Equipment characterization to mitigate risks during transfers of cell culture manufacturing processes

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Abstract The production of monoclonal antibodies by mammalian cell culture in bioreactors up to 25,000 L is state of the art technology in the biotech industry. During the lifecycle of a product, several scale up activities and technology transfers are typically executed to enable the supply chain strategy of a global pharmaceutical company. Given the sensitivity of mammalian cells to physicochemical culture conditions, process and equipment knowledge are critical to avoid impacts on timelines, product quantity and quality. Especially, the fluid dynamics of large scale bioreactors versus small scale models need to be described, and similarity demonstrated, in light of the Quality by Design approach promoted by the *FDA*. This approach comprises an associated design space which is established during process characterization and validation in bench scale bioreactors. Therefore the establishment of predictive models and simulation tools for major operating conditions of stirred vessels (mixing, mass transfer, and shear force.), based on fundamental engineering principles, have experienced a renaissance in the recent years. This work illustrates the systematic characterization of a large variety of bioreactor designs deployed in a global manufacturing network ranging from small bench scale equipment to large scale production equipment (25,000 L). Several

traditional methods to determine power input, mixing, mass transfer and shear force have been used to create a data base and identify differences for various impeller types and configurations in operating ranges typically applied in cell culture processes at manufacturing scale. In addition, extrapolation of different empirical models, e.g. Cooke et al. (Paper presented at the proceedings of the 2nd international conference of bioreactor fluid dynamics, Cranfield, UK, 1988), have been assessed for their validity in these operational ranges. Results for selected designs are shown and serve as examples of structured characterization to enable fast and agile process transfers, scale up and troubleshooting.

Keywords Mixing · Oxygen mass transfer · Cell culture · Large scale bioreactors · Scale up

List of symbols

A	Correlation parameter for empirical mixing correlations (–)
$app.$	Apparent volumetric mass transfer
$k_{La}(CO_2)$	coefficient of carbon dioxide (1/h)
b	Correlation parameter for reference floc diameter (–)
c_H	Mixing number (–)
$c_{O_2,L}$	Current oxygen concentration (%-sat)
$c_{O_2,L}^*$	Maximum dissolved oxygen concentration (%-sat)
$c_{CO_2,L}$	Current carbon dioxide concentration (%-sat)

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$C_{CO_2,L}^*$	Maximum dissolved carbon dioxide concentration (%-sat)
D	Impeller diameter (m)
d_{FV}	Reference floc diameter (μm)
d_{p50}	50th percentile of measured chord lengths of flocs (μm)
G	Correlation parameter for reference floc diameter (–)
H	Liquid height at tank (m)
h	Impeller build in height (m)
iVCD	Integrated viable cell density (10^5 cells $\cdot$ h/ml)
K	Parameter of mass transfer correlation (–)
$k_{La}(O_2)$	Volumetric mass transfer coefficient of oxygen (1/h)
M	Torque (Nm)
m	Correlation parameter for reference floc diameter (–)
n	Impeller speed (s^{-1} or rpm)
OTR	Oxygen transfer rate [mol(O_2)/L/h]
P	Power (W)
P/V	Specific power input to liquid volume (W/m^3)
$P_{\text{effective}}$	Effective power input (W)
P_{drained}	Power input into a drained vessel (W)
P_{load}	Power input into a liquid filled vessel (W)
Po	Power number ($Po = P/(\rho_L \cdot n^3 \cdot D^5)$) (–)
Re	Stirrer Reynold's number ($Re = \rho_L \cdot n^2 \cdot D/\eta$) (–)
T	Inner tank diameter (m)
t	Time (s)
u	Stirrer tip speed (m/s)
V	Volume (L or m^3)
v_{sg}	Superficial gas velocity (m/s)
w	Baffle width (m)
z_{blades}	Number of impeller blades (–)
z_l	Number of impellers (–)
α	Parameter of mass transfer correlation (–)
β	Parameter of mass transfer correlation (–)
ε	Energy dissipation rate (W/kg)
$\bar{\varepsilon}$	Mean energy dissipation rate (W/kg)
η	Dynamic viscosity (Pa/s)
ν	Kinematic viscosity (m^2/s)
θ	Mixing time (s)

λ	Kolmogorov scale (m)
ρ_L	Density of the liquid (kg/m^3)
τ	Shear force (Pa)
q	Sparging rate (L/min)

Abbreviations

A315	Lightnin A315 impeller
A510	Lightnin A510 impeller
CO_2	Carbon dioxide
CHO	Chinese hamster ovary cells
EE	Elephant ear impeller
EMA	European Medicines Agency
FDA	US Food and Drug Administration
MFC	Mass flow controller
NaCl	Sodium chloride
O_2	Oxygen
PB	Pitched blade turbine
QbD	Quality by Design
rpm	Round per minute
RT	Rushton turbine
sLpm	Standard liter per minute
vvm	Volume per volume and minute

Introduction

A large number of monoclonal antibodies and therapeutic proteins have been approved and are expected to be the major source of revenue in the upcoming years for the biotech industry (Reichert and Pavlou 2004; Reichert 2001, 2002). Due to their ability to properly glycosylate and process these complex large molecules, mammalian cells in suspension are the expression system of choice in the industry (Qi et al. 2003; Sethuraman and Stadheim 2006; Wurm 2004; Xie and Wang 1997). Since the early 1980s process yields and manufacturing scales have increased tremendously due to a large extent of research and development work. Manufacturing scales up to 25,000 L operated in a batch, fed batch or repeated batch mode followed by sequence of chromatography, filtration and concentration steps represent the state of the art technology (Heath and Kiss 2007; Su 2010). The successful execution of bioprocess scale-up and technology transfer activities are key enablers for the acceleration of time to market, supply chain risk mitigations, and market access, and have become necessary in large biopharmaceutical companies with a global footprint (Pohlscheidt et al.

2013). From a regulatory perspective, the Quality by Design (*QbD*) approach promoted in the recent years by the *EMA* and *FDA* requires detailed process and product understanding and clear definitions of critical quality attributes and critical process parameters. Within this approach multivariate studies and characterizations are performed in bench scale equipment to define a design space and establish proven operating ranges and control strategies. It is essential to understand and demonstrate similar fluid dynamics for the large scale manufacturing equipment to achieve similar performance within the design space. Recent publications demonstrate the need for enhanced understanding of the fluid dynamics and the impact on cell culture performance for several unit operations in the context of *QbD* and the associated design space (Broly 2014; Sieck et al. 2013; Soos 2014).

In general, the cultivation of mammalian cells requires specific process design and equipment layout due to their unique properties (e.g. shear sensitivity, intolerance of large concentration gradients, low oxygen demand, and sensitivity to non-physiological carbon dioxide concentrations). Therefore, the design of bioreactors and the scale up of different operating conditions is of crucial importance to maintain product quality and quantity throughout the entire product lifecycle. (Hu and Wiltberger 2014) Appropriate mixing, sufficient oxygen transfer, and adequate stripping of carbon dioxide have to be realized at low shear force for animal cell cultures (Aunins and Henzler 2001; Bailey and Ollis 1986; Henzler and Kauling 1993; Hu and Aunins 1997; Nienow 2000, 2006, 2010). Detailed process knowledge and extensive characterization of process equipment are absolutely necessary for the determination of the optimal operating window of mammalian cell culture processes. Most companies (especially those with a heterogeneous manufacturing network encompassing a large variety of bioreactor designs) have created equipment data bases to enable early assessment of risks and determination of operating conditions including structured and data-driven process adaptations without utilizing costly time in the manufacturing equipment. Knowledge of physical characteristics of bioreactors with regard to mixing and power input in conjunction with powerful tools such as scale down models and simulation software (e.g. *Computational Fluid Dynamics*) have enabled avoidance of pitfalls during technology transfers while minimizing plant downtime and impact to cost base (Jenzsch et al. 2009; Pohlscheidt et al. 2013).

Looking back to the “origins” of cell culture manufacturing, the first cell culture bioreactors were “retrofitted” from prokaryotic reactor designs. Today, several different impeller types and aeration systems have been developed and implemented. Several authors have performed studies with mammalian cells or representative model systems at various scales to characterize and optimize mixing, mass transfer, and shear force in order to minimize impacts to cell culture performance and product quality. (Biedermann 1994; Chalmers 1994; Godoy-Silva et al. 2009, 2010; Mollet et al. 2004; Nienow 1998, 2010). While several empirical correlations and data exist for operational ranges used in prokaryotic cultivations (Cooke et al. 1988; Grenville 1992; Henzler and Biedermann 1996; Van’t Riet 1979), very little data exist for operating ranges of mammalian cell culture, which in most case utilize power inputs around 10–50 W/m³ in pilot and production scale equipped with multi stage impeller systems.

This work systematically presents a large variety of bioreactor designs deployed in a global manufacturing network from small bench scale equipment (10 L) to large scale production vessels (25,000 L). Several traditional methods to determine power input, mixing, and shear force have been used to create a data base and identify differences for various impeller types and configurations in operating ranges between 5 and 250 W/m³. In addition, extrapolation of different empirical models, e.g. Cooke et al. (1988) have been assessed for their validity in these operational ranges. Results for selected designs are shown and serve as examples of structured characterization to enable fast and agile process transfers.

Materials and methods

Stirrer and vessels

For the characterization, both test systems (10 L up to 1000 L) and production bioreactors (10 L up to 25,000 L) with various impeller configurations, baffle numbers, and sparger types were used. Figure 1 shows exemplary vessel geometries and impeller types that are used in the Roche Biotech Drug Substance Production network.

Aspect ratios of liquid height to tank diameter (*H/T*) as well as relative impeller diameters (*D/T*) are given in addition to bioreactor scales.

Fig. 1 Some examples of vessel and stirrer geometries of mammalian cell culture bioreactors that were used for characterization experiments across the Roche Biotech Production Network

Power characteristics

In general, experimental determination of power input ($P_{\text{effective}}$ [W]) can be accomplished using frequency converters or torque sensors. For both methods the power introduced into the loaded vessel (P_{loaded} [W]) must be subtracted by the power introduced into a drained vessel (P_{drained} [W]). This enables consideration of friction and bearing losses of the agitation system. A minimal amount of liquid was added to avoid increased friction due to missing lubrication of the seal at the friction points.

$$P_{\text{effective}} = P_{\text{loaded}} - P_{\text{drained}} \quad (1)$$

Data obtained as effective power input were used to calculate the corresponding Power Number (Po) by following Eq. 2.

$$Po = \frac{P_{\text{effective}}}{\rho_L \cdot n^3 \cdot D^5} = \frac{2 \cdot \pi \cdot n \cdot (M_{\text{loaded}} - M_{\text{drained}})}{\rho_L \cdot n^3 \cdot D^5} \quad (2)$$

where ρ_L [kg/m³] is the liquid density, n [s⁻¹] is the stirrer speed, and M [Nm] is the measured torque. More details about determination of power characteristics can be found elsewhere (Kraume 2005; Storhas 1994; Zlokarnik 2001).

Mass transfer measurements

A basic limitation of most aerobic fermentation processes is the extremely low solubility of oxygen in fermentation media (Henzler and Kauling 1993;

Nienow 2000). To solve this requirement for bacterial fermentation processes the gas throughput or the stirrer speed can be enhanced. By these two measures the specific surface for oxygen transfer can be increased. But for industrial cell culture processes, due to shear force sensitivity of animal cells due to bubble bursting and/or stirring, the issue is often alternatively solved by increasing the oxygen fraction in the air to increase the driving force for oxygen-mass transfer. However, while feeding oxygen enriched gas may solve the oxygen supply problem, it is not at all a practicable solution as it exacerbates the CO₂-stripping problem for larger cell culture reactors (<300 L scale). While at lower volumetric gas throughput the rates of oxygen consumption and CO₂ production are comparable, the dissolved CO₂ may accumulate. More details about this stripping problem for large scale cell culture reactors can be found by Sieblist et al. (2011a). To understand the role of mass transfer in cell culture processes, it is also necessary to characterize the stripping qualities of carbon dioxide. Therefore, $k_L a(\text{CO}_2)$ values have to be determined as well.

For all mass transfer measurements presented in this paper, an aqueous electrolyte solution at 37 °C was used. The osmotic pressure of this solution was adjusted with sodium chloride to 300 mOsmol. This osmolality is a typical value prevailing in animal-cell culture media.

The dynamic gassing in/gassing out method was used to determine volumetric mass transfer coefficients. This method is widely used for determining of $k_L a$ -values in production vessels due to its ease of application (Bandyopadhyay et al. 1967; Yang et al.

2007). The method relies on the measurement of the absorption or desorption rate of a gas in a medium.

The flow rates of all gases used for the mass transfer experiments were controlled by calibrated thermal mass flow controllers. With these devices a specific gassing rate q [Ln/min] can be realized. The superficial gas velocity was calculated according to the following equation:

$$v_{sg} = \frac{q}{A} = \frac{4 \cdot q}{\pi \cdot T^2} \quad (3)$$

In this equation A [m²] is the cross sectional area of reactor.

For oxygen mass transfer, all oxygen in the medium must initially be stripped out by nitrogen to start the experiment. Next, the oxygen-free medium is gassed with air thereby allowing recording of the oxygen saturation kinetics. The k_{La} value [h⁻¹] can then be determined from the recorded oxygen saturation curves. The mathematical description, neglecting the consumption term (OUR), can be realized with following equation.

$$OTR = \frac{dc_L}{dt} = k_{La}(O_2) \cdot (c_{O_2,L}^* - c_{O_2,L}) \quad (4)$$

where $c_{O_2,L}^*$ [%-sat.] is the maximum dissolved oxygen solubility and $c_{O_2,L}$ [%-sat.] is current measured concentration level of oxygen in the liquid. After integration, the mass transfer coefficient can be determined by:

$$k_{La}(O_2) = \frac{\ln\left(\frac{c_{O_2,L}^* - c_{O_2,L}}{c_{O_2,L}^* - c_{O_2,L,0}}\right)}{\Delta t} \quad (5)$$

An oxygen saturation range between 20 and 80 % was used, as recommended by Liepe, for the evaluation of the measured data (Liepe et al. 1988). More details of this method can be found elsewhere (Linek et al. 1987; Tribe et al. 1995).

In principle, mass transfer of carbon dioxide within a bioreactor from the continuous liquid phase into the dispersed gas phase can be described by the same relationships as the oxygen-mass transfer. For CO_2 the volumetric carbon dioxide mass transfer rate CTR then reads

$$CTR = k_{La}(CO_2) \cdot (c_{CO_2,L}^* - c_{CO_2,L}) \quad (6)$$

where $c_{CO_2,L}$ is the dissolved carbon dioxide concentration in the liquid and $c_{CO_2,L}^*$ is the saturation

concentration in the fluid. The determination of the carbon dioxide concentration in the liquid is more complex, however, due to the dynamic conversion of CO_2 as follows:

More details to calculate the dissolved carbon dioxide concentration can be found by Minkevich and Neubert (1985), Contreras (2007), and Sieblist et al. (2011a). As shown therein, the dissolved carbon dioxide content CO_2^* directly correlates with the pH value of the medium via $H^+ = 10^{-pH}$. Therefore, only the pH value need be measured during the stripping of carbon dioxide for the determination of the k_{La} value.

For the carbon dioxide stripping experiments the medium was saturated with 10 vol% carbon dioxide and 90 vol% nitrogen. As a next step, the CO_2 can be stripped out with compressed air while the pH-value has to be measured. For the $k_{La}(CO_2)$ evaluation the measured pH data between 4.5 and 5.2 were used.

Mixing measurements

Liquid–liquid mixing is one of the most important physical phenomena in a bioreactor. For example, timely decrease of concentration gradients is critical for pH control and substrate distribution. According to Henzler (1998), a degree of mixing of 95 % was chosen to assure sufficient uniformity in the stirred vessel.

Two different methods were applied to determine the mixing time for data presented in this paper. A dye decolorization measurement method (iodine–starch system) was used for transparent lab scale vessels from 10 L up to 1000 L scale. A detailed description of this method can be found elsewhere (Henzler 1998; Hiby 1979; Käppel 1973, 1979; Storhas 1994). The process of homogenization can be visualized over the whole reaction vessel using this chemical method.

To prepare the reactor for the decolorization method a volume of 0.2 % of the reactor liquid derived from a 1 wt% starch solution should initially be added to the reactor liquid to enhance the colorization of the iodine system. The liquid solution inside the vessel should then be colorized using a 1 M iodine–potassium iodide solution. Subsequently

Iodine can be then reduced by adding a sodium thiosulphate solution (1 M) causing decolorization of the liquid.

The time between adding the sodium thiosulphate solution and the complete decolorization of the liquid should be measured. This time is defined as mixing time (θ). The decolorization reaction runs stepwise from dark blue to colorless and is recognizable even at high coat thicknesses, which is advantageous for this method (Hiby 1979; Zlokarnik 2001). Moreover, the point of last homogenization and thus the point of worst mixing within the system can be localized very easily. The determination of last point of mix, however, is operator dependent and qualitative in nature.

The color change method is not applicable for production vessels due to constraints of GMP regulations such as cleanability and tank opaqueness. Therefore, a physical method of increasing conductivity was used for these reactors. This determination of mixing times is more complicated and requires sensors as a method of mixing time determination, since only one physical measurement value is detected in the immediate vicinity of the probe.

Conductivity probes (TetraCon 325 from WTW GmbH, Weilheim) were used as sensors, and the signal was recorded 20 times per second. For the mixing time measurements in production vessels a 20 wt % sodium chloride solution was used as tracer material. For each experiment a tracer volume of 0.015 % of the reactor liquid volume was added. The mixing time in this case is defined as the period of time until the measured conductivity signal has reached the range of ± 5 % of final conductivity.

The mixing time (θ), however, largely depends on localization of the probes within the reactor because mixing process and fluid pattern depends on the type of impeller system used. The positions of sensors for the production vessels were derived from decolorization experiments in geometric related lab scale vessels at 300 or 1000 L scale. Probes were positioned at places where the decolorization most sensitively changed upon changes in the operational conditions.

In all mixing time experiments the tracer solution was added at the liquid surface near to the stirrer shaft by an addition funnel which could be sealed with a valve. To safeguard with respect to statistical

fluctuations, the decolorization experiments were repeated at least five times for each stirrer speed. For conductivity experiments, however, the experiments were repeated at least seven times.

Shear force measurements

In this article, an objective was to show data for comparison of different mixing systems with respect to the shear force. Some model systems for the determination of shear forces induced by stirring can be found in literature (Biedermann 1994; Wollny et al. 2007; Wollny 2010; Wollny and Sperling 2007). The model particle system of blue clay and polymer, described by Hoffmann et al. (1992), Henzler and Colleagues (Henzler 2000; Henzler and Biedermann 1994a, b, 1996), applies to data presented in this paper.

To use this model particle system of blue clay and polymer a mixture of 1 g/L NaCl and 5 g/L Wittschlicker blue clay (H. J. Braun Tonbergbau, Alfter-Witterschlick) must be added to reactor liquid to initially create a blue clay suspension. This solution should be homogenized for about 10 min to ensure complete dissolution of the particles in the liquid.

The flocculation process can be started then by adding 5 ml/L Praestol^{TM} BC 650 (Ashland Water Technologies, Columbus, OH, USA) from a stock solution with a concentration of 2 g/L. The impeller speed should be less than that to be examined later during the flocculation. If nearly equal floc sizes are generated the impeller speed could be increased to the target value after 5 min. Due to acting hydrodynamic shear force the flocs are destroyed and the destruction kinetics can be measured in situ by a Focused Beam Reflectance Measurement probe (FBRM®, Lasentec D600L, Mettler Toledo, Albstadt, Germany). Details can be found elsewhere (Kempkes et al. 2008; Kougoulos et al. 2005).

The average particle diameter (d_{p50}) of the model-particle system can be used as a surrogate measure for the acting shear force. The following criteria was used to determine the floc diameter since there is no state of equilibrium (Bücher 1993).

$$\frac{d(d_{p50})}{dt} \leq 0.0055 [\mu\text{m/s}] \rightarrow d'_{p50} \quad (8)$$

A reference floc diameter (d_{FV} [μm]) according to Pohlscheidt (2005) can be calculated to achieve comparability of measured floc diameters at different

specific power inputs (P/V [W/m^3]) and various impeller combinations:

$$d_{FV} = m \cdot d_{P50}' - b$$

$$m = 1.3 \cdot 10^{-6} \cdot \left(\frac{P}{V}\right)^2 + 1.37 \cdot 10^{-3} \cdot \left(\frac{P}{V}\right) + 2.46$$

$$b = 8.12 \cdot 10^{-5} \cdot \left(\frac{P}{V}\right)^2 + 6.48 \cdot 10^{-3} \cdot \left(\frac{P}{V}\right) + 76.9$$
(9)

To fit the reference floc diameters, the correlation function from Henzler (2000) was used.

$$d_{FV,calc} = G \cdot \left(\frac{1}{F} \cdot \frac{P}{\rho \cdot V}\right)^{-1/3}$$

$$F = \left(\frac{D}{T}\right)^2 \cdot \left(\frac{h_{blades}}{D}\right)^{2/3} \cdot z_I^{0.6} \cdot \sin \alpha^{0.6} \cdot z_{blades}^{2/3} \cdot \left(\frac{H}{T}\right)^{-2/3}$$
(10)

In this function the parameter z_I is the number of impellers, z_{blades} is the number of impeller blades and h_{blades} is the vertical height of the impeller blades. The adjustable parameter for this function is the factor G .

To relate data from turbulent shear force measurements to shear theories from Kolmogorov (1941), Henzler (2000) has published the following function:

$$\tau = 0.0676 \cdot \rho_L \cdot d_{P50}'^2 \cdot \frac{\varepsilon}{u}$$
(11)

It must be noted, however, that the maximum power dissipation as the location of the maximum shear stress cannot be determined by these measurements. According to Henzler (2000), however, reliable conclusions in relation to the maximum stress intensity in different reactors settings regarding to the disintegration process can be drawn from the overall results.

Results and discussions

Power number

One central element in the characterization of bioreactors and impeller is the power number (Po). For

aerated and unaerated systems the power number allows a scale independent calculation of power inputs during process transfers and scale up.

Several operating conditions are influenced by the power input (see Table 1). The constant power input per volume (P/V [W/m^3]) is one of the most common scale-up principles in cell culture in order to achieve adequate mixing as well as mass transfer. However, this scale up is criterion only a first assumption. Some other parameters such as carbon dioxide stripping or heat transfer are also very important parameters for cell culture processes and could not scaled up in this way. Therefore, an iterative process will follow to determine the optimal operating conditions for a particular cell line. There is not a universally agreed-upon scaling criterion that leads to success for cell cultures in all cases. Each cell line and each product requires customization of process parameters to reach an optimal cultivation process.

The influence of impeller design on power input has been described elsewhere (Bates et al. 1963; Do et al. 2001; Houcine et al. 2000). In this case, several systems were tested and compared to literature data and to each other. A short overview of the determined power numbers for the 320 L vessel is depicted in Fig. 2.

These numbers and the power numbers from Fig. 3 were used in the subsequent experiments to calculate the power input per volume.

In general, these power numbers are in line with previously published numbers from other authors (Wilke et al. 1989; Zlokarnik 2001). Clear differences according power numbers among the various systems shown in Fig. 2 are readily apparent. As can be seen in Fig. 3, a large scale dependency was not found between 320 and 12,000 L, as also described by Junker (2004) for yeast and *E. coli* processes. The small differences are due to minor variability in geometric designs.

The behavior is different for scales below 100 L. At these scales slight geometrical differences can affect the power number significantly. Another reason for the differences can be found in the development of turbulence eddies. Therefore, the development of flow cascades can be changed, as described by Nienow (Nienow 1968; Nienow and Miles 1978) and Bujalski et al. (1987) for radial flow impellers. This issue does not exist in the case of two impellers and clearance of >1 impeller diameter or axial flow impellers. This

Table 1 Expected course for different operating conditions during scale up with P/V and $v_{SG} \sim \text{const}$

Operating condition	Determining criteria	Relation/function	Expected behavior
Suspending	Tip speed (u)	$u = \pi \cdot n \cdot D = T^{1/9}$	Rising with increasing scale
Micro Mixing	Energy dissipation (ε)	$\varepsilon = \frac{P}{V \cdot \rho_L}$	Equal
Macro Mixing	Mixing time (θ)	$\theta \approx \frac{T}{u} \approx V^{2/9}$	Rising with increasing scale
Mass Transfer	Mass transfer coefficient ($k_L a$)	$k_L a = K \cdot \left(\frac{P}{V}\right)^\alpha \cdot (v_{sg})^\beta$	Comparable
Shear Force	Turbulent shear force (τ)	$\tau = 0.0676 \cdot \rho_L \cdot d_{p50}^2 \cdot \frac{\varepsilon}{u}$	Comparable
	Kolmogorov eddy size (λ)	$\lambda = \left(\frac{\varepsilon}{\nu}\right)^{1/4}$	Comparable

Fig. 2 Po-Numbers for some different stirrer configurations set up in a 320 L vessel. All results in this diagram were determined with a torque transducer **Fig. 3** Comparison of power numbers on different scales. All experiments were carried out with an H/T ratio of 1.6. The impellers were evenly distributed over the fill level

phenomenon could be well visualized in *CFD* (Computational Fluid Dynamics) simulations and was clearly seen in mixing experiments. Here the known segmental mixing was observed and clearly attributed to the middle Rushton impeller creating a flow barrier and non-uniform flow patterns (not shown). The data are mainly shown since power input per volume or energy dissipation rates are used in the following studies.

Mixing

Mixing is needed in a cell culture bioreactor to establish a homogeneous liquid solution and dampen gradients introduced by feed media and base addition for *pH* control, for example. The impact of insufficient mixing has been discussed elsewhere (Amanullah et al. 1994, 2001; Godoy-Silva et al. 2010; Lara et al. 2006; Nienow 2006, 2010; Varley and Birch 1999). Mixing times increase with increasing scale and therefore specific attention to influencing factors such as impeller type, geometry, and baffle configuration during scale up and tech transfer is required. One way to improve mixing is to increase impeller speed. A significant increase of impeller speed and associated power inputs can, however, lead to negative impact on cell culture performance and potentially product quality due to increased hydrodynamic shear force caused by velocity gradients (Kolmogorov 1941, 1991). On the other hand, poor mixing and areas of no mixing have to be avoided (Lara et al. 2006). Next to impeller speed minor changes of impeller systems (e.g. increase spacing, exchange of single impellers) can improve mixing behaviors dramatically without significantly impacting other phenomena such as aeration or acting shear force at constant power inputs. Figure 4 shows the typical mixing pattern of three different stirrer systems tested in a 320 L vessel at constant volumetric power input of 100 W/m^3 . The decolorization method described in 2.4 was used for these experiments.

In this case, three standard Rushton turbines ($D/T = 0.33$) were compared to three pitched blade stirrers ($D/T = 0.33$, $\alpha = 45^\circ$) and a system of two Rushton and a pitched blade impeller installed at clearance $h/D = 0.4$ at a power input of 100 W/m^3 . The mixing pattern and times show essential differences. For Rushton turbines the mixing occurs in segments from top-to-bottom as described by other

authors (Jahoda and Machon 1994; Machon and Jahoda 2000). A clear axial flow is visible and the last point of mixing occurs at the top in the three pitched blade-impeller system operated in the down-pumping mode. Significantly enhanced mixing behavior and reduced mixing times could be achieved without increasing power input only by combining a Pitched blade impeller with the two Rushton Turbines. The decrease of mixing time from 182 to 75 s is a substantial improvement and might be of special interest at larger scales to enhance *pH* control, for example. Remarkable differences become apparent for these and other systems tested upon analysis of the mixing time versus energy dissipation behavior at different power inputs.

Figure 5 shows the mixing times at different power inputs for several impeller systems (2- and 3-stage) and *H/T* ratios. Within the *H/T* ratio of 1.6 the Rushton turbines show the longest mixing times. The best performance of a three stage impeller system with respect to mixing times at equal power input is achieved with the combination of two Rushtons and one pitched blade on top in the down-pumping mode. A significant difference can be observed between $2 \times A510$ impeller installed in a 320 L as well as in a 1000 L tank at $H/D = 1.3$ compared to an *A310* and *A510* setting. In this case, comparable mixing times require a *tenfold* increase in power input. The two elephant ear-impeller system showed the highest mixing numbers due to the high *H/T* fill ratio of the reactor. Figure 5 also illustrates how simple design changes or standardization of bioreactor design across a production network can help to improve mixing behaviors.

The published empirical correlation by Cooke et al. (1988) and novel equation (see Table 2) were fitted and applied to measure mixing times in energy dissipation ranges from 0.005 up to 0.1 W/kg , as shown in Fig. 5. Also the fitted constants (*A*) for each of the correlations are presented in Fig. 5.

The correlation of Cooke does not consider the ratio of vessel-to-agitator diameter, nor the number of agitators. The new correlation, however, includes the numbers of possible circulation zones which can be induced by the impellers. Also the tank-to-impeller diameters (T/D) as well as the height-to-diameter ratio of the reactor (H/T) are taken into account.

The evaluated parameter *A* allows a detailed assessment of mixing times under different operating

Fig. 4 Mixing characteristics for three different stirrer settings at the same physical stirrer power input of 100 W/m^3 upon addition of thiosulfate to the top surface near the shaft. The experiments were conducted in a 320 L acrylic glass vessel

Fig. 5 Mixing times determined in a 320 L vessel as function of the mean energy dissipation. The *dashed lines* are model fits indicated in Table 2. All experiments were carried out with decolorization method. The impellers were equally distributed over the filling height for these experiments

Table 2 Empirical correlations used to fit the ascended mixing times

Author	Empirical correlation
Correlation 1: (Cooke et al. 1988)	$\theta_m = A \cdot Po^{-1/3} \cdot n^{-1} \cdot \left(\frac{D}{H}\right)^{-2.43}$
Correlation 2: (This paper)	$\theta_m = A \cdot \left(\frac{T}{D} \cdot \varepsilon\right)^{-1/3} \cdot \left(\frac{H}{T}\right)^{2/3} \cdot (Z_l + 1)^{2/3}$

conditions. The determined mixing times for all tested impeller systems can be described empirically with different correlations such as shown in Table 2 in the same order of magnitude with respect to model fit quality. However, no single correlation like Cooke et al. (1988), to fit all measurement data for different impeller configurations could be determined. The maximum deviation between optimal fit and fit with *Correlation1* with one constant was 102 % ($A = 263.9$). For the new presented correlation in this paper, the maximum deviation was 54 % ($A = 0.935$). In addition, comparison of different scales was performed using the dimensionless mixing number (c_H) which can be calculated by:

$$c_H = \theta_{95\%} \cdot n \quad (12)$$

The mixing number should be constant for full turbulent and fully baffled systems, according to literature (Zlokarnik 1967). This mixing number can be used for direct comparison of mixing behaviors and yields the number of revolutions to achieve 95 %

homogeneity of the system. Figure 6 shows the results of the 320 L scale by plotting dimensionless mixing number versus the impeller Reynolds number.

Different impeller systems are expected to show a scale dependency for mixing as has been reported elsewhere (Wilke et al. 1989; Zlokarnik 1967). Therefore, laboratory small scale bioreactors might show a different behavior relative to large scale. This might be of importance when using a non-validated scale down system as an initial model for a new cell line and attempting to assess equipment differences between sites in the case of process scale-up or tech transfer. Figure 7 illustrates the mixing number c_H for three stirrer systems from 10 L up to production scales.

These results illustrate a clear scale dependency, and the mixing number becomes nearly constant at scales >100 L. Additionally, these results are comparable to results for *E. coli* and yeast fermentation process published by Junkers (2004). Impacts to cell culture performance due to mixing or shear force might not be apparent at smaller scales (2 or 10 L) and can yield unexpected results upon direct scale up to full commercial scale. Even the use of 100 L bioreactors might not give a representative picture. Hence, detailed characterization in combination with pilot scale test runs and established simulation tools is absolutely necessary and might avoid lost production time and/or cost-intensive setup (Junker 2004). However, single sets of experiments at large production

Fig. 6 Depiction of the mixing numbers (c_H) as a function of the stirrer Reynolds number. All experiments were conducted in 320 L vessel with decolorization method

Fig. 7 Mixing numbers for three different impellers setting used in various scales

Fig. 8 Comparison of mixing numbers from different impellers setting determined using two different methods. Open symbols are results from decolorization measurements from experimental vessels, the full symbols indicate those from conductivity measurements in production vessels

equipment should be considered to verify results of experimental tanks. As discussed in 2.4, different methods might be needed and should be compared with each other. As shown in Fig. 8, data across multiple scales analyzed with different methods yield in comparable results.

These results indicate that differences in mixing behavior across scales must be understood to enable cell culture process transfer, scale up, and troubleshooting. This is especially true for sensitive cell

lines and products or processes that rely upon fast mixing at low power inputs.

As an example, the impact of different bioreactor configurations on mixing and, thus, cell culture performance can be seen in the following case study. During the evaluation of an appropriate bioreactor configuration to scale down a 12,000 L cell culture manufacturing process into a 10 L system mixing studies have been performed. In comparison to a 3-stage impeller configuration (2× Rushton + 1×

Fig. 9 Mixing studies in a 10 L bioreactor - Impact of impeller configuration: Two impeller configurations have been compared based on results obtained from CDF calculations

pitched-blade impeller) a 2-stage system (1× Rushton + 1× pitched-blade impeller) was investigated. Mixing characteristics obtained from experiments as well as from computational fluid dynamic (CFD) calculations indicated a 3 times faster mixing in the 2-stage compared to the 3-stage system, as can be seen in Fig. 9.

These data have also been confirmed in real experiments (data not shown). In this case a faster top-to-bottom mixing could be achieved when using the 2-stage impeller system.

Both bioreactor configurations have been used to run scale down cultivations in parallel (see Fig. 10). The differences in top-to bottom mixing using the two impeller configurations led to considerably different amounts of base addition during *pH* control in both bioreactors.

It has been observed that the bioreactor with better mixing used less corrective agent (sodium carbonate solution) for *pH* control. Differences in base addition resulted in differences in osmolality. The increased base consumption from configuration #1 led to higher osmolality levels which had a negative impact on cell growth and finally on product titer. The change to the impeller configuration finally resulted in a significantly higher product titer of around 35 %.

As demonstrated in this case study, the standardization and optimization of bioreactor designs to optimize mixing can be advantageous, especially at large scale.

Mass transfer O_2/CO_2

In addition to mixing, mass transfer of O_2 and removal of CO_2 is of crucial importance in cell culture bioreactors. Enabling techniques include surface aeration, membrane aeration and—especially in large scale manufacturing—bubble aeration. Several sparger systems have been established to satisfy the oxygen demand at large scale and with low power inputs. While oxygen supply is generally not an issue at high cell densities, with increasing scales up to 25,000 L and further advancement of cell densities $>5.0E^{07}$ cells/ml sufficient oxygen supply at low power input might be an area of concern. In addition, several authors have reported performance dependency on CO_2 levels and therefore stripping of CO_2 becomes a critical issue (deZengotita et al. 1998, 2002; Gray et al. 1996; Mostafa and Gu 2003; Royce and Thornhill 1991).

Next to power input, tank geometry, and impeller design, several sparger systems have been tested. These include open-pipe sparger (Mostafa and Gu 2003), micro sparger (e.g. glass or stainless steel sinter spargers) (Czermak et al. 2005; Mostafa and Gu 2003; Sieblist et al. 2010) and ring spargers in large-scale manufacturing. Gas inlet pressure and fluid flow influence primary bubble size (at the sparger), while secondary bubble dispersion occurs at turbulent flow fields in the near of the impellers. However, at low power inputs of 5–50 W/m³ the impact of dispersion

Fig. 10 Impact of bioreactor mixing on cell culture performance. **a** Differences in base consumption, **b** observed osmolalities in the culture broth, **c** measured integrated viable cell density, **d** comparison of achieved product titer

by the impeller is not as dominant in mammalian cell culture systems as compared to bacterial fermentations.

In this work, several sparger systems were tested and mass transfer coefficients determined as a function of scale, superficial gas velocity (v_{sg}), and specific

power input (P/V). Figure 11 shows $k_L a$ -values determined for a ring and jet sparger (20 sparging holes a $\varnothing$ 0.2 mm) installed in an 320 L vessel. A three-stage Rushton turbine setting was used as the stirrer system.

These data suggest that the sparging characteristics of these two distinct sparger types can be nearly

Fig. 11 Comparison of ring and jet sparger installed in a 320 L tank ($H/T = 1.6$), **a** at same superficial gas velocity (v_{sg}) without stirring, **b** At the same pneumatic and mechanical power input (P/V). As stirring system three Rushton turbines were used

equalized by this stirrer configuration. This phenomenon could not be seen, however, if only axial impellers are used. The reason for this difference is the formation of gas cavities behind the stirrer blades of a Rushton turbine (Riet and Smith 1975; Sieblist et al. 2011b).

Figure 11b indicates the fitted mass transfer correlation for stirred vessels:

$$k_L a = k \cdot (P/V)^\alpha \cdot v_{sg}^\beta, \quad (13)$$

presented originally by Van't Riet (1979) and other authors, as dashed lines. This correlation or modified versions of it are broadly used to describe mass transfer in microbial reactors. Figure 12 shows two examples of determined $k_L a(O_2)$ -data from 12,000 L cell culture reactor.

As can be seen from these examples, the correlation works quite well for power input (P/V) and superficial gas velocity (v_{sg}) values lower than those described by Van't Riet in his review (Van't Riet 1979). Similar results have been shown already by Xing et al. (2009) via the example of a 5,000 L cell culture reactor. However, as recognized by the factors α and β from the original Van't Riet correlation for ionic solutions ($K = 0.002$, $\alpha = 0.7$, $\beta = 0.2$), the influence of impeller system on $k_L a(O_2)$ is typically much lower for cell culture bioreactors relative to microbial reactors. In contrary, the power input derived from the superficial gas velocity becomes the dominating factor to influence the $k_L a(O_2)$. This can be recognized

from the fitted mass transfer correlation factors α and β shown in Fig. 12.

Availability of oxygen from pressurized air is generally not the limiting factor for mass transfer in large scale cell culture processes. Were there to be a limitation in terms of oxygen, this could be overcome by utilization of pure oxygen or increasing aeration rates. However, the latter would cause an increased shear stress on the cells via bubbles bursting at the liquid surface, as well as significant foaming issues (especially in high protein containing media). These negative effects could be reduced by the use of *Pluronic*® F68 and/or antifoam substances. However, the use of *Pluronic*® F68 should be done with caution. As shown by Sieblist et al. (2013) *Pluronic*® F68 can significantly influence mass transfer.

In contrast, if the driving force is increased by the addition of pure oxygen and the v_{vm}/v_{sg} is reduced, to the removal of CO_2 might become an issue. The effect is demonstrated in Fig. 13.

These data suggest that the power input of the impeller system does not influence the CO_2 stripping characteristics at typical cell culture sparging rates. It can therefore be concluded that the specific mass transfer area is no longer important for CO_2 discharge. This fact naturally limits the creation of scale-down models for large cell culture bioreactors. More details about CO_2 -depletion phenomenon and the limitation of carbon dioxide stripping for larger cell culture reactors (>200 L) can be found in Sieblist et al. (2011a).

Fig. 12 $k_L a(O_2)$ -values measured for an impeller configuration of **a** two Elephant ear impellers (2x EE; $D/T = 0.33$; $H/T = 1.8$) and **b** two Rushton turbines and one pitched blade

turbine (2x RT + 1x PB; $D/T = 0.34$; $H/T = 1.6$) installed in an 12,000 L tank. The impellers were equally distributed over the filling height. The dashed lines are the fitted $k_L a$ -correlations

Fig. 13 Apparent k_{LA} of carbon dioxide determined in 12,000 L reactor ($H/T = 1.6$) equipped with $2 \times RT + 1xPB$ and sparged with pressurized air

Shear Force

Shear force and the impact to cell culture have been discussed in literature by several authors (Cherry 1993; Elias et al. 1995; Godoy-Silva et al. 2010; Joshi et al. 1996; Kretzmer and Schügerl 1991; Nienow 1998; van der Pol and Tramper 1998; Sieck et al. 2013; Sorg et al. 2011; Yang and Wang 1992). The influence of shear stress and appropriate scaling strategies are as well discussed by Nienow and colleagues (Nienow 2014; Nienow et al. 2013).

In this work a blue clay system (see [Shear force measurements](#)) was used to describe the acting shear force from the stirrer system in a 320 L system. The shear forces caused by bursting of the bubble are not considered here. For some of the mixing systems shown here, the same relations were also carried out with an oil/water emulsion system according to Wollny and colleagues (Wollny et al. 2007; Wollny 2010). The determined relative dependencies, however, were nearly identical to the results shown here. Therefore, only the blue clay data are presented here. As shown by many authors the method is in general suitable to describe relative acting shear force caused by the agitation system and densities, viscosities and surface tensions in stirred tank reactors (Henzler 2000). However, the impact on the biology still is not solved.

Several power inputs and impeller systems were tested. Figure 14 shows exemplary results from two and three multi-stage impeller settings. Results from

one Rushton and a pitched blade turbine are included as reference data. The combination of different multi-stage impeller systems and the impact of shear force have not been described in literature. However, the general tendency is comparable to results of Henzler and colleagues (Henzler 2000; Henzler and Biedermann 1994a, b) and Wollny and colleagues (Wollny et al. 2007; Wollny and Sperling 2007).

Flakes from the model particle system of blue clay and polymer become shredded by the shearing forces. These forces are generated by the fluid turbulences which are induced by the agitator. Figure 14 shows determined reference floc diameter d_{FV} related to the physical power input. The bigger the floc diameter the lower the shearing forces acting on the flakes.

The highest shear force, represented by a low d_{FV} , in relation to the specific power input was yielded by the single stage systems and three pitched blade setting followed by three Rushton turbines. The lowest shear loading was created by combination of two Rushton and one pitched blade turbine. The often so-called “low shear” impellers like *Lightnin A510* ($2 \times A510$) and *Elephant ear* ($2 \times EE$) were multi stage systems that yielded the highest shear load.

These results are in general in line with the observation by Henzler and colleagues (Henzler 2000; Henzler and Biedermann 1994a, b, 1996). Impellers with linear blades, high D/T ratios, and high power numbers generally yield lower shear force in a fully turbulent and fully baffled system.

Fig. 14 Comparison of reference floc diameters vs. power input estimated for some impeller settings tested in a 320 L stirred vessel (4 baffles; $w/T = 0.1$). The dashed lines are the fitted correlations according to Henzler(2000)

Fig. 15 Comparison of reference floc diameters vs. impeller tip speed estimated for some impeller settings in the 320 L vessel ($T = 640$ mm; 4 baffles; $w/T = 0.1$). The dashed lines are the fitted correlations according to Henzler(2000)

The cause of this effect is the tip speed of the stirrer system. The lower the power number of the impeller system the higher must be the stirrer speed to realize the same specific power input.

Figure 15 illustrates that the so called low shear impellers induce the lowest shear forces at the same stirrer tip speed. Overall, these results substantiate that acting shear force is a function of the impeller system at constant power input as proposed by Henzler, for example, or at impeller speed proposed by Wollny (Wollny et al. 2007; Wollny 2010).

It should be noted, however, that the most damage of mammalian cells induced by shear forces comes from the collapse of bubbles on the liquid surface. Nevertheless, this influence is suppressed in most cell culture media by the addition of *Pluronic® F68*. Therefore, only the acting shear forces induced by the impeller were discussed in this paper.

In general, the impact of shear forces is expected to be both cell line and process dependent, and should not be generally excluded in a technical assessment. Comparable shear forces induced from the stirring

Table 3 Comparison of different impeller system performances at equal power input of 100 W/m³

	3×RT	3×PB	2×RT + 1×PB	2×EE	2×A510
Mixing	++	+++	++++	+	+++++
Mass transfer	++++	++	+++	+	+++
shear force	++++	+++	+++++	++	+

+++++, best case; +, worst case

system can be achieved by both—changing the impeller speed and/or optimizing the impeller system. More important, higher power inputs can be delivered for certain systems thereby enhancing mixing and mass transfer toward more robust process control, for example (Soos 2014).

Conclusions

The results of this work illustrate that thorough characterization of bioreactors with respect to mixing and mass transfer characteristics is essential for process understanding. This understanding subsequently can mitigate business risks and accelerate timelines associated with bioprocess scale up, tech transfer, and trouble shooting. The knowledge of critical unit operations for process performance combined with detailed equipment characteristics is essential—especially in the context of *QbD* and the described Design Space. As a result, classical engineering technology is experiencing a renaissance in the industry.

Simple modifications and standardization of systems can lead to a more uniform hydrodynamic condition from pilot scale to large scale manufacturing. However, the definition of a common standard requires detailed analysis and impact assessments. Comparison across the different reactor settings tested in this work illustrates significant differences and highlights the need for an end to end view across the three phenomena: mixing, mass transfer, and shear force. The example of 2× *RT* and 1× *PB* compared to other systems shows in most cases superior performance as can be seen in Table 3.

In the biopharmaceutical industry, however, it is often not preferred to change reactor configurations due to qualification and approval status of an established GMP facility. In such cases the process engineers have to calculate operating conditions of the different systems based on the detailed process knowledge and data

obtained from characterization studies. Depending on the crucial unit operation of the production process, the scaling criteria can be different.

This work further illustrates that published correlations for microbial reactors can be used for operating conditions of cell culture processes from laboratory up to full commercial scale with minor adaptations of correlation parameters. The work also shows that some conclusion of Junkers (2004) can be extrapolated to cell culture operating conditions and should be considered during development and process validation and/or characterization work.

The combination of experimental vessels, the verification of data in production equipment, and the establishment of relevant correlations allows fast assessment of risk, data-driven design of operational parameters, and accelerated trouble shooting. Furthermore, the parallel implementation of *CFD* models and validated scale down models will help to mitigate risks even further. The work also suggests that a standardization of equipment across a manufacturing network might help to avoid process and/or product quality issues related to mixing, mass transfer and shear.

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