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The efficient development of a novel recombinant adenovirus zoster vaccine perfusion production process

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Abstract
The adenovirus vector vaccines induce humoral and cellular immune responses and have been used to develop vaccines for effective prevention of life-threatening viruses, such as Ebola and Coronavirus. High demand of vaccines worldwide requires optimization of the production process. Perfusion process increases cell concentration and volumetric productivity, so that it becomes the commonly used strategy in vaccine production. In this study, we optimized and developed a perfusion process for the adenovirus-based zoster vaccine production efficiently. We first tested different perfusion strategies in shake flasks, showing semi-continuous strategies for optimal HEK 293 cell growth. We then evaluated three empirical key parameters (cell concentration at the time of infection (VCC), multiplicity of infection (MOI), and virus production pH) by the design of experiment (DoE) method, from which the robust setpoint (VCC 7 cells/mL, MOI 9, and virus production pH 7.17) was confirmed in both shake flask and 2 L bioreactor. In the bioreactor, we compared the performances of two perfusion systems, the commercially-available Xcell ATF® system and a novel peristaltic pump-driven alternating tangential flow perfusion system (PATFP system) that we developed. During cell cultivation stage, both perfusion systems have comparable performances regarding viable cell concentration and cell viability. At 2 dpi, the PATFP system resulted in an adenovirus titer of $2.1 \times 10^{10}$ IFU/mL and cell-specific virus yield of 2,062 IFU/cell, reaching 75% and 77% of values for Xcell ATF® system. This study demonstrates the perfusion process to be superior strategy for adenovirus-based vaccine production compared to the batch-mode strategy ($1,467$ IFU/cell). Furthermore, our PATFP system shows potential to be comparable to the Xcell ATF® system, and it would become an alternative perfusion strategy for the vaccine production.

1. Introduction
Herpes zoster (HZ, shingles) is an infectious skin disease caused by the varicella-zoster virus, a member of the family of the herpes viruses [1]. HZ causes painful, blistering skin eruption, and its sequelae of postherpetic neuralgia severely impacts patient’s life quality [2]. Approximately one in three persons are infected with HZ in their whole life, and vaccines are still the most effective way for prevention [3]. The live attenuated virus vaccine from Merck (Zostavax®) and the recombinant zoster vaccine from GlaxoSmithKline (Shingrix®) are two primary commercially available herpes zoster vaccines.

Adenoviruses are a type of double-stranded DNA virus with genome sizes between ~ 34–43 kb [4]. Compared with other viruses, they are able to replicate to high titers in complementing cell lines, such as HEK 293 cells [5]. Furthermore, compared with other types of vaccines (such as live attenuated virus vaccine and recombinant protein vaccine), adenovirus vector vaccine is able to induce humoral and cellular immune responses [6]. Thus, adenoviruses hold the potential to be developed as an alternative HZ vaccine.

Batch process and perfusion process are the two main strategies for mammalian cell cultivation and vaccine production. The former has the merit of easy operation, while the latter yields higher cell concentration and virus titer, suitable for process scale-up.
(Shen, Jacob, et al. 2016). The ATF is used to retain cells during the perfusion cultivation process. The XCell ATF® system (Repligen, USA) has been widely used in cultivations of CHO cells and avian cells [7,8]. The issue of membrane fouling in XCell ATF® system can be addressed by a bidirectional flow in hollow fiber module and backflush action generated by a diaphragm pump [9]. Bidirectional flow can be easily generated by several pumps, such as peristaltic pump and gear pump. However, this brings about the issue of shear force from the pump, which needs to be considered for an optimized ATF system [10].

In this study, we optimized and developed a perfusion process for production of adenovirus-based zoster vaccine by HEK 293 cells efficiently. Key process parameters were evaluated with the design of experiment (DoE) approach to study their effect on adenovirus titer and determine the robust setpoint. The process with robust setpoint was scaled up to a 2 L benchtop bioreactor with perfusion strategy, using either the XCell ATF® system or a novel PATFP (peristaltic pump-driven alternating tangential flow perfusion culture) system we developed, respectively. Such cultivation process could also be applied for production of other biologics for human health.

2. Materials and methods

2.1. Cell line, culture medium and virus strain

HEK 293 cell line, adapted to suspension and serum-free culture, was used for adenovirus production in this study. The cells were pre-cultured in baffled shake flasks with HEK 293 SFM medium (Laboratory customization) at 37 °C, 5% CO2 atmosphere and 120 rpm. The recombinant adenovirus vector used for infection was an adenovirus containing glycoprotein E of herpes zoster (Ad-HER). Viral stock titers were prepared at 1.1 × 10^12 IFU/mL and aliquots were stored at −80 °C.

2.2. Cell culture in shake flasks

Cells were cultured with 50 mL HEK 293 SFM medium in 250 mL baffled shake flasks (Corning, USA), with an initial cell concentration of 0.3 × 10^6 cells/mL. The semi-continuous operation was conducted after the 4rd day, with perfusion rates of 0.5 RV/day, 1 RV/day, or 2 RV/day, respectively. In detail, the cells were transferred into 50 mL centrifugal tube and centrifuged at 800 rpm for 5 min. The cell-free supernatant was removed (for 0.5 RV/day, 50% cultivation volume was removed, for 1 RV/day and 2 RV/day, all supernatant was removed). Then equal amount of warm fresh medium was added to resuspend the cell pellets for continuous cultivation. Samples were taken daily to measure cell concentration and metabolites in medium (glucose, glutamine, lactate and ammonia).

2.3. Reduced combinatorial design based on DoE

Three key process parameters for Ad-HER production, the viable cell concentration at the time of infection (VCC), the multiplicity of infection (MOI), and the virus production pH (pH), were tested using DoE method. The parameter values set were shown in Table 1. The experiments were designed based on the model of reduced combinatorial design recommended by MODDE software (MKS Data Analytics Solutions, Umea, Sweden), with the adenovirus titer and cell-specific virus yield (CSVY) during 3 days post infection (dpi) as the responses. The experimental matrix was shown in Table 2. The pH was controlled by manually supplemented with 7.5% w/v NaHCO3 or 1 M HCl.

2.4. Bioreactor cultivation in perfusion mode

The bioreactor used in this study was a 5 L benchtop STR bioreactor (Applikon, Holland) with 2 L working volume. Cells were proliferated in flasks and inoculated into the bioreaction with initial cell concentration of 0.3 × 10^6 cells/mL. The culture conditions were controlled by control tower to 37 °C, pH 7.2 (regulated by injection of CO2 or addition of 7.5% w/v NaHCO3), stirring speed of 120 rpm, pO2 40% air-saturation (by injecting pure oxygen).

Two perfusion systems (PATFP system and XCell ATF® system) were evaluated in this work. The PATFP system (peristaltic pump-driven alternating tangential flow perfusion system, made in our laboratory) consists of one hollow fiber module, three peristaltic pumps and two balances (Fig. 1). The hollow fiber module was connected to the bioreactor via two ports. A peristaltic pump (recirculation pump) was used for the cycle of medium in bioreactor and hollow fiber module. The recirculation pump direction change frequency was set at 20 min. The other two peristaltic pumps were used for feed and harvest, respectively. The accuracy of perfusion rate was ensured by modifying the rotation speed of feed and harvest pump with the feedback from balances of feed bottle and harvest bottle. The cell suspension flow rate within the hollow fiber module was set at 0.7 L/min. The self-balancing mode of PATFP system was used. Every 3 h, the data of balances were collected and the weight change of medium in bioreactor and the actual flow rate of feed and harvest pump were calculated. When the cumulative difference of two balances was higher than 8 g, the bioreactor weight was modified by adding or withdrawing medium with high speed. When the ratio of practical flow rate and setpoint flow rate is higher than 1.02 or lower than 0.98, the rotation speed of feed and/or harvest pump is adjusted automatically. In comparison, the XCell ATF® (ATF2) perfusion system (Repligen, USA) was connected to the bioreactor via the top port and the cell suspension flow rate within the hollow fiber was set at 0.7 L/min with the C24U-V2.0 controller. Meanwhile, two additional peristaltic pumps (MasterFlex, USA) with 14# peristaltic pump tubing (Saint-Gobain, France) were used for feed and harvest, respectively. The hollow fiber module in both systems were composed of PES fibers with 1 mm lumen diameters, 60 cm length and 0.2 μm pore size (Repligen, P/N F2:RF02PES, USA).

2.5. Analysis of samples

Viable cell concentration and cell viability were determined by the trypan blue exclusion method. Glucose and glutamine were measured by the Cedex Bio analyzer (Roche Diagnostics, Switzerland). The pH of the shake flasks were measured by the gas sensors of BioProfile Flex2 Analyser (Nova Biomedical, USA). The titer (infectious units, IFUs) of adenovirus was quantified by the QuickTiter™ Adenovirus Titer Immunoassay Kit (Cell BioLabs, USA). The CSVY was calculated by dividing the adenovirus titer by the viable cell concentration at the time of infection.

3. Results

3.1. Cell culture in shake flasks

Before perfusion cultivation in bioreactors, we first tested semi-continuous cultivation in shake flasks. As shown in Fig. 2a, the cell
concentration reached the highest of $6.1 \times 10^6$ cells/mL at day 5 in the batch mode. In comparison, the semi-continuous operations resulted in significantly higher cell densities. Meanwhile, the cell densities continue to increase throughout nine days. Especially for 1RV/day and 2 RV/day, the cell concentration reached $2.4 \times 10^7$ cells/mL at day 9. Thus, 1 RV/day was recommended. The different growth behaviors in Fig. 2a could be interpreted by metabolite profiles in Fig. 2b-2c. Depletion of glutamine could account for cease of cell growth in the batch mode after day 5. In contrast, the semi-continuous operation allows refreshment of the medium, so that the cells continued to grow. The glutamine concentration was higher than 0.5 mM at day 4 to 5 with semi-continuous operation of 1 RV/d rather than 0.5 RV/d. However, the glutamine concentration was lower than 0.5 mM when cell concentration was higher than $8.0 \times 10^6$ cells/mL (day 5) even the medium in shake flask was changed twice a day. In all, based on the growth curves and metabolite profiles, the perfusion strategy in the bioreactor was set as: perfusion started when cell concentration reached $5.0 \times 10^6$ cells/mL, initial perfusion rate of 1 RV/day, then 2 RV/day when cell concentration reached $8.0 \times 10^6$ cells/mL (“1RV/day to 2 RV/day” in Fig. 2).

3.2. Characterization of Ad-HER production process

The Ad-HER titer and CSVY within 3 days post infection of each run were collected (Table 2). There’s a trend that virus titer decreases from 2 dpi to 3 dpi, while increases from 1 dpi to 2 dpi. An increase of MOI resulted in decrease of fold expansion. By considering the replication cycle of adenovirus (maximal viral titer obtained at 36–48 h post infection (hpi) [5]) and evaluating the data in Table 2, the adenovirus titer and CSVY at 2 dpi were used to build the response model. The quantitative relationships between Ad-HER production (titer, CSVY) and key process parameters (VCC, MOI and virus production pH) were built by multiple linear regression, using MODDE software:

$$
\text{Titer}_{2\text{dpi}} = -1.5 \times 10^{11} - 3.9 \times 10^8 \cdot \text{MOI} + 9.2 \times 10^9 \cdot \text{VCC} + 1.9 \times 10^{10} \cdot \text{pH} - 1.7 \times 10^8 \cdot \text{VCC}^2 - 2.4 \times 10^7 \cdot \text{MOI} \cdot \text{VCC} + 6.2 \times 10^8 \cdot \text{MOI} \cdot \text{pH} - 7.0 \times 10^8 \cdot \text{VCC} \cdot \text{pH}
$$

Table 2
Reduced combinatorial design and experimental data of responses.

| No | MOI | VCC at infection | Titer (IFU/mL) 1 dpi | 2 dpi | 3 dpi | CSVY(IFU/cell) 1 dpi | 2 dpi | 3 dpi | Fold expansion
|-----|-----|------------------|---------------------|-------|-------|-------------------------|-------|-------|----------------
| 1   | 1   | 4                | 7.2                 | 5.4   |       |                         |       |       |                
| 2   | 5   | 4                | 7.2                 | 5.9   |       |                         |       |       |                
| 3   | 10  | 4                | 6.9                 | 5.9   |       |                         |       |       |                
| 4   | 1   | 4                | 6.9                 | 6.4   |       |                         |       |       |                
| 5   | 5   | 8                | 6.9                 | 8.8   |       |                         |       |       |                
| 6   | 10  | 8                | 7.2                 | 8.2   |       |                         |       |       |                
| 7   | 1   | 8                | 7.2                 | 7.7   |       |                         |       |       |                
| 8   | 5   | 12               | 6.9                 | 12.5  |       |                         |       |       |                
| 9   | 10  | 12               | 7.2                 | 11.9  |       |                         |       |       |                
| 10  | 1   | 12               | 7.2                 | 11.6  |       |                         |       |       |                
| 11  | 5   | 16               | 7.2                 | 15.3  |       |                         |       |       |                
| 12  | 10  | 16               | 6.9                 | 15.3  |       |                         |       |       |                
| 13  | 1   | 16               | 6.9                 | 16.3  |       |                         |       |       |                
| 14  | 5   | 12               | 6.9                 | 11.5  |       |                         |       |       |                
| 15  | 10  | 16               | 6.9                 | 15.5  |       |                         |       |       |                
| 16  | 10  | 4                | 6.9                 | 5.9   |       |                         |       |       |                

$^a$ The CSVY was calculated by dividing the adenovirus titer (column 6, 7, 8) by the viable cell concentration at infection (column 5).

$^b$ The fold expansion was calculated by dividing the adenovirus particles in the harvest (column 6, 7, 8) by adenovirus particles for infection (data in column 2 multiplied by data in column 5).

Fig. 1. Diagram of the PATFP system for cell perfusion cultivation.
Model analysis was shown in Table 3 and Fig. 3. The models well explained the response variations with $R^2 = 0.914$ and $Q^2 = 0.514$ for adenovirus titer, $R^2 = 0.975$ and $Q^2 = 0.930$ for CSVY. Both models had no lack of fit (validity > 0.25). The replicate plot indicated the variability of the replicates (blue point connected by a line) was small. The coefficient plots (Fig. 3b and Fig. 3d) indicated the VCC and virus production pH were significant model terms ($P < 0.05$). The virus production pH had positive effect on both titer and CSVY. The VCC had positive effect on adenovirus titer.

3.3. Determination of the robust setpoint and design space

According to the ICH guidance documents, the design space is defined as the multi-dimensional combination and interaction of process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change and not subjected to regulatory notification [11]. The robust setpoint and design space were determined based on the predictive model established in Section 3.2. Titer $> 1.2 \times 10^{10}$ IFU/mL, CSVY $> 10^3$ IFU/cell and probability of failure $< 1\%$ were set as criteria. The design space with a robust setpoint for Ad-HER production was shown in Fig. 3e. The robust setpoint was determined to be VCC of $1.04 \times 10^7$ cells/mL, MOI of 9 and virus production pH of 7.17. The design space hypercube was: VCC from $0.88$ to $1.28 \times 10^7$ cells/mL, MOI from 6 to 10, and virus production pH from 7.15 to 7.20.

3.4. Ad-HER production in bioreactor with XCell ATF<sup>®</sup> system and PATFP system

The robust setpoints of process parameters determined in Section 3.3 were tested in shake flasks (Fig. 4a). The adenovirus titer was increased from $1.2 \times 10^{10}$ IFU/mL to $1.7 \times 10^{10}$ IFU/mL with a shift of perfusion rate form 1 RV/day to 2 RV/day (Fig. 4d), though no increase of viable cell concentration was observed. Also, the adenovirus titer in shake flask was within the prediction of the mathematical model ($1.3 \times 10^{10}$ IFU/mL to $1.8 \times 10^{10}$ IFU/mL).

The process was scaled up to a 2 L benchtop bioreactor, with the perfusion process achieved by either XCell ATF<sup>®</sup> system or the PATFP system we developed. Both perfusion systems have comparable performances during the cell growth phase, with viable cell concentration approximately $1.0 \times 10^7$ cells/mL and cell viability above 95% (Fig. 4b). After infection (MOI = 9), viable cell concentration and cell viability dropped for both systems (Fig. 4b). The viable cell concentration and cell viability dropped slower in XCell ATF<sup>®</sup> system than in PATFP system. We suspect this may be due to the lower shear force in XCell ATF<sup>®</sup>, since the HEK 293 cells infected with virus seem more sensitive to shear force, based on our observation. The metabolite profiles showed a sharp decrease of glucose and glutamine during the adenovirus replication (Fig. 4c). The adenovirus titer at 2 dpi reached $2.1 \times 10^{10}$ IFU/mL with CSVY of 2,062.
IFU/cell and fold expansion of 239 for PATFP system and 2.8/C210 IFU/mL with CSVY of 2,684 IFU/cell and fold expansion of 318 for XCell ATF/C210 system (Fig. 4d). The CSVYs in both perfusion systems were higher than batch cultivation in shake flask (1,467 IFU/cell, data not shown), indicating "cell density effect" was not observed. When using XCell ATF/C210 system, the increase of cumulative medium weight change caused by the feed and harvest pump was observed (Fig. 4e). This phenomenon was caused by the deformation of peristaltic pump and/or increase of medium viscosity. The tube position on the pump head was changed manually at 117.5 h (-7h at Fig. 4e) due to the large deviation of the two balances. However, when using PATFP system with self-balancing mode, the change of medium weight in bioreactor was maintained lower than 20 g.

### Table 3
The analysis of variance (ANOVA) results for reduced combinatorial model.

| Terms | Titer | CSVY |
|-------|-------|------|
|       | Coefficient | P value | Coefficient | P value |
|       | (scaled and centered) | | (raw data) | |
|       | Coefficient | P value | Coefficient | P value |
|       | (scaled and centered) | | (raw data) | |
| Constant | 1.3E + 10 | < 0.0001 | 1.5E + 11 | |
| MOI | 8.8E + 08 | 0.1142 | 3.9E + 09 | |
| VCC | 4.3E + 09 | 0.0003 | 9.2E + 09 | |
| pH | 2.4E + 09 | 0.0005 | 1.9E + 10 | |
| MOI*MROI | | | | |
| VCC*VCC | -6.2E + 09 | 0.0025 | -1.7E + 08 | |
| MOI*VCC | -6.4E + 08 | 0.4293 | -2.4E + 07 | |
| MOI*pH | 4.2E + 08 | 0.4488 | 6.2E + 08 | |
| VCC*pH | -6.3E + 08 | 0.4063 | -7.0E + 08 | |
| Lack of fit | | | | |
| R² | 0.301 | 0.314 | |
| Adjusted R² | 0.839 | 0.914 | |
| Q² | 0.514 | 0.930 | |

**Fig. 3.** Model evaluations and design space exploration. with adenovirus titer as the response. (a) The replicate index and (b) the coefficient plot (scaled and centered) for adenovirus titer. (c) The replicate index and (d) the coefficient plot (scaled and centered) for CSVY. (e) The design space with a robust setpoint for Ad-HER production. The criteria are: titer > 1.2E + 10 IFU/mL and CSVY > 1,000 IFU/cell.
4. Discussion

Adenovirus-based vectors are important tools for production of vaccines because of their immunogenicity, safety, and high production efficiency. The adenovirus type 5 (Ad5) has been used for production of Ebola vaccine and Coronavirus vaccine [6,12]. The recombinant virus we produced in this study was constructed by inserting the glycoprotein E gene of herpes zoster into adenovirus.

In order to handle emergent diseases using immune strategies, efficient process tools are essential so as to shorten the development cycle. Among them, the DoE approach is an outstanding one, which allows pharmaceutical scientists to systematically evaluate factors based on a prespecified design [11]. In a recent study, we used the DoE approach to study the interactions of process parameters and identified the robust setpoint and control space of process parameters to produce live attenuated pseudorabies virus vaccine [13]. In this study, we further used DoE to investigate the interactions of process parameters and the relationships between process parameters and product attributes, then an adenovirus-based vaccine production process was developed efficiently.
The high through-put parallel bioreactor systems, such as Ambr® 250 bioreactor system (Sartorius, Germany) and DASGIP® parallel bioreactor system (Eppendorf, Germany), which enable comparable process parameter control as the benchtop bioreactor, would be the best choice for process development. However, shake flask is still the scale-down model most extensively used to provide primary process data, due to its convenience and cost-effectiveness. Thus, we built up the process model based on the DoE method, using the data from shake flask cultivation. We first evaluated three process parameters (VCC, MOI, and virus production pH) in shake flasks by DoE. The DoE table was generated with a reduced combinatorial design recommended by MODDE software, which worked well in our previous study [13]. According to virus yield models, VCC and the virus production pH were significant terms. The positive effect of VCC (Fig. 3b) indicates that the adenovirus yield could be increased with viable cell concentration increased. Higher pH (7.2) was more suitable for adenovirus expansion than pH 6.9. Similarly, pH of 7.2 was beneficial for GFP encoding recombinant adenovirus production [14]. The robust set-point and design space were generated based on the model with 8192 simulated points and criteria of titer > 1.2 × 10^10 IFU/mL, CSVY > 10^3 IFU/cell, probability of failure < 1%. Fortunately, a robust setpoint and design space were identified (Fig. 3e), which will be used to define the operating space of bioreactor process parameters during the process scale-up.

During shake-flask cultivation, we observed semi-continuous process with medium exchange resulted in higher viable cell concentration and virus yield, compared to the batch-mode process (Fig. 2). However, in general, the semi-continuous operations in shake flasks generate high fluctuation of nutrition concentrations during medium exchange. In contrast, the perfusion process yields higher cell concentration and virus titer due to precise control of process parameters, such as pH and DO. The semi-continuous operation in shake flasks allows optimization of HEK 293 cells growth to high cell concentration (~2 × 10^7 cells/mL, Fig. 2a), so that virus expansion can be performed at high cell concentration. When cells cultured in bioreactor with perfusion systems, cell concentration of 1.0 × 10^7 cells/mL with high cell viability was reached (Fig. 4b). The volumetric productivity and CSVY in perfusion process were higher than that of a batch process, with infection at low cell concentration of 1.5 × 10^6 cells/mL (titer of 2.2 × 10^9 IFU/mL, CSVY of 1467 IFU/cell). This indicates there was no “cell density effect”, caused by nutrient depletion and/or metabolite inhibition in the medium [15].

To develop an economical perfusion process, several parameters need to be optimized, such as perfusion beginning time, perfusion rate, the type of perfusion system used, etc. Different perfusion strategies, such as constant cell-specific perfusion rate (CSPR) [16] and constant glucose concentration [17], were used to reach high cell concentration or virus titer. Lactate and ammonia are known to be major byproducts, which affect the cell growth when their accumulations reach certain levels [18]. Thus, the lactate and ammonia should be controlled at sub-growth inhibitory levels throughout the perfusion cultivation process. The perfusion approach enables continuous addition of fresh medium and removal of by-products, so that high cell concentration can be achieved. Thus, it has been used for production of recombinant therapeutics proteins (CHO cells), virus-like particles (HEK 293 cells), and vaccines (avian cells) [7,8,19].

The TFF system and the ATF system are the most common perfusion systems applied in biologics production processes [7,8]. The TFF system is driven by a peristaltic pump or a centrifugal pump only with unidirectional flow [8,10], so that an additional declogging technique is needed to remove cell debris and other materials might plug the membrane [20]. Also, the peristaltic pump used in TFF system was the major contributor to shear force, so that low shear centrifugal pump was used as an alternative [10]. In contrast, the XCell ATF® system is driven by a diaphragm pump, which minimizes generation of shear force. The commercially-available XCell ATF® system has outstanding membrane sieving performance with high membrane flux, and have alternate shear flow and backflush at the filter surface to remove foulings, which make it superior over the TFF system [7,9,10]. The flow rate in PATFP system was the same as that in XCell ATF® system, according to the membrane flux at the harvest (data not shown), we didn’t observe the issue of membrane fouling in the hollow fiber module. Meanwhile, recycle of cell-containing medium can be easily achieved between the bioreactor and hollow fibers in our PATFP system, driven by peristaltic pump. There would be a risk of deformation and abrasion of the peristaltic pump tubing after long-term running, although we didn’t observe this issue after continuous working with peristaltic pump for 5 days.

In the XCell ATF® system, the shear force from diaphragm pump could be neglected, so that the fluid flow in fiber module was the main contributor of shear stress. The crossflow shear rate reaches 1500 s⁻¹ at a flow rate 0.7 L/min, which was still below the threshold of 3000 s⁻¹ for damaging mammalian cells [21]. In PATFP system, the peristaltic pump would be a major source of shear stress, as shown in a previous study that use of the peristaltic pump in TFF perfusion systems resulted in lower cell growth rate and viability compared with XCell ATF® system [10].

In this study, we observed comparable viable cell concentration and cell viability in PATFP system and XCell ATF® system during the cell cultivation stage. However, these two parameters dropped more dramatically post infection in PATFP system, possibly owing to the higher shear force generated from the peristaltic pump (Fig. 4b). Actually, there have been reports showing that the infected cells are more sensitive to shear stress [22]. In fact, the XCell ATF® system is a cell-retention module and additional peristaltic pumps should be introduced for medium exchange. However, the wear of pipe wall or clogging of the membrane could lead to the inaccurate feed rate and removal rate, and the change of culture volume (Fig. 4e). Thus, the pump speed is usually adjusted manually to obtain accurate perfusion rate. According to our data, the daily medium weight change in the bioreactor without adjustment of pump rotation speed reached 60 g (Fig. 4e), accounting for 3 % of the culture volume. In comparison, the PATFP system enables automatic adjustment of the pump rotation speed based on the feedback from balances, so that the volume variation can be limited within 20 g per day.

In PATFP system, availability of oxygen in the recirculation loop would be another concern to prevent its application. By estimation, a volume of 200 ml in recirculation loop with a flow rate of 0.7 L/min results a residence time of 17 s. The average residence time in the XCell ATF® (ATF2) system of cells could be 50 s, though the pump cycle time was a few second [23]. Such value is significantly lower than the oxygen depletion time of 179 s at cell concentration 1.5 × 10^7 cells/mL, according to published formula [23,24]. This suggests that the cells were exposed to sufficient oxygen phenomenon inside the recirculation loop.

The pore size mainly impacts product retention [25], and careful selection of the pore size allows increased membrane fluxes and prolonged lifetime of perfusion process. Larger pores (0.5 μm or larger) in hollow fiber membranes prevent membrane plug, while small pores (0.2 μm) benefit retention of virus particles [19,25,26]. There also have reports about the impact of pore size on cell viability and cell-specific yield [26]. In this study, we didn’t observe membrane plug when we used the standard hollow fiber with pore size 0.2 μm and inner lumen diameter 1 mm, indicating membrane capacity was higher than 100 L/m² with the fluxes of 15.4 L/m²/day and 30.8 L/m²/day. However, we should note that the filtration fluxes used in this study was lower than other reports [20,27,28].
and the effect of PATFP system on membrane capacity should be further confirmed.

Finally, two perfusion systems (XCell ATF® and PATFP) may result in changes of other parameters in regard to product quality, such as ratio of infectious virus particle units over total number of virus particles (IFU/VP) and the replication competent adenovirus (RCA), which would impact the downstream purification process. These impacts were not evaluated in this work.

5. Conclusion

In this study, we developed an optimized perfusion process for adenovirus-based zoster vaccine production using the DoE approach and a PATFP system we developed. The cell growth process was optimized in shake flasks with semi-continuous operations. Three key process parameters (VCC, MOI, the virus production pH) of Ad-HER production were selected and evaluated by the DoE approach, from which the robust setpoint was obtained (VCC of $1.04 \times 10^5$ cells/mL, MOI of 9 and virus production pH of 7.17). Finally, the robust setpoint was tested in a 2 L benchtop bioreactor in XCell ATF® system and PATFP system, which confirmed the success of such perfusion process for adenovirus-based vaccine. Compared with XCell ATF® system, PATFP system resulted in similar adenovirus titer and cell-specific virus yield, and reached 75% and 77%. It implies the PATFP system as an alternative perfusion system to XCell ATF® system for adenovirus production in future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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