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Production of adeno-associated virus (AAV) serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery

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A B S T R A C T

Adeno-associated virus (AAV) is being used successfully in gene therapy. Different serotypes of AAV target specific organs and tissues with high efficiency. There exists an increasing demand to manufacture various AAV serotypes in large quantities for pre-clinical and clinical trials. A generic and scalable method has been described in this study to efficiently produce AAV serotypes (AAV1-9) by transfection of a fully characterized cGMP HEK293SF cell line grown in suspension and serum-free medium. First, the production parameters were evaluated using AAV2 as a model serotype. Second, all nine AAV serotypes were produced successfully with yields of 10^{13} Vg/L cell culture. Subsequently, AAV2 and AAV6 serotypes were produced in 3-L controlled bioreactors where productions yielded up to 10^{13} Vg/L similar to the yields obtained in shake-flasks. For example, for AAV2 10^{13} Vg/L cell culture (6.8×10^{11} IVP/L) were measured between 48 and 64 h post transfection (hpt). During this period, the average cell specific AAV2 yields of 6800 Vg per cell and 460 IVP per cell were obtained with a Vg to IVP ratio of less than 20. Successful operations in bioreactors demonstrated the potential for scale-up and industrialization of this generic process for manufacturing AAV serotypes efficiently.

1. Introduction

Viral vectors are highly evolved gene delivery systems for therapeutic interventions. Over the last ten years, adeno-associated viruses (AAV) emerged as one of the most promising vectors for gene therapy and vaccine. Mueller and Flotte (2008) have extensively reviewed clinical trials. Phase I and Phase II clinical trials utilizing AAV have been carried out worldwide that have jumped to 92 in 2012 (http://www.wiley.com//legacy/wileychi/genmed/clinical, access date: December 4, 2012) from 47 in 2007 (Edelstein et al., 2007). Safety and efficacy of AAV vectors (AAV serotype 1, 2, 6 and 8) have been evaluated in clinical trials for the treatment of different diseases, including Parkinson's disease (Eberling et al., 2008; LeWitt et al., 2011), cystic fibrosis (Moss et al., 2007; Wagner et al., 2002), hemophilia B (Manno et al., 2006, 2003), rheumatoid arthritis (Mease et al., 2010), Leber's congenital amaurosis (Hauswirth et al., 2008; Maguire et al., 2008; Simonelli et al., 2010) and treatment of lipoprotein lipase deficiency (Mingozzi et al., 2009; Stroes et al., 2008). Recently, the European Medicines Agency recommended approval of the Western world's first AAV-mediated gene therapy product (Yla-Herttuala, 2012). In animal studies, AAV has been shown to have potential to be used as a vaccine either by having attached antigen on the AAV capsids (Nieto et al., 2012; Rybniker et al., 2012) or by introducing a gene for a particular antigen (Sipo et al., 2011; Xin et al., 2001) to induce humoral response. Logan and Alexander (2012) have reviewed the potential of AAV to be used as a tool for the manipulation of immune system response. Mice challenged with human papillomavirus HPV16PsV were protected by vaccinating them with adeno-associated virus like particles (AAVLPs) consisting of VP3 containing L2 epitopes from HPV16 and HPV31 inserted at position 587 and 453, respectively (Nieto et al., 2012). AAV-HIV vector expressing HIV-1 env, tat and rev genes showed positive results when used in BALB/c mice when given as HIV vaccination (Xin et al., 2001). A vaccine against SARS coronavirus (SARS-CoV) has been developed and tested in BALB/c mice by cloning receptor binding domain in AAV vector (Du et al., 2006).

Over 100 different variant (naturally occurring or synthesized) capsid sequences of AAV have been reported. However, the most commonly studied are the eleven AAV serotypes (AAV1 to AAV11) isolated from human, simian, and rhesus and cynomolgus monkeys. Serotype AAV6 (Rutledge et al., 1998; Xiao et al., 1999) is believed to be a recombinant product of AAV2 and AAV1. AAV serotype 2 has been extensively studied for delivering genes to treat a variety of diseases. They are unable to replicate in the absence of helper viruses and to date, no known human pathogenicity have been associated with wild-type AAV infections. Also persistent long-term transgene expression levels are achieved (Xiao et al., 1996) when transducing dividing and non-dividing cells. It has

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been reported that humoral responses of infection by AAV2 is common and neutralizing AAV2 antibodies are present in the range of 35–80% of the human population throughout the world depending on the age group and the country, suggesting the limitation on the potential of AAV2 as delivery system (Chirmule et al., 1999; Erles et al., 1999; Moskalenko et al., 2000). Boutin et al. (2010) investigated humoral responses to natural exposure of AAV types 1, 2, 5, 6, 8 and 9 and reported that all four IgG subclasses were produced. They concluded that vectors based on AAV5, AAV8 and AAV9 are the best candidates for gene therapy in humans. Many researchers considered the use of different serotypes of AAV to circumvent the pre-existing immune response. More importantly, it has been observed that serotypes of AAV transduce cells from different organs with various efficiencies. Therefore, a specific AAV serotype could be selected for efficient gene delivery into target tissues. Van Vliet et al. (2008) have summarized the tissue specific transduction efficiencies. Asokan et al. (2012) reviewed advances in the use of AAV for gene therapy application. They summarized the best combinations of AAV serotypes with organs, e.g., liver (AAV8), cardiac and musculoskeletal (AAV1, AAV6 and AAV9), central nervous system (e.g., for Parkinson, Alzheimer's Batten's epilepsy, lysosome storage disorder: AAV5 and AAV9), and eye (AAV8). The serotypes AAV1 to 8 were investigated to transduce rat myocardium (Palomeque et al., 2007) out of which AAV1 and AAV6 were identified as the best candidates; the efficacy of AAV1, 2 and 5 was compared for the delivery of gene to different regions of the nervous system (Burger et al., 2004); for gene delivery to heart when compared to AAV1, 6, 7, and 8, AAV9 was the most efficient (Bish et al., 2008); and AAV6 was shown to be more efficient in delivering gene to cornea in vitro when compared to AAV8 and AAV9 (Sharma et al., 2010). AAV serotypes 1, 5, 7 and 8 among the serotypes 1–8 tested showed most effective cell mediated immunity response in muscle when compared to AAV2 (Xin et al., 2006). Attempts have been made in constructing laboratory-derived serotypes to target single tissue type to minimize the AAV dosage required for a treatment. For example, Asokan et al. (2010) engineered AAV2i8 from AAV2 and AAV8 to change the properties of the new virus construct. This construct reduced the gene expression in mouse liver by 40 folds (in contrast to AAV8) while high levels of expressions were observed in the muscle cells (in contrast to AAV2). Relatively very low levels of expressions were observed in Brain. Lin et al. (2009) showed that recombining different capsids increased the responses to HIV-1 gag which is a further advancement to the use of AAV as vaccine. This showed that by mixing different serotypes, the properties of AAV could be engineered to maximize the expression in the target tissue. The delivery of AAV vaccine by selecting intranasal, subcutaneous or muscular route has different effect on immune response (Xin et al., 2001).

Major advancements have been realized in the molecular engineering, structural analyses and processing of AAV vectors. Grieger et al. (2006) published a detailed protocol on the production and characterization of AAV vectors. The use of serum-free medium has also advanced the commercial manufacturing of the AAV vectors for human use. Aside from these improvements, the supply of AAV vectors for clinical studies for the proof of concept remains challenging, thus limiting the number and duration of clinical trials. The need for different serotypes to target specific tissues has further added complexity to the production, purification and analytical procedures.

There are different modes of AAV production. AAV has been produced successfully in producer cell lines and packaging cell lines (Chadeuf et al., 2000; Clark et al., 1995; Farson et al., 2004; Gao et al., 2002; Liu et al., 2000; Thorne et al., 2009; Yuan et al., 2007) where the cells could be anchorage dependent or in suspension. Producer cell lines contain all AAV production components (e.g., vector genome and AAV Rep & Cap) and helper virus (e.g., Adenovirus type 5) is used to induce. Packaging cell lines use two viruses, one to induce the AAV rep-cap genes present in the packaging cell line and the other to supply the gene of interest to be encapsidated. Stable cell lines (Clark et al., 1995; Liu et al., 2000) are more practical for large-scale manufacturing with consistently high yields after the success of the proof of concept. Transient transfection technology, however, offers a rapid generation of material for the proof of concept. For transient transfection the components required for AAV production are supplied by plasmids, or in a combination of plasmids and a helper virus. Transient transfection has been done using adherent cells in cell plates (Salvetti et al., 1998; Zolotukhin et al., 1999), cell factories (Drittanti et al., 2001) or roller bottles (Liu et al., 2003). When the scaling up of the process is desired in a short period of time to generate pre-clinical/clinical material or material required for the proof of concept, methods using adherent cells may need more incubator space and are time-consuming and labor-intensive processes. Technology is advancing to support the anchorage dependent cell growth as well by providing up to 100 layered CellCube® units (Corning Life Sciences, Tewksbury, MA, USA) with liquid volume of 6000 mL which can support more than 10^{10} cells. Selecting cells that are able to grow in suspension is an alternative for scaling up in conventional bioreactors (Durocher et al., 2007; Park et al., 2006; Reed et al., 2006). A major advantage of transient transfection is that serotype-specific vectors of AAV can be easily generated in a short period of time by supplying a capsid/replication plasmid of choice, along with the transgene and helper plasmids. Therefore, transfection is the most chosen mode for AAV production to rapidly generate preclinical material. Another approach using the insect cell-baculovirus system for AAV manufacturing has recently attracted attention and has shown great potential for the large-scale manufacturing of clinical material (Meghrous et al., 2005; Mena et al., 2010; Urabe et al., 2002). The advantages and challenges of the different methods have been extensively reviewed elsewhere (Aucoin et al., 2008).

To scale-up the production of any clinical grade biological material, it is very important to have a cell line that maintains a high yield of the product. The cell line used for the production must be generated in a sufficient quantity as a Master Cell Bank (MCB) with full characterization. NRC-Montreal facility has generated and is maintaining such a cell line, the human embryonic kidney (HEK) 293SF-3F6 cell line, which meets the requirements of MCB for manufacturing under GMP guidelines. In this work, it is demonstrated that this cell line is capable of producing sufficient quantities of different AAV serotypes.

The plasmid/mammalian cell system described in the present study is based on transfection of HEK293SF-3F6 by three plasmids employing polyethylenimine (PEI) as the DNA transporter in a serum-free medium. The system was used for the production of different serotypes of AAV. Among the plasmids supplied to produce different serotypes of AAV, the AdV helper and transgene plasmids remained the same in all productions. The plasmid containing the capsid proteins supplied for the production of AAV dictated the serotype produced. Assuming that the production of different serotypes may be less dependent on the type of capsid plasmid used, selecting one serotype as a model has minimized the number of experiments necessary to evaluate parameter optimization; therefore, generalizing the production protocol, which would be suitable to produce all serotypes available. From the available serotypes, AAV2 was selected as a model AAV for the investigation of production parameters. The effects of plasmid DNA concentration at different cell densities on the transfection efficiency for the production of AAV were investigated. Using optimal transfection conditions, nine serotypes of AAV (AAV1-9) were produced in shake flasks to demonstrate the production of multiple serotypes in the cGMP HEK293SF-3F6 cell line. Scalability was evaluated by

producing two serotypes (AAV2 and AAV6) in three runs of 3-L bioreactor.

2. Materials and methods

2.1. Plasmid constructs

Triple plasmids used at a weight ratio of 1:1:1 contained the Rep/Cap plasmid of adeno-associated virus type 1–9, which comprised the gene sequences for replication and structural proteins. The transgene plasmid of ITRCMV-eGFP (single stranded), which harbored the transgene for enhanced green fluorescent protein (eGFP), was driven by the cytomegalovirus promoter (CMV) between AAV inverted terminal repeats (ITRs). The adenovirus helper-functions were supplied by pXX6-80 plasmid that contains genes for E1A, E1B, E4 and E2A proteins and VA RNAs. The AAV pseudotypes 1, 2, 3, 4, 5, 6, 7, 8 and 9 are pseudo-packages of rAAV-containing capsids of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 and AAV9, respectively, with the ITRs of AAV2. The transgene plasmids, pXX6-80 and serotype-specific plasmids (AAV1-6, 8 and 9) were provided by Jude Samulski from Samulski Laboratory, Gene Therapy Center, University of North Carolina at Chapel Hill (http://genetherapy.unc.edu/services.htm) with the exception of the plasmid for serotype 7 (pAAV2/7) that was obtained from The Penn Vector Core, University of Pennsylvania, Philadelphia, PA (http://www.med.upenn.edu/gtp/vectorcore/ServicesRates.shtml).

All transfections were done using 25-kDa linear polyethylenimine (PEI) from Polysciences Inc. (Warrington, PA, USA) as a DNA transporter.

2.2. Cell lines

HEK293SF-3F6 (cGMP cell line from National Research Council Canada, Montreal, Qc, Canada, www.nrc-cnrc.gc.ca/eng/rd/hht/index.html) used for AAV production, were grown and maintained as a suspension culture in serum-free HyClone Thermo Scientific HyQ SFM4Transfx-293 medium supplemented with 1 g/L Pluronic F-68, 4 mM L-glutamine and 2 g/L $NaHCO_3$. HeLa cells were maintained in DMEM containing 10% FBS. Both cell lines were propagated at 37 °C in 5% CO_2.

2.3. AAV production protocols

HEK293SF-3F6 cells were grown in 19 mL of serum-free HyQ SFM4Transfx293 medium and transfected with 1 mL transfection mixture in 125-mL shake flasks. After 48 h of production, 10% (v/v) of lysing buffer concentrate (20 mM $MgCl_2$, 1% Triton X-100 in 500 mM Tris buffered solution, pH 7.5) and 1% (v/v) of freshly diluted Benzonase® (Merck KGaA, Darmstadt, Germany) to supply a final concentration of 50 units benzonase/mL to digest host-cell DNA and unpackaged virus DNA were added to the harvested cell culture. After 1 h of incubation at 37 °C with agitation, concentrated $MgSO_4$ was added to obtain a final concentration of 37.5 mM; the solution was incubated further for 30 min (Chahal et al., 2007). The addition of $MgSO_4$ was necessary to avoid AAV aggregation and AAV binding to other cellular components released during lysis.

To study the effect of different media on AAV yield, when the cells under maintenance (in SFM4Transfx-293 medium) reached a cell density of 1×10^6 cells per mL, 20 mL of cell culture were centrifuged at $385 \times g$ for 10 min and then resuspended either in F17 (Invitrogen, Burlington, Ontario, Canada) or in SFM4Transfx-293 (Thermo Scientific Hyclone, Logan, UT, USA) fresh medium. Earlier experiments had indicated (data not shown) that SFM4Transfx-293 and F17 media were good for cell growth but the AAV yield was less in F17 medium. Therefore, it was decided to see if the cell cultures maintained under SFM4Transfx-293 medium and then replacing with F17 medium at the time of infection would be beneficial for AAV production. The harvested samples were purified by iodixanol gradient before assaying for genomic and infective viral particles.

To verify the effect of cell density and amount of DNA supplied, cells at different cell densities were transfected with either 1 μg/mL or 1 μg/million cells basis. Cells were grown up to about 1×10^6 cells/mL and appropriate volume was then centrifuged and cells were resuspended in fresh medium to obtain about 1, 2, 4 or 8 million cells/mL. These cell densities were transfected in 20 mL cell culture volume with PEI to DNA ratio of 2:1. Samples were taken every 12 h post transfection (hpt).

To evaluate the potential positive effect of histone deacetylase, sodium butyrate and butyric acid (neutralized by sodium hydroxide to a neutral pH; referred to as butyric acid) were added to the cell culture at a cell density of 1 million cells/mL. The final concentration of these additives was 0 (as control), 1, 2.5, 5, or 10 mM from 1 M stock solutions. After 48 hpt, a portion of the cell culture was monitored by FACS for percent transfected cells and relative amount of GFP signal from the GFP positive cells. The other portion of the cell culture was lysed to determine the concentrations of the infectious viral particles (IVP/mL).

2.4. Bioreactor production of AAV

A 3-L Chemap CF-2000 bioreactor (Mannedorf, Switzerland), equipped with two 45° pitched blade impellers and three vertical surface baffles, was used to investigate the scalability of the process through production of the following two serotypes: AAV2, and AAV6. The listed serotypes were selected because of subsequent studies required at larger scales. The final working volume was adjusted to 2.0 L. The agitation was set to 80 rpm, and the temperature was maintained at 37 °C. A pH of 7.2 was monitored and controlled by CO_2 supplementation during the experiment. Dissolved oxygen was maintained at 40% by supplementing the inlet gas with oxygen using computer-controlled mass-flow controllers and FIX-MMI software (Intellution, Norwood, MA). The bioreactor was inoculated at a cell density of approximately 0.5×10^6 cells/mL with viability greater than 95%. When the cell density reached approximately 1.0×10^6 cells/mL, the cells were transfected with the PEI/DNA complexes (polyplexes) with a PEI to DNA ratio of 2:1. At the time of harvest, AAV from the cell culture in the bioreactor was released using the Triton X-100 method. All solutions were added directly to the bioreactor, and the lysate was centrifuged at $4000 \times g$ for 20 min. The supernatant was stored at −80 °C for further processing.

2.5. Purification of AAV by iodixanol step gradient protocol

AAV samples (12.3 mL) were purified by overlaying them on top of series of step gradients using 15, 25, 40 and 54% iodixanol concentrations (adapted from Zolotukhin et al., 1999) containing 5, 5, 7 and 5 mL, respectively. The 15% iodixanol concentration also contained 1 M NaCl to avoid aggregation of AAV with other cellular proteins and negatively charged nuclear components. After the completion of centrifugation, 5 mL were withdrawn from 2 mm below the 40/54 interface marked before starting the ultracentrifugation at $385{,}000 \times g$ for 1 h 45 min in Sorvals T-865 rotor in Sorval Ultracentrifuge.

2.6. Quantitation of viral vectors

AAV infectivity was determined by the gene transfer assay (GTA) using GFP as a reporter gene in all cases. The procedure described by Cheng et al. (1997) was adapted for AAV infectivity assay where sample is diluted before addition to the cells to have the GFP

Table 1
Comparison of AAV productions in F17 and SFM4Transfx-293 media. The experiments were done in duplicates.

	AAV production in F17 medium	AAV production in SFM4Transfx-293 medium	*P* value
Viable cell density at 48 hpt	1.56E+06	2.13E+06	
% Cells expressing GFP	37	50	
Infective viral particles (IVP/mL)	1.28E+08 ± 0.13E+08 Std dev = 1.84E+07	3.33E+08 ± 0.82E+07 Std dev = 1.16E+07	0.006**
Genomic viral particles (VG/mL)	2.53E+09 ± 0.15E+09 Std dev = 2.12E+08	5.50E+09 ± 0.13E+09 Std dev = 1.83E+08	0.004**
Vg/IVP ratio	20	17	
IVP/cell	82	156	
Vg/cell	1620	2580	

** *P* value <0.01 indicates that IVP and VG concentrations obtained in F17 and SFM4Transfx-293 media are significantly different at 99% confidence level.

positive cells in the range of 2–20% to assure that only single virus has entered the cell for GFP expression. The lysed samples either were assayed with or without the purification by iodixanol step gradient ultracentrifugation protocol. Infectivity assay is independent of the contaminant DNA, but reveals the percent recovery from the iodixanol purification step. The GFP-positive cells were quantified by FACS using HEK293 cells in suspension as described by Chahal et al. (2007) or HeLa cells as anchorage dependent cells but with the similar protocol. Briefly, the HeLa cells were co-infected with wild-type Adenovirus similar to HEK293 cells. After 24 h of infection, the cells were washed with PBS and trypsinized for 15 min at 37 °C. The cells were suspended in medium containing 10% serum to inactivate the trypsin and the cells were centrifuged and resuspended in 2% formaldehyde solution in PBS. The GFP-positive cells were quantified by FACS.

AAV genomic particles were determined by real-time-qPCR. Lysed samples treated with Benzonase® were purified by iodixanol step-gradient protocol. The purified samples were diluted 100-fold and retreated with 50 units of Benzonase®/mL to ensure the removal of host-cell DNA and unpackaged virus DNA. The High Pure Viral Nucleic Acid kit (Roche Applied-Science, Indianapolis, USA), was used to purify viral nucleic acids from AAV samples before real-time polymerase chain reaction (RT-PCR). The samples were diluted in water and supplemented with Poly A and Proteinase K solutions. A standard curve was generated for each RT-PCR run. The reaction was performed in the LightCycler 3 instrument (Roche Diagnostics, Québec, Canada). The reactions were performed in a total volume of 20 μL containing 18 μL of PCR Mix, 12 μL H_2O, 1 μL forward primer 5 mM, 1 μL reverse primer 5 mM, 4 μL Master Mix LightCycler Fast-Start SYBR Green I (Roche Diagnostics, Québec, Canada) and 2 μL of template (serial dilution of standard from 10^3 to 10^8 molecules, unknowns or water for the negative control). The PCR conditions were as follows: pre-incubation at 95 °C for 10 min; denaturation at 95 °C for 15 s; annealing cycles at 55 °C for 35 s; and extension at 72 °C for 40 s. The CMV Forward primer (5′-CAAGTACGCCCCCTATTGAC-3′) and the CMV Reverse primer (5′-AAGTCCCGTTGATTTTGGTG-3′) were used. Data analysis was performed using LightCycler 3.5 software (Roche Diagnostics, Québec, Canada).

3. Results

3.1. Effect of type of culture medium

SFM4Transfx-293 medium ranked higher in all respects (Table 1). The AAV yields in terms of infective viral particles and viral genomic particles were higher in SFM4Transfx-293 medium than F17 medium when compared on volumetric and cell specific basis. Considering the percent error associated with infective viral particle assay (up to ±30%), the Vg/IVP ratio may be considered similar in both cases. SFM4Transfx-293 medium was selected as a medium of choice for AAV production because volumetric and cell specific infective viral particles produced were twice as much as obtained in F17 medium.

3.2. Effect of the amount of plasmid DNA on transfection efficiency and AAV yield

When the cells were transfected with 1 μg/mL on volumetric basis, except for 8.0E+06 cells/mL cell culture, the higher the initial cell density, the higher was the transduced cells reflected by GFP positive cells, whereas, IVP yields were consistently approximately 1.0E+08 IVP/mL cell culture (Fig. 1). The transfected GFP positive cells did not increase proportional to the increase in the initial cell density. When the DNA plasmid was supplied on the basis of 1 μg DNA/10^6 cells, percent cells transfected improved relative to 1×10^6 cells/mL cell culture (Fig. 2). However, again by proportionally increasing the plasmid DNA for higher cell density did not bring similar proportional increase in GFP positive cells as were obtained by 1×10^6 cells/mL cell culture set.

Genomic particles purified by iodixanol protocol and infective viral particles in the lysate were consistently approximately 6.0×10^9 Vg/mL cell culture and 7.0×10^8 IVP/mL cell culture, respectively, for all cell densities, even though, the cell densities at 48 hpt were higher for higher initial cell densities (Fig. 3). To reflect the efficiency of the production of AAV by total cells at 48 hpt, the cell specific yield was highest for the 1×10^6 cells/mL cell culture and decreased for higher initial cell densities. About 2500 Vg/cell

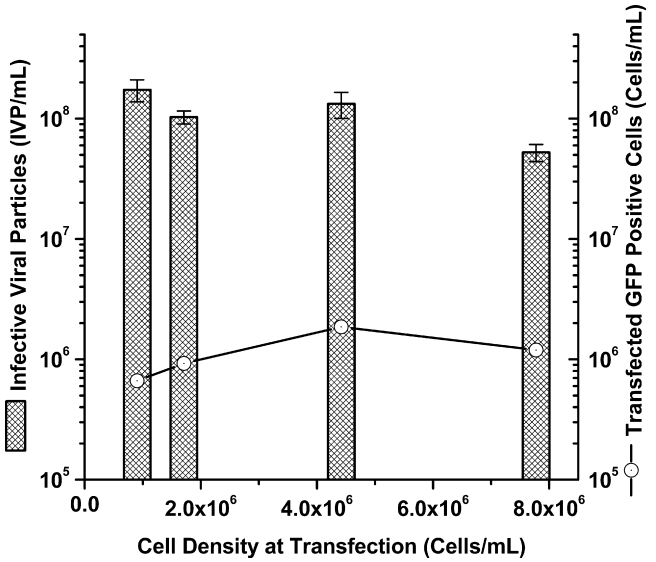

Fig. 1. Cells at various cell densities (1, 2, 4 or 8 million cells/mL) were transfected with 1 μg plasmid DNA/mL cell culture in fresh medium. These cell densities were transfected in 20 mL cell culture volume with PEI to DNA ratio of 2:1. The cell cultures were harvested 48 h post transfection (hpt). The infective viral particles are reported as IVP/mL cell culture.

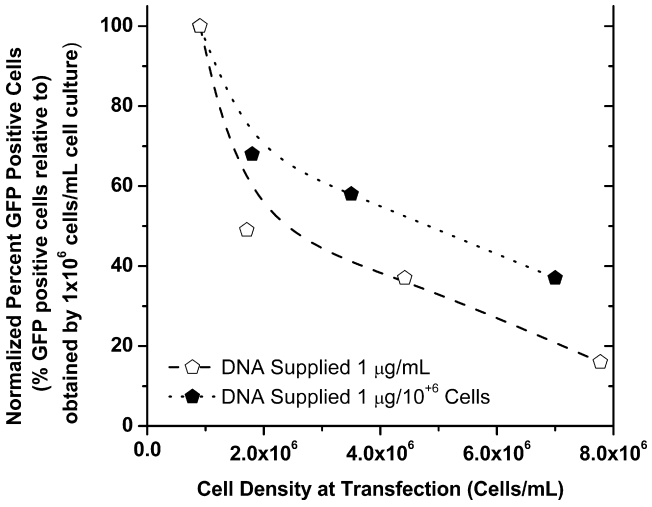

Fig. 2. Cells at various cell densities (1, 2, 4 or 8 million cells/mL) were transfected with 1 μg plasmid DNA/million cells in fresh medium. These cell densities were transfected in 20 mL cell culture volume with PEI to DNA ratio of 2:1. The cell cultures were harvested 48 h post transfection (hpt). Comparative data from Fig. 1 has been re-plotted to reflect percent GFP positive cells normalized to GFP positive cells obtained by transfecting at cell density of 1.00E+06 cells/mL.

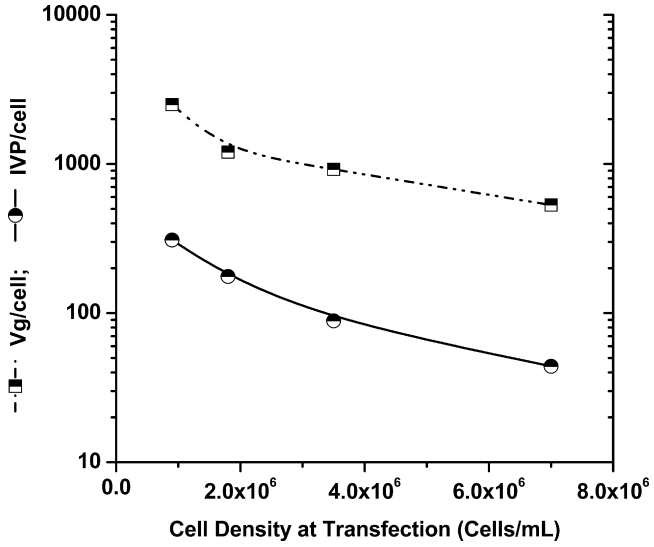

Fig. 4. Cell specific genomic and infective viral particle concentrations as a function of cell density at transfection. Production and harvesting conditions is same as in Fig. 1.

and 310 IVP/cell were obtained at cell density of 1×10^6 cells/mL (Fig. 4).

3.3. Effect of butyrates on transfection efficiency and AAV yield

No increase in the number of transfected cells by the addition of sodium butyrate or butyric acid was observed at the concentrations tested. The percentage of transfected cells measured by FACS (at least 10,000 counts) remained at approximately 40% (Fig. 5a). However, the transfected cells showed a higher amount of GFP expression at higher concentrations of sodium butyrate or butyric acid (Fig. 5b). No improvement in the yield of AAV production by the addition of sodium butyrate or butyric acid was observed. Infectious vector yields for AAV production experiments containing butyric acid or sodium butyrate were found to be lower when compared to the corresponding controls (Fig. 5c).

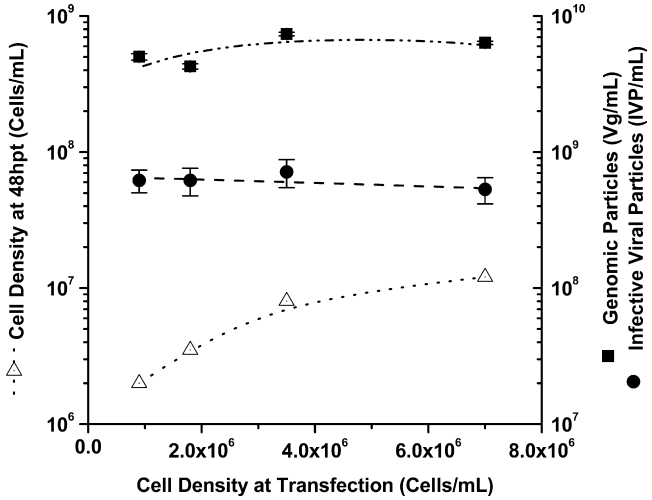

Fig. 3. Cell density and virus yield (in terms of genomic and infective viral particle concentrations) as a function of cell density at transfection. Production and harvesting conditions is same as in Fig. 1. The genomic particles and infective viral particles are reported as Vg and IVP per mL cell culture, respectively.

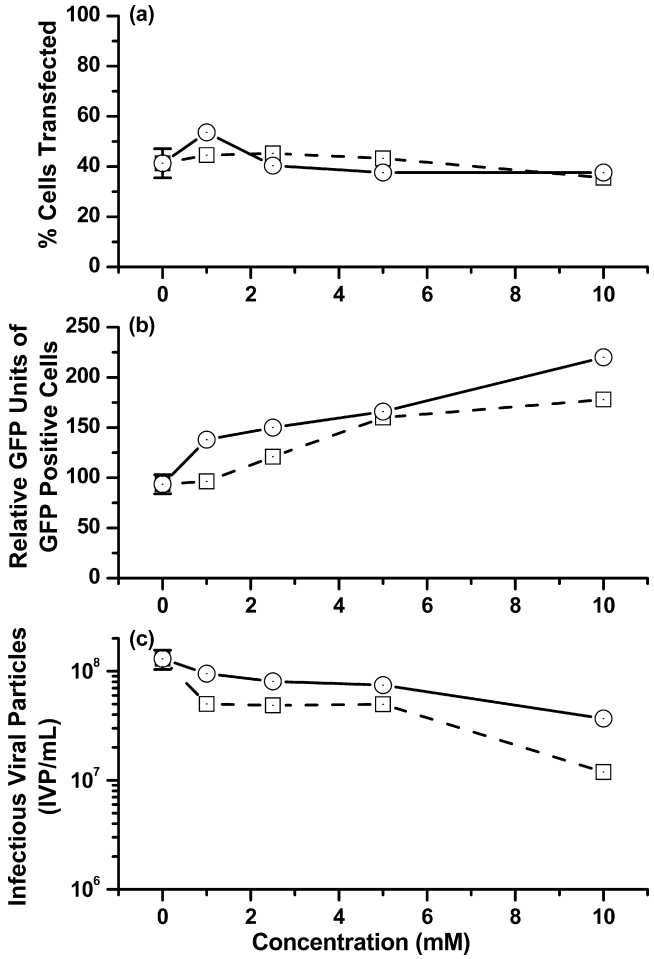

Fig. 5. Effect of butyric acid (circles) and sodium butyrate (squares) on the transfection efficiency (a), GFP expression level of transfected cells (b), and AAV2 yield represented by infective viral particles (c). The infective viral particles are reported as IVP/mL cell culture.

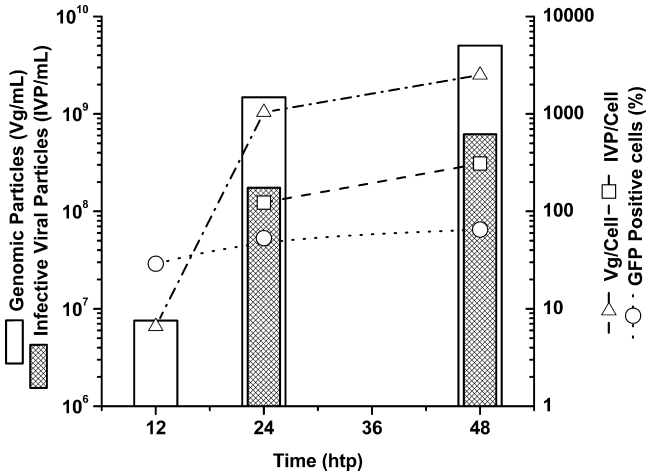

Fig. 6. The production kinetics of AAV2 includes transfection efficiency and AAV2 yield (in terms of volumetric and cell specific) as a function of time. The genomic particles and infective viral particles are reported as Vg and IVP per mL cell culture, respectively.

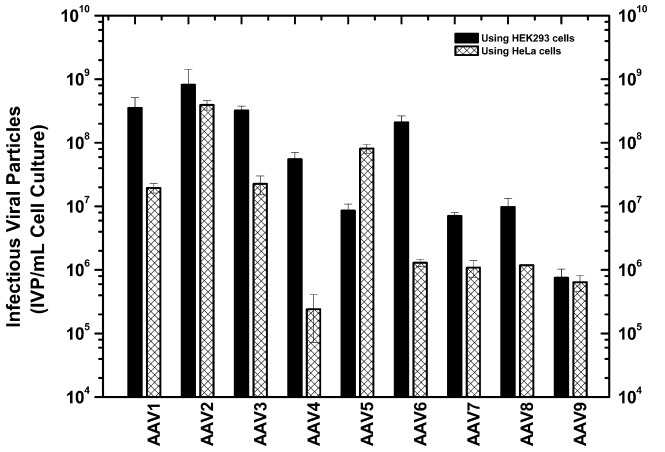

Fig. 7. AAV serotypes (AAV1-9) were produced using HEK293SF cells at cell density of 1.00E+06 cells/mL in 20 mL of serum-free medium in 150-mL shake flasks in duplicates. The cell cultures were harvested 48 h post transfection (hpt). Infective viral particles were assayed using HEK293 and HeLa cells.

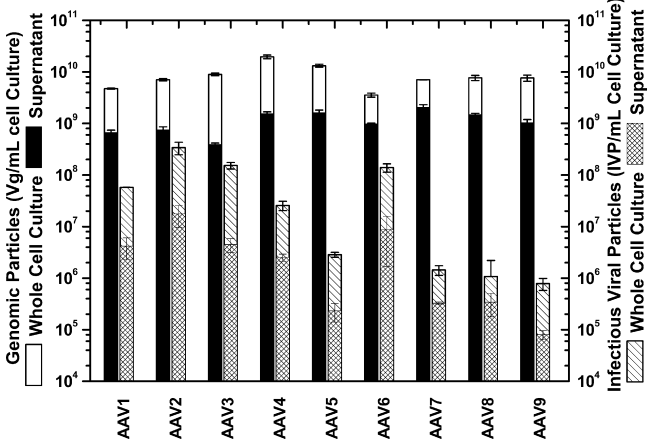

Fig. 8. AAV serotypes (AAV1-9) were produced using HEK293SF cells at cell density of 1.00E+06 cells/mL in 20 mL of serum-free medium in 150-mL shake flasks in duplicates. The cell cultures were harvested 48 h post transfection (hpt). The whole cell cultures and supernatant of cell cultures (by removing cell pellet by centrifugation) were purified by iodixanol protocol, and then assayed for genomic (by PCR) and infective viral particle (using HEK293 cells) concentrations.

3.4. Kinetics of AAV2 production

The production kinetics of AAV2 is shown in Fig. 6. Volumetric production of genomic and infective viral particles increased as a function of time. On average, iodixanol purification protocol resulted in about 80% recovery of Infective viral particles from cell lysate. Since it was not possible to estimate genomic particle concentration in cell lysate without purification, it is expected to have similar recovery from the viral genomic particles/mL of iodixanol purified AAV samples as obtained by the infective viral particle recovery by iodixanol purification method. However, the results in Fig. 6 are presented as of purified AAV samples without correcting for 80% recovery but backtracked to the volume of cell culture used. For example, 5 mL of Iodixanol purified AAV came from 12.3 mL of cell culture lysate, therefore a factor of 12.3/5 = 2.46 was used to calculate the nuclease resistant genomic particles of AAV produced in the cell culture.

3.5. Productions of AAV serotypes in shake flasks

To demonstrate that this production method is generic and applicable to any serotype, the same methodology was applied for the production of other AAV serotypes (AAV1-9) using HEK293SF cells by transfection in 20 mL of serum-free medium in 150-mL shake flasks in duplicate. Infective viral particles were assayed using HEK293 and HeLa cells. Fig. 7 showed that AAV yield varied depending on the serotypes but was independent to the type of cells (HEK293 or HeLa) used for infectivity assay.

The similar experiment was repeated but this time the genomic and infective viral particles were assayed from iodixanol purified cell lysates and culture supernatants to see the cell associated (intracellular) and released (extracellular) AAV. Fig. 8 showed that the genomic particles produced after 48 hpt were in the same order of magnitude (~10^{10} Vg/mL cell culture) for all AAV serotypes whereas 4–28% were present in supernatant. Table 2 shows percent AAV in the supernatants of different AAV serotypes production cultures at 48 hpt. Most of the AAV serotypes produced are cell associated. At 48 hpt, AAV6, AAV7 and AAV8 showed higher proportion of Vg in supernatant (28, 29 and 19%, respectively). AAV serotypes produced different levels of infectious viral particles. It should be noted that AAV infectivity assay is done using either HEK293 (or HeLa cells in Fig. 7) and because AAV serotypes are tissue specific therefore, infective assay does not reflect similar information for comparative purpose, but should be used only as an indicator that the vectors are functionally active to infect cells and are capable of expressing the gene.

Table 2
Percent genomic particles in the supernatants of different AAV serotype productions.

AAV serotypes	% Genomic particles in the supernatant[a]
AAV1	14
AAV2	11
AAV3	4
AAV4	8
AAV5	12
AAV6	28
AAV7	29
AAV8	19
AAV9	14

[a] The genomic particles obtained by real-time qPCR were back calculated for concentration in cell culture by diving by 2.46 factor (12.3 loaded on tube and 5 mL withdrawn). Percent genomic particles present in the supernatant were calculated by dividing the Vg in supernatant/mL cell culture by Vg in whole cell culture/mL (shown in Fig. 8) multiplied by 100%.

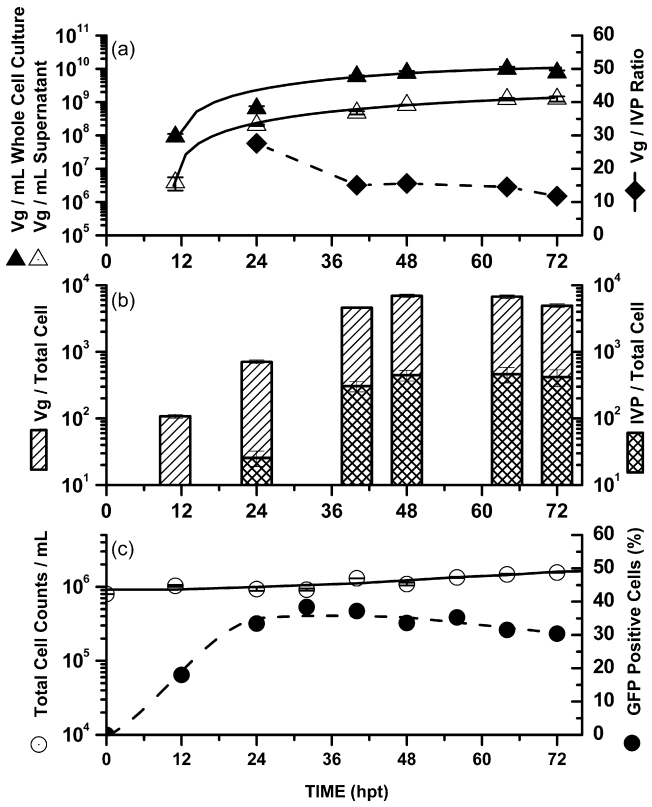

Fig. 9. Bioreactor was inoculated at a cell density of 0.35E+06 cells/mL with 97% viability. The cells were transfected with the PEI/DNA complexes (polyplexes) with PEI to DNA ratio of 2:1 in a serum-free medium to produce AAV2. The total cell culture volume at the start of transfection was 2000 mL. Samples were taken every 8 or 12 h intervals up to 96 hpt. This figure shows the genomic particle yield (in Vg/mL whole cell culture and Vg/mL supernatant of cell culture) and Vg/IVP ratio in (a), the cell specific yields (in terms of Vg/total cell and IVP/total cell) in (b), and total cell counts/mL culture and percent of GFP positive cells in (c).

3.6. Productions of AAV serotypes in bioreactors

To demonstrate scalability and potential industrialization of the process, the transfection was performed in a controlled bioreactor. The production kinetics were similar for both serotypes (AAV2 and AAV6); therefore, detailed kinetics have been reported only for the production of AAV2 (Fig. 9a, corresponding to Run#01 in Table 3). The samples were taken at 8–12-h intervals. The cells continued to grow to a density of 1.87×10^6 cells/mL, and the viability at 72 hpt was 82%. About 10–15% Vg and IVP were detected in the supernatant similar to shake flask experiments (Table 2 and Fig. 8). The cell specific production in terms of Vg/total cells and IVP/total cells (Fig. 9b) were also in the same order of magnitude as obtained in the shake flask experiments (Fig. 6). Transfection showed that 40% of the cells were expressing GFP. After 36 hpt even though the viability started to decrease, the total cell counts were still increasing, therefore, percent GFP positive cells started to show a decline (Fig. 9c).

Table 3
Yields of AAV2 and AAV6 obtained in 3-L bioreactors.

Serotypes	Vg/mL (RSD[a] = 3%)	IVP/mL (RSD[a] = 23%)	Vg/IVP
Run#01: AAV2	1.0E+10	6.8E+08	15 (std dev ±3)
Run#02: AAV2	1.2E+10	6.1E+08	20 (std dev ±5)
Run#03: AAV6	2.0E+09	1.7E+08	12 (std dev ±3)

[a] RSD = relative standard deviation (assayed from the same sample).

The whole cell culture broth was treated in the bioreactor to maximize the recovery of AAV produced and to streamline the overall process by eliminating steps such as harvesting, centrifugation (or microfiltration) and resuspension of the cell pellet to disrupt the cells for virus extraction. Concentrations of Triton X-100 lower than 0.4% did not affect the activity of Benzonase®, which was used to reduce non-viral DNA and non-encapsidated AAV DNA present in the lysate. Benzonase addition also resulted in a reduction of the medium viscosity. The Triton X-100-treated cell culture gave a similar infectious AAV yield compared to the yield by the freeze/thaw method (data not shown). A maximum of 2×10^{13} Vg and 1.3×10^{12} IVP of AAV2 were produced from 2 L of cell culture produced in 3 L bioreactor in less than 64 hpt. The yields of two runs of AAV2 and one run of AAV6 obtained in the bioreactor experiments are summarized in Table 3.

4. Discussion

Figs. 1 and 2 demonstrated that there was certain improvement in the overall %GFP positive cells for higher cell densities when the plasmid DNA was supplied on the basis of 1 μg/million cells, but improvement was not worth the cost of addition of extra plasmid. The cell cultures with higher DNA concentrations showed significantly larger number of aggregates. This result was probably due to excess plasmid DNA or a high concentration of PEI associated with DNA in polyplexes formed with a higher DNA load at the time of transfection. Polyplexes have a net positive charge. It is hypothesized that polyplexes, when present in excess, may act as bridges between the cells that are usually negatively charged and cause aggregation. Therefore, a PEI to DNA ratio of 2:1 was used in all the transfections done in this study based on earlier work in the author's laboratory. These observations and recommendations were dependent on the formulation of the serum-free medium selected. Because of the proprietary nature of the commercial serum-free medium used, it was difficult to speculate the effect of other macromolecules present in the cell culture medium. It was also difficult to assess the interactions with polyplexes and the mechanism of transfection. Park et al. (2006) showed that the quantity of DNA that was optimal for AAV production was dependent on the cell line, the mode of production (such as anchorage or suspension) and the type of the medium. Optimal DNA concentrations for suspension cells were 6–12 μg/mL for HEK293 and HEK293T (adapted for suspension) with yields of 24,000 and 115,000 Vg/cell, respectively. For the anchorage dependent cells, the optimal DNA concentration was 3 μg/mL for HEK293T with a yield of 65,000 Vg/cell (Park et al., 2006). It should be noted that methods for determining genomic particles are not standardized and vary from one laboratory to another (Aucoin et al., 2008). Among the different media tested, Hildinger et al. (2007) reported that optimal AAV yield was obtained with 1.25 μg DNA/mL with a PEI:DNA ratio of 3:1 in a medium composed of 1:1 mixture of RPMI and Ex-Cell media. It was also reported that cutting down the DNA to half reduced the yield by about 50% but by increasing the DNA did not have proportional gain in the yield.

The cell-specific yield at low cell densities has been reported between 100 and 17,000 Vg/cell (Chadeuf et al., 2005; Collaco et al., 1999; Drittanti et al., 2001; Feng et al., 2007; Okada et al., 2001) for GFP or lacZ reporter gene production by HEK293 cells using plasmid transfection technology. Volumetric production did not increase proportionally at cell densities higher than 1.0×10^6 cells/mL (Fig. 3), which was also reflected by the cell-specific yield (Fig. 4). Similar reduced transfection efficiency was observed by Hildinger et al. (2007) and they concluded that it could be because DNA-PEI polyplexes were added on a volumetric basis. The low yield in cell cultures at higher cell densities

could be due to the exhaustion of some key components from the medium necessary for AAV production and cell maintenance. These observations also indicated that there is a potential for improvement in the AAV production yield by optimizing the medium and designing some feeding strategies for high cell density cultures in a fed-batch mode. This problem remains largely unsolved and requires a more detailed analyses and a better understanding of the cell metabolism and kinetic mechanisms involved in the transfection and expression processes. The "cell density effect" is a metabolic limitation that has been observed in the author's laboratory with other expression systems. Henry et al. (2004) addressed this phenomenon by proposing a perfusion system for adenovirus production to alleviate nutrient limitations caused by high cell density cultures. For this study, it was decided to keep producing AAV by transfecting cells at the cell density of 1×10^6 cells/mL until other means of medium replacement or fed-batch techniques are developed or additives are found to improve the transfection efficiency and AAV yield.

Sodium butyrate and butyric acid, for the regulation of gene expression by keeping histones in the acetylated state (inhibition of histone deacetylase) for easy accessibility of regulatory factors to DNA (Vidali et al., 1978) were exploited to improve the production yield of AAV. In some cases, the use of sodium butyrate had increased (Backliwal et al., 2008; Hunt et al., 2002; Jiang and Sharfstein, 2008; Sena-Esteves et al., 2004) the yield of proteins and viruses, and in other cases, it had no effect (Sena-Esteves et al., 2004). In the present study, although the expression levels of the transgene in the transfected cells were directly proportional to the concentrations of sodium butyrate or butyric acid, their use did not contribute to increase the yield of AAV production. Based on the high expression of GFP protein, the addition of sodium butyrate or butyric acid would also have produced Rep proteins in excess. The Rep proteins have a toxic effect on HEK293 cells (Chang and Shenk, 1990; Chang et al., 1989; Clark et al., 1995; Li et al., 1997; Yang et al., 1994; Zhou and Trempe, 1999); therefore, the low AAV yield in the presence of sodium butyrate or butyric acid could be due to accumulation of other toxic proteins in the cell culture environment. Lentivirus production by HEK293 cells has been increased by the use of sodium butyrate (Ansorge et al., 2009; Karolewski et al., 2003) and caffeine (Ellis et al., 2011). AAV2 production was not increased by sodium butyrate (present study) or caffeine (Ellis et al., 2011) using HEK293 cells. However, an increase in AAV production has been shown by the addition of 5 mM sodium butyrate when the insect cells/baculovirus system was used (Huang et al., 2007) and baculovirus-mediated transduction of HEK 293 cells gave higher AAV yield by the addition of sodium butyrate (Huang et al., 2007).

Kinetics of AAV production showed that the infectious titers lagged behind the genomic particle titers most likely because their maturation into infectious particles took place after the formation of capsids and encapsidation of the viral genome (Fig. 6). Viral genomic (Vg) particles started to accumulate as early as 12 hpt however, IVP were not seen until 24 hpt. This could be due to 12 h sampling interval and sensitivity of assay. The genomic viral particles are produced earlier and infective viral particles are matured later. Myers and Carter (1980) showed by radioactive pulse chase labeling method that AAV capsid proteins assemble into empty capsids very quickly. Their experiment suggested that these empty capsids are later transferred to DNA-containing full particles or defective-interfering particles. They further showed that the maturation to stable full particles took much longer in terms of several hours whereas, empty capsids appeared very rapidly. The GFP positive cells remained same after 24 hpt suggesting that there was no secondary infection seen for AAV2 (Figs. 6 and 9c). Xiao (2010) argued that AAV2, AAV6 and AAV8RQNR (a mutant that binds strongly to HSPG) would show less particles in free medium because of re-adsorption onto the cell membrane and re-entry into the live cells, resulting in a low AAV yield because of the un-coating and degradation of re-entered viruses. This argument implies that there should be at least a small increase in the number of transduced cells at a later stage of AAV production because of an equal probability of adsorption by non-transfected cells. An increase in GFP positive cells was not observed (Figs. 6 and 9c). The low Vg/IVP ratio of less than 20 at 48 hpt in cell culture indicated acceptable encapsidation of AAV2 vectors achieved by transfection technology using HEK293SF cells.

In this study, it has been shown that different serotypes of AAV (1–9) can be produced using PEI with transfection technology in a serum-free medium with yields close to 10^{13} Vg/L cell culture (Fig. 8) using 1×10^6 cells/mL and 1 μg total DNA/mL cell culture, determined by first purifying by iodixanol protocol to remove plasmid and non-encapsulated viral DNA present in the samples. The infectious viral particle assay used HEK293 cells, but the AAV serotypes had different specificities for this cell line. The infectious viral particle productions reflected by the infectivity assay are provided only to demonstrate that different AAV serotypes produced using this scalable process are functional in terms of infectivity. The results indicated that the HEK293 or HeLa cell lines used for the transduction (infectivity) assay had variable "permissiveness" to different AAV serotypes. The titers published should be carefully evaluated because they are highly dependent on the type of assays used to determine the total or functional titers. A cell-based assay should be used only when the titers from the same serotypes are compared to determine functionality loss during the vector processing, and genomic particles by PCR based assay may be used when titers from the same or different serotypes are compared (Grieger et al., 2006). The quantitation of AAV has been reviewed by Aucoin et al. (2008). It is strongly suggested that any comparison with the data published in the literature should be done with great care considering different expression systems and different quantitation methods used.

The 3-L bioreactor experiments for the production of AAV2 (two runs), and AAV6 (one run) demonstrated the scalability of the process (Table 3). Although the total cells continued to increase, after 40 hpt, the cell viability started to decrease. The bioreactor experiment provided similar yields as observed in the shake flask experiments for similar conditions demonstrating scalability of the process. The time of harvest may be set preferentially between 48 and 64 hpt (Fig. 9). The volumetric production of infectious and genomic viral particles remained constant around 6.8×10^{11} IVP/L and 1.0×10^{13} Vg/L, respectively, between 48 and 72 hpt. The cell specific yields of 6800 Vg/cell and 460 IVP/cell were obtained for AAV2. Using different plasmid constructs and medium selection providing better conditions, an improvement from earlier yields (Durocher et al., 2007) has been reported with better encapsidation (Vg/IVP ratio ~15) at a higher cell density of 1×10^6 cells/mL and without any medium exchange. The HEK293T cells adapted to a "loose" suspension in a 2-L bioreactor at a cell density of 1×10^6 cells/mL with FBS gave a cell-specific yield of 345 ± 154 IVP/cell; the genomic particles per cell were not reported (Park et al., 2006). Generally, the HEK293T produced higher cell-specific infectious particles than HEK293 cells (261 IVP/HEK293T cells vs. 44 IVP/HEK293 cells) (Park et al., 2006). Yuan et al. (2011) produced single stranded (ssAAV) and self-complementing (scAAV) AAV vectors of serotypes AAV2, AAV8, and AAV9 by the AAV producer cell line and triple transfection. The group reported $5–8 \times 10^{13}$ vector genomic particles of AAV2 per Nunc Cell Factory and 1.3×10^5 genomic particles per cell. Their cell line produced AAV2 vectors with Vg/transducing units (TU) ratio of 85 and Vg/TU ratio of 480 by triple transfection method. Lock et al. (2010) produced AAV1, 6, 7, 8 and 9 by transient transfection at small scale using anchorage dependent cells with yields in the range of $0.5–3 \times 10^5$ genome

copies/cell. Their ten liter productions of AAV6, 8 and 9 gave yields in the range of 3×10^{13} to 1×10^{15} genome copies by anchorage dependent cells. Although the cells during production were grown in serum free medium, but were kept in 10% fetal bovine serum prior to the transfection. Grieger and Samulski (2012) reported high AAV yields using a HEK293 cell line that was selected for high transfection efficiency and high AAV production. This cell line was able to produce $>10^5$ Vg/cell in a serum-free suspension cell culture. This shows that there is a great potential yet to be explored to optimize the cell lines for better optimization of transfection efficiencies.

In author's laboratory, the baculovirus/insect cell system is a well optimized process to support high cell density production of AAV vectors. Combining low MOI and a fed-batch production strategy, it was shown that the insect cells/baculovirus system AAV yield can be increased seven-fold. Approximately 2×10^{14} Vg/L and 2×10^{12} IVP/mL cell culture were obtained in the bioreactor experiment at a harvest cell density of 9.5×10^6 cells/mL (Mena et al., 2010), which is proportionally similar to cell specific production by transfection at low cell density reported in this manuscript. From a process standpoint, it is remarkable that despite limited process improvements and operation at low cell density, the large scale transient transfection technology performs as well as the baculovirus/insect cell technology for production of AAV serotypes. Again, this underlines the yet non-exploited potential of the transient transfection in HEK293 cell suspension cultures for high yield and large scale industrial production of AAV 1–9 serotypes evaluated in this study, which is further supported by recent work by Grieger and Samulski (2012). Also, based on the data from this study it can be said with confidence that other serotypes can be easily produced at larger scales in the cGMP HEK293SF cell line using the presented generic protocol.

5. Conclusions

It has been demonstrated that the human embryonic kidney (HEK) 293SF-3F6 cell line, a fully characterized GMP Master Cell Bank, is capable of producing different serotypes of AAV (AAV 1–9) in suspension cultures by transient transfection in a serum-free medium without medium exchange. Three bioreactor runs showed that the yields are comparable to shake-flask runs for the corresponding serotypes and therefore shows the potential to scale-up and industrialization of this generic process for manufacturing efficiently AAV serotypes.

Conflict of interests

The authors declare that no competing interests exist.

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