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# High-cell-density cultivations to increase MVA virus production

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ABSTRACT

Increasing the yield and the productivity in cell culture-based vaccine manufacturing using high-cell-density (HCD) cultivations faces a number of challenges. For example, medium consumption should be low to obtain a very high concentration of viable host cells in an economical way but must be balanced against the requirement that accumulation of toxic metabolites and limitation of nutrients have to be avoided. HCD cultivations should also be optimized to avoid unwanted induction of apoptosis or autophagy during the early phase of virus infection. To realize the full potential of HCD cultivations, a rational analysis of the cultivation conditions of the appropriate host cell line together with the optimal infection conditions for the chosen viral vaccine strain needs to be performed for each particular manufacturing process.

We here illustrate our strategy for production of the modified vaccinia Ankara (MVA) virus isolate MVA-CR19 in the avian suspension cell line AGE1.CR.pIX at HCD. As a first step we demonstrate that the adjustment of the perfusion rate strictly based on the measured cell concentration and the glucose consumption rate of cells enables optimal growth in a 0.8 L bioreactor equipped with an ATF2 system. Concentrations up to $57 \times 10^6$ cells/mL (before infection) were obtained with a viability exceeding 95%, and a maximum specific cell growth rate of 0.019 $h^{-1}$ (doubling time = 36.5 h). However, not only the cell-specific MVA-CR19 virus yield but also the volumetric productivity was reduced compared to infections at conventional-cell-density (CCD).

To facilitate optimization of the virus propagation phase at HCD, a larger set of feeding strategies was analyzed in small-scale cultivations using shake flasks. Densities up to $63 \times 10^6$ cells/mL were obtained at the end of the cell growth phase applying a discontinuous perfusion mode (semi-perfusion) with the same cell-specific perfusion rate as in the bioreactor (0.060 nL/(cell d)). At this cell concentration, a medium exchange at time of infection was required to obtain expected virus yields during the first 24 h after infection. Applying an additional fed-batch feeding strategy during the whole virus replication phase resulted in a faster virus titer increase during the first 36 h after infection. In contrast, a semi-continuous virus harvest scheme improved virus accumulation and recovery at a rather later stage of infection. Overall, a combination of both fed-batch and medium exchange strategies resulted in similar cell-specific virus yields as those obtained for CCD processes but 10-fold higher MVA-CR19 titers, and four times higher volumetric productivity.



## 1. Introduction

Modern recombinant vector vaccines combine the advantages of an attenuated or even host-restricted infection with a highly immunogenic expression of an antigen of choice. Especially promising vectors are highly attenuated poxviruses, including modified vaccinia virus Ankara (MVA) [1]. MVA has been attenuated by repeated passaging in chicken fibroblast cultures [2], and its properties as potential human vector vaccine have been well characterized [1,3–11]. Various MVA recombinants that express different viral heterologous antigens have been generated and extensively tested in pre-clinical and clinical trials as candidate vaccine against diseases such as AIDS, influenza, severe acute respiratory syndrome (SARS) and human respiratory syncytial virus (RSV) infection [7,9–11]. The use of MVA as a vector-based vaccine, however, is predicted to require highly concentrated doses of about $10^8$ infectious units (IU) per mL [1,6], preferably produced in media free of animal-derived components.

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MVA (similar to other viruses such as influenza A virus or yellow fever virus) is being produced in material obtained from embryonated chicken eggs or primary chicken embryo fibroblasts (CEF) [8]. However, the use of primary animal-derived material that is continuously fed into vaccine production processes is not an optimal scenario. To overcome this problem, two fully permissive avian suspension cell lines, AGE1.CR (in the following CR) and AGE1.CR.pIX (in the following CR.pIX) were developed [12] and adapted to proliferation in a chemically defined medium to enable the establishment of robust high-yield production processes [13–15]. Because MVA spreads preferably via cell-to-cell contact a culture format was developed where cell agglomerates of infected and non-infected cells are being induced for production of vaccine preparations. A MVA strain (MVA-CR19) was recently obtained with this system that propagates also in non-agglomerated CR.pIX suspension cells [16]. With this new isolate that represents a different genotype of MVA, titers in the order of $10^8$ IU/mL can be obtained at conventional-cell-densities (CCD) of about $2.0 \times 10^6$ cells/mL. As a higher fraction of MVA-CR19 is released into the supernatant, harvest of infectious units does not require whole-cell lysates anymore, facilitating the subsequent downstream processing [16].

To further increase virus titers, high-cell-density (HCD) processes can be established [17]. While virus propagation in HCD often results either in a decrease in cell-specific yields [18,19] – the so-called "cell-density effect" [20,21] – or in low volumetric productivity [22], there are reports on positive effects on virus titers and cell-specific yields if certain medium feeding/exchange strategies prior to or during virus propagation are being applied. The described strategies can involve medium recirculation, medium perfusion, and periodic virus harvesting [17]. However, in most of these approaches adherent cell lines were used with limited options for scale-up and process intensification. A few authors described the use of suspension cells at $10^7$ cells/mL [22–26], but without addressing details of optimization.

Here, an analysis regarding the effect of various medium feeding strategies before and after infection on the yields for production of MVA-CR19 in suspension CR.pIX cells at HCD ($50–63 \times 10^6$ cells/mL) is presented. Cultivations were performed in perfusion bioreactor and shake flasks as a small-scale model. We demonstrate that by applying optimized feeding strategies at HCD, similar cell-specific virus yields can be obtained while maximum virus titers as well as volumetric productivity can be increased significantly compared to CCD cultivations.

## 2. Materials and methods

### 2.1. Cells and medium

Suspension CR.pIX cells [12] were cultivated in chemically defined CD-U3 medium (Biochrom GmbH) with a glucose concentration of 33–40 mM, supplemented with glutamine, alanine (both 2 mM final concentration, Sigma), and recombinant insulin-like growth factor (LONG-R3IGF, 10 ng/mL final concentration, Sigma). Cells were passaged twice a week at a seed concentration of $0.8 \times 10^6$ cells/mL.

### 2.2. Cultivation in bioreactor

CR.pIX cells were cultivated in a lab-scale bioreactor (BIOSTAT®B plus, Sartorius AG) with a working volume of 0.8 L. The bioreactor was inoculated at $0.8 \times 10^6$ cells/mL with pre-cultures expanded in 250 mL shake flasks and operated at 37 °C, pH 7.2, and a stirring speed of 120–160 rpm. Dissolved oxygen concentration (DO) was controlled at 40% by pulsed aeration with pure oxygen through a 20 $\mu$m pore size micro-sparger to a maximum of 0.048 vvm, representing a volumetric oxygen transfer coefficient ($k_La$) of 10.85 $h^{-1}$ at 142 rpm. Cells were initially cultivated in batch until a glucose concentration of 14–17 mM (60–72 h after inoculation) was reached. At that point, perfusion was started using an ATF2 perfusion system controlled by the C24U–V2.0 controller from Refine Technology and a polysulfone 500 kDa hollow fiber cartridge (UFP-500-E-4X2MA, GE Healthcare). Cell suspension was pumped through the hollow fiber with a recirculation rate of 1.0 L/min and fed with defined perfusion rates to achieve a minimum concentration of $50 \times 10^6$ cells/mL.

Feeding was based on a constant cell-specific perfusion rate (CSPR) [27] taking into account the steady state mass balance for substrates as described by Kompala and Ozturk [28] with glucose as the major energy source for CR.pIX cells [29] as:

$$CSPR = D/x = q_{g/x}/(C_{gM} - C_{gR}) \quad (1)$$

with the dilution rate (D, $h^{-1}$), the viable cell concentration (x, cells/mL), a cell-specific glucose consumption rate ($q_{g/x}$) of $8.54 \times 10^{-11}$ mmol/(cell h) (based on previous data, not shown), a glucose concentration in CD-U3 medium ($C_{gM}$) of 33–40 mmol/mL, and a glucose concentration in the bioreactor ($C_{gR}$) of 6 mmol/mL.

As D is the ratio between the perfusion rate Q (mL/h) and the reactor's working volume $V_w$ (mL), the perfusion rate Q as a function of time can be expressed as:

$$Q = x_i\, e^{\mu t}\, V_w\, CSPR \quad (2)$$

Q was adjusted manually according to the initial cell concentration $x_i$ to cover the increasing nutrient demand (i.e. glucose) until the next sampling time point (for simplicity reason either 12 or 24 h). Therefore, the cascade control of the BIOSTAT®B plus module was used. For all calculations a constant cell-specific growth rate $\mu$ = 0.026 $h^{-1}$ was considered.

Three hours before infection, the perfusion rate was set to 2.5 times its calculated value to achieve a total medium exchange of 0.85 reactor volumes to further reduce the risk of nutrient limitation, and to dilute unwanted by-products that could negatively affect virus propagation. After infection, the perfusion rate was adjusted to the calculated values, applying the same $\mu$ as for the cell growth phase. From 36 hours post infection (hpi) a specific cell death rate of 0.028 $h^{-1}$ was taken into account to compensate for increasing cell losses due to progress of infection (based on previous data, not shown).

### 2.3. Shake flask cultivations

Small-scale cultivations were carried out in shake flasks with baffles (#215-2273, 125 mL (50 mL $V_w$) or #215-2277, 250 mL (110 mL $V_w$), VWR International, LLC) at 37 °C, 5% $CO_2$ and 185 rpm agitation speed in a Multitron incubation shaker (Infors AG) with 5 cm orbit. For experiments at CCD, shake flasks were inoculated to $0.8–0.9 \times 10^6$ cells/mL and cultivated for 72 h to reach about $4.0 \times 10^6$ cells/mL before infection. For experiments at HCD, the same inoculation procedure was performed and cells were cultivated in batch for 72 h before starting semi-perfusion.

Semi-perfusion was carried out by exchanging periodically enough medium to cover the glucose demand between two sampling time points. Assuming that the medium exchange volume ($V_E$) should equal the amount of medium exchanged in a continuous perfusion process for the same time period, $V_E$ can be derived solving Eq. (2) for $d(V_E) / dt = x_i\, e^{\mu t}\, V_w\, CSPR$ to obtain:

$$V_E = \mu^{-1}\, x_i\, (e^{\mu\, \Delta t} - 1)\, V_w CSPR \quad (3)$$

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