Vaccine 37 (2019) 7019–7028

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Efficient influenza A virus production in high cell density using the novel porcine suspension cell line PBG.PK2.1

Gwendal Gränicher [a,*], Juliana Coronel [a], Alexander Pralow [a], Pavel Marichal-Gallardo [a], Michael Wolff [a,b], Erdmann Rapp [a], Alexander Karlas [c], Volker Sandig [c], Yvonne Genzel [a], Udo Reichl [a,d]

[a] Max Planck Institute for Dynamics of Complex Technical Systems, Bioprocess Engineering, Sandtorstr. 1, 39106 Magdeburg, Germany
[b] Institute of Bioprocess Engineering and Pharmaceutical Technology, University of Applied Sciences Mittelhessen, Wiesenstrasse 14, 35390 Gießen, Germany
[c] ProBioGen AG, Goethestr. 54, 13086 Berlin, Germany
[d] Chair for Bioprocess Engineering, Otto-von-Guericke-University Magdeburg, Universitätsplatz 2, 39106 Magdeburg, Germany

ARTICLE INFO

Article history:
Available online 17 April 2019

Keywords:
High titer influenza virus
Cell metabolism
Porcine suspension cells
Bioreactor
hollow-fiber ATF perfusion

ABSTRACT

Seasonal and pandemic influenza respiratory infections are still a major public health issue. Vaccination is the most efficient way to prevent influenza infection. One option to produce influenza vaccines is cell-culture based virus propagation. Different host cell lines, such as MDCK, Vero, AGE1.CR or PER.C6 cells have been shown to be a good substrate for influenza virus production. With respect to the ease of scale-up, suspension cells should be preferred over adherent cells. Ideally, they should replicate different influenza virus strains with high cell-specific yields. Evaluation of new cell lines and further development of processes is of considerable interest, as this increases the number of options regarding the design of manufacturing processes, flexibility of vaccine production and efficiency.

Here, PBG.PK2.1, a new mammalian cell line that was developed by ProBioGen AG (Germany) for virus production is presented. The cells derived from immortal porcine kidney cells were previously adapted to growth in suspension in a chemically-defined medium. Influenza virus production was improved after virus adaptation to PBG.PK2.1 cells and optimization of infection conditions, namely multiplicity of infection and trypsin concentration. Hemagglutinin titers up to 3.24 $\log_{10}$(HA units/100 μL) were obtained in fed-batch mode in bioreactors (700 mL working volume). Evaluation of virus propagation in high cell density culture using a hollow-fiber based system (ATF2) demonstrated promising performance: Cell concentrations of up to 50×10^6 cells/mL with viabilities exceeding 95%, and a maximum HA titer of 3.93 $\log_{10}$(HA units/100 μL). Analysis of glycosylation of the viral HA antigen expressed showed clear differences compared to HA produced in MDCK or Vero cell lines. With an average cell-specific productivity of 5000 virions/cell, we believe that PBG.PK2.1 cells are a very promising candidate to be considered for next-generation influenza virus vaccine production.

1. Introduction

Influenza virus type A can cause acute respiratory illnesses and constitutes a severe threat for global public health. Vaccination is the major measure to prevent and control influenza virus infections [1]. Inactivated influenza vaccines are still mainly produced in embryonated chicken eggs [2]. In case of pandemics, influenza vaccine production capacity using this technology would be limited by a lack of manufacturing infrastructure and egg availability [3]. As an alternative, cell-culture based production of viral vaccines was established as a platform technology. By using MDCK or Vero cells, influenza vaccines have been developed and licensed, namely Influvac® (Solvay Pharmaceuticals Inc.), FluMist® (MedImmune), Influjet® (Baxter Vaccines) [4] and Flucelvax® (Seqirus) [5]. Other host-cell lines such as AGE1.CR®, PER.C6® [4], EB66® [6] or DuckCelt®-T17 [7] have also been evaluated for influenza vaccine production. Highest reported influenza virus yields were obtained in MDCK suspension cells with titers of 3.9 $\log_{10}$(HA units/100 μL) for influenza A/PR/8/34 virus [8]. Although several cell lines have been tested, there are still significant efforts to develop more potent cell lines. Ideally, a host cell line should have a doubling time of 20–30 h with high viability, allow easy scale-up, and enable fast virus production to high titers with low protein and DNA concentration in the virus harvest to facilitate purification [4]. Moreover, safety aspects should be assessed. In particular, the cell line should be free of any adventitious agents and have low tumorigenic and oncogenic potential [9]. As influenza pandemics

* Corresponding author.
E-mail address: graenicher@mpi-magdeburg.mpg.de (G. Gränicher).

https://doi.org/10.1016/j.vaccine.2019.04.030
0264-410X/

can arise from different animal reservoirs, having a larger choice of cell substrates from different species for production is beneficial [4]. Also, differences in productivity can be observed depending on influenza virus strain and cell line origin.

In order to answer increasing demands in influenza vaccines, innovative cell culture bioprocesses have been developed. Volume-expanded fed-batch cultures [10], continuous bioprocesses [11] or high cell density perfusion process [12] have been evaluated for the production of various viral vaccines. The latter technology seems most promising, but was up to now only shown for less suitable cell substrates. Therefore, evaluating a high cell density process with a new potential cell line could boost influenza vaccine manufacturing.

Here, PBG.PK2.1, a novel suspension cell line derived from immortal porcine kidney cells and growing in chemically-defined CD-U5 medium (ProBioGen AG) is presented. For the first time, influenza virus was propagated in porcine suspension cells, which could be of interest in case of an influenza pandemic from porcine origin. In order to assess the efficiency of the production process established, product quality, upstream and downstream processing aspects were considered. PBG.PK2.1 cells grew with high viability to cell concentrations up to 50×10^6 cells/mL in perfusion mode. High influenza A/PR/8/34 (H1N1) virus yields were obtained at different scales and different operational mode (fed-batch and perfusion). Maximum titers achieved were up to 3.93 $\log_{10}$(HA units/100 μL). Glycosylation analyses of the viral HA antigen showed significant differences compared to the same influenza virus strains propagated in other host cell lines or chicken embryos [13–17]. Low protein and DNA contamination levels were measured for bioreactor harvests from fed-batch and perfusion mode.

2. Material and methods

2.1. Cell line generation

PBG.PK2.1 cells were derived from immortal adherent porcine kidney cells and adapted to suspension growth in a chemically-defined medium CD-U5 by ProBioGen AG (Germany). Initially, this cell line was not suitable for vaccine manufacturing due to a chronic infection with porcine circovirus 1. Following suppression of virus replication followed by single cell cloning, PBG.PK2.1 cells were cured from this virus.

2.2. Cell culture and medium

Chemically-defined CD-U5 (ProBioGen AG) basal medium was supplemented with 2 mM L-glutamine (Sigma) and recombinant insulin-like growth factor (LONG-R^3 IGF, 50 ng/mL final concentration, Sigma). Cells were incubated in orbital shaker (Multitron Pro, Infors HT) at 7.5% CO_2 with a shaking speed of 150 rpm and 50 mm shaking diameter. Baffled shake flasks with 50 mL working volume (wv) were used. Cells were inoculated at a cell concentration of 0.8–1.0 $\times 10^6$ cells/mL and passaged every 3 days, until passage 40.

For larger scale cultivations, a DASGIP bioreactor system (Eppendorf) was used with 450–700 mL wv. The bioreactor was inoculated from shake flasks pre-cultures at a cell concentration of 0.8–1.0 $\times 10^6$ cells/mL. The system was agitated with a pitched blade impeller at 110 rpm (upflow) with aeration by an L-sparger (1 mm pore size). The temperature was maintained at 37 °C. The pH value was set at 7.2 by sparging CO_2. For aeration at a DO level at 40%, O_2 and N_2 flow rates were controlled between 3 L/h and 9 L/h. During the virus production phase, pH was increased from 7.2 to 7.4 to mimic virus production in shake flasks. Cell cultures were infected at a multiplicity of infection (MOI) of 10^{-5} infectious virions/cell. Trypsin (Gibco, # 27250–018) was added at 0 and 16 h post infection (hpi) at an activity of 10^{-6} trypsin units/cell (U/cell) to facilitate infection. Starting 24 hpi, the cell culture volume was increased with supplemented CD-U5 medium from 550 mL to 710 mL to avoid substrate limitation.

For the batch cultivation mode, the cell-specific growth rate (μ), the cell-specific substrate consumption rate and by-product production rate (q_s) were determined using the following equations:

$$\mu = \frac{\ln\left(x(t_{n+1})/x(t_n)\right)}{t_{n+1} - t_n} \quad (1)$$

$$Y_{x/s} = \frac{x(t_{n+1}) - x(t_n)}{c_s(t_n) - c_s(t_{n+1})} \quad (2)$$

$$q_s = \frac{\mu}{Y_{x/s}} \quad (3)$$

With x, viable cell concentration;
t, cultivation time;
n, sampling time point; and
c_s, cell culture compound concentration.

2.3. Perfusion culture in bioreactor

An ATF2 cell retention system (Repligen) coupled to a DASGIP bioreactor was used for perfusion culture, using a PES hollow-fiber membrane (0.2 μm pore size, 470 cm^2, Spectrum). By using a diaphragm, the cell culture broth was pumped in and out through the membrane filter at an exchange flow rate of 0.9 L/min. DO, pH and temperature were set as described in Section 2.2. A micro-sparger (10 μm pore size) was used for aeration (flow rate varying from 0.3 to 4 L/h). Perfusion was started when a glucose concentration of 18 mM was reached. In order to shorten the time to perfusion, the bioreactor was inoculated at a cell concentration of 3×10^6 cells/mL from pre-cultures grown in shake flasks. Supplemented CD-U5 (Section 2.2) was used as perfusion medium. The cell-specific perfusion rate (CSPR) was calculated following cell-specific glucose consumption rate determined in batch mode. With a constant CSPR of 0.07 nL/cell/day, the perfusion rate (Q) was manually adjusted using the following equations [18]:

$$CSPR = \frac{q_g}{c_{gm} - c_{gb}} \quad (4)$$

$$Q = x_i \cdot e^{\mu \cdot t} \cdot wv \cdot CSPR \quad (5)$$

With q_g, cell specific glucose consumption rate;
c_{gm}, glucose concentration in the medium (35 mM);
c_{gb}, target glucose concentration in the bioreactor (6 mM);
x_i, initial cell concentration; and
wv, bioreactor working volume.

During the three hours before infection at a MOI of 10^{-5}, 0.8–0.9 bioreactor volume was replaced with fresh medium by applying a perfusion rate of 150–200 mL/h. At 1 hpi, the bioreactor working volume was increased from 510 mL to 660 mL. At the time of infection (TOI), perfusion medium was additionally supplemented with 22 trypsin units/L and the pH increased from 7.2 to 7.4 (parameters defined based on previous optimization in shake flasks at high cell density).

2.4. Virus origin and quantification

A MDCK cell-derived virus seed (human influenza virus A/PR/8/34 H1N1: Robert Koch Institute, Amp. 3138) was used for adaptation to porcine cells. Additionally, porcine influenza virus A/Bakum/1832/00 H1N2 (Impstoffwerke Dessau-Tornau) and

influenza virus B/Brisbane/60/2068 (National Institute for Biological Standards and Control, Amp. 09/168) were tested in shake flasks. To facilitate virus infection, trypsin (Gibco, # 27250-018) was prepared in PBS with 5000 units/mL according to the activity given by the manufacturer. A/PR/8/34 influenza virus was adapted to porcine cells by three passages in 50 mL wv baffled shake flasks. For each passage, the cell concentration was set to 5×10^6 cells/mL at TOI with a trypsin concentration of 10^{-6} trypsin U/cell. The cell culture supernatant was collected 36 hpi. For infection, 0.0004% v/v of the supernatant from the previous passage was transferred to the fresh cell culture medium. Before addition, cell cultures were centrifuged at 150*g* for 10 min to remove spent medium and resuspended in fresh medium containing the virus to prevent any substrate limitation. After adaptation, a seed virus with a $TCID_{50}$ of 2.02×10^9 infectious virions/cell was obtained.

Three different methods were used for virus particle titration. Hemagglutinin (HA) titer was determined as described earlier by Kalbfuss et al. [19] and quantified in $\log_{10}$(HA units/100 μL) with a discretization measurement error of ±0.15 log units. The infectious virus titer was measured according to Genzel and Reichl [20] by $TCID_{50}$. In addition, the HA content was quantified in μg/mL by a single radial immuno-diffusion (SRID) assay as described previously [21].

Viral particles concentration (c_{vir}), cell-specific virus yield (CSVY) and culture medium productivity (P_V) were calculated using the following equations (based on Vazquez et al. 2018 [22])

$$c_{vir} = 2 \cdot 10^7 \cdot 10^{(\log_{10}(HAunits/100\mu L))} \quad (6)$$

$$CSVY = \frac{C_{vir,max} \cdot wv_{t1}}{x_{v,max} \cdot wv_{t2}} \quad (7)$$

$$P_v = \frac{C_{vir,max} \cdot wv_{t1}}{V_{tot} \cdot t_{tot}} \quad (8)$$

With $x_{v,max}$, maximum concentration of viable cells obtained until highest virus titer was reached;
wv_{t1}, cell culture working volume at maximal virus titer;
wv_{t2}, cell culture working volume at highest viable cell concentration;
V_{tot}, total spent medium during cell growth and virus production phases; and
t_{tot}, total time from cell culture start until highest virus titer time point.

2.5. Determination of the viable cells, cell metabolites, amino acids, dsDNA and total protein concentrations

Cell concentration and viability were determined using a cell counter (Vicell, Beckman Coulter) with trypan blue staining. As cells were forming aggregates (around 5 cells) when cultivated in bioreactor, samples were first incubated for 5 min at 37 °C with a concentration of 60–80 trypsin units/mL to disaggregate cells before using the cell counter. Viability was measured without using trypsin. Glucose, glutamine, lactate and ammonium concentrations were determined using a Bioprofile 100 plus (Nova biomedical). Amino acid concentrations were measured using an Acquity H-Class UPLC instrument (Waters). Total protein and dsDNA concentrations were assessed through respectively a Bradford and a PicoGreen assay following methods described previously [21].

2.6. Imaging flow cytometry

Samples with 2×10^6 cells were collected and fixed in 2% paraformaldehyde (Morphisto GmbH) during 30 min at 4 °C. After centrifugation (10 min, 300*g*, 4 °C), the pellets were resuspended in 1 mL of cold PBS and transferred to 15 mL falcon tubes containing 4.5 mL of cold 70% ethanol (v/v). The samples were stored at −20 °C until use. Antibody staining for viral nucleoprotein (NP) was done following Frensing et al. [23], with small changes in the protocol. Briefly, the wv for blocking and antibody incubations was reduced to 25 μL and the number of washing steps was reduced to 1–2 times. Cells were then resuspended in the remaining 30–50 μL and DAPI was added (approx. 5 μg/mL). Using the ImageStream X mark II (Amnis, EMD Millipore), 10,000 single cells per sample were collected. The IDEAS software (v. 6.2) was used to analyze the data. Cells positive for NP were determined as infected and apoptotic cells were measured using the DAPI signal and brightfield images [23].

2.7. Influenza virus glyco-analysis

Influenza virus was harvested using g-force step-gradient centrifugation as described previously [15,16]. Site-specific glycopeptide analysis was performed according to Pralow et al. [24]. Briefly, influenza virus glycoproteins were sequentially digested using trypsin (Trypsin Sequencing Grade Modified, V5111, Promega) and Flavastacin (AspN, P8104S, New England Biolabs) using a modified version of the filter-aided sample preparation method of Wisnievski et al. [24–26]. Glycopeptide enrichment was performed using hydrophilic interaction liquid chromatography solid phase extraction according to the modified workflow of Selman et al. [27], recently published by Hoffmann et al. [25]. Enriched glycopeptides were separated and measured on a reversed-phase liquid chromatography system coupled online to an LTQ Orbitrap Elite mass spectrometer. Data analysis was performed manually and semi-automated using glyXtool^{MS}, an in-house developed software for the analysis of glycopeptide mass spectrometry data, published by Pioch et al. [28].

3. Results and discussion

In order to efficiently evaluate a new cell line for the production of influenza vaccines, not only cell growth and virus properties, but also key process parameters regarding upstream and downstream processing should be assessed. In a first step, cell growth and metabolism of the PBG.PK2.1 cell line were evaluated without virus infection. Subsequently, influenza virus production was characterized and optimized. Finally, viral hemagglutinin glycosylation was analyzed, and protein and DNA impurity levels of crude harvests assessed.

3.1. Cell growth and metabolism

Cultivations in shake flasks (100 mL wv) and in DASGIP bioreactors (550 mL wv) were compared. PBG.PK2.1 cells showed similar viabilities above 97% with an exponential growth phase from 0 to 120 h for both scales (Fig. 1A). Cell concentrations and growth rate were slightly higher in shake flasks (Table 1). In bioreactors, small aggregates of about 5 cells were observed. Most likely, this was due to a too low agitation speed, as aggregates were previously described for poorly agitated cell cultures [29,30]. Such aggregates have been reported to create heterogeneity and to increase shear stress, which could affect maximum cell concentration and doubling time [29]. When comparing lowest doubling time in batch mode with MDCK suspension cells (36 h [31]), HEK293 suspension cells (33 h [32]) and AGE1.CR cells (25 h [12]), PBG.PK2.1 cell line had a relatively high value of 38 ± 11 h in CD-U5. As the CD-U5 medium was developed initially for AGE1.CR cell culture, the composition of the medium might be

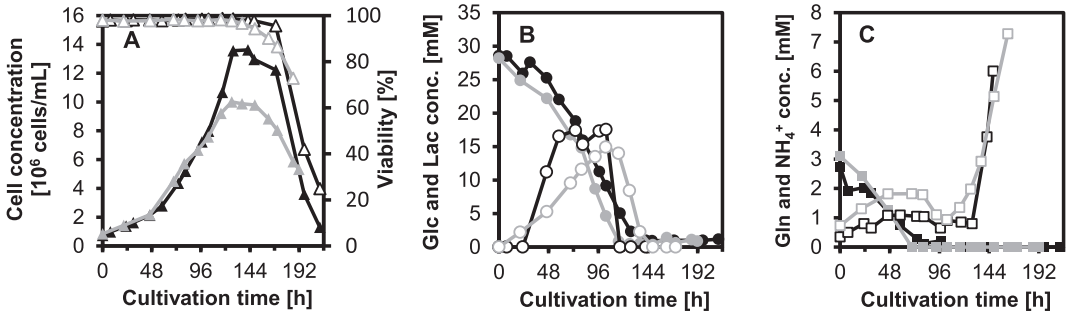

Fig. 1. Growth of PBG.PK2.1 cells in CD-U5 medium in one representative run (of n = 2) in an 100 mL wv shake flask (black) and in one representative run (of n = 3) in a 550 mL wv stirred tank bioreactor (grey) – (A) viable cell concentration (▲), cell viability (△) – (B) glucose (●) and lactate (○) concentrations – (C) glutamine (■) and ammonium (□) concentrations.

Table 1
Growth parameters of PBG.PK2.1 cells in chemically-defined CD-U5 medium in batch mode for shake flasks and bioreactors.

	Cultivation time range [h]	Shake flask, n = 2	Bioreactor, n = 3
Cell-specific growth rate [h^{-1}]	20–120	0.021 ± 0.004 [a]	0.020 ± 0.006
Doubling time [h]	20–120	34 ± 8	38 ± 11
Maximum cell concentration [10^6 cells/mL]		13.39 ± 0.04	9.86 ± 0.10
Cell diameter [μm]	20–120	15.19 ± 0.25	14.48 ± 0.46
q_{Glc} [10^{-11} · mmol/cell · h]	20–120	−6.64 ± 3.06	−6.64 ± 0.94
q_{Gln} [10^{-11} · mmol/cell · h]	20–70	−1.59 ± 0.37	−2.32 ± 0.65
q_{Lac} [10^{-11} · mmol/cell · h]	20–110	8.61 ± 3.19	3.79 ± 2.52
q_{NH4+} [10^{-11} · mmol/cell · h]	20–70	1.04 ± 0.85	1.40 ± 0.06

q_{Glc}, cell-specific glucose consumption rate; q_{Gln}, cell-specific glutamine consumption rate; q_{Lac}, cell-specific lactate production rate; q_{NH4+}, cell-specific ammonium production rate.
[a] Mean and standard deviation.

further improved specifically for PBG.PK2.1 cells to enable higher growth rates.

As shown in Table 1, the production and consumption rate of the main cell culture metabolites were similar in shake flasks and bioreactors.

Lactate and ammonium are well-known by-products. Lactate concentrations above 20 mM and ammonium levels as low as 2–3 mM have been shown not only to have adverse effects on growth for many mammalian cell lines [33–35], but also on virus vaccine production [36]. However, these limits were not exceeded during the exponential cell growth phase (Fig. 1B and C). A decrease in lactate concentration was observed after 105 h cultivation time, once glucose level was less than 10 mM. This suggests the ability of the PBG.PK2.1 cells to use lactate as a carbon source after glucose depletion. Interestingly, porcine cells continued growth even after exhaustion of glutamine after about 80 h cultivation time (Fig. 1A and C). As glutamine has been reported to be the main source of ammonium accumulation [36], medium without or a low glutamine content could be considered for PBG.PK2.1 cells for recombinant protein and virus manufacturing. This is similar to the avian cell line AGE1.CR growing in a comparable chemically-defined medium (CD-U2) without glutamine [37]. Finally, as glutamine was shown not to be necessary for PBG.PK2.1 cell growth, glucose was used to determine the CSPR for process optimization and intensification.

3.2. Screening of virus propagation in shake flasks

As the influenza A/PR/8/34 seed virus was generated in MDCK host cells, the virus was first adapted to propagate more efficiently in PBG.PK.2.1 cells following the method described in Section 2.4 (Fig. 2A). Interestingly, neither higher $TCID_{50}$ nor HA titers were observed with increased number of passage. However, maximum titers were reached 12 hpi earlier for later passages compared with the first passage. This was similar to other mammalian cell lines such as Vero [38] or HEK293 cells [39], where an earlier onset of virus release has also been observed during virus adaptation. For all further experiments of this study, the seed virus adapted after three passages in PBG.PK2.1 cells was used. In a next step, MOIs between 10^{-2} and 10^{-5} and trypsin activities varying between 10^{-5} U/cell and 10^{-7} U/cell were tested to further improve influenza virus production. As shown in Fig. 2D, optimum influenza A/PR/8/34 production was obtained using a MOI of 10^{-5} infectious virions/cell with a trypsin activity of 10^{-6} U/cell. The low MOI was in accordance with previous studies showing that MOIs equal or lower than 0.001 infectious virions/cell allowed higher titers for cell-based influenza A production [38,39]. Under these optimized conditions, a maximum titer of 3.4 $\log_{10}$(HA units/100 μL) with a CSVY equal to 5375 virions/cells (Table 2) was obtained.

Further screenings were also performed regarding porcine influenza A H1N2 (A/Bakum/1832/00) and influenza B (B/Brisbane/60/2068) virus production in shake flasks. Taking into account the optimized settings for influenza A/PR/8/34, maximum titers of 3.37 $\log_{10}$(HA units/100 μL) and 2.89 $\log_{10}$(HA units/100 μL) were obtained for porcine influenza A and influenza B virus, respectively (without prior virus adaptation and with optimized amount of seed virus). PBG.PK2.1 cells thus were permissive for at least three different influenza virus strains. Moreover, the high titers achieved for porcine influenza A H1N2 (A/Bakum/1832/00) could be of interest for the porcine vaccination market. The same strain was tested before in CAP cells, but resulted in a lower titer of 3.0 $\log_{10}$(HA units/100 μL) [40]. While comparing HA titers from different groups is difficult, due to assay limitations and differences in cultivation platforms, it seems that the use of PBG.PK2.1 cells can result in higher influenza virus titers compared to, for example, PER.C6 or HEK293 cells (Table 3). Furthermore, as also reported in literature, the production of influenza B virus strains can result in lower titers compared to high-producer

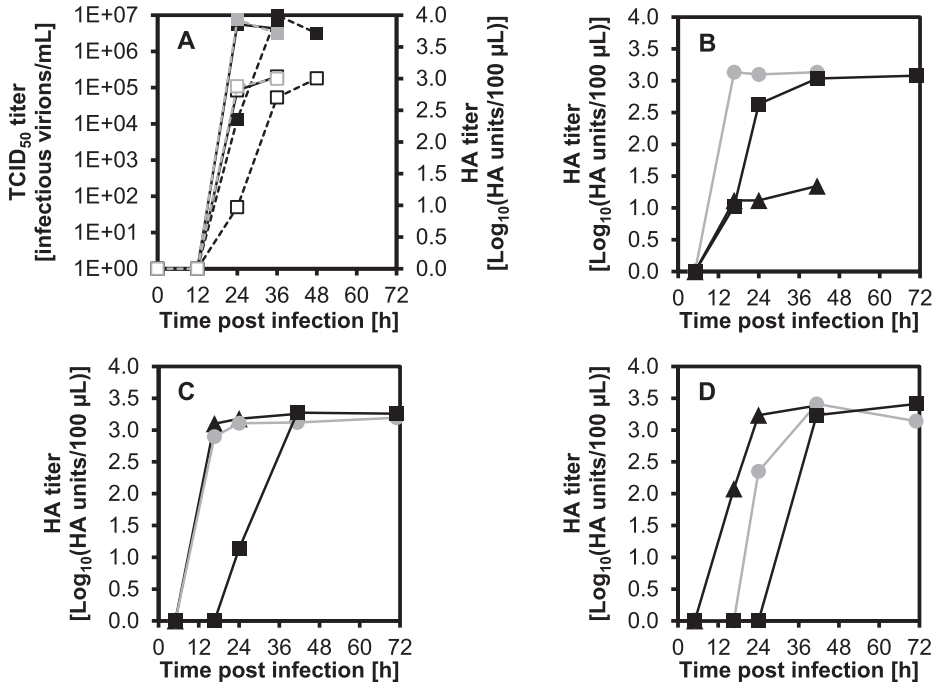

Fig. 2. Optimization of influenza A/PR/8/34 virus production in PBG.PK2.1 cells cultivated in CD-U5 medium in shake flasks. (A) Virus adaptation: Influenza A/PR/8/34 virus passaged in PBG.PK2.1 cells. Passage 1 (black dotted line), passage 2 (black line) and passage 3 (grey dotted line) are presented. $TCID_{50}$ titer (■) and HA titer (□) are compared for each passage. (B) HA titer at MOI = 10^{-2} and trypsin activity of 10^{-5} U/cell (▲), 10^{-6} U/cell (●) and 10^{-7} U/cell (■). (C) HA titer at MOI = 10^{-3} and trypsin activity of 10^{-5} U/cell (▲), 10^{-6} U/cell (●) and 10^{-7} U/cell (■). (D) HA titer at MOI = 10^{-5} and trypsin activity of 10^{-5} U/cell (▲), 10^{-6} U/cell (●) and 10^{-7} U/cell (■).

Table 2
Influenza A/PR/8/34 virus production in PBG.PK2.1 cells considering key parameters for upstream and downstream processing.

	Optimized condition[a]	Fed-batch mode	Perfusion mode
	Shake flask, n = 5	Bioreactor, n = 3	Bioreactor, n = 2
Process time [b]	n.d.	144 ± 6[c]	198 ± 7
Cell concentration at TOI [10^6 cells/mL]	5.0 ± 0.1	5.0 ± 0.1	46.0 ± 4.2
Maximum cell concentration [10^6 cells/mL]	9.0 ± 0.3	7.1 ± 0.3[d]	44.4 ± 4.4[d]
Maximum HA titer [$\log_{10}$(HA units/100 µL)]	3.38 ± 0.03	3.24 ± 0.04[d]	3.93 ± 0.05[d]
CSVY [virions/cell]	5375 ± 273	5006 ± 540	3929 ± 876
Culture medium productivity [10^9 virions/L/day]	n.d.	5.88 ± 0.21	1.87 ± 0.29
Maximum $TCID_{50}$ titer [10^8 infectious virions/mL]	n.d.	4.4 ± 1.2[d]	32.0 ± 0.0[d]
Max. HA content [µg/mL]	n.d.	5.62 ± 1.16[d,e]	20.21 ± 2.42[d]
dsDNA impurity level per HA dose at optimal harvest time point [ng] [f]	n.d.	18170 ± 2460[d,e]	18960 ± 1860[d]
Protein impurity level per HA dose at optimal harvest time point [µg] [f]	n.d.	520 ± 100[d,e]	379 ± 29[d]

TOI, time of infection; HA, hemagglutinin; CSVY, cell-specific virus yield; dsDNA, double stranded DNA; n.d., not determined; hpi., hours post infection.
[a] Optimized conditions: MOI = 10^{-5}, trypsin = 10^{-6} trypsin U/cell, total medium replacement at TOI (batch mode).
[b] Process time is from cell culture bioreactor inoculation until maximal reached HA titer.
[c] Mean and standard deviation.
[d] Bioreactor working volume increased by 30% after infection.
[e] Values determined only for 2 bioreactor.
[f] One HA dose = 15 µg, best harvest time point in fed-batch mode = 48 hpi, best harvest time point in perfusion mode = 36 hpi.

influenza A virus strains [7,40]. Nevertheless, more influenza virus strains from different animal origin should be tested in PBG.PK2.1 cells, to fully evaluate its permissiveness.

3.3. Virus production in bioreactor

For larger scale influenza A/PR/8/34 virus production, cells were cultivated in DASGIP bioreactors with 550 mL wv using the optimized MOI and trypsin activity (Section 3.2). The medium was not replaced at the TOI to simplify the process for larger scales. Consequently, higher host cell protein was to be expected. Trypsin protease was added again after 16 hpi at 10^{-6} U/cell to ensure complete virus infection. The cells were cultivated until a cell concentration of 6.8×10^6 cells/mL was achieved and diluted to 5×10^6 cells/mL before infection. The cell concentration before bioreactor dilution was selected following previous optimization studies performed in shake flasks (data not shown). To avoid glucose limitation, the bioreactor working volume was increased by 30% with supplemented CD-U5 medium at 24 hpi. A maximum HA titer of 3.24 ± 0.04 $\log_{10}$(HA units/100 µL) was obtained between 36 and 48 hpi (Fig. 3A). No limitation in glucose and glutamine was observed during the virus production phase (Fig. 3C and D). Moreover, no toxic levels were reached for lactate (20 mM) and ammonium (2–3 mM) during the first 48 hpi. Compared to the optimized process in shake flasks, a similar CSVY of 5006 virions/cell was observed in bioreactors (Table 2), suggesting that this process is scalable to higher bioreactor volumes. Almost 100% of the cells were infected at the point of maximum titer

Table 3
Comparison of maximum HA titer and cell-specific virus yields for different cell lines and bioprocess modes.

Cell line	Batch/Fed-batch process		Perfusion process			Ref.
	Max. HA titer [$\log_{10}$(HAU/100 µL)]	CSVY [virions/cell]	Max. HA titer [$\log_{10}$(HAU/100 µL]	CSVY [virions/cell]	Cell conc. at TOI [10^6 cells/mL]	
Vero, adh.	2.6	4976	n.d.	n.d.	n.d.	[38]
MDCK, adh.	3.0	33,255	3.9[a]	19,000	16	[38,54]
DuckCelt–T17, sus.	1.8	n.d.	n.d.	n.d.	n.d.	[7]
AGE1.CR, sus.	2.5	1292	3.5	1266	48	[12]
CAP, sus.	2.9	3883	3.7	4086	27	[12]
PER.C6, sus.	3.0	n.d.	n.d.	n.d.	n.d.	[55]
HEK293, sus.	3.0	4683	3.3[b]	3960	6	[12,32,39]
MDCK, sus.	3.9	40,000	n.d.	n.d.	n.d.	[8]
PBG.PK2.1, sus.	3.2–3.4	4466–5648	3.9–4.0	3053–4805	40–49	Pres. work

HA, hemagglutinin; U, units; CSVY, cell-specific virus yield; Max., maximum; conc., concentration; TOI, time of infection; adh., adherent; sus., suspension; n.d., not determined; Ref., reference; Pres., presented.
[a] cell culture time = 72 h was taken as harvest point.
[b] cell culture time = 168 h was taken as harvest point.

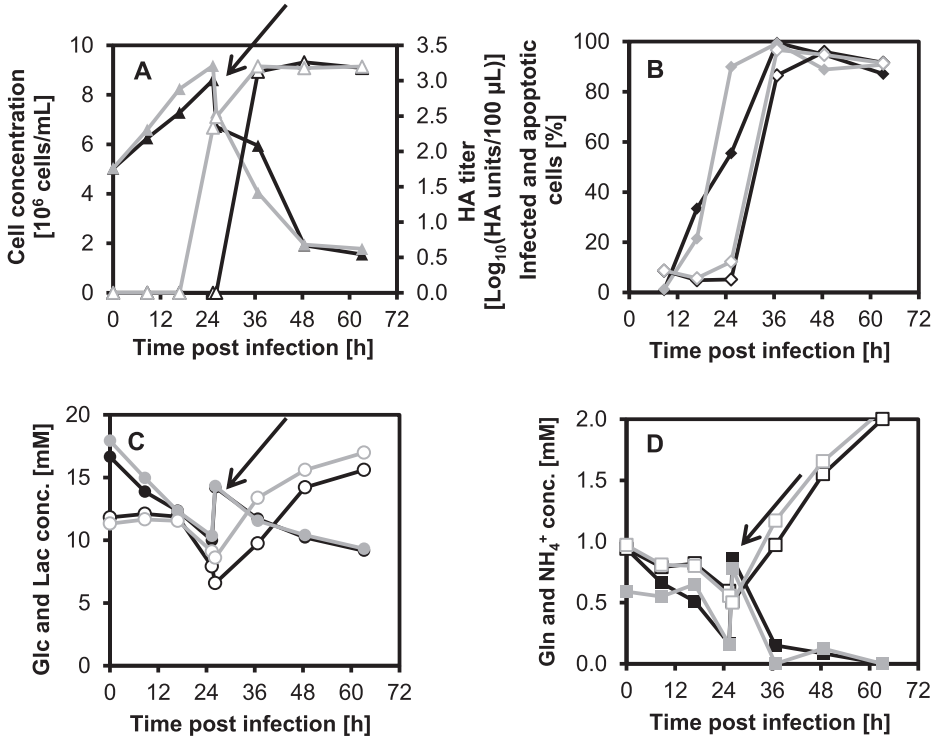

Fig. 3. Influenza A/PR/8/34 virus production of run 1 (black) and run 2 (grey) in bioreactor in fed-batch mode using PBG.PK2.1 cell line growing in CD-U5 medium. Both cell cultures were coming from the same pre-culture. MOI of 10^{-5} with a trypsin concentration of 10^{-6} U/cell were used at the time of infection. (A) Viable cell concentration (▲), HA titer (△). (B) Percentage of infected cells (◆), percentage of apoptotic cells (◇). (C) Glucose (●) and lactate (○) concentrations. (D) glutamine (■) and ammonium (□) concentrations. Arrows indicate cell culture volume increase (from 550 to 710 mL) with fresh CD-U5 medium.

(36–48 hpi) (Fig. 3B). This might indicate that the virus efficiently infected all cells of the cultures with current infection parameters. Eventually, trypsin addition could be reduced to a single addition at the TOI with an activity higher than 10^{-6} U/cell.

Influenza A virus produced in PBG.PK2.1 cells resulted in higher HA titers and CSVYs compared to adherent Vero cells, human or avian cells (Table 3). However, MDCK cells still outperform the porcine cells in terms of CSVY (40000 virions/cell) and maximal titer (3.9 $\log_{10}$(HA units/100 µL)) (Table 3). Nevertheless very high $TCID_{50}$ titers ($4.4 \pm 1.2 \times 10^8$ infectious virions/mL) were obtained with PBG.PK2.1 cells, which is of high interest regarding the production of life-attenuated influenza vaccines. This was also true for $TCID_{50}$ values reported for other high-producer cell lines such as HEK293 [32] or AGE1.CR [12] cells.

3.4. Process intensification

To further intensify influenza virus production, two cultivations were performed in perfusion mode using an ATF2 system and cells infected at a concentration of around 46×10^6 cells/mL. Cell viability exceeding 97% and consistent growth during the exponential growth phase were observed before infection (Fig. 4A). The fact that similar doubling times were obtained as for batch cultivations in bioreactors (Table 1) suggests that cell growth was not impaired by the ATF system (and the high cell concentrations). Using a cell-specific glucose consumption rate taking into account cell concentration avoided glucose limitations for both runs (Fig. 4C). Glutamine levels were close or equal to zero for both runs (Fig. 4D), but are not considered critical for this cell line (see

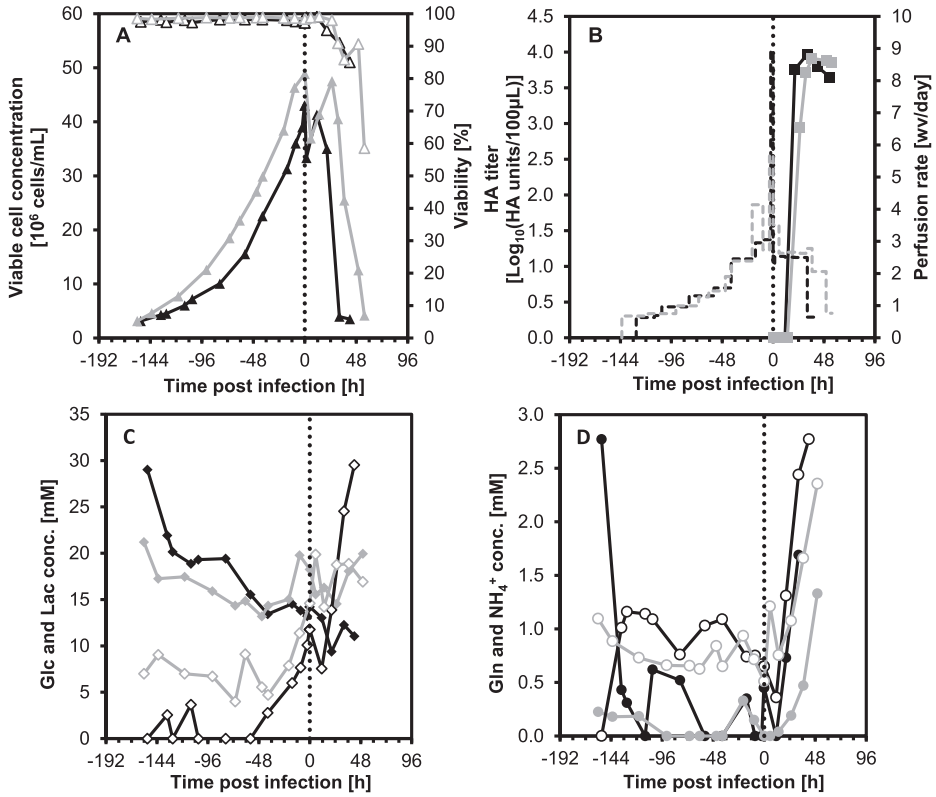

Fig. 4. PBG.PK2.1 cell growth and influenza A/PR/8/34 virus production of run 1 (black) and run 2 (grey) in bioreactor in perfusion mode. Cell cultures were coming from different pre-culture. CD-U5 medium was used for the whole run. A DASGIP system coupled to an ATF2 system (0.2 μm membrane cut-off) was used for the perfusion runs. A trypsin concentration of 22 U/mL in perfused media and a MOI of 10^{-5} was applied at the time of infection. (A) viable cell concentration (▲), viability (△). (B) HA titer (■), perfusion rate (dashed lines). (C) glucose (◆) and lactate (◇) concentrations. (D) glutamine (●) and ammonium (○) concentrations. Dotted vertical lines correspond to the time of infection.

Section 3.1). Correspondingly, low ammonium concentrations below 1.5 mM were observed during the cell growth phase before infection. While at least 13 amino acids have been reported to be essential for mammalian cell growth [41] no limitations were observed for 12 of them during the whole cultivation period (data not shown). However, tryptophan levels close or equal to 0 mM were measured during the cell growth phase. As very low tryptophan concentrations of 0.05 mM are often recommended for mammalian cell culture [41], and the limit of quantification of the HPLC method used here was 0.25 mM (data not shown), it is not clear whether this had an impact on growth performance.

With higher cell concentrations, higher levels of non-quantified virus production inhibitors and limitation in other substrates can decrease the CSVY and titers [42,43]. To avoid such limitations, fresh cell culture medium was added continuously by increasing the perfusion rate 3 h before infection. This resulted in an increase in the working volume by 30% after virus infection. Overall, a maximum titer of 3.93 ± 0.05 $\log_{10}$(HA units/100 μL) (Fig. 4B) was achieved in the bioreactor for both runs at 36 hpi. As previously reported [12,18], the virus particles generally accumulate in the bioreactor when an ATF system is used for cell retention even with membrane pore sizes as large as 0.5 μm. Unspecific virus binding to the membrane and membrane fouling due to cell debris and DNA accumulation could explain this effect. A CSVY of 3929 ± 876 virions/cell was obtained in perfusion (Table 2), corresponding to a decrease of 30% compared to optimal virus infection conditions in batch mode (shaker). With the continuous supply of fresh medium, no limitation in glucose, and ammonium as well as lactate below critical values during the virus production phase, this reduction could be due to the presence of other, non-quantified inhibitors of virus production. Nevertheless, results are still very promising for high cell density influenza virus production compared to other high cell density processes using either CAP cells [12] (CSVY = 1883 virions/cell) or AGE1.CR cells [22] (CSVY = 1266 virions/cell) at concentrations above 30×10^6 cells/mL. A similar CSVY (3960 virions/cell) was also reported for influenza production in perfusion mode using HEK cells: however, the cell culture was infected at lower cell densities (6×10^6 cells/mL) [32]. Our obtained titers have the potential to be further increased through a DOE approach. The maximal HA titer could be increased with medium optimization (based on a detailed metabolic characterization) [37,44] as the CD-U5 medium was first designed for avian cell culture. Concerning $TCID_{50}$, high titer of 3.2×10^9 infectious virions/mL were obtained in the perfusion cultures, showing a positive signal for life-attenuated influenza vaccine production in high cell density.

The culture medium productivity in perfusion mode was equal to $1.9 \pm 0.3 \times 10^9$ virions/L/day, which was around three times lower compared to the fed-batch processes performed at the same scale (Table 2). Similar to recombinant protein production in perfusion mode, one strategy to increase economic competitiveness of perfusion processes is to reduce their perfusion rates. An iterative stepwise decrease of the perfusion rate as a medium development strategy has been shown to efficiently increase productivity for mammalian cell culture using an ATF system [45]. However, such a strategy should not compromise CSVY and cell growth by potentially increasing the concentration of inhibiting components in the cell culture medium. Another way to decrease the amount of spent medium is the better control of the perfusion rate. Manual adjustments of the perfusion rate did not always fit cell growth and led to

temporary overfeeding during the cell growth phase (Fig. 4B). For example in Fig. 4C glucose concentration clearly exceeded the determined minimum glucose concentration of 6 mM (Section 2.3). One way to control the perfusion rate is to use an on-line capacitance probe for determination of cell concentration taking into account cell-specific medium demand [46]. Another solution to reach higher productivity could be continuous harvesting avoiding virus degradation. The ATF systems only partially allow continuous harvest and accumulate virus as well as possible virus deactivating components inside of the bioreactor. An alternative might be the use of acoustic settlers, which has already been shown for influenza virus production in perfusion mode using HEK293 cells for concentrations up to 18×10^6 cells/mL [32].

3.5. Influenza A/PR/8/34 hemagglutinin glycosylation

Glycosylation of recombinant proteins (i.e. monoclonals) is a critical quality attribute. For cell-culture-based viral vaccines, yet no glycoanalysis of antigens is required by the authorities [47,48]. Considering the use of different host cells for vaccine production with their significant impact on glycosylation it seems natural to equally consider this point. Different studies have shown the importance of influenza A virus glycosylation in terms of immunogenicity [49,50]. Here, for the analysis of the multiple potential glycosylation sites located on HA and NA, state-of-the-art mass spectrometry-based site-specific glycopeptide analysis was necessary.

An overview for the site-specific glycopeptide analysis of influenza A virus antigen HA propagated in the porcine cell line is given in Table 4. Surprisingly, exclusively high-mannose-type (Man) *N*-glycans were identified on the HA1 *N*-glycosylation sites N285 (Man7 and Man8) and N303 (Man8). Two potential *N*-glycosylation sites (N27/28 and N40) were not found to be glycosylated. *N*-glycosylation site N497, located at the HA2 domain, was also identified to carry high-mannose type *N*-glycans (Man6 and Man8), together with a potential hybrid-type *N*-glycan Hex7-HexNAc3 (Hexose (Hex), *N*-acetylhexosamine (HexNAc)). All fragment ion spectra of the detected *N*-glycopeptides are shown in the supplementary information. In contrast to the HA antigen, no glycopeptides were detected for NA. Furthermore, no *O*-glycopeptides of HA or NA were identified (data not shown). Although glycosylation sites located at the head region of HA have been shown to influence virulence, studies suggest there is no clear specific glycosylation site which has a crucial effect on immunogenicity as HA glycosylation can modulate humoral responses focused on different HA regions [51].

Compared to the glycosylation pattern of HA expressed in other host cell systems (i.e., chicken eggs, MDCK or Vero cells), influenza A virus propagated in PBG.PK2.1 cells seems not to have complex-type *N*-glycosylations [13–17]. The high mannose glycosylation pattern identified resembles more the glycosylation of recombinant HA produced in Sf9 cells [17,51]. It is an unexpected finding as porcine cells are normally distinguished by highly complex *N*- and *O*- linked glycans. This finding could indicate different properties in terms of virulence and immunogenicity that need to be further elucidated by performing glycoimmunological experiments including animal trials.

3.6. Analysis for downstream processing

Specifications for inactivated cell-culture-derived whole-virion influenza vaccines are set by the European Pharmacopoeia Commission in terms of antigen content, protein levels, impurities such as DNA and endotoxins, residual infectivity, and others [52]. In order to determine the potential burden regarding downstream processing of the virus harvest produced, the impurities that are the most challenging in subsequent virus purification were measured: total protein and host cell DNA. According to the current European Pharmacopoeia Commission, cell-culture-based influenza vaccines should have 15 μg of HA antigen per strain, <10 ng DNA and the protein content should be <6 × HA antigen content and <100 μg per strain (final product).

HA contents up to 6.4 μg/mL and 21.9 μg/mL were obtained from bioreactor cultivations in fed-batch and in perfusion mode, respectively. (Fig. 5A). Following total protein (Fig. 5B) and DNA

Table 4
Site-specific glycopeptide analysis of the influenza A virus glycoprotein hemagglutinin. ManX – high-mannose type *N*-glycan (X = number of mannoses), Hybrid – hybrid-type *N*-glycan [Hexose (Hex), *N*-acteylhexosamine (HexNAc)]. Red indicates the glycosylated asparagine.

Site	Sequence	*N*-glycan composition	Enzyme	Fragment ion spectrum
N285	GFGSGIITS**N**	Man7	Trypsin + Flavastacin	Supplementary Figure 1
		Man8	Trypsin + Flavastacin	Supplementary Figure 2
N303	CQTPLGAI**N**	Man8	Trypsin + Flavastacin	Supplementary Figure 3
N497	**N**GTYDYPK	Man6	Trypsin	Supplementary Figure 4
		Man8	Trypsin	Supplementary Figure 5
		Hybrid Hex7HexNAc3	Trypsin	Supplementary Figure 6

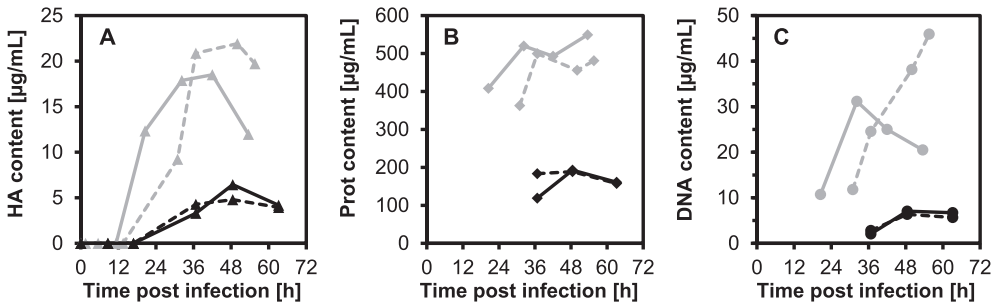

Fig. 5. Influenza A/PR/8/34 virus production parameters, using the PBG.PK2.1 cell line, in fed-batch mode for run 1 (black line) and run 2 (dotted black line) and in perfusion mode for run 1 (grey line) and run 2 (dotted grey line) to be considered for further downstream processing. (A) HA antigen content determined by SRID assay (▲). (B) Total protein concentration in the cell culture broth determined by Bradford assay (◆). (C) Host cell dsDNA concentration in the cell culture broth determined by PicoGreen assay (●).

(Fig. 5C) levels over time, we determined the best time of harvest (highest ratio of HA antigen per content of DNA (μg HA/ng DNA) and total protein (μg HA/μg total protein)) to be 48 hpi for fed-batch mode and 36 hpi for perfusion mode. At these time points, around 18,500 ng dsDNA and up to 600 μg total protein were measured per HA dose for both modes (Table 2). Similar DNA and protein contents in influenza virus harvests have been reported in the past and tackled by different purification techniques, most recently single-use steric exclusion chromatography [21] and pseudo affinity chromatography with sulfated cellulose membrane adsorbers [53].

Overall, this indicates that perfusion processes with their higher productivity do not necessarily put an additional burden to subsequent downstream processing compared to fed-batch modes. It is evident that additional parameters analysis (such as the viscosity of virus harvests and virion size distributions) and a proper assessment on purification performance are needed, but were out of scope here and will be tested in future work.

4. Conclusion

An ideal host cell line for influenza vaccine production should display robust growth in suspension with high viability, easy scale-up, fast virus production to high titers and low total protein and DNA concentrations in virus harvest broths to facilitate purification [4]. PBG.PK2.1 cells showed relatively high cell growth with high viability. More importantly, a low production rate of the metabolic by-products lactate and ammonium was observed in batch mode. A maximum cell density of 10^7 cells/mL was reached in bioreactor batch mode. Scaling up from shake flask to bioreactor scale was shown to be efficient, as similar doubling times and substrate consumption rates were observed.

PBG.PK2.1 cells have shown to be permissive for at least three influenza virus strains. Using this cell line could be also of interest for veterinary use, as a high porcine influenza virus titer (3.37 $\log_{10}$(HA units/100 μL)) was obtained. Concerning human influenza A/PR/8/34 virus titers, similar or higher CSVYs were obtained in fed-batch mode, compared to other processes using suspension cells such as CAP, PER.C6 or HEK293 cells. However, CSVY (5375 virions/cell) was found to be lower compared to the highest CSVY reported in literature (up to 40,000 virions/cell in MDCK cells [8]). In addition, PBG.PK2.1 cells showed to be a good candidate for high cell density processes, since cell growth to high cell concentrations maintaining high viability was achieved in perfusion mode. Most importantly, CSVY was maintained high, which allowed reaching HA titers of 3.93 ± 0.05 $\log_{10}$(HAU/100 μL). Such a titer using a suspension cell line at bioreactor scale is to our knowledge among the highest reported in literature. Furthermore, high $TCID_{50}$ titers were obtained in fed-batch (4.4×10^8 infectious virions/mL) and perfusion mode (3.2×10^9 infectious virions/mL), which could be of interest for life-attenuated influenza virus production. Exclusively high-mannose-type *N*-glycans were detected on different HA sites, which is very different compared to influenza A virus strains produced in MDCK or Vero cells. Whether this is related to differences in immunogenicity and/or virulence should be further investigated in follow-up studies. Regarding downstream processing, dsDNA and protein contamination levels of 18500 ng and 600 μg were found in fed-batch and perfusion mode at the optimal harvest time point. Such impurity levels have been reported to be handled successfully in chromatography-based purification regimes including steric exclusion chromatography [21].

Taking into account the high maximum cell concentrations obtained in batch mode, cell growth in a chemically-defined medium, suitability of cells for up-scaling and process intensification, and the high influenza virus titers obtained makes PBG.PK2.1 cells a promising candidate for next-generation influenza virus vaccine manufacturing.

Acknowledgment

The authors would like to thank A. Bastian, C. Best, L. Fichtmüller, S. König and N. Wynserski for excellent technical support. Additionally, the authors would like to acknowledge I. Jordan, as he was involved at the early stage of development of the cell line at ProBioGen.

Conflict of interest

G.G., J. C., A. P., P.M., M. W., E. R., Y. G., U. R. declare that they have no conflict of interest. A.K., V.S. are employees of ProBioGen where PBG.PK2.1 and CD-U5 have been developed.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.030.

References

[1] Organization WH. Vaccines against influenza WHO position paper. Wkly Epidemiol Rec 2012;87:461–76.
[2] Krammer F, Palese P. Advances in the development of influenza virus vaccines. Nat Rev Drug Discov 2015;14:167–82.
[3] Ulmer JB, Valley U, Rappuoli R. Vaccine manufacturing: challenges and solutions. Nat Biotechnol 2006;24:1377–83.
[4] Genzel Y, Reichl U. Continuous cell lines as a production system for influenza vaccines. Expert Rev Vaccines 2009;8:1681–92.
[5] Harding AT, Heaton NS. Efforts to Improve the Seasonal Influenza Vaccine. Vaccines (Basel) 2018;6.
[6] Milian E, Kamen AA. Current and emerging cell culture manufacturing technologies for influenza vaccines. Biomed Res Int 2015;2015:11.
[7] Petiot E, Proust A, Traversier A, Durous L, Dappozze F, Gras M, et al. Influenza viruses production: evaluation of a novel avian cell line DuckCelt(R)-T17. Vaccine 2018;36:3101–11.
[8] Huang D, Peng WJ, Ye Q, Liu XP, Zhao L, Fan L, et al. Serum-Free Suspension Culture of MDCK Cells for Production of Influenza H1N1 Vaccines. PLoS One 2015;10:e0141686.
[9] Aubrit F, Perugi F, Leon A, Guehenneux F, Champion-Arnaud P, Lahmar M, et al. Cell substrates for the production of viral vaccines. Vaccine 2015;33:5905–12.
[10] Pohlscheidt M, Langer U, Minuth T, Bodeker B, Apeler H, Horlein HD, et al. Development and optimisation of a procedure for the production of Parapoxvirus ovis by large-scale microcarrier cell culture in a non-animal, non-human and non-plant-derived medium. Vaccine 2008;26:1552–65.
[11] Tapia F, Jordan I, Genzel Y, Reichl U. Efficient and stable production of Modified Vaccinia Ankara virus in two-stage semi-continuous and in continuous stirred tank cultivation systems. PLoS One 2017;12:17.
[12] Genzel Y, Vogel T, Buck J, Behrendt I, Ramirez DV, Schiedner G, et al. High cell density cultivations by alternating tangential flow (ATF) perfusion for influenza A virus production using suspension cells. Vaccine 2014;32:2770–81.
[13] Harvey DJ. Mass spectrometric analysis of glycosylated viral proteins. Expert Rev Proteomics 2018;15:391–412.
[14] Schwarzer J, Rapp E, Hennig R, Genzel Y, Jordan I, Sandig V, et al. Glycan analysis in cell culture-based influenza vaccine production: Influence of host cell line and virus strain on the glycosylation pattern of viral hemagglutinin. Vaccine 2009;27:4325–36.
[15] Hennig R, Rapp E, Kottler R, Cajic S, Borowiak M, Reichl U. N-glycosylation fingerprinting of viral glycoproteins by xCGE-LIF. In: Lepenies B, editor. Carbohydrate-Based Vaccines: Methods and Protocols. Springer, New York: New York, NY; 2015. p. 123–43.
[16] Schwarzer J, Rapp E, Reichl U. N-glycan analysis by CGE–LIF: Profiling influenza A virus hemagglutinin N-glycosylation during vaccine production. Electrophoresis 2008;29:4203–14.
[17] Yanming A, Parsons LM, Jankowska E, Melnyk D, Joshi M, Cipollo JF. N-Glycosylation of Seasonal Influenza Vaccine Hemagglutinins: Implication for Potency Testing and Immune Processing. J Virol 2019;93:e01693–e1718.
[18] Vazquez-Ramirez D, Genzel Y, Jordan I, Sandig V, Reichl U. High-cell-density cultivations to increase MVA virus production. Vaccine 2018;36:3124–33.
[19] Kalbfuss B, Knöchlein A, Kröber T, Reichl U. Monitoring influenza virus content in vaccine production: precise assays for the quantitation of hemagglutination and neuraminidase activity. Biologicals 2008;36:145–61.

[20] Genzel Y, Reichl U. Vaccine Production. In: Poertner R, editor. Animal cell biotechnology: methods and protocols. Totowa, NJ: Humana Press; 2007. p. 457–73.
[21] Marichal-Gallardo P, Pieler MM, Wolff MW, Reichl U. Steric exclusion chromatography for purification of cell culture-derived influenza A virus using regenerated cellulose membranes and polyethylene glycol. J Chromatogr A 2017;1483:110–9.
[22] Vazquez-Ramirez D, Jordan I, Sandig V, Genzel Y, Reichl U. High titer MVA and influenza A virus production using a hybrid fed-batch/perfusion strategy with an ATF system. Appl Microbiol Biotechnol 2019.
[23] Frensing T, Kupke SY, Bachmann M, Fritzsche S, Gallo-Ramirez LE, Reichl U. Influenza virus intracellular replication dynamics, release kinetics, and particle morphology during propagation in MDCK cells. Appl Microbiol Biotechnol 2016;100:7181–92.
[24] Pralow A, Hoffmann M, Nguyen-Khuong T, Rapp E, Reichl U. Improvement of the glycoproteomic toolbox with the discovery of a unique C-terminal cleavage specificity of flavastacin for N-glycosylated asparagine. Sci Rep 2017;7:11419.
[25] Hoffmann M, Pioch M, Pralow A, Hennig R, Kottler R, Reichl U, et al. The fine art of destruction: a guide to in-depth glycoproteomic analyses-exploiting the diagnostic potential of fragment ions. Proteomics 2018.
[26] Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods 2009;6:359–62.
[27] Selman MH, Hemayatkar M, Deelder AM, Wuhrer M. Cotton HILIC SPE microtips for microscale purification and enrichment of glycans and glycopeptides. Anal Chem 2011;83:2492–9.
[28] Pioch M, Hoffmann M, Pralow A, Reichl U, Rapp E. glyXtoolMS: An open-source pipeline for semiautomated analysis of glycopeptide mass spectrometry data. Anal Chem 2018.
[29] Marks DM. Equipment design considerations for large scale cell culture. Cytotechnology 2003;42:21–33.
[30] King JA, Miller WM. Bioreactor development for stem cell expansion and controlled differentiation. Curr Opin Chem Biol 2007;11:394–8.
[31] Lohr V, Genzel Y, Behrendt I, Scharfenberg K, Reichl U. A new MDCK suspension line cultivated in a fully defined medium in stirred-tank and wave bioreactor. Vaccine 2010;28:6256–64.
[32] Petiot E, Jacob D, Lanthier S, Lohr V, Ansorge S, Kamen AA. Metabolic and Kinetic analyses of influenza production in perfusion HEK293 cell culture. BMC Biotech 2011;11:12.
[33] Hassel T, Gleave S, Butler M. Growth inhibition in animal cell culture. The effect of lactate and ammonia. Appl Biochem Biotechnol 1991;30:29–41.
[34] Ozturk SS, Riley MR, Palsson BO. Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production. Biotechnol Bioeng 1992;39:418–31.
[35] Cruz HJ, Freitas CM, Alves PM, Moreira JL, Carrondo MJT. Effects of ammonia and lactate on growth, metabolism, and productivity of BHK cells. Enzyme Microb Technol 2000;27:43–52.
[36] Schneider M, Marison IW, von Stockar U. The importance of ammonia in mammalian cell culture. J Biotechnol 1996;46:161–85.
[37] Lohr V, Hädicke O, Genzel Y, Jordan I, Büntemeyer H, Klamt S, et al. The avian cell line AGE1.CR.pIX characterized by metabolic flux analysis. BMC Biotech 2014;14:72.
[38] Genzel Y, Dietzsch C, Rapp E, Schwarzer J, Reichl U. MDCK and Vero cells for influenza virus vaccine production: a one-to-one comparison up to lab-scale bioreactor cultivation. Appl Microbiol Biotechnol 2010;88:461–75.
[39] Le Ru A, Jacob D, Transfiguracion J, Ansorge S, Henry O, Kamen AA. Scalable production of influenza virus in HEK-293 cells for efficient vaccine manufacturing. Vaccine 2010;28:3661–71.
[40] Genzel Y, Behrendt I, Rodig J, Rapp E, Kueppers C, Kochanek S, et al. CAP, a new human suspension cell line for influenza virus production. Appl Microbiol Biotechnol 2013;97:111–22.
[41] Eagle H. Amino acid metabolism in mammalian cell cultures. Science 1959;130:432–7.
[42] Ferreira TB, Ferreira AL, Carrondo MJ, Alves PM. Effect of re-feed strategies and non-ammoniagenic medium on adenovirus production at high cell densities. J Biotechnol 2005;119:272–80.
[43] Lindsay DA, Betenbaugh MJ. Quantification of cell culture factors affecting recombinant protein yields in baculovirus-infected insect cells. Biotechnol Bioeng 1992;39:614–8.
[44] Huang D, Xia-Hou K, Liu XP, Zhao L, Fan L, Ye Z, et al. Rational design of medium supplementation strategy for improved influenza viruses production based on analyzing nutritional requirements of MDCK Cells. Vaccine 2014;32:7091–7.
[45] Konstantinov K, Goudar C, Ng M, Meneses R, Thrift J, Chuppa S, et al. The "Push-to-Low" Approach for Optimization of High-Density Perfusion Cultures of Animal Cells. In: Hu W-S, editor. Cell Culture Engineering. Springer, Berlin Heidelberg: Berlin, Heidelberg; 2006. p. 75–98.
[46] Nikolay A, Leon A, Schwamborn K, Genzel Y, Reichl U. Process intensification of EB66(R) cell cultivations leads to high-yield yellow fever and Zika virus production. Appl Microbiol Biotechnol 2018.
[47] EMA – Guideline on development, production, characterisation and specifications for monoclonal antibodies and related products - draft (2009); 2013.
[48] EMA – Guideline on Influenza Vaccines - Quality module (2017); 2017.
[49] Hutter J, Rodig JV, Hoper D, Seeberger PH, Reichl U, Rapp E, et al. Toward animal cell culture-based influenza vaccine design: viral hemagglutinin N-glycosylation markedly impacts immunogenicity. J Immunol 2013;190:220–30.
[50] de Vries RP, Smit CH, de Bruin E, Rigter A, de Vries E, Cornelissen LA, et al. Glycan-dependent immunogenicity of recombinant soluble trimeric hemagglutinin. J Virol 2012;JVI:01084–1112.
[51] Tate M, Job E, Deng Y-M, Gunalan V, Maurer-Stroh S, Reading P. Playing hide and seek: how glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection. Viruses. 2014;6:1294.
[52] Kalbfuss B, Reichl U. Viral vaccines purification. In: Wen E, Ellis R, Pujar N, editors. Vaccine development and manufacturing. John Wiley & Sons, Inc.; 2014. p. 97–180.
[53] Fortuna AR, Taft F, Villain L, Wolff MW, Reichl U. Optimization of cell culture-derived influenza A virus particles purification using sulfated cellulose membrane adsorbers. Eng Life Sci 2018;18:29–39.
[54] Tapia F, Vogel T, Genzel Y, Behrendt I, Hirschel M, Gangemi JD, et al. Production of high-titer human influenza A virus with adherent and suspension MDCK cells cultured in a single-use hollow fiber bioreactor. Vaccine 2014;32:1003–11.
[55] Pau MG, Ophorst C, Koldijk MH, Schouten G, Mehtali M, Uytdehaag F. The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. Vaccine 2001;19:2716–21.