Vaccine 37 (2019) 7003–7010

Contents lists available at ScienceDirect

Vaccine

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

Semi-perfusion cultures of suspension MDCK cells enable high cell concentrations and efficient influenza A virus production

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ARTICLE INFO

Article history:
Available online 29 April 2019

Keywords:
MDCK suspension
High cell density
Influenza A virus production
Process intensification
Semi-perfusion
Cell culture-based vaccine manufacturing

ABSTRACT

Control and prevention of rapid influenza spread among humans depend on the availability of efficient and safe seasonal and pandemic vaccines, made primarily from inactivated influenza virus particles. Current influenza virus production processes rely heavily on embryonated chicken eggs or on cell culture as substrate for virus propagation. Today's efforts towards process intensification in animal cell culture could innovate viral vaccine manufacturing using high-yield suspension cells in high cell density perfusion processes. In this work, we present a MDCK cell line adapted to grow as single cell suspension with a doubling time of less than 20 h, achieving cell concentrations over 1×10^7 cells/mL in batch mode. Influenza A virus titer obtained in batch infections were 3.6 $\log_{10}$(HAU/100 μL) for total- and 10^9 virions/mL for infectious virus particles ($TCID_{50}$), respectively. In semi-perfusion mode concentrations up to 6×10^7 cells/mL, accumulated virus titer of 4.5 $\log_{10}$(HAU/100 μL) and infectious titer of almost 10^{10} virions/mL ($TCID_{50}$) were possible. This exceeds results reported previously for cell culture-based influenza virus propagation by far and suggests perfusion cultures as the preferred method in viral vaccine manufacturing.

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1. Introduction

Influenza virus infections are responsible for millions of flu patients and associated with 290,000–650,000 annual deaths worldwide [1]. Additionally, outbreaks of pandemic influenza virus strains have caused millions of deaths in the last century, and still are a threat for the future [2]. To minimize influenza spread, limit health risks and reduce its economic burden, vaccination campaigns pose a huge demand with 500 million vaccine doses to be manufactured and distributed each year (2015) [3,4]. Besides traditional egg-based vaccine manufacturing, production platforms based on cell culture contribute increasingly to an overall growing market. In contrast to eggs, animal cells can be propagated in chemically defined media lacking potential allergens, are independent from egg supply chains, enable fast scale-up and large scale manufacturing with a relatively small foot print [5,6]. For influenza vaccine manufacturing, animal cells are either used for recombinant expression of viral antigens or as a host cell substrate for the propagation of whole virions [7,8]. The first recombinant influenza vaccine (Flublok®) was commercialized in 2014, using insect cells for the expression of hemagglutinin (HA) antigens [9,10]. For viral human influenza vaccines, only the mammalian Vero and Madin-Darby canine kidney (MDCK) cells have been used commercially (Influvac®, Optaflu®/Flucelvax®, Preflucel®) [7,11]. Additionally, HEK293 and other cell lines like Per.C6®, EB66®, CAP®, AGE1.CR® were evaluated as a cell substrate for influenza virus propagation [11–14]. High specific growth rates, high cell concentrations and growth in single cell suspension in chemically defined media make these cell lines very interesting for influenza virus production. However, with a cell-specific virus yield (CSVY) exceeding 10,000 virions/cell (5×10^{-4} HAU/cell), MDCK cells remain the most productive cell line for influenza viruses [15]. Initially, MDCK cells were cultivated as adherent cells on microcarriers [16–19]. Further cell line and media development led to MDCK suspension cell lines, used both in academia [20–23] and industry [24]. These cell lines, however, typically have a lower specific growth rate, grow only to rather low cell densities and/or as cell aggregates or display lower specific virus yields. Only recently, advances in medium development led to fast growing MDCK suspension cells

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https://doi.org/10.1016/j.vaccine.2019.04.054
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reaching high cell concentrations [25,26]. In addition to cell line and medium development, process intensification towards high cell density (HCD) perfusion culture can increase virus titers or overall process productivity [27,28]. Perfusion technologies are already applied successfully to intensify CHO cell-based manufacturing of recombinant proteins [29]. More recently, this technology was also evaluated in more detail for cell culture-based virus propagation [30–33]. Finding the best perfusion strategy for both cell growth and virus propagation phase is needed, to prevent medium limitations and maintain cell-specific productivity avoiding the so called "cell density effect" [34,35]. Depending on specific demands of the cell line and cultivation media available, the cell specific perfusion rate (CSPR) has to be optimized towards optimal cell growth with minimal medium consumption [36]. To mimic lab-scale perfusion processes, shaker experiments in semi-perfusion can be applied as a scale-down model [27]. First experiments with HEK293, AGE1.CR and CAP cells showed the potential of influenza virus production at high cell density. However, low CSVY didn't lead to significantly higher virus titers than those obtained with MDCK cell-based processes [30,31].

In the presented study, we demonstrate the adaptation of a MDCK suspension cell line to a new cultivation medium (Xeno™), which enables growth in single cell suspension, high specific growth rate and high cell concentrations. Additionally to batch experiments, we evaluate the potential of perfusion cultures for the manufacturing of influenza virus vaccines. We demonstrate the possibility to combine the high CSVY of MDCK cells with a high cell concentration in perfusion systems to maximize influenza virus titers.

2. Material and methods

2.1. Cell lines & cell culture

The MDCK suspension cell line, here referred as MDCK.SUS2 (P43), was previously adapted from adherent MDCK cells (ECACC, #84121903) to grow in suspension in chemically defined medium (Smif8 medium, Gibco, acquired through K. Scharfenberg, University of Applied Sciences, Emden/Leer, Germany, supplemented with 4 mM glutamine, and 4 mM pyruvate) [23]. MDCK.SUS2 cells adapted to the animal component free medium (Xeno™-S001S, #FG0100402, Shanghai BioEngine Sci-Tech) were established as a new cell line, here referred as MDCK.Xeno (see below). Both MDCK suspension cell lines were cultivated in 50 mL working volume in shaker flasks (125 mL baffled polycarbonate Erlenmeyer Flask, Corning®, #431405) at 37 °C in a 5% CO_2 atmosphere with a shaking frequency of 185 rpm (Multitron Pro, Infors HT; 50 mm shaking throw). Cells were passaged every 3–4 days with seeding cell density of 0.5×10^6 cells/mL. Cell concentration, diameter and viability were measured with a cell counter (Vi-CELL XR, Beckman Coulter, #731050), cell concentrations over 10×10^6 cells/mL were diluted before measurements with PBS. Due to cell aggregates, MDCK.SUS2 cells were trypsinized (10 min, 0.9 × trypsin, 37 °C) prior to cell counting. Extracellular metabolite concentrations were measured from the cell free supernatant with a BioProfile 100 Plus analyzer (Nova Biomedical).

2.2. Cell adaptation

For the adaptation of the MDCK.SUS2 (P43) cells to Xeno medium, a step-wise medium change was applied. Over the first adaptation period, the content of Xeno medium was increased by 10% steps. During adaptation, MDCK cells were passaged by spinning down cells (300*g*, 5 min, RT) to adjust to 1×10^6 cells/mL final inoculation concentration. Cells were resuspended in the new medium mixture (10% carry over) and cultivated for 3 days. In cases of low cell growth, additional passages with the same medium mixture were performed to stabilize adaptation.

2.3. Influenza virus infection

All infections were carried out with an influenza A seed virus strain A/PR/8/34 of the subtype H1N1 (Robert Koch Institute, Berlin, Germany), in the following called influenza A virus or IAV. The original influenza A virus strain obtained from RKI (Amp. 3138) was propagated in adherent MDCK cells (ECACC, #84121903). The infectious titer of the final seed virus was 1.1×10^9 virions/mL ($TCID_{50}$). Trypsin (Gibco, #27250-018; 5000 U/mL in PBS) was added at time of infection with a final activity of 20–30 U/mL (5–50×10^{-7} U/cell). For infections in semi-perfusion culture, trypsin was added to the feed at the respective concentration. Immediately before infection, cells were spun down (300*g*, 5 min, RT) and 50% (90% for semi-perfusion) of cell free medium was replaced with fresh medium.

2.4. Semi-perfusion culture

Semi-perfusion cultures were established to reach high cell density conditions in shaker flasks [27]. In each perfusion step, cells are pelleted by centrifugation (400*g*, 10 min, RT) and up to two third (33 mL) of the cultivation volume was removed and replaced with warm, fresh cultivation medium. With increasing cell concentration, more medium had to be replaced or the time interval between medium replacements was decreased to realize an overall constant CSPR. For the calculation of the time of the perfusion step (Eq. (1)) or the perfusion volume (Eq. (2)), a constant specific growth rate of 0.027 h^{-1} and a CSPR of 2.5 pL/(cell h) was used.

$$\Delta t = \frac{\ln\left(\frac{V_P}{V_W}\frac{\mu}{CSPR}\frac{1}{[X]} + 1\right)}{\mu} \quad (1)$$

$$\frac{V_P}{V_W} = \frac{[X]CSPR(e^{\mu\Delta t} - 1)}{\mu} \quad (2)$$

Δt: time between perfusion steps (h)
V_W: working volume (mL)
[X]: cell concentration (cells/mL)
V_P: perfusion volume (mL)
μ: specific growth rate (1/h)
CSPR: cell specific perfusion rate (pL/(cell h))

2.5. Hemagglutination assay

In order to quantify the total number of influenza virus particles, the hemagglutination assay was used as described in detail by Kalbfuss et al. [37]. Virus containing cell suspension was centrifuged to remove cells and cell debris (2000*g*, 5 min, RT) and the supernatant was stored at −80 °C until measurement. Virus samples and HA standard (undiluted and $1{:}2^{0.5}$ predilution) were serially diluted (0.5^n with n = 1–12) with PBS in 96 round bottom wells. 100 μL of chicken erythrocyte solution was added (2×10^7 erythrocytes/mL) to diluted samples (100 μL) and incubated for 3–8 h at RT. The HA activity was evaluated using a plate reader (infinite® M200 microplate reader, Tecan) measuring the extinction at 700 nm and the final titer was calculated by a curve fitting function of the resulting extinction data. The virus titer is expressed as the common logarithm ($\log_{10}$) of the HA unit (HAU) per analysis volume (100 μL): $\log_{10}$(HAU/100 μL). The corresponding total number of virus particles was calculated by multiplying HAU and erythrocyte concentration:

$$\frac{Virus_{total}}{mL} = 2E7\,\frac{1}{mL}\cdot HAU = 2E7\,\frac{1}{mL}\cdot 10^{\log_{10}(HAU/100\mu L)} \quad (3)$$

2.6. $TCID_{50}$ assay

For the quantification of infectious influenza virus particles the $TCID_{50}$ assay was used as described in detail by Genzel and Reichl [38]. The virus samples were collected from cell culture and spun down to remove cells and cell debris (2000*g*, 5 min, RT). Sterile supernatant was stored until measurement at −80 °C. Confluent MDCK cells in 96 well plates were infected with a serial dilution of the virus sample and stained after 24 h with an HA specific primary antibody (anti-influenza A/PR/8/34 H1N1 HA serum, #03/242, NIBSC) and a fluorescence labeled secondary antibody (Alexa Fluor donkey anti-sheep IgG antibody, #A11015, Thermo Fisher Scientific). Fluorescence positive and negative wells were counted and the infectious virus titer was calculated from eight replicates according to the Spearman-Kärber method [39,40].

2.7. Accumulated virus titer

The accumulated virus titer ($Titer_{AC}$) was calculated to allow a comparison of viral titers in a multiple harvest process (semi-perfusion) with the batch experiments. For this, the virus titer in each harvest/perfusion step ($titer_h$) was multiplied by the respective harvest volume (V_h), summed up and divided by the working volume (V_W) (Eq. (4)).

$$Titer_{Ac} = \frac{\sum(V_h \cdot Titer_h)}{V_W} \quad (4)$$

For the calculation of the accumulated HA-titer (HA_{AC}) it has to be considered that Eq. (4) can only be applied for HA-units (HAU). Therefore, logarithmic version of the HA-titer was calculated using Eq. (5):

$$HA_{Ac}\left[\log_{10}\left(\frac{HAU}{100\mu L}\right)\right] = \log_{10}\left(\frac{\sum\left(V_h \cdot HA_h\left[\frac{HAU}{100\mu L}\right]\right)}{V_W}\right) \quad (5)$$

3. Results and discussion

3.1. MDCK.SUS2 adaptation to Xeno medium

The original MDCK.SUS2 cell line cultivated in Smif8 medium had an average doubling time of 24–26 h (Fig. 1 AI), growing in small cell aggregates with variable size (Fig. 1BI). A direct adaptation of this cell line to the Xeno medium failed. Therefore the fraction of Xeno medium was slowly increased or kept constant until MDCK cells were able to grow in pure Xeno medium with a promising growth performance. Over the whole adaptation period, the viability of MDCK cells stayed above 90%. It seemed that the adaptation had a strong effect on the specific growth rate, but only minor effect on the overall health of the cell population. The whole adaptation process was divided into three phases. In the first adaptation phase (0–31 days) cell growth was similar or better compared to pure Smif8 cultivations (Fig. 1AII), the size of cell aggregates increased and a higher maximum cell concentration was reached (Fig. 1BII). In the second adaptation phase (31–66 days), cell growth was dramatically decreased with increasing Xeno content (Fig. 1AIII). Additionally, cellular aggregates disappeared and MDCK cells grew as single cells (Fig. 1BIII&IV). After the second adaptation phase, MDCK cells were growing in pure Xeno medium with a lower doubling time compared to the original culture (24 vs 34 h). In the third adaptation phase (66–180 days), cells were cultivated over multiple passages in Xeno medium to generate the cell line finally selected for process intensification studies (after 180 days) (Fig. 1AIV). During this adaptation phase, no morphological changes were observed, but cell metabolism seemed improved, leading to better specific growth rate, higher cell concentrations and lower lactate as well as ammonium accumulation. The last adaptation phase could also be considered a selection phase, where a (sub)population of cells was selected for more efficient and faster growth. Fully adapted cells (passage 60) were used to create a cell bank for further experiments. A re-adaptation of the MDCK.Xeno cell line to Smif8 medium reversed all the adaptation effects and cells were growing comparable to the original cell line (data not shown). The long adaptation time (>50 passages, 180 days) of the MDCK.SUS2 cell line to a stable MDCK.Xeno cell line with optimal growth in Xeno medium was rather surprising. In particular, the time period required for cell adaptation was in a comparable range as establishment of the original suspension cell line from adherent MDCK cells (>40 passages) [23,41]. As both media were developed for suspension cell growth, we expected a rather fast adaptation of the MDCK.SUS2 cell, but metabolic and morphological changes, as well as a (sub)population selection seemed to have increased the adaptation time. We confirmed canine origin of the cell line by proteomic analysis (data not shown), but additional genetic characterization would be necessary for commercial use. Tumorigenicity, which is often a concern for suspension cell lines, should also be tested if commercial use is anticipated. As other MDCK suspension cells have already

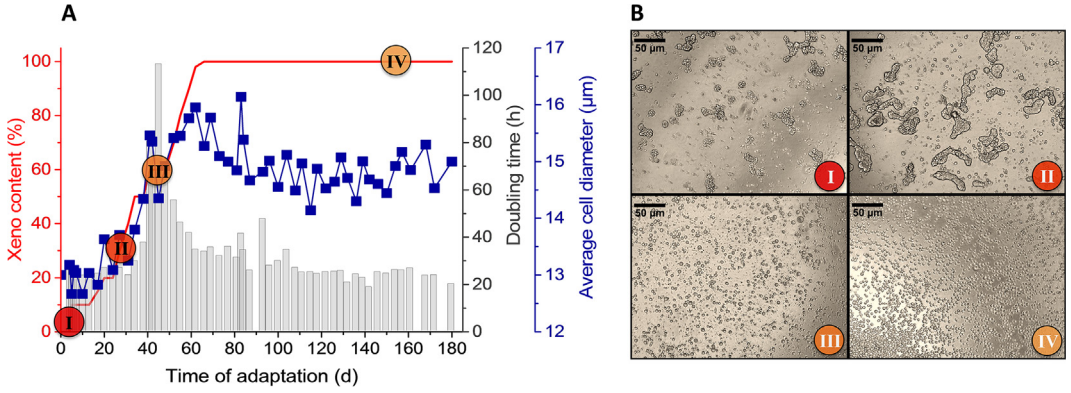

Fig. 1. Adaptation of MDCK.SUS2 cells from Smif8 to Xeno medium (MDCK.Xeno). MDCK.SUS2 cells were monitored over 60 passages during the adaptation to Xeno medium. A: average doubling time (grey bars: ■) and average cell diameter (■) was analyzed over the adaptation time and with increasing Xeno medium content (▬). B: Phase contrast microscopy pictures of MDCK suspension cells for morphological evaluation in different medium composition (I: 0% Xeno; II: 30% Xeno; III: 60% Xeno; IV: 100% Xeno).

been approved for commercial use, we strongly believe that the presented MDCK.Xeno cell line could be a good candidate for influenza vaccine production [42,43].

3.2. Cell growth and metabolism: Smif8 vs Xeno medium

Fully adapted MDCK.Xeno cells were able to grow in Xeno medium to cell concentrations above 13 × 10^6 cells/mL in shaker flasks (Fig. 2D). This was a significant improvement compared to MDCK.SUS2cells growing in Smif8 medium, where cells usually reach maximum cell densities between 6 and 8 × 10^6 cells/mL (Fig. 2A). Additionally, MDCK.Xeno cells were able grow with a much higher specific growth rate (μ_{max}: 0.036 1/h) compared to the MDCK.SUS2 cells (μ_{max}: 0.026 1/h).For both cell lines, the viability (95%) was stable over the cell growth phase and only decreased after a short stationary phase together with the viable cell concentration (Fig. 2A&D). Due to a higher concentration of the main energy metabolites, glucose and glutamine in the Xeno medium (Fig. 2B&E), an increase in cell concentrations was not very surprising. However, in the Xeno medium, single cell MDCK cells could utilize the available metabolites more efficiently to fuel growth demands. Interestingly it seemed that higher growth rate of MDCK.Xeno cells was mainly due to an overall higher consumption of glucose. Over 96 h of cultivation, the cell-specific consumption rate of glutamine was quite similar for both cell lines (17 vs 18 fmol/(cell h)), but the consumption rate of glucose was almost three times higher for MDCK.Xeno cells (26 vs 74 fmol/(cell h)) (Fig. 2C&F). Differences in consumption rates of these primary energy metabolites were much more visible in the early stage of cultivation. For MDCK.SUS2 cells, rates of glutamine and glucose consumption were quite similar over cultivation period of 96 h (Fig. 2C). For MDCK.Xeno cultivations, however, rates of glutamine and glucose consumption were highly elevated in the beginning (0–48 h) and later decreased dramatically (Fig. 2F). This could be explained partly by a metabolic shift in the middle (~72 h) of the MDCK.Xeno cultivation with a shift from from lactate production to lactate consumption (Fig. 2E). This effect was not observed for MDCK.SUS2 cells, where lactate accumulated to a similar level, but was not consumed later (Fig. 2B). A higher glutamine concentration in the Xeno medium led to a much higher accumulation of ammonium of up to 6 mM at 72 h of cultivation (Fig. 2E). For both cell lines, the cell diameter decreased after 48 h of cultivation. For the MDCK.Xeno cells there was an additional increase of average cell diameter during the initial lag phase of the cultivation (Fig. 2A&D). Here, changes in substrate concentration and decrease in osmolality (from 320 to 270 mOsm/kg) could have led to variations in the average cell diameter. Due to their better specific growth rate, higher maximum cell concentration and single cell growth, MDCK.Xeno cells easily outperformed not only MDCK.SUS2 cells, but also other MDCK suspension cell lines [20–23,26,44]. The only disadvantage was the production of high amounts of ammonium, which potentially can have a negative influence on virus replication [45–47].

Overall, these observations demonstrate the impact of medium development, where without any genetic manipulation massive changes of cell line performance are possible. Unfortunately, it still unclear what medium component(s) are linked to the adaptation to this specific MDCK.Xeno cell phenotype since the medium composition is not disclosed and detailed studies regarding uptake and release of medium compounds and metabolic by-products are still missing.

3.3. Influenza A virus production in batch mode

To evaluate differences between both MDCK suspension cells with respect to influenza A virus production, each cell line was inoculated in three shaker flasks with 0.5 × 10^6 cells/mL (Xeno) or 0.8 × 10^6 cells/mL (Smif8) respectively, and cultivated for 72 h. Same infection conditions (MOI 10^{-3}, 37 °C) were chosen with a trypsin activity of 20 U/mL. The previously used trypsin amount based on cell concentration (10^{-5} U/cell) [23] was not applicable for the MDCK.Xeno cells due to higher cell concentrations. Trypsin concentrations over 50 U/mL led to cell lysis, visible by a fast decrease in cell concentration with stable cell viability. With the

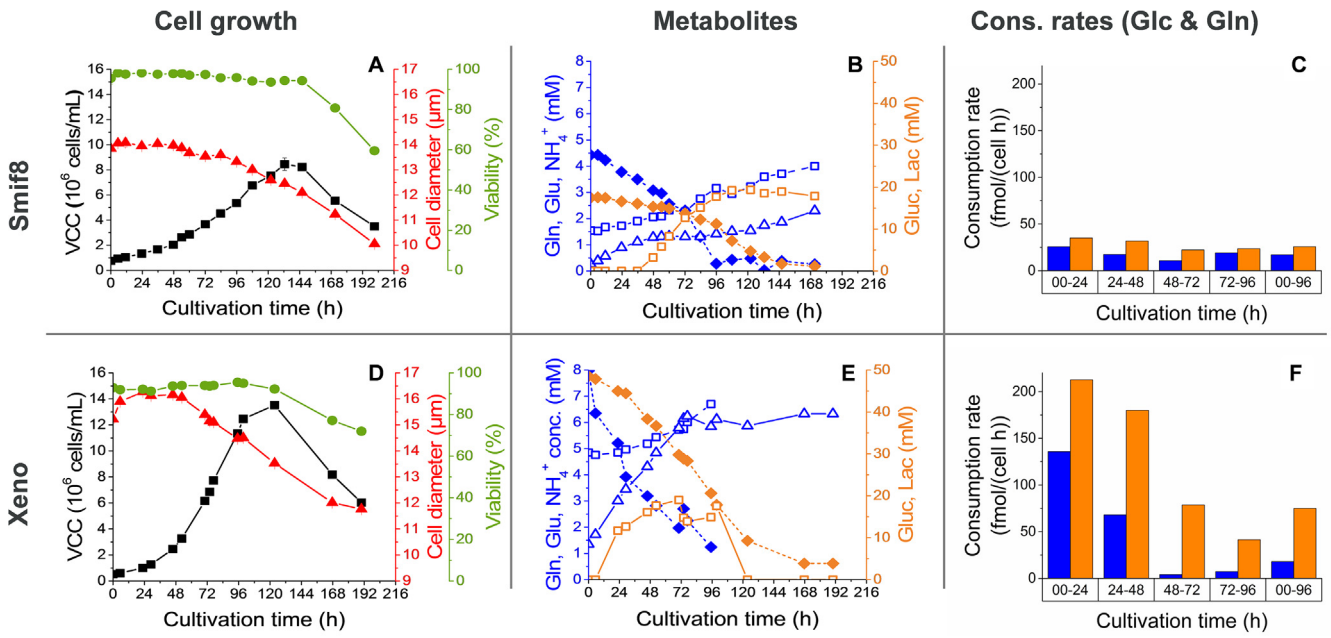

Fig. 2. Growth and metabolism of MDCK.SUS2 and MDCK.Xeno cells. MDCK cells were cultivated in shake flasks in Smif8 (A–C) and Xeno medium (D–F). Cell growth (A & D) and extracellular metabolites (B & E) were monitored during batch cultivation. Consumption rates (C & F) were calculated for 24 h intervals until 96 h for glutamine (■) and glucose (■); ■ viable cell concentration, ● viability, ▲ average cell diameter, ♦ glutamine (Gln), □glutamate (Glu), △ ammonium (NH_4^+), ♦ glucose (Glc), □ lactate (Lac), VCC: viable cell concentration.

chosen trypsin activity both cell lines continued to grow for the first 24 h post infection (hpi), reaching 5.5×10^6 cells/mL and 11.5×10^6 cells/mL, followed by a fast drop in cell concentration (Fig. 3). With the start of virus accumulation at 24 hpi, the average cell diameter decreased by 3–4 μm until the end of the infection. With MDCK.SUS2 cells a virus titer of 3.4 $\log_{10}$(HAU/100 μL) (5×10^{10} virions/mL) was reached at 36 hpi (Fig. 3A) compared to MDCK.Xeno, where virus production continued to increase to a maximum titer of 3.6 $\log_{10}$(HAU/100 μL) (8×10^{10} virions/mL) at 48 hpi (Fig. 3B). Considering the higher cell concentration of MDCK.Xeno cells, MDCK.SUS2 cells had a higher cell-specific virus titer (9100 ± 700 virions/cell) compared to MDCK.Xeno cells (7200 ± 400 virions/cell). In an attempt to further optimize infection conditions for MDCK.Xeno cells, different multiplicities of infection (MOI), trypsin amounts and infection temperatures were tested. However, all tested conditions had limited effect on the final HA titer (data not shown) and therefore infection conditions (MOI 10^{-3}, 37 °C) were chosen as before but with a slightly higher trypsin activity of 30 U/mL. For a more detailed analysis of the infection dynamics with MDCK.Xeno cells, three independent infection experiments were performed (cultivation conditions as described before). Additionally to the HA titer, the $TCID_{50}$ titer was evaluated. Similar to the previous experiments (Fig. 3) the results showed a robust infection at the chosen conditions. In the three independent experiments, very consistent cell concentrations and HA titer (3.6 $\log_{10}$(HAU/100 μL)) were reached (Fig. 4). High infectious virus titers exceeding 10^9 infectious virions/mL ($TCID_{50}$) were detected as soon as 24 hpi with a maximum at 30 hpi (2.7×10^9 infectious virions/mL). Afterwards, the infectious virus titer declined and was finally reduced by three orders of magnitude at 60 hpi (Fig. 4B). Both dynamics for infectious titer and total number of virus particles (based on HA) were very reproducible between the experiments, higher variations were observed for cell concentration, viability and cell diameter during cell death after virus production (>24 hpi) (Fig. 4A). For these independent experiments, the mean CSVY was slightly higher (8200 ± 1100 virions/cell) compared to the previous experiment (Fig. 3), but with higher variability due to variations of biological replicates. Previously reported CSVY for this cell line fell in the range between 7000–10,000 virions/cell [15,23] and correspond to our findings. Even though higher virus titers have been reported already for MDCK cell lines [26,48], an influenza virus A titer of 3.6 $\log_{10}$ (HAU/100 μL) was the highest titer we have reached with batch or fed-batch experiments, so far.

3.4. Influenza A virus production with MDCK.Xeno cells in semi-perfusion

In a next step, a semi-perfusion strategy was evaluated in shake flasks to achieve even higher cell concentrations (>15 × 10^6 cells/mL) and to investigate options regarding the establishment of bioreactor processes in perfusion mode. In particular, we wanted to verify that influenza virus production with MDCK.Xeno cells was possible at high cell densities without a reduction in CSVY (the so-called "high cell density effect"). In first attempts, the feeding strategy was optimized towards the extension of the exponential cell growth phase with high specific growth rates. In preliminary experiments a CSPR of 2.5 pL/(cell h) was determined

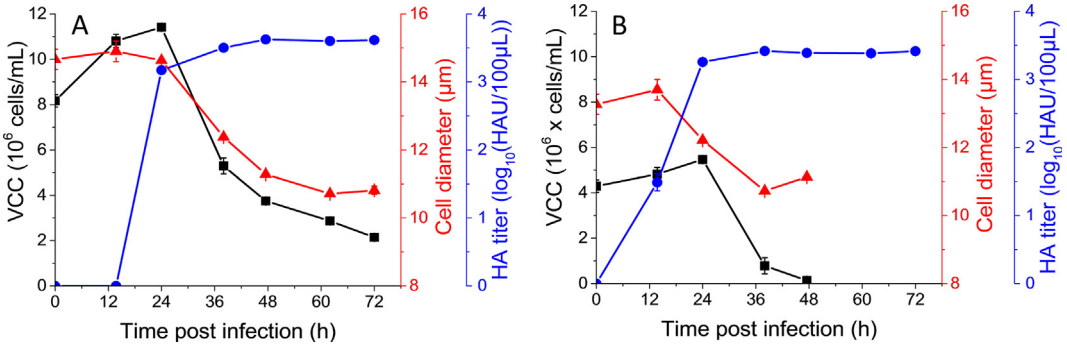

Fig. 3. Influenza A virus production of MDCK.Xeno and MDCK.SUS2 cells in batch culture. MDCK.Xeno (A) and MDCK.SUS2 (B) cells were cultivated in three parallel shake flasks for 72 h and infected with influenza A virus. Cell diameter, viable cell concentration (VCC) and virus titer (based on HA assay) were monitored for 72 h post infection. MOI: 10^{-3}, 37 °C, trypsin: 20 U/mL (2.5×10^{-6} (Xeno) & 5×10^{-6} (SUS2) U/cell); ■ viable cell concentration (VCC), ▲ average cell diameter, ● total virus titer (HA titer); Error bars: SD of three parallel infections.

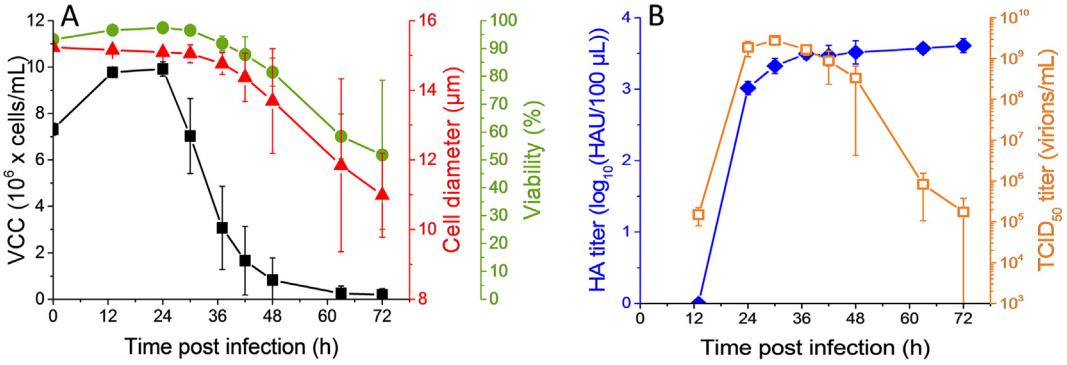

Fig. 4. Optimized influenza A virus production of MDCK.Xeno cells in batch culture. In three independent experiments, MDCK.Xeno cells were cultivated for 72 h and infected with influenza A virus. After infection, the cell concentration, viability and cell diameter (VCC) (A) as well as HA and $TCID_{50}$ (B) was monitored. MOI: 10^{-3}, 37 °C, trypsin: 30 U/mL (4×10^{-6} U/cell); ■ viable cell concentration, ▲ average cell diameter, ● viability, ♦ HA titer, ● infectious titer ($TCID_{50}$); Error bars: SD of three independent experiments.

to allow high cell densites with MDCK.Xeno cells. Cell growth was reduced in semi-perfusion compared to batch ($\mu_{sp} < \mu_{max}$), therefore a constant specific growth rate of 0.027 1/h was assumed. Applying this feeding strategy, it was possible to reach cell concentrations of 40×10^6 cells/mL in 7 days (0.5×10^6 cells/mL seeding cell concentration) (Fig. 5 B). Over the whole perfusion process, 4–5 times of the working volume (200–250 mL) of Xeno medium was needed (Fig. 5A). By continued semi-perfusion even higher cell concentration were possible (>60×10^6 cells/mL), but this was not pursued due to process instability (lower specific growth rate, viability) and handling issues. With higher cell concentrations, the time interval between perfusion steps decreased, becoming limiting at a certain time ($\Delta t < 4$ h). Variations of medium temperature, pH and osmolality could potentially have created cell stress and reduced the cell growth and viability. For these reasons, a cell density of 40×10^6 cells/mL was considered as optimal, to investigate influenza A virus infection in high cell density conditions. Accordingly, in another set of three experiments, MDCK.Xeno cells were cultivated to 40×10^6 cells/mL and infected with influenza A virus with a MOI of 10^{-3} (HCD1) and 10^{-1} (HCD2 and HCD3) (Fig. 5). The higher MOI was chosen to limit cell growth post infection, and to reduce the effect of perfusion (virus dilution) in the early infection phase. Using low MOI infection conditions (MOI 10^{-3}) (Fig. 5 black circles), similar infection dynamics concerning maximum HA and $TCID_{50}$ titer were observed as for HCD2 and HCD3 performed at a MOI of 10^{-1}. Using the lower MOI, cells continued to grow post infection to a maximum cell concentration of 60×10^6 cells/mL and started to die with the onset of virus accumulation (24 hpi). For higher MOI infections, virus release started earlier but cells died rapidly after infection (<12 hpi), which resulted in fast virus accumulation and lower maximum cell concentrations (Fig. 5B). All infections showed very high virus titers (>4 $\log_{10}$(HAU/100 μL). Considering the multiple harvests performed in each perfusion step, the calculated accumulated titer exceeded 4.3 $\log_{10}$ (HAU/100 μL), reaching the maximum at 30 hpi (MOI 10^{-1}) and 48 hpi (MOI 10^{-3}), respectively. For the best performing experiment (HCD 2), a HA titer of 4.2 $\log_{10}$(HAU/100 μL) was reached, which corresponded to an accumulated titer of almost 4.5 $\log_{10}$ (HAU/100 μL). For the same cultivation, an accumulated titer of 10^{10} infectious virions/mL ($TCID_{50}$) was obtained. Regarding HA titers, these are the highest values reported for influenza A virus production in animal cell culture, so far. Neither with other MDCK cell-based processes [15,21,26,48] nor with other cell lines cultivated in high cell density culture [30,31], HA titers over 10,000 HAU (4 $\log_{10}$(HAU)) were achieved. Only the combination of high cell density cultivation and high cell-specific productivity of MDCK.Xeno cells allowed the improvement of virus titers by this extent. The two infection experiments performed at a MOI of

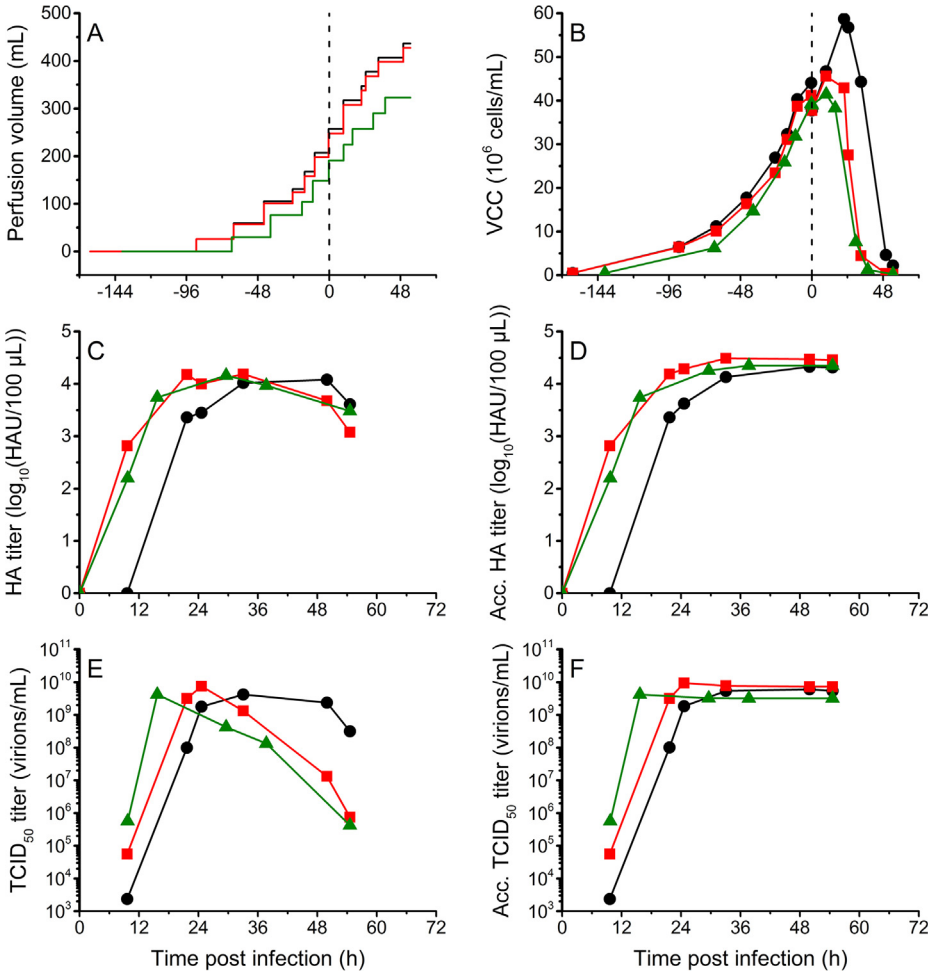

Fig. 5. Influenza A virus production with MDCK.Xeno cells in high cell density culture. MDCK.Xeno cells were cultivated in semi-perfusion shaker experiments to 40×10^6 cells/mL and evaluated for the production of influenza A virus. Accumulated virus titers were determined from total virus titers produced in perfusion steps based on the fixed working volume (50 mL). A: total volume of Xeno medium used for perfusion; B: viable cell concentration (VCC); C: virus titer (HA) in cell suspension; D: accumulated virus titer of multiple harvests (HA); E: infectious virus titer ($TCID_{50}$) in cell suspension; F: accumulated infectious virus titer of multiple harvests ($TCID_{50}$) ● HCD1 (MOI 10^{-3}), ■ HCD2 and ▲ HCD3 (MOI 10^{-1}); 37 °C, trypsin: 20 U/mL (5×10^{-7} U/cell).

Table 1
Comparison of influenza A virus production with MDCK.Xeno cells in batch and semi-perfusion culture.

	VCC_{max}	V_{medium}	max. virus titer		acc. virus titer		t_{max}	CSVY	Vol. productivity	Cell yield
			HA	$TCID_{50}$	HA	$TCID_{50}$		(HA based)	(HA based)	
	10^6 cells/mL	mL	$\log_{10}$(HAU/100 μL)	10^9 virions/mL	$\log_{10}$(HAU/100 μL)	10^9 virions/mL	h	virions/cell	10^{12} virions/(L d)	10^6 cells/mL
Batch	9.3	75	3.60	2.2	3.60	2.2	140	8200	9.1	6.2
HCD 1	58.7	487	4.08	4.2	4.33	6.0	210	7300	5.0	6.0
HCD 2	45.6	448	4.19	7.5	4.49	9.4	193	13,600	8.6	5.1
HCD 3	41.5	373	4.16	4.2	4.35	4.3	177	10,800	8.2	5.6

HCD1-3 Cultivations of MDCK.Xeno in high cell density; HCD1: MOI 10^{-3}, HCD2&3: MOI 10^{-1} (see Fig. 5).
VCC: viable cell concentration; V_{medium}: overall volume of used cultivation medium; t_{max}: process time until maximum HA titer.
SCVY: cell-specific virus yield; volumetric productivity: total number of virions per day and V_{medium}.
Cell yield: maximum number of cells per V_{medium}.

10^{-1} not only showed the highest virus titers but also a better overall performance compared to the low MOI infection. With this strategy it was possible to improve CSVY, compared to conventional batch experiments, to over 10,000 virions/cell (8000 virions/mL for batch). Additionally, the volumetric productivity of these two experiments was similar to the batch experiment (Table 1), proving this approach was not only valuable for increasing virus titers but also commercially feasible with respect to medium consumption. The lower productivity and cell-specific virus titer for the low MOI infection (10^{-3}) might be caused by feeding issues (medium limitations) at cell concentrations of 60×10^6 cells/mL as already described earlier. Future experiments will focus on scalable "real" perfusion systems using filtration retention devices, where the virus can be retained in the cultivation broth, and optimized feeding strategies can be applied both for cell growth and virus production. Additionally, even higher cell concentration might be feasible using a continuous controlled perfusion system [28,31,32].

4. Conclusion

Optimization of MDCK cell-based influenza vaccine manufacturing has often focused on advanced reactor technology to increase productivity. Only limited research, however, was dedicated to medium development. Nevertheless, Huang et al., introduced a new cell culture medium for the cultivation of MDCK suspension cells, which enabled very promising influenza virus titers [26]. In a long process (>180 days), we adapted our MDCK.SUS2 cell line to the Xeno medium to generate the MDCK.Xeno cell line, growing in single cell suspension. This newly established cell line grew faster and to higher cell concentration than other MDCK suspension cells [20–23,26,44], and showed various changes in metabolism as well as in morphology compared to the original cell line. Due to an improvement in cell concentrations, a higher virus titer (3.6 $\log_{10}$(HAU/100 μL) was achieved in batch infections with influenza A virus, compared to MDCK.SUS2 cells. For process intensification, semi-perfusion enabled us to cultivate and infect this MDCK.Xeno cell line in a high cell density environment. Here, we were able to show cell concentrations up to 6×10^7 cells/mL, producing an influenza A virus titer of up 4.2 $\log_{10}$(HAU/100 μL). Both cell concentration (for MDCK.Xeno) and influenza A virus titer are the highest reported for conventional MDCK cultivations [15,26,31] and intensified processes [30,31,48]. A short process time in semi-perfusion (<8 days), led to a process with similar productivity compared to batch culture, despite high medium consumption (5–7 × volume of batch cultivations). Further optimization of perfusion strategy should allow an even more efficient utilization of perfusion medium and a further increase in process productivity for both cells and viruses [29–32,49]. In particular, with use of capacitance sensors for on line measurement of cell concentrations and feeding control the implementation of highly productive continuous perfusion cultures should be feasible. With the virus yields achieved, so far, very competitive cell culture-based influenza vaccine manufacturing processes can be implemented that help to overcome limitations of egg-based production systems and contribute significantly to reduce time for pandemic preparedness in case of an influenza epidemic.

Acknowledgements

The authors would like to thank Claudia Best and Nancy Wynserski for their excellent technical support regarding general lab work, cell culture, and analyses of virus samples, i.e. the time consuming and laborious $TCID_{50}$ assays. The authors greatly appreciate the contribution of Shanghai BioEngine Sci-Tech for providing the Xeno medium.

Conflict of interest

Wen-Song Tan and Xuping Liu are affiliated as directors with Shanghai BioEngine Sci-Tech and were involved in the development of the Xeno™ medium both for scientific and commercial purposes. The remaining authors declare no conflict of interest.

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