment and AdV infection (Fig. 3a). We found HepG2 viability after AdVRSVβgal 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 ± 5.06 % and 93.25 ± 0.63 % viability with PEG 1000/K$_2$HPO$_4$ treatments. PEG 8000/K$_2$HPO$_4$ treatments showed between 87.25 ± 4.40 % and 92.20 ± 2.99 % viability.

Fig. 1. Infection of HepG2 cells by AdVRSVβgal. (a). HepG2 infection by CsCl prepared vector after partitioning by 3 ATPS formulations. Positive staining for β-galactosidase activity was identified in each ATPS upper phase, with highest infection by PEG 600/(NH$_4$)$_2$SO$_4$. Uninfected HepG2 cells appear negative for enzyme activity. (b). Quantification of cell infection: PEG1000/K$_2$HPO$_4$; 55.15 ± 2.74 %, PEG8000/K$_2$HPO$_4$; 59.85 ± 9.84 %, PEG600/(NH$_4$)$_2$SO$_4$; 97.00 ± 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 AdRSVβgal. IMRS2 infection was: $10^{11}: 100 \pm 0\%$, $10^{10}: 80 \pm 3.84\%$, $10^{9}: 60 \pm 1.15\%$, $10^{8}: 46 \pm 2.19\%$, $10^{7}: 15 \pm 1.76\%$ and $10^{6}: 1 \pm 0.88\%$ (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/K2HPO4, 91.7 ± 2.4 % and 30.0 ± 1.2 % cells stained positive for AdV infection using PEG 8000/K2HPO4. 91.3 ± 1.5 % and 93.7 ± 2.2 % cells were infected using PEG 600/(NH4)2SO4 (n = 3).
PEG 600/(NH₄)₂SO₄ 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 cytototoxicity due to ATPS treatment or Ad 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 (Albertson, Andersson et al. 1982; Oelmeier, Dismér 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, Dismér 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 AdVs with differing complex surface topologies will require suitable methodology for rapid scale-up of AdV preparations, whilst retaining its molecular weight. When combined with a kosmotropic salt, such as sodium phosphate ATPS, highly infectious AdV partitioning occurs in comparison to untreated cells (P < 0.05). This suggests no cytotoxicity via ATPS subsequent to AdV purification and recovery (n = ≥ 4).

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). 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 (Albertson, 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
be precipitated from virus preparations (Negrete, Ling et al. 2007a, b; Oelmeier, Dismer 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 % (NH$_4$)$_2$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 - K$_2$HPO$_4$, and 14 % - 14 % w/w PEG8000 - K$_2$HPO$_4$ and 20 % - 20 % w/w PEG 600 - (NH$_4$)$_2$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- (NH$_4$)$_2$SO$_4$. Using this optimal system, the quantities required for early phase clinical trials is estimated at 1 × 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).

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/ (NH$_4$)$_2$SO$_4$ partitioned vector being the highest. When using PEG 600/ (NH$_4$)$_2$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

![Fig. 5. Application of PEG 600/ (NH$_4$)$_2$SO$_4$ ATPS purified AdVLuc in vivo. AdVLuc virus was purified using PEG 600/(NH$_4$)$_2$SO$_4$ ATPS or CsCl ultracentrifugation. 1 × 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).](image-url)
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/(NH₄)₂SO₄ 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 - (NH₄)₂SO₄ 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 Ad 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 Schrjabu: 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.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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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/(NH₄)₂SO₄ 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 - (NH₄)₂SO₄ 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 Ad 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 Schrjabu: 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.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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