Criteria for scale-up and scale-down of bioreactors for cultivation of mammalian cells

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Invictus

Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the horror of the shade,
And yet the menace of the years
Finds, and shall find, me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul.

From William Ernest Henley (1849 – 1903)
Abstract

Application of quality by design (QbD) requires identification of the maximum operating range for parameters affecting the cell culture process. These include hydrodynamic stress, mass transfer or gradients in dissolved oxygen, carbon dioxide and pH. Since most of these are affected by the impeller design and speed, the main goal of the first part of this work was to identify the maximum operating range for hydrodynamic stress, where no variation of cell growth, productivity and product quality can be ensured. To properly represent the oscillation stress exposure of cells in large-scale bioreactors, two scale-down models were developed operating under laminar and turbulent condition, generating repetitive oscillating hydrodynamic stress with maximum stress values ranging from 0.4 to 420 Pa. Two manufacturing cell lines (CHO and Sp2/0) used for the synthesis of therapeutic proteins were employed. For both cell lines multiple process outputs were used such as cell growth, morphology, metabolism and productivity, to determine the threshold values of hydrodynamic stress resulting in values equal to 32.4±4.4 Pa and 25.2±2.4 Pa for CHO and Sp2/0, respectively. Below the measured thresholds both cell lines did not show any appreciable effect to hydrodynamic stress on any critical quality attribute, while above, cells responded negatively to elevated stress. To confirm the applicability of the proposed method, the obtained results were compared with data generated from classical small-scale reactors with a working volume of 3L.

Although several scaling models are suggested and described in the literature, they mostly lack reasonable validation or comparison at pilot or manufacturing-scale. Therefore, the second part of this work is dedicated to a validation of the before developed oscillating shear system. A 300 L pilot scale bioreactor was used for the validation, operated at either safe agitation conditions (7 Pa) or at 28 Pa, corresponding to the edge of failure (25 Pa) of the used Sp2/0 cell line. In order to consider the simultaneous action of stirring and sparging the
maximum stress values for the used bioreactors were determined using a stress sensitive particulate system. Pilot scale data were compared with historical data from classical 3 L cultivations and cultivations from the oscillating stress loop model. Results for the growth behavior, analyzed metabolites, productivity and product quality showed a dependency on the different environmental stress conditions but not on reactor size. Pilot scale conditions were very similar to those generated in the oscillating stress loop model confirming its predictive capabilities, including conditions beyond the edge of failure.

The final part of this work extends the investigation to multiple scale dependent parameters as observed from the oscillatory environment of a large-scale bioreactor. Parameters studied were increasing dissolved carbon dioxide over time, oscillation of hydrodynamic stress and dissolved oxygen plus the perturbation of the pH by a basic feed. Parameters were first examined individually resulting in the corresponding threshold values. A two zone model consisting of two differently sized interconnected bioreactors was developed to perform combinatorial experiments. With this model the most realistic large-scale environment was simulated and the validity of the before determined thresholds was reevaluated. Using different bubble sizes, elevated dCO$_2$ levels were simulated resulting in a critical value of 100 mmHg, which inhibited growth completely. Combination experiments in the two zone model showed that the oscillation of dissolved oxygen between 40 and 60 % had no negative impact on growth or metabolism. In contrary, the combination of stress and elevated dCO$_2$ levels both being below their individual threshold values, results in a combined negative effect on growth and productivity. This clearly indicates that only studies with combined effects of several parameters would represent the proper strategy when constructing a design space for a cell cultivation process.
Zusammenfassung


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First of all I would like to thank Prof. Massimo Morbidelli for giving me the opportunity to do my thesis in his group and all the trust he gave me in this project. His great vision to turn a Biologist into and Engineering was a hard task but finally successful and I am grateful for the experiences that came along with it.

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I want to thank my collaboration partner Merck Serono for supporting the thesis and including me into their work environment. Special thanks at this point go to Matthieu Stettler, the USP group manager, for his supervisions and ongoing unconditional support of the project. He taught me that unconventional ideas and approaches also have value in an industrial environment, although they might seem to be too much, off business. The same accounts for Hervé Broly, VP Biotech Process Development and David Beatty, Senior Director EMD Millipore, who both highly supported the strategy behind the project and the collaboration.

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Final thanks go to my family being my strongest back bone and everlasting motivator. My grandma Meta was always there with her experience and helped me and still does, to grow a better person every day. I thank my brother Fabian, with his wife Simone for all the moral support when times got rough. Thanks to Anna and Luis, their children, for all your priceless smiles. At the very last thanks to my mother and my father to support me generously from my youngest days and encouraging me to follow my dreams wherever they may lead me.

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Chapter 1

Introduction

Modern society is growing older and older and with increased live span the risk to develop chronicle illness like arthritis, cancer or diabetes is getting bigger with each year of one’s life. The only medications know against such illnesses nowadays is the use of monoclonal antibodies (mAb) mostly produced by mammalian cells (Walsh, 2010). To support this increasing needs bioreactors of increased volumes are being used currently reaching volumes of 20000 Liters (Birch and Racher, 2006; De Jesus and Wurm, 2011; Varley and Birch, 1999). To meet the demand of the marked it is therefore required to scale new or existing processes from the laboratory milliliter scale to GMP plants with several thousands of liters. Due to the limited lifetime of a commercial patent of such products and lengthy clinical trial, time is usually limited for the biochemical engineer to perform the challenging tasks of process scale up and validation (Willoughby, 2006). Additional complications occur through the multitude of cultivation devices used for mammalian cell culture. In the early development of a bioprocess uncontrolled systems like static t-flasks, agitated roller bottles or shake flasks are used to grow cells. With increasing the cell culture volume single used bag based systems (i.e. Wave bioreactor), orbitally shaken systems (i.e. Shake flasks, TubeSpin) and stirred bioreactor are commonly used for cell cultivation (Levine et al., 2012; Stettler et al., 2007). Mixing dynamics in between those devices can be
completely different. Whereas in the stirred tank reactor the impeller causes homogenization of the system it is the wall itself that is used in an orbitally shaken bioreactor. The wave system uses a single use plastic bag on a horizontally shaken plate generating a wave that mixes the cell culture. The cultivation environment of these conceptually very different systems is tried to be compared and understood with detailed characterizations for mixing, gas mass transfer, heat transfer and hydrodynamic stress (Li et al., 2006; Mollet et al., 2004; Sieblist et al., 2011a, 2011b). Although all of those systems find application in a development stage, the system of choice for manufacturing-scale has clearly become the stirred tank reactor as it can be seen from its use in all major pharmaceutical companies (Chu and Robinson, 2001; Marks, 2003; Nienow, 2006; Xing et al., 2009).

The Quality by Design (QbD) concept introduced by the US Food and Drug Administration (2009) expects from the producers a profound knowledge of their production process and a detailed characterization of the relationship between process conditions and critical quality attributes (CQA). This should include the understanding of the impact of raw material variability and different production scales on the final product (Rathore, 2009; Seely and Seely, 2003). Therefore, typical process optimization strategies are used already during early process development by the screening of key operating parameters like temperature, pH or dissolved oxygen (Heath and Kiss, 2007; Looby et al., 2011; Rouiller et al., 2012). The influence of different scales however is rather seldom addressed, mostly due to the relative small amount of data available from large scale and the limited possibilities for experimentation at manufacturing-scale. The adverse effects of scale depend parameters, like hydrostatic pressure, suboptimal gas mass transfer and a broad distribution of hydrodynamic stress, nevertheless is often observed (Eon-duval et al., 2014; Gray et al., 1996; Mostafa and Gu, 2003; Tsang et al., 2013) but for the systematic analysis of them, the development and application of suitable downscale models is needed. The here presented work aims to review
the possibilities and develop methodologies to better understand inhomogeneity’s typical for large scale.

1.1 Outline

This work pursues the following objectives:

- **Development of a downscale device to investigate oscillating hydrodynamic stress on mammalian cells**

  The oscillatory nature of hydrodynamic stress is well known from stirred tank reactors where the distribution of the hydrodynamic stress covers several orders of magnitudes. A small-scale bioreactor device is to be developed being capable of mimicking the high and low stress regions, while giving the possibility to determine a cell specific threshold. Furthermore, the decoupling of stress magnitude and stress frequency is aimed to allow the simulation of different reactor scales and mixing conditions. Ideally the device is tested with several cell lines and processes to evaluate its general applicability. Typical performance parameters like cellular growth and metabolism will be used to classify the results. A special emphasis is moreover taken on the productivity and product quality.

- **Verification of such a downscale device using pilot scale and manufacturing-scale data**

  The before developed downscale model will be validated using especially designed pilot scale experiments to show its predictive capabilities. After the determination of the cell specific threshold for oscillating shear the verification will be performed by running an experiment at pilot scale beyond the before determined threshold. The comparison with large scale data obtained from manufacturing-scale should further support the applicability of the model.
• Development and application of a two zone bioreactor downscale model to simultaneously simulate multiple heterogeneities known from manufacturing-scale

Due to the presence of multiple heterogeneities in large scale a two zone model consisting of two interconnected bioreactors is to be developed, which is capable of simulating several scale dependent parameters simultaneously. The combination of parameters will be (1) shear heterogeneity, (2) increasing dissolved carbon dioxide, (3) feed based pH excursions and (4) oscillating dissolved oxygen. Growth behavior and metabolism will be used to interpret the results, while single parameter results will be compared to those obtained from combinatorial experiments.
Chapter 2

A hydrodynamic stress scale down model

2.1 Introduction

Production of biopharmaceuticals is a delicate process considering the risks connected to the administration of the product into human beings and the possibility of life-threatening side effects (Attarwala, 2010; Schneider et al., 2006). To minimize these risks, the Quality by Design (QbD) concept (US Food and Drug Administration, 2009) aims at increasing the knowledge of the production process and characterizing the relationship between process conditions and critical quality attributes (CQA). This includes the understanding of the impact of raw materials variability and different production scales on the final product (Rathore, 2009; Seely and Seely, 2003). Key component of this concept is the design space that needs to be characterized for each process step resulting in a maximum operating range, which describes the overall process operational limits for which a defined product quality can be ensured. Mostly, this evaluation is performed at development scale using design of experiments (DoE), which raises the question how valid the results are at manufacturing scale (Eon-duval et al., 2014). The micro environment at manufacturing scale can vary substantially.

1 Original paper title: “Determination of the maximum operating range of hydrodynamic stress in mammalian cell culture”
due to variations in mixing time, possibly inducing pH, oxygen and temperature gradients as well as differences for oxygen transfer and carbon dioxide removal (Amanullah et al., 2001; Li et al., 2010; Sieblist et al., 2011a). A maximum operating range established at small scale (Eon-duval et al., 2012; Rouiller et al., 2012) needs to be proven valid across other scales. Therefore options are limited. Application of a similar strategy at large-scale is possible; however, it requires very high investment for the manufacturer. The alternative would be to define a maximum operating range of scale independent parameters, e.g. O$_2$ transfer, CO$_2$ removal, temperature or maximum hydrodynamic stress (Eon-duval et al., 2014; Sieck et al., 2014), instead of using scale dependent parameters like stirring speed or gas flow rate. In this way some process parameters will be different at different scales, but can be defined as unique scale independent parameters. This implies the use of scale independent parameters in a DoE approach, to define a maximum operating range valid for multiple scales.

Process productivity is nowadays achieved by increasing cell densities beyond 20 $\times$ 10$^6$ cells/mL, optimizing specific productivity and culture duration. In these conditions oxygen supply of state-of-the-art suspension cell cultures is challenging due to the combination of high cell density, increased specific oxygen uptake rate and reduced mass transfer caused by media components, i.e. surfactants. Compensation can be achieved by increased aeration combined with the proper level of agitation. Although the effect of hydrodynamic stress on mammalian cells is well-researched (Chisti, 2010, 2001) and mammalian cells show good resistance against stress, as shown by several authors (Al-Rubeai et al., 1995; Godoy-Silva et al., 2009b; Nienow, 2006; Nienow et al., 2013; Tanzeglock et al., 2009), different thresholds of hydrodynamic stress are tolerated by various cell lines. Due to this, any new process development within a QbD framework has to contain the response study of a culture to the above mentioned scale independent parameters. In particular, the lower limit will be determined by minimum O$_2$ transfer and CO$_2$ removal rates, and mixing
performance of the bioreactor, while the upper limit of the maximum operating range will be
defined by the threshold values for lethal or sub-lethal effects due to stress. In classical single
vessel scale down models high stirring speed is used to reach the edge of failure for the
hydodynamic stress (Nienow, 2009; Nienow et al., 2013). While this is the simplest possible
approach the application of high agitation drastically reduces the exposure period of cells to
highest stress values present closed to the impeller. Furthermore, depending on the robustness
of the investigated cells, to reach the stress threshold values it could require agitation rates
outside of the technical limit of the used motor. Additionally vortex formation becomes very
likely with the consequence of introducing air bubbles into the culture, a highly undesired
effect because it can cause cell damage (Nienow, 1998).

Therefore, the goal of this study was to develop a scale-down system allowing us to
cover a broad range of hydrodynamic stress avoiding the above mentioned limitations. After a
detailed characterization of the proposed system it was used to investigate the effect of
oscillating hydrodynamic stress on cell growth, productivity and product quality using two
recombinant manufacturing cell lines. The first was derived from a Chinese hamster ovary
(CHO) host cell line and the second from a mouse hybridoma (Sp2/0) host cell line. Since
various approaches were applied previously in the literature, where cells were exposed to
hydodynamic stress, using laminar or turbulent conditions (Godoy-Silva et al., 2009b; Keane
et al., 2003; Kunas and Papoutsakis, 1990; Oh et al., 1989; Sieck et al., 2013; Tanzeglock et
al., 2009), both flow regimes were applied in the present study to identify an impact of the
flow type on cells performance.
2.2 Materials and Methods

2.2.1 Cell lines and bioreactor setup

Two model cell lines were used in this study. The first derived from a CHO host cell line producing a fusion protein, which is in clinical development. The second was derived from a Sp2/0 host cell line producing a commercialized monoclonal antibody.

Inoculum preparation for the CHO cells was started by thawing a cell bank vial and directly diluting it into proprietary animal-derived component free expansion media. The culture was kept at 37 °C and 90 % humidity. Cell density after thawing was $0.5 \pm 0.1 \times 10^6$ cells/mL and at least three sub-cultivation steps were performed every week to keep the cell density below $2.5 \times 10^6$ cells/mL. Due to relatively high cell densities used for bioreactor seeding, cells were diluted into a second nutritionally rich expansion media, during the last 5 days of the inoculation preparation, to grow them to a cell density of more than $5 \times 10^6$ cells/mL. Independent of the bioreactor size, the duration of the expansion culture was always kept at 14 days. Cells were seeded with a density equal to $1.4 \pm 0.1 \times 10^6$ cells/mL into a bioreactor prefilled with animal-derived component free proprietary media. For this cell line a 3 L bioreactor (Sartorius Stedim, Germany) was used equipped with online temperature, pH and dissolved oxygen (DO) measurement probes (Mettler-Toledo, Switzerland) and one elephant ear impeller inclined by 45° from horizontal plane, pumping the liquid downwards (see Figure 2.1). Agitation speed during the culture was set to 150 rpm and the volume average energy dissipation rate was calculated according to (Perry et al., 1997):

$$\langle \varepsilon \rangle = \frac{N_p D^4 N}{V}$$

(2.1)

where $N_p$ is the power number of the impeller, $D$ the impeller diameter, $N$ the agitation rate and $V$ the bioreactor volume.
Considering $N_p$ equal to 2.35 this results in $\langle \varepsilon \rangle$ being equal to $9.5 \times 10^{-3}$ W/kg. Temperature was controlled at 37 °C for the whole cultivation process. Due to lactate production of the growing culture, the pH profile was characterized by a drift from the initial value of 7.2 down to 7.0 over the first 2 days. After this reduction, the pH was kept constant until the end of the culture by the addition of acid (lactic acid, 1 M) and base (NaOH, 0.5 M) when needed. To keep the dissolved oxygen (DO) at the set-point of 50 % air saturation, a constant air flow of 5 mL/min combined with an oxygen on demand strategy was applied resulting in a total volumetric gas flow being below 40 mL/min (0.013 vvm). Glucose was fed daily from day 3 in order to keep the glucose concentration in the bioreactor between 2 g/L and 4 g/L. Additionally, a chemically defined bolus feed was added on day 6.

For the expansion of the Sp2/0 culture, cells were thawed into a complex proprietary media and were first cultured in orbitally shaken T-flasks and then, when an appropriate
volume was reached, in spinner flasks (Integra, Switzerland). The culture was incubated at 37 °C and 90 % humidity. Initial cell density was $0.5 \pm 0.1 \times 10^6$ cells/mL and sub cultivation was performed every 2 days to keep the cell density below $1.5 \pm 0.1 \times 10^6$ cells/mL. Cells were kept in expansion for 29 days. Seeding into the bioreactor was performed using the same media with a cell density of $0.3 \pm 0.1 \times 10^6$ cells/mL. Geometric details of the reactor were the same as mentioned before but for agitation a radially pumping Rushton impeller was mounted in the bottom part of the vessel and an elephant ear impeller, inclined by 45° from horizontal plane pumping the liquid upwards, was mounted 8 cm above the Rushton impeller. Agitation was set to 110 rpm ($1.1 \times 10^{-2}$ W/kg) and the DO was controlled at 70 % air saturation using a constant air flow of 10 mL/min combined with oxygen on demand. The total gas flow rate during the whole culture did not exceed 40 mL/min corresponding to 0.013 vvm. The pH was controlled with CO2 addition at 7.1 until day 3 when a step change down to 6.9 was applied and kept constant for the rest of the process. A single concentrated nutritional bolus feed was added on day 2 containing a complex mixture of glucose, amino acids and proteins.

It is worth noting that preliminary experiments, for both cell lines, performed under elevated gas flow rate confirmed that under the applied conditions damage of the cells by aeration is negligible (Chisti, 2000). In all runs replicates were conducted to evaluate process robustness. Error bars in the presented results represent one standard deviation obtained from at least two independent runs.

2.2.2 Stress exposure device

To expose the cells to a well-defined hydrodynamic stress, two different experimental setups were used. The first one is conceptually similar to that used by the Chalmers group (Godoy-Silva et al., 2009a, 2009b; Hu et al., 2011; Mollet et al., 2007) and consists of a standard 3 L bioreactor described above which was equipped with an external loop driven by a contact-free centrifugal pump (BPS-200, Levitronix, Switzerland) as depicted in Figure 2.1.
By pumping the culture liquid through an external loop containing a nozzle an oscillating hydrodynamic stress similar to the one present in stirred bioreactors was generated, with the high stresses in the nozzle representing the region in the impeller vicinity and the lower stresses those in the bioreactor bulk zone. To generate various magnitudes of the hydrodynamic stress, nozzles of various diameters were mounted at the outlet of the centrifugal pump. A large inner diameter tube equal to 10 mm was used to minimize the effect of wall shear stress in the loop. The loop system was activated before inoculation so that the stress exposure was applied from the very beginning of the culture. Besides the hydrodynamic stress magnitude, the exposure period is another parameter which has to be properly controlled (Sieck et al., 2013). It can be estimated from the mixing times ($t_{\text{mix}}$) which are reported, for a 5000 L bioreactor in the order of 1 to 2 minutes, depending on the conditions (Xing et al., 2009). The measured $t_{\text{mix}}$ of an in house 5000 L bioreactor used for both cells at manufacturing scale was equal to 45 seconds, at culture operating conditions. When considering the circulation time ($t_c$) being 1/5 of $t_{\text{mix}}$ (Khang and Levenspiel, 1976; Nienow, 1997) a lower estimate of the cell exposure period would be 12 to 24 seconds. However, as it was shown by Soos et al. (2013), liquid is pumped through zones with largest values of the hydrodynamic stress with much higher periods, in the order of several minutes. Therefore, to approximate the oscillatory cell path in a large-scale bioreactor, an estimation of the exposure period equal to two times the mixing time was used, i.e. 90 seconds. Considering the working volume of the used bioreactor, i.e. 3L for all tested conditions the pump rotation speed was adjusted to deliver a constant flow rate of 2 L/min through the external loop (Table 2.1) resulting in exposure period of 90 seconds.

Although correlations to estimate the averaged hydrodynamic stress inside a vessel, as a function of the stirring speed are available in the literature, see for example Sánchez Pérez et al. (2006), they cannot be applied to evaluate this quantity in the used external loop.
Table 2.1: Maximum hydrodynamic stress characterization of the used loop system shown in Figure 2.1 through CFD calculations (see Appendix Figure 8.2 and Appendix text information) and using the shear sensitive particulate system described by (Villiger et al., 2014).

<table>
<thead>
<tr>
<th>$D_{\text{nozzle}}$ (mm)</th>
<th>$v_{\text{pump, agit}}$ (rpm)</th>
<th>$Q_{\text{loop}}$ (L/min)</th>
<th>$Re_{\text{nozzle}}$</th>
<th>$\tau_{\text{max}}^{\text{CFD}}$ (Pa)</th>
<th>$\sigma_{\text{max}}^{\text{CFD}}$ (m²/s³)</th>
<th>$\tau_{\text{max}}^{\text{EXP}}$ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>800</td>
<td>2</td>
<td>6769</td>
<td>17 / 1.6</td>
<td>417 / 21</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>900</td>
<td>2</td>
<td>10154</td>
<td>21 / 13</td>
<td>637 / 1250</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>1100</td>
<td>2</td>
<td>12184</td>
<td>24 / 22</td>
<td>832 / 1909</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>1300</td>
<td>2</td>
<td>15231</td>
<td>35 / 59</td>
<td>1769 / 3694</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>2400</td>
<td>2</td>
<td>20307</td>
<td>92 / 135</td>
<td>12222 / 11286</td>
<td>83</td>
</tr>
<tr>
<td>2.5</td>
<td>2800</td>
<td>2</td>
<td>24369</td>
<td></td>
<td></td>
<td>103</td>
</tr>
</tbody>
</table>

Therefore, to quantify the magnitude of the hydrodynamic stress in the external loop to which the cells were exposed, a detailed CFD characterization of the used pump and nozzle was performed. Detailed information on this can be found in the supplementary information.

Furthermore, the system was experimentally characterized by a shear sensitive particulate system (Villiger et al., 2014). The maximum hydrodynamic stress values present in the external loop obtained from both methods are summarized in Table 2.1. As can be seen, both methods result in very similar values of the maximum hydrodynamic stress covering a range from 15 Pa ($3.3 \times 10^2$ W/kg) to 103 Pa ($1.5 \times 10^4$ W/kg), which is similar to the values reported by the Chalmers group (Godoy-Silva et al., 2009a, 2009b; Hu et al., 2011; Mollet et al., 2007). Furthermore, under all investigated conditions the flow in the external loop was turbulent with $Re_{\text{nozzle}} = Q D / \nu A$ being always above 6800 (Table 2.1), with $Q$ referring to the liquid flow rate, $D$ and $A$ being the nozzle diameter and its cross sectional area and $\nu$ corresponding to the fluid kinematic viscosity.

To investigate the effect of the flow type a simple batch cultivation device generating a laminar extensional flow at the entrance of a sudden contraction was employed as well
(Tanzeglock et al., 2009). Cells were cultured as described above for 2 days without any feeding and exposed to the stress right after inoculation. The device consists of a gently agitated thermostated reservoir containing the cell culture connected through tubing to a syringe. To obtain the same exposure period as in the first system the speed of the syringe pump was adjusted such that under all conditions, the cells were exposed to the high hydrodynamic stress on average every 90 seconds. In between the syringe and the reservoir, nozzles of variable sizes were installed to generate different stress values (see Figure 2.2) ranging from 0.4 to 420 Pa.

![Simple shear device](image)

Figure 2.2: Simple shear device used to generate oscillating stresses. A syringe pump containing cell culture fluid is attached to a temperature-controlled vessel via a capillary. The capillary diameter is varied to generate various magnitudes of the hydrodynamic stress.

Similar to the previous setup also in this case cells were periodically exposed to various levels of the hydrodynamic stress. The magnitudes of the hydrodynamic stress were calculated by computational fluid dynamics (CFD) as discussed previously (Soos et al., 2010; Tanzeglock et al., 2009). A summary of the obtained values is reported in Table 2.2. As can be seen under all investigated conditions, the flow was laminar or transitional with $Re_{\text{nozzle}}$, being always below 1200. Thus, it was possible to compare the response of the studied cells to similar stress magnitudes using both laminar and turbulent conditions. It is worth noting that for both setups the exposure period can be easily changed and adapted to the mixing
conditions of any other sized bioreactor, simply by varying the flow rate generated by the pump. Therefore, using this setup it is possible to independently tune magnitude and period of the hydrodynamic stress, while covering a broad range of stresses as reported in the literature.

Table 2.2: Hydrodynamic stress characterization of the simple batch cultivation device (see Figure 2.2).

<table>
<thead>
<tr>
<th>$D_{\text{nozzle}}$ (mm)</th>
<th>$Q_{\text{nozzle}}$ (mL/min)</th>
<th>$V_{\text{culture}}$ (mL)</th>
<th>$Re_{\text{nozzle}}$ (-)</th>
<th>$\tau_{\text{CFD}}^{\text{max}}$ (Pa)</th>
<th>$\epsilon_{\text{CFD}}^{\text{max}}$ (m$^2$/s$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20</td>
<td>30</td>
<td>222</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
<td>30</td>
<td>887</td>
<td>40</td>
<td>921</td>
</tr>
<tr>
<td>0.5</td>
<td>27</td>
<td>40.5</td>
<td>1175</td>
<td>56</td>
<td>1795</td>
</tr>
<tr>
<td>0.3</td>
<td>10.6</td>
<td>15.9</td>
<td>784</td>
<td>109</td>
<td>6780</td>
</tr>
<tr>
<td>0.2</td>
<td>10.6</td>
<td>15.9</td>
<td>1175</td>
<td>420</td>
<td>101402</td>
</tr>
</tbody>
</table>

Compared to previous works (Godoy-Silva et al., 2009a, 2009b; Nienow et al., 2013) where stress exposure was applied during or after the exponential phase, in the present work the loop system was activated before inoculation so that the oscillating stress was applied from the very beginning of the culture. We believe that stress exposure needs to be applied throughout the whole culture, especially during the initial cultivation phase when the cells tend to be more sensitive to any change of the environment (Sieck et al., 2013). Additionally, compared to previous studies (Godoy-Silva et al., 2009a, 2009b) where the exposure period needed to be changed (11 up to 333 minutes) to apply various stress levels, in this work the exposure period, using both laminar and turbulent conditions, was kept constant allowing a better comparison among various experiments.

### 2.2.3 Offline Data Analysis

Daily samples were taken from all cultures to follow the growth behavior and the production and consumption of nutrients and metabolic products. The samples were used to monitor cell density and viability (Vi CELL, Beckman Coulter, USA), pH (Seven Multi,
Mettler-Toledo, Germany), pO$_2$ and pCO$_2$ (ABL5 blood gas analyzer, Radiometer, Switzerland), metabolites (Nova CRT, Nova Biomedical, USA), turbidity (Turb 550, WTW, Germany) and osmolality (Micro Osmometer, Advanced Instruments, USA). The amount of produced protein was analyzed by standard HPLC analysis using Protein A. Product quality was analyzed after Protein A capture by size exclusion chromatography for protein aggregates (Berridge et al., 2009) and glycosylation analysis was performed via gel electrophoresis (CGE-LIF) using the procedures published in the literature (Papac et al., 1998; Rapp et al., 2011).

Specific consumption and production rates were calculated according to the following equation (Adams et al., 2007):

$$ q_i = \frac{\Delta c_i \cdot 2}{\Delta t(X_t + X_{t+1})} \quad (2.2) $$

where $q_i$ is the specific metabolic rate, $\Delta c_i$ the concentration difference of the corresponding metabolite, $\Delta t$ the time difference in between two consecutive culture samples and $X$ the cell density. According to several published studies (Croughan and Wang, 1989; Lakhotia et al., 1992; Senger and Karim, 2003; Tanzeglock et al., 2009), the measure of DNA was used to quantify the amount of cells damaged due to necrosis. Therefore, the supernatant was analyzed with Quant-iT dsDNA High-sensitivity assay kit (Life Technologies, Switzerland) according to the instruction of the supplier.

2.3 Results

2.3.1 Effect of oscillating stress exposure on cell growth

Two different mammalian cell lines with significantly different processes were used to investigate a cell line specific response to elevated hydrodynamic stress. Both cell lines were cultivated in the 3L bioreactor equipped with the external loop that was used to expose cells
to various values of hydrodynamic stress. Nozzles of various diameters were applied to generate different magnitudes of the hydrodynamic stress ranging from 15 Pa to 103 Pa. The exposure period was set to 90 sec by applying the pumping speed through the loop equal to 2L/min. All data were compared to classical bioreactor cultivations without the external loop attached. Student’s t-test was used to identify differences between cultivations performed at different stress values. P-values above 0.1 were considered as equivalent to the control culture. P-values below 0.1 were considered as different (indicated with * in Figures) and values lower than 0.05 were considered as significantly different (indicated with ** in Figures).

Time evolution of the measured viable cell density, corresponding viability and product titer is presented in Figure 2.3a-f. The presented error bars represent two standard deviations calculated at least from two independent cultivations. In cases where no repetition of the sample was available the presented error bar represents the largest value measured for standard conditions at a given time point. As can be seen in Figure 2.3a, time evolution of the viable cell density is characterized by rather large variation leading to very comparable results when considering stresses lower than 83 Pa. However, when increasing the stress values above this level differences can be observed. Considering the fact that largest differences were observed at the end of the culture a statistical significance between various stresses was evaluated at the harvest time point. Calculated p-values with respect to the control conditions using Student’s t-test confirmed the difference of the cultures performed at the stress level of 83 Pa and 103 Pa resulting in p-values of 0.01 and 0.04, respectively. The other stress levels resulted in p-values above 0.3, i.e. no significant difference compared to the control conditions. From the viability data presented in
Figure 2.3b, it can be seen that for all conditions the most significant variation was observed on day 2.

Figure 2.3: Time profile of the viable cell density (a, d), cell viability (b, e) and product titer (c, f) measured for CHO cell line (a, b, c) and for Sp2/0 cell line (d, e, f). Symbols correspond to various magnitudes of the hydrodynamic stress introduced with a classical single vessel, 1.2 Pa (□, Sp2/0 control), 2.2 Pa (○, CHO control) or the oscillating hydrodynamic stress loop systems 15 Pa (▲), 21 Pa (△), 38 Pa (▽), 83 Pa (◇) and 103 Pa (◇). Error bars represent two standard deviations from at least 2 independent experiments. Error bars shown for Sp2/0 cells represent variation calculated for standard conditions.
Although cells recover after the viability drop a systematic reduced viability is present while increasing the hydrodynamic stress, with lowest viability measured for highest stresses (see Figure 2.3b). This was confirmed by p-values calculated on day 2, which resulted in values above 0.5 for stress below 83 Pa. In contrast p-values evaluated for hydrodynamic stress equal to 83 Pa and 103 Pa were equal to 0.1 and 0.02, again confirming statistical difference with respect to the standard cultivation conditions. The last quantity presented in Figure 2.3c is the time evolution of the measured product titer. Differences can be seen already from the time evolution and were further confirmed by Student’s t-test, with calculated p-values of 0.05 and 0.002 for stresses equal to 83 and 103 Pa, respectively. Lower stresses showed no statistical difference, with p-values above 0.15. It is worth noting that for all above mentioned parameters statistical difference obtained from Student’s t-test is in closed agreement with the comparison of error bars derived from two standard deviations which therefore can be used for direct comparison of the measured quantities.

Results measured for the other cell line (Sp2/0) are presented in Figure 2.3d to f. Since for elevated stresses no experimental repetitions were performed statistical analysis was not possible for this cell line. Instead, for better visual comparison error bars calculated from standard runs, corresponding to two standard deviations, were used for all other conditions. As can be seen, the qualitative response of these cells to elevated stress was similar to that measured for CHO cells even though Sp2/0 cells showed slightly reduced growth already at 15 Pa and a strong lethal response was observed for stress equal to 83 Pa (see Figure 2.3d). Moreover, a clear difference can be seen at the end of the culture, where for intermediate stress levels (15 or 21 Pa) viable cell density was almost 100% higher than
that measured for the standard conditions where no external loop was used. When comparing the profiles of cell viability measured for CHO and Sp2/0 cells shown in Figure 2.3b and e, respectively, it can be seen that in contrast to CHO cells only a small drop in cell viability was observed during the first day. The profile is characterized by an initially constant value of cell viability followed by a monotonic decline at the later stage of the culture (see Figure 2.3e). When higher stress values were applied, i.e. 38 Pa, the viability started to decay earlier, while in the case of 83 Pa the cell viability decayed immediately after inoculation. It is worth noting that an increased longevity with higher viability at the end of the culture was observed for data measured in the small-scale loop model applying stress values of 15 or 21 Pa. This is also reflected in the product amount, shown in Figure 2.3f with highest titers at the harvest. This indicates that the effect of stress could even positively enhance cell growth, viability and productivity, supporting the presented methodology with oscillating stress (Lakhotia et al., 1992; Senger and Karim, 2003).

Since the measurement of viability based on trypan blue staining determines only the viable and dead (necrotic) cells, which represent the total amount of cells used to calculate the culture viability, these values could be affected by the missing information of lysed cells. To overcome this limitation and to detect the total amount of lysed and necrotic cells the analysis of double stranded DNA was performed on the supernatant of the cultures (Tanzeglock et al., 2009). Due to differences in viable cell density between two studied processes the increase of DNA amount released between working day 0 and 2 was considered. Obtained results measured for different stress values are plotted in Figure 2.4.
Figure 2.4: Change in DNA concentration in culture supernatant for CHO (●) and Sp2/0 cells (□) exposed to oscillating stress form day 0 to day 2. Error bars represent two standard deviations (N = 2) and p-values are indicated with stars (* for p < 0.1, ** for p < 0.05). Error bars shown for Sp2/0 cells represent variation calculated for standard conditions.

It can be seen that higher values of DNA amount were measured for Sp2/0 cells compared to CHO cells. By increasing the stress level in both cases there in an increase in the DNA amount in the supernatant with a more than 2 fold increase for the Sp2/0 culture while for the CHO culture an approximately 5 fold increase was measured when applying highest stress values. Calculated p-values for the CHO cell line where 0.005 for the 83 Pa condition and 0.07 for the 103 Pa condition while for other stresses the p-values were above 0.1. When considering lower seeding cell density for Sp2/0 (approximately one order of magnitude with respect to CHO cell culture) measured higher amount of DNA indicates that Sp2/0 cell undergo substantial lysis. This would explain the difference in the viability profile shown in Figure 2.3b and e, where in the case of Sp2/0 cells initially no significant drop was observed. In this case weak cells were directly lysed without being detected as dead cells resulting in almost constant viability over the first three days of the culture. In the case of CHO cells, lysis was not that severe and therefore dead cells can be still detected resulting in a drop of cell viability. This is a further indication that Sp2/0 cells are more fragile compare to CHO cell. Presented results clearly show that, depending on the environment, the cells can react with increased growth, as seen for the CHO cells (Stress [Pa]

<table>
<thead>
<tr>
<th>Stress [Pa]</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA [mg/L]</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
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<td>0.8</td>
<td>1.0</td>
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</tr>
</tbody>
</table>

* for p < 0.1, ** for p < 0.05.
Figure 2.3a) or with decreased growth or even death, as observed for the Sp2/0 cells (Figure 2.3c). Similar observations made by others (Davies et al., 1986; Lakhotia et al., 1992) indicate that great care has to be taken when evaluating the response of cells to elevated stress.

### 2.3.2 Cell Metabolism, Productivity and Amount of cell aggregates

Cell culture samples were measured every day for glucose (GLC), lactate (LAC), glutamine (GLN), glutamate (GLU) and ammonia (NH4) as described in the materials and methods section. From the obtained values, specific rates were calculated according to Eq. (2.2). When considering 95% confidence interval it was found that no significant changes were observed for the different stress values applied (see Appendix Figure 8.8). Except the conditions when the culture was dying immediately after inoculation, similar effects of the hydrodynamic stress were observed on the amino acid metabolism (see Appendix Figure 8.9 and Figure 8.10) with all values lying within the experimental error (two standard deviations representing a 95% confidence interval) of the standard condition. This clearly indicates that for both cell lines their metabolism was unchanged even though crossing the threshold values for cell growth and productivity.

Another parameter which could be affected by the applied stress is the cell productivity. The specific productivity ($q_{Titer}$) measured for both cells as a function of time are plotted in Figure 2.5.
Figure 2.5: Time profile of the specific productivity for the CHO cells (a) and the Sp2/0 cells (b). Symbols correspond to various magnitudes of the hydrodynamic stress introduced with a classical single vessel, 1.2 Pa (□, Sp2/0 control), 2.2 Pa (○, CHO control) or the oscillating hydrodynamic stress loop systems 15 Pa (△), 21 Pa (△), 38 Pa (▽), 83 Pa (▷) and 103 Pa (◇). Error bars represent two standard deviations from at least 2 independent experiments. Error bars shown for Sp2/0 cells represent variation calculated for standard conditions.

As can be seen for the CHO cell line, similar $q_{Titer}$ were measured for stress values up to 38 Pa. Statistics revealed that the apparent decrease in $q_{Titer}$ at 83 and 103 Pa is significant with p-values below 0.1. In comparison, the specific productivity of the Sp2/0 cell line was similar for all conditions until day 6. After this point, a reduction of $q_{Titer}$ is observed for the control culture while almost constant $q_{Titer}$ (except on the last day) was measured for stress values below 38 Pa (see Figure 2.5b). This represents an increase of about 30 – 50 % with respect to the control culture. It is worth noting that for the highest stress value, no growth was observed (see
Figure 2.3c). As a result, no protein was produced, hence the absence of data in Figure 2.5b.

Furthermore, the cell size and the amount of cell aggregates, present during the culture, were measured. As can be seen from Figure 8.11a and c in the Appendix, cell size varies during the cultivation, increasing for CHO cells while an opposite trend was measured for Sp2/0 cells. Student’s t-test for the CHO cell cultivations with p-values of 0.08 for 21 Pa and 0.1 for 38 Pa confirmed the observed increase of the cell diameter. A significant statistical difference for reduced cell size, with p-values lower than 0.01, were found for values of 83 and 103 Pa. In the case of Sp2/0 there is small decrease of cell size for 38 Pa and a substantial reduction for stress equal to 83 Pa. Furthermore, from Figure 8.11b and d in the Appendix it can be seen that for both cell lines a substantial amount of cell aggregates is present during the culture, which is a phenomena observed previously by several authors (Borys and Papoutsakis, 1992; Renner et al., 1993). However, this amount becomes systematically lower when the applied stress increases, reaching almost no aggregates present at highest stress values.

### 2.3.3 Stress threshold determination

To determine the upper threshold for a viable and productive culture and to identify its cell line dependency, we further analyzed the available data. The obtained data are summarized for both cell lines in Figure 2.6 using the various parameters discussed above. In the case of statistical difference, i.e. p-values evaluated from Student’s t-test below 0.1 or 0.05, values were indicated with one or two starts. Stress values for the reactors without external loop system attached were estimated using the correlation developed by Villiger et al. (2012). It can be seen that in all cases a rather similar trends are obtained, which is characterized by a region where an increase of the hydrodynamic stress has no effect (horizontal lines in
Figure 2.6), and a region where the stress has a rather strong impact. Due to the limited number of experiments performed at high stress values trends of the presented quantities were obtained by a linear fit of the points showing statistical difference (p-value < 0.1 or non-overlapping error bars) plus the one before. The demarcation line represents the stress threshold value and characterizes a maximum operating range of the hydrodynamic stress for a given cell line. Interestingly, as can be seen from Figure 2.6, the threshold is dependent on the measured parameter. In particular, the initial decrease of the viability during the first 24 h (for detailed evaluation see Appendix Figure 8.7) is very similar for both cell lines resulting in a stress threshold around 25 Pa. Applying higher stress resulted in a large decay of the viability (see Figure 2.6a and e).

The results from the laminar shear device are shown in Figure 2.6a (crosses). As can be seen cell viability drops down at slightly higher values of the hydrodynamic stress compared to the loop operated under turbulent conditions, indicating different responses of the cells to the flow type. This is in agreement with Nienow (2006) who suggested to apply turbulent conditions already in the scale down models when aiming to simulate a large scale bioreactors (Nienow, 2006).
Figure 2.6: Hydrodynamic stress threshold determination (dashed lines) based on the viability drop during the first 24 h of the culture (a, e), the average size of the cells during exponential phase from day 1 to 4 (b, f), the specific DNA amount in the culture on day 4 (c, g) and the specific productivity of the cells during exponential phase from day 2 to 6 (d, h). Data obtained from the simple batch device operated under laminar conditions (×) is shown in (a), while fed-batch data is shown with open circles (O). Error bars represent two standard deviations from at least 2 independent experiments. Error bars shown for Sp2/0 cells represent the variation calculated for standard conditions. For the CHO cell line p-values were calculated for all data and visualized with stars (* for $p < 0.1$, ** for $p < 0.05$).
Similar observations were made when different devices have been used to measure the cell response, e.g. rheometer vs. contracting nozzle, since cells respond differently when exposed to steady simple shear or to extensional flow patterns (Tanzeglock et al., 2009).

The same methodology to determine the stress threshold value was applied for other parameters such as the cell size evaluated during exponential phase (Figure 2.6b, f), the specific DNA content in the supernatant calculated on day 4 (Figure 2.6c, g) and the specific productivity evaluated during exponential phase (Figure 2.6d, h). In these cases p-values were calculated for all points against corresponding control conditions and are shown in Figure 2.6 when they were smaller than 0.1. From these four process parameters an average value and a corresponding standard deviation was calculated per cell line, resulting in an average hydrodynamic stress threshold for the CHO cell line of 32.4±4.4 Pa and for the Sp2/0 of 25.2±2.4 Pa. As already indicated by the growth profiles and confirmed also by the results presented in Figure 2.6, the studied CHO cell line is more robust than the Sp2/0 cell line.

When producing a monoclonal antibody, the quality of the target molecule is even more important than the growth and metabolic profiles of the culture. Therefore, similarly to the cell behavior we investigated the critical quality attributes (CQA) such as the amount of protein aggregates and the glycosylation of the secreted proteins as a function of the applied stress. The fraction of aggregates was determined via size exclusion chromatography on three different days, during exponential phase, at peak cell density and during stationary phase. Although an increase of protein aggregates over time could be observed (see Appendix Figure 8.12a and b) the difference at a given day in between the applied conditions was negligible. The fusion protein produced by the CHO cells showed relatively high amounts of aggregates of about 18 %, while negligible amounts of protein aggregates (below 1 %) were measured
for the Sp2/0 cells. Such large amounts for a non-purified fusion protein are not surprising and agree with studies published in the literature (Rouiller et al., 2012; Shukla et al., 2007).

The percent change of the major glycoforms at the applied stress levels for both cell lines is shown in Figure 2.7.

![Figure 2.7: Comparison of the major glycoforms of the produced proteins, analyzed at different oscillating hydrodynamic stresses on day 10. Values were normalized with respect to the control bioreactor. Controls are shown as zero value with corresponding error bars, which represent two standard deviations (N = 2). Error bars shown for Sp2/0 cells represent the variation calculated for standard conditions. Control stress for the CHO culture (a) was 2.2 Pa, for the Sp2/0 culture (b) 1.2 Pa. Values were normalized to the control condition (3L bioreactor without external loop attached) and sorted in ascending order. The first bar is always zero and represents the control with the corresponding error bars obtained from at least two non-parallel independent cultivations and therefore corresponding to a 95% confidence interval. As can be seen from]
Figure 2.7a the variation of the various glycoforms is not significant since under all tested stresses the error bars overlap. In the case of the Sp2/0 cell line the variation is even less pronounced (see Figure 2.7b). Contrary to other reports (Godoy-Silva et al., 2009a) we can conclude that a change in the glycosylation pattern without a change in the growth behavior is very minimal, which is a finding similar to that recently reported by Nienow et al. (2013) indicating values below 5%.

2.4 Conclusion

In the present work, two production cell lines were exposed to oscillatory hydrodynamic stress of various magnitudes using two systems mimicking large scale maximum stress via laminar and turbulent flow. Both tested cell lines showed good resistance against hydrodynamic stress with threshold values equal to 32.4±4.4 Pa for the CHO derived cell line, while slightly lower values equal to 25.2±2.4 Pa were obtained for Sp2/0 derived cell line. Slightly higher value of the stress threshold determined for laminar flow indicates the necessity to apply turbulent conditions for the correct representation of a bioreactor. With stress close but beyond the threshold, the CHO cells reacted with better growth but reduced productivity. The Sp2/0 cells showed a decrease in growth when crossing the threshold but increased longevity in the end of the culture resulting in slightly higher productivity. The validation of the proposed method, comparing it to large-scale data will be part of future studies. The different behavior of the cells shows the necessity to investigate the stress threshold independently for each cell line. The applied procedure enables the prediction of the maximum operating range, where no adverse effect on productivity and product quality is observed, using laboratory-scale bioreactor equipment.

Quality by design needs the inclusion of scale, via scale independent variables, resulting in a design space that is not limited to one reactor type and size. We suggest the use of stress as one of these variables, because of its mechanistic connection to the cell response.
Inclusion of stressors resulting from heterogeneities in O\textsubscript{2} and CO\textsubscript{2} as well as pH fluctuations can complete such a scale independent design space.
Chapter 3

Oscillating hydrodynamic stress at pilot scale

3.1 Introduction

Bioprocess scale-up in cell culture industry is a task, still based on historical experience than scientific knowledge. Even though performed regularly, differences in process performance are commonly observed in-between scales which are mainly resulting from intrinsic differences between laboratory and production scales (Humphrey, 1998; Li et al., 2006; Mostafa and Gu, 2003; Xing et al., 2009; Yang et al., 2007). Classical scaling rules are applied by keeping various reactor characteristic such as power input or tip speed constant to decide on the applied operating parameters in larger scales (Glacken et al., 1983; Ju and Chase, 1992; Marks, 2003; Nienow, 2006). However, it is never possible to full fill all the criteria because they are partially contradicting (Schmidt, 2005) often resulting in a compromised solution. Further complication comes from non-unique determination of the maximum values of shear rate or hydrodynamic stress tolerated by cells.

Pioneering works in bubble free systems with classical single vessel bioreactors were conducted by Oh et al. (1989) reporting a agitation tip speed ($v_{tip}$) of 1.4 m/s, corresponding

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2 Original paper title: “Pilot scale verification of an oscillating hydrodynamic stress model used for mammalian cell culture”
to a power input of $1.9 \times 10^{-1}$ W/kg, to ensure no cell damage. In contrast, Leist et al. (1986) reports tip speeds of 3.5 m/s (1.5 W/kg) and Kunas and Papoutsakis (1990) of 2.6 m/s (2.7 W/kg) to be safe for cell culture, while Al-Rubeai et al. (1995) report $v_{\text{tip}}$ of 1.9 m/s ($3.1 \times 10^{-1}$ W/kg) to be above the cell damage threshold. This disagreement between various authors is further multiplied by the presence of bubbles. Under such conditions and without the presence of shear protective agents, like Pluronic F-68, critical tip speeds around 0.5 ($3.3 \times 10^{-2}$ W/kg) and maximum gas flow rates between 0.02 and 0.03 vvm have been reported (Cruz et al., 1998; Oh et al., 1992). Although addition of Pluronic can minimize the effect of bubbles (Chalmers and Bavarian, 1991; Jöbses et al., 1991; Ma et al., 2004; Murhammer and Goochee, 1990; Oh et al., 1992), it results in reduction of mass transfer (Murhammer and Pfalzgraf, 1992; Sieblist et al., 2013), therefore higher stirring speeds need to be applied to meet the oxygen requirements of current production cell lines. Furthermore, it might be impossible to reach the cell specific threshold using high agitation in classical single vessel bioreactors due to very robust cells, equipment limitations or vortex formation resulting in bubble entrapment and foam formation. Reactor runs without head space might be a solution but can be operational challenging. The change of environmental parameters like the substantially reduced exposure time and the increased mass transfer complicate the comparison with other scales. Moreover, the above mentioned approaches don’t address the heterogeneous nature of large scale reactors, i.e. spacial variations in stress (Soos et al., 2013), pH (Osman et al., 2002) or $O_2$ (Amanullah et al., 1993; Serrato et al., 2004).

To simulate oscillating hydrodynamic stress a laminar extensional flow device was developed by the group of Chalmers (Godoy-Silva et al., 2009a, 2009b; Ma et al., 2002; Mollet et al., 2007) using an external loop forcing the culture broth through a well-defined nozzle, exposing the cells to laminar stress values ranging from $1.2 \times 10^{-2}$ W/kg to $6.4 \times 10^{3}$ W/kg. However, the device is not capable to fully decouple exposure frequency and
exposure magnitude, limiting its applicability when aiming to mimic different large scale conditions. Additionally, as discussed by Nienow et al. (Nienow, 2006; Nienow et al., 2013) the application of laminar flow represent an intrinsic difference when comparing to the turbulent flow of manufacturing-scale bioreactors. Building on this argument Sieck et al. (2013) proposed a procedure to oscillate hydrodynamic stress in a turbulent operated classical single stirred bioreactor, by changing the agitation speed periodically according to the mixing conditions present in the manufacturing-scale. Although this approach gets closer to the reality it accepts the fact that due to perfect mixing cells are exposed to the high stress values at much higher frequencies than observed at manufacturing-scale. To employ turbulent flow and decoupling exposure period and hydrodynamic stress magnitude, Neunstoecklin et al. (2014a) recently proposed a scale-down model, where a 3 L bioreactor is equipped with an external loop capable of generating a broad range of maximum hydrodynamic stresses (15 Pa to 103 Pa). The proposed system was used to determine the stress threshold values for a Sp2/0 and a CHO cell line using a fed-batch cultivation process. The measured quantities, including growth, metabolism, product quantity and quality, agreed well with those measured in large scale bioreactors, thus supporting the proposed methodology. Despite this agreement the applied stresses in large scale bioreactors never exceed the determined threshold values so the true validity of the proposed method was never demonstrated beyond the edge of failure. Furthermore, no data of this kind for direct comparison exist in the open literature.

Therefore, the goal of this work is to provide such a validation, targeting stresses above the previously measured threshold value for the Sp2/0 cells (Neunstoecklin et al., 2014a) using a 300 L pilot scale bioreactor. In order to apply comparable operating conditions to those used in the scale-down model, the maximum hydrodynamic stress in the 300 L pilot scale bioreactor was characterized using a shear sensitive particulate system (Villiger et al., 2014). Measured growth behavior, metabolism, productivity and critical quality attributes
from the 300 L scale were compared to historical data from the oscillating hydrodynamic stress loop model.

3.2 Materials and Methods

3.2.1 Bioreactor scales and setup

Two different bioreactor sizes were used to culture the above mentioned cells. The development scale was a 3L bioreactor (Sartorius Stedim, Germany), followed by a pilot scale bioreactor with a working volume of 300 L (New MBR, Switzerland). All reactors were equipped with baffles and a combination of two impellers. One Rushton impeller mounted at the bottom of the vessel and an elephant ear impeller mounted above, inclined by 45° from horizontal plane pumping the liquid upwards. Geometric details of all scales are given in Figure 3.1. The basic reactor operating parameters are compared in Table 3.1. Calculation of the volume average energy dissipation rate for all scales was performed according to the following equation (Perry et al., 1997):

\[
\langle \varepsilon \rangle = \frac{N_p D^5 N^3}{V}
\]

(3.1)

where \( N_p \) is the power number of the impeller, \( D \) the impeller diameter in meter, \( N \) the agitation rate in 1/s and \( V \) the bioreactor volume in m³. Impeller Reynolds numbers \( Re_{imp} = ND^2 \delta / \mu \) are given to verify turbulent conditions.
Figure 3.1: Dimensions of bioreactors used in this study. Values refer to bioreactors with a volume equal to 3 L and 300 L (all values in cm).

Table 3.1: Applied operating conditions for the presented bioreactor cultivations.

<table>
<thead>
<tr>
<th>$V_{Bo}$ (L)</th>
<th>$N$ (rpm)</th>
<th>$D$ (m)</th>
<th>$v_{tip}$ (m/s)</th>
<th>$N_p$ (-)</th>
<th>$Re_{imp}$ (-)</th>
<th>$\langle \varepsilon \rangle$ (W/kg)</th>
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<tr>
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<td>1.806</td>
<td>3.8</td>
<td>189838</td>
<td>$1.27 \times 10^{-1}$</td>
</tr>
</tbody>
</table>
3.2.2 Hydrodynamic stress characterization

Maximum hydrodynamic stress values in the bioreactors used for this study were determined using a non-cellular based shear sensitive system (Villiger et al., 2014). It is based on the measurement of the maximum stable size of aggregates composed out of polymeric nanoparticles. When exposed to certain hydrodynamic stress these aggregates undergo breakup until steady state size is reached, which is controlled by the maximum stress present in the system (Soos et al., 2013). A detailed description of the method and the results are provided in the supplementary material.

3.2.3 Cell line and inoculum preparation

The cell line used in this study was derived from a Sp2/0 host cell line producing a commercialized monoclonal antibody. A Cell bank vial was directly thawed into complex proprietary media for the expansion of the cells. Density after thawing was $0.5 \pm 0.1 \times 10^6$ cells/mL and sub cultivation of the cells was performed every other day to keep the cell density below $1.5 \pm 0.1 \times 10^6$ cells/mL. Initially orbitally shaken T-flasks were used and after reaching an appropriate volume glass spinner flasks (Integra, Switzerland) were used to culture the cells at 37 °C with 90 % humidity. Spinner flasks were aerated through an open pipe sparger using air supplemented with 5 % CO$_2$. Duration of the expansion was always 29 days before cells were used for inoculation. Seeding was performed into a prefilled bioreactor using the same media, with a cell density of $0.3 \pm 0.1 \times 10^6$ cells/mL.

3.2.4 Cell cultivation and offline data analysis

In the 3L system the agitation rate was set to 110 rpm and the DO was controlled at 70 % air saturation with a constant air flow of 10 mL/min combined with oxygen on demand. The total gas flow rate during the whole culture never exceeded 40 mL/min, corresponding to 0.013 vvm. The overall power number for the two mounted impellers was assumed by the
addition of the individual ones, being 4.96 for the Rushton turbine (Sieblist et al., 2011b) and 1.7 for the up pumping marine impeller (Zhu et al., 2009). With a total $N_P$ being equal to 6.66 the average power input results in $\langle \varepsilon \rangle$ equal to $1.06 \times 10^{-2}$ W/kg. For the determination of the hydrodynamic stress threshold for the given cell line an external loop was attached to this reactor enabling the introduction of oscillating stress, ranging from 15 to 103 Pa. For more details readers should refer to our previous work (Neunstoecklin et al., 2014a). The 300 L bioreactor was controlled at an agitation speed of 71 rpm and a DO set point of 60 % air saturation. The latter was first controlled with air until a flow rate of 0.4 L/min was reached. After that oxygen was added to the gas stream on demand resulting in maximum total volumetric gas flow rates of 2 L/min, corresponding to 0.0067 vvm. At this scale $N_P$ was determined from CFD studies being equal to 3.8 giving an $\langle \varepsilon \rangle$ of $1.35 \times 10^{-2}$ W/kg. To expose the culture to stress values beyond the threshold, this reactor was furthermore operated at an agitation speed of 150 rpm corresponding to $\langle \varepsilon \rangle$ equal to $1.27 \times 10^{-1}$ W/kg with $\tau_{\text{max}}$ equal to 28 Pa. All other parameters were kept as mentioned before. The pH was controlled at all scales via CO$_2$ with a set point of 7.1 until day 3 when a step change down to 6.9 was applied. This value was kept constant for the rest of the process. A concentrated nutritional bolus feed was added when a cell density of $2 \times 10^6$ cells/mL was reached, usually occurring on day 2 or 3, containing a complex mixture of glucose, amino acids and proteins.

Daily samples were taken from all cultures to follow the growth behavior and the production and consumption of nutrients and metabolic products. The samples were used to monitor cell density and viability (Vi CELL, Beckman Coulter, USA), pH (Seven Multi, Mettler-Toledo, Germany), pO$_2$ and pCO$_2$ (ABL5 blood gas analyzer, Radiometer, Switzerland), metabolites (Nova CRT, Nova Biomedical, USA), turbidity (Turb 550, WTW, Germany) and osmolality (Micro Osmometer, Advanced Instruments, USA). The amount of
produced protein was analyzed by standard HPLC analysis using Protein A. Product quality was analyzed after Protein A capture by size exclusion chromatography for protein aggregates (Berridge et al., 2009). Glycosylation analysis was performed via gel electrophoresis (CGE-LIF) using the procedures published in the literature (Papac et al., 1998; Rapp et al., 2011). Charged variants were analyzed via capillary isoelectric focusing (cIEF) using an iCE280 system (ProteinSimple, USA).

Specific consumption and production rates were calculated according to the following equation:

\[
q_i = \frac{\Delta c_i \cdot 2}{\Delta t (X_t + X_{t+1})}
\]  

(3.2)

where \( q_i \) is the specific metabolic rate, \( \Delta c_i \) the concentration difference of the corresponding metabolite, \( \Delta t \) the time difference in between two consecutive culture samples and \( X \) the viable cell density (Adams et al., 2007).

### 3.3 Results

#### 3.3.1 Shear characterization of the used bioreactor systems

When using stirred and sparged bioreactors the cell culture broth is exposed to various sources of hydrodynamic stress originating from turbulent flow, gas jet present during bubble formation, bubble rise or bubble burst (Chisti, 2000; Cruz et al., 1998; Liu et al., 2013; Michaels et al., 1996; Oh et al., 1992; Zhu et al., 2008). To evaluate the relative contribution of stirring and sparging to the maximum hydrodynamic stress present in a 3 L and 300 L bioreactor, both were characterize by the shear sensitive system described above (Villiger et al., 2014). Effects from bubble burst were prevented by the use of Pluronic F-68 at same concentrations as used for subsequent culture experiments (Boulton-Stone and Blake, 1993;
Clincke et al., 2010; Jordan et al., 1994). For the 3 L bioreactor the obtained values measured for stirring, acting alone and its combination with sparging are presented in Figure 3.2a.

![Figure 3.2: Analysis of the maximum hydrodynamic stress, $\tau_{\text{max}}$, analyzed by the shear sensitive particulate system developed by Villiger et al. (2014). Two vessel sizes, 3 L (a) and 300 L (b), were investigated using various agitation speeds, gas flow rates and two types of spargers. Open circles ($\bigcirc$) correspond to the non-aerate single phase condition. The applied gas flow rates, for an open pipe sparger with a hole opening of 1 mm, were 0.02 vvm ($\blacktriangledown$), 0.03 vvm ($\blacksquare$) and 0.09 vvm ($\triangle$). In the 300 L scale additionally a 50 µm sparger using 0.03 vvm ($\blacklozenge$) was analyzed because it was used during cultivation. Arrows indicate the cultivation conditions used for the cell culture experiments.](image)

As can be seen in the case of the non-aerated system the maximum hydrodynamic stress ($\tau_{\text{max}}$) increases from 1 to 20 Pa with increasing impeller Reynolds numbers ($Re_{\text{imp}}$) covering a range from 7000 up to 50000. When gas bubbles are introduced into the bioreactor
in the presence of stirring it is shown that at low $Re_{imp}$ (around 10000) the level of the hydrodynamic stress is solely controlled by the applied gas flow rate. In particular, when low gas flow rates are used, corresponding to 0.02 vvm, the measured $\tau_{\text{max}}$ is only slightly higher than that measured for single phase. On the other hand, when the gas flow rate is increased to 0.09 vvm, stresses as high as 4 Pa can be measured over a rather broad range of $Re_{imp}$ up to about 18000 (see Figure 3.2a). By further increase of $Re_{imp}$ the effect of the gas flow rate becomes negligible and the measured $\tau_{\text{max}}$ for all conditions is equal to that measured for single phase. This indicates that depending on the applied $Re_{imp}$ two regimes can be observed, one dominated by the gas flow rate, while the second one is controlled by the stirring. In the gas dominated regime the different mechanisms, i.e. (1) bubble inlet gas velocity (inlet gas jet), (2) bubble coalescence and break up, (3) bubble rise and (4) bubble burst, acting alone or in combination, are responsible for higher stress values (Nienow, 2006; Oh et al., 1992; Villiger et al., 2014). The arrow in Figure 3.2a indicates the cultivation conditions used for all 3 L systems applying 0.013 vvm using a perforated pipe with 1 mm openings where the maximum stress is originating from stirring.

In a similar way also for the 300 L bioreactor $\tau_{\text{max}}$ was measured for various combinations of stirring and sparging. Furthermore, two types of ring spargers, located under the bottom impeller were investigated, equipped with either 8 sintered spargers (average pore diameter equal to 50 µm) or 8 nozzles with a diameter of 1 mm. The obtained results are present in Figure 3.2b. Similar to the 3 L bioreactor a monotonic increase of $\tau_{\text{max}}$ with $Re_{imp}$ was found for non-aerated circumstances. When considering intermediate gas flow rates, corresponding to 0.03 vvm, values of $\tau_{\text{max}}$ measured for the sinter sparger are very similar to those measured for single phase conditions, indicating negligible contribution of small bubbles. In contrast, when openings with 1 mm diameter were used, generating larger
Results

Chapter 3

bubbles, the level of $\tau_{\text{max}}$ is clearly visible for low $Re_{\text{imp}}$ being approximately 5 Pa with gas flow rates of 0.03 vvm and about 8 Pa at 0.09 vvm (see Figure 3.2b). By further increase of $Re_{\text{imp}}$ this effect becomes negligible indicating that at high $Re_{\text{imp}}$ the maximum hydrodynamic stress is dominated by the turbulence generated from the impeller. Arrows in Figure 3.2b indicate the cultivation conditions used applying 0.03 vvm and sintered spargers. Moreover, in all cultivations the effect of bubble burst was prevented, due to the use of Pluronic F-68, which is known to inhibit the attachment of cells to bubbles (Gigout et al., 2008; Handa-Corrigan et al., 1989; Meier et al., 1999; Trinh et al., 1994; Wu, 1995).

3.3.2 Proliferation behavior of the cells

Small-scale cultivations were performed at 110 rpm ($Re_{\text{imp}} \approx 10800$) corresponding to a $\tau_{\text{max}}$ equal to 1.7 Pa. Prior to this study the described cell line was characterized upon its hydrodynamic stress resistance using a specially designed shear loop system described earlier (Neunstoecklin et al., 2014a). This setup uses a simple loop connected to a fully controlled 3 L bioreactor using a contracting nozzle to introduce a well-defined hydrodynamic stress. A bearing less centrifugal pump is used to force the cell culture broth continuously through the nozzle. By tuning the rotation speed of the pump and selecting several nozzle sizes, the exposure period and stress magnitude can be varied independently to mimic the desired large scale situation. In Figure 3.3 the determination of the threshold as measured by Neunstoecklin et al. (2014a) is shown using the initial viability decrease of the culture (a), the specific titer (b), the maximum cell density (c) and the cell size during exponential phase (d). Depending on the quantity used for the threshold determination the result can vary. The overall average value for the here investigated Sp2/0 cell resulted in 25.2±2.4 Pa. It is worth noting that an alternative to the applied loop system, would be to use of a non-loop containing single bioreactor, operated under elevated stirring speed and thus resulting in higher stresses.
(Nienow et al., 2013). However, due to the robustness of the studied industrial cell line, the very high stirring speeds that would be required are above the maximum operating limit of the motor and therefore not reachable. Furthermore, even before reaching the maximum stirring speed formation of vortex was observed resulting in a non-desired secondary effect of bubble entrapment and consequent breakup (Chisti, 2000; Emery et al., 1995; Hua et al., 1993; Kunas and Papoutsakis, 1990). Finally, when using bench top bioreactor operated at elevated stirring speed an exposure period, by which cells would be exposed to highest values of the stress, becomes very short not being representative of the manufacturing conditions.

Figure 3.3: Threshold determination of the studied Sp2/0 cell line as described by Neunstoecklin et al. (2014a). Quantities used were the viability decrease during the initial 24 h of the culture (a), the specific titer in the exponential growth phase (b), the maximum cell density (c) and the cell size during exponential phase (d). Vertical dashed lines represent the threshold for the corresponding quantity. The overall average value results in 25.2±2.4 Pa. For comparison pilot scale data is plotted with solid symbols, 300 L at 71 rpm (●) and 300 L at 150 rpm (◆).
To show the validity of the proposed methodology the same cell line was cultivated at conditions below and above the threshold, using a 300 L pilot scale vessel. In order to compensate for run to run variability’s all data were aligned to the time point of the initial bolus feed, performed after reaching a cell density of $2 \times 10^6$ cells/mL, as described in the material and method section. In particular, cultivations were performed at 71 rpm ($Re_{imp} \approx 90000$) corresponding to a stress of 7 Pa being well below the threshold and at the maximum agitation speed possible for the given reactor which is 150 rpm ($Re_{imp} \approx 190000$) corresponding to 28 Pa (see Figure 3.2b). Vortex formation was not observed, due to the upwards pumping marine impeller. In Figure 3.4a the growth profiles of these cultures are compared with historical data obtained from either 3 L cultivations in classical single vessels, applying stress values of 1.7 Pa or 3 L systems equipped with an external loop applying various values of hydrodynamic stress. As can be seen the standard, low stress, 300 L condition closely follows the growth and viability profile of the 3 L system at 1.7 Pa (Figure 3.4a, b) with an exception on day five where the 300 L culture shows a higher cell density. With the overlapping standard errors at this point this can be interpreted as an experimental error. This aligned trend is furthermore valid for the evolution of the cell size and the cell aggregates in the culture (Figure 3.4c, d). Furthermore, results are very alike when comparing data from the 300 L run at 28 Pa with the 3 L oscillating shear model applying 21 Pa. For both, the maximum cell density is decreased from $5 \times 10^6$ cells/mL down to $4 \times 10^6$ cells/mL and the viability profile is first comparable with the standard but stays higher from day 7 on. Cell sizes appears to be not affected but the cellular aggregation rate is considerably reduced especially between day 2 and 7 verifying the higher hydrodynamic stress values present in both systems. Interestingly, when comparing to manufacturing-scale (data not shown), the characteristically higher viability values after day 7 are much better fitted with either the 3 L oscillating shear model or the pilot scale experiment with high agitation.
Figure 3.4: Comparison of the growth behavior (a, b) and the morphology (c, d) of the Sp2/0 cell line cultured at 3 L scale using maximum stress values of 1.7 Pa (▁), 21 Pa (△) and 38 Pa (▽), together with data measured in 300 L bioreactors using maximum stress values of 7 Pa (●) and 28 Pa (●). Error bars represent one standard deviation and are obtained from at least two independent cultivations.

3.3.3 Cell metabolism and productivity

Cell culture samples were measured every day for glucose (GLC), lactate (LAC), glutamine (GLN), glutamate (GLU) and ammonia (NH4) as described in the materials and methods section. From the obtained values, specific rates were calculated according to Eq. (2.2). As can be seen from Appendix Figure 8.14 no significant difference of specific rates was observed between the applied conditions. With Lactate being one of the most important metabolite stress indicators, usually used to interpret the wellbeing of the culture (Le et al., 2012; Li et al., 2010, 2012; Zagari et al., 2012) it can be seen in Figure 3.5a, that lactate concentrations are lower for cultures with higher stress values, accompanied with lower growth, resulting in specific rates that are very similar.
Figure 3.5: Lactate (a) and product titer (b) production for the discussed cultivations. Maximum stress values applied at 3 L were 1.7 Pa (▲), 21 Pa (▲) and 38 Pa (▽), while at 300 L they were 7 Pa (●) and 28 Pa (◆). Error bars represent one standard deviation and are obtained from at least two independent cultivations.

The specific productivity however shows a decreasing trend from values around 37 to 10 pg/cell/day, when conditions beyond the threshold of 25.2±2.4 Pa were applied (Figure 3.3b). This can also be seen in Figure 3.5b where the total amount of product at the day of harvest is lowest, for the 300 L experiment performed at 28 Pa. Interestingly, highest titers could be observed for the conditions applying 21 Pa in the 3 L system with about 15 % more final product than obtained for the control culture. This can be explained by the increased longevity of this culture with an approximately 10 % higher viability from day 6 on compared to the control. Similar findings are reported by Senger et al. (2003) who realized increased production of total recombinant tissue-type plasminogen activator protein when applying
moderate shear. These findings indicate the possibility, to increase productivity through hydrodynamic stress, as long as the critical threshold is not trespassed.

### 3.3.4 Effect on key product quality attributes

Several authors discussed the effect of laminar or turbulent flow on the behavior of mammalian cells in culture and their effect on the final product. Particular interest is given into the influence of such stressor onto the quality of the protein. Most reports conclude only minor impact on quality attributes like glycosylation and or charged variants as long as no change in the growth behavior and the basic metabolite can be observed (Neunstoecklin et al., 2014a; Nienow et al., 2013; Scott et al., 2012; Senger and Karim, 2003; Sieck et al., 2013). Only one study exists that claims to observe significant changes in the glycosylation at hydrodynamic conditions below the threshold effecting growth and metabolism (Godoy-Silva et al., 2009a). In this study, the effect of hydrodynamic stress on the measured quality attributes (nine major glycoforms) for sub lethal conditions is negligible (Figure 3.6a). It can be seen that for conditions below the threshold (black 1.2 Pa, forward slash 15 Pa and back slash 7.8 Pa), independent on the vessel size, the distributions are very similar. Values above 25 Pa (vertical line 38 Pa and horizontal line 28 Pa) show a shift towards more complex glyco structures as shown especially for the abundance of the bi-antennary form FA2G2aG2 (Figure 3.6a structure 8), being distinctly higher than that of the control. A very similar trend is visible for the charged variants profiles (Figure 3.6b). For stress values below the threshold only minor changes are observed, while increasing the stress above the threshold results in a distribution shift to the right, towards more alkaline forms. The distribution for both, the glycosylation and charged variants is dependent on the applied hydrodynamic stress beyond the threshold, and not on the reactor size. This becomes clear when comparing the patterns of the 3 L run at 15 Pa (forward slash) and the 300 L run at 7 Pa (back slash). Results in between the two are very similar.
Figure 3.6: Comparison of glycosylation (a) and charged variants (b) profiles at day 11 measured in 3 L and in 300 L bioreactors for various values of the maximum hydrodynamic stress. Error bars represent one standard deviation and are obtained from at least two independent cultivations.

The same applies to the 3 L run at 38 Pa (vertical line) and the 300 L run at 28 Pa (horizontal line). Generally spoken one can say that the response to the applied environmental condition, i.e. hydrodynamic stress, results in sub optimal growth and therefore in the same change in quality, which in reverse can be used as a fingerprint representing the operating conditions. In Appendix Figure 8.15 the glycosylation and charged variants profiles are again displayed during late exponential phase at day 5. Total values at this day are different but the overall trends are the same as discussed above. These results clearly indicate the strong dependency of quality on the growth behavior of the culture. Furthermore, the obtained data in the 300 L pilot scale reactor fully support the predictive capabilities of the earlier developed small-scale model (Neunstoecklin et al., 2014a) and indicate that maximum
hydrodynamic stress should be the scaling criteria of choice when agitation is considered as the main source of hydrodynamic stress.

3.4 Conclusion

Determination of a scale independent safe operating range by scale down models described in literature is limited, because available published models are not validated using pilot or manufacture scale conditions. In this work, a validation of an earlier developed oscillating hydrodynamic stress scale down model was pursued using a 300 L pilot scale bioreactor. Maximum hydrodynamic stress values at this scale were determined at various operating parameters with a shear sensitive particulate system based on the breakage of aggregates composed of polymer nanoparticles. With this knowledge the operating parameters could be adapted to the same conditions as previously simulated in the stress loop system. Using the measured hydrodynamic stress threshold of the investigated Sp2/0 cell line equal to 25 Pa the operating conditions for the pilot scale were set either well below (7 Pa) or above (28 Pa) this value. The obtained results were compared with historical data from either classical 3 L cultivations and with data obtained from the oscillating stress loop system. The growth behavior of the cells at high agitation was reduced but showed a better longevity expressed through a higher viability at the end of the cultivation. Very similar results were obtained from the oscillating hydrodynamic stress loop scale-down model applying comparable hydrodynamic conditions. Furthermore, the productivity and product quality were very much aligned throughout the scales when comparing similar stress values. A shift to more complex glycosylation forms as well as a shift to more basic charged variants could be observed with increasing stresses beyond the edge of failure being rather dependent on the hydrodynamic stress environment than on the actual reactor scale. The here presented data show for the first time a validation study for a scale down model using an edge of failure approach at pilot scale and proves the applicability and predictability of the discussed scale...
down model. This knowledge assures better confidence when applying it in a quality by design frame work, when determining the maximum agitation operating range of a process.
Chapter 4

Comparison to production scale data

4.1 Introduction

The interest in biopharmaceuticals rose significantly within the past decades resulting in a continuously growing market (Walsh, 2010). Especially monoclonal Antibodies (mAbs) are of great interest and importance to the industry considering their seemingly infinite therapeutic potential, which was discovered in the mid-1970s (Köhler and Milstein, 1975). Since then the number of diseases that can be cured or stopped from becoming worse such as rheumatoid Arthritis or cancer has grown continuously. Along with the high therapeutic potential of mAbs their high demand has forced the industry to develop faster and more yielding processes, higher titers with more productive cell lines using larger bioreactors (Jordan et al., 2013; Rouiller et al., 2013). At the moment bioreactors of 25000 L are the biggest in the industry for mammalian cell culture and titers of 5 g/L and more have been reported (Kim et al., 2012; Nienow, 2006; Varley and Birch, 1999).

The biggest challenge of such scales is the presence of in homogeneities which are hard to address during the development (Lara et al., 2006; Ozturk, 1996) but cannot be avoided when scaling bioprocesses to such scales. Hydrostatic pressure influences the gas solubility, slow mixing can generate suboptimal pH perturbations and nutritional gradients.
Furthermore, the fluid dynamics in-between scales vary substantially causing a different hydrodynamic environment for the cultured cells. Hydrodynamic stress is an often discussed parameter when scale up is performed, with the concern of fragile mammalian cells that could be harmed by the large scale environment. Several authors address this topic using scale down models of various forms to investigate the robustness of mammalian cells against hydrodynamic stress (Godoy-Silva et al., 2009b; Keane et al., 2003; Kunas and Papoutsakis, 1990; Neunstoecklin et al., 2014a, 2014b; Oh et al., 1989; Sieck et al., 2013; Tanzeglock et al., 2009). A rheometer producing uniform steady simple shear and a contracting nozzle device generating oscillating extensional flow was used by Tanzeglock et al. (2009) to investigate the robustness of CHO and HEK cells, concluding that laminar simple shear quickly induces necrosis while oscillating extensional flow at the same amplitude results in an apoptotic response of the cells. Other authors using a similar oscillating contracting nozzle approach, attached to a bioreactor, could show the response of CHO cells to very high stress values ($6.4 \times 10^6 \text{ W/m}^3$) including the influence on the product quality at sublethal conditions (Godoy-Silva et al., 2009a). This however is the only report showing stress as a modifier of product quality when growth is not effected. It appears more general that growth is influenced by hydrodynamic stress and as a consequence of this product quality is modified as reported by Scott et al. (2012) or Neunstoecklin et al. (2014a). The representation of such systems against large scale is however difficult and very scarce. As it was shown by Neunstoecklin et al. (2014b) the developed 3L scale down model can well represents a 300 L pilot scale bioreactor. According to our knowledge this is the only study where such comparison was shown, although, a comparison to manufacturing-scales, with several thousand liters of working volume, is still missing. Such a comparison using cultivation data from 5000 L and 15000 L is presented in this chapter. The stress values present in the large scale reactors were estimated using the correlation provided by Villiger et al. (2012).
4.2 Results

4.2.1 Growth and productivity in large scale compared to oscillating hydrodynamic stress

The two before discussed cell lines (CHO and Sp2/0) having significantly different processes were cultivated at manufacturing-scale with volumes of 5000 L and 15000 L. The stress thresholds were $32.4\pm4.4 \text{ Pa}$ for the CHO derived cell line, while a slightly lower value of $25.2\pm2.4 \text{ Pa}$ was found for the Sp2/0 derived cell line (Neunstoecklin et al., 2014a). The hydrodynamic stress generated by the different vessels used, applying the corresponding operating parameters, typical for the individual process, is compared in Table 4.1 and geometric details of the vessels can be found in Figure 4.1.

Table 4.1: Maximum stress present in the different bioreactor scales studied at the corresponding agitation rate, estimated according to Villiger et al. (2012).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>$V$ (L)</th>
<th>$N$ (rpm)</th>
<th>$v_{tip}$ (m/s)</th>
<th>$\tau_{exp}$ (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>3</td>
<td>150</td>
<td>0.471</td>
<td>2.2</td>
</tr>
<tr>
<td>CHO</td>
<td>5000</td>
<td>71</td>
<td>1.859</td>
<td>30.2</td>
</tr>
<tr>
<td>SP2/0</td>
<td>3</td>
<td>110</td>
<td>0.346</td>
<td>1.2</td>
</tr>
<tr>
<td>SP2/0</td>
<td>5000</td>
<td>42</td>
<td>1.100</td>
<td>11.0</td>
</tr>
<tr>
<td>SP2/0</td>
<td>15000</td>
<td>35</td>
<td>1.466</td>
<td>19.1</td>
</tr>
</tbody>
</table>

As can be seen in Figure 4.2a, no significant effect of the applied hydrodynamic stresses was measurable for the CHO cell line when stress levels below 83 Pa were applied. An even increased peak cell density compared to the control was observed at this stress level. Further increase to 103 Pa resulted in a substantially prolonged lag phase and peak cell
density was delayed by three days. Comparison of data obtained from 5000 L bioreactors indicated good agreement of cell density to conditions with a hydrodynamic stress of 21 Pa.

![Figure 4.1: Geometry of the studied bioreactors (all values in cm). Basic geometry of the 5000 L / 15000 L vessel used for the Sp2/0 culture. The CHO culture was only cultivated in the 5000 L scale.](image)

From the viability data presented in Figure 4.2b, it can be seen that for all conditions a drop in cell viability on day two is observed. Although cells recovered after the viability drop, a systematic trend is shown while increasing the hydrodynamic stress, with lowest viability measured for highest stresses (see Figure 4.2b). The observed differences in the cell viability profile during the first three days, with approximately constant values measured for the 5000 L bioreactor, were most probably related to the fact that for large-scale cultivations the inoculum preparation was already done in stirred tanks resulting in more resistant cells. In contrast, in the case of the presented scale-down model the inoculum was prepared in roller bottles with substantially lower stress values (Peter et al., 2006). Due to this difference, the cells’ response to elevated stress was probably more pronounced for the small-scale system compared to large-scale where no adaption was required.
Figure 4.2: Time evolution of the viable cell density (a, c) and cell viability (b, d) measured for CHO cell line (a, b) and for Sp2/0 cell line (c, d). Open symbols correspond to various magnitudes of the hydrodynamic stress introduced with a classical single vessels, 1.2 Pa (□, Sp2/0 control), 2.2 Pa (○, CHO control) or the oscillating hydrodynamic stress loop systems 15 Pa (◇), 21 Pa (△), 38 Pa (▽), 83 Pa (▷) and 103 Pa (◊). While close symbols refer to large-scale bioreactors: 5000 L (■) and 15000 L (●).

Results measured for the other cell line (Sp2/0) are presented in Figure 4.2c and d. As can be seen, the qualitative response of the cells to elevated stress was similar to that measured for CHO cells even though these cells showed slightly reduced growth already at 15 Pa and a strong lethal response was observed for stress equal to 83 Pa (see Figure 4.2c). Moreover, a clear difference can be seen at the end of the culture, where for intermediate stress levels (15 or 21 Pa) viable cell density was almost 100% higher than that measured for the standard conditions where no external loop was used. Profiles measured in 5000 L and 15000 L also had a higher cell density as well as cell viability, at the later stage of the cultivation in both large-scale bioreactors, closely agreeing with the data measured in the small-scale loop model applying stress values of 15 or 21 Pa (see Figure 4.2c and d).
Substantially lower values were measured for standard conditions using classical 3 L bioreactor without an external loop. This indicates that the effect of stress could even positively enhance cell growth and viability, supporting the presented methodology with oscillating stress (Lakhotia et al., 1992; Senger and Karim, 2003). Furthermore, these data also clearly indicate that the classical small-scale model, composed of a single stirred bioreactor, is not properly mimicking the environment which cells experience, considering manufacturing-scale bioreactors.

The specific productivity ($q_{Titer}$) measured for both cell types and all scales is plotted in Figure 4.3.

Figure 4.3: Time evolution of the specific productivity for the CHO cells (a) and the Sp2/0 cells (b). Open symbols correspond to various magnitudes of the hydrodynamic stress introduced with a classical single vessels, 1.2 Pa (□, Sp2/0 control), 2.2 Pa (○, CHO control) or the oscillating hydrodynamic stress loop systems 15 Pa (◁), 21 Pa (△), 38 Pa (▽), 83 Pa (▶) and 103 Pa (◇). While close symbols refer to large-scale bioreactors: 5000 L (●) and 15000 L (■).
As can be seen for the CHO cell line, similar $q_{\text{Titer}}$ were measured for stress values below 21 Pa, while further increase resulted in a systematic decrease of $q_{\text{Titer}}$. Similar to the cell growth, large-scale data from 5000 L bioreactor agrees well with the small-scale data when the stress level was below 38 Pa. A substantial deviation is observed when the applied stress in the small-scale system exceeded this value.

In comparison, the specific productivity of the Sp2/0 cell line was similar for all conditions until day 6. After this point, a reduction of $q_{\text{Titer}}$ is observed for the control culture while almost constant $q_{\text{Titer}}$ (except on the last day) was measured for stress values below 38 Pa (see Figure 4.3b). This represents an increase of about 30 – 50 % with respect to the control culture. When considering $q_{\text{Titer}}$ evaluated for the 5000 L bioreactor as shown in Figure 4.3b, the values closely agree with results obtained in the small-scale loop system with elevated stress. Data obtained from the 15000 L bioreactor agree within the experimental error with the other data measured at 3 L as well as in 5000 L bioreactors until day 6. The slightly lower specific productivity observed during the later stage can be explained by the higher cell density in the 15000 L after day six compared to the 5000 L data or the classical single reactor scale-down model (Figure 4.2c). A possible cause for this could be cell adaption to higher stress values present at the 15000 L scale, similar to the results observed in the small-scale loop model at elevated stress.

### 4.2.2 Large scale data comparison to individual stress thresholds

In Figure 4.4 the threshold determination for individual process quantities is shown as described by Neunstoecklin et al. (2014a). When comparing the data obtained from large-scale bioreactors, it can be seen that they closely agree with those measured in the small-scale system supporting the applicability of the presented method (see closed circles corresponding to the 5000 L scale and closed squares for the 15000 L scale in Figure 4.4).
Figure 4.4: Hydrodynamic stress thresholds (dashed lines) in comparison with manufacturing data. Shown is the viability drop during the first 24 h of the culture (a, e), the average size of the cells during exponential phase from day one to four (b, f), the specific DNA amount in the culture on day four (c, g) and the specific productivity of the cells during exponential phase from day two to six (d, h). Available large-scale data from 5000 L (●) and 15000 L (■) are shown for comparison.
Based on the obtained data, it can be seen that the chosen stirring speed for large-scale cultivations is always below the here determined threshold value (Figure 4.4), where no adverse effect on the cell performance was observed. Although the maximum hydrodynamic stress changes in between the scales, its obtained range is scale independent and therefore should be used as one of the design space parameters when applying a Quality by Design framework.

4.3 Conclusion

Comparison of available large scale data with results used before for the determination of the hydrodynamic stress threshold, for two different mammalian cell lines, could reveal the predictive strength of the oscillating loop scale down model. Better agreement with large-scale was obtained using this model indicating the limitation of commonly used classical single vessel models to study the effect of hydrodynamic stress. Especially the increased longevity of the Sp2/0 cell line, in the end of the culture, observed at both manufacturing-scales (5000 L and 15000 L), could be reproduced applying comparable oscillating stress with the discussed model. The good agreement across various scales qualifies this methodology to be applied during development for the determination of the maximum operating range for agitation.
Chapter 5

Simulation of large scale heterogeneity

5.1 Introduction

Therapeutic proteins such as monoclonal antibodies (mAb) need to be properly glycosylated to have a desired biological activity. Nowadays, this can be efficiently achieved using mammalian cells. To ensure optimal cellular growth conditions stirred bioreactors are used with volumes of several thousand liters. Despite the success when using these large vessels differences in cell behavior are often observed when comparing them to lab scale bioreactors. Reasons therefore are differences in mixing characteristics (Chisti, 2001; Nienow, 2006) and consequently inhomogeneous mass transfer of O₂ and CO₂ (Mostafa and Gu, 2003; Xing et al., 2008).

Of particular importance is the accumulation of dissolved carbon dioxide (dCO₂) in mammalian cell culture, an often observed phenomenon in large scale culture. Sieblist et al. (Sieblist et al., 2011a) discuss CO₂ accumulation in gas bubbles due to their increased residence time in the reactor, either through recirculation or due to the large scale liquid height, resulting in very low CO₂ removal rates and therefore the possibility of dCO₂

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3 Original paper title: “Simulation of the large scale specific heterogeneous environment for mammalian cell culture”
accumulation during culture. Due to these different environmental conditions in between several scales, the removal rates for CO$_2$ are much lower for large scale than for development scale. This difference becomes even more severe with continuously increasing cell densities often resulting in un-physiologic levels of dCO$_2$ being higher than 100 mmHg (Mostafa and Gu, 2003; Xing et al., 2008). Most approaches to investigate these conditions already at development scale, use different buffer conditions to change the dCO$_2$ level either throughout the whole process or at a distinct time point (Zhu et al., 2005). These fixed elevated set-points however do not represent the usually observed increasing dCO$_2$ profiles as described in literature for large scale bioreactors (Gray et al., 1996; Mostafa and Gu, 2003).

An increased concentration of dissolved gases at the bottom of a vessel, due to hydrostatic pressure, is another phenomena well documented in literature (Rhiel and Murhammer, 1995; Serrato et al., 2004; Xing et al., 2009). As a consequence cells will be periodically exposed to the various values of dissolved oxygen (DO). Although investigations of different set-points of DO at development scale is common practice (Heidemann et al., 1998; Jan et al., 1997; Kilburn and Webb, 1968; Li et al., 2006; Meilhoc et al., 1990; Restelli et al., 2006), the simulation of gas gradients, as they are seen by cells in a manufacturing-scale bioreactor, is so far only studied for bacterial cultures or insect cells. Two compartment models are here for used, circulating the cells through two different dissolved oxygen environments (Amanullah et al., 1993; Rhiel and Murhammer, 1995). For mammalian cells studies with simulated oscillatory DO conditions are very rear. Serrato et al. (2004) for example used a simple one compartment model with a step change of the DO set-point, periodically every 800 to 12800 sec.

In addition to the above mentioned variations of concentration of dissolved gasses, perturbations in pH can be observed, when pH is controlled via acid/base addition or when special feeds with high or low pH are used. This can further affect cell growth, metabolism,
productivity and most importantly the quality of the produced mAbs, such as glycosylation (Butler, 2006; del Val et al., 2010; Hossler et al., 2009; Kimura and Miller, 1996; Restelli et al., 2006; Scott et al., 2012; Zanghi et al., 1999).

To cover all above mentioned parameters and their interplay during a single cultivation, in this study a two zone scale down model was developed and tested. The used system is composed out of two interconnected stirred and sparged bioreactors allowing the periodic exposure to perturbations of DO and hydrodynamic stress, upon the studied industrial CHO cell line. Due to high pH of the applied feed, pH perturbations were introduced using a fed-batch cultivation. To mimic dCO₂ accumulation in the system comparable to that present in a large scale bioreactor, the CO₂ removal rate was controlled by the combination of gas flow rate and size of bubbles introduced into the system. The resulting decrease of the pH during accumulation situations was accepted to a certain extend because the applied process didn’t foresee base addition due to known adverse effects base can have on the lactate metabolism (Le et al., 2012). Obtained results are compared to the effect of single parameters.

5.2 Materials and Methods

5.2.1 Cell line and process description

Cells used in this study were CHO cells provided by Merck Serono producing an IgG1 antibody and were cultured in a fed batch process with chemically defined culture media and feeds. For inoculum preparation cells were thawed from a working cell bank vial, washed once with pre heated media and diluted with fresh media to a concentration of 0.3±0.1 × 10⁶ cells/mL. Cells were incubated in a shaking incubator (Kuhner AG, Switzerland) at 37 °C, 90 % humidity and 5 % CO₂ using orbitally shaken TubeSpin bioreactors (TPP, Switzerland) for 7 days, followed by vertically shaken roller bottles
(Corning, USA) for additional 7 days. Three sub cultivation steps with a dilution down to $0.3 \pm 0.1 \times 10^6$ cells/mL were performed every week to keep the cell density below $2.5 \times 10^6$ cells/mL. The orbital of the shaker was set to 25 mm, agitation for TubeSpin bioreactors was set to 320 rpm and for roller bottles to 130 rpm. Seeding into a 3 L bioreactor (DASGIP, Germany) prefilled with proprietary chemically defined production media was performed at a cell density equal to $0.2 \pm 0.1 \times 10^6$ cells/mL. The duration of the fed batch culture process was 7 or 14 days with three different feeds being Glucose, a chemically defined amino acid feed and two amino acids dissolved in 1 M NaOH. Glucose was fed daily from working day 3 on, to keep the concentration between 2 g/L and 4.5 g/L. The two other feeds were added on working days 3, 5, 7, and 10.

5.2.2 Bioreactor setups and control

Three different bioreactor configurations were used in this work. The first was a classical single vessel stirred tank reactor with working volume 3L. The second was a modified version of the first one equipped with an external shear loop to introduce oscillating hydrodynamic stress as described by Neunstoecklin et al. (2014a). The third was a further modification where two reactors were interconnected to introduce simultaneous oscillation of dissolved oxygen (DO) and hydrodynamic stress. In this setup a 3 L reactor was interconnected within a loop, with a 0.3 L reactor driven by a bearingless centrifugal pump (BPS-200, Levitronix, Switzerland) as depicted in Figure 5.1. The integration of the smaller bioreactor was implemented without headspace to avoid vortex formation and bubble entrapment. Both reactors were fully controllable, with the 3 L reactor representing the larger bulk region, the 0.3 L reactor corresponding to the region around the sparger of a large scale reactor and the pump together with a contracting nozzle corresponding to high stress zones near the impeller blades. Adjustment of the centrifugal pump speed allowed the adaption of the system to different circulation times. All reactors were equipped with online temperature,
pH and DO measurement probes (Mettler Toledo, Switzerland). In the 3 L vessels two pitched blade impellers both inclined by 30° from horizontal plane and pumping upwards operated at 250 rpm, were installed.

![Bioreactor dimensions of the two zone model employed for the simulation of large scale heterogeneity using a modified DASGIP vessel. All dimensions in mm.](image)

Figure 5.1: Bioreactor dimensions of the two zone model employed for the simulation of large scale heterogeneity using a modified DASGIP vessel. All dimensions in mm.

Power input of these impellers was measured at different agitation rates using a torque meter (Messtechnik Schaffhausen, Germany) and the Power number of the combined impellers was found to be constant at 1.6±0.2 for Reynolds numbers $Re_{imp}$ larger than 12000 (see Figure 8.16). According to Hudcova et al. (1989) the power number of individual impellers can be added to obtain the combined power number in a multi impeller setup. One of the here used 30° pitched blade impellers therefore would have a power number of 0.8 which is in agreement with the measured data from Shaw (1994) reporting 0.6 for a similar pitched blade. In the 0.3 L reactor a Rushton turbine operated at 200 rpm ($Re_{imp} \approx 9700$) was used for better mass transfer. In all cases temperature was controlled at 37 °C, DO at 50 % air saturation (if not stated differently) and pH control was achieved with a one sided PI
controller keeping the pH below 7.1 using CO₂ in the inlet gas stream. Due to lactate production a downwards drift of the pH was allowed which never exceeded values of lower than pH 6.75. Various aeration strategies were applied to change the CO₂ removal rate (CRR), using different sparger openings. Two different strategies were followed to keep the DO at the set point. The first one was based on O₂ on demand, where the O₂ gas flow rate was increased during the cell culture, while in the second approach a fixed total gas flow rate with an adjustable oxygen fraction was applied. The utilized gas flow rates according to the corresponding sparger are summarized in Table 5.1.

Table 5.1: Sparger sizes, their corresponding bubble sizes and volumetric gas flow rates used to influence the CO₂ removal rate ($d_b =$ bubble diameter, $d_{p, Sp} =$ pore size of the sparger, $Q_{gas} =$ volumetric gas flow rate, $V_{Liquid} =$ bioreactor liquid volume, $v_s =$ bubble rise velocity according to Clift et al. (1978)).

<table>
<thead>
<tr>
<th>$d_b$ [mm]</th>
<th>$d_{p, Sp}$ [µm]</th>
<th>$Q_{gas, min}$ [L/h]</th>
<th>$Q_{gas, max}$ [L/h]</th>
<th>$Q_{gas}/V_{Liquid}$ [vvm]</th>
<th>$v_s$ [m/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.61</td>
<td>1000</td>
<td>0.3</td>
<td>33</td>
<td>0.0018 - 0.183</td>
<td>7.12 × 10⁻⁴</td>
</tr>
<tr>
<td>0.43</td>
<td>0.5</td>
<td>0.3</td>
<td>1.2</td>
<td>0.0018 - 0.007</td>
<td>2.59 × 10⁻⁵</td>
</tr>
<tr>
<td>0.26</td>
<td>10</td>
<td>0.3</td>
<td>2</td>
<td>0.0018 - 0.011</td>
<td>4.32 × 10⁻⁵</td>
</tr>
</tbody>
</table>

5.2.3 Offline data analysis

During cultivation in the bioreactor, daily sampling was performed. Cell density and viability measurements were performed using the automated cell counter Cedex XS (Innovatis, Switzerland). Furthermore, values for cell diameter, average compactness and aggregation rate were obtained from the device. Measured values were used to calculate specific growth rates and doubling times using the following equations:

$$\mu = \frac{1}{X} \frac{dX}{dt}$$  (5.1)
\[ t_d = \frac{\ln 2}{\mu} \]  

(5.2)

where \( \mu \) is the specific growth rate, \( t_d \) the doubling time and \( X \) the viable cell density. Values for \( t_d \) in animal cell culture range from 15 to 60 hours depending on grow conditions and cell line (Adams et al., 2007). The here described CHO cells had an average doubling time of approximately 22 h. Basic metabolites were measured with a Super GL compact (Hitado, Germany) using enzymatic sensors for the detection of glucose and lactate. Titer determination was performed via standard HPLC techniques using a Protein A column (POROS® A20 Analytical HPLC column). Dissolved CO\(_2\) was measure right after sampling using a dCO\(_2\) dipping probe (PreSens, Germany).

**5.2.4 Bubble Size Analysis**

To better understand the influence of the sparger type on gas mass transfer and hydrodynamic stress a detailed bubble size analysis in the cultivation media was performed, for each employed sparger at different volumetric gas flow rates. Pictures were taken from the rising bubbles close to the sparger and analyzed using image analysis software (ImageJ 1.45s). To obtain a statistically relevant data set, for every condition more than 100 bubbles were analyzed. Preliminary experiments indicate, that in the whole range of the investigated conditions the produced bubbles can be well approximated as spherical and therefore the sphere equivalent diameter (SED) was used to characterize different sparger types. The SED is defined as:

\[ SED = \sqrt[4]{\frac{4A_b}{\pi}} \]  

(5.3)

where \( A_b \) is the area of the bubble in the image, determined by the image analysis software. This analysis was performed for several different spargers and flow rates as summarized in
Table 5.1. Furthermore, for every condition the rising time was estimated using the correlation given by (Clift et al., 1978).

### 5.2.5 Characterization of mass transfer rates for O₂ and CO₂

The apparent oxygen mass transfer coefficient \( k_{LCA_{O_{2}}} \) was first determined in plain culture media using Clark electrodes (Mettler Toledo, Switzerland) for several bubble sizes, aeration rates and surfactant conditions using the dynamic gassing in method, described by Bandyopadhyay and Humphrey (1967). During all experiments the headspace of the reactor was gassed with the same gas as used for the sparger, at a relative high flow rate of 1 L/min. The change in oxygen concentration over time is described as the \( k_{L}a \) multiplied with the oxygen gradient between liquid and gas phase:

\[
\frac{dC}{dt} = k_{L}a \left( C^*_{L} - C_{L} \right)
\]

where \( C^*_{L} \) is the oxygen concentration in liquid phase at saturation and \( C_{L} \) is the measured oxygen concentration. Assuming perfect mixing conditions and a constant \( C^*_{L} \) the integration of Eq. (5.4) for two discrete time points’ results in:

\[
\ln \left[ \frac{C^*_{L} - C_{L,0}}{C^*_{L} - C_{L,i}} \right] = k_{L}a \left( t_i - t_0 \right)
\]

where \( C_{L,0} \) is the oxygen concentration at \( t_0 \) and \( C_{L,i} \) the measured oxygen concentration at \( t_i \), respectively. The \( k_{L}a \) can be estimated from the slope of the curve, when plotting the logarithmic term on the left hand side of Eq. (5.5) as a function of time.

Due to concerns that this measurement is not fully representative for the culture conditions, were cells and surfactants are present, for selected conditions the \( k_{L}a \) was measured during the culture. In this case Eq. (5.4) needs to be extended with the oxygen uptake rate (OUR) of the cells resulting in:
\[
\frac{dC}{dt} = k_{l,a}(C_L^* - C_L) - qOUR \cdot X
\]  
\hspace{1cm} (5.6)

with \(qOUR\) being the specific oxygen uptake rate of the cells and \(X\) the viable cell density. Due to the fact that \(qOUR\) can vary over the culture time this value was determined each time immediately before the \(k_{l,a}\) measurement (Bandyopadhyay and Humphrey, 1967; Ducommun et al., 2000).

When assuming \(C_L\) to be the oxygen concentration of the bulk average dissolved oxygen (DO) and \(C_L^*\) can be represented by a mean value, Eq. (5.6) can be rearranged as follows:

\[
C_L = -\frac{1}{k_{l,a}} \left( \frac{dC}{dt} + qOUR \cdot X \right) + C_L^* \hspace{1cm} (5.7)
\]

Applying a linear regression of \(C_L\) as a function of the term in parenthesis, the \(-\frac{1}{k_{l,a}}\) is equal to the slope of the resulting linear fit while \(C_L^*\) being its intercept (Bandyopadhyay and Humphrey, 1967). Comparison of both approaches i.e. measurement in media with a given concentration of antifoam and the measurement in cultivation broth resulted in very similar results as can be seen from Appendix Figure 8.17b - d.

Dissolved carbon dioxide (dCO\(_2\)) was measured using a fluorescence online probe (PreSens, Germany) to determination the apparent mass transfer coefficient for carbon dioxide \((k_{l,a_{CO_2}})\) applying the same methodology as described above for dissolved oxygen.

5.3 Results

The heterogeneous environment in manufacturing-scales beyond several thousand liters is a well-known fact and discussed in literature for mammalian systems as well as for microbial systems (Bylund et al., 1998; Hewitt et al., 2000; Lara et al., 2006; Ozturk, 1996; Willoughby, 2006). For a realistic down scale model ideally all scale dependent parameters
need to be considered and applied simultaneously. Parameters such as hydrostatic pressure, influencing gas solubility, increased maximum stress and reduced mixing are regularly discussed. Emulating them at development scale is either not possible or only hard to achieve. Multi compartment models were proposed by several authors aiming to simulate the heterogeneous environment through plug flow loops or two compartments with different conditions. Furthermore, commonly those models focus only on one parameter like pH perturbation (Amanullah et al., 2001), O2 and nutrient gradients (Serrato et al., 2004) or hydrodynamic stress oscillations (Godoy-Silva et al., 2009a; Neunstoecklin et al., 2014a, 2014b; Palomares et al., 2010; Scott et al., 2012). However, a combined effect of multiple parameters using all of them, below the single parameter threshold value, on mammalian cell culture was not presented until now.

5.3.1 Simulation of elevated dissolved carbon dioxide profiles

To achieve various dCO2 accumulation profiles a combination of three spargers and various gas flow rates was used in this study. Examples of two conditions using a sinter sparger with an average pore size of 10 µm and open pipe sparger with seven openings of 1 mm are presented in Figure 5.2a together with the corresponding bubble size distribution. As can be seen from Figure 5.2a the 10 µm sparger generates very small bubbles with diameters ranging from 0.15 to 0.4 mm with a mean of 0.26 mm. In contrast, the 1 mm sparger generates bubbles covering a range from 2 to 9 mm with an average bubble size of 5.61 mm. For other conditions bubbles were in between the above mentioned sizes. Comparison of the different flow rates showed a slight shift to smaller bubbles for the 10 µm sparger and a slight shift to larger bubbles for the 1 mm sparger when applying higher gas flow. Comparison of the fitted distributions showed very similar results and therefore the further used mean bubble sizes were considered as flow rate independent for the applied flow rate ranges.
Figure 5.2: Bubble pictures of different spargers and flow rates. A porous sparger with an average porosity of 10µm (A) was employed plus a perforated pipe sparger, with seven openings of 1mm (B). Corresponding mean bubble sizes were 0.26 mm (A) and 5.61 mm (B) evaluated by photographic analysis. In row 1 each sparger is shown at a volumetric flow rate of 0.3 L/h and in row 2 each is shown with its maximum applied flow rate as stated in Table 5.1. The bubble size distribution of the porous sparger is compared in (a) at volumetric flow rates of 0.3 L/h (black) and 2 L/h (forward slash). The open pipe sparger is shown in (b) applying flow rates of 0.3 L/h (black) and 33 L/h (forward slash). Data were fitted with normal distribution curves and peak comparison showed only a minor influence of the applied volumetric gas flow rates on the average bubble size.
Furthermore, the operating conditions for gassing were selected avoiding any negative effect of hydrodynamic stress generated by the bubbles on cells. In particular, the entrance velocity or corresponding gas Reynolds jet were kept well below the critical values reported in the literature (Villiger et al., 2014; Zhu et al., 2008). To avoid high values of the hydrodynamic stress due to the bubble burst at the gas-liquid interface all cultivations were conducted using 2 g/L Pluronic F-68. As it was shown in our previous work (Villiger et al., 2014) as well as by other authors (Chalmers and Bavarian, 1991; Jöbses et al., 1991; Ma et al., 2004; Murhammer and Goochee, 1990; Oh et al., 1992) the addition of Pluronic F-68 at such concentration is sufficient to prevent cell entrapment by bubbles and therefore avoids their damage by bursting bubbles. A summary of the calculated bubble sizes obtained for different sparger types and flow rates is presented in Table 5.1.

Characterization of the apparent mass transfer coefficients for oxygen and carbon dioxide were conducted in culture media for standard culture conditions, applying 250 rpm agitation and gas flow rates from 0.3 L/h to 33 L/h, depending on the used sparger (see Table 5.1). It was found that the mass transfer coefficient is a strong function of the bubble size and volumetric gas flow rate and its value increases with bubble size reduction, while an increase of gas flow rate results in an increase of the $k_{L}a$ (see Figure 5.3 and Appendix Figure 8.17a).

To evaluate the effect of antifoam on the $k_{L}a$ additional measurements were conducted using various antifoam concentrations and in culture measurements at the end of the cultivation (see Appendix Figure 8.17b-d). The influence of the power input was not investigated because all cultivation experiments were conducted at the same stirring speed of 250 rpm. As can be seen from Figure 5.3 the apparent $k_{L}a_{\text{CO}_2}$ is lower for both investigated spargers than that for oxygen, a phenomena previously reported by Gray et al. (1996) who measured the apparent $k_{L}a$ for CO$_2$ about 95% smaller than the one for oxygen. Furthermore, as can be seen from Figure 5.3, the decay of the $k_{L}a_{\text{CO}_2}$ is much higher for the porous sparger ($d_b = 0.26\text{ mm} / d_{p,sp}$)
10 µm / △ than for the perforated pipe sparger (d_b 5.61 mm / d_p,sp 1000 µm / ○). The corresponding \( k_L a_{CO_2} \) for the perforated pipe sparger and the 10 µm porous sparger were equal to 1.53 h\(^{-1}\) and 0.28 h\(^{-1}\), respectively, even though for both conditions a \( k_L a_{O_2} \) was approximately equal to 5 h\(^{-1}\).

![Figure 5.3: Apparent mass transfer coefficients for oxygen (**k_L a_{O_2}** ) and carbon dioxide (**k_L a_{CO_2}** ) in culture media for two different spargers. Closed symbols represent the **k_L a_{O_2}** for oxygen, while open symbols show the **k_L a_{CO_2}** for carbon dioxide. Diamonds (△) are measurements from the 10 µm sparger, while circles (○) are from the 1 mm sparger. All lines represent power law fits to the corresponding data.](image)

A detail inspection of the flow of individual bubbles, revealed substantial recirculation of smallest bubbles following the liquid flow, while large bubbles were not dominated by the liquid and only rose upwards. This would have direct consequence on the CO_2 concentration in the bubbles, and therefore, reduced the removal rate of CO_2 (Sieblist et al., 2011a). As a consequence this results in a reduced apparent \( k_L a_{CO_2} \). This phenomenon was subsequently used to generate various pCO_2 profiles comparable to those reported in the literature form large scale bioreactors (Gray et al., 1996; Mostafa and Gu, 2003; Xing et al., 2009; Zhu et al., 2005).
5.3.2 Effect of elevated dCO₂ on cell cultivation

To simulate buildup of dCO₂ due to cell metabolism during the cell cultivation similar to that present in the large scale bioreactors, was in the presented work achieved by affecting the carbon dioxide removal rates (CRR). Various combinations of the used sparger and gas flow rates were used to achieve different maximum levels of dCO₂ during the cell culture. It is worth noting that this strategy does not require any modification of the cultivation media (e.g. buffer composition) or any predefined dCO₂ profiles which could be unknown for processes in development.

Figure 5.4 shows the results of five cultivations for various profiles of dCO₂ covering a range from 20 to 160 mmHg. For cultures aerated with large bubbles (\(d_b = 5.61\) mm) two different aeration strategies were followed which were either on demand with increasing oxygen gas flow up to 33 L/h or fixed airflow of 33 L/h with an increasing oxygen fraction (Figure 5.4d, □ and ○). The \(k_{l,a}\) and therefore the removal rate of CO₂ is a function of the gas flow rate (Mostafa and Gu, 2003) and it was expected that for the first aeration strategy the CO₂ removal will be less efficient than for the second. This can be seen in Figure 5.4c where the dCO₂ measurements show higher values for the on demand strategy (○) until approximately day 5 where the gas flow becomes equal to the fixed gas flow strategy (□) and therefore also the dCO₂ values become very similar. Independent on the applied strategy dCO₂ levels were well below 50 mmHg during the whole culture time resulting in good growth, with peak cell densities at day 7 about \(16 \times 10^6\) cells/mL, and similar growth rates. In the case of elevated dCO₂ values two porous spargers with average pore diameters of 0.5 and 10 microns, generating bubbles with sizes of 0.43 mm (◇) and 0.26 mm (▽) were used, resulting in peak dCO₂ concentrations of 110 mmHg and 160 mmHg, respectively (Figure 5.4c). For the first condition dCO₂ was constant at about 40 mmHg until day 3 and then rose linearly until day 6 to a value of 110 mmHg and stayed there until the end of the culture.
Figure 5.4: CHO cultured with various dCO₂ time evolution profiles generated using different bubble sizes and aeration strategies to change the corresponding carbon dioxide removal rates (see Figure 5.2 and Table 5.1). A perforated pipe sparger with a pore size of 1000 µm (\(d_b = 5.61\) mm) was used applying either an on demand aeration strategy (○) or a constant aeration (□) with 33 L/h (0.18 vvm). Elevated dCO₂ conditions were generated with two different porous spargers and gas flow rates. A 10 µm sparger (▽, \(d_b = 0.26\) mm) with a constant gas flow of 0.3 L/h generated highest dCO₂ levels up to 160 mmHg and a 0.5 µm sparger (◇, \(d_b = 0.43\) mm) with a constant gas flow of 1 L/h generated final dCO₂ levels of 110 mmHg. A rescue condition using the 0.5 µm sparger (△, \(d_b = 0.43\) mm) with 1 L/h gas flow through the sparger and additional 32 L/h of air through the headspace of the reactor resulted in a normal dCO₂ profile and growth. Graph (a) shows the viable cell density, (b) the accumulating product titer, (c) the evolution of dissolved CO₂ and (d) the applied gas flows. Error bars represent one standard deviation (\(N = 2\)).

The second condition started with a concentration of 30 mmHg and rose immediately to 80 mmHg at day 3 followed by a further increase to 160 mmHg on day 6. The last condition (△) represents an experiment where the sparger size was equal to 0.5 µm and to avoid buildup of dCO₂ the CRR was increased by an additional air flow of 32 L/h through the bioreactors head space. Herewith the dCO₂ profile could be recovered into a normal
physiological range and growth as well as productivity where restored (Figure 5.4a-c). The pH profiles of these experiments shown in Figure 5.5 are well correlated with the evolution of the dCO₂, with lowest pH values for experiments with highest dCO₂.

![Figure 5.5: pH profiles of the cultivations with elevated dCO₂. Symbols are the same as in Figure 5.4.](image)

With these results it is explicit that the decreased performance of the cultures is due to the dCO₂ accumulation, while other effects like stress due to bubble rise can be considered as not harmful for the applied conditions. The elevated dCO₂ environment has a significant influence on the growth rate and peak cell density of the cultures. When values of about 110 mmHg are reached growth is stopped and the cell density stays at the current value (Figure 5.4a) which in our case was around 10 × 10⁶ cells/mL. For even smaller bubbles this happens on day 4 and peak cell density reaches not more than 6 × 10⁶ cells/mL. This reduced amount of cells furthermore explicitly impacts the total productivity of the process with titers being half or three times smaller than those obtained from a process with a low dCO₂ profile (Figure 5.4b). These findings are well supported by literature where increased sparging lowers dCO₂ and increases the product titer (Mostafa and Gu, 2003). Replacement of bicarbonate buffer with MOPS-histidine buffer is reported to reduce dCO₂ concentrations resulting in increased growth and a corresponding titer being almost twice the one for conditions with dCO₂ accumulation (Goudar et al., 2007). The here presented method provides a quick and
easy approach to investigate the possible situation of manufacturing-scale without modification of the media composition and gives the ability to determine a critical value of dCO₂ for the given cell line and uncovers the key parameters needed to be considered for best CO₂ removal. Obtained results clearly indicates that larger bubbles are preferred as long as oxygen deliver can still be achieved and high overall gas flow rates have a great power to increase CO₂ removal as long as hydrodynamic stress can be omitted (Villiger et al., 2014).

5.3.3 Determination of the maximum hydrodynamic stress threshold

Before the investigation of the combined effect of several operating parameters on the cells behavior using the experimental setup shown in Figure 5.1 the effect of hydrodynamic stress was investigated in a separate experiment. Following the experimental procedure developed earlier by our group (Neunstoecklin et al., 2014a) the studied cell line was exposed to oscillatory hydrodynamic stress of various magnitudes using a 3 L bioreactor equipped with an external loop driven by a centrifugal pump. Several nozzle sizes right behind the pump enable the tuning of the maximum stress value, while the pump speed can adjust the exposure frequency. Conditions applied here were the same as described by Neunstoecklin et al. (2014a) with a liquid flow rate through the loop equal to 2 L/min, corresponding to an exposure period of cell to peak stress values equal to 90 seconds with maximum stress values ranging from 2.2 Pa to 103 Pa (Neunstoecklin et al., 2014a). Figure 5.6a compares the growth profiles of the cultures applying various values of hydrodynamic stress. It can be seen that for stresses up to 38 Pa the growth is very comparable to the standard conditions, while at 38 Pa the growth is even slightly increased with a better longevity at the end of the culture. With a stress value of 103 Pa a strong decrease of the viable cell density is observed, with a peak cell density being only half compared to the standard condition. The same trend was also visible in the productivity of the corresponding experiments (data not shown).
Figure 5.6: Stress threshold determination of the studied cell line using cell density and fraction of cell aggregates. In (a) the cell density is shown and in (c) the fraction of cell aggregates. The applied stress values applied were 2.2 Pa (○), 15 Pa (△), 38 Pa (▽) and 103 Pa (◊) using the shear loop system described by Neunstoecklin et al. (2014a). In (b) the integral viable cell density at day 7 (IVC) was used to determine the stress threshold, while in (d) the average aggregation fraction during stationary phase was used. Lines were obtained by linear regression of the first three points and the last two. The dashed lines represent the individual threshold for the investigated quantity. The average of the two was defined as the cell specific threshold with a value of 36±3.7 Pa.

The used CHO cells were very prone to aggregation especially during the end of the culture, with aggregation rates of up to 50 %, which can be seen in Figure 5.6c. With increased hydrodynamic stress it was expected that cells will be more singularized, which was confirmed by the conducted experiments. The time profile of the fraction of aggregates for the experiment applying 15 Pa is almost identical to the standard, while with 38 Pa a decrease of the aggregate fraction can be seen in the end of the culture. With 103 Pa a significant reduction is observed with an aggregate fraction never being higher than 12 %. For the determination of a stress threshold the integral viable cell density (IVC) at day 7 and the
aggregation rate during stationary phase was used. Both were plotted over the applied stress and due to the limited amount of data available linear regressions were applied to the first three and the last two points (see Figure 5.6) similar as described by Cruz et al. (1998). The intersection of the lines was defined as the threshold for the individual quantity (dashed line) and the average of these two as the threshold for the given cell line, resulting in a value of $36 \pm 3.7$ Pa.

### 5.3.4 The two zone model for oscillating oxygen conditions

Embossed from the historical anxiety of shear stress, very gentle agitation rates are usually applied at manufacturing-scale in mammalian cell culture. A direct impact of slow mixing is the pressure gradient along the vertical axes of the reactor resulting in different concentrations of dissolved gases (Manfredini et al., 1983; Oosterhuis and Kossen, 1984). When considering the standard operating conditions for mammalian cell culture in a 15000 L bioreactor with a liquid height of 3.4 m, using air as gas, Oosterhuis and Kossen (1984) reported gradients in DO covering ranges from 10 to 15%. Similar results for such a gradient in different liquids are also described by Manfredini et al. (1983) and Steel et al. (1966). Although a considerable amount of publications is available studying the effect of dissolved oxygen on mammalian cell culture (Li et al., 2006; Ozturk and Palsson, 1991, 1990; Restelli et al., 2006; Shi et al., 1993), mostly agreeing that DO values between 10% and 90% are suitable for cell cultivation, only few works exist that investigate the oscillation of dissolved oxygen. Serrato et al. (2004), being to our knowledge the only authors reporting results on oscillating oxygen concentration for mammalian cell culture, describe the effect of heterogeneous oxygen conditions on mAb N-glycosylation from hybridoma cells. By a periodic change of the DO set-point an oscillation was achieved between 17% and 3% with a minimum period (limited by the gas mass transfer) of 800 seconds. Due to the difficult controllability of dissolved oxygen at the low level (3%) because of the slow polarographic
Clark electrodes a hypoxic situation is very probable and could be the most reasonable explanation of the described results. In addition, the used oscillation period of 800 second is rather long. Therefore, to investigate the cell behavior to oscillating values of DO, in this study we use a two zone model (TZM) composed out of two interconnected bioreactors (see Figure 5.1). The recirculation of the culture broth between the two bioreactors is realized by a centrifugal pump. In this way cells will be exposed to the oxygen oscillation with a shorter period, which in this case was equal to 90 seconds. Furthermore, to avoid a possible hypoxic situation the oxygen tension was varied between 60 % and 40 % and compared to a control situation where both reactors were set to 50 %. Having characterized the effects of the above mentioned centrifugal pump the two zone model could be used with high confidence neglecting the effects introduce by the pump driving the system. In Figure 5.7 the online dissolved oxygen measurement is shown for the two interconnected reactors of the two zone model over the whole cultivation.

![online oxygen measurement](image)

**Figure 5.7**: Online dissolved oxygen signal from the two zone model during culture. Top line measured in the small compartment (0.3 L) and bottom line measured in the large compartment (3 L). The desired difference of 20 % DO between the compartments could be achieved and maintained throughout the whole culture.

It can be seen that the two different set-points could be maintained from the beginning throughout the whole time of the culture. The top line with 60 % DO represents the smaller reactor mainly aerated with pure oxygen. The lower line represents the larger reactor initially
aerated with nitrogen to remove the incoming oxygen rich liquid. With increasing culture time this gas was enriched with oxygen to compensate for the increasing oxygen demand generated by the growing culture.

The first experiments were conducted under the above described standard conditions using the 1 mm perforated pipe sparger in both vessels generating large bubbles. Flow rates were set to 33 L/h in the big vessel and to an on demand flow rate of pure oxygen (~0.1 L/h) in the small vessel. The pump flow rate for the recirculation was set to 1 L/h to increase the liquid residence time in the large vessel, which was needed to achieve the 20 % DO difference between the two vessels. A shear characterization using the above described shear sensitive system (Villiger et al., 2014) resulted in a maximum hydrodynamic stress of approximately 25 Pa, being well below the before determined threshold for this cell line. It is worth noting that the following experiments investigating oscillating oxygen conditions can be considered as combination experiments of oscillating stress and oxygen resulting from the stress introduced by the pump nozzle combination and the recirculation through the different environments of the two vessels. Due to the availability of two identical systems two experiments were conducted in parallel using one as a control with DO set-points set to 50 % in both vessels (indicated with solid up-triangles in Figure 5.8), while in the second system the DO set-points were set to 40 % in the large vessel and to 60 % in the small (indicated with open up-triangles in Figure 5.8). The difference between the two vessels was chosen as a worst case scenario based on the above mentioned literature, reporting DO gradients from 10 to 15 % in 15000 L vessels with a liquid height of 3.4 m (Manfredini et al., 1983; Oosterhuis and Kossen, 1984; Steel and Maxon, 1966). The results of both experiments are compared in Figure 5.8 with a standard cultivation in a simple single vessel bioreactor (open squares). It can be seen that growth, productivity, dCO2 evolution and lactate profile are very similar for
those conditions, concluding that the oscillating DO behavior between 40 and 60% in combination with the elevated hydrodynamic stress had no impact on the culture.

Figure 5.8: Cultivation results from the two zone model (▲, 1 mm sparger, \(d_b = 5.61 \text{ mm}, Q_{\text{gas}} = 33 \text{ L/h}, \text{DO} 50\% - 50\%\); △, 1 mm sparger, \(d_b = 5.61 \text{ mm}, Q_{\text{gas}} = 33 \text{ L/h}, \text{DO} 40\% - 60\%\); ▼, 10 µm sparger, \(d_b = 0.26 \text{ mm}, Q_{\text{gas}} = 0.3 \text{ L/h}, \text{DO} 50\% - 50\%\); ▽, 10 µm sparger, \(d_b = 0.26 \text{ mm}, Q_{\text{gas}} = 0.3 \text{ L/h}, \text{DO} 40\% - 60\%\)) in comparison with a classical 3 L cultivation (□, 1 mm sparger, \(d_b = 5.61 \text{ mm}, Q_{\text{gas}} = 33 \text{ L/h}, \text{DO} 50\%\)) and a cultivation with elevated dCO₂ (◇, 0.5 µm sparger, \(d_b = 0.43 \text{ mm}, Q_{\text{gas}} = 1 \text{ L/h}, \text{DO} 50\%\)). Shown is the viable cell density (a), the dissolved CO₂ (b), the product titer (c) and the lactate formation (d).

It is worth noting that the used device is not limited to the above mentioned DO gradient and could also be used to investigate other DO variations being lower or higher than the here mentioned. Furthermore, the possibility to achieve very small oscillation periods, being close to the circulation time of manufacturing-scales, represents an advantage compared to single vessel systems as describes by Serrato et al. (2004). In the last campaign combination of all investigated parameters was studied using the same conditions as above.
however combined with lower CRR applying smaller bubbles ($d_b = 0.26 \text{ mm}$) generated by a 10 µm sparger and reducing the gas flow rate to 1.3 L/h until day 5. To avoid further dCO$_2$ accumulation the gas flow was increased for about 15 % after day 5 to 1.5 L/h. Again in one TZM the DO set-point was set to 50 % for both vessels (solid down-triangles) and two different DO set-points equal to 40 % in the big vessel and to 60 % in the small (open down-triangles). With those conditions it was possible to increase the dCO$_2$ levels to values around 80 mmHg (Figure 5.8b) being higher than the control but still below the critical value of 100 mmHg as discussed above. Results show drastically reduced viable cell density (Figure 5.8a), a 50 % reduced final titer (Figure 5.8c) and a very different Lactate profile (Figure 5.8d). However the direct comparison between fixed and oscillating dissolved oxygen conditions showed no difference. For comparison in Figure 5.8 an elevated dCO$_2$ condition conducted in a classical single vessel bioreactor is shown (open diamond). As can be seen from Figure 5.8b the dCO$_2$ profile is analogous to the TZM condition and is even more elevated after day 5. Still the growth of this culture is better aligned with the control until day 5 (Figure 5.8a), shows a more expected Lactate profile (Figure 5.8d) and better productivity (Figure 5.8b). This data clearly indicates that combination of several parameters, in this particular case hydrodynamic stress and dCO$_2$ where both are below their individual threshold values, can lead to synergistically negative effects. This has direct implication on the design space of the process being narrower compared to that obtained using individual parameters.

5.4 Conclusion

The development and application of a two compartment scale down model, capable of investigating the combined effect of several process parameters was developed. The system composed of two stirred tank bioreactors of different volume interconnected by a loop allows the investigation of hydrodynamic stress, DO gradients, pCO$_2$ accumulation and pH perturbations on cell behavior. Applicability of this system was demonstrated using a
fed-batch process cultivating and industrial CHO cell covering ranges of stress from 2.2 to 103 Pa, DO oscillations from 40 to 60 %, pCO₂ accumulation up to 150 mmHg and pH perturbations between 6.7 and 7.4. It was found that application of hydrodynamic stress well below the determined CHO cell threshold (36±3.7 Pa) combined with pCO₂ accumulation up to 80 mmHg results in a significant reduction of cell growth and consequently also the amount of produced mAb. In contrast, elevated stress combined with DO oscillation between 40 and 60 % and or pH perturbations have no impact on cell growth and productivity. This can have a strong impact when determining a process design space using single parameters without considering the simultaneous action of several process parameters.
Chapter 6

Concluding remarks

A detailed study of multiple large scale specific heterogeneities has been studied in this work by the development of physical bioreactor based down scale models being capable of simulating (1) oscillating hydrodynamic shear, (2) increasing dissolved carbon dioxide over time, (3) feed based pH excursions and (4) oscillating dissolved oxygen. For these scale dependent parameters threshold values could be defined which are suggested to be used when up-scaling bioprocesses.

The first part of this work focused on the robustness of two different mammalian cells against oscillating hydrodynamic stress. A bioreactor based scale down model was developed being capable of simulating high and low hydrodynamic stress as it is common for stirred tank reactors. An external loop was connected to a 3 L development bioreactor driven by a centrifugal pump. Inside this loop right after the pump a contracting nozzles of various size could be installed to expose the cells, circulating through the loop, to a well-defined turbulent flow regime. The bioreactor was therefore considered as the bulk region of a manufacturing-scale reactor and the nozzle as the region around the impeller. Hence, the described system decouples the hydrodynamic stress magnitude (nozzle size) and frequency (recirculation flow rate) enabling the simulation of various bioreactor sizes by adapting those quantities to the ones present in the system of interest. Here the recirculation flow rate and the
resulting average exposure period, was based on the mixing time of a known manufacturing bioreactor and was set to 2 L/min, resulting in an exposure time of 90 seconds. A broad range of maximum hydrodynamic stress was applied ranging from 1.2 Pa up to 103 Pa to determine the cell specific threshold for two different cell lines. These thresholds were determined based on several quantities like growth, productivity, product amount and quality. Generally both cell lines were robust against the applied stress values with threshold values of 32.4±4.4 Pa for the CHO cell lines and 25.2±2.4 Pa for the Sp2/0 cell line.

To validate this system and to show its predictive capability, pilot scale experiments at 300 L scale were conducted below and above the before determined stress threshold. The determination of the maximum stress used for this analysis was conducted for the pilot scale bioreactor as well as for the loop system using a shear sensitive particulate system based on the aggregation and breakup behavior of PMMA particles. Therefore, a direct comparison of the two systems was possible. Results from the pilot scale runs were well in agreement with the expected hypothesis. At low hydrodynamic stress (7.8 Pa), introduced through normal agitation (71 rpm), pilot scale data follows the expected growth and metabolite profiles know from development scale below the threshold. The same is true for high stress (33 Pa) in pilot scale (150 rpm) which resulted in a reduced cell growth pattern, very similar to the one measured in the scale-down model equipped with the external loop. Comparison with large scale data from manufacturing-scales of 5000 L and 15000 L could further confirm these findings.

Heterogeneities in large scale are not only limited to hydrodynamic stress but include dissolved gas gradients derived from hydrostatic pressure. This can results in DO gradients and dCO₂ accumulation as observed previously in the literature. To include these effects into the before developed scale-down model, it was further developed into a two zone model consisting of two differently sized bioreactors interconnected with a loop. The constructed
two zone model consisted of a 3 L bioreactor, representing the manufacturing-scale bulk region and a 300 mL bioreactor representing the impeller vicinity. Both vessel had individual control for DO and pH and could therefore be operated at different set points. The application of the already before used centrifugal pump enabled the circulation of the culture broth through both environments while simultaneously the exposure to oscillating hydrodynamic stress was possible. To include the accumulation of dCO\textsubscript{2} the removal rate of the carbon dioxide was modified by changing the aeration bubble size and rate. With this technique various profiles of accumulating dCO\textsubscript{2} were achieved, with a peak value covering a range from 50 mmHg to 150 mmHg. To show the applicability of this system experiments were conducted where the culture was exposed to all above mentioned heterogeneities to identify their interaction onto the growth and productivity of the culture. It was found that the interaction of hydrodynamic stress and elevated dCO\textsubscript{2} has a negative impact on the culture although both values were not beyond the before determine thresholds. The oscillation of dissolved oxygen between 40 and 60 % was not found to be harmful for the culture, either as single parameter or in combination with elevated stress or dCO\textsubscript{2}. 
Chapter 7

Outlook

The special conditions present in large-scale bioreactors have been thoroughly investigated in this work and scale-down models have been developed to better understand the important criteria needed for scale-up and scale-down. It was possible to present a methodology to evaluate scale dependent parameters already at development scale to avoid suboptimal process conditions at manufacturing-scale. However, there are several interesting things where further investigation would be needed.

The generality of the presented systems should be validated using other cellular systems including different mammalian species’ and even yeast strains. In some cases surprising results were observed, like positive effects on cellular productivity caused by unusually high hydrodynamic stresses. A follow up on these observations could reveal a simple and easy tool for process optimization. The validity and predictability of the developed two zone model was not yet confirmed. Interesting would be, if a classical design of experiments based process optimization, varying several operating parameters, would give the same results when using either a classical single vessel reactor or the developed two zone model. Scaling these results to large scale should show the real benefits of the system. Miniaturization and atomization of such a system could have a great applicability for clone
screening, where the predictability of the right cell clone highly depends on the process conditions that ideally should be as close as possible to the final manufacturing scale.

For all experiments a more detail analysis on the molecular and genetic level could give a better understanding on the underlying mechanism for the observed results. Quantitative PCR could be used to look deeper into the protein pathways involved to break down the different responses of the cell culture on a molecular level. The application of nowadays affordable protein chip analysis would help to analyze the metabolic state of the cell and give the opportunity to develop metabolic network models, decoding the cellular behavior. Moreover, these chip analysis would give a characteristic fingerprint of the process, which could be used to validate batch to batch data. Different analysis methods facing multi variant procedures, versus the here mostly univariant approaches, surely would help to give better understanding on the interconnection of the variables. Moreover the detailed characterization of large-scale vessels using experimental and computational techniques would help to better understand the specific environments cells are exposed to, depending on the vessels scale.
Chapter 8

Appendix

8.1 A hydrodynamic stress scale down model

8.1.1 Hydrodynamic stress characterization

The fluid flow in the centrifugal pump as well as in the contracting nozzles was characterized by computational fluid dynamics (CFD) simulations using commercial software, ANSYS Fluent v12.1 (Ansys, 2009). Geometry of the centrifugal pump (see Appendix Figure 8.1) was imported from CAD drawing provided by the pump vendor (Levitronix, Switzerland) into the meshing software GAMBIT 2.4 (ANSYS Inc., USA). Computational domain, containing entrance tube, pump housing, pump rotor and outlet tube, was used for the simulations and it was meshed with a combination of hexahedral and tetrahedral elements. Rotation of the rotor with respect to the pump housing was simulated using the multiple reference frame model (Ansys, 2009). The turbulence inside the pump was modeled through a RNG $k$-$\varepsilon$ model (Ansys, 2009) combined with a standard wall function. Several refinement steps were performed to obtain a grid independent solution resulting in a final mesh size ranging from $3.2 \times 10^6$ up to $4.3 \times 10^6$ computational elements (condition dependent).
Particular attention was taken when refining the zone close to the rotor edges as well as to the narrow gap region in-between rotor and pump housing (see Appendix Figure 8.1). Simulations were running as steady state using COUPLED scheme to model Pressure-Velocity coupling and SECOND order spatial discretization scheme for pressure, momentum, turbulent kinetic energy and dissipation rate. Properties of the fluid were those measured for
media at 37 °C with a density of 1010 kg/m³ and dynamic viscosity of 0.6974 mPa.s. Solution convergence was monitored by calculating the torque acting on the pump rotor as well as the magnitude of the residuals for solved quantities. Once convergence was reached calculations were made for pump-averaged values as well as the evaluation for local quantities. Calculated torque was compared with values provided by the pump vendor which agree within 10% error, supporting the applied approach. Furthermore, validation of the applied computational approach in terms of pressure drop-flow rate curves can be found in Blaschczok et al. (Blaschczok et al., 2013)

Since this pump was used to pump the cell culture through the external loop, values of the stress to which cells were exposed were calculated using an approach develop previously by Soos et al. (2010) and Tanzeglock et al. (2009). Briefly, in the case of the mean velocity gradient the corresponding stress can be calculated according to Blaser (1998) and Soos et al. (2010):

\[
\tau_L = \frac{\mu}{2} \alpha_L \tag{7.1}
\]

with \( \alpha_L \) being the maximum positive eigenvalue of the mean velocity rate of strain (Bird et al., 2002; Pope, 2000).

In the case of turbulent flow the stress originating from the turbulent fluctuations could become dominant. Its magnitude depends on the relative size of a cell with respect to the smallest eddies present in the flow which are characterized by Kolmogorov micro scale, \( \eta_K = (v^3/\varepsilon)^{1/4} \) where \( \varepsilon \) is the energy dissipation rate of a turbulent flow. Below this length scale the flow is approximately linear and accordingly, the hydrodynamic stress due to local turbulent fluctuations, \( \tau_{vs} \), is approximated through Eq. (7.1) where \( \alpha_L \) is substituted for its turbulent counterpart \( \alpha_T = \sqrt{\varepsilon/(6v)} \) (for more details see Soos et al. (2010)). This results in:
On the other hand, when the cell size, \( d_{\text{cell}} \), is larger than \( \eta_k \), i.e., when the cell is in the inertial sub range of turbulence, the hydrodynamic stress to which the cell is exposed to, results from the difference in the pressure on opposite locations of the cell (Hinze, 1955). Under these conditions the hydrodynamic stress due to turbulent fluctuations equates to:

\[
\tau_{\text{IS}} \approx \rho \left( \varepsilon d_{\text{cell}} \right)^{\frac{3}{\alpha}}
\]

Evaluating all mentioned stress values it was found that \( \tau_{\text{VS}} \) was reaching the highest values (see Appendix Figure 8.2). Due to a complicated flow pattern in the pump, cells can follow different trajectories. To take this into account several hundred trajectories were analyses and the maximum value of \( \tau_{\text{VS}} \) was extracted from these trajectories (see Appendix Figure 8.3). It was found that cells are exposed to high values of the hydrodynamic stress over very short period of time. When comparing various rotation speeds of the rotor corresponding to various nozzle diameters, it was found that normalized stress distributions are rather similar and cover approximately 1.5 orders of magnitude (see Appendix Figure 8.3c). A summary of maximum values of the hydrodynamic stress (\( \tau_{\text{VS}} \)) calculated for various rotation speeds is presented in Table 2.1 of the main text.
Figure 8.2: Comparison of the various values of the hydrodynamic stress present in the centrifugal pump. Pictures (a), (b) and (c) were generated using Eqs. (7.1), (7.2) and (7.3) respectively.
Figure 8.3: Example of the several hundred trajectories of massless particles used to evaluate the maximum hydrodynamic stress present during a single pass through the pump (a) together with a variation of the hydrodynamic stress magnitude along a single trajectory (b). Pump rotation speed was equal to 900 rpm in the simulations presented in (a) and (b). Comparison of the normalized maximum hydrodynamic stress, with respect to the pump averaged value, evaluated for several conditions, corresponding to various nozzle diameters, is given in Table 2.1 in the main text.
A similar approach was applied also for the contracting nozzle (see Appendix Figure 8.4). In particular, due to the axial symmetry of the nozzle and because the flow was turbulent over the whole range of the studied conditions (see Table 2.1), 2D axisymmetric conditions were used for the simulations.

Turbulence was modeled by employing RNG $k$-$\varepsilon$ model (Ansys, 2006) combined with enhanced wall treatment at the wall proximity. To properly resolve gradients of pressure, velocity as well as turbulent quantities the narrowest part of the nozzle was meshed with 50 computational nodes in radial direction, while 60 computational nodes were used in axial direction. Furthermore, the section upstream and downstream was included in the
computational domain. Comparable sizes of the nodes were used in the nozzle vicinity followed by the mesh expansion using a factor of 1.05 resulting in a total mesh size around 21000 elements. Also in this case COUPLED scheme was used for pressure-velocity coupling and combined with SECOND order spatial discretization for the pressure, momentum, turbulent kinetic energy and its dissipation rate (Ansys, 2006). After simulation convergence, i.e. when residuals of all modeled quantities reached $10^{-5}$, hydrodynamic stress along several trajectories was analyzed. Examples of three trajectories are presented in Appendix Figure 8.5.

![Diagram of trajectories](image)

Figure 8.5: Example of trajectories used to evaluate the level of hydrodynamic stress to which cells will be exposed to. Values are calculated from equations (7.1), (7.2) and (7.3) for two trajectories, one starting at the nozzle axis (number 3 in panel a) while the other one starts closet to the wall (number 1 in panel a).

As can be seen there is substantial variation of the hydrodynamic stress at the nozzle entrance as well as its exit, however as can be seen from Appendix Figure 8.5b highest values of the hydrodynamic stress are due to the mean velocity gradient and are located near the wall at the nozzle entrance. Taking into consideration our previous work (Harshe et al., 2011; Soos et al., 2010) and the fact that the complete bioreactor volume will pass under the investigated
conditions, the nozzle approximately 1000 times over the cell doubling period, it is assumed that every cell will experience several times the zone with highest stress. Therefore, this value should be used when evaluating the effect of the hydrodynamic stress on the cell culture. Comparing the distribution of the maximum hydrodynamic stress, evaluated using the above described trajectory analysis and presented in Appendix Figure 8.6, it can be seen that the narrower distribution of the stress maxima is obtained in the pump compared to the nozzle.

Figure 8.6: Comparison of the distributions of the hydrodynamic stress calculated for both pump and nozzle applying a nozzle diameter of 6 mm and a pump speed of 900 rpm.

However, in both cases the maximum values present in the whole system are very comparable. A summary of such maximum values calculated for various nozzle diameters using a constant flow rate of 2 L/min is presented in Table 2.1.

To validate such obtained stress values in parallel to the CFD characterization the maximum hydrodynamic stress present in the whole system (i.e. bioreactor combined with an external loop) was measured also experimentally using a shear sensitive particulate system (Villiger et al., 2014). In a typical breakage experiment, the initial suspension of PMMA nanoparticles was aggregated in a 2 L beaker with a latex mass fraction, $\phi$, of $1 \times 10^{-2}$. A 0.2 mol/L sodium chloride solution, well above the critical coagulation concentration (CCC), and a 40 mm pitch blade impeller operated at 50 rpm were used for the initial aggregation of primary PMMA nanoparticles. As soon as the strength of the growing clusters is smaller than
the applied stress, i.e. when their size becomes large enough (Bäbler et al., 2010; Ehrl et al., 2010), the aggregates break leading to the formation of aggregate cluster fragments. Subsequently, these fragments can re-aggregate until a dynamic equilibrium between aggregation and breakage is reached, resulting in a steady state aggregate size (Moussa et al., 2007).

The initial aggregates were diluted in a studied system (bioreactor alone or bioreactor equipped with loops system) filled with deionized water resulting in a final particle mass fraction of $5 \times 10^{-3}$. This high dilution factor of 200 reduces the salt concentration to 1 mM, preventing any significant re-aggregation as it is well below the CCC of the nanoparticles. Consequently, breakage becomes the only mechanism controlling the aggregate size. As soon as the hydrodynamic stress exceeds the aggregates’ strength, they will break until a new steady state size is reached (Kusters, 1991; Soos et al., 2013).

Since bursting bubbles at the air-liquid interface can generate very high hydrodynamic stress (Boulton-Stone and Blake, 1993; Garcia-Briones et al., 1994; Ma et al., 2004), surfactant (0.5 g/l of Pluronic® F-68) was added to prevent the attachment of aggregates to rising bubbles (Ma et al., 2004; Mollet et al., 2004).

Once steady state cluster size was reached, an off-line sample of 25 mL was gently withdrawn and measured by small angle light scattering (SALS). This procedure was repeated for nozzles of various sizes combined with appropriate pump speed to ensure flow rate through the nozzle equal to 2 L/min. To ensure that no restructuring occurs during breakage experiments the fractal dimension of the aggregates, $d_f$, was determined from light scattering data as discussed in the previous work (Ehrl et al., 2008; Soos et al., 2008).

Following an experimental procedure developed earlier (Soos et al., 2010), the relation between the steady state aggregate size and the applied maximum hydrodynamic stress was constructed by breaking the initial aggregates in contracting nozzles of various diameters and
applying different flow rates. The aggregate suspension was pumped several hundred times through the nozzle in order to guarantee complete aggregate breakage (Harshe et al., 2011; Soos et al., 2010). The evolution of the CMD was monitored by small-angle light scattering and the steady state aggregate size was correlated to the maximum hydrodynamic stress calculated from direct numerical simulations (Soos et al., 2013).

For more details we refer to the original papers where this method as well as its application to single phase and multiphase flow was applied (Soos et al., 2013; Villiger et al., 2014). Comparison of both methods is presented in Table 2.1 of the main text. As it can be seen both approaches, CFD as well as shear sensitive particulate system, are resulting in very comparable values of the maximum hydrodynamic stress supporting the CFD characterization discussed above.
Figure 8.7: Viability decay over 24 h for CHO cells. In (a) results from the simple shear device as described in the material and methods section. Applied stress values calculated via CFD where 0.4 Pa (●), 40 Pa (▲), 56 Pa (▼), 109 Pa (●) and 420 Pa (▲) – also see Table 2.2. The same methodology was used for the bioreactor runs equipped with external loop and results are shown in (b). Regression was performed over the first 24 h, the points at working day 2 show the adaption of the cells to the hydrodynamic stress. Stress values are derived from the system characterization describe in the material and method section and were 2.2 Pa (○), 15 Pa (◇), 21 Pa (△), 28 Pa (◇), 38 Pa (▼), 83 Pa (◇) and 103 Pa (◇). Error bars represent two standard deviations from at least 2 independent experiments.
Figure 8.8: Time evolution of specific consumption and production rates of glucose (GLC), lactate (LAC), glutamine (GLN), glutamate (GLU) and ammonia (NH4) during the fed-batch cultivation measured for CHO cells (a – e) and Sp2/0 cells (f – j). Symbols correspond to various magnitudes of the hydrodynamic stress introduced with a classical single vessels, 1.2 Pa (□, Sp2/0 control), 2.2 Pa (○, CHO control) or the oscillating hydrodynamic stress loop systems 15 Pa (△), 21 Pa (▲), 38 Pa (▽), 83 Pa (◇) and 103 Pa (◇). Error bars represent two standard deviations from at least 2 independent runs.
Figure 8.9: Amino acid profiles from CHO culture. Meaning of the symbols is the same as in Figure 2.3 or Appendix Figure 8.8 and error bars represent two standard deviations ($N = 2$).
Figure 8.10: Amino acid profiles from SP2/0 culture. Meaning of the symbols is the same as in Figure 2.3 or Appendix Figure 8.8 and error bars represent two standard deviations (N = 2).
Figure 8.11: Cell size over culture time and aggregation behavior as a function of various hydrodynamic stress values. CHO culture (a, b), Sp2/0 culture (c, d), symbols show various magnitudes of hydrodynamic stress introduced with a classical single vessels or the oscillating hydrodynamic stress loop system. Values for (a) were 2.2 Pa (○, CHO control), 21 Pa (△), 38 Pa (▽), 83 Pa (◧) and 103 Pa (◊). Values for (b) were 1.2 Pa (□, Sp2/0 control), 15 Pa (◁), 21 Pa (△), 38 Pa (▽) and 83 Pa (◧). Error bars represent two standard deviations from at least 2 independent runs.
Figure 8.12: Aggregation behavior of the final products over time (a). Symbols show various magnitudes of hydrodynamic stress, 1.2 Pa (□, Sp2/0 control), 2.2 Pa (○, CHO control), 15 Pa (△), 21 Pa (▲), 38 Pa (▽), 83 Pa (►) and 103 Pa (◇). Error bars represent two standard deviations from at least 2 independent runs. In (b) the product aggregation at peak cell density is shown over the applied stress for CHO cells (○) and SP2/0 cells (□).
8.2 Oscillating hydrodynamic stress at pilot scale

8.2.1 Determination of the maximum hydrodynamic stress of the used stirred tank bioreactors

To determine the maximum hydrodynamic stress values of the reactors used in this work, poly(methyl methacrylate) (PMMA) nanoparticles were synthesized by a monomer-starved semi batch emulsion polymerization (Sajjadi and Yianneskis, 2003; Villiger et al., 2014). Particles with a mean diameter of 60 nm were produced and monodispersity was shown by dynamic light scattering (DLS) with a polydispersity index (PDI) of 0.04. Initial aggregates were prepared in a gently stirred 2 L beaker using a 0.2 M sodium chloride solution with a latex mass fraction of $1 \times 10^{-2}$. At this condition the particles grow due to aggregation until their strength becomes lower than the maximum applied stress, $\tau_{\text{max}}$ (Ehrl et al., 2010; Soos et al., 2008). Multiple re-aggregation and breakup events occur until a steady state aggregate size is reached. Subsequent dilution of these particles (200 x) into the vessel to be analyzed filled with deionized water reduces the salt concentration to 1 mM, well below the critical coagulation concentration, preventing further aggregation and breakup becomes the dominant mechanism controlling the aggregates size. To ensure steady state conditions the time evolution of the aggregate sizes was monitored by a small-angle light scattering instrument, Mastersizer 2000 (Malvern Instruments, UK). Using the previously determined calibration from Villiger et al. (Villiger et al., 2014) for stress and aggregate size, the measured steady state size was used to calculate the maximum stress present in the system of interest.

The steady state size of the particles is reached after 90 minutes as shown in Appendix Figure 8.12a, where $R_g$, the size of the particles, is not changing anymore for aerated and non-aerated conditions for both reactor scales. The comparison of the structure factor $S(q)$
measured for PMMA aggregates under various conditions, plotted as a function of the scattering wave vector $q$, showed a reduction of aggregate sizes, indicating an increase of the hydrodynamic stress (data not shown). To identify any differences in the internal structure of the formed aggregates $S(q)$ was plotted as a function of the normalized particle size $q\langle R_g \rangle$ (see Appendix Figure 8.12b). All data fall on the same curve with a slope (fractal dimension) of a power law equal to -2.7, indicating very compact aggregates whose internal structure is independent whether aerated or non-aerated conditions were applied.

Figure 8.13: The breakdown behavior of the PMMA particle sizes over time is given in (a) to verify their steady state size after 90 min, for the 3 L (■) and 300 L bioreactors (□ no aeration, ◇ 0.09 vvm aeration). In (b) the structure factor $S(q)$ is plotted over the normalized particle size $q\langle R_g \rangle$, to show the particles structural independency on aerated or non-aerated conditions, for the 3 L bioreactor operated at 75 rpm without (■) and with aeration (◇ 0.09vvm) as well as for the 300 L bioreactor at 40 rpm without (□) and with (◇ 0.09vvm) aeration.
Figure 8.14: Progression of the specific production and consumption of the basic metabolites Glucose (a), Lactate (b), Glutamine (c), Glutamate (d) and Ammonia (e) plus the product titer (f). Symbols refer to maximum stress values of 1.7 Pa (), 21 Pa () and 38 Pa () at 3 L scale, while at 300 L scale they symbolize 7 Pa () and 28 Pa (). Error bars represent one standard deviation and are obtained from at least two independent cultivations.
Figure 8.15: Glycosylation (a) and charged variants (b) profile measured at day 5 using 3L and 300L bioreactors operated at various maximum hydrodynamic stresses.
8.3 Simulation of large-scale heterogeneities

Figure 8.16: Determination of the power number ($N_p$) for the used reactor setup, with two pitched blade impellers inclined by 30° from horizontal plane, pumping upwards. Measured data is shown with closed circles ($\bullet$). Horizontal lines indicate the determined $N_p$ (1.6±0.2) and the corresponding error region, derived from fully turbulent measurements with $Re > 12000$. 
Figure 8.17: Measurement of the apparent oxygen mass transfer coefficient ($k_L a_{O_2}$) in culture media for different gas flow rates and bubble sizes, generated with diverse sparger types (a). Agitation was fixed to 250 rpm in all cases. Bubble sizes and corresponding sparger pore openings were, $d_b = 0.26 \text{ mm} / d_{p,sp} = 10 \mu\text{m}$ (○), $d_b = 0.43 \text{ mm} / d_{p,sp} = 0.5 \mu\text{m}$ (▽), $d_b = 0.67 \text{ mm} / d_{p,sp} = 50 \mu\text{m}$ (△), $d_b = 1.00 \text{ mm} / d_{p,sp} = 100 \mu\text{m}$ (□), $d_b = 5.61 \text{ mm} / d_{p,sp} = 1000 \mu\text{m}$ (★). Measurements with different concentrations of antifoam agent are shown in (b), (c) and (d) for fixed superficial gas flow rates of $8.63 \times 10^{-5}$ m/s, $1.08 \times 10^{-4}$ m/s and $7.12 \times 10^{-4}$ m/s, respectively. Closed symbols correspond to measurements during the culture (see materials and methods section in the main text) and serve as comparison to verify, that non culture based $k_L a_{O_2}$ measurements are representative for culture conditions.
### Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
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<tbody>
<tr>
<td>$X_t$</td>
<td>viable cell concentration at time $t$</td>
<td>$[10^6 \text{ cells/mL}]$</td>
</tr>
<tr>
<td>$q_{\text{Titer}}$</td>
<td>specific mAb production rate</td>
<td>$[\text{pg/cell*day}]$</td>
</tr>
<tr>
<td>$q_{\text{DNA}}$</td>
<td>specific DNA release rate</td>
<td>$[\text{pg/cell*day}]$</td>
</tr>
<tr>
<td>$q_{\text{GLC}}$</td>
<td>specific glucose consumption rate</td>
<td>$[\text{pmol/cell*day}]$</td>
</tr>
<tr>
<td>$q_{\text{LAC}}$</td>
<td>specific lactate production rate</td>
<td>$[\text{pmol/cell*day}]$</td>
</tr>
<tr>
<td>$q_{\text{GLN}}$</td>
<td>specific glutamine consumption rate</td>
<td>$[\text{pmol/cell*day}]$</td>
</tr>
<tr>
<td>$q_{\text{GLU}}$</td>
<td>specific glutamate consumption rate</td>
<td>$[\text{pmol/cell*day}]$</td>
</tr>
<tr>
<td>$q_{\text{NH}_4}$</td>
<td>specific ammonia production rate</td>
<td>$[\text{pmol/cell*day}]$</td>
</tr>
<tr>
<td>$\tau_{\text{max}}$</td>
<td>maximum hydrodynamic stress</td>
<td>$[\text{Pa}]$</td>
</tr>
<tr>
<td>$\tau_{\text{exp}}$</td>
<td>experimentally determined $\tau_{\text{max}}$</td>
<td>$[\text{Pa}]$</td>
</tr>
<tr>
<td>$\tau_L$</td>
<td>laminar hydrodynamic stress</td>
<td>$[\text{Pa}]$</td>
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<tr>
<td>$\tau_{\text{YS}}$</td>
<td>turbulent hydrodynamic stress</td>
<td>$[\text{Pa}]$</td>
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<tr>
<td>$\tau_{\text{JS}}$</td>
<td>inertial sub range of turbulent hydrodynamic stress</td>
<td>$[\text{Pa}]$</td>
</tr>
<tr>
<td>$\mathcal{E}$</td>
<td>energy dissipation rate of turbulent flow</td>
<td>$[\text{W/kg}]$</td>
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<tr>
<td>$\langle \mathcal{E} \rangle$</td>
<td>mean energy dissipation rate</td>
<td>$[\text{W/kg}]$</td>
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<td>$Re_{\text{nozzle}}$</td>
<td>Reynolds number of the nozzle</td>
<td>$[]$</td>
</tr>
<tr>
<td>$N_p$</td>
<td>power number of the impeller</td>
<td>$[]$</td>
</tr>
<tr>
<td>$N$</td>
<td>agitation rate of the impeller</td>
<td>$[1/\text{s}]$</td>
</tr>
<tr>
<td>$D$</td>
<td>diameter of the impeller</td>
<td>$[\text{m}]$</td>
</tr>
<tr>
<td>$D_{\text{nozzle}}$</td>
<td>diameter of the nozzle</td>
<td>$[\text{mm}]$</td>
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<tr>
<td>$V_{\text{Bio}}$</td>
<td>volume of the bioreactor</td>
<td>$[\text{L}]$</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Unit</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>$Q_{\text{nozzle}}$</td>
<td>flow through the nozzle</td>
<td>ml/min</td>
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<tr>
<td>$Q_{\text{loop}}$</td>
<td>flow through the loop</td>
<td>L/min</td>
</tr>
<tr>
<td>$v_{\text{pump agit}}$</td>
<td>agitation rate of the centrifugal pump</td>
<td>rpm</td>
</tr>
<tr>
<td>$v_{\text{Agit}}$</td>
<td>agitation rate of the impeller</td>
<td>rpm</td>
</tr>
<tr>
<td>$v_{\text{Tip}}$</td>
<td>tip speed of the impeller</td>
<td>m/s</td>
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doi:10.1080/20013891081692


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Bibliography


Curriculum vitae

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Benjamin Neunstoecklin
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Practical Experience
May 09 – present Doctoral student at ETH Zurich in the group of Prof. M. Morbidelli in the field of Production of Recombinant Proteins, Zurich, Switzerland
PhD project title:
Development of criteria for scale-up and scale-down of bioreactors for cultivation of mammalian cells
Nov 09 – Apr 12 Doctoral student in collaboration with Merck Serono S.A., Biotech Process Sciences, Corsier-sur-Vevey, Switzerland
Jul 08 – Nov 08 Internship at the industrial life science company Lonza Biologics Inc., Portsmouth, NH, USA
Operator in manufacturing department – Upstream Manufacturing
Personal objective: Studying the functions and processes of large scale protein fermentation
Sep 07 – May 08 Diploma student at the German Cancer Research Center (DKFZ), Heidelberg, Germany
Master’s thesis:
Functional analysis of Defensin proteins and their influence on tumor growth
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May 06 Internship at the Institute of Human Genetics, Heidelberg, Germany
Screening of the gene HTR3E in anorexia patients
Jan 06 Internship at the Institute of Zoology, Heidelberg, Germany
Analysis of the structures and functions in signal molecules
May 05 Internship at the Institute of Human Genetics, Heidelberg, Germany
Mutation analysis, chromosome preparation, functional analysis in Xenopus-Eybroths

Education
Sep 02 – Jun 08 Student of biology at the University of Heidelberg, Germany
Major subject: Molecular biology
Minor subjects: Cell biology and business administration
Degree: Diploma in biology (equivalent to Master of science)
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Sep 01 – Jun 02 Community service at Theresienkrankenhaus, Mannheim, Germany
Nursing service in intensive care
Aug 92 – Jun 01 Werner Heisenberg Gymnasium, Weinheim, Germany
(German High School equivalent)
Major subjects: Biology and Math
Degree: “Abitur” – general qualification for university entrance
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Skills
Specialist in up- and downscaling of bioprocesses including bioreactor characterization and control
Cultivation of mammalian cells like CHO or human and mouse derived cell lines
Expert knowledge in different platform processes including various media compositions
Excellent knowledge of reactor control for batch, fed batch and continuous mammalian cell culture operations
Profound knowledge of analytical tools for the determination of product amount and product quality like aggregation, glycosylation or charged variants using ELISA, HPLC or CE-LIF
Excellent knowledge of versatile bioprocess equipment (DASGIP, Infors, Sartorius, Nova, PreSens) and its interoperability with automated control software via OPC
Experience in large scale mammalian cell fermentation up to 20,000 liters
Tissue culture with cervical cancer cells (HeLa) and intestine cancer cells
PCR, DNA-Sequencing, DNA-Cloning
Gel electrophoresis with Agarose and Polyacrylamid
Blotting techniques (Northern-, Southern- and Western-Blotting)
Chromosome preparation, Micro-injection in Xenopus-Embryos

Language Skills
German: native language
English: fluent (speaking, reading, writing)
French: conversational (speaking, reading)

IT Knowledge
Excellent knowledge of MS Windows and all MS Office applications
Good programming knowledge in Perl, VBA, MatLab, MySQL, JavaScript and HTML

Zurich, February 2nd, 2014

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Publications


Conference proceedings

Oral Presentations

Benjamin Neunstoecklin, Maximilian Lularevic, Massimo Morbidelli, Miroslav Soos (2013) "Effect of heterogeneous cultivation conditions on mammalian cell productivity and product quality"  
AIChE Annual Meeting, 2013, San Francisco, USA

Benjamin Neunstoecklin, Miroslav Soos, Massimo Morbidelli (2012) "Facing heterogeneities in bioprocesses, a downscale approach tailored to suit large scale conditions"  
AIChE Annual Meeting, 2012, Pittsburgh, USA

Benjamin Neunstoecklin, Miroslav Soos, Massimo Morbidelli (2012) "Characterizing heterogeneity of environmental conditions in various bioreactor scales used for cell cultivation"  
15th European Congress on Biotechnology, 2012, Istanbul, Turkey

Poster Presentations

Benjamin Neunstoecklin, Thomas Villiger, Massimo Morbidelli, Miroslav Soos (2013) "A scale down approach focusing large scale heterogeneity"  
23rd European Society for Animal Cell Technology Meeting, 2013, Lille, France

Benjamin Neunstoecklin, Miroslav Soos, Massimo Morbidelli (2012) "Facing heterogeneities in large scale bioprocesses"  
15th European Congress on Biotechnology, 2012, Istanbul, Turkey

Benjamin Neunstoecklin, Matthieu Stettler, Miroslav Soos, Massimo Morbidelli (2010) "Heterogeneity in bioreactors - Why different conditions change performance"  
Merck Serono Biotech and Manufacturing Excellence Forum, 2010, Montreux, Swiss

Benjamin Neunstoecklin, Miroslav Soos, Massimo Morbidelli (2009) "Study on the mammalian cell behavior and metabolism in the presence of shear stress"  
14th European Congress on Biotechnology, 2009, Barcelona, Spain

Awards

Winner of the ACTIP Fellowship Award 2013-2014