Advances in Biochemical Engineering/Biotechnology 138
Series Editor: T. Scheper

Dieter Eibl
Regine Eibl *Editors*

Disposable Bioreactors II

Springer

138
Advances in Biochemical Engineering/Biotechnology

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Volumes are organized topically and provide a comprehensive discussion of developments in the field over the past 3–5 years. The series also discusses new discoveries and applications. Special volumes are dedicated to selected topics which focus on new biotechnological products and new processes for their synthesis and purification.

In general, volumes are edited by well-known guest editors. The series editor and publisher will, however, always be pleased to receive suggestions and supplementary information. Manuscripts are accepted in English.

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Disposable Bioreactors II

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ISSN 0724-6145 ISSN 1616-8542 (electronic)
ISBN 978-3-642-45157-7 ISBN 978-3-642-45158-4 (eBook)
DOI 10.1007/978-3-642-45158-4
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2009935692

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Single-use (disposable) systems that are discarded after one-time usage have become well-established in modern biopharmaceutical production processes. In addition to disposable bags for storage and transport, as well as disposable filters and mixers, disposable bioreactors in particular are replacing their reusable counterparts. However, the application of disposable bioreactors has led to technological innovations, resulting in an increase in cell densities, product titers and process safety, and has contributed to further reductions in time and costs for development and manufacturing of new products. Examples include, amongst others, large-scale cell banking in bags, one-step inoculation, the omission of intermediate cultivation steps and an increase in the scale-up factor.

These trends have also been influenced by further developments in hollow fiber technology. The availability of improved hollow fiber systems such as the ATF module from Refine Technologies has increased the focus on perfusion and concentrated fed batch processes among users. This is no surprise since it has been demonstrated that these strategies are accompanied by more efficient and more controllable operations for reduced bioreactor volumes.

In addition, published data using insect, stem and plant cells as well as microorganisms has shown that the beneficial usage of disposable bioreactors can exceed mammalian cell-based cultivations. Besides wave-mixed and stirred disposable bioreactors, orbitally shaken systems, which have recently become available for up to pilot-scale, are now dominant.

This volume, entitled "Disposable Bioreactors II", contains ten chapters, written by renowned experts, and presents novel developments in and applications for disposable bioreactors. Starting with a summary of engineering parameters for the most often used disposable bioreactors, for the first time, methods for their characterization are recommended. Furthermore, the availability of suitable disposable bioreactors for production of cell therapeutics based on human mesenchymal stem cells is discussed, and the suitability of wave-mixed bioreactors for rapid virus-like particle vaccine production processes with insect cells and the baculovirus expression vector system is highlighted.

Three chapters alone deal with the cultivation of microorganisms (bacteria, yeasts, microalgae) and allow us to conclude that there is future potential for the development of disposable bioreactors. Using the example of the Process4Success approach from Sartorius Stedim Biotech, the advantages of flexible production

facilities based on process platforms using disposables, which are becoming increasingly accepted for antibody production processes using mammalian cells, are presented. Finally, aspects of quality management, including risk analyses prepared by users and manufacturers, make a major contribution to the success of disposable bioreactors.

We are grateful to all the authors, the series editor, Prof. Dr. Thomas Scheper, and the publisher for their support, and hope that this volume will be helpful for your studies, research and production processes.

Wädenswil, Summer 2013

Dieter Eibl
Regine Eibl

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Adv Biochem Eng Biotechnol (2014) 138: 1–44
DOI: 10.1007/10_2013_187

Published Online: 23 April 2013

Dynamic Single-Use Bioreactors Used in Modern Liter- and m^3- Scale Biotechnological Processes: Engineering Characteristics and Scaling Up

Christian Löffelholz, Stephan C. Kaiser, Matthias Kraume, Regine Eibl and Dieter Eibl

Abstract During the past 10 years, single-use bioreactors have been well accepted in modern biopharmaceutical production processes targeting high-value products. Up to now, such processes have mainly been small- or medium-scale mammalian cell culture-based seed inoculum, vaccine or antibody productions. However, recently first attempts have been made to modify existing single-use bioreactors for the cultivation of plant cells and tissue cultures, and microorganisms. This has even led to the development of new single-use bioreactor types. Moreover, due to safety issues it has become clear that single-use bioreactors are the "must have" for expanding human stem cells delivering cell therapeutics, the biopharmaceuticals of the next generation. So it comes as no surprise that numerous different dynamic single-use bioreactor types, which are suitable for a wide range of applications, already dominate the market today. Bioreactor working principles, main applications, and bioengineering data are presented in this review, based on a current overview of greater than milliliter-scale, commercially available, dynamic single-use bioreactors. The focus is on stirred versions, which are omnipresent in R&D and manufacturing, and in particular Sartorius Stedim's BIOSTAT family. Finally, we examine development trends for single-use bioreactors, after discussing proven approaches for fast scaling-up processes.

Keywords Computational fluid dynamics · Engineering characteristics · Main applications · Scale-up · Single-use bioreactor types

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Abbreviations

1-D	1-dimensional
2-D	2-dimensional
3-D	3-dimensional
$A_{o,G}$	Interfacial area of gas bubbles
a	Specific interfacial area
B	Width of the bag
Bo	Bond number
c_m	Dimensionless mixing number
c_{o2}	Actual concentration of the oxygen in the liquid
c^*_{o2}	Saturation concentration of the oxygen in the liquid
c_s	Distance between stirrers
c_s/d_s	Ratio of stirrer distance to stirrer diameter
cv	Culture volume
C	Correlation factor
CFD	Computational fluid dynamics
CHO	Chinese hamster ovary cells
d_0	Shaking diameter
d_{32}	Sauter diameter
d_B	Bubble diameter
$d_{m,SF}$	Maximal shake flask diameter
d_s	Stirrer diameter
d_s/D	Ratio of the stirrer and bioreactor diameter
d_{SF}	Diameter shake flask
d_x	Cell diameter
D	Vessel diameter
D_I	Inner diameter of the container
DO	Dissolved oxygen
Do_2	Diffusion coefficient of oxygen
FDA	Food and Drug Administration
Fr	Froude number
FVM	Finite volume method
hMSC	Human mesenchymal stem cells
h	Stirrer height
h/D	Ratio of stirrer height and bioreactor diameter
h/H	Ratio of stirrer and liquid height
H	Height of the liquid
Ha	Hatta number
HCD	High cell density
H/D	Ratio of liquid height and bioreactor diameter
HTS	High-throughput screening
k	Rocking rate
k_H	Henry coefficient
k_L	Mass transfer coefficient

k_aL	Volumetric mass transfer coefficient
k_n	Reaction coefficient
L	Length of the bag
LBM	Lattice–Boltzmann method
LDA	Laser–Doppler anemometry
m	Slope in (26)
M	Torque
M_d	Dead weight torque (measured without liquid, representing only the bearing torque)
n	Reaction order
Ne	Newton number
N_S	Rotation frequency
OD	Optical density
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
P	Power input
P/V	Specific power input
PIV	Particle image velocimetry
PMP	Plant-made protein
p_{O2}	Oxygen partial pressure
PTV	Particle tracking velocimetry
q_{02}	Specific oxygen uptake rate
Re	Reynolds number
$\mathrm{Re}_{\mathrm{crit}}$	Critical Reynolds number
RT	Rushton turbine
S.U.B.	Single-use bioreactor from ThermoFisher scientific
SBI	Segment blade impeller
Sc	Schmidt number
t	Time
u_G	Superficial gas velocity
$u_{\max}$	Maximum velocity
u_{Tip}	Tip speed
V	Working volume
WIM	Wave-induced motion
X	Living cell density
x	Radial coordinate
x_1 and x_2	Empirical constants
XD	Extreme density
α	Volume fraction
β	Coefficient
γ_{NT}	Local shear gradients
$\gamma_{\mathrm{NT,\ m}}$	Mean local shear gradients
ε_{T}	Local energy dissipation rate
η	Viscosity

θ_m	Mixing time
λ	Kolmogorov length scale
ρ	Density

Contents

1 Introduction

The term "single-use" bioreactor (or "disposable" bioreactor) refers to the fact that the cultivation container is made of FDA-approved plastics (e.g., polyethylen, ethylenevinylacetate, polycarbonate, polystyrene) [1], and is only used once [2]. The cultivation container is typically provided in a sterile state and already pre-assembled, so that it can be used directly without further preparation. After finishing the bioprocess it is decontaminated and discarded. The resulting absence of sterilization and cleaning procedures allows products to be changed and new production campaigns to be started very quickly [3]. This finally leads to higher process flexibility, savings in time and costs, improvements in biosafety, and reduced environmental impact and waste, as has already been demonstrated in different studies with single-use bioreactors [4–6]. Sensor techniques [2], leachables/extractables (which can be secreted from plastic material and decrease product quality) [7], stability of the plastic material, and vendor dependence are among the main drawbacks of single-use bioreactors [8].

Nevertheless, advantages prevail, and single-use bioreactors have reached annual growth rates of 11 % [8] during recent years. This continuous growth can be ascribed to the international demand for rapid development, increased

manufacturing of new biotherapeutics (such as antibodies, hormones, enzymes, and vaccines), and an up to tenfold increase in the quantities of product titers that can be reached in these types of bioreactors [9, 10]. This last achievement explains the reduction of bioreactor size from 20 and 10 m^3 to 2 and 1 m^3 respectively, and the availability of single-use bioreactors in these smaller sizes.

Whereas single-use cultivation containers up to 50-L culture volume are rigid plastic vessels, cultivation containers for larger culture volumes are flexible, multi-layered plastic bags. These bags differ in their shapes and are 2-D pillowlike, 3-D cylindrical, 3-D cubes, 3-D with asymmetrical geometry or 3-D U-shaped. It is worth mentioning that trouble-free use of a flexible bag always requires a support container (dike or vessel often made from stainless steel) which supports the bag and keeps it in shape.

With the exception of single-use bioreactors developed for high-throughput screening (HTS), such as the Advanced MicroBioReactor System from TAP Biosystems [11, 12] or the Biolector from m2p-labs [13], and hollow fiber-based stem cell bioreactors such as the Quantum Cell Expansion System from CaridianBCT [14, 15], single-use bioreactors for milliliter-scale applications are normally not instrumented. These types of single-use bioreactors are not included in this chapter. In contrast, liter- and cubic meter-scale single-use bioreactors are equipped with standard or single-use sensors (installed either in situ or ex situ) to measure and control main process parameters such as pH and DO [16, 17]. However, single-use bioreactors are characterized by a lower level of instrumentation in comparison to reusable versions. In single-use bioreactors standard sensors are only regarded as a compromise inasmuch as their application requires the availability of 12-mm ports and aseptic connectors, or other special solutions. In addition, standard sensors need to be calibrated and sterilized before use, increasing the contamination risk. For this reason, single-use noninvasive and optical sensors are preferred by the majority of users.

2 Single-Use Bioreactor Types

The way for single-use bioreactors was paved by Fenwal‘s invention of the plastic blood bag in 1953 [18]. The first hollow fiber reactor was developed by Knazek et al. [19] in the early 1970s, followed by the replacement of CellFactories [20] and roller flasks. However, the development of further single-use bioreactors finally led to wave-mixed bioreactors [21, 22], which have increasingly displaced spinner flasks in seed inoculum productions since the early 2000s. Today the user can choose among a multitude of single-use bioreactors provided by different vendors. A systematization of single-use bioreactors that is analogous to that of their reusable counterparts was recommended by Eibl et al. [23, 24] and is based on the type of power input. Due to improved energy and mass transfer, dynamic single-use bioreactors have gained importance as scale has increased.

2.1 Current Overview

It can be clearly seen from Table 1, which summarizes commercially available liter- and cubic meter-scale dynamic single-use bioreactors, that mechanically driven wave-mixed and stirred systems represent the largest groups among the listed types. This development can be explained by the long-term experience of using stirred reusable bioreactors and the available knowledge in this area. The Wave was the first scalable single-use system that became accepted, despite its new mixing principle, wave-induced motion (WIM).

The WIM is caused by rocking or raising the platform containing the differently shaped single-use bags and is dependent on the bioreactor type (e.g. BIOSTAT CultiBag RM or AppliFlex). To date, six single-use bioreactor configurations from different vendors (the Wave Bioreactor, the BIOSTAT CultiBag RM, the Smart-Bag, the AppliFlex, the CELL-tainer and the XRS Bioreactor System) are in use for a wide range of production organisms including microorganisms [25], algae [26], plant cells [27], animal cells [28–30], bioactive T-cells [31, 32] and human mesenchymal stem cells (hMSCs) [33, 34]. As described by Ref. [27], power input and mass transfer are mainly influenced by the rocking rate, rocking angle, culture volume, culture broth viscosity and aeration rate. Along with WIM, bubble-free surface aeration in wave-mixed bioreactors results in more homogeneous energy dissipation and reduced foaming and flotation compared to stirred cell culture bioreactors. This is particularly the case for 1-D moving bags, which (in contrast to 2-D or 3-D moving bags) can exhibit limited power input and oxygen transfer for non-Newtonian fluids (e.g., plant cell suspensions) and aerobic microbial high cell density (HCD) cultivations. However, this problem is of less significance in animal cell-derived productions, where Newtonian fluid flow behavior is assumed. Scaling up of geometrically dissimilar wave-mixed single-use bioreactors (see also Sect. 5) is more problematic (maximum scale is limited; less knowledge of scale-up criteria exists), despite the fact that the sales literature highlights easy scalability as one advantage of this type of bioreactor.

As with wave-mixed bioreactors, mechanically driven, rotatory oscillating (BayShake Bioreactor [35]) and orbitally shaken single-use bioreactors (Current Bioreactor, OrbShake Bioreactor) contain no moving parts in the bag. They are surface-aerated and characterized by homogeneous energy dissipation and negligible foaming or flotation. Because they only recently entered cell culture labs, there are fewer application data available, even though bioengineering data have been determined and scale-up criteria proposed [4, 34, 36–38].

In Meissner‘s mechanically driven, oscillating, single-use Saltus Bioreactor (formerly VibroMix), the power input is adjustable through regulation of the motor amplitude and frequency [39]. An axial flow that mixes and aerates the cells in a cylindrical bag is caused by the movement of one or more perforated disks, which are fixed to an oscillating hollow shaft. The Saltus Bioreactor can generate high local power inputs and is designed for applications that are suited to medium to high shear conditions and culture broths with high viscosity. This means that this

Table 1 Current overview of commercially available liter- and cubic meter-scale single-use bioreactors

Working principle	Bioreactor brand	Max. cv	Vendor	References
Mechanically driven, oscillating movement, wave-mixed				
1-D WIM by rocking a pillowlike 2-D culture bag	Wave Bioreactor	500 L	GE Healthcare	[22, 44–49]
	BIOSTAT CultiBag RM	300 L	Sartorius Stedim Biotech	[25–28, 50–58]
	SmartBag	25 L	Finesse	[59]
1-D WIM by rocking a 3-D culture bag	AppliFlex	25 L	Applikon Biotech	[54, 60, 61]
2-D WIM by rocking a 3-D culture bag	CELL-tainer	125 L	Cellution Biotech	[56, 62– 64]
3-D WIM by rocking a 3-D culture bag	XRS Bioreactor System	25 L	Pall Life Sciences	[65]
Mechanically driven, rotatory oscillating				
3-D culture bag (cube-shaped)	BayShake Bioreactor	1 m^3	Bayer Technology Services	[4, 35, 66]
Mechanically driven, oscillation motion				
3-D culture bag with one or more vibrating disks	Saltus Bioreactor (in the past VibroMix)	100 L	Meissner Filtration Productions, Incorporation	[24, 39]
Mechanically driven, orbitally shaken				
3-D culture bag	CURRENT Bioreactor	300 L	AmProtein	[67–69]
	OrbShake Bioreactor	250 L	Satorius Stedim Biotech/Kühner	[36, 70–73]
Mechanically driven, stirred				
Rotating stirrer, magnetically coupled, 3-D culture bag	XDR	2 m^3	Xcellerex (now part of GE Healthcare)	[74–78]
	Mobius CellReady	200 L	Merck Millipore	[79–81]
	BIOSTAT CultiBag STR	1 m^3	Sartorius Stedim Biotech	[57, 82–86]
Rotating stirrer, mechanically coupled, rigid cylindrical vessel	UniVessel SU	2 L	Sartorius Stedim Biotech	[87, 88]
	Mobius CellReady	2.4 L	Merck Millipore	[21, 34, 89, 90]
	CelliGEN BLU Single-Use Bioreactor	50 L	Eppendorf	[91]
Rotating stirrer, mechanically coupled, 3-D culture bag	Single-Use Bioreactor (S.U.B.)	2 m^3	Thermo Fisher Scientific	[54, 64, 92–94]

(continued)

Table 1 (continued)

Working principle	Bioreactor brand	Max. cv	Vendor	References
Tumbling stirrer, magnetically coupled, 3-D culture bag (cube-shaped)	Nucleo Bioreactor	1 m^3	ATMI	[93, 95, 96]
Pneumatically driven				
Air Wheel design, 3-D culture bag (U-shape)	PBS Bioreactor	2 m^3	PBS Biotech	[40, 97]
Bubble column type, 3-D culture bag (asymmetrical geometry)	CellMaker Regular	8 L	Cellexus	[41–43]
Pneumatically driven and mechanically driven (hybrid)				
Combination of bubble column and stirred type, 3-D culture bag (asymmetrical geometry)	CellMaker Plus	50 L	Cellexus	[41]
Hydraulically driven				
Fixed bed type, 3-D culture bag	iCELLis	55 L $\approx$ 666 m^2	ATMI	[98–101]

Remark If not specially specified, the bag is cylindrical
cv culture volume

single-use bioreactor is not recommended for animal cell cultivations and processes with shear-sensitive plant cells.

Aeration and mixing in stirred single-use bioreactors, for which animal and microbial versions exist, is ensured by the aeration device (which is in most cases static) and rotating or tumbling stirrer(s) installed in the bag. A stirrer must be aseptically sealed, if it is not magnetically coupled, as is the case in the Mobius CellReady 3 L from Merck Millipore and the S.U.B. versions from ThermoFisher Scientific. Nevertheless, scaling-up of stirred single-use bioreactors is easier due to the geometrical similarity within a bioreactor family, which is normally based on reusable stirred cell culture bioreactors (compare also Sects. 3 and 4).

Pneumatically driven single-use bioreactors (Table 1) are applied for animal [40] and microbial [41–43] cultivations. They operate on the bubble column principle and provide homogeneous energy dissipation and high-efficiency mass transfer.

Disadvantages of bubble column bioreactors principally include bubble coalescence, strong foaming, and flotation. The currently available single-use, pneumatically driven bioreactors differ mainly in bag scale, shape and method of bubble generation. More detailed information about their working principles and characteristics are provided by Refs. [24 and 39].

Bubble column and stirring principles are combined in Cellexus's CellMaker Plus, a hybrid, single-use bioreactor, suitable for microorganisms, algae, and animal cells [41, 102]. The first hydraulically driven bioreactor with a bag is the ATMI's iCELLis, a single-use fixed bed bioreactor. The iCELLis uses medical-grade polyester microfiber macrocarriers, which provide capacity for HCDs, leading to high product titers in mammalian cell-based vaccine productions [98, 99]. As described by Prieels and Hambor [100, 101], this single-use bioreactor also allows successful expansion of hMSCs, where the scale is defined by the height of the bed.

2.2 Scale-Dependent, Potential Fields of Application

If focusing on potential fields of application for the previously described dynamic, single-use bioreactors, seven scale- and production-organism-dependent fields become evident (see Fig. 1). Production organisms are either grown as free or immobilized (bound to a carrier) cells. In most cases the bioreactors produce animal cell-derived products used in prophylaxis, diagnosis and therapy on a medium volume scale. They are mainly operated in fed batch (feeding) mode or, if HCDs and high-level protein titers are required, in perfusion mode [103]. For example, DSM Biologic's XD process [104] is a perfusion process that guarantees cell densities of around 1×10^8 cells/mL and antibody titers of around 25 g/L.

In seed inoculum productions, the wave-mixed BIOSTAT CultiBag RM and Wave Bioreactor have become widely accepted, whereas stirred, single-use bioreactors up to 1 m^3 are the systems of choice if mammalian cell-derived

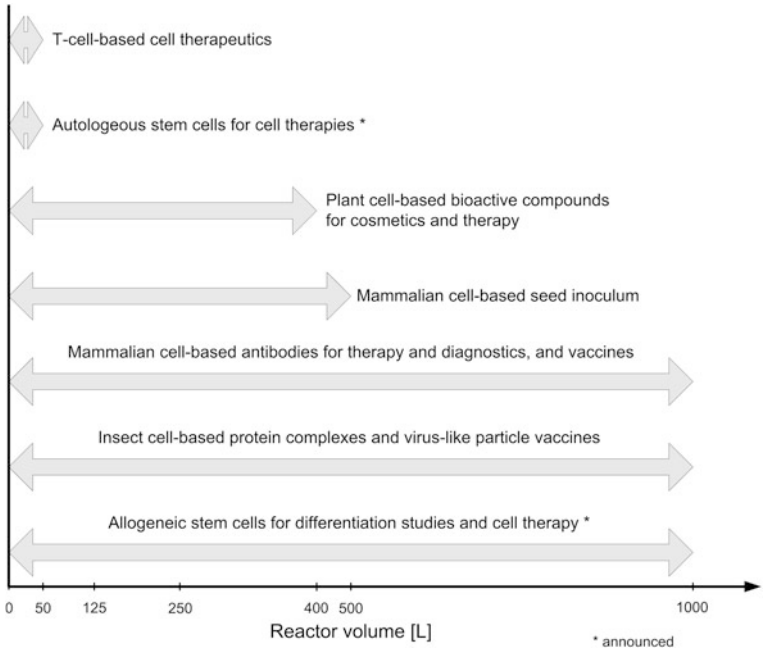

Fig. 1 Potential fields of application for dynamic single-use bioreactors exceeding mL-scale

therapeutics are the targeted products. Even though stirred single-use systems are already available up to 2 m^3, they have rarely been used. This is closely related to the more extensive operating procedures and training for staff, which both increase as culture volumes in single-use bioreactor bags rise.

If shear-sensitive cells (such as T-cells, bone marrow, or adipose tissue derived hMSCs) need to be grown, or if processes have to be realized, in which extensive foaming can occur (e.g., insect cell-based processes, where no chemically defined culture medium exists), wave-mixed single-use bioreactors should be chosen. As already mentioned in Sect. 2.1, they are characterized by homogeneous energy dissipation and low foam formation.

Wave-mixed bag bioreactors (up to a culture volume of 300 L) are also successfully used for the commercial production of plant-cell-derived secondary metabolites for cosmetics. Prominent product examples include the PhytoCELL-Tec products (*Malus domestica* Uttwiler Spätlauber, *Vitis vinifera* Gamay Teinturier Fréaux Grap, and *Arganium spinosum*) from Mibelle Biochemistry [105] and RESISTEM from Sederma [106]. As described by [26], existing photobioreactor versions are also suitable for microalgae cultivations. For the manufacturing of so-called plant-made proteins (PMPs) single-use bioreactors have rarely been used.

Nevertheless, there will be a demand in the future for single-use bioreactors for plant and microbial cell-derived high-value products that are not limited by mass transfer (energy and oxygen). Specially designed microbial versions of the CELL-

tainer and the XDR have proven themselves for HCD cultivations of *Escherichia coli, Pichia pastoris* and *Aspergilli* [63, 75]. ODs between 100 and 140 are also achievable for microorganisms grown in standard versions of the BIOSTAT CultiBag RM, as demonstrated by different groups [25, 82, 107]. In these cases there was either a low culture volume of 20 % or special feeding strategies were applied.

3 Bioengineering Characterization of Single-Use Bioreactors: Methods and Parameters

Knowledge of principal single-use bioreactor engineering parameters (such as mixing time, mass transfer coefficient, power input, fluid flow etc.) enables fast process optimization, scaling-up, and comparison of different types of bioreactors. The same methods were used to determine the bioengineering characteristics of the single-use bioreactors as for their reusable counterparts. Taking this into account, there is a differentiation between fundamental and advanced engineering methods. Methods that have been established and proven for single-use bioreactors and their resulting parameters are subsequently summarized and discussed.

3.1 Fundamental Engineering Characterization

In general, the flow regime in bioreactors can be characterized as laminar, transitional, or turbulent, depending on the dominance of viscous or inertial forces. This is characterized by the Reynolds number (Re), which is defined for stirred systems by (1) and depends on the stirrer diameter (d_S), speed (N_S) and liquid properties: liquid density (ρ_L) and viscosity (μ_L). It is well-known that the flow becomes fully turbulent above a critical Reynolds number that was found to be in the order of 1–10 $\times$ 10^4 for small- and medium-scale stirred bioreactors [88, 90, 108, 109], which is comparable to standard stirrer systems [59].

$$\mathrm{Re} = \frac{N_S \cdot d_S \cdot \rho_L}{\mu_L} \tag{1}$$

Similar relationships were introduced for orbitally shaken (e.g., shake flasks) [111] and rotatory oscillating bioreactors (i.e., the BAYSHAKE bioreactor) [35, 66], where Re is determined using the averaged rotational frequency and the maximum diameter of the mixing device. For shake flasks a critical Reynolds number of $\mathrm{Re} > 6 \times 10^4$ was found [112]. Wave-mixed systems with 2-D motion can be characterized by a modified Reynolds number given by (2), which is determined by the working volume (V), the width of the culture bag (B), the liquid level (H), the rocking rate (k) and an empirical constant that depends on the bag

type (*C*). This definition was derived from channel flows, providing a critical Re (Re_{crit}) of 1,000 [29].

$$Re_{mod} = \frac{V \cdot k \cdot C \cdot \rho_L}{\mu_L \cdot (2H + B)} \tag{2}$$

In addition to turbulence, the fluid flow in orbitally shaken bioreactors can be described by the "in-phase" and "out-of-phase" phenomenon, where the latter is characterized by liquid not moving in phase with the rotation of the shaker table [111]. A further parameter, which can often be easily related to the stirrer or vessel dimensions, is the maximum observable fluid velocity (u_{max}). Although, significantly higher tangential peak velocities were found in the stirrer wake region of Rushton turbines [113, 114] with regard to conventional stirrers, the maximum velocity in a number of different stirred single-use systems was found to correspond well to the stirrer tip speed (u_{Tip}) defined by (3) [90, 108, 109, 115].

$$u_{tip} = \pi \cdot d_S \cdot N_S \tag{3}$$

Furthermore, the tip speed of stirred systems is directly related to the impeller Reynolds number (4), which provides a first approximation of the maximum velocity at a desired turbulence. Because of shear sensitivity, a critical value of 1.0–2.0 m/s has been proposed [102].

$$Re \propto u_{Tip} \cdot d_S \tag{4}$$

In addition to these general criteria, the most important parameter for an engineering characterization is the volume-specific power input (*P*/*V*). Two approaches were developed for its determination: the torque method and the temperature method. The torque method, where the effective stirrer torque (difference in torque for stirring with liquid *M* and dead torque, M_d) is determined using a torque sensor (5), has become the standard method for conventional stirred vessels [116]. Consequently, this method was used for the measurement of the power input in the small-scale Mobius CellReady 3 L [90] as well as in the medium-scale BIOSTAT CultiBag STR 50 L [85, 86] and the S.U.B. Hyclone [108, 109]. The torque measurement was also shown to be feasible for shake flasks [117] and orbitally shaken cylindrical vessels [118, 119]. Therefore, the highest local energy input ε_{max}, which is often related to mechanical stress, is proportional to the specific power input under turbulent conditions (6).

$$P/V = \frac{(M - M_d) \cdot 2 * \pi \cdot N_S}{V_L} \tag{5}$$

$$P/V \propto \varepsilon_{max} \propto \frac{u_{Tip}^3}{d_S} \tag{6}$$

As an alternative, the temperature method was developed, where the power input is obtained from the heat balance given by (7), where $c_P \cdot w \cdot dT_F/dt$,

$U{\cdot}A{\cdot}(T_F{-}T_0)$ and $\mathrm{d}Q_0/\mathrm{d}t$ represent the change in temperature, the overall heat transfer rate through the vessel walls, and the heat-generating rate by the power consumption, respectively.

$$-c_p \cdot w \cdot \frac{\mathrm{d}T_F}{\mathrm{d}t} = U \cdot A \cdot (T_F - T_0) - \frac{\mathrm{d}Q_0}{\mathrm{d}t} \tag{7}$$

Although the torque method was expected to provide greater accuracy in terms of power consumption [118], because the power consumption can be obtained directly from the measured torque, results from both methods correlated reasonably well [119]. However, it should be emphasized that the power inputs investigated in these studies were in the range of 0.5 and 8 kW/m^3, which is much higher than typical values used for cell cultures in stirred systems, which are typically in the range of 0.01–0.25 kW/m^3 [120]. Thus, the temperature method may not be feasible for stirred or wave-mixed single-use systems, because of the much lower power input. Furthermore, when measuring the power consumption in small vessels, heat insulation is required, inasmuch as the temperature change from heat loss is relatively quick and leads to higher inaccuracies [118].

The power input of pneumatically driven bioreactors can be estimated from the superficial gas velocity (u_g) according to (8) if the isothermal expansion of gas is the predominant source of power [121]. However, no published data about specific power inputs for the pneumatically driven bioreactors were found.

$$\frac{P_G}{V_L} = \rho_L \cdot g \cdot u_G \tag{8}$$

Based on the power input, mixing and oxygen mass transfer can be estimated. Mixing (θ_m) is mostly characterized by the mixing time, defined as the duration required to achieve a defined degree of homogeneity after disturbance of the system (e.g., by change of temperature, concentration, conductivity, color, and/or density). In the majority of cases, 95 % homogeneity is accepted as adequate mixing performance. To determine mixing times two main approaches were applied: (de-)colorization methods and sensor methods.

Although the latter have the drawback of only measuring the mixing at specific locations, potentially leaving dead and rest zones hidden, these methods have been used to characterize different single-use bioreactors from benchtop to large-scale [80, 97, 122–125]. The advantages of the sensor methods are the precise data they deliver, reducing interobserver differences, and the fact that no optical accessibility is required, as is the case for (de-)colorization methods [116]. The main disadvantage of colorimetric methods is their inherent subjectivity, due to the personal view of the investigator. This may be overcome by automated image analysis, which has been used in mixing analysis of stirred single-use bioreactors [126].

For standard stirred bioreactors, it is well-known that the dimensionless mixing number (c_m), which represents the stirrer rotations required for the desired homogeneity (9), becomes constant under fully turbulent conditions [110]. It is not entirely surprising that the same relationship was confirmed for small- and

medium-scale single-use stirred vessels [90, 115]. Typical mixing numbers (c_m) for those systems are in the range of 20–40, leading to mixing times below 30 s for meaningful stirrer speeds in mammalian cell cultures.

$$c_m = N_S \cdot \theta_m \tag{9}$$

Based on turbulence theory, it was found that mixing time is inversely proportional to the third root of the specific power input under turbulent flow conditions. Furthermore, the mixing time is related to geometrical parameters leading to (10) which is valid for bioreactors where $H = D$ [120, 127].

$$\theta_m \propto \left(\frac{P}{V}\right)^{-1/3} \cdot \left(\frac{d_S}{D}\right)^{-1/3} \cdot D^{2/3} \tag{10}$$

For bioreactors with higher aspect ratios ($H/D > 1$), an additional term is introduced [i.e. $(H/D)^{2.43}$], which was originally developed for multiple impellers but has been shown also to take the influence of the filling height in single impeller systems into account [120]. This is represented by (11), which was shown to correlate well with mixing times in stirred single-use bioreactors predicted by CFD [115].

$$\theta_m \propto \left(\frac{P}{V}\right)^{-1/3} \cdot \left(\frac{d_S}{D}\right)^{-1/3} \cdot \left(\frac{H}{D}\right)^{2.43} \cdot D^{2/3} \tag{11}$$

Mixing in orbitally shaken bioreactors was found to be scalable by keeping the ratio of the inner diameter of the container (D_I) to the shaking diameter (d_{SF}) and the Froude number (Fr), defined by (12), constant [73]. Depending on the shaking speed, shaking amplitude, filling volume and vessel diameter, mixing numbers are between 5 and 80 for vessels of up to 1,500 L [73].

$$\mathrm{Fr} = \frac{2 \cdot \pi \cdot N \cdot (D_I + d_{SF})/2}{\sqrt{g \cdot D_I}} \tag{12}$$

In addition to mixing, oxygen mass transfer is considered to be the most important process during aerobic cultivation. The overall oxygen demand of the cells throughout the cultivation (OUR) must be met by the oxygen transfer rate (OTR). This demand is influenced by the specific oxygen uptake rate (q_{o2}) and increases as long as the number of viable cells (X) is also increasing, where c_{o2} and c^*_{o2} represent the actual and the saturated oxygen concentration respectively (13).

$$q_{o2} \cdot X = k_L \cdot a \cdot \left(c^*_{o2} - c_{o2}\right) \tag{13}$$

Oxygen transfer is mostly characterized by the overall volumetric mass transfer coefficient (k_La), which represents the product of the liquid mass transfer coefficient (k_L) and the specific interfacial area (a). For submerged aerated systems, the interfacial area depends on the local gas volume fraction (α) and the local bubble size represented by the Sauter mean diameter (d_{32}; 14).

$$a = \frac{A_{o,G}}{V_L} = \frac{6 \cdot \alpha}{(1-\alpha) \cdot d_{32}} \tag{14}$$

An approximate estimate of the gas–liquid interfacial area in surface-aerated, circular or cube-shaped vessels may be obtained from (15):

$$a = \frac{\pi \cdot D^2}{V_L} \text{ or } a = \frac{L \cdot B}{V_L} \tag{15}$$

However, it is notable that the bioreactor or surface motion will increase the interfacial area. For shake flasks, the interfacial area has been predicted to follow (16) for a fixed shaking diameter [128].

$$a \propto N^{0.6} \tag{16}$$

Although separate determination of the interfacial area in surface aerated systems was performed by image analysis [129], using an estimation of the evaporation rate [130], a chemical model system [131], and computational fluid dynamics (CFD) [128, 132], this remains difficult for submerged aeration because of the various factors affecting the local bubble size (aeration system, gas dispersion, media properties, etc.). Therefore, usually the k_La value is measured using either the gassing-out method or the sulphite method. Other methods, such as the respiratory gassing-out method, are of minor importance for engineering characterization in single-use bioreactors and are, therefore, not discussed in detail here (for more information please refer to Ref. [133]).

According to the definition, given in [116], in the gassing-out method the oxygen in the liquid is depleted by the introduction of nitrogen. After complete depletion, air is introduced leading to an increase in the oxygen concentration, where the rate of the concentration increase is determined by the k_La. Thus, the k_La can be obtained from the oxygen mass balance in the liquid (dc_{o2}/dt), which may be written for a totally mixed system by (17).

$$\frac{dc_{o2}}{dt} = k_La \cdot \left(c^*_{o2} - c_{o2}\right) \tag{17}$$

The gassing-out method is most often used in single-use systems above mL-scale independent of the type of power input (see Table 2). Typical k_La values achieved in stirred single-use bioreactors from benchtop to large scale are in the order of 5–40 1/h, depending on the scale, aeration rate and agitation. For example, k_La values of up to 35 1/h were achieved at typical cell culture agitation rates in the Mobius CellReady 3 L bioreactor with the microsparger [90, 134]. Similar values were found for the medium-scale BIOSTAT CultiBag STR 50 L at specific power inputs of 90 W/m^3 and an aeration rate of 0.1 vvm (see Sect. 4.5). In general, the k_La values in stirred bioreactors can be calculated by (18), where x_1, x_2 and C are bioreactor-dependent empirical constants. For the above-mentioned bioreactors, it was found that the influence of the superficial gas velocity was more pronounced than the specific power input P/V (i.e. $x_1 < x_2$). This is clearly

different from standard bioreactors used for microbial fermentations [135] and may be explained by the low gas dispersion capacities of the stirrers operated under typical cell culture conditions.

$$k_L a = C \cdot (P/V)^{x_1} \cdot u_G^{x_2} \tag{18}$$

Lower $k_L a$ values of between 4 and 20 1/h were reported in rocker-type wave-mixed systems with water and cell culture medium using typical process parameters for mammalian cells (6–10° rocking angle, 25–30 rpm, 0.25 vvm, 40–50 % filling level) [29]. In contrast, much higher $k_L a$ values of up to 700 1/h were reported for the CELLtainer, which is characterized by an additional horizontal displacement enabling much higher specific power inputs of up to 3.8 kW/m^3 [63]. As a result of such high oxygen transfer capacities, the cultivation of high oxygen demanding cultures may be realized without oxygen limitation.

However, it should be emphasised that results in surface-aerated systems obtained by the classical gassing-out method should be treated with caution because of the significant effect of the headspace gas composition. After introduction of nitrogen, the headspace is (nearly) free of oxygen, then increases continuously as the air supply is switched on again. During this process, the liquid saturation concentration changes with time according to Henry's Law ($p\mathrm{o}_2 = k_H * c^*_{\mathrm{o}2}$). Assuming a time-independent $c^*_{\mathrm{o}2}$, as given in (17), may lead to $k_L a$ being affected and to an erroneous effect of the aeration rate, which was confirmed by our own measurements [136] and is supported by data given in [137].

In the sulphite method, the depletion of oxygen is achieved by oxidation of sulphite ions to sulphate ions in the presence of a catalyst, such as copper, ferric, cobalt or manganese ions (19).

$$2\mathrm{SO}_3^{2-} + \mathrm{O}_2 \xrightarrow{\mathrm{Co}^{2+};\mathrm{Fe}^{-};\mathrm{Mn}^{+};\mathrm{Cu}^{+}} 2\mathrm{SO}_4^{2-} \tag{19}$$

Because the mass transfer phenomenon is coupled with a chemical reaction when using the dynamic sulphite method, the different sulphite oxidation regimes should be taken into account [138]. These can be classified by the Hatta number (Ha) defined by (20), where n, k_n and $D_{\mathrm{O}2}$ denote the reaction order for oxygen, the reaction constant, and the diffusion coefficient in the solution.

$$\mathrm{Ha} = \frac{\text{reaction rate}}{\text{mass transfer rate}} = \frac{\sqrt{\frac{2}{n+1} \cdot k_n \cdot \left(c^*_{\mathrm{o}2}\right)^{n-1} \cdot D_{\mathrm{O}2}}}{k_L} \tag{20}$$

Catalyst concentration, pH, temperature, and even light irradiation are the primary parameters that influence the reaction rate [139]. By using the film model, a reasonable approximation of the exact solution of the stationary mass balance can be derived for the absorption process (21) [140]. Measurements of the $k_L a$ have to be conducted in a nonaccelerated sulphite oxidation reaction regime, where Ha < 0.3 and the term α in (21; [131]) can be neglected [131].

Table 2 Overall oxygen mass transfer coefficients obtained by gassing-out method in different selected single-use bioreactor systems

System	Scale [L]	Agitation	Aeration rate [vvm]	k_oL [1/h]	References
Mechanically driven, stirred					
Mobius CellReady	3[a]	250 rpm	0.25	35	[90]
	200[a]	140 rpm	0.05	60	[80]
BIOSTAT UniVessel SU	3[a]	435 W/m^3	0.2	100	[146]
BIOSTAT CultiBag STR	50[b]	240 rpm	0.1	47	[85, 86]
	200[b]	240 rpm	0.1	37	[86]
Hyclone S.U.B.	50[b]	200 rpm	0.1	8.3	[125]
	200[b]	100 rpm	0.01	15	[23]
XDR-1000	1000[a]	132 rpm	0.015	9	[147]
Mechanically driven, oscillation motion					
BayShake Bioreactor	32[b]	120°, 210 W/m^3	n.a.	20	[35]
Pneumatically driven					
PBS bioreactor	n.a.			20	[148]
Mechanically driven, oscillating movement, wave-mixed					
AppliFlex Bioreactor	2.5[b]	6°, 24 rpm	0.5	14.6	[61]
	5[b]	11°, 25 rpm	0.5	24	[61]
	10[b]	4°, 20 rpm	0.025	4.4	[29]
Biostat CultiBag RM	1[b]	n.a.	n.a.	22	[57]
	10[b]	n.a.	n.a.	6	[57]
BioWave Bioreactor	1[b]	6°, 30 rpm	0.25	10	[29]
	10[b]	5°, 30 rpm	0.25	9.3	[29]
	100 L[b]	10°, 24 rpm	0.25	5.6	[29]
CELL-tainer	10[b]	20°[c]	n.a.	≈700	[63]
Mechanically driven, orbitally shaken					
OrbShake bioreactor SB200-X	100[b]	70 rpm	n.a.	27	[70]

[a] total volume, [b] working volume, [c] 20-cm displacement, n.a. not available

$$\mathrm{OTR} = \sqrt{\underbrace{\frac{2}{n+1} \cdot k_n \cdot \left(c^*_{o2}\right)^{n-1} \cdot D_{O2}}_{\alpha} + \underbrace{k_L^2}_{\beta}} \cdot a \cdot \left(c^*_{o2} - c_{o2}\right) \tag{21}$$

The sulphite method was primarily used in noninstrumented, small-scale systems, such as microtiter plates [131, 138], Tubespin reactors [141], shake flasks [142], and the BioLector [143]. Volumetric oxygen transfer coefficients of up to 200 1/h have been observed in 96-well microplates operated at high shaking frequencies (n_{SF}) of over 1000 rpm [130]. Correlating k_La by dimensionless groups, (22) was obtained, where Sc and Bo denote the Schmidt number $(\mu/(\rho \cdot D))$ and the Bond number $(\rho \cdot d^2 \cdot g/\sigma)$ respectively, and x and y are dependent on the microplate geometry.

$$k_L a = 31.35 \cdot D_{O2} \cdot a \cdot \mathrm{Re}^{0.68} \cdot \mathrm{Sc}^{0.36} \cdot \mathrm{Fr}^{x} \cdot \mathrm{Bo}^{y} \quad (22)$$

Lower values for $k_L a$ of up to 104 1/h were found for shake flasks with 200 mL working volumes at agitation rates of 210 rpm [141]. Comprehensive investigations of oxygen mass transfer have been carried out by Büchs and his co-workers, who found that the "out-of-phase" phenomenon (see above) has an adverse effect on the oxygen mass transfer [144]. The data could be correlated to the maximum oxygen transfer capacity (mmol/L/h) by (23), where the rotational speed n is given in rpm, the shaking diameter d_0 and the flask diameter in cm and the working volume in mL [142].

$$\mathrm{OTR}_{\max} \propto n_{\mathrm{SF}}^{0.84} \cdot V_L^{-0.84} \cdot d_0^{0.27} \cdot d_{m,\mathrm{SF}}^{-1.25} \quad (23)$$

Further engineering characterization, such as determination of residence time [29, 145] and heat transfer [118], are of minor importance for single-use bioreactors and only few reports are available in the literature. This may be explained by the fact, that single-use bioreactors are primarily used for cell culture applications, where low feeding rates are used, leading to significantly longer residence times compared to mixing time, and almost no heating or cooling limitations. Nevertheless, heating and cooling may become problematic in a microbial process performed in single-use bioreactors.

3.2 Advanced Engineering Characterization

Advanced engineering methods are applied to gain local and instantaneous values for the fluid flow, which, in addition to the previously described criteria that are considered as volume-averaged parameters, can be used for bioreactor characterization and scaling-up. Keeping in mind that the fluid flow in bioreactors can be very heterogeneous, the "global" parameters may not be sufficient for an advanced characterization. For example, it is well-known that the stirrer power is only dissipated in a small fraction of the bioreactors and the maximum dissipation rate is 100–200 times higher than the volume-average [149]. This may have an intrinsic effect on shear-sensitive organisms.

Some attempts have been made to characterize local fluid flow in single-use bioreactors by measuring fluid velocities using particle image velocimetry (PIV) [108], particle tracking velocimetry (PTV) [150], laser Doppler anemometry (LDA) [109], and hot-film anemometry [151]. The latter method has the drawback of affecting the flow, because the probe must be positioned inside the liquid. Therefore, the contactless, laser-based PIV and PTV methods are preferred. However, although these experimental methods are reliable, they are too time consuming to characterize the complete 3-D fluid flow within a typical bioreactor [152]. Thus, numerical methods are used to overcome this limitation and, of these methods, CFD has become the most important approach in recent years.

The fundamentals of CFD are based on mass, momentum and energy conservation equations, which are second-order partial differential equations that, for the most part, cannot be solved analytically. Thus, different numerical methods, such as the finite volume method (FVM) or the Lattice–Boltzmann method (LBM) are used to render the flow field. In each case, the fluid domain is divided into a discrete number of elements and the conservation equations are solved for each volume element. Details of the numerical basics and the algorithms used are provided elsewhere [153–157].

CFD has been applied for characterization of fluid flows in different stirred [88, 90, 108, 109, 158] and wave-mixed bioreactors [29, 151], orbitally shaken flasks [128], microtiter plates [159], the rotatory oscillating BayShake Bioreactor [35], and the pneumatically driven PBS Bioreactor [109]. The main advantage of CFD is that local and time-resolved data about the fluid flow (e.g., pressure, turbulence, shear stress) as well as physical and chemical properties (e.g., concentrations, viscosity, density, gas hold-up) can be obtained.

The local specific energy dissipation rate (ε) is considered to be an important parameter that can be predicted using CFD. This can be used to calculate the Kolmogorov micro-scale of turbulence (λ) in turbulent flows defined by (24). The turbulence micro-scale defines the size of the smallest turbulent eddies. Various studies have proposed that cell damage occurs in bioreactors, if the size of these eddies is comparable to the biological entity (i.e. $\lambda/d_X \approx 1$) [160–162]. However, this theory is yet to be proven and there are some doubts inasmuch as it does not take the physical properties of the cells into account [120, 127].

$$\lambda = \left(\frac{\mu^3}{\rho_L^3 \cdot \varepsilon}\right)^{1/4} \tag{24}$$

Furthermore, ε is often used to predict oxygen mass transfer, based on Higbie's penetration theory [163, 164]. Here, the liquid oxygen mass transfer coefficient is related to the surface renewable rate resulting in (25).

$$k_L = \frac{2}{\sqrt{\pi}} \cdot \sqrt{D_{O2}} \cdot \left(\frac{\varepsilon \cdot \rho_L}{\mu_L}\right)^{1/4} \tag{25}$$

Together with the specific surface area a, the k_La, which has been shown to be spatially heterogeneous, not only in large-, but also in small-scale bioreactors, can be predicted using CFD [88, 90]. However, special two-phase models that are not discussed here in detail, which take momentum exchange of the continuous and dispersed phases into account, are required. Detailed information is provided in Ref. [156].

4 Bioengineering Data of the UniVessel SU and BIOSTAT CultiBag STR

The following case study presents a possible approach for the engineering characterization of small- to medium-scale, stirred, single-use bioreactors. For this purpose, the two single-use bioreactor systems UniVessel SU and BIOSTAT CultiBag STR from Sartorius Stedim Biotech were chosen because they are as close as possible to the design and instrumentation of conventional glass or stainless steel cell culture bioreactors. The rigid UniVessel SU (Fig. 2a) is equipped with two-stage segment blade impellers with a blade angle of 30°. The impellers have a diameter of 0.055 m and are mounted with an impeller distance (c_s) of 0.07 m. The diameter of the cylindrical vessel (D) increases towards the top (from 0.118 to 0.126 m), caused by the validated manufacturing process. The ratio of liquid height to vessel diameter (H/D) is 1.3 and ensures a maximum working volume of 2 L. The bioreactor can be equipped either with conventional probes or optical sensors and is unbaffled.

In the case of the BIOSTAT CultiBag STR product line, the cultivation vessel is a flexible 3-D bag, which has to be mounted in a stainless steel support housing (Fig. 2b). Two different top-driven stirrer configurations are available: a combination of a Rushton turbine (RT) and SBI or two SBIs. Irrespective of the configuration, the stirrer diameters are 0.37, 0.23, 0.31, and 0.38 m for the corresponding working volumes of 50, 200, 500, and 1,000 L, respectively. However, the BIOSTAT CultiBag STR line primarily differs from the smaller-scale series in the differently shaped bottoms of the cultivation vessels. The other geometric parameters (e.g. d_s/D, H/D, c_s/d_s etc.) are similar for all the different size bags in the unbaffled BIOSTAT CultiBag STR line (for details see Refs. [85, 86]) and, therefore, fulfill the scale-up criteria. The measurement of pH and dissolved oxygen are realized using small optical sensors, which have no significant influence on the fluid flow, and no additional elements are installed.

In order to characterize the UniVessel SU and the BIOSTAT CultiBag STR line using CFD, the ANSYS Fluent commercial software package was used.

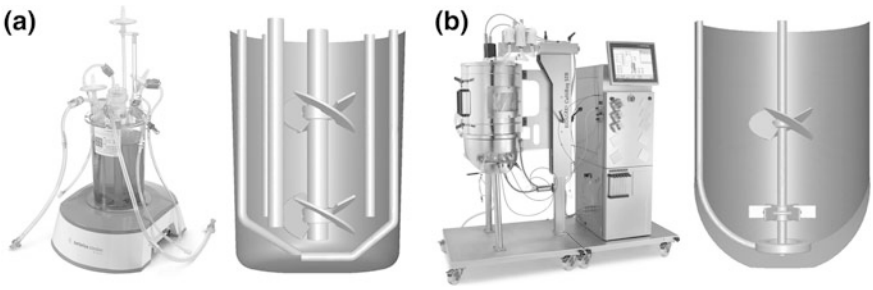

Fig. 2 The stirred single-use cell culture bioreactors from Sartorius Stedim with the UniVessel SU (**a**) and the BIOSTAT CultiBag STR with a working volume of 50 L (**b**)

Depending on the maximum working volume, the fluid domains of the nine different bioreactor configurations were divided into unstructured, body-fitted grids with up to approximately 5-mio volume elements. PIV was used for experimental measurement of the fluid flow in a 50 L vessel. In the systems with volumes of up to 200 L, the power input was determined using the torque method and the mixing time was predicted by the decolorization method.

4.1 Fluid Flow Pattern and Velocity Distribution

In Fig. 3, the ungassed fluid flow pattern obtained using CFD and PIV are compared for the mid-vessel plane (x–y-plane). The velocity components in the x- and y-directions are considered and the values are normalized by the tip speed (u_{Tip}). The highest fluid velocity magnitudes are found near the impeller tips and correspond well with the calculated tip speed (u_{Tip}) of 1.05 m/s using (3) (data not shown), which creates fully turbulent conditions (see Sect. 4.2).

In the BIOSTAT CultiBag STR 50 L with two SBIs (Fig. 3a, b), the highest relative velocity of 0.45 u_{Tip} occurs at the outer blade edges. Along the blade discharge the relative velocities decrease down to 0.3 u_{Tip} and a swirl in the axial direction can clearly be seen. This results in fluid recirculation along the vessel wall towards the fluid surface and the stirrer shaft. Here, the relative velocities range between 0.03 and 0.15 u_{Tip}. Due to prevailing axial swirls next to the impeller discharge of the two segment blade impellers, an axial fluid flow pattern and a similar velocity distribution can be observed for both the numerical and experimental methods. In summary, the CFD model can be considered as valid, because the numerically and experimentally obtained results visually agree [165–167]. Furthermore, the obtained fluid flow pattern corresponds well to patterns seen for conventional stirrers.

In the case of the RT + SBI stirrer configuration (Fig. 3c, d), the upper SBI clearly shows an axial flow profile in down-pumping mode with a maximum velocity of 0.45 u_{Tip}, whereas the lower RT discharges the fluid radially towards the vessel wall. The CFD simulation shows a maximum relative velocity of 0.51 u_{Tip} (x- and y-direction), which develops close to the impeller. In the predicted fluid flow pattern, the upward-forming loop represents the major flow, caused by the downward-inclining blade discharge. This results in a less pronounced swirl along the bottom wall. The experimental investigations reveal that the fluid is discharged radially, impinges on the outer wall, splits, and moves up and down, forming two recirculating loops in each vessel half. The interaction of the two impellers can be ignored if the c_s/d_s ratio exceeds 1.25 [110], which is true in the present case. In contrast, a slight downward tendency in the impeller discharge was observed in different experimental [168, 169] and numerical investigations [170] using RT in fully baffled and unbaffled vessels under turbulent conditions.

A more quantitative analysis is given in Fig. 3e, where radial profiles of the normalized fluid velocities for different heights in the BIOSTAT CultiBag STR

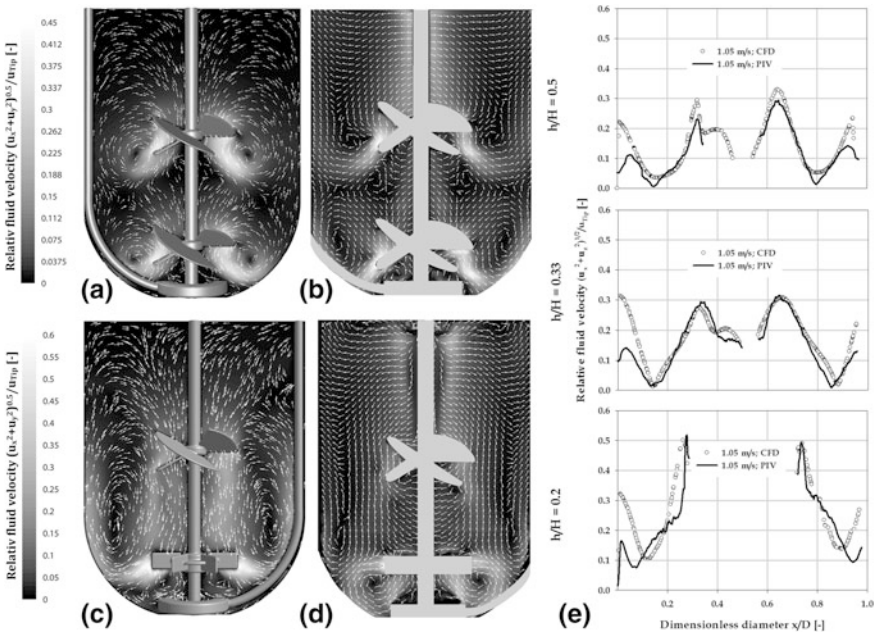

Fig. 3 Evaluation of the BIOSTAT CultiBag STR 50 L for both stirrer configurations. The numerical and experimental fluid flow patterns for the 2 × SBI configuration is shown in **a** and **b**, and for the RT + SBI configuration in **c** and **d**. A quantitative analysis of the relative velocity as a function of dimensionless diameter is provided in **e** for different dimensionless heights (mid-plane of RT and SBI as well as in the middle of both stirrers)

50 L with RT and SBI (Fig. 3e) are presented. Almost identical velocity distributions were found a third and half-way up the vessel height ($h/H = 0.33$; $h/H = 0.5$), with deviations towards the vessel walls. This may be explained by both numerical and experimental uncertainties resulting from optical distortions. The up to 60 % lower relative velocities were obtained in the experimental approach. However, the maximum and minimum velocities that were predicted for the impeller discharge and the point of flow reversal (where up- and downward flow meet) were well captured by PIV, with deviations below 10 %. For the rest of the velocity profile, the two different methods correlate sufficiently, with a maximum deviation of 10 %. However, the two graphs do not correlate if h/H is 0.5 or x/D is 0.35 (Fig. 3e). This inconsistency is based on the experimental measurement technique where the SBI covers the fluid and thus prevents determination. The above-mentioned deviating circulation flow is clearly shown in the velocity distribution profile of $h/H = 0.2$. Here, the maximum relative velocity of 0.51 u_{Tip} decreases similarly along the blade discharge towards the vessel wall ($0.1 < d_s/D < 0.3$ and $0.7 < x/D < 0.9$). Compared to the experimental investigation, the CFD simulation predicts a 0.05 x/D reduced discharge, causing a downward fluid flow (Fig. 3e). In addition, the velocities near the vessel wall differ by up to 45 %

when comparing the two methods. However, further experimental investigations of power input and mixing times are recommended in order to compare the methods and assess the influence of different fluid flows on biochemical engineering parameters.

4.2 Power Input

The power input represents the most important criterion for scaling-up of bioreactor systems [171]. Consequently, determining this was one of the major tasks of this case study to characterize and compare the UniVessel SU and the BIOSTAT CultiBag STR versions. By using CFD, the power input (P) in the BIOSTAT CultiBag STR 50 L was determined by the torque at the stirrer elements (1 and 5). It was demonstrated that, while exceeding a critical Reynolds number (Re_{crit}) of 10^4, a turbulent flow regime is reached when the Newton number (Ne) becomes a constant with values of 1.1 for the 2 × SBI configuration and 3.1 for the RT + SBI configuration [88]. Thus, the values obtained for Re_{crit} and Ne are comparable to those for conventional glass or stainless steel bioreactors [110, 172, 173]. Furthermore, the CFD predicted and experimentally determined power inputs were very similar [85, 86] with a maximum deviation below 15 %.

The tip speed was increased up to a maximum u_{Tip} of 1.8 m/s, which has been proposed as the maximum tolerable fluid velocity for mammalian cells [160]. At such high tip speeds, the maximum P/V of about 86 and 240 W/m^3 were obtained for the 2 × SBI configuration and the RT + SBI configuration, respectively (Fig. 4a). Henzler and Eibl and Eibl propose a maximum specific power input of 100 W/m^3 to avoid any cell damage [102, 174], although, a higher range of up to 250 W/m^3 is suggested by Nienow [120].

An additional analysis of the power input was carried out for aerated conditions. Although the effect of aeration is normally negligible, due to the low gassing rates used for mammalian cell cultures, the total power input (P/V) is usually applied to make a comparison or to scale up bioreactors [120, 175]. For the two stirrer configurations, the determined aerated P/V values obtained for an aeration rate of 0.02 vvm follow the same trend as the unaerated power input (see Fig. 4a), with a mean increase of 15 %. This could be verified by experimental data (not shown). The numerically and experimentally obtained results for the BIOSTAT CultiBag STR 50 L correlated well, therefore determination of P/V was also carried out for the UniVessel SU and the BIOSTAT CultiBag STR 200, 500 and 1000 L, for both stirrer configurations. In Fig. 4b the results are presented as a function of u_{Tip}. If u_{Tip} is used as a scale-up or scale-down criterion, P/V increases significantly for smaller volumes, but decreases for large volumes and is described by the relation in (6). Therefore, in the UniVessel SU the maximum P/V is about 435 W/m^3, which is unreasonably high for cell culture applications, but sufficient for microbial fermentations. Reasonable power inputs for cell culture applications of up to 150 W/m^3 have already been achieved with medium values of u_{Tip} in the range of

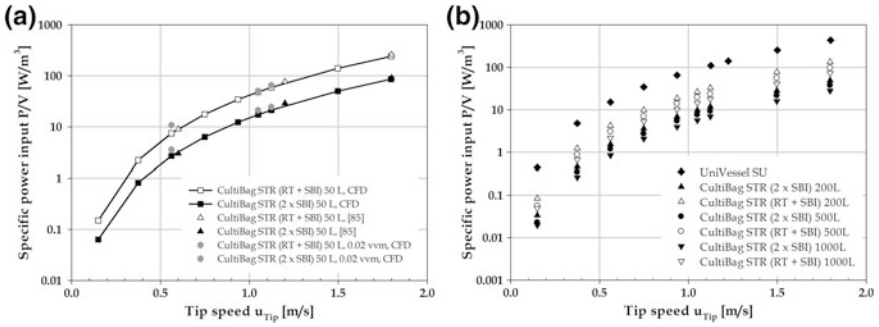

Fig. 4 Comparison of numerical and experimental investigations of the specific power input as a function of tip speed in the BIOSTAT CultiBag STR 50 L under unaerated and sparged conditions (**a**). Comparison of the CFD-predicted specific power input as a function of tip speed for all bioreactor sizes and configurations (**b**)

0.5–1.25 m/s. For these values, Re is between 0.8 and 2×10^4, indicating turbulent fluid flow, for a constant Newton number of 1.5.

In the case of the BIOSTAT CultiBag STR with working volumes of 200, 500, and 1,000 L (Fig. 4b), the *P/V* behaves according to (6). Considering the maximum *P/V* for the 50 L bioreactor with $\sim$86 W/m^3 ($2 \times$ SBI) and $\sim$240 W/m^3 (RT + SBI), the power inputs in the 200-L bioreactors are only $\sim$48 W/m^3 ($2 \times$ SBI) and 133 W/m^3 (RT + SBI). P/V decreases even further in the 1,000-L bioreactors so that $\sim$28 W/m^3 ($2 \times$ SBI) and $\sim$73 W/m^3 (RT + SBI) are achieved. The numerically determined Ne numbers of 1.1 and 2.8 ($2 \times$ SBI/RT + SBI) diverge only slightly from the values for the 50-L bioreactors, which can be explained by the minor differences of the dimensionless ratios of the reactor geometry for the various sizes.

In summary, it could be demonstrated that the numerically determined engineering parameters (Re_{crit}, Ne, *P/V* range) agree excellently with the experimentally obtained results. Furthermore, it could be shown that the characteristics of the investigated single-use bioreactors are comparable to those of conventional cell culture bioreactors.

4.3 Mixing Time

The characterization of the mixing behavior in the investigated single-use bioreactors was performed dependent on u_{Tip} using an identical range of up to 1.8 m/s (see Sect. 4.2). For this purpose, the concentration was determined, after addition of an inert tracer with identical fluid properties to the vessel contents. The tracer concentration was predicted transiently using a numerical method, however, the flow field of the steady simulation was "frozen". The CFD predicted mixing times were validated by comparing experimental data. The iodometrical decolorization

[116] (UniVessel SU) and conductivity methods [85] (BIOSTAT CultiBag STR) were applied, and 95 % mixing was assumed.

Figure 5a depicts the experimentally and numerically obtained mixing times (θ_m) for the UniVessel SU and the BIOSTAT CultiBag STR 50 and 200 L, which are presented as a function of P/V. Turbulence increases as power input rises, therefore this directly leads to a decrease in the mixing time [172]. Although about 100 s are required to achieve the desired 95 % homogeneity in the UniVessel SU at lowest power input (0.5 W/m^3), only about 3 s are required at the maximum power input (435 W/m^3). In the BIOSTAT CultiBag STR (2 x SBI), the numerical mixing times range between 10 and 60 s for the 50-L bioreactor (0.8–86 W/m^3), and between 20 and 60 s for the 200-L scale (1.5–49 W/m^3). Therefore, the mixing times of the BIOSTAT CultiBag STR are in the same range as for the UniVessel SU. Although it was found that fluctuations in the tracer concentrations were underpredicted by the applied RANS approach using the k-ε turbulence model, the mixing times correlate fairly well [176, 177]. Comparison of the numerically and experimentally determined mixing times (Fig. 5a) shows a maximum deviation below 4 % for the UniVessel SU. Higher deviations were found for the BIOSTAT CultiBag STR systems, where mixing times differ by up to 18 %, which can be ascribed to uncertainties in both experimental and CFD predicted data.

Based on similar results obtained for the UniVessel SU, the mixing times in the BIOSTAT CultiBag STR line with RT + SBI (Fig. 5b) and SBIs (data not shown) were analyzed. As shown in Fig. 5b, the mixing time decreases with increasing working volume and decreasing specific power input. This results in a mixing time ranging between 13 s (50 L; 240 W/m^3) and 200 s (1,000 L; 0.05 W/m^3) for the stirrer configuration of RT + SBI. However, in cell culture processes a power input in the range of 1 up to 150 W/m^3 is required and here the mixing times are between 15 and 75 s. The numerically determined mixing number (c_m) specifies the number of rotations required to achieve the desired homogeneity and is 29 ± 5. In addition, the exponent of −0.32 of the regression line (Fig. 5 B) is roughly equivalent to the theoretically cited exponent of −0.33, which is obtained under turbulent flow conditions [178]. In the BIOSTAT CultiBag STR (2 × SBI), the determined mixing times are on average 30 % higher considering the entire operation range. According to this, the homogenization number is 34 ± 1, whereas the exponent of the regression line is identical to the theoretical value (data not shown). In summary, the comparison of the results from the CFD simulation and the experimental measurements agree well ($c_H > 95$ %, 80 % $< \theta_m <$ 100 %).

4.4 Mechanical Stress

The spatially resolved data obtained by CFD can also be used to evaluate mechanical stress that can potentially damage cells. The turbulence, the formation of eddies [179, 180], and velocity gradients [160, 181] are considered potential

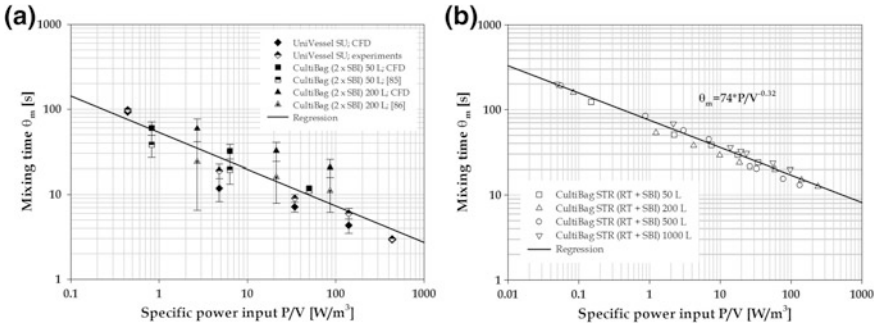

Fig. 5 Comparison of numerical and experimental mixing times as a function of specific power input in the UniVessel SU and the BIOSTAT CultiBag STR 50 L and 200 L (**a**). The error bars indicate the simple standard deviation of the mixing times. Additionally, the mixing time as a function of specific power input for the BIOSTAT CultiBag STR (RT + SBI) is shown (**b**)

sources of mechanical stress. In the following sections, methods to determine mechanical stress for the UniVessel SU are discussed. The maximum power input is proportional to the mechanical stress (24).

4.4.1 Kolmogorov Length Scale

According to the theory that cells are damaged by eddies of comparable size, the size of the smallest turbulent eddies (λ), also referred to as the Kolmogorov microscale of turbulence, was determined assuming local isotopic turbulence according to (24). Smaller eddies do not possess the energy to harm the cells, and cells follow larger eddies convectively. The volume-averaged turbulence microscales predicted for the UniVessel SU are summarized in Fig. 6a, where the static fluid zone, the rotating zone of the stirrer, and the stirrer surface are shown.

The investigation of the volume-weighted averaged Kolmogorov length scale in the fluid domain and in the stirrer zone revealed values of between 50 and 400 μm. The minimum Kolmogorov length scales are determined directly at the stirrer sites and range between 32 and 9 μm (0.4 W/m^3 < P/V < 435 W/m^3). Furthermore, it was found that λ (24) correlates well with P/V as follows (26):

$$\lambda \propto (P/V)^{-m} \tag{26}$$

where m is 0.2 ± 0.01, a value that is near the theoretical value of 0.25 provided by (24). This agrees well with the literature and is almost identical to other single-use bioreactors [108]. Comparable values were also obtained for the BIOSTAT CultiBag STR product line, where the regions close to the stirrer and the rotating stirrer zone (as well as in the fluid zone, data not shown) were investigated (Fig. 6b). In addition, the Kolmogorov length scale increases if the scale increases, and thus the power input declines.

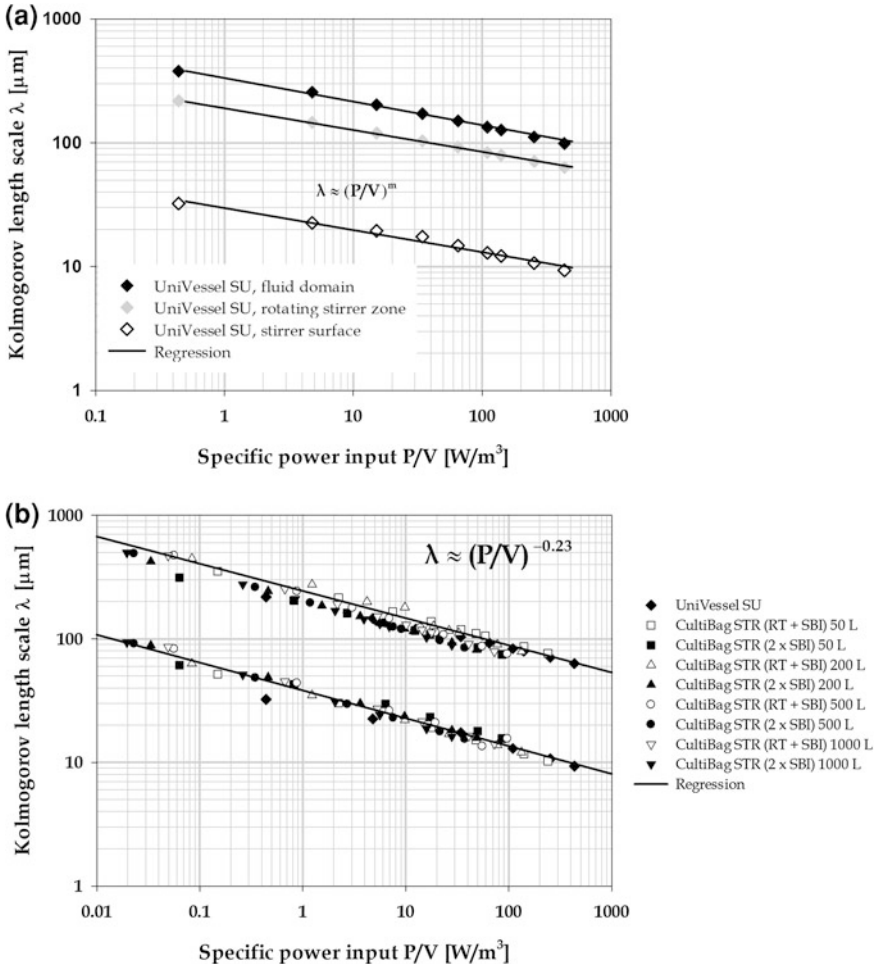

Fig. 6 Numerically predicted Kolmogorov length scale for the overall fluid domain, the rotating stirrer zone, and close to the stirrer surface as a function of specific power input in the UniVessel SU (**a**). The length scale close to the stirrer surface and in the rotating zone for all bioreactor sizes and configurations is shown in (**b**). The stirrer zone comprises the fluid that is directly in contact with the stirrer surface, whereas the rotating stirrer zone is made up of the stirrer movement volume. For all scales, the rotating stirrer volume is approximately 7 % of the total volume. The fluid domain represents the overall fluid without the rotating stirrer zones

When considering the size of CHO cells (10 µm $< d <$ 20 µm), no cellular damage is expected for either bioreactor type at meaningful power inputs, because the smallest eddies are significantly larger than the cells and, therefore, the cells will follow the eddies in a convective manner. In contrast, exceeding a *P/V* of 10 W/m^3 is expected to damage the cells in the region close to the stirrer,

inasmuch as the minimum Kolmogorov length scale is below 20 µm. However, this theory has not been proven thus far and describes a hypothesis (see Sect. 3.2).

4.4.2 Velocity Gradients

In addition to turbulence, cells are thought to be affected by velocity gradients, where shear and normal gradients (experimentally and numerically determined) can be distinguished [88, 108, 114]. Shear gradients were found to be dominant in stirred bioreactors [88, 114], and predominantly responsible for cell damage [181], therefore normal gradients have not been considered in the present study. The shear stress distribution, as given in (Fig. 7a), was obtained by discretizing the shear stress values into 250 bins and summing the volume elements where the shear stress occurred.

This volume-weighted distribution can be described by a logarithmic normal function, providing a maximum volume fraction of about 4.7 % in the UniVessel SU, irrespective of the specific power input (Fig. 7a). The local shear gradients (γ_{NT}) obtained for a maximum volume fraction (median value), increase proportionally to the third root of P/V from 2.4 up to 28 1/s [see (27); Fig. 7b]. The resultant exponent of 0.33 has already been published for the Single-Use Bioreactor (S.U.B.) and the BIOSTAT CultiBag STR 50 L (both stirrer configurations) in earlier experiments [108].

$$\gamma_{NT} \propto (P/V)^{1/3} \tag{27}$$

For the maximum P/V ($\approx$435 W/m^3), a maximum local shear gradient of $\sim$1,000 1/s was determined. According to Yim and Shamlou [182], the range of local shear gradients affecting the physiological state of the cells is between 500 and 5,000 1/s. Taking this into consideration, damage to the cells cultivated in the UniVessel SU at a maximum P/V cannot be excluded. Nevertheless, the numerically obtained mean and maximum shear gradient values are far beyond the critical values of 1–3 $\times$ 10^5 1/s which are known to damage the cells irreversibly [160].

4.5 Volumetric Mass Transfer Coefficient

The investigation of the volumetric mass transfer coefficient k_La was performed numerically using the Euler–Euler approach. As mentioned above, the k_La depends on, among other things, the (local) gas-holdup, the properties of the medium, and the size of the gas bubbles. These factors are primarily affected by the sparger and influenced by bubble coalescence and break-up processes. The experimental determination of the size of the gas bubbles was performed using photography [88] and sophisticated Shadowgraphy (Fig. 8a) as described in Ref. [207]. In the CFD models, a unique bubble size was assumed, although the prediction of bubble size

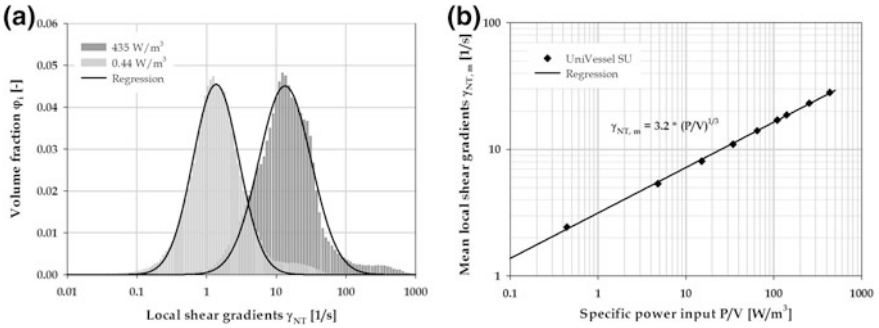

Fig. 7 Evaluation of the frequency distribution for the local shear gradients as a function of volume fraction in the UniVessel SU for a specific power input of 0.4 and 435 W/m^3 (**a**). Comparison of the mean local shear gradients as a function of specific power input in the UniVessel SU under transient ($0.4 < P/V < 34$ W/m^3) and turbulent ($34 < P/V < 435$ W/m^3) flow conditions (**b**)

distribution by population balance equation models has been shown to improve the accuracy of k_La determination. However, these models result in much higher computational demands. Depending on the cell line, high aeration rates or strong sparging can immediately damage the cells, however, this can be prevented if the aeration rate is below 0.1 vvm [183]. CHO cells, which represent the most often used production organism in the modern biopharmaceutical industry, have specific oxygen uptake rates on the order of 0.25–0.35 $\times$ 10^{-12} mol/cell/h [38, 184]. More detailed data for specific oxygen uptake rates of various cell lines is provided in Refs. [185, 186]. Due to the low oxygen requirements, k_La values on the order of 2–37 1/h are mostly sufficient to reach medium to high cell densities, as shown in Ref. [187].

In the UniVessel SU two-phase simulations were performed for P/Vs from 0.4 to 435 W/m^3, assuming an air bubble size of 1 mm. In order to analyze the k_La (18), superficial gas velocities of 2.8 $\times$ 10^{-4} and 5.7 $\times$ 10^{-4} m/s (corresponding to 0.1 and 0.2 vvm) were used (Fig. 8b). According to Zhu, the flow regimes resulting from these aeration rates and bubble diameters are not significantly altered [188]. The numerically determined k_La values (14 and 25) for the UniVessel SU at maximum working volume were plotted as a function of P/V (Fig. 8b). Depending on the aeration rate, k_La values ranging from 10–60 1/h (0.1 vvm) and 20–100 1/h (0.2 vvm) were found. Comparing the experimental results to the numerically obtained results, the latter deliver lower k_La values, which are, nevertheless, on the same order of magnitude [189]. However, these values are very high, meaning they are sufficient for aerobic microbial fermentations, requiring higher specific power inputs and aeration rates.

Increasing the P/V results in lower oxygen transfer resistance, due to the higher surface renewal rate of the bubbles [190] and, therefore, leads to higher mass transfer coefficient values of k_L and k_La, respectively. In both bioreactor types, the liquid mass transfer coefficient k_L ranges from 1.25–2.65 $\times$ 10^{-4} m/s (25). Based on these results, the maximum required k_La of 37 1/h mentioned above, has

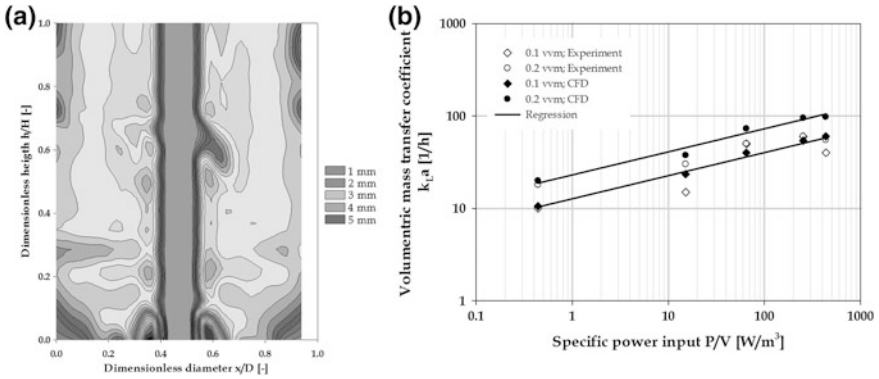

Fig. 8 Determination of the mean bubble diameter in the BIOSTAT CultiBag STR (RT + SBI) 50 L using Shadowgraphy (**a**). Experimentally and numerically-predicted volumetric oxygen mass transfer coefficients as a function of specific power input for the aeration rates of 0.1 and 0.2 vvm in the UniVessel SU (**b**)

already been achieved with a *P/V* of 90 W/m^3 at an aeration rate of 0.1 vvm, and an even lower *P/V* of 10 W/m^3 at 0.2 vvm. In the BIOSTAT CultiBag STR 50 L (both configurations; Fig. 8a), the mean bubble diameter was 5 mm and was measured using the Shadowgraphy technique at a P/V of 1.05 W/m^3 and an aeration rate of 0.02 vvm. In this case, a maximum k_La value of 35 1/h was determined at an aeration rate of 0.1 vvm [88] using the gassing-out method.

Using the correlation suggested by van't Riet in (18), which represents the k_La as a function of the specific power input and the superficial gas velocity, the coefficients C, x_1, and x_2 were found to be 0.4, 0.25, and 0.78, respectively (28). Here, the superficial gas velocity has a strong influence on the k_La value, whereas the specific power input is of only minor importance. This may be explained by the low dispersion capacity of the stirrer when operated at low agitation rates. These results were also found for other single-use bioreactors such as the Mobius CellReady 3 L bioreactor or a prototype of the UniVessel SU with a Rushton turbine and a segment blade impeller [88, 90].

$$k_La = 0.4 \cdot (P/V)^{0.25} \cdot u_G^{0.78} \tag{28}$$

5 Scale-up of Single-Use Bioreactors

A key element in the biopharmaceutical industry is the transfer of the cultivation process from lab to production scale (scale-up), while ensuring identical process characteristics [191]. The most often applied scale-up approach is based on geometric similarity (height to diameter ratio) and/or engineering parameters (e.g., u_{Tip}, P/V, θ_m, k_La, λ, γ_{NT}) of the bioreactor [171, 172, 183, 192].

In order to determine a bioreactor's engineering parameters, a fundamental engineering characterization is required (see Sect. 3), where the specific power input, mixing time, and volumetric mass transfer coefficient represent the most often used scale-up criteria [192, 193]. For microbial fermentations, the heat exchange surface has proven itself as a reliable scale-up factor [120] whereas in microcarrier-based stem cell cultivations the suspension criterion, where the microcarriers are homogeneously dispersed, has been successfully introduced [33, 194]. However, it is not possible to keep all parameters constant when scaling up [38, 192] and, therefore, a compromise must often be found under consideration of critical process parameters (e.g., oxygen transfer or specific power input), which have to be identified in advance [178].

The impeller speed, which is often used for scaling up in the pharmaceutical industry [88] is not a major parameter for scaling up [120, 172] and results in a decreasing of the specific power input. Based on a tip speed of 0.9 m/s, the specific power input is 118 W/m^3 in the UniVessel SU and decreases in the BIOSTAT CultiBag STR 1,000 L to 5 W/m^3. For bioreactors larger than benchtop scale, this leads to decreased mixing and mass transfer and results in unacceptable cell growth.

To prevent the formation of concentration gradients, mixing time represents a further criterion that can cause issues when scaling up. A relationship between the mixing time and bioreactor/stirrer type is provided in Eq. (11), based on the specific power input and the reactor as well as the stirrer geometry [120, 127, 195]. As shown in Fig. 9, the mixing times predicted for the different single-use bioreactors and sizes investigated in this study were well correlated by this single equation (with $R^2 = 0.97$). The determined proportional factor is 3.5, which is in the same range as the predicted value of 5.9 [195].

$$\theta_m = 3.5 \cdot (P/V)^{-1/3} \cdot (d_s/D)^{-1/3} \cdot (H/D)^{2.43} \cdot D^{2/3} \tag{29}$$

Especially at larger scales, inhomogeneous mixing contributes to the formation of pH and nutrient gradients as a result of local hydrodynamics [196], which may result in a reduction of cell growth and protein expression [127, 197, 198]. However, keeping mixing time constant when scaling up leads to a significant increase in the specific power input at larger scales [178, 199]. If the mixing time in the UniVessel SU is estimated to be 34 s (approximately 1 W/m^3), specific power inputs of 22 W/m^3 for the BIOSTAT CultiBag STR (2 × SBI) 500 L and 28 W/m^3 for the 1,000-L scale are required. According to (24), the Kolmogorov length scale in the stirrer zone and close to the stirrer decreases to 89 and 16 µm, respectively. In addition, an increase in the shear gradients can be observed due to the rising specific power input. When considering the working volume ($\gamma_{NT} \propto V^{-0.16}$) and the ratio of impeller diameter to vessel diameter ($\gamma_{NT} \propto d_s/D^{-2.7}$) (data generated by own studies, but not shown), correlation (30) results, showing a linear graph with a single proportional factor C for the UniVessel SU and the BIOSTAT CultiBag STR across the different scales (see Fig. 10). In the present study, C was found to be 0.05 (31). Considering the mixing time, as mentioned above, the mean local shear gradients are approximately 1.5 1/s (500 L,

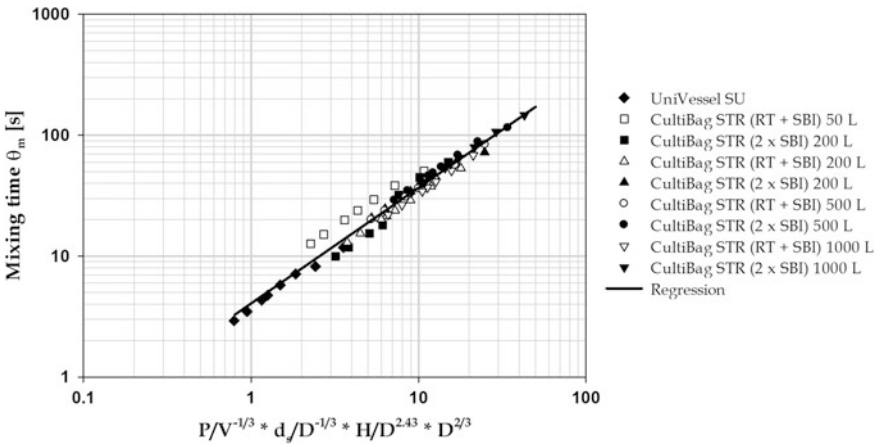

Fig. 9 Comparison of the CFD-predicted mixing times correlated by (11) for all bioreactor configurations investigated

21 W/m^3) and 1.4 1/s (1,000 L, 28 W/m^3), respectively. The maximum local shear gradients are below a value of 1,000 1/s (data not shown) and the Kolmogorov microscale is in a typical range for cell culture processes where no cell damage is expected (see Sect. 4.1, [160, 182]).

$$\gamma_{\mathrm{NT},m} = C \cdot (P/V)^{1/3} \cdot V^{-0.16} \cdot (d_s/D)^{-2.7} \tag{30}$$

$$\gamma_{\mathrm{NT},m} = 0.05 \cdot (P/V)^{1/3} \cdot V^{-0.16} \cdot (d_s/D)^{-2.7} \tag{31}$$

Specific power input has the largest influence on mass transfer and represents a successful compromise for scaling-up a bioreactor according to the Büche theorem [172]. Therefore, it is suggested that specific power input should be kept constant during scale-up, a technique that has been successfully applied in microbial fermentations and animal cell cultivations [38]. However, the scale-up with a constant specific power input results in an increase in mixing time, the Reynolds number and tip speed, whereas the stirrer speed, Froude number, and shear gradient, are decreased under turbulent flow conditions. In contrast, the eddy length scale remains constant according to (24).

In addition to the power input, oxygen mass transfer is a further scale-up criterion for aerobic processes. As already mentioned, animal cells have lower metabolic rates and oxygen demands than yeast and bacteria, but in high cell density processes, or in cases where aeration is limited by lack of mechanical stress tolerance, oxygen mass transfer can become a limiting factor [200]. If direct bubble aeration is applied, the risk of damaging the cells as a result of the bubbles bursting increases [160, 201–203, 147]. This risk also increases as the bubble diameter decreases [120].

The difficulty in scaling-up cell culture-based processes results from a lack of preservation of local flow structures as the reactor vessels are scaled-up [193]. It is

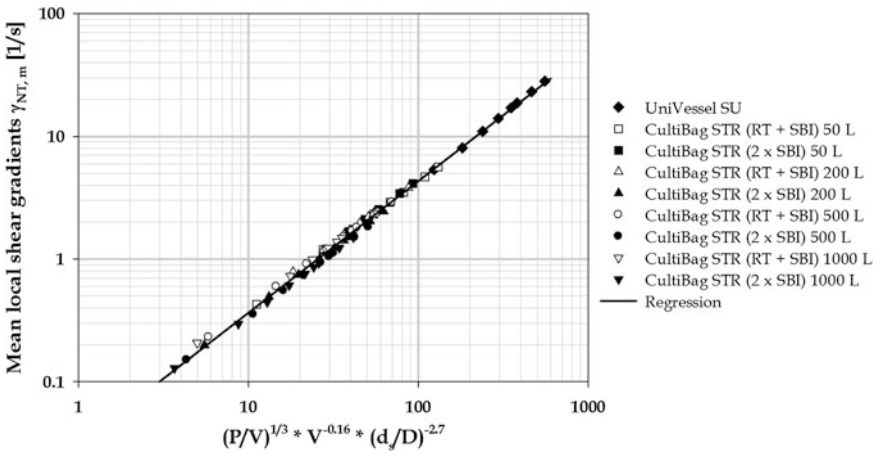

Fig. 10 Mean local shear gradients as a function of (30) for all single-use bioreactors investigated

well known that highly localized regions of high-energy dissipation exist and that local flow structures strongly depend on the vessel geometry and operating conditions. These local flow characteristics cannot be described adequately by global scale-up parameters. Therefore, engineering characterization is required, which consists of spatially resolved data obtained from experimental [193] and numerical methods. In order to turn the scaling-up of single-use bioreactors into a describable and understandable process, numerical techniques are increasingly being introduced in scale-up studies [204]. However, scaling up of bioreactors and processes remains a challenge and is "as much an art as a science" [205] and therefore, extensive know-how is presupposed [206].

6 Conclusions and Outlook

In this review, instrumented, commercially available single-use bioreactors from benchtop up to m^3 scale have been presented. Single-use bioreactors with entirely new working and aeration principles, such as wave-mixed, orbitally shaken, vibrating disk and rotatory oscillating systems, have established themselves during the past decade. However, the trend is moving more towards the development of bioreactors that are similar to conventional glass or stainless steel bioreactors and take mass transfer and power input from stirrers into account. This trend is independent of application and includes cell expansions, antibody and vaccine production, and manufacturing of secondary metabolites used in cosmetics.

The availability of bioengineering data speeds up the processes of selecting the most suitable single-use bioreactor type, defining process optimization parameters and scaling-up. In addition, it makes comparison with other single-use bioreactors

and their traditional counterparts possible, as shown for the UniVessel SU and the BIOSTAT CultiBag STR versions. In addition to established experimental methods, modern techniques such as CFD and PIV have become increasingly important. Their application has led to a reduction in experimental effort, time, and costs as well as ultimately contributing to more rapid product development and manufacturing.

Acknowledgments The results presented are part of a PhD thesis. The authors are grateful to Dipl.-Ing. Ute Husemann, Dr. Gerhard Greller, Dipl.-Ing. Jacqueline Herrman and Dr. Alexander Tappe, from Sartorius Stedim Biotech for providing geometric details for the bioreactors under investigation and experimental results for the BIOSTAT CultiBag STR, as well as for their participation in many helpful discussions.

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Adv Biochem Eng Biotechnol (2014) 138: 45–60
DOI: 10.1007/10_2013_188

Published Online: 20 April 2013

Orbitally Shaken Single-Use Bioreactors

Wolf Klöckner, Sylvia Diederichs and Jochen Büchs

Abstract Orbitally shaken single-use reactors are promising reactors for upstream processing, because they fulfill three general requirements for single-use equipment. First, the design of the disposable parts is inherently simple and cost-efficient, because no complex built-in elements such as baffles or rotating stirrers are required. Second, the liquid distribution induced by orbital shaking is well-defined and accurately predictable. Third, the scale-up from small-scale systems, where shaken bioreactors are commonly applied, is simple and has been successfully proven up to the cubic meter scale. However, orbitally shaken single-use reactors are only suitable for certain applications such as cultivating animal or plant cells with low oxygen demand. Thus, detailed knowledge about the performance of such systems on different scales is essential to exploit their full potential. This article presents an overview about opportunities and limitations of shaken single-use reactors.

Keywords Animal cell culture · Hydromechanical stress · Orbitally shaken · Out-of-phase · Oxygen transfer · Power input · Scale-up

Contents

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1 Introduction

Orbitally shaken bioreactors are widely used for small-scale screening and process optimization. Complex mechanical and electronic parts such as the shaker drive, the power train, and the control unit are integrated in the shaker and, therefore, separated from the reactor vessel. This allows a simple and cost-efficient reactor design, important not only for many parallel experiments on a small scale but also crucial for single-use applications in general. Unlike shaken systems, stirred single-use reactors require a complex sealing of the stirrer shaft or a magnetic clutch for energy transmission that is disposed of with the bag-reactor after each cultivation [1].

The simple design and handling of shaken bioreactors has led to their wide acceptance for screening and process optimization. After the detection of optimized conditions and suitable strains on a small scale, the cultivation conditions have to be transferred to a larger production scale. However, the scale-up from a shaken bioreactor to a bubble-aerated stirred tank reactor requires detailed knowledge about the basic engineering characteristics of both systems. Consequently, extensive research has been conducted to determine suitable methods for the transfer of culture conditions from shaken to stirred tank reactors [2–5]. Nonetheless, problems may still occur due to differences in oxygen transfer, hydromechanical stress, aeration, mixing, power input and temperature control between both reactor types. These problems are partly avoided by using orbitally shaken large-scale bioreactors. It is obvious that a transfer of culture conditions is highly simplified when the same basic principle for mixing, aeration, and power input is applied during scale-up. But even if the same working principle is applied, changes in culture conditions resulting from an increased reactor scale need to be carefully considered. In particular, the maximum oxygen transfer capacity is reduced with increasing reactor size due to a reduced volumetric oxygen transfer area. The basic engineering parameters for utilizing orbitally shaken disposable bioreactors with volumes ranging from 50 mL up to 1,000 L are described in the following chapter.

2 Types and Scales of Orbitally Shaken Single-Use Reactor Systems

Different types of orbitally shaken single-use reactors for different scales are currently available on the market. Figure 1 illustrates the various sizes and shapes of some commonly used small-scale reactors. The TubeSpin® system (Techno Plastic Products AG), available for reactor volumes of 15, 50, and 600 mL, has been developed for optimizing cell culture processes. Small tubes with a volume of 15 or 50 mL allow more parallel experiments to be performed on one shaker compared to single-use Erlenmeyer flasks. In addition, the conical tube can be directly used for centrifugation during sample preparation. A comparison between

results obtained with CHO cells cultivated in 50 mL TubeSpin® reactors and conventional glass reactors was first described by Jesus et al. [6]. A comparable growth and antibody production was reported with both reactor types.

Single-use Erlenmeyer flasks made out of polycarbonate or polypropylene have a similar geometry to that of conventional glass flasks (see Fig. 1). However, the material properties of single-use flasks differ from the properties of borosilicate glass, commonly used for conventional flasks. The impact of material properties on the maximum oxygen transfer capacity is discussed in Sect. 3.2.

Cylindrical orbitally shaken vessels consisting of polycarbonate or polypropylene and with volumes of 5 to 50 L have been used to cultivate mammalian, plant and insect cells. These vessels usually have tube connectors on top for active aeration. Figure 2 depicts various rigid vessels and bag reactors that are used in scales from 10 to 200 L, whereby the recommended shaking parameters depend on culture requirements as described in Sect. 3.2.

Bag-reactors for orbitally shaken platforms are so far available with nominal volumes of 50 and 200 L (Sartorius Stedim Biotech). As Fig. 3 shows, shakers with special bag holders are required to operate them. The OrbShake SB 200-X bioreactor system was developed by the company Kühner, Birsfelden, Switzerland, in cooperation with Lausanne. Engineering parameters for the application of orbitally shaken bag-reactors are discussed in Sect. 3.

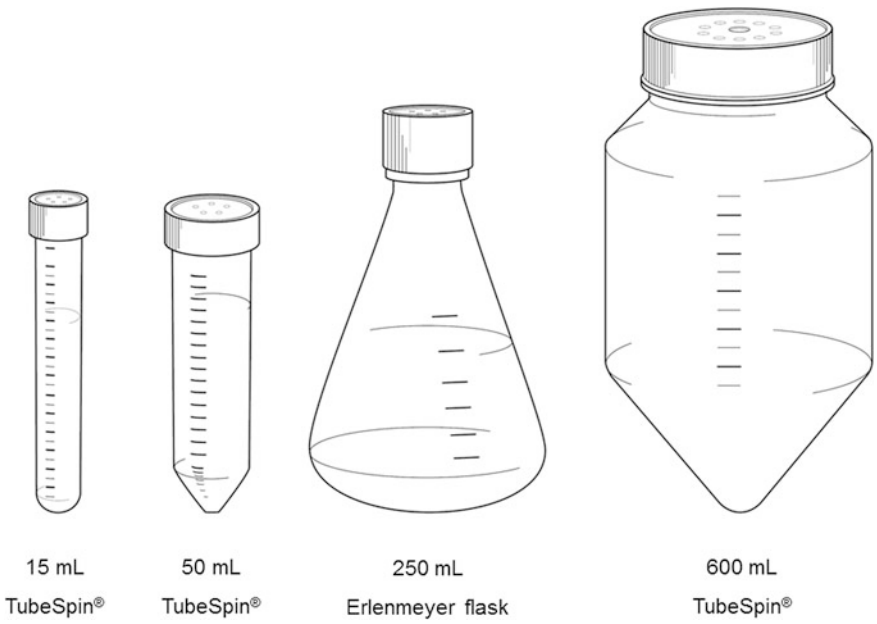

Fig. 1 Examples for orbitally shaken single-use reactor systems with volumes from 15 to 600 mL

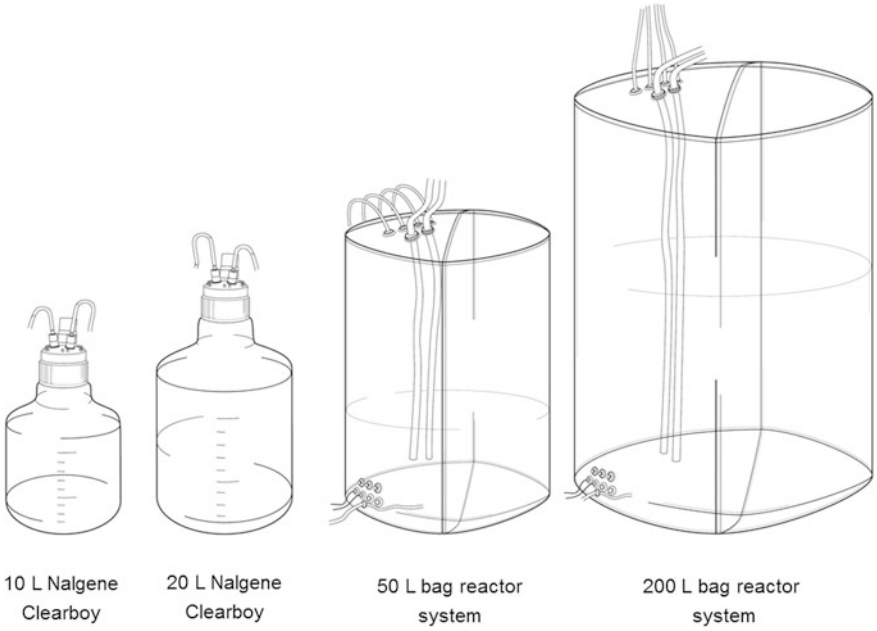

Fig. 2 Examples for orbitally shaken single-use reactor systems with volumes from 10 to 200 L

3 Engineering Parameters of Orbitally Shaken Single-Use Reactors

To choose the right cultivation conditions, it is important to know the fundamental engineering parameters of a bioreactor such as power input, hydromechanical stress, oxygen transfer and mixing performance. The following sections discuss the parameters for correctly applying orbitally shaken reactors.

3.1 Liquid Distribution, Power Input, and Hydromechanical Stress

The well-defined and homogeneous liquid distribution during shaking is a key benefit of orbitally shaken single-use reactors. Reproducible flow conditions are required for a detailed characterization and application of the system. A circulating liquid flow is induced during the shaking process, whereby the liquid follows the direction of the centrifugal force during one in-phase rotation. A balance between centrifugal force and gravitational force leads to a liquid distribution with the shape of a rotational paraboloid as previously described for shake flasks [7]. The typical liquid distribution for water-like viscosities in a cylindrical shaken reactor is shown in Fig. 4.

Fig. 3 Commercially available OrbShake SB 200-X and SB 50-X bioreactors (with kind permission of Kühner AG, Birsfelden)

By assuming that frictional forces are negligible for liquids with water-like viscosities, it follows that the pressure in the liquid is isotropic. This implies that the hydrostatic pressure p_{hydr} in the liquid is equal to the pressure induced by centrifugal acceleration p_{cent}:

$$p_{\mathrm{hydr}} = \int_{h_0}^{h} g \cdot \mathrm{dz} = p_{\mathrm{cent}} = \int_{0}^{r} r \cdot (2 \cdot \pi \cdot n)^2 \cdot \mathrm{dr}. \tag{1}$$

The gravitational acceleration in Eq. (1) is denoted by g and the shaking frequency by n. Geometric variables in Eq. (1) are defined as shown in Fig. 4. The liquid height in a cylindrical shaken bioreactor [7] follows from Eq. (1):

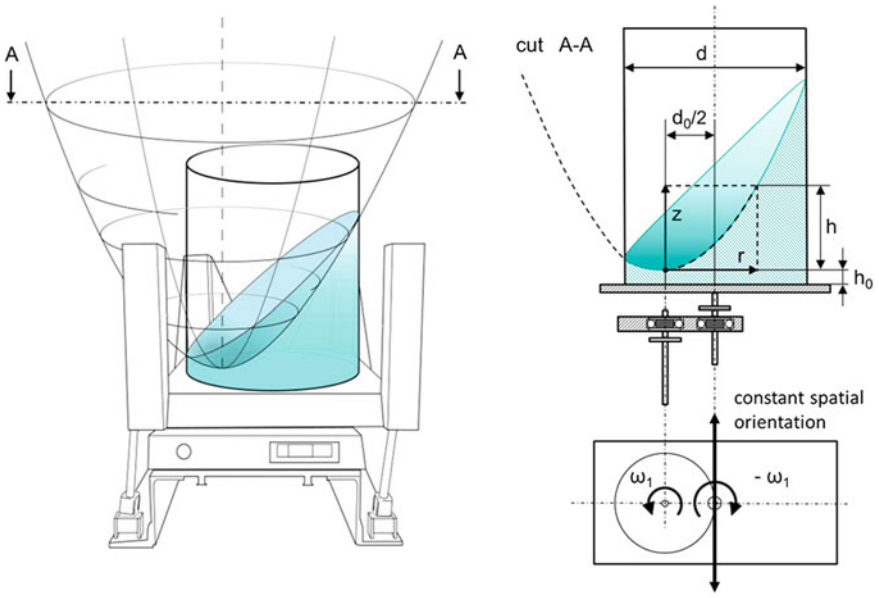

Fig. 4 Liquid distribution of a liquid with water-like viscosity during orbital shaking in a cylindrical single-use reactor

$$h = \frac{r^2 \cdot (2 \cdot \pi \cdot n)^2}{2 \cdot g} + h_0 \tag{2}$$

An accurate calculation of the power transfer and gas transfer areas during shaking can be realized with a mathematical model on the basis of Eq. (2) (manuscript in preparation).

Power is transferred during shaking due to friction between the rotating liquid bulk and the cylindrical reactor wall. Different measurement systems for determining the average power input ($P/V_Ø$) in cylindrical bioreactors have been described. A simple and effective determination of $P/V_Ø$ can be realized with a torque sensor integrated in the shaker drive [8, 9]. This method also allows online monitoring of $P/V_Ø$ during biological cultivations. However, integrating a torque sensor in the shaker drive is complex and requires a redesign of the shaker drive. This can be avoided by using a temperature method for determining $P/V_Ø$ in large-scale bioreactors. Moreover, this method only requires online monitoring of the liquid and surrounding air temperature during a cooling-down process [10]. A comparison between values measured with a torque sensor and values calculated with the temperature method showed comparable results for both techniques [11]. An extension of the temperature method allows one to consider the influence of viscosity changes on heat losses over the reactor wall [12]. Values for $P/V_Ø$ in cylindrical shaken single-use reactors range from 50 W/m^3 to 2 kW/m^3 depending on the filling volume, shaking frequency, and liquid viscosity. Figure 5 presents

the measurement values for $P/V_{Ø}$ in a 10 and 20 L vessel. In addition, a scale- and volume-independent correlation for $P/V_{Ø}$ in orbitally shaken reactors was recently developed for reactor volumes of up to 2,000 L [9].

Furthermore, hydromechanical stress in shaken bioreactors was investigated in several research studies using Erlenmeyer flasks [13, 14]. Here, maximum stable drop-size measurements were conducted in coalescence inhibited liquid–liquid two-phase systems in order to determine the ratio of maximum local energy dissipation (ε_{max}) to volumetric energy dissipation ($\varepsilon_{Ø}$). Values for $\varepsilon_{max}/\varepsilon_{Ø}$ ratios in Erlenmeyer flasks were between one and seven and therefore about ten times lower compared to the ratios determined in stirred tank reactors [13, 14]. The evenly distributed energy dissipation in orbitally shaken bioreactors leads to lower levels of hydromechanical stress compared to that of stirred tank reactors at the same level of volumetric power input. The evenly distributed energy dissipation is attributed to the fact that the size of the reactor wall (that acts as a power introducing element in orbitally shaken reactors) is much larger than the size of a stirrer in conventional bioreactors relative to the reactor liquid volume [13]. However, differences between the conical glass wall in Erlenmeyer flasks and the cylindrical plastic wall in single-use bags might lead to differences in hydromechanical stress between both systems. This influence has not been described in the literature up to now.

3.2 Aeration and Maximum Oxygen Transfer Capacity

The applicability of orbitally shaken single-use reactors for aerobic bioprocesses highly depends on their potential to deliver a sufficient amount of oxygen to the

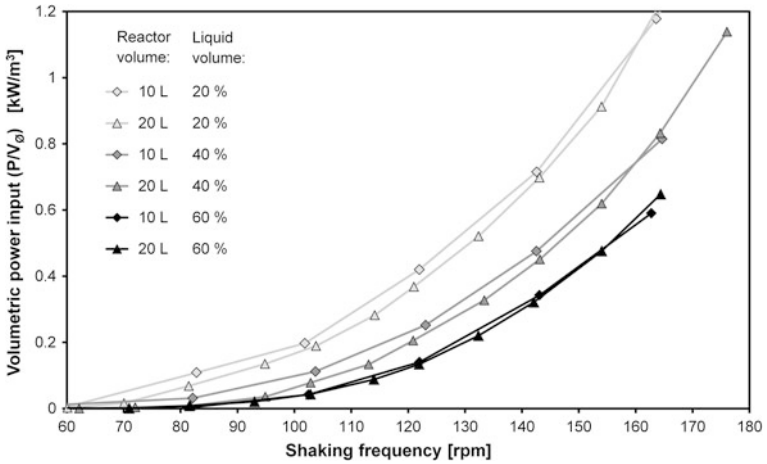

Fig. 5 Volumetric power input ($P/V_{Ø}$) in cylindrical orbitally shaken reactors (Nalgene Clearboy); measured with a torque sensor using water at 25 °C; diameter of the 10 L reactor = 25 cm; diameter of the 20 L reactor = 28.6 cm; shaking diameter = 5 cm

cells. The first quantitative characterization of the oxygen transfer in orbitally shaken single-use reactors was reported in 2008 [15]. Comparatively low values for the oxygen transfer coefficient (k_La) of between 1 and 30 1/h were reported for conventional cylindrical vessels. A three- to five-fold increase in the oxygen transfer was achieved with a helical track attached to the inner wall of the cylindrical reactor [15]. However, there is a trade-off between the benefit of a higher oxygen transfer rate and the increased production costs for reactors with integrated helical track. Increased values for k_La were also reported for square and baffled reactor systems compared to non-baffled cylindrical reactors, but the improved mass transfer characteristics were accompanied by an inhomogeneous and undefined liquid flow that might hamper scale-up [15]. The influence of different shaking frequencies and filling volumes on k_La values in cylindrical orbitally shaken reactors was investigated in scales from 0.05 to 1,000 L. Sufficient oxygen transfer for cultivating mammalian cells was realized in culture volumes of up to 1,000 L [16]. The influence of different filling volumes, shaking frequencies and liquid properties on k_La values in cylindrical orbitally shaken reactors was recently investigated in scales from 50 mL to 200 L (manuscript in preparation).

Values for k_La of between 7 and 10 1/h are regarded as necessary in order that a sufficient amount of oxygen be delivered for cultivating mammalian cells [17, 18]. The dissolved oxygen tension (DOT) in the liquid phase changes during a normal batch-cultivation with constant aeration and agitation according to the cell density of the culture. A changing DOT has no influence on aerobic cell growth as long as oxygen is available in the liquid phase at a sufficient level and diffusion between liquid phase and cell wall is not hampered (e.g. due to cell aggregation, biopolymer production or filamentous growth). Consequently, the k_La value has no influence on cell growth as long as oxygen is available in the liquid phase at a nonlimiting level. However, a constant k_La value has been recently reported as an adequate means to keep the pH level constant during scale-up [18, 19]. The described effect is most likely caused by similar levels of dissolved carbon dioxide (CO_2) and not related to oxygen transfer. CO_2 transfer between the gas and liquid phase is much faster than oxygen transfer due to the higher solubility of CO_2 in aqueous solutions. In contrast to oxygen transfer, equilibrium conditions between gas and liquid phases usually prevail for CO_2. Thus, the dissolved CO_2 concentration is mainly affected by the ventilation rate and not by the k_La value. This was recently shown in large-scale reactors for CHO cell cultivation where three- to four-fold increased CO_2 removal rates were achieved at a constant k_La value only by increasing the ventilation rate [20]. Hence, it is advisable to use a constant volumetric ventilation rate and not a constant k_La value to avoid pH shifts during scale-up of mammalian cell cultivations. Nevertheless, it is important to ensure that the k_La value is high enough during scale-up to prevent oxygen limitations, but solely a constant k_La is not a sufficient scale-up criterion.

In small-scale cultivations, k_La values for the 50 mL TubeSpin® system were reported in a range from 2 to 40 1/h depending on the filling volume and shaking frequency [21]. Figure 6 shows the pattern of the oxygen transfer rate (OTR) during a cultivation of *Nicotiana tabacum* BY-2 cells in 50 mL TubeSpin®

reactors using RAMOS [22, 23]. In the graph, the OTR in a conventional 250 mL shake flask was added as reference cultivation. Different filling volumes (V_L) were used in the TubeSpin® system to investigate different levels for the maximum oxygen transfer capacity (OTR_{max}). All cultures reached an oxygen limitation with the applied shaking frequency of 180 rpm as indicated with the dotted lines in Fig. 6. OTR_{max} levels are in the expected range according to reported k_La values for TubeSpin® reactor system [21]. Even if the oxygen supply at 180 rpm is not high enough to cultivate plant cells at the adjusted filling volumes without oxygen limitation, the OTR_{max} is still sufficient for cultivating mammalian cells. The results show that a detailed characterization of small-scale single-use systems is necessary to prevent unsuitable cultivation conditions during screening.

The oxygen transfer characteristics of conventional Erlenmeyer flasks were characterized in detail in different studies [24–26]. The rotating bulk liquid generates a thin liquid film on the hydrophilic glass wall that strongly contributes to the total oxygen transfer capacity [25]. This effect is reduced in single-use flasks made of polypropylene or polycarbonate due to the hydrophobic surface properties of the materials. Figure 7 shows a comparison between k_La values measured in flasks made of polycarbonate and those measured in conventional glass flasks. Values were determined by employing the Respiration Activity Monitoing System (RAMOS) using a sulfite oxidation reaction in the liquid phase to reduce the dissolved oxygen concentration [27]. Measured k_La values at 37 °C with the sulfite oxidation reaction are higher than values measured with medium for insect cell cultivation at 27 °C [28]. This was expected, as the diffusion coefficient for oxygen and thereby the k_La increases with increasing cultivation temperature. Oxygen supply in single-use flasks was about 30 % lower compared to oxygen transfer in conventional glass flasks (see Fig. 7). These significantly reduced k_La values in

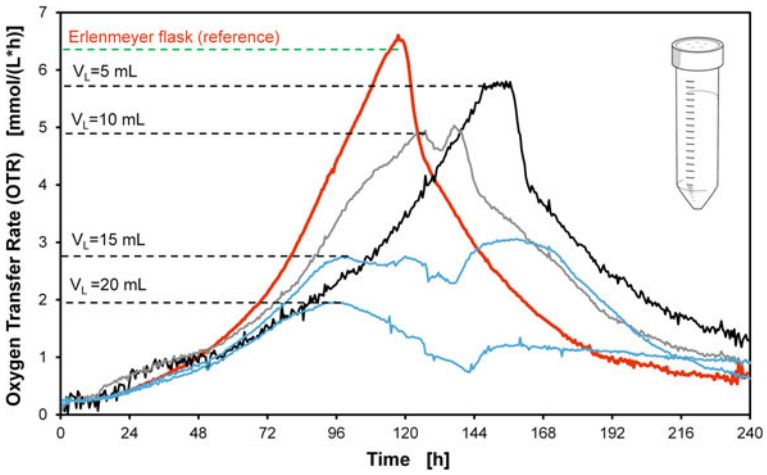

Fig. 6 Cultivation of *Nicotiana tabacum* BY-2 cells in Murashige and Skoog medium in 50 mL TubeSpin® reactors at a shaking frequency of 180 rpm with a shaking diameter of 5 cm

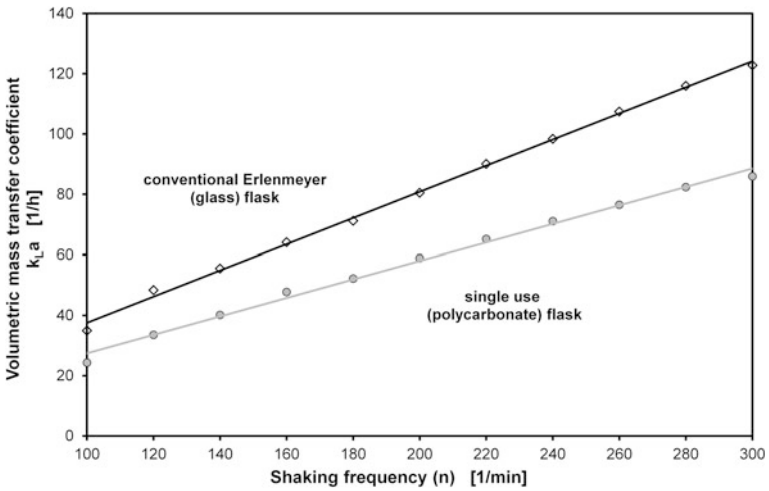

Fig. 7 Comparison of the volumetric mass transfer coefficient (k_La) in conventional glass flasks and single-use polycarbonate flasks; flask volume = 250 mL; filling volume = 50 mL; temperature = 37 °C; shaking diameter = 5 cm; sulfite solution with 0.5 M Na_2SO_3; 10^{-7} m $CoSO_4$; 0.1 M phosphate buffer; initial pH = 8; oxygen solubility Lo_2 = 0.65 mmol/(L · bar)

single-use flasks make them unsuitable for cultivating aerobic yeast or bacteria cells with a high oxygen demand. However, k_La values in single-use flasks are still high enough for cultivating mammalian or insect cells even at moderate shaking frequencies.

3.3 Mixing Performance and Out-of-Phase Operation

The first detailed characterization of the mixing performance in cylindrical orbitally shaken bioreactors with volumes ranging from 2 to 1,500 L was reported by Tissot et al. [29]. A calorimetric method was used to determine mixing times for different shaking frequencies and filling volumes. The best mixing efficiency was generated close to the reactor wall. Longer mixing times were reported in the center of the bulk liquid [29]. These findings concur with the fact that power transfer in shaken bioreactors occurs between the liquid bulk and the reactor wall. Consequently, local power input and mixing properties are at a maximum close to the wall and decrease with increasing distance from the wall. A comparison of mixing times in a cylindrical 30 L reactor using shaking diameters of 2.5 and 5 cm showed significantly higher mixing times with the smaller shaking diameter of 2.5 cm below frequencies of 115 rpm. Similar mixing times were detected for both shaking diameters for frequencies over 115 rpm [29]. This observation can be attributed to the critical shaking frequency that has to be exceeded to induce a circulating liquid flow in the reactor. The critical frequency describes the minimal

required shaking frequency to overcome inertial forces and to provoke liquid motion in the reactor [9]. An increase in the shaking diameter leads to a decrease in the critical frequency. This phenomenon is not comparable with the occurrence of out-of-phase operation conditions. In contrast to the critical frequency, the out-of-phase phenomenon occurs during the shaking process (also at high shaking frequencies) and leads to a breakdown of liquid motion. This phenomenon has been extensively characterized for shake flask bioreactors [30, 31]. Out-of-phase operation in orbitally shaken reactors is associated with a strong decrease in mixing performance, oxygen transfer, and power input. A comparison between the liquid distribution during in-phase and out-of-phase operation in cylindrical bioreactors is shown in Fig. 8.

As shown in Fig. 8, different shaking diameters were used with otherwise equal conditions. A metal ball rotating in a glass flask on the right side of the shaker thereby indicates the direction of the centrifugal force. With a shaking diameter of 2.5 cm, the liquid is oriented in the direction of the centrifugal force, thereby indicating in-phase operation (Fig. 8a). Here the liquid is evenly distributed, providing a large mass transfer area between gas and liquid phase. By contrast, with a shaking diameter of 1.25 cm and otherwise equal operating conditions, out-of-phase operation was observed (Fig. 8b). In this case, the liquid is no longer oriented in the direction of the centrifugal force, as indicated by the black rotating ball on the shaker. The strong reduction in the mass transfer and power transfer area triggers significantly lower mixing, power input, and oxygen transfer properties.

Out-of-phase operation occurs when frictional forces exceed centrifugal forces during shaking [30]. The most effective way to prevent out-of-phase operation is to

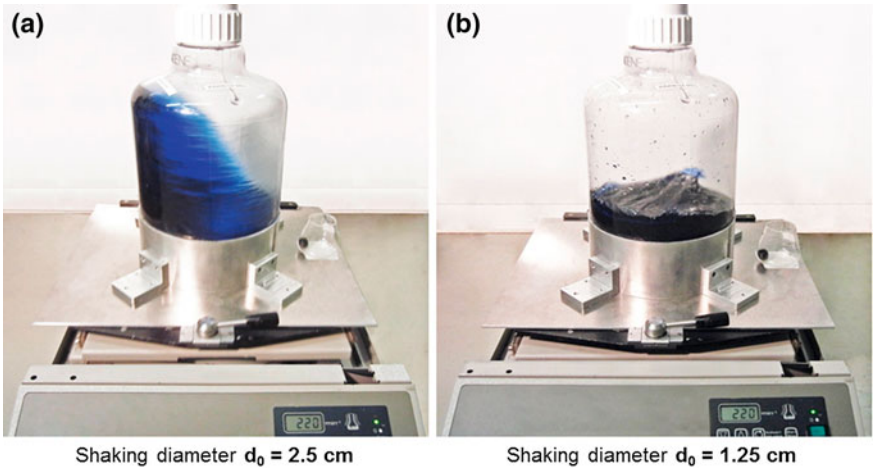

Fig. 8 Comparison between "in-phase" and "out-of-phase" operation in shaken cylindrical single-use reactors; reactor volume = 10 L; reactor diameter = 25 cm; filling volume = 2.5 L; shaking frequency = 220 rpm; dynamic viscosity = 0.984 mPas

increase the ratio between shaking and reactor diameter as demonstrated in Fig. 8. The influence of out-of-phase operation on volumetric power input ($P/V_{Ø}$) and mass transfer (k_La) is presented in Fig. 9. Please notice that a filling volume of 5 L was used for the measurements in Fig. 9 instead of the 2.5 L that were used in Fig. 8. Values for k_La were determined with a RAMOS for cylindrical reactors using a sulfite oxidation reaction in the liquid phase to reduce the dissolved oxygen concentration [27]. Furthermore, power input was measured with a torque sensor integrated in the shaker drive [9]. An abrupt decrease in the $P/V_{Ø}$ and k_La values was detected when the reactor with a shaking diameter of 1.25 cm reached out-of-phase conditions at 220 rpm. As previously described for shake flasks, a higher filling volume leads to a later occurrence of out-of-phase conditions [30]. Thus, at a shaking diameter of 1.25 cm and a shaking frequency of 220 rpm the reactor system with 5-L filling volume is still in-phase (Fig. 9), whereas the bioreactor with a 2.5 L filling volume is already out-of-phase (Fig. 8b). Reduced power input, mixing performance, and oxygen transfer combined with chaotic and non-reproducible cultivation conditions are all well-known characteristics of out-of-phase operation conditions in shake flasks [32]. Therefore, an adequate dimensioning of the shaking diameter according to the reactor scale is essential to prevent out-of-phase operation in orbitally shaken bioreactors.

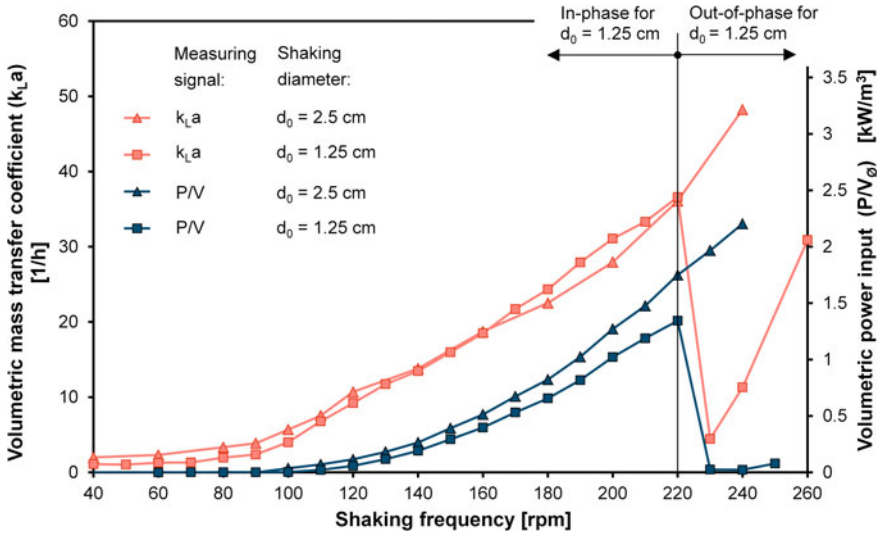

Fig. 9 Values for $P/V_{Ø}$ and k_La during "in-phase" and "out-of-phase" operation in a shaken single-use reactor; reactor volume = 10 L; reactor diameter = 25 cm; filling volume = 5 L; temperature = 25 °C; dynamic viscosity = 1.554 mPas; sulfite solution with 1 M Na_2SO_3; 10^{-7} M $CoSO_4$; 0.012 M phosphate buffer; initial pH = 8; oxygen solubility Lo_2 = 0.56 mmol/(L · bar)

4 Applications of Orbitally Shaken Single-Use Reactors

The first application of orbitally shaken single-use reactors was reported by Liu and Hong [33] for cultivating insect and animal cells. They monitored the number of viable cells during cultivation using orbitally shaken vessels in different scales and compared the results with values from a stirred tank reactor. For the first time, the scale-up from shake flasks to cylindrical shaken single-use bioreactors with culture volumes of up to 36 L had been successfully proven in this work.

The general suitability of orbitally shaken bioreactors for cultivating *Nicotiana tabacum* BY-2 cells growing in suspension was proven in cylindrical reactors with volumes of 20 and 50 L [34]. The successful utilization of square bottles for cultivating mammalian cells was first described by Muller et al. [35]. Comparable yields were reported between cultivations in square bottles of different size and cultivations in spinner flasks [35]. The first application and validation of the 50 mL TubeSpin® system for cultivating animal cells was reported by Jesus et al. [6]. Experiments were conducted with sealed and open ventilation membranes to investigate the influence of different ventilation rates on evaporation, pH and dissolved oxygen concentration. A sufficient oxygen supply and CO_2 removal rate was reported even for tubes that were entirely closed during a cultivation time of 4 days [6].

The successful application of the TubeSpin® system for the cultivation of mammalian cells was proven in several studies [36, 37]. Characteristics of the reactor system such as the cost-efficient design and easy handling make them suitable for a large number of parallel screening experiments. Consequently, the influence of 29 different cultivation media and 20 protein hydrolysates on growth and productivity of a CHO cell culture was investigated with the TubeSpin® system [37]. The effective application of the system for transient gene expression with CHO cells was also recently proven. Similar protein yields in the TubeSpin® system compared to standard stirred tank reactors were reported [36].

5 Conclusion and Outlook

Within the past 10 years, orbitally shaken single-use reactors have developed from the first proof of concept to established systems for upstream processing. Today, reactors are available in volumes ranging from 15 mL to 200 L, and the basic working principle has been substantiated up to reactor volumes of 2,000 L. Fundamental engineering parameters such as oxygen transfer, power input, mixing performance and hydromechanical stress have been investigated in several research studies. In addition, the applicability of orbitally shaken single-use reactors for cultivating animal, insect and plant suspension cells has been demonstrated on different scales. A major advantage of shaken single-use reactors compared to systems with a wave, rocking or stirred agitation is the very well-

defined liquid movement in the reactor and the fact that orbitally shaken bioreactors are commonly applied for screening and media optimization in small-scale systems. Transferring culture conditions from shake flasks or microtiter plates to orbitally shaken single-use reactors is greatly simplified due to similar characteristics with respect to hydromechanical stress, mixing and oxygen supply. The commonly accepted advantages of shaken bioreactors for small-scale systems such as simple and cost-efficient reactor design, easy handling and low hydromechanical stress are also essential requirements of single-use reactors. Despite the effort that has already been expended on characterizing shaken single-use reactors, further investigations are needed to exploit their full potential. In particular, a more detailed description of the fluid flow properties during shaking would be advantageous to allow a precise characterization of hydromechanical stress and out-of-phase operation. Nevertheless, orbitally shaken single-use reactors are already today a serious option.

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Adv Biochem Eng Biotechnol (2014) 138: 61–97
DOI: 10.1007/10_2013_233

Published Online: 10 August 2013

Therapeutic Human Cells: Manufacture for Cell Therapy/Regenerative Medicine

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Abstract Human primary cells (e.g. adult stem cells) as well as differentiated cells, including those of the immune system, have been found to be therapeutically useful and free of ethical concerns. Several products have received market authorization and numerous promising clinical trials are underway. We believe that such primary therapeutic cells will dominate the market for cell therapy applications for the foreseeable future. Consequently, production of such cellular products warrants attention and needs to be a fully controlled pharmaceutical process. Thus, where possible, such production should change from manufacture towards a truly scalable industrialized process for both allogeneic and autologous products. Here, we discuss manufacturing aspects of both autogeneic and allogeneic products, review the field, and provide historical context.

Keywords Allogeneic · Autologous · Immunity · Manufacture · Stem cells · Therapeutic cells

Abbreviations

ADSCs	Adipose-derived stem cells
ATMPs	Advanced therapeutic medicinal products
API	Active pharmaceutical ingredient
BSE	Bovine spongiform encephalopathy
CF	Cell factory

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CQAs	Critical quality attributes
CPD	Cumulative population doublings
CF	Cell factory
CS	Cell stack
CTP	Cell therapy product
cGMP	Current good manufacturing practice
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
flt-3L	Fms-related tyrosine kinase 3 ligand
GFs	Growth factors
GMCSF	Granulocyte monocyte colony stimulating factor
GMP	Good manufacturing practice
HSA	Human serum albumin
HS	Hyperstack
HSCs	Hematopoietic stem cells
hMSCs	Human mesenchymal stem cells
IDO	Indole amine oxygenase
IL	Interleukin
IPC	In process control
LOD	Limits of detection
MLR	Mixed lymphocyte reaction
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
MoA	Mode of action
mRNA	Messenger ribonucleic acid
NK	Natural killer cell
PGE	Prostaglandin
QC	Quality control
RPM	Revolutions per minute
SCF	Stem cell factor
TCR	T cell receptor
TFF	Tangential flow filtration
TILS	Tumor infiltrating lymphocytes
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TREGS	T regulatory cells

Contents

1 Introduction

The public debate around stem cells and their medical application focuses largely on embryonic stem cells and their assumed potential in therapeutic settings. Similarly, the field of induced pluripotent stem cells has recently been highlighted by the award of the Nobel Prize in Medicine [1]. Comparatively little attention is directed towards the field of adult stem cells or other primary cells used for therapeutic purposes. This is somewhat puzzling because human primary cells—such as adult stem cells; endothelium, muscle, and skin cells; and cells of the immune system—have been found to be useful, easy to generate, and free of ethical concerns. In addition, several products have received market authorization

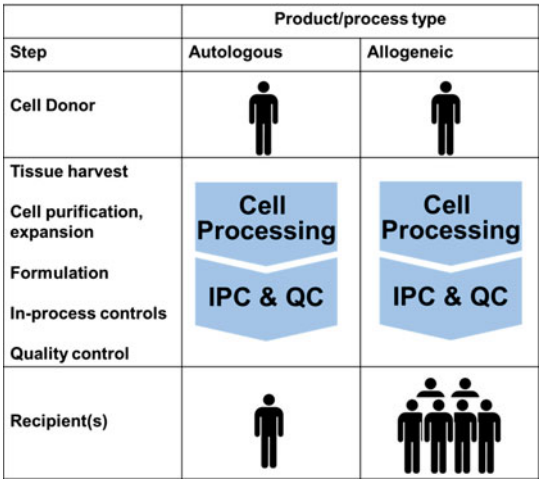

Fig. 1 Allogeneic/universal donor approaches permit the administration of the same product lot to many individuals. Hence, these approaches require large batches and, in turn, distribute in-process testing (IPC) and quality control (QC) expenditure across a large number of product doses, thus rendering this approach economically favorable. In contrast, autologous/patient specific approaches are subject to very similar testing but have to absorb this expenditure into every single product dose. For the latter, the tissue donor and recipient of the final product are the same individual and the batch size equals 1. However, allogeneic/universal donor products are derived from one donor and may be administered to hundreds or thousands of recipients. Certain cells, such as human mesenchymal stem cells, appear to permit allogeneic treatment; others cells, such as certain products based on immunocells, do not. Manipulation steps for an autologous therapy may be similar in scope as for an allogeneic therapy; however, because they target only a single recipient, they are much smaller in scale. The similar testing requirements for the individual dose and for an entire batch of doses makes autologous products more expensive (per dose) than allogeneic products, for which economies of scale apply and testing of representative doses occurs instead of consuming part of each dose

and numerous promising clinical trials are underway. Hence, we believe that primary therapeutic cells will dominate the market for cell therapy applications for the foreseeable future and therefore make such cells the focus of this review.

In cell therapy and, in the wider sense, within regenerative medicine, living human cells are applied to specific disease scenarios and are found to provide clinical benefits. To convert early approaches into true pharmaceutical manufacture, significant quantities of cells have to be manufactured under suitable conditions (Fig. 1). Because the vitality of cells administered is crucial, the final active pharmaceutical ingredient (cells) cannot be terminally sterilized. Thus, as with most biological therapeutics, the entire manufacturing process requires developing aseptic procedures. A further complication arises from the fact that cell expansion technologies developed for bioproduction of antibodies and proteins cannot be translated immediately. First, the biology of therapeutically used cells exerts requirements different from those of industrial production cell lines. In addition, at the end of a bioproduction process, cells are typically considered waste; therefore,

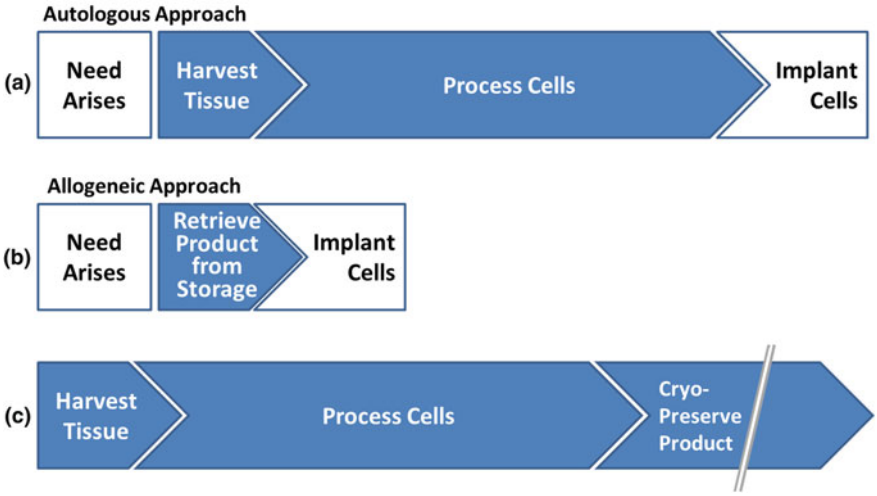

Fig. 2 Allogeneic products are available virtually instantaneously, whereas autologous ones are not. Consequently, the former can be applied in emergency situations—something that might be difficult for the latter. Autologous products typically are manufactured once a need arises, hence manufacture/processing is in the critical path (**a**). In contrast, allogeneic products have to be merely retrieved from storage and thus are available virtually instantaneously (**b**). Allogeneic products, because the recipient does not have to be known in advance, can be manufactured and stored when convenient; hence, manufacture/processing can be uncoupled from need/use (**c**)

downstream processing steps are designed to dispose of them rather than to preserve them. Finally, certain therapeutic indications may require highly specialized cells that, in turn, require highly specific processes. Thus, the manufacture of living cells as pharmaceuticals provides numerous challenges, many of which differ substantially from those posed by bioproduction processes discussed elsewhere in this volume.

One might distinguish two approaches: (1) cell therapy based on mass produced allogeneic cells and (2) therapies based on autologous cells, which are typically harvested, manipulated, and administered to the same individual (Fig. 2).

2 Autologous Therapies

For autologous therapies, cells from one individual are removed, treated, expanded in culture when needed, and finally returned to the same individual (i.e. donor and recipient are one and the same individual). Approaches can use cells from many tissues (both healthy and even cancerous). New therapies for battling cancer in an individual use a patients' biopsy and cell culture expansion; the cells are then killed but the process protects their ability to stimulate the patients' immune system to target and eliminate cancer cells still in body tissues [2]. Therapies with

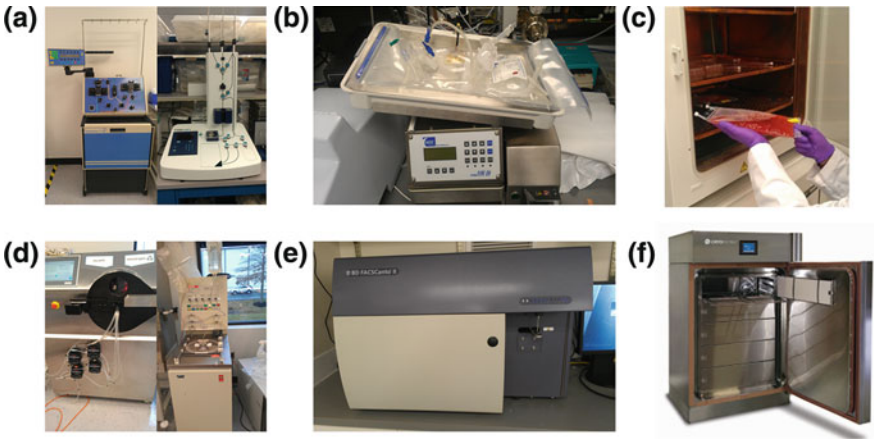

Fig. 3 Manufacturing equipment commonly used in autologous cell therapy. With each patient being its own "lot", manufacturing processes cannot be scaled up, only scaled out more in the same equipment. **a** Isolation using the closed systems Terumo Elutra (*left*) and Miltenyi CliniMACS® (*right*); **b** Closed culturing using GE wave system cellbags; **c** Closed culturing with AFC VueLife bags, grown in an incubator; **d** Downstream cell concentration with a KSEP (*left*) or Terumo COBE (*right*) centrifuge; **e** Analytical methods using the BD fluorescence activated cell sorting Canto II; **f** Cryopreservation with controlled rate freezer

healthy cells intended to restore system functionality in the patient have a longer history in medical practice and are better known. For historical reasons, transplanting blood systems or parts thereof is particularly well understood; hence, we provide examples of autologous approaches focusing on cells of the blood system.

A difficulty for autologous therapies is economics, because any production-associated costs (e.g. quality control testing), cannot be dispersed over large batches. By their very nature, batch sizes of autologous therapies are small ($n = 1$). Thus conventional culture equipment may suffice quantitatively; however, the biology of the cells in question may well pose specific challenges (e.g. potency). Like allogeneic cell manufacturing, which can be scaled up, these cells have limited lifespans. However, because the cells are limited to reinfusion back into the same individual, they therefore can only be scaled out. In other words, producing iterations of smaller batches and associated challenges is the key to successful commercial autologous manufacturing. The most frequently used cells in clinical manufacturing of hematopoietic lineage are the progenitor $CD34^+$ hematopoietic stem cells (HSCs), dendritic cells (DCs), T-cells, and natural killer (NK) cells [3, 4]. Each subset has its own advantages and challenges, discussed below. In each of these cell types, manufacturing methods (Fig. 3) in early-stage clinical development are frequently expensive because legacy protocols from academic laboratories tend to not be focused on cost. Open manufacturing systems (well plates) are labor intensive, and the risk of contamination (failed lots), serum-containing culture medium (supply risk), and cumbersome processing equipment is not supportive of commercialization (e.g. centrifugation).

2.1 T-Cells

T-cells are one of the most versatile and powerful of the autologous cell therapy candidates. As the natural cell type responsible for host defense and eliminating cancers, they are also a clear choice as therapeutic products [4]. T-cells have antigen-specific receptors that can be modified or selected for, can expand rapidly, and can develop memory to be retained indefinitely in vivo. Of the various T-cell subsets, CD4 helper, CD8 killer, and T-regulatory cells (TREGs) are the most prominent in the field, although not exclusively. Although it is thought that CD8 killer T-cells are the most effective cell type for eliminating cancers, it appears that a mix of CD4 and CD8 has the best long-term potential [5]. TREGs (also $CD4^+$) are used not to destroy cells but to dampen the immune system in the case of autoimmunity [4].

Manufacturing methods for T-cell therapeutic products vary, depending on the requirements of dose and whether or not the cells are genetically modified. The Adoptive Cell Therapy method of Rosenberg involves the expansion of tumor-derived tumor infiltrating lymphocytes (TILs) to very large numbers using high doses of interleukin (IL)-2 (>1,000 IU/ml). These therapies have resulted in final cell product doses of 50–100 billion T-cells, expanded in culture over an average of 35 days [6]. In contrast, gene-modified T-cells have been able to achieve results with far fewer cells, such as those used by June [4]. A typical patient infusion in these cases may be 300 million, of which 15 million are positive for the transgene [7]. T-cells are not adherent and thus do not require trypsin to harvest, but they do require cell contact and are generally amenable to two-dimensional culture.

The culture of T-cells has most often been performed in plastic culture flasks, sometimes starting in well plates and then scaling up. Hyperflasks (Corning) and Cell Factories (Nalge) have also been used with success. These vessels are limited as to the amount of nutrients and space that can be provided, imposing constraints on the density of cells per milliliter that can be achieved, generally 2–3 million/ml but no more than 5 million/ml before cell viability suffers. An alternative culture vessel is recently developed is the GREX (Wilson Wolf), a cylindrical culture vessel with a gas permeable bottom, allowing the cells on its floor to receive adequate gas exchange while adding sufficient media to support prolonged growth [8]. Closed system alternatives suitable for Good Manufacturing Practice (GMP) and commercial manufacturing include gas permeable VueLife Teflon bags (American Fluorosis), and Wave Systems from GE Healthcare and Sartorius. These vessels are ideal for the culture of T-cells because they support rapid cell division with minimal manipulation. Feeding and splitting is not critical due to the higher volumes of media allowed; in addition, sterile sampling is possible through Lure lock ports. Wave Systems in particular can support very high levels of T-cell concentrations, even approaching 10 million/ml with proper perfusion and cell mixing through rocking, with 1- to 5-L cultures typical, although options up to 100 L are available. Mixing T-cells at rates above the 10 rocks per minute used in Wave System decreases viability possibly due to shear forces. Therefore,

traditional stir-tank bioreactors are not a feasible option for this cell type. Furthermore, primary T-cells have a limited lifespan and capacity to expand, imposing a constraint in the number of population doublings attainable from each patient. The maximum starting number of cells from apheresis is about 10^9 T-lymphocytes, whereas cells removed from a tissue such as a tumor may be as low as 100–1000. Culture times vary from 7–10 days to more than 5 weeks, depending on the type and number of starting cells. For unmodified cells, sufficient time is required to select antigen-specific cells or to deprogram anergy, tolerance, or quiescence. For gene-modified cells, fewer cells and less time are required, but there is also a complicated manipulation involved (e.g. retroviral transduction). For these cell ranges, multiple T-flasks or their multilayer versions, such as Cell Factories or Hyperflasks, can be used.

For the culture of T-cells, serum-free media have been demonstrated to be acceptable alternatives to the traditional Roswell Park Memorial Institute (RPMI) medium with 10 % serum; examples include XVIVO-15 and -20 (Lonza), Optimizer, AIMV (Life Tech), Stemline (Sigma), and TexMACS (Miltenyi). Removal of serum dramatically reduces overall cost (through reduced washing and release testing requirements) and regulatory risk; it also improves the supply chain risks. Additional large cost drivers include growth factors, for which GMP versions can be prohibitively expensive. Although GMP interleukin (IL)-2 Proleukin (Prometheus Labs) is used in stand-alone patient therapy, it has also been used as a culture supplement at high levels (3,000–6,000 IU/ml) that can exceed 50 % of the cost of the product. Current IL-2 levels are 100 IU/mL for moderate or gene-modified cells T-cell expansion. It is the unmodified cells such as TILS that require the much higher levels of growth factors (GFs; 3,000–6,000 IU/mL) to be able to expand cells to the enormous numbers required for clinical efficacy [9]. Effective downward titration of IL-2 has been successful ex vivo, but it has not been thoroughly explored clinically.

Two methods of cell activation are currently in widespread use: CD3/CD28-bound beads (Dynabeads, Life Tech), or feeder cells. Dynabeads are extremely potent T-cell activators that can induce considerable expansion of the population [10]. However, the beads must be removed before patient administration, and additional tests should be implemented to confirm this. In addition, there are cost, availability, and licensing considerations with the beads. Alternatively, feeder cells may be used to promote large-scale T-cell cultures. These feeders consist of a mixture of irradiated, allogeneic, and mononuclear cells pooled from 3–5 donors; they act to provide soluble and cell-contact dependent growth signals lasting for no more than a week. At that point, the T-cell culture can be transferred to a large-scale system, such as the Wave Bioreactor, and media can be perfused at a rate that can support rapid expansion [9]. Methods to generate artificial feeders are being explored. These cell lines can be modified to express customized growth factors and cell surface co-stimulators. If these are proven to be equivalent to the primary cells, they could be generated in large numbers and banked as a universal reagent, greatly reducing the cost of labor and supply. Addition of IL-7, -15, or -21 may impact the phenotype of the T-cell product, such that reduced overall levels of IL-2

or feeders can be used [11]. The target population will determine the exact cytokine cocktail and whether CD8, CD4, TREGs, or mixtures will in large measure determine the makeup of the media.

Tracking certain culture parameters, such as nutrient levels, can be helpful in monitoring the health and metabolism of the cell population, which is particularly critical given that donor-to-donor variability of autologous cell products increases process variability. Such monitoring might allow for real-time adjustments to media feeds or might drive decisions about harvest times. Closed system culturing allows for sterile and simple sampling to monitor such parameters including cell number, viability, and phenotype.

Downstream processing of T-cells shares many of the same challenges of other cell therapies and will be addressed generally and in greater detail below in Sect. 4. The formulation of T-cells has typically been performed by centrifugation, either traditional or continuous, incurring cell loss, scale-out bottlenecks, and contamination risk. Although many hematopoietic cell preparations have been prepared as fresh products (with a limited shelf life of hours to days and cool storage), there is increasing success with frozen storage formulation with many cell types. Most T-cell products have tolerated freezing upon harvest, and 5 % dimethyl sulfoxide (DMSO) as a commonly used cryoprotectant does not seem to negatively affect the quality of the phenotype. GMP formulations containing DMSO, such as Cryostor5 and 10 (Biolife Solutions), have improved cell health upon thaw after cryopreservation.

2.2 Dendritic Cells

Dendritic cell culture is both simpler than that of T-cells and yet more challenging. Culture times are shorter and less variable, with either 3 or 7 days as the industry standard. However, the cells differentiate and mature but do not expand. In addition, the required cell dose is much lower than for T-cell therapies, at 1×10^7 per dose on average and 3–5 individual doses administered [12]. Therefore, enough cells can be harvested from a single apheresis collection to satisfy a complete vaccination campaign as well as product release testing. Typically, peripheral blood mononuclear cells are obtained and used to isolate $CD14^+$ monocytes. A 5-day differentiation from these precursors in the presence of IL-4 and granulocyte monocyte colony stimulating factor (GM-CSF; typically 250–1,000 IU/ml each) is used to produce immature dendritic cells [13]. This is followed by 24 h of activation with one of several maturation "cocktails" (e.g. tumor necrosis factor [TNF]-alpha, IL-6, and prostaglandin 2), during or after which the antigen loading occurs. Tumor antigens may be universal or derived from patient biopsies; they may consist of peptides loaded directly into the major histocompatibility complex (MHC) grooves or proteins internalized, processed, and presented on the MHC. These "foreign" tumor antigens act in vivo to activate T-cell subsets following migration of the dendritic cell to the regional lymph

nodes. Following antigen pulsing, the dendritic cells are concentrated, formulated (with DMSO) and cryopreserved, stored, shipped, and administered as multiple vaccine doses.

Traditional culture methods rely on adherence of the monocyte precursors to plastic using media (RPMI) that contained 5–10 % serum. Nonmonocytes (i.e. lymphocytes and granulocytes) are removed after a 2-hour incubation, and the monocytes naturally detach as they differentiate into dendritic cells. Two-stack cell factories have been used most frequently, with about 1×10^6/ml starting $CD14^+$ monocytes in a volume of 300 mL. More recent, advanced clinical and commercial products have used closed system gas permeable bags, leveraging the lack of adherence requirements, and also typically start at 1 million cells/ml. Monocytes cultured in Teflon-coated bags will differentiate at high efficiency, with a yield of immature DCs exceeding 50 % and very low cell loss to the culture bag. The addition of maturation factors and antigen for the final 24 h are procedural variables that present opportunities for optimization. Several new protocols have been used to harvest mature DC after a 3-day combination culture, termed "Fast DC" [14]. It is not yet established whether these cells are as potent as traditional methods. Most advanced clinical and commercial DC products have switched to serum-free media (AIM V, X-VIVO 15, CellGro), which is not in all cases superior to RPMI/10 % serum; however, it is sufficient for manufacturing to achieve the required doses and quality parameters, while reducing testing costs and improving regulatory profile.

Optimization of DC manufacturing is challenging because measuring the phenotype (i.e. potency) involves elaborate methods. Downregulation of CD14 and upregulation of CD209, CD83, CD80, and CD86 indicate the identity, but a potency marker to determine whether the cells can initiate a T-cell response in vivo is more difficult. The flow phenotype of a mature DC is dramatically different from its monocyte precursor, and a robust method of analysis is required for accurate discrimination of manufacturing process change effects. The only licensed autologous product, Dendreon's DC product Provenge, uses expression of CD54 for potency. IL-12 or TNF-alpha secretion measured by enzyme-linked immunosorbent assay (ELISA) is a common measure of potency, but the ability to induce T-cell proliferation in vitro via a mixed leukocyte reaction (MLR) is also indicative of functional maturity [15]. MLR typically requires the use of T-cell responders from patients with cancer and access to an irradiator. Protocols for the addition of the antigen to the final cell product is also an evolving area. Traditional peptide loading has worked well to induce T-cell responses in vivo and is the most common method in traditional DC vaccines. However, newer methods of transfecting DNA or RNA have the potential to dramatically lower cost because GMP nucleic acid is much cheaper than GMP protein. The drawback is the open system nature of transfection and nucleofection. Advances in automation may help address this bottleneck and dramatically lower the cost of labor for this step [16].

2.3 Natural Killer Cells

Natural killer (NK) cells are another attractive cell type for autologous products [17]. NK cells are derived from peripheral blood and cultured at 0.5×10^6/ml in Wave Bags or other suitable vessels. The culture and expansion techniques of the NK cell are similar to those used for T-cells. However, NK cells lack the T-cell receptor (TCR) and cannot be expanded by CD3/CD28 ligation (i.e. stimulation with Dynabeads). Cytokines and growth factors seem to be sufficient to support their expansion. The precise combination of GFs should be carefully selected and tested because there are possible downregulatory or immunopathological aspects to the cell phenotype and effector functions. NK cells can be used allogeneically or autologously because they are not MHC-restricted and do not require a tissue type match to be safe and effective. The use of serum-free media (e.g. CellGro) has been reported, although this is often supplemented with 5 % serum. The addition of 500 IU/ml of IL-2 is usually sufficient to achieve up to 100-fold expansion. Additionally, supplementation of the culture with feeder layer cells can increase the expansion up to 500-fold.

2.4 Hematopoietic Stem Cells

$CD34^+$ hematopoietic stem cells (HSCs) are also an attractive target for cell therapy [11]. These cells are the progenitors of all blood cells and can be used with great effect. These cells have been used to repair damaged tissues, can differentiate into MSCs, and also can be modified to express defective genes or antisense payload to then be expressed in all 16 lineages. They are self-renewing and can be found in the bone marrow and the umbilical cord. Growth of the cells is difficult because they differentiate as they divide. However, Fms-related tyrosine kinase 3 ligand, granulocyte monocyte colony-stimulating factor, stem cell factor, and thrombopoietin are used to culture the cells for short durations (typically days).

The era of personalized cell therapy has arrived. It is no longer in a nascent stage. Efficacy is being established, and there are promising products in late-stage clinical testing, with a large number of products at an early stage as well (Table 1). Now that these technologies provide efficacious alternatives to diseases lacking countermeasures, secondary challenges such as logistics, price per dose, insurance coverage and reimbursement, profit margins, and novel regulatory challenges may be addressed. Paramount among solutions to these issues is streamlining manufacturing methods, including release testing, traceability, and automation, so that the clinical effect of process changes will be interpreted correctly. Also, establishing validated potency assays is an important step towards obtaining a Biologics License from the U.S. Food and Drug Administration (FDA). Fortunately, these issues are coming into focus and being implemented and accepted throughout the field, as industrial standards are incorporated in stride with clinical development.

Table 1 Several product candidates fill the pipeline of autologous cell therapies (from company websites and www.clinicaltrials.gov 2013)

Cell type	Company	Target indication	Phase
Dendritic cell	Argos	Renal cell carcinoma	III
	Northwest biotherapeutics	Glioblastoma	III
	Prima biomed	Ovarian cancer	IIb
	Immunocellular therapeutics	Glioblastoma	II
T-cell	Novartis	Leukemia	I
	Kite pharma	Leukemia	I
	TxCell	Crohn's disease	II

3 Allogeneic Therapies

For allogeneic therapies, cells are removed from an individual donor, then culture-expanded, processed, and stored under appropriate controlled conditions and administered to multiple recipients once needed. Thus, cells from one individual are used to treat a multitude of genetically unrelated individuals. Preserved allogeneic cells offer a major therapeutic advantage of having cells available for acute needs. Additionally, they offer a manufacturing advantage in that costs associated with both manufacturing and product testing are distributed over many doses and thus reduced on a per-dose basis compared to an equivalent autologous therapy. At the same time, they also pose a challenge to manufacturing because conventional/historic stem cell culture procedures are not geared towards producing large quantities of cells, particularly under accepted GMP rules for pharmaceutical manufacture. In many cases, cell culture expansion processes progress through as many as three development stages: (1) often beginning on a small scale to determine specific needs of obtaining cells for preclinical use (2) proceeding to a medium-size stage in which tens to hundreds of pharmaceutical-grade doses are needed to support clinical trials, and finally (3) finishing with process scale adaptions to achieve thousands of doses for true commercial production. Multiple cycles of significant process revision require additional time, comparability testing, FDA/European Medicines Agency (EMA) review, and occasionally additional clinical trial confirmations. These events add cost and often time delay to the product's commercial launch. A development program that, from the outset, foresees and avoids such obstacles and quickly achieves a manufacturing technology that is scalable, robust, and consistent will likely be the first to cross the finish line achieving licensure for commercialization.

3.1 Background

During the late 1960s and early 1970s, Friedenstein and coworkers discovered and described a population of adherent cells from bone marrow to which stem cell properties were subsequently attributed [18–20]. Due to their origin, these cells

were later referred to as mesenchymal stem cells (MSCs) [21, 22]. Thus, bone marrow began to be viewed not only as a source of hematopoietic stem cells but also as a source of cells capable of addressing connective tissue maladies. Cells sharing certain features with MSCs—somatic stem cells—have since been detected in many tissues [23]. However, MSCs have been characterized particularly well and hence will be used in this chapter as a model.

Consistent with considering MSCs as a source of material for rectifying diseases related to connective tissue, preparations of MSCs were tested for their abilities to generate various connective tissues in vitro (typically bone, cartilage, and fat), as well as to regenerate tissue defects in vivo [24, 25]. Common to these analyses and animal models is the notion that cells implanted may replace/regenerate tissue previously diseased or destroyed.

3.2 Current Definition

Following a flurry of initial characterizations, potency claims, and general descriptions, certain standardized tests were agreed upon in order to define MSCs. In particular, the International Society for Cell Therapy put forth a set of characteristics defining MSCs [26]. Three criteria were agreed upon: (1) the ability to adhere to plastic growth surfaces; (2) the expression of surface markers as detected by fluorescence activated cell sorting (FACS) comprising CD105, CD73 and CD90, lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules; and (3) the ability of cells to differentiate into osteoblasts, adipocytes, and chondrocytes in vitro. Of note, work by McGonagle and coworkers has recently highlighted the marker CD 271 as a possible tool to uniquely identify MSCs [27].

3.3 Activity

As discussed below in some detail, oftentimes cell therapy products exert positive effects without the cause being fully understood. Consequently, designing potency assays is fraught with difficulty (e.g. see the discussion on immunomodulation). We referred to the ability to differentiate into lineages of adipocytes, chondrocytes, and osteoblasts as a characteristic used in defining MSCs. This ability is sometimes also discussed in the context of potency assays and, indeed, sometimes required by regulatory bodies [28]. In our view, however, this connection seems speculative and suggests that the connection between potency and stemness to should be shown on a case-by-case basis rather than being presumed.

This association may also have historic reasons because, originally, stem cells had been thought to replace degraded tissue by means of serving as spare parts, similar perhaps to certain tissue engineering concepts [29]. It has since become

clear that this does not need to be the mode of action and that stem cells infused or otherwise applied to the body may have only a transient presence. Because the therapeutic benefit is clearly evident, a current hypothesis aiming to explain the beneficial effects observed in the context of temporary presence suggests that it may be the ability of stem cells to release substances and factors guiding the body towards regeneration that provides for their potency [30, 31]. Consequently, secreted molecules such as growth factors have become an important target for attempts to characterize MSCs [32].

3.4 Animal Models

Animal models have been the tool of choice to explore the therapeutic potential of MSCs. These may be roughly divided into those serving to delineate the potency of cells in particular disease scenarios and models exploratory in nature. The former group includes models demonstrating repair of connective tissues such as cartilage or bone, such as the rat critical bone gap model for bone regeneration [24, 33], the goat destabilized joint model for osteoarthritis [34], the mouse hind-limb ischemia model for regeneration of perfusion [35–37], as well as models for diseases such as Parkinson's [38, 39] and diabetes [40]. The latter group includes models in which the location of cells infused systemically is explored as well as their general distribution, tissue integration and, to some degree, potency. Locating cells after administration has been achieved with tracing techniques, such as being human in an animal model, by being derived from a male and implanted into a female animal (using the Y-chromosome as a tracer) or being labeled with radioactive DNA precursors, before infusion into rodents [41, 42]. Apart from the expected accumulation in small diameter vasculature, accumulating cells were specifically detected at sites of injury. For example, in a rodent infarct model, cells have been detected at the damaged heart muscle area as well as at the incisions required for the procedure [43].

For the purpose of exploring the general ability of MSCs to integrate into the body/tissues, the fetal sheep integration model has been used; here, human MSCs are administered to fetal sheep in utero at a pre-immune-competent stage and their distribution is analyzed postnatally. Somewhat surprisingly, the distribution or integration of MSCs in these models was nearly ubiquitous [44], which is perhaps counterintuitive given the proven ability of MSCs to differentiate into a limited number of tissues [22]. In general, the question arises whether to use either human MSCs in an animal model or to use isogeneic cells (i.e. those derived from the animal strain used). In the former scenario, cells used are the most relevant for further development; however, outside of immuno-incompetent animals such as nude mice or rats, they do require sometimes severe immune suppression, which potentially affects results. In the latter scenario, no such suppression is required; however, cells tested are derived from the respective animal strain rather than from humans, and hence they may exhibit different properties.

3.5 Safety

At this time, therapies based on MSCs have accumulated an outstanding safety profile. Remarkably, thousands of patients have received MSCs to date and no significant hazards have been reported [45, 46]. Before becoming overconfident on this point, however, it is prudent to recall that MSCs are relatively large cells, sometimes up to 20 um in diameter, and they are capable of undesired aggregation events triggered by processing steps or formulation. Comparing dimensions of multicell aggregates to those of arterioles, there appears to be a potential for trapping cells and hence blocking blood flow. Clearly, a product consisting of uniformly suspended single cells is preferred. Because individual processes likely differ regarding the propensity of cells to aggregate, individual development work (e.g. for appropriate formulation) is needed. Further safety data are needed to better understand the limits of this therapy as well as adjusting manufacturing practices to maximize patient safety.

3.6 Lack of Rejection

A wealth of studies carried out in vitro and in animals, as well as results from human applications, are indicative of a general absence of immune responses/rejection towards MSCs [44, 47–49]. This was certainly an unexpected finding and one of fundamental importance regarding the ability to convert cell therapy based on MSCs into a true pharmaceutical. One theory attempting to explain these findings focuses on the ability of MSCs to actively modulate immune responses by the mechanisms discussed above. An experimental assessment of this attribute is described in Sect. 3.17.

Another contributor to the lack of rejection may be the transient nature of MSC presence. For instance, it has been observed that, following the systemic infusion of human cord blood–derived somatic stem cells into immune-suppressed swine, the signal for human DNA in swine heart muscle nuclei was strong soon after infusion and weak at a later time point (C. van den Bos, personal observation). Possible explanations put forward include limited lifespans or fusion events [50, 51]; an explanation consistent with this observation might be an initial fusion event followed by successive elimination of human DNA in fused nuclei.

3.7 Immunity and Manufacturing

Although scientifically exciting, the finding that MSCs do not seem to provoke immune responses also has the immediate practical consequence of creating the opportunity for the concept of universal donors. Thus, production schemes for

MSCs are not restricted to the path of autologous products. Because there is no known limitation on the number of recipients for allogeneic products, large quantities of cells may now be produced from single donors and the production of MSCs may follow production patterns similar to those of biologic drugs such as antibodies. Early on, antibodies were manufactured from cells grown in laboratory scale in mice and then in perfusion chambers and similar small scale devices [52, 53]; currently, highly developed production cell lines and processes are used, in which production occurs in stirred and bubble column bioreactors at cubic meter scale. MSC production has followed a similar growth pattern.

3.8 Manufacturing and Technology Transitions

One might perceive that production of therapeutic doses of MSCs is at a similar inflection point in its development as antibodies were in the 1990s: currently, the gold standard for producing MSCs is to use devices, processes, and technologies derived from laboratory cell culture work—much like those employed by Friedenstein more than 40 years ago [18]. This approach has its merits, particularly in its versatility benefiting exploratory work. However, its inherent properties render high-quality mass production difficult. Examples for inherent issues include minimally controlled (often oscillating) culture conditions such as cell density and nutrient levels. Consequently, the industry is moving towards systems able to overcome these inherent limitations and which are truly scalable—namely, bioreactors. However, MSC biology comes with certain requirements. The needs and limitations of such primary cells in culture expansion (typically harvested at or before the fifth or sixth passage from their isolation) may restrict direct implementation of advances seen for transformed (immortalized) cells used in antibody production.

3.9 Challenges to Manufacturing

The clinical safety of stem cell therapy seems to be well established while scientific advances into new medical applications continue at an impressive rate. Due in part to these successes, the greatest challenge to the field of therapeutic cell therapy in today's financial and political environment may well be an economic one. Can cell therapies more effectively manage human illnesses than the current pharmaceutical offerings? Traditional pharmaceutical therapies provide symptomatic relief and often require chronic dosing to achieve this. Stem cell therapy may well be focusing on curative outcomes. Understanding the differences in per-dose manufacturing costs between small molecules or proteins versus those of cell therapies is essential. Also, understanding how to value the difference in their respective treatment outcomes will be crucial for the commercial success of cell

therapies compared to current pharmaceutical offerings. In the sections that follow, we elaborate on current manufacturing options and strategies for MSC therapies.

From a purely manufacturing perspective, there are opportunities to improve economics of cell therapies, but most of these have their own technical and economic aspects to consider. Closed system cell culture manufacturing makes economic (and safety) sense as a risk mitigation to reduce product lot loss due to contamination. Eventually, we may see closed systems operating under less stringent clean room standards (thus reducing facility costs) without impacting overall product safety. In another example, a typical cell culture procedure from an academic laboratory uses a somewhat subjective assessment of confluence to decide the time for harvest (e.g. 4–7 days per passage). From a scheduling perspective, moving a manufacturing process from a culture confluence-based harvest to a fixed-day harvest allows much more efficient use of human and facility resources and correspondingly reduces costs. Developing a defined medium (free of products of animal origin) should assure consistency of harvest output and improve safety (eliminating safety risks and associated expensive testing for some adventitious agents). At the same time, removing animal serum as a culture medium supplement avoids anticipated global supply limitations or the possibility that bovine spongiform encephalopathy-infected animals may appear in the few countries still able to supply fetal bovine serum (FBS) [54].

3.9.1 Dosing

Early dosing expectations for many allogeneic cell therapies seemed to fall between 1–10 million cells per patient, but now many clinical trials are exploring doses of 100 million cells or more. As culture scale must grow to meet this need, the industry will find that making more concentrated dosing formulations poses challenges for conventional cell processing equipment. Even more of a concern is that, for medical indications where patient populations are estimated as 100,000 per year, annual production needs could reach a level of trillions of cells (10^5 doses $\times$ 10^8/dose $=$ 10^{13} total cells/year). At this scale, planar culture is easily overwhelmed and the only manufacturing technology we have in hand that is likely to succeed will use scalable, large-volume bioreactors. This requires new infrastructure and investments to support this shift in platform technology. Several key questions come to mind that need answers quickly to enable this paradigm shift. Will stem cells grown under such conditions perform the same as those grown in the static flask environment in which they were first identified? Do we have the analytical tools ready to assess this?

3.9.2 Biological Limitations to Culture Expansion Yields

At some point in the expansion scheme, there is a limit to how many expansion cycles or passages the original donor cells can undergo without becoming

senescent or perhaps losing key functionality. This apparent senescence may well be due to the influence of in vitro culture conditions and not reflect the cell's capability in vivo. Currently, typical MSC processes do not exceed six passages, where each passage is loosely defined as the number of cycles of seeding and harvest on planar culture devices that a batch of cells has experienced; this, in turn, is related to the number of cell divisions the batch has gone through. This is similar but distinct from the case of a continuous bioreactor culture where the cumulative (or total number of) population doublings is calculated from direct cell counts. In all cases, the total number of divisions a primary cell population can undergo is limited; hence, the degree of expansion a primary cell can experience is one process limitation. An overall balance is needed between the number of passages the MSCs from any one donor can undergo and the availability of qualified (e.g. marrow) donors to meet total product demand. A larger final production batch size offers some economy of scale in its testing and manufacturing costs but requires more confidence in having qualified the donor in the first place—assuring that age, gender, ethnicity, and personal genetics do not create batch-to-batch variability that compromise successful manufacturing (e.g. abnormal growth rate), analytics (e.g. nonuniform potency test results), or variable clinical effectiveness.

3.9.3 Regulatory Expectations

The questions and rigorous guidance coming for regulatory agencies serve both public safety and ultimately commercial success, helping the therapeutic product reach the right patient population and establish achievable expectations for its efficacy. Agency perspectives will evolve as we gain new insights into the therapeutic and scientific aspects of cell-based therapies. Although most academic culturing of MSCs employs FBS-supplemented media, there is a perception that agencies will encourage sponsors to make efforts to decrease or eliminate the use of animal products due to concerns about BSE and adventitious agents. Use of recombinant growth factors in culture medium to replace serum would improve the product safety. However, introducing new raw materials brings new costs and may become problematic if potency assay results or other critical quality attributes (CQAs) change. Another example can be seen in that many stem cell therapies may act via more than one biological mode of action (e.g. MSCs release multiple bioactive molecules). The fate of the cells (longer-term engraftment or shorter-term residency as cytokine factories) may be hard to assess, yet product potency tests must be based on such information. As we learn more about a given product candidate, this could mean that its analytical support expenses continue to expand. The challenge will be to find the right balance of resources needed to achieve the necessary product characterization.

3.10 Markers Versus Process

As discussed above, MSCs have been defined by means of their ability to adhere to plastic, to differentiate into certain lineages, and to comply with a set of surface markers determined by FACS. These features have been chosen for their robust presence on MSCs and, indeed, we find these markers displayed by MSCs in virtually all situations. As these features do not tend to change, their value for in-process or quality controls is rather limited. Consequently, the production process used historically for MSCs becomes one of the key defining parameters. Moving to a truly scalable production system inherently changes this process; hence, much work will be directed at characterizing MSCs and or MSC-like cells derived from such altered production processes.

3.11 Current Solutions

MSCs have the fortuitous property of adhering strongly to plastic surfaces. Thus, researchers are able to grow cells in a wide array of culture vessels to suit their needs. Isolations, assays, and process optimizations can be done in well plates (e.g. 6- to 96-well formats). As experimental success drives the need for more cells, the culture scale can be increased by transition into T-flasks of increasing size (T-25 to T-225). After several passages, confluent cultures of MSCs can yield as much as 50,000 cells/cm^2; for a T-225 flask, this generates 10 million cells.

Culture scale-up can continue to ever larger collections of repeating planar surfaces. Multilayer, stacked Cell Stacks (Corning) and Cell Factories (Nalge) are a significant scale-up in culture surface area with thin, rectangle single layers ($10 \times 20 \times 3$ cm), which can then be built into multiple layer units (2, 4, and 10 layers) with sonic welding process or an adhesive. Units up to 10-layer stacks can fit in standard incubators. Further scale-up can occur with multiple 10-layer units (e.g. 50 in a production campaign); for potentially commercially relevant scale, the culturing can occur in larger blocks of layers (currently a 40-layer single block unit) or even larger scale units (e.g. 120-layer units are under development). These cell stack (CS) and cell factory (CF) units have dual ports for adding media and cells and allow a minimal passive gas exchange. Active gassing of larger planar units like the CF40 may be beneficial. Typically 150–200 ml of media per layer is used, which then allows sufficient airspace over the medium for gas exchange. Corning also offers a 12- and 36-layer product known as Hyperstack (HS). The bottom surface of each layer in these products is gas permeable and there is a thin open space between each layer, such that virtually no head space is required atop the medium. In this way, a 36-layer HS vessel occupies the same footprint as a more conventional 10-layer vessel. As the culture vessels grow in size, density (layers), and weight, they will need reinforced incubators and often mechanical assists to support media loading and removal. The CF40 and HS36 weigh about

11 kg when filled with media (often 100–150 mL per layer). The 40-layer units can be moved with what is essentially a forklift assembly, an automated cell factory manipulator (ACFM), and a large floor incubator.

Yields from scaled-up cultures are expected to maintain those reached in small scale, often at or above 40,000 cells/cm^2. The single-layer CS and CF units described above (with respective surface areas of 632 and 636 cm^2) can yield 25 million cells or more; correspondingly, the 10-layer units can yield 200 million or more cells. Multilayer vessel yields often are not exact multiples of the layer number, with some losses experienced due to transfer tubing dead volumes and manipulation challenges to recover all cell harvest fluid. Clinical production needs often tally in the low billions of cells and can be achieved by harvesting tens of CF10s or several CF40 of HS36 containers. Estimated commercial needs can run into hundreds of billions or up to several trillion cells, which will require a corresponding scale consideration for liquid handling and cell harvest and subsequent downstream processing (cell concentration and some kind of buffer exchange or diafiltration before final formulation).

The costs of cultured cells are understandably a significant concern for cell therapy companies aiming for scale-up and eventual commercialization. Primary cost drivers are (in decreasing order) culture media followed by facility and labor. Scaling up from a 10- to 40-layer vessel improves labor and testing utilization. However, as discussed previously, the need for trillions of cells will likely require an even more scalable technology. If 1 trillion cells requires 4,000 CF 10 units (each yielding 250 million cells) this could become quite a logistical challenge. A more appealing scale-up solution might be a microcarrier-based bioreactor system achieving 1 million cells/ml, which then would need four production runs in a 250-L reactor to achieve the same trillion cell objective.

3.12 Forthcoming Solutions and Lessons from Bioproduction Versus MSC Biology

Current bioproduction bioreactors often belong to either the group of stirred tank bioreactors or to that of wave-type bioreactors. Earlier models of stirred tank bioreactors tended to be laid out as stainless steel tanks of up to 20 m^3 to be cleaned and sterilized in situ, whereas current developments reflect increases in productivity and are towards smaller sizes and single-use bioreactor types described elsewhere in detail (see Chap. 1). The layout is typically such that an outer shell made from stainless steel encases a sterile single-use insert bag coming in contact with cells and product. For cells to be exposed to a homogeneous concentration of nutrients as well as to limit local accumulation of waste products, such reactors are agitated by stirring mechanisms typically comprising one or more impellers driven by a shaft. Thus, the tank's contents are continuously in motion.

If MSCs were to be fed into such a system and would not be provided with growth surfaces to adhere to, their survival would be impeded. So, although existing bioreactors provide excellent technology in regard to single-use materials and control of cultures, they are devoid of adequate growth surfaces for MSCs. One solution to this problem is to completely redesign bioreactors and to derive sometimes rather complicated devices providing such growth surfaces. Another solution to this problem, and perhaps a more economical one, is to involve a bridging technology hitherto used in vaccine production, namely microcarriers. Microcarriers are typically spherical particles of 100- to 250-μm in diameter, made from various materials with surfaces modified such that cell adhesion is facilitated [55]. They can provide qualitatively and quantitatively adequate growth surfaces and may be added to cultures over time so that growth surfaces may be expanded during a culture. Indeed, certain microcarriers exhibit a higher affinity for cells than conventional growth surfaces.

The material used for microcarriers varies widely, ranging from polymeric carbohydrates to solid plastics to biologics/proteinaceous matter. This provides for a wide array of features important in expanding cells—namely surface characteristics facilitating cell attachment and materials suitable for easy agitation, but also for harvesting and separating cells from such microcarriers by virtue of differences in size and density.

Culture medium within a bioreactor is agitated so that cells are exposed to uniform and controlled environments. This agitation may be achieved by different means and it appears that currently the market is dominated by systems either employing internal stirrers (stirred tank bioreactors) or by rocking the liquid volume (wave-type bioreactors). In either case, systems are set up as disposable/single-use systems such that product does not come in contact with reusable surfaces. This is principally accomplished by providing sterile disposable bags containing connections and inserts for either system.

In wave-type systems, bags are placed in a suitable tray and are provided with heating, environmental controls, and sensory systems. Such trays are then moved along their longitudinal axes in slow oscillating patterns so that a wave oscillates through the liquid volume, causing agitation. Devices and bags are supplied in various sizes so that volumes ranging from tens of milliliters to 500 L can currently be employed.

Stirred tank bioreactors comprise essentially a column of culture medium into which an impeller attached to a shaft reaches; the shaft is connected to a driver outside of the bag in such a fashion that its operation does not breach sterility. There is a wealth of know-how derived from both microbial as well as mammalian fermentation; one area of the science of hydrodynamics is focused upon is facilitating sufficient agitation so to permit the transfer of oxygen and nutrients while removing wastes and CO_2 at the same time—that is, to permit sufficient mass transfer [56]. Particularly in microbial cultures, enormous cell densities of metabolically highly active cells can be reached [57]. Microbial cells are mechanically robust so that the requirement for high exchange rates can be met by intense agitation. In contrast, mammalian cells are large, lack the bacterial cell wall, hence

are comparatively fragile so that there are narrow limits to agitation intensity. Despite such limitations, bioproduction protocols have managed to reach impressive productivities over time—a feat to which high cell densities contribute.

3.13 Adaptation/Directed Evolution of Industrial Cell Lines

Bioproduction processes have evolved to produce high cell densities and hence require high mass transfer rates. This need has been met by manipulating industrial production cell lines such as CHO cells [58] such that they withstand the comparably harsh conditions within a bioproduction process. Besides the obvious desire for maximal bioproductivity, this includes the ability to survive in the absence of attachment and the ability to withstand moderate to vigorous agitation. Thus, industrial production cell lines have been manipulated/selected for productivity and for compatibility with production processes and equipment resulting in today's highly efficient production cell lines [59]. This evolution took more than 20 years and resulted in cells that are very much different from the original primary animal cells [60].

3.14 Therapeutic Cells Should NOT be Adapted

Cells produced for therapeutic use need to be available on time and in sufficient quantities and quality. Because cells, once produced and formulated, can be stored cryogenically for extended periods of time–decades, if need be [61]—timeliness of production might not be the limiting factor. The quality of such cells, however, is a vastly more complex issue. As discussed throughout this chapter, characterization of therapeutic cells is not comprehensive. This is due partly for reasons of not fully understanding mechanisms of action, but also partly due to the inherent complexity of a mammalian cell. If size serves as a proxy of complexity, it might be helpful to consider the example of a fully characterized active pharmaceutical ingredient (API), such as a small molecule, and compare this to that of a mammalian cell. The molecular weight of a small-molecule API might be around 650 Dalton or 650 g/mol [62], whereas a mammalian cell, estimated to be around 2.5 ng [63], would have an apparent molecular weight of more than 1 quadrillion (1.5×10^{15}) g/mol—that is, 2×10^{12} times as large. For comparison, this ratio is similar to the volumetric ratio between the contents of a teaspoon and that of the great pyramid of Giza [64]. Thus, we consider the complexity of a mammalian cell to be very high indeed and, in turn, the current ability to characterize it to be rather limited.

Therapeutic cells are intended for application to the human body without causing harm. The safety profile for unaltered MSCs thus far is excellent [45, 46] and it is entirely unclear as to how significant changes/adaptations might affect the

safety of MSCs. For instance, loss of need for adhesion is sometimes discussed in the context of cancer; therefore, any permanent measures aimed at relieving MSCs of this need are likely to be undesirable. Also, as discussed above, primary cells such as MSCs can accumulate a limited number of population doublings only (i.e. they do not expand beyond a certain point), which further impedes lengthy manipulations or in vitro evolution.

Consequently, the production of primary therapeutic cells requires the adaptation of bioproduction equipment to the biological needs and limitations of such cells rather than the other way around.

3.15 Providing Scalable Adhesion Surfaces in Stirred Tank Bioreactors: Microcarrier-Based Bioreactor Processes

Early bioproduction cell lines were not yet adhesion independent and hence had similar requirements as we observe them now for primary therapeutic cells. A solution found at the time were particles made from cell culture-compatible materials providing adhesion surfaces [55]. Such particles are available in great variety and from multiple vendors.

Developing a microcarrier-based MSC expansion protocol comprises identifying useful combinations of specific cells, culture media, microcarriers, and agitation conditions. Although MSCs and their variances from various tissues appear similar, they are by no means identical. Consequently, specific cells require specific combinations of the above. To elucidate such combinations, it is useful to employ small-scale systems capable of evaluating the performance of individual combinations of the above with reasonable throughput. In our experience, shortcuts such as exploring combinations of microcarriers in 96-well formats or similar tend to be different from bioreactor conditions to such a degree that the information obtained is of limited use. Therefore, useful small-scale systems should resemble bioreactors as much as possible. There are certain devices on the market that provide arrays of small-scale spinners/bioreactors, such as the AMBR system (TAP Biosystems, Hertfordshire, UK). Also, small, disposable and manually operated spinner type devices may well serve the purpose. Although they possibly do not provide the same degree/ease of throughput, they do not require significant hardware investments outside of spinner platforms and focus the operation on the use of disposables and closely resemble bioreactors.

With this tool at hand, suitable processes, experimental settings, and combinations of the above ingredients and factors can be explored for maximum efficiency. The readout of such explorations is primarily proliferation, ideally determined directly by viable cell counts. To allow for this, the system (i.e. the combination of experimental culture setup, devices, cells, microcarriers, and other ingredients) needs to be able to provide sufficient differences between those combinations of parameters not suitable and those deemed suitable (i.e. a sufficient

dynamic range). The number of cell divisions can be calculated from cell counts and be expressed as cumulative population doublings (CPD). In our experience, a system providing for a CPD of at least six in favorable conditions tends to be sufficiently sensitive and, with a view on throughput, a cell culture duration of 1–2 weeks might suffice to attain this. Using sufficient multiples ($n \geq 4$), this provides for reliable data.

We feel that, due to their flexibility, comparatively simple setups at the early stages of such exploration are preferable over more sophisticated systems. The latter (e.g. fully controlled small-scale bioreactors systems) may be employed productively once the above basic parameters have been established. For instance, the above exploration may be carried out by addressing nutritional requirements simply by media exchanges. This is suitable for small-scale work but certainly not economical for production cultures. Consequently, in the second step, media exchanges may be replaced by specific feeds and the parameters for these can be explored using small-scale fully controlled bioreactors.

Expanding adult somatic stem cells: A medium-scale bioreactor example

Adipose tissue is a rich source of somatic stem cells; these have been termed adipose-derived somatic stem cells (ADSCs). Although somatic stem cells share many features regardless of their tissue of origin, differences exist and ADSCs have been recognized for their vigorous growth and resilience. The latter features make them good candidates for exploring potentially challenging processes. Hence, we used them here to provide an example of somatic stem cell expansion in a stirred tank type bioreactor (Fig. 4).

Data provided are from early development runs and by no means represent the maximum cell yield achievable. In addressing key parameters, such as agitation intensity, we consider yields of more than 10^6/ml to be quite feasible. Considering the dose size currently envisioned (i.e. between 10 and 200 $\times$ 10^6 cells/dose), it appears that this will render industrial production of somatic stem cells feasible and hence facilitate the economically viable production regenerative medicine products (Table 2).

3.16 Critical Quality Attributes for Therapeutic Cells

All cell therapy products (allogeneic, autologous, and pluripotent) require definition and characterization of the following four CQAs to assure regulatory acceptance and both clinical and commercial success.

- Safety: Demonstrating that the product is not contaminated with any adventitious agents and, if appropriate, is not tumorigenic
- Identity: Demonstrating that the product contains the intended cellular and noncellular (excipient) components

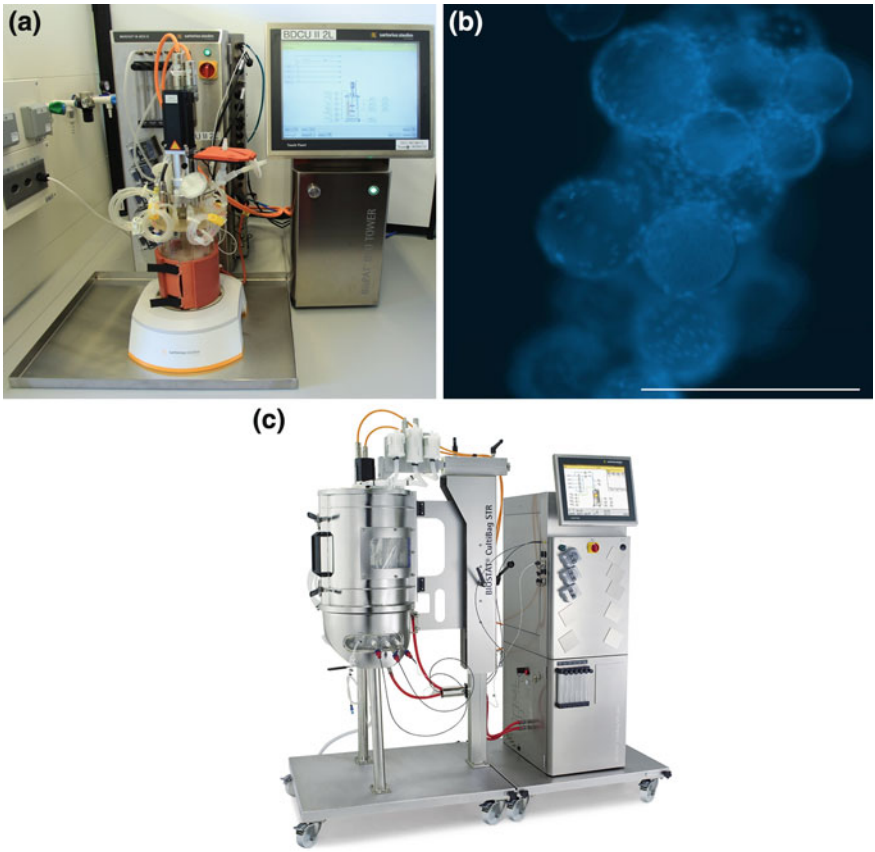

Fig. 4 Adult stem cells from adipose tissue can be expanded in stirred tank bioreactors to yields that are consistent with commercial production. **a** View of the bioreactor employed, including supporting equipment; **b** Fluorescent-microscopic image of microcarriers at time of harvest. Cells were visualized and localized using nuclear stain (DAPI). The bar indicates 400 μm; **c** A stirred 50-L bioreactor as a possible next scale-up step. In one example, we employed standard Sartorius Univessel SU 2-L systems using a total cell culture medium volume of up to 2 L. adipose-derived somatic stem cells (ADSCs) were from Lonza and culture expanded as per the vendor's suggestions in planar culture. To initiate the bioreactor culture, the vessel was filled with 0.8 L of medium containing 10–22 g of micro-carriers (SoloHill) treated according to the manufacturer's recommendations and equilibrated overnight at 37 °C, pH 7.2, and 100 RPM. The culture process was started by adding between 11 and 23 million ADSCs suspended in a volume of 30 ml. Agitation was halted after cell addition to allow for a 3- to 4-h seeding period. Medium was then added to a total volume of 1.5–2 L and agitation was set to 95–125 RPM—the minimum RPM required to maintain suspension throughout the culture. Then 20- to 30-ml samples were taken daily to follow cell proliferation and a 50 % medium exchange was carried out between days 4 and 7. The culture was harvested at day 10 and yielded between 500–700 million cells corresponding to expansion factors of 30–50 and cell yields of 0.25–0.35 million cells/ml. Cells appeared to be well distributed over micro-carriers and also to aggregate micro-carriers

Table 2 Commercial scale production of therapeutic stem cells appears to be quantitatively feasible

	Dose size (1×10^6)			
Cell yield (1×10^6/ml)	10	50	100	200
0.1	10	50	100	200
0.2	5	25	50	100
0.5	2	10	20	40
1	1	5	10	20

In this model, we assume that 100,000 doses are required per year, that production occurs in 1000-L bioreactors, and that 100 such lots per year are feasible. Based on these assumptions, we calculated the number of lots required to achieve 100,000 doses depending on productivity (cell yield per volume) and dose size (cell number per dose). For example, to produce 100,000 doses of 200×10^6 cells/dose with 100 or fewer lots, a minimal cell yield of 0.2×10^6 cells/ml is required

- Purity: Demonstrating that the product is within specifications regarding undesired components, such as contaminating cell types or residual process reagents
- Potency: Demonstrating that the product possesses the inherent biological properties that relate to the mechanism of action intended to support treating the medical indication.

Decades of technological advances in the biochemical sciences (e.g. high-performance liquid chromatography, polymerase chain reaction, mass spectrometry) have helped establish straightforward, well-accepted methods to assess the first three CQAs of this list. This has been aided by the fact that most biological (antibody and protein-based) therapeutics also use these now common tools in their testing. Correspondingly, the collective learning from one type of therapeutic is often quite relevant to many others. As mentioned in Sect. 3.14, the complexity of cells and accordingly their mechanism of therapeutic action represent significant challenges to current characterization tools.

3.17 Potency

In contrast to the other three CQAs mentioned in the Sect. 3.16, a potency assay is a very product-specific challenge and, thus, is worthy of some special discussion. Process development of a potential stem cell product whose precise mechanism of action is not well characterized, yet has proven effective in key preclinical evaluations, need not wait for the perfect potency assay to be discovered. Rather, one should proceed by collecting a matrix of more general measures of biological activity (such as the several concepts described here) with the intent to track and correlate these measurable properties with the clinical efficacy testing of current and future batches. Conclusive data will be needed eventually to show that a batch of cells that underperform in vivo can also be shown to have a different in vitro

assay result profile than batches of cells that do perform as expected. Such a correlation (both positive and negative) is also a requirement for a stability-indicating assay, of which potency is a logical one, to link to the manufacturing process and ultimate shelf-life determination of the final product.

Although early clinical trials may proceed with preliminary assays, efforts should be made to track such data and seek trends and patterns that will support establishment of acceptance ranges. Later-phase (pivotal) clinical trials and FDA approval of a stem cell therapy will likely need a compelling data set to show evidence of manufacturing consistency and relevance to product performance. Eventually, well-established assays with defined acceptance limits are required to serve as release criteria for the commercial product potency tests. Failure to define such assays and associated specifications puts reliable manufacture at great risk—not the sort of message with which patients (customers), reviewers (FDA, EMA), or investors are inclined to be satisfied.

The development of a reliable potency assay for a new cell therapy product would ideally be based upon a general hypothesis for the mechanism of action of the product. Research and clinical study of stem cells may often start with observations of benefits seen at a relatively high or complex biological levels, such as cardiovascular tissue repair, angiogenesis, tissue regeneration, and spinal crush repair. It is this sort of noteworthy result that often drives the development of a new therapeutic candidate. The definition of an in vitro, robust, and relevant bioassay as a surrogate for this organismal benefit can be quite challenging in its own right and often struggles to keep pace with more straightforward efforts like cell culture scale-up. As manufacturing practices become more standardized, the adaptation of a new research stem cell process to a robust clinical process with straightforward commercial development pathway may occur in less time than it takes to develop a new and complex bioassay for potency and then show its correlation to other product characterization attributes. For production of material for early clinical trials, potential assays should be tracked and acceptance range targeted. For later-phase clinical trials in which efficacy is being demonstrated, evidence of manufacturing consistency will need to be shown via established assays with defined acceptance limits serving as release criteria for the potency tests. Stem cells are likely to exert their therapeutic effects by one or more mechanisms, which could include the following: their secretion of paracrine factors, such as cytokines and growth factors, to induce changes in whatever new environment they find themselves in following their administration or by a more direct differentiation into a new cell type. A defining attribute of freshly isolated MSCs is their ability to differentiate (under controlled conditions) into osteoblasts, adipocytes, and chondrocytes. Both qualitative and quantitative assays are available for each of these cell-type end products (i.e. their trilineage potential). However, unless the in vivo cell differentiation into one of these cell types is essential as part of their therapeutic mechanism of action, then trilineage potentiality may not be the most relevant potency test for an MSC-based therapy.

Designing the appropriate potency assay for a stem cell product could involve developing one or more ELISAs targeting detection of secreted factors. In Table 3,

Table 3 Mesenchymal stem cells (MSCs) are thought to affect their benefits by secreting active factors.

Factors detected at >100 pg/ml	Factors detected at >10 pg/ml	Undetected factors <10 pg/ml or assay limit of detection
IL-6	GM-CSF	IL-1 α and IL-1β
IL-8	GRO-1	IL-4 and IL-5
MCP-1	IL-2	IL-10IL and IL-12p70
SDF-1α	RANTES	IL-13 and IFNγ
VEGF		MIP-1α and MIP-1β
		MMP-9 and TNFα

Such factors are grouped here according to prevalence. MSCs were cultured at confluence in T-25 flasks in αMEM+ 10 % fetal bovine serum for 72 h. Culture supernatant was centrifuged and then assayed for bioactive factors using commercial enzyme-linked immunosorbent assay kits or antibody reagents

we show a panel of specific immunoassays applied to a representative MSC product. Of the 21 cytokines and factors examined in this array, we consistently see readily measurable levels of 9 factors and undetectable levels of the 12 others. This gives immediate credence to the idea that a secretion array of cytokines might be very useful in characterizing biopotency of MSCs. From this panel, it is apparent that five factors (IL-6, IL-8, MCP-1, SDF, and VEGF) may be quantitatively important and hence may be useful probes of changes in MSCs, such as in cellular responses to changes in culture medium, process steps, or perhaps as a potency or stability indicator. The values reported for the next four lowest reporting factors (GRO-1, IL-2, IL-5, and Rantes) are near the limit of quantitation for their respective assays, so the numbers reported are perhaps more useful in defining only the order of magnitude of their secreted concentrations as compared to others more readily quantitated. It should be noted, however, that it is likely that the conditions under which cells are grown for generating test samples will also play a significant role in the amount of factors secreted.

Another concern for a product-specific potency assay design is the extent to which there needs to be systemic or localized persistence of the MSCs following their therapeutic delivery. If cells are short-term residents, then transient cytokine production, as described in Sect. 3.17, may be key to their performance. If they home to a specific location (e.g. a point of inflammation or a specific organ) and attempt to become established, then another MSC attribute, T-cell suppression, may be essential for their prolonged survival and therapeutic effect.

Relevant to this, the enzyme indoleamine 2,3-dioxygenase (IDO) has been described convincingly as a key mediator of immune modulation and as a lymphocyte suppressor [65, 66]. The enzyme catabolizes tryptophan and thus diminishes the concentration of this amino acid deemed vital for the activity of cells of the immune system and, additionally, produces substances termed kynurenines which further diminish the activation of lymphocytes. MSCs can be induced with interferon (IFN)-γ to stimulate production of IDO. Thus, measurement of IDO levels or kynurenines in the supernatant of IFN-γ-stimulated MSCs

could be considered as an invitro measure of induced biological function; it might serve as a surrogate measure of their ability to persist in the patient and be considered important in the potency testing of an MSC therapy in which long-lasting MSCs are deemed important.

As mentioned above, animal models may provide the best insights into physiological outcomes linked to a cell therapy (e.g. restored blood flow in a mouse hind limb occlusion where angiogenesis-related markers can be investigated). This could provide the necessary foundation from which secondary or surrogate assays (which one hopes will be easier, faster, and less expensive to perform than animal work) can be developed and applied to process development and commercial manufacture.

3.18 Practical Challenges

Although the measurement of potency plays a central role in the control of product quality (Fig. 1), it remains one of the most complex aspects of cell therapy product development. This principle often results in the assay or assays that define potency being finalized very late in the product development. This mismatch in time between need and availability can pose a risk, for example, to implementing successful efforts to improve cell yields arising from culture medium optimization (e.g. converting to a serum-free medium), or other changes that impact cell growth rate and expose the cells to new stresses during growth or processing. Should any of these have a significant (positive or negative) impact on the potency assay readout, it is important to have such knowledge before committing to a process change. Also, maintaining consistent product potency through later-stage development is clearly essential to building strong links between various clinical studies. A phase I safety trial for one product form may not be accepted as meeting regulatory benchmarks for a modified product whose unit potency is multiples higher or fractions of that of the original. Although in vitro based comparability testing is regularly an outcome of significant process improvement efforts, it is sometimes necessary to consider additional clinical study evaluations as well.

3.19 Future Directions for Cell Testing

MSCs and related cells are subject to intense scrutiny using an array of modern tools, including microarrays to analyze snapshots of mRNA expression patterns by cells in certain situations. Commercial services involving microarrays are affordable tools today. Beginning with comprehensive gene array profile data may then allow genes of interest to be identified (much as was described above for cytokine screening) and subsequent analyses may be focused on a limited set of gene products providing a set of targets, which may lend itself to standard quality

control tests. By their very nature, microarrays analyze mRNA expression levels and hence are a step removed from analyzing expression levels of proteins. Although most of today's potency assays focus on the analysis of the actual expressed and/or secreted proteins with flow cytometry and immune-assay read-outs, there is great value in pursuing mRNA expression profiling and perhaps even epigenetic-oriented analysis. Despite the potential complexity of the information provided by these powerful analyses, they may become quite useful in exploring key regulatory and response pathways of MSCs and may be of special importance for cell therapy indications for which current research has not identified a straightforward mechanism of action link to therapeutic performance. Although complex by virtue of generating data for most known gene products, such assays have become comparatively cheap and advances in bioinformatics permit a degree of automation. Thus, such assays might be considered useful IPCs, identity tests, or indeed potency assays.

4 Key Factors Towards Economic Success

Current in vitro information as well as animal study-related information is available to support a vast array of claims regarding the beneficial effects of cell therapies and/or stem cells. These claims seek confirmation with clinical trials with clear and unambiguous positive outcomes to assure the economic success of cell therapies as a whole. Few market applications, however, have been approved following rigorous full-fledged clinical trials. One recent example of an approved allogeneic product, Prochymal (developed and marketed by Osiris Therapeutics, Maryland, USA) was approved in Canada and New Zealand in 2012 but has yet to make a significant market impact.

Expectations for more product approvals will drive the need for a step-change in demand for high-quality therapeutic cell products, and the industry is preparing for this. Part of this preparation is the current transition from laboratory-scale production systems and their industrialized derivatives to truly scalable systems involving dynamic bioreactors (hollow fiber bioreactors, fixed bed bioreactors, fluidized bed bioreactors, stirred bioreactors, Wave bioreactors). Moving from planar culture platforms to dynamic bioreactors may sound straightforward. With the above in mind as well as the yet-to-be-discussed regulatory requirements, this endeavor becomes nontrivial and will require several years to fully advance. Particularly, developing such scalable processes with built-in reliability will pose a challenge. Similarly, the integration of technologies capable of not only generating large quantities of cells but also processing, formulating, and cryopreserving them in such quantities will all require significant new efforts.

New assay technologies should come into play and support better product characterization and help de-risk process change implementation. Applying these assays—the development of which may be faster, and which themselves may be

cheaper and more relevant—will also help the economics of cell therapy manufacturing.

Extensive cost modeling and optimization are likely to be needed to assure market success of the first large market allogeneic therapeutic. This is true for both allogeneic and autologous manufacturing scenarios. Although these two therapeutic approaches are not yet facing head-to-head competition, both have a need for cost reductions in order to become well received in the market place.

5 Downstream Processing

The primary drivers for downstream processing for any cell-based therapy involve decisions around the need to (1) collect cells in a suspension, then (2) perform medium exchanges to remove unwanted carryover (process residuals) and replace this with formulation medium and (3) concentrate the cells to the density needed for the intended therapeutic dose. While achieving these objectives, it is also important to keep the scale of downstream processing well matched to the upstream platform to which it is linked. These tasks pertain to both allogeneic and autologous approaches.

The final harvest of cells can be triggered by either achieving a target confluence or upon reaching a fixed day for the final passage. Although a confluence-based culture harvest is typical of a small-scale cell culture (academic and experimental settings), there are significant incentives to move large-scale cultures (clinical and commercial) to a fixed-day harvest schedule. Although an individual may have a flexible schedule and easily harvest several T-flasks in a short time, the logistics and staffing required to harvest the likes of 60 ten-layer CFs in a GMP cleanroom is significant and involves arranging work schedules for upwards of 15 individuals for a process that may require eight or more hours. Thus, as a stem cell process moves into a scaled-up scenario, there will be opportunity and need to transition the process to a predictable and set manufacturing schedule. This is complicated by biological variation: individual cell batches may not proliferate at the exact same rate and hence reach the specified degree of confluence in planar systems at different times. Because the latter is perhaps the prime indicator of the need to passage, variations in harvest time are likely and need to be factored into any scheduling.

The first action to be taken in downstream processing is often a rinse of cells to remove medium and any debris before starting a trypsinization (or other enzyme digestion such as collagenase) to release the cells from the growth surface and initiate a liquid/cell suspension collection step. There are several enzyme options available to do so, including animal-derived trypsin (porcine or bovine pancreatic extracts), recombinant porcine trypsin, and a "trypsin-like" microbial enzyme (TrypLE). Enzyme cost, speed of cell release, and efficiency of release are factors to be considered. The level of trypsin activity used in a cell harvest is often considered to be a risk to cell health if prolonged exposure occurs during

subsequent cell handling. For larger-scale cultures, the trypsinization may be performed for a 10–20 min incubation to facilitate handling of multiple containers. Typically, cell culture procedures then call for a trypsin inactivation step by incubating or "quenching" trypsin activity with FBS, which contains serum anti-protease inhibitors.

If the quenching step is viewed as an essential step, then some analytical confirmation should be sought. When bovine serum is added to quench trypsin activity, we have observed that the quenching potential with FBS is variable between manufacturers and even different commercial lots and should be titered for this application. Some reports indicate that human serum albumin (HSA) can be added to protect cells (and quench trypsin). However, in our hands, we find that trypsin enzymatic activity is in no way inhibited by HSA nor do we find evidence of albumin serving as a substrate for trypsin (data not shown). At the clinical/commercial level, this needs to be monitored.

Usually, the next step after collecting a single cell suspension is to begin volume reduction to concentrate the cells and at the same time remove the trypsin, serum, and residual culture medium. There are several methods to achieve this: the simplest and most common technology is a standard laboratory benchtop centrifugation. For larger volumes, there are larger-scale centrifuges (e.g. Unifuge, Centritech). Small-scale cultures (<2 L) are well suited to centrifugation, but as harvest volumes increase to >10 L (e.g. 250 mL of trypsin plus quench for each of >40 ten-layer CFs) and cell yields approach 5–10 billion, then other options become more efficient.

Two alternative and scalable technologies gaining credibility for MSC processing are tangential flow filtration (TFF) and a high-speed, large capacity elutriation processing via kSep. A TFF setup with a cartridge membrane with a 100-micron pore size and 0.5 sq ft surface area can easily process up to a 5-L harvest and then support diafiltration and a 25-fold volume reduction in less than 4 h with excellent recovery and maintenance of viability. More importantly, the TFF system is a closed operating system and unlike bottle centrifugation requires no open container or manipulations in a constraining Biosafety cabinet setting. There are likely some shear stresses on the cells during the recirculation of retentate (cell suspension), but our experience has not shown a significant impact on cell viability or stability. The overall benefits of effective rinsing of the cells, clearance of process residuals, and concentrating cells to levels above 25 million/ml make the TFF procedure effective for MSC processing. The unit has, for instance, a practical total cell capacity limit of 5–10 billion cells.

For larger scale cell harvests (10–40 billion), there is an elutriation technology known as kSep (KBI Biopharma), which concentrates cells in a free solution setting within a collection chamber when the flow rate of the incoming cell stream is balanced by centrifugal forces of the unit. This closed system unit achieves cell collection with a single pass of the feed stream without compression against a hard surface (like a cell pellet from centrifugation); therefore, it is gentler and also

maintains cells at very high viabilities. Follow-on rinsing with buffer or diafiltration medium through the cell suspension allows rapid and effective removal of process impurities. Processing times are usually shorter than for TFF processing at equivalent scale. The smallest kSep model handles up to 40 billion cells and a larger kSep unit could handle up to 400 billion cells in a single processing cycle. Both systems have fully disposable tubing and centrifuge chambers sets, making this ideal for supporting larger bioreactor harvests in a current GMP-compliant manner.

Final product container options for cell therapies typically include Mylar-lined bags, glass vials, or a sterile, sealed polymeric vial. The bags can be filled with a cell suspension transferred by syringe. Larger scale fills can be done with open glass vials typically used in pharmaceutical applications, but they require an appropriate controlled environment for this open system manipulation. Another alternative that has a more closed system character employs Crystal Vials (Aseptic Technologies), which are presterilized and then filled by a needle puncturing of the vials septum that can be automated. The septum is then resealed with a laser, assuring a tight seal and secure container ready for cryopreservation.

Concentrated cells are usually formulated in well-defined solutions (compatible with direct patient administration, often by dilution into saline for intravenous administration). For short-term refrigerated storage (24–72 h), one may use a storage solution like Hypothermasol (BioLife Solutions). Often, cells can be stored for periods of years in a frozen state using appropriate solutions (e.g. CryoStor [BioLife] or ProFreeze [Lonza]) that are also supplemented with DMSO from 3–10 % final concentration as a cryopreservation agent. A programmed freeze rate of 1 $^{\circ}$C per minute is a standard procedure, followed by long-term storage in a vapor-phase liquid nitrogen dewar.

6 Regulatory Considerations

Similar to antibody APIs, the end product of this process needs to be sterile. The final product itself cannot be terminally sterilized because it is a viable entity. Consequently, the entire process needs to be executed aseptically. This places the entire production run into clean rooms and closed systems and emphasizes the need for single-use material.

Countries regulate such processes individually. For the European Union, Regulation 1384 (the Advanced Therapeutic Medicinal Products Regulation) has been in force since December 2008 and details procedures for market authorization for a cell therapy, gene therapy, and tissue engineering products summarized as advanced therapy medicinal products [67]; similar regulations apply in other countries, including the United States.

7 Summary and Outlook

A wealth of evidence exists for the beneficial effects attributed to therapeutic cell treatments, for stem cells and others as well. First market authorizations have been granted and promising clinical trials are ongoing. Hence, it is likely that the industry will see the development of groundbreaking products based on cells as active pharmaceutical ingredients in the coming years. Currently, production processes for these APIs are at a transition phase between traditional cell culture expansion processes, their derivatives, and fully scalable bioreactor processes. Based on promising early steps documented here and elsewhere [68], we expect these processes and tools to be in place within the coming years so that the stage is set for supporting the needs of a thriving cell therapy industry in both quantity and quality of production processes.

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Adv Biochem Eng Biotechnol (2014) 138: 99–125
DOI: 10.1007/10_2013_186

Published Online: 18 April 2013

Fast Single-Use VLP Vaccine Productions Based on Insect Cells and the Baculovirus Expression Vector System: Influenza as Case Study

Regine Eibl, Nina Steiger, Sabine Wellnitz, Tiago Vicente, Corinne John and Dieter Eibl

Abstract During the last few years virus like particles (VLPs) have become increasingly interesting for the production of vaccines. This development is explained by their excellent safety profile as well as a significant number of clinical studies showing strong and long-lasting protection. A further reason is the possibility of speeding up VLP vaccine manufacturing by implementing single-use (SU) technology in the case of mammalian and insect-cell-based processes, for which a multitude of SU devices up to middle-volume scale already exist. After briefly introducing the vaccine types and expression systems currently in use, this chapter turns to VLP vaccines and the insect cell/baculovirus expression vector system (IC/BEVS). Based on the main process characteristics and typical process flow of IC/BEVS-based VLP vaccine productions, suitable SU devices and their implementation are addressed. We subsequently report on the successful development of a fast, scalable benchtop production process generating a four-protein component influenza A H1N1 VLP vaccine candidate. This process is based on *Spodoptera frugiperda* (*Sf*)-9 cells and combines Redbiotec's rePAX^{TM} technology with obtainable SU devices for upstream (USP) and downstream processing (DSP).

Keywords DSP · Influenza VLP vaccine · *Sf*-9/BEVS · USP · Wave-mixed bioreactor

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Abbreviations

ABS	Absorption spectrum
AEX	Anion exchange chromatography
AGE1.CR	Cell line from Muscovy duck embryos designed by specialists at Probiogen
A. thaliana	*Arabidopsis thaliana*
BV	Baculovirus
BEVS	Baculovirus expression vector system
BHK-21	Baby hamster kidney cell line
CBS	Custom biogenic system
CCI	Cell concentration at infection: NB CCI is also referred to as time of infection (TOI)
CD4	Cluster of differentiation 4 (glycoprotein exposed on the surface of immune cells)
CEFs	Chicken embryonic fibroblasts
DCs	Dendritic cells presenting antigens, used to produce therapeutic cancer vaccines such as Provenge
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DSM	Dutch-based life and material sciences company
DSP	Downstream processing
EB14	Cell line derived from chicken embryonic stem cells
E. coli	*Escherichia coli*
ELISPOT	Enzyme-linked immunospot
FDA	Food and Drug Administration
FEP	Fluorinated ethylene propylene
GMT	Geometric mean titer
GSK	GlaxoSmithKline
HA	Hemagglutinin
HCD	High cell density
HEK293	Cell line derived from human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	Hemagglutinin inhibition
Hi-5	Cell line (also called BTI-TN-5B1-4) originating from the ovarian cells of the cabbage looper, *Trichoplusia* ni
HPV	Human papilloma virus
IC	Insect cell
IgG	Immunoglobuline G
HTS	High-throughput screening
IEX	Ion exchange chromatography
MDCK	Madin–Darby canine kidney cell line, cells derived from kidney of a cocker spaniel
MES	2-(N-morpholino) ethanolsulfonic acid

MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MRC-5	Fetal lung cell line
MWCO	Molecular weight cut-off
M1	Matrix M1
M2	Matrix M2
NA	Neuraminidase
N. benthanmiana	*Nicotiana benthamiana*
N. tabacum	*Nicotiana tabacum*
P	Statistical significance
PBS	Phosphate buffered saline
PBS-1	Patented cell line from HepaLife Technologies, cells derived from an immortalized chicken embryo cell
PCR	Polymerase chain reaction
PERC.-6	Human retinal cell line (owned by Crucell Holland B.V.), that guarantees high product yields (8 g L^{-1} in fed batch productions of antibodies and 27 g L^{-1} in antibody production processes based on DSM's XD technology)
P. pastoris	*Pichia pastoris*
POH	Point of harvest, also called time of harvest (TOH)
pfu	Plaque-forming units
p.i.	Post infection
RM	Rocking motion
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS-Page	Sodium dodecyl sulfate polyacrylamide
SEC	Size-exclusion chromatography
SRD	Single radial immune diffusion
SU	Single-use
Sf	*Spodoptera frugiperda*
S2 cells	Schneider 2 cells, representing one of the most commonly used *Drosophila melanogaster* cell lines
$TCID_{50}$	50 % tissue culture infection dose
TMP	Transmembrane pressure
TOH	Time of harvest
TOI	Time of infection
USP	Upstream processing
Vero	Cell line derived from the kidney of an American green monkey
VLP	Virus like particle
V0	Recombinant seed virus rBVH1N1-PR834
V1	Master seed virus
V2	Working seed virus used to generate the WVB
vvm	Volume per volume per minute

WCB	Working cell bank
WVB	Working virus bank
XD technology	Highly intensified cell culture process with titers up to 15 times higher than current standard production processes for biotherapeutics
ZHAW	Zurich University of Applied Sciences

Contents

1 Introduction

Driven by the advances in biotechnology, the vaccine industry has undergone remarkable development since the FDA approval of the first human vaccine, a smallpox virus, in 1931 [1, 2]. The types of vaccines used today, for which an overview is given in Fig. 1, have expanded from traditional live, attenuated, or inactivated whole organisms to include toxoid vaccines, subunit vaccines, conjugated vaccines, DNA vaccines, and vector-based vaccines [3–5]. The vast majority of these vaccines are prophylactic and were developed to provide efficient protection against infectious diseases such as smallpox, hepatitis, measles, mumps, tetanus, cholera, and influenza. Because preventive vaccines are products for healthy patients, they display characteristics of typical bulk products and should therefore be (1) cheap to produce in large quantities, (2) stable during storage, (3)

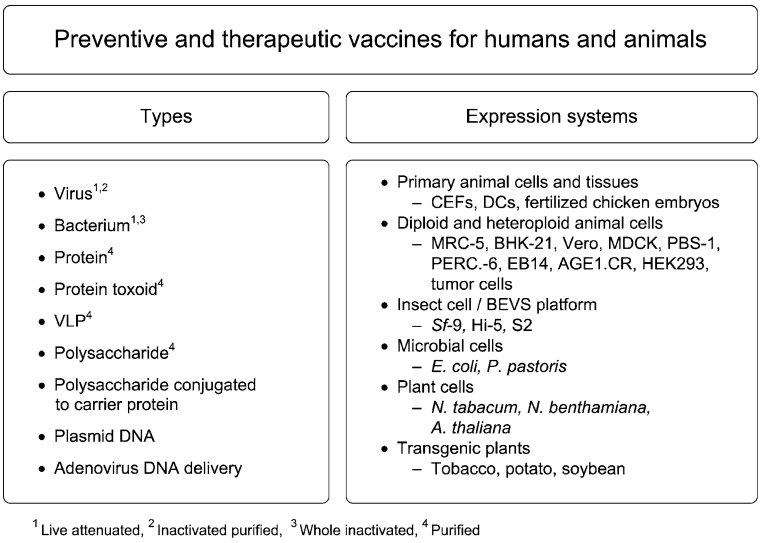

Fig. 1 Overview of today's vaccine types and expression systems

easy to apply, and (4) free of side effects. They stand in contrast to therapeutic cancer vaccines inducing immunity against tumor-associated antigens, which are given to seriously ill cancer patients. Although there is an urgent need for therapeutic cancer vaccines, only a few licensed products (e.g., Dendreon's Provenge against human prostate cancer and Vical's Oncept, a canine melanoma vaccine) are currently available on the market [6–9].

It is worth mentioning that the development and manufacture of new generation vaccines has entailed a change in the originally used expression systems (Fig. 1). In virus-based vaccine productions, primary tissues were partly replaced by primary animal cells, diploid finite and heteroploid continuous animal cell lines. Where possible, serum, hydrolysates, and further animal components were eliminated from the vaccine production process. Hence, different cell lines were adapted to grow in suspension, simplifying their handling and allowing an easier and faster process scale-up. Nevertheless, the use of animal products as well as serum and production with adherent cells remains relevant in current vaccine manufacturing [10].

For VLP vaccines a wide range of expression systems (including microbial cells, plant cells, whole transgenic plants, and mammalian and insect cells) has proven to be suitable [11]. VLPs are a specific class of subunit vaccines that mimic the organization and conformation of authentic native viruses. They contain no infectious genetic material (VLPs completely lack DNA or RNA genomes) and are therefore nonreplicating and nonpathogenic [12]. Because VLP structures are very similar to infectious viral capsid proteins, a lower dose of VLP antigen relative to subunit vaccine is sufficient to provoke similar protective immunity [13]. In addition to their ability to induce strong B cell responses, VLPs have been shown

to stimulate CD4 cell proliferative and cytotoxic T lymphocyte responses against pathogens [14–16]. This feature, together with the fact that multivalent VLPs can be engineered by the simultaneous presence of multiple viral epitopes on a single particle, makes VLPs very effective and attractive vaccine candidates. The diversity of VLPs in terms of virus structure is also worthy of note. More than 30 different viruses that infect humans and animals have already been used to generate VLPs [5, 11]. Among them are viruses with a single capsid protein, multiple capsid proteins, and viruses with and without lipid envelopes. However, not all of the viruses described were found to be appropriate vaccine targets.

Merck's Recombivax HB for hepatitis B protection was the first successfully introduced VLP vaccine. Soon after its FDA-approval in 1986, GSK's Engerix-B, the second VLP vaccine against hepatitis B, was licensed [17]. Both vaccines were made by inserting the gene for a hepatitis B surface antigen into engineered *Saccharomyces cerevisiae* strains [18]. About 20 years later two variants of human papilloma virus (HPV) vaccine entered the market. Gardasil from Merck, which is also produced in *S. cerevisiae*, is a tetravalent VLP (HPV types 6, 11, 16, and 18) made of recombinant papilloma virus major capsid protein L1 [19]. GSK's Cervarix, a bivalent HPV vaccine against types 16 and 18, is the first commercial VLP vaccine based on the IC/BEVS [20]. Meanwhile, further VLP vaccine candidates are in the stages of pre- and clinical trials, for example, influenza virus, respiratory syncytial virus, cytomegalovirus, parvovirus B19, and Norwalk virus [21, 22].

The most promising results (VLP yields up to hundreds of milligrams per liter) were reported for expressions in yeasts, bacteria, and insect cells [11]. However, the complexity of the VLPs produced with the IC/BEVS is highest (VLP vaccine candidates formed with up to four proteins are already in clinical trials [11]). Roldão [11] and Palomares et al. [23] ascribe this efficiency and versatility to the baculovirus' ability to shut off transcription of early host genes and to allocate the cellular transcriptional and translational apparatus for the expression of heterologous genes.

2 VLP Productions with the IC/BEVS Process Platform

VLP productions based on the IC/BEVS platform are two-phase processes run in a biosafety level 1 environment. They consist of the growth phase (ca. 2 days) of noninfected insect cells and the production phase (ca. 4 days), which is induced by adding recombinant baculovirus suspension at an exponential cell growth stage. Baculoviruses derived from the *Autographa californica* nuclear polyhedrosis virus are preferred, because of its straightforward target gene integration [24]. During the production phase, the cell diameter of the infected insect cells increases (from ca. 13 μm up to ca. 20 μm), whereas cell proliferation progressively stops. This is the result of successful virus replication.

2.1 Factors Influencing Product Yield and Quality

Product quantity and quality in IC/BEVS-based VLP productions are strongly influenced by: (1) the recombinant baculovirus (passage number, stability), (2) the insect cell line (passage number, growth, and production behavior), (3) the culture medium and its additives, (4) the bioreactor and its bioengineering parameters, and (5) the growth, infection, and production parameters used. Along with the working cell bank (WCB), the working virus bank (WVB) forms the cornerstone of a VLP production process, which is delineated in Fig. 2 and discussed in Sect. 2.2. Recombinant baculovirus stocks with titers exceeding 10^8 plaque-forming units (pfu) mL^{-1} are preferred. To avoid the presence of defective interfering particles, they should be retitered every half-year and passaged fewer than five times [17]. In the face of genetic variations affecting VLP yield, the insect cells should be subcultivated a maximum of 30–50 times [25, 26].

The routinely applied insect cell lines include the *Spodoptera frugiperda*-derived ovarian cell lines *Sf*-21 (which are only important for baculovirus isolation and propagation) and *Sf*-9, and the *Trichoplusia* ni egg cell homogenate-derived cell line BI-TN-5B1-4, commonly referred to as the Hi-5 cell line. These insect cell lines grow at temperatures between 27° and 28 °C, and the bulk pH ranges between 6.1 and 6.5 [27, 28]. Unlike mammalian cells, they are not dependent on a humidified CO_2 balanced atmosphere. In addition, insect cell lines tolerate higher levels of nutrients (glucose, free amino acids). The main energy source in their culture medium is glucose [29], which is contained in concentrations of between 7 and 10 g L^{-1}. The growth of *Sf*-9 and Hi-5 cells is not limited by lactate, pyruvate, and ammonium [30, 31]. In contrast to mammalian cell cultures, lactate is normally negligible or only produced at very low levels by insect cells. Higher lactate levels indicate that the insect cells are oxygen-limited or stressed by shear [24, 32, 33]. When using serum- or protein-free culture media, insect cells grow in suspension. Indeed, this simplifies DSP, but may entail the addition of medium additives (polymers such as Pluronic F68) to protect cells against shear stress in dynamic bioreactors [34]. On the other hand, such polymers can hinder the interaction between virus and host, and may finally lower the quantity as well as the quality of the product [35]. A further possible limitation coming from serum absence is an increased VLP degradation by cell and viral proteases (protease activity is inhibited by serum).

The already mentioned shear stress caused by a bioreactor and its design can also inhibit VLP production. In this context, it should be pointed out that early VLP generation after cell infection is a critical point. The freshly infected cells are more sensitive to shear and require more oxygen than noninfected ones in the growth phase [36, 37]. Thus, phase-dependent adjustment of the main operating parameters of the bioreactor (such as aeration rate, DO level, tip speed) is important. In addition, the optimum infection parameters covering adequate multiplicity of infection (MOI), the cell concentration at infection (CCI), and the point of harvest (POH) have to be determined [28].

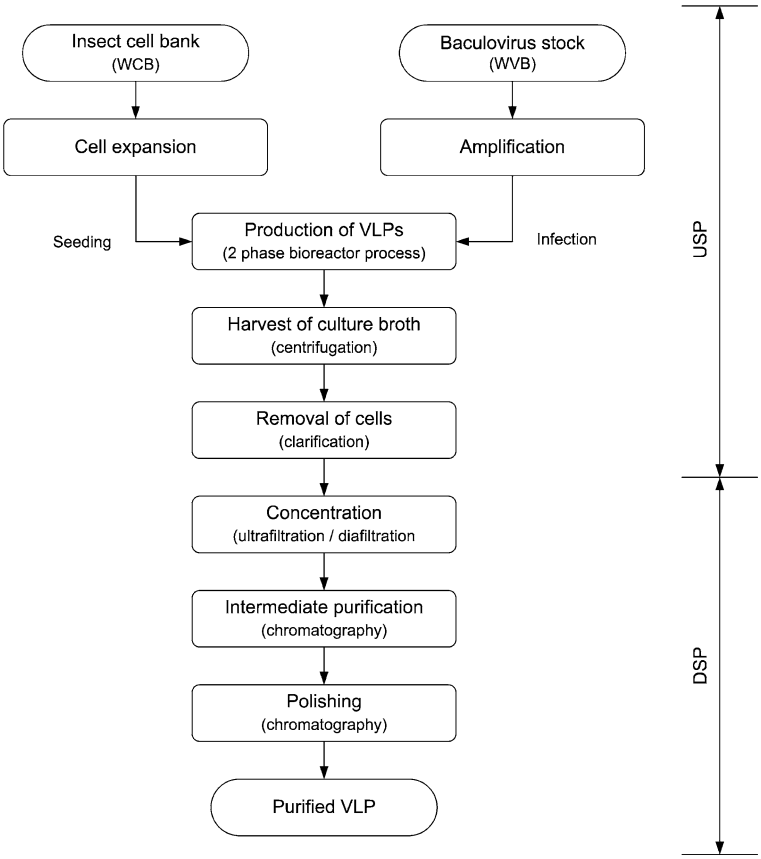

Fig. 2 Schematic diagram for IC/BEVS-based productions of secreted VLPs

2.2 Process Flow

In Fig. 2 a schematic diagram for typical IC/BEVS-based VLP vaccine production with secreted VLPs is presented. After expanding cells (viability over 95 %) and amplifying the virus in parallel, the production bioreactor (which is generally stirred or wave-mixed) [38–42] is seeded with noninfected cells. The cells are propagated until the desired CCI is reached. Subsequently, virus infection at the defined MOI is carried out. At optimum POH, which is often the instant when the peak cell diameter is obtained, the bioreactor broth is harvested. The cells are removed by centrifugation and/or depth-filtration, and the VLPs (contaminated by baculovirus particles, host cell protein, host cell DNA, and potentially endotoxins due to any buffer addition or nonaseptic step) are submitted to a purification process. VLP purification includes concentration by ultrafiltration, intermediate chromatographic purification, chromatographic polishing, and chemical virus

inactivation [43, 44]. The latter step is not shown in Fig. 2. If the VLPs are not secreted, a cell disruption method is stringently required before the clarification step indicated.

A number of scalable purification strategies [such as ion exchange chromatography (IEX), size-exclusion chromatography (SEC), and affinity chromatography] have been successfully established for VLPs [21, 45–48] in recent years. During the production of recombinant VLPs, baculoviruses are coproduced. In the case of enveloped VLP products, both the VLP and the baculovirus share the same original cell membrane. If they exhibit a similar particle size, DSP becomes even more complicated as both particles have for the most part very similar biophysical properties. Process design therefore becomes very challenging, for instance, in electrostatics-based unit operations such as IEX [43]. Other biophysical features such as very specific tagging need to be used to allow efficient separation of the two virus particle types.

3 SU Technology for IC/BEVS-Based Production Processes: State of the Art

In spite of frequently discussed issues connected with leachables and extractables [49–52], SU equipment is currently well accepted in USP and DSP of insect cell-based processes. The SU systems available on the market allow smaller, cheaper, safer, and faster process developments and productions when used and handled correctly [53]. In a first instance, this applies to small and medium-sized processes aimed at biotherapeutics, where vaccines represent an important product category. It therefore comes as no surprise that many manufacturers of human and veterinary vaccines (e.g., Baxter, Crucell, GSK, Sanofi Pasteur, Virbac) are increasingly using SU systems for their campaignwise production by reason of time savings, higher flexibility, and reduced risk of cross-contamination. As shown in Fig. 3, these SU systems include expendable laboratory items, simple peripheral elements, and equipment for unit operations and platform technologies [54, 55].

3.1 Upstream Processing

The availability of SU connectors, SU sampling and transfer systems, SU mixers, SU bioprocess containers, SU bioreactors, suitable sensors, SU pumps, and biomass separation systems enables the realization of complete SU-USP up to 2 m^3 working volume [56, 57]. Maximum selection diversity exists for bioreactors (see also “Semi- and commercial VLP vaccine products”), but generally only wave-mixed types such as Wave Bioreactor or BIOSTAT CultiBag RM and stirred types such as S.U.B., XDR, Mobius CellReady, or BIOSTAT CultiBag STR are utilized

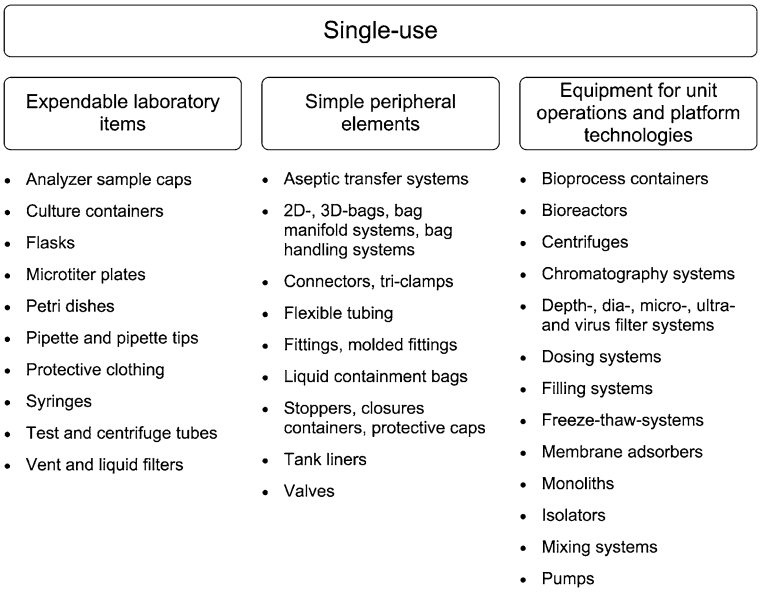

Fig. 3 Categorization of available single-use devices. (This material is reproduced from Ref. [54] with the kind permission of John Wiley & Sons, Inc.)

[10, 58]. It is interesting that descriptions of process developments and productions (batch, fed-batch, perfusion mode) of preclinical samples, which are all based on insect cells, mainly refer to wave-mixed bioreactors [21, 39–42, 59–63].

To perform continuously repeating USP operations (e.g., mixing, storage, transport, inoculum production, fermentation, and biomass separation), process platforms that combine these unit operations have proven to be beneficial. There are USP platforms from different vendors (e.g., Sartorius Stedim Biotech, Merck Millipore, GE Healthcare), characterized by different sizes as well as different numbers and sequences of the individual process steps for medium production, fermentation, and biomass separation [63, 64].

The technical limitations of available SU systems for USP result from the mechanical stability and scalability (max. 30-inch filter cartidges, max. 3,000-L bags) of the plastics. If SU–USP facilities are required that exceed the maximum size, this can be accomplished by parallelization of the SU devices.

3.2 Downstream Processing

Although the increasing implementation of SU technology for USP has also led to the development of SU systems for DSP, the latter has not yet achieved the importance of USP–SU processes. Nonetheless, all important process steps such as

virus decrease, ultra- and microfiltration, diafiltration, virus filtration, and bulk handling (filling, thawing, freezing) can currently be performed with SU systems [65–67]. In the instance of chromatography, SU systems have begun to catch up. Advantages such as flexibility, as well as reduced time and costs (which come from prepacked columns being ready to use) are, however, accompanied by the major drawback of the high, and sometimes prohibitive, expense of the chromatographic steps required. In addition, ready-to-use solutions for chromatography systems are limited to column bed volumes up to 20 L. This means that harvests at 1-kL scale and above would require multiple purification cycles. But the slow-paced development of SU systems for bead-based chromatography has given rise to alternative purification concepts [68].

Porous DSP chromatographic matrices are being used with great success for both viral clearance of biologicals or for the purification of large biopharmaceuticals. Disposable membrane adsorbers and monoliths have shown to be excellent alternatives to packed-bed chromatography in this context. Even though the dynamic binding capacity is typically lower than for bead-based matrices, membrane adsorbers (which are always designed as SU devices) and monoliths (recently also available as SU units) can be operated with higher flow rates. This was demonstrated in numerous reports for the purification of various virus particles [69–74].

4 Case Study: Fast Benchtop Influenza VLP Production Process Combining Redbiotec's rePAX™ with Single-Use Technology

Usage of SU systems goes hand-in-hand with intensified efforts to save additional time and costs by establishing novel USP concepts. This becomes urgent when seasonal and pandemic vaccines, such as for influenza, have to be manufactured. For example, in the event of a pandemic, the vaccine demand will be five- to tenfold that of the current global seasonal influenza vaccine production capacity [75]. Approaches to solving this problem include increasing the scale-up ratio (>1:5 steps) and omitting intermediate cultivation steps in shake flasks and spinners [60, 76–78]. Instead of cells that have already been expanded in shake flasks or spinners inoculated with cells from a vial, the bioreactor is directly inoculated with thawed cells from a cryobag. This method is also referred to as one-step inoculation and presupposes a large-volume WCB. Cells at high cell density (HCD, $>2 \times 10^7$ cells mL^{-1}) and of large volume are required, which can be guaranteed either by a large-volume cell expansion with subsequent centrifugation or continuous perfusion.

Due to its bioengineering characteristics (homogeneous energy dissipation, sufficient oxygen transfer for HCDs, no antifoam agent required, scalability up to 300-L culture volume), wave-mixed SU bioreactors are the most suitable for insect cell-based VLP productions [79].

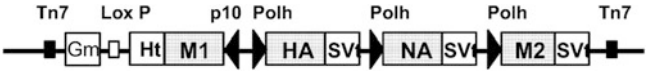

Fig. 4 Schematic representation of the transgene construct coding for all proteins of influenza A/H1N1/Puerto Rico/8/34 VLPs

As a case study, an influenza VLP production process completely based on SU technology was designed. The BIOSTAT CultiBag RM 20/50 was used for (1) production of the large-volume WCB stored in cryobags at −196 °C, (2) generation of the working seed virus stored in vials at −80 °C, (3) cell expansion (cryobag-to-CultiBag inoculation and repeated fed-batch expansion), and (4) influenza VLP stock production (1 and 10-L working volumes).

The primary aim was to generate, within the shortest period of time, preclinical influenza A/H1N1/Puerto Rico/8/34 VLPs (Fig. 4), assembled by four proteins [hemagglutinin (HA), neuraminidase (NA), and matrix M2, and M1 as capsid protein] and designed to express antigen-specific immune responses in a challenge experiment (see Sect. 4.7). The VLPs were produced by a single baculovirus infection with rePAX^{TM} coexpression technology (see also Sect. 4.2 and www.redbiotec.com).

Based on antecedent high-throughput screening (HTS) experiments, *Sf*-9 cells were chosen as the production cell line. HTS was performed in two single-use bioreactors, the CultiFlask 50 Disposable Bioreactor from Sartorius Stedim Biotech (which is also available under the trade name TubeSpin Bioreactor 50 from TPP) [80, 81] and the BioLector from m2p labs [82]. Taking the quantity as well as the quality of HA and NA into account, recombinant baculovirus of generation V1 (see also Sect. 4.3) was applied. From expressions verified by Dot blot and Western blot analyses, it was clear that an infection at low MOI (MOI < 1) was optimal and a temperature shift post infection (p.i.) should be avoided.

The *Sf*-9 cells (originally obtained from Gibco Invitrogen and from which a vial-WCB was available) were grown in Sf900-III SFM. Cell density, cell viability, cell size, cell morphology, and medium nutrient as well as metabolite concentration were observed daily during experimental runs. Baculovirus quantity (virus titer) was determined by plaque assay, and SDS-Page and Western blot were used to estimate baculovirus quality. In addition, transgene stability was checked by PCR (Fig. 5). Additional methods used to control quantity and quality of secreted influenza VLPs and their efficacy are summarized in Sects. 4.6 and 4.7.

4.1 Large-Volume WCB

Figure 6 shows the flowchart of the large-volume WCB creation covering HCD expansion, as well as filling, freezing, and storage of cryobags. A perfusion process was established that delivered HCDs ($>3 \times 10^7$ cells mL^{-1}) and viabilities exceeding 97 % within 5 days. This cell amount makes seeding of 30-L working volume with 1×10^6 cells mL^{-1} possible for subsequent VLP productions. If

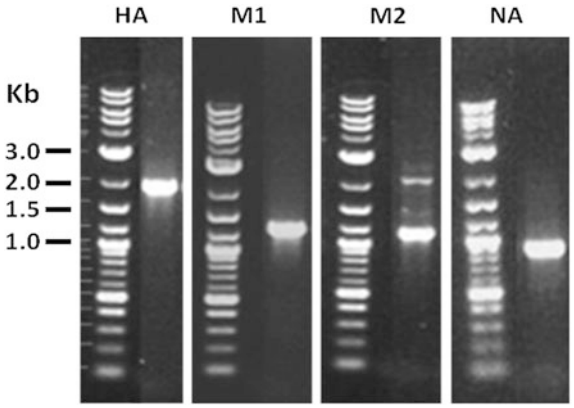

Fig. 5 PCR analysis on the presence of transgenes in the baculovirus genome. For HA, M1, and M2 transgenes, upstream primers were designed to bind on intergenic regions and the downstream primer to bind on transgene sequence. For NA, both primers bind to the transgene sequence

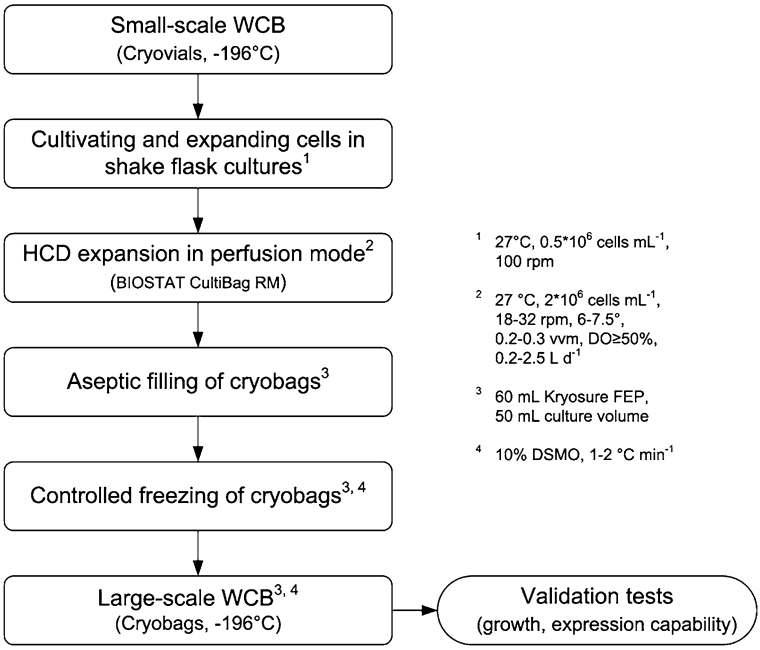

Fig. 6 Flowchart of the large-volume WCB creation

seeding with vial-based cells is performed and intermediate cultivation steps in shake flasks or spinners occur, a twofold increase in time (i.e., the time normally required to produce this amount of seed inoculum) is expected.

Maximal growth rates of 0.027 h^{-1} were calculated for the *Sf*-9 cells grown in 2-L bags containing 1.2-μm polyethersulfone membranes. These perfusion bags, which were operated with 1-L working volume, allowed monitoring and regulation of pH and DO. To maintain DO levels at a minimum of 50 %, the rocking rate (18–32 rpm) and rocking angle (6°–7.5°) were increased by a cascade control. At higher rocking angles and rates, pure oxygen was added (instead of raising the rocking rate and rocking angle further) in order to avoid strong foam formation. During continuous perfusion, glucose levels were maintained at between 8 and 10 g L^{-1} by exponentially increasing the initial perfusion rate (0.2 L d^{-1}) to 2×10^7 cells mL^{-1}. The perfusion rate was then linearly increased up to 2.5 L d^{-1}, and on day 5 a total of 3×10^{10} cells were harvested from one 2-L perfusion bag.

One 2-L perfusion bag guaranteed the preparation of fifteen 60-mL KryoSure FEP bags (50-mL working volume) from American Fluoroseal. This type of cryobag showed the most convincing results in an extensive test series that aimed to investigate the handling, disruption, and leakage behavior of different cryobag types at storage temperatures between −170 and −190 °C. But before filling the cryobags with cell stock, the *Sf*-9 cell suspension broth had to be aseptically transferred to storage bags (e.g., Flexboys) into which the cryoadditive (dimethyl sulfoxide, DMSO) was fed.

Filling was facilitated by placing the cryobags in metal storage boxes and keeping them on ice. The freezing procedure (freezing rates between 1 and 2 °C min^{-1}) was developed for a controlled rate freezer from Custom BioGenic system (CBS). Until the cryobags were used for one-step cell inoculation, they were kept in two independent cryovessels and stored in their liquid nitrogen vapor phases. The procedure of large-scale cell banking was successfully validated by periodically performing comparative growth studies with cryovial- and cryobag-derived cells in shake flasks and wave-mixed bags. Independent of the WCB type and the cultivation system, the cells doubled within 23.5 h, and showed similar growth, substrate, and metabolite courses. In addition, the expression capability of the *Sf*-9 cells was selectively verified. For Hi-5 cells, the suitability of a comparable but slightly modified approach was recently presented by Bögli et al. [61].

4.2 Cryobag-to-CultiBag Inoculation and Cell Expansion

For the direct cultivation of cryobag-derived cells in a wave-mixed CultiBag, a dilution of cell suspension and culture medium in a ratio of 1:10 is suggested. Moreover, initial cell density should not fall below 1.5×10^6 cells mL^{-1} for *Sf*-9 cells, and initial working volume should not be below 0.5 L. By rocking the bag at an identical rocking rate and rocking angle but with a lower cell suspension volume, a higher specific power input and, thus, more intense shear forces (which may cause cell damage) are generated [83]. The BIOSTAT CultiBag RM-based cell expansions were started at 27 °C, 6°, 16 rpm, 0.2 vvm and DO ≥ 50 %.

As soon as the cells achieved the exponential phase (between 24 and 48 h after inoculation), a first dilution was carried out. For this purpose, about half the cell culture broth was removed and the start conditions (initial cell density and culture volume) were re-established. As a consequence of the subsequent dilution step (normally after 72 h, only feed), the maximum working volume of 1 L was reached. The cells were then propagated (2–3 days) until peak cell densities of around 7×10^6 cells mL^{-1} were achieved. The expanded cells were used either for three parallel influenza VLP productions at 1-L scale or one VLP production at 10-L scale, with an initial working volume of 5 L (see also Sect. 4.4).

4.3 Virus Generation, Amplification, and Banking

The recombinant rBVH1N1-PR834 seed virus, V0, was engineered by transfecting *Sf*-21 cells cultivated in Sf900-III SFM. Assembly of four expression cassettes into the vector pRBT 136 (Fig. 4), transposition of the transfer vector into DH10MultiBac competent cells [84], selection and expansion of four correct clones, and DNA isolation [85] were successfully performed. The master seed virus, V1, was generated by amplification in shake flasks. By performing a subsequent amplification in the BIOSTAT CultiBag RM (1-L working volume), the working seed virus, V2, was produced. A CultiBag 2 L was filled with 0.5-L culture medium and inoculated with 1×10^6 cells mL^{-1}. Then 24 h post inoculation the infection (MOI = 0.1, CCI = 1×10^6 cells mL^{-1}) was carried out after culture medium feeding to maximum working volume.

Infection courses in the BIOSTAT CultiBag RM correlate well with those found in 250-mL shake flasks. Cell proliferation stop (48 h p.i.) and virus harvest (POH = 92 h p.i.) were achieved at the same time, and virus titers ranged between 1 and 1.3×10^8 pfu mL^{-1}. The four desired transgenes were detected independently of the cultivation system. Assuming a MOI of 0.01, CCI of 2×10^6 cells mL^{-1}, and virus titer of 1×10^8 pfu mL^{-1}, one CultiBag 2 L delivers working seed virus for 16 preclinical VLP productions at 300-L scale.

After separating cells (centrifugation up to 1 L, depth filtration at a scale >1 L), the V2 virus broth was aseptically filled into vials. Long-term storage of the virus stock in Sf900-III SFM supplemented with 10 % glycerol at −80 °C was performed by controlling the freezing rate at 1 °C min^{-1} until reaching −80 °C. However, decreasing virus titers and infection capabilities (due to possible stability issues) have to be considered. Figure 7 shows that, after 8 months storage, the virus titers decreased by a factor of between five and six under the best storage conditions. Because virus titers had already decreased by 80 % after 4 months storage time, long-term storage at 4 months and −80 °C is not recommended without additives. Jorio et al. [86] explain the loss of infection capability with aggregate formation.

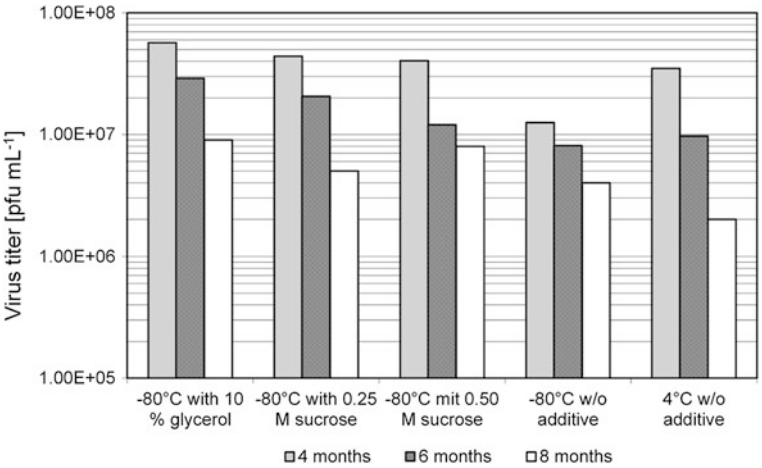

Fig. 7 Virus titers (V2) as a function of selected storage conditions (additives, storage time). The dashed line indicates the original virus titer (6 × 107 cells Ml^{-1}) after separation and purification

4.4 VLP Stock Production at 1-L and 10-L Scale

The VLP productions at 1- and 10-L scale were performed with *Sf*-9 cells from 60-mL KryoSure FEP bags and V2 virus stored in cryovials. As described in Sect. 4.2, the cell inoculum (5–6 × 10^6 cells mL^{-1}) for the 10-L stock productions had previously been generated in CultiBags 2 L (1-L working volume). The seed cells were then transferred to the production bag and further propagated (between 1 and 2 days) in feeding mode. Whereas the rocking angle, gas volume flow per unit of liquid volume per minute, and DO level for starting the BIOSTAT CultiBag RM were identical at 1- and 10-L scale, the initial rocking rates were set differently. Due to the nongeometrical similarity of the CultiBags at 1- and 10-L scale, the initial rocking rate needed to be adjusted to ensure a comparable fluid flow regardless of scale.

After completing the final feeds in the production bags, cell densities of 2 × 10^6 cells mL^{-1} (corresponding to the CCI at maximum working bag volume) were reached and the infections were initiated with the working virus stock (MOI = 0.01). At 88 h p.i. the VLP stocks were harvested.

All BIOSTAT CultiBag RM-based production experiments delivered similar results, which were also in good agreement with those obtained in parallel shake flask runs. Figure 8 exemplifies the infection kinetics measured in VLP productions using shake flasks and the BIOSTAT CultiBag RM at 1-L scale. The cells achieved maximum exponential growth rates between 0.03 and 0.032 h^{-1} at 24 h p.i. The maximum cell density (5.5–6 × 10^6 cells mL^{-1}) was detected at 48 h p.i.

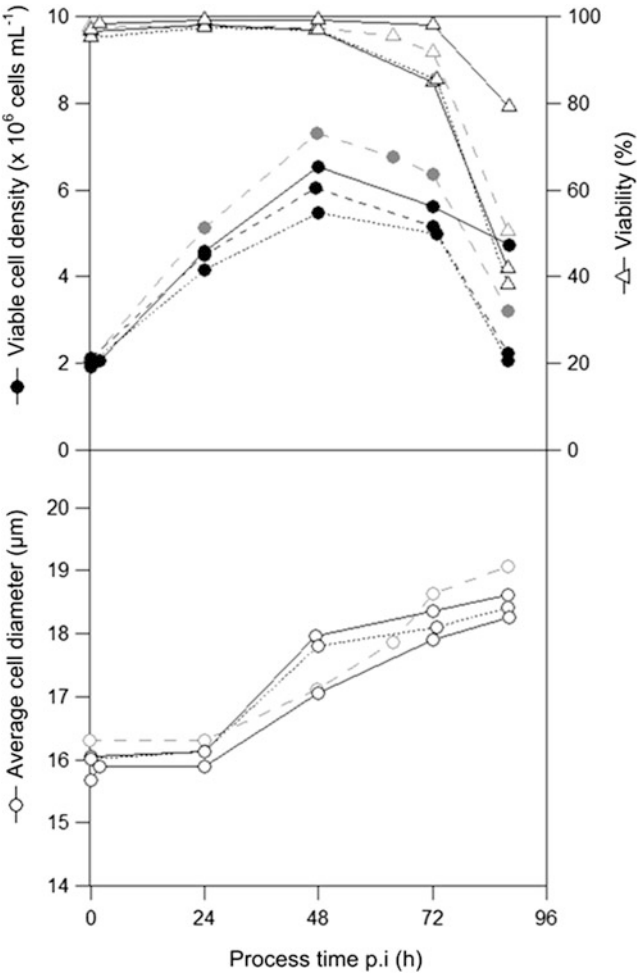

Fig. 8 Preclinical influenza VLP productions at mL and 1-L scale. Shake flasks are shown in grey; the continuous and dashed lines represent data from two BIOSTAT CultiBag RM-based productions

4.5 DSP of the VLP Stock

Recovery and purification of the VLPs produced were performed as described in [43] and [87]. Clarification at benchtop-scale was carried out with depth-filter capsules having either 0.65-µm (Sartopure PP2 from Sartorius Stedim Biotech) or 0.5-µm (Mini Profile filter capsule from Pall) pore size. Both are SU/disposable polypropylene depth-filter membranes. The clarification process developed gave consistently high recovery yields exceeding 90 % based on hemagglutination assay.

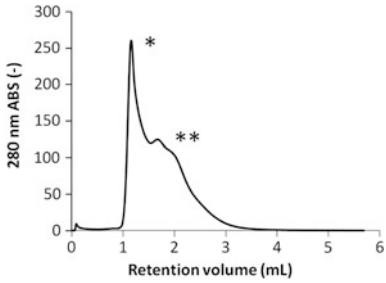

Fig. 9 SEC of the final VLP stock

As an intermediate concentration step, ultrafiltration combined with diafiltration (tangential crossflow filtration, concentration factor of 10, two diafiltration volumes for buffer exchange) was implemented. A 500-kDa molecular weight cutoff (MWCO) Centramate membrane cassette of 0.1 m^2 (Pall) was used with a transmembrane pressure (TMP) of 0.8–1.1 bar, adjusted using the retentate side valve. Over a number of different concentration trials, 75 % recovery was achieved based on hemagglutination assay. An additional capture step relying on a bind-elute chromatography step improved product purity. Membrane adsorbers (Sartobind Q from Sartorius Stedim Biotech) and monoliths (CIM QA from Bia Separations) were evaluated in terms of their suitability for purifying large particles (especially for binding and eluting). Both matrices were coupled to either an Äktapurifier 100 or an Äkta avant 150 chromatography system controlled with UNICORN software (GE Healthcare). Equilibration buffers comprised 50-mM MES (pH 6.5) for cation exchange or 50-mM HEPES (pH 7.2) for anion exchange. Elution was carried out using a linear elution gradient up to 1 M NaCl. As a final concentration step, the product elution pools were subjected to ultrafiltration/diafiltration using a 750-kDa MWCO hollow fiber cartridge (GE Healthcare). Bind-elute anion exchange chromatography (AEX) with membrane adsorbers or monoliths resulted in over 80 % recovery yields based on hemagglutination assay. Special attention was given to the total protein concentration upon loading the matrix in order to prevent breakthrough and, thus, product loss. As a conservative measure, the load of material to the matrix was set to less than 80 % of the binding capacity. A final concentration step (using a 750-kDa MWCO hollow fiber cartridge) yielded up to 100 % recovery based on hemagglutination assay. Furthermore, it was observed that more than five diafiltration volumes did not remove additional low molecular weight protein/lower molecular weight protein further.

Although 3 $\log_{10}$ reduction of baculoviruses was obtained using this DSP approach, the final product still contained a titer of 5×10^8 pfu mL^{-1}. As observed by SEC, the final material shows a sharp void volume peak (Fig. 9), which is caused by VLPs and baculoviruses (*), and a further peak (**) illustrating residual host cell proteins. Preparative SEC and a combination of IEX steps are currently being developed to improve host-cell protein and baculovirus removal.

Table 1 Main methods used to control quantity and quality of influenza VLPs

Parameter	Method
Quantity	
Total protein	Bradford
HA protein	Dot blot, Western blot, and comparative HA assay with commercial vaccine and known amount of HA
Quality	
Presence and size of VLPs	Electron microscopy
VLP purity	SDS-Page
Presence and functionality of NA	NA assay (fluorescence measurement)
Presence and functionality of HA	HA assay and single radial immune diffusion (SRD)
Presence of M1 and M2 proteins	Western blot and pool of sera from mice immunized with PR8/34 VLPs

4.6 Basic Characterization of the VLPs

Table 1 provides an overview of the main methods applied to control the quantity and quality of the VLPs routinely. These methods can be used to establish that (1) the VLPs have been successfully expressed, (2) all four cloned transgenes have been coexpressed, and (3) the corresponding proteins have been assembled into the final VLP candidate.

HA concentrations between 1 and 3 mg L^{-1} were determined for the VLP stock generated in the BIOSTAT CultiBag RM-based productions at 1- and 10-L scale. These concentrations are in accordance with data published by other groups [43]. Electron microscopy images showed VLPs of 90–120 nm in size (Fig. 10). They resembled the physical shape of the parental virus and indicated the correct assembly of the M1 protein together with the envelope harboring NA, HA, and M2. Purified VLP stocks were found to be NA and HA active (Fig. 11). Antibodies from immunized mice detected protein bands (with molecular weights of around 27 and 11 kDa) only in the VLP samples, which confirmed the presence of M1 and M2.

4.7 Mouse Studies

The basic characterization of the VLPs was supplemented by mouse studies. They were conducted at the University of Wisconsin–Madison animal facilities and approved by its Interinstitutional Animal Care and Use Committee. The mouse studies were aimed at the detection of humoral and cellular immune responses caused by the purified influenza A H1N1 VLPs from VLP productions at 1- and 10-L scale. Three groups of 6- to 8-week-old BALB/c mice were immunized with prime and boost doses of 100 μL PR8/34 VLPs containing 3 μg HA, empty baculovirus, or phosphate buffered saline (PBS) solution. Bleedings to analyze the humoral immune responses were performed on days 0, 21 (prime), and 42 (boost).

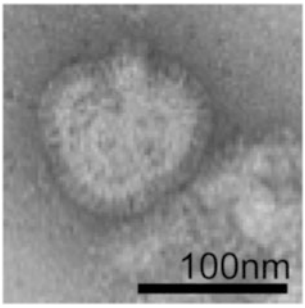

Fig. 10 Exemplary electron microscopy of generated influenza A H1N1 VLPs

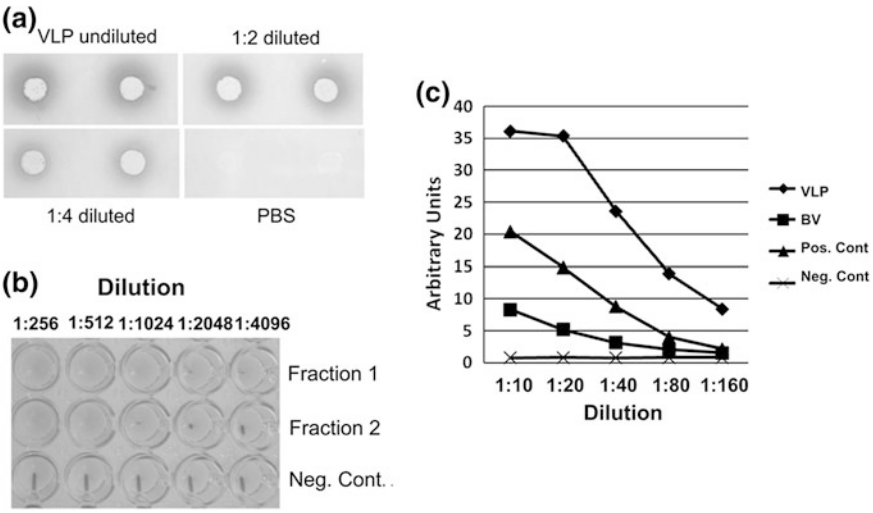

Fig. 11 Immune and functional assays for VLP characterization. SRD analysis of VLP dilutions to detect HA (**a**). HA assay indicating the functionality of HA of two different VLP fractions after SEC in comparison to a PBS negative control (**b**). NA assay showing functionality of the VLP-NA protein (**c**)

On experimental day 42, all three mice groups were challenged intranasally with 10^4 $TCID_{50}$ units of PR8/34 influenza virus. Percentage mean weight and survival rates of the experimental groups were recorded for 2 weeks post challenge. Three days following challenge, three mice from each group were euthanized and their lungs harvested for viral load titration. Collected serum samples were tested for: (1) total immunoglobuline G (IgG) by enzyme- linked immunostaining assay, (2) PR8/34 specific HA inhibition by HA assay, (3) neutralizing antibody titers against PR8/34 and A/Aichi/2/68 by microneutralization assay, (4) viral titers by quantification on $TCID_{50}$ on MDCK cells, and (5) interferon-gamma (IFN-γ) by enzyme-linked immunospot (ELISPOT).

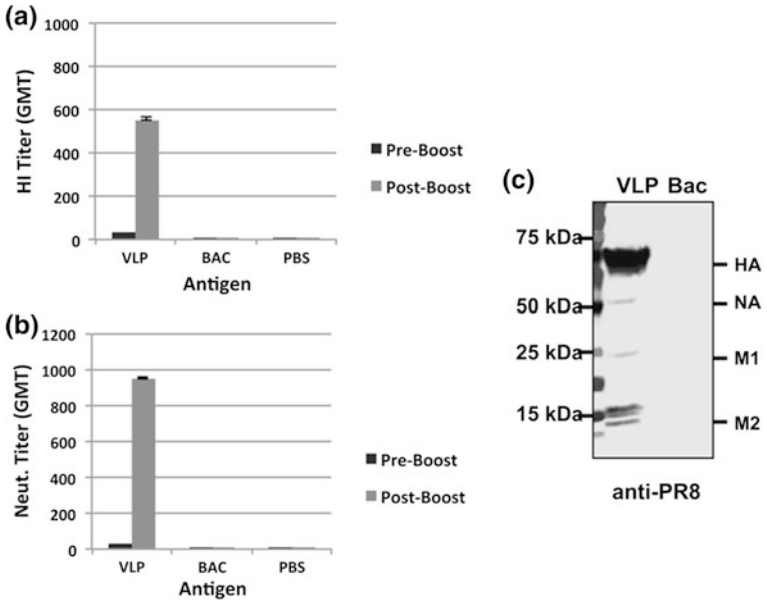

Fig. 12 Humoral immune response and cross-reactivity of the purified influenza VLPs. Sera from VLP-immunized mice inhibited hemagglutination capacity (**a**) and neutralized the parental PR8/34 virus (**b**), whereas no effect was observed with samples of empty baculovirus (BV) or PBS. PR8/34 VLPs were analyzed by Western blot using sera from mice infected with influenza A H1N1 PR8/34 virus (**c**)

Geometric mean titers (GMTs) of 2,560 and 10,240 of total IgG were recorded for mice immunized with the VLPs after prime and boost, respectively. In the empty baculovirus and PBS control groups, no IgG was detected. As can be seen in Fig. 12a, hemagglutinin inhibition (HI) tests resulted in a specific antibody increase of 17 times after boost. Postboost antibody titers were significantly higher ($P < 0.05$) than preboost titers. A statistically significant increase in neutralizing antibodies of 30 times after boost was found in the microneutralization assay (Fig. 12b). In Western blot analyses, immune serum against H1N1/PR8/34 reacted with VLP samples and detected four bands corresponding to the four proteins of the VLPs (Fig. 12c). No cross-reactivity was found in empty baculoviruses.

Figure 13a shows that pre- and postboost IFN-γ production of mice immunized with the VLPs were higher ($P < 0.05$) than those of mice immunized with the empty baculoviruses or PBS. A sevenfold increase in the number of spots was found (from 35 to 243 spots). In contrast, no significant differences were observed between mice immunized with the controls (empty baculovirus or PBS). However, due to the fact that the whole parental virus was used for restimulation, it is not clear at which protein(s) in the influenza VLPs the cellular immune response was directed.

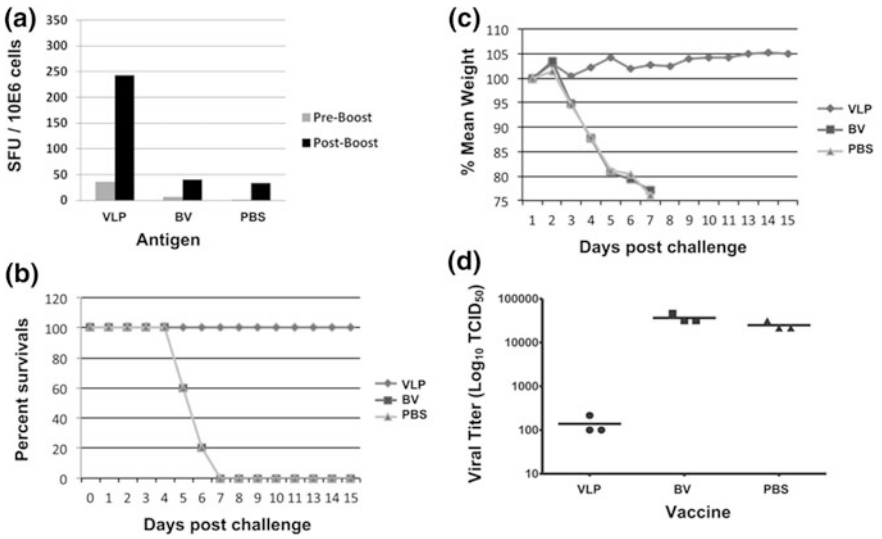

Fig. 13 Cellular immune response and challenge experiment. Pre- and postboost IFN-γ production measured by ELISPOT (**a**). Weight loss (**b**) and mortality (**c**) observed after day 6 in control mice. Infectious virus titers in lungs of mice immunized with VLPs, baculovirus, and PBS (**d**)

Whereas mice immunized with the VLP candidate gained weight of about 5 % over the two-week period, the mice in the control groups consistently lost about 20 % of their body weight (Fig. 13b). All mice immunized with VLPs survived the challenge with the influenza A/PR8/34 H1N1 virus (Fig. 13c) but the control groups succumbed to challenge and were euthanized by day 7 postchallenge. The median time to death of the mice in the control groups was 6 days. Figure 13d illustrates that the viral titer for the VLP group was lower ($P \leq 0.05$) than those estimated for the control groups. The baculovirus group recorded a slightly higher mean titer (3.65×10^4 $TCID_{50}$ units) than the PBS group (2.49×10^4 $TCID_{50}$ units) although the titers were not significantly different ($P > 0.05$). Overall, these results provide convincing evidence of the vaccine candidate potential of our VLP and SU technology.

5 Summary and Outlook

In this review, the implementation of SU devices in production facilities for vaccines and, in particular, for VLP vaccines based on the IC/BEVS has been discussed. When correctly used, currently available SU systems not only allow for safer development and manufacturing of vaccines, but also guarantee additional time and cost savings based on new USP and DSP concepts [61]. This is an

enormous advantage for VLP vaccine candidates aimed at the prevention of seasonal and pandemic infections, for instance, influenza. It is not surprising that different vaccine manufacturers are currently using similar approaches to the one presented in this case study. For example, Novavax (www.novavax.com) has an influenza IC/BEVS-based VLP vaccine candidate in the pipeline, which expresses only three of the four proteins used in our VLP vaccine candidate.

Irrespective of the product, it is possible to shorten the manufacturing timeline of an influenza VLP vaccine by at least 50 % when compared to a classical influenza vaccine manufactured in eggs. Moreover, there is the potential to reach the same degree of protection without adding any adjuvants. The final goal is a flexible vaccine factory consisting wholly of single-use devices that can be integrated in advanced, well-prepared containers. This would make vaccine manufacturing in a short timeframe possible almost anywhere in the world. Innovative solutions such as Sartorius Stedim Biotech's FlexMoSys concept are paving the way for the realization of this vision.

Acknowledgments We would like to thank Nathalie Riesen and Christoph Ries (ZHAW, Wädenswil, CH) for their assistance regarding design and analysis of the VLP production experiments as well as Nicole Imseng (ZHAW, Wädenswil, CH) for her practical support and proofreading. Armando Zuniga (Redbiotec AG, Schlieren, CH), Brock Kingstad-Bakke, Joseph Brewoo, and Jorge Osorio (all University of Wisconsin–Madison, USA) are acknowledged for the execution and interpretation of the mouse preclinical studies. Julia Friesen, Matthias Meier, and Dorothea Karrer (all Redbiotec AG, Schlieren, CH) receive our gratitude for their excellent laboratory support. We also thank Christian Schaub for taking overall responsibility and providing support for the project at Redbiotec AG. This work was supported by the Commission for Technology and Innovation based in Switzerland.

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Adv Biochem Eng Biotechnol (2014) 138: 127–147
DOI: 10.1007/10_2013_189

Published Online: 20 April 2013

Microbial High Cell Density Fermentations in a Stirred Single-Use Bioreactor

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Abstract Microbial fermentations are of major importance in the field of biotechnology. The range of applications is rather extensive, for example, the production of vaccines, recombinant proteins, and plasmids. During the past decades single-use bioreactors have become widely accepted in the biopharmaceutical industry. This acceptance is due to the several advantages these bioreactors offer, such as reduced operational and investment costs. Although this technology is attractive for microbial applications, its usage is rarely found. The main limitations are a relatively low oxygen transfer rate and cooling capacity. The aim of this study was to examine a stirred single-use bioreactor for its microbial suitability. Therefore, the important process engineering parameters volumetric mass transfer coefficient (k_La), mixing time, and the heat transfer coefficient were determined. Based on the k_La characteristics a mathematical model was established that was used with the other process engineering parameters to create a control space. For a further verification of the control space for microbial suitability, *Escherichia coli* and *Pichia pastoris* high cell density fermentations were carried out. The achieved cell density for the *E. coli* fermentation was $OD_{600} = 175$ (DCW = 60.8 g/L). For the *P. pastoris* cultivation a wet cell weight of 381 g/L was reached. The achieved cell densities were comparable to fermentations in stainless steel bioreactors. Furthermore, the expression of recombinant proteins with titers up to 9 g/L was guaranteed.

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Keywords *Escherichia coli* · Fed batch · Heat transfer coefficient · k_La · Mixing time · Nanobodies® · *Pichia pastoris* · Process engineering characterization · Stirred single-use bioreactor

Abbreviation

a	Empirical value for mathematical k_La description
A	Heat transfer area
AOX	Alcohol oxidase
b	Empirical value for mathematical k_La description
Δc	Concentration difference
Δc_0	Initial concentration difference
$C(t)$	Dissolved oxygen concentration
C^*	Oxygen saturation concentration
CQA	Critical quality attributes
d_1	Vessel diameter
d_2	Impeller diameter
DCU	Digital control unit
DCW	Dry cell weight
E. coli	*Escherichia coli*
$F(t)$	Feed flow rate
F_G	Gas flow rate
HCDF	High cell density fermentation
IPTG	Isopropyl-β-D-thiogalactopyranoside
k	Heat transfer coefficient
k_La	Volumetric mass transfer coefficient
KPP	Key process parameters
M	Goodness of mixture
n	Stirrer speed
Ne	Newton number
OD_{600}	Optical density measured at 600 nm
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
P/V	Power input per volume
P. pastoris	*Pichia pastoris*
pO_2	Oxygen partial pressure
q_{O_2}	Specific oxygen uptake rate
Q	Heat flow
Q_{prod}	Released heat flow
QbD	Quality by design
RM	Rocking motion
RO	Reverse osmoses
s	Thickness of the reactor wall
S	Correlation factor for the heat generation
S_{Feed}	Concentration of the feed solution

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STR	Stirred tank reactor
t	Time
ΔT	Temperature difference
u	Tip speed
V	Filling volume of the bioreactor
V_0	Initial volume
WCW	Wet cell weight
X	Cell density (dry cell weight)
X_0	Initial cell density
Y_{X/O_2}	Oxygen yield coefficient
$Y_{X/S}$	Yield coefficient
Z	Empirical value for mathematical k_La description
α_1	Convective heat transfer of the cooling liquid inside the double wall
α_2	Convective heat transfer of the reactor wall into the medium
λ	Wall thermal conductivity
μ	Specific growth rate
μ_{set}	Selected specific growth rate for the fed batch
ρ	Density
θ	Mixing time
v	Superficial air velocity

Contents

1 Introduction

The use of microorganisms has great importance for the production of pharmaceuticals in biotechnology. The fast and undemanding growth of these organisms makes them cost-efficient expression systems. These organisms produce nowadays 58 % of recombinant proteins and enzymes in industry [1]. Therefore, microorganisms are of great financial interest because the market has US $99 billion total sales [2]. Typical products are vaccines, plasmids, and recombinant proteins produced by bacterial expression systems such as *Escherichia coli* or yeasts such as *Pichia pastoris* [3, 4]. Economic pressure leads to the development of new microbial production platforms, which allow posttranslational modifications [5] or secretion of the target protein [6]. These features were the main limitation of microorganisms for the production of recombinant proteins in the past.

Nowadays, stainless steel fermenters are the gold standard cultivation devices for microbial cells. During the last decades the use of single-use bioreactors has become more and more attractive for applications with mammalian and insect cells [7] due to the many advantages these bioreactors offer. These are, for example, the reduced investment and operational cost, the improved time-to-market by the short lead times, reduced qualification efforts, as well as a maximized facility output by a reduced operational downtime [8, 9]. Based on this, processes in single-use systems have the opportunity to be very cost efficient. Furthermore, these bioreactors offer higher flexibility and, because the cultivation chamber is discarded after the fermentation, the risk of cross-contaminations is significantly reduced [10].

Microbial processes are subjected to the same cost pressure as mammalian cell applications. Therefore, single-use bioreactors represent an interesting technology for this field. Although these bioreactors offer manifold advantages and opportunities microbial cultivations are seldom reported and in general the achieved cell densities are lower compared to fermentations in stainless steel bioreactors [11].

Many different single-use bioreactors are now available on the market. They differ in terms of shape, power input, and gassing strategy. The first available bioreactor was the rocking motion–type bioreactor in the late 1990s [12]. For this device, mixing and oxygen transfer are achieved by wave-induced motion. Back and forth movement of a platform generates waves at the liquid/gas interface. These systems are only aerated by overlay [13]. Because of the gentle mixing device and the bubble-free aeration, these systems are mainly used for the cultivation of shear-sensitive cell lines such as mammalian, plant, and insect cells [7, 12]. There have been some published attempts to cultivate fast-growing microorganisms in such bioreactors. The yeast *Saccaromyces cerevisae* was successfully cultivated to a DCW of 9 g/L. This is approximately two times higher compared to shake flask cultures [14]. By using a substrate-limiting fed batch mode for an *E. coli* fermentation, cell densities of $OD_{600} = 60$ (DCW = 20 g/L) were achieved [11]. With an adapted linear feeding strategy it was possible to achieve cell densities comparable to reusable fermenters [15]. For these trials the BIOSTAT® RM by Sartorius Stedim Biotech was used, which had a heating and

Table 1 Different single-use bioreactors for microbial applications

Bioreactor	Vendor	Working principle	Reference
CELL-tainer®	Cellution	Mechanically driven, wave mixed	[16, 17]
BIOSTAT® RM	Sartorius Stedim Biotech	Mechanically driven, wave mixed	[15]
XDR	Xcellerex (now part of GE Helthcare)	Magnetic coupling, stirring	[18]
BIOSTAT® STR 50	Sartorius Stedim Biotech	Magnetic coupling, stirring	[15, 19]

cooling coil to remove the heat generated by the cells. To improve the rocking-motion–type bioreactor regarding the oxygen transfer rate, mixing efficiency devices were developed, which are characterized by an additional horizontal displacement [16]. Although k_Las above 300 h^{-1} have been published for such a device, the reached cell densities for *E. coli* fermentations were lower than these high oxygen transfer rates let suggest [17].

Another type of bioreactor is the stirred system where the mixing and the power input are achieved by impellers and in general they are actively aerated. Different types are available on the market [13]. The impeller design and position, as well as the sparger design differ from conventional stainless steel fermenters, hence, a direct scale-up and process transfer can be challenging. With a new developed and technically adapted bioreactor for mammalian cells by Xcellerex (XDR-50 turbo) successful tests with different microorganisms were performed. During an *E. coli* fermentation an OD_{600} of 145 was achieved [18]. Table 1 displays some single-use bioreactors for microbial fermentations.

In general the main limitations of single-use bioreactors for microbial cultivations are low oxygen transfer rate, inefficient mixing, or inadequate heat transfer. To by pass these limitations a substrate-limiting fed batch mode can be used. By this approach the specific growth rate can be controlled, which can be followed by the Monod-kinetic [20]. As a consequence, parameters such as the specific oxygen uptake rate, nutrient uptake rates, and heat generation are influenced.

2 Stirred Single-Use Bioreactor BIOSTAT® STR

A bioreactor where the design relates to those from reusable fermenters is the BIOSTAT® STR by Sartorius Stedim Biotech. It has a cylindrical cultivation chamber, two impellers mounted on a rigid shaft, and the gassing is carried out by a submerged sparger. The holes of the sparger have a diameter of 0.8 mm. Because the impeller shaft is connected to the motor via a magnetic coupling, the culture system remains closed and sterile. It is designed with a height-to-diameter ratio of 2:1, a convex bottom (see Fig. 1a), and an impeller-to-bag diameter ratio of 0.38 [19]. The STR family was designed for the cultivation of mammalian cells up to 2,000 L scale.

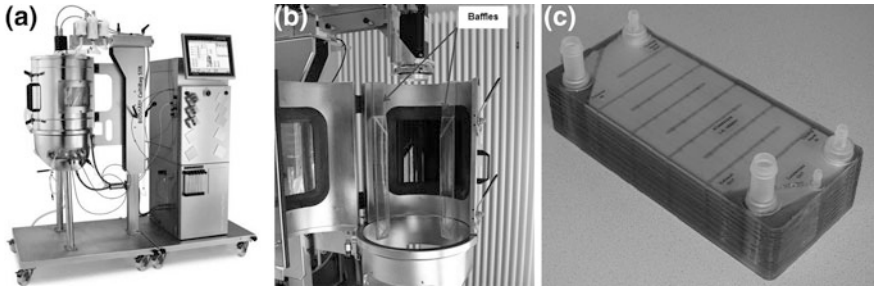

Fig. 1 **a** The BIOSTAT® STR 50 with digital control unit (DCU-tower). **b** Used baffles, which are installed at the outside of the cultivation chamber. **c** A single-use plate heat exchanger used as exhaust cooler

The main limitation for microbial use is the oxygen transfer rate. Modifications were necessary to perform microbial cultivations in the BIOSTAT® STR 50 cell culture version. In order to improve the oxygen transfer rate of the stirred single-use bioreactor, the power input per volume and the gas flow rate were increased. The power input per volume was improved by increasing the stirrer speed. To avoid poor flow patterns (vortexing) at higher stirrer speeds four baffles were installed at the outside of the cultivation chamber (see Fig. 1b) to increase turbulence. The baffles displace the cultivation chamber as a result of the bag shape alteration. To guarantee sufficient head space, the maximal filling volume was reduced to 45 L. The gas flow rate was increased to 20 Lpm (0.44 vvm). Due to the higher gas flow rate, a larger amount of water vapor enters the exhaust filter and may cause a blockage. To avoid this, a single-use exhaust cooler was installed (see Fig. 1c), which was designed as a plate heat exchanger. Water vapor passes through the exhaust cooler and condenses before it enters the exhaust gas filter. The condensed water is redirected into the cultivation chamber. A digital controlling unit (DCU) from the reusable fermenters is adjusted to the single-use cultivation platform. The controlling unit utilizes fluorescence-based single-use sensors for pO_2 and pH control. Furthermore, there is the opportunity to install classical probes for the pH and pO_2 measurements. For microbial fermentations the impeller configuration 6-blade-disk impeller (bottom) and 3-blade-segment impeller (top) was used. This configuration was selected because it guarantees the highest power input per volume for the given torque that can be transferred by the magnetic coupling (2 Nm). The Newton number for this configuration is 3.2 (data Sartorius Stedim Biotech).

3 Control Space Approach

An essential part of the pharmaceutical industry is quality by design (QbD) to ensure stable product quality. This is a concept that suggests the quality can be planned and controlled [21]. This is accomplished by the adjustment of process-

specific critical quality attributes (CQA). In order to achieve this detailed knowledge of the fermentation process and the bioreactor, CQAs are necessary. For fermentation processes typical CQAs can be product glycosylation or purity, for example [22]. This allows the definition of parameters where the process performance is optimal (key process parameters, KPP) [23]. Typical KPPs are the oxygen transfer rate, mixing efficiency/homogeneity, or the feeding strategy. To evaluate if the stirred single-use bioreactor fulfills the KPPs for microbial applications, the mixing time, volumetric mass transfer coefficient (k_La), and heat transfer coefficient were determined by process-engineering characterization. It is well known that for optimal microbial growth the parameter oxygen transfer rate and temperature control are important [24]. From these values a control space model for the oxygen transfer was developed.

3.1 Process Engineering Characterization

Microorganisms can have high substrate consumption rates [24]. Hence, it is possible that concentration gradients occur in the medium, which can have disadvantageous effects on the growth behavior. Based on this, a bioreactor is needed that ensures sufficient mixing efficiency. The mixing behavior of a bioreactor can be described by two parameters: the goodness of mixture and the mixing time [25]. These parameters are influenced by the reactor geometry, the power input, and the impeller design. The goodness of mixture is the degree of homogeneity after inhomogeneity occurs in a solution (1).

$$M = 1 - \frac{\Delta c}{\Delta c_0} \tag{1}$$

where M is the goodness of mixture (–), Δc is the concentration difference (mol/L), and Δc_0 is the initial concentration difference (mol/L). The mixing time defines the time needed to completely homogenize a solution after the occurrence of inhomogeneity. Commonly, homogenization is regarded as complete when a goodness of mixture of 0.95 is achieved [26]. For microbial fermentations mixing times below 10 s are regarded as suitable [24].

The mixing time is influenced by the stirrer speed of a bioreactor, which can be characterized by the tip speed (2). For microorganisms in general, tip speeds between 2 and 6 m/s are used [27].

$$u = \pi \cdot n \cdot d_2 \tag{2}$$

where u is the tip speed (m/s), n the stirrer speed (s^{-1}), and d_2 is impeller diameter (m).

For aerobic growing organisms oxygen is a key nutrient. Oxygen is important for growth, maintenance, and metabolic production [28]. The consumed amount of oxygen by the organisms is given by the oxygen uptake rate (OUR [mol/(L · h)];

(3)). It depends on the specific growth rate [μ (h^{-1})] and the cell density [X (g_{DCW}/L)]. It is further characterized by the oxygen yield coefficient [$Y_{X/O2}$ (g_{DCW}/mol_{O2})], which depends on the cultivated organism and the carbon source. A typical value for of $Y_{X/O2}$ for *E. coli* is 39 (g_{DCW}/mol_{O2}) [29], which corresponds to a specific oxygen uptake rate (q_{O2}) of 17.5 mmol/(g_{DCW} · h). Compared to other organisms this is fairly high and an OUR of 350 mmol/(L · h) has been reported [30] for *E. coli* fermentation, which can be challenging for bioreactors.

$$OUR = q_{O_2} \cdot X = \frac{\mu}{Y_{X/O_2}} \cdot X \tag{3}$$

During cultivation a sufficient oxygen amount must be transferred into the medium. The transferred oxygen is given by the oxygen transfer rate (4). It depends on the difference between the oxygen saturation concentration and the dissolved oxygen concentration [27]. This difference can be increased, for example, by aeration with oxygen enriched air. The *OTR* is also influenced by the volumetric mass transfer coefficient, which describes the efficiency of the oxygen transfer for a bioreactor. It is influenced by the bioreactor and impeller design, aeration strategy, and the power input.

$$OTR = k_L a \cdot (C^* - C) \tag{4}$$

where *OTR* is the oxygen transfer rate [mol/(L · h)], $k_L a$ the volumetric mass transfer coefficient (h^{-1}), C^* oxygen saturation concentration (mol/L), and C is the dissolved oxygen concentration (mol/L).

To ensure aerobic conditions during a fermentation the *OTR* has to be at least equal to the *OUR*. Based on empirical relationships it is possible to describe the $k_L a$ mathematically. One description was given by Van't Riet where the $k_L a$ is correlated with the power input per volume *P/V* and the superficial air velocity v [31]. *P/V* is influenced by the stirrer speed, impeller geometry, and installation height of the impeller (see 5) [27]. For microbial fermentations *P/V* above 3,750 W/m^3 can be found [32].

$$P/V = \frac{Ne \cdot \rho \cdot n^3 \cdot d_2^5}{V} \tag{5}$$

where *P/V* is power input per volume W/m^3, ρ is the density of the liquid kg/m^3, and V is the filling volume of the bioreactor m^3. *Ne* is the dimensionless Newton number (–), which depends on the impeller geometry. To describe the influence of the gas flow rate, the superficial air velocity is used (6).

$$v = \frac{F_G}{\frac{\pi}{4} d_1^2} \tag{6}$$

where v is the superficial air velocity m/s, F_G is the gas flow rate m^3/s, and d_1 is the vessel diameter m. One empirical relationship to calculate the $k_L a$ is given by (7) [31].

$$k_L a = Z \cdot P^a \cdot v^b \tag{7}$$

where Z, a, and b are empirical determined values.

During the fermentation processes heat is produced by the cells. Because only 40–50 % of the stored energy in the carbon source is used for biomass production [33], the residual amount is released as heat. For aerobic organisms the produced heat correlates with the OUR (8) because oxygen is the final electron acceptor [34].

$$Q_{prod} = S \cdot OUR \cdot V \tag{8}$$

with Q_{prod} is the released heat flow W, V is the filling volume of the bioreactor L, and S is a constant correlation factor with the value 0.46 kJ/m mol_{O2} [34]. Due to this relationship the estimation of the produced heat during fermentation is possible.

To guarantee reproducible processes, it is necessary to control the cultivation temperature. In bioreactors this is arranged by cooling walls or cooling coils [27]. For the single-use bioreactor used for this investigation, the temperature control is carried out by a double-wall system. If the double wall is regarded as a plane wall the heat transfer of the system is given by (9).

$$Q = k \cdot A \cdot \Delta T \tag{9}$$

where Q is the heat flow [W], k is the heat transfer coefficient [W/(m^2 · K)], A is the heat transfer area [m^2], and ΔT is the temperature difference between the reactor content and the heating/cooling wall [K]. The heat transfer coefficient is the sum of the overall thermal resistances (10). It is composed of the convective heat transfer of the cooling liquid inside the double wall (α_1 [W/(m^2 · K)]) and the convective heat transfer of the reactor wall into the medium (α_2 [W/(m^2 · K)]). Furthermore, it is influenced by the heat transfer through the reactor wall, which depends on the wall thermal conductivity λ [W/(m · K)] and the wall thickness [m].

$$k = \frac{1}{\frac{1}{\alpha_1} + \frac{s}{\lambda} + \frac{1}{\alpha_2}} \tag{10}$$

To avoid a temperature increase during fermentation, the heat flow has to be at least equal to the heat flow released by the organisms.

3.2 Mixing Efficiency of the Stirred Single-Use Bioreactor

In order to characterize the mixing efficiency of the single-use bioreactor the mixing time (θ) was measured by the decolorization method. The cultivation chamber was filled with RO-water. The RO-water was colored with potassium iodide and starch (dark blue). Afterwards, sodium thiosulfate was added, leading to a decolorization of the solution. The time between the addition of $Na_2S_2O_3$ to the

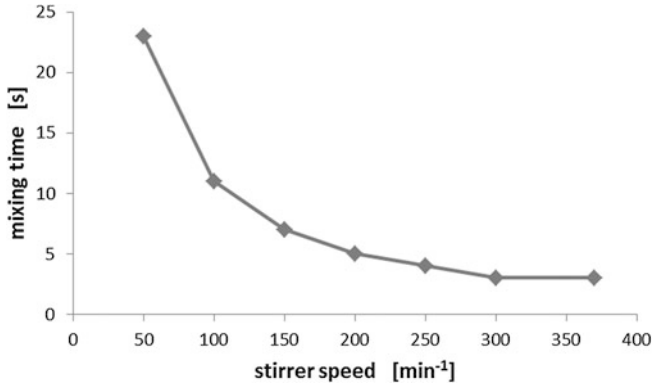

Fig. 2 Mixing time characteristics for the configurations 1 × 6-blade-disk (*bottom*) + 1 × 3-blade-segment (*top*) impeller with baffles dependent on the stirrer speed

complete decolorization is defined as mixing time (θ). This method has the advantage, when compared to other common methods (e.g., concentration method), that zones of poor mixing can be detected. The mixing times were determined as a function of the stirrer speed for the impeller configuration 1 × 6-blade-disk (bottom) + 1 × 3-blade-segment impeller (top) (see Fig. 2). The mixing times decrease with increasing agitation rate due to the increased power input per volume. For microbial cultivations mixing times below 10 s can be regarded as suitable [24]. This is the case for stirrer speeds above 150 min^{-1} ($u = 1.1$ m/s, $P/V = 66$ W/m^3).

3.3 Heat Transfer of the Stirred Single-Use Bioreactor

To quantify the cooling capacity of the stirred bioreactor, the heat transfer (k-value) was examined. Cooling curves were determined and then used to calculate the k-value of the system. This parameter is influenced by the heat transfer of the cooling liquid inside the double wall, of the stainless steel housing, of the bag material, and of the liquid inside the bioreactor. For the cell culture version of the BIOSTAT® STR 50 the k-value was calculated from the slope of cooling curves by nonlinear curve fitting and was 248 W/($m^2 \cdot$ K). This value allows the calculation of the overall heat flow/cooling capacity of the bioreactor. For microbial stainless steel fermenters heat transfer coefficients of 1,200 W/($m^2 \cdot$ K) [35] are common. Based on this, the cooling capacity of the stirred single-use system is approximately five times lower compared to a conventional stainless steel fermenter.

Figure 3 shows the cooling curve for the BIOSTAT® STR 50 at 370 min^{-1} ($u = 2.7$ m/s, $P/V = 934$ W/m^3) with the impeller configuration 6-blade-disk impeller (bottom) and 3-blade-segment impeller (top) with four baffles. The

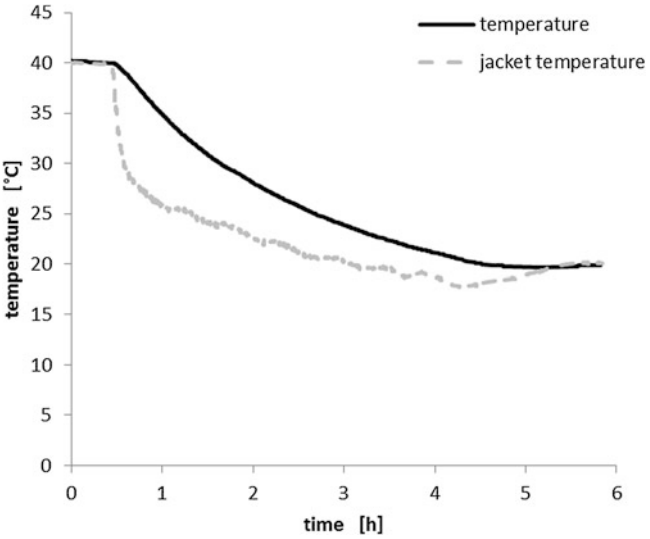

Fig. 3 Cooling curves for the stirred single-use fermenter. The medium inside the bioreactor was cooled down from 40 °C to 20 °C at a stirrer speed of 370 min^{-1}. The black lines indicate the temperature inside the bioreactor, and the dashed grey line the temperature of the double wall

cooling-down time was 4.2 h, which is two times higher compared to the measurements without baffles. This can be explained by the decreased contact area of the bag with the double wall.

3.4 k_La *Model of the Stirred Single-Use Bioreactor*

To quantify the oxygen transfer rate of the stirred single-use bioreactor the k_La was determined by the gassing-out method using 1 × PBS-buffer [36]. The measurement of the dissolved oxygen concentration (pO_2) was carried out with optochemical probes (PreSens). The liquid phase was saturated with air and the probes were calibrated. Afterwards, the PBS-buffer was deoxygenated by the supplementation of nitrogen to decrease the pO_2 below 10 %. After N_2 addition, air was sparged to the system and the pO_2 increase was recorded. The k_La was calculated from the slope of the mass balance (11).

$$\ln\left(\frac{C^* - C_1}{C^* - C_2}\right) = k_La \cdot (t_2 - t_1) \tag{11}$$

where C^* is the oxygen saturation concentration ($mmol_{O2}/L$), C_1 and C_2 ($mmol_{O2}/L$) are the dissolved oxygen concentrations at the time t_1 and t_2 (h).

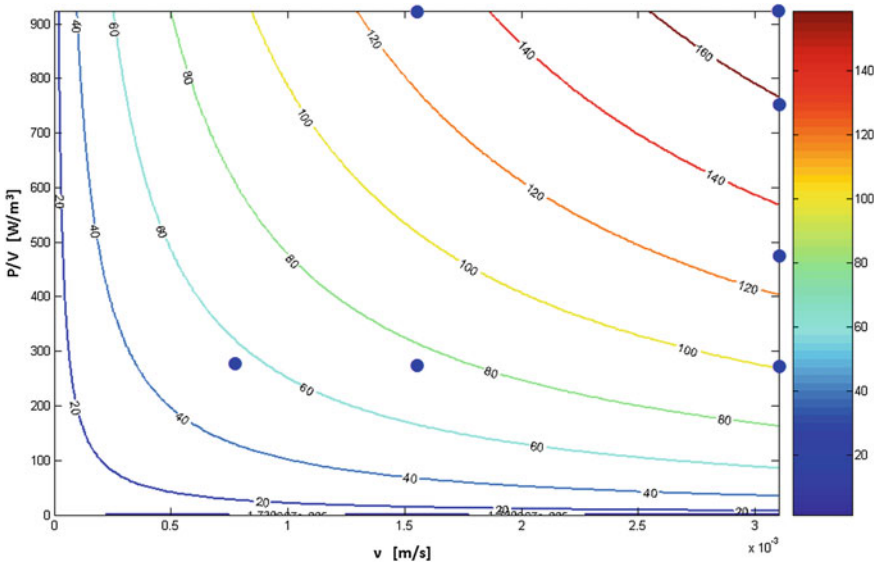

Fig. 4 Contour plots of the k_La characteristics calculated by the empirical relationship $k_La = Z \cdot P^a \cdot v^b$. The characteristics for 1 × 6-blade-disk + 1 × segment impeller with baffles are shown. Lower k_La-values are indicated by blue and higher k_La-values by red. The points where the measurements were performed are indicated by blue spots

With the results of the determined k_La characteristics a model was established for its calculation. For the trials, gas flow rates between 5 Lpm ($v = 0.71 \cdot 10^{-3}$ m/s; (6)) and 20 Lpm ($v = 3.1 \cdot 10^{-3}$ m/s) were used. The stirrer speed was varied between 250 min^{-1} ($P/V = 276$ W/m^3; see (5)) and 370 min^{-1} ($P/V = 934$ W/m^3). By employing a nonlinear curve fitting the empirical values of the Van't Riet relationship were calculated (see Sect. 3.1). Figure 4 shows the calculated k_La and the measured values. The k_La increase with increasing power input per volume and superficial air velocity.

The calculated correlation by Van't Riet is given by (12). At a superficial air velocity of $3.1 \cdot 10^{-3}$ m/s (gas flow rate = 20 Lpm) and a P/V of 934 W/m^3 (stirrer speed = 370 min^{-1}) a maximal k_La of 175 h^{-1} was obtained. Conventional stainless steel fermenters of this scale have k_La-values of approximately 500 h^{-1} [32]. Therefore, the k_La of the BIOSTAT® STR 50 is three times lower. The model equation had a standard deviation of 5 % and therefore can be regarded as accurate and allowing the calculation of k_La-values for a specific set of parameters.

$$k_La = 95.25 \cdot (P/V)^{0.448} \cdot v^{0.425} \tag{12}$$

With this model and the relationship for the oxygen transfer rate (4) it is possible to calculate the important process parameter *OTR*. With this knowledge, process strategies can be designed with regard to the quality attributes.

4 Microbial Cultivations in the Stirred Single-Use Bioreactor BIOSTAT® STR

To challenge the developed model based on the process engineering characterization, cultivations of two fast-growing microorganisms were performed in the stirred single-use bioreactor. To define a suitable control space for microbial applications, the process engineering parameters must be discussed in detail. The results of the mixing time, which were comparable to stainless steel fermenters, indicate that the KPP homogeneity is suitable for fast-growing microorganisms. Furthermore, the results are comparable to stirred stainless steel fermenters. Volumetric mass transfer coefficients of 175 h^{-1} were determined; compared to stirred stainless steel fermenters, this is a factor of two to three lower [32]. Because aerobic conditions are necessary, the gassing with oxygen enriched air can be used to achieve similar *OTR*s as with a conventional bioreactor. The maximal *OTR* is consequently 180 mmol/(L · h) if the gassing is carried out with pure oxygen.

A typical value for the heat transfer coefficient of stainless steel fermenters is 1,200 W/(m · K) [35]; the heat flow/cooling capacity of the stirred single-use fermenter is five times lower. Based on the correlation given by (8), a constant temperature can be guaranteed up to an *OUR* of 380 mmol/(L · h), if a cultivation temperature of 37 °C (used for the *E. coli* fermentation, Sect. 4.1) is assumed. For the *P. pastoris* (see Sect. 4.2) fermentation a cultivation temperature of 30 °C was used. As a consequence, a constant temperature can be ensured up to an *OUR* of 285 mmol/(L · h). The values are rather high [30] and far above the *OTR* of the system. As a consequence, the heat transfer is not regarded as the most critical factor. It should be kept in mind that the influences of the baffles were not considered for the *k*-value determination. Therefore, it is suggested that the cooling capacity of the bioreactor is lower.

This process engineering characterization suggests that limitations might occur regarding the oxygen transfer rate. Nevertheless, to achieve high cell densities in a stirred single-use bioreactor without any limitation the process strategy can be adapted. The oxygen uptake rate and the heat production depend on the growth rate of the organisms (3, 8). The specific growth rate can be lowered by cultivating in a substrate limiting fed batch mode. There are two common fed batch strategies used for microbial fermentations. The first is an exponential increasing feeding strategy, and the other one a constant substrate feeding strategy. The flow rate characteristics of the exponential feeding strategy are given by (13).

$$F(t) = \frac{\mu_{set} \cdot X_0 \cdot V_0}{Y_{X/S} \cdot S_{Feed}} \exp(\mu_{set} \cdot t) \tag{13}$$

where $F(t)$ is the feed flow rate at t [L/h], μ_{set} is the selected specific growth rate (1/h), $Y_{X/S}$ is the yield coefficient [$g_{DCW}/g_{substrate}$], and S_{Feed} is the C-source concentration of the feed [$g_{substrate}$/L].

For this type of fed batch strategy, μ is controlled to a fixed value usually below μ_{max} to avoid overflow metabolism. Therefore, the oxygen uptake rate and the heat generation increase exponentially during the fermentation. This feeding profile was utilized for the *E. coli* fermentations (see Sect. 4.1).

For the cultivation of the yeast *P. pastoris* (see Sect. 4.2) a constant feeding profile was used (14).

$$F(t) = c \tag{14}$$

This profile has the advantage of a slower *OUR* increase, heat production, and a lower automation effort. The disadvantages are an extended cultivation time and a lower biomass and production yield.

4.1 Escherichia coli High Cell Density Fermentation

By performing an *E. coli* fermentation the stirred single-use bioreactor was verified during microbial applications. In addition to a high cell density fermentation the possibility of protein expression was tested, therefore the strain *E. coli* BL21 (DE3) ProKaDo pET 15b (University of Bielefeld, Germany) was used. The expressed target protein ProKaDo is a catalytic domain of the metalloproteinase 12 [37].

For the cultivation, an exponential feeding profile was performed with a μ_{set} of 0.1 h^{-1}. The cultivation conditions were $pO_{2,set} = 20$ %, $pH_{set} = 6.8$ and temperature = 37 °C. A chemically defined medium was used [30] and an exponential feeding strategy was employed to control μ to 0.1 h^{-1}. In Fig. 5a the characteristics of the cell density μ and the pH value are shown. During the batch phase the cell density increased exponentially from an OD_{600} of 0.2 to 14. After the feed start ($t = 6$ h) the cell density further increased exponentially with a lower slope due to the substrate limiting glucose supplementation. The final OD_{600} was 175 (DCW = 60.8 g/L). The pH measured by a classical probe was kept in the desired range. A constant μ of 0.7 h^{-1} was determined during the batch phase, which corresponds to the maximal specific growth rate for 37 °C (own measurement). During the fed batch phase it was possible to control μ to 0.1 h^{-1}.

With the pO_2-feedback control loop including stirrer speed, gas flow rate, and pure oxygen gassing, the pO_2 was controlled close to or above the set point (Fig. 5b). The fluctuations after $t = 30$ h were due to antifoam addition. With increasing oxygen demand the stirrer speed increased from 250 to 370 min^{-1} ($u = 1.84 - 2.7$ m/s, $P/V = 276 - 934$ W/m^3). Due to the decreased μ resulting from the substrate limiting feed supplementation the slope of the stirrer speed alteration was lower. It remained constant at 370 min^{-1} for $t > 12$ h until the end of the fermentation. At the end of the batch phase ($t > 5$ h) and at a cell density of $OD_{600} = 7.6$ (DCW = 2.2) oxygen was supplied at an exponentially increasing rate, consequently, the gas flow rate of air decreased. The calculated *OUR* (3) at this time was 39.6 mmol/(L · h). The *OTR* determined by the model equation (12)

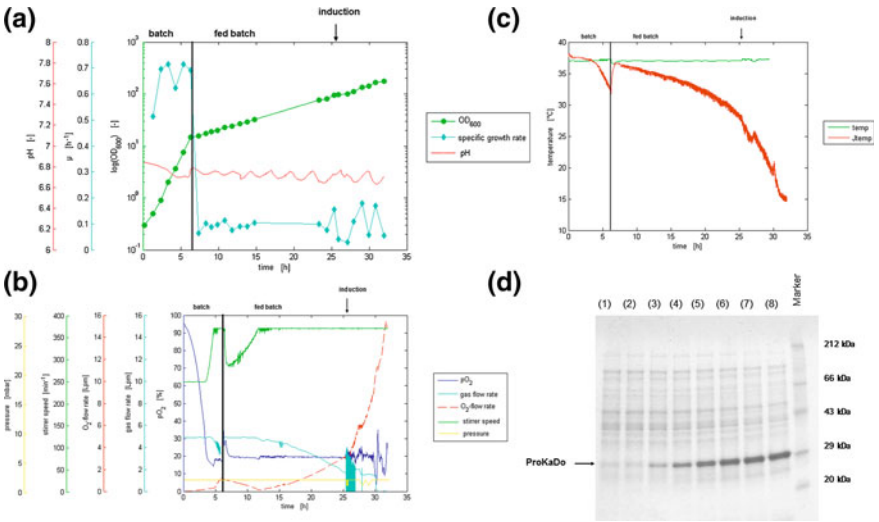

Fig. 5 Fed batch with exponentially feeding rate of *E. coli* BL21 (DE3) ProKaDo pET 15b in the BIOSTAT® STR 50. **a** Optical density (OD_{600}; *green circles*), the specific growth rate μ (*cyan squares*), and pH (*red line*). **b** Oxygen partial pressure (pO_2; *blue line*), gas flow rate (*cyan line*), stirrer speed (*green line*), O_2-flow (*red line*), and pressure (*yellow line*). **c** Cultivation temperature (*green line*) and the double wall temperature (Jtemp; *red line*). **d** Comassie Brilliant blue stained SDS–polyacrylamide gel, lane (1) before induction, (2) directly after induction, (3) after 1 h, (4) after 2 h, (5) after 3 h, (6) after 4 h, (7) after 5 h, and (8) after 6 h. Cultivation parameters BIOSTAT® STR 50: stirrer speed = 250 – 370 min^{-1}, aeration rate = 5 – 20 Lpm, temperature = 37 °C, $pH_{setpoint}$ = 6.8, $pO_{2,setpoint}$ = 20 %, μ_{set} = 0.1 h^{-1}

is 37.6 mmol/(L · h), therefore it can be concluded that calculated k_Las are in good agreement with the oxygen transfer efficiency of the bioreactor during a fermentation.

After the feed start the slope of the O_2-gas flow rate decreased, which was caused by the decreased specific growth rate and the therefore lower *OUR* (3). The O_2-gas flow rate increased exponentially showing that the *OTR* have to be further increased up to 158.94 mmol/(L · h) [(4) and (12)]. At the point of harvest, the gas flow rate was 15 Lpm (0.33 vvm). The strategy to increase the gas flow rate at the end of the fermentation was selected to minimize foam generation. The pressure was below 5 mbar for the entire cultivation. It can be concluded that the exhaust cooler was able to condense the water in the exhaust air in the expected way.

To evaluate if the cooling capacity was suitable during the fermentation, the cultivation temperature and the temperature of the double wall are shown in Fig. 5c. For the entire fermentation the jacket temperature decreased to 15 °C, due to the increased heat production by the cells. With the used cooling unit the double wall can be further decreased to 10 °C. The cultivation temperature remained constant indicating the suitability of the single-use bioreactor for the temperature control.

The protein expression was analyzed by a Comassie Brilliant blue stained SDS-PAGE (Fig. 5d). The protein expression was induced at $t = 26$ h by the addition of IPTG to a final concentration of 0.1 mM. At times prior to the induction, no expression of the target protein (molecular weight 29 kDa) was detectable. After the induction, the band intensity at 29 kDa increased, showing the possibility of an *E. coli* high cell density fermentation with protein expression.

4.2 Pichia pastoris High Cell Density Fermentation

Another microorganism that recently became interesting for expression of recombinant proteins is the methylotrophic yeast *P. pastoris*. The use of this host has become popular due to the ease with which it can be genetically transformed and the fact that it can be easily grown to high cell densities. Another important advantage of this yeast is the fact that it can produce soluble, correctly folded recombinant proteins that have undergone all the posttranslational modifications required for the functionality of certain therapeutic proteins [38]. The foreign DNA coding for the recombinant protein of interest can be inserted with high efficiency and in multiple copies in the genome of *P. pastoris* via homologous recombination. The use of the strong AOX promoter results in a high expression of the recombinant protein, which results in a low cost of goods.

Pichia pastoris was used as a second microorganism for a further evaluation of a microbial application of the stirred single-use bioreactor BIOSTAT® STR 50. As a case study, expression of a Nanobody® via *P. pastoris* was evaluated. Nanobodies® are a novel class of antibody-derived therapeutic proteins based on immunoglobulin single variable domains. The Belgian company Ablynx is focused on the discovery and development of these Nanobodies® for a range of serious human diseases including inflammation, hematology, oncology, and pulmonary disease.

A high cell density fed batch fermentation protocol was performed: a constant flow rate of glycerol for biomass build up, followed by a constant MeOH feed for Nanobody® expression. The cultivation conditions were $pO_{2,set} = 30$ %, $pH_{set} = 5$, and temperature = 30 °C. A complex medium containing yeast extract was used. The used strain (X33) expressed a trivalent Nanobody®, which was secreted into the extracellular medium.

During the biomass production phase (batch phase followed by a glycerol fed batch phase), the cells grew to a wet cell weight of 381 g/L (Fig. 6a). The specific growth rate at the end of the batch phase was 0.24 h^{-1}, which corresponds to the maximal specific growth rate at 30 °C (exponential growth). During the glycerol fed batch phase (which was initiated after depletion of the glycerol in the batch medium), the μ decreased due to the constant glycerol flow rate, which was applied during this phase (no exponential growth). During the induction phase, no additional biomass was built up and the Nanobody® was expressed by feeding MeOH into the medium.

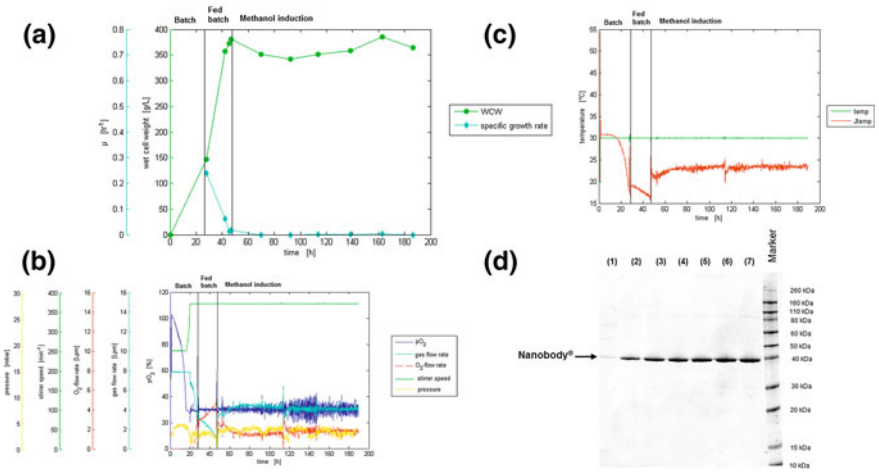

Fig. 6 Expression profile of a trivalent Nanobody® via *Pichia pastoris* using a high cell density fed batch protocol with constant feeding rates in the BIOSTAT® STR 50. **a** The evolution of the wet cell weight (WCW; *green circles*) and the specific growth rate (*cyan squares*). **b** Oxygen partial pressure (pO_2; *blue line*), gas flow rate (*cyan line*), stirrer speed (*green line*), O_2-flow (*red line*), and pressure (*yellow line*). **c** The cultivation temperature (*green line*) and the temperature in the double wall (Jtemp, *red line*). **d** Instant blue stained SDS–polyacrylamide gel, lane (1) prior induction, (2) after 23 h, (3) after 46 h, (4) after 67 h, (5) after 92, (6) after 116 h, and (7) after 140 h of induction. Cultivation parameters BIOSTAT® STR 50: stirrer speed = 250 – 370 min^{-1}, aeration rate = 5 – 20 Lpm, temperature = 30 °C, $pO_{2,setpoint}$ = 30 %

The pO_2 decreased to the setpoint of 30 % within 16 h after inoculation due to cell growth (Fig. 6b). This setpoint was maintained during the rest of the fermentation. To control the pO_2, the stirrer speed was increased from 250 to 370 min^{-1} (u = 1.84 – 2.7 m/s, P/V = 276 – 934 W/m^3). After 20 h of fermentation, the maximum stirrer speed was reached (u = 2.7 m/s). To further maintain the pO_2 setpoint, the gassing was performed with oxygen enriched air. The amount sparged into the bioreactor (O_2 – gas flow rate) increased exponentially to a maximum value of 3.1 Lpm at end of the glycerol fed batch phase. During the entire glycerol fed batch phase (t = 24 – 46 h) it was possible to control the pO_2 at 30 %. After the peak cell density of WCW = 381 g/L (t = 46 h) was achieved, the induction was started by continuous supplementation of methanol. No further cell growth was detectable during the induction phase. The oxygen uptake rate remained constant, which can be concluded by the O_2-flow (constant at approximately 1 Lpm during complete induction phase). It was therefore possible to control the pO_2 to its setpoint until the end of fermentation. The pressure in the bag was below 5 mbar during the whole run.

Figure 6c shows the characteristics of the cultivation temperature and the temperature of the double wall. During the batch and fed batch phases a significant decrease of the double wall temperature was observed. This indicates a high heat

production during these phases. After start of the induction, the jacket temperature increased again, which is a consequence of the fact that there being no further biomass build up during this phase and the lower metabolic activity during Nanobody® expression. The lowest value of the double wall temperature was 16.5 °C and therefore the cooling capacity was not exhausted. A temperature control at the setpoint of 30 °C was possible for the entire fermentation.

The Nanobody® production was analyzed via SDS-PAGE (see Fig. 6d). During the induction phase ($t > 46$ h) the Nanobody® titer in the extracellular medium increased. In the supernatant of the fermentation broth a final Nanobody® yield of 9 g/L was detected. Taking the biomass into account, this corresponds to a yield of 5.7 g/L in the total cell broth.

5 Conclusion and Outlook

This chapter presented tests of a stirred single-use cell culture bioreactor (BIOSTAT® STR 50) for its microbial suitability. The bioreactor was evaluated for microbial use by the determination of important process engineering parameters, using them to establish a mathematical model. It can be assumed that the mixing time is suitable for microbial applications [24]. The determined k_La, and consequently the efficiency of oxygen transfer, is according to the few reports about microbial single-use bioreactors, significantly improved [14]. Nevertheless, the k_La is lower than that of stainless steel fermenters designed for microorganisms [32]. This implies that for cultivations with high oxygen demand, pure oxygen must be supplied to ensure the necessary *OTR*. Furthermore, the selection of an appropriate feeding strategy is essential to avoid oxygen limitations. Another important issue for microbial applications is the removal of produced heat. The heat transfer coefficient of the single-use bioreactor is approximately five times lower compared to conventional stainless steel bioreactors [35]. Based on this, the cooling capacity might be a limiting factor for microbial high cell density fermentation. With an adapted feeding strategy and the supplementation of pure oxygen, HCDFs of *E. coli* and *P. pastoris* were successfully performed. The achieved cell densities of $OD_{600} = 175$ (DCW = 60.8 g/L) for *E. coli* and WCW = 381 g/L for the *P. pastoris* fermentation, respectively, were comparable to results obtained during fermentations in stainless steel and glass bioreactors [3]. Furthermore, the cell densities were significantly higher compared to results reported by other investigators, which used single-use bioreactors for microbial cultivations [11]. Oxygen consumption and maximum gas flow rate needed during the cultivations suggest the potential of using these types of single-use bioreactors for higher oxygen-demanding processes. *E. coli* fermentation processes with even higher cell densities exist ($OD_{600} = 200 - 250$) [39], which still have to be carried out in reusable systems. The expression of recombinant proteins was possible up to a titer of 9 g/L. The pressure inside the cultivation chamber remained constantly

low, hence it can be concluded that the exhaust cooler was able to condense the water vapor in the exhaust air.

Although successful cultivations were performed, it was obvious that the *OTR* and the heat transfer were nearly exhausted. Nevertheless, for seed train fermentations, the used stirred single-use bioreactor is an attractive alternative. If HCDFs should be performed, a process adaptation by gassing with oxygen-enriched air as well as decreased feeding profiles might be necessary to guarantee optimal growth conditions.

To achieve the same performance as stainless steel bioreactors, the k_La of the single-use fermenter should be increased. This can be arranged by increasing the stirrer speed, which is possible by modifications of the drive unit. It can be concluded by the Van't Riet relationship (12) that this has the greatest potential on the *OTR* because the exponent for *P/V* is 5 % higher compared to the exponent of the superficial gas velocity. The model further indicates that a higher superficial gas velocity contributes to a k_La increase.

Although the cooling capacity was sufficient for the presented processes, it is significantly lower compared to conventional bioreactors, which may cause a temperature increase for processes with a higher heat generation. One way to increase the cooling capacity is to increase the heat transfer area. For future studies, different organisms should be cultivated in the stirred single-use bioreactor. This allows the verification of different cultivation and process strategies. In this way, further examples of microbial fermentation in single-use systems can be gained and possible limitations can be detected. The results indicate the potential of single-use bioreactors for microbial applications at laboratory and pilot scale therefore offers the advantages of single-use technologies to these processes.

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Adv Biochem Eng Biotechnol (2014) 138: 149–177
DOI: 10.1007/10_2013_238

Published Online: 4 August 2013

Quorus Bioreactor: A New Perfusion-Based Technology for Microbial Cultivation

Sheena J. Fraser and Christian Endres

Abstract This chapter briefly reviews perfusion-based cultivation solutions used in biomanufacturing. It further introduces the innovative single-use Quorus Bioreactor, which was designed for the efficient cultivation of nontraditional production cell types. The Quorus Bioreactor design, process control, and productivity are described. Case studies are presented using *Aspergillus niger* and *Lactococcus lactis* as model organisms to demonstrate process flexibility, efficiency, and scalability.

Keywords *Aspergillus niger* · Biofilm · *Lactococcus lactis* · Microorganism · Perfusion · Quorus Bioreactor · Single use

Abbreviations

CFD	Computational fluid dynamics
CHO	Chinese hamster ovary
DO	Dissolved oxygen
DSP	Downstream processing
ECS	Extra-capillary space
GLS	Gas–liquid–solid
GRAS	Generally regarded as safe
HF	Hollow fiber
ICS	Intra-capillary space
LS	Liquid–solid
MGB	Membrane gradostat bioreactor
MWCO	Molecular weight cutoff
SFR	Single fiber reactor
STR	Stirred tank reactor
UF/MF	Ultrafiltration/microfiltration

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QB	Quorus Bioreactor
$L_{reactor}$	Reactor capacity in liters
Vol_{ECS}	ECS capacity
∅	Diameter

Contents

1 Introduction

Bioprocess sources report renewed interest in perfusion-based cultivation solutions [1–3] as the biomanufacturing industry continues to adopt single-use production technologies [4, 5]. This industry is largely driven by an increased focus on adopting innovative strategies to improve process productivity and efficiency, lower operating costs, and ensuring regulatory compliance and product quality [6]. Perfusion is a particularly effective means of intensifying bioprocess conditions to obtain high cell densities and productivities in a low- to no-shear growth environment, which—when combined with the advantages of using single-use technologies—may offer more flexible, smaller volume, and more cost-effective production processes [7] (Table 1).

Perfusion processes can be broadly defined as a process wherein new medium is continuously delivered to cells at a rate sufficiently high enough to maintain cell growth at high cell densities, while exhausted medium is continuously removed.

Table 1 Summary of the advantages and limitations of perfusion cultures

Pros	Cons
High biomass yields	*Requires more development time*
Cell retention and concentration	High media requirements
Continuous dilution of excreted metabolic waste	Constant feed rate to dynamic culture requires complex operation
Smaller batches	*More susceptible to operational upsets*
Higher yields, smaller systems	Including contamination
Risk of operational upsets mitigated by continuous product harvesting	Filter fouling and plugging of cell retention device
High productivity	*Regulatory*
Simplified downstream processing, continuous recovery of secreted product	Varying cell densities, environmental conditions, and cell stability can alter product quality
Approximately 10-fold more productive than batch culture	Must show comparable product quality throughout perfusion cycle
Less cell damage, purer product	
Single-use equipment	*Single-use equipment*
Faster, safer, cleaner	Limited automation level
Overall process is cost- and time-saving	Restricted by available sensors and sampling solutions
Flexible	
Modular scale-up	Scalability and cost of single-use devices

Perfusion is typically used in the cultivation of shear-sensitive cell types to high densities with enhanced productivity.

Perfusion in hollow-fiber (HF) systems was first introduced in the early 1970s in an effort to cultivate mammalian cells in a more natural growth environment [8]. The technique has since been adapted to tissue culture [9], organ regeneration [10], stem cell proliferation [11] and high cell density growth of mammalian, plant, insect, and microbial production cells [12–17]. HF and membrane devices still dominate the perfusion reactor market, with two distinct modes of operation.

2 Cell Retention Systems

Mechanically agitated (stirred, vibrated, platform tipping) or pneumatically mixed (airlift, bubbles) cultivation vessels can be operated in batch, fed batch, continuous, or perfusion modes. In perfusion mode, cells are maintained in suspension within the cultivation vessel, while a cell retention device (spin filter, cell settling, centrifugation, filtration module) serves to selectively retain cells as nutrients are replenished. Simultaneously, secreted products are harvested and/or toxic metabolic waste is removed. The scalability and efficiency of different cell retention devices are discussed in detail elsewhere [1, 17]. Factors influencing perfusion efficiency include the following: (1) choice of perfusion system; (2) cell type, product type, and process scalability; (3) perfusion filter type, surface area, and pore size; and (4) filtrate flow rate and recirculation rate.

Perfusion cultures in single-use bioreactors have been demonstrated for Chinese hamster ovary and other conventional cell types [17, 18]. Filtration modules offered by companies such as Refine Technology (AFT system) and Spectrum Labs (CellFlo) can be added on to most cultivation systems. Single-use bioreactor products with perfusion options are now offered by AmProtein, Applikon, ATMI, PBS, Sartorius, Thermo-Fisher, Wave and Xcellerex (both part of GE Healthcare).

3 Classic Perfusion Systems

In classic perfusion, cells are adhered to a solid substrate (e.g. fixed bed particles, Sponceram disks, hollow fibers, membranes) with nutrients perfused through the substrate to the attached cells. These reactors have largely been applied to adherent mammalian cell cultivation (Table 2).

In the 1980s and 1990s, HF reactors were considered to be state of the art in the manufacture of monoclonal antibodies from mammalian cells [19]. In recent years, perfusion system application in biomanufacturing has rather been limited to specialized applications, such as for culturing stem cells and serving as feeder bioreactors.

Recent advances in mammalian production strain development report that 2 g/L product titers are now readily achievable in fed-batch mode [20]. With a theoretical >10-fold increase in productivity promising even higher product titers [21], smaller yet more efficient perfusion technologies are once again being thrust into the spotlight.

Solid-substrate perfusion systems offer additional advantages over cell retention devices linked to conventional bioreactors, including higher cell densities, low or no shear, lower apoptosis rates, and higher product purity. In many instances, negative perceptions remain around heterogeneity in high cell density cultivations, including channeling, temperature gradients, non-uniform cell growth, and transfer gradients. Concerns are primarily addressed at the effects of culture heterogeneity on cell stability, product quality, and scalability of mammalian cell culture bioreactors.

3.1 Packed-Bed Reactors

Single-use applications of solid-state reactors in the form of perfused (Cellcube) or packed bed (Fibrastage, CellTank, iCellis) bioreactors offer low-shear solutions for the cultivation of adherent mammalian cell types.

In packed-bed reactors, anchorage-dependent cells are seeded within immobilized carrier material, which is packed and retained in a cylindrical housing. Oxygenated medium is recirculated through the packed bed and exhausted, or product-containing medium can either be exchanged batch-wise or continuously.

Table 2 Short list of classic solid-substrate perfusion systems

Single-use bioreactors	Company	Largest scale	Cell application
Fixed and rotating bed			
Cell cube	Corning (Tewksbury, MA, USA)	4 × 100 cubes setup, 85,000 m^2 surface area, ~6 L working volume	Mammalian, plant, insect
CellTank	CerCell (Holte, Denmark)	CellDream 38 series 15,000 mL three-dimensional matrix	Mammalian, plant, insect
Integrity iCellis	ATMI (Danburry, CT, USA)	500 m^2 surface area, 25 L working volume	Mammalian, plant, insect
Z RP Bioreactor	Zellwerk/Glen Mills (Oberkrämer, Germany)	GMP Breeder 200–5000 mL vessel	Mammalian, plant, insect
Hollow fiber			
AcuSyst systems	BioVest Interational (Minneapolis, MN, USA)	Xcellerator, 20 cartridges ~2.5 L culture volume	Mammalian, plant, insect
AutovaxID	BioVest International (Minneapolis, MN, USA)	One cartridge, ~ 110 mL culture volume	Mammalian, plant, insect
FiberCell	FiberCell Systems (Frederick, MD, USA)	FibreCellDuet, 2 × 150 mL cartridges	Mammalian, plant, insect
Membrane bioreactor			
CELLine	Integra Biosciences (Zizers, Switzerland)	CELLine 1000, 20 mL culture volume	Mammalian, plant, insect
miniPERM	In Vitro Systems/Greiner Bio-One (Kremsmünster, Austria)	miniPERM classis, 30–50 mL culture vol ume	Mammalian, plant, insect
Quorus GLS bioreactor	Quorus Biotech (Cape Town, South Africa)	Single module, ~2 L culture volume	Filamentous microbes
Quorus LS bioreactor	Quorus Biotech (Cape Town, South Africa)	Single module, ~100 mL culture volume	Gram-positive bacteria

The distinct advantage of this three-dimensional environment is the better mimicking of in vivo conditions and therefore the possibility of generating high cell densities, while the concentration of free cells growing in suspension is limited [22]. Further advantages include simple system configuration and geometry, as well as low carrier material deterioration by friction or collision [23].

However, direct monitoring of cell density or viability during cultivation is hindered and spatial concentration gradients [24], temperature gradients [25], or blocked channels may occur. Integrity iCellis is currently the largest working volume single-use packed-bed reactor on the market, alongside the new entrant into the single-use bioreactor market, the CerCells CellDream series.

3.2 Hollow-Fiber Reactors

HF reactors are ultrafiltration/microfiltration (UF/MF) filtration type modules mainly applied to high density cell cultivation of anchorage-dependent mammalian cells.

Reactor modules typically comprise bundles of 160- to 200-μm HF membrane filters potted on either end into a cylindrical housing such that the reactor is separated into two compartments: The intracapillary space (ICS) compartment with a flow path from end to end through the membrane lumen and the extracapillary space (ECS) compartment accessed through side ports on opposite ends of the shell side of the cylindrical housing. In cross section, adjacent membranes within an HF bundle are in contact (Fig. 4).

In HF reactors, the ECS compartment serves as the cell cultivation chamber wherein cells are adhered to an outer surface of HF and perfused with nutrients/oxygen from the lumen side. A high membrane surface area in a low ECS volume is used to maintain high cell densities in a low- to no-shear growth environment. Nutrients and waste are exchanged across the membrane wall, in much the same way as originally envisaged by Knazec et al. in the early 1970s [8]. HF reactors were the first single-use bioreactors developed, favored for the in vitro production of hybridoma-derived monoclonal antibodies on a small scale in the 1980s to 1990s. Companies such as Endotronics, Cellex, and Biosyn that offered HF reactor products in the 1970s and 1980s no longer exist. Today, commercial versions are available from FiberCell Systems and Biovest (previously Unisyn/Biosyn).

There has been little change to HF reactor design in the last 40 years. Technological improvements are limited to advances in process control and polymer science. HF material type and molecular weight cutoff (MWCO) are selected to suit specific modes of operation and process criteria. In perfusion, there is an efficient exchange of nutrients and waste across the membrane wall, while the membrane type determines both the selectivity and efficiency of this process. Similarly, product can either be concentrated in the ECS filled with cells or exchanged across the membrane wall and harvested from the ICS.

One of the key limitations reported for HF systems remains their diffusion-limited mass transport to cells cultivated at high cell densities, aggravated by axial and radial gradients that affect cell stability and limit bioreactor scalability. The recent addition of a pressure cycling regimen to Biovests AcuSyst systems has gone a long way to offsetting this limitation [26]. HF reactor applications are typically favored over flat-sheet systems due to their larger surface-to-volume ratio and lower cost.

3.3 Membrane Bioreactors

Membrane bioreactors have been widely applied at very large scales in wastewater treatment [27], yet their use in biomanufacturing is largely limited to HF-type reactors [28]. This is attributed to instability of poorly regulated heterogenous cell growth and its impact on product quality.

CellLine and miniPerm bioreactors are among the first single-use membrane-based bioreactors developed, used in cell culture laboratories for more long-term cell expansions, screening experiments, and sample production at milliliter scale. In these devices, cells are immobilized in a compartment separate from the nutrient feed. Nutrients selectively diffuse from a medium supply chamber to the cell culture compartment through a polymer membrane. Different MWCOs of the membrane allow for the retention of cells between medium changes and can also act to concentrate secreted products within the cell culture chamber. Mass transfer is relatively good on a small scale, but it requires a large head-space volume for sufficient aeration and diffusion of nutrients/oxygen to cell culture space.

Commercially available single-use membrane reactors remain focused on mammalian applications and are unsuited to microbial biomanufacturing applications. An exception to this is the recently introduced Quorus Bioreactor (QB), which is a tubular membrane bioreactor designed and engineered for the cultivation of stable productive microbial biofilms and manufacture of high value products, discussed in detail herein.

4 Quorus Bioreactor

In early 1990s South African researchers developed and patented a membrane bioreactor process for the production of secondary metabolites derived from microbial biofilms [29, 30], subsequently described in the literature as the membrane gradostat bioreactor (MGB) and bioprocess [16]. Over the following decade, MGB research focused on demonstrating this bioreactor as a promising technology for use in bioremediation and biotransformation applications [16, 31, 32]. Improved productivity of MGB biofilms over suspension cultures has been reported for the production of oxidative enzymes, recombinant proteins, and bioactive small molecules (Table 3).

Table 3 Cultivation of filamentous microbes using membrane gradostat bioreactor technology

Organism	Product	Reference
Phanerochaete chrysosporium ME446, BKMF1767	Peroxidases	[33–35]
Neurospora crassa	Polyphenoloxidase, laccase	[31]
Aspergillus niger Xyn2	Recombinant xylanase	[36]
Streptomyces coelicolor A3(2)	Actinorhodin	[36]
Penicillium sp.	Wortmannin	Unpublished
Streptomyces sp.	Rapamycin	Unpublished

Fig. 1 Modular scale-up. *Streptomyces* sp. (*left*) and *Penicillium* sp. (*right*) cultivated in reusable Quorus Bioreactor for the production of bioactive small molecules under Synexa Life Sciences in the mid-2000s

In 2003, MGB process technology was licensed to the startup company Synexa Life Sciences (Cape Town, South Africa) for the manufacture of high-value, low-volume active pharmaceutical ingredients for early-stage clinical and diagnostic research (Fig. 1). As co-inventor of MGB technology and co-founder of Synexa, the late Dr. Winston Leukes, together with a small team of researchers, saw MGB technology evolve into the QB, described herein.

At Synexa, the commercial potential of the QB was demonstrated through the modular scale-up of *Penicillium* sp., which was cultivated using reusable 2-L Quorus GLS (gas-liquid-solid) bioreactor systems for the production of wortmannin. In a 30-day process, the productivity of wortmannin ($g/L_{reactor}$ per day) was equivalent to conventional stirred tanks. However, ease of downstream processing (DSP) was significantly improved through capture of product from an essentially cell-free permeate rather than whole-cell extracts. The reduced time, lower expense, and higher purity of product obtained from Quorus GLS cultivations resulted in more reliable and cost-efficient production of wortmannin.

In 2006, Quorus engineers redesigned QB modules using single-use materials for QB manufacture due to their ease of manufacture and improved safety. Over the same period, integration of single-use technologies into upstream processes

became a strategic priority for both bioreactor suppliers and biopharmaceutical manufacturers [37, 38]. In 2011, Quorus Biotech (Cape Town, South Africa) was established to focus commercialization efforts on introducing one of the first single-use tubular membrane bioreactors on the market, designed and engineered for the production of value-added products from stable, productive microbial biofilms.

4.1 Quorus GLS Bioprocess

The GLS bioprocess is an aerated process [30, 39] developed to better mimic the natural growth environment in which terrestrial biofilm-forming microbes exist. The GLS bioprocess is best suited to the cultivation of filamentous biofilms in which a network of interconnected hyphae (fungal or bacterial) anchor the biofilm onto the outer surface of a capillary membrane, thus stabilizing the biofilm and allowing higher permeation rates during QB operation [40]. Biofilm growth occurs at the GLS interface. Biofilm growth is supported by a constant feed of soluble nutrients supplied convectively from the membrane lumen, while a constant stream of air flows across the biofilm surface (Fig. 2).

GLS biofilm growth is self-regulating. Under optimal nutrient conditions, a given nutrient feed rate sustains the biofilm at a constant thickness. Initial GLS biofilm growth kinetics are similar to those described in batch cultivation processes [41–43]. Steady-state biofilms are observed after 10–14 days, although this is largely isolate and application dependent. Steady-state biofilms show spatiotemporal distribution of the different phases of cell growth. The first phase comprises a thin zone of cells at the membrane surface closest to nutrient feed, which is maintained in exponential growth. As nutrients are depleted, cells enter into and are maintained in stationary (idiophasic) phase. Lastly, a thin layer of cells furthest from the membrane enter a decline phase and subsequent sporulation. At higher air flow rates, cell debris and spores are sloughed off the surface of a biofilm in decline phase [16]. Spatiotemporal distribution of growth is maintained by radial nutrient gradients established across the biofilm as perfused nutrients are metabolized. The zone of stationary growth is induced under nutrient limiting conditions, as is secondary metabolism and the production of value-added products [44, 45].

Dissolved oxygen (DO) transport to cells occurs at the outer liquid boundary layer of the biofilm and is aided by convective flow of a humidified air stream across the biofilm surface, typically maintained at an air flow rate of approximately 1 vol vol_{ECS}^{-1} min^{-1}. The rate of solute transport into the biofilm is determined by linking the convective mass transfer rate to the diffusive mass transport rate. Under atmospheric pressure, Ntwampe et al. reported oxygen flux values of 0.27–0.7 g/m^2 per hour for single-capillary MGB cultivations [42]. Optimized airflow distribution within the QB design (Sect. 4.3) reduces dead spots and dissolved oxygen (DO) limitations observed with MGB operation. These authors also showed that DO transport into the biofilm could be improved through the addition of

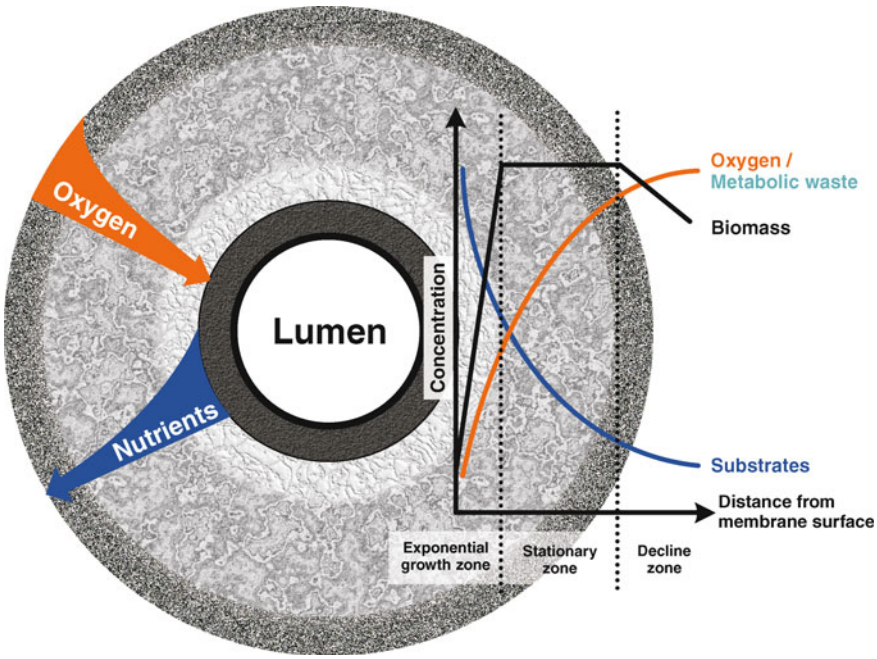

Fig. 2 Quorus GLS bioprocess is an aerated solid-state based biofilm process [30, 39], also described as the membrane gradostat process [16]. Biofilm growth kinetics are spatiotemporal, regulated by opposed radial nutrient and oxygen gradients established across the stable biofilm

surfactants to the nutrient feed [46]. Radial DO gradients are established across the biofilm, with highest DO concentrations at the outer aerated biofilm surface and an average penetration depth of 306–530 μm [42]. Later-stage biofilm cultures showed significantly higher DO penetration depths, attributed to varying biofilm morphology (density) and metabolism (cells more metabolically active closer to nutrient feed) as biofilm thickness increased and progressed towards steady state [42].

QB process control is pneumatic. Nutrient feed rate is regulated by the transmembrane pressure between ICS and ECS compartments [41]. During Quorus GLS operation, the ECS is maintained at a constant pressure, while the ICS pressure is adjusted to support and sustain a volumetric nutrient feed rate of 0.01–0.05 vol vol_{ECS}^{-1} h^{-1}, determined by isolate type and its growth parameters.

The constant perfusion of nutrients from the ICS to the ECS not only renews nutrient supply to cells within the biofilm, but it also serves to dilute metabolic waste excreted and accumulate any value-added products in the permeate. Furthermore, the constant air stream through the ECS not only replenishes oxygen at the biofilm surface, but it also facilitates the continuous removal and collection of permeate from the ECS for sampling, analysis, and downstream processing.

The Quorus GLS bioprocess is distinguished from other perfusion processes by the maintenance of immobilized cells in a constantly aerated environment; improved mass transfer of both nutrients and oxygen by convective-diffusive flow within a biofilm; and the formation of opposing gradients across the biofilm radius. Biofilm growth occurs in a low-shear environment, is maintained in steady-state for extended periods of time, and is achieved using a fully automated and controlled membrane bioreactor system (Sect. 4.6).

Lastly, the Quorus GLS bioprocess is ideally suited to the continuous removal of value added products that are (1) toxic to continued cell growth and productivity, (2) labile and easily transformed or degraded if left to accumulate, and (3) volatile and can be directly captured from the constant air stream from Quorus GLS cultivations.

4.2 Quorus LS Bioprocess

The Quorus LS (liquid-solid) bioprocess [47] is a submerged process best suited to extended-batch cultivation of shear-sensitive microbes for the production of secreted products. The LS bioprocess favors cultivation of cell types with efficient protein secretion mechanisms [48–50], particularly Gram-positive bacteria [51, 52]. Growth occurs at the LS interface of a capillary membrane as cells accumulate at the outer membrane surface to high cell density as a compact cell-cake or biofilm. A constant flow of soluble nutrients is supplied from the ECS, across the membrane wall towards the membrane lumen, at a rate sufficiently high to maintain cell growth and metabolism in an actively growing biofilm (Fig. 3).

Quorus LS biofilm growth shows spatiotemporal distribution of the different growth phases. Cells furthest from the membrane surface are sustained in exponential growth by higher nutrient concentrations on the feed side. As cell growth continues, biofilm thickness increases and perfused nutrients are depleted by actively growing cells (Fig. 3). This results in radial nutrient gradients, established across the biofilm radius. Under nutrient-limiting conditions, stationary growth and secondary metabolism are induced, as is the production of value-added products [53, 54]. At a constant nutrient feed rate, biofilm thickness would continue to increase and high nutrient uptake by the cells closest to the feed would result in nutrient exhaustion and anoxic conditions furthest from the nutrient feed, along with a zone of cell decline and death. In contrast, the Quorus LS bioprocess aims to limit the formation of a decline zone, sustaining biofilm productivity at increasing cell densities and biofilm thickness.

Quorus LS bioprocess innovation is enabled by a fully automated, pneumatically regulated fluid delivery system. Nutrient feed rate is regulated by the pressure differential established across the membrane, between ECS and ICS compartments. A form of chemostatic control [55] is used to ensure that the nutrient feed rate is maintained at levels sufficiently high to prevent nutrient exhaustion, thereby

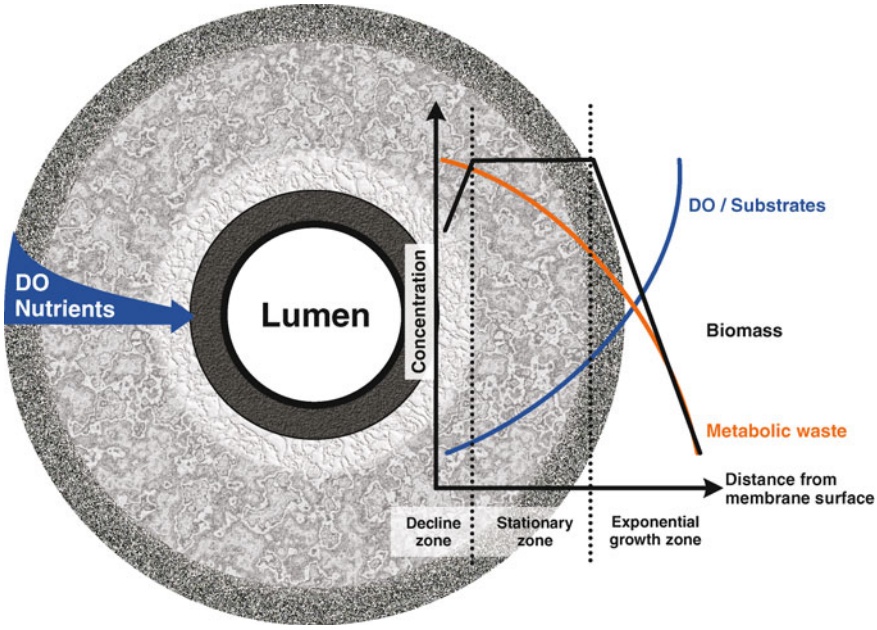

Fig. 3 The Quorus LS bioprocess is a submerged biofilm process. Biofilm growth kinetics are spatiotemporal, regulated by unidirectional nutrient and dissolved oxygen (DO) gradients established across the stable biofilm

limiting cells entering decline phase. Using pH measurements as an indicator of cell growth and metabolism, the nutrient feed rate is proportionately increased to maintain a pH setpoint-defined metabolic activity as cell densities increase. Increased feed rate is achieved through a controlled increase in pneumatic pressure applied to the nutrient supply vessel, to a maximum of 1 bar. Flow rates in excess of 1–2 vol vol_{ECS}^{-1} h^{-1} have been sustained for extended periods, resulting in significantly higher product rates than observed with alternate cultivation methods [36]. A convective-diffusive model for solute mass transfer into a metabolically active biofilm at elevated pressures and flow rates has not yet been defined.

Constant perfusion of nutrients from the ECS to the ICS not only renews nutrient supply to metabolically active cells within the biofilm, but it also serves to dilute and eliminate metabolic waste excreted and accumulate secreted value-added products. Cell-free permeate from Quorus LS can be directly integrated into downstream capturing and purification processes. Lastly, the Quorus LS bioprocess is ideally suited to the continuous removal of value-added products that are (1) toxic to continued cell growth and productivity, (2) labile and easily transformed or degraded if left to accumulate, and (3) secreted protein produced by autoinducible expression systems [53, 54].

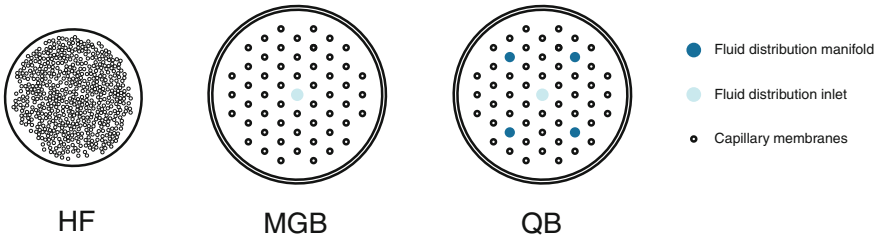

Fig. 4 Typical membrane distribution within a hollow-fiber (HF) module, membrane gradostat bioreactor (MGB), and Quorus Bioreactor (QB)

4.3 Efficiency of Bioreactor Design

A key differentiator of Quorus module design and operation in comparison to standard HF perfusion modules lies in a more uniform and consistent fluid delivery to immobilized cells with increasing reactor size. HF modules comprising bundles of 160- to 200-μm diameter polymer membranes provide a high surface area for mammalian cell attachment. However, mass transfer limitations make the HF module design unsuitable for more metabolically active microbial cultivation [28]. The equidistant capillaries within MGB or QB modules ensure sufficient space for uniform radial biofilm growth. Under optimal cultivation conditions, biofilms are maintained in steady state at an isolate-dependent radial thickness without contacting the adjacent biofilm and disrupting fluid distribution to each capillary (Fig. 4).

MGB were initially manufactured with novel externally skinned polymer capillary membranes for improved biofilm attachment [29, 56]. Despite significant advances in HF polymer durability, ceramic capillaries were determined to be better suited to commercial QB design [40]. By using this more rigid and inert material, problems associated with membrane integrity failure through convective flow at higher operating pressures, mechanical stress of penetrative cell growth, biodegradation of certain membrane polymers [57], and polymer swelling and distortion [58] are eliminated.

QB modules are manufactured with equidistant 2- to 4-mm ø ceramic UF/MF capillary membranes (Fig. 5), sealed end-to-end within the reactor manifold. Although ICS and ECS compartmentalization is consistent with previous HF and MGB designs, the QB module design [59] further incorporates a perforated fluid distribution manifold integrated between capillary membranes, ensuring uniform fluid delivery within the bioreactor's ECS between capillary membranes with lower residence time and limited dead spots (Fig. 6). In the Quorus GLS process, perforated distributors are used to aerate the biofilm; however, in the Quorus LS process, perforated distributors feed nutrients to the biofilm. Further design features include a tapered reactor base for improved draining of the ECS during operation.

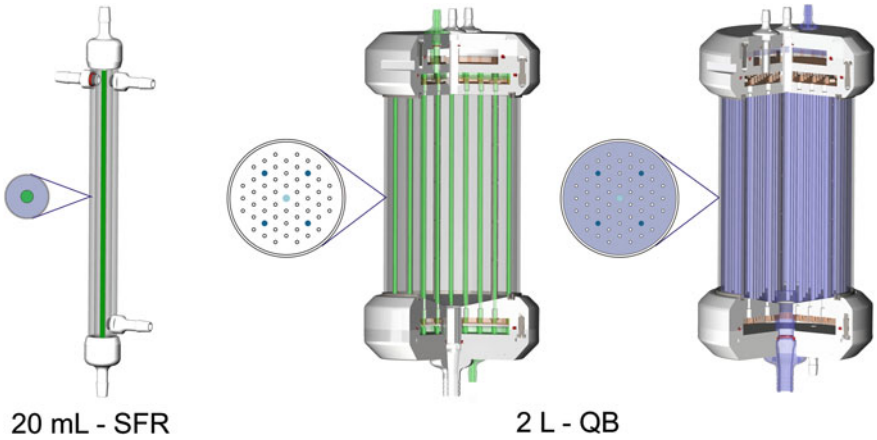

Fig. 5 The Quorus Bioreactor design [59] with intracapillary space (*green*) and extracapillary space (*blue*) fluid flow paths illustrated for 20-mL single-fiber reactor (SFR) and 2-L Quorus Bioreactor (QB) module designs

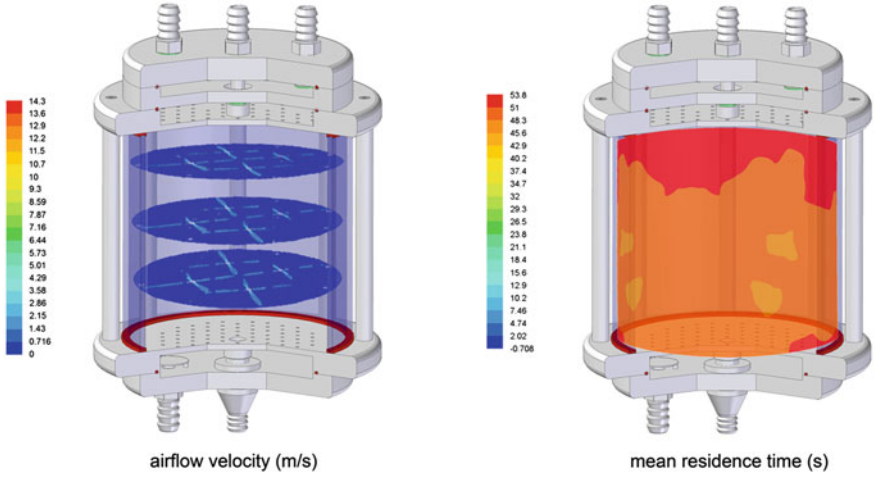

Fig. 6 The Quorus Bioreactor fluid distribution manifold with perforated distributors promotes uniform fluid delivery throughout the extracapillary space between capillary membranes. Computational fluid dynamics at 60 kPa, 1 vvm airflow (unpublished data)

Vertical orientation of QB prevents droplet formation observed in horizontal capillaries, limits fluid collection and biofilm growth at the reactor chamber wall, and allows for more uniform biofilm growth along the capillary length [16, 33]. QBs are operated in dead-end mode for improved mass transport by convective diffusion [60, 61].

4.4 Bioreactor Scalability

Biofilm and bioprocess research and development studies are performed using Quorus single-fiber reactor (SFR) modules, in which a single UF/MF membrane is housed within a 20-mL reactor module (Fig. 5). SFRs are operated in parallel using experimental design in order to assess key process conditions required for optimal QB operation [41, 62]. Process conditions including incubation temperature, operating pressure, nutrient type, feed rate, and aeration rate can be easily adjusted during operation in order to determine their effects on biofilm growth and productivity [34]. Process conditions developed for a single capillary are directly scalable to multifiber QB processes.

Scale-up of module design based on the number of capillaries housed within the reactor chamber is linear (Sects. 4.6 and 4.8). Scale-up from Quorus SFRs to 2-L Quorus GLS bioreactors showed a 7-fold increase in enzyme production with a 10-fold increase in bioreactor size [63].

4.5 Bioreactor Setup and Operation

QB configuration includes a selection of ancillary components required for operation. Nutrient supply vessel (NSV), prime vessel (PV), and product collection vessels (PCV) are standard ancillaries for all Quorus processes. The Quorus GLS bioprocess additionally includes a humidification vessel (HV) to facilitate aeration of the biofilm and an optional condensate vessel (CV) to trap condensate in the outbound airflow during GLS operation (Fig. 7b). The Quorus LS bioprocess additionally includes an inline pH probe connected to the permeate outlet (Fig. 7a).

A single benchtop incubator is adapted to house and operate 20 mL to 2 L Quorus SFR, LS, and GLS setups (Fig. 8). On-board control software allows the user to define the process type, adjust configuration settings, and monitor process performance during operation. QB process control regulates fluid delivery based on transmembrane pressure within the QB membrane module either by manual pressure adjustment, automated control of specified nutrient flux rates, or chemostatic control of fluid delivery to maintain a pH setpoint. Larger-scale QBs are operated as standalone systems.

4.6 GLS Case Study: Recombinant Xylanase Production *by* Aspergillus niger *D15*

Filamentous fungi are increasingly being developed as expression systems for the production of complex heterologous proteins. These fungi have well-developed

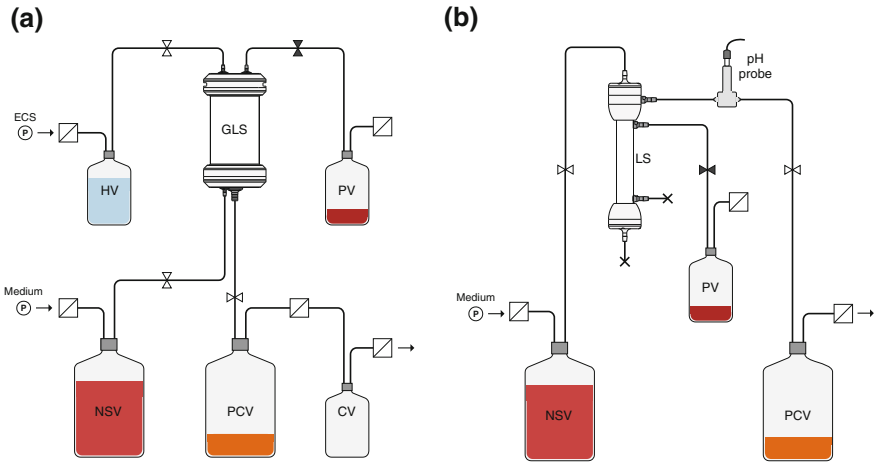

Fig. 7 Bioreactor configuration for **a** Quorus (GLS) bioprocess and **b** Quorus LS bioprocess. All components are housed within the Quorus Bioreactor Benchtop Console, including bioreactor module (GLS/LS), nutrient supply vessel (NSV), permeate collection vessel (PCV), prime collection vessel (PV), humidification vessel (HV), and condensate vessel (CV)

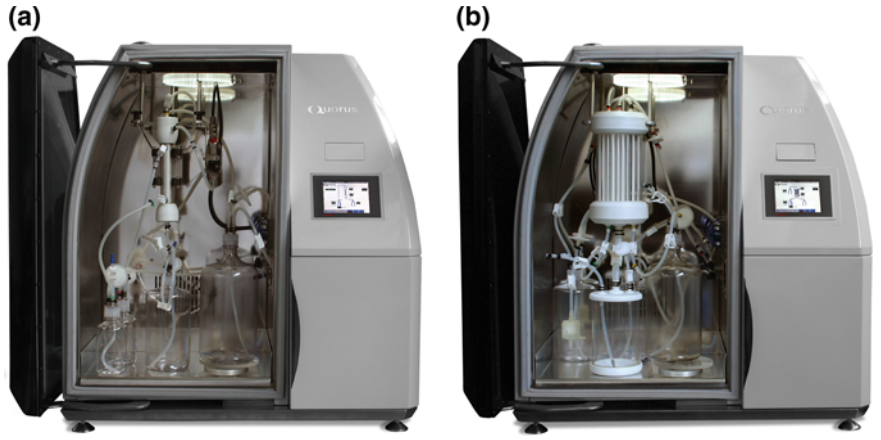

Fig. 8 The Quorus Bioreactor Benchtop Console is a standalone incubator with onboard automated process control, easily adapted to different Quorus Bioreactor setups and operations. **a** Quorus LS bioreactor and **b** Quorus GLS bioreactor setups

secretory pathways and are capable of producing large quantities of specific proteins, which may be easily recovered from the cell culture fluid [64–67]. *A. niger* is one of the best studied fungi with Generally Recognized as Safe (GRAS) status, It is used for the efficient expression of industrial enzymes, antibodies, and antibody fragments [68–70]. In this case study, recombinant xylanase

production from *A. niger* D15[1] [71] is observed using single-use 20-mL Quorus SFR and 2-L Quorus GLS bioreactors. Productivity in comparison to batch stirred-tank cultivation was also assessed.

SFR operating conditions were defined through preliminary screening experiments to determine optimal growth conditions for scale-up (unpublished). Three 20-mL SFRs were operated in parallel, each inoculated with 1×10^7 fungal spores and cultured with 2× minimal growth medium [36, 71]. Standard operating conditions were set at 30 °C, 0.02 L/min airflow with an ECS (backpressure) set at 30 kPa and nutrient flux regulated to approximately 1.0 ml/h. During screening experiments, SFRs were operated in pressure control mode, wherein nutrient flow rates were manually maintained using incremental increases in transmembrane pressure to compensate for the effect of increased biofilm resistance on flux rate. In SFR culture, pressure control mode is used to better control biofilm growth until an optimal biofilm thickness is reached, which can result in an extended lag phase. The 2-L Quorus GLS bioreactor was comprised of 50 capillary membranes with the same physical dimensions and characteristics as in the SFR. Scale-up of ECS aeration rate is linear (2 L/min), whereas nutrient feed rate is determined by the number of capillaries (50 ml/h). QuorusGLS bioreactors were operated in flux control mode, ensuring a more stable cultivation environment.

In both SFR and 2 L Quorus GLS bioreactor cultivations, *A. niger* D15 biofilm thickness stabilized after 10–15 days, following which steady-state conditions were maintained until each experiment was stopped. The *A. niger* D15 biofilm was more corrugated and gradostat-induced growth zones were more distinct than previously described with *P. chrysoporium* MGB biofilms [29, 33, 40]. Steady-state *A. niger* D15 biofilms averaged 1.5 cm and showed characteristic morphological differentiation (Fig. 9) into primary (exponential), secondary (stationary), and tertiary (decline and sporulation) growth zones (Fig. 2).

Stable xylanase production was achieved with continuous operation (Fig. 10). SFR experiments yielded $8.7 \pm 0.1 \times 10^5$ U of xylanase activity in an 18-day cultivation experiment, with an average production rate of $4.9 \pm 0.1 \times 10^2$ U/day. Direct scale-up of SFR process parameters to a 50-capillary 2-L Quorus GLS bioreactor yielded $9.2 \pm 0.3 \times 10^5$ U of xylanase activity in a 40-day cultivation experiment with a production rate of 2.3×10^4 U/day. Scale-up criteria for Quorus processes are defined by the number of capillaries in each module rather than reactor capacity. Taking into consideration slight differences in reactor startup conditions, the scale-up factor from SFR to a multifiber QB can be considered as linear.

QB productivity was benchmarked against stirred-tank cultivations. In these experiments, xylanase production was demonstrated to be 2–3 times higher in 2 L Quorus GLS cultivations than batch cultivations (Table 4). Furthermore, excessive

[1] This modified strain was provided to Synexa Life Sciences (Pty) Ltd (Cape Town, South Africa), to whom it was given by Wilhelm Willem van Zyl, Stellenbosch University, South Africa, for demonstration purposes only.

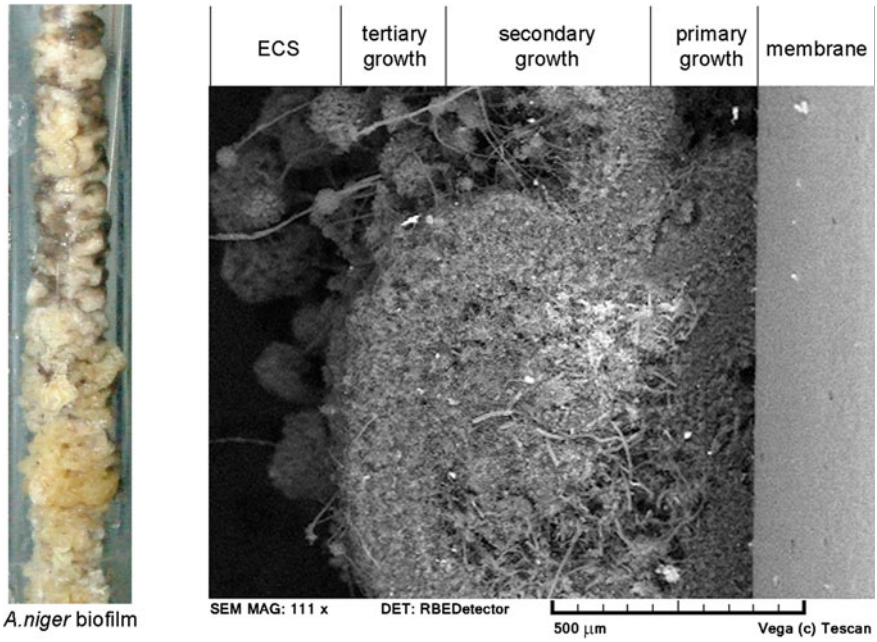

Fig. 9 *A. niger* biofilm cultivated using Quorus SFR, with scanning electron micrograph of *A. niger* biofilm in cross-section. A zone of exponential (primary) growth is found at the membrane surface. A central, less dense zone of hyphae in stationary (secondary) growth forms the largest portion of an established biofilm. Tertiary biofilm structure results from a decline in growth and differentiation of the biofilm into spore-forming aerial hyphae at the biofilm-air interface (ECS)

foaming and volume loss in *A. niger* STR cultivations necessitated the addition of antifoam agents, which are either costly, harmful to cell growth, or cause problems with downstream product separation.

4.7 GLS Case Study: Advantages and Challenges

The Quorus GLS bioprocess serves to provide a stable, productive solution for the cultivation of filamentous microbes such as *A. niger* in a low-shear growth environment. In Quorus GLS bioreactors, humidification of air supply, higher aeration rate across the biofilm surface, and higher operating pressures are all used to improve oxygen mass transfer into the biofilm with low to no shear. Nutrients are supplied by convective-diffusion for improved mass transfer to perfused cells. Cell damage caused by mechanical agitation and invasive aeration in traditional cultivation systems (as required to sustain culture growth, facilitate DO transfer, distribute nutrients, and maintain culture fluidity [72]) is eliminated. Furthermore,

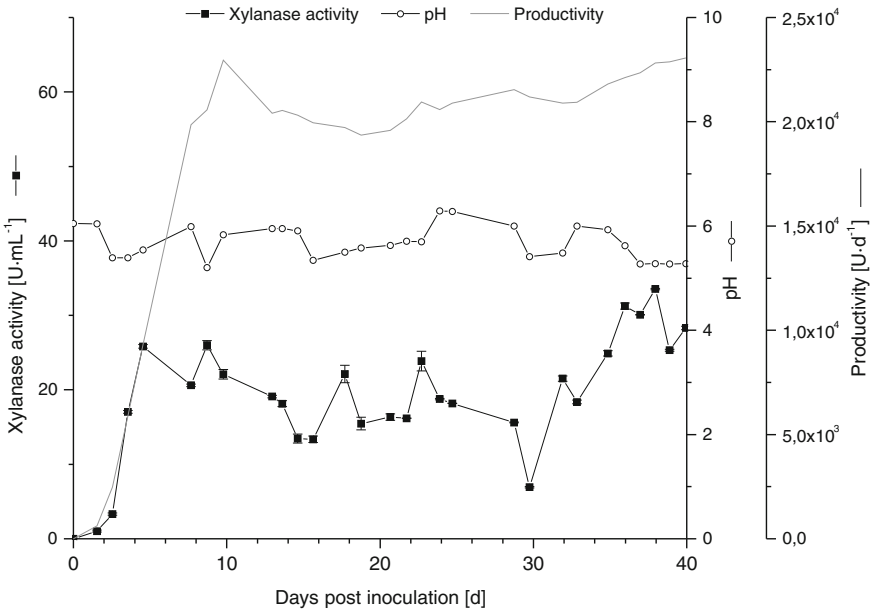

Fig. 10 Continuous production of xylanase from *A. niger* D15 (xyn2) using a 50-membrane 2 L Quorus GLS bioreactor. Displayed are xylansse activity, pH, and productivity [36]

there is no need for the addition of antifoam, used to prevent volume and product loss in conventional cultivations.

Uniform capillary distribution and improved fluid delivery promote stable biofilm growth and metabolism. Microbial biofilms are slower growing, more resistant to fluctuating environmental conditions, and more genetically stable than their planktonic counterparts [73]. The continuous renewal of nutrients, efficient dilution of metabolic waste, and removal of value-added products maintains the metabolic efficiency of the biofilm. This feature makes Quorus GLS ideally suited to the continuous production and harvesting of value-added products that might otherwise inhibit further metabolite production, become toxic to cells, or be susceptible to enzymatic degradation or transformation.

Overgrowth of cells and poor mass transfer at high cell density is a problem associated with traditional cultivation and perfusion systems alike [72]. Cultivation of filamentous fungi in a submerged culture is particularly problematic [74]. Filamentous cell types readily attach to all surfaces, forming heterogenous tissues on baffles, impellors, probes, sampling ports, and tubing. In such cases, uncontrolled biofilm growth has a negative impact on process efficiency. In contrast, Quorus GLS bioreactor productivity is enhanced through the stable, controlled formation of complex differentiated filamentous biofilms. Under optimal conditions, steady-state formation is achieved and productive biofilms are maintained for extended periods, resulting in improved reactor performance. However, cell

Table 4 *A. niger* xylanase productivity with different reactor types [36]

Cultivation type	Yield (activity, units/day)	Normalized yield (activity, units/$L_{reactor}$/day)
Quorus SFR	$4.9 \pm 0.1 \times 10^2$	–
2-L Quorus GLS[a]	$2.3 \pm 0.1 \times 10^4$	$1.1 \pm 0.1 \times 10^4$
2-L Fermenter[b]	$1.2 \pm 0.1 \times 10^4$	$3.9 \pm 0.2 \times 10^3$
10-L Fermenter[b]	$5.9 \pm 0.1 \times 10^4$	$3.9 \pm 0.1 \times 10^3$

[a] 50-membrane GLS module
[b] Batch and stirred

Table 5 Advantages and limitations of Quorus GLS cultivations

Pros	Cons
Biofilm growth	*Overgrowth*
Designed for filamentous microbes	Uniform biofilm growth disrupted
More stable growth and metabolism	Blockage of outlet ports
Continuous dilution of excreted metabolic waste	Research describing key factors regulating growth currently limited to model organism
Sustained production	
Productivity	*Lower titers*
Continuous recovery of secreted products	Not suited to intracellular products
Higher yields, smaller systems	Secreted product accumulated, not concentrated
>3-fold more productive than batch culture	*Regulatory*
Biofilm retention, simplified downstream processing	Good manufacturing practice compliance not yet demonstrated
Less cell damage, purer product	
Single-use	*Single-use equipment*
Flexible	Scalability and cost of single-use devices
Scale-up is linear	Restricted by available sensors and sampling solutions
Faster, safer, cleaner	
Overall process is cost- and time-saving	

overgrowth in QB can be process-limiting, particularly when bioreactor outlet ports become blocked.

At higher aeration rates, spores and aerial hyphae are sloughed off from the biofilm and removed from the QB along with product containing permeate. This cell debris will grow in the product collection vessel if nutrients in spent medium are not completely depleted. Regular removal and clarification of permeate would limit the effects of growth in the product bottle on crude product. Permeate can be collected, stored, and processed in batches or continuously harvested and transferred to DSP. Further advantages and limitations of Quorus GLS technology are summarized in Table 5.

4.8 LS Case Study: Process Intensification of Lactococcus lactis cultivation using the Quorus LS Bioprocess

L. lactis is a well-researched Gram-positive fermentative bacterium with GRAS status that has been developed for the efficient expression and extracellular secretion of recombinant products [52, 75, 76]. In this case study, the *L. lactis* P170 expression system is used as a model organism to demonstrate Quorus LS bioprocess efficiency. This autoinducible expression system regulates gene expression through glucose depletion and the accumulation of lactic acid within the cell culture environment. A signal peptide sequence enables the enhanced secretion of recombinant product into the culture broth. Optimal expression occurs in stationary phase at pH 5–6.5 [77]. *L. lactis* PRA290 (strain supplied by Bioneer A/S, Denmark) is engineered with the *Escherichia coli* β-lactamase gene under the control of the P170 promotor with an erythromycin selective marker. *L. lactis* PRA290 was cultivated in single-use 20-mL Quorus SFRs and 0.2-L Quorus LS bioreactors.

SFR operating conditions were defined through preliminary screening experiments (data unpublished). Three SFRs were each inoculated with 0.1 mL *L. lactis* PRA290 cultured at 30 °C in M17 broth for 16 h. An initial flux rate of approximately 1 vol/$\text{vol}_{\text{reactor}}$ per hour was used. SFRs were operated using Quorus pressure control mode, wherein incremental increases in ECS pressure were used to facilitate Quorus LS chemostatic control. During the SFR operation, permeate pH was maintained above pH 5 with a maximum flux rate of 3 vol/vol_{ECS} per hour, recorded after 30 h. In comparison to a 15-h STR batch cultivation, SFR cultivations were extended to 53 h.

Improved control was observed with multifiber Quorus LS bioreactor operation using fully-automated Quorus LS chemostatic control to maintain a minimum flux setpoint of 0.25 vol/$\text{vol}_{\text{reactor}}$ per hour and pH setpoint of pH 6. A lower initial flux setpoint was selected for multifiber Quorus LS bioreactor experiments to minimize medium usage during culture lag phase. In 0.2-L Quorus LS bioreactors, a maximum flux of approximately 5 vol/vol_{ECS} per hour was observed at 12 h postinoculation. The 0.2-L Quorus LS cultivation was stopped after 43.3 h.

High-density biofilm growth was observed as a thick cell-cake on the outer surface of capillaries (Fig. 11). In each case, a 10- to 12-h lag phase was observed before pH levels dropped sufficiently to induce P170 regulated gene expression and β-lactamase was detected in the permeate. High β-lactamase production rates were maintained for the remainder of the cultivation (Fig. 12).

SFR experiments yielded a total of $2.2 \pm 0.6 \times 10^3$ units of β-lactamase in 40 h with an average production rate of 48 ± 1.1 units/hour. Scale-up of SFR conditions to a 37-capillary 0.2-L Quorus LS bioreactor yielded a total of $8.6 \pm 0.1 \times 10^4$ units of β-lactamase in 43.3 h with an average production rate of $1.9 \pm 0.02 \times 10^3$ U/h. Quorus scale-up criteria is based on the number of capillaries in each module rather than reactor capacity. The scale-up factor for Quorus LS bioprocess is therefore considered to be linear.

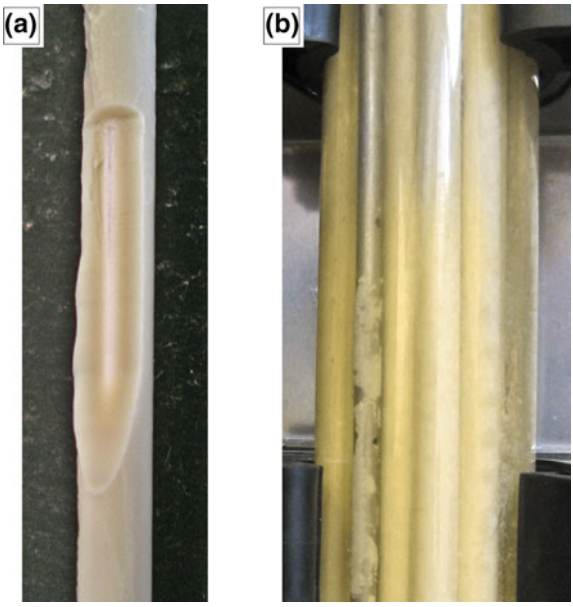

Fig. 11 *L. lactis* PRA290 biofilm. **a** Biofilm growth in single-fiber reactors at 71 h postinoculation yielded an 8-mm biofilm on a 4-mm capillary. A gradient in cell density is observed, where cells closest to the capillary surface are more densely packed than on the extracapillary space side. **b** Biofilm growth in a multifiber Quorus Bioreactor module

In a benchmarking study (Table 5), the 0.2-L Quorus LS bioreactor showed similar titers (U/L) and production rates (U/h) to fermenters. In normalizing productivity according to reactor size used ($U/L_{reactor}$ per hour), the Quorus LS bioreactor outperformed batch cultivation in fermenters by more than 25 times (Table 6).

4.9 LS Case Study: Advantages and Challenges

Cell retention systems are particularly advantageous in the concentration and maintenance of cells cultured to high densities. Perfusion using cell retention devices has been shown to improve productivity by at least 10-fold. These systems use a separate cell cultivation vessel and perfusion module. Furthermore, factors impacting cell growth, including mechanical agitation and aeration, remain problematic, whereas high circulation rates through perfusion modules can cause additional damage to shear-sensitive cells. In contrast, the Quorus LS bioreactor and bioprocess integrate cell growth and retention into a single device. This not only eliminates the need for a separate cultivation chamber but also minimizes the shear stress to which cells are exposed.

Quorus LS technology offers the additional advantage of integrating cell removal and clarification of value-added product into the same device in which cells are cultivated. Cell retention on the surface of ultrafiltration membranes

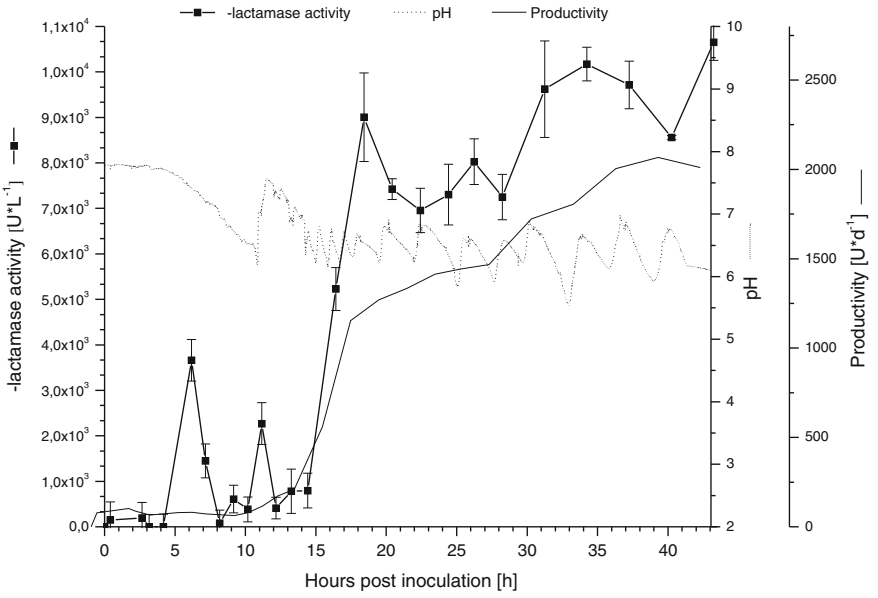

Fig. 12 Extended production of β-lactamase from *L. lactis* PRA290 in a 0.2-L Quorus LS bioreactor. Displayed are β-lactamase activity, pH, and productivity [36]

Table 6 *L. lactis* β-lactamase productivity in different reactor types [36]

Cultivation type	Titer (activity, U/L)	Yield (activity, U/h)	Normalized Yield (activity, $U/L_{reactor}$ per hour)
Quorus SFR[a]	$4.9 \pm 0.9 \times 10^3$	48 ± 1.1	$2.4 \pm 0.5 \times 10^3$
0.2-L Quorus LS	$5.5 \pm 0.6 \times 10^3$	$2.0 \pm 1 \times 10^3$	$9.1 \pm 1.0 \times 10^3$
2-L fermenter[b]	$7.3 \pm 0.01 \times 10^3$	$9.6 \pm 0.1 \times 10^2$	325 ± 5
10-L fermenter[b]	$7.2 \pm 0.2 \times 10^3$	$3.9 \pm 0.3 \times 10^3$	263 ± 9

[a] Unpublished
[b] Batch and stirred

results in the collection of crude product as a cell-free permeate that can be transferred directly to or integrated into product capture and purification processes.

Using the Quorus LS system, cells are grown to high cell densities as a compact cell cake or biofilm, maximizing productivity in a relatively small cultivation space. The Quorus LS bioreactor was shown to be as productive as a stirred-tank reactor at least 25 times its size. Savings are further evidenced in the integration of three separate devices into one, including the cell cultivation chamber, perfusion device, and cell harvesting device. Further advantages and limitations of Quorus LS technology are summarized in Table 7.

Table 7 Advantages and limitations of Quorus LS cultivations

Pros	Cons
Biofilm growth	*Excessive growth*
Very high cell density	Membrane fouling and upper pressure safety limit are process limiting
Favors facultative anaerobes	Research describing key factors regulating growth limited to model organism
Continuous dilution of excreted metabolic waste	
Productivity	*Production*
Continuous recovery of cell-free secreted products	Not suited to intracellular products
Higher yields, smaller systems	Secreted product accumulated, not concentrated
>3-fold more productive than batch culture	Regulatory
Cell retention, simplified downstream processing	Good manufacturing practice compliance not yet demonstrated
Less cell damage, purer product	
Single-use	*Single-use equipment*
Flexible	Scalability and cost of single-use devices
Faster, safer, cleaner	Restricted by available sensors and sampling solutions
Overall process is cost- and time-saving	

5 Conclusion and Outlook

In an industry in which established production strains [78] and traditional cultivation technologies [79, 80] have dominated for decades, there is little wonder that despite the breakthrough of single-use bioreactor technologies, commercial bioreactor innovation has largely been limited to incremental improvement of existing bioreactor designs. Yet a question remains: Has limited innovation in bioreactor design contributed to the slow adoption of newer expression systems and unconventional production strains by industry?

As researchers continue to unravel factors influencing protein expression, folding, and conformational stress in foreign hosts [48, 75, 81, 82] and drug discovery increasingly reverts to microbial natural products as a source of new drug leads [83–86], the commercial potential of unconventional cell types and the number and variety of unconventional expression systems is set to increase. Lower productivity and extended development timelines observed when working with cells poorly suited to traditional cultivation technologies render production unaffordable, resulting in potentially valuable products being shelved from commercial pipelines. An opportunity exists for innovative bioreactor designs that offer a more natural growth environment for unconventional cell types, thereby providing a more efficient and cost-effective manufacturing solution for their products.

Amidst renewed interest in perfusion systems, few single-use technologies are as well suited to microbial cultivation as the QB, a pneumatically operated single-use membrane bioreactor system developed for the cultivation of nontraditional cell types. The advantages and limitations of Quorus design and operation can be summarized according to the following distinct niche applications:

- Quorus GLS bioprocess: ideally suited to the continuous production and harvest of secreted, toxic, labile or volatile products produced by complex microbial biofilms formed by filamentous fungi and actinobacteria
- Quorus LS bioprocess: ideally suited to the extended production and harvest of toxic, labile or process-inhibiting products secreted by Gram-positive bacteria and other cell types.

Knowledge of mass transfer is essential for understanding and controlling perfusion processes. Quantifying the nutrient gradients regulating stable, differentiated biofilm formation in Quorus bioprocesses remains a key focus.

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Adv Biochem Eng Biotechnol (2014) 138: 179–206
DOI: 10.1007/10_2013_219

Published Online: 27 July 2013

Cultivation of Marine Microorganisms in Single-Use Systems

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Abstract Marine cultures are an important source of novel substances and enzymes. As efforts to isolate strains from (deep) sea environments increase, the demand for methodology platforms to cultivate these organisms is also rising. Due to the high salt concentration and the shear sensitivity exhibited by some heterotrophic microalgae, single-use systems originally designed for the cultivation of mammalian cell lines can be a valuable alternative. Using the cultivation of the heterotrophic marine microalgae *Crypthecodinium cohnii* as an example, this chapter makes suggestions for experimental design, for improving process development by integrating parallel experiments, and for scaling-up and scaling-down methodologies. It describes how to identify suitable single-use systems and how to integrate a two-layer system with perfluordecalin to improve the gas transfer in deep-well plates. The process is also scaled up in several single-use systems. We also describe challenges in the process development to achieve sufficient oxygen transfer, monitoring, and control, and we discuss limitations such as corrosion, long-term stability, and leachables in single-use systems. Finally, we demonstrate a method for cheap, fast, and consistent process development for marine microorganisms.

Keywords Crypthecodinium cohnii · Marine heterotrophic microalgae · Perfluorodecalin · Polyunsaturated fatty acid · Scale-up

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Abbreviations

CDW	Cell dry weight
CHO	Chinese hamster ovary
DHA	Docosahexanoic acid
DWP	Deep-well plate
DO	Dissolved oxygen
FSC	Forward scatter channel
k_La	Volumetric oxygen transfer coefficient
OD	Optical density
PFC	Perfluorochemicals
PFD	Perfluorodecalin
PVC	Polyvinyl chloride
PI	Propidium iodide
SSC	Side scatter channel
SUB	Single-use bioreactor
UYF	Ultra Yield flask

Contents

1 Introduction

The intensive development of single-use bioreactors (SUBs)—also referred to as disposable bioreactors—has increased the application of single-use systems in bioprocesses in recent years. Whereas the majority of past research focused on the design of SUBs for mammalian cell culture, the focus in research has shifted more towards investigations of how to adapt SUBs for novel fields of application, such as plant cells [1–3], phototrophic microalgae [4, 5], and anaerobic bacteria [6]. The successful application of SUBs for aerobic bacteria [7–11] and for yeasts [9, 12] has also been described in the literature. However, examples of microbial cultivations in SUBs are still rare, mainly due to the lower mass transfer coefficient compared to common stainless steel stirred-tank reactors. In contrast, small-scale systems such as deep-well plates (DWP) are an opportunity for the conduction of many parallel experiments for automated bioprocess optimization. Therefore, these polymer-based tools are widespread in bacteria and yeast process development [13].

The advantages of the application of SUBs for bioprocesses are significant: (i) the initial investment costs are lower; (ii) a scale-up can be performed very efficiently in some cases simply by increasing the bag size; and (iii) cleaning and sterilization steps are minimized, which saves time and money [14–17]. It has also been demonstrated that the environmental impact of single-use technology is lower compared to conventional stainless steel equipment, mainly due to the larger amounts of water and manpower needed for cleaning traditional systems [18]. According to an economic comparison by Sinclair and Monge, savings in running costs would amount to approximately 6 % when applying single-use technologies compared to stainless steel reactors, and, if capital costs are included, 17 % can be saved in running costs [19]. Different types of SUBs are currently available on the market. The most often used systems can be grouped into the categories wave-mixed, orbital shaken, and stirred bioreactors. Detailed descriptions of these reactor types are found in several recent reviews [14, 20].

The interest in bioactive compounds from marine microorganisms has increased in recent decades and is now a major branch of modern marine (blue) biotechnology. In several studies, marine microorganisms have been exposed to extreme conditions, for example, to varying degrees of salinity, pressure, and temperature; they have been subjected to wave forces, ultraviolet radiation, and various nutrient limitations [21, 22]. Enzymes from these organisms are important resources for use as biocatalysts in the synthesis of fine and bulk chemicals due to their extraordinary stability and activity under extreme reaction conditions [21]. However, marine microorganisms require a water activity at the level of seawater ($a_W = 0.98$) [23] and additional sodium ions for growth [23–25]. Sodium ions are needed for active transport, which is dependent on the sodium motive force, flagellar rotation, and the stability or activation of membrane and periplasmatic components in marine bacteria [25]. The majority of studies with marine microorganisms were conducted in shake flasks [23]. For cultivations carried out at atmospheric pressure, glassware is widely used to prevent corrosion problems [26].

2 Examples of Enzyme Production Processes with Marine Bacteria

Enzymes and products from marine microorganisms offer a wide range of benefits. One example is Salinosporamide A from the marine bacterium *Salinispora tropica*. Salinosporamide A is a proteasome inhibitor with a broad spectrum of applications for treating various tumors [27]. Tsueng et al. tried to exchange the chloride ions in the media with sulfate ions to avoid corrosion in the process, as patented by Barcley [28] for the heterotrophic microalgae *Shizochytrium* and *Traustochytrium*. It was observed that a concentration of 86 mM of chloride ions was still necessary to obtain maximal growth [27]. It should be remarked that stainless steel corrodes at a concentration of 8.5 mM sodium chloride [29], and thus a process development based on such microorganisms is not trivial.

The widely applied host *Bacillus licheniformis* is halotolerant and grows best if seawater is used instead of tap water [30]. When a 1 M NaCl solution is added to a tap water medium, the maximum growth reaches 75 % of the growth in seawater and the productivity is 25 % higher than without NaCl. The authors suggested using seawater for process development in order to save freshwater resources. Optimal growth was reported for the alkalophilic strain *Beauveria bassiana* BTMF S10, which produces extracellular glutaminase with NaCl concentrations higher than that in seawater (9 % NaCl), and thus presents an even greater challenge with regard to corrosion [31]. Other examples of valuable products from marine organisms include the sulfite oxidase production for biosensor systems with the marine bacterium *Sulfitobacter pontiacus* in a marine broth fermentation medium [32], and the production of extracellular protease with the marine bacterium *Vibrio harveyi* in a solution supplemented with seawater [33].

The review by Sarkar et al. [34] is recommended for a complete overview of marine enzyme production in bioreactor processes. The examples in this review clearly demonstrate the benefit of using SUBs for whole cell processes with marine microorganisms, as long as systems with sufficiently high oxygen transfer rates are available. Moreover, employing SUBs in process development can circumvent corrosion problems and limitations in growth and production rates and avoid issues arising from patents covering the replacement of chloride ions.

3 Cultivation of Marine Microorganisms in Single-Use Bioreactors

The industrial cultivation of marine microorganisms such as marine bacteria and heterotrophic microalgae presents several challenges: (i) the oxygen demand is high compared to mammalian cell culture processes due to the higher growth rates of microorganisms; (ii) the marine medium facilitates corrosion of common stainless steel bioreactors; (iii) reliable sensor systems are necessary for optimized

process control; and (iv) the single-use bags must exhibit long-term stability in order to carry out long-term cultivations and to save costs when applying repeated fed-batch procedures or similar strategies.

3.1 Cultivation of Microbial Cells with High Oxygen Demand in Single-Use Systems

Today, the application of SUBs is mainly restricted to production processes using mammalian and insect cells [14]. Nevertheless, there have been attempts to use SUBs for the cultivation of microbial cells with higher oxygen demands. Mikola et al. employed the WAVE Bioreactor® introduced to the market by Wave Biotech LCC (Bridgewater, NJ, USA) for the cultivation of *Saccharomyces cerevisiae* [12]. The bioreactor was equipped with a frit sparger to enhance the oxygen transfer rate. A maximum k_La-value of 38 h^{-1} was achieved for a 5-L bag when sparging with air. It was increased to 60 h^{-1} by raising the O_2 content of the inlet gas to over 90 % v/v. Ullah et al. described the application of the BIOSTAT® CultiBag RM (Sartorius-Stedim Biotech, Göttingen, Germany) for the cultivation of *Corynebacterium diphtheria* for vaccine production. The measured k_La values were in a range between 6 h^{-1} (air sparging) and 12.9 h^{-1} (oxygen sparging) [7]. Hitchcock described the production of a recombinant *Listeria monocytogenes* vaccine for Phase 2 clinical trials [35]. To comply with validation standards, the BIOSTAT CultiBag RM SUB was applied instead of traditional stirred-tank reactors. When the filling volume was reduced to 20 % of the total volume, a suitable gas transfer rate was achieved. A final optical density of $OD_{600} = 12$ was obtained in a total liquid volume of 5 L.

In microbial cultivations, the fed-batch method is usually used to avoid oxygen limitation during the cultivation, because the oxygen uptake rate correlates with the substrate consumption rate [36]. This strategy was applied successfully to compensate for limited oxygen transfer in SUBs. Glazyrina et al. demonstrated the feasibility of using the BIOSTAT CultiBag RM system for the cultivation of recombinant *Escherichia coli*. A dry cell weight of 10 g L^{-1} was obtained when using an internal feeding system (EnBase®, BioSilta Oy, Oulu, Finland) [8]. A twofold increase in the cell density was achieved by employing a fed-batch procedure with additional oxygen sparging. Further improvements in process control resulted in a dry cell weight of 49.4 g L^{-1} in the rocking-motion-type bioreactor [10]. In a similar process, the researchers even managed to increase the cell density to 60 g L^{-1} in the BIOSTAT CultiBag STR system.

Galliher et al. described cultivations of *Escherichia coli* in a 50-L single-use stirred bioreactor (XDR-50, Xcellerex, GE Healthcare Inc., USA) at cell densities of OD = 120 (corresponding to approximately 40 g L^{-1} dry cell weight) [9]. Furthermore, in *Pseudomonas fluorescens* cultivations, a dry cell weight of over 100 g L^{-1} was achieved as described in the same report. The growth was comparable to results obtained in a conventional 2 L bioreactor.

The CELL-tainer® (CELLution Biotech BV, Assen, Netherlands) exhibits a two-dimensional rocking motion in the horizontal and vertical directions, which results in k_La-values up to 300 h^{-1} [37]. Junne et al. demonstrated the application of the CELL-tainer for the cultivation of *Escherichia coli* [11]. In these cultivations, a glucose-limited fed-batch process was successfully employed to prevent oxygen limitation. By using partly oxygen sparging, a maximum dry cell weight of over 40 g L^{-1} was achieved within 32 h in 12-L liquid volume and 45 g L^{-1} was achieved within 29 h in 120-L liquid volume.

3.2 Cultivation of Phototrophic Microalgae in Single-Use Bioreactors

An example of a single-use screening system for the cultivation of phototrophic microalgae is described by Menke et al. [5]. They employed an airlift and a see-saw bioreactor made of tubelike bags that are usually used for commercial packaging. The authors used this system with a dimension of 100 mL in order to screen various phototrophic algae strains for the treatment of wastewater. Other SUBs used for the cultivation of phototrophic microalgae are made of polymer foil. NOVAgreen offers ready-to-use solutions for greenhouses to convert CO_2 from biogas plants to algae biomass, which can be used as a feedstock for aquaculture. Bergmann et al. suggested changing the material of the flat panel airlift photobioreactor developed by Subitec (Stuttgart, Germany) from PVC to other single-use materials when it is to be utilized as a single-use technology. Cultivations with *Phaeodactylum tricornutum* (*Bacillariophyceae,* eicosapentaenoic acid), *Haematococcus pluvialis* (*Chlorophyceae, astaxanthin*), and *Nannochloropsis oculata* (*Eustigmatophyceae,* polyunsaturated fatty acids) have been performed successfully in flat panel reactors specifically designed for cultivation of phototrophic microalgae [43]. Lehman et al. applied the wave-mixed BIOSTAT CultiBag RM (Satorius Stedim Biotech) equipped with red and white LEDs, the wave-mixed AppliFlex (Applikon Biotechnology) with white light LEDs, and the orbital shaken CultiBag RM operated in the Multitron Cell shaker (Infors HT) and equipped with cool white fluorescent tubes. In all three systems, cell densities were achieved that were comparable to those in reusable stirred, helical tubular, and airlift photobioreactors [4].

3.3 Sensors in Single-Use Bioreactors for Marine Processes

Sensors in SUBs have to be cost-efficient and reliable. A long service life is not required, as long as they are fully disposable [44]. Employing optical sensors is a very common technique. The principle of this measurement is based on embedding a fluorescent indicator, which is immobilized in a polymeric matrix. The optical detector is connected to the transducer via a glass fiber through a transparent window in the bag. An excitation light source is connected to one end of the glass

fiber and the change in the intensity or decay time of the reflected fluorescence light is measured. The signal is proportional to the concentration of the analyte [44–46]. Optical pH and oxygen sensors utilize different dyes for the measurements. For oxygen sensors, metal complexes are immobilized within polymers, for example, ruthenium-tris-(diphenyl-1,10-phenanthroline) in silicone. These ruthenium–ligand complexes have luminescence decay times on the order of 1–5 μs, they are only moderately quenched by oxygen, and they can be used to determine oxygen concentrations between air saturation and full oxygen depletion [45]. Typical dyes for pH sensors include fluorescein derivatives combined with 8-hydroxy-1,3,6-pyrene trisulfonic acids [44]. The sensors determine the pH by measuring the ionic strength. Usually, the range is limited to three pH units [45, 47].

The main advantages of these precalibrated optical sensors is that they can be easily miniaturized, they are simple to apply, and the costs are low in comparison to amperometric Clark electrodes [44, 47]. The main disadvantage is the reduced long-term stability due to photobleaching [44]. Optical sensors are usually mounted in the bag before gamma sterilization is performed. When SUBs are used for phototrophic applications, bleaching due to LEDs decreases the sensor stability [4].

Our own experiments have proved the general applicability of disposable sensors for use in marine media, however, the sensors differed in their performance. The SB200-X orbital shaker (Kuhner, Birsfelden, Switzerland) was used for the cultivation of *C. cohnii* at a total liquid volume of up to 120 L. The bioreactor was equipped with optical online DO and pH sensors (PreSens, Regensburg, Germany). The sensors were applied within a range of 0–100 % oxygen saturation and a pH between 5.5 and 8.5 [48]. The pH was adjusted manually because no method for direct control had yet been established, thus variations in the pH occurred (Fig. 1). The DO measurement in the SB200-X reactor was reliable over the cultivation time of approximately 40 days, but a large discrepancy was observed between online and offline measurement of the pH values. Optical sensors are described as being highly dependent on the ionic strength of the solution [45], which is particularly high in algae processes in sea-salt media (an ionic strength of at least 0.5 M). Changes in osmolality during the cultivation might have caused problems in the measurement. For an optical pH sensor (Fluorometrix, Stow, MA, USA), Hanson et al. reported a change in the pH measurement of 0.05 units when the osmolality was increased from 320 to 450 mOsm kg^{-1}. Such a change is caused by feeding [49].

In contrast to the Kuhner reactor, the pH was controlled for cultivations in the CELL-tainer and in the Integrity™ PadReactor (ATMI, Hoegaarden, Belgium; see Fig. 1). The CELL-tainer is equipped with traditional electrochemical pH and polarographic DO electrodes, which are customized for the single-use application. They are mounted in small cups at the bottom of the bag, which offers the advantage that they are covered with liquid at all times, even at low filling volumes [37, 50]. The pH sensor is completely disposable, whereas the DO sensor consists of a membrane, which is already incorporated into the bag upon delivery, and a reusable electrode. The electrolyte is added to cover the membrane before the electrode is mounted. The measurement range for the pH value is not restricted

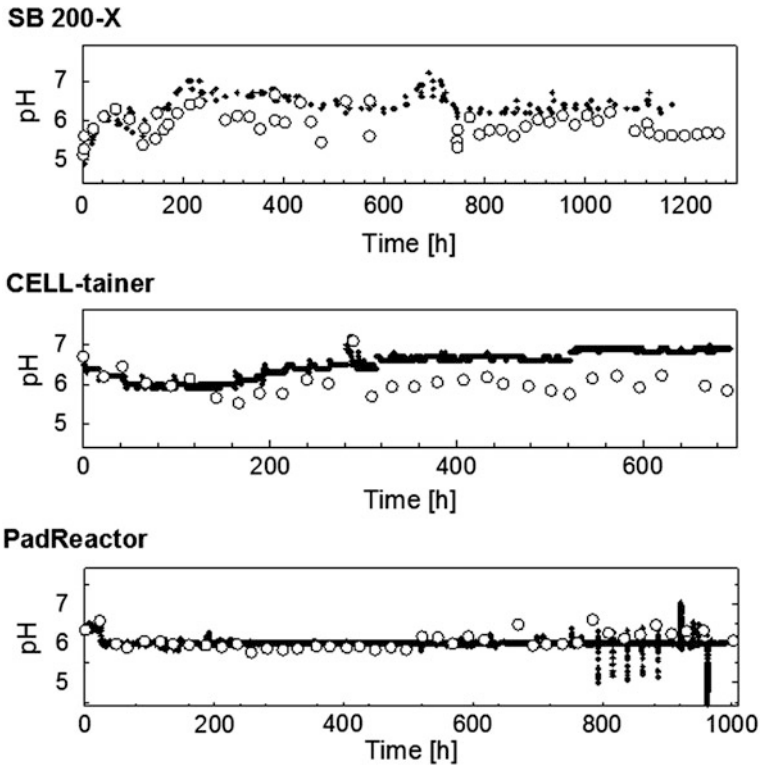

Fig. 1 Differences between *online* (*black dots*) and *offline* (*open circles*) measurements with different sensors in single-use bioreactors

(pH 0–14) [50]. Figure 1 depicts the pH measurements at which a drift is observed. The setpoint was adjusted accordingly to compensate for these deviations in the measurement.

In the PadReactor, in contrast, conventional electrodes are used that are designed for use in stainless steel stirred-tank reactors. These electrodes can be calibrated, autoclaved, and connected via aseptic Kleenpak™ connectors (Pall Corp., Port Washington, NY, USA). They are completely reusable and offer the same accuracy and long-term stability as when used in conventional stirred-tank reactors. The pH values obtained from the PadReactor were the most reliable in this study (Fig. 1). Unfortunately, the reactor system does not provide a sufficient gas transfer rate for the cultivation of *C. cohnii* (data not shown). This observation reinforces the benefit of implementing standard electrodes for process control in marine bioprocesses.

3.4 Long-Term Stability of Bags

When single-use bags are applied in cultivation processes, there is a higher risk of leakage compared to stainless steel bioreactors. However, our studies on different SUB systems have shown that these bags can withstand cultivation times of at least 1,000 h (Fig. 1).

One major concern in using polymer-based bioreactors is the presence of extractables and leachables [20, 51]. Rader and Langer [52] reported that components in the "tie layer" of multilayer laminated bags or the labels on the outside of the bag can result in appreciable leaching into the process. The definitions for extractables and leachables are given by the Bio-Process Systems Alliance (BPSA). According to these definitions, extractables are "chemical compounds that migrate from any product-contact material (including elastomeric, plastic, glass, stainless steel, or coating components) when exposed to an appropriate solvent under exaggerated conditions of time and temperature," whereas leachables are "chemical compounds, typically a subset of extractables, that migrate into a drug formulation from any product-contact material (including elastomeric, plastic, glass, stainless steel, or coating components) as a result of direct contact under usual process conditions or accelerated storage conditions" [53].

Factors that can influence the prevalence of extractables and leachables include (i) the composition of the product fluid; (ii) the contact time and temperature, which influence the kinetics and thermodynamics of the leaching process; (iii) the size of the interface between the product and the single-use material; and (iv) any pretreatment of the material, for example, by gamma sterilization, which can alter its properties [51, 53]. Ding showed that extractables and leachables exist in single-use systems and should be taken into account for process development [51]. He describes an approach to determine the prevalence of extractables and leachables and to evaluate their influence on the specific process.

The DECHEMA e.V. working group, "Single-Use Technology in Biopharmaceutical Manufacturing," carried out a test across different laboratories. Bags from different vendors were incubated for 7 days with sterile water. The growth of different cell lines was examined in chemically defined media prepared with this incubated water. Two bag materials were shown to have an influence on growth [54]. Unpublished results from our lab proved that the growth performance can be altered by leachables and that these effects disappeared when the complete culture was transferred to another culture device. This is probably due to volatile leachables evaporating. In the new cultivation device, the concentration dropped below toxic levels and growth rates recovered. This observation indicated that leachables do matter for applications involving marine cultures and that this could be due to the properties of the medium. However, no systematic studies have been conducted on this issue and knowledge in this area is sparse. In any case, applications involving marine cultures require that care be taken with respect to the occurrence of leachables. The influence of leachables might be tested by applying different

single-use systems as it is unlikely that the same toxic components are released at the same rate for different plastic materials.

4 Case Study: Bioprocess Development in Single-Use Bioreactors with the Heterotrophic Marine Microalgae *Crypthecodinium cohnii*

The marine heterotrophic microalgae *Crypthecodinium cohnii* synthesizes the polyunsaturated fatty acid docosahexaenoic acid (DHA) in large amounts, which makes this organism attractive for industrial applications. DHA has a positive influence on human health, because it protects against heart disease, cancer, diabetes, and depression [55–57]. The traditional source of DHA for humans is fatty fish, but fish are notable to synthesize DHA on their own, instead obtaining this fatty acid via the food chain, predominantly by consuming microalgae [55, 56, 58].

C. cohnii is a eukaryotic microorganism, belonging to the group dinoflagellates. It is equipped with two flagella for locomotion. During growth, two different morphological forms appear: motile swimming cells and cysts, which are cells that have shed their flagella [59]. The cells appear cyst-like during the stationary phase and accumulate DHA when nutrition sources other than the carbon source are missing [56]. Microscopic images of the two morphological forms are shown in Fig. 2.

Hu et al. [60] estimated that a volumetric oxygen transfer coefficient ($k_L a$) of at least 33 h^{-1} is necessary for the growth of *C. cohnii* cells. The bioreactor experiments in stirred bioreactors described in the literature maintained $k_L a$ values of approximately 600–800 h^{-1} in order to ensure a sufficient oxygen supply at lab scale [61]. One reason for the high demand for oxygen is the fact that this process cannot be operated in a nutrient-limited fed-batch mode during those process phases where the limited availability of substrate could reduce the respiratory

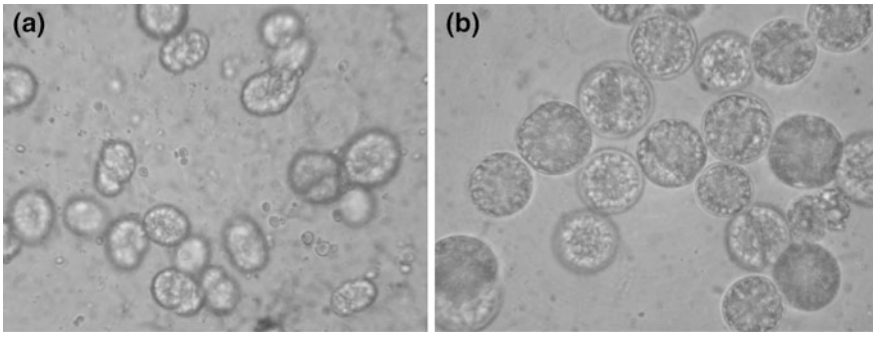

Fig. 2 Microscopic images of *C. cohnii* suspended in the growth (**a**) and production (**b**) phases (magnification 1:630)

activity of the culture. In addition, the desaturases, which play a key role in fatty acid biosynthesis, use oxygen as a co-factor in eukaryotic cells. Hence, their activity depends on the availability of oxygen [62–64] and thus oxygen limitation may reduce the product yield. Nevertheless, this pathway has still not been verified in *C. cohnii*, as no polyunsaturated fatty acids aside from DHA have been found and no oxygen-dependent desaturases have been identified [65].

Different opinions are discussed in the literature as to the shear sensitivity of *C. cohnii* cells. Hu et al. reported that high shear forces reduced the mobility of the cells, due to the loss of their flagella. They observed that this damage is reversible and that cells undergo a recovery process in which they first begin to spin and then move in a straight line once the shear forces have ceased [64]. It was demonstrated that the algae are not harmed by shear stress that occurs in shake flasks [60]. However, Yeung et al. showed that cell proliferation is inhibited by the occurrence of high shear stress but that the culture is able to recover [66]. Furthermore, it was proven using propidium iodide (PI) staining that the viability of the cells was not reduced as a result of shaking [66, 67]. Experiments in our own laboratory revealed that shear stress occurs due to the mechanical forces within stirred-tank reactors and that the resulting loss of the flagella is problematic (unpublished data). The sensitivity to shear stress is even higher under oxygen-limited conditions. We conclude that it is beneficial to employ SUBs that exhibit low shear forces. However, the detailed mechanisms that lead to higher shear sensitivity and the loss of flagella are not yet fully understood.

In the marine habitat in which *C. cohnii* grows, the required chloride ion concentration is at least 1 % w/w [56, 68]. At these high chloride concentrations, it is advantageous to use polymer-based systems, as the corrosion problems common to stainless steel bioreactors can be avoided. Custom-made solutions such as resistant types of stainless steel or special coatings require high investment costs [29] and often are not even applicable (e.g., on seals or moving parts such as the stirrer shaft). These challenges provided the motivation to employ SUBs in each process development stage in a case study of a process development strategy for marine cultures.

4.1 Cultivation in Deep-Well Plates

In media and bioprocess development, a larger number of influencing parameters have to be analyzed and parallel experiments must be carried out to obtain reliable and statistically significant data. This is especially important for slowly growing organisms, where the reproducibility is often poor due to the prolonged cultivation time. In order to fulfill these requirements, small-scale systems must provide comparable conditions across parallel cultivations and these should be scalable to the pilot and production scales. Deep-well and micro-well plates are widely used in process development, but these systems typically lack a sufficient oxygen supply [69, 70]. When the oxygen demand of the cells exceeds the oxygen transfer

capacity in the medium, oxygen limitation occurs and the observed growth rate is only a function of the mass transfer rate in the medium [71, 72].

One way to overcome such limitations is to employ a liquid two-layer system, with one layer characterized by increased gas solubility. If this (primarily hydrophobic) layer is mixed with the hydrophilic cultivation layer, the surface area in such a suspension can be greatly increased compared to the gas–liquid interface in shaken systems without direct sparging. The same effect is achieved when the two-layer system is not mixed, but the hydrophobic layer remains at the bottom of the well. In such systems, oxygen is provided not only by the top gas layer but also by the liquid hydrophobic bottom layer. It is "reloaded" when it comes into contact with the gas layer at the edges of the spinning liquid section. Usually, a water spout is formed. When the edges of the hydrophobic layer cover the walls of the well, a film is formed which increases the transfer of components from the gas into the hydrophobic layer and vice versa.

Liquid perfluorochemicals (PFCs) are fully synthetic derivatives of saturated hydrocarbons, in which all hydrogen atoms have been replaced by fluorine atoms. The chemical bonds between carbon and fluoride ions are strong (approximately 487 kJ mol^{-1}). Therefore, PFCs are inert compounds with high resistance to heat [73]. One of the unique physiochemical properties of liquid PFCs is their high solubility with respect to polar gases. The solubility of respiratory gases (O_2 and CO_2) in perfluorinated liquids is higher than in water. The solubility of oxygen in liquid PFCs is about 35.5 mM compared to 2.2 mM in water; the solubility of carbon dioxide is 125 mM in PFC compared to 57 mM in water [73, 74]. Therefore, liquid PFCs can be employed as liquid carriers (vectors) of respiratory gases and also as scavengers for gaseous by-products of cellular metabolism. They have attracted interest as suitable additives, having been confirmed by experimental results and clinical investigations [73, 75, 76].

Over the past 30 years, many studies have shown that the application of oxygenated liquid PFCs can improve the oxygenation of *E. coli* [69, 76–78] and other microbial [75, 80, 81], plant cell [73, 76, 81], and animal cell cultures [82–84]. In contrast to biological O_2/CO_2 carriers (i.e., myoglobin and hemoglobin), there is no chemical attraction between gas molecules and the PFCs, because perfluorinated liquids dissolve gases according to Henry's law. The gas transfer rate into PFCs increases linearly with the partial pressure of particular gaseous components. The gas molecules occupy cavities between PFC molecules, which facilitate rapid release of the gas molecules to the water layer [85]. Due to the high density of PFCs, which is about 1.9 g cm^{-3} [86], and their strong hydrophobicity, a two-layer system is formed with PFC at the bottom layer (see Fig. 3). For biotechnological applications, it is noteworthy to mention that liquid PFCs added to the culture medium do not change the concentration of the medium components [75].

The possibility of increasing the gas transfer rates with a suitable gas carrier even at small scales was previously reported by Pilarek et al. for *E. coli* [69, 78] and by Meyer et al. for CHO cells [87]. Pilarek and colleagues showed that the cell density of *E. coli* increased by 40 %. An increase was also observed for the amount of a heterologous alcohol dehydrogenase [69] and plasmid concentration [78]. Meyer

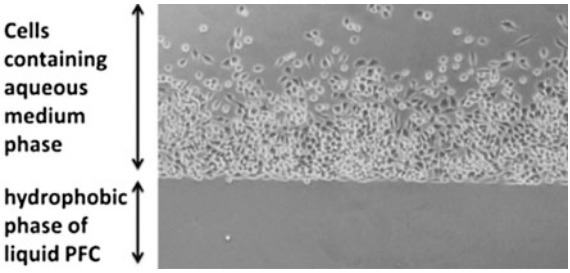

Fig. 3 Adherent BHK-21-cell fibroblasts cultured in a liquid–liquid culture at a flexible interfacial area between culture medium and perfluorinated oxygen carriers

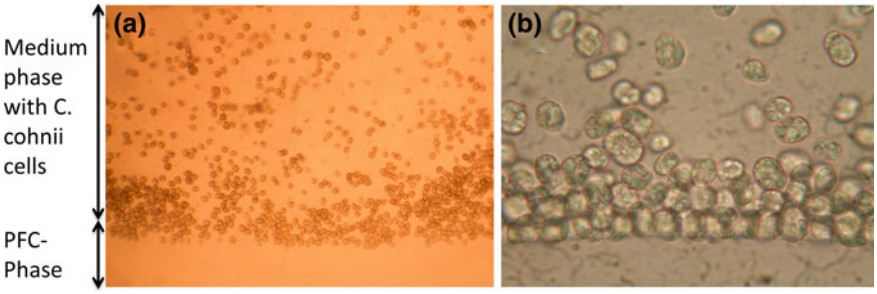

Fig. 4 Microscopic image of *C. cohnii* cells at the liquid–liquid interface between PFD and aqueous media (magnification: **a** 1:100, **b** 1:630)

et al. achieved the same cell densities for CHO cells and the produced recombinant monoclonal antibodies in a PFC-supported 96-deepwell plate culture system with poor mixing compared to cultures in highly ventilated shake flasks [87].

The two-layer cultivation system containing perfluorodecalin (PFD) was also tested for the heterotrophic microalgae *C. cohnii* (see Fig. 4). The cells were cultivated for 7 days in 24-well DWP. The cultures contained 3 mL of culture broth and 3 mL of PFD, which was saturated with pure oxygen before application. The amount of cells and the DHA content were measured at the end of the experiment, and the specific DHA content in the culture was determined. The O_2 concentration was measured with an amperometric DO sensor (Medorex, Noerten–Hardenberg, Germany) directly in the wells at several timepoints throughout the cultivation. The amperometric DO sensor measures O_2 in an aqueous layer. Therefore, the measured concentration refers to the concentration of O_2 that can be released to an aqueous layer in an equilibrium state when deoxygenated water and the PFD-sample are mixed. To determine the CO_2 content in the culture broth and in the PFD layer, each layer was poured into airtight sealed vials, and the culture broth was diluted with an equal amount of methanol in order to stop metabolic activity. The CO_2 content was determined with an optical sensor (PreSens, Regensburg, Germany).

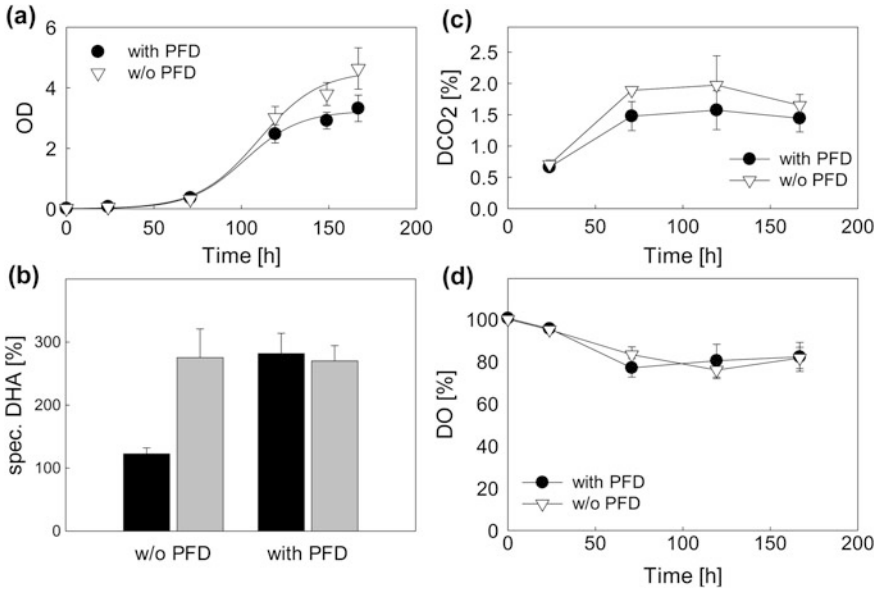

Fig. 5 *C. cohnii* cultivation in DWPs with or without oxygenated PFD. **a** Growth curve; **b** specific DHA concentration (*black* cultivation with several breaks for sampling; published in Ref. [88], *gray* without longer breaks, 100 % equals the results obtained in an Erlenmeyer flask); and **c**, **d** dissolved CO_2 and dissolved O_2 in the culture broth with or without PFD. The PFD was saturated with O_2 before the experiment by sparging with pure oxygen

Surprisingly, the positive influence of oxygenated PFD on DHA production, which was described in [88], was not visible when only a few samples were taken manually within a very short time (Fig. 5). The application of the two-layer system with PFD as a screening agent allows for many parallel cultivations when samples are collected with a liquid handling system. Due to the longer time required for this type of sampling, oxygen limitation occurred during the sampling period, which caused a decrease in DHA production. This is in agreement with earlier shake flask and bioreactor cultivations in which we observed that even small interruptions in the oxygen supply lowered the metabolic activity of the cells or even caused cell lysis. Such interruptions in the oxygen supply were partially avoided when PFD was added, although the sampling times are still critical and should be kept as short as possible. These practical handling factors might help to explain studies [62–64] that reported the dependency of DHA synthesis and cell viability on the availability of oxygen. In our case, the measurement of the O_2 concentration revealed that the cells were not subjected to oxygen limitation in the PFD screening system. This finding enables us to observe the influence of different additives on a small scale. An important aspect is the composition of the cell culture medium, especially the amount of carbon sources. When *C. cohnii* was cultivated with the same medium (i.e., glucose and yeast extract) used in larger

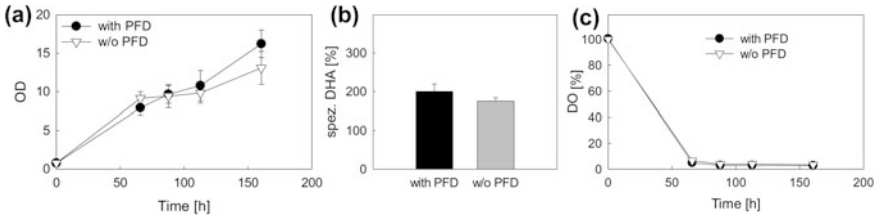

Fig. 6 Cultivation in DWP with and without oxygenated PFD with modified media to achieve higher cell densities. **a** Growth curve, **b** specific DHA concentration (100 % equals the results obtained in an Erlenmeyer flask), and **c** dissolved O_2 in the culture broth with and without PFD

systems, the cell densities continued to increase, however, due to the lower oxygen transfer rate even in the PFD-based system, oxygen limitation appeared as early as 66 h (see Fig. 6).

The measurement of dissolved CO_2 demonstrated for these cultures that the amount of CO_2 in the cultivations without PFD was higher compared to cultures with PFD addition. Because an increase in the dissolved CO_2 concentration was also visible in the PFD layer (data not shown), it is likely that, aside from the higher supply of oxygen, the removal of CO_2 from the aqueous cultivation layer facilitated the cultivation. The DO concentration was similar in the aqueous cultivation and PFD layer in all experiments (see Fig. 6).

4.2 Microalgae Cultivation on a Shake Flask Scale

Shake flasks were employed to close the gap between screening in multiwell plates and fermentations on a pilot scale. Although plates are beneficial because of the high number of parallel experiments that can be carried out on the μL or mL scales, the cell densities have to be restricted when other methods of growth control, such as those in the fed-batch mode, are not applicable. In contrast, higher cell densities can be achieved on a pilot scale due to the higher oxygen transfer rates, the ability to control process parameters (e.g., pH), and the possibility of continuous operation without breaks for aeration; however, the number of parallel experiments is limited.

4.3 Integration of Flow Cytometry in Process Development

Stress due to oxygen limitation and shear stress have an influence on cell physiology and morphology. Flow cytometry was used in order to examine these influences within different flask geometries. The cell size and granularity increase when *C. cohnii* cells rest in the G_1-phase of the cell cycle [66, 67] and when they

Table 1 Volumetric oxygen transfer coefficients in the various single-use bioreactor systems discussed in this study

Cultivation system	Supplier	Volume (L)	Total working volume (L)	Max k_La (h^{-1})	Source
Pad reactor	ATMI		50–100	<20	[38]
CELL-tainer	CELLution Biotech	15	15	300	[37]
		115	150	450	[11]
TubeSpin 50	TPP	0.02	0.05	45	[16]
TubeSpin 600	TPP	0.3	0.6	45	[39]
Ultra Yield Flasks	Thomson Scientific	0.5	2.5	114	[40]
SB200-X	Kuhner	100	200	25	[41]
		100	200	11–13	Own calculations
		200	200	8	[42]
Prototype	Kuhner	2,000	2,000	3	[42]

accumulate DHA in the stationary phase. In addition, de la Jara et al. [89] demonstrated the linear correlation between Nile Red staining intensity and DHA content in *C. cohnii* cells. This methodology was applied in our lab with similar outcomes [89].

In order to monitor cell membrane integrity, PI staining was applied. PI binds to DNA but cannot cross the intact cell membrane, and therefore only stains non-viable cells. The application of PI to detect non-viable *C. cohnii* cells was described by Yeung and Wong [66, 67]. Da Silva et al. [90] used PI to show that applying an oxygen vector in bioreactor cultivations has no influence on the integrity of the cell membrane (Table 1).

In our study, a concentration of 1 μg L^{-1} was chosen because this concentration was sufficient to stain more than 98 % of cells in a positive control experiment (cells were exposed for 10 min to a 70 % v/v ethanol solution). The percentage of cells stained with PI always remained below 3 %, which shows that there was no direct negative effect on cell membrane integrity in the different systems, even though the cells were not able to grow. This is in agreement with results by Wong et al., who reported that shaking had a negative effect on cell proliferation, but that cell viability was not negatively affected [66, 67].

4.4 Microalgae Cultivation in Ultra Yield Flasks

As *C. cohnii* exhibits a high demand for oxygen [60], Ultra Yield™ flasks (UYF, Thomson Instrument Company, Oceanside, CA, USA) were used because they are characterized by comparably high k_La values of up to 400 h^{-1} [40]. A microscopic examination of the culture revealed a high number of oil droplets, which we believe to be an indicator of cell lysis. The lysis is possibly due to the high turbulence caused by the baffles at the bottom of these flasks (see Fig. 7).

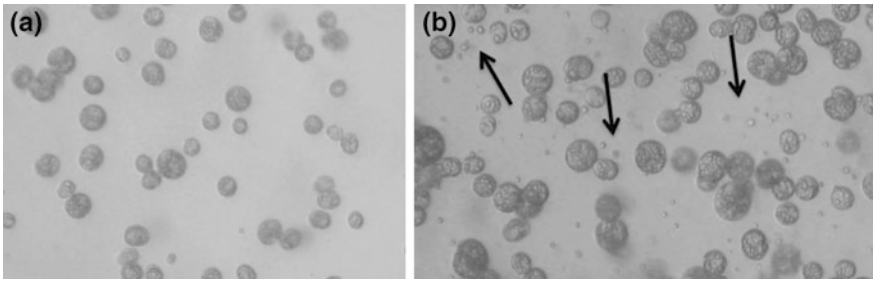

Fig. 7 Microscopic image of *C. cohnii* cells grown in TubeSpin bioreactors (**a**) and UYFs (**b**) (65 h of cultivation). *Arrows* indicate lipid droplets in the culture (magnification: 1:630)

Table 2 Comparison of the results for cell number and for volumetric and specific DHA content obtained in the Ultra Yield flasks at different shaking speeds

Shaking speed (rpm)	10^7 Cells mL^{-1}	Vol. DHA (%)[a]	Spec. DHA (%)[a]
180	2.88 ± 0.21	450 ± 81	276 ± 32
230	2.51 ± 0.24	325 ± 10	231 ± 29
315	1.70 ± 0.22	29 ± 2	294 ± 40

Orbital shaker, amplitude: 2.5 cm

[a] 100 % relates to the production of DHA in Erlenmeyer flasks with the same shaking conditions, as published in Ref. [88]

In order to investigate the influence of shear stress, the shaking speed was increased in steps. Cultivations were performed with a working volume of 100 mL in 500 mL UYFs. The volumetric and specific DHA concentrations were calculated relative to the results obtained in the Erlenmeyer flask control experiments (see Table 2).

When the shaking speed was increased from 180 to 230 rpm, the cell number as well as the volumetric and specific amounts of DHA decreased. When the shaking speed was set to 315 rpm, cell growth ceased, likely due to excessive shear forces (Table 2). Nevertheless, the specific DHA content was highest at 315 rpm, although with a lower number of cells. This can be explained by the fact that when growth ceased, more substrate was available per cell for maintenance and DHA production, leading to a higher specific production even though the volumetric yield was low.

Flow cytometric analysis confirmed these results. Nile Red staining intensity was highest at 180 rpm, and the distribution of the cells was narrow compared to results obtained at 315 rpm (Figure 8). This finding indicated that shear stress had a negative influence on the DHA content of the cells [88]. Hence, although a higher power input is achieved, which increases the gas transfer rate, the high shear forces act counter to the goal of increasing the product yield. Experiments should be designed to identify the most suitable conditions for shake flask experiments.

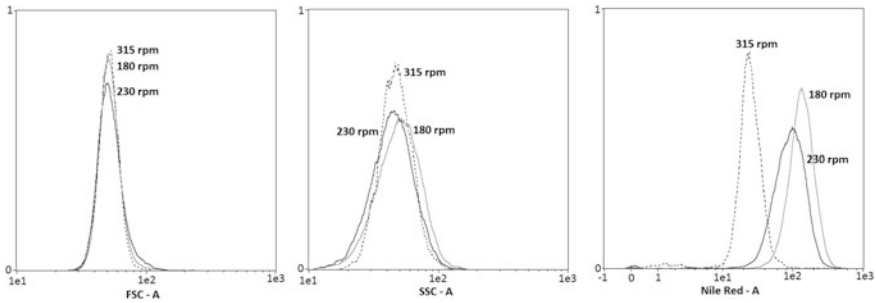

Fig. 8 Flow cytometer measurements of cells grown in UYFs at different shaking speeds (180 rpm: *grey*, 230 rpm: *black*, 315 rpm: *dashed line*). FSC refers to the cell size, SSC to the cell granularity, and Nile Red to the DHA content [88]

4.5 Microalgae Cultivation in TubeSpin® Flasks

Several studies have proven the suitability of TubeSpin® bioreactors for the cultivation of mammalian [16, 91–93] and insect cells [94, 95] up to a scale of 50 mL. De Jesus et al. showed the advantages offered by TubeSpin bioreactors 50 for process development with CHO cell cultures [91]. No oxygen limitations were observed, and CO_2 was stripped because of the large interface in the headspace. Zhang et al. [16] demonstrated that it was possible to conduct parallel cultivations in TubeSpin bioreactors 50 and measured k_La values of up to 45 h^{-1}. Strnad et al. [92] applied a D-optimal design approach in order to optimize process parameters for the cultivation of CHO cells in a TubeSpin bioreactor 50. The highest product titer was obtained at a high shaking rate and a low filling volume. The authors assumed that the higher mass transfer rate at these settings is responsible for the increased product titer.

Similar results were obtained with *C. cohnii* in the TubeSpin bioreactor 600 in our own experiments. The shaking speed was varied between 130 and 230 rpm and the filling volume between 100 and 300 mL. In this study, the highest cell number and the highest DHA content were measured at the highest shaking speed and with the lowest filling volume. However, for the specific amount of DHA, a second optimum was observed at a low shaking speed and high filling volume, which was probably caused by reduced shear forces at these settings. The specific DHA concentration under these conditions was high because older cells, which are usually rich in DHA, were not destroyed [88].

In order to obtain a high product titer, a speed of 230 rpm and a filling volume of 100 mL were chosen for a comparison between the standard Erlenmeyer flask and the TubeSpin bioreactor. The benefits provided by the TubeSpin bioreactor were clearly visible (see Table 3 and Fig. 9).

The number of cells and the volumetric and specific DHA contents were higher in the TubeSpin bioreactors than in the Erlenmeyer flasks (Table 3). The cells stained with Nile Red lay in a narrow range and exhibited high intensity. The

Table 3 Results for cell number, volumetric, and specific DHA content obtained in the TubeSpin bioreactor compared to conventional Erlenmeyer flasks

Shake flask	10^7 Cells mL^{-1}	Vol. DHA (%)	Spec. DHA (%)
Erlenmeyer flask	1.79 ± 0.21	100 ± 2	100 ± 11
TubeSpin bioreactor	3.20 ± 0.15	401 ± 27	223 ± 16

Cultivations were performed with liquid volumes of 100 mL (total volume: 600 mL in TubeSpin bioreactors, 500 mL in Erlenmeyer flasks, orbital shaker, amplitude: 2.5 cm, 230 rpm). The volumetric and specific DHA concentrations were calculated as a percentage of the results in the Erlenmeyer flasks. Experiments in TubeSpin bioreactors were performed in triplicate [88]

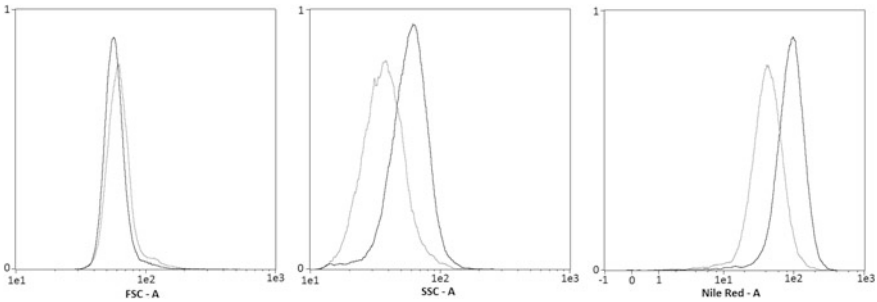

Fig. 9 Flow cytometer measurements for cells grown in TubeSpin bioreactors (*black lines*) compared to cells grown in conventional Erlenmeyer flasks (*grey lines*). FSC refers to the cell size, SSC to the cell granularity, and Nile Red to the DHA content [88]

granularity of the cells was higher, which is due to the higher number of lipid droplets inside the cells [88]. The average percentage of cells stained with PI was 1.05 % in the TubeSpin bioreactor and 1.58 % in the Erlenmeyer flask, which indicates that the cell membrane integrity is not harmed in either flask geometry.

The oxygen transfer rate might be higher in TubeSpin bioreactors than in Erlenmeyer flasks due to the geometry of these flasks. Zhang et al. [16] claimed that the highly dynamic interface generated in the TubeSpin system leads to a large surface area of the culture broth, which increases the k_La values. Werner et al. [96] complement this hypothesis with the theory that the adjacent gas layer is accelerated by the high velocity of the media, which increases the gas transfer rate in the head space and therefore also the gas transfer rate in the medium. Additionally, Jia et al. [97] suggest that the frusticonical bottom is preferable to a flat bottom because of the larger area which is exposed to air.

Film formation on the bag wall during shaking plays a role in increasing the rate of oxygen transfer to the liquid. A thin film is formed on the wall, which is quickly saturated with oxygen from the headspace, thereby increasing the total amount of oxygen in the bulk liquid layer. [98] During experiments in our laboratory, we measured a k_La value of 100 h^{-1} using the sulfite method [40]. These results indicate that the TubeSpin bioreactor is a suitable tool for process development for marine heterotrophic microalgae on the shake flask scale because it exhibits low shear forces and comparably high gas transfer rates.

4.6 Microalgae Cultivation on the Liter Scale in Single-Use Bioreactors

A method for the consistent scale-up from small to large volumes was developed in TubeSpin bioreactors up to the m^3 scale for an orbital shaking bioreactor system [16]. The TubeSpin design on a small scale and on a large scale in the SB200-X bioreactor both offer a cylindrical shape without any additional moving parts, which is an advantage common to orbital shaken systems [98]. The shear forces are low and a large gas–liquid interface develops during shaking [42, 99]. Furthermore, foam formation is reduced in these systems in contrast to directly aerated and stirred bioreactors [99]. Tissot et al. have demonstrated the scalability of mixing mechanism and surface development, as long as the d/d_s (inner container diameter divided by the shaking diameter) and the Froude number are kept constant [100]. Liu et al. [101] optimized mammalian and insect cell processes on the 20-500 mL scale and performed a scale-up to 8–36 L. Zhang et al. [16] achieved sufficient cell numbers and cell viability in a 200 L cylindrical vessel on an orbital shaker with both 25 and 100 L working volumes. The results were comparable to the results obtained with 50 L in a common 150 L stainless steel reactor. Stettler [102] attempted to show that the system could be scaled up to 1,500 L, demonstrating an initial attempt with a 750 L working volume. The cell densities were lower than in a 200 L reactor, but we can still conclude that orbital shaken systems are suitable for production scale. Cell titers from a CHODG44 cell line obtained in 50 mL tubes, 1.5 L shaken bottles, a 200 L orbital shaker, and a 2 L stirred-tank reactor were found to be similar [41].

The number of *C. cohnii* cells cultivated in TubeSpin 600 flasks and in the SB200-X were comparable [88]. These results indicate that the TubeSpin concept can be scaled up to at least a volume of 120 L for the microalgae process. The DO concentration in the culture broth was measured near the bottom of the SB200-X bioreactor. Full DO depletion was detected over long periods of the cultivation time. Nevertheless, cell density increased despite the critical dependence of the algae on oxygen as previously discussed [88]. Broekhuizen emphasized that dinoflagellates have a higher mobility than other algae. He pointed out that they can move to either the surface of the ocean to obtain sunlight or to deeper sections of the ocean if there is a lack of nutrients at the surface [103]. If this is also true for *C. cohnii* cells, they might be able to swim to the large gas–liquid interface, which is formed in the Kuhner reactor. There they could absorb oxygen and drop into deeper sections of the reactor when nutrition becomes limited, leaving space for other cells that are suffering from oxygen depletion. This storage feature would also be advantageous considering the film formation in orbital shaken systems. The cells absorb oxygen in the film layer and store it when they are reunited with the main liquid layer, where oxygen is limited. Thus, the specific growth behavior in orbital shaking systems could be explained by such a storage feature. However, it is not clear which of these phenomena is most relevant for the growth of the

Fig. 10 Scale-up from deep-well plates to TubeSpin 600 bioreactors and finally to Kuhner SB200-X orbital shaking reactors

algae. Additionally, these results suggest that DO measurements near the bottom of the reactor might not be representative of the whole liquid layer.

The goal of a consistent process development from a small to a large scale was achieved for heterotrophic marine microalgae and can be seen as a model for other shear-sensitive marine microorganisms. However, the small-scale experiment was carried out in rectangular DWPs as shown in Fig. 10, because the oxygen transfer rates were critically low on the small scale. The oxygen transfer rate is doubled in square wells compared to round wells [104], therefore the chosen DWPs are advantageous.

5 Summary, Conclusions, and Outlook

Employing single-use equipment to develop a process for the cultivation of marine microorganisms, demonstrated for the example of the heterotrophic microalgae *C. cohnii*, is beneficial. In this case, this equipment leads to lower shear stress and ensures sufficient oxygen input for growth and production of DHA. In addition, corrosion is avoided and investment costs at the process development stage are generally reduced. Our calculations indicate a 15 % reduction in investment and running costs over the first 5 years at a scale of up to 200 L. The reason for this is the elimination of investment costs for coatings (usually 10 % of the total investment costs for stainless steel reactors) and additional maintenance costs for seals and spare parts. Common coatings for stainless steel reactors include polytetrafluorethylene (PTFE) and perfluorvinylmethylether copolymers such as perfluoralkoxylalkane (PFA), or enamel coatings. Replacing common steel with less corrosive alloys can increase the total bioreactor investment costs by about 25 %.

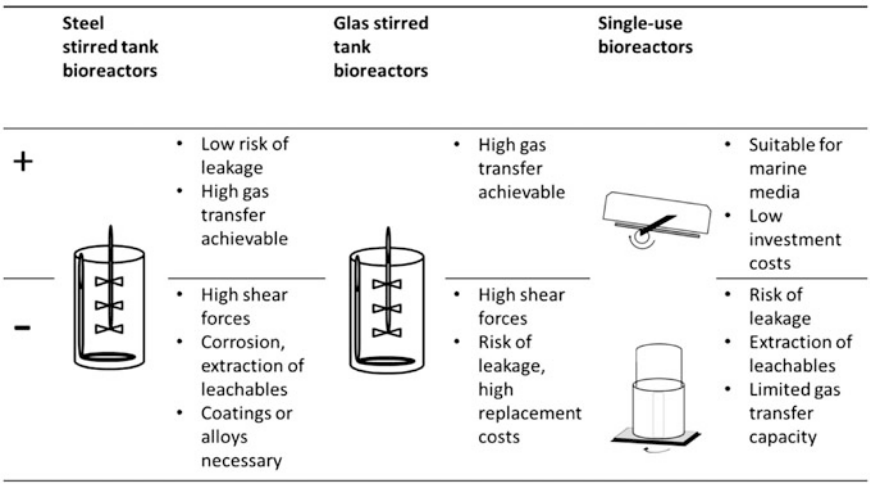

Fig. 11 Overview of advantages and disadvantages of different types of bioreactors for application in marine bioprocesses

Abrasion also causes additional maintenance costs. (Additional costs for supplying steam in in situ autoclavable reactors are not considered.)

Using glass reactors lowers the investment costs but drastically increases the risk of damage during operation, which would result in increased maintenance costs for repair and replacement. Hence, aside from the economic benefits, employing single-use systems for marine bioprocess development also decreases the risk of material-dependent delays in process development (leaching of corrosive materials, repair times). Because single-use systems are available up to the m^3 scale, they are suitable for the scales typically used in development. They represent a suitable alternative for integrating marine bioprocesses in typical process development strategies, because they are not affected by the high salt concentrations in marine media. Although monitoring installations are still not fully compatible with those installed in steel stirred-tank reactors, the solutions currently available allow for sufficient monitoring over the short fermentation time scales that are usually relevant in bacterial fermentation. An overview of the advantages and disadvantages is provided in Fig. 11.

For cultivation of *C. cohnii*, we have proven that DWPs can be scaled up to the 120 L scale while still providing adequate DHA production. Several promising approaches, such as cultivation in the CELL-tainer system, have been scaled up to near 150 L [11]. Cultivations with heterotrophic marine microorganisms were successful on the 15 L scale [105], therefore cultivations on this larger scale are envisaged. A parallel cultivation strategy seems to be beneficial, as the cultivation lasts several days, and therefore parallel cultivations with time shifts would save manpower and downstream capacities. This approach would also compensate for the limited scales of SUBs currently on the market.

In contrast to heterotrophic microalgae, most marine bacterial processes can be performed in a carbon-source–limiting fed-batch mode. Thus, the feed can be controlled to compensate for the lower oxygen transfer rates in SUBs compared to stirred-tank steel reactors. Because SUBs can be employed for nearly all bacterial cultivations, they are therefore especially suited for use in marine bioprocess development in the laboratory and on a pilot scale. The cost reductions associated with these systems might lead to more intensive research in this field and wider application of these species.

Acknowledgments We thank the Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz, Bundesanstalt für Landwirtschaft und Ernährung (Project: FENA: Fischmehl- und öl Ersatzstoffe für eine nachhaltige Aquakultur, No.: 511-06.01-28-1-73.026-10) and MareNutrica (Niendorf, Germany) for financial support. Furthermore, we thank the companies CELLution (Assen, The Netherlands), Kuhner (Birsfelden, Switzerland), and ATMI (Hoegaarden, Belgium) for providing the SUBs for experiments, and PreSens (Regensburg, Germany) for donation of the CO_2 sensor. Furthermore, we thank Agnieszka Niedziolka, Magda Chmielewska, and Steffi Annemüller for conducting experiments with PFD and TubeSpin bioreactors.

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Adv Biochem Eng Biotechnol (2014) 138: 207–237
DOI: 10.1007/10_2013_220

Published Online: 15 August 2013

Flexible Biomanufacturing Processes that Address the Needs of the Future

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Abstract As the age of the blockbuster drug recedes, the business model for the biopharmaceutical industry is evolving at an ever-increasing pace. The personalization of medicine, the emergence of biosimilars and biobetters, and the need to provide vaccines globally are just some of the factors forcing biomanufacturers to rethink how future manufacturing capability is implemented. One thing is clear: the traditional manufacturing strategy of constructing large-scale, purpose-built, capital-intensive facilities will no longer meet the industry's emerging production and economic requirements. Therefore, the authors of this chapter describe the new approach for designing and implementing flexible production processes for monoclonal antibodies and focus on the points to consider as well as the lessons learned from past experience in engineering such systems. A conceptual integrated design is presented that can be used as a blueprint for next-generation biomanufacturing facilities. In addition, this chapter discusses the benefits of the new approach with respect to flexibility, cost, and schedule. The concept presented here can be applied to other biopharmaceutical manufacturing processes and facilities, including—but not limited to—vaccine manufacturing, multiproduct and/or multiprocess capability, clinical manufacturing, and so on.

Keywords Cost · Flexibility · Monoclonal antibody · Process platforms · Time-to market

Abbreviations

AEX	Anion exchange
BP	Buffer preparation
CAPEX	Capital expenditures
CEX	Cation exchange
CMO	Contract manufacturing organization
CNC	Controlled but not classified
COG	Cost of goods
CPFR	Collaborative planning forecast replenishment
cGMP	Current good manufacturing practice

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DSP	Downstream processing
ETO	Engineer to order
FAT	Factory acceptance test
FDA	Food and drug administration
LRV	Log reduction value
mAb	Monoclonal antibody
MUS	Multiple use system(s)
PQP	Project qualification plan
SAT	Site acceptance test
SCADA	Supervisory control and data acquisition
SU	Single use
SUS	Single-use system(s)
SUT	Single-use technology
SUB	Single-use bioreactors
URS	User requirement
USP	Upstream processing specification

Contents

1 Introduction

Time-to-market is one of the most important factors for new biopharmaceutical drugs. Global players often have dedicated resources and process platforms to move from clinical development to commercial supply. Depending on the size of a particular market, type of application. and the availability of manufacturing capacities, they can export their process to different regions. However, manufacturing capacities are concentrated in developed countries, especially North America (36.6 %) and Europe (25.9 %) [1]. If a target market is located somewhere else (e.g., Asia or South America), expensive cold-chain logistic concepts are required. The development of process platforms for small- to mid-scale production, which can be exported as a whole, is another alternative. Predefined, configurable unit operations, or even generic platform processes, will reduce time and engineering efforts during design and construction of such plants. With the

Process4Success initiative, Sartorius Stedim Biotech covers the engineering and equipment needs of antibody manufacturing processes and various vaccine production operations by incorporating a "fit-for-purpose" design.

Process platforms for different applications under this Process4Success initiative are designed for flexible manufacturing operations. Consequently, fast changeover to other processes is an important task that this initiative takes into consideration. The Process4Success approach intends to benefit not only contract manufacturing organizations (CMOs) but also the industrialization departments of originators [2]. This initiative aims to reduce engineering efforts during planning and execution and also during equipment qualification. By this approach, we have shown that a complete 1,000-L single-use mAb greenfield production plant (two USP lines and one DSP line) for Phase III material can be designed, executed, and tested within less than 2 years.

Switching over to single-use manufacturing has a major impact on the financials of such an investment, significantly reducing capital expenditures. For our generic 1,000-L mAb platform, we have evidence that only ~40 % of a comparable stainless steel plant is required. Although operational expenditures are higher because of single-use consumables, manufacturers will reach the break-even point faster. Assuming a capacity utilization of 20 batches per year, this point is estimated at approximately 10 years.

2 General Considerations When Planning to Establish a Single-Use Process

Within the recent decade, contract manufacturers have been confronted with major changes in their markets. The well-known rationales for single-use technologies (SUT) are as follows.

- Capital expenditure (CAPEX)savings
- Faster setup of SUS production facilities
- Less SIP/CIP validation effort
- Cost and flexibility benefits in preclinical and clinical drug development phases.

Another major challenge in the biopharmaceutical industry is the increasing trend toward outsourcing manufacturing capacities of pharmaceutical companies involving both small- and large-scale processes. In line with this trend, contract manufacturers need to cover the demand for outsourcing of process development and of GMP-compliant production by providing integrated services in combination with multiproduct facilities. Nowadays, these facilities must be highly adaptable in order to respond to changing upstream and downstream processing requirements [3]. More than ever, the key factors in this contract manufacturer business are timelines, fast product changeovers, high process flexibility, and low running costs. However, financial risks and expiring patents combined with the appearance

of biosimilars and follow-up drugs are increasing the competitive pressure on the biopharmaceutical industry. This situation is leading to more and more flexible, low-cost platform manufacturing technologies. The main intention is to achieve significant time and cost savings by utilizing new and innovative single-use equipment and consumables [4].

Basically, everybody understands the beauty of the SU concepts. Less capital investment, high flexibility, and reduced complexity by eliminating the need for cleaning and cleaning validation processes, combined with faster execution of such concepts, are appealing to all who are exploring new ways of biopharmaceutical manufacturing.

Of course, there are technical limitations, but these are receding at the moment. For this reason, this chapter does not focus on the current status of SUT, but rather only on the challenges that the general decision of going with the SUT concept entails.

All advantages mentioned above are valid only as long as SU components are available in a reliable and reproducible quality for the entire lifespan of the process considered. This is a challenge not only for a CMO supply chain manager who will have to make sure that SU components are in stock when they are needed but also for the supplier of these parts as well. After all, a CMO does not know whether a supplier who has the best technology today will be able to supply the same technology 10 years from now. This means that deciding on a specific technology is quite often linked to making a bet on the future development of a specific supplier. This is not the case for traditional stainless steel technology. Even if the company that sells conventional stainless steel equipment goes bankrupt or is no longer able to provide maintenance or other services, it will still be easier to work around this situation than to find a different supplier for a highly specific bag assembly, which may include sensor and mixing technologies not available from other companies. For stainless steel equipment, the production systems need to be qualified only once, whereas with SUT, you have to rely on the quality system and the sustainability of your supplier.

There are many important aspects to consider in assessing potential suppliers for SU processes. One has already been mentioned: how established is the supplier of the company from which you intend to buy consumables. Do you trust that this company will not just have the best solution for you today but tomorrow as well? Is this company an innovation driver that will stay ahead of the curve so you can capitalize on next-generation developments in SUT as well? Does the company have a proven track record with you based on partnership and trust? Can you be sure that this company will still be interested in your business once you have made the big capital investment and once daily business with training, service, and on-time supply of critical SU components starts?

Picking the right supplier is essential for the success of your project. Fortunately, there are several suppliers that offer entire process solutions covering all or most of the process steps and technologies from upstream to downstream. All of these potential suppliers incorporate proprietary technologies into their products, making them unique and incompatible with the systems of other suppliers. Such

unique technologies are often the very reason you consider using them. So when you are evaluating the technologies of different suppliers, you will find solutions that you like from each of them, which results in a short list of equipment for your process from different suppliers. This is a very common procedure for conventional stainless steel equipment. However, in the case of sourcing single-use technologies, having several suppliers entails significant challenges and risks as different solutions from different suppliers do not fit with one another. Such incompatibilities originate beside others in the different sterile connectors, tubing materials and diameters, different films and contact layer materials and, quite often, in the different SCADA communication protocols used by the controllers of the systems you are working with, and so on. You can request manufacturers to use only components that you specify, which will result in highly customized bag assemblies. This would allow you to create your own internal standards within your process.

A good approach is to standardize a specific type of sterile connector. Such an approach would enable you to be flexible in connecting your equipment in different ways to adapt to the changing needs of your process design. This is essential for single-use technology when flexibility is key. Most of the suppliers of these technologies are willing to modify their existing standard assemblies, but introducing such changes comes at a price. It is not just that now one company has to buy your specified connectors from another company, which is more likely than not a competitor in this field. This also means that the resulting cost for you and other users will be higher as this third-party connector will be more expensive than the original supplier's own brand solution. Any customization involves additional effort in manufacturing and quality control, which pushes the cost even higher. Typically, this cost will be passed on to the end customer, you, who requested the changes and will have to deal with the consequences such as higher price. In addition, such customer-specific designs are not readily available off the shelf. To overcome long delivery times for these components, long-term contracts and specific delivery forecasts for customized designs must be established to deal with these issues. In particular, for CMOs, these forecasts can become a liability when they have to respond swiftly to new opportunities or situations as they arise.

Manufacturers of single-use components and technologies are often hesitant to agree on long-term contracts for customized designs. The reason is the high risk entailed, that their own subsuppliers could increase the prices or experience a shortage in the other brand components that are to be incorporated into the customized designs. So what looks nice on paper upon the creation of your own standard may cause many issues in the aftermath and may very well affect the security of supply of such critical designs. Sometimes you will not have a choice as your biological process may have requirements that cannot be met with standard products. But even in these cases, you will realize that it is better from the stance of risk mitigation and prudent economics to pick just one supplier and to stick to already existing designs as much as possible.

This strategy is advisable only if the predesigned solutions available on the market have been proven to be "fit for purpose." This is only possible if your

supplier has a good process understanding and regulatory expertise and if your supplier owns all the critical components so that he has direct control over the supply, quality, and cost. The result is that all of the established companies that offer SU solutions to the biopharmaceutical industry are trying to close gaps that they are identifying in their portfolios so that they do not become dependent on components they would have to buy from competitors. The supplier with the best concept will have clear advantages as security of supply is so critical for utilization of SUT. As a result, relatively small and inexpensive suppliers will find it increasingly difficult to keep up with the pace of change. In the ensuing shake-out, only a few suppliers will ultimately be left that are able to fulfill the industry needs in terms of pricing, quality, products, and security of supply.

Finally, the decision of which technology you are going to use in your process is not so much a question of finding the best individual technology, but rather a decision for choosing a supplier with the best overall concept. This holds true for many decisions that you need to make and is not limited to single-use technology. However, for SUT, such a decision will have far greater consequences.

In any case the party who is going to make the decision will have to realize that there are overall and significant differences, which become apparent when classic production scale equipment or full MUS are compared with SUS. The following differences become apparent when classic production-scale equipment is compared with single-use plants.

The classic process is more strongly driven by systems that predominantly incorporate process-scale stainless steel technology. By nature, such systems are hardly subject to any limitations regarding scale-up of process volumes. However, the considerable degree of hard-piped stainless steel constructions in these systems logically results in their limited flexibility when it comes to changing volumes, altering the sequence of process steps, or otherwise adapting them to specific process needs. Typically, once the design specifications of such reusable systems have been defined and these systems have been built, they can be reconfigured only at a relatively high cost in a labor-intensive and time-consuming re-engineering project that entails considerable validation effort.

By contrast, due to market development and the ever-increasing efficiency of biotech processes involving high-titer concentrations, the requirements regarding new production systems have substantially evolved over the past years in the direction of relatively small manufacturing capacity that affords a high degree of flexibility. This approach focuses much more strongly on functionality and, therefore, has inevitably led to SUS whose stainless steel components serve as tools and control elements, whereas its elements for conveying a product are predominantly, if not entirely, made of plastic.

The SU products and SUS currently available on the market do not yet cover all requests that the pharmaceutical market has addressed to plant engineering companies. For this reason, SUS still have some limitations compared with conventional system technologies in many areas. In the following, a few of these momentary restrictions are mentioned, on which suppliers of single-use solutions are continuously working to resolve.

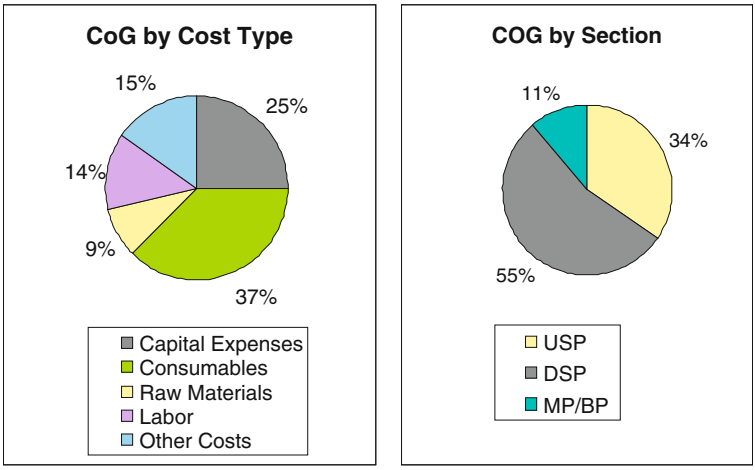

Fig. 1 Typical overall cost structure of a mAb-based SU process platform (estimates = 1,000 L/ titer of 5 g/l)

- High-volume single-use bioreactors (SUB) of >2,000 L
- Efficient high-volume SU mixing solutions for bulk production of buffer and media (>2,000 L)
- Movable high-volume SU containers (e.g., >1,000 L)
- Disposable lenticular and cross-flow filtration systems for high volumes
- Efficient SU cell removal systems (e.g., SU centrifuges)
- SU chromatography matrices with high binding capacity for bind and elute applications
- Availability of SU sensor technology
- Connector/disconnector technologies for large inner diameter tubing
- SU components and operational units to be used under high pressure and at high flow rates >1 bar (14.5 psi; bags, tubes, sensors, pumps).

When this list of areas is considered, it can be seen that the majority of the restrictions involve a cost issue or technical limitations, particularly with respect to the maximum volume that SUS can provide. However, it is often the case that both factors are relevant. There is thus a maximum process size that governs the choice of whether to implement an SU process that will still make economic sense.

Technically, it is indeed possible to overcome momentary volume limitations by multiplying smaller (technically feasible) unit operations, but there are economic constraints in this case, which are reflected by the higher cost of goods (COG) per batch. The actual maximum process size that can still be implemented cost-effectively using SU technology depends on many factors, such as the price that the particular product can command on the market, the number of batches per year, the competitive edge to be gained, if any, by launching the product as early as possible, and so on (Fig. 1).

3 Implementation of a "Fully Single-Use mAb Production Line"

In this section, we talk about a real-life project that was executed in cooperation with a CMO. It illustrates the key drivers from a customer perspective, provides a good example of how such a project can be executed, and the challenges that must be dealt with are reviewed.

The decision to design a fully single-use clinical material manufacturing plant was made very early on in the customer's decision-making process. The main reason to opt for a single-use concept was that the CMO had an idea about what mAbs he wanted to manufacture, although no contract was in place at that point. This meant that he knew he had to invest in equipment to attract clients to use his CMO services, but did not know what this first process would look like in detail. As single-use technologies allow both to be rearranged when the process needs change and are less expensive, the decision was easy. These were only two reasons but most probably the most important ones.

The CMO quickly realized that he did not just need a supplier, but a partner that would help him to turn his plans and ideas into reality efficiently. The decision as to which supplier of SU products to choose was based on a specific type of "ecosystem." Such a system means that each supplier has proprietary technologies or formats of their products, which set them apart from other suppliers in terms of incompatibility. Of course, within a specific ecosystem, all individual technologies and components must work together smoothly. The customer has to be careful only when planning to implement technologies from other ecosystems, as this may create problems.

Having only one supplier or one main supplier may be seen as a drawback, as you would be relying heavily on the supplier not to let you down when you need process-critical components or other support and services from them. However, this issue does not go away by picking multiple vendors for the equipment of the different process steps.

What therefore, is the best way to create win–win situations and real partnerships? This is obviously easier when you have some buying power and when your own business model is linked to that of the supplier. This was the case in the situation at hand, and the supplier as owner of the consumable manufacturing company was strongly interested in such a long-term partnership as well as the manufacturing of the required systems. Indeed, this business model was linked to the long-term and sustainable business generated by single-use components continuously used for the process equipment. This business model enabled the company to leverage its long-term business in consumables by offering attractive pricing for their systems. In a nutshell, the supplier will be successful with his business if the user is successful as well. This explains why suppliers are motivated to support users as much as possible.

Limiting the number of suppliers to a minimum of one or two can even make sense in the case where certain technologies do not perform as well as others.

These technologies may constitute products such as filters that do not feature the desired throughput or bags that are not available in the preferred size. In such instances, instead of changing suppliers, it may be more reasonable simply to ask the preferred supplier to compensate for the lack of his product's or technology's performance, for example, by increasing the filter area or supplying more bags of a smaller size for the process. This solution allows the customer to minimize the number of suppliers.

Additional reasons for limiting the number of suppliers are:

- Even well-thought-out supply concepts will require changes over time, resulting in change notifications. Accordingly, you need a consistent way of dealing with such change notifications, which is easier with just one or a very limited number of suppliers.
- Upgrading existing SUS designs with new technologies such as sensors or mixing systems is easier.

Of course, it is essential that the supplier company understand its responsibility to assign dedicated resources and support the user in all aspects over the entire lifetime of the manufacturing process.

In addition to the discussion about which supplier to work with, the main topics prior to the execution of the project were:

- Is "single-use" going to be the right solution?
- What are the economic benefits? After how many batches or years of operation will the SU process become more expensive than if the customer had opted for the traditional stainless steel option instead?

The only way to inject some rationale into this discussion and decision-making process was to address the issue of cost for the SU components necessary per batch as early as possible. This was enabled by using a generic mAb process in which preconfigured unit operations were aligned (Fig. 2). This template, called Process4Success made it possible to discuss in detail which components would become necessary for a mAb process at a given volume and product titer, and helped us to gain an understanding of the CAPEX and the COG. The Sartorius Stedim Biotech Process4Success templates were highly useful at this early conceptual stage of the project, as the CMO was not able to present his own process at that time.

The Process4Success platform consists, in addition to other SU solutions, of several FlexAct® systems that can be configured to meet the needs of varying scale operations and volumes. All process platforms are off-the-shelf solutions and do not require additional engineering. These platforms are designed to fulfill the process needs of the specific steps within the Process4Success platforms. The preconfigured bag assemblies are designed to meet the requirements of a certain process volume and product titer. This means that the probes, tubes, filters, and the like, are already sized and tested for a real-life process. FlexAct® is fit for the purpose as long as the customer's process is close to that of the Process4Success template.

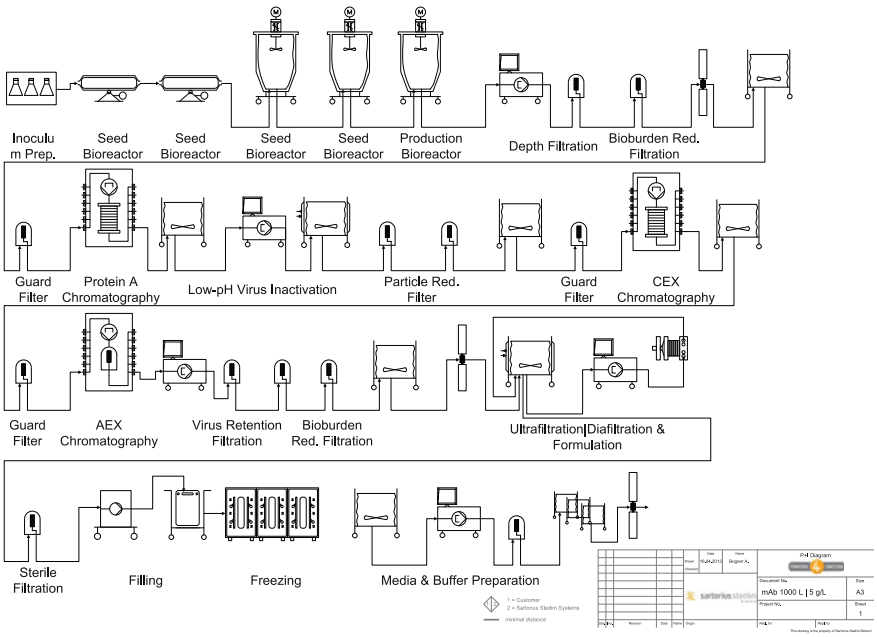

Fig. 2 Process flow diagram to illustrate a fully SU monoclonal antibody process (created with SuperPro Designer®)

As part of this process platform concept, this makes FlexAct® the perfect starting point for all conceptual designs of processes that are not identical but close to the template. Equipment and component lists just have to be adapted to the specific needs. FlexAct® solutions are configurable.

Sartorius Stedim Biotech offers Process4Success templates for different processes, volumes, and titers. Questionnaires are used to find the template that is the closest to the process for which the user is looking. This template process can be used in the next step as a basis for a conceptual design.

The entire equipment list is not just linked to CAPEX but to the footprint of the equipment as well, allowing the size of the necessary cleanrooms to be easily calculated. Because installing and handling of SUT such as Palletanks® requires some space, the layout of an SUT-based facility looks different from that of a conventional multiuse technology-based facility.

RAFT® are rapid aseptic fluid transfer ports. They can aseptically connect large-footprint process components such as Palletanks®, for example, for buffer storage in chromatography steps in a controlled but not classified (CNC) area, with the classified process area.

When fully single-use processes are discussed, one of the main technical challenges is how to deal with large volumes of buffer, media, and product intermediates. Bags for mixing and storage of liquids are available up to 2–3,000 L,

but at this size they can no longer be moved around very easily, which limits the flexibility of their use in changing process designs.

Some companies use casters to manually move around Palletanks® up to 1,000 L volumes. This can be done with special casters, but it takes a minimum of three operators to do so and the facility has to be spacious enough to allow room for maneuvering and storage. Another challenge is to determine the filling level of the movable bags. A common method is to determine volume by weight with load cells that are mounted between the bins, holding the bag with the liquid and the movable rig. A local display on the rig will then show the weight or filling level. This method is very useful as long as the rigs are not moved around when the Palletank is filled. The load cells can handle vertical loads, but are sensitive if exposed to horizontal stress. In practice, the load cells can be mechanically decoupled from the rig when moved around, but as this is not convenient, the use of platform balances has become the standard solution.

Platform balances are about 2.5–5 cm high. This height can be overcome with small Palletanks® only, as their weight does allow for manual operation. Filled large-scale bags can be positioned on a pit scale that is flush with the floor. Bags in Palletanks® can be moved nearly effortlessly onto such pit scales. But the position of this type of scale has to be defined very early on, as they cannot be moved around like most platform balances can. Under cGMPs, platform and pit-mounted scales must allow for regular cleaning under their weighing platforms. To meet this requirement, floor platform scales can either be moved around or have a lift deck platform that can be lifted into an upright position to allow for thorough cleaning (Figs. 3 and 4). Palletanks® with a capacity of 1,000 L or larger will quite often be stationary installations and not moved around at all.

These are just a few examples of the specific requirements and current limitations of SUT that need to be taken into consideration early on in the process development phase. During process development, it is crucial that the final process does not exceed 3,000 L of in-process storage volume. Effective mixing technologies are available up to a certain volume only. This is a key fact as mixing is not just necessary for homogenization; it is also indispensable when cooling, heating, or adjustments of pH, conductivity, and so on become necessary. This adds another limitation to consider at the outset. The same holds true for cooling and heating rates. The heat transfer from a stainless steel jacket through a polymer film into the medium is not as good as if there were no film in between. The films are validated up to a certain contact temperature, which naturally limits the temperature of the inner jacket surface toward the bag film. The limitation of the maximum heating or cooling rates becomes even more severe with increasing volumes, as the potential heat exchange surface of a cubical container does not increase linearly with volume.

Even more important, not all small-scale SUT can be scaled down or up. It is well known that some SUB technologies are not only linearly volumetric scalable within their own product family, but also to a larger-scale multiuse stainless steel bioreactor. This linear scalability of SUB technology to conventional stainless steel bioreactors is important whenever commercial large-scale production is

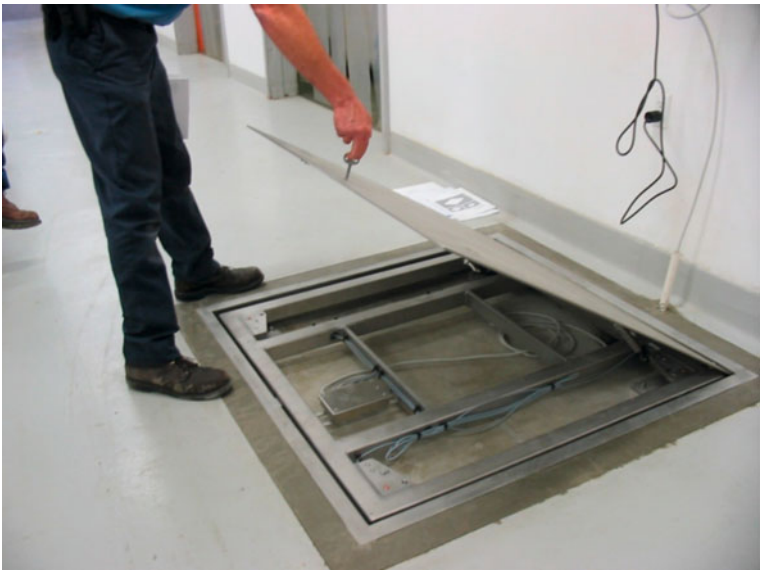

Fig. 3 Sartorius pit-mounted scale, flush with the floor, platform lifted for cleaning

Fig. 4 Common solution: Palletank® on Sartorius IFS4 flat-bed scale with a ramp

planned for a conventional stainless steel plant. Choosing a SUB technology for clinical phase material that cannot be easily scaled up to traditional stainless steel systems would limit the value of data generated for later scale-up. To allow direct

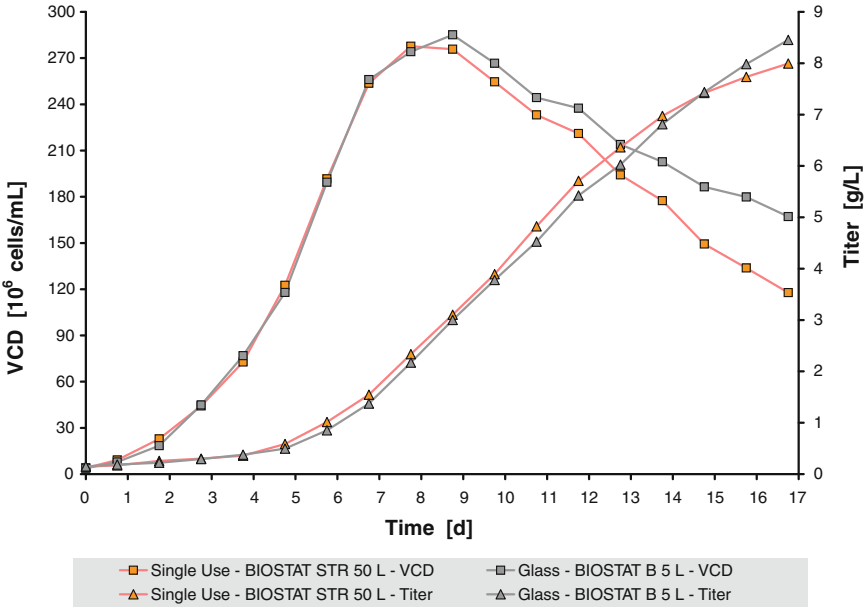

Fig. 5 Scale-up comparison trials of shake flask with a conventional glass bioreactor to a SUB

comparability of data and fast and easy process transfer, the design criteria for SUB should most closely approximate those of conventional large-scale stainless steel bioreactors (Fig. 5).

A manufacturing process designed for SUT should fulfill the following minimum criteria.

- Minimized number of process steps
- No need for organic solvents
- Small volumes (higher product concentrations, higher capacity buffers, eliminate or avoid dilution steps, etc.)
- Minimized number of different buffer systems in the process to reduce the number of bags necessary in production
- Highly dynamic binding capacity resins used in bind-and-elute applications ultimately to reduce the column volumes and the buffer volume needed
- Minimized number of different brand polymers/plastics/films to reduce validation efforts and supply chain issues
- Presterilized and closed loop assemblies used whenever possible to minimize contamination risk
- Based on technologies with a sound scale-up (and scale-down) design available in multiple scales to allow later scale-up of the manufacturing concept.

Fig. 6 Presterilized aseptic FlexAct® UD single-use cross-flow system

Very specific challenges are associated with high flow rate and high-pressure operations as those touch on scalability, as well as on process and operator safety. As stated many times, one major advantage of SU manufacturing is the high operational variability and flexibility. No stainless steel piping or permanently mounted equipment impairs modifications or prevents new technologies from being introduced into manufacturing. Thermoplastic tubing can be cut or welded anywhere and enhances flexibility. This is generally true of low-pressure operations only, as thermoplastic tubing proves to be soft and incompatible with high-pressure applications. The maximum pressure compliance with tubing depends on its material, wall thickness, inner diameter, and connection design.

Reinforced tubing can withstand higher pressure, but is not thermally weldable. This is why mechanical sterile connectors are the technology of choice for high-pressure and high flow rate applications. The connectors have to be available in different inner diameter sizes and be validated for use at the necessary pressure rates. Given these technical limitations, the key to the success of each project is to analyze and simulate each single process step in detail and check it against flow, pressure, and sterility requirements. An overall tubing and connection design is best created when the methodical evaluation is performed together with the user.

FlexAct® UD—a fully single-use cross-flow or tangential flow filtration system—is a good example for this discussion (Figs. 6 and 7). Characteristically, a cross-flow application is run at high inlet pressure and at a high flow rate. These two parameters are essential for the performance of this filtration technology. The preconfigured and tested assembly developed for the FlexAct® UD fulfills these requirements. Proof was provided by extensive testing of the preconfigured single-use loop with all connectors, probes, pumps, and cross-flow filtration cassettes. Such complex assemblies should only be modified outside the high-flow and high-pressure loop, to limit the risk of malfunction. The recirculation bag can be

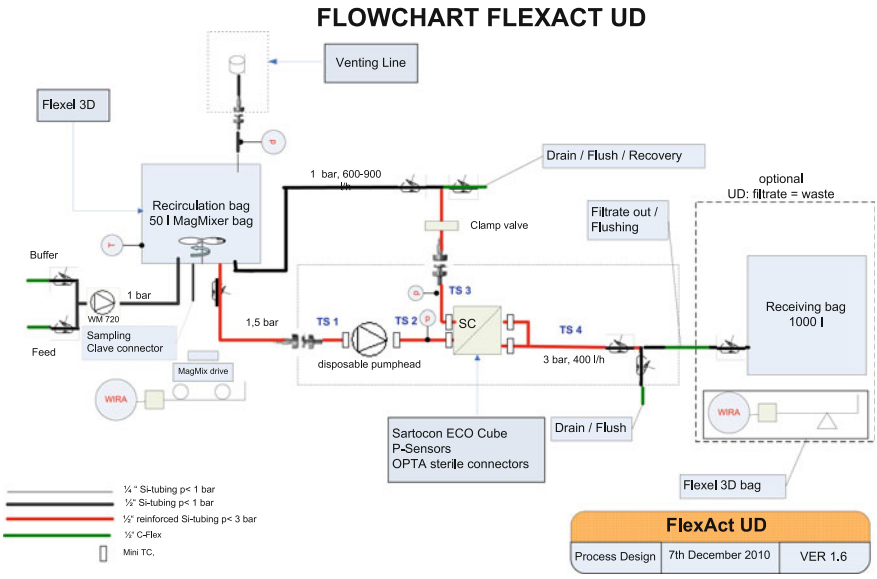

Fig. 7 Preflushed and presterilized SU flow path of a FlexAct® UD cross-flow system

changed within certain limits, but any modification of the assembly between the pump and the cross-flow cassette would require new tests to ensure that the assembly will finally work as expected. To avoid malfunction, any changes should be discussed in greater detail with the provider of the SU cross-flow system and the single-use loop.

Another example of a critical process step is nanofiltration. This application, which is used to remove viruses from mammalian expression system-based processes is a highly critical step in virus risk mitigation strategy. Usually, nanofiltration has to be operated at a specific constant pressure (1 or 2 bar).

In nanofiltration, most virus retention studies are performed at a static pressure that is specific to the type of filter and that is also the pressure for this production-scale process step. In SUT, pressure overlay on a tank cannot be applied to achieve static pressure as the bags are not resistant to pressure. However, the more common peristaltic pumps generate considerable pulsation. Such pulsation can cause pressure peaks that exceed the validated pressure range. This will ultimately lead to the question of whether the nanofilter retains the log reduction value (LRV) demonstrated by a lab-scale system with static process-scale pressure. The supplier of the virus retentive filter should be able to prove that peristaltic pump pulsation does not affect the LRV performance of the nanofilter. This proof should be provided in combination with the SU filtration system and assemblies intended for later use in the process.

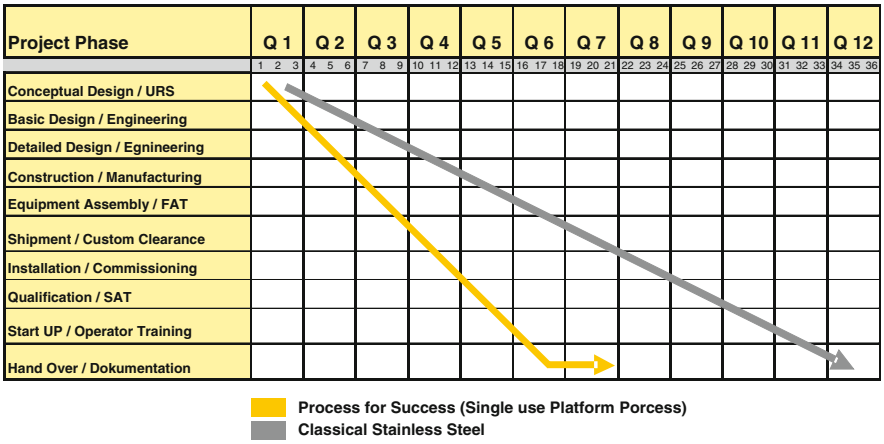

Fig. 8 Gantt chart to illustrate the timeline for a 1,000 L batch size single-use project

4 Project Execution Showcase

The following section describes how a fully SU greenfield CMO production facility for mAb based on mammalian cell cultivation was put into practice. As is often seen on the market, one major driver from the customer's perspective here was also time-to-market. The overriding aim was to implement the entire project including the preconceptual and conceptual design phases, within a mere 18 months (Fig. 8).

We illustrate how we designed and started up a new facility from scratch. The challenge was not only to create a brand new SU production flow path supplying clinical Phase I and II material. We also wanted to help the client obtain US Food and Drug Administration (FDA) approval for manufacturing and providing clinical Phase III products and design the production line according to the cGMP guidelines. As is often typical for new CMOs in the described situation, there was no profound and specific process knowledge available at the time the entire project was executed. This fact and the clear requirement to keep the process design as flexible as possible for multiproduct purposes make it understandable that the customer's task was to design the process based on a generic production platform concept like that shown in Fig. 9.

Process4Success, the proven SU platform process simulation tool, was employed to determine and develop the conceptual design framework together with the client. This approach helped both partners accelerate the key criteria for this project: time-to-market.

With Process4Success, it is possible to provide highly predefined but further configurable unit operations while reducing engineering effort. Process4Success not only helps during the design and construction phase, but also during the qualification phase of such SU plant projects. By applying this approach, we

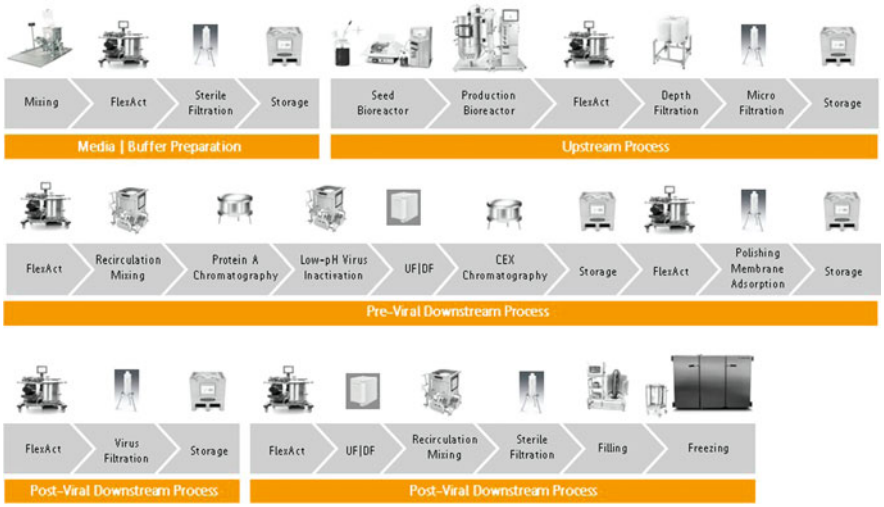

Fig. 9 Example of a generic single-use SU platform for bulk production of mAb

implemented a complete 500 L and 1,000 L SU mAb greenfield production facility with two upstream lines and one downstream line in less than 20 months. The customer's main focus at this stage was to prevent handling and contamination risks as much as possible and develop and build an advanced greenfield factory comprising a fully (100 %) SU process production plant. The intention was to use fully closed bag systems and unit operations, including preinstalled sterile filters, sampling manifolds, and single-use components wherever possible. The capability of sterile sealing and welding operations at several process interfaces were major driving forces. To reach this high safety level with regard to contamination and cross-contamination, several standard single-use items had to be slightly modified and thereby became customer-specific designs.

It is important to know that the majority of such specific modifications are reversible and have no effect whatsoever on the design and automation of single-use equipment design platforms such as FlexAct COM (central operating modules).

By now, it will have become perfectly obvious that this flexible approach significantly strengthens and benefits SU projects. It gives planners the ability to start the basic design phase even without full knowledge of all process details. This approach allows continuous project planning and implementation and leads to significant time savings. Design closure, as is the nature of classical stainless steel projects, is always a critical milestone of a "one-way nature." In conventional stainless steel projects, the user requirement (URS) clarification, design definition, and start of construction work are interdependent process steps that require more detailed and time-consuming assessment at an earlier stage compared to SU projects.

During the SU project described, many specific bag and transfer set designs were set up in preliminary or intermediate project phases. This enabled the client to postpone the final design decisions on SU items and parts of the flow path to later project phases.

By decoupling the detailed design of SU components from the associated hardware design to a certain degree, the supplier gains an early opportunity to start production of the equipment and systems, even though not all details are exactly defined as fixed. This indeed provides significant advances when fast project cycles are desired. Using the Process4Success approach, we first drafted the process layout according to technical aspects. Particularly in such cases, practical experience has shown that a significant and, for many customers, decisive advantage can be gained when designing SU processes. This not only applies to the areas of process development and material production for clinical Phases I and II, but especially to companies who, as CMOs, intend to manufacture biopharmaceutical products on the process scale.

We started the project under discussion here by defining a process chain based on the generic design approach. Because this approach renders it impossible to make the large number of individual SU parts mutually compatible, the individual connections and interfaces between the various unit operations require manual sealing and welding processes at many sites. In the case described, however, no concrete process specifications were in place at the time the project was planned. That was why we aimed systematically to reduce the number precisely of such manual operational steps. As a result, this approach led to an essentially contained process line designed to have a highly minimized contamination potential. At this point, the focus was placed more on technical aspects than on cost-effectiveness. At several sites, customer-specific modifications that deviated from the standard design of many key components, such as bioreactor bags and mixing bags, were initially made.

Such modified SU configurations have technical advantages including:

- Fewer manual steps/sealing and welding =>labor costs
- Lower risk of improper operation or installation
- Low contamination risk
- Easy and safe removal of sterile samples through preinstalled manifolds
- Low risk of getting individual SU component groups/products mixed up
- Ready-to-use parts/fit for purpose
- Quick setup times/plug and play.

On the other hand, there are also disadvantages that have to be differentiated in relation to the user's situation. Some of the most important should indeed be mentioned at this juncture.

- Less flexibility for replacing components
- Less flexibility in switching the process step sequence
- Higher overall costs because of more engineer-to-order (ETO) products
- More complex installations of products/manifolds/handling/risk to fail, and so on

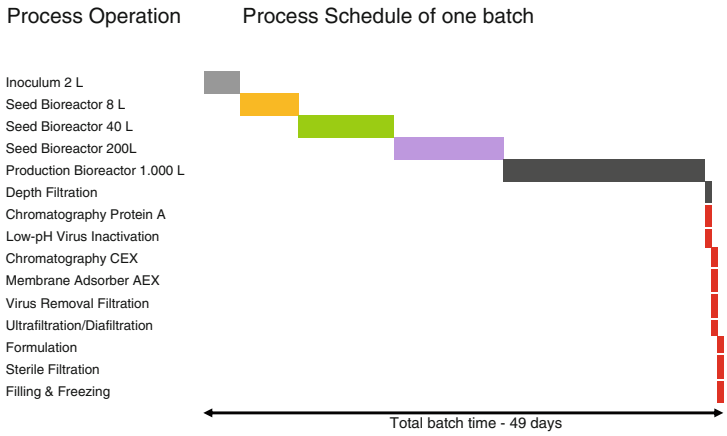

Fig. 10 Production time for a single batch based on a SU mAb process

- Longer delivery times because the product is not stocked
- Stricter robustness requirements for the supply chain.

In the project described above, all the modifications meant that the percentage of products not included in the standard portfolio went up to 80 % and was thus relatively high. In the cost-effectiveness analysis, this increased the overhead for SU materials when budgeting overall expenses and ultimately exceeded the customer's expectations. Although the much-touted flexibility of SUS is essential, particularly for CMOs, to keep pace with the ongoing advancement and increasing market globalization of the biopharmaceutical industry, such systems can be seen only in conjunction with the benefits of cost-effectiveness and competitiveness that they bring.

As already indicated, SU processes use a high number of consumables and, therefore, entail a relatively high proportion of variable costs. However, this proportion can be leveraged from process to process based on the interrelationships stated above.

Specifically, this opens avenues for a CMO to develop individual concepts with the contract customer, for example, in order to custom-engineer processes that accommodate the latter's needs. Each process step can be individually assessed and optimized based on a cost and risk analysis. For instance, it may make sense to convert a noncritical buffer preparation step from its original closed design into a semi-open or a completely open process step and use less expensive standard products. As a result, the entire process and its individual process steps can be engineered to be less expensive or more expensive but with higher safety. This possibility for making such adjustments is one of the outstanding advantages only provided in conjunction with SU processes.

It is generally known that the process times implemented in systems have a decisive impact on the overhead cost structure. This applies to conventional stainless steel plants and to advanced SUS alike. It is also undisputed that the

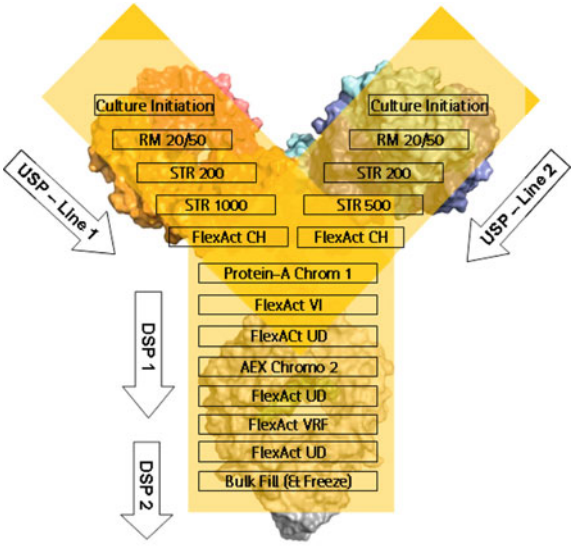

Fig. 11 Consolidation of two upstream production lines into one downstream line to expand the total production capacity of a single-use mAb plant

burden of fixed costs is lower for SUS than for conventional equipment because the CAPEX proportion is lower for the latter systems. In the present showcase example, this was an essential criterion that prompted the CMO to opt for SUT.

If the system usage times are considered for one complete process run consisting of individual unit operations for upstream and downstream processing (Fig. 10), it can be seen that the capacity usage of bioreactor systems employed in upstream processing can run as high as 90 % of the total equipment usage time. By contrast, the proportion of total usage times for systems that perform downstream processing accounts for only a small percentage of the total batch production time. Therefore, the process obviously needs to be designed for a higher rate of utilization of such downstream processing equipment. This was achieved by expanding the bioreactor capacity of the entire system by adding a second upstream line with a capacity of 500 L. In the actual process, this means that two upstream processing lines were combined into a single downstream line (Fig. 11).

From an economic viewpoint, engineering such a SUS is much more attractive than a conventional production line as the CAPEX for a SUS is only around 25 % (see Fig. 1). In an ideal case, this enables the usage capacity of a downstream production line to be doubled or even tripled as required. It is generally known that currently a large number of biopharmaceutical products are in the development pipeline so that over the short and medium term, an increased number of new products submitted for approval can be expected. Precisely because of this, the engineering process described above offers tremendous potential for increasing a CMO's ability to respond flexibly and rapidly to the growing and fast-changing requirements in this market.

After the SU process flow path and all related unit operations were defined, a detailed list of equipment was drawn up. This was the basis for designing the

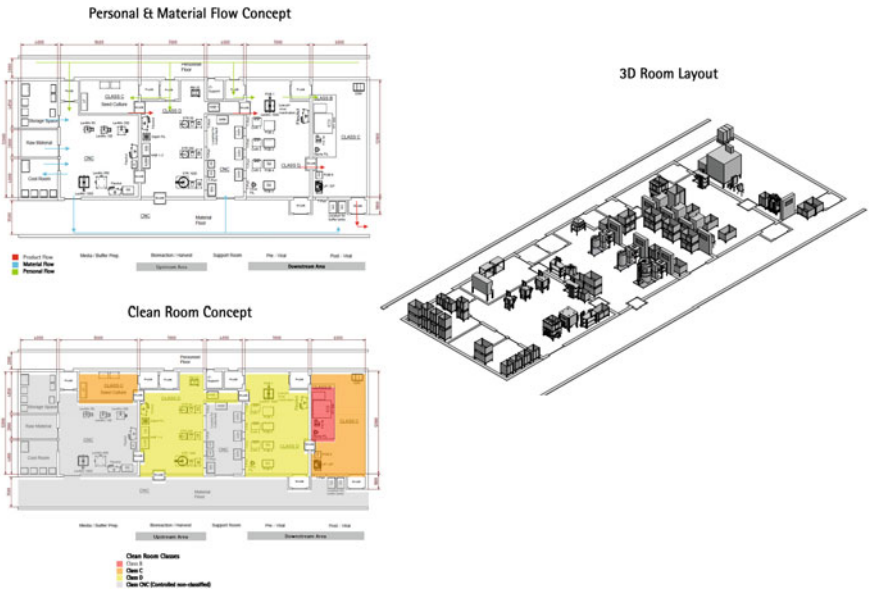

Fig. 12 Example of a facility layout concept to adapted to a single-use mAb process

cleanroom layout of the SU facility. Here again the Process4Success platform approach constitutes the perfect tool to support the groundwork in creating appropriate cleanroom layout concepts. Such room layout plans helped the client not only to set up the right cleanroom class, but also a conceptual design for personnel and material flows during production. Furthermore, the compilation of related 3D layouts supported a better understanding of the entire production room design and architecture.

Detailed scale drawings of equipment for each unit operation were generated first, then appropriately arranged in the floor plan. Figure 12 shows a few drawing examples for such a layout design. Even the general layout provided by Process4Success enabled engineers to estimate footprint requirements quite accurately, which perfectly supported the facility planning phase.

In the given example, only production areas were considered, whereas adjacent supporting areas, such as laboratories and intermediate CMC warehouses, were not included.

The manufacturing process designed starts with media and buffer preparation in rooms which are CNC. FlexAct media preparation and buffer preparation systems in different scale-up levels were implemented as standardized configurable single-use solutions. These unit operations are provided as purpose-built solutions customized to perform and control each required application, such as for mixing, transfer, filtration, and storage of media or buffers. Only single-use bags contained in stainless steel Palletanks® are used for both mixing and storage purposes. The media and buffer preparation facility also allows for additional storage space to accommodate consumables such as bags and filters and provides sufficient space

for other raw materials and a cold room for intermediate storage of media and buffers. Storage container bags with buffer and media for upstream and downstream processing are designed, in principle, as mobile components to be transferred into or out of the adjacent production area. However, in the design provided as an example, these storage containers were placed in a separate support room until needed. Aseptic transfer ports were installed to facilitate sufficient fluid transfer between different classes of cleanroom process areas through tubes if cGMP guidelines require such transfer. This specific arrangement helped to reduce the size of expensive cleanroom space significantly.

Except for the seed culture area, the entire upstream processing room was designed to achieve cleanroom class D conditions and contain the production bioreactors as well as a FlexAct CH, which is a SU depth filtration system for cell harvesting. In the design depicted, the downstream processing area is divided into two different room sections. The first section was designed as a Class D area, also referred to as the previrus clearance section. In this, purification steps, such as protein A, CEX, and AEX, as well as virus inactivation and virus filtration, are conducted.

Accordingly, the second section in the downstream area, a so-called postvirus area, was designed and classified to achieve Class C conditions. This room consists of a final concentration stage, with diafiltration steps performed using a SU crossflow filtration system called FlexAct UD. Furthermore, the final formulation steps, including pH and conductivity adjustments of the final bulk substance, are conducted in this Class C cleanroom. The final step for filling the product is carried out in an isolator classified as a Class B cleanroom.

Parallel to the definition of a cleanroom layout and the equipment to be used, the foundation for qualification of systems and the entire process lines was already laid in the basic engineering phase by specification of the user requirements profile (URS).The differences between SU and conventional projects, which have already been described to some extent in the previous sections, explain why the terms URS, factory acceptance test (FAT), and site acceptance test (SAT), as they are understood in the classical sense, take on new meanings as soon as they are employed in terms of SUS or entire SU processes.

Although conventional systems usually use only a few other materials in addition to pure stainless steel as product-contact components, the situation is quite the opposite in the case of hybrid systems and, above all, for pure SUS. In such systems, the equipment serves only as the frame and supporting structure to enable use of disposables. By contrast, the plastics that come in contact with the product consist basically of filter elements, tubing, bags or tanks, sensors, and combined transfer units, called “transfer sets.”

Naturally, during qualification of SU systems, both the equipment and the corresponding SU components must be defined along with their technical specifications, and their basic functions must be tested as part of FAT and SAT. SU platform systems are designed to be used in different multiple applications and cleanroom classes and are therefore often built as a mobile unit; that is, set up on castors. The flexibility afforded by their transportable design is desirable and essential, but also simultaneously poses a new challenge regarding the definition of

the design of matching SU products. In the qualification of such processes, it is precisely this flexibility in the product's design that confronts both the equipment manufacturer and the customer with a completely new set of challenges.

For example, it is quite possible that the specific locational requirements at a customer's facility dictate changes to the product design during an on-site FAT test. Compared with a classic process run, the need for making such modifications arises considerably later and must therefore be allowed for in the overall design concept. In individual cases, the changes to the process design can also make it necessary to revalidate the system. Implementing such changes in SU processes and revalidating SUS understandably entail less labor and expense than is the case with conventional stainless steel manufacturing equipment.

Generally, SUT can be regarded as relatively young. As soon as biopharmaceutical companies move beyond the R&D stage to scale up their processes for commercial manufacture of clinical material by employing SUS, the issue of validation comes to the fore. Even though validation of biopharmaceutical processes with SUS is considerably easier and less cost intensive than those using conventional multiuse systems, this step still is expensive and time-consuming. For suppliers of SUS, equipment qualification is both a challenge and an opportunity as a result of recent developments. Meanwhile, manufacturers of biopharmaceutical systems and products have been fiercely competing with one another to offer customers support in obtaining regulatory approval of their production processes. Such system manufacturers vie for this business by focusing their best efforts on providing technical specifications to expedite (promote) their customers' validation procedures.

Ultimately, this entails making qualification documents available, which are as comprehensive as possible, and offering easy-to-adapt qualification concepts. As a result, this part of validation services provided by suppliers is gaining completely new and ever-increasing significance as the generally recognizable trend is gravitating toward SUT.

To maintain the right balance in offering complete, yet economically viable, qualification services of the type and scope required, a so-called project qualification plan (PQP) was drawn up together with the customer in the showcase project. At this point, attention must be drawn to the fact that the regulatory requirements and guidelines have not yet been fully established down to the last detail for qualification of SUS. For this reason, individual specific concepts and auxiliary equipment must be developed to meet the requirements of each particular situation.

This is why in the showcase example described, a preliminary test, called an "impact assessment," was defined as the initial basis for establishing the URS to be qualified.

Following the impact assessment of all unit operations included in a process, a detailed qualification plan subdivided into four basic elements was drawn up (Fig. 13). In the qualification plan of our showcase example we, together with our project partner, defined the technical specifications, which were relevant to FAT, before outbound shipment of the SUS, and to SAT following installation of the

	Criteria	Assessment
1	Does the system have Direct contact with the product?	☐ Yes / ☐ No
2	Does the system provide an excipient or does it produce an ingredient or solvent?	☐ Yes / ☐ No
3	Will normal opera tion or failure have impact on product quality?	☐ Yes / ☐ No
4	Does the control of the system affect product quality?	☐ Yes / ☐ No
5	Does the system produce data which are used to accept or reject product?	☐ Yes / ☐ No
6	Does the system preserve product status?	☐ Yes / ☐ No
7	Are the equipment components data-generating or data-handling units?	☐ Yes / ☐ No
8	Will the equipment be used for storage of media, buffer, reagents and /or product (s) where storage conditions are controlled?	☐ Yes / ☐ No

Fig. 13 Example of an impact assessment per unit operation to evaluate the scope of qualification

system at the customer's location. The four basic elements upon which the qualification concept is based can be described as follows.

- URS for Equipment
  - Specifies critical/noncritical components including automation and reusable instrumentation (e.g., transmitters)
  - Describes and defines the scope of qualification activities (FAT/SAT)
  - Recommendation of verification scope for process qualification
- URS for SU Components/Assemblies (Standardized)
  - Specifies general requirements, such as FDA/SFDA/EP/USP, and so on
  - Defines the scope of qualification activities
- URS Automation Systems Specifies requirements for monitoring and SCADA systems
- List and Definition of Standardized and Prequalified Instruments and Accessories.

Standardized instrument systems are therefore qualified before they are shipped out to the customer and as such are no longer part of FAT or SAT.

The particular URS of the components that have not been qualified up to this point therefore comprise the decisive document, which essentially describes the acceptance criteria and, moreover, the traceability of tested technical characteristics and test methods. The total scope of qualification of SUS is thus characterized and defined both by the URS and by the impact assessment. It makes sense in this case to structure and/or classify URS according to requirements and test criteria, which are important for qualification in compliance with GMP standards,

No.	Requirement	Impact on Product Quality		Classification		Verification in			Qualification in			Remarks
		Crit.	Non-crit.	GMP	GEP	DR	FAT	SAT	IQ	OQ	PQ	
1	**Design Requirements**											
1.1	The Media Preparation container (MPB 22) shall be suitable to hold single use bags with a minimum working volume of 30 L and a maximum working volume of 50 L		x		x	x						
1.2	The outer surface shall be easy to clean and resistant against wiping with industry standard cleaning solutions like alcohol and soap		x		x	x						
2	**Technical Requirements**											
2.1	The Media Preparation container (MPB 21) shall be equipped with a holding device for powder bags	x		x			x		x			
2.2	The Media Preparation container (MPB 22) shall be equipped with a holding device for powder bags	x	x	x			x		x			
2.3	The Media Preparation container (MPB 21) shall be suitable to hold one external drive unit for mixing	x		x			x	x	x			
2.4	The Media Preparation container (MPB 22) shall be suitable to hold one external drive unit for mixing	x		x			x	x	x			
2.5	The Media Preparation unit shall have one pump for transfer of WFI into the Media Preparation containers (MPB 21) and (MPB 22) and for transfer of media into disposable storage bags	x		x			x		x			
2.6	The Media Preparation container (MPB 21) shall not have sharp edges that may tear the bag or injure personnel.		x		x		x					

Fig. 14 Abstract of a URS for SU equipment and components

and to those that are significant according to good engineering practice. An example is shown in Fig. 14.

Compared with engineering of conventional manufacturing systems, there are still well-known restrictions in the world of SU that may be both of a technical and an economical nature. To cite one example, the availability of SU sensors with comparably long-term and high accuracy needed for gamma sterilization is a critical issue.

Although ongoing improvements and new developments can be observed in SUT, there is still a lack of a wide spectrum of technically sophisticated and cost-efficient sensors in some areas that enable the same degree of automation to be implemented as do conventional stainless steel systems. As a consequence, the degree of automation used for SU processes is usually considerably lower than for conventional equipment due to either a lack of technical SU solutions or to their growing complexity. For example, this has resulted in the trend that the majority of complex preconfigured SU products are equipped with simple pinch valves. This, in turn, results in many operations that are strictly manual by nature. In other words, SU solutions are often automated only in specific partial steps or can be automated to a limited extent based on a strictly economical viewpoint. At the same time, market requirements on the validation of biopharmaceutical processes for compliance with cGMP standards are at the same level as is the case for conventional process systems from a regulatory stance.

Here it becomes clear that the benefit of the high flexibility of a SU process is leveraged by more manual, errorprone actions. This can only be overcome by ongoing training, which is sometimes challenging within a cGMP environment.

Standardization of bag sizes, connections, and assemblies are necessary to reduce the complexity of supply chain issues as well as operator mistakes. To make the human factor more predictable and to be able to control risks posed regarding this factor, both the supplier and the operator of SUS must focus on instructing and training the people who work with such systems.

Specifically, this means that considerable care must be devoted to the preparation of documents for standard operating procedures and workflows as part of the qualification process of SUS. The requirements on companies that rely on SU solutions can be summarized as follows.

- Skilled operating personnel that has been trained at the highest technical level to work with SUS
- Complete and 100 % traceable documentation and updating of SOPs (standard operating procedures)
- Extensive training in theory and practice for personnel, who do not directly work with SU products; that is, training on how to handle polymer consumables ranging from incoming goods, storage, in-house transportation, unpacking, all the way to disassembly and disposal
- Regular post and refresher training courses of all employees who use or operate SUS
- Overall policy for ensuring such training sessions are held and documented.

Complete and professional training is of crucial importance in working with SUS because manual and semi-automatic systems by nature have fewer process safety mechanisms. As the locational and spatial conditions of aseptic bags and tubing subassemblies are especially important in SUS, operators receive only initial general instructions during FAT. More detailed training of operating personnel is then held inside the customer's production facility once the SU units have been set up and assembled. Because of the special situation described above, the key to success in operating SUS lies in extensive and professional user training.

In the present showcase project, a holistic training program was designed and implemented. First, personnel received intensive training on performing just the maintenance and operating steps on the SUS. Moreover, hands-on training that involved process simulations and test runs accompanied by upstream and downstream application experts were conducted for each unit operation.

In the previous sections, we distinguished between standardization and customer-specific adaptation of SU products. As this subject also plays a role in logistics and in supply chain operations, this is discussed once again in detail.

The general rule is that complex SU products are currently in a ramp-up phase of technical development and are therefore undergoing frequent change and innovation. This gives rise not only to technical challenges, but also logistics issues for the manufacturer and user of SU products alike. A technical change to an SU subassembly frequently has consequences affecting the logistics, which must be considered.

These developmental changes entail the following challenges, among others.

- Changes to the type of materials/composition of materials
- New SU components/sensors/connectors, and the like
- Evolving complexity/designs/configuration of SU assemblies
- Varying logistics requirements, flow and transport of materials/storage/packaging
- Changing manufacturing and assembly processes
- Qualification and requalification of labor and expenses
- (functionality, shelf life, sterility, etc.).

The essential components and steps of a mAb process are encountered in every manufacturing procedure of this nature. As a result, it can be represented as a generic overall process. Despite this, however, it can be observed that when it comes to the actual definition and implementation of technical requirements, individual changes are repeatedly required. This makes it necessary to diverge from the existing standardized approach and to make customer-specific adaptations. This especially applies to CMOs, as they as a rule need to change over processes using their existing equipment and process lines. Apart from the constant introduction of newly enhanced individual components, this change-over requirement is therefore also a phenomenon that runs counter toward the effort to standardize products or product groups and to maintain continuity.

For suppliers of SU products, this means by increasing customization the total number of nonstandardized product variations is steadily increasing as well. In consequence the suppliers' internal workflows and business processes are getting more complex which has a negative impact on the service level with regard to supply chain capabilities.

As the issue of security of supply plays a major role for SU products in any case, it is certainly easy to understand that the trend toward adapted products moves this issue even more sharply into focus. For this reason, in the present project, the procurement departments of both partners had to work out a security-of-supply strategy. A well-known term in this context is collaborative planning forecast replenishment (CPFR). An important prerequisite for devising such a strategy is to have a functioning relationship between the customer and the supplier.

A fundamental basis for establishing such a relationship is to have comprehensive knowledge of the entire manufacturing planning and of that related to one's own SUS and to have classified such SU products into critical and less critical process steps. Each product must be individually classified and, in the broadest sense, involves risk analysis on the customer's part. Classification based on risk analysis is thus the basis for establishing a joint CPFR strategy in which the following points must be considered.

- Forecast planning
- Production planning
- Replenishment strategy
- Consignment
- Safety stock per item.

Extensive and well-thought-out planning provides leverage in achieving security of supply. Ideally, the objective in this case as well should be to decrease the number of variants or to keep this quantity as low as possible in order to reduce the complexity of logistics workflows to the fullest extent feasible. As mentioned earlier, customer-specific adaptations in SUS usually have a stronger impact on SU components than they do on the equipment itself. Exactly the opposite is true for conventional manufacturing systems. Depending on the particular perspective, this has both advantages and disadvantages.

Although customer-specific adaptations for SUS may occasionally have substantial long-term effects on the structure of current costs for SU components, this is not the case in engineering stainless steel systems. For the operator of SUS, this means that as she increases the proportion of modified, nonstandardized SU assemblies and products, she can expect the running production costs—variable expenses—to rise significantly as well. The economic attractiveness of SUT will thus diminish in proportion compared with that of conventional technologies and systems.

Against this backdrop, the customer in the showcase project described conducted risk analysis at a later project stage, that is, after successful engineering runs had been conducted. The objective of this analysis was to examine critically the customer-specific design layout planned in light of the actual local circumstances. An ideal time for such a risk assessment has proved to be the phase between installation and SAT. This design review thus prompted the customer to reverse a few modifications previously decided upon and return to standardized components that were less complex. In addition to reducing operating overhead per production run, this also had a positive impact to some extent on simplifying the complexity of the design and logistics of SU assemblies and products. The increased number of standardized, consistently designed components in the overall portfolio as a result of this review benefited both the customer and the supplier.

Aside from the cost-related aspects mentioned, the shelf life of SU assemblies is also briefly discussed in the following. The lifetime of SU subassemblies depends on how long the chemical–physical properties of consumables will last during storage. Particularly for innovative, complex SU assemblies, the specified functionality of individual components within the entire assembled SU unit also plays a major part, however.

As a rule, the lifetime of a unit is qualified and identified in a long-time storage study of the time a product is manufactured and gamma sterilized. Both customers and suppliers aim to achieve a product lifetime of three years, even though this is not always possible. The shelf life of a complex SU product can only be as long as the demonstrated and qualified shelf life of the components that were last added to it. Precisely because functional SU components and sensors are further developed at such a rapid pace, it is only natural that a customer will want to use a bioreactor featuring the latest SUT as quickly as possible.

However, as soon as new components that are added change the construction of an SU assembly, this may make it necessary to re-assess its defined shelf-life

specifications. Under certain circumstances, this may call for a completely new shelf-life study to be conducted.

Therefore, the desire to implement new configurations rapidly conflicts with the need to qualify the minimum shelf life of an SU assembly, as such storage studies take considerable time. For this reason, complex SU assemblies are frequently qualified in steps based on their components and, therefore, initially indicate a temporary shelf life of only one to 2 years.

Depending on how far away a customer is geographically located from the manufacturer, such SU assemblies may have only a short shelf life remaining until they are finally used. This is due to their supply chain, which includes the time it takes for transportation, customs clearance, intermediate storage, and the like. In exceptional cases, such as production runs that are off schedule, the customer may even face the risk that individual SU assemblies can no longer be used. This leads to logistics difficulties that need to be solved, especially for young companies that are still in the ramp-up phase so they cannot yet reliably plan their needs for equipment and materials with sufficient accuracy based on empirical data. The showcase project described indicates that there are still challenges ahead that need to be addressed, but also that the appropriately engineered SUT helps CMOs respond to the fast-changing market requirements of the advanced biopharmaceutical industry.

5 Factory of the Future

In the majority of drug-producing companies there are currently initiatives such as "factory of the future" or "manufacturing on demand" with the goal of identifying and describing the needs of the next generation of biological drug manufacture. In some industry organizations, such as the International Society for Pharmaceutical Engineering, working groups are summarizing these needs [5]. The primary goals are to simplify the production process, make the process stable and reliable under current and future GMP requirements, and, last but not least, to reduce waste.

As discussed, new facilities will need to meet four basic requirements:

- Lower CAPEX
- Provide a platform for rapid transition between product development and commercialization
- Ensure rapid start-up and ease of compliance
- Be reusable and flexible as process and products do change.

Flexible environments and SUS have eliminated many of the classical constraints on biopharmaceutical processes and have enabled designers to engineer modular bioprocesses and easily house them in properly classified environments. The ability to repurpose, improve, change, and reuse these core elements of a facility affects the industry from large pharma to small biotech companies. Whether planning new facilities, establishing first-time manufacturing capabilities,

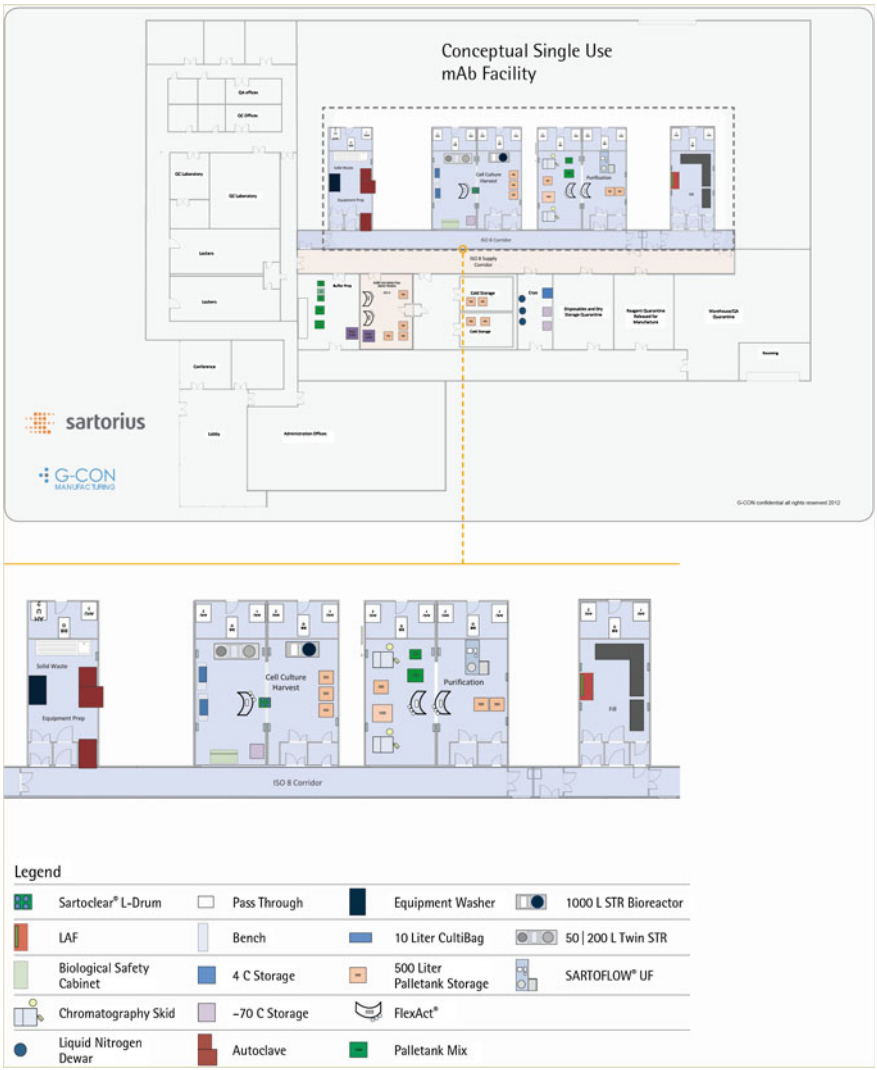

Fig. 15 SU mAb production facility

or executing new supply chain strategies by decentralizing manufacturing, companies now have new options to consider. And as suppliers continue to develop innovative products and collaborate effectively, the closer the biopharmaceutical industry will approach their goal of implementing their future manufacturing strategies.

Sartorius and G-Con Manufacturing have developed a modular facility concept "FlexMoSys" which is a perfect example of how to enable the industry to cope with the challenges of the future. The mobile cleanroom unit named POD

(trademark application ongoing) has been equipped with air bearings and can be moved into place effortlessly as they "float" on a layer of compressed air. No special rigging is required to move the facilities into place within the gray space. Process piping is completed at the factory, and process equipment can be preinstalled at a factory or at the customer's site. The utilities are connected via umbilicals with quick connectors to a service chassis. Services, such as water for injection and USP water, are prepiped into the PODs and provide the required zero-dead volume. The cleanrooms are typically connected to an access corridor in order to provide an interface to the building and to provide another level of pressure cascade for containment. PODs have onboard inlet and outlet filters in addition to high-efficiency particulate air filters inside the workspace, which effectively isolate the cleanroom from the gray space and permit the POD to be used in either a positive or a negative pressure mode and in a constant volume or a variable volume mode.

PODs have on-board fire suppression so that hard-piped connections to building sprinkler systems are not necessary. Construction materials are consistent for use with and resistant to the major disinfectants and decontamination systems including vapor-phase hydrogen peroxide. PODs are also equipped with a complete and robust control system. All sensors and control systems are Internet protocol addressable. Each POD is connected to the local area network via Ethernet and a single cable connection. Installation/operation protocols are provided to the recipient and facilitate the integration of the environmental monitoring and control. With an array of features designed into the POD, innovative facility designs can be realized to address most pharmaceutical process challenges. Figure 15 illustrates the application of such technology to a typical mAb facility equipped with Sartorius' Process4Success single-use process platform technologies as described in the sections above.

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Adv Biochem Eng Biotechnol (2014) 138: 239–272
DOI: 10.1007/10_2013_198

Published Online: 23 June 2013

An Approach to Quality and Security of Supply for Single-Use Bioreactors

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Abstract Single-use systems (also referred to as disposables) have become a huge part of the bioprocessing industry, which raised concern in the industry regarding quality and security of supply. Processes must be in place to assure the supply and control of outsourced activities and quality of purchased materials along the product life cycle. Quality and security of supply for single-use bioreactors (SUBs) are based on a multidisciplinary approach. Developing a state-of-the-art SUB-system based on quality by design (QbD) principles requires broad expertise and know-how including the cell culture application, polymer chemistry, regulatory requirements, and a deep understanding of the biopharmaceutical industry. Using standardized products reduces the complexity and strengthens the robustness of the supply chain. Well-established supplier relations including risk mitigation strategies are the basis for achieving long-term security of supply. Well-developed quality systems including change control approaches aligned with the requirements of the biopharmaceutical industry are a key factor in supporting long-term product availability. This chapter outlines the approach to security of supply for key materials used in single-use production processes for biopharmaceuticals from a supplier perspective.

Keywords Change control · Qualification · Quality by design · Risk management · Security of supply · Single-use bioreactor · Sourcing strategies · Standardization

Abbreviations

CPP	Critical process parameter
CQA	Critical quality attribute
DoE	Design of experiments
EP	European pharmacopeia
FDA	Food and Drug Administration
FMEA	Failure mode effect analysis
GMP	Good manufacturing practice

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ICH	International Committee Harmonization
LLDPE	Linear low-density polyethylene
PAT	Process analytical technology
PE	Polyethylene
QbD	Quality by design
SUB	Single-use bioreactor
URS	User requirements specifications
USP	US pharmacopeia

Contents

1 Introduction

The assurance of a continuous and robust security of supply is a key requirement specifically for single-use materials in biopharmaceutical processing. Any shortage or interruption will finally interrupt or at least be a threat to the constant delivery to patients of their needed medication.

To control the supply chain security is now identified as a challenge that needs a holistic approach [1]. Each quality system for production of pharmaceuticals ultimately must make sure that procedures are in place to assure the uninterrupted supply and quality of the final drug product. This involves not only the production process itself but also covers purchased materials and control of outsourced activities.

Single-use systems have become of major importance for the bioprocessing industry. Their reliable and timely supply can make or break a production run which in turn raises concerns regarding security of supply in the industry [2]. It is the aim of each quality system to limit the risk of any supply interruptions with appropriate measures. Robust security of supply can be achieved by numerous measures and is a multidisciplinary approach covering the full lifecycle of a product starting from the early phase design, material selection, qualification, production process, and supply chain with subsuppliers. From an end-user perspective, the increased use of standardized single-use products positively affects reliability and timely delivery of single-use systems.

For all components in contact with process fluids, either during transfer to and from the bioreactor, and during the cell culture, the major concern of the industry is the impact of interactions between the plastic parts composing the single-use bioreactor (SUB) and the process fluids. Indeed, a change of fluid contact material due to resin or component discontinuation requires an assessment of qualification needs. An appropriate change management procedure, as described in the quality section of this chapter is therefore a must.

Security of supply of polymers and all plastic parts must be integrated as a key target at the very beginning of the development of the single-use bioreactor. Indeed, procedures are essential that ensure functionality of a component, for example, by using QbD principles, long-term availability through sourcing strategies, and maintenance of the product by robust quality systems. Depending on the criticality of a component, typically assessed during the design phase, "make or buy" options are evaluated and the sourcing strategy is defined, based on a risk management approach. Selection of suppliers for resins, films, and other plastic parts is an expected output of the design phase. A robust supplier relationship management will support supply chain robustness during the product life (Sect. 3).

2 QbD Principles as a Platform for Quality and Security of Supply

In this section, an introduction to QbD is given, and illustrated through an example: the film that constitutes the bag chamber of the SUB. Of course, this is only an example and QbD principles must not be restricted to the film and should be extended to all components that constitute the SUB, and to the SUB itself.

2.1 Purpose and Scope

A single-use bioreactor, depending on its design, is composed of many parts such as film, tube, connectors, filters, sparging device, and stirrer, among others. For

SUB vendors the depth of in-house production can significantly vary and very often, they are assemblers of components. Some of these components are in contact with the cell culture, others with media or alkaline/acidic solutions added to control the pH. Each part of a SUB must be designed and selected from a form, fit, and function point of view, to meet the application requirements.

Single-use bioreactors are becoming more and more technically advanced and widely used both for clinical and commercial supply of biologics. This in turn demands new technologies and technical polymers that have to be selected carefully and qualified by the SUB vendors to meet application requirements. In order to ensure suitability for a wide range of cell lines and applications, a close collaboration between vendors and users is necessary to ensure appropriate design and selection of raw materials.

The market share of medical applications of the overall polymer market is very low, hence a very limited volume and variety of medical grade polymers are available. For instance, polyethylene, which is one of the most common polymers in the biopharmaceutical industry, represents less than 1 % of the global polyethylene market. As the commercial importance of medical polymers is very low compared to other applications for leading polymer suppliers, there is an inherent risk that certain resin grades may be discontinued or changed. Therefore security of supply of polymers and all plastic parts must be integrated as a key requirement at the very beginning of the development of the single-use bioreactor.To address this challenge, the SUB designer might select and qualify a resin grade that is not marketed as medical grade but is compliant with all relevant standards. In this case, one of the risk-mitigating factors is to select a blockbuster resin out of the supplier portfolio, based on the resin technical requirements and specifications, and to establish the appropriate quality plans and supply agreement. Another risk-mitigating factor would be to identify and qualify a second source that meets the same specifications.

2.2 Introduction to Quality by Design

The QbD approach and associated International Committee of Harmonization guidelines ICH Q8 [3], Q9 [4], and Q10 [5] are increasingly being adopted by the biopharmaceutical industry in order to ensure consistent quality of a product through the design of its manufacturing process to deliver the intended performance of the product consistently. Quality is built in and not ensured by controls and inspection, even if controls and inspection are necessary. Indeed, a robust process design together with process understanding and a sound quality system with appropriate quality controls assures quality throughout the product life cycle. Process analytical technology (PAT) has been defined by the US Food and Drug Administration (FDA) as a mechanism to design, analyze, and control pharmaceutical manufacturing processes through the measurement of critical process parameters (CPP) that affect critical quality attributes (CQA). This approach,

followed by the biopharmaceutical manufacturing industry [6], can be easily derived and extended to any product development process, as described below.

2.2.1 Quality Target Product Profile and CQAs

The first step consists in translating the application need, expressed in user requirements specifications (URS), into product technical specifications and into a product. A functional analysis is usually performed to set film, connectors, tubing, and final product specifications. For instance, robustness, a key criterion for single-use bags, is achieved through not only one but many properties such as film flexibility, seal strength resistance, and puncture and tear resistance. Knowing that some of these properties may be antagonistic, the definition of technical specifications requests a good knowledge of the application. As an example, a stiff film is highly resistant to puncture, but shows poor flexibility and resistance to fatigue. Depending on the condition of use of the SUB, either stiffness or flexibility might be a critical criterion for the performance. Based on the functional analysis, a risk analysis is performed to identify and quantify all possible failure modes of the product along its full life cycle. ICH Q9 [4] describes various tools for risk analysis. The most common tool to perform a risk analysis is called failure mode effect analysis (FMEA), however, for meaningful execution the risk analysis must be performed by a multifunctional team. This analysis will allow the establishment of a control plan, which will describe all the tests that have to be performed to qualify the product, in our case the single-use bioreactor. The quality target product profile forms the basis of design for the development of the product and is one of the expected outputs of the functional analysis. In our case of interest, the quality target profile of a SUB would include robustness of the system, comparability to classical bioreactors with regard to its geometrical design parameters, compatibility with a wide range of cell lines, and guaranteeing the best security of supply. Then, the quality target product profile must be linked to measurable critical quality attributes. CQA is officially defined by the ICH Q8 as a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality. Again, using the film example, one of the CQAs for robustness could be the film elongation at break as defined by the ASTM D882. Often, one property is related to more than one quality attribute. Next to functional and performance attributes, regulatory considerations must also be included, to select appropriate materials and to integrate relevant tests in the control plan. Last but not least, a good control plan must follow a testing strategy relevant for the application. The security of supply of critical components has to be integrated into the risk analysis step. It may be disconnected from the technical risk assessment process and addressed in a risk assessment from a sourcing perspective including questions such as: what is the risk of having only one supplier for this component? Is there any risk related to the location of this critical component's supplier? Do we have a

contract in place? Next to identifying risks, risk mitigation strategies are essential. These aspects are covered in the supply chain section (Sect. 3) of this chapter.

2.2.2 Critical Process Parameters and Design Space

Once the specifications and CQAs have been defined, it must be proven that the product manufacturing process is robust enough to achieve the target product profile. Therefore, the second step addresses the manufacturing process optimization and qualification. As a starting point, the manufacturing process must be analyzed, not only the manufacturing process parameters for in-house component assembly, but also for better control, the manufacturing process parameters of critical sourced components. This is where a strong partnership between SUB manufacturers and the suppliers is necessary. This analysis will allow understanding the impact of raw material variations and manufacturing process parameters on product performance and quality as defined in the first step of the QbD. To do so, another risk assessment is performed to identify the CPPs (e.g., the parameters that must be controlled) in order to ensure CQAs will stay in the appropriate range and ultimately to assure product quality. It is recommended to use good science, knowledge, and experience to understand the impact of raw materials and process parameters on CQAs. Raw materials can be polymer resins in the case of a component manufactured in-house by the SUB supplier, or a plastic part, in the case of a supplied component.

This manufacturing process risk assessment should again be performed by a multidisciplinary team including manufacturing process experts, scientists, and application specialists to identify the CPPs and their impact on CQAs. The design space is constituted of the authorized variations of the CPPs, it demonstrates understanding of parameter interactions, and provides manufacturing flexibility. It also provides an effective basis for change control and ensures long-term quality and security of supply. Design of experiments (DoE) is the most commonly used tool to define a design space. The risk analysis of a supplier manufacturing process must naturally be performed together with the supplier. If the manufacturing process is highly manual, operator competences can be identified as a CPP.

2.2.3 Product Qualification

Qualification is integrated along the development phase. The material qualification strategy depends on the SUB vendor: it is possible to qualify at the component level or alternatively at the finished product level. Qualification at the component level means that each single component will be tested according to relevant standards as described in the quality section of this chapter (Sect. 4). To qualify the component, the SUB manufacturer may decide to test the component itself or work with the supplier to perform the testing. Qualification at the finished product level means that materials are qualified during the performance qualification of the

finished product. Even though strategies may vary, the overall target is to prove performance of the SUB in its application. Although testing of components is crucial for selection of components to assess the readiness for use, only testing on finished SUBs can finally confirm the functionality and performance of the SUB in all its CQAs including relevant mechanical and physical parameters for a cell culture application such as mixing performance, cell growth, and the like. Therefore a combined approach of qualification on the component level as well as on the finished product level might be the most meaningful and efficient way to qualify such complex systems.

As for any production process, validation also comprises the qualification of critical production equipment and production steps. Finally, critical production steps have to have controls involving inspection with the goal of ensuring that the finished products can be manufactured reliably and reproducibly and with the desired quality. The product (or component) can only be qualified when its manufacturing process is proven to be robust. Section 2.3.4 describes, as an example, the qualification options for the film.

2.2.4 Control Strategy, Product Life-Cycle Management, and Continuous Improvement

Once the product and its related manufacturing process are developed and qualified, a control and batch release strategy has to be designed and implemented on critical raw materials and process parameters that affect the CQAs. The product and process life cycle must be managed to ensure continuous improvement and an efficient change management strategy.

The continuous improvement of the manufacturing process is supported by a relevant process analytical approach. The target is to monitor CPPs and CQAs, preferably inline, and thus improve testing efficiency and manufacturing consistency. One of the process analytical tools is DoE, which supports the multivariate data analysis, typically a software tool that conducts the statistical analysis of raw data related to monitored and controlled parameters.

Robust change control procedures covering the entire supply chain of raw materials and the production process support reliable and reproducible performance of the SUB during its life cycle and help to support unexpected side effects of changes. Good knowledge of the application and understanding the potential impact that a change might have, support efficient assessment of a change and help to determine the related qualification activities necessary at the manufacturer's site. Moreover, it provides a good basis for the assessment of revalidation needs at the end user of the SUB. Standardization of the SUB and its related components as well as limitation of design variations especially if SUBs are used in GMP applications, allow effective and robust quality and supply chain strategies by reducing the complexity and therefore limiting the validation needs for all involved partners.

2.3 Discussion QbD Principles Using the Film as an Example

In the sections below, we use a SUB film to illustrate the approach described in the above sections. Our intention is to give practical examples to ease understanding of the QbD approach, in particular to give examples of CQAs and CPPs relevant to the film. The discussion of the approach, however, does not cover all aspects. Indeed, CQAs and CPPs cannot be considered universally applicable and depend, in this specific case of film formulation and manufacturing process, on SUBs suppliers and their partners.

2.3.1 Film General Considerations and Definitions

Monolayer film structures such as polyvinyl chloride (PVC) and ethylene vinyl acetate (EVA) have been widely used for many years for storage and administration of blood products and parenteral nutrition. However, the properties required today of polymeric film structures for biopharmaceutical applications cannot always be achieved by a monolayer structure. The minimum barrier structure used for biopharmaceutical bags today features a backbone (see Fig. 1), itself composed of one or more layers, which determines the overall mechanical behavior and barrier properties of the structure and the contact layer, which must combine inertness and good sealing properties.

To achieve a multilayer film structure, the layers can either be laminated or coextruded. When laminated, all film layers are extruded separately and are bonded together by a thermoset adhesive. When coextruded, the film is manufactured in a one-shot operation and the film layers are bonded together by a tacky thermoplastic polymer. In addition, these two manufacturing techniques can be combined (Fig. 2).

What is commonly called plastic material is typically composed of polymers and additives. Indeed, very few polymers can be used without additives. To allow a robust transformation of the resin granulates in films and to aid further manufacturing to finished products, additives are needed to adjust the characteristics of the resin [7]. These additives have the purpose of ensuring usability of the material during the different manufacturing steps as well as to ensure performance of the film structure as such.

Particularly in medical applications, most polymers require the use of additives in order to achieve the targeted properties for a given application: PVC is softened by plasticizers to achieve flexibility, antioxidants are added to polypropylene to withstand sterilization by gamma irradiation, or antistatic agents might be added to polyethylene bags used for powder containment and transfer. The most widely used additives in plastic film applications are antioxidants, slipping agents, and antiblocking agents. Antioxidants are organic compounds that slow down the

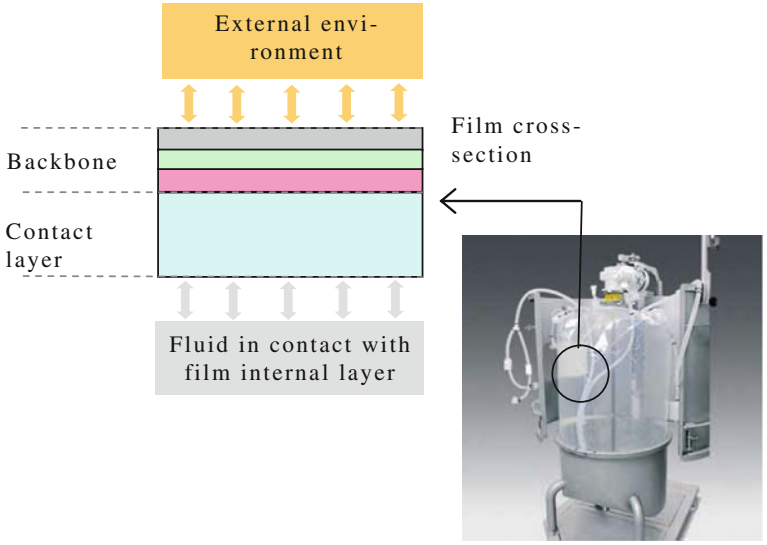

Fig. 1 Example of film structure

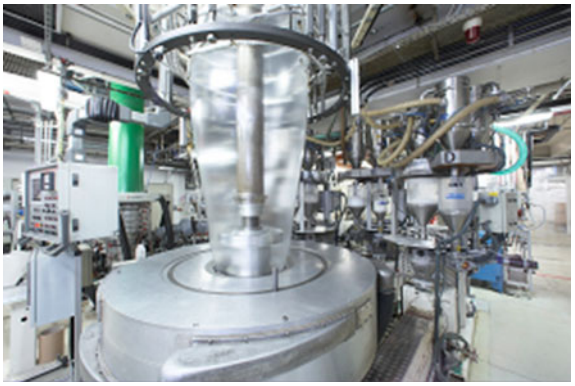

Fig. 2 Blown film extrusion (Courtesy of Südpack GmbH)

oxidative degradation of polymers. Primary antioxidants protect the plastic film and give the final product resistance to ageing so that it maintains its properties throughout its shelf-life. Secondary antioxidants are needed during the processing of plastics. Slipping and antiblocking agents are added to the polymer during the extrusion process. They are very often used together in order to improve processing behavior and end-performance of polymers. Additives will degrade into by-products during the resin-to-film extrusion processing, during the film-to-bag manufacturing steps, and during gamma irradiation.

2.3.2 Discussing Quality Target Product Profile and CQAs Using Film as an Example

The challenge for the development of a new film is to define its quality target profile based on end-user needs and to transform the URS into technical specifications. Indeed, end-user concerns related to a film used in SUBs are expressed from the application perspective. Examples are listed below.

Interactions between the film and contained solutions must be as limited as possible. This means limiting extractable and leachable compounds that could migrate from the film and affect cell growth, but also limiting the adsorption of media ingredients, such as growth factors and other critical additives to the film. The film must be resistant to acid and alkali solutions that are used for pH control.

A SUB must be mechanically robust. Size and capacity of SUBs may vary from 1 L to 2,000 L. A SUB is exposed to mechanical stress which is highly dependent on the shape of the SUB, the mixing strategy, and its implementation. The rocking motion of pillow-type bags requires a film that is fatigue resistant, whereas 3D-shaped stirred bioreactors need a stiffer film. In both cases, robustness is achieved not only through the film but also through strong welds.

A SUB should be free of contaminants that might interfere with, if not suppress, cell growth or compromise the safety of the intermediate. Contaminants can be of chemical nature, particulate matter, or of biological nature. Today, there are no regulatory requirements (FDA, EU, ISO, etc.) that mandate a certain clean-room classification for component manufacture such as the film. The requirement instead is that the production environment shall be controlled in order to ensure that the final SUB, which in turn is used for production of biopharmaceuticals meets the requirements for its application, that is, supports cell growth and meets the specification of the bulk harvest with regard to bioburden and endotoxins. Also, control of biological contamination is important as it determines the gamma irradiation process parameters for successful sterilization. In this regard, it is important to understand that the level of bioburden is typically related to the manual assembly procedures of the components and not to the film manufacturing process itself. Due to the high temperature and automation of the film extrusion process, microbiological contamination is unlikely to occur. Potential chemical contaminants are controlled by extractable and leachable studies and ideally confirmed by cell growth studies. Further considerations should be given to the cleaning procedure of equipment for manufacturing of the film but also assembly of the SUB. Appropriate cleaning and controls ensure absence of contaminants.

Last but not least, security of supply of the SUB is a concern, as the discontinuation of supply is a major threat in terms of drug supply to the patients. Therefore, the pharmaceutical industry requests long-term security of supply.

These requirements have to be transformed into technical specifications and definition of critical attributes for the polymer and films. Interactions between the film and process solutions are controlled by the specification of the polymer resins, their additive packages, and the film extrusion process. Robustness is controlled by specifying the mechanical properties of the film, such as elongation and strength.

Furthermore, using polymers with a large sealing window supports the robustness and reliability of the bag manufacturing process. Security of supply of the film is achieved through appropriate polymer selection in partnership with the resin suppliers and film manufacturing processes control.

Selection of Polymers and Additive Packages

A review of different disposable bags used in the biopharmaceutical industry shows that a wide variety of polymer materials is applied [8]. During the early days of single-use bioreactors, SUB suppliers used technology platforms established in other application areas such as medical devices. Today, SUB manufacturers strive to adapt the polymer and film properties to the cell culture application and select from a toolbox of resin parameters that would support the application-driven needs. As mentioned in the introduction, targeted film properties might only be achieved by developing a multilayer structure, using the following resin property toolbox.

- Molecular architecture (linear, branched, cross-linked, thermoplastic, thermoset, etc.)
- Copolymer structure (random, block, alternating, graft, combination) or blends (alloy)
- Polymerization (free radical, addition, step, condensation, stereochemistry)
- Molecular weight(s) and distribution (degree of polymerization, molecular weight, viscosity, free chains, etc.)
- Polymer morphology (crystallinity, amorphous, orientation)
- Thermal properties (melting temperature, glass transition temperature, conductivity, expansion, stability, heat capacity, and fusion)
- Mechanical properties (tensile strength, tear resistance, mechanical impact/penetration resistance, burst, pinhole flex resistance, etc.)
- Barrier properties and mass transfer characteristics (permeability, migration, adsorption, etc.)
- Optical characteristics (gloss, haze, transparency, etc.)
- Surface and adhesion (related to additives: surface tension, adhesive bond strength, blocking, sealing, friction, etc.)
- Electrical properties
- Biocompatibility, compliance with pharmacopeia monographies when available.

In the following, an example is given of the selection of polymers used for barrier and contact layers.

Barrier Layer

Typically, in bioprocesses the gas exchange and especially the DOT (dissolved oxygen tension) are controlled. Therefore, it is not necessary to integrate a barrier layer into a film for a SUB. However, the integration of a barrier layer is sensible as the biopharmaceutical industry strives to limit the number of different films used throughout upstream and downstream processing. Ideally, if possible, one film should cover all applications throughout upstream and downstream processing

until final filling of the drug product. However, today this is not yet possible due to the differences in requirements for the various single-use bag applications where some applications require gas impermeability or specific resistance to low temperatures. At the least, SUB films should cover the requirements for media and buffer storage, where a barrier layer is needed to avoid excessive exposure to air. Furthermore, a barrier layer limits the potential migration of compounds from the external layers. Barrier properties can be achieved by different materials: ethylene vinyl alcohol (EVOH), vinylidene chloride copolymers (PVDC), or aluminum. Contribution of the barrier layer to overall mechanical strength of the film structure must be carefully assessed, inasmuch as it increases film stiffness and therefore decreases flexibility which may limit fatigue resistance. Therefore thickness of the barrier layer, film oxygen transmission rate, and elongation at break of the overall film belong to the film critical attributes, linked to the barrier layer.

Contact Layer

The contact layer directly affects sealing resistance and interactions between film and cell culture. Sealing properties are linked to the thermal properties of the polymer, its morphology, its molecular structure, and so on. Examples of critical attributes for the sealing polymer are: melting temperature, density, melt flow index, and, at the film level, the seal strength resistance as measured by ASTM F88.

Interactions between film and cell culture are more complex to characterize. Indeed, film and cell growth are connected in two dimensions:

- Adsorption of media components such as lipids or proteins, which will negatively influence cell growth by limited availability of these compounds [9]
- Release of compounds from the film materials which might also directly influence the biological behavior of the cells

The most frequent approach used by SUB vendors for characterizing interactions is to perform extractable tests with preselected solvents [10, 11] and provide data compiled in so-called extractable guides. However, it is strongly recommended that SUB designers use a robust biological test system for the assessment of the film materials with regard to cell growth of different cell lines. These extractable analysis and biological tests are the starting point for the definition of quantifiable CQA of the film and relevant to cell culture.

Supplier Selection

Resin and film suppliers have to be carefully selected. Due to the criticality of these components, partnering or alliances with suppliers is strongly recommended. Critical aspects for the selection of external strategic partners for film and resins are:

- Access to the formulation of the resin and additive package, as the knowledge is key to defining the relevant extractable test-related analytical methods and relevant solvents. Good support from resin suppliers is necessary to identify the

resin CQAs for the bioreactor application, which might be different from other application areas of single-use films. Definition of good specifications between resin suppliers and SUB manufacturers is a critical success factor for long-term security of supply

- Control of the extrusion process parameters (see Sect. 2.3.3) and more specifically:
  - Defined process design space with identified sources of variability and routine control of critical process parameters to achieve consistent control of CQA
  - Reliable change control procedures
  - Freedom to work with other subcontractors or transfer of film extrusion in the case of change of ownership or bankruptcy, contract cancellation, and the like
- Contractually guaranteed security of supply, quality, cost, and service through supplier assessment and quality assurance agreement
- Codevelopment capabilities for continuous improvement of product and process.

2.3.3 Single-Use Bioreactor Film CPPs and Design Space

Once the CQAs have been identified, sources of variability must be identified and the CPPs and design space must be defined. Figure 3 schematically shows the film extrusion process. (NB: In the case of a multilayer structure, the number of extruders increases, but the approach is the same.)

Some of the CPPs most often identified for the extrusion process are listed below.

- Raw material variations: The same polymer can be synthesized using a different catalyst process. Polymerization is a statistical continuous process and the consistency of resin parameters such as molecular weight is controlled by,

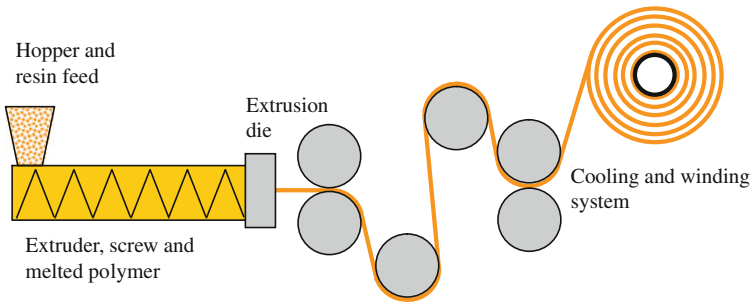

Fig. 3 Schematic representation of extrusion process

among other parameters, the stoichiometry of raw materials and temperature. Batch definition varies from one resin supplier to another. Within the polymerization process itself chemical substances such as polymerization initiators, catalyst, and solvents are added to the monomers to control the polymerization process. These chemicals may also be found at trace level in the extractable analysis. Metals especially often come from the formulation of the polymer. Additives may be added in the resin during the pelletizing process or during film extrusion. Resin and additives' specification range and controls must be defined to meet extrusion process robustness and application requirements

- Melt temperature: If the melt temperature is too low, it's not possible to transform polymers into films. If it is too high, there is a risk of polymer degradation. Potential polymer degradation leads to black specs, oxidation of the polymer, chain scission, and the like. The degradation profile of additives, which are added to the polymer on purpose to protect the molecular chains during the resin-to-film extrusion step, is directly linked to the temperature profile and history. This means that variations of residual additives and by-products are expected as a batch-to-batch variation and must be characterized
- Screw speed: The screw speed leads to shear stress exposed to the polymer and auto heating. The impact and risk profiles associated with different screw speeds are the same as for the melt temperature variation
- Cooling temperature: Variation of the cooling temperature of the chilled rolls has an impact on the crystallinity of the polymers, its transparency, and film surface roughness
- Winding rolls speed and tensions: These parameters control the film thickness and film dogleg.

For more efficiency, CPPs must be identified and discussed with film and resin suppliers. Using design of experiments will allow identification of parameters that affect film quality attributes, their potential interactions, and the optimization of parameters to set the manufacturing process parameters in the center of the design space.

2.3.4 SUB Film Qualification

Qualification approaches of a SUB were introduced in Sect. 2.2.3. We use the film here as an example to illustrate the qualification part of the QbD approach. As discussed in previous sections, qualification can be performed at the component level. In that case, the film is considered to be a component. It is considered as qualified, together with its manufacturing process, when predefined criteria are consistently passed. Some standards [8, 12] usually used in the packaging industry can be utilized to characterize the film, such as

- ASTM D882 or ISO 527-3 to characterize tensile properties
- ASTM D1004, ASTM D1922, ISO 6383-1, or ISO 6383-2 to characterize tear resistance

- ASTM D3985 to characterize oxygen gas transmission rate through plastic film.

Using this approach, the output of the film qualification is a supplier film fact sheet which can be provided as a stand-alone document or integrated in a document where SUB suppliers compile product performance data. However, the above standards give a methodology to follow and not specifications or criteria. Therefore, the main drawback of this approach is the difficulty for the end user to link film properties to its final process application and predict suitability of the SUB for the intended use as a cell culture chamber. Therefore, a combined film and SUB performance qualification approach based on the final customer application is preferred.

Choosing a qualification approach based on film robustness and cell growth comparability to stainless steel or glass vessels is dependent on the SUB vendors' ability to:

- Establish worse case trial conditions to evaluate the robustness of the film regarding hydrostatic pressure and mechanical forces generated during the application
- Establish a robust film resistant to bag manufacturing, packaging, shipping, and installation conditions
- Development of a predictive biological test system to assess different film materials regarding cell growth of different cell lines.

Moreover, data have to be collected from biological application conditions in order to check that all sources of variability have been identified and their impact on product performance, including cell growth, assessed and characterized prior to releasing the product design and specification.

More information on qualification of material, SUBs' manufacturing process and suppliers are given in the quality section (Sect. 3) of this chapter.

2.3.5 SUB Film Continuous Improvement and Change Management

When CQAs, CPPs, and design space have been defined and qualified, a life-cycle approach for continuous process improvement and change control is defined. The approach for the film and resins does not differ from other components. Therefore, the reader might go directly to the quality section (Sect. 3) of this chapter for more information on this topic.

2.4 Conclusion

Quality, assurance of supply, and change control of SUBs have to be integrated in a quality by design approach, based on a risk analysis performed by multidisciplinary teams, in which strategic suppliers and end users have to be involved as much as possible. Expected outputs of the approach are:

- Defining critical process parameters and design spaces to ensure that CQAs are controlled within specified ranges. Identifying sources of variability and routinely controlling them to ensure CQAs are controlled within specified ranges
- Controlling the entire SUBs manufacturing process.

A QbD approach for film development was presented in this section. Critical quality attributes and critical process parameters were discussed, however, this list is not exhaustive and must be adapted to the film formulation and its manufacturing process, which may differ from one supplier to another. The QbD approach is not limited to film and a similar approach must be followed for tubing and connectors.

3 Security of Supply, Sourcing, and Supply Chain Based on a Risk Management Approach

In the introduction to this chapter it was highlighted why the importance of maintaining a continuous and robust security of supply is a key requirement, specifically for single-use materials in biopharmaceutical processing. This section continues this discussion from a sourcing and supply chain perspective, while continually monitoring and managing any potential interruption of the delivery of the SUB to the end user. As of today there is no official definition of supply chain risks [17]. For the purpose of this section we define supply risk as a threat that could result in the inability of the vendor to supply its current and future customers with products or jeopardize the current or future demand of its products [17]. To better understand the risk, a classical risk assessment approach seems to be the most appropriate way to address this topic.

Historically, the burden of risk management was borne by finance departments as economical risks and insurable risks. Recently, the understanding has changed. Today, it is considered that only a holistic and enterprisewide risk management approach will lead to an efficient way of dealing with risk. The risk management process consists of four main phases: (1) risk identification, (2) risk analysis and evaluation, (3) risk treatment, and (4) risk monitoring. Although current risk management literature contains a multitude of methods and instruments that apply to finance and economic departments, we can only find sporadic references in the literature of how to implement these methods and instruments in the procurement/supply chain environment [17].

Following the risk management approach, the risk treatment can be divided in six different steps:

(a) Risk avoidance:

The strategy of risk avoidance consists of deliberately circumventing or eliminating activities associated with risk. An example of this strategy is when a company chooses to abandon the procurement of components from vendors

located in "crisis" areas to avoid supply disruptions that may arise as a result of the political situation.

(b) Risk mitigation:

Risk mitigation is the strategy that aims to reduce the occurrence probability of a supply interruption or to reduce the scale of its damage. Examples of the implementation of this strategy include maintaining contingency inventory, multiple and redundant production locations, duplicate stores of mission-critical tools, and establishing multiple suppliers per component. Other known strategies include the signing of purchasing and quality contracts as well as the QbD approach. Successful establishment of these strategies requires competences and related organization. This is related to efforts and investments in resources and competences to fulfill all these strategies.

(c) Risk shifting:

Risk shifting connotes that the transfer of risk or the financial consequences of a devastating event are passed onto another risk carrier before it occurs. In general the risk-shifting strategy is fulfilled by using insurance companies; however, in the context of this chapter, risks are shifted to suppliers. One example here is to sign purchasing contracts.

(d) Risk acceptance:

The strategy of the risk acceptance is also called passive risk policy. This involves a conscious acceptance of risk. In this case, the occurrence of a disruptive event is low or can be easily absorbed by reserves or with a calculated charge on the selling price. When this strategy is employed the company chooses to bear the risks itself.

(e) Risk monitoring:

The last phase in the risk management process is risk monitoring. Risk monitoring is a continuous process and is designed to make a comparison between the actual and the desired risk position.

(f) Risk assessment:

Once risks have been identified, they must then be assessed as to their potential severity of impact (generally a negative impact, such as damage or loss) and to the probability of occurrence. The fundamental difficulty in risk assessment is determining the rate of occurrence because statistical information is not available on all kinds of past or future incidents. Furthermore, evaluating the severity of the consequences (impact) is often quite difficult for intangible assets [16]. Due to this complexity a detailed risk assessment approach would go beyond the scope of this chapter and is therefore not part of it.

When identifying the potential risks, we are able to divide them into four different pillars [17]:

1. Internal
2. Suppliers
3. Customers
4. Business environment

When doing a risk assessment on these four different pillars, the following single risks can be identified.

1. Internal
(a) Production locations
(b) Production processes
(c) Selection of materials
(d) Employees (strike)
(e) Financial risks
(f) Warehousing
2. Suppliers
(a) Mono (sole)/multi suppliers
(b) Location of suppliers
(c) Contracts with suppliers
(d) Supply processes from second-tier suppliers
(e) Employees
(f) Financial risks
3. Customers
(a) Transportation towards customers
4. Business environment
(a) Regulatory affairs
(b) Competition
(c) Political environment
(d) Financial markets

For the purpose of this chapter, we only discuss the following risks.

1. Internal
(a) Production locations
(b) Production processes: This is covered in the QbD section
(c) Selection of materials: This is covered in the QbD section
(d) Warehousing
2. Suppliers including second-tier suppliers
(a) Mono (sole)/multi suppliers
(b) Location of suppliers
(c) Contracts with suppliers: Contract fraud
(d) Financial risks
3. Customers
(a) Transportation towards customers: Warehouse contingency, transportation fraud
4. Business environment
(a) Regulatory affairs. This is covered in the section on quality assurance.

3.1 The Risk Matrix

Table 1 shows an example of appropriate strategies in the four different areas of risk treatment for each identified risk.

Within the context of this section it is not possible to give extensive details for each strategy from the table shown above. In order to outline the importance of signing contracts with suppliers and the significance of managing the supplier relationship, additional information on this topic is given here.

Within the growing market for single-use products, and the increasing complexity in the business with the vendors for these products, it is recommended to develop a specific approach for contracting suppliers. The approach used for contracting suppliers is connected with a top-management business relationship management approach (down to the second- and third-tier suppliers). Please see Fig. 4.

Contracts would usually cover the following topics.

- Change clause/assurance of supply
- What is a change?
- Quality assurance, specifications
- Commercial topics: pricing, safety stocks
- Legal topics.

Being aware that the more complex contractual regulations are/will be—especially the ones dealing with complex and sometimes even future business topics as we are discussing in this chapter—the less they can be regulated or the more inclined the parties are to act "outside" of the contract.

To reduce opportunistic behavior two options can be chosen: applying rules that drive enhanced compliance and control as stipulated in the principal agent theory or by trust. Trust is not a given; it needs to be developed, systematically reinforced, and networked. The relational contract theory has two models by which long-term trust can be attained from relationship management: partnering and alliances. Any partnering or alliance model works well only with top management involvement on both sides [18]. Please see Fig. 5.

With regard to the most complex part of bioreactors, the film used to form the cell culture vessel, and the knowledge about polymer markets, we see the exact situation described above. Only a partnering or alliance model can significantly reduce the risks that can occur throughout the entire supply chain. For this reason it is highly recommended to form an alliance with the most critical suppliers for the components of a single-use bioreactor, based on a long-term alliance model approach and further supported by a long-term contract.

In order to follow this risk management approach in its entirety (and only the full approach would guarantee control over supply chain and sourcing aspects) all other strategies should be based on long-term market experience of a supplier of bioreactors and as well historically be developed to the same extent as shown here for the contract negotiation strategy.

Table 1 Risk matrix

Risk									
Risk treatment	Internal			Suppliers				Customers	
	Production locations	Selection of materials	Warehousing	Mono/multi suppliers	Location of suppliers	Contract fraud	Financial risks	Transportation	Transportation fraud
Risk avoidance	2 production facilities available	2 materials per component no materials from emerging markets, use standard commodities	Multiple warehouses	Supplier evaluation (commercial, technical, quality)	2 locations of supplier per component mandatory	N/A	Continuous monitoring of the financial strength of a supplier	Multiple warehouses	N/A
Risk mitigation	Additional safety stocks	QBD	Additional safety stocks	Supplier contracts (Commercial and Quality), supplier performance review	Additional safety stocks (down to second tier)	Long term relation, supplier relationship management by top management level	Dedicated prepayments if necessary	Only using first class forwarders	Tamper evident packaging, only using first class forwarders
Risk shifting	N/A	Contracts with suppliers including second tier to hold 2 years	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Risk acceptance	No	Yes	No	Yes	No	Yes	No	Yes	Yes

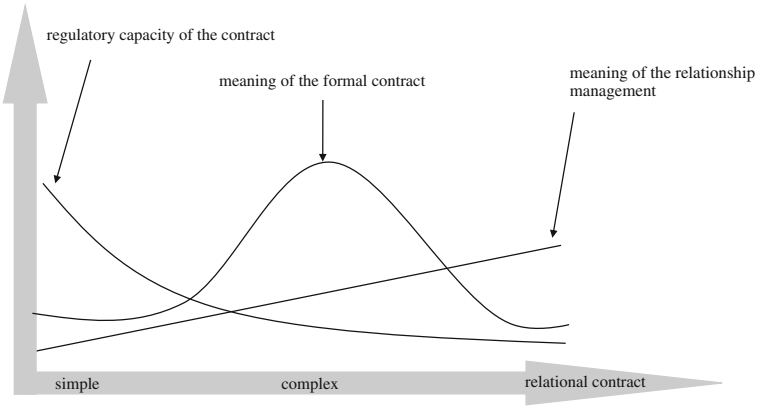

Fig. 4 Contract and relationship management [18]

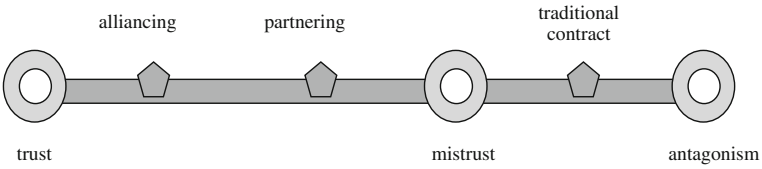

Fig. 5 Relational base of contracts [18]

3.2 Risk Monitoring over the Entire Product Lifecycle

The last phase in the risk management process is risk monitoring. Risk monitoring is a continuous process and is designed to make a comparison between the actual and the desired risk position. The main task of risk monitoring is the monitoring of the risk treatment strategy.

The effectiveness and the status of the implementation, including timetables of the strategies defined in the risk matrix, have to be monitored in defined intervals. The management of supply chain and sourcing departments should lead the company in reducing any deviations from the current status to the desired strategy.

The supplier performance monitoring, from a commercial and a quality perspective, is an integral part of the risk monitoring and should take place in defined time intervals (i.e., annually, quarterly).

3.3 Outlook

In order to fulfill the aforementioned task of guaranteeing a continuous and robust security of supply, several actions need to take place on behalf of a supplier of a single-use bioreactor. The key factor is a risk assessment based on the previously

mentioned matrix approach. This risk assessment is a complex topic and has to be performed in close collaboration among R+D, quality, production, supply chain management, and sourcing on behalf of a manufacturer of a SUB. Few companies have experience in establishing and managing long-lasting complex supply chain and sourcing relations in the supplier landscape. This approach is necessary for those companies manufacturing SUBs. The methods and strategies that need to be developed internally should be based on many years of experience in serving the single-use market for biopharmaceutical manufacturing. These strategies have gone, and will continue to go, through many steps of fine-tuning and improvement together with all stakeholders involved in this process.

4 Quality Assurance Concept to Support Security of Supply Along the Supply Chain

As outlined earlier, risk management and appropriate risk mitigation strategies are essential from a design perspective as well as from a sourcing perspective. In both dimensions quality assurance is an integral part.

Quality risk management (ICH Q9) [4] is an essential tool to identify potential risks and to determine processes and procedures to anticipate risks from a quality perspective. This risk management approach needs to cover the entire product life cycle. The intensity of the involvement of different functions may vary during the different phases of the product life cycle, however, quality involvement during the different phases helps to support security of supply. All quality assurance driven measures have the ultimate objective of robustly ensuring that a product constantly performs according to its initial specification and thus can be reliably delivered within the expected delivery time to the market. Quality involvement of security of supply concepts is driven by the fundamental concept that any quality issue within the supply chain may interrupt the delivery of the product. Based on that principle, robust quality systems are a basic requirement within a security of supply concept. The nature of the quality involvement during the phases of product life cycle is changing. During the design phase, for instance, it is to work with the design functions in order to establish a proper risk assessment from a technical perspective. These risk assessments are the basis for the qualification plans that have to cover technical aspects as well as regulatory. This requires detailed understanding of the application to define the CQAs and experience on the manufacturing process for raw materials and the assembly process to determine the critical control parameters. When establishing the qualification plan it is the responsibility of the quality function to ensure that regulatory needs for GMP applications are considered and addressed.

From a sourcing perspective, the quality function is involved in terms of supplier qualification, establishing contracts, and auditing. However, due to the availability of polymeric materials or specific technology needs, a manufacturer of

a SUB might have to use components and/or raw materials from a supplier with limited experience of biopharmaceutical applications. Especially in such cases, building relationships to key functions at the supplier from a quality perspective is as essential as the contract, audits, and the formal qualification of a supplier. Also, from a quality point of view, the same statement can be made as outlined in Fig. 4 from a sourcing perspective. Likewise, the more critical the material is, the more complex the technology, and the more complex the underlying supply chain is, the more quality involvement is needed from the beginning. Strong partnering on the quality level helps the partners understand the demand from the biopharma customers, especially regarding their quality and regulatory perspective and to establish appropriate quality systems.

Once the design phase is completed, control plans are established and suppliers are qualified and approved, the tasks for the quality function change. During the routine production phase of the SUB it is now the task to maintain and improve the product and process performance constantly to meet the initially defined CQAs and to monitor the performance of the critical raw material suppliers. In addition, close market surveillance is needed to anticipate any trend, application need, or quality issue to support continuous improvement of the SUB. Market surveillance and detailed analysis of customer feedback is especially key although SUBs—compared to classical bioreactors—are still a quite new technology, continuously requiring some adaptations to market needs. Robust change control procedures are a must to ensure that the needs of regulated processes and GMP applications are met but also to support continuous improvement of the product.

From a quality perspective, security of supply during the design phase has to consider aspects such as material and product qualification and supplier qualification.

During routine production, quality involvement is required to ensure performance consistency and to initiate continuous improvement activities regarding the following.

1. Production process
2. Continuous control and release of raw materials and finished products
3. Supplier performance monitoring
4. Change control
5. Continuous performance review.

4.1 The Design Phase from a Quality Perspective

Developing a SUB means assembling various components of mostly polymeric nature from different subsuppliers. Even though SUBs are based on established technologies such as disposable bags and mixing and sensor technologies, designing a bioreactor with performance parameters as close as possible to classical stainless steel stirred-tank bioreactors requires the development of numerous

new specific components and sometimes even technologies and materials. At a minimum, optimization of established technology platforms such as the film is required to meet the specific demands of a SUB in terms of robustness and reproducible cell growth.

Robust performance and quality are therefore highly dependant on the qualification of the material for its intended use, the qualification of the supplier, and the quality systems to ensure the assembly process. However, due to the involved complexity, intelligent qualification approaches are to be established on the material level as well as on the finished product level. As outlined earlier, material qualification for the film is a key attribute. The full functionality of the SUB regarding physical parameters, robustness, cell culture performance, and, last but not least, regarding extractable or potential contamination of a cell culture, can be finally confirmed only by testing on fully equipped SUBs using the actual conditions of use as well as worst-case scenarios. Careful assessment and review are needed to define which attributes can be effectively tested on a material level, component level, or on a finished product level.

Application-relevant aspects are published by manufacturers of SUBs in so-called *validation guides*. These documents provide the user with an overview of application-relevant data in support of the material selection and the design of their process validation strategy.

The specifications and resulting control parameters are set based on QbD principles. Ideally, the control parameters are set to confirm that the product performs according to expectations. Control strategies do not test quality into product but confirm that products are assembled according to defined procedures and they perform as expected. Quality controls have the purpose of ensuring lot-to-lot consistency. Furthermore, quality controls such as for raw materials can result from the risk assessment as part of a risk mitigation strategy. As outlined earlier, manufacturing a SUB means assembly of numerous components consisting of different materials, having their tolerances. The development status and detailed characterization of a component and impact on the performance can vary by the type of component [19]. Quality control procedures help to control lot-to-lot variability and to identify inconsistencies. Early detection of any inconsistency allows anticipating any impact on material availability. For some materials there might be a very well defined design space and a well-established understanding of the performance attributes related to bioprocess applications. There might be other components, already established for a long time in biopharmaceutical processes; however, in some cases the knowledge has not yet achieved a level of maturity desirable for cell culture applications. Especially in such cases, control plans, data analysis, and market surveillance are essential to gather the relevant information leading to a better understanding. Therefore control plans have to be constantly reviewed and adjusted to achieve the same level of knowledge for these raw materials and components.

4.1.1 Qualification

As already outlined in Sect. 2.3.4, the qualification as part of the design phase should confirm the suitability of a material for its intended use. Clear URSs describe the intended use. A risk assessment versus this URS helps to identify aspects. The risk assessment should not be limited to purely technical or functional aspects but also right from the start include aspects of security of supply.

Typically, raw materials for single-use bioprocess applications are of a polymeric nature. However, there is a lack of definition to determine unambiguous requirements related to raw materials for bioprocess applications [19]. Even for standard chemical raw materials, compendial standards may not focus on quality attributes that are relevant for quality assurance of biotechnology processes and specifically for cell culture applications as relevant for SUBs.

For a first assessment based on regulatory aspects it is recommended to include classical requirements for polymeric materials in contact with pharmaceutical products. Orientation can be given by using requirements and expectations as established for pharmaceutical packaging. Also food industry standards and medical device-related standards may help to assess the usability of a material for biopharma applications. However, all these standards and attributes have to be assessed carefully in terms of relevance for a cell culture application and are not necessarily release criteria for a raw material or the SUB. The following criteria may give some overview of relevant standards and regulations to take into account when qualifying raw materials of a polymeric nature for single-use applications.

Table 2 provides an overview of applicable USP or EP or other standards from a chemical perspective.

Next to standards describing the chemical and biological relevant properties of a material in use for pharmaceutical applications, it is essential to expand such a review to attributes coming from other regulated areas such as Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; EC Regulation1907/2006 or Restriction of Hazardous Substances ROHS (EU Directive 2011/65EU) which may interfere with the availability of a polymeric material or additives as well. Table 3 provides an overview of regulatory requirements relevant for assessing the material from a biological perspective.

The fulfillment of the requirements is mostly related to the polymeric material. However, the raw materials are subject to numerous transformations during the subsequent manufacturing process of the components, for example, extrusion or irradiation. These transformations may interfere with the chemical properties of the material. Therefore it has to be ensured that such requirements are reliably maintained during the entire manufacturing process from resin raw materials through extrusion or mould injection, assembly, and final sterilization.

Next to initial assessments, strategies have to be in place to review the compliance status of the material repeatedly. Furthermore, changes in existing requirements or new requirements should be closely observed to assess a potential impact on the regulatory status of a material. It is recommended to include in this

Table 2 Overview of standards that can be considered for qualification from a chemical perspective

	References	Title
Extractables	EP 3.1.x.	"Material qualification"
	EP 3.1.5	PE with additives for containers
	USP <643>	Total Organic Carbon;
	USP <661>	Containers, Physicochemical
	USP <645>	Conductivity
	USP <788>, EP 2.9.19	Particle release
	EP/USP Monography for "Sterile water for injections"	Stability of water for injection
Particulate matter	USP <788>	
Product compatibility	ASTM D 543-06	Standard practices for evaluating the resistance of plastics to chemical reagents

Table 3 Overview of standards that can be considered for qualification from a biological perspective

	References	Title
Sterility	ISO 11137	Sterilization of health care products—radiation
Endotoxins	USP <85> and EP 2.6.14	Bacterial endotoxins test
Biocompatibility	ISO 10993-5	Biosafety cytotox. Test.
	USP <87>	Cytox. Test
	USP <88>	Plastic class VI test
	ISO 10993-4	Selection of tests for interactions with blood
Bioburden	ISO 11737	Sterilization of medical devices
TSE/BSE or ADCF	EMEA/410/01	Note for guidance on minimizing risks of transmitting animal spongiform encephalopathy agents via human and veterinary products

regular review not only the raw material as such but also any additives needed during processing of a polymeric material.

During the phase of initial qualification, this review of regulatory requirements would ensure the choice of a material that is compliant with existing and coming requirements. By this, long-term availability can be supported and the risk of shortage of supply can be successfully anticipated.

4.1.2 Supplier Qualification

Current regulatory requirements and standards such as described in ISO 9001 [20] request manufacturers to control products and services obtained from subsuppliers.

The process for supplier selection and qualification together with relevant criteria are outlined in standards and guidance documents [21]. With regard to security of supply of a bioreactor this becomes even more relevant due to the number of involved materials and components and the typically low market share of such applications compared to the overall polymer market. Close cooperation with the sourcing function also forms the platform for partnering regarding quality assurance. To confirm the initial material assessment and to ensure long-term ability of a supplier to maintain the quality level, detailed and repeated supplier assessments from a quality perspective are essential. This initial supplier qualification should cover aspects such as the supplier′s quality system and experience in offering material for pharmaceutical or medical applications and reliability.

This information can be collected by questionnaires detailing the exact expectations. However, visits, or better, formal audits are tools to support and confirm the provided information as well as to develop a close relationship and cooperation the better to understand and meet the demands.

4.1.3 Supplier Quality System

Any supplier of raw materials in direct contact with process fluids during a bioprocess should have established a quality system meeting international standards such as ISO 9001, ISO 13485, or CFR 21 Part 820. Ideally, the compliance with such standards is confirmed through an audit conducted by a supervisory agency. An ISO 9001 certificate or other certifications relevant to the quality system of the supplier provide a first guidance in assessing the supplier's capabilities to support biopharmaceutical applications.

4.1.4 Supplier Auditing

Supplier auditing is a tool to confirm impressions and information collected via initial questionnaires. For key materials in direct contact with process fluids it is a good practice to perform supplier audits to verify if the quality system in place is robust and meets the demands for pharmaceutical application of the raw materials or components. This is especially relevant as in many cases the suppliers of polymeric material or other raw materials have their main market in applications other than single-use materials for bioprocess applications. During auditing special focus should be set on all quality systems in place that may have a direct impact on product quality as well as an impact on security of supply. Related to security of supply, focus should be set on the supplier's approach to securing its raw material supply, change control, and related notification procedures.

4.1.5 Quality Agreement

Quality agreements should be negotiated with any key supplier of critical raw materials for bioprocess applications. Such quality agreements should cover the general expectations for the quality system. Related to security of supply, special focus should be set on agreed information procedures and timelines for product nonconformities and change notifications. To minimize the risk of any shortage of material due to product nonconformities, mitigation plans should be in place and contingency plans should be established in close cooperation. Regarding change notifications, timelines for notification are essential. The timelines for notification and acceptance of a change need to cover potential requalification needs at the "assembler" and at the end user. Therefore scenarios have to be defined and agreed upon well in advance to ensure that unchanged materials are available for an appropriate timespan.

4.1.6 Continuous Performance Reviews of the Supplier

Procedures should be established to review the performance of a supplier regularly to meet the initially set requirements. Quality indicators agreed with the supplier allow a facts-based assessment of the supplier performance. For critical raw materials of SUBs, repeated regular audits can support such assessments. These critical raw materials should be defined during the initial risk assessment. Typical criteria defining such critical materials are:

1. Direct contact of the material with the process fluid and exposure of a high surface area to these process fluids
2. Long contact time of the material with the process fluid
3. Single-source material
4. The material is critical for the performance of the product.

4.2 The Routine Production Phase from a Quality Perspective

During the routine production phase the role of the quality function changes from establishing CQA and test plans to continuous monitoring. The quality function supports that all defined product and process parameters are reliably met, to detect any deviation, and to establish corrective and preventive actions if needed. Even though it is the ultimate goal to achieve a high level of quality by design, regular inspection and testing of raw materials as well as close monitoring of the production process and appropriate quality control tests of the products are required to ensure that the SUB reliably meets the defined performance criteria. Changes might be

required to support continuous improvement activities. Robust engineering change control processes including procedures for customer and market notification are essential to meet the requirements of biopharma applications.

4.2.1 Incoming Inspection

Based on the agreed specification for any supplied material, incoming inspections have to be established to confirm the conformance of the supplied material with its specification. Incoming goods inspections should include supplier certificate, conformity of material, dimensional checks, and material verification tests by infra-red (IR) or other methods ensuring a clear material identification. If possible, the incoming goods inspection should include tests predictive of critical performance of the material in the final application. Typical tests can be tensile strength for films and weldings or pressure hold tests for connections.

4.2.2 Production Process

SUBs are produced by combining different components and materials of mostly polymeric nature. Typical assembly technologies are the welding of one or more polymeric material or alternatively gluing of such components. The critical quality attribute is integrity and functionality of the final assembly.

A robust final assembly process of the materials and components ensures reliable product performance and limits the risk of interruption of the supply chain due to quality issues. Good understanding of the production process and its variability as well as the variability of the raw materials, for example, achieved by design of experiments, support the robustness of the process and mitigate quality issues. For this purpose the assembly process and its parameters have to be well characterized. A clear understanding of the CPPs together with established control plans for the CPPs, ensure a robust production process.

For example, polymeric raw materials are typically connected by welding. The welding process is basically characterized by temperature, pressure, and time. These parameters are set based on the physical characteristics of the polymeric material. A good understanding of the correlation of the process parameters and well-characterized tolerances support the reliability of the welding process, resulting in highly reliable connections. A subsequent process validation confirms the chosen parameters including the given tolerances. In-process controls have to be set on critical production steps to detect nonconformities. However, prevention of nonconformities by a robust process rather than detection through testing and control is clearly preferred to support security of supply.

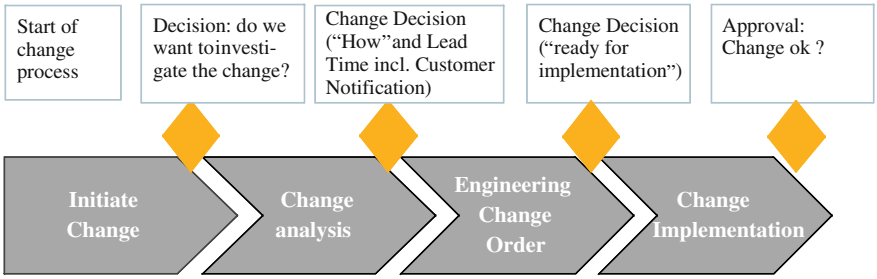

Fig. 6 Principal phases of an engineering change process

4.3 Change Control

Robust change control procedures along the entire process chain are a key requirement to ensure long-term security of supply and regulatory compliance. An engineering change process as shown in Fig. 6 can be divided into different phases of initiation of a change throughout analysis initiating an engineering change order and finally change implementation. Engineering changes can be triggered by numerous events such as the initiative of a supplier, the internal need of product optimization, preventive and corrective actions, or by customer needs.

Detailed analysis of a change needs to be done to evaluate if all initially validated attributes of the finished products will remain unchanged by the intended change. Furthermore, the impact on the application has to be evaluated. Unexpected side effects have to be excluded by qualification. An initially defined specification and understanding of the critical quality attributes allow a better understanding of the potential impact of any change either at the raw material supplier level or during assembly of the components.

Once the decision is taken to implement a change, a detailed execution plan is needed. This also includes a robust notification procedure of the users. Appropriate notification timelines to end users have to be established allowing the end user appropriate time to assess the impact of the change on the processes and product quality and if needed to allow appropriate timelines for validation. Timelines may vary depending on the expected impact of the change, for example, from typically 3 months for changes on the drawing level involving no new materials. Material changes on the raw material level might require up to 24 months, for example, changes of resins that are the raw material for films in contact with process fluids. Precise material specifications resulting from the initial material characterization and qualification will simplify assessing the impact of a change on the application. A good practice is to assess and document such events in advance.

Decision trees or matrices can be useful tools to support reliable and reproducible assessments. These decision matrices or trees are based on generic types of technical changes assessing the potential impact on the product and its application. The matrix is established by a competent team of experts covering the different

relevant functions (applications needs and technical needs, as well as regulatory and quality needs). It structures the changes into different categories by using generic descriptions. Furthermore, it supports the assessment of the need to notify the customer and the timeline for such notification. Such a decision tree or matrix should give guidance to the organization to assess the impact of the change and unify the approach for customer notification. Even though there might be events that require case-by-case decisions or consideration of specific user or application needs, this approach provides guidance to clarify expectations and needs of all involved parties including the end users and suppliers.

4.3.1 Change Control with Suppliers

Change control notification and clear requirements need to be agreed upon with all suppliers of critical raw materials or components. It is important to define clearly and understand the nature and the impact of changes. Classical change control requirements in contracts refer to changes in form, fit, or function. However, this definition might leave too much room for interpretation for a single-use material used in bioprocess applications. Therefore a more precise definition of technical events that may finally interfere with the performance or regulatory requirements of the material is essential. An upfront assessment of potential events that might change the material is helpful. This limits the room for interpretation. Within security of supply concepts it is essential to anticipate the potential impact of a change well in advance to limit if not avoid any effect on the delivery of the product. A risk mitigation strategy is typically established in the supply and quality agreements and can consist of long-term notification periods, defined inventory levels, and last-call options of larger amounts of materials.

4.3.2 Internal Change Control Procedures

Next to change control on the raw material supplier side, change control on the in-house production process is essential. Similar approaches with predefined matrices identifying potentially relevant changes limit the impact on delivery performance.

4.3.3 Effect of Customer-Driven Changes on Standard Products

The need of implementing changes is not only driven by continuous improvement, optimization, and adjustments from a manufacturer point of view. There might also be the desire of end-user driven changes. These changes typically refer to specific requests in design such as specific components. It is the task of a SUB manufacturer to design the SUB to meet the needs of the application as described in Sect. 2. Therefore such demands for adjustment have to be reviewed carefully by the SUB manufacturer to decide if such demands are implemented on the full

product range. However, there might be the demand of users for application-specific adjustments of the SUB design. These requests may vary per end user and lead to a high number of customer-specific designs with specific components. Application-specific adjustments can be implemented by using configurable designs based on standard options. From a security of supply and quality perspective using established standard products or variations based on configurable designs are the preferred option.

Standardization limits the complexity and the related impact of any change on all levels including raw materials, suppliers, qualification, in-house production processes, control of drawing revisions, and finally change notification and drawing approval to end users. A high level of security of supply can be better maintained if a high level of standardization is achieved. Standardization allows appropriate sourcing volumes per components and limits the number of suppliers. Reduction of complexity allows efficient focus on key suppliers and key material. Especially on a contractual level as well as on a partnership level higher volumes are beneficial to increase the willingness of all partners to invest time in the relationship. Furthermore, standardization supports focusing on key material and supports detailed characterization and qualification. Even though standardization means less flexibility in establishing user-specific changes, standardization or alternatively configurable solutions support robustness of the security of supply.

5 Concluding Remarks

Quality and security of supply for SUBs are based on a multidisciplinary approach requiring well-established specifications, set-based on QbD principles for raw materials, components, and the final SUB itself. Due to the application, SUBs show a higher level of complexity in terms of involved materials, components, and management of the supply chain. Complexity of the single-use system including the bag-holder and control and supply tower can be reduced by using standard configurations and limiting as much as possible customization and variation of bag design and bespoke configurations. Expertise of the final application of the SUB on polymers and other technologies such as sensors and long-term experience in biopharmaceutical applications is a prerequisite to developing a state-of-the-art SUB system.

Long-term security of supply requires well-established supplier relations and cooperation through partnering and alliances beyond the level achieved with long-term contracts. Risk mitigation strategies need to be adopted. Although dual sourcing is a well-established practice to secure long-term supply, there might be cases where single sourcing combined with long-term partnerships, cooperation, and aligned inventory levels is the appropriate alternative to limit the risk of discontinuity of supply and the impact of raw material variations. Finally, robust change control procedures along the entire supply chain, starting from resin supply up to the end-user level, is a must to limit and manage the burden of product

changes driven by product improvements as required by the market and to deliver process improvements.

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Adv Biochem Eng Biotechnol (2014) 138: 273–288
DOI: 10.1007/10_2013_244

Published Online: 7 September 2103

A Risk Analysis for Production Processes with Disposable Bioreactors

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Abstract Quality management systems are, as a rule, tightly defined systems that conserve existing processes and therefore guarantee compliance with quality standards. But maintaining quality also includes introducing new enhanced production methods and making use of the latest findings of bioscience. The advances in biotechnology and single-use manufacturing methods for producing new drugs especially impose new challenges on quality management, as quality standards have not yet been set. New methods to ensure patient safety have to be established, as it is insufficient to rely only on current rules. A concept of qualification, validation, and manufacturing procedures based on risk management needs to be established and realized in pharmaceutical production. The chapter starts with an introduction to the regulatory background of the manufacture of medicinal products. It then continues with key methods of risk management. Hazards associated with the production of medicinal products with single-use equipment are described with a focus on bioreactors, storage containers, and connecting devices. The hazards are subsequently evaluated and criteria for risk evaluation are presented. This chapter concludes with aspects of industrial application of quality risk management.

Keywords Disposable bioreactor · Extractables · GMP production · Hazard analysis · Leachables · Risk management

Abbreviations

API	Active pharmaceutical ingredient
ASTM	American society for testing and materials

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BPSA	Bio-process systems alliance
BSE	Bovine spongiform encephalopathy
CCP	Critical control point
CFR	Code of federal regulation
cGMP	Current good manufacturing practice
EFTA	European free trade association
EMA	European medicines agency
EP	European pharmacopeia
EU	European union
FDA	Food and drug administration
FMEA	Failure mode and effects analysis
FTA	Fault tree analysis
GMP	Good manufacturing practices
HACCP	Hazard analysis and critical control points
ICH	International conference on harmonisation
IEC	International electrotechnical commission
ISO	International organization for standardization
ISPE	International society for pharmaceutical engineering
LAL	Limulus amebocyte lysate
NASA	National aeronautics and space administration
PDA	Parenteral drug association
PQRI	Product quality research institute
PW	Purified water
QM	Quality management
QRM	Quality risk management
SOP	Standard operating procedure
SUS	Single-use system
TOC	Total organic carbon
USA	United States of America
USP	United States Pharmacopeia
WFI	Water for injection

Contents

1 Introduction

Traditionally, quality management and control systems in the pharmaceutical industry have relied considerably on defined rules and well-established standards and methods. The rules were laid down by government authorities in the form of good manufacturing practices (GMP), and established methods were defined by qualification and validation processes in pharmaceutical companies. This has led to a very conservative approach to new manufacturing methods and has serious limitations when it comes to innovative biological production systems. The European Medicines Agency (EMA) follows the guidelines given in Annex 2 of the EU–GMP regulations [1] that biological methods may "display inherent variability, so that the range and nature of by-products may be variable. As a result, quality risk management (QRM) principles are particularly important for this class of materials…." In addition to the inherent variability of biological systems, the use of the latest flexible production systems using single-use manufacturing tools has profound implications for the quality control of such production processes.

Risk management is now an integral part of new GMP regulations, both in the European Union and the United States. The production of pharmaceuticals in single-use systems by biological means is no longer controlled by applying standard GMP rules but is extended to methods of risk management as defined by guidelines such as the ICH Q9 "Quality Risk Management" [2] and ICH Q10 "Pharmaceutical Quality System" [3], which have been approved by the pharmaceutical authorities in the United States, European Union, and Japan. This approach has the advantage of being flexible enough for future development in both biological production systems and innovative single-use solutions. But there is also a certain shift of responsibility from the authorities to the pharmaceutical companies involved, which brings challenges to both parties. Not only do inspectors check compliance issues using defined lists, companies are also obliged to create specific rules and specifications based on a scientific rationale for their processes and systems. As there is a wide degree of discretion, risk analysis tools are essential to document evaluations as well as the scientific rationale behind decisions made during risk evaluation processes.

A definition of terms for this chapter is shown in Table 1 and is based on the ICH Q9 guidelines [2], which follow (among others) the definitions given by several ISO norms such as ISO 14971 "Application of risk management to medical devices" [4] and ISO 31000 "Risk management—principles and guidelines" [5].

Table 1 Definition of terms used in risk analysis

Hazard	"The potential source of harm" [4]. Hazards are therefore qualitative descriptions of sources and may lead to hazardous situations or harm from different risks
Severity	"A measure of the possible consequences of a hazard" [2]
Harm	"Damage to health, including the damage that can occur from loss of product quality or availability" [2]
Risk	"The combination of the probability of occurrence of harm and the severity of that harm". Risks are in most cases quantitative and serve as the basis of risk management systems. This system allows the "assessment, control, communication and review of risks" with the aim of mitigating risk for patient safety within the whole life cycle of medicinal products. [4]
Risk analysis	"The estimation of the risk associated with the identified hazards" [2]

2 Regulatory Background for the Production of Medicinal Products

The production of pharmaceuticals is based on GMP, which provides a framework for the manufacture of safe effective drugs at a constant quality level. GMPs are published and controlled by pharmaceutical authorities, and are justified by the potentially devastating impacts on the health of patients. Health authorities act on two levels to ensure patient safety: first by the registration process for new drugs and second with the application process for a production license. The relevant regulatory authority is always the official body of a country in which the medicinal product is marketed and distributed to patients. For most countries in Europe, this is the European Medicines Agency (EMA) in collaboration with the authorities of the EU member states; for the United States it is the Federal Drug Administration (FDA). Both authorities are connected through several harmonization agencies, of which the most important is the International Conference on Harmonisation (ICH).

In the European Union the EMA follows the basic GMP guidelines set out by the European Commission. The guidelines consist of three parts and 19 annexes. The first part is titled "Basic requirement for medicinal products" [6], and the second part is based on ICH Q7 [7, 8] and deals with specific issues for the manufacturing of active pharmaceutical ingredients (API). The third part, "GMP-related documents," cites ICH Q9 [2] as the basis for quality risk management in Europe. Although Switzerland is not a member state of the European Union, all three parts of the GMP guidelines are applicable, as Switzerland has a fully operational mutual recognition agreement with the European Union as well as access to ICH as a representative of the European Free Trade Association (EFTA).

For the United States, the basic set of GMPs comparable to the EU–GMP part I is given by the Federal Code of Regulation CFR Part 211 "Current good manufacturing practices for finished pharmaceuticals" (cGMP [9]). This regulation is supported by documents entitled "Guidance to the industry," which cover specific topics of pharmaceutical manufacturing. The basis of ICH Q9 in the United States is given by the "Guidance for Industry Q9 Quality Risk Management" [10]. GMPs

in both the United States and European Union are supplemented by the United States Pharmacopeia (USP, [11]) and the European Pharmacopeia, respectively (EP [12]), which contain specifications and descriptions of standard pharmaceutical ingredients as well as basic requirements for methods in pharmaceutical analytics and production. They also include acceptance criteria for extractables for product-contact materials [13].

In addition to regulations issued by governmental authorities and the ICH, additional information and best practices are provided by organizations such as the International Society for Pharmaceutical Engineering (ISPE), the International Organisation for Standardisation (ISO), and the Parenteral Drug Association (PDA). They publish information in the form of handbooks or technical reports, which are considered to be state-of-the-art when it comes to the transfer of regulatory requirements to manufacturing processes.

3 Regulations and Guidelines for Single-Use Equipment

Containers, tubing, and connectors used for single-use manufacturing are made out of polymers. The USP and the EP define the use and quality of these polymers. Sect. 3.1 in EP [12] sets tests for the physical and chemical properties of polymers such as acidity and alkalinity, soluble substances in hexane, additives, extractable metals, antioxidants, and many others. The USP [11] describes methods for the characterization of the polymers < 661 > and their biological reactivity < 87 > and < 88 > as well as limits for residues of heavy metals < 661 > and nonvolatiles < 661 >. Although these documents were originally written in regard to the quality of primary packaging, they are also used for single-use systems in production processes.

4 Key Methods of Risk Management

There are many risk management tools described in the literature [14] and listed in ICH Q9 [2]. There is no tool suitable for every task, and it is not always necessary to follow a specific method. The common basis for all tools is a clearly described process founded on standard operating procedures (SOP), using flowcharts to illustrate them graphically. Manufacturing processes are subsequently validated according to critical process parameters, which are established by QRM teams based on scientific rationales. The parameters are controlled by protocols and checklists for each production batch, and the results of risk assessment, risk control, and risk review have to be retained in written records. These written records not only form the specific knowledge base for a manufacturing company carrying out risk control [15] but also serve as a basis for inspections by regulatory authorities.

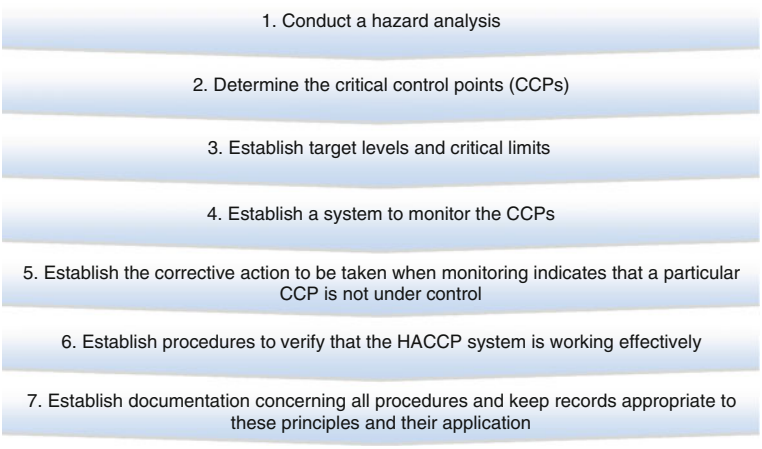

Fig. 1 The seven principles of HACCP [20]

The most widely used tool in the pharmaceutical industry is the failure mode and effect analysis (FMEA, IEC 60812 [16]). This method enables extensive listing of all potential failure modes for every process step and subsequently the classifying of severity, probability of occurrence, and chance of detection of a failure mode before the customer or patient is affected. The product of severity, probability, and chance of detection is defined as the "risk priority number." This number serves to prioritize risks and the subsequent mitigation strategy to reduce the overall risk of a process. FMEA is a powerful tool for complex biopharmaceutical processes [17] as it breaks down the complexity into manageable steps and results in structured written tables, which record the rationale behind the risk evaluation process. FMEA methodology is well established, and hands-on guidelines are available [18].

The Fault Tree Analysis (FTA, IEC 61025, [19]) links multiple possible causes of failures. It enables a systematic approach to all possible failures, and graphically depicts the root causes and their interconnection. It is therefore a perfect complement to FMEA as it shows how the failure modes used in FMEA have been established. There are many similar methods used such as Ishikawa diagrams or fishbone diagrams.

The hazard analysis and critical control points (HACCP) concept was established during the Apollo mission by the Pillsbury Company and NASA to ensure the safety of food for astronauts. The method consists of seven principles, which are shown in Fig. 1 [20]. The whole system is safe if the critical control points are set in the correct position and kept well under control. This is the case if the critical control points are measurable in a timely fashion, the parameters measured are clear quality indicators of the finished product quality, and the acceptable range at the critical control points is clearly defined and validated.

5 Hazards of Single-Use Manufacturing

Risk management for single-use equipment starts with the evaluation of potential hazards as possible causes of patient risk. These hazards are related to the material of the single-use equipment, to the specific process design, and to the product produced. Supply chain and lifecycle management for disposable processes are very different from traditional single-use processes in biomanufacturing. Quality risk management issues must therefore not only cover the manufacturing site but also include suppliers, contract partners, and internal departments from development to product discontinuation over the whole product lifecycle [21].

5.1 Material-Related Hazards

The material used for single-use equipment should show as little interaction with biological material as possible or as specified for the intended use. Specific tests are available for testing bioactivity either by placing material or extracts of the material in contact with mammalian cells and looking for changes in cell morphology USP < 87 > or by injecting extracts into mice and rabbits USP < 88 > [11]. The results of such tests should show no uncontrolled interaction with biological material and the FDA usually expects the polymers to belong to class VI USP [11].

Mechanical stability of production equipment is a key prerequisite for use in pharmaceutical production. On a smaller scale this issue is normally well under control, but on a larger scale mechanical stability is a limiting factor for the employment of single-use equipment. Different mechanical properties may be tested, depending on the use of the material. For containers and films, tests for puncture and impact resistance (ASTM D1709 [22], ISO 7765-2 [23]), tear resistance (ASTM D1004 [24]), tensile strength (ASTM D882 [25], ISO 527-3 [26]), seal integrity (ASTM F88 [27]), temperature at which polymers show brittleness (ASTM D1709 [22], D746 [28], ISO 8570 [29]), and resistance to chemical reagents such as solvents, acids, bases, or metals (ASTM D543 [30]) are available. Tubing and connectors are tested for compression (ASTM D395 [31]), resistance to penetration and impact (ASTM D2240 [32], D256 [33]), tear resistance (ASTM D624 [34]), elongation and tensile strength (ASTM D412 [35]), burst resistance and pressure rating (ASTM D1599 [36], ISO 7241-2 [37], EN 12266-1 [38]), and integrity (ASTM D4991 [39], E515 [40]).

Gas transmission rates are an additional factor and influence the fitness for use of polymer material for films and containers. Permeability of oxygen and water vapor is of special interest and can be measured by the method described in ASTM D3985 [41].

The origin of polymeric material, its additives, lubricants, and cleaning agents may also cause problems. In particular, materials that may transmit animal

spongiform encephalopathy agents must be avoided as they may impose a severe risk to patients. Sect. 5.2.8. of the EP [12] deals with this problem and CFR part 94.18 lists countries in which BSE has been identified [42]. In addition, material of animal origin may be the cause of virus transmission or allergenic substances. It is therefore essential that the origin of all material can be traced to the source for risk evaluation purposes.

The material used for single-use equipment should also be free from endotoxins as many of the products are for parenteral use. Testing for endotoxins by the Limulus amebocyte lysate (LAL) test is described in USP < 85 > [11] and EP 2.6.14 [12]. Validated absence of endotoxins together with validated sterility must be included in a product specification and therefore form part of the supplier qualification.

5.2 Process-Related Hazards

The most important hazards are leachables and extractables from polymer material as they may migrate into the drug formulation. A definition of the terms [43] is given in Fig. 2.

Assessment of the chemical interaction of container material and pharmaceutical content is in three phases: material screening and selection, simulation study including worst-case evaluation, and product assessment of the actual case [44]. This approach is also described by organizations such as the FDA [45], EMA [46], Product Quality Research Institute (PQRI) [47], and the Bio-Process Systems Alliance (BPSA) [48]. In order to ensure good quality of the final drug product or drug substance it is important to consider not only the risk to patients but also the risk to the drug-producing organism [49].

Studies of extractables will create a design space for the types and concentrations of substances that may, depending on the material processed in single-use containers, contaminate a product. The bandwidth of testing conditions such as temperature, pH-value, or surfactant concentration need to be extended, when compared to standard use, to include all possible extraction conditions for substances from container material. However, reasonable test conditions should be used to generate relevant data for worst case, but not impossible, process conditions [50].

Solvents used for extractables studies should be of the polar (e.g., water, ethanol) and nonpolar (e.g., hexane, isopropanol) types. Solvents tested based on systems with water should include different pH-values, ionic strengths, and detergent concentrations. Extraction conditions should vary in temperature (e.g., 20–80 °C) and contact time (e.g., hours to days). An in-depth example of an extractables study has been published, starting with a complete list of product-contact material before clinical phase-1 and finishing with quality assurance approved test results before phase-3 clinical trials [13].

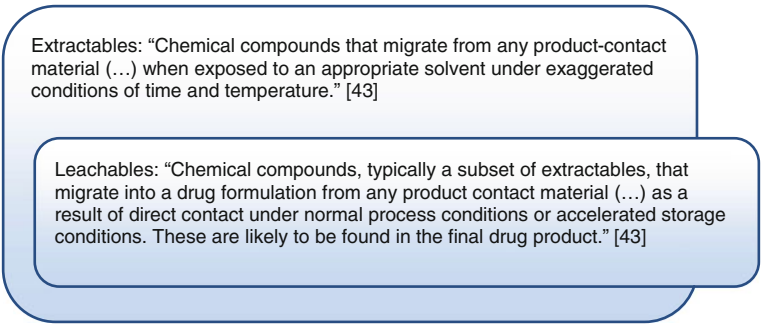

Fig. 2 Definition of extractables and leachables

This sort of test should be supplemented by leachables studies, which show migration of substances under actual process conditions such as temperatures, times applied, and media used. The results of such leachables studies are normally a subset of the data covered by extractables studies. Examples of programs for extractables and leachables studies are given in the literature [51].

Other than the solvent power of the contents of single-use containers, the equipment used for pharmaceutical production should not add any substance to the process. This is usually checked by determining the total organic carbon (TOC) in rinse water of pharmaceutical grade, such as water for injection (WFI) or purified water (PW). In both cases only a residue of 0.5 mg l^{-1} TOC is allowed [12]. This means that a polymer container should not release a higher quantity of substances contributing to TOC after gamma sterilization when measured with a method such as USP < 643 > [11].

Sterility is a prerequisite for producing medicinal products for parenteral or ophthalmic use. For traditional multiuse equipment, the pharmaceutical manufacturer carries out the validation of sterilization processes. However, with single-use equipment delivered as sterilized components, the supplier has to guarantee sterility. Most equipment is made from polymers and thus sterilized by radiation. This process is covered by the ISO 11137 [52] series and well described in the literature [53]. However, sterility is not only about sterile manufacturing components, it is also concerned with aseptic manufacturing processes, which are covered by Annex 1 [54], Sect. 5.1 in EP [12] for Europe and specific guidance for industry “Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice” [55], and USP [11] Chapter < 71 > for the United States. These documents set out specific requirements for production areas in which processing steps for sterile drug products are performed. Single-use equipment allows completely closed system processing, which may be performed in areas with lower cleanroom classifications if producing drug substances (API) [56].

Connected to the sterility issue is the need for container closure integrity. The aim is separation of content from the environment by reliable containment

allowing exchange only through a dedicated port. This is measured by using gaseous or liquid tracers, or by applying a vacuum or pressure to the container and measuring the retention time of the state applied. Microbial challenge tests may be supplemented with test microbiological containment, USP < 71 > [11].

During operation particulates may be produced either by the content itself (through aggregation) or by crystallization. In addition, tears caused by mechanical stress may lead to additional particle forming. This is especially the case when peristaltic pumps are used.

Conductivity and defined pH-values are of high importance for biological processes such as biotechnological production of active pharmaceutical ingredients. Materials for single-use equipment should not change pH-values of the enclosed liquids and should allow stable pH-values even in low-buffered liquids such as WFI or PW.

Quality management systems are crucial for safe processes. This is particularly true for supply chain management and change control systems. In the case of typical single-use systems, the responsibility for the process is shared in a different way from traditional stainless steel multiuse systems. For example, the pharmaceutical company involved must be informed of changes in the polymers used for the equipment. Hence, thorough qualification of a SUS-supplier needs to be an integral part of the QM system.

5.3 Product-Related Hazards

Many products manufactured with single-use equipment contain proteins such as monoclonal antibodies or cytokines as pharmaceutical active substances. It is important that the container material does not reduce the activity of these substances. Due to the chemical nature of polymers and proteins, possible interaction can lead to adsorption and thus loss of pharmaceutical activity. Variation in the amount of adsorbed protein is highly dependent on container material and protein type and therefore proteins need to be individually evaluated [57]. Besides adsorption, inactivation of protein by leachables from the container material as well as interaction with components of the growth media such as polysorbates or preservatives has to be considered.

Specific hazards have to be considered when producing viral vaccine material and a biosafety level 2 or 3 environment may be required. In many processes multiple cell lines are used, which further increases the complexity of risk evaluation. Changing from stainless steel containers to disposable equipment weakens the primary containment barrier and thereby increases the probability of operator contamination and/or product loss. It therefore creates additional requirements for secondary containment reliability [58]. Retention vessels need to be installed, as well as overpressure and leakage detection. An additional issue is the safe disposal of the bags after their use.

The anticipated use of the drug product is also worth consideration [59]. There is the intended therapeutic dose, which can be calculated to accepted residue levels of impurities. On the other hand, drugs addressing specific patient populations with their specific vulnerabilities (such as immune-compromised, infant, or elderly patients) have to be taken into account.

6 Risk Evaluation Criteria

After determining hazards connected with the use of disposable bioreactors, containers, fittings, and connections as part of risk identification and analysis, the risk evaluation phase follows [5]. It aims to set priorities for the subsequent risk mitigation process and to establish a basis for risk acceptance decisions. Both are essential to performing the qualification and validation process needed for the approval of new equipment or processes [60]. The basic criteria for this evaluation process in manufacturing are proximity to active pharmaceutical ingredients (API), extraction capability of growth medium or buffer, duration of contact, product contact surface area, toxicity of extractables, temperature, and inherent material resistance to extraction [12].

On the basis of material, process, and product-related hazards, impurity concentrations in the final product can be calculated for different scenarios [61]. In the worst-case scenario, purification steps do not cause any decrease in impurities; however, in reality, protein purification steps serve to decrease impurities of low molecular weight, which are typical leachable substances. For the final risk evaluation, residual concentrations have to be evaluated in the process validation step based on toxicological expertise. The ultimate goal of the risk process is always the safety of the patient in all its facets; it is not a tool for cost reduction in production processes.

7 Industrial Application

The goal of the industrial application of risk analysis is to set priorities for taking effective measures in order to maximize safety by optimum use of the means available. The basis is the evaluation of separate risk dimensions such as probability and severity, as well as creating a risk matrix. This matrix is subsequently reduced to one single risk number, which is then used as a basis for management decisions. This step has to be performed with caution, because information can be lost and different dimensions need to be weighted against each other [62].

The single risk dimension should not be zero, as the product of the risk dimension would then also be zero. In addition, the risk levels for each dimension should be kept at a minimum to provide an unambiguous assignment of each situation to a well-defined level. This minimizes the chance of a risk analysis being

Table 2 Definition of the risk values

Risk	Risk description	Risk value
Pharmaceutical application	Inhalation, injection, nasal, rectal	10
	Transdermal	5
	Topic, oral	1
Distance to the patient	Final filling	10
	Production of final API	5
	Production of API intermediate	1
Time of exposition	More than 7 days	10
	48 h to 7 days	5
	Less than 48 h	1
Surface to volume ratio	More than 0.01 cm^2 mL^{-1}	10
	0.01–0.001 cm^2 mL^{-1}	5
	Less than 0.001 cm^2 mL^{-1}	1

Table 3 Possible risk scores and their classification to the risk levels for a specific application at current date

100	500	1000	2500	5000	10000
50	250	500	1250	2500	5000
25	125	250	625	1250	2500
10	50	100	250	500	1000
5	25	50	125	250	500
1	5	10	25	50	100

Green low risk level; *yellow* medium risk level; *red* high risk level

Table 4 Measures based on the risk levels

Measures	Risk levels		
	low	medium	high
Leak, pressure, crack verification	Y	Y	Y
Tear evaluation	Y	Y	Y
pH-value: change evaluation	N	N	Y
Sorption test	N	Y	Y
Leachable test	N	N	Y
Particulate evaluation	N	N	Y
Sterility evaluation	Y	Y	Y
Depyrogenisation evaluation	Y	Y	Y
Spallation test for peristaltic pump tubing	N	Y	Y
Filter integrity test	Y	Y	Y

Y Measures have to be taken, *N* Measures usually not necessary

manipulated to achieve a favored outcome. The following example from industry does not seek to provide a recipe as in a cookery book, but rather to give a starting point for the development of a specific procedure depending on the actual process.

For the industrial production of an API, Werthenstein BioPharma (MSD) defined the four risk dimensions as pharmaceutical application (A), distance to the patient (B), time of exposition of the API to the polymeric material (C), and surface-to-volume ratio of the container (D). The risk values (see Table 2) of the four dimensions are then multiplied to a risk score number.

$$\text{Risk score number} = A \times B \times C \times D$$

This number is used to categorize each hazard to one of three risk levels for the process reviewed. Based on this risk level, the measures that need to be taken are determined. Because just three levels are used per risk dimension, only the values shown in Table 3 can be calculated.

The risk levels determine the measures that need to be taken to evaluate a process. The range of measures to be evaluated for process safety is given in Table 4. Using this procedure, the rationale for a decision is well defined and the process can be traced from the initial position to the final steps. This not only helps to allocate financial and personnel resources in a company, but also to perform an inspection or customer audit successfully.

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Adv Biochem Eng Biotechnol (2014) 138: 289–292
DOI: 10.1007/978-3-642-45158-4

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