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Analyzing the Impact of Heterogeneity in Genetically Engineered Cell Lines for Influenza Vaccine Production Using Population Balance Modeling

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Abstract: Engineering of novel cell lines for biotechnological processes, e.g. influenza virus vaccine production, can be achieved by the genetic modification of host cell gene expression. Therefore, versatile genome editing methods such as lentiviral transduction can be applied to improve the production process. However, due to random integration of lentiviral-delivered genes in the host cell genome, nonuniform, i.e. heterogeneous, gene expression within the host cell population is expected. Within this contribution we investigate the influence of this cell-to-cell variability on important process variables like the maximum virus yield. Therefore, a multi dimensional population balance model is proposed which, on the one hand comprises a detailed description of the intracellular viral replication cycle and, on the other, also accounts for the expected heterogeneity in the host cell population. The results indicate that the overall vaccine production process can be improved by enhancement or inhibition of certain steps in the viral replication cycle. Furthermore, the achieved improvements show robustness against moderate degrees of cell-to-cell variability from genetic modification of host cells via transduction.

Keywords: population balance modeling, influenza vaccine production, multiscale modeling, cell-to-cell variability

1. INTRODUCTION

Influenza is a highly contagious disease of the respiratory tract which is mostly caused by the influenza A virus. It does not only occur in seasonal epidemics, but also occasionally in off seasonal pandemics affecting several million people world wide. Vaccination is the principle measure to counteract influenza. Up to date, the majority of influenza vaccines is produced in embryonated chicken eggs. This process is well-established and relatively cheap, but it also has several disadvantages which can be overcome by cell culture-based production processes (Nichols and LeDuc, 2009; Genzel and Reichl, 2009). These are already widely-used for other viruses and become increasingly relevant for influenza vaccines as well. However, cell culture based production processes need optimization.

Besides optimization of the process setup itself, optimized cell lines can be used to improve the overall production process. One approach is to modify gene expression of host cell factors important for viral replication to establish a high yield production platform. Here, genetic modifications, e.g. by lentiviral transduction, are used in order to achieve an overexpression or knockdown of selected host cell factors to increase the cell specific viral production rate. It was formerly shown that modifications of host cell factors can be used to influence the the activity of viral polymerase (Tafforeau et al., 2011). However, upon lentiviral transduction the degree of overexpression or knockdown of host cell genes varies within the modified cell population (Bushman et al., 2005). Thus a considerable cell-to-cell variability in the viral production rates is expected. This may have an significant impact on the vaccine production process and requires a detailed analysis.

Besides thorough experimental characterization, mathematical modeling and model based analysis of the process on multiple scales plays a key role for a deeper understanding of the underlying biological mechanisms and the development of suitable technical realizations. Recently, a detailed description of the intracelluar viral replication cycle has been developed (Heldt et al., 2012). In Heldt et al. (2013) this single cell kinetics was used within an age-structured model formulation to analyze the effects of direct acting antivirals within a multiple cycle infection. As a consequence of the age-structured formulation, all cells with the same infection age exhibit the same intracellular composition. Thus, cell-to-cell variability by means of, for instance, heterogeneous production rates is not taken into account. Any observed heterogeneity stems from a more or less slow progression of multiple cycles of infection. Alternatively, the framework of population balance modeling (Ramkrishna, 2000) can be used to account for cell-to-cell variability, as presented in (Müller et al., 2013).

In this manuscript, we will present an extension of the

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previous modeling approaches. Thereby, the modification of intracellular reaction rates resulting from knockdown and overexpression and heterogeneity of the modified host cell population is taken into account. The corresponding population balance equation represents a multi dimensional partial differential equation, which can be analyzed efficiently using a recently developed approximate moment method (Dürr and Kienle, 2014; Dürr et al., 2015b,a). Cell-to-cell variability as result of different degrees of knockdown/overexpression is incorporated by assuming that kinetic parameters vary within the host cell population. At first, a model based sensitivity analysis will be used to identify the most promising modifications for an improvement of the overall virus yield. Next, the effect of the cell-to-cell variability on this modifications is investigated. Therefore, different levels of heterogeneity are represented by broadening variability of the corresponding parameter distributions within the modified cell population.

2. MATHEMATICAL MODEL

Recently, a detailed single cell model describing the replication of influenza A virus within mammalian cells was presented (Heldt et al., 2012). This model accounts for major steps of the viral life cycle with a focus on viral RNA replication and its regulation. The basic scheme of the involved reactions is depicted in Fig. 1. We will abstain from the full presentation of the single cell model at this point and refer the interested reader to the corresponding publications (Heldt et al., 2012, 2013) for a detailed presentation. Instead, we will focus on the description of the model on the cell population level, which uses the framework of population balance modeling (Ramkrishna, 2000). The dynamics of the infected cell number density distribution i_c is given by the following multi dimensional population balance equation

$$\frac{\partial i_c}{\partial t} + \nabla_{\mathbf{x}^*}\left(\mathbf{h}^*\ i_c\right) = -(k_T^{\mathrm{Apo}} + k_i^{\mathrm{Apo}}) i_c + r^{\mathrm{inf}}\ T \qquad (1)$$

where $\mathbf{h}^*$ represents the single cell dynamics $\mathbf{h}^* = [\mathbf{h}, \mathbf{0}]^T$ of the extended state vector $\mathbf{x}^* = [\mathbf{x},\ \mathbf{k}]^T$. In the definition of the latter $\mathbf{x}$ and $\mathbf{h}$ are the single cell state vector and the single cell dynamics according to Heldt et al. (2013), respectively. The vector $\mathbf{k}$ denotes the vector of kinetic parameters which varies within the cell population. In addition to infected cells, which are born with rate r^{inf} and die with rate $(k_T^{\mathrm{Apo}} + k_i^{\mathrm{Apo}})$, apoptotic infected cells I_a are considered. Apoptotic infected cells lyse with the rate k^{Lys}. In contrast to the infected cells, these are assumed non-distributed, i.e. uniform. Their concentration is described by the following ordinary differential equation

$$\frac{\mathrm{d}I_a}{\mathrm{d}t} = \int_{\mathbf{X}} \left(k_T^{\mathrm{Apo}} + k_I^{\mathrm{Apo}}\right) i_c\ \mathrm{d}\mathbf{x} + r^{\mathrm{Inf}}\ T_a - k^{\mathrm{Lys}}\ I_a\,. \qquad (2)$$

Furthermore, the concentration of uninfected target cells T and their apoptotic counterparts T_a are characterized by the following equation

$$\begin{aligned}\frac{\mathrm{d}T}{\mathrm{d}t} &= g\ T - r^{\mathrm{inf}}\ T - k_T^{\mathrm{Apo}}\ T\\ \frac{\mathrm{d}T_a}{\mathrm{d}t} &= k_T^{\mathrm{Apo}}\ T - r^{\mathrm{inf}}\ T_a - k^{\mathrm{Lys}}\ T_a\,.\end{aligned} \qquad (3)$$

Target cells grow with rate g, become apoptotic with rate k_T^{Apo} and lyse with rate k^{Lys}. The growth rate g is defined by

$$g = \left[\frac{g_{\max}}{T_{\max}}\left(T_{\max} - T - \int_{\mathbf{X}} i_c\ \mathrm{d}\mathbf{x}\right)\right]\,. \qquad (4)$$

Virus particles are distinguished depending on their location. There are free active virus particles V (located in the extracellular medium), virus particles attached to the surface of target cells V^{Att} and virions located in endosomes of target cells V^{En}. The respective dynamics of the virus particle concentration are given by

$$\begin{aligned}\frac{\mathrm{d}V}{\mathrm{d}t} &= \int_{\mathbf{X}} r^{\mathrm{Rel}}\ i_c\ \mathrm{d}\mathbf{x} - k_V^{Deg}\ V + \sum_k \left[k_n^{\mathrm{Dis}}\ V_n^{\mathrm{Att}} - k_{c,n}^{\mathrm{Att}} B_n\ V\right]\\ \frac{\mathrm{d}V_n^{\mathrm{Att}}}{\mathrm{d}t} &= k_{c,n}^{\mathrm{Att}}\ B_n\ V - \left(k_n^{\mathrm{Dis}} + k^{\mathrm{En}}\right)\ V_n^{\mathrm{Att}} - \left(r^{\mathrm{inf}} + r^{\mathrm{lys}}\right)\ V_n^{\mathrm{Att}}\\ \frac{\mathrm{d}V^{\mathrm{En}}}{\mathrm{d}t} &= k^{\mathrm{En}}\left(V_{hi}^{\mathrm{Att}} + V_{lo}^{\mathrm{Att}}\right) - k^{\mathrm{Fus}}\ V^{\mathrm{En}} - \left(r^{\mathrm{inf}} + r^{\mathrm{lys}}\right)\ V^{\mathrm{En}}\end{aligned} \qquad (5)$$

with

$$\begin{aligned}B_n &= B_n^{\mathrm{tot}}\,(T + T_a) - V_n^{\mathrm{Att}}\,, \quad k_n^{\mathrm{Dis}} = \frac{k_{c,n}^{\mathrm{Att}}}{k_{c,n}^{\mathrm{Equ}}}\\ n &\in \{lo,\, hi\}\,.\end{aligned} \qquad (6)$$

Infected cells release active virus (r^{Rel}) that can attach (k_n^{Att}) or dissociate (k_n^{Dis}) from binding sites B_n of target cells, or get degraded (k_V^{Deg}). Two types of binding sites B_n for the virus particles on the surface are considered: low affinity (lo) and high affinity (hi). The cell internalizes attached virions V_n^{Att} via endocytosis (k^{En}). Upon fusion of the viral membrane of virions inside endosomes V_{En} and the endosomal membrane (k^{Fus}), the viral genome is released into the cytoplasm. A detailed description of the involved kinetic processes can be found in Heldt et al. (2012). The infection rate r^{inf} and lysis rate r^{lys} are defined as

$$\begin{aligned}r^{\mathrm{inf}} &= \frac{F_{\mathrm{inf}} k^{\mathrm{Fus}} V^{\mathrm{En}}}{T + T_a}\\ r^{\mathrm{lys}} &= \frac{k^{\mathrm{lys}} T_a}{T + T_a}\,.\end{aligned} \qquad (7)$$

The viral release rate $r^{\mathrm{Rel}}(\mathrm{x})$ depends on the amounts of viral compounds in the cells and is given by

$$r^{\mathrm{Rel}}(\mathbf{x}) = k^{\mathrm{Rel}} \frac{V p_{M1}^{cyt}}{V p_{M1}^{cyt} + 8\ K_{Vrel}} \prod_j \frac{P_j}{P_j + N_{P_j}\ K_{Vrel}}\,,$$
$$P_j \in \{RdRp,\ HA,\ NP,\ NA,\ M1,\ M2,\ NEP\}\,. \qquad (8)$$

The initial conditions for virus and target cell concentrations are set to 6.9 10^4 virions/ml and 4.9 10^5 cells/ml, respectively (Heldt et al., 2013). The remaining extracellular initial conditions are zero. All additional model parameters can be found in Heldt et al. (2013). As described in the reference, virus entry is considered on the macroscopic scale and newly infected cells are initialized with a complete set of 8 vRNP segments.

3. MODEL SOLUTION

The population balance equation (1) represents a multi dimensional partial differential equation which is coupled to a set of ordinary differential equations (2) - (5).

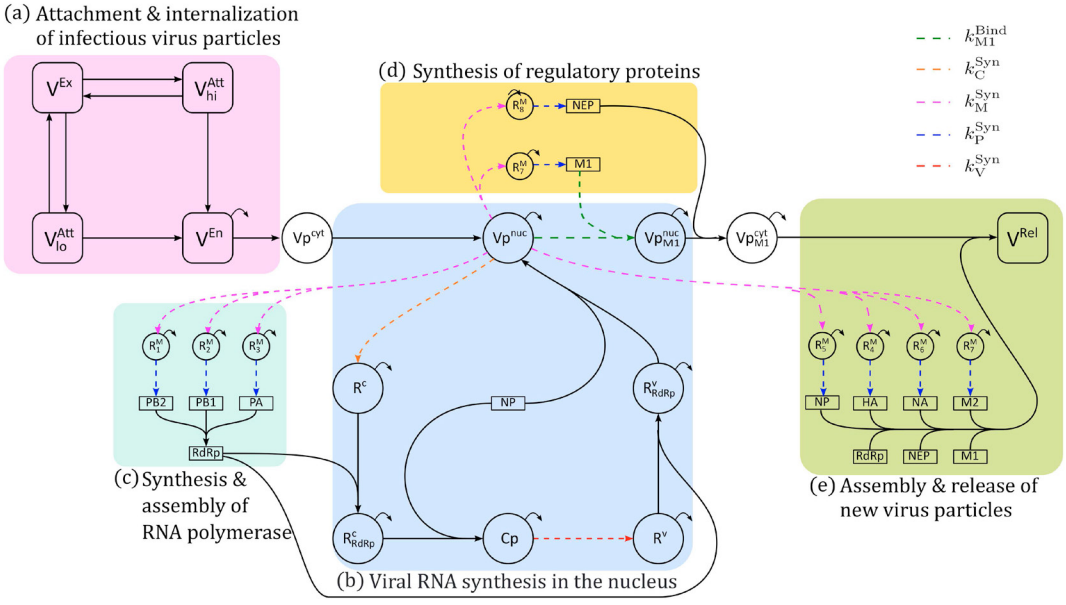

Fig. 1. *Model of the single cell replication cycle of influenza virus based on Heldt et al. (2012). After attachment and internalization of infectious virus particles* ***(a)*** *a set of 8 vRNP segments (Vp^{cyt}) is released into the cytoplasm to enter the nucleus (Vp^{nuc}). In the nucleus, synthesis of viral mRNA, cRNA and vRNA takes place. While viral mRNAs are exported from the nucleus, cRNA and vRNA are encapsidated by the heterotrimeric viral polymerase (RdRp) and several copies of NP to form cRNPs (Cp) and vRNPs (V_p^{nuc})* ***(b)****. In the cytoplasm, viral mRNAs (R_i^M) are translated into viral proteins* ***(c-e)****. Three of the viral proteins assemble to RdRp, which is essential to form new vRNPs (Vp^{nuc}) in the nucleus* ***(c)****. The synthesis of M1 and NEP is required to facilitate nuclear export of vRNPs (Vp_{M1}^{cyt}) into the cytoplasm* ***(d)****. In the last step of the influenza virus replication cycle all viral proteins and the NEP-M1-vRNP complex assemble at the plasma membrane and form a new virus particle. Progeny virions (V^{Rel}) are released into the extracellular medium* ***(e)****. Investigated parameters are marked with different colored and dashed pathways.*

In general, the dimension of the partial differential equation corresponds to the dimension of the extended state vector $\mathbf{x}^*$ which comprises the intracellular viral compounds ($dim(\mathbf{x}) = 27$) and the distributed kinetic parameters. As standard numerical methods for full solution suffer from an enormous numerical effort, we decided to use our recently developed approximate moment method (Dürr and Kienle, 2014; Dürr et al., 2015a,b) to come up with a numerical solution. In contrast to the aforementioned classical discretization based methods, the technique relies on the solution of only a small number of ordinary differential equations characterizing integral quantities of the full number density distribution like means and variances with respect to the intracellular states. The technique combines the direct quadrature method of moments (Marchisio and Fox, 2005) with an efficient choice of quadrature abscissas based on monomial cubatures (see e.g. Stroud (1971)). Due to space restriction we will abstain from a detailed description of the method. Details are found in the corresponding contributions (Dürr and Kienle, 2014; Dürr et al., 2015a,b).

4. SENSITIVITY ANALYSIS

First, a sensitivity analysis of the single cell model will be carried out to determine the most promising targets for an improvement of the vaccine production. Simulations are evaluated with respect to maximum concentration of the extracellular virus particles and the respective peak time. It is assumed, that genetic modifications of the cells can be mapped directly to a modification of corresponding intracellular reaction rate parameters. Furthermore, in this section it is assumed that all cells are modified with the same degree of knockdown/overexpression and thereby exhibit the same modified parameter set.

A comprehensive analysis of host cell factors affecting influenza virus polymerase activity demonstrate the importance of RNA synthesis rates in the mathematical model (Tafforeau et al., 2011). Furthermore, the viral matrix protein 1 (M1) is known to promote the nuclear export (Martin and Helenius, 1991). For this reason, in the following, focus is on modifications of the synthesis rates of viral mRNA, vRNA, cRNA, viral protein translation and the binding rate of viral protein M1 which mark very important limiting steps during the viral replication cycle. The affected kinetic reactions are colored in the basic scheme Fig. 1. The corresponding kinetic parameters are modified in a biological reasonable range by a factor of 0.2 or 5, respectively.

At first, only one parameter is changed while the others are kept unmodified. Thus 10 independent combinations have to be analyzed in view of the desired improvements stated above. Here, an inhibition of the binding rate of M1 k_{Bind}^{M1} has the largest effect on the virus dynamics (Fig. 2)

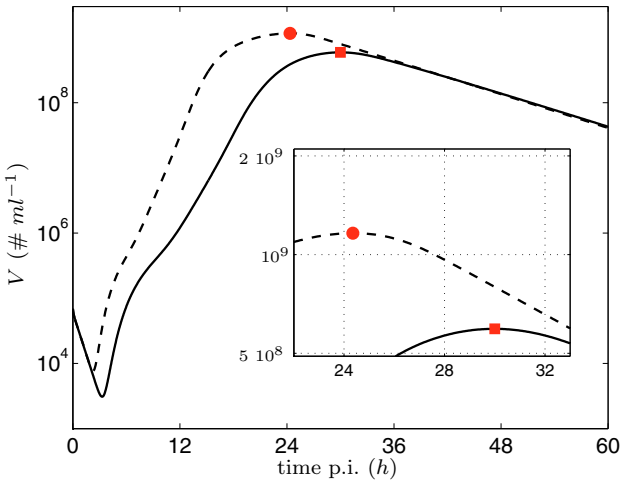

Fig. 2. *Virus concentration for inhibition of binding rate of M1 (dashed) compared to parental cell line (solid); filled red symbols mark the maximum concentrations*

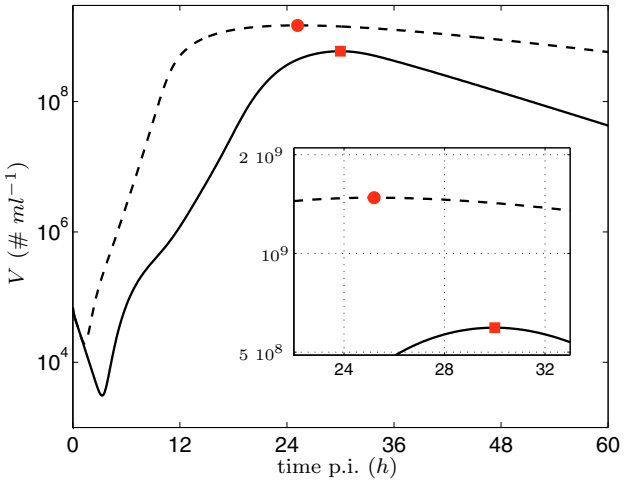

Fig. 3. *Virus concentration for modification of 5 rates* $\left[k_{M1}^{\text{Bind}} \downarrow,\ k_C^{\text{Syn}} \rightarrow,\ k_M^{\text{Syn}} \uparrow,\ k_P^{\text{Syn}} \uparrow,\ k_V^{\text{Syn}} \uparrow\right]$ *(dashed) compared to parental cell line (solid); filled red symbols mark the maximum concentrations*

as this causes a delayed shutdown of viral replication. In contrast to the parental cell line (solid), the maximum virus yield approximately doubles from $5.94 \cdot 10^8$ to $1.16 \cdot 10^9$ # ml^{-1}. Furthermore, the peak time has decreased from approximately 30 to 24.4 h post infection (p.i.).
In the second case, all reaction rates are subject to modification. Thus, an overall amount of $3^5 = 243$ combinations has to be analyzed. The best improvement is found for the combination, in which the synthesis rates for vRNA, mRNA and viral proteins are increased, the M1 binding rate is reduced while the cRNA synthesis rate is kept unmodified. In Fig. 3 the corresponding virus dynamics is depicted (dashed) in comparison to the unmodified cell population (solid). The maximum virus concentration of the modified cells increases by a factor of 2.49 to $1.48 \cdot 10^9$ # ml^{-1}. Furthermore, also the peak time decreases to 25.2 h p.i. It can be seen that the virus concentration does not decrease as fast as in case of the previous modification. This suggests that the cells produce virions for a longer time period which may be also advantageous for the overall vaccine production process as harvesting at later time points still results in an considerably higher virus yield.

5. EFFECTS OF CELL-TO-CELL VARIABILITY

In the previous section it was assumed that all modified cells show the same level of gene expression. However, when cells are modified with the help of lentiviral vectors, the degree of overexpression and knockdown varies from cell to cell. Thus, the modified cell population will exhibit some degree of heterogeneity in their ability to propagate the influenza virus. In the following, it is assumed that this heterogeneity can be mapped to a distribution of the corresponding kinetic parameters within the modified cell population. A desirable transduction would result in a cell population with parameters distributed narrowly around the desired modification. Different degrees of heterogeneity resulting from lentiviral transduction can now be represented by broadening distributions of the kinetic parameters. In the following, the parameter distribution within the cell population is approximated by a weighted sum of five logarithmic Gaussian distributions to account for asymmetric distributions as illustrated in Fig. 4.

5.1 Modification of one reaction rate

At first, the inhibition of the M1 binding to viral genomes is considered while the other rates are kept unmodified. As mentioned above, it is assumed that a prior transduction results in a distribution of the parameter k_{Bind}^{M1} within the cell population which is described by a weighted sum of five logarithmic Gaussians

$$k_{\text{Bind}}^{M1} \sim \sum_{l=1}^{5} a_l \ \mu_l \ e^{\mathcal{N}(0,\sigma_l)} \,. \tag{9}$$

The mean values μ_l are varied uniformly in a logarithmic scale between the nominal value k_{Bind}^{M1} and 0.2 k_{Bind}^{M1} and the variances are chosen as $\sigma_l = 0.05$, $l = 1, \ldots, 5$. By using different values for the coefficients a_l, different scenarios can be simulated, which relate to different host gene expression pattern. For the following simulation study, three different scenarios are investigated (see Fig. 4). These approximate an increasing heterogeneity resulting from the transduction process. The results of the numerical simulations are shown in Fig. 5. It can be seen that the maximum virus concentrations (red markers in Fig. 5) decrease with increasing degree of heterogeneity. For the analyzed scenarios (symbols corresponding to the ones in Fig. 4) the maximum virus concentrations are $1.13 \cdot 10^9$ # ml^{-1} for scenario (a), $1.02 \cdot 10^9$ # ml^{-1} for (b) and $9.03 \cdot 10^8$ # ml^{-1} for (c). The peak time increases to 24.6 (a), 25.2 (b) and 26.1 h p.i.(c). The simulation results suggest that the overall production process using modified cell lines is relatively robust against host cell heterogeneity and an increase of the maximum virus titers is possible. However, for larger degrees of heterogeneity the desired aim of increasing the maximum virus concentration is not obtained.

5.2 Modification of multiple reaction rates

In this section the influence of heterogeneity is investigated, when up to five reaction rates are modi-

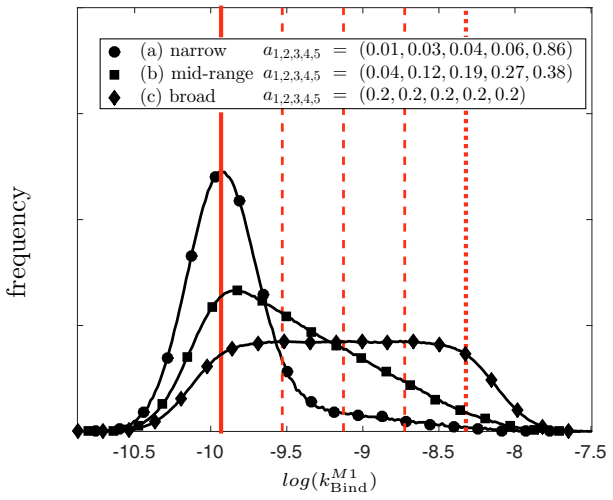

Fig. 4. *Parameter distributions for investigated scenarios representing different degrees of cell-to-cell variability; mean values of the underlying logarithmic normal distribution are highlighted red*

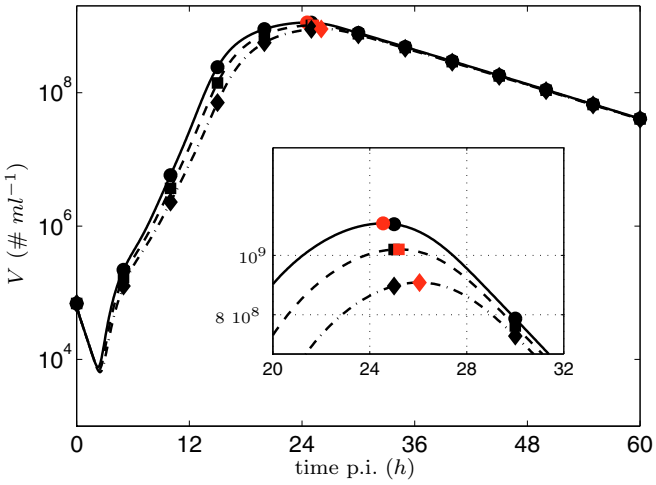

Fig. 5. *Virus concentration for different levels of host cell heterogeneity if the M1 binding rate is modified; the maxima are highlighted red for each scenario*

fied simultaneously. The best combination for a knockdown/overexpression of five reaction rates was already found from the sensitivity analysis. Here, enhancement of mRNA, vRNA and protein synthesis rates in combination with a reduced M1 binding rate has shown the best improvement in maximum virus yield and peak time. Similar to the previous investigation, it is assumed that cell-to-cell variability resulting from transduction can be represented by a weighted sum of five logarithmic Gaussians. Those are now five dimensional, as five reaction rates are considered

$$\sum_{l=1}^{5} a_l \; \mu_{\mathbf{l}} \cdot e^{\mathcal{N}(\mathbf{0},\mathbf{diag}(\sigma_{\mathbf{l}}))} \,. \tag{10}$$

The mean vectors of the distributions $\mu_{\mathbf{l}}$ are varied uniformly in a logarithmic scale between the nominal parameter vector μ_1 and the best combination μ_5

$$\mu_1 = \left[k_C^{\text{Syn}},\; k_M^{\text{Syn}},\; k_P^{\text{Syn}},\; k_V^{\text{Syn}},\; k_{M1}^{\text{Bind}}\right]^T$$
$$\mu_5 = \left[k_C^{\text{Syn}},\; 5\; k_M^{\text{Syn}},\; 5\; k_P^{\text{Syn}},\; 5\; k_V^{\text{Syn}},\; 0.2\; k_{M1}^{\text{Bind}}\right]^T . \tag{11}$$

The variances are chosen as $\sigma_l = 0.05$. Furthermore, for the different scenarios, the weighting coefficients a_l are kept at the values reported in Fig. 4. It is worth mentioning that the cell population exhibits heterogeneity with respect to all considered reaction rates, even if they are not modified (the mean value of k_C^{Syn} remains at the original value). Thereby, a more general effect of genetic modifications is incorporated: viral and cellular factors are interconnected in a complex virus host cell interaction network. Consequently, the genetic modification of one host cell factor is likely to affect multiple steps of the viral replication cycle. Thus, it can be expected that an overexpression or knockdown of one host cell factor aiming on the enhancement or inhibition of a certain kinetic reaction rate also introduces some degree of heterogeneity in other reaction rates.
Three different scenarios are used to analyze the effects of heterogeneity within the cell population on the overall vaccine production process. The simulation results are depicted in Fig. 6. As in the previously analyzed case, a broader parameter distribution correlates with a decreased maximum virus concentration and an increased peak time. The resulting values for the maximum virus concentrations (locations are highlighted red in Fig. 6) are $1.44 \cdot 10^9$ # ml^{-1} (a), $1.32 \cdot 10^9$ # ml^{-1} (b) and $1.11 \cdot 10^9$ # ml^{-1} (c). The corresponding values for the peak times are 25.5, 25.9 and 26.9 h p.i. In comparison to the simulation results for a homogeneous population as computed in section 4, only around 40% of the increase in virus concentration is achieved which again emphasizes the impact of cell-to-cell variability on the overall process. However, even for the worst assumed scenario (c)

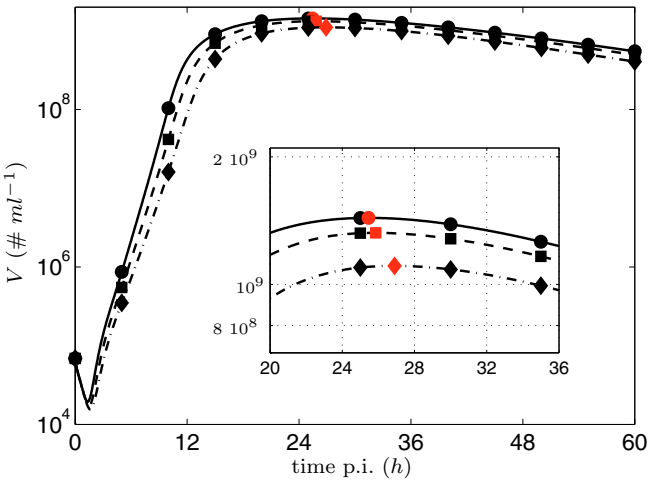

Fig. 6. *Virus concentration for different levels of host cell heterogeneity if five kinetic parameters are modified (see Eq. 11); the maxima are highlighted in red for each scenario*

a significant increase in the virus yield is predicted which suggests that the modification of certain reaction rates by knockdown/overexpression of host cell factors can be a suitable tool to overcome limitations in the overall process.

6. SUMMARY AND OUTLOOK

In this contribution, we presented a model based approach to describe the influence of host cell factor modifications on a influenza vaccine production process. Therefore, a previously developed model was extended to account for both, the modifications of the intracellular viral replication kinetics and cell-to-cell variability resulting from different levels of host cell factor expression upon transduction. It was assumed that modifications of specific host cell factors by means of overexpression and knockdown can be represented as an increase or decrease of certain kinetic model parameters. Furthermore, heterogeneity within the modified cell population, as expected from random events during transduction, was incorporated using parameter distributions. First, possible candidates for modifications of single or multiple host cell factors were identified using a sensitivity analysis. Simulation results of the corresponding modifications show an improved process performance by means of increased virus titer and reduced peak time. Afterwards, the influence of cell-to-cell variability upon transduction was investigated. Therefor, the model was solved for different levels of heterogeneity with respect to the corresponding kinetic parameters.

The simulation results suggest that with modified production cell lines both, an increase in the maximal virus concentration as well as a reduction in peak time can be achieved. The best candidate to reach this goal hypothesize an enhanced RNA and protein synthesis together with a reduced M1 binding to vRNP in the nucleus as most sensible factors. Furthermore, model predictions show that improvements are robust against moderate degrees of cell-to-cell variability. For this reason, our model based analysis indicates that the use of genetically modified cell lines is a promising option to overcome limitations in vaccine production processes.

In the present study, it is assumed that each successive host cell factor modification results in the same degree of cell-to-cell variability within the modified cell population. In a more realistic setting, the knockdown and overexpression of host cell factors is likely to increase the cell-to-cell variability the more host cell factors are modified. This may have a stronger effect on the overall process performance. Note, that predictions in this study are preliminary and require experimental validation. A currently ongoing thorough characterization of the influence of gene expression levels on influenza virus replication will enable a realistic characterization of the model parameter distributions.

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